Exploring and Exploiting DNA Repair Mechanisms to Improve Suicide Gene Therapy with Ganciclovir

by

Brendon Paul Ladd

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in The University of Michigan
2012

Doctoral Committee:

Professor Donna S. Shewach, Chair
Professor William D. Ensminger
Professor Wendell W. Weber
Associate Professor Thomas E. Wilson
Assistant Professor Christine E. Canman

To my wife Lyndsey

For all of her love and support

Acknowledgements

I would like to thank Donna Shewach for five years of outstanding mentorship. Her guidance and patience got me to where I am today. I am extremely grateful for time she spent helping me finishing my last experiments and the writing of this dissertation. I would also like to thank the rest of the former and current lab members (Jess, Sheryl, Mike, Paul). Especially Sheryl, who taught me valuable lab techniques, helped with writing, analyzing data and helping me develop early ideas into actual hypotheses. I would also like to thank the members of my committee for providing valuable advice on my project. In particular I would like to thank Dr. Wilson for his collaboration and letting me perform experiments in his lab and Dr. Canman for providing reagents and for all of her efforts as the chair of my qualifying exam.

I would also like to thank my parents, family, and friends for all of their support. Finally, I would like to thank my wife Lyndsey for moving with me to Michigan and believing in me throughout graduate school and supporting me in my career and everything else!

Table of Contents

List of Figures

List of Abbreviations

4-PB – 4-Phenylbutyrate a KDAC inhibitor

53BP1 – A protein involved in double strand break repair

5-FC – 5-fluorocytosine

5-FdUMP – 5-fluorodeoxyuridine monophosphate

5-FU – 5-fluorouracil

ACV – Acyclovir

araA – 9-β-D-ribofuranosyladenine

araT – 1-β-D-arabinofuranosylthymine

ATM – A kinase involved in sensing DNA damage, particularly double strand breaks

ATR – A kinase involved in sensing DNA damage

BER – Base excision repair

BLM – Blooms Helicase, a helicase involved in DNA resection during DNA repair

BRCA1 – A protein involved in HR, particularly in facilitating rad51 activity

CAR – Coxsackievirus and adenovirus receptor (used by adenoviruses to infect cells)

CHK1 – Involved in checkpoint activation. Chk1 is activated by ATR kinase after DNA damage

CMV – Cytomegalovirus

CtIP – C-terminus Interacting Protein, an exonuclease that resects DNA during HR

dGTP – deoxyguanosine triphosphate

DNA2 – An exonuclease that resects DNA during HR

dNTP – The triphosphate form of any nucleotide

DSB – Double stand break

EXO1 – An exonuclease that resects DNA during HR

GCV – Ganciclovir

GCVMP – Ganciclovir monophosphate

GCVTP – Ganciclovir triphosphate

HR – Homologous Recombination

HSV-TK – Herpes simplex virus thymidine kinase

HU – Hydroxyurea

IR – Ionizing radiation

KDAC – Lysine deacetylase

MDR4 – Multidrug resistance protein

MDR5 – Multidrug resistance protein

MRE11 – An endonuclease involved in sensing DNA damage, part of the MRN complex

MRN – A protein complex containing MRE11, RAD50 and NBS1

NBS1 – A protein involved in sensing DNA damage, part of the MRN complex

NHEJ – Non-homologous end joining

PEPT1 – A protein transporter involved in the absorption of amino acids in the intestine

PEPT2 – A protein transporter involved in the absorption of amino acids in the intestine

Polβ – polymerase β (involved in base excision repair)

Rad50 – A protein involved in sensing DNA damage, part of the MRN complex

RAD51 – A protein that mediates strand invasion during homologous recombination

RAD52 – A protein involved in HR, particularly in facilitating rad51 activity

RPA – A protein that binds ssDNA often generated from stalled DNA replication or resection

SAHA – Suberoylanilide hydroxamic acid, a KDAC inhibitor also known as vorinostat

SCE – Sister Chromatid Exchange, a deleterious event occurring during failed HR

SGS1 – The yeast ortholog to mammalian CtIP, an exonuclease used during HR

ssDNA – single stranded DNA

TTP – Thymidine triphosphate

VPA – Valproic acid, a KDAC inhibitor

yCD – Yeast cytosine deaminase (deaminates 5-FC to 5-FU)

γH2AX – The phosphorylated histone variant H2AX (which is phosphorylated upon DNA damage)

ABSTRACT

Exploring and Exploiting DNA Repair Mechanisms to Improve Suicide Gene Therapy with Ganciclovir

By

Brendon Paul Ladd

Chair: Donna S. Shewach

Exploring the unique mechanisms of anticancer drugs can provide the opportunity to identify novel targets for future drug development. Suicide gene therapy with the herpes simplex virus thymidine kinase (HSV-TK) and ganciclovir (GCV) is a selective approach for the treatment of cancer. Only the cells containing HSV-TK can activate GCV to a toxic metabolite, thus sparing normal dividing tissues. Upon activation by HSV-TK and further metabolism by host cell enzymes, GCV becomes incorporated into the DNA of dividing tumor cells resulting in cell death by a unique mechanism compared to other HSV-TK substrates. However, the underlying mechanistic differences that confer high anticancer activity for GCV are still unknown. The studies described in this dissertation identify differences in the magnitude of DNA damage and the DNA repair pathways activated by GCV and a less toxic HSV-TK substrate, 1-β-D-arabinofuranosylthymine. Furthermore, the DNA repair pathway of homologous recombination (HR) is identified as a critical repair mechanism to survive GCV exposure.

These observations suggest that inhibition of HR will improve GCV mediated tumor cell kill. While there are no inhibitors specific for HR, this dissertation demonstrates that the lysine deacetylase inhibitor Vorinostat (SAHA) inhibits HR in response to GCV resulting in synergistic tumor cell kill. Importantly, this synergy occurs only in cells proficient in HR demonstrating that the mechanism of synergy between GCV and SAHA is specifically due to inhibition of HR after GCV induced DNA damage. Collectively, these studies reveal that tumor cells activate the DNA repair pathway of HR in response to GCV and identify SAHA as a novel, mechanism based drug to enhance HSV-TK/GCV gene therapy.

Chapter I

Introduction

The indiscriminate nature of traditional cancer chemotherapy, which targets all dividing cells, has initiated a search for more selective approaches such as suicide gene therapy. With this strategy, only the cells containing the suicide gene are capable of activating a prodrug to a toxic metabolite, thus conferring selectivity for tumor cells while sparing normal dividing tissues. One of the most commonly used suicide gene therapy strategies transfers the cDNA for herpes simplex virus thymidine kinase (HSV-TK) into tumor cells followed by treatment with the antiviral drug ganciclovir (GCV). This approach has demonstrated exquisite tumor cell kill both in cell culture models and xenograft studies. However, the efficacy of this strategy in clinical trials has been limited, primarily due to low gene transfer. To improve this gene therapy strategy, several mechanistic studies have been conducted to determine the mechanism of GCV. Although progress has been reported, the exact mechanism by which GCV mediates tumor cell death has remained elusive. The results presented in this dissertation demonstrate that cells

require the DNA repair pathway homologous recombination (HR) to survive exposure to GCV, and if repair fails, cell death occurs. Furthermore, the data reveal that pharmacological inhibition of HR can synergistically increase tumor cell killing with HSV-TK/GCV.

History of nucleoside analogs and introduction to suicide gene therapy

Nucleoside analogs are small molecules that are structurally related to the endogenous nucleosides. Similar to endogenous nucleosides, many nucleoside analogs are phosphorylated to the triphosphate form which can compete with endogenous nucleotides for incorporation into DNA. Although the exact mechanism by which nucleoside analogs exert their cytotoxic effects remains unclear, many nucleoside analogs can inhibit the growth of tumors and viruses. For this reason, nucleoside analogs are a mainstay in the treatment of viral infections and cancer.

The first approved systemic antiviral drug was the nucleoside analog vidarabine (Adenine Arabinoside, araA) (Fig. 1.1), the use of which was restricted to life threatening infections due to accompanying toxicities such as nausea, vomiting, and bone marrow suppression(1). Before the discovery of araA, the purine analog 2,6-diaminopurine (Fig. 1.1) was developed by Burroughs Wellcome company which was used for the treatment of chronic granulocytic leukemia in adults(2-4). Although it was known that 2,6-diaminopurine was also active against vaccinia virus, the toxicity of the drug limited its use to cancer chemotherapy(4). Gertrude Elion at Burroughs Wellcome hypothesized that diaminopurine arabinoside, which

2

contains the sugar group from araA, together with its base 2,6-diaminopurine (Fig. 1.1) would be a superior antiviral compared to araA because the modified sugar would confer the same mild selectivity as araA while the modified base would be a poor substrate for metabolism by adenosine deaminase thus increasing the half life of the drug(4). They observed that diaminopurine arabinoside was less cytotoxic than araA and the major metabolite, guanine arabinoside, had antiviral activity as well(4). Although diaminopurine arabinoside appeared to produce promising results, Elion *et al* were unsure if this was sufficient to warrant full scale production of diaminopurine(4). At this time, Schaeffer *et al* at the University of Buffalo demonstrated that nucleoside analogs of adenosine that were comprised of acyclic carbohydrate moieties lacking the 2' and 3' carbons (Fig. 1.2) were recognized by adenosine deaminase(5). This work led Elion and colleagues at Burroughs Wellcome to hypothesize that other enzymes may recognize these analogs as well(4). Further, others demonstrated that the herpes virus genome contained a thymidine kinase(6) and that another emerging compound, thymine arabinoside (araT) (Fig. 1.3) had strong antiviral activity(7) without inhibiting DNA replication in normal cells(8) presumably due to selective activation by HSV-TK(9) and selective interaction with the viral polymerase(8). Based on these observations, Elion and colleagues screened for acyclic nucleoside analogs lacking both the 2' and 3' carbons and possessing antiviral activity(4). They identified 2 compounds, (1) acyclic adenosine (Fig. 1.2), which had antiviral activity but required high concentrations to inhibit viral replication relative to araA(4) and (2) acyclic-2,6-

diaminopurine riboside (Fig. 1.2)(4;10). Upon further study of acyclic-2,6-diaminopurine riboside metabolism, it was revealed that the base was metabolized into guanine to produce acycloguanosine (Acyclovir; ACV) (Fig. 1.2)(10) and that ACV was the active antiviral compound and 100 times more active than the parent compound(11). Fyfe *et al* at Burroughs Wellcome identified HSV-TK as the viral enzyme that activates ACV(10;12) and ACV became the first truly selective antiviral drug. It remains the front line therapy for herpes simplex virus (HSV) infections today(13).

The requirement for activation of ACV by a viral enzyme provided a mechanism for selectivity to HSV infected cells(14;15). In addition, following activation by HSV-TK, ACV is a 3000-fold better substrate for viral polymerase compared to human polymerase, thus providing another mechanism of selectivity for inhibiting viral replication(14-17). In light of the remarkable selectivity of ACV for inhibiting the herpes virus, similar compounds were synthesized and tested for inhibition of a broader range of viral species and for selectivity for virally infected cells only. One of these compounds was ganciclovir (GCV) (Fig. 1.3). In addition to inhibiting herpes simplex virus (18-22) GCV is 100-fold more effective at inhibiting cytomegalovirus (CMV)(22-26) than ACV due to its selective activation by the CMV kinase UL97(27;28). While GCV is more selective for the viral DNA polymerase compared to the human DNA polymerase, the magnitude of this polymerase selectivity is less than that observed with ACV(29). However, GCV is still considered

to have excellent selectivity for virally infected cells due to the requirement for activation by a virally delivered kinase(20).

Compared to deoxyguanosine, ACV lacks the 2' and 3' carbons on the sugar ring and GCV lacks only the 2' carbon (Fig 1.2, 1.3, respectively). Similar to endogenous nucleosides, GCV and ACV require phosphorylation to the mono, di, and then triphosphate forms to be activated(20-22). The active triphosphate form can then be used as a substrate for DNA synthesis(14).

Several of the preliminary studies demonstrating selectivity of ACV and GCV for inhibiting HSV versus host cell replication did so by measuring cell growth in cells with or without HSV infection(14-17). These studies demonstrated that GCV and ACV are selective due to their requirement for HSV-TK to perform the initial phosphorylation to activate these compounds(30) and that cells containing HSV-TK are capable of phosphorylating GCV to levels that can inhibit cellular DNA synthesis. Moolten *et al* hypothesized that this selective cytotoxicity could be exploited to target cancer cells specifically and demonstrated that GCV could arrest clonal expansion of HSV-TK expressing cells in HSV-TK chimeric mice(31-36). Although this approach has evolved significantly over time, many consider these studies to mark the beginning of the pursuit of HSV-TK/GCV as a gene therapy strategy for the treatment of cancer. Currently, tumor cells are selectively targeted to express HSV-TK, thus acting as a "suicide gene". Therefore, only the tumor cells containing the "suicide gene" are capable of activating a prodrug to a toxic metabolite, thus conferring selectivity for tumor cells while sparing normally dividing tissues.

5

Importantly, this approach has demonstrated excellent tumor cell kill in animal models in which strong tumor growth inhibition and complete tumor regressions have been observed(37;38).

Pharmacology of GCV

At the cellular level, functional studies have suggested that GCV can be transported across the membrane by a purine nucleobase carrier and nucleoside transporter(39-42). Efflux of phosphorylated metabolites of GCV occurs by the multidrug resistance protein 4 (MDR4) and MDR5(43-45). For GCV to be cytotoxic, it must first be activated to its monophosphate form by a viral kinase(20-22) such as HSV-TK then further phosphorylated to the di and triphosphate forms by guanylate kinase and nucleoside diphosphokinase, respectively(22;23;46-48). The triphosphate form of GCV (GCVTP) is considered the active metabolite (21) and competes with dGTP for incorporation into viral DNA which inhibits viral replication(20-22).

ACV is exquisitely selective for inhibiting HSV 1 and 2 replication therefore ACV is primarily used for the treatment of HSV 1 and 2 infections(49). Ganciclovir inhibits HSV 1 and 2 at concentrations similar to acyclovir (ACV)(18;50) despite the fact that GCV is a better substrate for HSV-TK (Km=66μM vs. 426μM, respectively)(22). In addition to inhibiting HSV 1 and 2, GCV can inhibit HSV 6(51;52), varicella zoster virus(18), Epstein-Barr virus(18;53), and cytomegalovirus (CMV) (27;28) at lower concentrations than ACV(25;48). Clinically, the primary use

for GCV is in the front line treatment of life threatening CMV infections in immune compromised patients and for prevention of such infections in solid organ transplant recipients(54-56). CMV does not contain a thymidine kinase, however the superior efficacy of GCV compared to ACV in inhibiting CMV is due to the fact that GCV can be activated by the CMV kinase UL97 (27;28). While the concentration of ACV required to inhibit CMV replication are above clinically achievable plasma concentrations, the concentrations which GCV inhibits CMV are 10 to 100 times lower (0.2-2.8ug/ml) than what is required with ACV(26). Therefore GCV is the superior antiviral for the treatment of CMV infections(13). The dose limiting toxicities associated with GCV treatment include neutropenia (15-40%) and thrombocytopenia (5-20%)(13;54;57). Other side effects include gastrointestinal disturbance (nausea, pain and diarrhea)(5-15%), headache and more rarely other CNS effects such as behavioral changes and convulsions(13;54). At therapeutic doses, the toxicities associated with ACV are relatively mild compared to GCV making ACV the frontline therapy for HSV 1 and 2 infections. However, ACV has little efficacy in patients with CMV infections. GCV is an efficacious treatment for CMV infections and therefore is used as a frontline therapy despite the associated toxicities(13).

Due to the poor oral absorption of GCV (6-9%)(13;54), the drug is administered as a 1 hour intravenous infusion of 2.5mg/kg every 8 hours or 5mg/kg every 12 hours for 14-21 days(54). After an IV bolus, GCV achieves peak and trough plasma concentrations of 8-11ug/ml and 0.6-1.2ug/ml, respectively, with a half life

of 2-4hr in patients with normal renal function(13;54;58;59). Additionally, 90% is excreted by glomerular filtration as unchanged drug(13;56). To circumvent the requirement for IV injection of GCV, valganciclovir was developed. Valganciclovir is similar in structure to ganciclovir with the exception that valganciclovir contains a valine conjugated to the carbohydrate moiety by an ester linkage. The conjugated valine allows uptake by both PEPT1 and PEPT2 transporters in the intestine(60-62). Following absorption, valganciclovir is rapidly and completely hydrolyzed into GCV by liver and intestinal esterases. The bioavailability of valganciclovir is approximately 61% with peak GCV plasma concentrations of approximately 6.1µg/ml. After an oral dose of GCV, the maximal concentration of GCV achieved in plasma is 59-67% of that achieved with intravenous GCV(63) and peak plasma concentrations of valganciclovir after an oral dose occur 1-2.5 hours post administration(55;62;64). Importantly, valganciclovir was as equally as efficacious as intravenous GCV for the treatment of CMV retinitis in clinical trials(63).

GCV in Gene therapy: Targeting Tumors

The first step in HSV-TK/GCV gene therapy is delivery of the HSV-TK cDNA to the tumor cells. This is often accomplished by direct intratumoral injection of an adenovirus containing the cDNA for HSV-TK(65). Although many viral vectors have been used for gene therapy, adenoviral vectors are commonly used because they have demonstrated superior transduction and suicide gene expression relative to other viruses such as retroviruses(65). Furthermore, retroviral vectors integrate

their genome into the host cell DNA which has resulted in adverse events such as leukemia in previous gene therapy clinical trials(66). The adenoviral genome does not integrate into the DNA of the host cells, thus eliminating the potential safety issue associated with integrating viruses such as retroviruses. Finally, adenoviruses infect cells by docking to the transmembrane Coxsackievirus and adenovirus receptor (CAR) which is ubiquitously expressed(67) and therefore can be used to infect virtually any tumor cells.

Due to the difficulty of targeting all of the tumor cells within a tumor, the use of gene therapy in the treatment of cancer is limited by the inability to transduce enough tumor cells to completely eliminate a tumor. In fact, it has been reported that fewer than 1% of cells within a tumor are successfully transduced via gene therapy protocols in humans(68). Therefore, suicide gene therapy depends heavily on improved methods to transduce more tumor cells. To circumvent the problem of low tumor cell transduction with intratumoral injection approaches, viruses carrying genes under tumor specific promoters have been generated. Although systemic delivery of these viruses has the potential to transduce normal and tumor cells, it is considered selective for tumor cells because a tumor specific promoter will not result in transcription of the suicide gene in normal cells. For example, DiMaio *et al* generated retroviral vectors carrying HSV-TK under the carcinoembryonic antigen (CEA) promoter to target pancreatic carcinoma(68;69). CEA is reactivated in pancreatic cancer cells, therefore only tumor cells will express proteins under the CEA promoter resulting in the expression of HSV-TK only in

tumor cells. A limitation with this approach is the tumor specific promoters drive relatively weak expression of the gene of interest compared to that achieved with a viral promoter(68;70). Therefore, this approach is limited by poor expression of the suicide gene(71). As a result, intratumoral injection with a stronger ubiquitously expressing promoter is still widely used. One such promoter is that of cytomegalovirus (CMV). The CMV promoter is highly effective at driving high expression of exogenously delivered genes and therefore commonly used to drive protein expression in gene therapy strategies(68;70;72). In an effort to improve transduction and transgene expression, several studies have delivered suicide genes via adenoviruses, which have the ability to infect any cell using the strong CMV promoter to drive expression of the suicide gene. To minimize the expression of the suicide gene in normal host tissues the use of adenoviral vectors delivering CMV driven genes is best suited for direct intratumoral injection. Thus this approach is most useful for tumors in which local progression is the major clinical problem(65;73-75).

The use of replication competent (oncolytic) adenoviruses to further improve gene transduction has produced some success(73;74). With this approach, adenoviral replication results in the eventual lysis of the host cell which then releases additional viral particles within the tumor which in turn can infect and kill surrounding tumor cells. When administered alone, this approach has demonstrated some positive results(68). However, this approach has not increased tumor cell transduction enough to be considered an efficacious treatment(68). The

co-delivery of HSV-TK with cytokines known to produce tumor immunity, such as IL2 has also been attempted in an effort to circumvent transduction limitations(76). With this approach, more immune cells are recruited to the tumor thus creating an immune response against tumor cells, or "tumor immunity," which results in greater tumor cell death despite low tumor cell transduction(68). While such suicide gene/cytokine combinations have demonstrated some promising results both in animal models and human studies, the level of success has not been sufficient to advance these approaches beyond clinical trials(68).

GCV in Gene therapy: Bystander Effect

The required activation of GCV to a phosphorylated form results in charged molecules that cannot easily traverse the cell membrane. Despite this, phosphorylated metabolites of GCV are transferred to neighboring non-HSV-TK containing cells by a process referred to as the "bystander effect"(32;77). The primary mechanism by which bystander cells receive GCV metabolites and subsequently die occurs primarily via gap junctional intercellular communication using a class of proteins called connexins(78-80). Connexins are transmembrane proteins that form a hemichannel that aligns with a connexin hemichannel on an adjacent cell resulting in unique pore structure or gap junction that connects the cytoplasm of both cells(81). The transfer of phosphorylated GCV to bystander cells facilitates the accumulation of active GCVTP metabolites and cell death in bystander cells despite the absence of HSV-TK in these cells. As expected, several

reports demonstrate the extent of gap junctional communication correlates with bystander cell killing and that there is a lack of bystander cell killing in the absence of gap junctional communication(78;79;81-85). In light of the fact that connexins mediate bystander cell killing, it was hypothesized that pharmacologic agents that increase or activate connexin expression would increase bystander cell killing in tumors either deficient or low in expression levels of connexins. Indeed, this principal was demonstrated by Touraine *et al* using the flavonoid-like compound apigenin and the cholesterol lowering drug lovastatin, in a mouse xenograft model(86).

Several studies demonstrate bystander killing in cells that were thought to be deficient in gap junctional communication, such as SW620 colon carcinoma cells(87). HeLa cells are also thought to be devoid of connexin proteins. However, while Gentry *et al* demonstrated that there was no rapid transfer of fluorescent dye between HeLa cells suggesting these cells lacked gap junctional communication, a more sensitive flow cytometric technique revealed a detectable level of communication over a prolonged time period.   Furthermore, the authors demonstrated that phosphorylated GCV was transferred to bystander cells(88). Studies performed in mouse models demonstrated that combining HSV-TK/GCV gene therapy with pharmacological agents that enhance the cytotoxicity of GCV resulted in prolonged survival, strong tumor growth inhibition and some complete regressions when only a fraction of the tumor contained HSV-TK expressing cells(38;75;88). It has recently been reported in a number of studies that lysine

deacetylase inhibitors (KDACs) increase gap junctional communication by increasing the expression of connexin 43(89-91). Due to the limited ability to transduce tumor cells with exogenous genes such as connexins, increasing the expression of endogenous connexin proteins with KDAC inhibitors such as Vorinostat (SAHA) may prove to be a superior method of increasing gap junctional communication.

GCV in Gene Therapy: Enhancement of HSV-TK Activity

The cytotoxicity of GCV is dependent on the amount of GCVTP within the cell, which is dependent on the initial activation by HSV-TK(92). Previous studies have demonstrated the ability to alter thymidine metabolism of HSV-TK by random mutagenesis of the enzyme(93). This led Black *et al* to hypothesize that altering HSV-TK will yield an enzyme that preferentially metabolizes GCV. To test this hypothesis, they used semi-random mutagenesis of the active site of HSV-TK to isolate mutant enzymes that enhance the cytotoxicity of GCV relative to WT HSV-TK as measured by growth inhibition in C6 glioma cells. (94-97). These studies yielded one HSV-TK mutant referred to as the SR39 variant. SR39 contains five amino acid changes within the catalytic site of HSV-TK that together result in a decrease of the affinity of the enzyme for thymidine, a direct competitor to GCV for binding to the active site(97). This decrease in competition between GCV and thymidine for SR39 HSV-TK resulted in an 83 fold kinetic advantage for activating GCV. In cell culture models, this increased kinetic advantage corresponded to an almost 300 fold increase in GCV sensitivity of rat C6 glioma cells expressing SR39 HSV-TK compared

to cells expressing WT HSV-TK. Furthermore, mouse xenograft studies revealed growth inhibition in tumors expressing the SR39 HSV-TK variant at doses of GCV that did not inhibit tumor growth in tumors expressing WT HSV-TK(97). The enhancement of GCV activation by mutant TK also resulted in an increase the number of activated GCV molecules to bystander cells, thus enhancing bystander killing(95). Therefore, the generation of mutant HSV-TK enzymes that are capable of enhancing GCV activation compared to wild type is a valuable contribution to the improvement of the efficacy of HSV-TK/GCV gene therapy.

GCV in Gene Therapy: Summary

Each of the approaches to improve gene therapy discussed above has demonstrated promising results. However, it appears that the most promising approach for improving gene therapy will be achieved by combining several approaches that improve tumor transduction in addition to pharmacological approaches that exploit the biology of tumor cells and the mechanism of GCV mediated cytotoxicity. For example, Freytag *et al* demonstrated excellent tumor cell kill in preclinical studies combining ionizing radiation (IR) with use of a replication competent adenovirus to deliver a fusion protein containing two suicide enzymes, followed by the administration of two prodrugs. The suicide enzyme fusion protein is the SR-39 HSV-TK variant fused to the yeast cytosine deaminase (yCD) enzyme, which metabolizes 5-fluorocytosine to the chemotherapeutic agent 5-fluorouracil(73;74). This double suicide gene therapy and IR combination

resulted in a doubling of tumor growth delay compared to IR alone in mouse xenograft models. Furthermore, when the double suicide enzyme was delivered by intratumoral injection with a replication competent adenovirus followed by IR, a 25% cure rate in DU145 xenografts and a 44% cure rate in LnCaP xenografts was noted compared to a 0% cure rate with either cell line with IR alone. Importantly, these studies noted that there was no additional toxicity in the combination therapy groups versus the IR alone group(98;99).

Based on the above results, clinical trials evaluating the combination of IR and gene therapy using a replication competent adenovirus to deliver yCD/HSV-TK(SR-39), followed by GCV and 5-FC treatment, were initiated in men with prostate cancer who had previously failed standard therapy. This combined approach produced an increase in the average prostate specific antigen doubling time from 17 to 31 months at the five year follow-up, which subsequently delayed the projected androgen suppression therapy by an average of two years(100). Importantly, 94% of all treatment related adverse events were considered mild to moderate in this trial whereas toxicities associated with most traditional chemotherapeutic regimens are generally severe(13). Therefore, the delay in tumor growth combined with the lack of severe toxicity observed during this clinical trial demonstrates the potential of gene therapy as a selective cancer treatment(73).

The successful approach described above combined several methods that had previously demonstrated promising results: (1) use of a replication competent

adenovirus to increase the transduction efficiency of tumor cells and to increase the duration of detectable expression of the virally delivered proteins(101;102), (2) use of the SR-39 variant of HSV-TK to increase GCV phosphorylation and incorporation into DNA(97), (3) synergistic cytotoxicity with GCV and 5-FU (103-105) , (4) at least additive cytotoxicity with replication competent adenoviruses and IR(106;107), and (5) synergistic cytotoxicity with 5-FU and, potentially, GCV when combined with IR(106-109). Collectively, these studies demonstrate that combining several approaches to enhance gene therapy results in clinically beneficial treatments. Combining this gene therapy approach with pharmacological enhancement of gap junctional communication and pharmacological enhancement of GCV cytotoxicity in tumor cells could provide additional efficacy of gene therapy approaches for the treatment of cancer.

Mechanism Based Enhancement of GCV Cytotoxicity

GCV is structurally similar to the endogenous nucleoside deoxyguanosine. GCV triphosphate (GCVTP) competes with dGTP for incorporation into DNA, an event that is required for GCV mediated cytotoxicity(92). Previously, Boucher *et al* hypothesized that decreasing intracellular dGTP levels would decrease the competition for GCVTP and result in increased GCVMP incorporation into DNA and synergistically enhance GCV mediated cytotoxicity. In support of their hypothesis, they demonstrated that decreasing dGTP with the antimetabolites hydroxyurea (HU)(38;75), gemcitabine (difluorodeoxycytidine; dFdCyd)(110), or 5-fluorouracil (5-

16

FU)(105) produced a synergistic increase in GCV mediated cytotoxicity. However, the mechanism by which these antimetabolites decreased dGTP differed. HU and dFdCyd caused a decrease in dNTP pools by direct inhibition of ribonucleotide reductase (RR)(111;112), an enzyme responsible for the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates for subsequent incorporation into DNA. While dFdCyd is useful clinically, decreases dNTPs, and results in strong synergistic cell kill when combined with GCV, dFdCyd can also be incorporated into DNA(112). The multiple mechanisms of dFdCyd mediated cell kill makes it difficult to evaluate the mechanism underlying its contribution to the synergistic increase in cell kill observed when combined with GCV. Conversely, HU affects primarily dNTPs thereby making it a valuable research tool for evaluating the role of deoxynucleotide pool imbalances in eliciting an increase in GCV mediated cell death.

HSV-TK/GCV gene therapy is synergistically enhanced when combined with yCD/5-FC gene therapy (73;74;113), however the contribution of dNTP pool imbalance as a result of 5-FC treatment was not initially appreciated. yCD converts 5-FC to 5-FU providing a selective method for delivering 5-FU to tumors while simultaneously avoiding normal tissue toxicity(114). 5-FU is metabolized by intracellular enzymes to 5-FdUMP which inhibits thymidylate synthase, an enzyme required to convert dUMP to dTMP, ultimately resulting in depleted thymidine triphosphate (dTTP) levels within the cell(115). Through allosteric regulation of ribonucleotide reductase, the decrease in dTTP resulted in the intracellular

depletion of dGTP. In addition to altering dNTP pools, 5-FU can be metabolized to nucleotides which are incorporated into both DNA and RNA, thereby making it difficult to conclude whether 5-FU mediated changes in dNTP pools or incorporation of the drug is responsible for the enhancement of GCV mediated cytotoxicity(115). To address this question, Boucher *et al* demonstrated that supplementation with deoxyguanosine during 5-FC incubation and prior to GCV incubation decreased the incorporation of GCVMP into DNA and subsequent cytotoxicity compared to concurrent 5-FC/GCV treatment(105). These studies support the hypothesis that the increase in GCV cytotoxicity is the result of the 5-FC mediated decrease in dGTP and increased incorporation of GCVMP into DNA. Furthermore, these studies demonstrated that mechanistic approaches can enhance efficacy of gene therapy strategies, an observation that will be considered for improving future gene therapy clinical trials.

Current Mechanistic Understanding of GCV Mediated Cell Killing in HSV-TK Expressing Tumor Cells

While it is known that incorporation of GCVTP into DNA is required for cytotoxicity, the exact mechanism by which this event causes cell death remains largely unknown.   Studies in B16 murine melanoma cells documented a change in cellular morphology, due to the reorganization of components of the cytoskeletal components, and an accumulation of cells in G2/M of the cell cycle following exposure to GCV(116).   GCV also initiates apoptosis as evidenced by a decline in

Bcl-2 levels and activation of caspases, following treatment(117). While these studies provide details of the downstream consequences of GCV induced cell death, the mechanisms by which cytoskeletal components become rearranged and apoptosis is initiated have not been addressed.

GCV, as well as the related compound ACV, inhibit DNA polymerases α, δ and ε(118). While both GCV and ACV preferentially inhibited DNA polymerase δ, GCV was 40 times more potent than ACV(118). GCV lacks the 2' carbon in the carbohydrate moiety, however the presence of the 3' carboxy-group allows extension of the DNA chain. Therefore, despite inhibition of DNA polymerases during incorporation into the nascent strand of DNA, GCV is not an obligatory chain terminator(48;119;120) as is the related compound acyclovir (ACV)(118), which lacks the 2' carbon of deoxyguanosine as well as the 3' carbon necessary for DNA chain elongation(121). Overexpression of DNA polymerase β (polβ) confers resistance to GCV while polβ null fibroblasts are hypersensitive to GCV(122). Polβ plays a role in the DNA repair pathway of base excision repair (BER) suggesting BER is initiated in response to GCV. Furthermore, inhibition of DNA polβ results in approximately 1.5-fold increase in GCVTP incorporation suggesting BER removes a portion of the incorporated GCVMP into DNA(122). Despite the removal of GCVMP by BER, studies have demonstrated that BER proficient cells retain GCVMP in DNA for as long as 48hr post drug washout(92). This retention of GCVMP in DNA suggests that although BER promotes survival in response to GCV by removing it from DNA before it can elicit cytotoxicity, the majority of incorporated GCVMP is

not recognized as a faulty nucleotide and remains in DNA where it eventually kills the cell.

To begin addressing the consequences of the presence of GCVMP in DNA, Thust *et al* demonstrated that GCV caused both single and double strand DNA breaks at clinically achievable concentrations which resulted in apoptosis(123). Additionally, they demonstrated that sub-cytotoxic GCV concentrations induced sister chromatid exchanges, chromosome breaks, chromosomal translocations, and other aberrations during the second cell cycle following GCV treatment whereas ACV did not(123-125). These types of chromosomal abnormalities result from aberrant or failed DNA repair suggesting that the DNA damage pathways and consequences of DNA repair differ significantly between GCV and ACV. In light of the fact that sister chromatid exchanges arise as a consequence of HR repair(126), these results also suggest HR is activated in response to GCV. In support of this hypothesis, studies evaluating yeast strains deficient in DNA repair proteins demonstrated that strains deficient in HR proteins are more sensitive to GCV indicating that HR is promoting survival in response to GCV(127). Rubsam *et al* evaluated the metabolism and cell cycle effects of GCV compared to less cytotoxic HSV-TK analogs acyclovir (ACV) and 1-β-D-arabinofuranosyl thymine (araT) (Fig. 1.3). The comparison of GCV to ACV revealed that the inferior cytotoxicity of ACV could be attributed to lower phosphorylation to the triphosphate form compared to GCV resulting in lower amounts of the active metabolite and subsequently lower incorporation into DNA. However, compared to GCVTP, araT triphosphate

accumulated to a greater extent while its elimination occurred more slowly. This

difference in kinetics resulted in a ~500-fold increase in incorporation of araTMP

into DNA compared to GCVMP at equitoxic concentrations(92). These results

suggested a unique mechanism of GCV mediated cytotoxicity compared to araT.

Rubsam *et al* also demonstrated that GCV elicits a unique cell cycle

disruption compared to other nucleoside analogs. Upon incorporation into DNA,

most nucleoside analogs including araT interfere strongly with DNA synthesis in the

first cell cycle after drug addition, however, surviving cells progress through S-phase

and return to a normal cell cycle distribution(92;128). This cell cycle pattern implies

that, while cells have difficulty incorporating analogs such as araT, if they succeed

the incorporated araT will serve as an adequate substrate for DNA synthesis in later

cell cycles(92). In contrast, GCVMP incorporation produces only moderate DNA

synthesis inhibition thereby permitting cells to eventually complete S-phase and

undergo cell division. Upon entry into the second S-phase, cells arrest permanently

and die(92) suggesting GCVMP is a poor substrate for DNA replication when in the

template strand of DNA.

Collectively, these studies have led to the following model for GCV mediated

cytotoxicity: GCVTP competes with dGTP for incorporation into DNA. Despite the

ability to inhibit DNA polymerases(118), GCVMP becomes incorporated into DNA in

internucleotide linkages(48;92). The DNA repair pathway of BER removes a fraction

of GCVMP from DNA which promotes survival(122), while GCV does not activate

nucleotide excision repair(122) thus demonstrating the specificity of repair

pathways activated by GCV. Following exposure to GCV during S-phase, cells subsequently complete DNA replication and the remainder of the cell cycle with GCVMP in DNA. Upon entry into the second S-phase cells permanently arrest and eventually undergo cell death by apoptosis(92). The DNA repair pathway of HR attempts to repair GCV induced DNA damage in the second cell cycle, however at cytotoxic concentrations HR fails to promote cell survival. This suggests targeting HR pharmacologically will be a novel, mechanism based approach to enhancing the cytotoxicity of GCV. Furthermore, the observation that GCV causes cell death despite lower incorporation into DNA relative to other HSV-TK substrates suggests a better understanding of HR in the context of repairing GCV induced DNA damage may reveal the mechanism of the superior cytotoxicity of GCV relative to other HSV-TK substrates.

DNA Repair and HR

Every day a cell in the human body receives an average of over 10,000 DNA lesions(129-131). The cause of this damage occurs from natural processes such as metabolism and normal DNA replication as well as from exogenous sources such as IR, sunlight, and chemicals such as chemotherapeutic agents. Of all the possible types of lesions to DNA, double strand breaks (DSBs) are the most deleterious(132). In order to repair DNA DSBs, the lesion must first be detected by cellular sensors such as the MRN complex(133), a heterotrimer consisting of MRE11, RAD50 and NBS1. After detection of a DSB, the MRN complex recruits and activates signal

transducers, such as ATM and ATR, which subsequently go on to activate a large number proteins involved in cell cycle checkpoints and DNA repair. Upon activation, repair proteins then localize to the DNA strand break and facilitate repair.

Although both ATM and ATR are required for a full checkpoint response(134), ATR is not directly activated by the DSB per se, but rather it is activated by single stranded DNA generated resection of DNA(134). Stalling of DNA replication also results in formation of single stranded DNA and thus activation of ATR(135), however the downstream targets of ATR such as chk1(136) are required for the full DNA damage response(136).

After detection of a DSB and activation of cell cycle checkpoint proteins, cells use two major repair pathways to repair DNA DSBs: non-homologous end joining (NHEJ) and HR(132). NHEJ is a repair pathway that occurs in all phases of the cell cycle and repairs DSBs by ligating the broken strands of DNA back together. NHEJ is considered a low fidelity DNA repair pathway because any sequence that is lost at the break when the damage occurs can be deleted when the strands are ligated back together. HR is a high fidelity DNA repair process that repairs DSBs during S and G2 phases of the cell cycle(137) and re-establishes stalled or collapsed replication forks (Fig. 1.4)(138). HR is considered a high fidelity DNA repair process because it uses the other chromosome or "sister chromatid" to resynthesize the damaged area which prevents any loss of genetic material (Fig. 1.4).

After activation of HR in response to a DSB in S-phase, DNA surrounding the break undergoes 5' nucleolytic resection creating a 3' ssDNA overhang. The

resection of the 5' end of DNA occurs in two phases. The first phase is resection

initiation, which is a slow process involving the MRN(139) complex and the

exonuclease CtIP(140-142). The second phase occurs more quickly and involves

BLM helicase(143) and the exonucleases Sgs1, Dna2, and exo1(139;144). Several

studies in yeast evaluating the exonucleases involved in both initiation and

elongation of DNA resection have demonstrated that if one or multiple

exonucleases are deleted, resection still occurs due to compensation by redundant

exonucleases(139;140;144;145). In human cells, however it was demonstrated that

the deletion or depletion of CtIP and Exo1 results in genomic instability(146),

thereby demonstrating the importance of resection during HR in mammalian cells.

After the formation of the ssDNA by resection, the 3' overhang is coated by

RPA to prevent the DNA from undergoing nonspecific annealing to other

ssDNA(138). In human cells BRCA2 then mediates the polymerization of the HR

required protein rad51 into a filament that replaces RPA on ssDNA. In yeast, the

replacement of RPA with rad51 is mediated by another ssDNA binding protein,

rad52. BRCA2 in human cells or rad52 in yeast then facilitate rad51 mediated

strand invasion of the 3' overhang into the sister chromatid(138). The invading

DNA is then free to anneal to the complementary sequence of the sister chromatid

which facilitates the resynthesis of any lost DNA sequence due to the break(138).

The crossover into the sister chromatid and subsequent annealing of the non-

displaced DNA to the sister chromatid results in two DNA structures referred to as

Holliday junctions which must be resolved correctly after HR is completed. Failure

to properly resolve both Holliday Junctions results in sister chromatid exchanges(126), a phenomenon observed in surviving cells treated with GCV thus implicating that HR is initiated in response to GCV.

This dissertation demonstrates that GCV produces more DNA damage than the less cytotoxic analog araT. GCV induced a biphasic DNA damage response with the second increase persisting until cell death suggesting the induction of unrepairable DNA damage. Further, GCV and araT activated the DNA damage response through different pathways: GCV primarily activated a double strand break response where araT primarily activated a stalled replication response. Finally, activation of the double strand break response after GCV treatment results in the activation of HR which promotes survival in response to GCV. Collectively, these observations suggest that GCV produces unrepairable DNA damage when in template DNA leading to the induction of a double strand break response. In an attempt to rescue the cell, HR is initiated to repair this damage. At sub-toxic concentrations of GCV, HR succeeds at rescuing the cell, however it causes SCEs(147) in exchange for repairing the DNA damage and resuming DNA replication(148). At cytotoxic concentrations, HR fails to repair the GCV induced DNA damage and cell death ensues. The results of these studies implicate HR in the mechanism of GCV mediated cell death, therefore, I proposed that inhibition of HR would enhance cytotoxicity of GCV.

Rationale for KDAC Inhibitors to Improve HSV-TK/GCV Gene Therapy

HSV-TK is delivered via adenovirus which infects cells by initially binding to CAR(67). Expression levels of CAR correlate with the number of cells transduced and cell survival achieved in cell culture models(149;150). Additionally, phosphorylated GCV metabolites are transferred to bystander cells by connexin mediated intercellular gap junctional communication(78-80). The overexpression of connexin proteins results in increased sensitivity to GCV(78;79;81). Therefore, simultaneously enhancing the expression of CAR and connexins pharmacologically will result in even greater cell tumor cell kill compared to enhancing either alone. The presence of SCEs following GCV indicates that the DNA repair pathway of HR is activated in response to GCV(123;124;126). Thus, although improving any of the above mentioned aspects of gene therapy would increase tumor cell kill in response to GCV, the pharmacological inhibition of HR combined with overexpression of both CAR and connexin proteins would provide a multipronged approach for improving gene therapy. Many of the genes involved in the above mentioned processes are regulated by lysine deacetylases (KDACs). KDACs are enzymes that remove an acetyl group from lysines and are most known for their deacetylation of histones, a modification associated with transcriptional repression. In addition to deacetylating histones it is now appreciated that KDACs deacetylate a wide variety of non-histone proteins.

Background of KDAC Enzymes

There are 18 KDAC enzymes grouped into 4 classes based on their sequence homology in yeast where they were first identified. Classes I, II and IV have a structurally similar catalytic domain and require a zinc ion for their function(151). Class I KDACs include KDAC1-3 and KDAC7 which share sequence homology with the yeast protein RPD3(152). Class I KDACs are ubiquitously expressed, nuclear proteins that are thought to be relatively specific to deacetylating histones compared to other KDAC classes(152). Class II KDACs include KDAC4, 6, 7, 9 and 10 and are homologous with the yeast protein HDA1(152). These enzymes exhibit tissue specific expression, are present in both the nucleus and cytoplasm, and are thought to be the KDACs associated with deacetylating many of the known non-histone KDAC targets. Class IV KDACs include only KDAC11 which has no yeast counterpart and shares sequence homology to both classes I and II KDACs(152). Class III KDACs include 7 enzymes referred to as the sirtulins (sirt1-7). They are homologous to the yeast protein Sir2 and differ significantly from the other classes of KDAC enzymes in that they depend on NAD+ (nicotinamide adenine dinucleotide) to catalyze the deacetylation of lysine(152).

In addition to changes in gene expression, activity of the KDACs has been linked to cell cycle control(153), cell motility(154), metabolism(155), DNA damage repair(153;156), and genomic stability(157). Therefore, it is not surprising that aberrant KDAC activity or mutation of KDAC enzymes is associated with many

cancers, and that an intensive search for inhibitors of these enzymes has commenced(158).

One of the first compounds demonstrated to inhibit KDAC enzymes was Trichostatin A, which is a natural anti-fungal compound isolated from *Streptomyces hygroscopicus.* Trichostatin A inhibited the growth of murine erythroleukemia cells by inducing their differentiation(159). While investigating the mechanism by which this compound inhibited cell growth, an increase in the acetylation of histones was observed(160). Due to high reactivity and instability, Trichostatin A was not evaluated clinically for the treatment of cancer(158), however it remains a valuable research tool. Around the same period of time as the discovery of the mechanism for Trichostatin A, Paul Marks *et al* were screening for non-toxic detergents generated by the lab of Ronald Breslow, both at the Memorial Sloan Kettering Cancer Center, that inhibited the growth of cancer cells. Following an exhaustive search, they discovered a promising compound, suberoylanilide hydroxamic acid (SAHA). Based on the strong antitumor activity demonstrated in clinical trials, SAHA received FDA approval in 2006 under the generic name Vorinostat for the treatment of patients with cutaneous T-cell lymphoma who have failed other treatments(161). SAHA is structurally similar to Trichostatin A and similar to Trichostatin A, the mechanism underlying SAHA mediated inhibition of tumor cell growth is via inhibition of class I and II KDAC enzymes(162).

Pharmacology of Vorinostat (SAHA)

SAHA became the first FDA approved KDAC inhibitor, which inhibits the enzymes through the reversible binding of the catalytic site of KDAC enzymes(158;163). Structurally, SAHA contains a phenyl group and a hydroxamic acid group connected by an aliphatic chain (Fig. 1.5). The phenyl group of SAHA binds the outside of the KDAC enzyme pocket while the aliphatic chain and hydroxamic acid moieties bind in the enzyme pocket. The hydroxamic acid moiety is positioned such that it can bind the zinc ion within the enzyme pocket which is used by KDAC enzymes to catalyze the deacetylation of lysines(158;163).

SAHA inhibits all class I KDACs (KDAC1, 2, 3, 8) and one class II KDAC (KDAC6)(164) at micromolar concentrations(158). SAHA is administered orally and is 43% bioavailable following 200 or 400mg doses(158;165). While the absorption of SAHA can be delayed in the presence of food high in fat, this does not appear to be clinically meaningful(166). The maximum tolerated dose of SAHA is 400mg/day whether given as a single 400mg dose or two. SAHA exhibits linear pharmacokinetics and a half life of approximately 91.6 to 127 minutes following an oral dose(165). SAHA is 71% bound to plasma proteins with serum concentrations ranging from 2 to 200 µmol/L(167) and is well distributed throughout the body even crossing the blood brain barrier in mouse models(168) where it can reach therapeutic concentrations within the CNS(169). SAHA is not metabolized by P450 enzymes therefore it is not expected to alter the metabolism of other pharmacological agents that inhibit or induce P450 enzymes. Instead, SAHA is

eliminated directly by phase II metabolism routes where it first undergoes glucuronidation followed by β-oxidation to the inactive metabolites vorinostat glucuronide and 4-anilino-4-oxobutanoic acid. Less than 1% of active SAHA is recovered in the urine, thus indicating that metabolism is the primary mechanism of elimination(165-167). Major dose limiting toxicities include anorexia, diarrhea, dehydration, and fatigue. Other side effects include vomiting, hyperglycemia, and hematological toxicities (anemia and thrombocytopenia). The only drugs contraindicated with SAHA are coumarin derived anticoagulants(165).

4-Phenylbutyrate (4-BP) and Valproic Acid (VPA) as KDAC Inhibitors

KDACs had already emerged as promising target for the treatment of cancer during the time that SAHA was being developed, thus a search ensued for existing natural products and pharmacologic agents that inhibited KDAC enzymes. Two such compounds are 4-phenylbutyrate (4-BP) and valproic acid (VPA). 4-BP is a natural product generated by anaerobic metabolism of fatty acids by intestinal flora and is hypothesized to be protective against colon cancer(170). VPA is used as an antiseizure medication where it works by slowing the recovery of sodium channels thereby preventing their seizure associated repeated firing(13). Since sodium channels have been implicated in processes such as cell adhesion in many cancer types(171), the activity of VPA on KDAC enzymes cannot be definitively implicated as the mechanism of antitumor activity observed. Chinnaiyan, *et al* hypothesized that VPA would be a valuable KDAC inhibitor for glioblastoma, a tumor type that is

protected from most chemotherapeutic agents by the blood brain barrier(172). The ability of antiepileptic medications such as VPA to cross the blood brain barrier is well established(13) and Chinnaiyan, *et al* hypothesized that CNS toxicities due to non-KDAC activities of VPA are a surrogate marker of VPA activity(172). It is now appreciated that SAHA readily crosses the blood brain barrier(168;169) and as such it provides a more potent and specific KDAC inhibitor for evaluation in CNS malignancies. Furthermore, SAHA is active at nanomolar and micromolar concentrations in cell culture whereas both 4-BP and VPA must be administered at high concentrations (milimolar range) in order to observe KDAC activity. Despite these limitations, studies performed with VPA and 4-PB have provided valuable information for the validation of KDAC enzymes as a target for the treatment of cancer, particularly in a gene therapy setting(89-91).

KDAC Inhibitors and Gene Therapy

KDAC inhibitors are widely known for their ability to inhibit the deacetylation of histones resulting in histone hyperacetylation and increased gene transcription(152;173). In light of the fact that several processes involved in determining the efficacy of HSV-TK/GCV gene therapy depend on transcription levels of key proteins such as CAR(67), connexins(78-80;82;84), and the virally delivered HSV-TK itself(68;174), coupled with the fact that KDAC inhibition leads to an increase in transcription of many genes, several groups have investigated KDAC inhibitors in combination with HSV-TK/GCV gene therapy. Kothari *et al*

demonstrated that VPA increased the expression of CAR and subsequently increased the transduction efficiency by adenovirus, substantially increased the expression of an HSV-TK/GFP fusion protein, and produced a reduction of tumor size in a mouse xenograft model(91). In addition to increasing the number of viral particles infecting each cell, Ammerpohl *et al* demonstrated that VPA also increases the expression of virally delivered genes under a CMV promoter(89). These studies demonstrate that VPA has the potential to increase HSV-TK expression by multiple mechanisms. Additionally, Asklund *et al* demonstrated that 4-phenylbutyrate increases gap junctional communication by increasing the expression of connexin 43(90). Collectively, these studies demonstrate that combination therapy with KDAC inhibitors and HSV-TK/GCV gene therapy is a promising approach for future studies both *in vitro*(89;90) and *in vivo*(91) and warrant further study. These studies did not however examine whether KDAC inhibitors are capable of proving any additional benefit to HSV-TK/GCV gene therapy by enhancing the cytotoxicity of GCV.

KDAC Inhibitors and DNA Damage Repair

GCV treatment in HSV-TK expressing cells results in DNA strand breaks(123) and studies in yeast demonstrate that HR promotes survival in response to GCV(127) suggesting HR may repair these breaks. Therefore, if KDAC inhibitors could pharmacologically inhibit HR they may provide yet another improvement of HSV-TK/GCV gene therapy approaches. Chinnaiyan et al demonstrated that the

accumulation of γH2AX and 53BP1 foci to sites of DNA damage persist in response to IR if cells were pretreated with VPA(172). Their continued presence could be explained by persistent DNA damage due to KDAC mediated inhibition of DNA repair mechanisms. While this study suggests DNA repair may be inhibited by KDAC inhibition, it does not directly demonstrate that DNA damage persists or provide insight as to which DNA repair pathway is inhibited.

To address the potential role of KDAC inhibition on inhibiting HR, Adimoolam *et al* evaluated the effects on HR after IR in cells that were pretreated with the KDAC inhibitor PCI-24781. These studies demonstrated that the inhibition of KDACs decreased mRNA levels of several required HR proteins including BRCA1, BRCA2 and rad51. A concentration and time dependent decrease in rad51 protein expression was also noted both in cell culture models and in mouse xenograft studies. Importantly, the authors noted that pre-incubation of cells with a KDAC inhibitor sensitized cells to IR, blocked rad51 foci formation after IR, and decreased the number of HR events measured in a cell based assay(156). Together these results suggest that KDAC inhibitors inhibit HR by decreasing the expression of HR required proteins and associated repair activity.

The inhibition of KDACs has also been demonstrated to affect HR by mechanisms other than altering transcription. KDAC inhibition with VPA has been demonstrated to counteract ATR activity, inhibit recruitment of HR proteins to an induced DNA DSB, and to slow DNA end resection during HR-mediated repair of an enzymatically induced DSB(175). Other studies have demonstrated that VPA

inhibits the deacetylation of CtIP, a required exonuclease for HR, which promotes its degradation(175). Furthermore, it has been demonstrated that the acetylation status of exo1, another exonuclease involved in HR, can alter the function of this protein(175). Therefore, KDAC enzymes perform several regulatory functions in DNA end resection during HR. Previous studies have demonstrated that the depletion of CtIP and exo1 results in genomic instability(146). The regulation of CtIP and exo1 by KDAC enzymes demonstrates a role for KDAC enzymes in the maintenance of genomic instability through regulation of HR.

To address which KDAC enzymes are involved in the regulation of genomic stability, Bhaskara *et al* demonstrated that knockout of KDAC3 in murine fibroblasts results in cells that undergo fewer HR and NHEJ events after an enzymatically induced DNA DSB. KDAC3 null cells also contain an increased number of chromosomal aberrations(153;157). Importantly, KDAC3 is a class I deacetylase and as such can be inhibited by SAHA(164). Therefore, SAHA may be capable of inhibiting DNA repair in tumor cells containing HSV-TK when combined with an agent such as GCV. Furthermore, previous reports evaluating DNA replication in the presence of SAHA noted a slowing of DNA replication, a phenomenon that can be recapitulated by depletion of KDAC3(176). GCV treatment results in moderate stalling of DNA synthesis during incorporation into DNA(92). Therefore, strong DNA replication inhibition may be observed by the combination of GCV and SAHA. Stalling of DNA replication activates an ATR mediated DNA damage response(135)

thus providing another mechanism of DNA repair activation in response to GCV and SAHA.

Collectively, these studies strongly suggest that KDAC inhibitors will synergistically enhance the efficacy of DNA damaging agents by inhibiting repair. Indeed, KDAC inhibitors have demonstrated synergistic tumor cell kill when combined with a variety of DNA damaging agents including doxorubicin(177;178), etoposide(178), 5-fluorouracil and fluorodeoxyuridine(179), cisplatin, and melphalan(180). Evidence that GCV may induce DNA damage that must be repaired by HR(127) suggests that KDAC inhibitors such as SAHA may synergize with GCV by inhibiting the repair of GCV induced DNA damage by HR. Furthermore, the potential for enhancing adenoviral HSV-TK gene delivery, HSV-TK expression, and gap junctional communication while simultaneously enhancing cell kill makes the combination of KDAC inhibitors with HSV-TK/GCV gene therapy an attractive approach for selectively targeting tumors.

The goal of chapter III of this dissertation is to determine whether the KDAC inhibitor SAHA can inhibit the HR repair of GCV induced DNA damage and elicit synergistic cell kill. Using U251 glioblastoma cells expressing HSV-TK, I demonstrate that coincubation with GCV and SAHA results in enhanced cytotoxicity. Using a cell based assay that detects HR events I demonstrate that SAHA inhibits HR activity. Additionally, SAHA decreases the protein levels of the HR required proteins rad51 and CtIP. Furthermore, SAHA completely blocks rad51 foci formation in response to GCV. Finally, I demonstrate that GCV and SAHA coincubation results in

synergistic cytotoxicity in HR proficient CHO cells while only additive cytotoxicity in HR deficient cells. Therefore, synergy observed between GCV and SAHA is due to the inhibition of HR by SAHA thereby preventing repair of GCV induced DNA lesions.

Conclusions

The overall goal of this dissertation is to investigate new approaches to improve HSV-TK/GCV gene therapy by enhancing the efficacy of GCV. These findings identify a novel drug combination with HSV-TK gene therapy and potentially identify new mechanisms for drug targeting in tumor cells. This information will ultimately provide novel approaches in animal studies and potentially clinical trials.

Previous studies have demonstrated that GCV is incorporated into DNA and induces chromosomal aberrations such as sister chromatid exchanges (SCEs) and chromosomal breaks(123-125). However, the cellular response preceding the generation of SCE's in response to GCV has not been determined. I hypothesize that GCV will activate DNA damage repair pathways differently than less cytotoxic analogs such as araT. Furthermore, I hypothesize that GCV will activate the DNA damage response to a greater extent than araT despite lower incorporation of GCV into DNA.

After DNA damage has occurred, the DNA damage response is activated by the kinases ATM and ATR. ATR is activated by single stranded DNA which often forms from stalled replication forks(135). ATM is activated primarily by DSBs which

can occur by damaging agents such as IR or collapsed replication forks(134), that result from the inability to resume DNA synthesis after stalling of DNA replication(181;182). Chapter II demonstrates that while both GCV and araT activate ATR and ATM, the kinetics of activation differ significantly. ATR activation in response to GCV is detected at a consistent level at all times evaluated during drug incubation and persists after the removal of GCV. Conversely, araT induces a strong, transient ATR response during drug incubation followed by a persistent low response after removal of araT. AraT also induces a weak persistent activation of ATM during drug incubation and after its removal. In response to GCV, ATM is strongly activated 24 hours following the addition of GCV, a time coincident to entry into the second cell cycle after the addition of drug where they subsequently arrest permanently and die. This is consistent with the hypothesis that, although both GCV and araT cause DNA replication stress, araT inhibits DNA replication while being incorporated into DNA. Conversely, GCV induces unrepairable DNA damage when in the DNA template.

   In addition to the ATM and ATR kinase pathways activated in response to GCV and araT chapter II demonstrates that both GCV and araT induce rad51 foci formation thus indicating HR is activated in response to both drugs. Additionally, there was no significant difference in the percent of rad51 positive cells or the number of rad51 foci per positive cell following GCV or araT treatment. Therefore, rad51 foci formation cannot explain the differences in cytotoxicity observed with GCV and araT. The observation that HR is activated in response to GCV supports

the suggestion that SCEs observed after low dose GCV occur as a result of HR. Therefore, the observation that araT activates HR was surprising given that previous studies have demonstrated that another less toxic substrate for HSV-TK, ACV, did not induce SCEs(123-125). This unexpected observation that both GCV and araT activate HR coupled with the observation that only GCV causes DSBs supports the hypothesis that GCV is inducing a unique, unrepairable DNA lesion whereas other HSV-TK substrates induce cell death by overwhelming the DNA damage response with potentially repairable DNA lesions.

The presence of rad51 foci and SCEs in response to GCV indicate the activation of HR, however, they do not indicate whether HR is promoting cell survival or cell death. Evidence of HR promoting cell survival in response to GCV has been documented in yeast(127). I hypothesize that HR promotes cell survival in response to GCV in mammalian cells. Furthermore, I hypothesize that pharmacologic inhibition of HR will produce synergistic cytotoxicity with GCV. To test the hypothesis that HR promotes survival in response to GCV, I have evaluated GCV sensitivity in matched CHO cell lines that are either proficient or deficient in HR. Using this approach, chapter III demonstrates that HR promotes cell survival in response to GCV suggesting that inhibition of HR will enhance the cytotoxicity of GCV. While there are no direct or specific pharmacologic inhibitors of HR, the KDAC inhibitor PCI-24781 was demonstrated to inhibit HR by decreasing the expression of HR required proteins such as rad51(156). Chapter III of this dissertation demonstrates that the FDA approved KDAC inhibitor SAHA also inhibits HR.

Furthermore, chapter III demonstrates that SAHA decreases the expression of HR required proteins such as rad51, inhibits HR activity in a cell based assay, and blocks rad51 foci formation in response to GCV. Finally, chapter III demonstrates that the combination of GCV and SAHA results in synergistic cytotoxicity in HR proficient cells and only additive cytotoxicity in HR deficient cells. These results suggest that HR is promoting cellular survival in response to GCV and pharmacological inhibition of HR with SAHA results in synergistic cell death.

Collectively, the results described within this dissertation demonstrate the mechanistic differences in activation of DNA damage repair pathways in response to GCV and a less cytotoxic drug, araT, which provides insight into the mechanism underlying the superior cytotoxicity of GCV compared to other HSV-TK substrates. Additionally, the results reveal the DNA repair pathway of HR is activated by GCV and the role of HR is to promote cell survival in response to GCV. The observation that HR promotes cell survival in response to GCV identifies HR inhibition as a unique mechanism based approach that may enhance gene therapy effectiveness. Furthermore, results described within this dissertation provide the field with a previously unappreciated role for the KDAC inhibitor SAHA and its ability to inhibit HR. Finally, the results uncover the novel mechanism based drug combination of GCV and SAHA. Based on the observations in this dissertation, future *in vivo* studies evaluating HSV-TK/GCV and SAHA could demonstrate a promising new direction for suicide gene therapy.
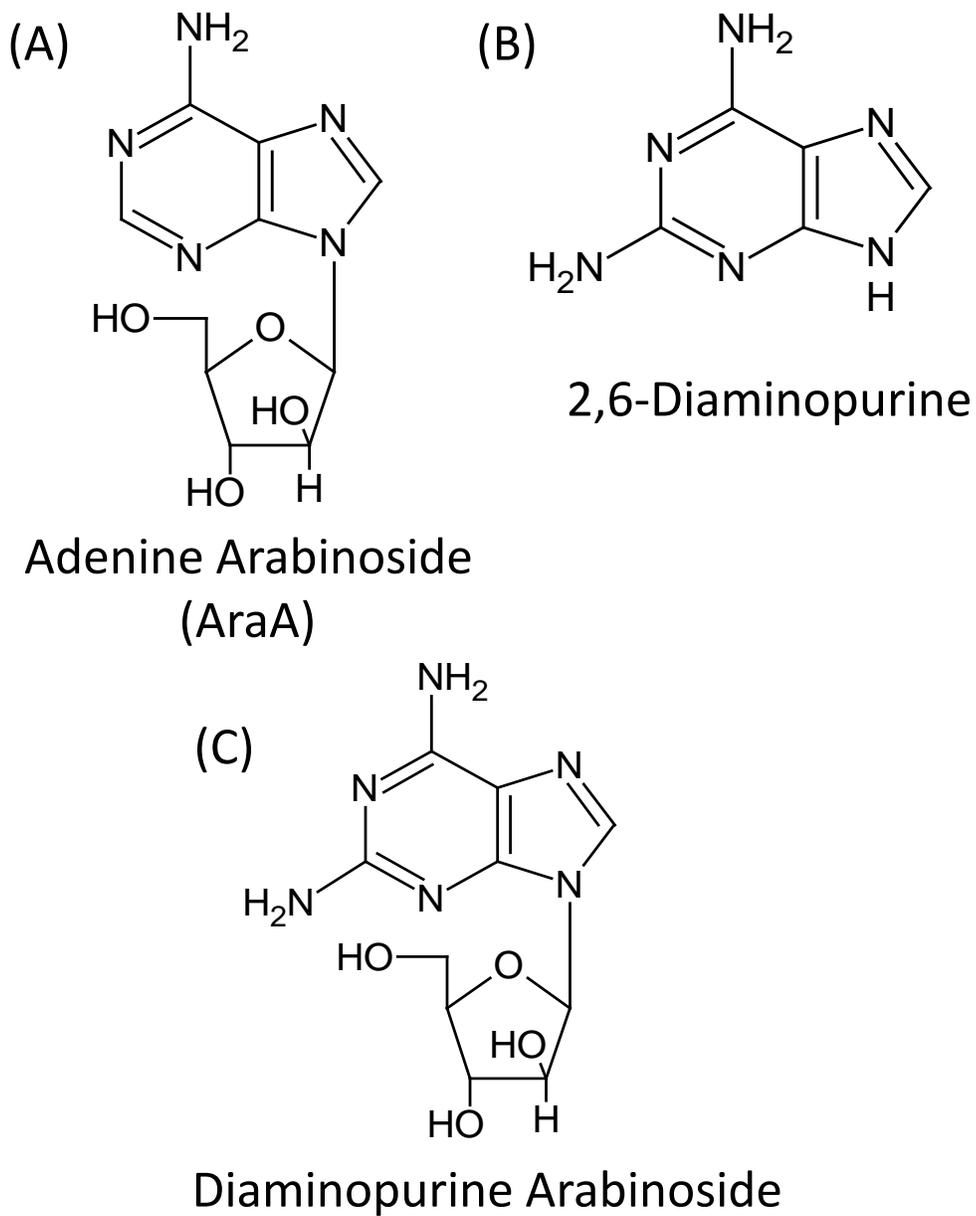
(A) Adenine Arabinoside (AraA)

(B) 2,6-Diaminopurine

(C) Diaminopurine Arabinoside

Figure 1.1 Development of ACV Pt. 1. A.) araA was the first antiviral marketed in the United States. B.) 2,6-diaminopurine was used to treat leukemia. It was known to have antivrial activity as well but was not used due to high toxicity. C.) Burroughs Wellcome Company combined the arabinose sugar with 2,6-diaminopurine (2,6-diaminopurine arabinoside) and observed it was more selective for inhibiting viruses compared to AraA but not as efficacious.
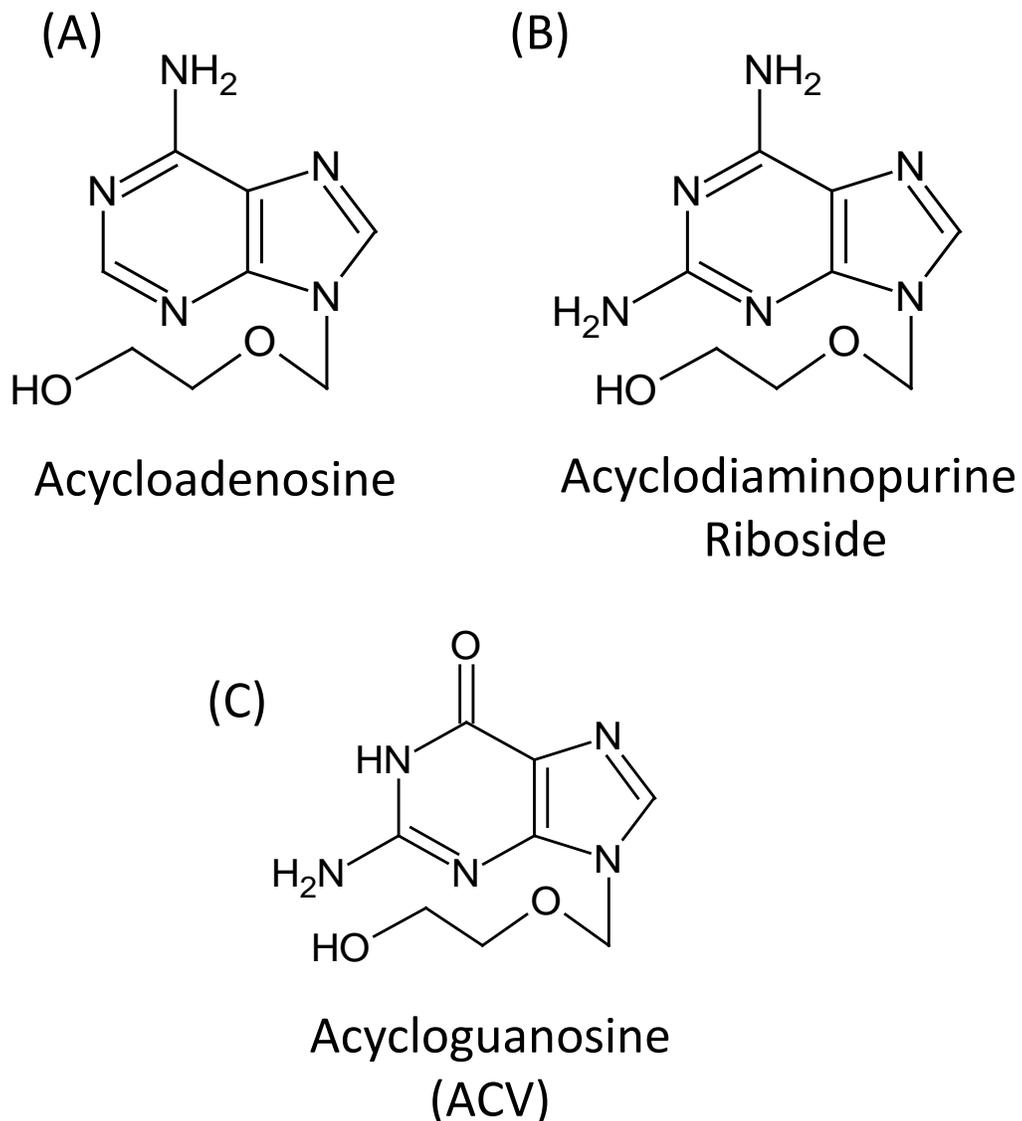
(A) Acycloadenosine

(B) Acyclodiaminopurine Riboside

(C) Acycloguanosine (ACV)

Figure 1.2 Development of ACV Pt. 2. A.) During the time 2,6-diaminopurine arabinoside was being developed, analogs with acyclic carbohydrates were demonstrated to be substrates for some cellular enzymes. B.) Burroughs Wellcome then made acyclic 2,6-diaminopurine riboside, which was selective and efficacious for inhibiting viral replication. C.) While studying the metabolism of acyclic 2,6-diaminopurine riboside it was discovered that the active antiviral was the guanosine metabolite (acycloguanosine, ACV)
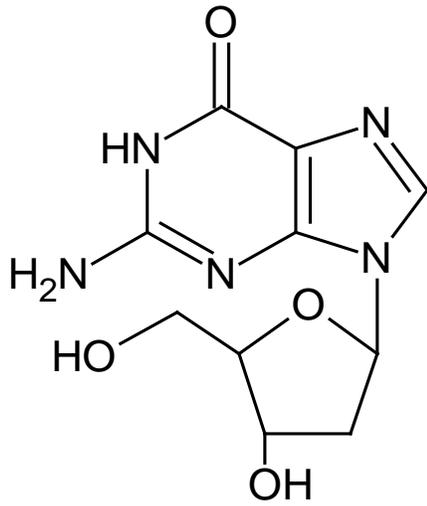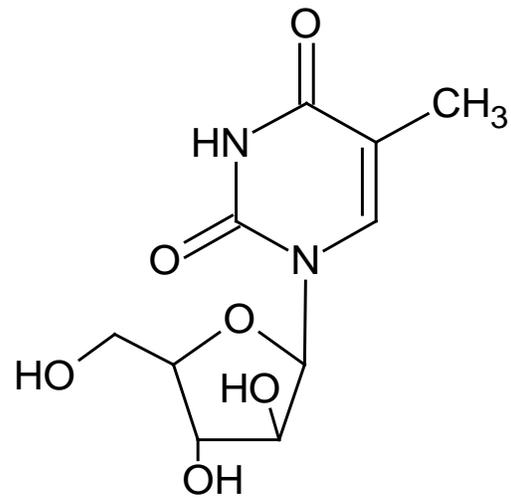
Deoxyguanosine
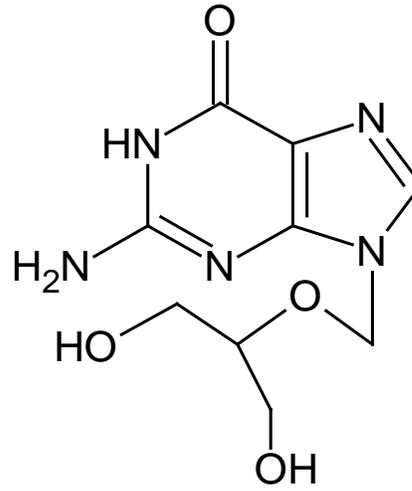
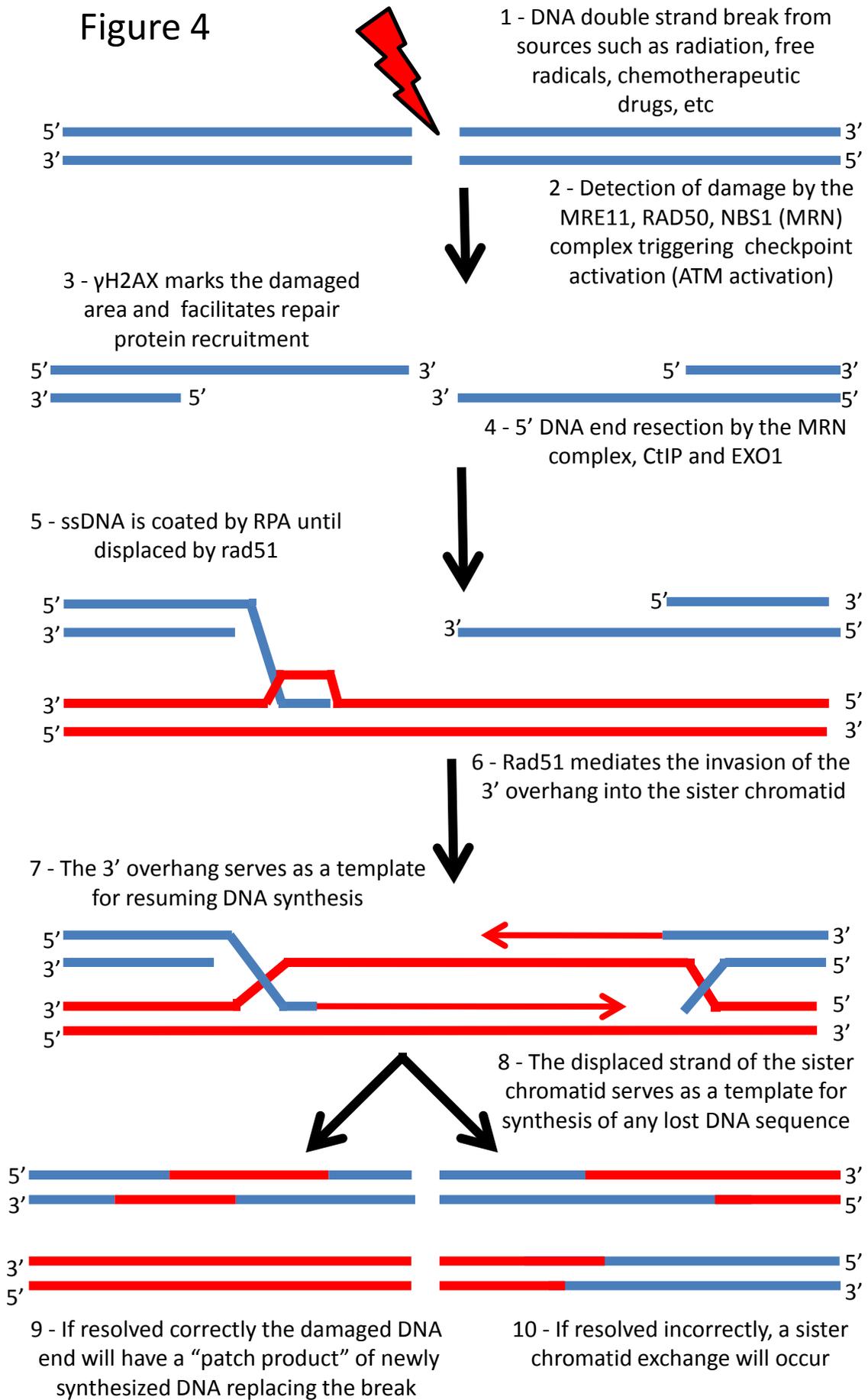**Ganciclovir (GCV)**
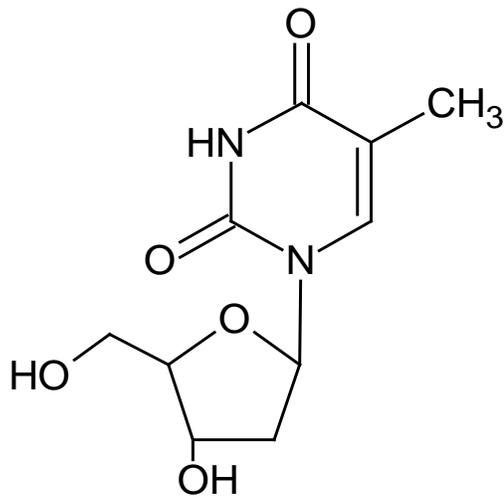
Thymidine

Thymine Arabinoside (AraT)

Figure 1.3 HSV-TK substrates. A.) GCV (right) is structurally similar to the endogenous nucleoside deoxyguanosine (left). B.) araT (right) is structurally similar to thymidine (left).

Figure 1.4 Homologous recombination. After a DNA double strand break, DNA is resected in a 5' to 3' direction. This creates 3' overhangs that invade the sister chromatid and are used as primers for DNA replication machinery to resynthesize the damaged region of DNA. After resynthesis of the damaged region, the sister chromatids must be properly resolved away from each other. If they are resolved incorrectly, sister chromatid exchanges occur.
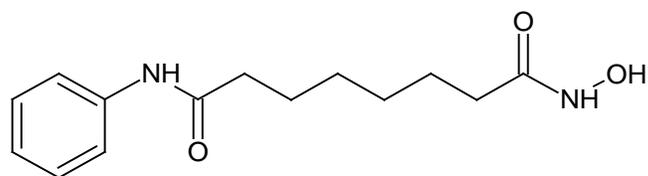
Figure 4

1 - DNA double strand break from sources such as radiation, free radicals, chemotherapeutic drugs, etc

2 - Detection of damage by the MRE11, RAD50, NBS1 (MRN) complex triggering checkpoint activation (ATM activation)

3 - γH2AX marks the damaged area and facilitates repair protein recruitment

4 - 5' DNA end resection by the MRN complex, CtIP and EXO1

5 - ssDNA is coated by RPA until displaced by rad51

6 - Rad51 mediates the invasion of the 3' overhang into the sister chromatid

7 - The 3' overhang serves as a template for resuming DNA synthesis

8 - The displaced strand of the sister chromatid serves as a template for synthesis of any lost DNA sequence

9 - If resolved correctly the damaged DNA end will have a "patch product" of newly synthesized DNA replacing the break

10 - If resolved incorrectly, a sister chromatid exchange will occur

44

Figure 1.5 Structure of SAHA

Reference List

1. Whitley, R., Alford, C., Hess, F., and Buchanan, R. (1980) *Drugs* **20,**

2. Thompson, R. L. (1949) *Science (New York, N.Y.)* **110,** 454

3. Thompson, R. L., Price, M. L., Minton, S. A., Elion, G. B., and Hitchings, G. H. (1950) *The Journal of Immunology* **65,** 529-534

4. Elion, G. B. (1989) *Science* **244,** 41-47

5. Schaeffer, H. J. (1971) *Journal of medicinal chemistry* **14,** 367-369

6. DUBBS, D. R. and KIT, S. (1965) *Virology* **25,** 256-270

7. UNDERWOOD, G. E., WISNER, C. A., and WEED, S. D. (1964) *Arch Ophthalmol* **72,** 505-512

8. Gentry, G. A. and Aswell, J. F. (1975) *Virology* **65,** 294-296

9. Kit, S., de Torres, R. A., and DUBBS, D. R. (1966) *Cancer Res* **26,** 1859-1866

10. Schaeffer, H. J. (1978) *Nature (London)* **272,** 583-585

11. Spector, T., Jones, T. E., and Beacham, L. M. (1983) *Biochemical Pharmacology* **32,** 2505-2509

12. Fyfe, J. A., Keller, P. M., Furman, P. A., Miller, R. L., and Elion, G. B. (1978) *Journal of Biological Chemistry* **253,** 8721-8727

13. Brunton, L. L., Lazo, J. S., and Parker, K. L. (2006) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 11th Ed., McGraw-Hill, New York

14. Elion, G. B., Furman, P. A., Fyfe, J. A., de Miranda, P., Beauchamp, L., and Schaeffer, H. J. (1977) *Proc.Natl.Acad.Sci.U.S.A* **74,** 5716-5720

15. Nishiyama, Y. and Rapp, F. (1979) *J Gen Virol* **45,** 227-230

16. Furman, P. A., McGuirt, P. V., Keller, P. M., Fyfe, J. A., and Elion, G. B. (1980) *Virology* **102,** 420-430

17. Davidson, R. L., Kaufman, E. R., Crumpacker, C. S., and Schnipper, L. E. (1981) *Virology* **113,** 9-19

18. Field, A. K., Davies, M. E., DeWitt, C., Perry, H. C., Liou, R., Germershausen, J., Karkas, J. D., Ashton, W. T., Johnston, D. B., and Tolman, R. L. (1983) *Proceedings of the National Academy of Sciences* **80,** 4139-4143

19. Chen, M. S., Walker, J., and Prusoff, W. H. (1979) *J Biol Chem.* **254,** 10747-10753

20. St Clair, M. H., Miller, W. H., Miller, R. L., Lambe, C. U., and Furman, P. A. (1984) *Antimicrob.Agents Chemother.* **25,** 191-194

21. Martin, J. C., Dvorak, C. A., Smee, D. F., Matthews, T. R., and Verheyden, J. P. (1983) *J.Med Chem.* **26,** 759-761

22. Ashton, W. T., Karkas, J. D., Field, A. K., and Tolman, R. L. (1982) *Biochemical and Biophysical Research Communications* **108,** 1716-1721

23. Smee, D. F., Martin, J. C., Verheyden, J. P., and Matthews, T. R. (1983) *Antimicrob.Agents Chemother.* **23,** 676-682

24. Mar, E. C., Cheng, Y. C., and Huang, E. S. (1983) *Antimicrob.Agents Chemother.* **24,** 518-521

25. Cheng, Y. C., Huang, E. S., Lin, J. C., Mar, E. C., Pagano, J. S., Dutschman, G. E., and Grill, S. P. (1983) *Proc.Natl.Acad.Sci.U.S.A* **80,** 2767-2770

26. Plotkin, S. A., Starr, S. E., and Bryan, C. K. (1982) *Am.J Med* **73,** 257-261

27. Littler, E., Stuart, A. D., and Chee, M. S. (1992) *Nature* **358,** 160-162

28. Sullivan, V., Talarico, C. L., Stanat, S. C., Davis, M., Coen, D. M., and Biron, K. K. (1992) *Nature* **358,** 162-164

29. Reid, R., Mar, E. C., Huang, E. S., and Topal, M. D. (1988) *J Biol.Chem.* **263,** 3898-3904

30. Keller, P. M., Fyfe, J. A., Beauchamp, L., Lubbers, C. M., Furman, P. A., Schaeffer, H. J., and Elion, G. B. (1981) *Biochemical Pharmacology* **30,** 3071-3077

31. Moolten, F. L. (1987) *Med Hypotheses* **24,** 43-51

32. Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. (1993) *Cancer Res.* **53,** 5274-5283

33. Moolten, F. L. and Wells, J. M. (1990) *J Natl.Cancer Inst.* **82,** 297-300

34. Moolten, F. L. (1986) *Cancer Res.* **46,** 5276-5281

35. Moolten, F. L. (1990) *Crit Rev Immunol.* **10,** 203-233

36. Moolten, F. L. (1994) *Cancer Gene Ther.* **1,** 279-287

37. Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M.
     (1992) *Science* **256,** 1550-1552

38. Boucher, P. D., Ostruszka, L. J., Murphy, P. J., and Shewach, D. S. (2002) *Gene
     Ther.* **9,** 1023-1030

39. Mahony, W. B., Domin, B. A., and Zimmerman, T. P. (1991)
     Biochem.Pharmacol. **41,** 263-271

40. Domin, B. A., Mahony, W. B., and Zimmerman, T. P. (1993) *Biochemical
     Pharmacology* **46,** 725-729

41. Buursma, A. R., van Dillen, I. J., van Waarde, A., Vaalburg, W., Hospers, G. A.
     P., Mulder, N. H., and de Vries, E. F. J. (0 AD) *Br J Cancer* **91,** 2079-2085

42. Haberkorn, U., Khazaie, K., Morr, I., Altmann, A., Mnller, M., and van Kaick, G.
     (1998) *Nuclear Medicine and Biology* **25,** 367-373

43. Adachi, M., Sampath, J., Lan, L. b., Sun, D., Hargrove, P., Flatley, R., Tatum, A.,
     Edwards, M. Z., Wezeman, M., Matherly, L., Drake, R., and Schuetz, J. (2002)
     Journal of Biological Chemistry **277,** 38998-39004

44. Schuetz, J. D., Connelly, M. C., Sun, D., Paibir, S. G., Flynn, P. M., Srinivas, R. V., Kumar, A., and Fridland, A. (1999) *Nat Med* **5,** 1048-1051

45. Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., Beijnen, J. H., Scheper, R. J., Hatse, S., De Clercq, E., Balzarini, J., and Borst, P. (2000) *Proc.Natl.Acad.Sci.U.S.A* **97,** 7476-7481

46. Biron, K. K., Stanat, S. C., Sorrell, J. B., Fyfe, J. A., Keller, P. M., Lambe, C. U., and Nelson, D. J. (1985) *Proceedings of the National Academy of Sciences* **82,** 2473-2477

47. Boehme, R. E. (1984) *Journal of Biological Chemistry* **259,** 12346-12349

48. Cheng, Y. C., Grill, S. P., Dutschman, G. E., Nakayama, K., and Bastow, K. F. (1983) *J.Biol.Chem.* **258,** 12460-12464

49. McGuffin, R. W., Shiota, F. M., and Meyers, J. D. (1980) *Antimicrob.Agents Chemother.* **18,** 471-473

50. Smith, K. O., Galloway, K. S., Kennell, W. L., Ogilvie, K. K., and Radatus, B. K. (1982) *Antimicrob.Agents Chemother.* **22,** 55-61

51. Agut, H., Collandre, H., Aubin, J. T., Guetard, D., Favier, V., Ingrand, D., Montagnier, L., and Huraux, J. M. (1989) *Res.Virol.* **140,** 219-228

52. Agut, H., Huraux, J. M., Collandre, H., and Montagnier, L. (1989) *Lancet* **2,** 626

53. Lin, J. C., Smith, M. C., and Pagano, J. S. (1984) *J Virol.* **50,** 50-55

54. Faulds, D. and Heel, R. C. (1990) *Drugs* **39,**

55. Jung, D. and Dorr, A. (1999) *The Journal of Clinical Pharmacology* **39,** 800-804

56. Noble, S. and Faulds, D. (1998) *Drugs* **56,** 115-146

57. Sommadossi, J. P. and Carlisle, R. (1987) *Antimicrob.Agents Chemother.* **31,** 452-454

58. Sommadossi, J. P. and Bevan, R. (1987) *J Chromatogr.* **414,** 429-433

59. Anderson, R. D., Griffy, K. G., Jung, D., Dorr, A., Hulse, J. D., and Smith, R. B. (1995) *Clin.Ther.* **17,** 425-432

60. Sugawara, M., Huang, W., Fei, Y. J., Leibach, F. H., Ganapathy, V., and Ganapathy, M. E. (2000) *J Pharm.Sci.* **89,** 781-789

61. Cocohoba, J. M. and McNicholl, I. R. (2002) *Ann.Pharmacother.* **36,** 1075-1079

62. Pescovitz, M. D., Rabkin, J., Merion, R. M., Paya, C. V., Pirsch, J., Freeman, R. B., O'Grady, J., Robinson, C., To, Z., Wren, K., Banken, L., Buhles, W., and Brown, F. (2000) *Antimicrob.Agents Chemother.* **44,** 2811-2815

63. Martin, D. F., Sierra-Madero, J., Walmsley, S., Wolitz, R. A., Macey, K., Georgiou, P., Robinson, C. A., and Stempien, M. J. (2002) *N Engl J Med* **346,** 1119-1126

64. Brown, F., Banken, L., Saywell, K., and Arum, I. (1999) *Clinical Pharmacokinetics* **37,**

65. Shewach, D. S., Zerbe, L. K., Hughes, T. L., Roessler, B. J., Breakefield, X. O., and Davidson, B. L. (1994) *Cancer Gene Ther.* **1,** 107-112

66. Rans, T. S. and England, R. (2009) *Annals of Allergy, Asthma & Immunology* **102,** 357-363

67. Yun, C. O., Yoon, A. R., Yoo, J. Y., Kim, H., Kim, M., Ha, T., Kim, G. E., Kim, H., and Kim, J. H. (2005) *Hum.Gene Ther.* **16,** 248-261

68. Roth, J. A. and Cristiano, R. J. (1997) *J.Natl.Cancer Inst.* **89,** 21-39

69. DiMaio, J. M., Clary, B. M., Via, D. F., Coveney, E., Pappas, T. N., and Lyerly, H. K. (1994) *Surgery* **116,** 205-213

70. Ponder, K. P., Dunbar, R. P., Wilson, D. R., Darlington, G. J., and Woo, S. L. (1991) *Hum.Gene Ther.* **2,** 41-52

71. Yoshimura, I., Suzuki, S., Tadakuma, T., and Hayakawa, M. (2001) *International Journal of Urology* **8,** S5-S8

72. Ghazal, P., Lubon, H., Fleckenstein, B., and Hennighausen, L. (1987) *Proc.Natl.Acad.Sci.U.S.A* **84,** 3658-3662

73. Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M., and Kim, J. H. (2003) *Cancer Res* **63,** 7497-7506

74. Freytag, S. O., Movsas, B., Aref, I., Stricker, H., Peabody, J., Pegg, J., Zhang, Y., Barton, K. N., Brown, S. L., Lu, M., Savera, A., and Kim, J. H. (2007) *Mol Ther* **15,** 1016-1023

75. Boucher, P. D., Ostruszka, L. J., and Shewach, D. S. (2000) *Cancer Res.* **60,** 1631-1636

76. Chen, S. H., Chen, X. H., Wang, Y., Kosai, K., Finegold, M. J., Rich, S. S., and Woo, S. L. (1995) *Proceedings of the National Academy of Sciences* **92,** 2577-2581

77. Bi, W. L., Parysek, L. M., Warnick, R., and Stambrook, P. J. (1993) *Hum.Gene Ther.* **4,** 725-731

78. Fick, J., Barker, F. G., Dazin, P., Westphale, E. M., Beyer, E. C., and Israel, M. A. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 11071-11075

79. Vrionis, F. D., Wu, J. K., Qi, P., Waltzman, M., Cherington, V., and Spray, D. C. (1997) *Gene Ther.* **4,** 577-585

80. Hamel, W., Magnelli, L., Chiarugi, V. P., and Israel, M. A. (1996) *Cancer Res.* **56,** 2697-2702

81. Mesnil, M. and Yamasaki, H. (2000) *Cancer Res.* **60,** 3989-3999

82. Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. (1996) *Proc.Natl.Acad.Sci.U.S.A* **93,** 1831-1835

83. Mesnil, M., Piccoli, C., and Yamasaki, H. (1997) *Cancer Res.* **57,** 2929-2932

84. Yang, L., Chiang, Y., Lenz, H. J., Danenberg, K. D., Spears, C. P., Gordon, E. M., Anderson, W. F., and Parekh, D. (1998) *Hum.Gene Ther.* **9,** 719-728

85. Touraine, R. L., Ishii-Morita, H., Ramsey, W. J., and Blaese, R. M. (1998) *Gene Ther.* **5,** 1705-1711

86. Touraine, R. L., Vahanian, N., Ramsey, W. J., and Blaese, R. M. (1998) *Hum.Gene Ther.* **9,** 2385-2391

87. Boucher, P. D., Ruch, R. J., and Shewach, D. S. (1998) *Hum.Gene Ther.* **9,** 801-814

88. Gentry, B. G., Im, M., Boucher, P. D., Ruch, R. J., and Shewach, D. S. (2005) *Gene Ther.* **12,** 1033-1041

89. Ammerpohl, O., Thormeyer, D., Khan, Z., Appelskog, I. B., Gojkovic, Z., Almqvist, P. M., and Ekstr÷m, T. J. (2004) *Biochemical and Biophysical Research Communications* **324,** 8-14

90. Asklund, T., Appelskog, I. B., Ammerpohl, O., Ekstrom, T. J., and Almqvist, P. M. (2004) *Eur.J.Cancer* **40,** 1073-1081

91. Kothari, V., Joshi, G., Nama, S., Somasundaram, K., and Mulherkar, R. (2010) *Int J Cancer* **126,** 733-742

92. Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. (1998) *Cancer Res* **58,** 3873-3882

93. Munir, K. M., French, D. C., Dube, D. K., and Loeb, L. A. (1994) *Protein Engineering* **7,** 83-89

94. Black, M. E., Newcomb, T. G., Wilson, H. M., and Loeb, L. A. (1996) *Proc.Natl.Acad.Sci.U.S.A* **93,** 3525-3529

95. Qiao, J., Black, M. E., and Caruso, M. (2000) *Hum.Gene Ther.* **11,** 1569-1576

96. Kokoris, M. S., Sabo, P., Adman, E. T., and Black, M. E. (1999) *Gene Ther.* **6,** 1415-1426

97. Black, M. E., Kokoris, M. S., and Sabo, P. (2001) *Cancer Res.* **61,** 3022-3026

98. Freytag, S. O., Paielli, D., Wing, M., Rogulski, K., Brown, S., Kolozsvary, A., Seely, J., Barton, K., Dragovic, A., and Kim, J. H. (2002) *International Journal of Radiation Oncology*Biology*Physics* **54,** 873-885

99. Barton, K. N., Paielli, D., Zhang, Y., Koul, S., Brown, S. L., Lu, M., Seely, J., Ho Kim, J., and Freytag, S. O. (2006) *Mol Ther* **13,** 347-356

100. Freytag, S. O., Stricker, H., Peabody, J., Pegg, J., Paielli, D., Movsas, B., Barton, K. N., Brown, S. L., Lu, M., and Kim, J. H. (2007) *Mol Ther* **15,** 636-642

101. Barton, K. N., Tyson, D., Stricker, H., Lew, Y. S., Heisey, G., Koul, S., de la, Z. A., Yin, F. F., Yan, H., Nagaraja, T. N., Randall, K. A., Jin, G. K., Fenstermacher, J. D., Jhiang, S., Ho, K. J., Freytag, S. O., and Brown, S. L. (2003) *Mol Ther.* **8,** 508-518

102. Barton, K. N., Stricker, H., Brown, S. L., Elshaikh, M., Aref, I., Lu, M., Pegg, J., Zhang, Y., Karvelis, K. C., Siddiqui, F., Kim, J. H., Freytag, S. O., and Movsas, B. (2008) *Mol Ther.* **16,** 1761-1769

103. Uckert, W., Kammertons, T., Haack, K., Qin, Z., Gebert, J., Schendel, D. J., and Blankenstein, T. (1998) *Hum.Gene Ther* **9,** 855-865

104. Wildner, O., Blaese, R. M., and Candotti, F. (1999) *Cancer Res.* **59,** 5233-5238

105. Boucher, P. D., Im, M. M., Freytag, S. O., and Shewach, D. S. (2006) *Cancer Res.* **66,** 3230-3237

106. Rogulski, K. R., Kim, J. H., Kim, S. H., and Freytag, S. O. (1997) *Hum.Gene Ther.* **8,** 73-85

107. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. (2000) *Hum.Gene Ther.* **11,** 67-76

108. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D., and Kim, J. H. (1998) *Hum.Gene Ther* **9,** 1323-1333

109. Rogulski, K. R., Zhang, K., Kolozsvary, A., Kim, J. H., and Freytag, S. O. (1997) *Clin.Cancer Res.* **3,** 2081-2088

110. Boucher, P. D. and Shewach, D. S. (2005) *Mol Ther* **12,** 1064-1071

111. Bianchi, V., Pontis, E., and Reichard, P. (1986) *Journal of Biological Chemistry* **261,** 16037-16042

112. Plunkett, W., Huang, P., Searcy, C. E., and Gandhi, V. (1996) *Semin.Oncol.* **23,** 3-15

113. Mullen, C. A., Kilstrup, M., and Blaese, R. M. (1992) *Proceedings of the National Academy of Sciences* **89,** 33-37

114. Moore, E. C. and Hurlbert, R. B. (1966) *J Biol Chem.* **241,** 4802-4809

115. Mader, R. M., Mnller, M., and Steger, G. G. (1998) *General Pharmacology: The Vascular System* **31,** 661-666

116. Halloran, P. J. and Fenton, R. G. (1998) *Cancer Res* **58,** 3855-3865

117. Tomicic, M. T., Thust, R., and Kaina, B. (2002) *Oncogene* **21,** 2141-2153

118. Ilsley, D. D., Lee, S. H., Miller, W. H., and Kuchta, R. D. (1995) *Biochemistry* **34,** 2504-2510

119. Hamzeh, F. M. and Lietman, P. S. (1991) *Antimicrob.Agents Chemother.* **35,** 1818-1823

120. Frank, K. B., Chiou, J. F., and Cheng, Y. C. (1984) *Journal of Biological Chemistry* **259,** 1566-1569

121. Reardon, J. E. (1989) *J Biol.Chem.* **264,** 19039-19044

122. Tomicic, M. T., Thust, R., Sobol, R. W., and Kaina, B. (2001) *Cancer Res* **61,** 7399-7403

123. Tomicic, M. T., Bey, E., Wutzler, P., Thust, R., and Kaina, B. (2002) *Mutat.Res.* **505,** 1-11

124. Thust, R., Tomicic, M., Klocking, R., Voutilainen, N., Wutzler, P., and Kaina, B. (2000) *Cancer Gene Ther.* **7,** 107-117

125. Thust, R., Tomicic, M., Klocking, R., Wutzler, P., and Kaina, B. (2000) *Mutagenesis* **15,** 177-184

126. Sonoda, E., Sasaki, M. S., Morrison, C., Yamaguchi-Iwai, Y., Takata, M., and Takeda, S. (1999) *Mol.Cell Biol.* **19,** 5166-5169

127. O'Konek, J. J., Boucher, P. D., Iacco, A. A., Wilson, T. E., and Shewach, D. S. (2009) *Cancer Gene Ther* **16,** 683-692

128. Sampath, D., Rao, V. A., and Plunkett, W. (0 AD) *Oncogene* **22,** 9063-9074

129. Peterson, C. L. and Cote, J. (2004) *Genes Dev.* **18,** 602-616

130. Lindahl, T. (1993) *Nature* **362,** 709-715

131. Lindahl, T. (2000) *Mutation Research/Reviews in Mutation Research* **462,** 129-135

132. Oza, P., Jaspersen, S. L., Miele, A., Dekker, J., and Peterson, C. L. (2009) *Genes Dev.* **23,** 912-927

133. Stracker, T. H. and Petrini, J. H. (2011) *Nat.Rev.Mol.Cell Biol.* **12,** 90-103

134. Shiotani, Bunsyo and Zou, Lee. Single-Stranded DNA Orchestrates an ATM-to-ATR Switch at DNA Breaks. Molecular Cell 33(5), 547-558. 3-13-2009.

135. Zou, L. and Elledge, S. J. (2003) *Science* **300,** 1542-1548

136. Zhao, H. and Piwnica-Worms, H. (2001) *Mol Cell Biol.* **21,** 4129-4139

137. Rothkamm, K. and Lobrich, M. (2003) *Proc.Natl.Acad.Sci.U.S.A* **100,** 5057-5062

138. West, S. C. (2003) *Nat Rev Mol Cell Biol* **4,** 435-445

139. Zhu, Z., Chung, W. H., Shim, E. Y., Lee, S. E., and Ira, G. (2008) *Cell* **134,** 981-994

140. Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A., Wittenberg, C., and Russell, P. (2007) *Mol.Cell* **28,** 134-146

141. You, Z., Shi, L. Z., Zhu, Q., Wu, P., Zhang, Y. W., Basilio, A., Tonnu, N., Verma, I. M., Berns, M. W., and Hunter, T. (2009) *Mol.Cell* **36,** 954-969

142. Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007) *Nature* **450,** 509-514

143. Nimonkar, A. V., Ozsoy, A. Z., Genschel, J., Modrich, P., and Kowalczykowski, S. C. (2008) *Proc.Natl.Acad.Sci.U.S.A* **105,** 16906-16911

144. Mimitou, E. P. and Symington, L. S. (2008) *Nature* **455,** 770-774

145. Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2006) *EMBO Rep.* **7,** 212-218

146. Eid, W., Steger, M., El Shemerly, M., Ferretti, L. P., Pena-Diaz, J., Konig, C., Valtorta, E., Sartori, A. A., and Ferrari, S. (2010) *EMBO Rep.* **11,** 962-968

147. Thust, R., Tomicic, M. T., Grabner, R., Friedrichs, C., Wutzler, P., and Kaina, B. (2004) *Mutagenesis* **19,** 27-33

148. Lambert, S., Mizuno, K., Blaisonneau, J., Martineau, S., Chanet, R., Frθon, K., Murray, J. M., Carr, A. M., and Baldacci, G. (2010) *Molecular Cell* **39,** 346-359

149. Fechner, H., Wang, X., Wang, H., Jansen, A., Pauschinger, M., Scherubl, H., Bergelson, J. M., Schultheiss, H. P., and Poller, W. (2000) *Gene Ther* **7,** 1954-1968

150. Li, D., Duan, L., Freimuth, P., and OΓÇÖMalley, B. W. (1999) *Clin Cancer Res* **5,** 4175-4181

151. Hodawadekar, S. C. and Marmorstein, R. (0 AD) *Oncogene* **26,** 5528-5540

152. Ropero, S. and Esteller, M. (2007) *Molecular Oncology* **1,** 19-25

153. Bhaskara, S., Chyla, B. J., Amann, J. M., Knutson, S. K., Cortez, D., Sun, Z. W., and Hiebert, S. W. (2008) *Mol Cell* **30,** 61-72

154. Whetstine, J. R., Ceron, J., Ladd, B., Dufourcq, P., Reinke, V., and Shi, Y. (2005) *Molecular Cell* **18,** 483-490

155. Kurdistani, S. K. and Grunstein, M. (2003) *Nat Rev Mol Cell Biol* **4,** 276-284

156. Adimoolam, S., Sirisawad, M., Chen, J., Thiemann, P., Ford, J. M., and Buggy, J. J. (2007) *Proceedings of the National Academy of Sciences* **104,** 19482-19487

157. Bhaskara, Srividya, Knutson, Sarah K., Jiang, Guochun, Chandrasekharan, Mahesh B., Wilson, Andrew J., Zheng, Siyuan, Yenamandra, Ashwini, Locke, Kimberly, Yuan, Jia ling, Bonine-Summers, Alyssa R., Wells, Christina E., Kaiser, Jonathan F., Washington, M. Kay, Zhao, Zhongming, Wagner, Florence F., Sun, Zu Wen, Xia, Fen, Holson, Edward B., Khabele, Dineo, and Hiebert, Scott W. Hdac3 Is Essential for the Maintenance of Chromatin Structure and Genome Stability. Cancer cell 18(5), 436-447. 11-16-2010.

158. Marks, P. A., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelly, W. K. (2001) *Nat Rev Cancer* **1,** 194-202

159. Yoshida, M., Nomura, S., and Beppu, T. (1987) *Cancer Res* **47,** 3688-3691

160. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990) *Journal of Biological Chemistry* **265,** 17174-17179

161. Willyard, C. (2010) *Nat Med* **16,** 18-21

162. Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A., and Marks, P. A. (1998) *Proceedings of the National Academy of Sciences* **95,** 3003-3007

163. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. (1999) *Nature* **401,** 188-193

164. Lee, J. H., Choy, M. L., Ngo, L., Foster, S. S., and Marks, P. A. (2010) *Proceedings of the National Academy of Sciences* **107,** 14639-14644

165. Kelly, W. K., O'Connor, O. A., Krug, L. M., Chiao, J. H., Heaney, M., Curley, T., MacGregore-Cortelli, B., Tong, W., Secrist, J. P., Schwartz, L., Richardson, S., Chu, E., Olgac, S., Marks, P. A., Scher, H., and Richon, V. M. (2005) *Journal of Clinical Oncology* **23,** 3923-3931

166. Rubin, E. H., Agrawal, N. G. B., Friedman, E. J., Scott, P., Mazina, K. E., Sun, L., Du, L., Ricker, J. L., Frankel, S. R., Gottesdiener, K. M., Wagner, J. A., and Iwamoto, M. (2006) *Clin Cancer Res* **12,** 7039-7045

167. Mann, B. S., Johnson, J. R., He, K., Sridhara, R., Abraham, S., Booth, B. P., Verbois, L., Morse, D. E., Jee, J. M., Pope, S., Harapanhalli, R. S., Dagher, R., Farrell, A., Justice, R., and Pazdur, R. (2007) *Clin Cancer Res* **13,** 2318-2322

168. Palmieri, D., Lockman, P. R., Thomas, F. C., Hua, E., Herring, J., Hargrave, E., Johnson, M., Flores, N., Qian, Y., Vega-Valle, E., Taskar, K. S., Rudraraju, V., Mittapalli, R. K., Gaasch, J. A., Bohn, K. A., Thorsheim, H. R., Liewehr, D. J., Davis, S., Reilly, J. F., Walker, R., Bronder, J. L., Feigenbaum, L., Steinberg, S.

M., Camphausen, K., Meltzer, P. S., Richon, V. M., Smith, Q. R., and Steeg, P. S. (2009) *Clin.Cancer Res.* **15,** 6148-6157

169. Shein, N. A. and Shohami, E. (2011) *Mol Med* **17,** 448-456

170. Davie, J. R. (2003) *J.Nutr.* **133,** 2485S-2493S

171. Chioni, A. M., Brackenbury, W. J., Calhoun, J. D., Isom, L. L., and Djamgoz, M. B. A. (2009) *The International Journal of Biochemistry & Cell Biology* **41,** 1216-1227

172. Chinnaiyan, P., Cerna, D., Burgan, W. E., Beam, K., Williams, E. S., Camphausen, K., and Tofilon, P. J. (2008) *Clin Cancer Res* **14,** 5410-5415

173. Vidanes, G. M., Bonilla, C. Y., and Toczyski, D. P. (2005) *Cell* **121,** 973-976

174. Chen, C. Y., Chang, Y. N., Ryan, P., Linscott, M., McGarrity, G. J., and Chiang, Y. L. (1995) *Hum.Gene Ther* **6,** 1467-1476

175. Robert, T., Vanoli, F., Chiolo, I., Shubassi, G., Bernstein, K. A., Rothstein, R., Botrugno, O. A., Parazzoli, D., Oldani, A., Minucci, S., and Foiani, M. (2011) *Nature* **471,** 74-79

176. Conti, C., Leo, E., Eichler, G. S., Sordet, O., Martin, M. M., Fan, A., Aladjem, M. I., and Pommier, Y. (2010) *Cancer Res.* **70,** 4470-4480

177. Maiso, P., Colado, E., Ocio, E. M., Garayoa, M., Martin, J., Atadja, P., Pandiella, A., and San Miguel, J. F. (2009) *Leukemia* **23,** 2265-2274

178. Namdar, M., Perez, G., Ngo, L., and Marks, P. A. (2010) *Proceedings of the National Academy of Sciences* **107,** 20003-20008

179. Fazzone, W., Wilson, P. M., Labonte, M. J., Lenz, H. J., and Ladner, R. D. (2009) *Int.J Cancer* **125,** 463-473

180. Lee, C. K., Wang, S., Huang, X., Ryder, J., and Liu, B. (2010) *Cancer Lett.* **296,** 233-240

181. Petermann, E. and Helleday, T. (2010) *Nat Rev Mol Cell Biol* **11,** 683-687

182. Saleh-Gohari, N., Bryant, H. E., Schultz, N., Parker, K. M., Cassel, T. N., and Helleday, T. (2005) *Mol.Cell.Biol.* **25,** 7158-7169

Chapter II

Unrepairable DNA Double Strand Breaks Initiate Cytotoxicity with HSV-TK/Ganciclovir

Summary

The herpes simplex virus thymidine kinase (HSV-TK) is the most widely used suicide gene in cancer gene therapy due to its superior anticancer activity with ganciclovir compared to other HSV-TK substrates, such as 1-β-D-arabinofuranosyl thymine (araT). We have evaluated the role of DNA damage as a mechanism for the superiority of GCV. Using γ-H2AX foci as an indicator of DNA damage, GCV induced $\geq$ 7-fold more foci than araT at similarly cytotoxic concentrations. The number of foci decreased after removal of either drug, followed by an increase in Rad51 foci indicating that (HRR) was used to repair this damage. Notably, only GCV produced a late and persistent increase in γ-H2AX foci demonstrating the induction of unrepairable DNA damage. Both drugs induced the ATR damage response pathway, as evidenced by Chk1 activation. However, GCV resulted in greater activation of ATM, which coincided with the late induction of γ-H2AX foci, demonstrating the presence of DNA double strand breaks (DSBs). The increase in DSBs after Rad51 induction suggested that they occurred as a result of a failed attempt at homologous recombination repair (HRR). These data demonstrate that the late and

unrepairable DSBs observed uniquely with GCV account for its superior cytotoxicity and further suggest that inhibition of HRR will enhance cytotoxicity with HSV-TK/GCV.

Introduction

While the initial goal of suicide gene therapy for cancer treatment was to maintain or increase tumor cell killing while sparing normal tissue toxicity, this approach also provided an opportunity to discover new drugs with potentially novel mechanisms of action that would lead to greater antitumor efficacy(1). In addition, identifying the mechanism by which drugs used in suicide gene therapy elicit cytotoxicity may provide new, novel drug combinations that can enhance gene therapy without compromising its selectivity. One of the most widely used and studied suicide gene therapy approaches utilizes the herpes simplex virus thymidine kinase (HSV-TK) to activate the antiviral drug ganciclovir (GCV) to produce a non-traditional toxic metabolite with the potential for a novel mechanism of action leading to greater cancer cell killing. Indeed HSV-TK/GCV exhibits unique kinetics of cell killing and a remarkably mild effect on DNA synthesis that distinguishes it from traditional nucleoside analogs. The resulting excellent antitumor activity in preclinical studies has prompted numerous clinical trials, with promising results in a combination approach in patients with prostate cancer(2-4).

HSV-TK/GCV is the most widely used suicide gene therapy approach both *in vitro* and *in vivo*. However, little attention has been focused on the mechanism by which it produces cell death. Similar to other nucleoside analogs, cytotoxicity requires activation of GCV (mediated by HSV-TK) to GCV 5'-triphosphate, which competes with dGTP for incorporation into DNA in internucleotide linkages(5;6). While GCV shares this basic

mechanism of cytotoxicity with other HSV-TK substrates, including the efficacious antivirals acyclovir (ACV) and 1-β-D-arabinofuranosylthymine (araT), GCV induces multi-log cell killing at sub-micromolar concentrations, whereas ACV and araT were weakly cytotoxic at concentrations >100 μM(5).   We have demonstrated previously that limited phosphorylation of ACV likely accounts for its poor cytotoxicity.   However, araT is phosphorylated and incorporated to a greater degree than GCV, thus the reason for the inferiority of araT is not clear.

A few studies have attempted to address the mechanism by which GCV causes cell death.   A study in B16 murine melanoma cells indicated GCV induced a morphological change in cells due to the reorganization of components of the cytoskeleton as well as an accumulation of cells in G2/M after a 48-72 hr incubation(7). It has also been reported that GCV treatment results in a decline in Bcl-2 levels and activation of caspases, leading to apoptosis(8).   While these studies highlight pathways utilized by GCV that lead to cell death, they do not address the mechanism by which GCV is many logs more cytotoxic than other HSV-TK substrates.   To begin addressing the consequences of GCV in DNA, Thust et. al demonstrated that GCV induced sister chromatid exchanges and chromosome breaks and translocations, whereas another substrate for HSV-TK, ACV, did not(9;10).   In light of the fact that sister chromatid exchanges arise as a consequence of HRR (HRR),(11) these results suggest that DNA damage and pathways involved in its repair differ significantly between these drugs.

In a comparison of the events that lead to cytotoxicity for GCV and araT, we reported a unique manner of delayed cell death in response to GCV(5).   Cells completed

one cell division after incubation with GCV. However, when they attempted to progress through the cell cycle for a second time, they were blocked in S phase where they remained until cell death occurred. In contrast, cells treated with araT accumulated in S phase and growth was inhibited for at least two days after drug washout, but subsequently cells progressed through the cell cycle and the cell number increased. This suggests that, with GCV treatment, an event occurring during this second round of DNA replication caused cells to permanently arrest in S phase, resulting in cell death whereas araT produced greater disruption during the first S-phase.

In order to further understand the mechanisms by which these drugs elicited cytotoxicity, we evaluated the consequences of DNA incorporation for GCV and araT. We hypothesized that the distinct cell cycle kinetics of cell death with GCV and araT would result in measurable differences in the induction of a DNA damage response. Therefore, we wished to measure the extent and time course of DNA damage and its repair following treatment with GCV compared to araT. In addition, we evaluated a role for HRR (HRR), as our previous studies in a yeast model indicated this repair pathway could rescue cells from GCV cytotoxicity,(12) and prior reports of sister chromatid exchanges promoted by GCV(9;10) suggested a role for HRR. Furthermore, we evaluated the extent to which each drug activated the two major DNA damage response pathways, mediated by ATR and ATM. Collectively, the results demonstrate a dramatic difference in the type and degree of DNA damage with GCV relative to araT, leading to distinct mechanisms of cell death.

Materials and Methods

Cell Culture

U251 human glioblastoma cells were maintained in exponential growth in RPMI 1640 medium supplemented with 10% calf serum (GIBCO, Grand Island, NY) and L-glutamine (Fisher Scientific, Pittsburgh, PA) in a humidified atmosphere at 37ºC with 5% CO_2. For stable expression of HSV-TK, U251 cells were transduced with a retroviral vector encoding the herpes simplex virus type 1 thymidine kinase, using the retrovirus long terminal repeat for a promoter, and the neomycin resistance gene for selection as previously described(5). HSV-TK-expressing cells were selected with G418, and individual clones were expanded and maintained in medium containing G418. HSV-TK expression was determined by incubating cells with GCV and measuring phosphorylated GCV metabolites in cell lysates.

Analysis of γ-H2AX foci formation by laser scanning confocal microscopy

Cells were grown on chambered slides for 48 hr prior to drug addition. After incubation with drug, the cells were washed with PBS and then fixed and permeabilized with acetone/methanol (50:50 v/v) for 10 min. The fixed cells were then washed with PBS, blocked with 10% goat serum for 1 h, incubated with γ-H2AX primary antibody (1:400 dilution; Upstate, Charlottesville, VA) for 1 h, washed, incubated with AlexaFluor 488 conjugated goat anti-rabbit secondary antibody (1:200 dilution; Molecular Probes, Eugene, OR) for 1 h, washed and mounted with ProLong antifade kit (Molecular Probes, Eugene, OR). Slides were imaged with a Zeiss LSM510 confocal microscope using a 60x

objective lens. Images of representative cell populations were captured, and γ-H2AX foci were counted visually. At least 5 - 16 cells per well were counted with triplicate wells per condition, and each experiment was performed at least three times.

Analysis of Rad51 foci formation by laser scanning confocal microscopy

Cells were grown on chambered slides for 48 hours prior to drug addition. Drug was added for 24 hours unless otherwise noted. At specified time points, cells were washed with PBS and permeabilized with Triton-X buffer (0.5% Triton, 20mM Hepes, 50 mM NaCl, 3 mM KCl, 300mM Sucrose) for 5 min. Permeabilized cells were then fixed with paraformaldehyde solution (3% PFA, 2% sucrose, 1X PBS) for 30 min, washed 3 times for 10 minutes in wash buffer (0.5% NP40, 0.3% Sodium Azide, 1X PBS), blocked with 10% goat serum for 1 hour, and incubated with rabbit anti-Rad51 primary antibody (1:1600 dilution; Calbiochem, La Jolla, CA) for 1.5 hours. Cells were then washed 3 times in wash buffer, incubated with AlexaFluor 488 conjugated goat anti-rabbit secondary antibody (1:2000 dilution; Molecular Probes, Eugene, OR) for 1 hour, washed 3 times in wash buffer then washed with DAPI (.1µg/ml DAPI in 1X PBS) and mounted with ProLong antifade kit (Molecular Probes, Eugene, OR). Slides were imaged with an Olympus FV500 confocal microscope using a 100x objective lens. Images of representative cell populations were captured, and Rad51 positive cells were scored visually (cells with $\geq$10 foci were considered positive). At least 63 - 260 cells per well were scored with triplicate wells per condition, and each experiment was performed at least three times. Statistical significance was determined using a t-test.

Analysis of γ-H2AX and BrdUrd immunostaining by laser scanning confocal microscopy

Cells were grown on chambered slides for 48 hr prior to drug addition. Cells were incubated with 30 μM BrdUrd for 30 minutes at the conclusion of drug incubation. Cells were fixed, permeabilized, and stained for γ-H2AX as described above, using AlexaFluor 594 conjugated goat anti-rabbit secondary antibody. After the final wash, antibody complexes were fixed with 3.7% paraformaldehyde in PBS for 10 minutes. Cells were treated with 2.5 N HCl for 30 minutes at 37°C and stained with AlexaFluor 488 mouse anti-BrdUrd conjugate (1:20 dilution, BD Pharmingen, San Jose, CA) for 1 hr. Slides were mounted and imaged as described above.   At least 14 - 58 cells per well were counted with triplicate wells for each condition, and the experiment was performed at least twice. Percent positive cells were calculated as the number of cells positive for the indicated marker (BrdUrd or γ-H2AX) divided by the total number of cells examined. Percent γ-H2AX positive cells that were also positive for BrdUrd was calculated as the number of cells positive for both markers divided by the number of BrdUrd positive cells.

Analysis of γ-H2AX expression by flow cytometry

After drug incubation, cells were harvested by trypsinization and washed with PBS. The pellets were resuspended in ice-cold PBS followed by the addition of cold 2% paraformaldehyde. Samples were then incubated at 4ºC for a minimum of 30 min. Fixed samples were centrifuged and the pellets were resuspended in PBS containing

0.5% Tween 20 and incubated at 3ºC for 15 min. PBS containing 0.5% Tween 20 and 5% serum (PBT) was added followed by centrifugation. Pellets were then resuspended in PBT. Anti-γ-H2AX antibody was added to each sample and incubated for 45 min at room temperature and then washed with PBT. The pellets were then resuspended in anti-rabbit phycoerythrin conjugate antibody (Sigma Chemical Co, St. Louis, MO) and incubated for 45 min at room temperature. Samples were washed with PBT and resuspended in 7-Amino Actinomycin D (7-AAD) (Molecular Probes, Eugene OR) and incubated at room temperature for at least 30 min prior to flow cytometric analysis. Analysis was performed on BD FacsCalibur at the University of Michigan Flow Cytometry Core Facility. At least 10,000 cells were evaluated for each condition, and the experiment was performed at least three times.

Western Blot

All western blots for Chk1(Cell Signaling), pChk1(Ser317)(Cell Signaling), and actin (Calbiochem) were performed on 10% polyacrylamide gels according to standard protocols. Western blots for ATM (Epitomics) and pATM(Ser1982) (Epitomics) were performed as described above with exceptions: resolving gels were 6% polyacrylamide, transfer buffer contained 10% methanol and transfers were carried out at 300 mAmps overnight at 4°C. All secondary antibodies were HRP conjugated and from Santa Cruz. Phospho-ATM bands were quantitated using Image J software from the NIH, version 1.41.

Results

γ-H2AX foci were used to identify sites of DNA damage, such as DNA double strand breaks (DSBs) or stalled replication forks(13-17). Measurement of γ-H2AX foci demonstrated a dose-dependent increase in γ-H2AX foci after a 24 hr incubation with GCV in U251tk cells, relative to untreated control cells (Fig. 2.1A and B). Incubation with the non-cytotoxic IC_{10} (0.03 µM) for GCV resulted in a 4.4-fold increase (±2.9) in γ-H2AX foci which was not significantly different from control (p = 0.3). Treatment with the IC_{50} (0.05 µM) or IC_{90} (0.2 µM) for GCV, however, significantly increased the number of γ-H2AX foci per cell (14.3 ± 6.3 fold and 24.4 ± 6.8 fold, respectively; p<0.001) indicating a substantial increase in DNA damage.

γ-H2AX expression was also assayed by flow cytometry in order to evaluate the effect of increasing drug concentrations on total γ-H2AX fluorescence. In untreated control cells, only 2% of the cells expressed detectable levels of γ-H2AX. Treatment with 0.2 and 1 µM GCV ($\geq IC_{90}$) for 24 hr significantly increased the percentage of cells expressing γ-H2AX to 20% (p<0.01) and 59% (p<0.001), respectively (Figs 2.2A and 2.2B). Thus, two different independent methods have demonstrated an increase in γ-H2AX with increasing GCV concentration. Because quantifying the number of sites of DNA damage per cell provided a more definitive assessment of the extent of DNA damage compared to measuring simply the percentage of cells positive for γ-H2AX, subsequent experiments measured DNA damage using *in situ* immunohistochemistry.

Previously we have demonstrated that, although cell cycle progression is slowed during incubation with GCV, cells completed S-phase and divided. The lethal insult

occurred during the second S-phase when cells were permanently arrested. Therefore we hypothesized that the DNA damage observed during GCV incubation (Fig. 2.1) was repaired enabling completion of the first S-phase, but additional DNA damage was incurred during the second S-phase. To test this hypothesis, U251tk cells were treated with either non-toxic (IC_{10}) or cytotoxic (IC_{50}, IC_{90}) concentrations of GCV for 24 hr and assayed for γ-H2AX foci formation (Fig 2.3). At each concentration of GCV tested, an increase in foci was apparent within 12 hr after drug addition, continued through the end of the incubation, and decreased by 12 hr after drug washout. At the IC_{10} for GCV, the number of foci was <5-fold greater than control levels throughout the 48 hr post-washout period. The two cytotoxic concentrations of GCV produced a considerably greater number of γ-H2AX foci, increasing to ~15 – 25-fold higher than control at the conclusion of the incubation. This high level of DNA damage appeared to be repaired, as the number of γ-H2AX foci decreased to ≤5-fold more than control by 12 hr after drug washout without a substantial decrease in cell number. However, after 24 hr following washout of GCV at the IC_{50} or IC_{90}, the number of foci increased to greater than 10-fold over control, at which point massive loss of cells was apparent.

In view of the fact that cells treated with GCV arrest permanently during the second round of DNA replication following drug incubation,(5) we wished to verify that the presence of DNA damage at that time, indicated by γ-H2AX foci, predominated in S phase cells. Cells were treated with either no drug (control) or GCV (IC_{10}, IC_{50} and IC_{90}) for 24 hr, then incubated with 5-bromo-2'-deoxyuridine (BrdUrd) for 30 min to identify cells actively replicating DNA, followed by staining for both BrdUrd in DNA and γ-H2AX.

At drug washout (0 hr), the majority of γ-H2AX positive cells were in S phase, as indicated by BrdUrd incorporation, with a decrease to approximately one-quarter to one-half of γ-H2AX positive cells in S-phase by 24 hr after GCV washout (Table 2.1). At 48 hr after washout of GCV at its IC_{50}, more than 70% of γ-H2AX labeled cells were in S-phase. Although cells treated with the IC_{90} for GCV were not positive for BrdUrd at this time point, previously we have demonstrated that these cells are in S phase (propidium iodide staining) but with DNA synthesis decreased by more than 80%(5;18). Thus, the large increases in γ-H2AX foci observed with cytotoxic concentrations of GCV occurred primarily in S-phase cells. In particular, cells dying in the second S-phase incurred significant DNA damage.

For comparison, we measured the effect of araT on γ-H2AX foci formation. After incubation of U251tk cells with the IC_{10}, IC_{50}, and IC_{80} for araT (1 μM, 11 μM, and 100 μM, respectively) for 24 hr, a concentration-dependent increase in γ-H2AX foci was observed (Fig 2.4A and 2.4B). However, the magnitude of foci formation was considerably less with araT (2 - 3.5-fold increase compared to control) relative to a similarly or less cytotoxic concentration of GCV (15 - 25-fold increase at IC_{50} and IC_{90}, respectively; Fig 2.1B).

Evaluation of the kinetics of foci formation with araT (IC_{50}) during a 24 hr incubation revealed a small increase in the number of γ-H2AX foci (2.25-fold greater than control). The number of foci decreased by 12 hr after drug washout and remained slightly higher (approximately 1.7-fold) compared to control cells. No further increase was observed for up to 96 hr after washout (Fig. 2.5B). Thus both the degree and

pattern of DNA damage was substantially different with araT relative to GCV. The number of foci in response to araT was not greater than that produced by the IC_{10} (non-cytotoxic) for GCV, suggesting that the damage indicated by γ-H2AX foci was not sufficient to account for the cytotoxicity of araT.

The kinetics of γ-H2AX foci formation observed with cytotoxic concentrations of GCV suggested that the initial drug-induced DNA damage was repaired, consistent with our finding that the cells completed progression through the cell cycle,(5) but the secondary onset of damage was not repaired (Fig. 2.3). In contrast, damage initiated by araT appeared to be repaired prior to drug washout without further evidence of DNA damage thereafter. Because we have previously demonstrated that araT and GCV produce S-phase accumulation and a slowing of DNA replication, we wished to determine whether HRR, the primary repair pathway for stalled replication forks and DNA DSBs during S-phase(19;20) was utilized to repair the damage. Following addition of GCV or araT, Rad51 positive cells were measured as an indicator of HRR(21)(Fig. 2.6). For both drugs, the number of Rad51 foci increased after drug addition and through 12 hr post drug washout, after which foci decreased but remained elevated for at least another 60 hr. Analysis of the number of foci per cell in positive cells revealed no significant difference between GCV and araT (Fig. 2.7). Thus, both drugs produced a similar activation of Rad51.

With evidence of DNA damage and its repair, we wished to determine the pathway responsible initiation of the γ-H2AX response. Thus, we evaluated the extent to which cells utilized the DNA damage response pathways initiated by ATR and/or ATM

following GCV or araT exposure. In response to replication stress, ATR kinase is activated, and its activity can be measured by phosphorylation of its downstream target, Chk1 on serine 317 (Fig. 2.8). Western blot analysis revealed that Chk1 phosphorylation was most pronounced during incubation with araT, whereas it decreased rapidly following drug washout and persisted at low levels at all subsequent time points evaluated. These results are consistent with the strong DNA replication block that occurs during araT incubation but is relieved following drug washout(5). GCV also induced an increase in Chk1 phosphorylation that was apparent both during and after drug incubation. These data indicate that, while both drugs initiated an ATR response, araT induced a more transient effect.

DNA damaging agents that produce DNA double strand breaks (DSBs) result in activation of ATM kinase, which can be detected by autophosphorylation at S1982 (Fig. 2.9). In response to araT (IC_{90}), there was less than a 6-fold increase in ATM phosphorylation which persisted throughout the time course evaluated. When GCV (IC_{90}) was added to cells, there was minimal activation of ATM during drug incubation. At 24 hr following drug washout, there was a dramatic increase in ATM phosphorylation, achieving an increase of nearly 20-fold by 72 hr post washout compared to control, indicating a strong DSB response. Together with the γ-H2AX data, ATM phosphorylation identifies the late-occurring DNA damage with GCV as DSBs, whereas cytotoxicity with araT is not due to a strong DSB response.

Discussion

Most nucleoside analogues elicit cytotoxicity through incorporation into DNA(22-24). However, the extent and mechanism of cell killing can differ between these drugs even though their primary event leading to cytotoxicity is similar. We have demonstrated previously that GCV was more cytotoxic than araT, despite the fact that U251 cells incorporated at least 5-fold more araTMP than GCVMP into DNA, suggesting that the functional consequences of incorporation induced by these nucleoside analogues are different(5). Here we have compared the extent and kinetics of DNA damage induced by exposure of tumor cells to GCV or araT, as well as the DNA damage response pathways utilized by these drugs. The results demonstrated that GCV induced significantly more DNA DSBs than araT at similarly cytotoxic concentrations as measured by γ-H2AX and ATM phosphorylation. The biphasic kinetics of DNA damage observed uniquely with GCV reflected the role of HRR in a failed attempt at DNA repair, leading to multi-log cytotoxicity. Taken together, these data support a distinct mechanism for cell death with GCV compared to araT.

Previous studies have demonstrated that treatment of cells with ionizing radiation or cytotoxic drugs induces γ-H2AX foci formation in a dose-dependent fashion(17;25-27). In the data presented here, we have used two different methods to demonstrate that induction of γ-H2AX increased in a dose-dependent manner with GCV. Following drug washout, the number of γ-H2AX foci decreased demonstrating that the cells were able to repair a portion of this damage. Time dependent resolution of foci formation has been demonstrated by others using ionizing radiation(15;25). The results presented

here differ in that we also observed an increase in γ-H2AX foci more than 24 hr after GCV washout, which to our knowledge has not been reported previously with other DNA damaging agents. This late increase in foci occurred only at the two cytotoxic concentrations of GCV (IC_{50} and IC_{90}), suggesting that this represents the lethal insult. Although the number of foci after GCV washout did not reach as high a level as observed during drug incubation, loss of cells due to cell death at this point interfered with our ability to quantify foci. Co-staining for γ-H2AX and BrdUrd demonstrated that most of the γ-H2AX foci were in S-phase cells, consistent with our previous data demonstrating an S-phase arrest at the times corresponding to the second increase in γ-H2AX foci. Association of the late increase in γ-H2AX foci at 48 hr after drug washout with cells in S-phase following induction of HRR suggests that the lethal insult occurred during attempted replication or repair of DNA. While many studies have focused on determining DNA damage during drug incubation, the studies presented here indicate that the critical events leading to cell death may occur long after drug washout.

Following exposure of cells to araT, γ-H2AX foci formation was strikingly different from that observed with GCV. While there was a dose-dependent increase in foci formation with araT, the maximum number of foci was at least 7-fold lower with araT despite the fact that more araTMP was incorporated into DNA(5). This demonstrates that it is not simply the absolute amount of nucleotide analog incorporated into DNA but the consequences of that incorporation that is important for cytotoxicity. Furthermore, only GCV produced a second increase in γ-H2AX following drug washout that was coincident with cell death, demonstrating a role for late DNA

83

damage in cytotoxicity. We have reported previously that apoptosis was induced similarly with both drugs, thus the increase in γ-H2AX foci following GCV treatment cannot be attributed to apoptosis(5). These findings and the fact that the γ-H2AX produced by araT was similar to that observed with a non-cytotoxic concentration of GCV implicates a different mechanism of cell death for araT vs. GCV.

Previous reports demonstrate that GCV induces sister chromatid exchanges, suggesting a role for HRR in responding to GCV-induced DNA damage(9;10). We investigated a role for HRR by analyzing Rad51 foci formation following treatment with GCV and araT. The results demonstrated that HRR was induced only after drug washout for both drugs. Because HRR responds to both stalled replication forks as well as DSBs, we further analyzed the DNA damage response pathways initiated by GCV and araT. The results demonstrated that both drugs activated ATR and ATM, though with strikingly different kinetics. The activation of ATR primarily during araT exposure indicated greater replicational stress induced by this drug, consistent with the greater inhibition of DNA synthesis by araT(5). The low activation of ATM during and after araT exposure suggests that this pathway was used to restart stalled replication forks. In contrast, GCV induced modest activation of ATR during and after drug exposure, consistent with its more moderate effect on DNA replication. GCV induced activation of ATM only after drug washout, as HRR declined. The concurrent increase in ATM activation and γ-H2AX foci after GCV indicates that the foci represent DSBs, consistent with reports by others of GCV-induced DSBs in other cell types(8). A recent report also observed an increase in Chk1 phosphorylation, a late increase in activation of a

downstream ATM substrate, Chk2, and an increase in γ-H2AX foci at a single late time point after addition of GCV and an adenovirus that transiently expressed HSV-TK(28). However, these studies evaluated only a single, high concentration of GCV with variable amounts of adenovirus for transduction. Furthermore, they did not report controls for the effect of the adenovirus alone and thus the relative contribution of adenovirus transduction vs. GCV to the checkpoint alterations cannot be determined.

Based on our findings, we propose the following model for GCV cytotoxicity: During the first cell cycle, GCVMP incorporation into DNA slows DNA replication resulting in activation of ATR/Chk1 and a subsequent increase in γ-H2AX foci formation as the cell attempts to replicate past or correct this lesion. Completion of DNA replication, as evidenced by progression through the cell cycle, allows γ-H2AX foci to resolve. During the next entry into S-phase, GCVMP in the DNA template either doesn't serve as a good substrate for replication, or it is recognized as fraudulent and the cell attempts to repair it. DNA replication is halted and HRR is used in an attempt to restart replication and/or repair the lesion as evidenced by an increase in Rad51. However, GCVMP blocks HRR from successfully completing repair, and strong activation of ATM concurrent with γ-H2AX foci indicates formation of DSBs that prevent completion of S-phase resulting in massive cell death. In contrast, araT produced a strong activation of ATR during drug incubation and a modest increase in ATR and ATM activation in the absence of γ-H2AX foci after drug washout, consistent with successful restarting of stalled replication forks. These data indicate that araTMP in DNA can stall replication but the cell can successfully resume synthesis. In contrast, GCVMP is accommodated

more readily in the nascent DNA, but it will not support replication when present in the DNA template.

In summary, the data demonstrate that the inability of HRR to repair GCV-mediated damage produced DSBs that resulted in cell death with GCV, whereas the mechanism of cell death with araT was distinctly different. Furthermore, at similarly cytotoxic concentrations DNA damage was less severe with araT and did not persist, whereas GCV induced greater DNA damage and it occurred in biphasic fashion. We suggest that GCVMP in the template blocked successful repair by HRR, leading to cell death. In contrast, we suggest that most of the DNA damage induced by araT was repaired, and cell effects other than direct DNA damage, such as signaling to cell death pathways,(29) results in cytotoxicity. These studies highlight that a novel mechanism accounts for the impressive antitumor activity of HSV-TK/GCV suicide gene therapy. These findings suggest that combining HSV-TK/GCV with approaches that compromise HRR will produce synergistic antitumor effects.

		% BrdUrd Positive	% γ-H2AX Positive	% of γ-H2AX Positive that are also BrdUrd Positive
0 hr	**C**	46 ± 9	26 ± 16	67 ± 31
	IC$_{10}$	57 ± 6	59 ± 20	64 ± 17
	IC$_{50}$	65 ± 6	80 ± 16	79 ± 12
	IC$_{90}$	85 ± 21	95 ± 3	86 ± 19
24 hr	**C**	38	15	75
	IC$_{10}$	77 ± 28	11 ± 4	25 ± 35
	IC$_{50}$	63 ± 5	20 ± 3	41 ± 8
	IC$_{90}$	56 ± 62	72 ± 22	57 ± 61
48 hr	**C**	n.d.	n.d.	n.d.
	IC$_{10}$	38 ± 3	8 ± 5	70 ± 42
	IC$_{50}$	62 ± 8	72 ± 21	79 ± 14
	IC$_{90}$	0	90	0

Table 2.1. Colocalization of γ-H2AX and BrdUrd in response to GCV. U251tk cells were incubated with GCV at the indicated concentrations (IC$_{10}$ = 0.03 mM, IC$_{50}$=0.05 mM, IC$_{90}$=0.2 mM) for 24 h followed by drug washout. Cells were assayed for γ-H2AX foci formation and bromodeoxyuridine (BrdUrd) staining at The indicated time points. Time = 0 represents the time of drug washout. Values represent the percentage of cells that stained positive for γ-H2AX (contained greater than 5 foci), BrdUrd, or both. At least 50 cells were counted at each time point. n.d.= not determined.
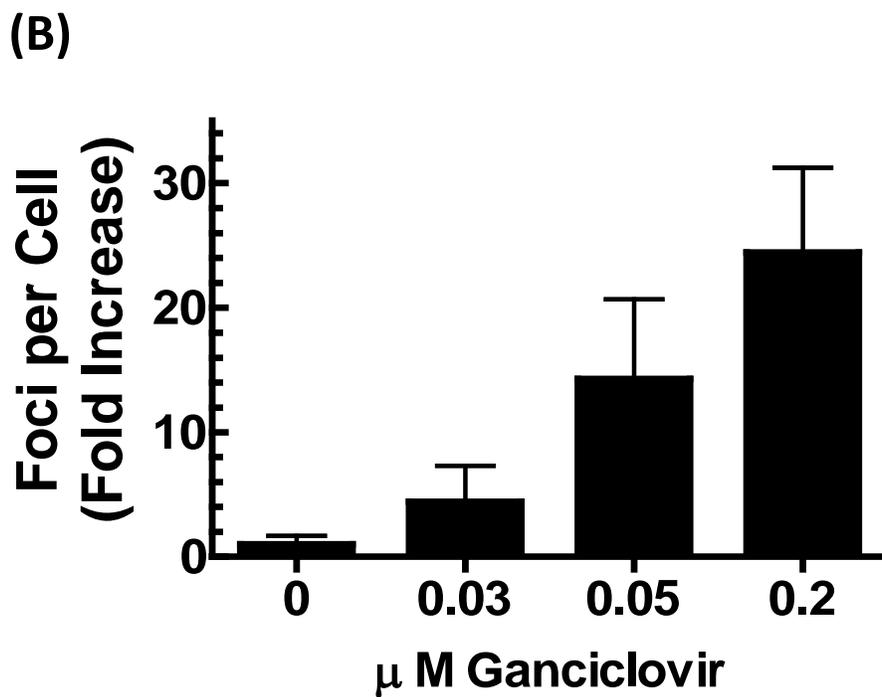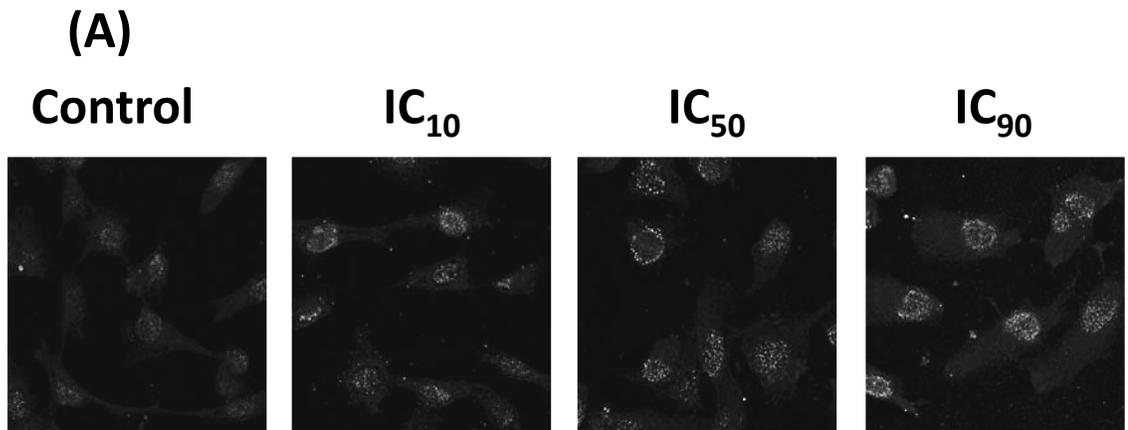
Figure 2.1 GCV induces a dose-dependent increase in γ-H2AX. U251tk cells were incubated with GCV for 24 hr and assayed for γ-H2AX foci formation. (A) representative cells as captured by confocal microscopy; (B) quantitation of the number γ-H2AX foci per cell. Points represent mean of triplicate experiments; bars represent standard error.

(A)

(B)
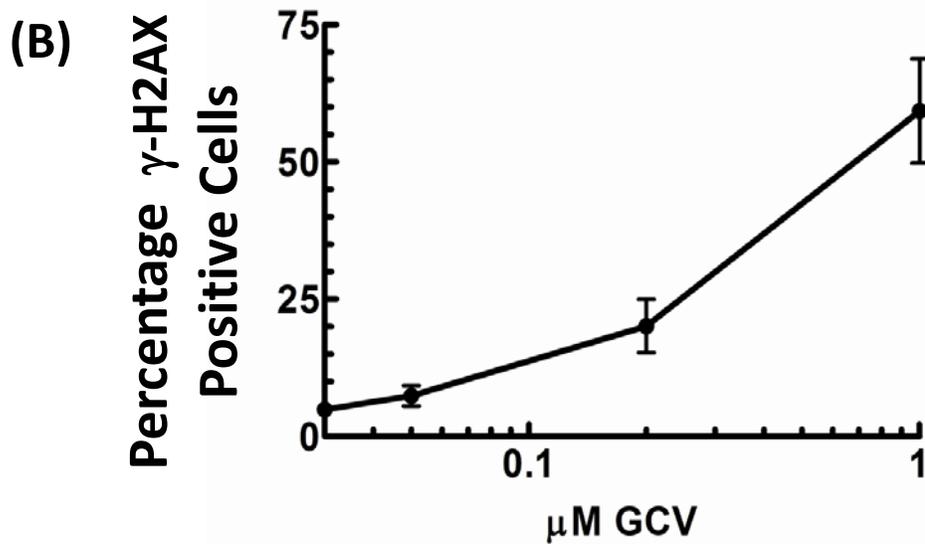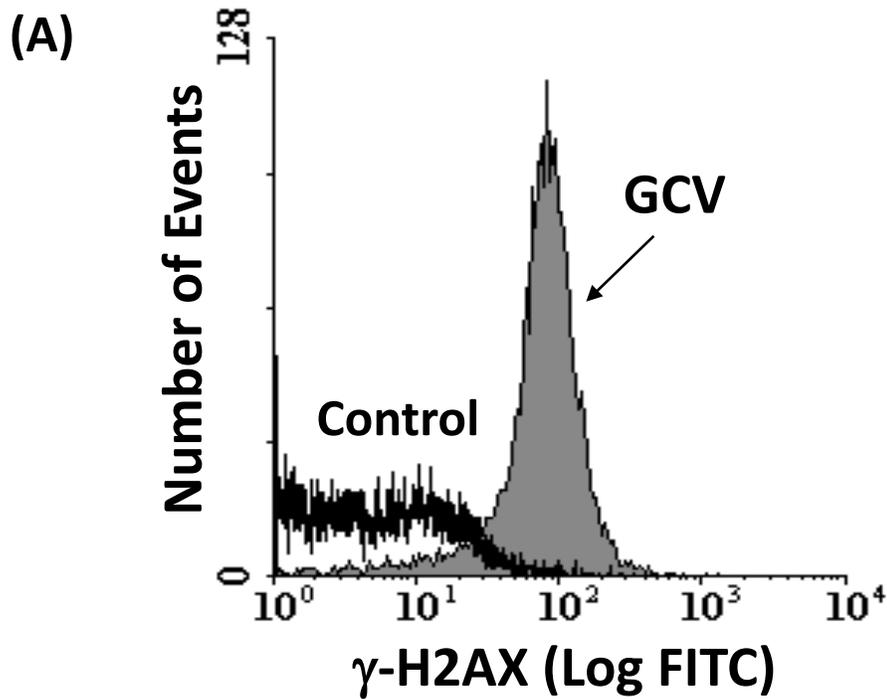
Figure 2.2 GCV induces a dose-dependent increase in γ-H2AX expression.
U251tk cells were incubated with GCV for 24 hr and assayed for γ-H2AX
expression by flow cytometry; (A) measurement of γ-H2AX expression by flow
cytometry after a 24 hr incubation with 1µM GCV (B) quantitation of
percentage of γ-H2AX expressing cells from flow cytometry. Points represent
mean of triplicate experiments; bars represent standard error.
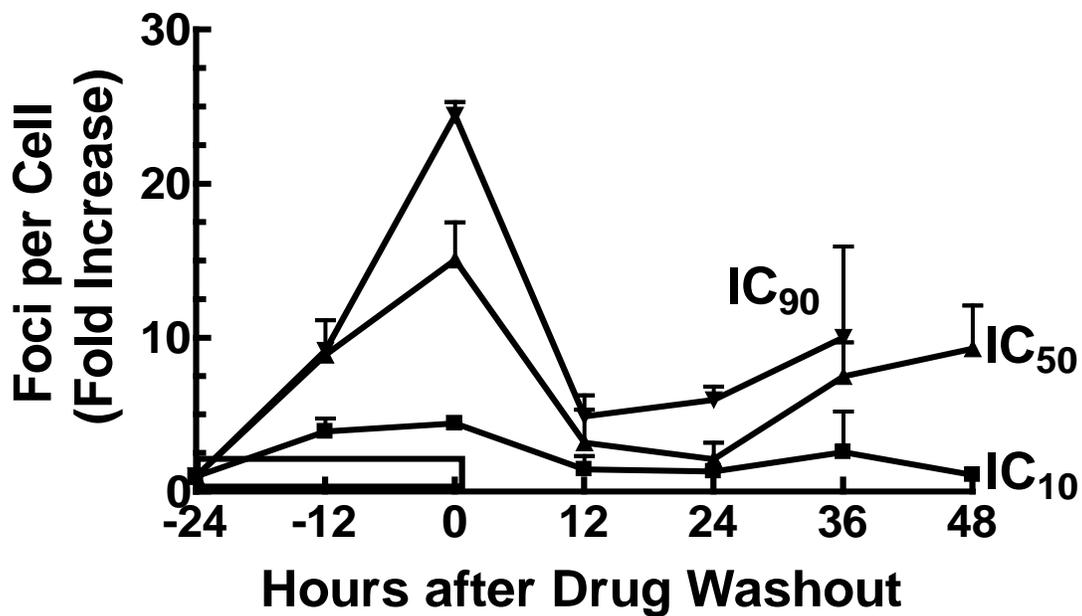
Figure 2.3 Time course of g-H2AX foci formation in response to GCV. U251tk cells were incubated with GCV at the IC_{10}, IC_{50} or IC_{90} for 24 h followed by drug washout. Cells were assayed by confocal microscopy for γ-H2AX foci formation at the indicated time points. Black bar indicates duration of drug incubation, points represent the mean of at least three experiments, bars represent standard error.

(A)

| Control | IC$_{10}$ | IC$_{50}$ | IC$_{80}$ |
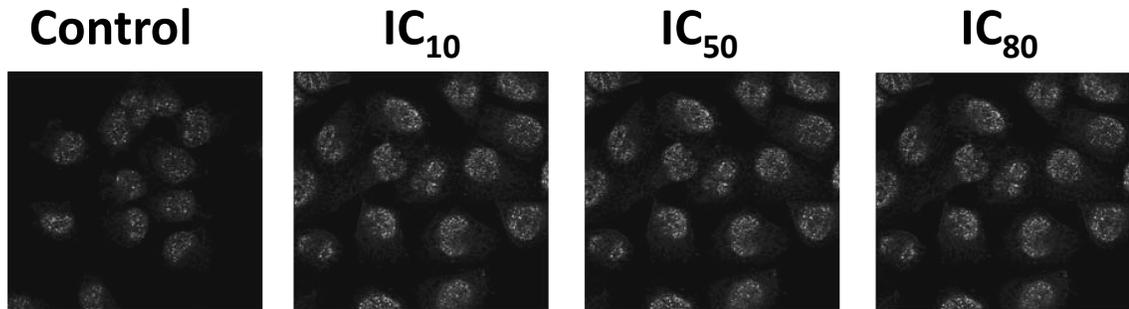
(B)
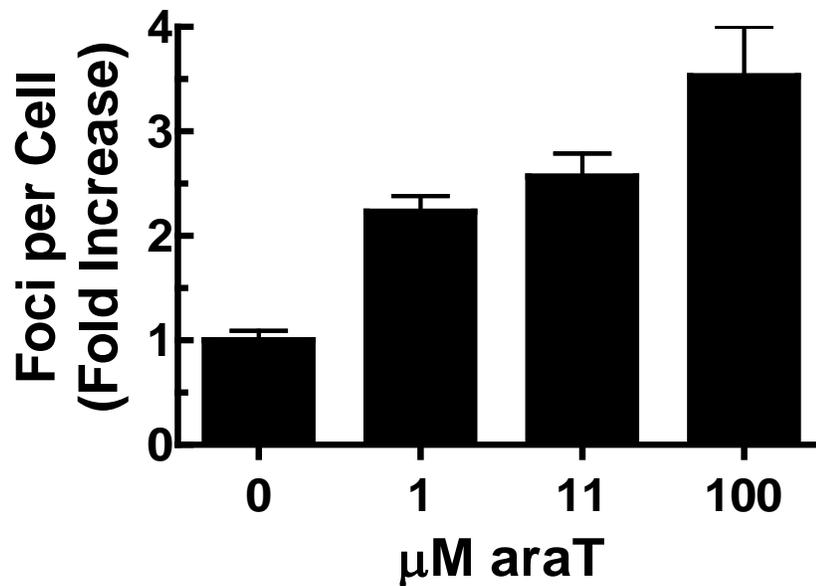
Figure 2.4 araT induces a dose-dependent increase in γ-H2AX. U251tk cells were incubated with araT for 24 hr and assayed for γ-H2AX foci formation. (A) representative cells as captured by confocal microscopy; (B) quantitation of the number γ-H2AX foci per cell. Columns, average of at least three separate experiments; bars, SE.
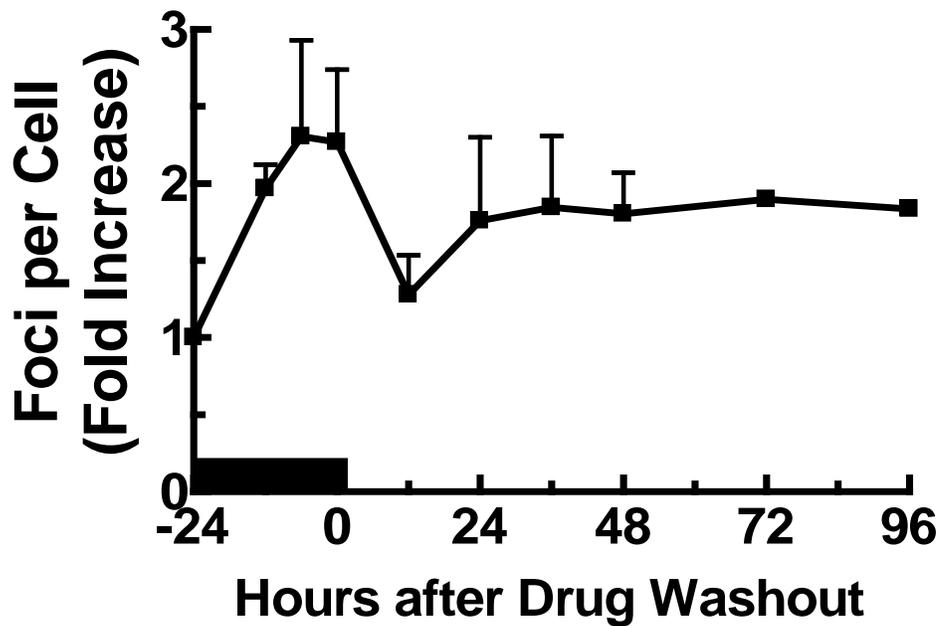
Figure 2.5 Time course of γ-H2AX foci formation in response to araT. U251tk cells were incubated with 100mM araT (IC$_{80}$) for 24 hr followed by drug washout. Cells were assayed for γ-H2AX foci formation by confocal microscopy at the indicated time points and the number of γ-H2AX foci per cell was determined. Black bar indicates duration of drug incubation. Points, mean of at least three experiments; bars, standard error.

(A)
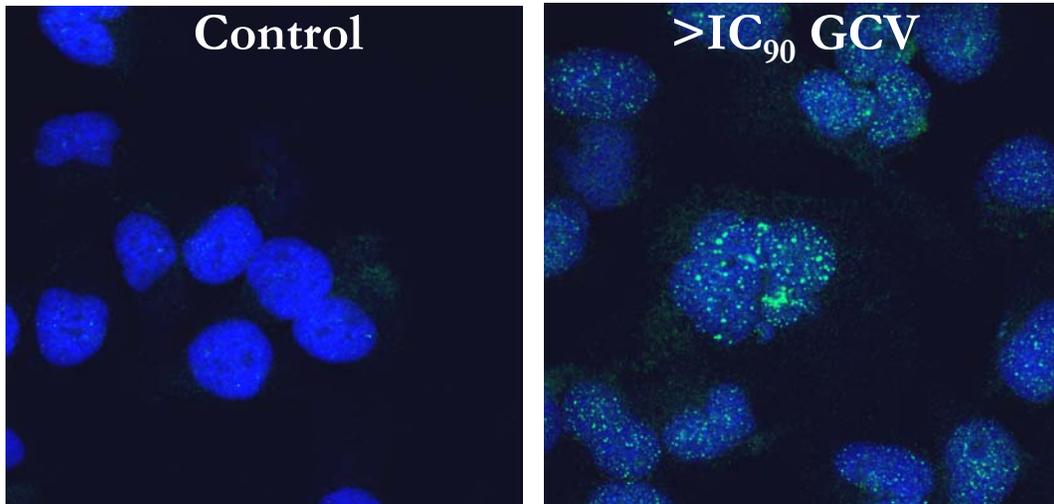
(B)
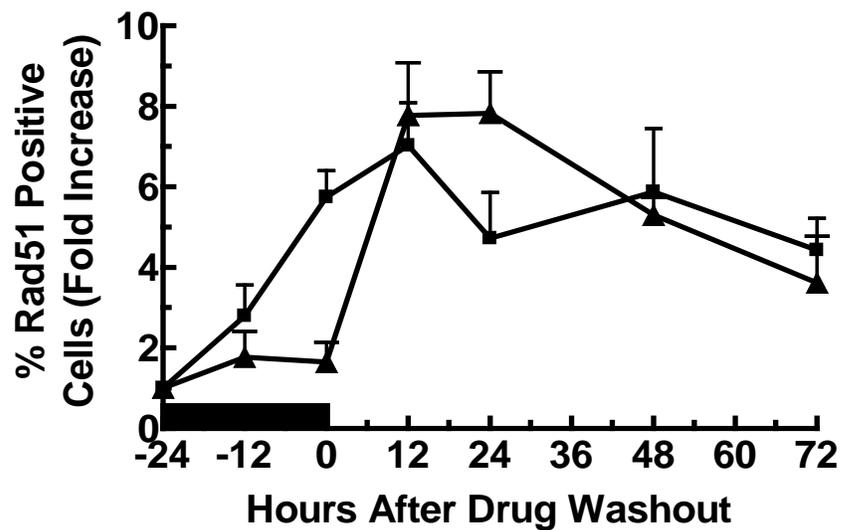
Figure 2.6 Time course of Rad51 foci formation in response to GCV or araT.
U251tk cells were incubated with (▲) IC_{90} GCV or (■) IC_{80} araT for 24 h
followed by drug washout. Cells were assayed by confocal microscopy for
Rad51 at the indicated time points (positive cell = $\geq$10 Rad51 foci). Black bar
indicates duration of drug incubation, points represent the mean of at least
three wells from a representative experiment, bars represent standard error.
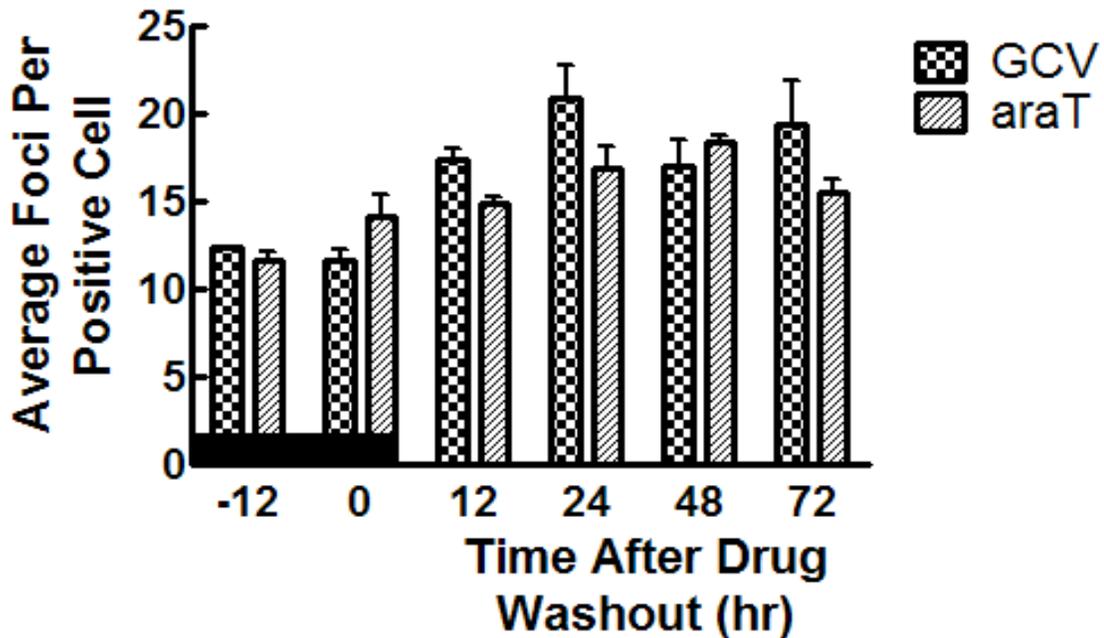
Figure. 2.7 Rad51 foci number in Rad51 positive cells in response to GCV or araT.
U251tk cells were incubated with IC_{90} GCV or IC_{80} araT for 24 h followed by drug
washout. Cells were assayed by confocal microscopy for Rad51 at the indicated
time points (positive cell = $\geq$10 Rad51 foci). Data represents the average foci per
cell in positive cells only. Black bar indicates duration of drug incubation, bars
represent the mean of at least three wells from a representative experiment, error
bars represent standard error.

(A) GCV
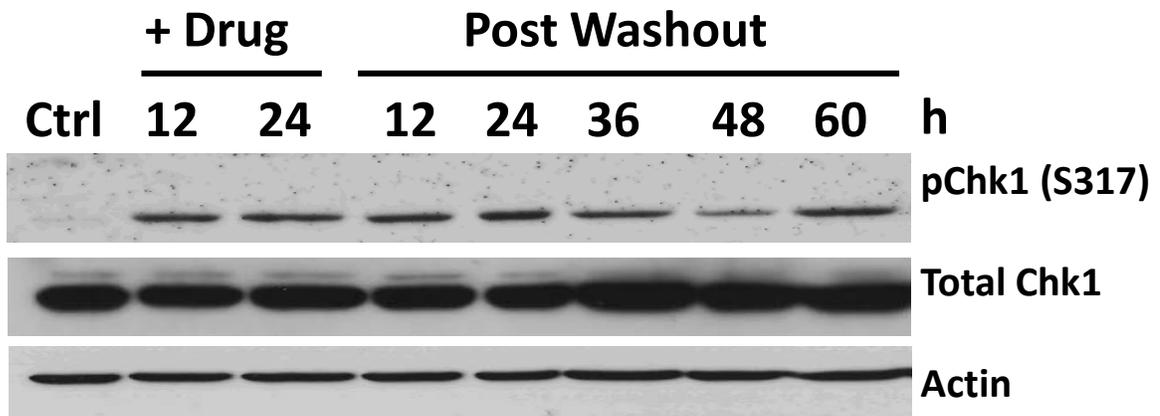
(B) araT
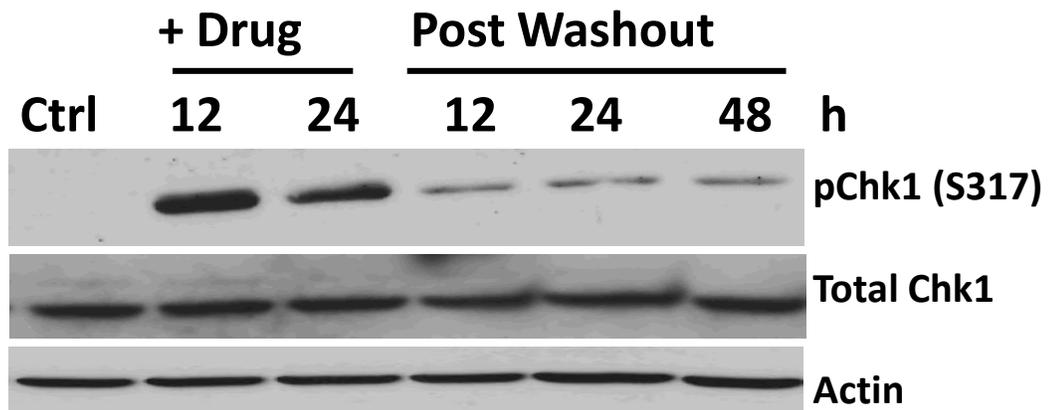
Figure 2.8 Time course of Chk1 phosphorylation in response to GCV or araT.
U251tk cells were incubated with (A) IC_{90} GCV or (B) IC_{90} araT for 24h followed by drug washout. pChk1(Ser317) was assayed by western blot at the indicated time points. Total Chk1 and actin were used as loading controls.

(A) GCV
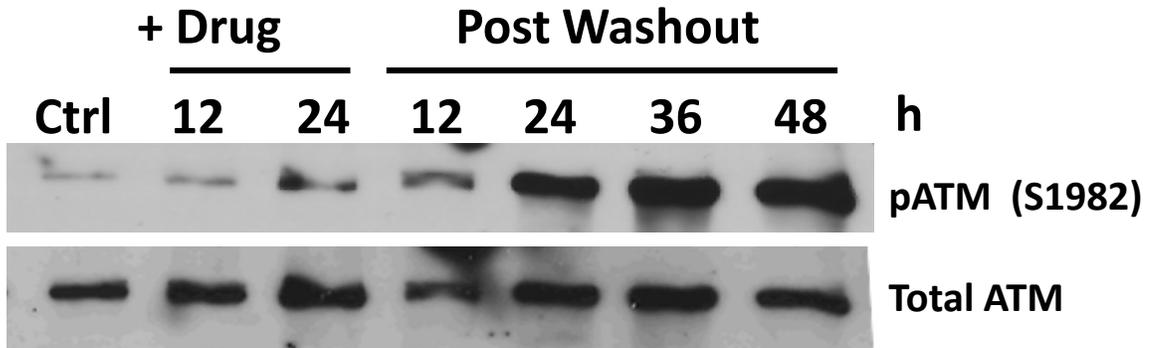
(B) araT
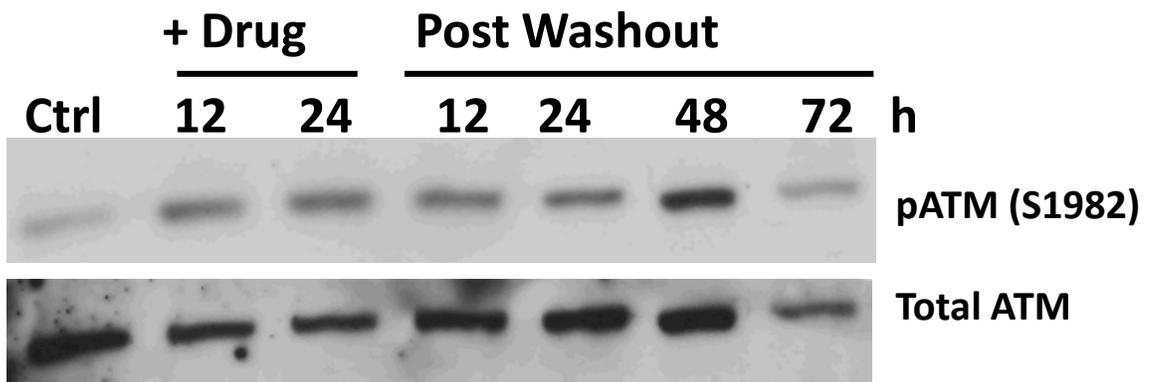
Figure 2.9 Time course of ATM activation in response to GCV or araT.
U251tk cells were incubated with (A) IC_{90} GCV or (B) IC_{90} araT for 24h
followed by drug washout. pATM (Ser1982) was assayed by western blot at
the indicated time points. Total ATM was used as a loading control.

Reference List

1. Moolten, F. L. (1994) *Cancer Gene Ther.* **1,** 279-287

2. Roth, J. A. and Cristiano, R. J. (1997) *J.Natl.Cancer Inst.* **89,** 21-39

3. Freytag, S. O., Khil, M., Stricker, H., Peabody, J., Menon, M., DePeralta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E., and Kim, J. H. (2002) *Cancer Res.* **62,** 4968-4976

4. Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M., and Kim, J. H. (2003) *Cancer Res* **63,** 7497-7506

5. Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. (1998) *Cancer Res* **58,** 3873-3882

6. Cheng, Y. C., Grill, S. P., Dutschman, G. E., Nakayama, K., and Bastow, K. F. (1983) *J.Biol.Chem.* **258,** 12460-12464

7. Halloran, P. J. and Fenton, R. G. (1998) *Cancer Res* **58,** 3855-3865

8. Tomicic, M. T., Thust, R., and Kaina, B. (2002) *Oncogene* **21,** 2141-2153

9. Thust, R., Tomicic, M., Klocking, R., Voutilainen, N., Wutzler, P., and Kaina, B. (2000) *Cancer Gene Ther.* **7,** 107-117

10. Thust, R., Tomicic, M., Klocking, R., Wutzler, P., and Kaina, B. (2000) *Mutagenesis* **15,** 177-184

11. Sonoda, E., Sasaki, M. S., Morrison, C., Yamaguchi-Iwai, Y., Takata, M., and Takeda, S. (1999) *Mol.Cell Biol.* **19,** 5166-5169

12. O'Konek, J. J., Boucher, P. D., Iacco, A. A., Wilson, T. E., and Shewach, D. S. (2009) *Cancer Gene Ther* **16,** 683-692

13. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) *J.Biol.Chem.* **273,** 5858-5868

14. Sedelnikova, O. A., Rogakou, E. P., Panyutin, I. G., and Bonner, W. M. (2002) *Radiat.Res.* **158,** 486-492

15. Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. (1999) *J.Cell Biol.* **146,** 905-916

16. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. (2000) *Curr.Biol.* **10,** 886-895

17. Robinson, B. W., Ostruszka, L., Im, M. M., and Shewach, D. S. (2004) *Semin.Oncol.* **31,** 2-12

18. O'Konek, J. J., Ladd, B., Flanagan, S. A., Im, M. M., Boucher, P. D., Thepsourinthone, T. S., Secrist III, J. A., and Shewach, D. S. (2010) *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **684,** 1-10

19. Rothkamm, K., Kruger, I., Thompson, L. H., and Lobrich, M. (2003) *Mol.Cell.Biol.* **23,** 5706-5715

20. Saleh-Gohari, N. and Helleday, T. (2004) *Nucleic Acids Res.* **32,** 3683-3688

21. Wyman, C., Ristic, D., and Kanaar, R. (2004) *DNA Repair (Amst)* **3,** 827-833

22. Plunkett, W., Chubb, S., Alexander, L., and Montgomery, J. A. (1980) *Cancer Res.* **40,** 2349-2355

23. Graham, F. L. and Whitmore, G. F. (1970) *Cancer Res.* **30,** 2636-2644

24. Parker, W. B., Shaddix, S. C., Chang, C. H., White, E. L., Rose, L. M., Brockman, R. W., Shortnacy, A. T., Montgomery, J. A., Secrist, J. A., III, and Bennett, L. L., Jr. (1991) *Cancer Res.* **51,** 2386-2394

25. Rothkamm, K. and Lobrich, M. (2003) *Proc.Natl.Acad.Sci.U.S.A* **100,** 5057-5062

26. Liu, J. S., Kuo, S. R., and Melendy, T. (2003) *Mutat.Res.* **532,** 215-226

27. Ha, L., Ceryak, S., and Patierno, S. R. (2004) *Carcinogenesis* **25,** 2265-2274

28. Abate-Daga, D., Garcia-RodrÆguez, L., Sumoy, L., and Fillat, C. (2010) *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1803,** 1175-1185

29. Tomicic, M. T., Bey, E., Wutzler, P., Thust, R., and Kaina, B. (2002) *Mutat.Res.* **505,** 1-11

Chapter III

Vorinostat Synergistically Enhances HSV-TK/Ganciclovir Gene Therapy by Inhibiting

Homologous Recombination

Summary

Many chemotherapeutic agents target DNA synthesis by either inhibiting DNA precursors or by direct incorporation into DNA. Among these compounds are nucleoside analogs, which resemble endogenous DNA substrates and can compete for incorporation into DNA resulting in the inhibition of DNA synthesis. In response to this inhibition, cells activate DNA repair mechanisms in order to complete DNA replication. However, the choice of DNA repair pathway, extent of repair activation, and the amount of cell kill can vary between different nucleoside analogs. Previous studies evaluating antiviral nucleoside analogs in a suicide gene (herpes simplex virus thymidine kinase) therapy approach to cancer treatment demonstrated that ganciclovir (GCV) incorporation into DNA causes a unique cellular response and elicits superior tumor cell killing compared to other nucleoside analogs used in the same gene therapy setting. Recently, we explored the mechanism by which GCV elicits superior cytotoxicity and

demonstrated a potential role for the DNA repair pathway of homologous recombination (HR) in response to GCV. Here, we extended these studies by evaluating GCV sensitivity in isogenic cell lines either proficient or deficient in HR. The results demonstrate that HR promotes survival in response to GCV. Based on this observation, we hypothesized that pharmacologic inhibition of HR will result in synergistic cell kill when combined with GCV. In light of previous reports demonstrating that inhibition of lysine deacetylases (KDACs) can inhibit HR, we evaluated the ability of suberoylanilide hydroxamic acid (SAHA; Vorinostat) to inhibit HR and enhance GCV cytotoxicity. In a cell based recombination reporter assay, SAHA (1 to 10µM) produced increasing inhibition (51 to 85%) of HR. Western blot analysis demonstrated a concentration-dependent decrease in the HR specific protein Rad51. At concentrations of SAHA that inhibited HR and decreased Rad51 expression, Rad51 foci formation was completely blocked with GCV treatment. SAHA also produced synergistic cytotoxicity in combination with GCV in HR proficient cells but only additive cell kill in HR deficient cells. Collectively, these data demonstrate that HR promotes survival in response to GCV and inhibition of this repair by SAHA results in synergistic cell kill. These studies suggest that inhibiting DNA repair prior to targeted DNA damaging approaches, such as suicide gene therapy, will provide a selective method for improving cancer therapy.

Introduction

Many nucleoside analogs inhibit DNA synthesis making them useful in the treatment of viral infections and certain cancers. In the context of cancer treatment,

the cellular responses, tumor cell kill, and patient toxicity varies depending the nucleoside analog used. Therefore a greater understanding of the mechanism of action of nucleoside analogs is required in order to rationally predict which nucleoside analog will be most beneficial to patients and identify the pathways for triggering cell death in tumor cells.

Ganciclovir (GCV) is an antiviral nucleoside analog used in suicide gene therapy approaches for the treatment of cancer. In order to be cytotoxic, GCV must be activated to its triphosphate, requiring herpes simplex virus thymidine kinase (HSV-TK) for the initial phosphorylation because mammalian kinases cannot use GCV as a substrate. Gene therapy approaches exploit this selectivity by delivering the HSV-TK cDNA as a "suicide gene" only to tumor cells followed by treatment with the substrate ganciclovir (GCV). With this strategy, only the cells containing the suicide gene are capable of activating a prodrug to a toxic metabolite, thus conferring selectivity for tumor cells while sparing normal dividing tissues. This therapy has demonstrated exquisite selectivity and excellent antitumor activity in many preclinical models which has resulted in several clinical trials including a combination approach in patients with prostate cancer(1-5).

Previous studies have demonstrated that GCV is more cytotoxic than other HSV-TK substrates such as 1-β-D-arabinofuranosylthymine (araT) despite the fact that GCV becomes incorporated into DNA to a lesser extent than araT(6), suggesting GCV has a unique mechanism of cell killing. Furthermore, at subcytotoxic concentrations, GCV induces sister chromatid exchanges (SCEs) whereas other HSV-TK substrates did not(7)

103

suggesting a potential role for the DNA repair pathway of homologous recombination (HR) in response to GCV. However, at cytotoxic concentrations SCEs were not observed potentially due to increased DNA synthesis inhibition by GCV(7). Using Rad51 foci as a direct indicator of HR events, we demonstrated a role for HR in response to GCV at cytotoxic concentrations(8). Although these studies demonstrate that HR is activated in response to GCV, they do not address the exact role of HR in promoting either cell survival or cell death. To address this question, O'Konek et al demonstrated that yeast strains deficient in HR are more sensitive to GCV than the corresponding WT strains(9). However, HR has a greater role in DNA repair in yeast compared to mammalian cells(10;11) therefore whether or not mammalian cells also utilize HR to survive GCV exposure is yet to be addressed.

Based on the observation that HR promotes cell survival in response to GCV in yeast, we hypothesized that HR promotes cellular survival in mammalian cells as well. Furthermore, we hypothesized that pharmacological inhibition of HR would result in synergistic tumor cell kill with GCV in HSV-TK expressing cells. While there are currently no inhibitors specifically for HR, Buggey et al demonstrated that the KDAC inhibitor PCI-24781 decreased the number of HR events in a cell based assay, abolished ionizing radiation (IR) induced Rad51 foci formation and decreased the expression of several proteins required for HR including Rad51(12). Furthermore it has been demonstrated that inhibition of KDAC enzymes results in persistent γH2AX and 53BP1 foci after IR(13), which accumulate at sites of DNA damage and recruit downstream repair proteins. Finally, KDAC inhibitors have recently been demonstrated to have excellent antitumor

activity when combined with DNA damaging agents prompting numerous clinical trials(14-19).

In addition to the potential increased GCV mediated cytotoxicity due to inhibition of HR, recent reports evaluating GCV in combination with the KDAC inhibitors noted improvements in suicide gene delivery and expression making these drugs an attractive combination with HSV-TK/GCV gene therapy(20;21). However, the role of HR repair in response to GCV in mammalian cells was previously unknown and therefore not considered in combination therapy strategies. Here, we demonstrate that HR promotes survival in response to GCV in mammalian cells providing another potential mechanism to be exploited pharmacologically to enhance GCV mediated cell kill. For our studies we evaluated the combination of the KDAC inhibitor Vorinostat (SAHA) with GCV in HSV-TK expressing cells because SAHA selectively affects cancer cells compared to normal non-transformed cells(22). The results demonstrate that SAHA inhibits HR and blocks HR repair of GCV induced DNA damage. Importantly, GCV and SAHA synergize only in HR proficient cells suggesting the synergy observed with GCV and SAHA is specifically due to inhibition of HR by SAHA.

Material and methods

Cell Culture

U251 human glioblastoma cells were maintained in RPMI 1640 medium supplemented with 10% calf serum (GIBCO, Grand Island, NY) and L-glutamine (Fisher Scientific, Pittsburgh, PA). Cells were maintained in exponential growth in a humidified

atmosphere at 37ºC and 5% CO_2. U251 cells stably expressing HSV-TK were generated as previously described(6). AA8 and irs1SF CHO cell lines were maintained in MEMα supplemented with 10% FBS (Gibco) and L-glutamine (Fisher Scientific) and maintained as described above. AA8 and irs1SF clonal cell lines were developed from parental cells using a retrovirus vector containing the cDNA for HSV-TK and LacZ(23). cDNAs were inserted into the pLKO.1-puro plasmid using EcoRI and BamHI. HeLa cells containing the DR-GFP construct (donated by Dr. Jasin) were maintained in DMEM containing 10% FBS and L-Glutamine were generated as previously described and maintained as described above. Individual clones expressing HSV-TK were generated as described above for the U251tk cell lines.

Western blots and immunohistochemistry:

Western blots were done according to standard protocol. Primary antibodies used were as follows: Rad51 (calbiochem), actin (calbiochem), histone 3, histone 3 acetyl-lysine 9, CtIP (cell signaling), HSV-TK (produced and donated by Dr. Black(24)). Rad51 foci formation was performed as previously described(8). Images of representative cell populations were captured, and Rad51 positive cells were scored visually (cells with 10+ foci were considered positive). For evaluating RPA (Lab Vision/NeoMarkers) foci formation after IR, U251tk cells were seeded on chambered slides. After 48hr, the indicated concentrations of GCV were added for 24hr followed by drug washout. 24hr after GCV washout cells were treated with 10Gy IR. 1hr post IR cells were fixed and stained for RPA and γ-H2AX (Trevagen). RPA and γ-H2AX staining was performed using

the same protocols as those used for evaluating Rad51 foci formation. Primary antibodies were mouse anti-RPA p34 (Lab Vision/NeoMarkers) and rabbit anti-γ-H2AX (Trevigen). Secondary antibodies were AlexaFluor 594-conjugated goat anti-mouse and AlexaFluor 488-conjugated goat anti-rabbit (each at 1:1000 dilution, Molecular Probes) for RPA and γ-H2AX, respectively.

Analysis of GCV metabolism and incorporation

Analysis of cellular deoxynucleotide triphosphates, GCV metabolism, and GCV incorporation was performed as previously described(6) with the following exceptions: Experiments measuring cellular deoxynucleotide triphosphates in U251tk cells were performed in cells treated with IC_{90} GCV (0.1µM) GCV for either 8 or 24hr. In AA8tk and irs1SFtk cells, IC_{90} (2µM or 0.2µM, respectively) for 8 or 16hr was used. Experiments measuring GCVTP and incorporation into DNA in U251tk cells were perfomed in cells treated with IC_{90} (0.1µM) [3H]GCV (Moravek Biochemicals, Inc., Brea, CA) for either 8 or 24hr. In AA8tk and irs1SFtk cells, each cell line was treated with 2µM or 0.2µM GCV (5% or 50% [3H]GCV, respectively) for 8 or 16hr.

Analysis of cell cycle

U251 cells expressing HSV-TK were grown in T25 flasks for 48hr prior to drug treatment. Cells were treated with GCV and/or SAHA at the indicated concentrations. All drug incubations were for 24hr unless otherwise noted. At the indicated times, cells were trypsinized and prepared for flow cytometry as previously described(6). Analysis was

performed on BD FacsCalibur at the University of Michigan Flow Cytometry Core Facility. At least 10,000 cells were evaluated for each condition, and the experiment was performed at least two times. Cell cycle phase was analyzed by Mod-fit LT (version 3.3.11, Verity Software House, Topsham, ME)

Clonogenic cell survival assay, isobologram analysis, and measurement of HR (DR-GFP assay)

Clonogenic cell survival assays were performed as previously described(1) with the exception that the AA8, AA8tk, irs1SF, and irs1SFtk cell lines were incubated in GCV and/or SAHA for 16hr. Isobologram analysis was applied to these dose-response curves as previously described(25). Each experiment was performed at least two times in triplicate. HeLa-DR-GFPtk cells were plated on 6-well plates at 1e5 cells/well. 48hr later, the indicated concentration of SAHA and/or adenovirus containing the ISceI cDNA was administered for 24hr. At the end of drug/virus incubation, fresh media was supplemented for 24hr. Cells were then trypsinized (0.25% trypsin, (Gibco)) for 5 min, pelleted by centrifugation (1000g for 5min), and resuspended in 0.5ml 1% formaldehyde (Fisher Scientific). Cells were analyzed for GFP expression by flow cytometry as described above. Data was analyzed using WinMDI software (version 2.9). Graph represents 3 individual experiments performed in triplicate.

Results

Homologous Recombination Promotes Survival in Response to Ganciclovir

Previously, we demonstrated a role for HR in response to GCV in mammalian cells(8) and that HR promotes survival in response to GCV in yeast(9). To determine the role of HR in response to GCV in mammalian cells, we evaluated GCV cytotoxicity in CHO cell lines either proficient (AA8) or deficient in HR (irs1SF) cell lines. Individual clones of each cell line stably expressing HSV-TK were generated using a retroviral vector. Cell lines stably expressing Lac-Z were used as controls for viral transduction. Using two separate clones of both HR proficient and deficient cell lines, the results demonstrated that HR-deficient irs1SFtk cells were >14-fold more sensitive (p = 0.013) to GCV than HR-proficient AA8tk cells indicating that HR promotes survival in response to GCV (IC_{50} = 0.08 $\pm$ 0.011 μM vs. 1.14 $\pm$ 0.694 μM, respectively)(Fig. 3.1). Importantly, the HR-deficient cells were more sensitive despite the fact that the AA8tk clones expressed similar levels of HSV-TK (Fig. 3.2) and accumulated similar levels of the active metabolite GCVTP after 8hr of [3H]GCV incubation (3.15 $\pm$ 0.07 and 2.96 $\pm$ 0.07 nMoles/10e7 cells after 0.2μM (p = 0.8515) and 27.91 $\pm$ 6.64 vs. 33.95 $\pm$ 3.26 nMoles/10e7 cells after 2μM (p = 0.4596) AA8tk6 and irs1SFtk22, respectively)(Fig. 3.4). Furthermore, dGTP, the endogenous competitor of GCVTP for incorporation into DNA, did not change significantly in response to GCV treatment in either cell line (Fig. 3.3). Finally, AA8tk cells incorporated as much or more GCV monophosphate (GCVMP) into DNA than irs1SFtk cells (2.84 fold $\pm$ 0.11 at 0.2μM (p < 0.0001) and 1.38 $\pm$ 0.41 at 2μM (p = 0.0853) after 8hr and 2.10 $\pm$ 0.18 at 0.2μM (p = 0.0053) and 2.31 $\pm$ 0.05 at 2μM (p < 0.0001)

after 16hr, respectively)(Fig. 3.5).   Collectively, these data demonstrate that altered metabolism of GCV, decreased dGTP, or GCVMP incorporation into DNA cannot explain the differences in cytotoxicity observed between the cell lines.   Thus, HR promotes survival in the AA8tk cells.

With the compelling data implicating a strong role for HR in promoting survival in the CHO cell lines, we wished to evaluate a role for HR in promoting survival with GCV in matched human cell lines with and without HR, utilizing shRNA mediated depletion of the HR required proteins Rad51 and XRCC3. Depletion of either Rad51 or XRCC3 in U251tk cell lines was toxic to the cells, thus sensitivity to GCV could not be measured (data not shown). To circumvent the toxicity associated with shRNA depletion of HR required proteins, we attempted to overexpress the HR required proteins Rad51, CtIP, and/or Exo1. Similar to what was observed with depletion of HR required proteins, overexpressing HR proteins also proved to be toxic (data not shown). Furthermore, we were not able to obtain cells with sustained overexpression of CtIP and/or Rad51 despite puromycin selection for the shRNA-transduced cells (data not shown) suggesting overexpression of HR required proteins confers a disadvantage for cellular proliferation resulting in selection of cells with normal expression of the HR required protein.

Cell cycle in AA8tk and irs1SFtk cells in response to GCV

To determine if HR deficiency resulted in altered cell cycle effects in response to GCV, cell growth and dual parameter cell cycle analysis was performed in both HR proficient and deficient cell lines. Similar to previous observations in human cell lines,

growth of AA8tk cells treated with IC_{90} GCV was inhibited until approximately 24hr after

GCV removal when cell number decreased due to cell death (Fig. 3.6). Cell cycle analysis

demonstrated an increase in early S-phase cells 4hr into GCV incubation (Fig. 3.7). As

time progressed, the number of cells in mid S, late S, and G_1 increased suggesting cells

were progressing through the cell cycle. Upon entry into the second cell cycle, cells

arrested in S and G_2/M suggesting permanent arrest in the second cell cycle until cell

death occurred. Conversely in the HR deficient irs1SFtk cell line, cell growth (Fig. 3.6)

and cell cycle analysis (Fig. 3.8) demonstrated that treatment with IC_{90} GCV resulted in

no cell cycle or growth perturbation, which was verified using a second, independent

irs1SFtk clone (data not shown). The HR deficiency in the irs1SF cell line is due to the

lack of the HR required protein XRCC3. To determine whether the lack of cell cycle

perturbation in response to GCV was the result of XRCC3 depletion or specific to the

irs1SF cell line, cell cycle analysis after incubation with GCV was evaluated in human

U251tk glioblastoma cells after shRNA of XRCC3 (Fig. 3.9, 3.10). Depletion of XRCC3

alone was toxic to cells in the colony formation assay performed to measure changes in

GCV sensitivity, however this assay requires trypsinizing and replating of cells at low

density. If cells are not trypsinized, they continued to grow until confluence suggesting

alterations in cell cycle could be measured after depletion of XRCC3. A non-specific

shRNA was used to control for viral infection. In control cells and non-specific shRNA

treated cells, the cell cycle of U251tk cells is moderately inhibited during GCV treatment

and permanent arrest occurs 24hr after removal of GCV. In XRCC3 depleted cells there

was no cell cycle perturbation until 48hr post GCV washout. At 48hr, cells arrested in S

and G$_2$/M of the cell cycle. Based on previous experiments with retroviral delivery of shRNAs to deplete proteins, the expression of XRCC3 may be restored 48hr after GCV removal (data not shown). These data suggest that HR is responding to GCVMP in template DNA in an XRCC3 dependent manner to prevent or repair lesions that will later result in cell death, however at cytotoxic concentrations HR fails to repair GCV induced DNA damage resulting in cell death.

DNA resection in the presence of GCVMP in template DNA

After treatment with GCV, we hypothesized that GCVMP in the template strand of DNA induces double strand breaks during replication(8). We further hypothesize that cells attempt to repair GCV induced DNA damage with HR, which involves DNA resection of the GCVMP containing template strand. Resection of GCVMP from DNA provides the only step in the repair pathway in which repair enzymes must chemically react with the modified carbohydrate moiety of GCVMP. Furthermore, previous studies have demonstrated that GCVMP remains incorporated in DNA for at least 96hr after removing GCV(6). Therefore, we hypothesize that GCV can inhibit DNA resection. To address this hypothesis we attempted to evaluate the ability of GCVMP in DNA to inhibit DNA resection during HR after IR. Previous reports have demonstrated that depletion of the HR required exonuclease CtIP prevents focal accumulation of RPA(26), a protein that coats single stranded DNA generated by resection, indicating resection does not occur in the absence of CtIP(26). In light of these findings, we attempted to evaluate RPA foci formation in cells pretreated with GCV and treated with IR, which induces DNA double

strand breaks, many of which are repaired by HR during S phase thus DNA must be resected in order for HR to occur(26)(Fig. 3.11, 3.12). To increase the probability of an IR induced DSB occurring in proximity of incorporated GCVMP, high concentrations of GCV and IR were used. The results demonstrated that after 10Gy IR, a decrease in the average RPA foci per cell from 44.3 $\pm$ 12.2 to 25.9 $\pm$ 4.6 and 16.5 $\pm$ 4.2 if cells were pretreated with 0.1µM (IC_{90}) or 1µM (IC_{99}) GCV, respectively. However, at 10µM we observed an increase in the average RPA foci per cell and variability between cells (61.2 $\pm$ 36.4) indicating that RPA foci formation after IR in cells pretreated with GCV may not be an adequate method for measuring resection through GCVMP-containing DNA during HR.

GCV inhibits HR

Results from the studies in the HR proficient and deficient CHO cell lines demonstrate that HR promotes survival in response to GCV, however, at cytotoxic concentrations HR fails to save the cell and cell death occurs. To determine whether GCV directly inhibits HR we measured HR events in a previously described cell based (DR-GFP) reporter assay(27) in the presence or absence of GCV. Briefly, a double strand break is enzymatically induced in a nonfunctional GFP (Fig. 3.14). If repaired by HR, a functional, nonexpressed GFP will be used to resynthesize the damaged area resulting in a functional GFP gene. The expression of GFP can be detected subsequently by flow cytometry. Using HeLa cells, we generated monoclonal populations of cells containing the DR-GFP reporter construct that also stably express HSV-TK (Fig. 3.13). Monoclonal cell lines were isolated and GCV sensitivity was assessed (Fig. 3.13). Hela-DR-GFPtk

clone 6 was used for HR studies. Using an adenovirus containing the cDNA for the I-SceI endonuclease, a DSB was generated and HR events were monitored by flow cytometery at the indicated concentrations of GCV (Fig. 3.15). The results demonstrated that GCV alone did not significantly increase the percentage of GFP positive cells in the absence of the I-SceI endonuclease. After induction of a double strand break with an adenovirally delivered I-SceI endonuclease, a concentration dependent decease in GFP positive cells was observed (Fig. 3.15) decreasing from 14.6% $\pm$ 0.26% in controls to 3.7% $\pm$ 0.18% with 10µM GCV (p<0.0001). The inhibition of HR required high concentrations of GCV, however this is likely attributed to low a frequency of GCVMP incorporation within the integrated reporter. Collectively, these data suggest that GCVMP in DNA can inhibit HR in this cell based assay.

Synergistic tumor cell kill with GCV and SAHA

Due to the increased sensitivity to GCV in HR deficient cells, we hypothesized that inhibition of HR would enhance cytotoxicity with GCV in HR-proficient cells resulting in synergy. KDAC inhibitors have recently been reported to inhibit HR(12;28), thus we evaluated the combination of GCV and the KDAC inhibitor SAHA on cellular survival. We chose to evaluate GCV and SAHA in U251tk glioblastoma cells due to the established role of HR in response to GCV in this cell line as indicated by the appearance of Rad51 foci after GCV treatment(8). To determine the concentrations of GCV and SAHA to evaluate drug synergy with we performed clonogenic cell survival assays with GCV and SAHA in U251tk cells to determine the IC_5 to IC_{90} concentrations of GCV and SAHA that

could then be used for testing synergistic cytotoxicity(Fig. 3.16). Based on these results, we evaluated the effects of GCV and SAHA at concentrations ranging from 0.3µM to 30µM of SAHA for our studies. Further, treatment of U251tk cells over this range of SAHA concentrations produced a concentration dependent increase in AceH3K9 at 8 hr after drug addition (Fig. 3.17). Next, we evaluated cytotoxicity with the combination of GCV and SAHA (Fig. 3.18). For GCV, the IC_{25}, IC_{50}, and IC_{90} were used. For SAHA, 0.3, 1, 3, and10µM were used. Isobologram analysis was used to determine the interaction between the two drugs, demonstrating that simultaneous addition of GCV and SAHA resulted in synergistic cell kill (Fig. 3.19).

Increased HSV-TK expression and decreased GCV Incorporation with SAHA

Previously it has been demonstrated that KDAC inhibition can increase mRNA of HSV-TK in stably expressing cells(21). However, the consequences on HSV-TK protein levels and GCV metabolism are yet to be determined. Therefore, we evaluated the expression of HSV-TK in U251tk cells stably expressing the enzyme. After 24hr of incubation, western blot analysis demonstrated a 1.5 fold increase in HSV-TK expression in response to SAHA alone and a 2.2 fold increase when GCV and SAHA were administered concurrently (Fig. 3.20). Further, we evaluated the effects on the phosphorylation and incorporation of [3H]GCV 8hr and 24hr post addition of [3H]GCV and SAHA (Fig. 3.21, 3.22, respectfully) and the effects of SAHA on dGTP levels, the endogenous competitor to GCV (Fig. 3.23). The results demonstrated a 2.2 fold decrease in the [3H]GCVTP after 8hr of incubation with SAHA and a maximal 1.8 fold

increase in amount of [3H]GCVTP after 24hr coincubation with 3µM SAHA. In addition, SAHA does not significantly alter dGTP levels in combination with GCV. Finally, measurement of incorporation of [3H]GCV into DNA revealed that SAHA did not alter incorporation after 8 hr, but after 24 hr there was a concentration-dependent decrease in incorporation, with a maximal reduction of 12-fold (22.3$\pm$ 0.81 to 1.88 $\pm$ 0.25 pmol/10e7 cells). Collectively, these data exclude the possibility that the synergistic cytotoxicity observed between GCV and SAHA is due to altered metabolism of endogenous nucleotides or GCV in response to SAHA.

Inhibition of HR with SAHA

Previously, we have demonstrated a role for the DNA damage response in the cytotoxicity of GCV(8). In particular, we demonstrated an increase in foci formation of the homologous recombination (HR) specific protein Rad51 during the second cell cycle after drug exposure. The Rad51 foci increase coincides with entry of cells into the second S-phase after the addition of GCV at which time cells permanently arrested and underwent apoptosis. Using a cell based assay, Buggy, *et al* demonstrated that the HDAC inhibitor PCI-24781 inhibits HR(12). Therefore, we evaluated the protein levels HR required proteins in response to SAHA including Rad51 and CtIP, a required exonuclease for DNA resection during HR. The results demonstrated SAHA alone didn't alter CtIP except at 10µM, whereas GCV produced a strong increase in CtIP that was blocked by SAHA in a concentration dependent manner, decreasing to 48%, 31% and 5%

of that observed with GCV alone with 1μM, 3μM and 10μM SAHA, respectively (Fig. 3.24).

Additionally, the results demonstrated a concentration dependent decrease in Rad51 to 39% of control at 3μM and 8% of control at 20μM of SAHA alone (Fig. 3.25). Notably, the combination of SAHA with an IC_{90} of GCV resulted in a decrease in Rad51 protein to less than 50% of GCV alone at 1μM SAHA and only 4% remained at the 20μM. We also evaluated Rad51 foci formation in response to GCV and SAHA (Fig. 3.26). The results demonstrated that the addition of 1μM SAHA decreased the percent of Rad51 positive cells with an IC_{90} of GCV from 16.5 to 0.33 fold increase over control 24hr post drug washout. Strikingly, there were no Rad51 positive cells after treatment and IC_{90} of GCV with 3 or 10μM SAHA or with any concentration of SAHA alone at any of the time points evaluated. Collectively, these results indicate that synergy between GCV and SAHA may be due to inhibition of HR by SAHA.

To further support the hypothesis that SAHA inhibits HR we measured HR events in a cell based DR-GFP assay in the presence or absence of SAHA similar to Fig. 3.15(27). The results demonstrated that SAHA reduced HR events in a concentration dependent manner with the percentage of GFP positive cells decreasing to 49% ±6.1%, 64% ±2.4%, and 15% ± 1.3% of controls at 1μM, 3μM, and 10μM, respectively (Fig. 3.27). Importantly, the concentrations used that inhibited HR were only mildly cytotoxic (Fig. 3.28). Taken together, with the data demonstrating a decrease in Rad51 protein levels and abolishment of GCV induced Rad51 foci, these experiments confirmed that SAHA

inhibits HR supporting the hypothesis that SAHA produces synergistic cytotoxicity with GCV by inhibiting HR.

Cell cycle effects with GCV and SAHA

Previously, we reported that GCV causes an arrest in the second S-phase of the cell cycle after drug addition(6). HR is used in S and G_2 of the cell cycle, therefore our results demonstrating decreased HR events in a cell based assay, decreased Rad51 expression and lack of Rad51 foci in response to GCV and SAHA could be explained by SAHA causing cell cycle arrest in G_1 of the second cell cycle after drug addition prior to entry into the second S phase where HR would occur. Therefore, we evaluated the cell cycle effects of SAHA alone and with GCV (Fig. 3.29). The results demonstrated that there are at least as many cells in S and G_2 with the combination of GCV and SAHA as with GCV alone, indicating that the combination does not cause a G_1 arrest. Thus, our findings that SAHA decreased Rad51 expression and HR events in a cell based assay cannot be explained simply by a G_1 arrest.

Synergistic cell kill only in HR proficient cells with GCV and SAHA

Based on our hypothesis that the synergy of GCV and SAHA is due to inhibition of HR, we further hypothesized that the combination of GCV and SAHA would be additive or antagonistic in cells lacking HR. To test this hypothesis, we evaluated the drug interaction in HR proficient and deficient CHO cells stably expressing HSV-TK. Four concentrations of both GCV and SAHA were used ranging from subcytotoxic

concentrations ($<IC_5$) to $\sim IC_{75}$ for each drug. The results demonstrated that irs1SFtk cells are more sensitive to SAHA as a single agent (data not shown). Further, using the isobologram analysis, we demonstrated that GCV and SAHA synergize in HR proficient cells but are only additive in HR deficient cells (Fig. 3.30). These data support the hypothesis that the drug synergy observed with GCV and SAHA is the result of inhibition of HR repair in response to GCV induced DNA damage by SAHA.

Discussion

A better understanding of the mechanistic differences in tumor cell killing by nucleoside analogs will identify which pathways are important for triggering cell death which can be used for future drug targeting strategies. Suicide gene therapy with HSV-TK/GCV is a promising approach for treating cancer while minimizing the toxicities associated with many traditional chemotherapeutic regimens. Importantly, GCV has superior cytotoxicity in HSV-TK expressing cells compared to other HSV-TK substrates(6) and has a unique mechanism of cell killing compared all nucleoside analogs studied to date. Here, we demonstrate that HR promotes survival in response to GCV and identify SAHA as an inhibitor of HR that enhances the cytotoxicity of GCV providing a novel drug combination for HSV-TK/GCV gene therapy.

Previous studies have demonstrated that after the addition of GCV to HSV-TK expressing cells, moderate DNA synthesis inhibition is observed in the first cell cycle followed by S-phase arrest in the second cell cycle(6). Immunohistochemical studies

demonstrated that cell cycle arrest is accompanied by an increase in DNA damage and activation of HR(8) which promotes cell survival in yeast(9). Here, using isogenic cell lines either proficient or deficient in HR, we demonstrate that HR promotes survival in response to GCV in mammalian cells. This observation suggests that inhibition of HR will result in enhanced GCV cytotoxicity. Using the KDAC inhibitor SAHA to inhibit HR, we demonstrate that GCV and SAHA synergistically kill HR proficient cells. Collectively, these studies provide a mechanistic rationale for evaluating the combination of GCV and SAHA *in vivo.*

While these studies demonstrate that inhibiting HR enhances the cytotoxicity, a better understanding of why HR fails to promote cell survival in response to GCV may uncover novel steps within the repair pathway that can be selectively targeted for enhancing the efficacy of DNA damaging agents. The observation that HR promotes survival in response to GCV raises the possibility that, at cytotoxic concentrations GCV, GCVMP incorporated into DNA inhibits HR resulting in cell death. In support of this hypothesis, we demonstrated that GCV inhibits the repair of a reporter construct by HR in a cell based assay. To address the specific step of HR that is inhibited by GCV, we attempted to evaluate whether GCVMP can inhibit the resection of HR resulting in failed repair. We chose to evaluate resection during HR for the following reasons: GCVMP causes stalling of DNA synthesis resulting in cell death when in the DNA template and when HR is used to restart DNA replication at a stalled replication fork, the template strand of DNA is resected. Therefore, in order for HR to resect the DNA template to

restart a stalled replication fork caused GCVMP in the DNA template, GCVMP must be resected by the HR machinery.

We attempted to address DNA resection during HR in human cell lines by measuring RPA foci formation after IR. RPA forms foci on single stranded DNA generated during HR and therefore was used in our studies as an indirect measurement of DNA resection(26). Although the results suggest GCV may be inhibiting DNA resection during HR, the variability observed in these experiments limited our ability to conclude GCV inhibited DNA resection during HR.

Based on our results in isogenic cell lines either proficient or deficient in HR suggesting that HR promotes survival in response to GCV, we hypothesized that pharmacologic inhibition of HR would result in synergistic cell kill with GCV. While there are no compounds that selectively inhibit HR, KDAC inhibitors have been demonstrated to decrease HR in cell culture and animal models(12).

Based on this observation, we evaluated the combination of GCV with the KDAC inhibitor and demonstrated that SAHA synergizes with GCV in U251tk cells. In light of the known role of HDACs in altering gene transcription and other cellular processes, we evaluated several metabolic parameters to determine if altered metabolism of GCV or dGTP, which GCV competes with for incorporation into DNA, is altered by SAHA. In these studies, we observed no significant changes that could contribute to the synergy observed with GCV and SAHA. Conversely, we observed that SAHA decreased Rad51 protein levels in a concentration dependent manner which was observed despite a decrease in the percent of G_1 cells where Rad51 is not expressed. Additionally, SAHA

treatment completely inhibited Rad51 foci formation in response to GCV and inhibited HR in a cell based assay. Finally, we observed synergistic cell kill in HR proficient cells and only additive cell kill in HR deficient cells. Together, these results indicate that synergistic tumor cell kill in HSV-TK expressing cells is due to inhibition of HR by SAHA.

Collectively, this study identifies a novel drug combination of SAHA with HSVTK/GCV gene therapy. The observation that HR promotes survival in response to GCV reveals that inhibiting HR pharmacologically with drugs such as SAHA enhances the cytotoxicity of GCV. In addition to these findings, recent reports evaluating GCV in combination with other KDAC inhibitors demonstrated increases the number of tumor cells infected by the adenovirus containing the HSV-TK cDNA(20) increased mRNA expression of HSV-TK in stably expressing cells(21) and increased the gap junction protein connexin 43, which increased bystander killing by increasing transfer of cytotoxic GCV metabolites from HSV-TK expressing cells to neighboring cells(20;21;29). In our studies, we also observed increases in HSV-TK, however we also observed a concentration dependent decrease in GCVMP incorporation suggesting increasing HSV-TK cannot explain the observed synergy with GCV and SAHA. Further, none of these studies considered the contribution of enhancing the cytotoxicity of GCVMP after incorporation into DNA with KDAC inhibition.

Together these studies demonstrate that KDAC inhibitors can improve HSV-TK/GCV gene therapy by multiple mechanisms. SAHA is an FDA approved KDAC inhibitor that is well tolerated in patients, an important aspect when considering the combination with a selective treatment such as gene therapy. Here, we demonstrate that SAHA can

improve gene therapy by a previously unappreciated mechanism: enhancing the cytotoxicity of GCV by inhibiting HR repair of GCV induced DNA damage. This observation is significant not only for enhancing HSV-TK/GCV gene therapy but potentially other targeted therapies such as ionizing radiation. While future studies evaluating the combination of SAHA with HSV-TK/GCV gene therapy in animal models are needed to determine if SAHA increases the efficacy of gene therapy sufficiently to warrant human trials, these results indicate a promising new direction for HSV-TK/GCV suicide gene therapy.
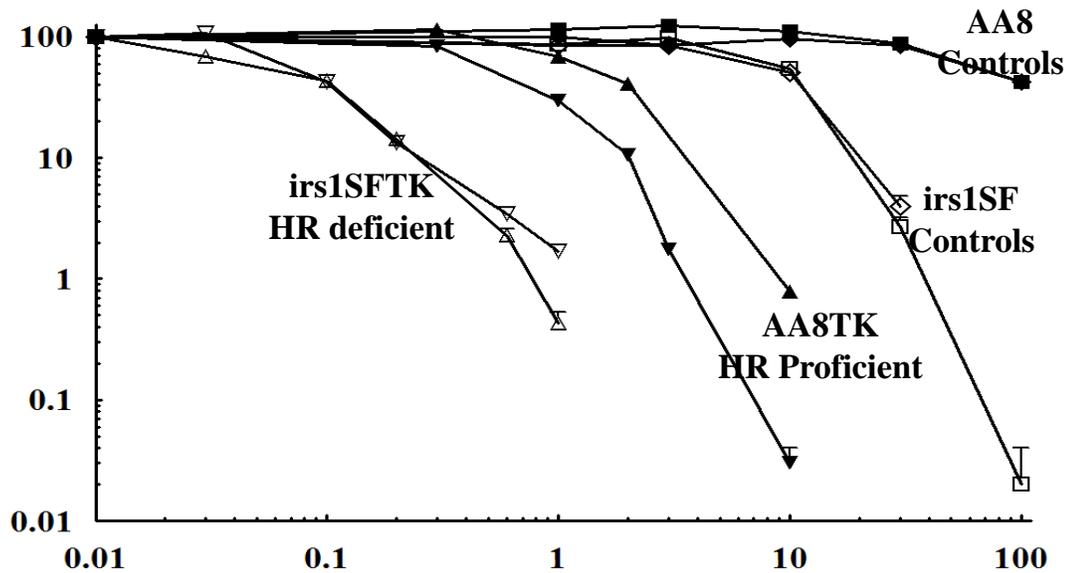
Figure 3.1 GCV cytotoxicity in HR proficent and deficient CHO cells. Individual clones of HR proficient (AA8) and HR deficient (irs1SF) were used to generate isogenic cell lines stably expressing HSV-TK or LacZ using a retrovirus. Exponentially growing cell lines indicated (♦AA8,■AA8LacZ, ▼AA8tk6, ▲AA8tk14, ◇irs1SF, □irs1SFLacZ, △irs1SFtk19, ▽irs1SFtk22) were exposed to increasing concentrations of GCV for 16 h. Survival was determined by a clonogenic cell survival assay and expressed as a fraction of plating efficiency for untreated cells. Points represent the mean of at least three wells from a representative experiment, error bars represent standard error.
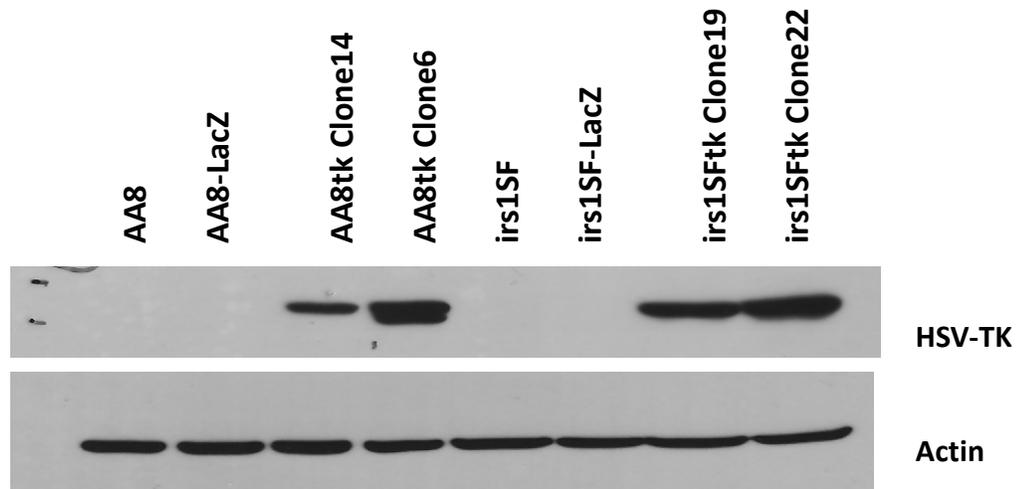
Figure 3.2 HSV-TK expression in HR proficent (AA8) and deficient (irs1SF) cells. Indicated cell lines were stably expressing LacZ (controls) or HSV-TK were subjected to western blot analysis. Total actin was used as a loading control.
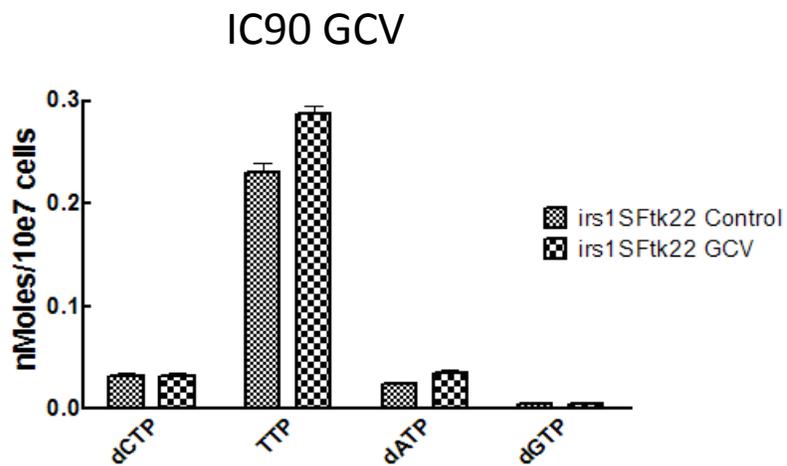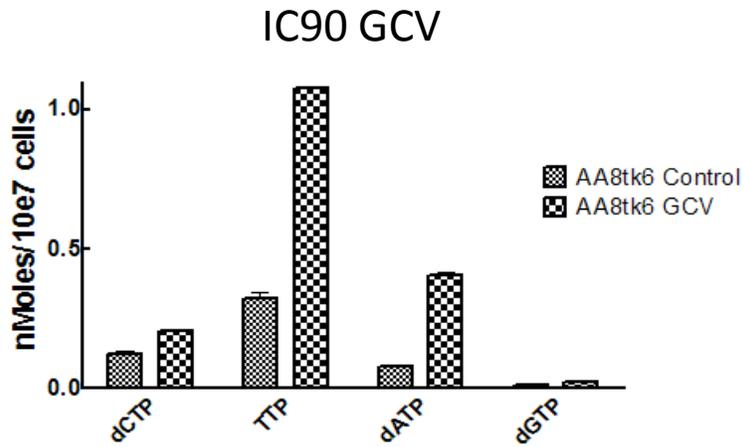
Figure 3.3 Effects of GCV on endogenous dNTP pools in AA8tk and irs1SFtk cells. AA8tk (top) or irs1SFtk (bottom) cells were incubated with IC$_{90}$ GCV (2μM or 0.2μM, respectively) for 16hr. At the end of drug incubation cells were harvested, nucleotides were purified by acid extraction endogenous dNTP pools were separated and measured by HPLC. Columns, mean; bars, SE

8hr Post Drug Addition
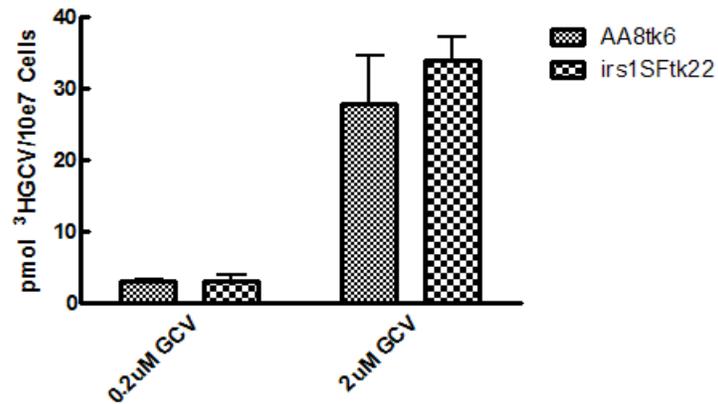
16hr Post Drug Addition
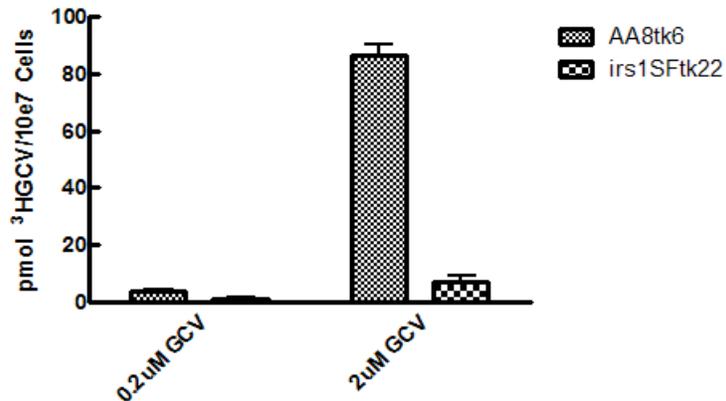
Figure 3.4 GCVTP levels in AA8tk and irs1SFtk cells. AA8tk or irs1SFtk cells were incubated with either 0.2µM [3H]GCV (IC_{90} irs1SFtk) or 2µM [3H]GCV (IC_{90} AA8) for either 8hr (top) or 16hr (bottom). At the end of drug incubation cells were harvested and nucleotides were purified by acid extraction. [3H]GCVTP metabolites were separated by HPLC and quantified by scintillation counting. Columns, mean; bars, SE

8hr Post Drug Addition
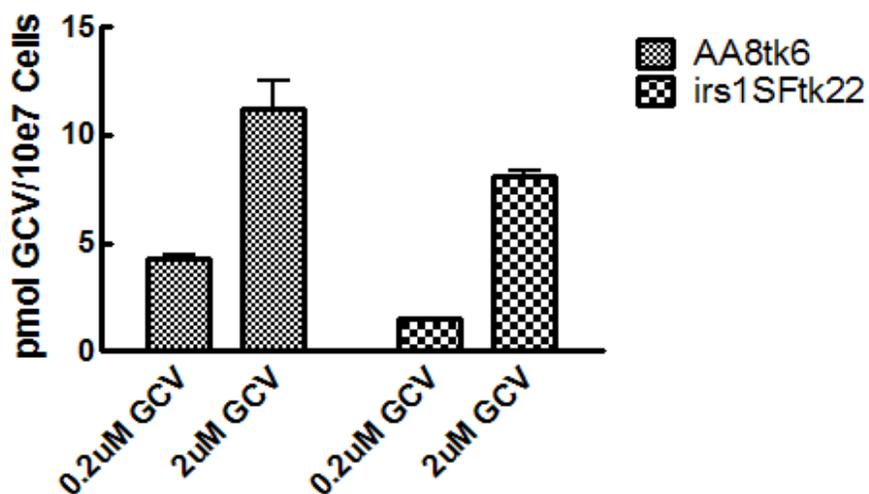
16hr Post Drug Addition
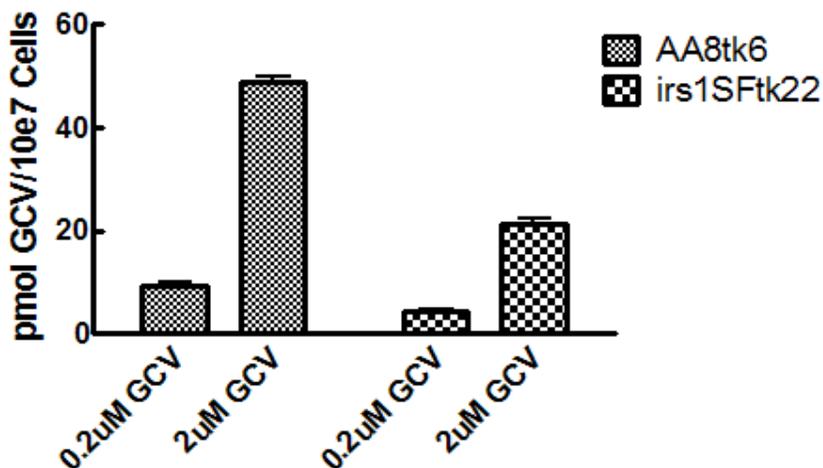
Figure 3.5 GCVMP incorporation into DNA in AA8tk and irs1SFtk cells.
AA8tk or irs1SFtk cells were incubated with either 0.2µM [3H]GCV (IC$_{90}$ irs1SFtk) or 2µM [3H]GCV (IC$_{90}$ AA8) for either 8hr (top) or 16hr (bottom). At the end of drug incubation cells were harvested. Nucleic acids were purified by acid extraction. DNA was pelleted by centrifugation and resuspended. Incorporated [3H]GCVMP was measured by scintillation counting. Columns, mean; bars, SE
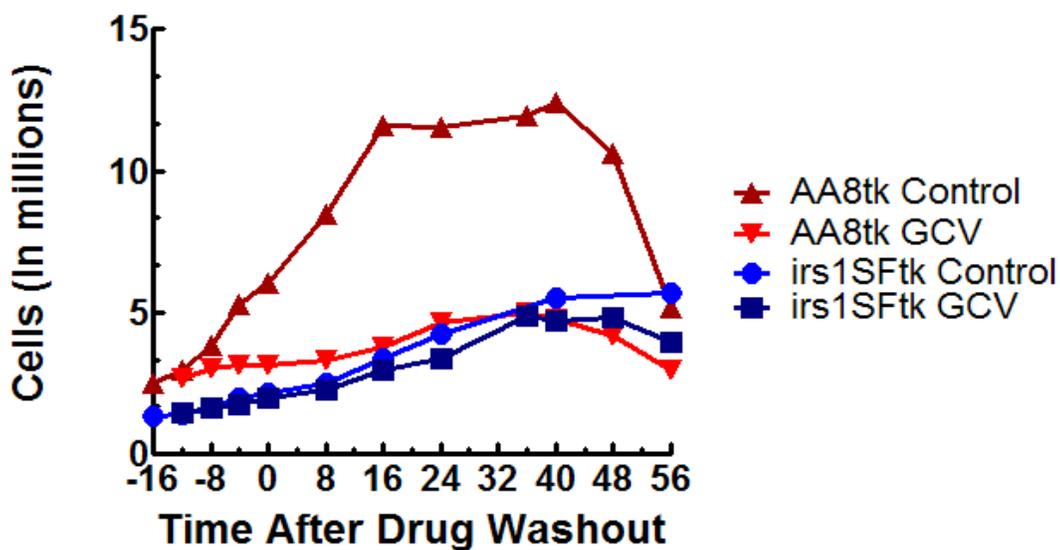
Figure 3.6 Growth inhibition in AA8tk and irs1SFtk cells in response to GCV. Cells were incubated with IC_{90} GCV for 16hr unless otherwise indicated. At the indicated time points cells were collected, counted, and prepared for dual parameter cell cycle analysis as described in materials and methods. Cell growth of a single reproducible experiment are shown.
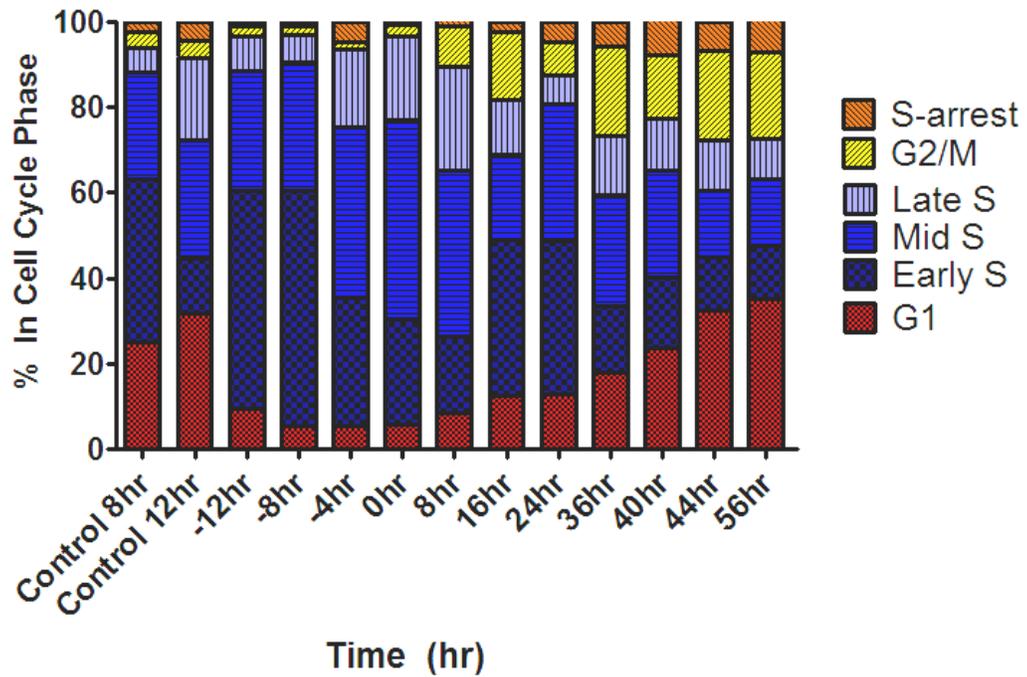
Figure 3.7 Cell cycle effects in AA8tk cells in response to GCV. AA8tk cells were incubated with IC_{90} GCV for 16hr unless otherwise indicated. At the indicated time points cells were incubated with 30 µM BrdU for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdU and DNA content as described in Materials and methods. Results of a single reproducible experiment are shown
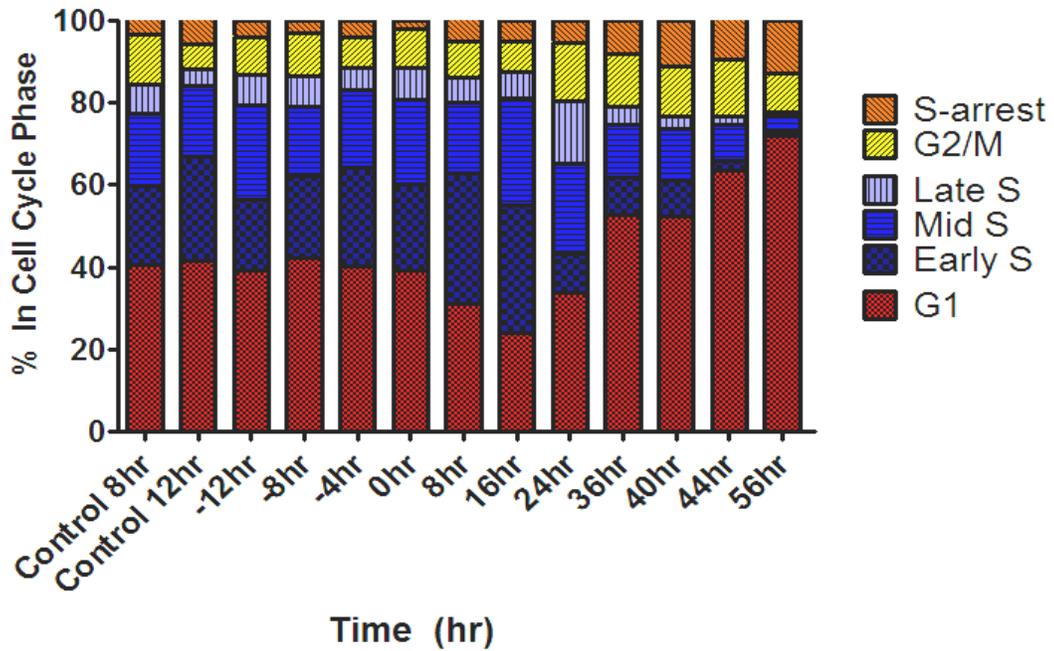
Figure 3.8 Cell cycle effects in irs1SFtk cells in response to GCV. irs1SFtk cells were incubated with IC_{90} GCV for 16hr unless otherwise indicated. At the indicated time points cells were incubated with 30 μM BrdU for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdU and DNA content as described in Materials and methods. Results of a single reproducible experiment are shown
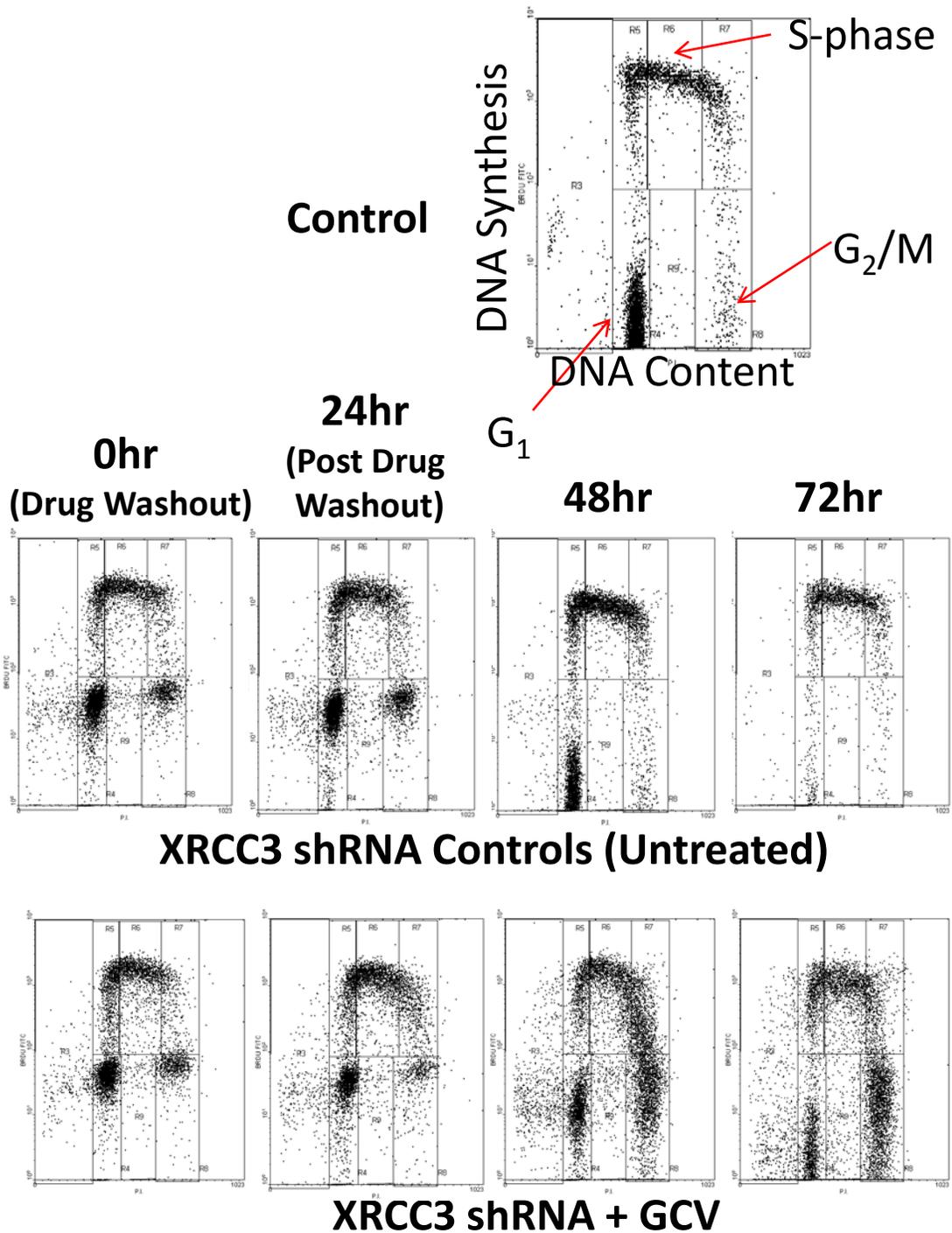
Figure 3.9 Cell cycle effects of XRCC3 depletion in U251tk cells in response to GCV. XRCC3 was depleted using an shRNA delivered by retrovirus in U251tk cells. Puromycin was used to select for virally infected cells. Cells were then replated and incubated with IC_{90} GCV for 24hr. At the indicated time points cells were incubated with 30 μM BrdU for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdU and DNA content as described in Materials and methods. Results of a single reproducible experiment are shown.
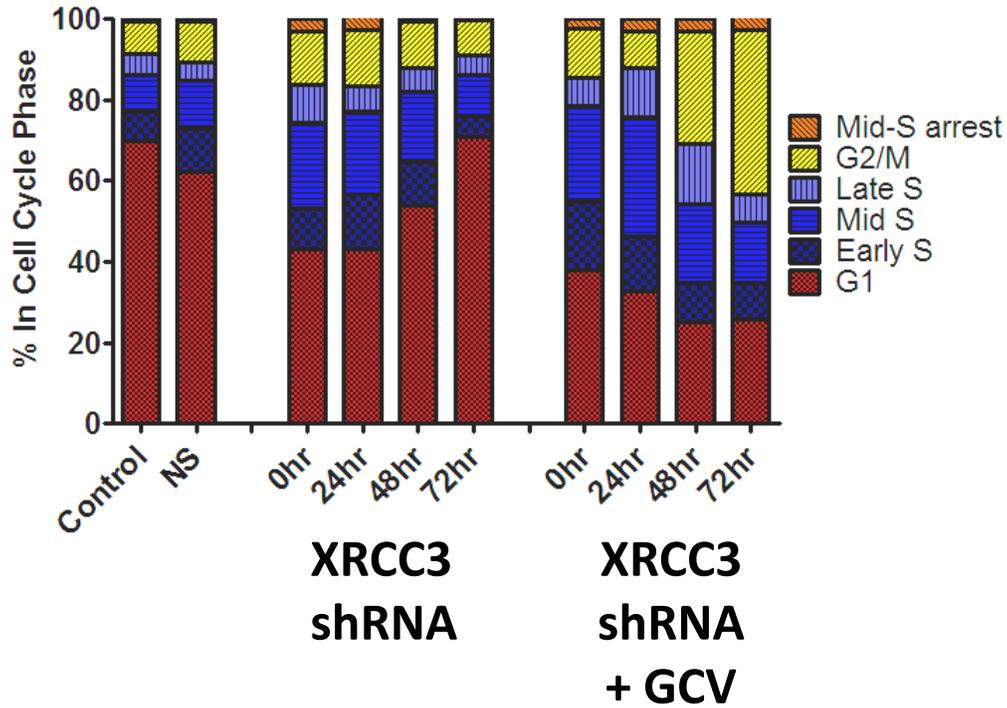
Figure 3.10 Cell cycle effects of XRCC3 depletion in U251tk cells in response to GCV. XRCC3 was depleted using an shRNA delivered by retrovirus in U251tk cells. Puromycin was used to select for virally infected cells. Cells were then replated and incubated with IC_{90} GCV for 24hr. At the indicated time points cells were incubated with 30 µM BrdU for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdU and DNA content as described in Materials and methods. Results of a single reproducible experiment are shown. NS = non-specific shRNA.

10Gy IR Alone

0.1µM GCV for 24hr
10Gy IR 24hr post GCV
washout
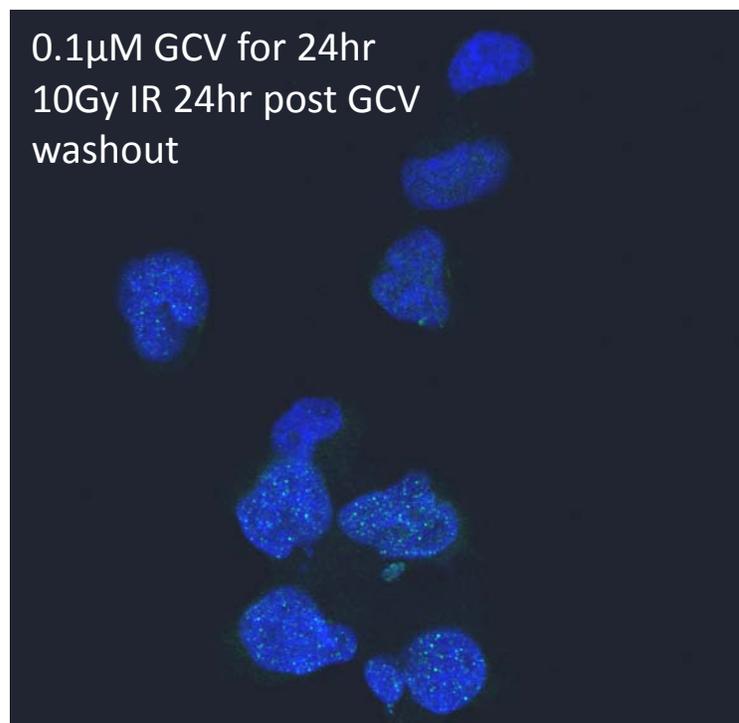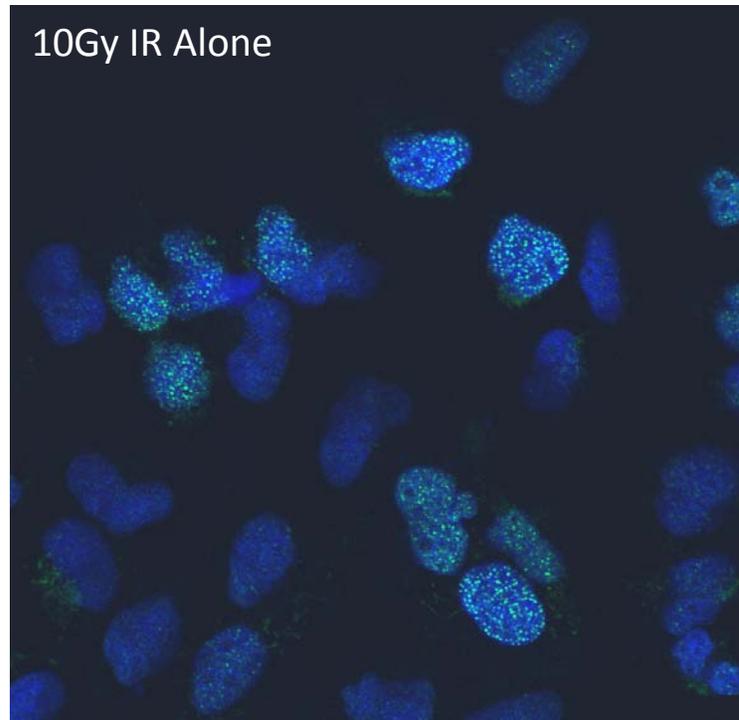
RPA – Green, Dapi - Blue

Figure 3.11 RPA foci formation after IR in cells pretreated with GCV.
U251tk cells were either untreated (Top) or incubated with GCV
(bottom) for 24hr followed by drug removal. 24hr post GCV washout
cells were treated with 10Gy IR and assayed for RPA foci formation.
Representative cells as captured by confocal microscopy.

Group:	Avg. Foci/Cell	+
IR Alone (10Gy)	44.29	12.15
IR+0.1µM GCV	25.88	4.58
IR+1µM GCV	16.45	4.18
IR+10µM GCV	61.21	36.35

Figure 3.12 RPA foci formation after IR in cells pretreated with GCV. U251tk cells were either untreated or incubated with the indicated concentration of GCV for 24hr followed by drug removal. 24hr post GCV washout cells were treated with 10Gy IR and assayed for RPA foci formation. Values represent mean of a single reproducible experiment; + represent standard deviation.
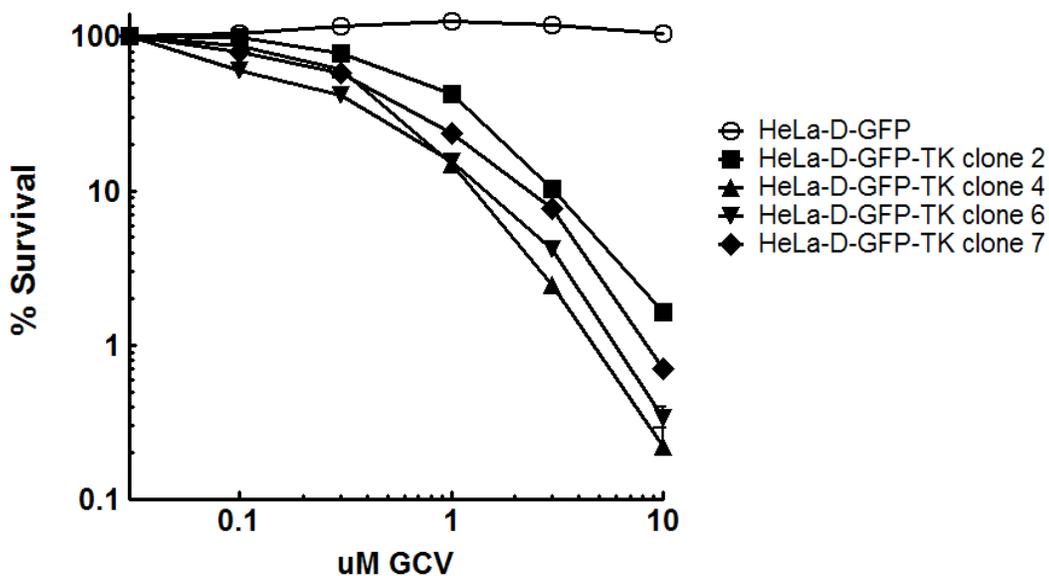
Figure 3.13 Sensitivity of HeLa-D-GFPtk cells to GCV. Exponentially growing HeLa-D-GFP non-HSV-TK-expressing cells (○) and HeLa-D-GFPtk cells stably expressing HSV-TK (♦,■, ▼, ▲) were exposed to increasing concentrations of GCV for 24hr. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error. Cell line HeLa-D-GFP-TK clone 6 was chosen for use in subsequent experiments.
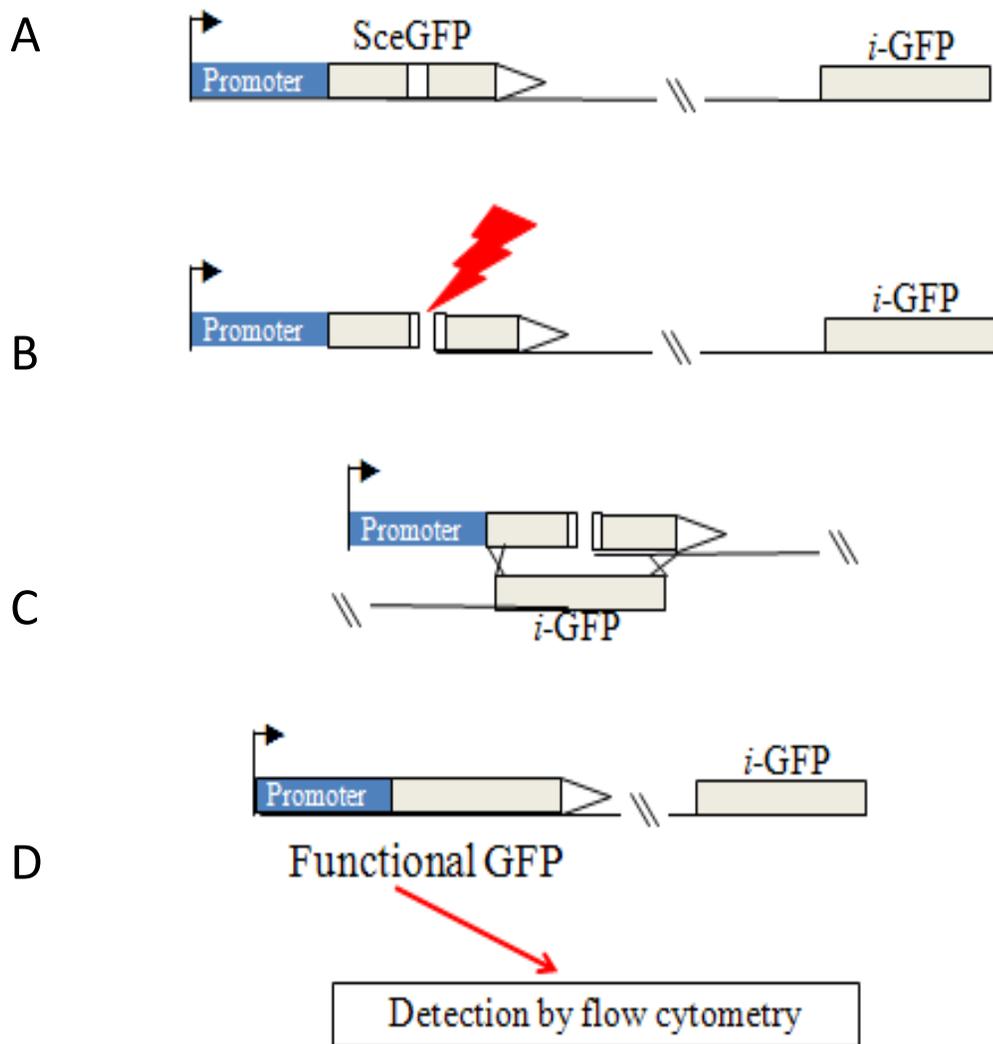
Figure 3.14 Schematic of HR repair of the D-GFP reporter construct. A.) SceGFP contains an insert encoding the recognition site of the I-SceI endonuclease resulting in a non-functional GFP. The i-GFP lacks a promoter and therefore is not expressed. B.) I-SceI site can be cleaved into a double strand break by the I-SceI endonuclease. C.) If repaired by HR, the i-GFP is used to resynthesize the SceGFP. D.)This deletes the insert resulting in a functional GFP which can be detected by flow cytometry
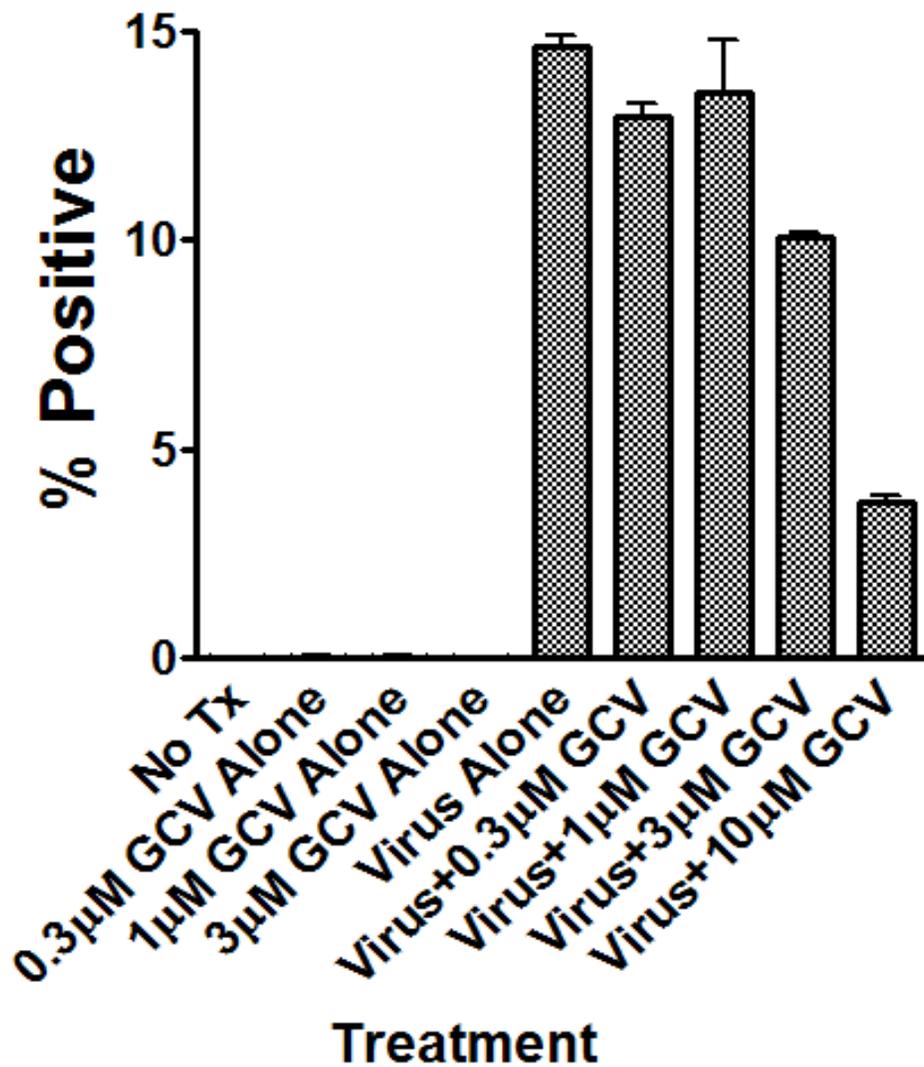
Figure 3.15 GCV inhibits HR repair. Hela-D-GFP-TK cells were incubated in the indicated concentration of GCV either with or without the AdNGUS24i adenovirus containing the cDNA for the I-SceI enzyme. 48hr later, cells were collected, fixed and assessed for GFP expression by flow cytometry. Bars represent a mean of at least three experiments run in triplicate, error bars represent standard error.
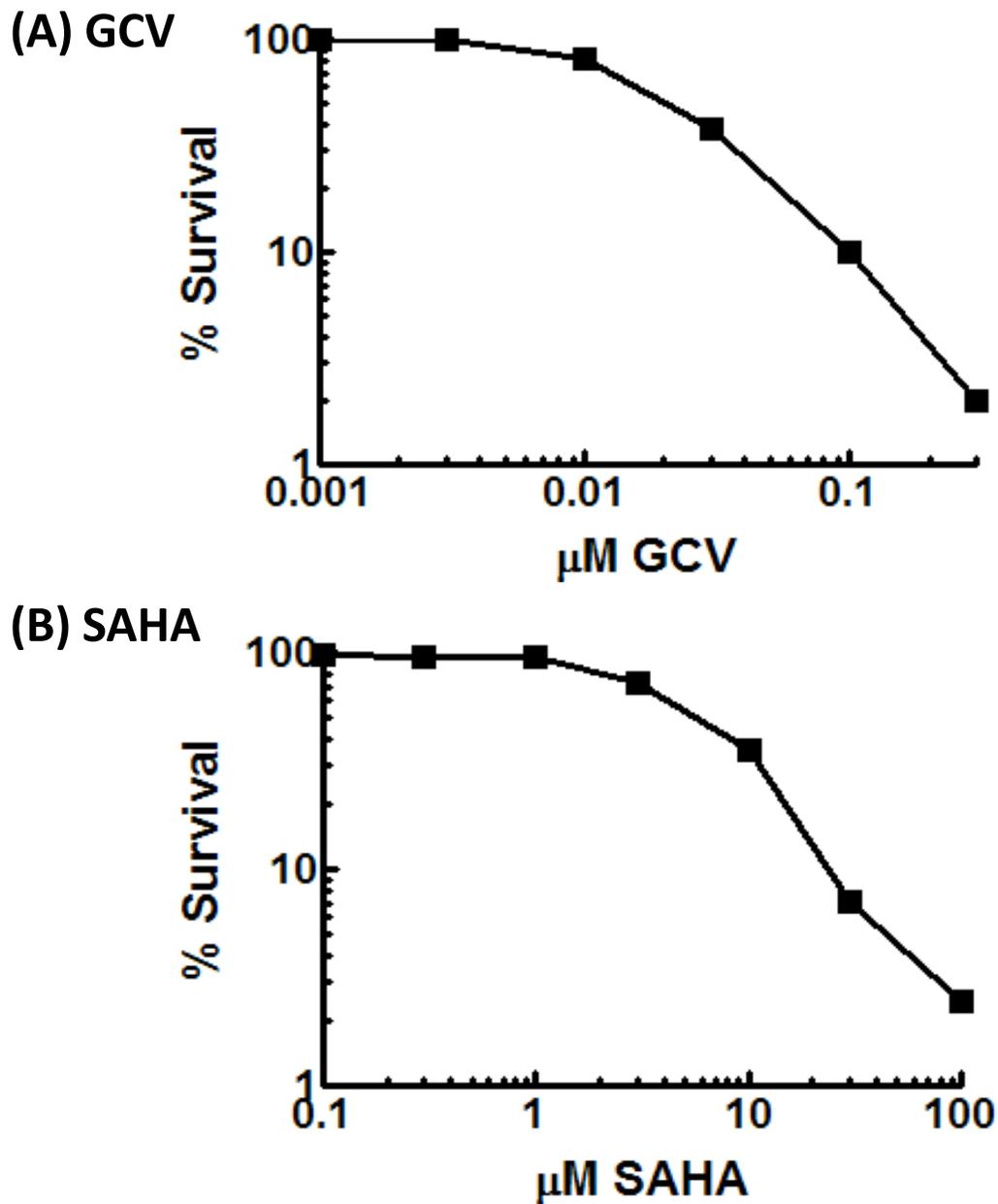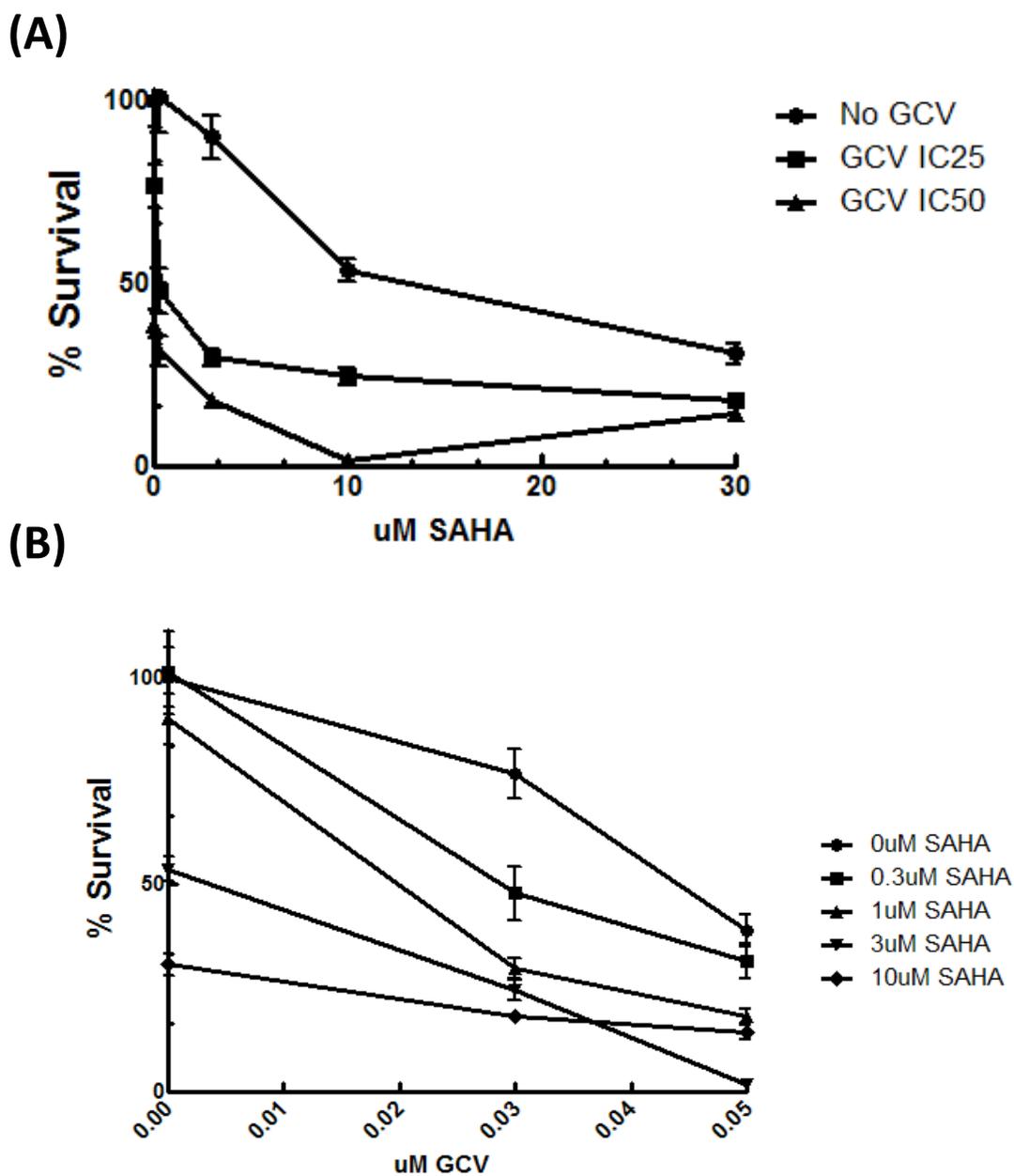
Figure 3.16 Sensitivity of U251tk cells to GCV or SAHA. Exponentially growing U251tk cells were exposed to increasing concentrations of (A) GCV or (B) SAHA for 24hr. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error.
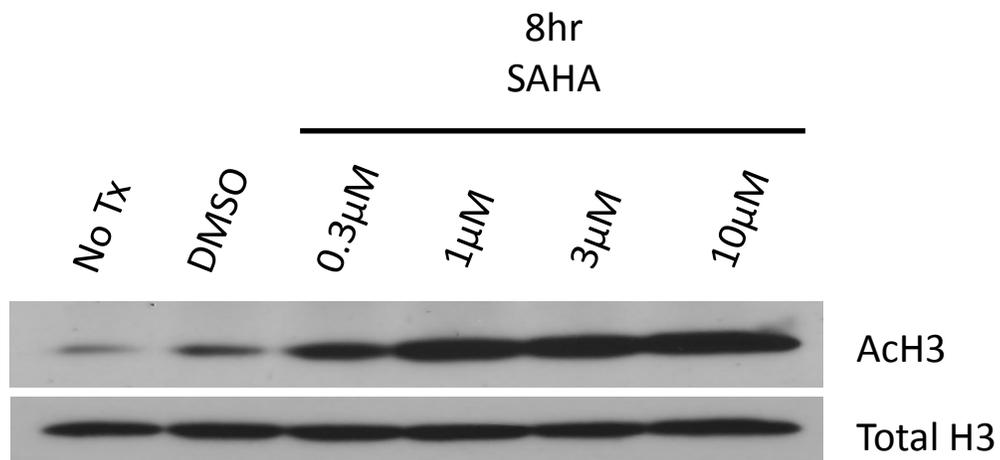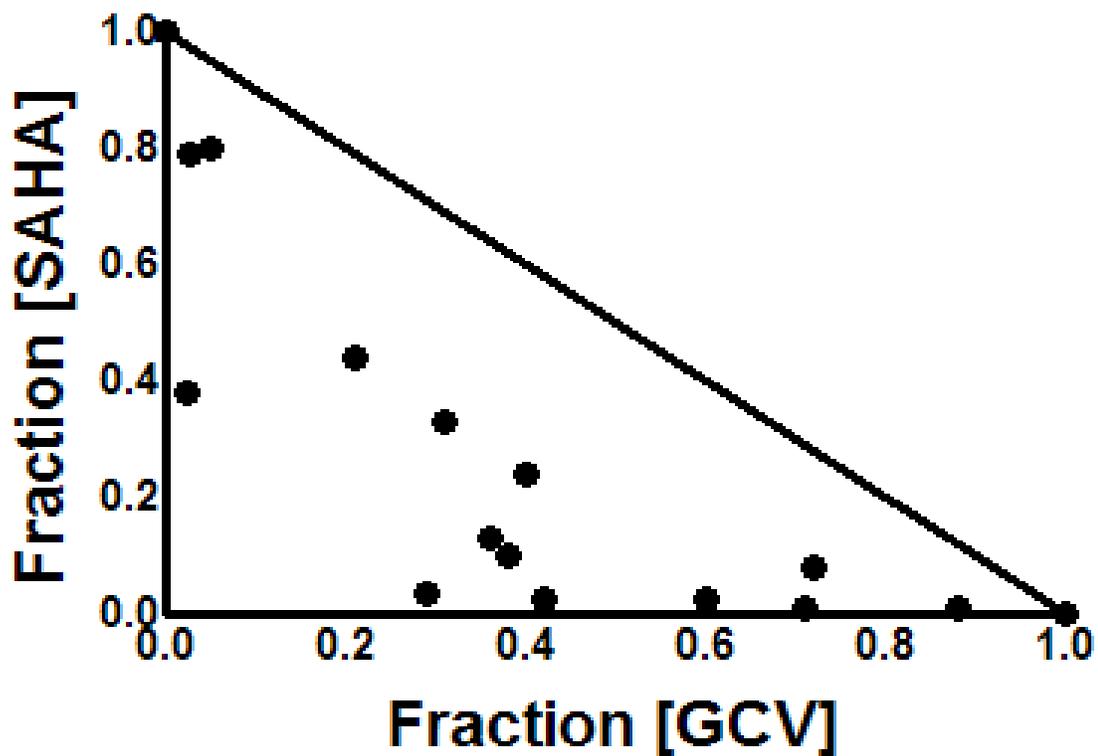
Figure 3.17 Histone 3 lysine 9 acetylation (AceH3K9) in response to SAHA.
U251tk cells were incubated with the indicated concentration of SAHA for 8hr.
AceH3K9 was assayed by western blot. Total H3 was used as a loading control.

(A)

(B)

Figure 3.18 Sensitivity of U251tk cells to GCV and SAHA. (A,B) Exponentially growing U251tk cells were exposed to increasing concentrations of GCV and SAHA for 24hr at the indicated concentrations. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error.

Figure 3.19 Isobologram analysis of GCV and SAHA in U251tk cells. Data from the clonogenic survival curves in Fig. 3.21 were used to generate isobolograms. The concentration of ganciclovir corresponding to IC25, IC50, and IC90 surviving fractions were used alone or in combination with 0.3, 1, 3, and 10µM SAHA. Fractions portray a representative experiment plated in triplicate. Diagonal line, isoeffective line of additivity.
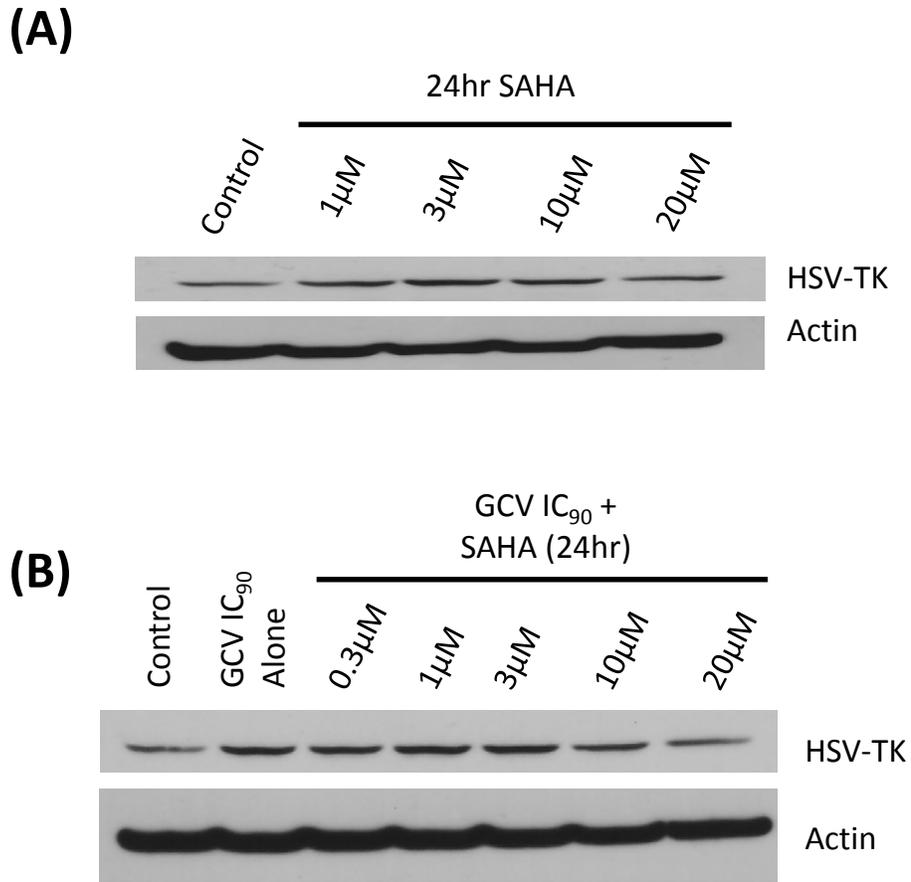
(A)

24hr SAHA

Control 1μM 3μM 10μM 20μM

HSV-TK

Actin

(B)

GCV IC$_{90}$ + SAHA (24hr)

Control GCV IC$_{90}$ Alone 0.3μM 1μM 3μM 10μM 20μM

HSV-TK

Actin

Figure 3.20 HSV-TK expression in response to SAHA. U251tk cells were incubated with the indicated concentration of SAHA alone (A) or SAHA and IC$_{90}$ GCV (B) for 24hr. HSV-TK was assessed by western blot. Actin was used as a loading control.
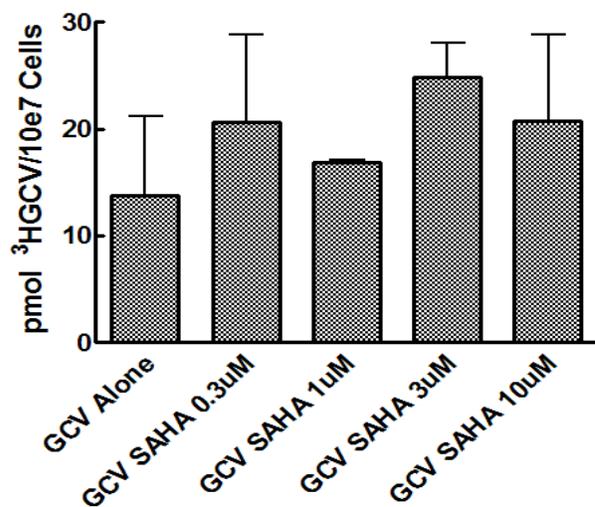
8hr Post Drug Addition
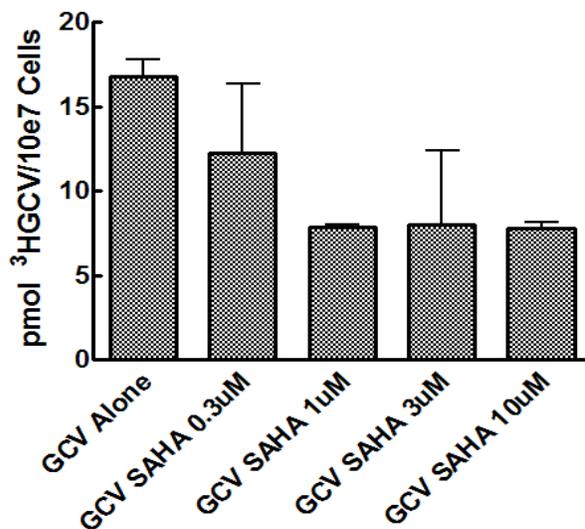
24hr Post Drug Addition

Figure 3.21 Effects of SAHA on GCVTP levels. U251tk cells were incubated with 0.1µM [3H]GCV (IC$_{90}$) for either 8hr (top) or 16hr (bottom). At the end of drug incubation cells were harvested and nucleotides were purified by acid extraction. [3H]GCVTP metabolites were separated by HPLC and quantified by scintillation counting. Columns, mean; bars, SE
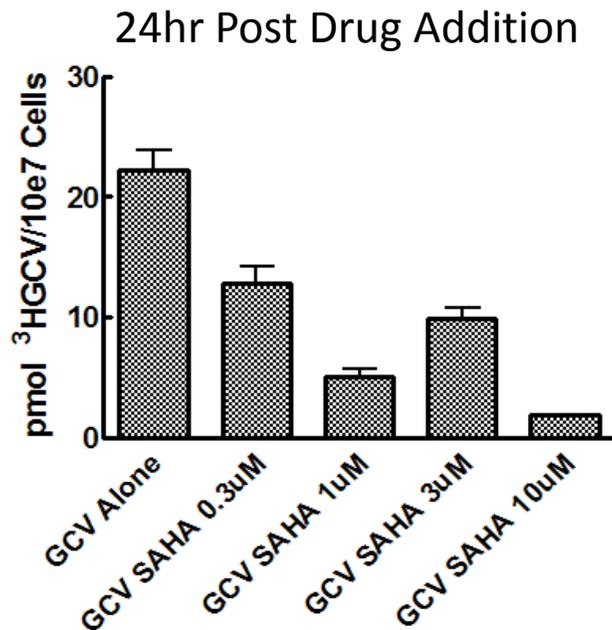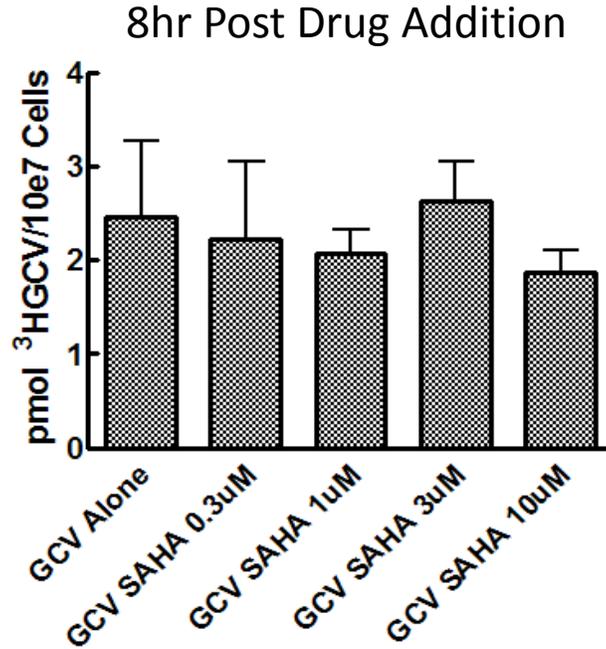
8hr Post Drug Addition

24hr Post Drug Addition

Figure 3.22 GCVMP incorporation into DNA. U251tk cells were incubated with either 0.1µM [3H]GCV (IC$_{90}$) for either 8hr (top) or 16hr (bottom). At the end of drug incubation cells were harvested. Nucleic acids were purified by acid extraction. DNA was pelleted by centrifugation and resuspended. Incorporated [3H]GCVMP was measured by scintillation counting. Columns, mean; bars, SE
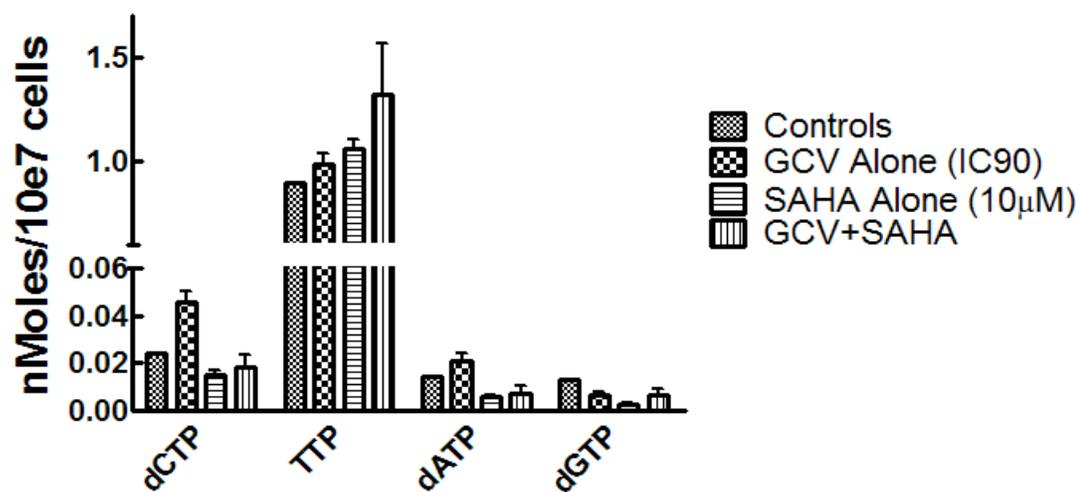
Figure 3.23 Effects of SAHA on endogenous dNTP pools. U251tk cells were incubated with either IC$_{90}$ GCV, 10μM SAHA alone or both IC$_{90}$ GCV and 10μM SAHA for 24h. At the end of drug incubation cells were harvested, nucleotides were purified by acid extraction endogenous dNTP pools were seperated and measured by HPLC. Columns, mean; bars, SE
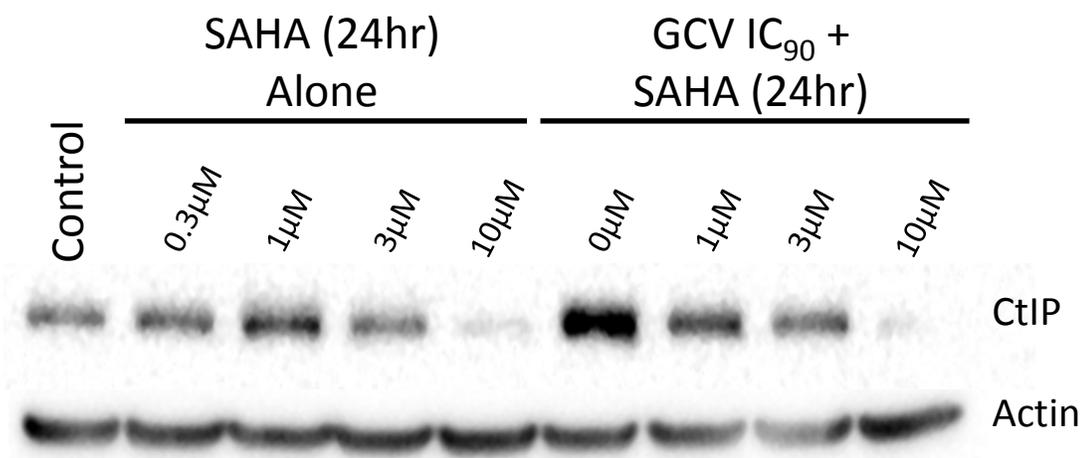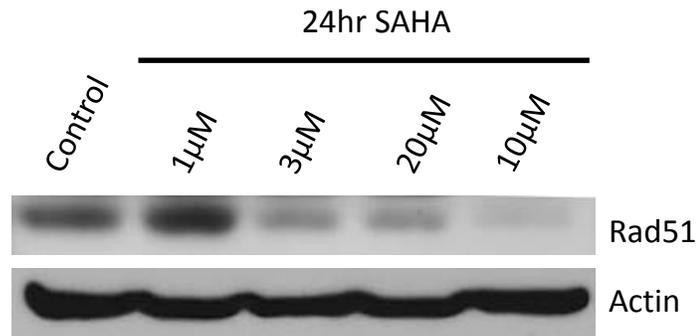
Figure 3.24 CtIP expression in response to SAHA. U251tk cells were incubated with the indicated concentration of SAHA alone (left) or SAHA and IC_{90} GCV (right) for 24hr. CtIP was assessed by western blot. Actin was used as a loading control.

(A)

24hr SAHA

Control 1μM 3μM 20μM 10μM

Rad51

Actin

(B)

GCV IC$_{90}$ +
SAHA
(24hr)

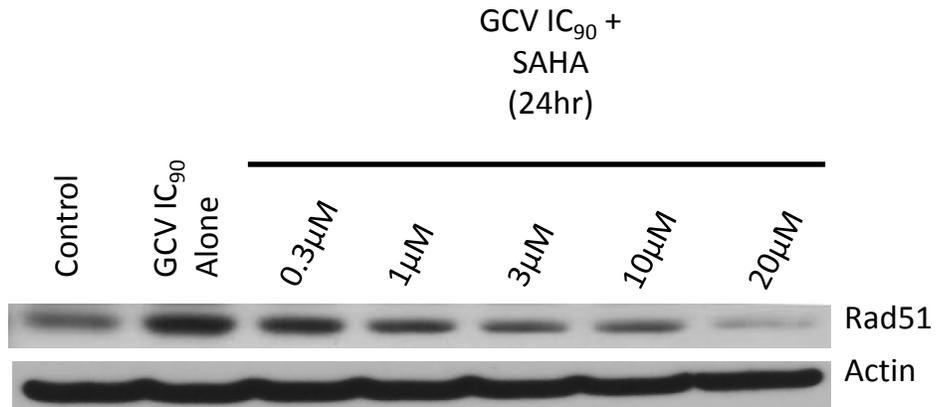Control | GCV IC$_{90}$ Alone | 0.3μM | 1μM | 3μM | 10μM | 20μM

Rad51

Actin

Figure 3.25 Rad51 expression in response to SAHA. U251tk cells were incubated with the indicated concentration of SAHA alone (A) or SAHA and IC$_{90}$ GCV (B) for 24hr. rad51 was assessed by western blot. Actin was used as a loading control.
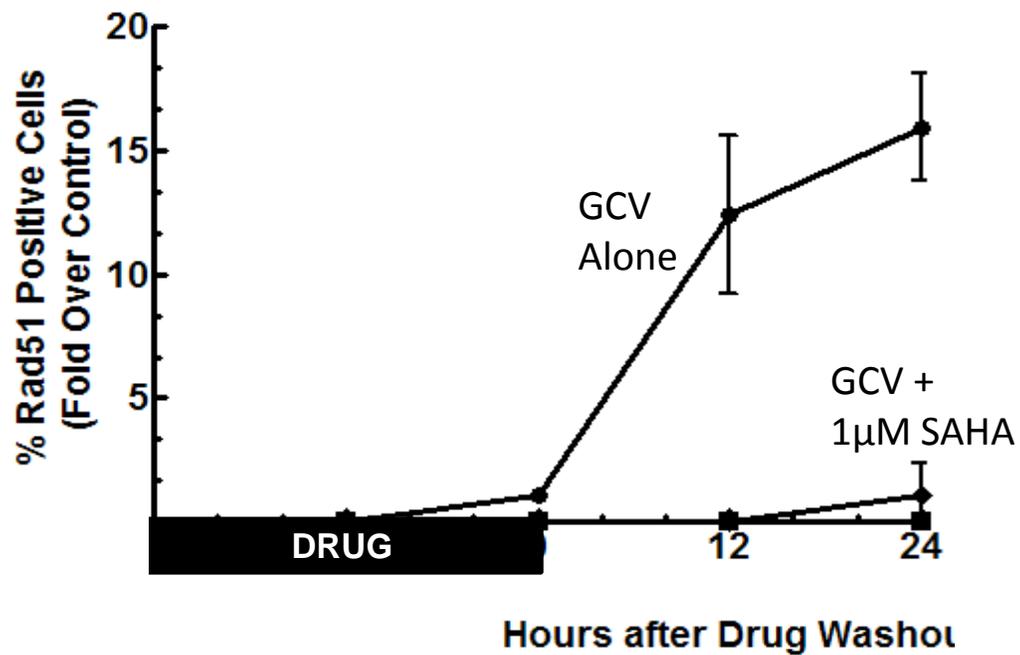
Figure 3.26 SAHA inhibits GCV induced Rad51 foci formation. U251tk cells were incubated with 1, 3, or 10µM of SAHA alone or with an IC_{90} GCV for the indicated times. Cells were assayed by confocal microscopy for Rad51 at the indicated time points (positive cell = $\geq$10 Rad51 foci). Black bar indicates duration of drug incubation, points represent the mean of at least two wells from a representative experiment, error bars represent standard error.
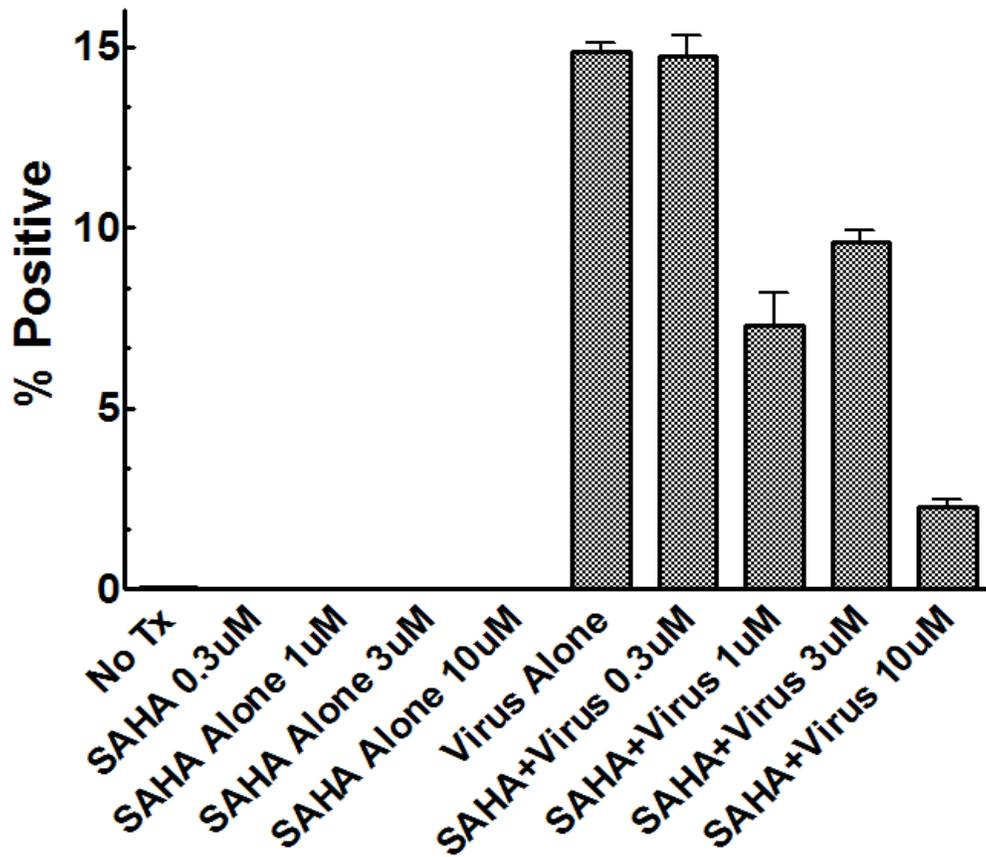
Figure 3.27 SAHA inhibits HR repair. Hela-D-GFP-TK cells were incubated in the indicated concentration of SAHA either with or without the AdNGUS24i adenovirus containing the cDNA for the I-SceI enzyme. 48hr later, cells were collected, fixed and assessed for GFP expression by flow cytometry. Bars represent a mean of at least three experiments run in triplicate, error bars represent standard error.

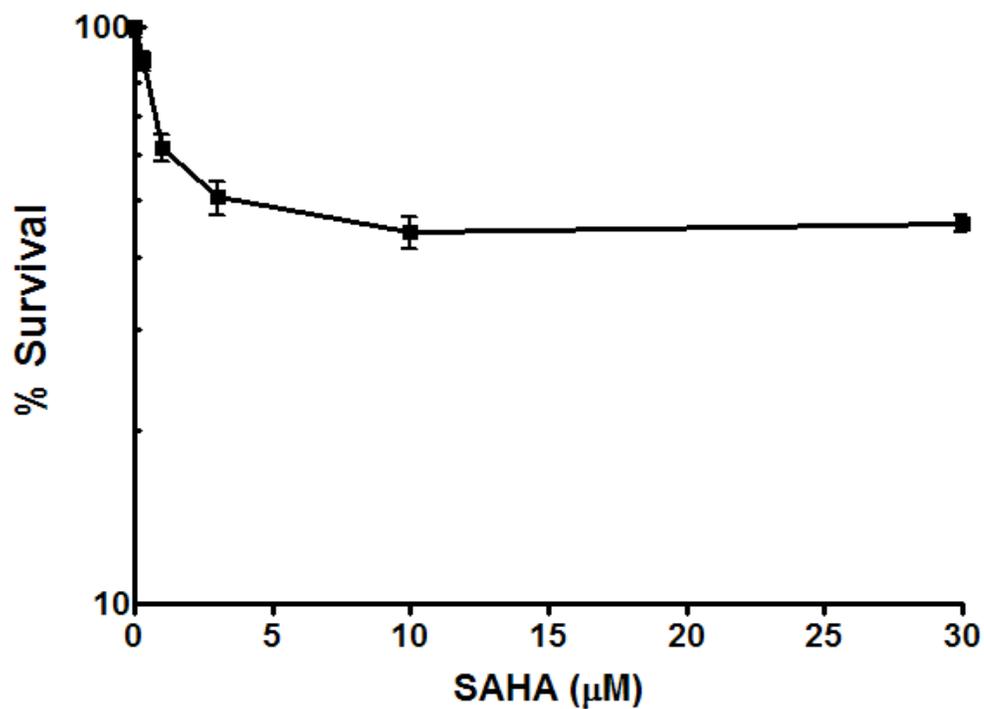**Figure 3.28 Sensitivity of HeLa-D-GFP-TK cells to SAHA.** Exponentially growing HeLa-D-GFPtk cells were exposed to increasing concentrations of SAHA for 24hr. Survival was determined by a clonogenic cell survival assay and expressed as a fraction of plating efficiency for untreated cells. Points represent the mean of at least three wells from a representative experiment, error bars represent standard error.
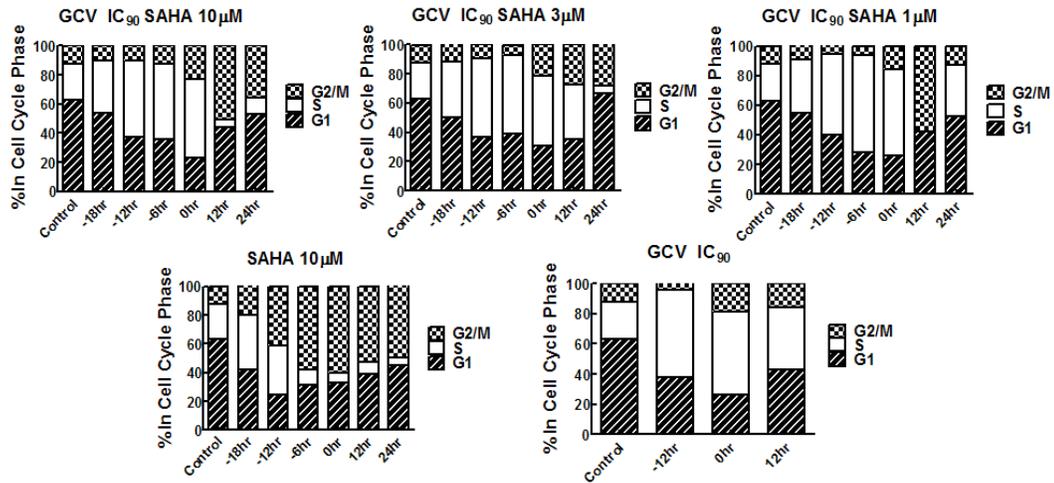
Figure 3.29 Cell cycle effects of GCV and SAHA. U251tk cells were incubated with IC90 GCV and/or the indicated concentrations of SAHA. At the indicated time points cells were collected, fixed, and cell cycle was analyzed by flow cytometry based on DNA content. Columns represent the cell cycle distribution of a representative sample at the indicated time point.
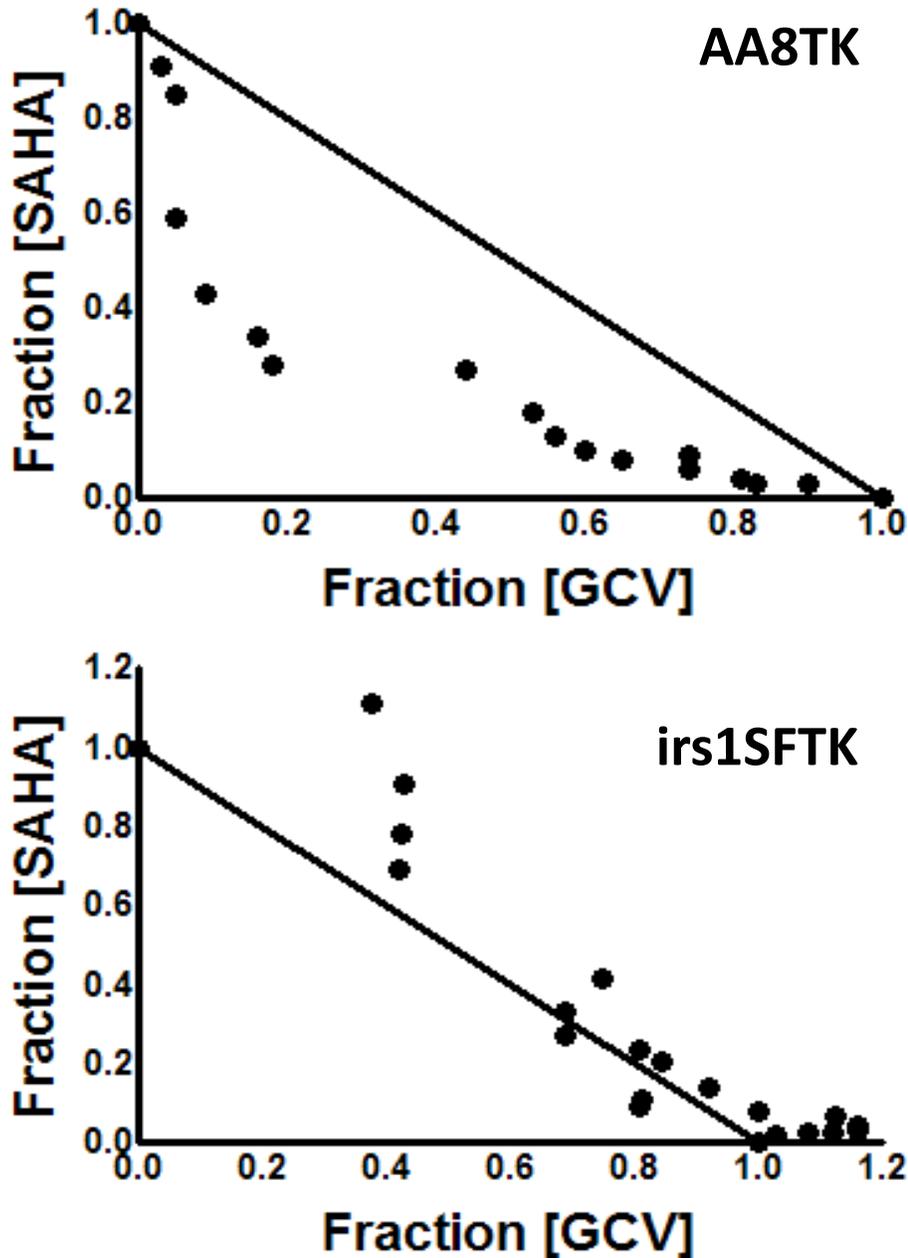
Figure 3.30 Isobologram Analysis of GCV and SAHA in AA8TK or irs1SFTK cells Exponentially growing AA8TK (top) or irs1SFTK (bottom) cells were exposed to increasing concentrations of GCV and/or SAHA for 16hr. Survival was determined by a clonogenic cell survival assay and expressed as a fraction of plating efficiency for untreated cells. Data from the clonogenic survival curves were used to generate isobolograms. The concentration of ganciclovir corresponding to IC_{10}, IC_{25}, IC_{50}, and IC_{90} surviving fractions were used alone or in combination with SAHA (1, 3, and 10, 30µM and 0.3, 1, 3, and 10µM for AA8tk and irs1SFtk, respectively). Fractions portray a representative experiment plated in triplicate. Diagonal line, isoeffective line of additivity.

Reference List

1. Boucher, P. D., Im, M. M., Freytag, S. O., and Shewach, D. S. (2006) *Cancer Res.* **66,** 3230-3237

2. Freytag, S. O., Movsas, B., Aref, I., Stricker, H., Peabody, J., Pegg, J., Zhang, Y., Barton, K. N., Brown, S. L., Lu, M., Savera, A., and Kim, J. H. (2007) *Mol Ther* **15,** 1016-1023

3. Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M., and Kim, J. H. (2003) *Cancer Res* **63,** 7497-7506

4. Freytag, S. O., Khil, M., Stricker, H., Peabody, J., Menon, M., DePeralta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E., and Kim, J. H. (2002) *Cancer Res.* **62,** 4968-4976

5. Freytag, S. O., Stricker, H., Peabody, J., Pegg, J., Paielli, D., Movsas, B., Barton, K. N., Brown, S. L., Lu, M., and Kim, J. H. (2007) *Mol Ther* **15,** 636-642

6. Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. (1998) *Cancer Res* **58,** 3873-3882

7. Tomicic, M. T., Bey, E., Wutzler, P., Thust, R., and Kaina, B. (2002) *Mutat.Res.* **505,** 1-11

8. Ladd, B., O'Konek, J. J., Ostruszka, L. J., and Shewach, D. S. (2011) *Cancer Gene Ther*

9. O'Konek, J. J., Boucher, P. D., Iacco, A. A., Wilson, T. E., and Shewach, D. S. (2009) *Cancer Gene Ther* **16,** 683-692

10. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. (1998) *EMBO J* **17,** 5497-5508

11. Sonoda, E., Sasaki, M. S., Morrison, C., Yamaguchi-Iwai, Y., Takata, M., and Takeda, S. (1999) *Mol.Cell Biol.* **19,** 5166-5169

12. Adimoolam, S., Sirisawad, M., Chen, J., Thiemann, P., Ford, J. M., and Buggy, J. J. (2007) *Proceedings of the National Academy of Sciences* **104,** 19482-19487

13. Chinnaiyan, P., Cerna, D., Burgan, W. E., Beam, K., Williams, E. S., Camphausen, K., and Tofilon, P. J. (2008) *Clin Cancer Res* **14,** 5410-5415

14. Ozaki, K. i., Kishikawa, F., Tanaka, M., Sakamoto, T., Tanimura, S., and Kohno, M. (2008) *Cancer Science* **99,** 376-384

15. Munster, P., Marchion, D., Bicaku, E., Schmitt, M., Lee, J. H., DeConti, R., Simon, G., Fishman, M., Minton, S., Garrett, C., Chiappori, A., Lush, R., Sullivan, D., and Daud, A. (2007) *Journal of Clinical Oncology* **25,** 1979-1985

16. Ramalingam, S. S., Parise, R. A., Ramananthan, R. K., Lagattuta, T. F., Musguire, L. A., Stoller, R. G., Potter, D. M., Argiris, A. E., Zwiebel, J. A., Egorin, M. J., and Belani, C. P. (2007) *Clin Cancer Res* **13,** 3605-3610

17. Ma, X., Ezzeldin, H. H., and Diasio, R. B. (2009) *Drugs* **69,**

18. Pauer, L. R., Olivares, J., Cunningham, C., Williams, A., Grove, W., Kraker, A., Olson, S., and Nemunaitis, J. (2004) *Cancer Invest* **22,** 886-896

19. SUNG, M. W. and WAXMAN, S. A. M. U. (2007) *Anticancer Research* **27,** 995-1001

20. Kothari, V., Joshi, G., Nama, S., Somasundaram, K., and Mulherkar, R. (2010) *Int J Cancer* **126,** 733-742

21. Ammerpohl, O., Thormeyer, D., Khan, Z., Appelskog, I. B., Gojkovic, Z., Almqvist, P. M., and Ekstr÷m, T. J. (2004) *Biochemical and Biophysical Research Communications* **324,** 8-14

22. Lee, J. H., Choy, M. L., Ngo, L., Foster, S. S., and Marks, P. A. (2010) *Proceedings of the National Academy of Sciences* **107,** 14639-14644

23. Flanagan, S. A., Krokosky, C. M., Mannava, S., Nikiforov, M. A., and Shewach, D. S. (2008) *Molecular Pharmacology* **74,** 863-871

24. Black, M. E., Kokoris, M. S., and Sabo, P. (2001) *Cancer Res.* **61,** 3022-3026

25. Leu, K. M., Ostruszka, L. J., Shewach, D., Zalupski, M., Sondak, V., Biermann, J. S., Lee, J. S.-J., Couwlier, C., Palazzolo, K., and Baker, L. H. (2004) *Journal of Clinical Oncology* **22,** 1706-1712

26. Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007) *Nature* **450,** 509-514

27. Weinstock, D. M., Nakanishi, K., Helgadottir, H. R., and Jasin, M. (2006) Assaying
Double[hyphen (true graphic)]Strand Break Repair Pathway Choice in Mammalian
Cells Using a Targeted Endonuclease or the RAG Recombinase. In Judith, L. C.,
editor. *Methods in Enzymology*
DNA Repair, Part B, Academic Press,

28. Robert, T., Vanoli, F., Chiolo, I., Shubassi, G., Bernstein, K. A., Rothstein, R.,
Botrugno, O. A., Parazzoli, D., Oldani, A., Minucci, S., and Foiani, M. (2011) *Nature*
471, 74-79

29. Asklund, T., Appelskog, I. B., Ammerpohl, O., Ekstrom, T. J., and Almqvist, P. M.
(2004) *Eur.J.Cancer* **40,** 1073-1081

Chapter IV

Conclusion

Suicide gene therapy is a selective approach for the treatment of cancer. HSV-TK/GCV suicide gene therapy is one of the most commonly used approaches due to its exquisite selectivity and tumor cell kill. Despite the fact that it has been almost twenty five years since the first publication with HSV-TK/GCV gene therapy, the exact mechanism by which GCV causes cell death is yet to be elucidated. However, it is well accepted that incorporation of GCVMP into DNA is a requirement for GCV to be cytotoxic. The pathways between incorporation and cell death still remain unclear. Elucidating this mechanism may uncover novel pathways important for killing tumor cells as well as identify new mechanism-based drug combinations with HSV-TK/GCV gene therapy. In light of this, the primary objectives of this dissertation were to 1) determine the cellular processes evoked in response to having GCVMP in the DNA template and 2) to pharmacologically exploit these processes to enhance GCV mediated cell killing.

Previous studies have demonstrated that GCV has a unique mechanism of cell killing compared to most nucleoside analogs such as the less cytotoxic compound

araT(1). Most analogs, including araT, inhibit DNA synthesis in the cell cycle after drug addition when being incorporated into nascent DNA resulting in cell death(1). GCV causes cell death in the second cell cycle after drug addition suggesting GCVMP in the template strand of DNA is the cytotoxic lesion in response to GCV.

In addition to a unique cell cycle disruption, previous reports demonstrate that GCV treatment results in sister chromatid exchanges (SCEs) where another HSV-TK substrate, acyclovir, did not. SCEs are thought to be the result of aberrant homologous recombination (HR). Using rad51 foci formation as an indicator of HR, we demonstrate that HR responds to GCV induced DNA damage in the second cell cycle after the addition of GCV when cell death occurs. In chapter III of this dissertation, I demonstrate that HR promotes survival in response to GCV in mammalian cell lines. Based on these observations, one may conclude that, of all HSV-TK substrates, only GCV activates the DNA repair pathway of HR. However, this is not the case as araT also activated HR in response to araT suggesting the activation of HR alone cannot explain the differences in cytotoxicity between GCV and araT. The observation that only GCV induces SCEs yet other analogs activate HR suggests attempting to repair GCVMP in the template results in unique disruption of HR repair.

One possible step of HR repair that could be inhibited by GCVMP in template DNA is the resection step. During DNA repair by HR, DNA is resected in a 5' to 3' direction around the damaged area. This generates a 3' overhang that invades the sister chromatid where it serves as a primer to resynthesize the damaged area preventing any

loss of genetic material. If the damage occurs at a replication fork, such as what might be expected from the DNA polymerase being unable to replicate DNA across from GCVMP, the template strand of DNA is resected so that extension of the nascent strand can continue from the sister chromatid. In the context of HR repair of GCV induced DNA damage, this indicates the resection machinery must remove GCVMP from the template DNA in order to complete repair. Interestingly, genetic studies have demonstrated that compromised DNA resection during HR results in deleterious chromosomal abnormalities such as chromosomal breaks(2), which also occur as a result of GCV treatment(3). Furthermore, previous studies have demonstrated that GCVMP remains in DNA for as long as 96hr after removal of GCV from cells(1). Collectively, these findings support the hypothesis that GCVMP in template DNA may inhibit DNA resection during repair. If this hypothesis is correct, that GCVMP is inhibiting the completion of HR repair, it would suggest that the unique mechanism of GCV cytotoxicity involves damaging DNA and then inhibiting the subsequent repair. This observation could also explain why GCV is more cytotoxic than araT despite lower incorporation of GCVMP into DNA compared to araTMP(1). Furthermore, in chapter II of this dissertation I demonstrate that GCV induces more ATM activation than araT suggesting more DNA double strand breaks (DSBs) are produced with GCV versus araT. Alternatively, the inhibition of resection by GCVMP may result in a persistent number of DNA DSBs that cannot be repaired resulting in persistent activation of ATM. Conversely, araT may induce more lesions than GCV that are repaired quickly allowing DNA replication to continue with subsequent inactivation of the DSB response. This would support the

hypothesis that GCV causes cell death by inducing unrepairable DNA damage whereas araT causes cell death by overwhelming cells with replication stress. Importantly, this suggests that selecting for drugs or drug combinations that induce DNA damage and inhibit the subsequent repair of this damage may be useful in future drug development.

A second possibility of the mechanism by which GCV may cause cell death in the second cell cycle is through the inhibition of the restart of DNA synthesis during HR. After GCV causes stalling of DNA synthesis, HR is used to restart replication from the newly synthesized sister chromatid. Due to the semi-conservative nature of DNA replication, the newly synthesized sister chromatid should not have GCVMP incorporated into DNA. However, GCV treatment results in sister chromatid exchanges(1) suggesting the GCVMP containing DNA template may have been exchanged with the newly synthesized sister chromatid. This gives rise to the possibility that, GCVMP containing sister chromatid is used DNA to restart DNA synthesis which could result in a second stalling of DNA synthesis and ultimately failed HR due to inability to restart DNA replication.

The observation that HR promotes survival after GCV treatment indicates pharmacological inhibition of HR will enhance the cytotoxicity of GCV. Previous studies have demonstrated that compounds which inhibit lysine deacetylase enzymes (KDACs) decrease HR required proteins(4) and block rad51 foci formation after IR in human cell lines(4). Further, KDAC inhibitors have been demonstrated to decrease resection during HR in yeast(5). In chapter III of this dissertation, we demonstrate that the KDAC

inhibitor SAHA synergistically kills HR proficient cells. Further, we demonstrate that SAHA decreases the protein levels of rad51 and decreases the number of HR events in a cell based assay. Strikingly, we observed synergistic tumor cell kill in HR proficient cell lines and only additive cell kill in a matched, HR deficient cell line. Collectively, these data suggest the synergistic cell kill observed with GCV and SAHA is due to SAHA inhibiting the HR mediated DNA repair of GCV induced DNA damage. Interestingly, we observed that SAHA treatment resulted in a decrease in protein levels of CtIP in combination with GCV. CtIP is an exonuclease responsible for resecting DNA during HR. The decrease in CtIP after SAHA treatment indicates both GCV and SAHA may inhibit HR by inhibiting DNA resection. The in vitro data provided in this dissertation warrants further studies to evaluate this novel combination in vivo. Additionally, these data support the hypothesis that selecting for drugs or drug combinations that induce DNA damage and inhibit the subsequent repair of this damage will be useful in future drug development.

HR is a multi-step DNA repair pathway and rationally deciding which step to inhibit may be difficult. The studies in this dissertation suggest that targeting DNA resection during HR may be a novel mechanism for increasing tumor cell kill. Genetic mutations that compromise HR results in increased susceptibility to cancer. Compromising DNA resection during HR results in genomic instability and chromosomal abnormalities(2), both of which occur in response to GCV treatment in HSV-TK expressing cells(3). The advantage to using GCV in a gene therapy strategy for the treatment of cancer is that only the tumor cells are capable of activating GCV to the

cytotoxic form. Therefore, normal tissues are spared not only from cell death but also from deleterious genomic insults that may result in secondary cancers. This suggests that, due to the potential toxicities and risk for secondary cancers, the best approach for exploiting this combination would be transient pharmacological inhibition of DNA repair with localized DNA damage, both of which are provided by HSV-TK/GCV gene therapy in combination with SAHA. Additionally, this has broader implications for other targeted approaches that damage DNA such as IR.

In conclusion, the results presented in this dissertation increase the knowledge of the mechanism of GCV mediated killing of tumor cells and identifies SAHA as a novel compound to enhance HSV-TK/GCV gene therapy. Additionally, the results identify DNA resection during HR as a potential target for future drug development. Finally, these studies demonstrate that investigations into pharmacological mechanisms of existing drugs can provide an opportunity to discover novel drug targets that may lead to new drugs with greater antitumor efficacy.

= GCVMP

1ˢᵗ cell
cycle

Due to semi-conservative DNA replication, GCVMP
is incorporated into one strand of DNA in each
daughter cell

GCV is removed from cells after the first cell
cycle and therefore should not be in newly
synthesized DNA in the second cell cycle

2ⁿᵈ cell
cycle

SCEs , however, result in GCVMP
containing DNA to be in the newly
synthesized sister chromatid

If HR attempts to restart DNA
replication stalling in response to GCV
by using the sister chromatid as a
template and the replication
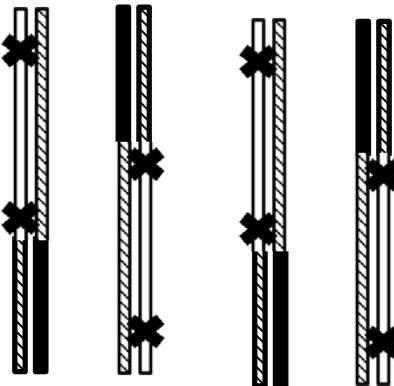machinery encounters another
GCVMP, HR may fail resulting in cell
death

Figure 4.1 GCV potentially inhibits DNA replication restart during HR. Schematic of a possible mechanism of GCV mediated cell death. If HR occurs in response to GCVMP in the DNA template downstream of a SCE, HR may encounter another GCVMP in the DNA template of the sister chromatid resulting in failed HR and cell death.

Reference List

1. Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. (1998) *Cancer Res* **58,** 3873-3882

2. Eid, W., Steger, M., El Shemerly, M., Ferretti, L. P., Pena-Diaz, J., Konig, C., Valtorta, E., Sartori, A. A., and Ferrari, S. (2010) *EMBO Rep.* **11,** 962-968

3. Tomicic, M. T., Bey, E., Wutzler, P., Thust, R., and Kaina, B. (2002) *Mutat.Res.* **505,** 1-11

4. Adimoolam, S., Sirisawad, M., Chen, J., Thiemann, P., Ford, J. M., and Buggy, J. J. (2007) *Proceedings of the National Academy of Sciences* **104,** 19482-19487

5. Robert, T., Vanoli, F., Chiolo, I., Shubassi, G., Bernstein, K. A., Rothstein, R., Botrugno, O. A., Parazzoli, D., Oldani, A., Minucci, S., and Foiani, M. (2011) *Nature* **471,** 74-79