THE ROLE OF DNA DAMAGE AND REPAIR IN GANCICLOVIR-MEDIATED CYTOTOXICITY

by

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To Nicholas,
for all his love and support

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ABSTRACT

Suicide gene therapy aims to improve selectivity of cancer treatment through expression of herpes simplex virus thymidine kinase (HSV-TK) in tumor cells, permitting phosphorylation of HSV-TK substrates with subsequent cytotoxic incorporation into DNA exclusively within the tumor. This approach results in significantly greater cytotoxicity to cancer cells in a novel delayed manner when applied with the antiviral drug GCV as compared to other HSV-TK substrates. To elucidate the mechanism for the unique cytotoxicity, DNA damage and the repair pathways involved in responding to this damage induced by GCV compared to related analogs were evaluated.

Using phosphorylated histone H2AX (γ-H2AX) as a marker of DNA damage, GCV induced >7-fold more damage than a different HSV-TK substrate, 1-β-D-arabinofuranosylthymine (araT), at equitoxic concentrations. Although the number of γ-H2AX foci decreased after removal of either drug, suggesting repair of these early lesions, only GCV produced a late and persistent increase in DNA damage indicating the induction of irreparable DNA damage. Rad51 foci formed primarily following the late increase in γ-H2AX foci, exposure, implicating homologous recombination repair (HRR) in responding to GCV-induced lesions.

A yeast-based screen of DNA damage response mutants was utilized to detect pathways nvolved in cytotoxicity with GCV. Yeast deficient in HRR, cell cycle checkpoint, and mismatch repair (MMR) proteins all exhibited increased sensitivity to

GCV. Survival studies in human cells confirmed greater GCV sensitivity in MMR deficient cells, primarily at high concentrations, validating the yeast assay. Thus, MMR and HRR may prevent cytotoxicity with ganciclovir.

Previous reports suggested that GCV is genotoxic. DNA mutations induced by GCV were characterized and compared to those resulting from treatment with two other structurally similar HSV-TK substrates, D-carbocyclic 2'-deoxyguanosine (CdG) and penciclovir (PCV). GCV induced a dose-dependent increase in mutation frequency, while highly cytotoxic concentrations of CdG and PCV failed to increase mutations. GCV predominantly induced GC→TA transversions which were significantly less frequent in control cells or those treated with PCV or CdG. Analysis of cell cycle progression revealed different mechanisms of cell cycle arrest for each of these drugs. Thus, alteration of the deoxyribose structure produced profound differences in DNA replication and its fidelity, resulting in striking differences in cytotoxicity. These data demonstrate that GCV induces significantly more DNA damage, which may not be repaired effectively by HRR and MMR, leading to multi-log cytotoxicity through mechanisms distinct from other structurally related HSV-TK substrates.

Chapter I

INTRODUCTION

Ganciclovir (GCV) is an anti-viral agent used clinically for herpesvirus infections that is also employed in a suicide gene therapy strategy for cancer. Compared to other structurally related compounds, GCV has a unique ability to induce multi-log cytotoxicity in human tumor cells *in vitro* at submicromolar doses. Although previous studies have contributed to the understanding of the mechanism by which GCV elicits cell killing, the mechanism of GCV's superior cytotoxicity has yet to be determined. The data presented here provide further insights into the mechanism of GCV-mediated cytotoxicity by characterizing the induction of DNA damage by GCV and repair pathways involved in responding to this damage.

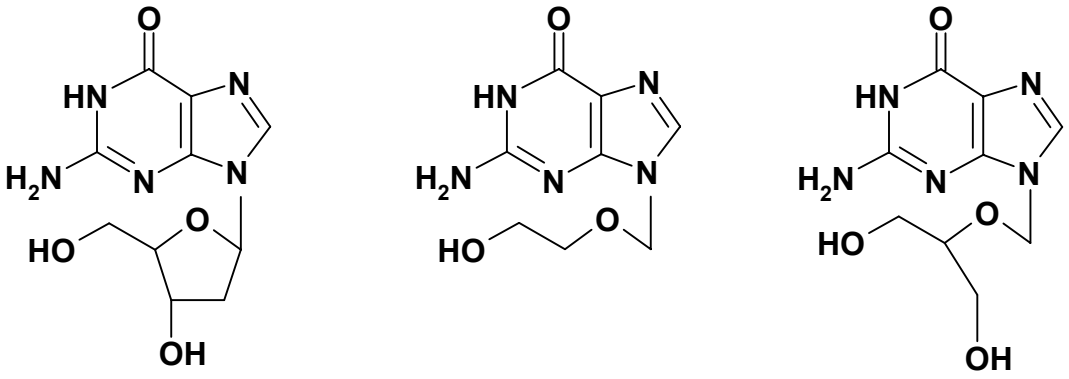
Ganciclovir as an Antiviral Agent

The herpesvirus family consists of eight members which infect humans and cause a variety of illnesses ranging from cold sores to chickenpox to encephalitis (1-3). Herpesvirus infected cells express a virally encoded thymidine kinase which can accelerate viral replication by maintaining high levels of phosphorylated thymidine metabolites which are necessary for replication (4). Compared to mammalian

thymidine kinase, herpes thymidine kinases have a broader range of substrate specificity, and can phosphorylate a variety of nucleoside analogs in addition to thymidine.

In 1977, vidarabine (9-β-D-ribofuranosyladenine, araA) was the first antiherpesvirus agent approved for systemic use, but high toxicities induced by the drug limited its use to life-threatening infections (5,6). Antiviral research seeking to develop new anti-herpetic drugs with low toxicity to host cells led to the discovery of a group of acyclic nucleoside analogs which are specifically phosphorylated by the viral thymidine kinase (7). An important drug discovered from this study, acyclovir (9-[2-hydroxyethoxymethyl]guanine; ACV), was the first truly selective drug with clinical activity against herpesvirus infections (8) and is currently in use today, where it is the drug of choice for a number of herpes infections (9). ACV is an analogue of 2'-deoxyguanosine with an acyclic sugar, lacking a 2'- and 3'-carbon and 3'-hydroxyl (Figure 1.1). A compound developed a few years after acyclovir to more closely resemble deoxyguanosine, ganciclovir (9-[(1,2-dihydroxy-2-propoxymethyl)-guanine], GCV), is similar in structure to ACV with the addition of a hydroxymethyl group at the 3′ position. This addition of a 3' carbon allows GCV to be internally incorporated into DNA (10,11), unlike ACV which is an obligate chain terminator (12).

The favorable therapeutic index for ACV and GCV is based on their selective activation in herpesvirus-infected cells, as these drugs undergo their requisite phosphorylation only in virally-infected cells expressing herpes simplex virus thymidine kinase (HSV-TK). GCV is a better substrate than acyclovir for HSV-TK (K_m= 66 μM and 426 μM, respectively) (13); however they are poorer substrates than thymidine (K_m= 0.2 μM). HSV-TK phosphorylates GCV to its 5'-monophosphate (GCVMP) (4,13,14).



2'deoxyguanosine **Acyclovir** **Ganciclovir**

Figure 1.1 Structure of 2'deoxyguanosine, acyclovir, and ganciclovir.

Subsequent phosphorylation by the cellular enzymes guanylate kinase and nucleoside diphosphokinase lead to the accumulation of GCV 5'-triphosphate (GCVTP) (10,14-17). GCVTP inhibits viral replication by interfering with viral DNA synthesis but unphosphorylated GCV does not interrupt DNA synthesis, thus uninfected cells which do not contain GCVTP are not affected (18). *In vitro*, GCV exhibits excellent antiviral activity against herpes simplex virus type 1 and 2 (13,19), human herpesvirus-6 (20,21), varicella-zoster virus (13), and Epstein-Barr virus (13,22) and is more potent than acyclovir against these viruses (10,23).

In 1988, GCV was the first antiviral drug to be approved for the treatment of cytomegalovirus (CMV) infections in humans (24). CMV lacks thymidine kinase, but phosphorylation to GCVMP is mediated by a protein kinase encoded by the CMV UL97 gene (25,26). Because ACV is poorly phosphorylated by UL97 kinase, CMV is 100-fold less sensitive to ACV than herpes simplex virus, and it is not possible to reach sufficient plasma concentrations required for therapeutic activity (27). While CMV is usually an innocuous infection in immunocompetent patients, CMV infections can result in vision loss or fatality in patients with compromised immune systems, such as transplant recipients (reviewed in (28,29)) and those with AIDS (30). Prophylaxis with anti-viral drugs, such as ACV and GCV, is commonly used in solid organ transplant patients to prevent CMV infections and the associated clinical syndrome. Without prophylaxis with GCV, evidence of CMV infections occurred in more than 50% of recipients of solid organ transplants (31) and 7-32% presented with significant CMV disease (32). GCV prophylaxis has reduced both the relative risk of CMV disease and mortality following organ transplant by 60% and 40%, respectively (reviewed in (33,34)).

Pharmacokinetics of GCV

Oral bioavailablity of GCV is poor, and thus it is usually administered by intravenous infusion (35). GCV has been given orally, although the very high doses required to achieve effective plasma concentrations has made this route of administration unattractive. The L-valyl ester prodrug of GCV, valganciclovir is transported into the bloodstream through peptide transporters PEPT1 and PEPT2 where it is rapidly and completely hydrolyzed to GCV by liver and intestinal esterases (36). Valganciclovir is significantly more bioavailable (60.9% versus 5.6% for oral ganciclovir) (37-39) with no new toxicities occurring following treatment with the prodrug. Clinically, plasma concentrations of 10-30 μM GCV have been reported (40-42). GCV concentrations in cerebrospinal fluid were 31 to 67% of plasma concentrations, and intraocular concentrations were similar to or higher than plasma levels. The primary route of elimination of GCV is through the kidney where almost 100% is excreted mainly as unchanged drug in the urine, with a terminal half-life of 4.5 hours (35).

Effective inhibitory concentrations of GCV for the herpes viruses range from 0.2 to 6.0 μM while much higher concentrations are required to cause toxicity to most normal host tissues (41). The notable exception is bone marrow cells, which are sensitive to concentrations of GCV similar to virus-infected cells, resulting in neutropenia in 15 - 40% of patients and thrombocytopenia in 5 - 20% (41,43). In addition to myelosuppression, CNS side effects ranging from headache to convulsions to coma occur in 5-15% of patients. Conversely, clinically relevant doses of ACV do not produce myelosuppression and, unlike GCV, ACV is generally well-tolerated with minimal and infrequently reported side effects, such as nausea, diarrhea, headache, or more

infrequently renal dysfunction (44). Because GCV has a much higher risk of causing dose-limiting bone marrow toxicity, it is typically reserved for treating life or sight threatening CMV infections (35,41).

Cancer Gene Therapy

Many traditional chemotherapeutic drugs to treat cancer have limited ability to distinguish cancerous cells from normal host cells. These therapies target cells based on their ability to proliferate, which does not allow distinction between neoplastic cells and many normal tissues in which cells are actively proliferating (bone marrow, gastrointestinal tract). This results in many deleterious effects associated with such treatments, such as bone marrow suppression, gastrointestinal toxicity, and neuropathy, which are responsible for significant morbidity and mortality and a very narrow therapeutic index. This limitation has led to the search for new therapies which increase the therapeutic index by selectively targeting tumor cells while sparing normal tissue from damage.

To improve the selectivity of cancer treatment, many cancer gene therapy strategies have been developed. Examples include transduction of drug-resistance genes into bone marrow stem cells to protect them from chemotherapy (45-49), immunotherapy using recombinant DNA tumor cell vaccines (50-54), tumor suppressor gene replacement (55,56), and inactivation of oncogenes (57,58). In suicide gene therapy, which are the most frequently studied strategies, tumor cells are engineered to express a foreign gene which encodes an enzyme that can convert a non-toxic pro-drug into a cytotoxic metabolite. Since the drugs used in these therapies have lower toxicity than traditional

chemotherapeutics, they are selective for the virally-transduced tumor cells while sparing host tissues. This allows the patient to be treated with higher doses of the agent to achieve tumor regression while limiting the side effects which normally occur in patients undergoing cancer chemotherapy. Some examples of enzyme/pro-drug gene therapy include cytosine deaminase/5-fluorocytosine (59-61), nitroreductase/CB1954 (59), purine nucleoside phosphorylase/6-methyl-purine-2'-deoxynucleoside (62), and herpes simplex virus-thymidine kinase/GCV.

Herpes Simplex Virus Type-1 Thymidine Kinase/Ganciclovir Gene Therapy

Perhaps the most widely studied suicide gene therapy strategy for cancer treatment utilizes the herpes simplex virus type 1 thymidine kinase (HSV-TK) with GCV. This strategy was first described by Frederic Moolten in 1986 and 1987 (63,64), and in 1990 initial studies of this gene therapy approach were reported (65,66). Studies have since progressed from *in vitro* evaluation to animal models and ultimately to many clinical trials.

GCV is transported into mammalian cells via the nucleoside transporter and purine nucleobase transporter in erythrocytes (67-70). Once inside HSV-TK-expressing cells, GCV is phosphorylated to GCVMP by HSV-TK (13). The subsequent phosphorylation events to GCV diphosphate (GCVDP) and GCVTP are performed by mammalian guanylate kinase and nucleoside diphosphokinase, respectively (10,14-17). GCVTP competes with dGTP for incorporation into DNA, and this incorporation leads to cell death (71).

An important feature of this therapy is that HSV-TK-expressing cells can induce GCV-mediated cytotoxicity in neighboring tumor cells that do not express the transgene. This phenomenon, known as the bystander effect, has been observed both *in vitro* and *in vivo* (72-75). Because currently it is only possible to transduce less than 1% of cells in patient tumors (76), the bystander effect is critical for the effectiveness of this approach.

Much research has focused on understanding the mechanism(s) of the bystander effect. Freeman et al performed co-culture experiment in which HSV-TK-expressing and non-HSV-TK expressing cells were cultured in a dish with two chambers separated by a membrane, so that they shared the same media, but did not have physical contact. Following exposure to GCV, the non-transduced cells did not die. However, when the same experiment allowed for contact between the HSV-TK expressing and non-HSV-TK expressing cells, it resulted in death of the non-transduced cells (77). Another study demonstrated that exposing non-HSV-TK expressing cells to conditioned media from cells expressing HSV-TK and treated with [^{3}H]GCV did not result in transfer of radioactivity or cytotoxicity to the non-HSV-TK expressing cells (78). These studies suggest that cell-cell contact and transfer of a GCV metabolic product is required for the bystander effect.

Phosphorylated GCV metabolites cannot freely diffuse through the cell membrane, however they can transfer between cells through gap junctions which are intercellular channels composed of two transmembrane hemichannels of connexin protein. When these channels from neighboring cells dock together, they form a central pore, allowing for the transfer of various molecules ≤1 kDa in molecular weight [reviewed in (79)]. The transfer of GCV phosphates between cells allows for

neighboring, non-transduced cells to accumulate the active GCV metabolite, GCVTP, and ultimately die. Although it has been reported that cells with good gap junction intracellular communication exhibit good bystander cytotoxicity (73,74,80,81), and cell lines that reportedly lack gap junctions do not exhibit bystander killing (82-85), bystander cytotoxicity has been observed in cells which do not express known connexin proteins, such as SW620 colon carcinoma cells (75). In HeLa cells, which also lack expression of known connexin proteins, GCV phosphates were transferred between cells via a mechanism that appeared to require cell contact. It was suggested that cells may express a low level of gap junctional intercellular communication that mediates transfer of small amounts of GCV phosphates to bystander cells (85).

The bystander effect also has been explained by other mechanisms. One hypothesis suggests that apoptotic vesicles generated during the death of HSV-TK expressing cells contain GCV metabolites and are phagocytosed by non-HSV-TK expressing cells (77). It has also been proposed that multidrug resistance proteins, MRP-4 and MRP-5, which can efflux phosphorylated nucleotides (86,87), might also efflux phosphorylated GCV metabolites (88), however this would require a mechanism able to transport phosphorylated compounds into cells. The wealth of data demonstrating that cell-to-cell contact is required for the bystander effect suggest that gap junctions are the most likely candidates responsible for the bystander effect.

Strategies to Improve HSV-TK/GCV Suicide Gene Therapy

Because current gene transfer efficiencies are inadequate for treatment with HSV-TK/GCV, many laboratories have focused on strategies to improve viral transduction by

modifying viral vectors and vector delivery (89-92). HSV-TK mutants have been engineered to increase specificity for GCV, such that tumor growth inhibition or regression can be achieved with lower doses of GCV. HSV-TK has a high affinity for thymidine (Km = 0.5μM) (93), while affinity for GCV is significantly lower (Km = 45 μM) (13,94). Black et al identified HSV-TK mutants with improved selectivity for GCV through random mutagenesis of the cDNA with selection in *E. coli*. When expressed in mammalian cells, these HSV-TK mutants enhanced sensitivity to GCV by increasing GCV phosphorylation and decreasing sensitivity of the enzyme to thymidine (95). One of the mutants (SR39) had an 83-fold higher specificity for GCV compared to thymidine, resulting in a 294-fold decrease in the IC_{50} for GCV (96). Subsequent work demonstrated that these mutants increased GCV-mediated cell killing in mammalian cells and tumor models (97-100). Mutants with increased specificity for GCV should allow for the administration of lower concentrations of GCV to achieve sufficient GCVTP levels in HSV-TK expressing tumor cells. This will not only allow for lower GCV concentrations to achieve higher tumor cell-killing but may also decrease GCV-associate side effects.

Since most cancers are typically treated with a combination of drugs and therapeutic modalities, other groups have focused on improving GCV cytotoxicity through combination therapies. Findings that demonstrated the existence of an anti-tumor immune response following HSV-TK/GCV therapy prompted studies combining the expression of cytokines, such as interleukin-2, to enhance this immune response with the enzyme-prodrug gene therapy resulting in increased tumor cell killing. Interleukin-2 has been combined with HSV-TK/GCV alone or with other cytokines and has resulted in

a 2- to 3-fold reduction in tumor volume in a variety of tumor models (101-105). The addition of interleukin-12 also enhanced anti-tumor immune response in combination with HSV-TK/GCV in animal models for colon and hepatocellular carcinoma (106-108). A clinical trial has shown that combining HSV-TK/GCV gene therapy and cytokines is safe, but the effectiveness and improvement over HSV-TK/GCV gene therapy alone has yet to be determined in humans (109).

Cell cycle effects of certain agents have been used to enhance GCV cytotoxicity. Pre-treatment with polyamine biosynthesis inhibitor 2-difluoromethylornithine resulted in accumulation of cells in S phase and addition of GCV when this compound is removed allows for increased GCV incorporation into DNA (110,111). Protein kinase C class inhibitor UCN-01 has been shown to abrogate camptothecin-induced S phase block, resulting in increased cytotoxicity and has also been shown to increase sensitivity to GCV (112).

Because GCV inhibits DNA polymerase δ (113), which is involved in DNA repair, it was hypothesized that the combination of GCV with other DNA damaging agents would result in synergistic cytotoxicity. Alkylating agent temozolomide, topoisomerase I inhibitor topotecan, and a dual suicide gene therapy strategy of cytochrome P450 2B1/cyclophosphamide have been reported to enhance cytotoxicity when combined with HSV-TK/GCV (114-118). While the data presented in these reports are statistically significant, they represent less than a 2-fold increase in cell killing and thus may not be clinically significant.

Other drugs which enhance GCV cytotoxicity include ribonucleotide reductase inhibitors hydroxyurea (75,119,120) and gemcitabine (121) which decrease dGTP pools,

resulting in increased GCVMP incorporation into DNA. Similarly, thymidylate synthase inhibition with (E)-5-(2-bromovinyl)-2'-deoxyuridine or 5-fluorouracil (122) decreases HSV-TK substrate thymidine and results in increased GCVTP. Several studies have combined cytosine deaminase/5-fluorocytosine, a 5-fluorouracil prodrug, and HSV-TK/GCV strategies and have shown that this is more effective compared with the use of either strategy alone (123-127), with optimal synergy occurring when 5-fluorocytosine was sequenced 24 hr prior to GCV (127). This therapy can be further enhanced by the addition of radiation (128,129).

The effectiveness of HSV-TK/GCV *in vitro* and in animal models has prompted many other clinical trials in the last two decades in a variety of malignancies, including glioblastoma (130-133), mesothelioma (134), ovarian (135,136), and prostate cancer which exhibited the most promising results (137-143). The major limiting factor for the effectiveness of this therapy is the ability to transduce a sufficient proportion of cells in the tumor. A phase III clinical trial for HSV-TK/GCV gene therapy for glioblastoma reported transduction efficiency was below 0.002% in all patients (144). Another study including 51 patients reported a maximum transduction efficiency of 2.6% in two patients with a value less than 0.03% in most patients (145).

One experimental protocol in clinical trials utilizes a three-pronged approach for treating prostate cancer which includes (a) an oncolytic, replication competent adenovirus, (b) combined cytosine deaminase/5-fluorocytosine and HSV-TK/GCV gene therapy, and (c) radiation therapy (125,139,141,142,146). Results from a preclinical model have been published recently extending this approach for the treatment of pancreatic cancer, and a Phase I trial in this malignancy is currently being conducted

(147). While the results from these studies have been promising, they have not been overwhelmingly successful, most likely due to the limitation of viral transduction.

A more recent application of the HSV-TK/GCV suicide gene therapy strategy is being developed as a means to control graft-versus-host disease (GVHD). GVHD is a T cell-mediated complication in patients with leukemia undergoing allogeneic bone marrow transplantation that is a major cause of transplant-related morbidity and mortality (148). GVHD is the result of the recognition of host antigens by donor T lymphocytes which results in activation of macrophages, secretion of inflammatory cytokines, and apoptosis of host cells (149). In this approach, retroviruses are used to stably transduce the HSV-TK gene into donor T lymphocytes *ex vivo* prior to donor lymphocyte infusion or transplantation. When GVHD develops, GCV is administered, resulting in the death of HSV-TK positive T lymphocytes. This approach has been shown to be very effective at suppressing GVHD in mouse models (150-154). Several small clinical trials have produced promising results, showing reduced GVHD after GCV administration (155-159) and a phase III clinical trial is scheduled to begin soon (160). Because it is possible to select for HSV-TK-expressing cells by cell sorting prior to transplantation, 100% of the transplanted T lymphocytes can be engineered to express HSV-TK, and viral transduction is not a limitation as is the case with solid tumors. If the selective killing of transduced T lymphocytes will be able to control GVHD without increasing rate of relapse, as has been observed in mouse models, this application of HSV-TK/GCV suicide gene therapy has potential to be successful in humans.

Mechanisms of HSV-TK/GCV-Mediated Cell Killing

Rubsam et al previously reported on the superior cytotoxicity of GCV as compared to other HSV-TK substrates (71). GCV induces >4 logs of cell kill whereas most other nucleoside analogues are only able to cause 1-2 logs of cell kill under similar conditions. It was determined that the superior cytotoxicity of GCV as compared with other HSV-TK substrates 1-β-D-arabinofuranosylthymine (araT) and acyclovir was not due to increased accumulation of active triphosphate metabolite, increased incorporation into DNA, or increased apoptosis. By staining DNA with propidium iodide, it was determined that GCV induced a unique manner of cell death where cells were able to complete one cell division cycle during and following GCV exposure; however, as they attempted to traverse S phase for a second time, they arrested and subsequently died. Conversely, cells treated with araT arrested in the first S phase during drug exposure. These data suggest that an event occurring during the second S phase following GCV exposure is responsible for cytotoxicity.

Because GCV elicits cell killing through incorporation into DNA in S phase, it was of interest to determine specific types of DNA damage induced by GCV. Thust et al have published reports showing that GCV induces sister chromatid exchanges (SCEs) and structural chromosome aberrations while acyclovir and penciclovir did not (161). SCEs can arise as a result of homologous recombination (162-164), so these data suggested that DNA damage induced by GCV induced homologous recombination repair (HRR) to repair this damage. Interestingly, SCE induction occurred during the second cell cycle following GCV treatment, similar timing to the S phase arrest Rubsam et al observed (71). Taken together, these data suggest an event occurring during this second

S phase is responsible for GCV's superior cytotoxicity. Because GCV elicits its action by incorporating into DNA, it is possible that as cells attempt DNA synthesis, the presence of GCVMP in template DNA causes irreparable damage which is responsible for GCV's high degree of cytotoxicity.

There have been a few studies reporting the effects of GCV exposure *in vitro*, aimed at determining the mechanism of GCV-mediated cytotoxicity. 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 (165). It has also been reported that GCV treatment results in a decline in Bcl-2 levels and activation of caspases, leading to apoptosis (166). However, GCV and araT induced apoptosis to a similar extent (71). Thus, while these studies highlight pathways utilized by GCV that lead to cell death, they do not address the mechanism(s) by which GCV is significantly more cytotoxic than most other HSV-TK substrates.

DNA Damage Response Pathways

In response to numerous DNA-damaging insults, cells have evolved complex mechanisms to monitor and repair DNA lesions in order to maintain genomic integrity. DNA damage is sensed by highly conserved signaling pathways which involve protein kinases such as ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) to recognize DNA lesions and activate cell cycle checkpoints which trigger cellular responses including activation of DNA repair machinery and cell-cycle arrest (167).

Dependent upon the type of damage, different protein repair complexes are involved in the overall cellular response (reviewed in (168)). DNA bases that are altered by small chemical modifications, such as oxidations, alkylations, or deaminations, are removed by base excision repair (BER), which excises 2-13 nucleotides containing the altered nucleotide. DNA polymerases then replace the removed nucleotides. In a method similar to that of BER, nucleotide excision repair (NER) is responsible for the repair of DNA bases containing bulky chemical adducts or cross-links. The mismatch repair (MMR) pathway is involved in the repair of mispaired nucleotides and small insertion/deletion loops created by DNA polymerase errors during replication.

Double-strand breaks (DSBs) are a particularly hazardous type of damage because they can lead to genome rearrangements, and two mechanisms exist to repair these lesions: non-homologous end joining (NHEJ) and HRR. A distinguishing feature between the DNA DSB pathways is the requirement of HRR for a sister chromatid present in the S/G_2 phase of replicating cells, implying that the repair of DSBs in non-replicating cells occurs via NHEJ. HRR is an error-free process that uses a sister chromatid as template DNA to achieve precise repair (169,170). In contrast to HR, NHEJ is an error-prone DSB repair mechanism that facilitates joining of broken DNA ends (171).

Translesion synthesis allows replication machinery to replicate past damaged DNA. This involves the use of specialized polymerases with more flexible base-pairing properties which take over for the blocked replicative polymerase, permitting translesion synthesis (172). Some mechanisms of translesion synthesis introduce mutations, but others do not. For example, Pol η mediates error-free bypass of lesions induced by UV

irradiation, whereas Pol ζ introduces mutations at these sites (173). The potential for introducing mutations during translesion synthesis may be less dangerous to the cell than completely arresting DNA synthesis or continuing the cell cycle with incompletely replicated DNA. The studies presented here investigate the nature of the lesions induced by GCV as well as the involvement of DNA repair pathways in responding to this damage.

Dissertation Research Rationale

The overall aim of this dissertation was to (1) investigate and characterize novel interactions of GCV with DNA, including the induction of DNA damage and mechanisms of DNA repair following GCV exposure, and (2) to provide a molecular explanation of the mechanism(s) by which GCV is more cytotoxic than other HSV-TK substrates. Although it had previously been determined that GCV induces cytotoxicity by incorporation into DNA and arrests cells in the second S phase following drug exposure, the nature of DNA damage induced had not been determined. It was my hypothesis that incorporation of GCVMP into DNA would cause more severe DNA damage, such as stalled or collapsed replication forks, DNA breaks, or mutations, than other HSV-TK substrates. Specific differences in DNA damage produced by GCV compared to other HSV-TK substrates have not been determined, and these differences will add to the understanding of GCV-mediated cell killing and may help to explain the superior cytotoxicity of GCV.

Not only is the nature of the GCV-induced DNA damage poorly understood, little is known about DNA repair pathways involved in responding to this damage. The only

report that provided evidence that GCVMP incorporated into DNA is subjected to repair implicated base excision repair because inhibition of DNA polymerase β sensitized cells to GCV (174). To date, no other data have been published which propose other mechanisms of DNA repair involved in responding to GCV incorporation into DNA.

Rubsam et al provided a direct comparison of the metabolism, DNA incorporation, and cell cycle effects of GCV and araT, but did not characterize the mechanism of the differing cell cycle progression induced by these agents (71). Chapter II extends the finding that GCV induces cell death at the second attempt to traverse S-phase by characterizing the extent and kinetics of DNA damage. By comparing DNA damage induced by GCV to that observed with araT, it was determined that GCV induces more DNA damage than araT and this damage is induced in a biphasic pattern, with a secondary induction of DNA damage occurring with similar timing to the S phase arrest observed following GCV treatment. The induction of Rad51 foci following GCV treatment further supported a role for homologous recombination in repairing GCV-mediated DNA damage.

The initial induction of DNA damage during GCV exposure decreased following drug removal, suggesting that repair had occurred. Studies included in Chapter III evaluated DNA repair mechanisms involved in responding to GCV-induced DNA damage. First, a role for mismatch repair was investigated. Second, a yeast-based assay was utilized to screen a panel of DNA repair mutants for increased sensitivity to GCV. This assay confirmed the MMR repair results and further demonstrated a role for homologous recombination and cell cycle checkpoints in responding to GCV-mediated

DNA damage. Similar experiments in mammalian cells would have taken considerably longer, and these results provide specific pathways to translate into human cells.

In order to more thoroughly understand the effects of GCVMP incorporation into DNA, in Chapter IV we characterized specific DNA mutations induced following treatment with GCV compared to other HSV-TK substrates. The presence of unique mutations in GCV-treated cells compared to control cells or those treated with other HSV-TK substrates suggests that the small changes in structures of these drugs results in dramatically different effects on the fidelity of DNA polymerases. This translated into different effects on cell cycle progression by the different HSV-TK substrates and suggests different mechanisms of cell killing for each substrate.

The research presented in this dissertation has provided the field with knowledge of GCV's ability to induce DNA damage as well as repair pathways involved in responding to this damage, leading to increased understanding of the unique ability of GCV to cause multi-log cell killing. Better understanding of this mechanism may lead to improvement of this gene therapy technique or to the development of new anti-cancer drugs which can induce similarly high degrees of cytotoxicity.

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Chapter II

DELAYED INDUCTION OF CELL DEATH BY GANCICLOVIR IS ASSOCIATED WITH γ-H2AX AND RAD51 FOCI

Summary

Transfer of the herpes simplex virus thymidine kinase (HSV-TK) suicide gene to malignant cells followed by treatment with ganciclovir (GCV) is one of the most widely used strategies for cancer gene therapy. Previously we demonstrated that GCV induced >3-logs more cell kill than HSV-TK substrates acyclovir and 1-β-D-arabinofuranosyl thymine (araT). GCV exhibited a delayed mode of action, in which cells traversed the first S-phase and completed cell division after GCV exposure, but arrested during the second S-phase. We have extended these studies by examining the role of DNA damage as a possible mechanism for the superior cytotoxicity of GCV. Using γ-H2AX foci formation as an indicator of DNA damage, GCV induced at least 7-fold more foci than that observed with araT at equitoxic concentrations. Although the number of foci decreased after removal of either drug suggesting repair of these early lesions, only GCV produced a late and persistent increase in foci indicating the induction of irreparable DNA damage. Furthermore, only the late induction of γ-H2AX was associated with formation of Rad51 foci, implicating homologous recombination repair in cytotoxicity with GCV. The appearance of these late lesions corresponded to the timing of the second S-phase arrest. These data demonstrate that GCV induces late DNA damage. The

inability to repair this damage by HRR may account for the superior cytotoxicity of GCV.

Introduction

Engineering tumor cells to express the herpes simplex virus type 1 thymidine kinase (HSV-TK) allows for the selective phosphorylation of ganciclovir (GCV) in transgene-expressing cells. Further phosphorylation by cellular kinases results in the accumulation of the toxic metabolite, GCV 5'-triphosphate (GCVTP), which competes with dGTP for incorporation into DNA (1). This approach has been successful in producing multi-log cell killing *in vitro* and strong tumor growth inhibition with some complete tumor regressions in animal models (2-6). These results have prompted clinical trials in patients with a variety of malignancies, including brain tumors, and a combination therapy approach in prostate cancer. Studies have demonstrated that HSV-TK/GCV therapy is well tolerated, with promising antitumor activity in prostate cancer (7-9).

Although the excellent cytotoxicity of HSV-TK/GCV has been documented in many different cell types, the mechanism by which cell death is induced is still not well understood. Similar to other nucleoside analogs, the primary mechanism of cytotoxicity is due to the incorporation of GCV monophosphate (GCVMP) into DNA, where the analog is readily incorporated into internucleotide linkages (10,11). While GCV shares this basic mechanism of cytotoxicity with other HSV-TK substrates, including acyclovir (ACV) and 1-β-D-arabinofuranosylthymine (araT), GCV induces multi-log cell killing at sub-micromolar concentrations, whereas ACV and araT are weakly cytotoxic at

concentrations >100 μM (10). Furthermore, treatment of cells with 0.1 μM GCV produced low GCVTP levels, similar to that of its endogenous competitor dGTP, yet this resulted in killing of 75% of the cells; however, accumulation of araT triphosphate was approximately 30 times higher and resulted in no significant cytotoxicity (IC_{10}) after treatment with 1 μM araT. At concentrations of 10 or 100 μM, accumulation of ACV triphosphate was 7-30 fold lower than GCVTP or araT triphosphate and incorporation of ACV monophosphate into DNA, where it acts as an obligate chain terminator (12,13), was below the limit of detection (10). These data suggest that the low cytotoxicity with ACV was most likely due to poor incorporation of ACV phosphates into DNA. However, there is not a clear explanation for the superior cytotoxicity of GCV compared to araT despite its much lower incorporation into DNA.

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 (14). It has also been reported that GCV treatment results in a decline in Bcl-2 levels and activation of caspases, leading to apoptosis (15). While these studies highlight pathways utilized by GCV that lead to cell death, this does not address the mechanism by which GCV is many logs more cytotoxic than other HSV-TK substrates. However, one study demonstrated that GCV induced sister chromatid exchanges and chromosome breaks and translocations, whereas ACV did not (16,17). Since sister chromatid exchanges arise as a consequence of homologous recombination repair (HRR) (18), 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 have reported a unique manner of delayed cell death in response to GCV (10). Cells were able to complete one cell division cycle 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 they underwent cell death. In contrast, cells treated with araT accumulated in S phase and growth was inhibited for at least two days after drug removal, but subsequently cells progressed through the cell cycle and cell number increased. This suggests that an event occurring during the second round of DNA replication following GCV treatment caused cells to arrest in S phase, resulting in cell death. We have proposed that, during the second round of DNA replication, the presence of GCVMP in the template produces DNA damage that cannot be repaired, resulting in a persistent lesion.

Taken together, these results suggest that GCV induces greater DNA damage with unique kinetics that results in multi-log cytotoxicity. In an effort to understand the differences in cytotoxicity between GCV and other HSV-TK substrates, we wished to measure the extent and time course of DNA damage induction by GCV. It has been reported previously that DNA damage results in the phosphorylation of histone H2AX on serine 139 by kinases such as Ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase (19-22). Accumulation of phosphorylated H2AX (γ-H2AX) at the sites of DNA damage induced by radiation and drugs results in formation of discrete foci which can be visualized using a phospho-

specific antibody (19,23-26). γ-H2AX was originally thought to be a marker of DNA double strand breaks (DSBs), but has also been shown to be phosphorylated as a result of DNA polymerase and ribonucleotide reductase inhibition by aphidicolin and hydroxyurea, respectively, suggesting formation of γ-H2AX foci at sites of stalled replication (27,28). Here we have characterized the formation of γ-H2AX foci following GCV treatment as a measure of DNA damage.

Reports of sister chromatid exchanges induced by GCV suggest a role for HRR, a process involved in the repair of DSBs and stalled replication forks (29). In order to characterize the involvement of this repair pathway, we measured the formation of foci containing Rad51, an essential protein mediating HRR (30). We have utilized these techniques to compare the kinetics and extent of DNA damage with GCV and araT in order to elucidate the differences that might result in the superior cytotoxicity observed with GCV.

Materials and Methods

Cell Culture. U251 human glioblastoma cell line was 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 were transduced with a retroviral vector encoding the herpes simplex virus type 1 thymidine kinase along with the neomycin resistance gene. Transgene expressing cells were selected with G418 and individual colonies were expanded and maintained in medium containing G418.

HSV-TK tranduction was confirmed by assaying lysates for phosphorylated GCV metabolites.

Analysis of γ-H2AX foci formation by laser scanning confocal microscopy. Exponentially growing U251 cells on 8-well coverslips were incubated with drug as indicated, 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.

Analysis of Rad51 foci formation by laser scanning confocal microscopy. Drug was added to exponentially growing U251 cells on 8-well coverslips for 24 hours unless otherwise noted. At specified timepoints, cells were washed with PBS and permeabilized with Triton-X buffer (0.5% Triton, 20mM Hepes, 50mM NaCl, 3mM 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 10+ foci were considered positive).

Analysis of γ-H2AX and BrdUrd immunostaining by laser scanning confocal microscopy. Exponentially growing U251 cells on 8-well coverslips were incubated with drug as indicated, followed by addition of 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.

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.

Results

In order to evaluate DNA damage induced by GCV, U251tk cells were incubated with GCV for 24 hr and then assayed for γ-H2AX foci formation. A dose-dependent increase in γ-H2AX foci was observed in cells treated with GCV compared to control cells (Figure 2.1A and B). In the absence of drug, control cells contained an average of 3.6 ± 2.5 γ-H2AX foci per cell. Treatment with the IC_{10} (0.03 μM) for GCV resulted in a 4.4-fold increase in γ-H2AX foci which was not significantly different from control ($p = 0.3$). Treatment with the IC_{50} (0.05 μM) and IC_{90} (0.3 μM) for GCV significantly increased the number of γ-H2AX foci per cell (14.3 $\pm$ 6.4 fold and 24.4 ± 6.8 fold, respectively; $p<0.001$).

γ-H2AX expression was then assayed by flow cytometry in order to evaluate the effect of increasing drug concentrations on total γ-H2AX fluorescence. In untreated

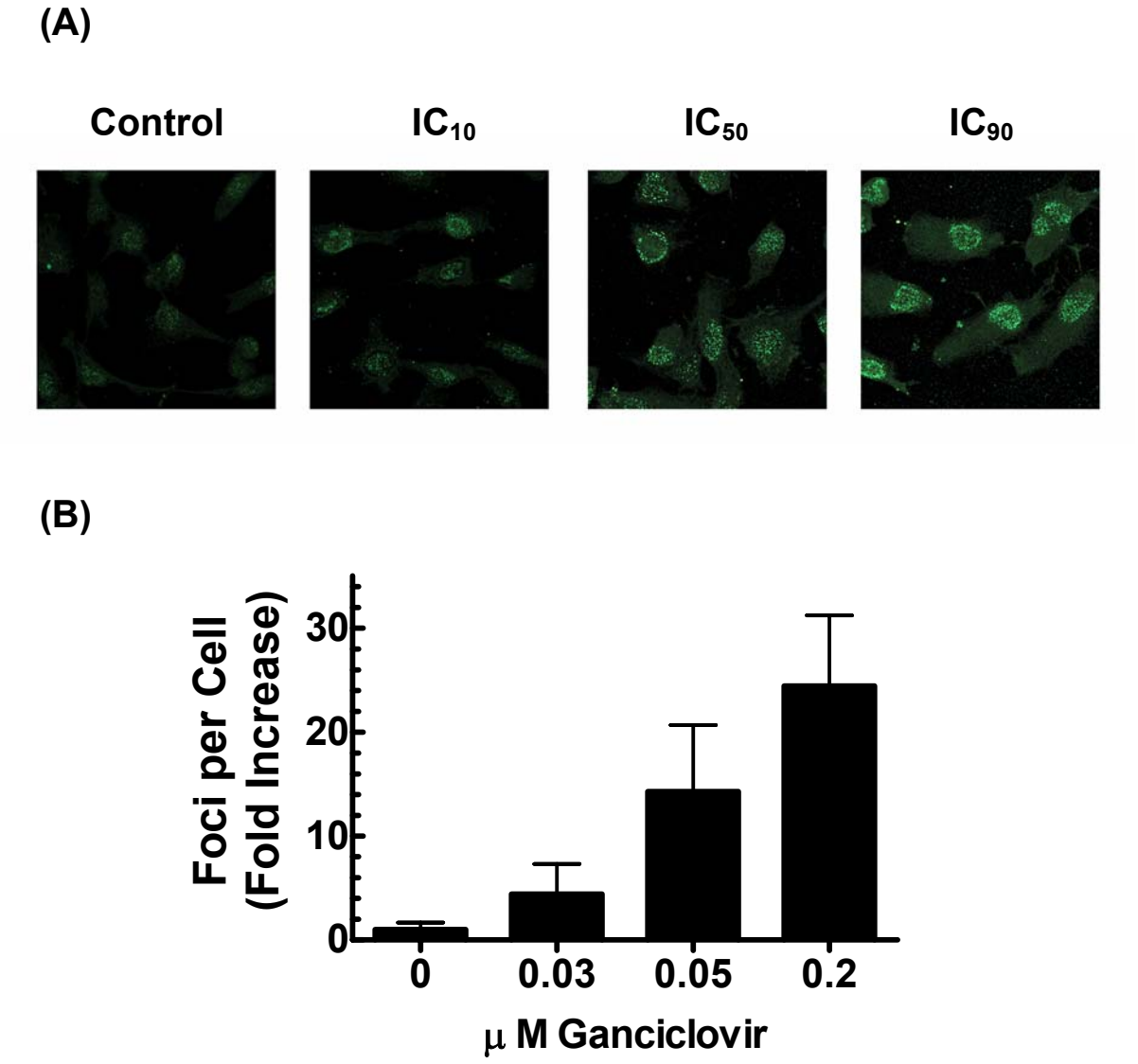

Figure 2.1. GCV induces a dose-dependent increase in γ-H2AX. U251tk cells were incubated with GCV for 24 hr and assayed for γ-H2AX foci formation. (A) representative cells as captured by confocal microscopy; (B) quantitation of the number γ-H2AX foci per cell. *Columns*, average of at least three separate experiments each consisting of at least 18 cells per condition; *bars*, SE.

control cells, only 2% of the cells expressed detectable levels of γ-H2AX. Treatment with 0.2 and 1 μM GCV for 24 hr significantly increased the percentage of cells expressing γ-H2AX to 20% ($p<0.01$) and 59% ($p<0.001$), respectively (Figures 2.2A and B). Thus, two different independent methods have demonstrated an increase in γ-H2AX fluorescence with increasing GCV concentration. Since we were interested in quantifying the number of sites of DNA damage per cell rather than just the percentage of cells positive for γ-H2AX, subsequent experiments measured DNA damage using the *in situ* assay.

Since DNA damage can be induced in cells but may be repaired, we wished to determine the kinetics of γ-H2AX foci formation during drug incubation and their persistence after drug washout. 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 (Figure 2.3). At each concentration of GCV tested, an increase in foci was apparent within 12 hr after drug addition which continued through the end of the incubation, but the number of foci decreased by 12 hr after drug washout. At the IC_{10} for GCV, the number of foci remained similar to control levels throughout the 48 hr post-washout period. In contrast, between 24 and 48 hr after washout of GCV at the IC_{50} or IC_{90}, the number of foci increased to greater than 10-fold over control.

Since cells treated with GCV arrest permanently during the second round of DNA replication following drug incubation (10), we have proposed that a lesion or other type of damage in DNA is encountered leading to cell death. Therefore we wished to determine if the presence of DNA damage, 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})

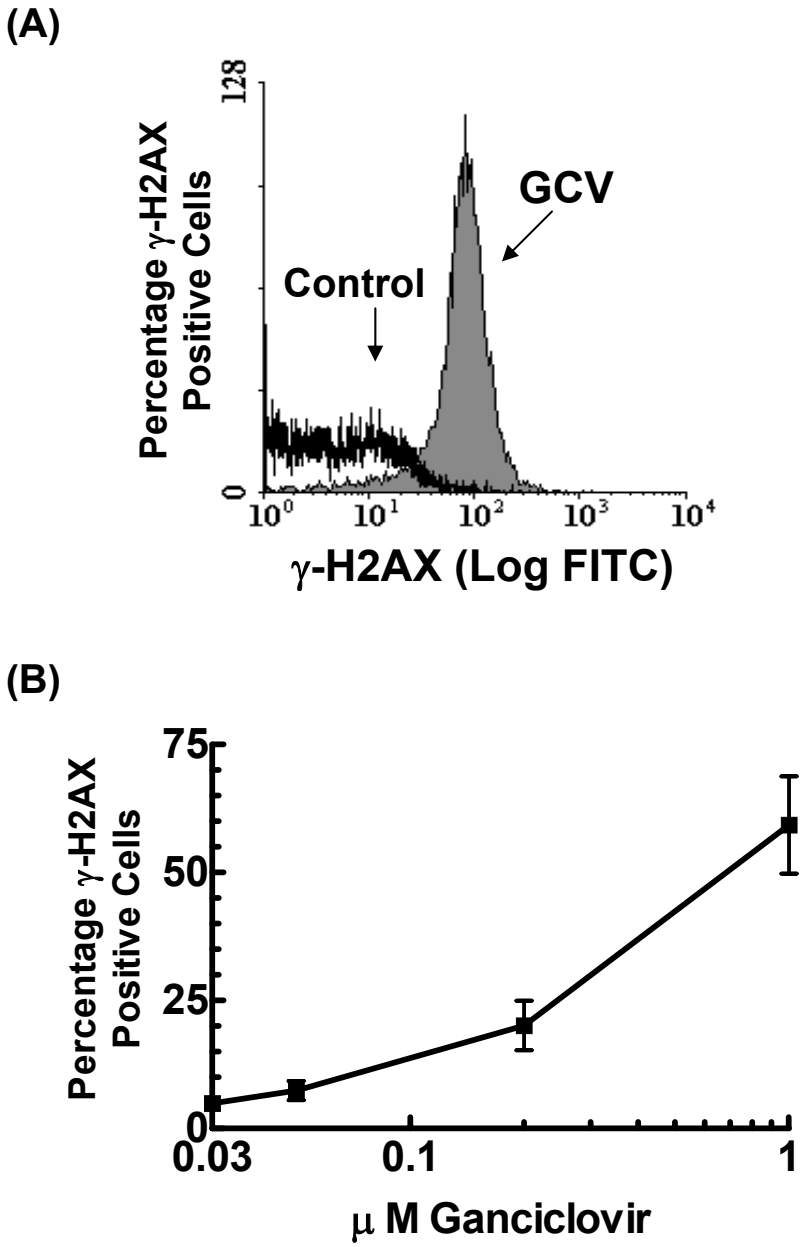

Figure 2.2. GCV induces a dose-dependent increase in γ-H2AX expression. U251tk cells were incubated with GCV for 24 hr and assayed for γ-H2AX expression by flow cytometry; (A) cytogram illustrating separation of control and GCV-treated cells by γ-H2AX fluorescence and (B) quantitation of percentage of γ-H2AX expressing cells from flow cytometry. *Points,* mean of triplicate experiments; *bars,* standard error.

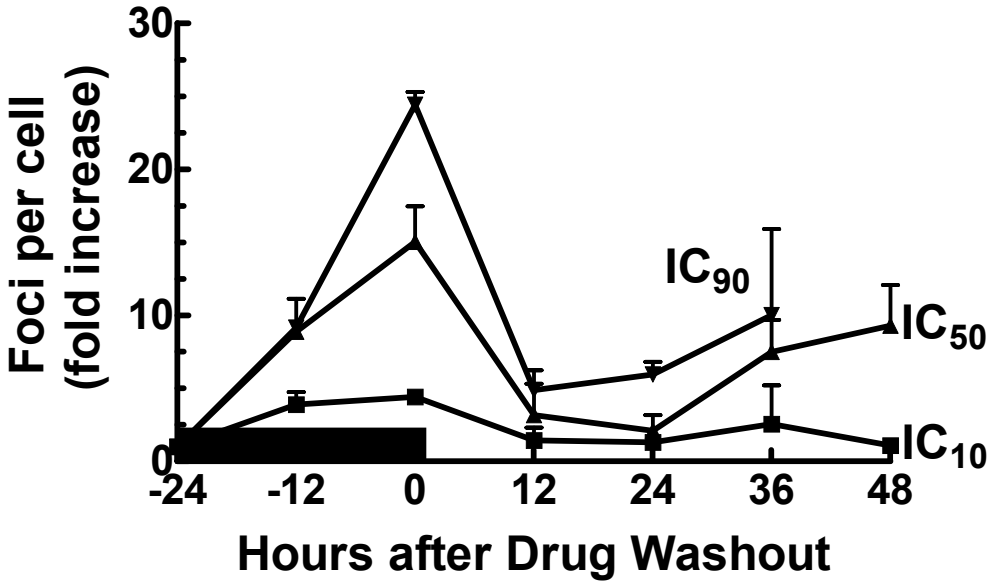

Figure 2.3. Time course of γ-H2AX foci formation in response to GCV. U251tk cells were incubated with GCV at the indicated concentrations (IC_{10}=0.03 μM, IC_{50}=0.05 μM, IC_{90}=0.3μM) for 24 h followed by drug washout. Cells were assayed by confocal microscopy for γ-H2AX foci formation at the indicated time points. Black bar indicates duration of drug incubation, points represent the mean of at least three experiments each consisting of at least 18 cells per time point and drug concentration, bars represent standard error.

for 24 hr, then incubated with 5-bromo-2'-deoxyuridine (BrdUrd) briefly followed by staining for both the thymidine analog in DNA and γ-H2AX. At drug washout, the majority of γ-H2AX positive cells were in S phase, as indicated by BrdUrd incorporation, with a decrease to approximately one-third of γ-H2AX positive cells in S-phase by 24 hr after GCV washout (Table 2.1). At 48 hr after IC_{50} washout, more than 80% of γ-H2AX labeled cells were in S-phase. At 48 hr following washout of the IC_{90} for GCV, no cells stained positive for BrdUrd incorporation. However, these cells exhibited massive cell death, with few intact cells remaining on the plate. A separate study using dual parameter flow cytometry to detect DNA content with propidium iodide and DNA replication with BrdUrd incorporation confirmed that, at 48 hr after GCV washout, the majority of cells were in S phase but had low levels of BrdUrd incorporation due to decreased DNA synthesis (data not shown).

In order to determine whether the amount of DNA damage could explain differences in cytotoxicity between GCV and araT, the induction of γ-H2AX foci by araT was evaluated. 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 dose-dependent increase in γ-H2AX foci was observed (Figure 2.4A and B). However, the magnitude of the increase in foci formation was considerably less with araT (2 - 3.5-fold increase compared to control) relative to GCV (15 - 25-fold increase, Figure 2.1B).

Since γ-H2AX foci formation exhibited a biphasic pattern after GCV treatment, we evaluated the kinetics of foci formation with araT. During a 24 hr incubation with 11 μM (IC_{50}) araT, there was a small increase in the number of γ-H2AX foci to

		% BrdU Positive	% γ-H2AX Positive	% of γ-H2AX Positive cells that were BrdU Positive
O h	**C**	40	23	34
	IC_{10}	56	56	63
	IC_{50}	65	76	80
	IC_{90}	79	96	80
24 h	**C**	38	15	31
	IC_{10}	60	12	33
	IC_{50}	59	21	33
	IC_{90}	55	73	45
48 h	**C**	n.d.	n.d.	n.d.
	IC_{10}	37	9	33
	IC_{50}	64	69	81
	IC_{90}	0	90	0

Table 2.1. γ-H2AX expression and BrdUrd incorporation in response to GCV. U251tk cells were incubated with GCV at the indicated concentrations (IC_{10}=0.03 μM, IC_{50}=0.05 μM, IC_{90}=0.3 μM) 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 removal. 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 indicated time point. n.d.=not determined.

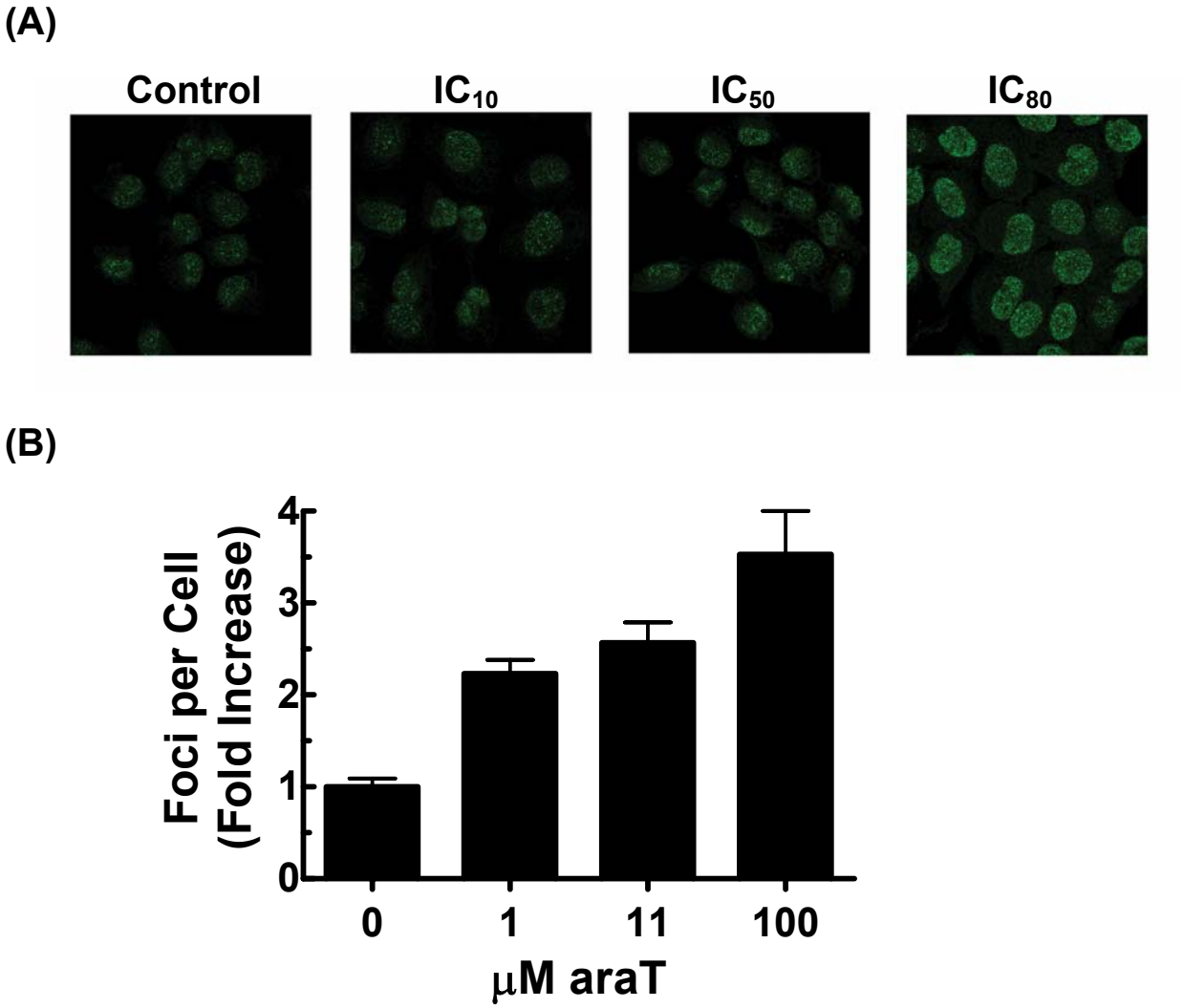

Figure 2.4. araT induces a dose-dependent increase in γ-H2AX. U251tk cells were incubated with araT for 24 hr and assayed for γ-H2AX foci formation. (A) representative cells as captured by confocal microscopy; (B) quantitation of the number γ-H2AX foci per cell. *Columns*, average of at least three separate experiments; *bars*, SE.

approximately 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. In contrast to the results with GCV, no further increase was observed for up to 96 hr after washout (Figure 2.5).

The kinetics of γ-H2AX foci formation observed with cytotoxic concentrations of GCV suggested that the drug-induced DNA damage was initially repaired, but the secondary onset of damage was not repaired (Figure 2.3). Since cytotoxic concentrations of GCV induce S-phase accumulation and a slowing of DNA replication, we wished to determine whether HRR was induced, since this is the primary repair pathway for DNA DSBs during S-phase (31,32). Cells were treated with IC_{90} GCV and assayed for formation of foci containing Rad51 at the indicated time points. Rad51 foci have been demonstrated to accumulate at sites of HRR and thus can be used as an indicator of this repair pathway following DNA damage (33). At 12 h after drug addition and 0 and 12 h after drug washout, there was a small increase (approximately 2-fold) in Rad51 positive cells compared to untreated controls. At 24 h after drug washout, the percentage of cells expressing Rad51 foci exhibited a large increase to 6-fold greater than control. The percentage of Rad51 positive cells decreased thereafter, and at 72 h after drug washout the percentage of Rad51 positive cells returned to a 2-fold increase over control cells (Figure 2.6). These data indicate that HRR was involved in repair of DNA damage primarily after GCV washout.

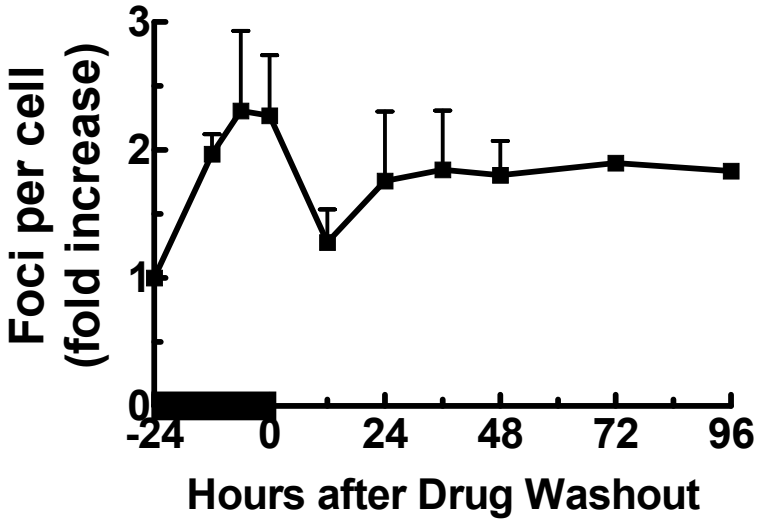

Figure 2.5. Time course of γ-H2AX foci formation in response to araT. U251tk cells were incubated with 11μM araT (IC_{50}) for 24 hr followed by drug washout. Cells were assayed for γ-H2AX foci formation by confocal microscopy at the indicated time points and the number of γ-H2AX foci per cell was determined. Black bar indicates duration of drug incubation. *Points,* mean of at least three experiments; *bars,* standard error.

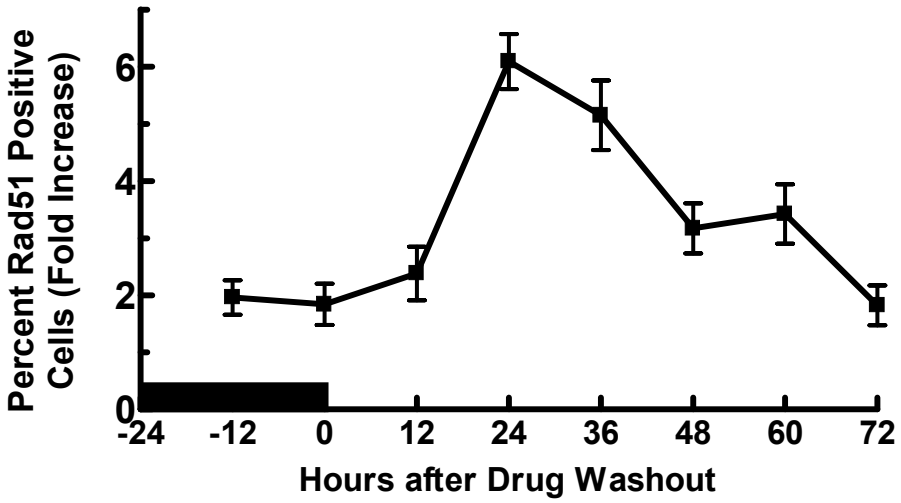

Figure 2.6 Time course of Rad51 foci formation in response to GCV. U251tk cells were incubated with 0.3 μM GCV (IC_{90}) for 24 h followed by drug washout. Cells were assayed by confocal microscopy for Rad51 (>10 Rad51 foci per cell) at the indicated time points. *Black bar,* duration of drug incubation; *points*, mean of at least three wells from representative experiments; *bars*, standard error of the mean.

Discussion

Most nucleoside analogues elicit cytotoxicity through incorporation into DNA (34-36). However, the extent of cell killing can differ between these drugs even though their primary mechanism of cytotoxicity is incorporation of the nucleotide analog into DNA. 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 is different (10). Here we have measured DNA damage induced by GCV and araT using γ-H2AX foci formation, which demonstrated that GCV induced significantly more DNA damage than araT at equitoxic concentrations. The biphasic kinetics of DNA damage observed uniquely with GCV likely reflect the role of HRR in a late but failed attempt at DNA repair, leading to multi-log cytotoxicity.

Previous studies have demonstrated that treatment of cells with ionizing radiation or cytotoxic drugs induces γ-H2AX foci formation in a dose-dependent fashion (23,37-39). It has also been reported that γ-H2AX foci are formed at stalled replication forks and do not solely represent DNA double strand breaks (40-42). Additionally, non-cytotoxic concentrations of the DNA polymerase inhibitor aphidicolin induced H2AX phosphorylation (28). In the data presented here, we have used two different methods to demonstrate that induction of γ-H2AX increased with increasing concentrations of GCV. Following drug washout, the number of γ-H2AX foci decreased demonstrating that the cells were able to repair a portion of this damage. The γ-H2AX foci present during drug incubation may also indicate replication fork stalling, and γ-H2AX diminishes after drug is removed and replication resumes. Time dependent resolution of foci formation has

been demonstrated by others using ionizing radiation (25,37). However, we also observed a subsequent increase in γ-H2AX foci formation 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 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 at drug washout. Furthermore, the late increase in γ-H2AX foci at 48 hr after drug washout also was also associated primarily with cells in S-phase, suggesting 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 significantly 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 (10). This indicates 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, the kinetics of foci formation and resolution was different from that observed with GCV. While the number of foci increased during incubation for both drugs, following araT washout foci formation decreased, whereas with GCV the number of foci increased by 36

hr post washout. 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 (10).

Previous reports demonstrate that GCV induces sister chromatid exchanges, suggesting a role for HRR in responding to GCV-induced DNA damage (16,43). We further investigated HRR by analyzing Rad51 foci formation following treatment with GCV. Unlike the pattern of γ-H2AX foci formation, only a 2-fold increase in Rad51 was observed during drug incubation while a single peak of cells with Rad51 foci was noted at 24 hr after drug washout. This suggests that the DNA damage signaled by the initial peak of γ-H2AX during drug incubation did not utilize HRR for repair. As the percentage of Rad51 positive cells declined, a second increase in γ-H2AX foci formation was evident. This suggests that, at 24 hr after GCV washout, cells are attempting to repair GCV-induced DNA damage through HRR. However, the subsequent increase in γ-H2AX suggests that HRR is not successful but rather it creates more DNA damage which does not appear to be repaired. This increase in irreparable DNA damage leads to cell cycle arrest in the second S phase post-GCV exposure, resulting in cell death.

The separable kinetics of γ-H2AX and Rad51 foci formation to our knowledge have not been reported before. Typically cells are stained for γ-H2AX or Rad51 at one or two time points after radiation or drug addition, whereas here we performed a time-dependent study over 96 hr. At any given time, both γ-H2AX and Rad51 foci could be observed, however they achieved their peak of presentation 24 hr apart. While γ-H2AX has been implicated in the formation of Rad51 foci (44), the 24 hr difference between the peak of γ-H2AX and Rad51 foci would argue against these events being associated.

Furthermore, cells progressed through the cell cycle and divided following drug washout, providing additional evidence that the initial γ-H2AX increase was not the signal for Rad51 foci formation.

In a previous report we demonstrated that, after treatment with GCV, cells were able to progress through one cell division cycle following drug removal and return to a normal cell cycle distribution. However, as cells attempted to progress through S-phase for a second time at approximately 48 hr after GCV washout, they arrested permanently followed by cell death. The results presented here support the cell cycle data. The decrease in number of γ-H2AX foci to control levels and increase in Rad51 foci at 12-24 hr following GCV washout occurred with similar timing to the return to a normal cell cycle distribution after drug treatment previously reported (10). Indeed our BrdUrd data confirm that control levels of S-phase cells are present at 24 hr post-washout for the IC_{10} and IC_{50} GCV. The subsequent increase in γ-H2AX foci occurred with similar timing to the cells' fatal attempt to progress through S-phase for the second time, as indicated by the increase in γ-H2AX/BrdUrd positive cells at 48 hr. This suggests that late-occurring DNA damage that the cell cannot repair is important for GCV cytotoxicity.

We suggest that this damage may be the result of GCVMP in the template strand during DNA synthesis. When DNA replication machinery encounters GCVMP in the template strand, the DNA polymerase may stall. This stalled replication fork may be sufficient for γ-H2AX induction and Rad51 foci may indicate HRR involvement in restarting the stalled replication fork. Alternatively, it has been proposed that a stalled replication fork may result in recognition of a specific endonuclease which generates a

nick in the template, resulting in the formation of a DSB, thus resulting in H2AX phosphorylation and induction of HRR for DSB repair (Figure 2.7 and (29,45)).

In summary, the patterns of γ-H2AX and Rad51 foci formation indicate that late-occurring DNA damage, unable to be repaired by HRR, resulted in cell death with GCV. Furthermore, at equitoxic concentrations DNA damage was less severe with araT and did not persist, whereas GCV induced greater DNA damage and it occurred in biphasic fashion. Although we do not know the nature of the persistent lesion with GCV, we suggest that DNA damage, possibly induced by attempted replication with GCVMP in the template, was unable to be repaired 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 (15,43,46,47), may account for its cytotoxicity.

Acknowledgments

I would like to thank Brendon Ladd for performing the experiments presented in this chapter characterizing Rad51 foci formation.

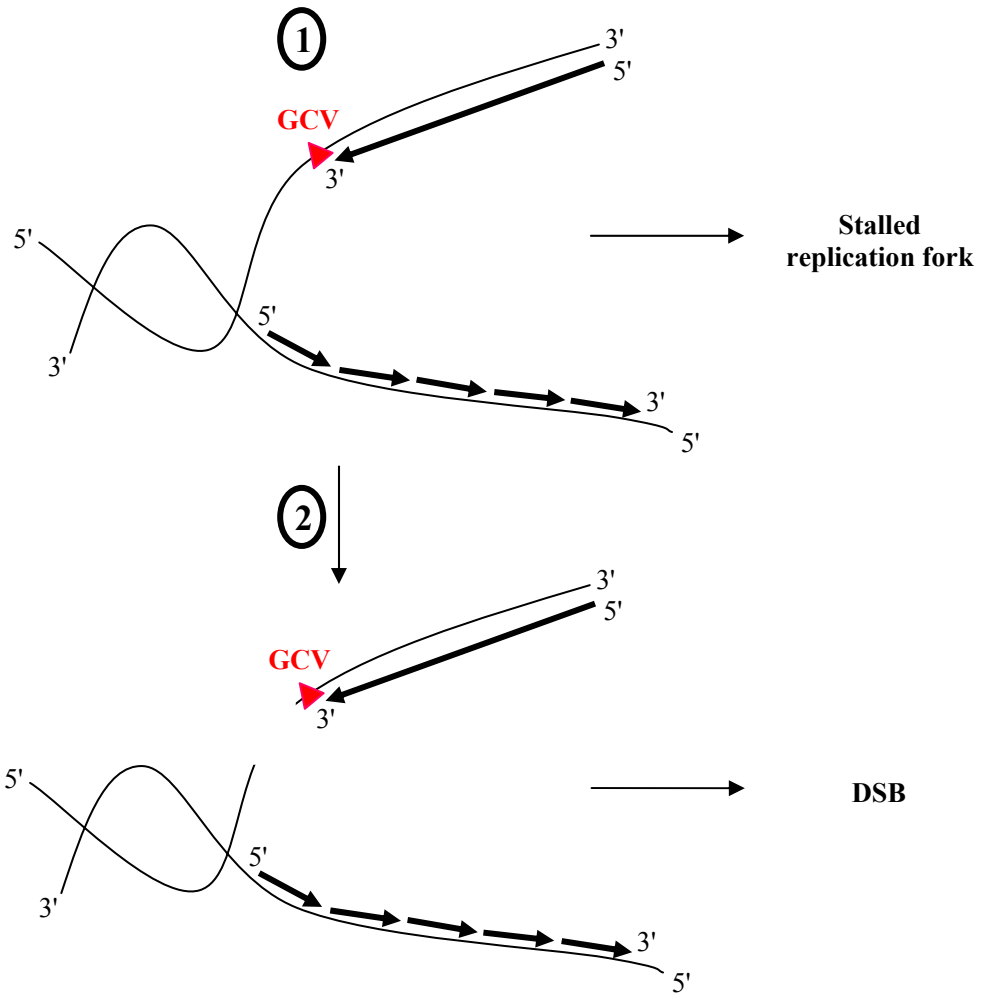

Figure 2.7. Proposed mechanism(s) of γ-H2AX and Rad51 foci formation in response to GCV. When DNA replication machinery encounters GCVMP (▲)in the template strand during DNA synthesis, the DNA polymerase is stalled (1). This stalled replication fork may be sufficient for γ-H2AX induction and Rad51 foci may indicate HRR involvement in restarting the stalled replication fork. Alternatively, the stalled replication fork may result in recognition of a specific endonuclease which generates a nick in the template (2), resulting in the formation of a DSB, thus resulting in H2AX phosphorylation and induction of HRR for DSB repair.

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Chapter III

EVALUATION OF DNA DAMAGE RESPONSE PATHWAYS IN SENSITIVITY TO GANCICLOVIR: MLH1 DEFICIENCY ENHANCES TUMOR CELL SENSITIVITY TO GCV

Summary

Suicide gene therapy with herpes simplex virus thymidine kinase and ganciclovir is notable for producing multi-log cytotoxicity in a unique pattern of delayed cytotoxicity in S-phase. Because hydroxyurea, a ribonucleotide reductase inhibitor that activates mismatch repair, can increase sensitivity to ganciclovir, we evaluated the role of MLH1, an essential mismatch repair protein, in ganciclovir cytotoxicity. Using isogenic HCT116TK (HSV-TK-expressing) colon carcinoma cells that express or lack MLH1, cell survival studies demonstrated greater ganciclovir sensitivity in the MLH1 deficient cells, primarily at high concentrations. This could not be explained by differences in ganciclovir metabolism, as the less sensitive MLH1-expresssing cells accumulated more ganciclovir triphosphate and incorporated more into DNA. Suppression of MLH1 in U251 glioblastoma or SW480 colon carcinoma cells also enhanced sensitivity to high concentrations of ganciclovir. Studies in yeast confirmed the results with MLH1, and further suggested a role for homologous recombination repair and several cell cycle checkpoint proteins in ganciclovir cytotoxicity. These data suggest that MLH1 can prevent cytotoxicity with ganciclovir, and enhancement of ganciclovir sensitivity with

hydroxyurea is likely due to increased incorporation of ganciclovir triphosphate into DNA and not activation of MLH1.

Introduction

In an effort to improve the selectivity of cancer chemotherapy, several suicide gene therapy strategies have been developed in which expression of a foreign gene in tumor cells activates a normally innocuous substrate to a cytotoxic metabolite (1). One of the most widely investigated strategies employs transfer of the cDNA for the herpes simplex virus thymidine kinase (HSV-TK), and expression of the enzyme facilitates phosphorylation of the antiviral drug ganciclovir (GCV) to its 5'-monophosphate, GCVMP. After subsequent phosphorylation by endogenous kinases to its 5'-triphosphate (GCVTP), this metabolite competes with dGTP for incorporation into DNA which leads to cell death (1). This approach has been successful in producing multi-log cell killing *in vitro* and strong tumor growth inhibition with some complete tumor regressions in animal models (2-5). These results have prompted clinical trials in patients with a variety of malignancies, and a combination therapy approach in prostate cancer. Clinical studies have demonstrated that HSV-TK/GCV therapy is well tolerated (6-8), with promising antitumor activity as part of a multimodality approach in prostate cancer (9).

HSV-TK/GCV is notable for its ability to cause high cytotoxicity through a unique manner of delayed cell death distinct from other antimetabolites. Previously we demonstrated that GCV induced >3-logs more cell kill than other HSV-TK substrates, such as 1-β-D-arabinofuranosyl thymine (araT), despite the fact that more araT was incorporated into DNA than GCV (10). U251 glioblastoma cells were able to complete

one cell division cycle after incubation with GCV for 24 hr. However, when they attempted to progress through the cell cycle for a second time, they were blocked in S phase where they remained until they died. In contrast, cells treated with araT accumulated in S phase and growth was inhibited for at least two days after drug removal, but subsequently surviving cells progressed through the cell cycle and cell number increased. This suggests that, with GCV treatment, an event occurring during this second round of DNA replication caused cells to arrest in S phase, resulting in cell death. Other reports demonstrated that, during a 48 – 72 hr continuous incubation in B16 murine melanoma cells, GCV induced a morphological change in cells due to the reorganization of components of the cytoskeleton (11) and an accumulation of cells in S or G2/M (12). In addition, GCV commonly induces an apoptotic cell death due to either a decline in Bcl-2 levels and activation of caspases (13,14), or through a CD95-dependent pathway (15).

While these studies have documented changes in cell cycle progression and induction of apoptosis induced by GCV, the mechanism by which drug incorporation into DNA leads to these consequences is not known. Based on our previous data demonstrating that treatment with GCV arrested cells in S phase, we hypothesized that attempted repair of GCV in the template leads to cell death. Tomicic et al have implicated base excision repair in removal of GCVMP from DNA in CHO cells (16). Previously we reported that GCV cytotoxicity can be enhanced by the addition of hydroxyurea (HU), a ribonucleotide reductase inhibitor that produces an imbalance in dNTP pools, resulting in additive cytotoxicity in HSV-TK-expressing cells and synergistic cytotoxicity in non-HSV-TK-expressing bystander cells across a wide variety

of solid tumor cell lines (17-19). Because HU causes an imbalance in dNTP pools which would lead to misincorporations and activation of the mismatch repair pathway (MMR) (20-22), this study aimed first to determine if MMR affects sensitivity to GCV. Further experiments in a yeast-based system investigated the role of MMR and other DNA repair pathways in GCV cytotoxicity, highlighting additional pathways which may be involved in sensing or repairing GCV-mediated DNA damage.

Materials and Methods

Cell Culture. HCT116 and SW480 human colon carcinoma and U251 human glioblastoma cell lines were maintained in Dulbecco's Modified Eagle medium, McCoy's, and RPMI (Invitrogen Life Technologies, Grand Island, NY); respectively. Media was supplemented with 2 mM L-glutamine (Fisher Scientific, Pittsburgh, PA) for all cell lines and 10% fetal bovine serum (Invitrogen) for HCT116 and SW480 and 10% bovine serum for U251 cells. All cells were maintained in exponential growth and kept in an atmosphere of 37°C and 5%CO_2.

Stable Gene-Expressing Cell Lines. HCT116 0-1, HCT116 1-2, SW480, and U251 cell lines were transduced with a retroviral vector encoding the herpes simplex virus type 1 thymidine kinase along with the neomycin resistance gene (4). Transgene expressing cells were selected with G418 and individual colonies were expanded and maintained in media containing G418 (Invitrogen). HSV-TK expression was confirmed by assaying lysates for phosphorylated GCV metabolites and immunoblotting for HSV-TK protein.

Clonogenic Cell Survival Assays. Exponentially growing cells were treated with GCV (Cytovene, Syntex, Palo Alto, CA) for 24 hr, trypsinized and diluted to approximately 100 viable cells per well in 6-well culture dishes. After 10-14 days, the cell colonies were fixed in methanol:acetic acid (3:1), stained with 0.4% crystal violet (Fisher Scientific), and visually counted. Cell survival is expressed as a fraction of the plating efficiency of control, non-drug treated cells. Each data point was plated in triplicate, and all assays were performed at least twice.

Cellular Nucleotide Analysis. After incubation with [8-^{3}H]GCV (Moravek Biochemicals, Inc., Brea, CA), cells were harvested by trypsinization and extracted with 0.4 N perchloric acid and neutralized following drug exposure. The acid-insoluble pellets containing radiolabled DNA were washed with 0.4 N perchloric acid and solubilized overnight in 1 N KOH. Incorporation of [8-^{3}H]GCV into DNA was quantitated by liquid scintillation spectrometry. For analysis of cellular GCV triphosphate, fractions containing [8-^{3}H]GCV triphosphate were collected and quantitated by liquid scintillation spectrometry based on the known specific activity of [8-^{3}H]GCV.

Depletion of MLH1 with small interfering RNA. Cells were plated on 6 well plates at a density of 1.0 x 10^5 cells/ml and incubated for 24 hours. Cells were transfected with 100 nM siRNA directed to MLH1 or non-specific siRNA (Dharmacon, Lafayette, CO) and Lipofectamine 2000 (Invitrogen, Grand Island, NY). At 24 hours post-transfection, media was replaced. Cells were expanded at 48 hours post-transfection and incubated for an additional 48 hours. Drug was added for 24 hours, and clonogenic cell survival assays

were performed. Cell lysates were collected at time of drug addition for Western Blot analysis of hMLH1 levels.

Immunoblot analysis. Whole-cell lysates were prepared in buffer [0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP40, 10 mM EDTA (pH 7.4)], with the addition of protease inhibitors (Complete Mini Protease Inhibitor Cocktail tablet, Roche, Indianapolis, IN). Proteins were separated by SDS-PAGE on 10% acrylamide gels and transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA). Blots were probed with hMLH1 polyclonal rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) or HSV-TK polyclonal rabbit antibodies and anti-rabbit horseradish peroxidase–linked antibodies. Proteins were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

***Saccharomyces cerevisiae* strains and expression constructs.** The base yeast strain used in these experiments, YW929 (*MAT*α, *ade2*::*STE3-MET15*, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0), was derived from the previously described suicide deletion strain YW798 by allowing the latter to undergo chromosome breakage and repair and selecting an *ade2* mutant product clone (23). HSV-TK and deoxycytidine kinase (dCK) expression constructs were created by amplifying the corresponding coding sequences with tailed primers so that the products could be ligated as a *Bam* HI-*Sal* I fragment into the previously described expression vector pTW300 (24). The product plasmids pTW382 and pTW383 express dCK and HSV-TK, respectively, from the strong constitutive *ADH1* promoter with a Myc epitope and His6 tag fused to the amino terminus. Chromosomal

expression constructs were then made by amplifying the *ADH1*-Myc-His6-dCK/HSV-TK cassettes by PCR using primers with tails homologous to the yeast *CAN1* gene. The fragments were transformed into YW929, canavanine-resistant *can1* colonies identified, and correct integration verified by PCR, α-Myc Western blot, and demonstration of drug-specific toxicity. The resulting yeast strains were YW967 (YW929 *can1*Δ::*ADH1*-dCK) and YW968 (YW929 *can1*Δ::*ADH1*-TK). Primer sequences are available on request.

Introduction of yeast gene deletion mutations was accomplished by a previously described mating strategy (25). Briefly, YW968 was mated in array format to a previously described single-plate array of 96 DNA damage response gene deletion mutants (25). Following selection of diploids and sporulation, recombinant haploids of the genotype *MAT*α, *ade2*::*STE3-MET15*, *can1*Δ::*ADH1*-TK, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0, xxxΔ::kanMX4 (where xxx refers to the various deleted genes) were identified by their growth as red (i.e. *ade2*) colonies on plates selective for methionine and containing canavanine and G418.

Measurement of GCV sensitivity in *Saccharomyces cerevisiae*. Overnight cultures were diluted 50-fold in synthetically defined media with glucose as the carbon source (24) and allowed to grow for 5 hr shaking at 280 rpm at 30° C. Cultures were then diluted to a calculated OD_{600} = 0.0005 in the same media containing varying concentrations of GCV. Growth was continued until the OD_{600} of the untreated control reached 0.5 ± 0.15 (~10 doublings). The OD_{600} of all cultures was then determined. Values are expressed as a fraction of the optical density of the corresponding untreated control sample.

Complementation of mutant *Saccharomyces cerevisiae* strains. PCR primers were designed to amplify the gene of interest (coding sequence plus 1000 bp upstream of the start codon) from wild-type yeast genomic DNA. Primers included tail regions (forward: 5'-TGGCGGCCGCTCTAGAACTAGTGGATCCCC, reverse: 5'-GATAAGCTTGATAT CGAATTCCTGCAGCCC) to allow gap repair of *Sma* I-digested vector pRS316 (*URA3*, CEN/ARS). Digested plasmid and PCR products were co-transformed into yeast strains containing the corresponding gene deletions to generate recombinant plasmids. Colonies were picked and tested for sensitivity to GCV as described above.

Results

These studies utilized the HCT116 0-1 cells, which are MMR deficient due to a truncated essential protein for MMR (MLH1) (26), and HCT116 1-2 cells which stably express MLH1 from its full length cDNA and are MMR proficient. Stably expressing HSV-TK clonal sublines were generated for both the MMR-deficient HCT116 0-1 and the MMR-proficient HCT116 1-2 cell lines. As illustrated in Figure 3.1, both of the HCT116 0-1tk clones (MMR deficient) were more sensitive to GCV than any of the HCT116 1-2tk clones (MMR proficient), especially at high GCV concentrations. One clonal subline from each cell line was chosen based on similar growth rates and sensitivity to GCV. Cytotoxicity of GCV in these two clonal sublines was similar at concentrations of 1 μM or less (% control survival at 1 μM = 3 ± 0.5% and 5.2 ± 1.4%, respectively); however, at 10 μM GCV, greater than one log more cell kill was observed in the MMR-deficient 0-1tk cells compared to the MMR-proficient 1-2tk cells (percent survival = 0.05 ± 0.03% & 0.72 ± 0.2 %, respectively; $p = 0.0046$).

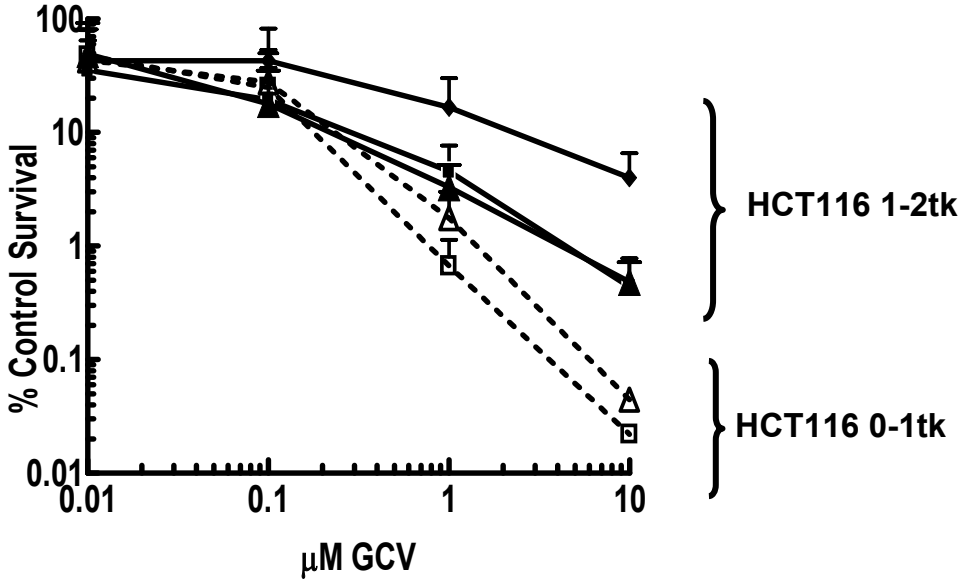

Figure 3.1. Sensitivity of HSV-TK-expressing HCT116 cells to GCV. Exponentially growing HCT116 0-1 (dashed line) and 1-2 cells (solid line) stably expressing HSV-TK were exposed to increasing concentrations of GCV for 24 hours. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error. Cell lines were chosen for use in subsequent experiments: HCT116 1-2tk (▲) and HCT116 0-1tk (Δ).

To determine whether the difference in cytotoxicity could be explained by differential metabolism of GCV in the two cell lines, we measured accumulation of GCVTP and its incorporation into DNA. In both cell lines, there was an increase in GCVTP during drug incubation, and a subsequent decrease in GCVTP levels following drug washout (Figure 3.2A). The HCT116 1-2tk clone accumulated approximately 3 times more GCVTP than the HCT116 0-1tk clone following treatment with 1 μM GCV (55.9 ± 3.9 pmol GCVTP/10^6 cells and 17.8 ± 1.4 pmol GCVTP/10^6 cells, respectively). There was an increase in the amount of GCVTP incorporated into DNA during drug incubation and for 6 to 8 hours following drug removal. The HCT116 1-2tk cells incorporated approximately two-fold more GCVMP into DNA than the 0-1tk cells (5.3 ± 0.3 pmol GCVMP/10^6 cells, 2.4 ± 0.01 pmol GCVMP/10^6 cells, respectively), consistent with the higher pool of GCVTP (Figure 3.2B). GCVMP was well-retained in DNA in both sublines for at least 48 hr after drug washout. The slight decrease detected in HCT116 0-1tk cells was accounted for by an increase in cell number (data not shown). Interestingly, 1 μM GCV was equitoxic in these two clones, despite the fact that there was twice as much GCVMP in the DNA of the 1-2tk clone. Similar results were obtained at 10 μM GCV in which the HCT116 1-2tk cells accumulated up to 4 times more GCVTP and up to 2 times more GCVMP in DNA compared to the HCT116 0-1tk cells (data not shown). Western blot analysis demonstrated that HCT116 1-2tk clone expressed substantially more HSV-TK than the HCT116 0-1tk clone, which accounts for the higher GCVTP accumulation and GCVMP incorporation into DNA observed in the HCT116 1-2tk clone (Figure 3.2C). Thus, reduced metabolism does not appear to account for the lower sensitivity to GCV of the HCT116 1-2tk cells.

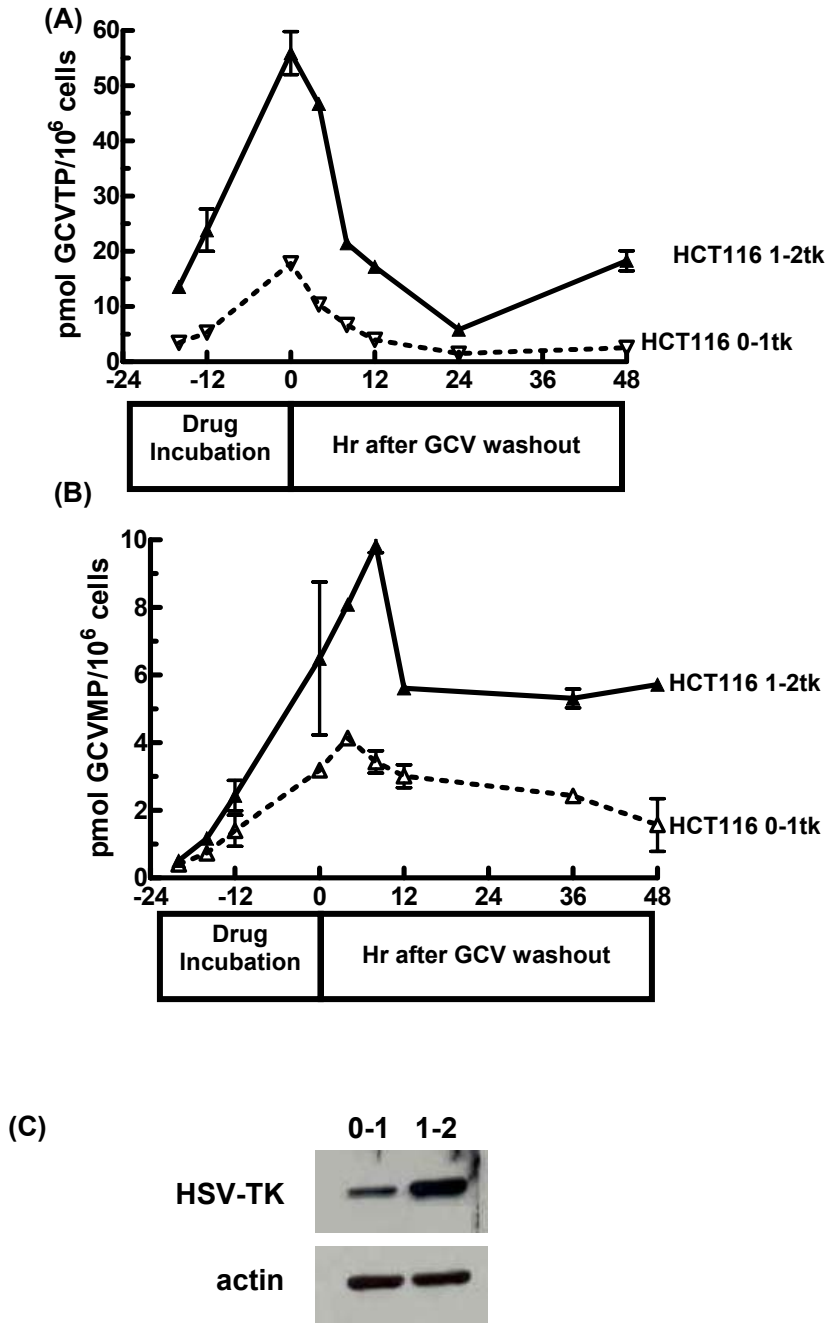

Figure 3.2 Metabolism of GCV in HCT116tk cell lines. (A) Accumulation of [^{3}H]GCVTP and (B) incorporation of [^{3}H]GCVMP into DNA were determined in HCT116 0-1tk (dashed line) and HCT116 1-2tk (solid line) treated with GCV for 24 hours. Points represent the mean of at least triplicate samples, bars represent SEM. (C) Whole cell lysates were analyzed by Western blotting for HSV-TK expression. Expression of actin was used as a loading control.

Because differential expression of HSV-TK in the two clones resulted in different levels of GCVTP, the cytotoxicity of GCV was tested in the parental (non-HSV-TK expressing) HCT116 cell lines. If cells that do not express HSV-TK are treated with high concentrations of GCV, the drug can be phosphorylated by cellular enzymes (27), and we assumed that this phosphorylation would be equivalent in the parental cell lines. Similar to the results in the HSV-TK-expressing cells, the MMR-deficient HCT116 0-1 cell line was more sensitive to GCV than the MMR-proficient HCT116 1-2 cell line (IC_{50} = 120 ± 5.8 μM and 477 ± 23.3 μM, respectively; p=0.0001) (Figure 3.3), with >1-log difference in survival at GCV concentrations ≥300 μM, suggesting that the higher sensitivity of the HSV-TK-expressing HCT116 0-1 cells was due to MMR deficiency.

Because MMR deficiency produces a mutator phenotype which may have affected sensitivity to GCV in the HCT116 0-1 cells, siRNA was used to suppress MLH1 expression in two other cell lines, U251tk human glioblastoma and SW480tk human colon carcinoma, both of which stably expressed HSV-TK and are considered MMR proficient (28). MLH1 decreased substantially by 72 hr post-siRNA transfection and remained suppressed for at least another 48 hr in both cell lines (Figures 3.4A and 3.5A). Suppression of MLH1 expression by siRNA increased the sensitivity of both cell lines to GCV, primarily at highly cytotoxic drug concentrations (>90% cell killing) (Figures 3.4B and 3.5B). Although there was not a complete deficiency of MLH1 in these studies, sensitivity to GCV was increased significantly, observed by a decrease in the IC_{99} for GCV from 6.25 ± 0.92 μM to 1.66 ± 0.11 μM in SW480tk (p = 0.02) and from 1.59 ± 0.09 μM to 0.44 ± 0.07 μM in U251tk (p = 0.0008).

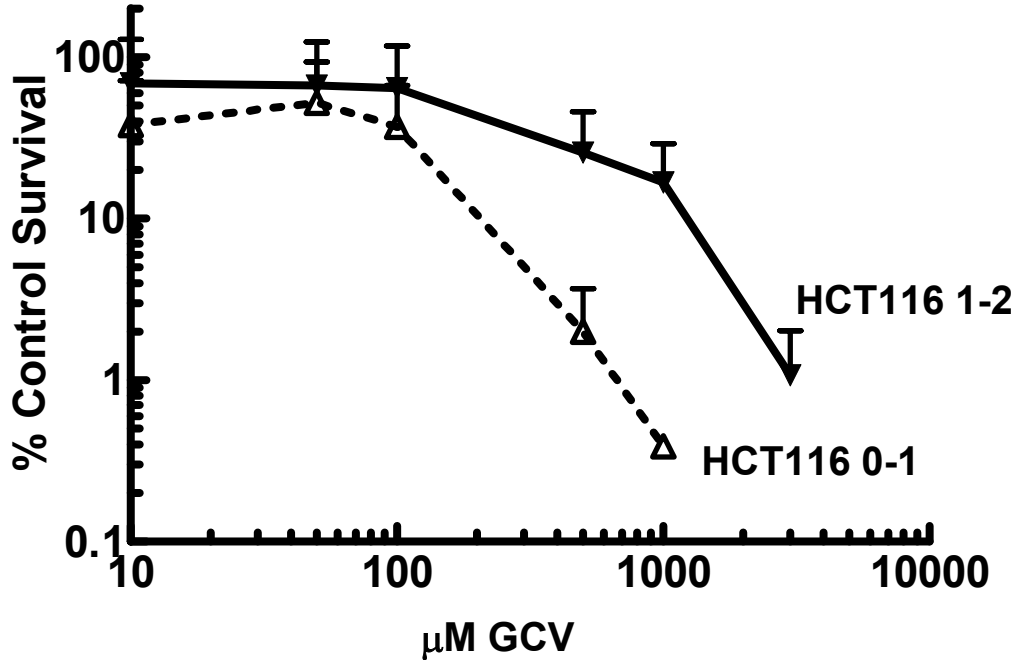

Figure 3.3. Sensitivity of non-HSV HCT116 cells to GCV. Exponentially growing HCT116 0-1 (dashed line) and 1-2 cells (solid line) non-HSV-TK expressing cells were exposed to increasing concentrations of GCV for 24 hours. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Points represent a mean of triplicate samples, bars represent standard error.

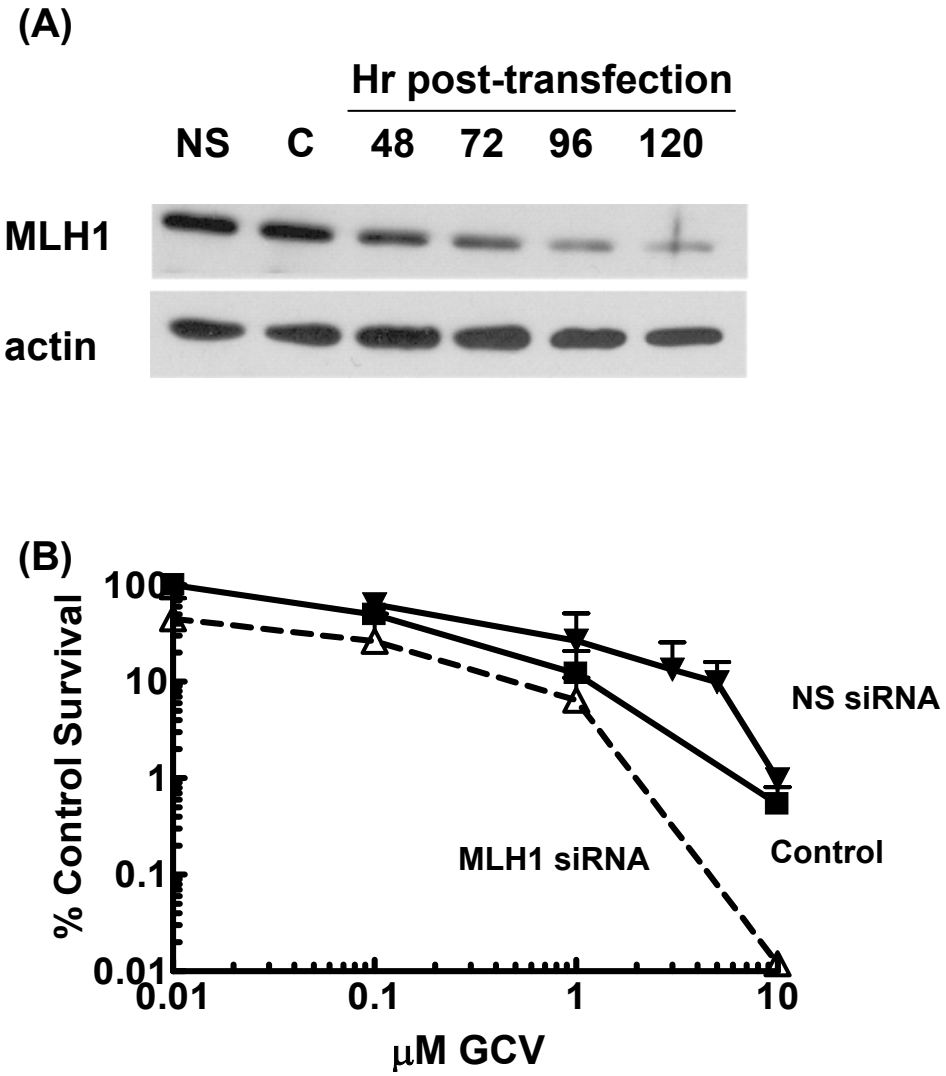

Figure 3.4. Depletion of MLH1 results in increased sensitivity to GCV in SW480tk cells. (A)Whole cell lysates were analyzed by Western blotting for MLH1 expression following no treatment (C), transfection with non-specific siRNA (NS) or MLH1 siRNA. Expression of actin was used as a loading control. (B) Sensitivity of cells treated with siRNA directed to MLH1 (dashed line), non-specific (NS) siRNA (solid line with triangles), or no siRNA (solid line with squares) was determined following exposure to increasing concentrations of GCV. Points represent the mean of triplicate experiments, bars represent SEM.

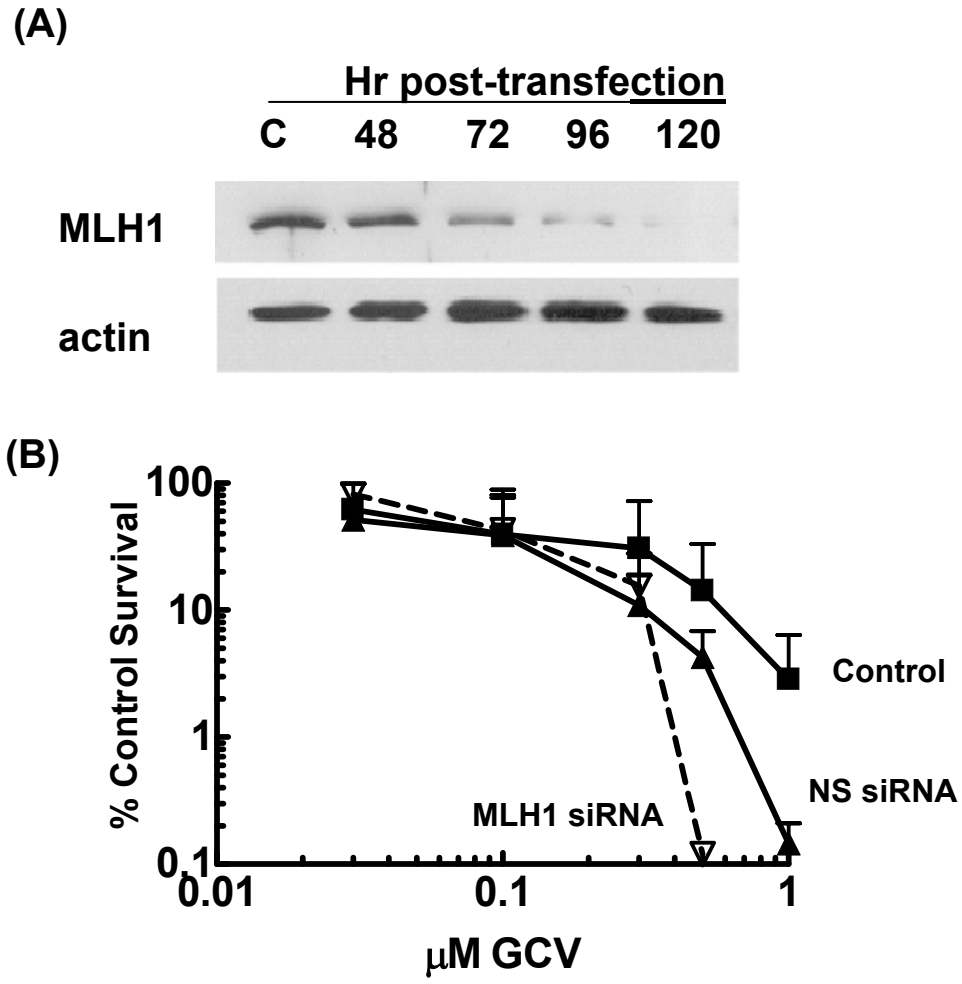

Figure 3.5. Depletion of MLH1 results in increased sensitivity to GCV in U251tk cells. (A) Whole cell lysates were analyzed by Western blotting for MLH1 expression following siRNA transfection. Expression of actin was used as a loading control. (B) Sensitivity of cells treated with siRNA directed to MLH1 (dashed line), non-specific siRNA (solid line with triangles), or no siRNA (solid line with squares) was determined following exposure to increasing concentrations of GCV. Points represent the mean of triplicate experiments, bars represent SEM.

In order to evaluate other DNA repair pathways that participate in repair of GCV-induced lesions, a *Saccharomyces cerevisiae*-based genetic screen was utilized to discover DNA damage response mutants with increased sensitivity to GCV. HSV-TK was placed under control of the strong constitutive yeast *ADH1* promoter and integrated into yeast chromosome V to stably express the gene. GCV conferred dose-dependent toxicity only in HSV-TK-expressing yeast and not in control yeast or those expressing deoxycytidine kinase (dCK) (Figure 3.6). Note that higher concentrations of GCV were required to affect growth of HSV-TK-expressing yeast compared to mammalian cells, which is typical in yeast likely due to poor transport properties for many drugs (29) and the high capacity of yeast for DNA repair, especially homologous recombination repair (30).

HSV-TK expression was next introduced into a panel of 96 DNA damage response yeast deletion mutants and the resulting strains screened for sensitivity to GCV. Table 3.1 indicates that MMR mutants exhibited a low to moderate increase in sensitivity to GCV at the concentrations tested. Mutants deficient in the MMR genes *MLH1* or *MSH2* were as sensitive as controls at a moderate concentration of GCV (0.3 mM) but exhibited significantly increased sensitivity at 5 mM (% control density = 62.4 ± 1.9 for the WT-HSV-TK strain and 46.2 ± 1.1 ($p<0.01$) and 50.1 ± 4.0 ($p<0.05$) for *mlh1* and *msh2* yeast, respectively). Yeast with deletions in homologous recombination repair or cell cycle checkpoint genes exhibited high sensitivity to GCV. Deletion mutants for the endonucleases MUS81 or MMS4 also showed high sensitivity to GCV, which may be due to their putative role in HRR (31). In addition, the increased sensitivity of the *asf1* mutant may be due to its role as a histone chaperone protein which may implicate it

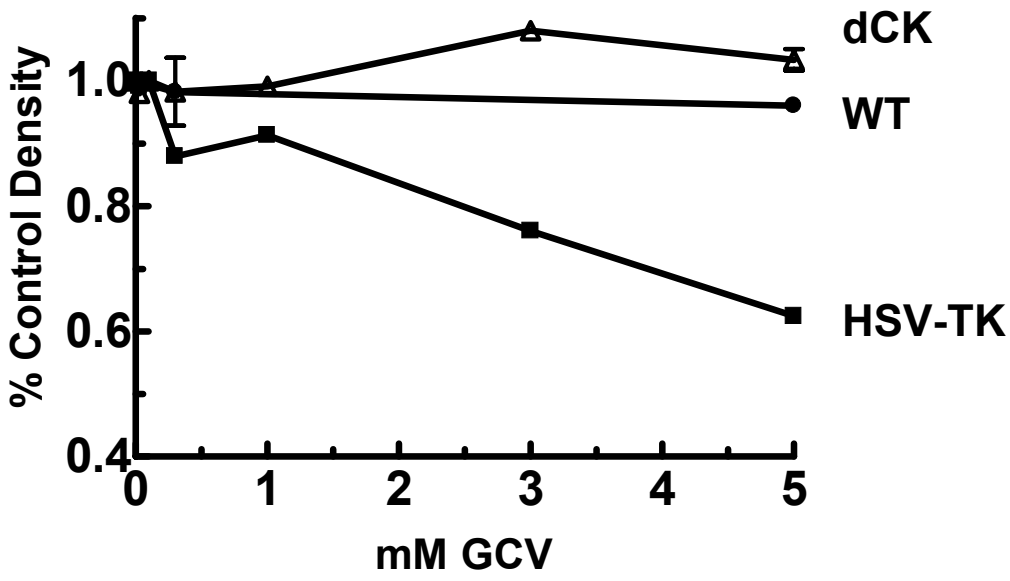

Figure 3.6. HSV-TK expression sensitizes *S. cerevisiae* to GCV. Exponentially growing liquid cultures of *S. cerevisiae* strains expressing HSV-TK, dCK, or no exogenous enzyme were treated with increasing concentrations of GCV. Cell density was determined and expressed as a fraction of the density of untreated control cultures. Points represent the mean of triplicate experiments, bars represent SEM.

Function	GeneName	GCV
RecQ/Topo III	TOP3	++
	SGS1	+++
helicase	HPR5	++
MRX complex	RAD50	+++
homologous recombination	RAD51	+++
	RAD55	+++
	RAD57	+++
	RAD54	+
	RAD52	++
	RAD59	+++
	RDH54	-
checkpoint	DUN1	+++
	RAD24	++
	DDC1	++
	MEC3	+++
	RAD9	+
	RAD17	++
	TEL1	-
endonuclease	MUS81	+++
	MMS4	+++
chromatin	CHD1	-
	ASF1	+++
mismatch repair	MLH1	+
	MSH1	++
	MSH2	+
	PMS1	+
post-replication repair	RAD6	-
	RAD18	-
	RAD5	+
replication	CTF4	-
	POL32	+
	RAD27	-
	DPB3	-
base excision repair	APN1	-

Table 3.1. Sensitivity of yeast strains to ganciclovir: +++, increase in sensitivity to 0.3 mM GCV (≤50% control density); ++, increase in sensitivity to 5 mM GCV (≤25% control density); +, modest increase in sensitivity to 5 mM GCV (≤50% control density); and - indicates no difference from control. The results were first determined by analysis of the array in 96 well plates and validated in triplicate in 2 ml liquid cultures.

in HRR or other DNA repair pathways (32).

In order to verify that the deleted genes were in fact responsible for the increased sensitivity to GCV, representative mutant strains (RecQ helicase, *sgs1*; MRX subunit involved in double strand break repair, *rad50*; homologous recombination protein, *rad52*; checkpoint protein, *dun1*; and 9-1-1 complex member, *rad17*) with moderate to high GCV sensitivity were complemented with the corresponding wild-type gene in a plasmid. In all cases, complementation resulted in decreased sensitivity to GCV, similar to wild-type levels (Figure 3.7A-E) as expected, thus verifying that the gene deletion was responsible for the increased drug sensitivity.

Discussion

Previously we have demonstrated a strong S-phase block associated with GCV cytotoxicity, suggesting that GCV produced irreparable DNA damage (10). However, the type of damage and the repair pathways that may be involved in repairing GCV-induced DNA damage have not been identified. Here we have evaluated the role of MLH1, a protein required for MMR, in the cytotoxicity of GCV using several human cell lines of varying sensitivity to GCV. Additional pathways that may be involved in GCV cytotoxicity were identified using a yeast deletion mutant assay. The results demonstrate that, at high concentrations of GCV, human or yeast cells that express MLH1 are less sensitive to GCV induced cytotoxicity. The yeast assay also implicated HRR in GCV cytotoxicity. These results suggest that GCV induces specific lesions that can be repaired by MMR or HRR, and impairment of these pathways leads to increased cytotoxicity.

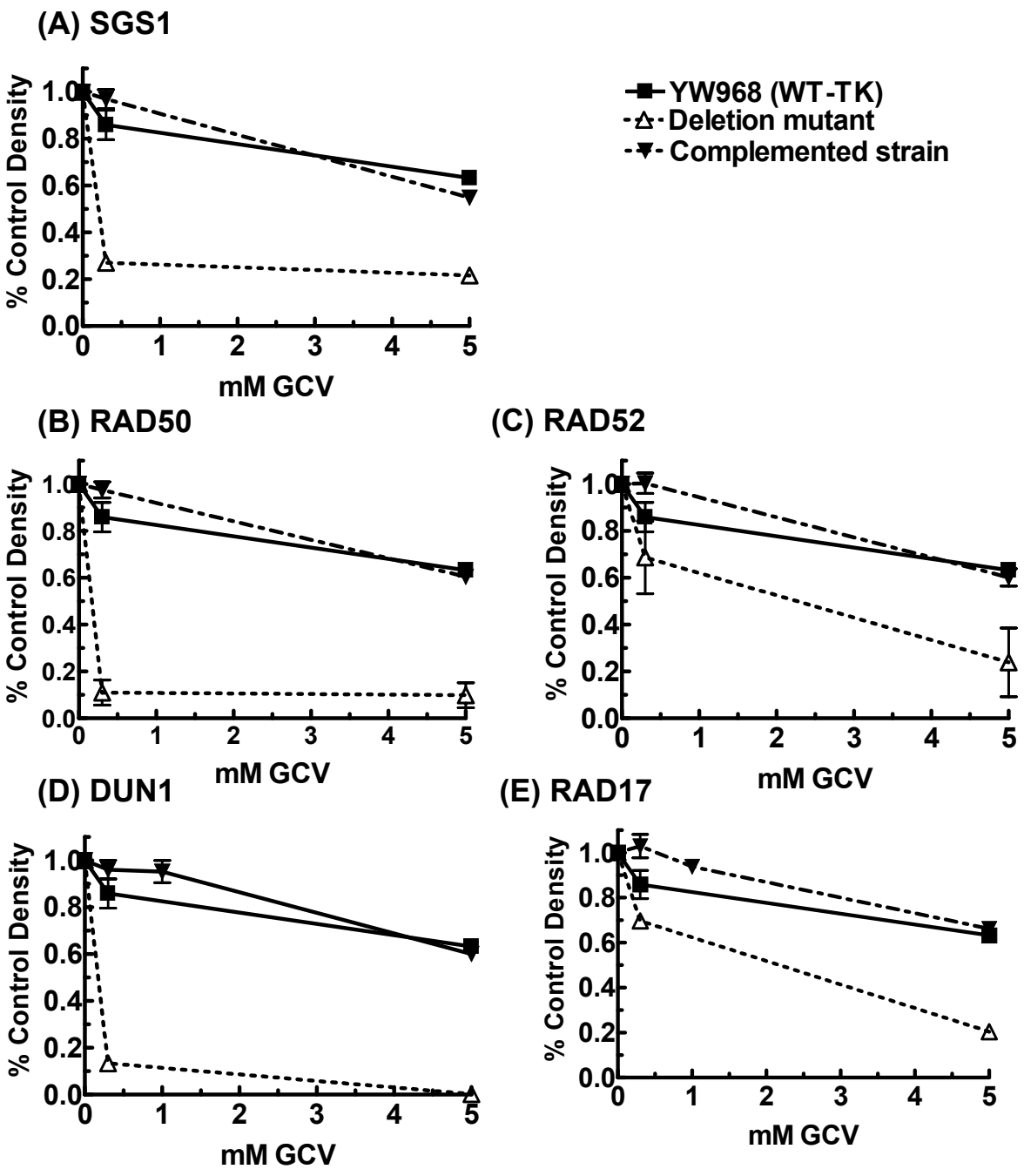

Figure 3.7. Complementation of *S. cerevisiae* mutants restores resistance to GCV. Exponentially growing liquid cultures of *S. cerevisiae* strains expressing HSV-TK and bearing specific gene deletion mutations [(**a**) *sgs2*, (**b**) *rad50*, (**c**) *rad52*, (**d**) *dun1*, (**e**) *rad17*] and their complemented counterparts were treated with increasing concentrations of GCV and cell density determined as in Figure 3.6. Solid lines and squares indicate wild-type yeast, dotted lines with open triangles indicate specific deletion mutants, and dashed and dotted lines with closed triangles indicate complemented strains. Points represent the mean of triplicate experiments, bars represent SEM.

The increase in cytotoxicity in HCT116 0-1 cells (deficient in MLH1) compared to HCT116 1-2 cells (expressing MLH1) was not due to an increase in GCVTP or its incorporation into DNA, since the HCT116 1-2 cells actually accumulated more GCVMP in the DNA than those deficient in MLH1. Further evidence for a direct role of MLH1 in cytotoxicity of GCV was demonstrated by the results in the U251 and SW480 cells using MLH1-specific siRNA, as well as the yeast assay which all demonstrated increased cytotoxicity of GCV in cells deficient in MLH1, primarily at high drug concentrations. Using siRNA to suppress MLH1 was important because it controlled for any differences the matched HCT116 cell lines may have accumulated, after many years of being cultured separately, that could affect GCV sensitivity. While the siRNA did not result in a complete reduction of MLH1, these cells still displayed an increase in sensitivity to GCV. Furthermore, the siRNA studies demonstrated that two different cell lines which differed in inherent sensitivity to GCV both exhibited increased sensitivity at $\geq IC_{90}$ for GCV when MLH1 expression was decreased.

There are several possibilities for the mechanism by which MLH1 deficiency enhances GCV cytotoxicity. Differential incorporation into DNA did not explain the decreased sensitivity of MMR proficient (MLH1 expressing) cells to GCV, raising the possibility that MLH1 is not recognizing or responding to the presence of GCVMP but rather other lesions in the DNA. MLH1 is a required protein for MMR, and therefore if high concentrations of GCV induce errors during DNA replication, such as mismatched nucleotides, deficiency of MLH1 would lead to more errors which may enhance cytotoxicity. Currently we are evaluating the possibility that GCV induces replication errors either during incorporation or as a result of GCVMP in the template during DNA

synthesis. Alternatively, MLH1 may protect cells from GCV-induced damage through downstream signaling, since MLH1 is known to participate in a variety of other pathways such as base excision repair, cell cycle checkpoints, and apoptosis (33-35).

Previously we have demonstrated that HU enhances cell killing with GCV (17-19), and we suggested that this occurred through the increased incorporation of GCVTP into DNA due to the HU-mediated decrease in dGTP. Alternatively, since HU-mediated dNTP pool imbalances activate MMR, it was also possible that HU enhanced cytotoxicity of GCV through activation of MMR which might increase incorporation of GCVMP into DNA as HU-induced mismatches were repaired, as suggested previously for the increased sensitivity of gemcitabine in MMR-proficient cells (36). However, direct evaluation in MMR-proficient and deficient cell lines here demonstrated that deletion or suppression of the required MMR protein, MLH1, actually enhanced cytotoxicity at high GCV concentrations. In contrast, most of the GCV/HU combination studies demonstrated strong synergy at concentrations of GCV below an IC_{90}. Taken together, these data suggest that the combination of GCV and HU elicit synergy by decreasing dGTP and thus increasing GCVMP in DNA rather than through activation of MMR.

We extended the results with MMR to screen a panel of yeast strains containing deletions in various DNA damage and repair genes to evaluate other pathways which may play a role at lower concentrations of GCV. DNA damage repair pathways and checkpoints in *S. cerevisiae* are conserved with those in humans (37). The yeast system allows for a rapid screen of many different mutants, a process which would be very difficult to conduct in mammalian cells due to the amount of time required to develop and test a large number of deletion mutants. Results in the yeast with deletions in MMR

genes *MLH1* or *MSH2* were similar to those observed in the human cell lines lacking MLH1, in which differences in sensitivity to GCV only occurred at a high concentration of GCV. Experiments in yeast have correctly predicted effects in human cells with other drugs as well. Previous work from the Wilson laboratory and others identified the major Tdp1-dependent pathway for resolution of aberrant topoisomerase complexes in yeast (25,38,39), findings which were subsequently confirmed in human cells (40). These data support the use of the yeast assay to discover other DNA damage response pathways that affect sensitivity to GCV.

The yeast assay demonstrated that deletion of genes involved in HRR and cell cycle checkpoints caused a significant increase in sensitivity to GCV. There are several mechanisms through which HRR could impact GCV cytotoxicity. Thust et al have demonstrated that GCV induces sister chromatid exchanges, which usually arise from HRR, during the second S phase after GCV exposure (41-43). In addition, HRR is required to restart a stalled replication fork (44), and we have also shown that, at concentrations $\geq IC_{50}$, GCV slows replication which likely is due to stalled replication forks (18). Thus, it will be important to determine the precise role of HRR in GCV cytotoxicity.

Other genes implicated in conferring sensitivity to GCV included *ASF1*, a histone chaperone involved in chromatin assembly (45). No increase in GCV sensitivity was observed in strains lacking *CHD1*, another chromatin remodeling factor, likely indicating differing roles of *ASF1* and *CHD1* in this process. Similarly, it has also been reported that deletion of *CHD1* did not modify sensitivity to HU, UV radiation, or methyl methanesulfonate, suggesting that this protein does not play a role in DNA repair (46).

Deletion of *SGS1*, a helicase involved in HRR, and *MMS4* or *MUS81*, which function together to cleave sites of stalled replication forks and lead to initiation of HRR also resulted in increased sensitivity to GCV. Together these data further support a role of HRR in responding to GCV-induced DNA damage. Interestingly, deletion of genes involved in postreplication repair and base excision repair did not confer sensitivity to GCV, suggesting that these pathways are not involved in protecting from GCV-mediated cytotoxicity. Although a study in Chinese hamster ovary cells suggested that base excision repair is involved in protection of cells to GCV (16), we have not observed excision of GCVMP from DNA in a variety of human cell lines (4,10,27).

Deletion of the gene for *DUN1*, a regulator of ribonucleotide reductase (47,48) which produces dNTPs for DNA replication and repair, also enhanced the sensitivity of yeast significantly to GCV. The absence of this protein would result in lower dNTP pools, impairing DNA replication and repair following GCV-induced DNA damage. In addition, lowered dNTP pools would likely result in an increase in GCVTP incorporation into DNA by decreasing the availability of its competitor, dGTP, another mechanism for increased cytotoxicity. Although a human homolog of *DUN1* has not been discovered, we have previously demonstrated a role for ribonucleotide reductase since its inhibition enhanced GCV cytotoxicity (17,18,49).

Proteins involved in promoting cell cycle checkpoints also appear to be involved in recognizing GCV-mediated damage, as deletion of Ddc1, Mec3, and Rad17, yeast homologs of the 9-1-1 complex that responds to DNA damage in mammalian cells, and Rad24, the clamp loader that loads the complex onto damaged DNA, rendered the yeast more sensitive to GCV (50). This complex is involved in facilitating activation of Chk1,

resulting in checkpoint activation and cell cycle arrest. This arrest allows time for cells to repair DNA damage and can lead to activation of signaling pathways ultimately leading to cell death if the damage is irreparable. These data suggest a role for this complex in responding to GCV-induced DNA damage, and will need to be confirmed in human cells.

These results suggest a variety of mechanisms to improve therapy with HSV-TK/GCV. The increased sensitivity of MLH1 deficient cells to high concentrations of GCV (0.5-10 μM) is within a clinically relevant range, as GCV typically achieves plasma concentrations of 10-30 μM in patients (51-53). Many human tumors are deficient in DNA damage response pathways, such as MMR (54,55). Perhaps the most successful clinical trials of this therapy have been in prostate cancer (56), a tumor type in which a significant percentage show loss of at least one MMR protein and reduced MMR capacity (55). The data presented here suggest that MMR deficient tumors would respond better to HSV-TK/GCV treatment than MMR proficient tumors since MMR appears to protect cells from GCV-mediated cytotoxicity. Based on the data from the yeast screen, tumors defective in certain checkpoint pathways would also be expected to respond better to GCV. Since normal tissues are generally proficient in these pathways, targeting MMR and checkpoint defective tumors would improve selectivity of this therapy. Furthermore, these studies suggest other pathways, such as HRR, which could be targeted in conjunction with GCV treatment to optimize therapeutic efficacy of this approach.

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Chapter IV

COMPARISON OF DNA MUTATIONS AND CELL CYCLE PROGRESSION INDUCED BY STRUCTURALLY RELATED DEOXYGUANOSINE ANALOGS

Summary

Ganciclovir (GCV) is an antiviral drug commonly used for cytomegaloviral infections in immunocompromised patients and is also in clinical trials in combination with herpes simplex virus thymidine kinase (HSV-TK) in a suicide gene therapy approach for cancer. Previous reports have suggested that GCV is genotoxic, but the exact nature of the induced DNA damage is not known. In this study, we examined the effects of GCV incorporation into DNA by characterizing the resulting DNA mutations and comparing the nature of these mutations to two other structurally related HSV-TK substrates, D-carbocyclic 2'-deoxyguanosine (CdG) and penciclovir (PCV). GCV and CdG are similarly toxic, and PCV is 1-2 logs less cytotoxic. GCV induced a dose-dependent increase in mutation frequency, while concentrations of CdG and PCV >IC_{90} failed to induce a significant increase in mutations. Interestingly, GCV predominantly induced specific GC→TA transversions which were not observed to as great an extent in control cells or those treated with PCV or CdG. This specific transversion occurred in both mismatch repair proficient and deficient cells, and was not affected by activation of mismatch repair with hydroxyurea. Analysis of cell cycle progression demonstrated that

GCV slowed progression through S-phase whereas CdG induced a greater G2/M block, but both GCV and CdG allowed cells to complete one cell cycle after drug treatment and divide followed by cell death in the second cell cycle. In contrast, cells treated with PCV exhibited a lengthy S-phase block due to profound depression of DNA synthesis, and some cell death occurred in the first cell cycle after drug treatment. These data suggest the inability to halt cell division after GCV or CdG treatment results in high cytotoxicity compared to the profound cell cycle inhibition observed with the less toxic PCV. Furthermore, the data demonstrate that alteration of the deoxyribose structure produced profound differences in DNA replication and its fidelity, resulting in striking differences in cytotoxicity.

Introduction

Ganciclovir (GCV) is a potent antiviral drug capable of eliciting multilog cytotoxicity at submicromolar concentrations in a variety of mammalian cell lines which have been engineered to express the herpes simplex virus thymidine kinase (HSV-TK) (1-7). In comparison, other HSV-TK substrates such as the structurally related acyclovir (ACV) and the thymidine analog, 1-β-D-arabinofuranosylthymine (araT), are only weakly cytotoxic to human cells expressing HSV-TK (1). However, D-carbocyclic 2'-deoxyguanosine (CdG), also induced multi-log cell killing at low concentrations (8).

HSV-TK facilitates phosphorylation of GCV to its 5'-monophosphate (GCVMP). After subsequent phosphorylation by endogenous kinases to its 5'-triphosphate (GCVTP), this metabolite competes with dGTP for incorporation into DNA which leads to cell death (9). It was reported previously that the superior cytotoxicity of GCV compared to

araT and acyclovir was not due to increased accumulation of active triphosphate metabolite, increased incorporation into DNA, or increased apoptosis (1). GCV induces a unique manner of cell death in which cells are able to complete one cell division cycle during and following GCV exposure; however, as they attempt to traverse S phase for a second time, they arrest and subsequently die. Conversely, cells treated with araT arrested in the first S phase during drug exposure.

Although these nucleoside analogs elicit their effects by incorporation into DNA, the resulting consequences of this incorporation, such as cell survival, differ significantly. Few studies have examined the events occurring downstream of GCVMP incorporation into DNA or characterized the specific nature of DNA damage induced by GCV. Thust et al have published reports showing that GCV induced sister chromatid exchanges (SCEs) and structural chromosome aberrations while acyclovir and the related penciclovir (PCV) did not (10-14). Interestingly, the observed SCE induction occurred during the second cell cycle following GCV treatment, with similar timing of the observed S phase arrest (1).

This study aimed to characterize the functional consequences of incorporation of these nucleoside analogs into DNA to determine if this can account for the differential cell killing with structurally related substrates. The ability of GCV to induce DNA mutations was characterized and compared to two other HSV-TK substrates, CdG and penciclovir (PCV). We present findings that, despite the structural similarities of GCV, CdG, and PCV, they have profoundly different effects on the fidelity of DNA replication and mechanism of cytotoxicity.

Materials and Methods

Cell Culture. HCT116 human colon carcinoma and U251 human glioblastoma cell lines were maintained in Dulbecco's Modified Eagle medium and RPMI (Invitrogen Life Technologies, Grand Island, NY); respectively. Media was supplemented with 2 mM L-glutamine (Fisher Scientific, Pittsburgh, PA) for all cell lines and 10% fetal bovine serum (Invitrogen) for HCT116 and 10% bovine serum for U251 cells. All cells were maintained in exponential growth and kept in an atmosphere of 37°C and 5%CO_2.

Stable Gene-Expressing Cell Lines. HCT116 0-1, HCT116 1-2, and U251 cell lines were transduced with a retroviral vector encoding for the Herpes Simplex Virus Type 1 Thymidine Kinase along with the neomycin resistance gene (1). Transgene expressing cells were selected and maintained with 1000 μg/mL and 400 μg/mL G418 (Invitrogen), respectively.

Clonogenic Cell Survival Assays. Exponentially growing cells were treated with drug for 24 h, trypsinized, counted with a Coulter electronic particle counter, and diluted to approximately 100 viable cells per well in 6-well culture dishes. After 10-14 days, the cell colonies were fixed in methanol:acetic acid (3:1), stained with 0.4% crystal violet (Fisher Scientific), and visually counted. Cell survival is expressed as a fraction of the plating efficiency of control, non-drug treated cells. All assays were performed at least twice with each data point plated in triplicate.

Shuttle Vector-based Mutation Assay. HCT116 and U251 cell lines were transfected with the pSP189 plasmid (which encodes the SV40 T antigen and mammalian origin of replication as well as the supF gene and an AMP resistance gene to aid in detection of the mutations and for growing on Amp plates, respectively (15)) using SuperFect transfection reagent (Qiagen). Medium was replaced after an overnight incubation with transfection complexes, and drug was added for 24 h. Following drug incubation, drug was removed and fresh media added. pSP189 plasmid was harvested 24 h after the conclusion of drug incubation and isolated using a Qiagen Miniprep kit, incubated with *Dpn*I (Invitrogen) to remove unreplicated plasmid DNA, and further purified by a phenol/chloroform extraction followed by precipitation with isopropanol/ethanol and dissolved in 0.5x TE (pH 7.5).

Transformation was accomplished via electroporation with 1 μL of TE containing plasmid DNA and 20 μL of electrocompetent MBM7070 *E. coli*. The transformation mixtures were plated onto agar plates containing 100 μg/mL ampicillin (Roche), 50 mg/mL isopropyl-L-thio-B-D-galactopyranoside (Invitrogen), and 20 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Roche). Colonies were counted, and mutation frequencies were calculated as number of white colonies / number of (white + blue) colonies. DNA from mutant clones was isolated and sequenced at the University of Michigan DNA Sequencing Core using the 20-mer primer (5'-GGCGACACGGAAATGTTGAA).

Cell cycle progression and DNA synthesis. Flow cytometric analysis was performed as previously described (16). Briefly, at the conclusion of the drug incubation, cells were

pulse labeled with 30 μM BrdUrd for 15 min, and then harvested by trypsinization, counted, and fixed in cold 70% ethanol. Fixed cells were resuspended in 0.5 mg/ml RNAse A (Boehringer Mannheim, Indianapolis, IN, USA) and incubated for 30 min at 37°C. Cells were then subjected to 0.1 N HCl containing 0.7% Triton X-100 (10 min on ice), followed by a 95°C incubation for 15 min in sterile water. One hundred μl of PBS containing 0.5% Tween 20 and 5% calf serum (PBT) was added to each cell pellet followed by the addition of 100 μl of anti-BrdUrd mouse IgG1 antibody (1:100 dilution; PharMingen, San Diego, CA, USA) and incubation for 30 min at room temperature. After centrifugation, 150 μl of FITC conjugated goat anti-mouse IgG antibody (1:20-35 dilution; Sigma Chemical, St Louis, MO, USA) was added to the pellet, mixed gently, and incubated for 30 min at room temperature. Samples were centrifuged and resuspended in 0.5 ml of 18 μg/ml propidium iodide containing 40 μg/ml RNAse A. Treated cells were placed in the dark for a 1 hr before cell cycle analysis using a Coulter EPICS Elite ESP flow cytometer (Coulter, Hialeah, FL, USA). Cell cycle data were further analyzed using WinMDI software (ver 2.8.8) provided by Joseph Trotter of The Scripps Research Institute (La Jolla, CA, USA). Percent DNA synthesis was determined by the change in the mean fluorescence intensity of BrdUrd incorporating cells.

Results

In order to determine the ability of GCV to induce mutations, a well characterized plasmid-based shuttle vector assay was employed (15). The pSP189 plasmid encodes the cDNA for supF which corrects an amber mutation in the β-galactosidase gene in *E coli*, and a mutation at nearly any site in the supF sequence prevents expression of β-

galactosidase. Replication of the plasmid in human tumor cells during drug incubation followed by transfer of the plasmid DNA to *E. coli* allows blue/white screening of supF mutations in bacterial colonies stained with X-gal. U251TK cells were transfected with the pSP189 plasmid, incubated with GCV for 24 hr, plasmid DNA was harvested 24 hr afer drug washout (to allow completion of DNA replication) followed by electroporation into *E. coli.* Using a broad range of GCV concentrations (IC_{10}-IC_{90}), a dose dependent increase in plasmid mutation frequency was observed (Figure 4.1). At concentrations of GCV $\geq$ 0.1 μM (IC_{75}), the increase in mutation frequency was significantly different from control, achieving a nearly 4-fold increase at a concentration of 1 μM. Analysis of the nature of the resulting mutations revealed that GCV induced a predominance of GC→TA transversions (Figure 4.2). Interestingly, at 0.03 and 0.05 μM GCV there was no significant increase in mutation frequency, yet 72% and 56% of the total mutations were GC→TA compared to 33% in control cells. At the higher concentrations of GCV, up to 81% of the mutations were GC→TA. The total increase in mutation frequency can be accounted for by the increase in GC→TA mutations, as there was no increase in any of the other mutations.

Further analysis of the mutations revealed two hot spots in the supF tRNA sequence where the majority of GCV-induced mutations GC→TA occurred (Figure 4.3). Following GCV exposure, the most frequent mutation was C→A at position 20 (C20A), accounting for 15-53% of total mutations. In addition, the prevalence of this mutation increased at higher GCV concentrations. The second most common mutation following treatment with GCV was C→A at position 48 (C48A). Although mutations at these sites were observed in control cells, they accounted for less than 5% of the total number of

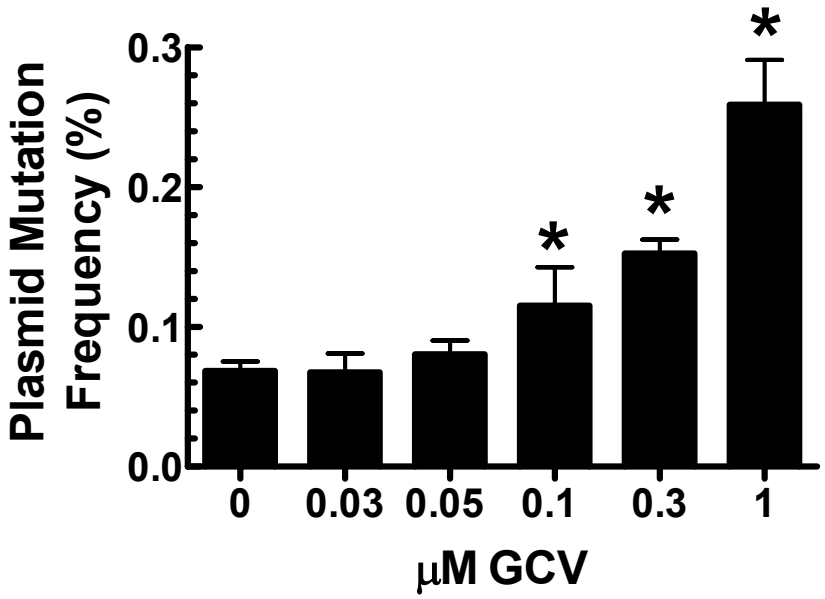

Figure 4.1. GCV induces a dose dependent increase in mutation frequency. pSP189 plasmid mutation frequency in U251tk cell line following exposure to increasing concentrations of GCV. Cells were transfected with the pSP189 plasmid overnight and incubated with 0.03 -1 μM GCV for 24 h. Plasmids were harvested 24 h after drug removal, and DNA from replicated plasmids was electroporated into MB7070 E. coli and mutations were determined. Mutation frequency was calculated as the number of white colonies / total number of colonies counted. *Columns*, average of at least three separate experiments; *bars*, SE; *asterisks*, significantly greater than the corresponding non-drug treated control.

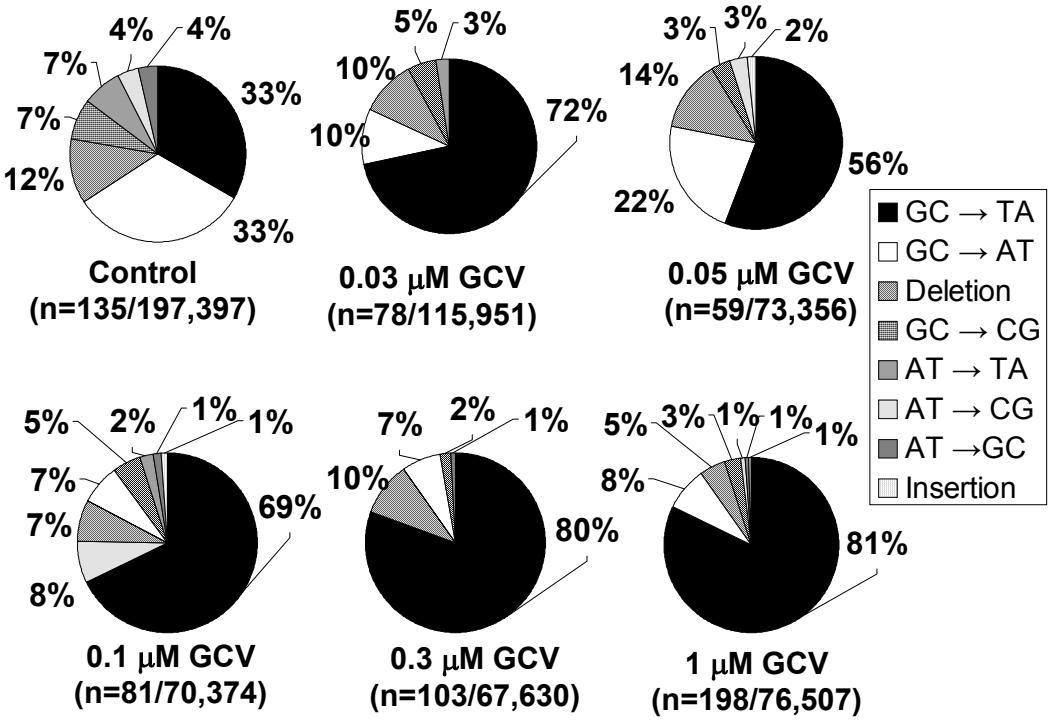

Figure 4.2. GCV induces a predominance of GC→TA transversions. pSP189 plasmid mutation frequency in U251tk cell line following exposure to increasing concentrations of GCV. Cells were transfected with the pSP189 plasmid overnight and incubated with 0.03 -1 μM GCV for 24 h. Plasmids were extracted 24 h after drug removal and mutations were determined. Plasmids were extracted from mutant colonies and submitted for DNA sequencing. n=total number of mutants sequenced/total number of colonies counted.

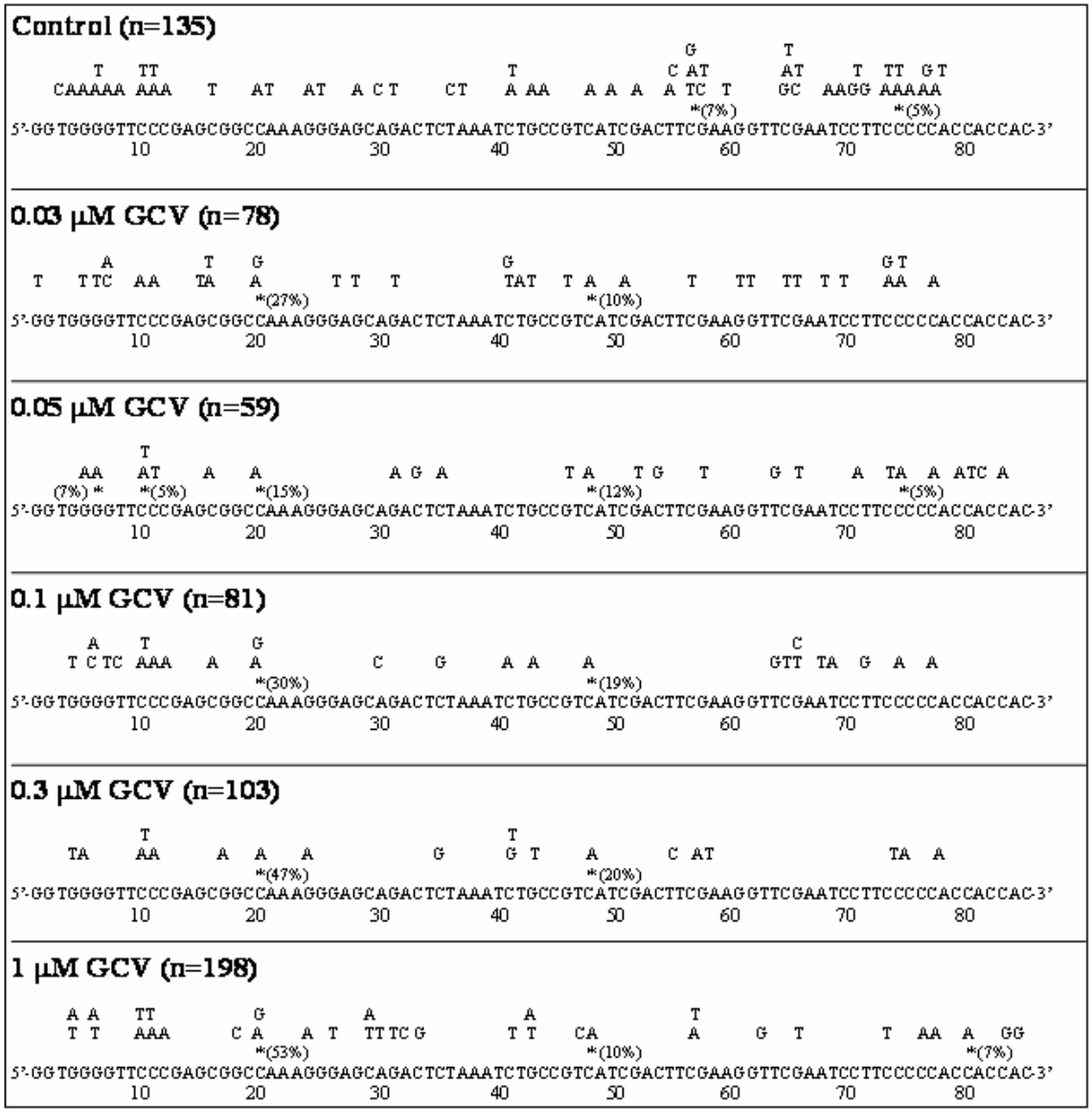

Figure 4.3. Distribution of single base substitutions in the *supF* cDNA replicated in U251tk cells. U251tk cells were transfected with pSP189 plasmid overnight and incubated with 0-1μM GCV for 24 h. Plasmids were extracted 24 h after drug removal and electroporated into *E. coli*. Plasmids were extracted from mutant colonies and submitted for DNA sequencing. The supF coding sequence is shown with the mutations at the individual sites. The asterisks denote frequently mutated positions with the percentage stating percentage of total base substitutions occurring at this point. n=total number of white colonies.

mutations. The pSP189 plasmid contains an 8-bp signature sequence, which provides over 65,000 possible signature sequences within the plasmid population. In order to determine that mutations occurring at the same location in supF tRNA were unique, the sequence of the 8 bp signature was analyzed and it was determined that each plasmid carrying the mutations had different signature sequences, and thus the predominance of the GC→TA mutations was not due to overrepresentation of a single plasmid.

Previously we have determined that the absence of a functional MMR pathway enhances cytotoxicity at high concentrations (>IC_{90}) of GCV. We wished to determine whether this difference in cytotoxicity was related to the nature or frequency of mutations induced. U251 cells are MMR-proficient, so we investigated the role of MMR status on mutations induced by GCV using HCT116TK colon carcinoma cells that are deficient (0-1TK) or proficient (1-2TK) in MMR. In addition, hydroxyurea (HU) was used to produce an imbalance in dNTP pools resulting in activation of MMR, allowing us to study the role of MMR activation on the nature and frequency of mutations. Cell survival studies demonstrated similar GCV sensitivity in the MMR-deficient 0-1TK cells compared to the MMR-proficient 1-2TK cells based on IC_{50} values (0.57 ± 0.04 and 0.39 ± 0.09, respectively; p=0.11) (Table 4.1). The addition of HU at 1 or 3 mM decreased the IC_{50} for GCV in the MMR-deficient 0-1TK (0.41 ± 0.06 and 0.34 $\pm$ 0.01 μM, respectively). In contrast, HU increased the IC_{50} for GCV by $\geq$2-fold in the MMR-proficient 1-2TK cells (0.77 ± 0.19 and >1.0 μM at 1 and 3 mM HU, respectively).

In order to determine if the enhanced cytotoxicity with GCV + HU in the HCT116 0-1TK (MMR-deficient) cells compared to the antagonism of cytotoxicity in the MMR-proficient HCT116 cell lines was due to MMR-mediated correction of mismatched

	mM HU	IC_{50} GCV (μM)
MMR-deficient HCT116 0-1TK	0	0.57 ± 0.04
	1	0.41 ± 0.06
	3	0.34 ± 0.01
MMR-proficient HCT116 1-2TK	0	0.39 ± 0.09
	1	0.77 ± 0.19
	3	>1.0*

Table 4.1. The addition hydroxyurea decreases the IC50 for GCV in MMR-deficient HCT116 0-1TK cells but increases the IC50 for GCV in the MMR-proficient HCT116 1-2TK cells. Exponentially growing U251tk cells were treated with GCV and/or HU for 24 hours. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Values represent Mean ± SE

*Survival =59.2 ± 8.6% control at 1 μM GCV

nucleotides in DNA, the mutation frequency was measured following treatment with GCV ± HU in both cell lines. The mutation frequency of untreated cells was significantly greater in the MMR-deficient 0-1TK cell line than in the MMR-proficient 1-2TK cell line (0.11 ± 0.01% and 0.06 ± 0.01%, respectively, p=0.03) (Figure 4.4). A 24 hr incubation with 1 μM GCV resulted in similar mutation frequencies in both the MMR-deficient 0-1TK and MMR-proficient 1-2TK cell lines (0.17 ± 0.02% and 0.14 ± 0.02%, respectively) that were significantly different from their corresponding controls ($p<0.05$). Treatment with 2 mM HU resulted in a significant increase in mutation frequency in the MMR-deficient 0-1TK cells (0.28 ± 0.03%; p=0.005), but not in the MMR-proficient 1-2TK cells (0.03 ± 0.01%; p=0.24) compared to untreated controls, as expected. The combined treatment with GCV and HU produced a further increase in mutation frequency in the MMR-deficient 0-1TK cell line (0.39 ± 0.06%). However, in the MMR-proficient 1-2TK cell line, the combination of GCV and HU resulted in a significant decrease in the mutation frequency (0.06 ± 0.01 %, p=0.01) compared to cells treated with GCV alone.

Further analysis of the nature of the resulting mutations again revealed that GCV induced a predominance of GC→TA transversions in both the MMR-deficient 0-1TK and MMR-proficient 1-2TK cell lines (49% and 88%, respectively) whereas this specific mutation accounted for only 29% and 12% of the total mutations in control cells from both cell lines. Furthermore, HU induced single base substitutions in which no single type of mutation accounted for more than 35% of the total mutations (Figures 4.5A and B). Interestingly, the combined treatment with GCV and HU in the MMR-deficient 0-1TK cell line resulted in a pattern of mutations more closely resembling those induced by HU than GCV alone. However, in the MMR-proficient 1-2TK cell line, despite the fact

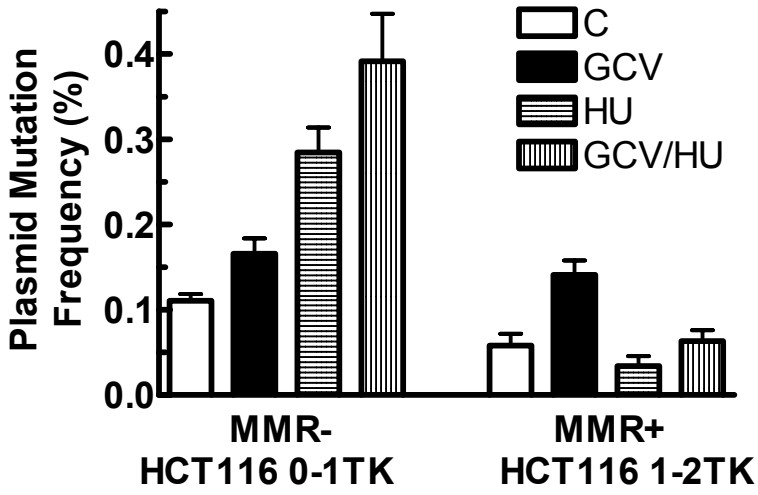

Figure 4.4. pSP189 plasmid mutation frequency in MMR-deficient and proficient HCT116tk cell lines following exposure to GCV and/or HU. Cells were transfected with the pSP189 plasmid overnight and incubated with 1μM GCV and/or 2 mM HU for 24 h. Plasmids were extracted 24 h after drug removal and mutations were determined. Mutation frequency was calculated as the number of white colonies / total number of colonies counted. *Columns*, average of at least three separate experiments; *bars*, SE; *asterisks*, significantly greater than the corresponding non-drug treated control.

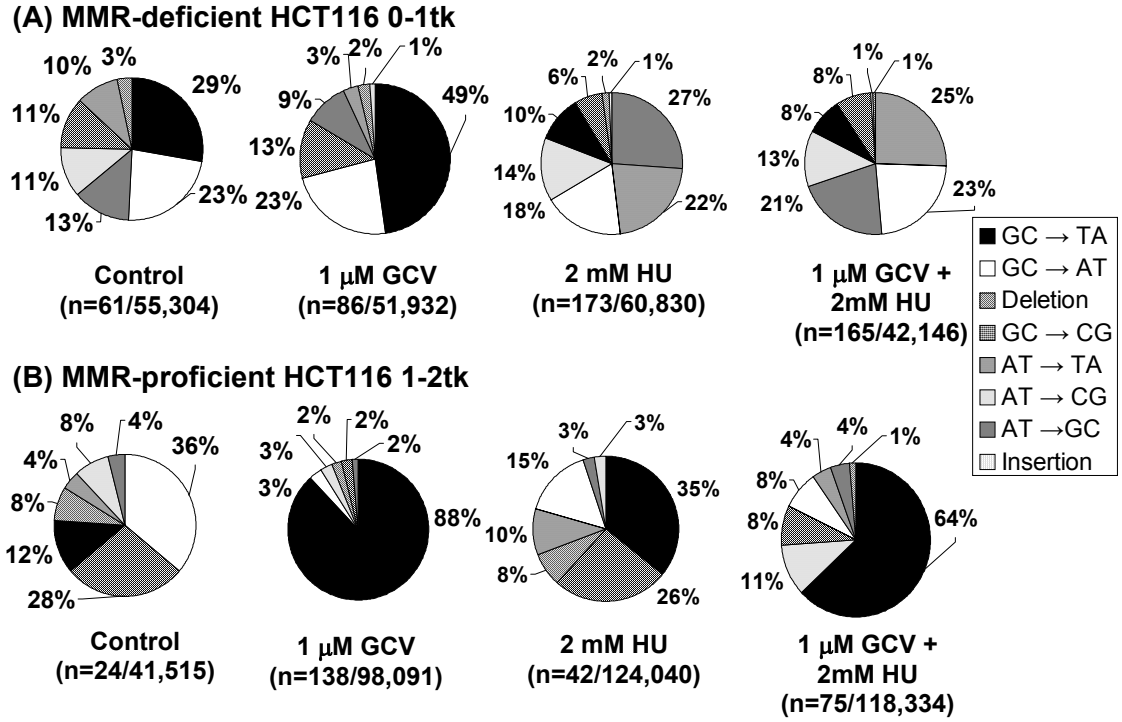

Figure 4.5. Nature of mutations in supF sequence of pSP189 plasmids replicated in MMR-deficient and proficient HCT116tk cell lines following exposure to GCV and/or HU. Nature of mutations in the supF sequence in pSP189 plasmids replicated in (A) MMR-deficient HCT116 0-1tk and (B) MMR-proficient HCT116 1-2tk. Cells were transfected with the pSP189 plasmid overnight and incubated with 1 μM GCV and/or 2 mM HU for 24 h. Plasmids were extracted 24 h after drug removal and mutations were determined. Plasmids were extracted from mutant colonies and submitted for DNA sequencing. n=total number of mutants sequenced/total number of colonies counted.

that the combination resulted in a decrease in the overall mutation frequency, GC→TA mutations still predominated. The C20A mutation predominated in the MMR-proficient 1-2TK cells (55%) (Figure 4.6) and but represented only 7% of total mutations in the MMR-deficient cells (Figure 4.7). The addition of HU resulted in a reduction of the C20A mutations in both cell lines; however they still represented 31% of total mutations in the MMR-proficient 1-2TK cell line, while they were absent in the MMR-deficient 0-1TK cell line.

In order to determine if the pattern of mutations induced by GCV were specific to deoxyguanosine analogs, mutations induced by two other structurally related compounds, CdG and PCV, were analyzed. The structures of these three drugs are similar as they all have changes to the deoxyribose moiety of deoxyguanosine with no alterations of the base (Figure 4.8A). GCV and CdG are similarly toxic in U251tk cells, as reported previously (8), whereas PCV was 1-2 logs less cytotoxic at equimolar concentrations (Figure 4.8B). Neither 1 μM CdG (IC_{99}) nor 10 μM PCV (IC_{93}) produced a significant increase in mutation frequencies compared to control (Figure 4.9A). In addition, there was not a predominance of any specific mutation following exposure to these drugs (Figure 4.9B) and neither the C20A or C48A mutation occurred with either of these two drugs (data not shown).

Because the changes in structure of these nucleoside analogs resulted in a distinct difference on induction of mutations, we wished to determine if these drugs differed with respect to the mechanism by which they cause cytotoxicity. Previously we have demonstrated a unique pattern of cell cycle progression after treatment with GCV, compared to ACV or araT, and we wished to determine whether CdG and PCV elicited

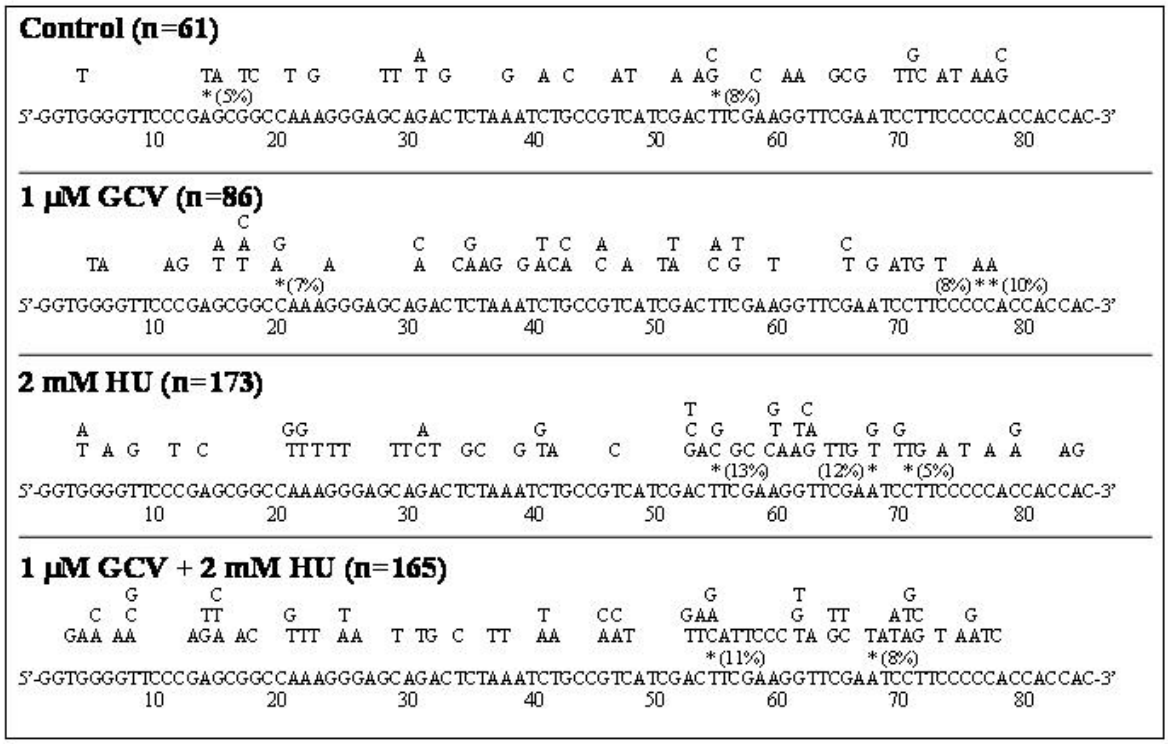

Figure 4.6. Distribution of single base substitutions in the *supF* cDNA replicated in MMR-deficient HCT116 0-1tk cells. MMR-deficient HCT116 0-1tk cells were transfected with pSP189 plasmid overnight and incubated 1 μM GCV and/or 2 mM HU for 24 h. Plasmids were harvested 24 h after drug removal and electroporated into *E. coli*. Plasmid DNA was extracted from mutant colonies and submitted for DNA sequencing. The supF coding sequence is shown with the mutations at the individual sites. The asterisks denote frequently mutated positions with the percentage indicating the percentage of total base substitutions occurring at this position. n=total number of white colonies.

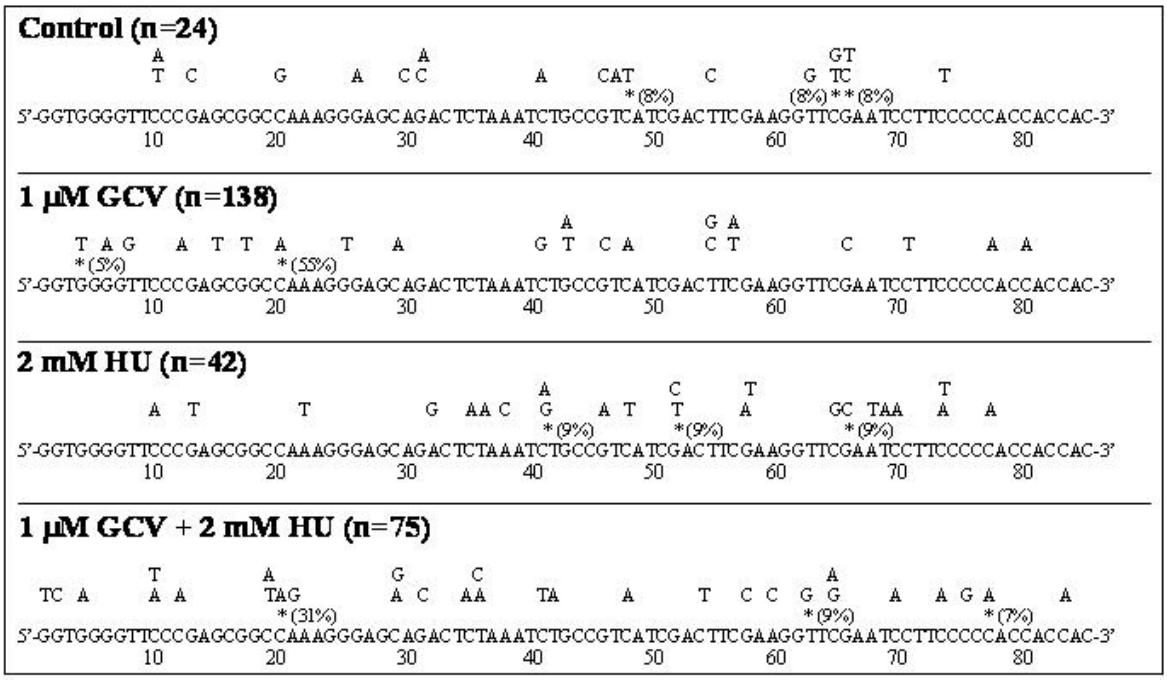

Figure 4.7. Distribution of single base substitutions in the *supF* cDNA replicated in MMR-proficient HCT116 1-2tk cells. MMR-proficient HCT116 1-2tk cells were transfected with pSP189 plasmid overnight and incubated with 1 μM GCV and/or 2 mM HU for 24 h. Plasmids were harvested 24 h after drug removal and electroporated into *E. coli*. Plasmids were extracted from mutant colonies and submitted for DNA sequencing. The supF coding sequence is shown with the mutations at the individual sites. The asterisks denote frequently mutated positions with the percentage indicating the percentage of total base substitutions occurring at this position. n=total number of white colonies.

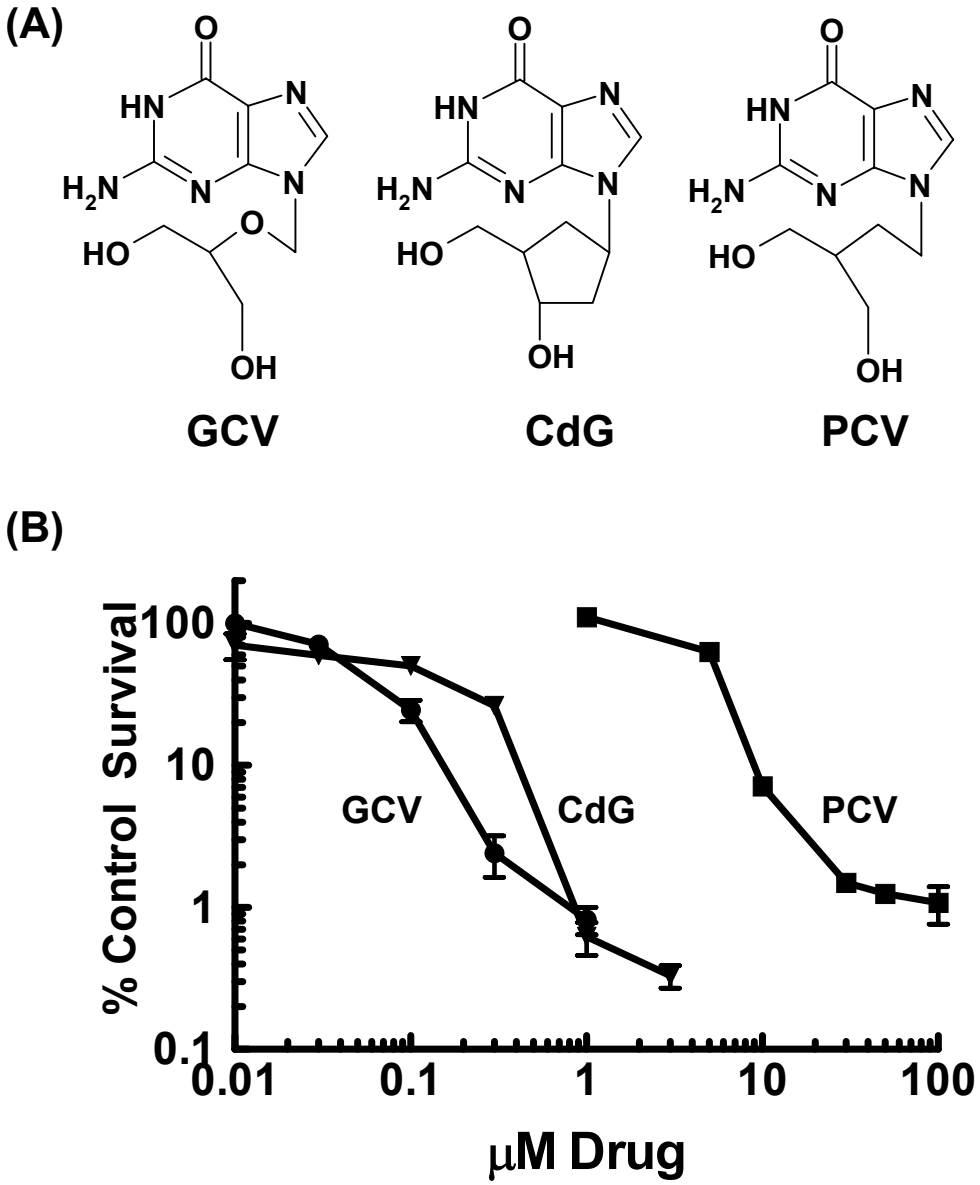

Figure 4.8 Sensitivity of U251tk cells to GCV, CdG, and PCV (A) Structures of GCV, CdG, and PCV. (B) Sensitivity of U251tk cells to GCV, CdG, and PCV. Exponentially growing U251tk cells were exposed to increasing concentrations of indicated drug for 24 hours. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. *Points,* mean of triplicate samples; *bars,* standard error.

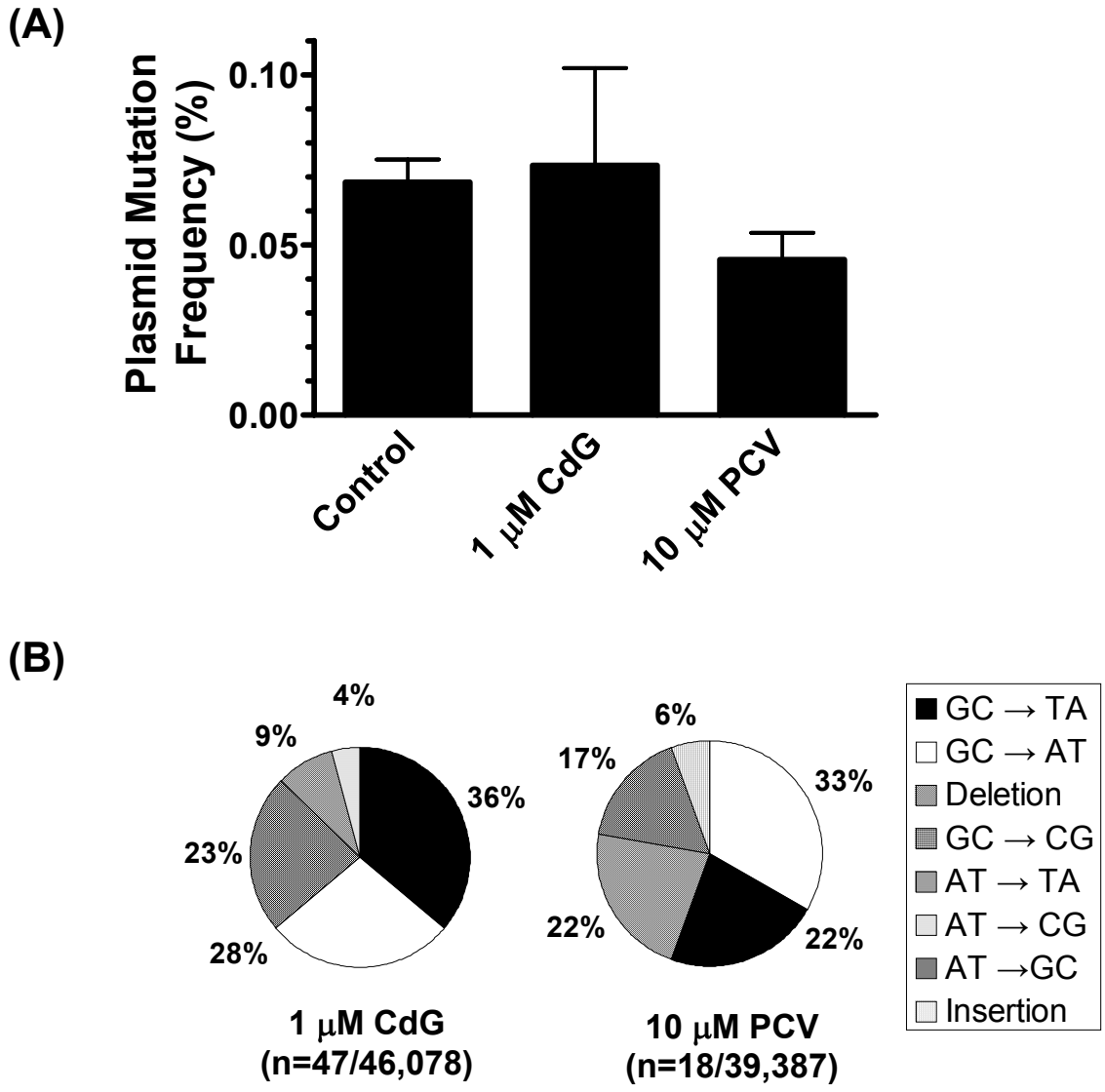

Figure 4.9. pSP189 plasmid mutation frequency in U251tk cell line following exposure to CdG and PCV. (A) pSP189 plasmid mutation frequency in U251tk cells following exposure to CdG or PCV. Cells were transfected with the pSP189 plasmid overnight and incubated with 1 μM CdG or 10 μM PCV for 24 h. Plasmids were harvested 24 h after drug removal and mutations were determined. Mutation frequency was calculated as the number of white colonies / total number of colonies counted. *Columns*, average of at least three separate experiments; *bars*, SE; *asterisks*, significantly greater than the corresponding non-drug treated control. (B) Nature of mutations in the supF sequence in pSP189 plasmids replicated in U251tk cells. Plasmid DNA was extracted from mutant colonies and submitted for DNA sequencing. n=total number of mutants sequenced/total number of colonies counted.

similar or different effects. Thus we evaluated the effect of these drugs on cell cycle progression by a dual parameter flow cytometry technique able to separate cells based both on DNA content (propidium iodide staining) and rate of DNA synthesis (amount of BrdUrd incorporated into DNA). Consistent with a previous report examining DNA content alone (1), cells treated with GCV were slowed in S phase during a 24 hour incubation with GCV and for 12 hours after drug removal (Figures 4.10 and 4.11 and Table 4.2). At 24 hours after drug removal, cells began to progress through the cell cycle, although there was still a high proportion in G_1 and early S phase. These data and the 2-fold increase in cell number demonstrated that these cells were completing the cell cycle and undergoing division. At 0 and 12 h after GCV washout, DNA synthesis, as measured by mean BrdUrd fluorescence, was similar to control cells. GCV-treated cells continued to accumulate in S phase through 72 hours following drug removal.

Similar to GCV-treated cells, following exposure to CdG cells were able to continue through the cell cycle one time following drug exposure, as indicated by an approximately 2-fold increase in cell number at 36 hr after drug washout (Table 4.2). However, they accumulated in G_2/M during drug incubation and beginning again at 24 hours after drug washout. In addition, DNA synthesis was lower compared to cells treated with GCV.

PCV-treated cells exhibited a pattern of cell cycle progression distinct from either GCV or CdG. Within 12 hr after drug addition, the cells accumulated in early S phase and remained there until at least 12 hr after drug washout, likely due to the strong inhibition of DNA synthesis. After drug washout, cells progressed from early to mid and late S phase through 48 hours after drug washout. There was a continuous decrease in

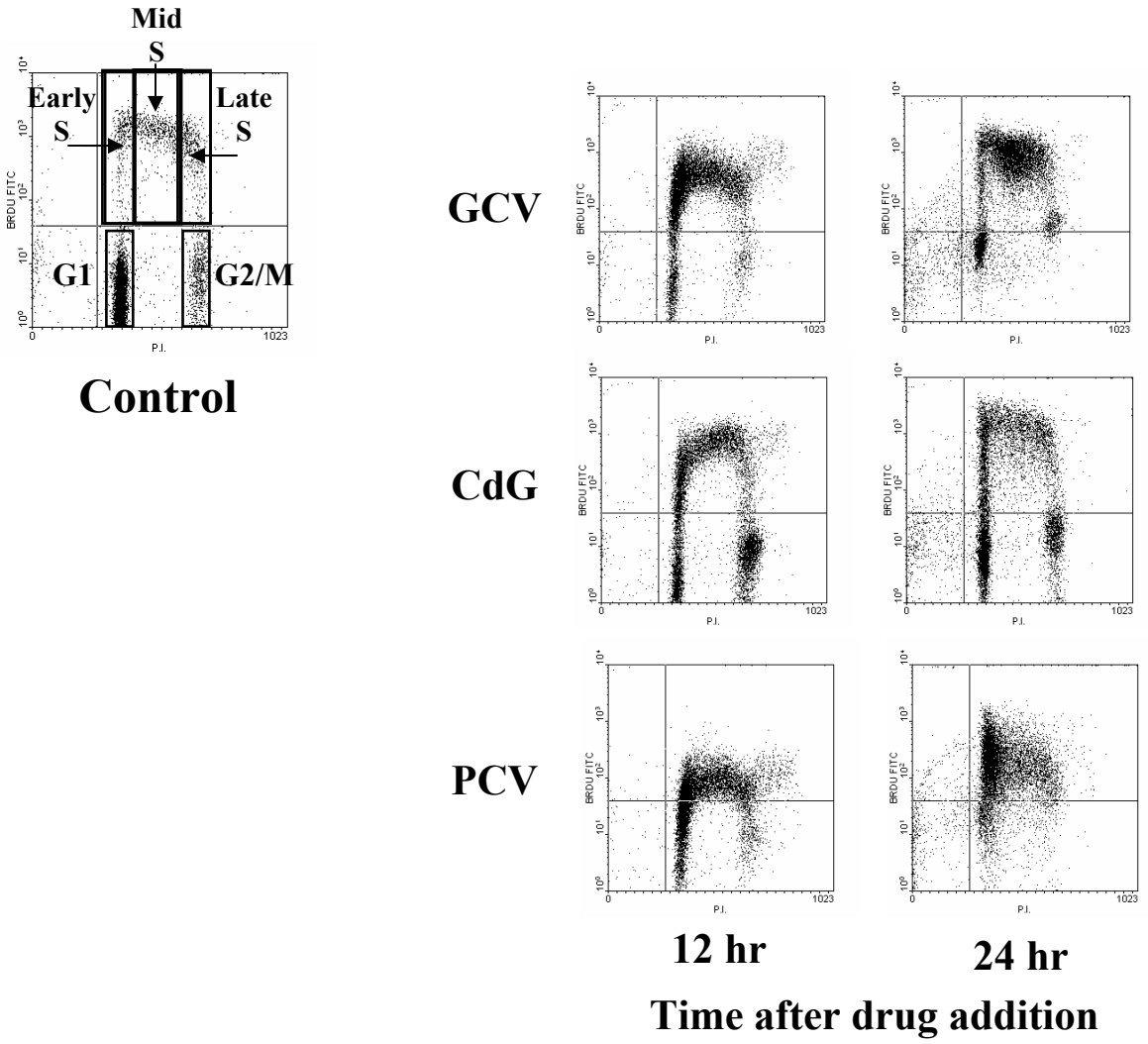

Figure 4.10. Effects of GCV, CdG, and PCV on the cell cycle distribution of U251tk cells during drug incubation. Cells were incubated with 1 μM GCV, 1 μM CdG, or 50 μM PCV for 24 h. Cells were analyzed at indicated time points during drug incubation Cells were incubated with 30 μM BrdUrd for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdUrd and DNA content as described in Materials and methods. Control represents a 24 h period without drug addition. Results of a single reproducible experiment are shown.

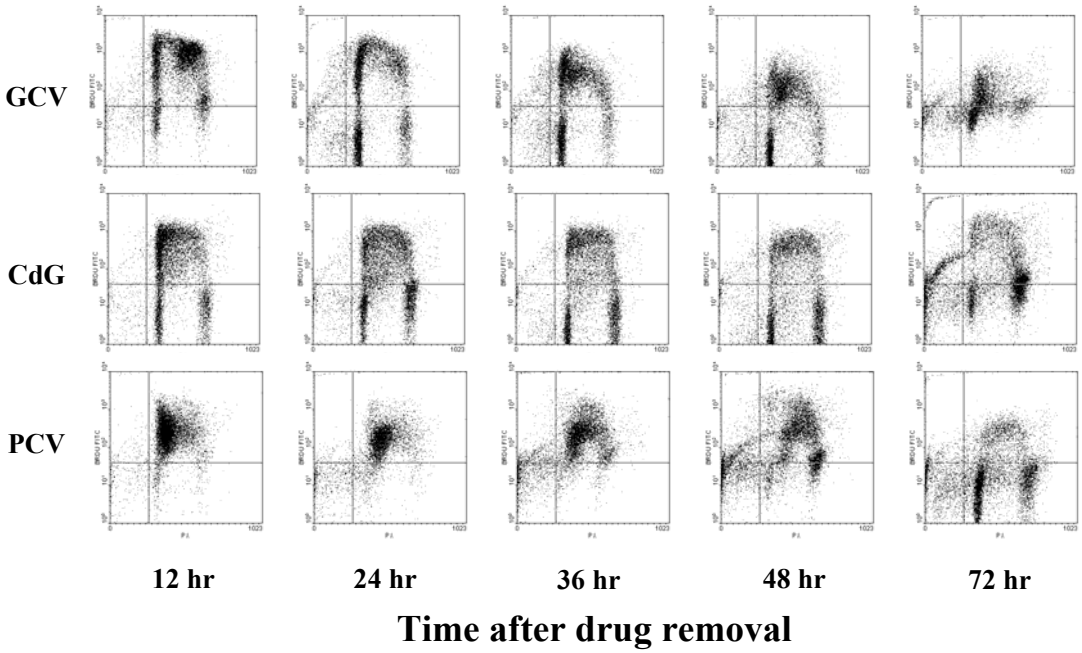

Figure 4.11. Effects of GCV, CdG, and PCV on the cell cycle distribution of U251tk cells following drug removal. Cells were incubated with 1 μM GCV, 1 μM CdG, or 50 μM PCV for 24 h. Drug containing medium was removed following the 24 h incubation and replaced with fresh drug-free medium. Cells were analyzed at indicated time points after drug removal. Cells were incubated with 30 μM BrdUrd for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdUrd and DNA content as described in Materials and methods. Control represents a 24 h period without drug addition. Results of a single reproducible experiment are shown.

Treatment	Time	G1 (%)	Early S (%)	Mid S (%)	Late S (%)	Total S (%)	G2/M (%)	Cell Number (x 10^6)	Mean BrdUrd Fluorescence (% Control)
Control	0 h	73.2	5.9	5.3	5.2	16.4	10.4	1.94	100
GCV	-12 h	10.7	55.8	19.0	11.2	86.1	3.2	0.96	39
	0 h	17.8	16.9	43.8	16.5	77.2	4.9	1.06	104
	12 h	10.0	23.9	28.8	28.1	80.8	9.2	1.19	103
	24 h	31.7	34.8	12.2	9.4	56.4	12.0	1.81	68
	36 h	31.6	40.7	14.0	7.0	61.7	6.7	1.6	35
	48 h	36.0	28.9	18.3	6.2	53.4	10.6	2.06	18
	72 h	22.2	47.1	15.7	12.1	74.9	2.9	0.74	12
CdG	-12 h	22.2	26.6	14.1	10.7	51.4	26.4	1.18	45
	0 h	33.6	27.8	13.0	10.8	51.5	14.8	1.49	62
	12 h	17.3	45.5	16.8	7.1	69.4	13.3	1.83	46
	24 h	23.3	17.6	21.1	15.5	54.2	22.4	1.84	52
	36 h	27.7	22.0	17.4	10.0	49.5	22.8	2.02	38
	48 h	31.1	16.5	15.3	9.9	41.7	27.2	1.78	36
	72 h	18.0	12.1	12.7	15.5	40.3	41.8	1.42	26
PCV	-12 h	27.3	39.9	17.9	10.2	68.0	4.7	0.93	11
	0 h	8.7	66.8	16.6	6.5	89.9	1.3	0.83	26
	12 h	2.6	77.2	15.7	3.8	96.7	0.7	0.86	37
	24 h	6.0	46.2	35.8	10.5	92.5	1.5	0.81	25
	36 h	2.7	56.8	31.4	8.7	97.0	0.3	0.62	30
	48 h	6.4	24.3	36.2	27.8	88.3	5.3	0.54	31
	72 h	40.6	12.7	12.5	11.7	36.9	22.5	0.29	16

Table 4.2. Effect of GCV, PCV, and CdG on the cell cycle distribution of U251tk cells. Effects of GCV, CdG, and PCV on the cell cycle distribution of U251tk cells. Cells were incubated with 1 μM GCV, 1 μM CdG, or 50 μM PCV 24 h. Drug containing medium was removed following the 24 h incubation (time=0 h) and replaced with fresh drug-free medium. Cells were analyzed at indicated time points during drug incubation (-12 h) and after drug removal. Cells were incubated with 30 μM BrdUrd for 15 min before harvest. Cells were then prepared for dual parameter flow cytometry to determine BrdUrd and DNA content as described in Materials and methods. Control represents a 24 h period without drug addition. Results of a single reproducible experiment are shown.

cell number during and after PCV-exposure, suggesting that the proportion of G_1 and G2/M cells observed at 72 hours after drug washout was higher because many of the S phase cells had died.

Discussion

Results presented here demonstrate that GCV induces a dose-dependent increase in mutation frequency with a predominance of GC→TA mutations. The unique pattern of mutations observed following exposure to GCV did not occur following treatment with two structurally similar HSV-TK substrates, CdG or PCV. Despite the fact that these nucleoside analogs all elicit cytotoxicity through incorporation into DNA, the consequences of this incorporation differ greatly, as demonstrated by distinct effects on DNA replication fidelity and cell cycle progression, resulting in significant differences in cytotoxicity.

We wished to determine whether the high cytotoxicity and delayed induction of DNA damage observed with GCV is due to the induction of excessive or unique mutations in DNA. Although GCV did induce a dose-dependent increase in mutation frequency, this alone is likely not responsible for the majority of GCV-induced cytotoxicity since there was not a significant increase in mutations at concentrations of GCV $<IC_{50}$ where significant cell death was observed. The induction of GC→TA mutations did occur at these lower drug concentrations. Although it may be possible that these specific mutations have especially deleterious effects, there are few scenarios in which mutations are sole contributors to cytotoxicity.

In previous studies characterizing structural effects of GCV incorporation into DNA, oligonucleotide duplexes were synthesized with and without GCVMP. Addition of GCVMP resulted in a decrease in melting temperature of 6.5°C decrease per drug residue (17), and disruption of the deoxyribose ring resulted in decreased stability of DNA. Determination of the solution structure of these GCVMP-containing duplexes demonstrated that the most significant distortions occurred at the site of GCVMP incorporation with a distinct kink in the sugar-phosphate backbone that extended two bases after GCVMP (Figure 4.12 and (18)). The authors hypothesized that this distortion would result in the pausing of DNA polymerases. It is intriguing to speculate that, with this pausing due to the presence of GCVMP, the DNA polymerases may insert incorrect nucleotides which would account for the induction of mutations observed in the studies presented here.

There are two possible explanations for the appearance of the more frequent C20A and C48A mutations. It is possible that insertion of GCVMP in a specific sequence prior to a required C residue alters the regional DNA conformation such that addition of an A is favored. This could be due to the acyclic nature of GCV which may allow more flexibility of the DNA structure, as suggested by Marshalko et al (17). Due to the small coding sequence of the supF cDNA, it was not possible to evaluate sequence-specific effects in this study, but would be of interest in future studies. In translesional synthesis in *Escherichia coli*, dAMP is preferentially incorporated opposite abasic sites in a phenomenon known as the "A rule" (19,20). If the A rule also applies to mammalian polymerases, the increased flexibility of GCV could result in the base being flipped out

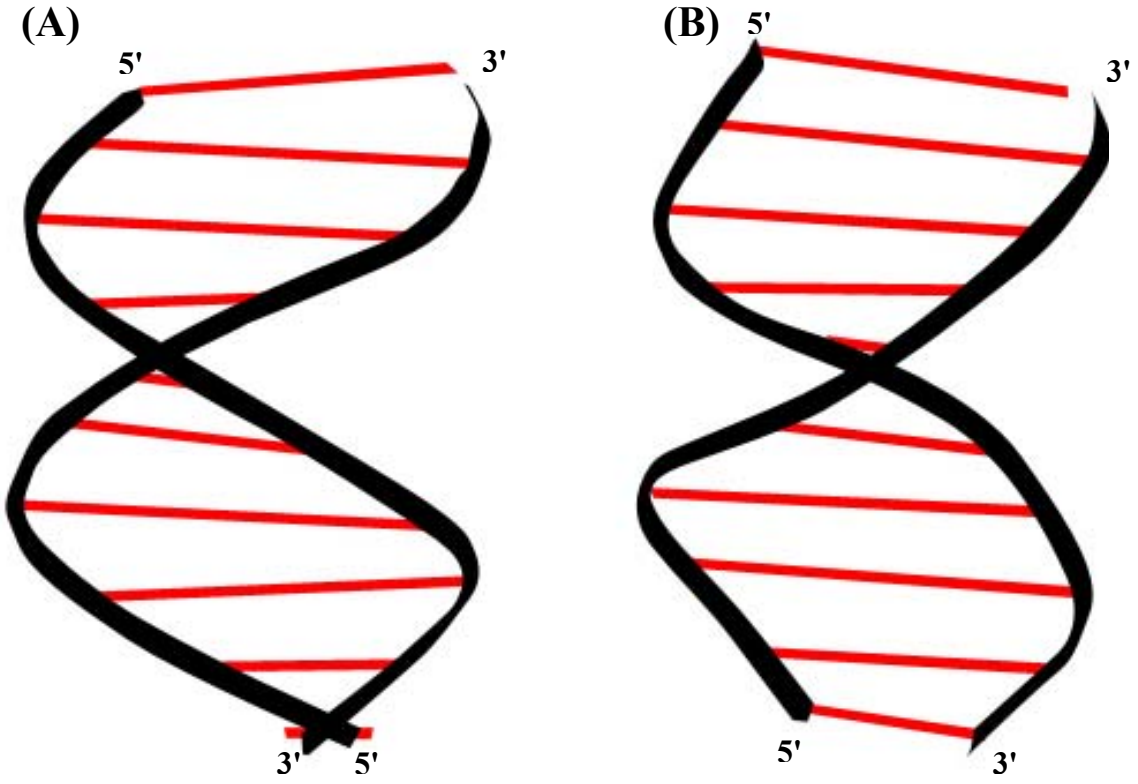

Figure 4.12. Effect of GCVMP incorporation on DNA structure. Structure of (A) control and (B) GCV-containing DNA duplexes. Control: 5'-CTGGATCCAG-3' GCV: 5'-CTGDATCCAG-3' (D=GCV) Adapted from Foti et al (18).

of the active site of the polymerase and an A being inserted by default, thus resulting in the predominance of C→A mutations.

The assay utilized in this study involves the replication of plasmid in both human cell lines and bacteria, and thus we cannot be certain that these mutations occurred during replication in the human cells or as a consequence of GCVMP incorporation in the nascent strand with subsequent mutation occurring in the bacteria. However, the likelihood that the mutations occurred during replication of the plasmids in human cells is supported by reports that GCV is genotoxic and carcinogenic in animal models which suggest a similar ability to induce mutations in replicating genomic DNA of mammalian cells.

The unique GCV-associated mutations were neither detected nor repaired by MMR, since the mutations predominated both in MMR-proficient U251TK and HCT116 1-2TK cell lines. A higher percentage of GC→TA mutations occurred in the MMR-proficient cell lines than in the MMR-deficient 0-1TK cell line, further demonstrating that these specific misincorporations are not repaired by MMR. The higher frequency of GC→TA mutations in the MMR-proficient cell lines did not translate into increased cell death, further demonstrating that these mutations cannot be solely responsible for the cytotoxicity of GCV. In addition, the data suggest that the enhancement of GCV cytotoxicity with HU in the MMR-deficient cells may be due to an increase in errors in DNA replication which are induced by HU. However, in the MMR-proficient HCT116 1-2TK cell line, the addition of HU activates MMR which decreases replication errors, resulting in antagonistic cytotoxicity.

We have shown in several different cell lines that GCVMP is well retained in DNA (1,4,6), suggesting that MMR is not capable of excising all of the fraudulent nucleotide. However, activation of MMR may result in the repair of other misincorporations. Although the mutation frequency with GCV is similar between MMR proficient and deficient cell lines, there is a greater predominance of GC→TA mutations in MMR proficient HCT116 and U251 cell lines, suggesting that these result from specific misincorporations which are not repaired by MMR. Alternatively, it is possible that attempted repair of GCVMP incorporation by MMR results in the production of GC→TA mutations by polymerase δ during the resynthesis step of the repair process. In addition to depletion of MMR, inhibition of DNA polymerase β, a gap-filling DNA repair polymerase, also sensitizes cells to GCV (21). These data demonstrate that there is a cellular repair response initiated by the presence of GCVMP in DNA. The presence of GCVMP in the template strand may cause polymerase δ or ε to pause at the site of incorporation, resulting in a stalled replication fork and the observed cell cycle arrest in S phase (Figure 4.13). A similar scenario could occur if the lesion was on the lagging strand, with inhibition of the polymerase α/DNA primase complex. Further research characterizing the interaction of GCVTP and GCVMP-containing DNA with DNA repair polymerases will increase our knowledge of the mechanism by which GCV induces cell death.

. Unlike GCV, neither CdG nor PCV induced a significant increase in mutation frequency, even at similarly toxic concentrations. CdG and PCV also did not alter the proportion of induced mutations. We have not observed this predominance of specific mutations with other drugs studied in this same system such as HU, 2',2'-difluoro-2'-

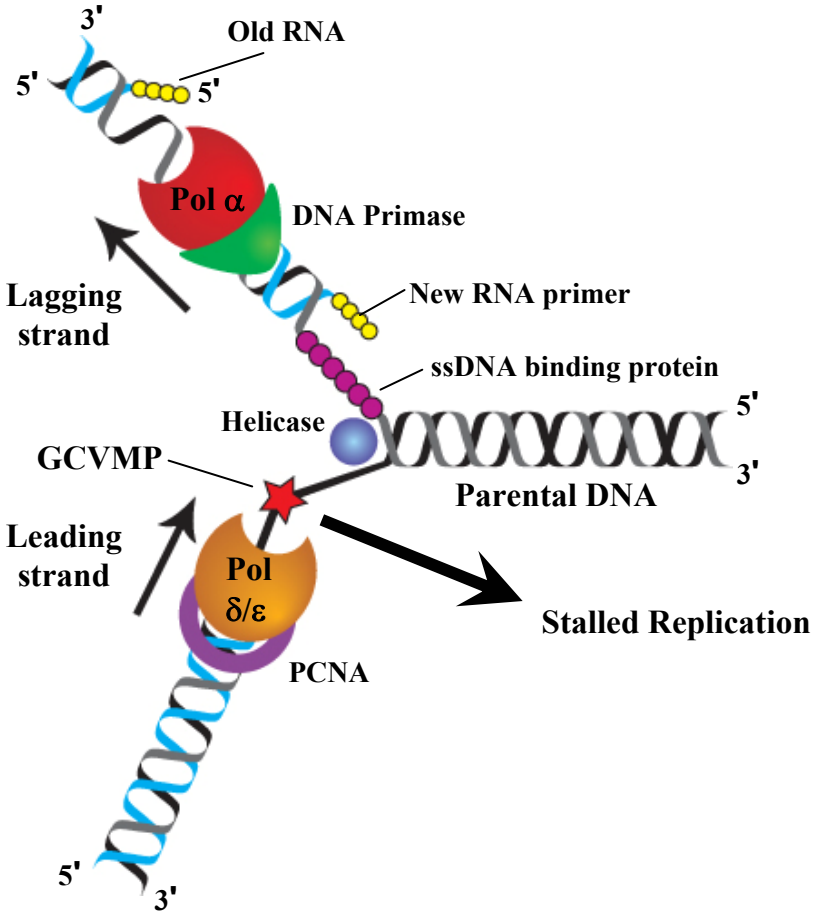

Figure 4.13. Proposed mechanism of cell cycle arrest in response to GCV. When DNA replication machinery encounters GCVMP (★) in the template strand during DNA synthesis, the DNA polymerase complex stalls. This stalled replication fork may be sufficient for S phase arrest and subsequent cell death.

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deoxycytidine, 1-β-D-arabinofuranosylcytosine, 5-fluoro-2'-deoxyuridine, and araT ((22); data not shown). Although very similar in structure to GCV, CdG and PCV did not induce this particular mutation nor did they produce an increase in other mutations under the conditions tested here.

Previous reports have demonstrated that GCV is more genotoxic than other HSV-TK substrates. ACV induced SCEs and chromosomal aberrations immediately after drug exposure, but only at very high concentrations, whereas GCV induced more SCEs and chromosomal aberrations at concentrations below IC_{50} and these events occurred during the second cell cycle after drug exposure (11,23). SCEs occur as a result of homologous recombination (24). This pathway may be activated in response to GCV-induced DNA damage, resulting in the formation of SCEs. PCV exposure resulted in significantly less plasmid mutations than GCV at both equimolar and equitoxic concentrations (12). Although there is no direct evidence that GCV is carcinogenic in humans, data presented here and in other reports describing the genotoxic properties of GCV indicate that the possible genetic risk of GCV should be considered. Studies of the long-term effects of GCV-induced genotoxic events in humans are warranted.

In addition to the differences in replication fidelity, the three deoxyguanosine analogs differed with respect to cell cycle progression. As we have reported previously, cells treated with GCV are able to complete one round of cell division following drug exposure and arrest in the subsequent S phase. This suggests that the observed S phase arrest is due to the presence of GCVMP in the template during DNA synthesis. Although GCV caused an S phase arrest and CdG produced more of a block in G_2/M, following exposure to either of these drugs cells were able to progress through the cell cycle. On

the other hand, the less cytotoxic PCV caused a more profound decrease in the rate of DNA synthesis compared to GCV and CdG with a strong delay in cell cycle progression. Similarly, araT, was previously reported to arrest cells in the first S phase during drug incubation but resulted in low cytotoxicity. This suggests that it is the ability of cells to divide with GCV or CdG present in their DNA which accounts for their greater cytotoxic effects.

While many nucleoside analogs elicit their effects by incorporating into DNA, the mechanism by which cells die and the extent of cytotoxicity is not always the same. The structurally related nucleoside analogs investigated here caused profoundly different interactions with DNA and distinct effects on cell cycle progression, resulting in differential cell killing. Further characterization of the consequences of DNA incorporation for nucleoside analogs will help in the development of more efficacious antitumor drugs.

Acknowledgments

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Chapter V

CONCLUSIONS

The major goal of this dissertation was to elucidate the mechanism by which GCV induces cell death in order to determine why GCV is more cytotoxic than other related drugs. Although HSV-TK/GCV gene therapy for cancer has been studied for nearly two decades, the mechanism of its cytotoxicity is still not completely understood. In recent years, the majority of published reports on this therapeutic approach involve methods of enhancing GCV cytotoxicity, mostly by the addition of other drugs or improvement of vector delivery of the HSV-TK gene, but mechanistic studies have been largely absent.

GCV is important because it is significantly more cytotoxic than other drugs: it has the ability to induce more cell death than most other nucleoside analogues under similar conditions (1). This powerful antitumor activity combined with a selective mode of delivery has great potential to improve the efficacy of cancer treatment while sparing normal tissue toxicity. The overall goal of this study was to elucidate the mechanism(s) for the superior cytotoxicity of GCV by characterizing the amount of DNA damage caused by GCV, its rate of repair, and the role of specific repair pathways. In addition, specific mutations in DNA induced by GCV were identified and compared to those

induced by other, less cytotoxic drugs. Understanding the lesions produced and the pathways that attempt to repair the GCV-induced lesions will allow us to improve this gene therapy approach to cancer treatment, as well as develop new drugs and targets to induce multi-log cell killing.

The studies presented here characterized DNA damage induced by GCV and repair pathways involved in responding to this damage. In Chapter II, we measured γ-H2AX foci as an indicator of GCV induced DNA damage. During GCV exposure, high levels of γ-H2AX foci are induced, however the subsequent decrease in this damage after drug removal suggests that this is repairable. Although there is not a complete cell cycle arrest during GCV incubation, cells are slowed in S phase (1). It has been reported that γ-H2AX foci are formed at stalled replication forks and do not solely represent DNA double strand breaks (2-4). This first increase in DNA damage may indicate slowing or stalling of replication machinery caused either by inhibition of DNA polymerases or reluctant extension of DNA following GCVMP incorporation, which is overcome after drug removal.

Coincident with the initial increase in γ-H2AX foci, only a small increase in Rad51 foci was also detected, suggesting that homologous recombination repair (HRR) is involved possibly to a minor degree in the repair of the initial damage. The second increase in DNA damage, measured by γ-H2AX foci, occurs after cells have divided and correlates with the timing of cell cycle arrest. This suggests that cells are not able to repair this damage, and this second onset of damage is what leads to cell death. It is possible that, when DNA replication machinery encounters GCVMP in template DNA, it causes replication to cease and replication forks to collapse. The large increase in Rad51

foci formed after drug washout and immediately preceding the second increase in γ-H2AX-detected DNA damage suggests that failed HRR may account for the lesions responsible for GCV cytotoxicity. The increased sensitivity of HRR-deficient yeast to GCV demonstrates that HRR is required to survive GCV-induced damage. Previous reports demonstrated that GCV induces sister chromatid exchanges, which also suggests a role for HRR in responding to GCV-induced DNA damage (5,6). HRR may be able to repair lesions or stalled replication forks during the first round of cell division during and following GCV exposure, thus sparing cells from death at this point. However, during the second cell cycle there is a strong induction of HRR which is unable to repair the second increase in DNA damage, resulting in cell cycle arrest and death. It will be interesting to directly measure the effect of inhibiting HRR on the induction of DNA damage with GCV to determine whether it is truly involved.

In contrast, araT, which is incorporated into DNA at >20 fold higher levels than GCV at similarly toxic concentrations (1), induces 7-fold less DNA damage than GCV as measured by γ-H2AX foci. These data demonstrate that cells are much more sensitive to GCVMP in DNA than araTMP, and the result of incorporation of these nucleoside analogs is quite different. Because it has a complete sugar ring, the presence of araTMP in DNA may have less of an effect on DNA stability than GCVMP which is acyclic. While both drugs are well retained in DNA after drug washout, surviving cells harboring araTMP resume DNA synthesis and cell cycle progression whereas GCVMP causes a permanent block in the second S-phase. This suggests that araTMP may serve as an adequate substrate in template DNA, but GCVMP does not. This could result in the

polymerase stalling or dissociating from the replication fork, activating cell signaling pathways leading to cell cycle arrest and ultimately death.

Because we wanted to focus on the consequences of GCV incorporation into DNA, it was logical to investigate repair pathways involved in responding to this damage. It had previously been reported that inhibition of DNA polymerase β sensitized cells to GCV (7). This polymerase has been implicated in DNA repair (8), suggesting that GCVMP incorporation is subject to repair. In order to more quickly and easily assay a wide variety of DNA repair pathways than would be possible in mammalian cells, in Chapter III we utilized a yeast-based screen of 96 DNA damage response mutants for increased sensitivity to GCV. Yeast deficient in genes involved in HRR, cell cycle checkpoints and, to a lesser extent, mismatch repair (MMR) exhibited significantly increased sensitivity to GCV, suggesting a role for these pathways in protecting from GCV-mediated cytotoxicity. We had already demonstrated a role for HRR in response to GCV in mammalian cells by showing that Rad51 foci were induced following exposure to GCV, and the results in yeast also implicated HRR in repairing GCV-induced lesions. Due to the inherent resistance of the yeast to GCV, we were unable to identify genes which conferred resistance to GCV when deleted. While the yeast assay allowed us to examine many DNA repair mutants for increased sensitivity to GCV and results in human cells have confirmed a role for MMR and HRR, the yeast results implicating cell cycle checkpoints in GCV cytotoxicity must be verified in mammalian cells.

In order to further characterize the role of MMR in responding to GCV-induced DNA damage, we characterized the sensitivity of HSV-TK-expressing HCT116 cell lines which are matched for MMR proficiency and deficiency and determined that the MMR-

deficient cell line was more sensitive to GCV, primarily at high concentrations. Interestingly, the proficient HCT116 cells actually accumulated more GCVMP in their DNA than their MMR-deficient counterparts at similarly toxic concentrations. Depletion of MLH1 by siRNA, a required MMR protein, sensitized two other cell lines, but only at high concentrations of GCV. siRNA resulted in depletion, but not complete elimination of MLH1, so basal MMR activity may still have been able to occur. The fact that the sensitization of MMR-deficient cells occurs primarily at high concentrations suggests that a threshold of damage exists where other pathways become overwhelmed and cell death is initiated. We must also consider the fact that while cells deficient in MLH1 are MMR deficient, MLH1 is known to participate in a variety of other pathways such as base excision repair, cell cycle checkpoints, and apoptosis (9-11). Future studies aimed at distinguishing the role of MLH1 in MMR vs. other DNA damage response pathways will help to elucidate the exact function of MLH1 in GCV cytotoxicity. In yeast, depletion of another required MMR protein, MSH2, conferred the same degree of sensitivity to GCV as deletion of MLH1, suggesting that the difference in sensitivity is indeed due to MMR.

We do not believe that MMR is able to protect cells from GCV cytotoxicity by removing GCVMP from DNA. We have shown that MMR-proficient cells had higher levels of GCVMP incorporation than the MMR-deficient cells at equitoxic concentrations. Excision of GCVMP from DNA has not been demonstrated directly, and any observed decrease in the amount of GCVMP in DNA of MMR-proficient HCT116 cells could be accounted for by an increase in cell number. While it is possible that rapid excision of some of the GCVMP residues from DNA by MMR during replication may occur, clearly a significant amount of GCVMP remains. This suggests that MMR

decreases GCV cytotoxicity by correcting some other GCV-induced DNA damage. MMR is responsible for correcting mismatched nucleotides in DNA which, if left unrepaired, could result in mutations.

In Chapter IV, we present studies of specific DNA lesions resulting from GCV treatment by characterizing the ability of GCV to induce mutations in DNA. GCV induced a dose-dependent increase in mutation frequency, with a predominance of GC→TA mutations. Because mutation frequency only increased at concentrations of GCV >IC_{50}, it appears unlikely that mutations are solely responsible for inducing cell death. Despite the fact that lower concentrations of GCV did not induce an increased total number of mutations, they did result in an increased percentage of specific GC→TA transversions, therefore cytotoxicity may be due to the nature of the induced mutations and not the overall number of mutations. These specific mutations were induced in both MMR proficient and deficient cell lines, and the MMR proficient cell lines (HCT116 1-2tk and U251tk) had a higher proportion of these mutations. These data suggest that these specific mutations are the result of misincorporations which are not readily repairable by MMR. The decrease in proportion of other types of mutations suggests that MMR repairs these, leaving a higher percentage of the GC→TA mutations. A study in NIH 3T3 cells determined relatively low repair rates for G:A (35%) and C:T (80%) mismatches as compared to G:T mismatches (100%) (12), so these misincorporations might be more prone to remain unrepaired.

A report of the solution structure of GCV in oligonucleotides illustrated that the phosphate-sugar backbone of GCV-containing DNA was kinked at the site of GCV incorporation (13). Because GCV is an acyclic deoxyguanosine analog, the open sugar

presumably would give GCV-containing DNA more flexibility, which may allow DNA to rotate in such a way that it promotes errors in polymerase insertion of nucleotides. We observed a high percentage of two specific C→A mutations (C20A and C48A) following GCV exposure. The high incidence of these specific mutations in three different cell lines suggests that the structure of these particular sequences of DNA make it more likely for the substitution of an adenosine in place of a cytosine. This could be due to the acyclic nature of GCV which may allow more flexibility of the DNA structure. Due to the small coding sequence of the supF cDNA, it was not possible to evaluate sequence-specific effects in this study, but would be of interest in future studies.

The oxidized purine 8-oxoguanine (8oxoG), a DNA lesion resulting from reactive oxygen species, is able to incorrectly base pair with A. If this mispair is not repaired, it also results in a GC→TA transversion. Oxidation of the guanine at C8 turns N7 into a hydrogen bond donor, and this allows a 8oxoG:A base pairing which does not disrupt either the polymerase active site or the DNA structure (14,15). Because there is not a major alteration in structure as would occur with a G:A base pair, 8oxoG:A mispairs readily evade proofreading. It is possible that the GC→TA mutations observed following treatment with GCV are a result of A being incorrectly inserted across from a GCV, as the loss of the structural constraint of a cyclic sugar ring may allow GCV to assume a different conformation which allows non-canonical basepairing capable of avoiding repair as well. This mechanism would require that the GC→TA mutations we detected were the result of GCVMP present in template DNA. While we believe the conditions favored incorporation of GCVMP into the nascent strand only, the possibility that the plasmids were replicated more than once in the human cells cannot be ruled out.

Although very similar in structure to GCV, neither CdG nor PCV were able to increase mutation frequency or the relative proportion of GC→TA or any other type of mutations. The specific C20A and C48A mutations did not occur following exposure to either CdG or PCV. CdG is not acyclic, supporting our hypothesis that it is the flexibility of the acyclic sugar which allows GCV to induce these mutations. PCV is also acyclic, however, it did not induce mutations. The only difference in structure between PCV and GCV is that PCV has a carbon instead of an oxygen on the acyclic ring, and it is possible that the removal of the oxygen decreases hydrogen bonding necessary for mutations to be induced. If increased flexibility is involved in GCV's formation of mismatches, the lack of an oxygen on the sugar ring of PCV may decrease flexibility and retain the structural constraint provided by an intact sugar ring and not allow for the interaction to occur. In addition, PCV causes significantly greater inhibition of DNA synthesis than GCV. In primer-template assays, PCVTP has been reported to be incorporated into DNA less efficiently than either ACVTP or GCVTP by DNA polymerases α, δ, and ε and, although PCV is not a chain terminator, further extension after incorporation of PCV is reluctant (16). PCVTP is a poor inhibitor of polymerases, so it likely inhibits DNA synthesis after incorporation into DNA. Since synthesis of DNA would be required for induction of mutations, this inhibition would result in a decreased mutation frequency as observed with PCV.

We must also consider the fact that this mutation assay involves the replication of plasmid in both human cell lines and bacteria, and we cannot determine in which organism these mutations were created. This pattern of mutations is unique to GCV and not simply an artifact of plasmid replication in bacteria, as we have performed this assay

with many other drugs and have never obtained similar results. Future experiments characterizing mutations in chromosomal DNA in human cells will verify these results and indicate where these mutations are being generated. Reports that GCV is genotoxic and carcinogenic in animal models support our findings that it can induce mutations in replicating plasmid DNA.

Although the studies presented in this dissertation are in the context of gene therapy for cancer, it is also important to note the GCV is widely used to treat cytomegaloviral infections in immunocompromised patients. Animal studies indicated that GCV is both mutagenic and carcinogenic, while rats and mice exposed to 2 to 8-fold more ACV or PCV than what is administered to humans did not have a significant increase in the incidence of tumors [reviewed in (17)]. It will be important to clarify how these experimental findings translate to the clinical setting in order to identify when the risks outweigh the benefits. In a small study of 7 AIDS patients who were treated with the anti-retroviral nucleoside analog azidothymidine, peripheral lymphocytes were examined for chromosomal aberrations and it was determined that 8% of lymphocytes had aberrations while the rate of aberrations was only 0.5% in untreated AIDS patients (18). Similar studies monitoring patients treated long-term with GCV for chromosomal aberrations, sister chromatid exchanges, gene mutations, and incidence of cancer should be conducted.

Not only do the small changes in structures of these drugs result in different effects on fidelity of DNA replication, but they all cause different patterns of cell cycle progression. Due to its strong inhibition of DNA synthesis, PCV arrests cells in S phase during drug incubation. In contrast, cells treated with either GCV or CdG are able to

complete one cell division cycle after drug exposure because they do not inhibit DNA synthesis, and they subsequently arrest in the second S or G2/M phases, respectively. Both GCV and CdG have the ability to induce multilog cytotoxicity, suggesting that it is the ability to complete one round of the cell cycle with the analog present in DNA which results in increased cell killing as compared to other chemotherapeutic agents. If cells continue to divide without recognizing the presence of nucleotide analogs in their DNA, the collision of the replication machinery with the analog may induce damage too severe for repair and make cell death inevitable. DNA repair varies depending upon whether the lesion is in the template or occurs during nucleotide insertion in the nascent strand, as most repair processes, such as MMR, repair mistakes in the newly synthesized strand and few others, such as transcription coupled repair, recognize lesions in the template (19). Frequency of misincorporations at modified nucleotides depends upon whether the modified nucleotide is in the template or as the incoming triphosphate (20,21). Because GCVMP is present in DNA of cells after they have completed one round of DNA replication, it is not completely excised by repair pathways involved in proofreading newly synthesized DNA. This would suggest that any repair which may occur is initiated following the recognition of GCVMP in the template strand. My studies demonstrating a second increase in γ-H2AX foci occurring with similar timing to the observed S phase arrest and immediately following an increase in Rad51 foci suggest that stalled replication forks are induced. DNA replication machinery may be unable to situate properly on DNA with GCVMP incorporated to continue replication past the lesion, resulting in a stalled replication fork. Rad51 is then recruited to re-start the stalled fork

but cannot get past the lesion, leading to the accumulation of lethal HRR intermediates and ultimately cell death.

The data presented here suggest a variety of mechanisms to modify clinical therapy with GCV. Perhaps the most successful clinical trials of HSV-TK/GCV gene therapy have been in prostate cancer (22), one of many human tumor types which are deficient in DNA damage response pathways, such as MMR (23,24). It has been reported that 2 to 50% of prostate cancers are defective in MMR (25). The data presented here suggest that MMR deficient tumors would respond better to HSV-TK/GCV treatment than MMR proficient tumors since MMR deficient cells are more sensitive to GCV. Although the data presented here demonstrate increased sensitivity in MMR deficient cells occurred with high concentrations of GCV, these concentrations represent clinically relevant doses. In addition, the yeast screen suggests that tumors defective in certain checkpoint pathways would also respond better to GCV. Since normal tissues are generally proficient in these pathways, targeting MMR and checkpoint defective tumors would improve selectivity of this therapy. These studies also suggest other pathways, such as HRR, could be targeted in combination with GCV treatment to enhance GCV cytotoxicity. Cells that lack BRCA1 or BRCA2 function are deficient in HRR and exhibit increased sensitivity to DNA damaging agents mytomycin C and etoposide and may also be more sensitive to GCV (26-29). Because overexpression of Rad51 is common in malignant cells (30), it will be interesting to explore the effect of this overexpression on GCV cytotoxicity in human cells.

In the future, it will be important to determine the extent and timing of DNA damage induced by CdG. It is my hypothesis that CdG will also induce high amounts of

DNA damage, and that this damage will occur similarly to that observed with GCV. If the multilog cytotoxicity is due to the fact that these analogs remain undetected in DNA until the second round of DNA replication following exposure, it will be interesting to characterize which, if any, repair pathways are initiated immediately preceding cell cycle arrest or if this is simply caused by failed DNA synthesis. Further characterization of the mechanism by which GCV and CdG cause multi-log cytotoxicity may provide the basis for development of new anti-tumor agents with the same ability which may be superior to those in use today.

Although the research presented here has added significantly to the knowledge of GCV's mechanism of action, there remain questions which will require further research to answer. It will be of interest to determine if GCV produces the same abundance of GC→TA mutations in mammalian chromosomal DNA, which could be accomplished by using an assay for mutations induced in the hypoxanthine-guanine phosphoribosyl transferase locus by evaluating resistance to 6-thioguanine. A much more complex issue will be to determine why these specific mutations are induced by GCV. It will be beneficial to more fully understand the consequence of GCV incorporation on DNA structure and characterize the interaction of GCV, in the triphosphate form as well as the monophosphate incorporated into DNA, with polymerases and other components of DNA replication machinery. This will help determine if these mutations arise from addition of the incorrect nucleotide opposite GCV, replication errors caused by changes in structure of GCV-containing DNA, or another mechanism.

In addition to understanding the nature of the lesions produced by GCV, it will also be of great interest to further investigate repair pathways involved in responding to

and repairing this damage. My research has generated preliminary data implicating several repair pathways, such as HRR, cell cycle checkpoints, and MMR, and future work will characterize the exact involvement of these pathways in responding to GCV-induced damage. For example, it will be important to determine if depletion of proteins essential for HRR in mammalian cells change sensitivity to GCV or induction of DNA damage.

There have been very few published reports of activation of DNA damage signaling pathways as a result of GCV exposure. The involvement of specific pathways can be determined by characterizing activation of proteins such as ATR, Chk2, p53, and caspases. Elucidation of these signaling pathways may aid in the determination of what recognizes the DNA lesions produced by GCV and the cause of the observed S phase arrest and will provide addition insight into the mechanism of action of GCV.

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