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Alteration of the carbohydrate for deoxyguanosine analogs markedly changes DNA replication fidelity, cell cycle progression and cytotoxicity

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Abstract

Nucleoside analogs are efficacious cancer chemotherapeutics due to their incorporation into tumor cell DNA. However, they exhibit vastly different antitumor efficacies, suggesting that incorporation produces divergent effects on DNA replication. Here we have evaluated the consequences of incorporation on DNA replication and its fidelity for three structurally related deoxyguanosine analogs: ganciclovir (GCV), currently in clinical trials in a suicide gene therapy approach for cancer, D-carbocyclic 2'-deoxyguanosine (CdG) and penciclovir (PCV). GCV and CdG elicited similar cytotoxicity at low concentrations, whereas PCV was 10–100-fold less cytotoxic in human tumor cells. DNA replication fidelity was evaluated using a supF plasmid-based mutation assay. Only GCV induced a dose-dependent increase in mutation frequency, predominantly GC→TA transversions, which contributed to cytotoxicity and implicated the ether oxygen in mutagenicity. Activation of mismatch repair with hydroxyurea decreased mutations but failed to repair the GC→TA transversions. GCV slowed S-phase progression and CdG also induced a G2/M block, but both drugs allowed completion of one cell cycle after drug treatment followed by cell death in the second cell cycle. In contrast, PCV induced a lengthy early S-phase block due to profound suppression of DNA synthesis, with cell death in the first cell cycle after drug treatment. These data suggest that GCV and CdG elicit superior cytotoxicity due to their effects in template DNA, whereas strong inhibition of nascent strand synthesis by PCV may protect against cytotoxicity. Nucleoside analogs based on the carbohydrate structures of GCV and CdG is a promising area for antitumor drug development.

Keywords

Ganciclovir; Penciclovir; Carbocyclic deoxyguanosine; Mutation frequency; DNA synthesis

1. Introduction

Nucleoside analogs are a mainstay of treatment for a variety of tumor types, including leukemias (e.g. cytosine arabinoside) and solid tumors (e.g. gemcitabine) [1]. In addition, antiviral nucleoside analogs, such as ganciclovir (GCV), are being evaluated in a suicide gene therapy approach to cancer [2]. These drugs all share a requirement for activation by intracellular phosphorylation, after which they serve as fraudulent substrates for DNA replication. Incorporation of these analogs into DNA correlates with cytotoxicity, and this event is thought to be the primary mechanism of antitumor activity for this class of drugs [3,4].

Despite their similarities, nucleoside analogs elicit vastly different effects on cell survival which cannot be explained simply by their relative amounts of incorporation into DNA [5]. While a great deal of information has been generated on the ability of this drug class to enter cells, undergo activation and incorporate into DNA, far less attention has been paid to the consequences of drug incorporation on subsequent DNA replication. Previous studies have evaluated the interactions between nucleoside analogs and the DNA replication machinery utilizing cell-free systems with primer templates, generally a single purified polymerase, and the analog triphosphate [6–9]. While these studies have provided some insight into the interaction between the analog and a single polymerase on a defined template, it does not accurately represent the situation in intact cells where several replicative and translesion polymerases work together with repair complexes to complete DNA replication [10]. For example, the triphosphates of cytosine arabinoside and gemcitabine produce strong inhibition or termination of replication on primer-templates with human DNA polymerases α , δ or ϵ , which would be expected to result in large deletions in DNA [7,9,11]. However in intact cells these drugs slow but do not halt completely DNA replication [12,13], likely due to the presence of additional polymerases and repair factors that function to assist the cell in correcting or bypassing DNA lesions [10,14]. While fluoroadenine arabinoside and adenine arabinoside are capable of causing DNA termination in intact cells to produce gene deletions [11,15], for other nucleoside analogs little is known about their effects on the fidelity of DNA replication in intact cells. We have demonstrated recently that replication of plasmid DNA in tumor cells in the presence of gemcitabine produces an increase in single base substitutions, whereas cytosine arabinoside does not which may contribute to the differences in cytotoxicity observed with these drugs [16].

Here we have extended our studies of the effects of nucleoside analogs on DNA replication using the deoxyguanosine analog ganciclovir (GCV). This drug was initially developed as an antiherpesvirus agent, and it is currently used for treatment or prevention of cytomegalovirus infection in immunocompromised individuals [17]. More recently, GCV has been used in a suicide gene therapy approach to cancer treatment, in which the herpes simplex virus thymidine kinase (HSV-TK) is introduced into tumor cells followed by systemic treatment with GCV [2]. HSV-TK/GCV suicide gene therapy is undergoing evaluation in a variety of malignancies, with promising results in clinical trials for prostate cancer and for treatment of graft vs. host disease following stem cell transplant [2,18].

We have reported that GCV induced a unique manner of delayed, S-phase specific cell death following its incorporation into DNA [5]. Thust et al. have demonstrated that GCV induced sister chromatid exchanges (SCEs) and structural chromosome aberrations, while acyclovir and the related penciclovir (PCV) did not [19–23]. Furthermore, Foti et al. have demonstrated that GCV in a decamer duplex distorted the duplex conformation 3' to the GCV residue. However, while these studies highlight the effects of GCV after its incorporation into DNA in an intact cell or oligonucleotide, they do not reveal the lesions in DNA that may occur in cells exposed to GCV. We wished to determine the effect of GCV

on the fidelity of DNA replication in intact cells to determine if it induces distinct aberrations that can account for the unique mode of cell killing. Since we have previously demonstrated a role for mismatch repair (MMR) in cytotoxicity with GCV [24], we also evaluated the effect of this repair pathway on the fidelity of DNA replication with GCV. In addition, we evaluated the role of the carbohydrate by comparing the effect of GCV to that of the structurally related acyclic PCV [25], as well as to D-carbocyclic 2'-deoxyguanosine (CdG) [26] which is much less flexible than either GCV or PCV. All three compounds contain guanine as the base. This series of analogs allowed us to evaluate the effect of small differences in carbohydrate structure on replication fidelity and cytotoxicity. Using a plasmid shuttle vector assay, the ability of GCV, PCV and CdG to induce DNA mutations was characterized [27]. The results demonstrate that, despite the similarities in structure and/or antiviral efficacy for GCV, CdG and PCV, they have profoundly different effects on the both the number and type of mutations that they induce, which may contribute to their differences in mechanism of cytotoxicity in tumor cells.

2. Materials and methods

2.1. Cell culture

HCT116 human colon carcinoma and U251 human glioblastoma cells were maintained in Dulbecco's Modified Eagle Medium or RPMI (Invitrogen Life Technologies, Grand Island, NY), respectively, in an atmosphere of 37 °C and 5% CO₂. Growth medium was supplemented with 2mM 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. HCT116 0–1tk, HCT116 1–2tk, and U251tk cell lines were derived after transduction with a retroviral vector encoding the cDNA for HSV-TK along with the neomycin resistance gene as previously described, which allowed phosphorylation of the deoxyguanosine analogs [5]. Transgene expressing cells were selected and maintained with 1000 and 400 µg/mL G418 (Invitrogen), respectively.

2.2. 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.

2.3. HPLC analysis of analog triphosphates

U251tk cells were incubated with GCV, CdG or PCV for 24 h, followed by drug washout and continued incubation in drug-free medium for 24 h. Cells were harvested at 4 and 24 h after drug addition and 24 h after drug washout for analysis of the analog 5'-triphosphates. Nucleotides were extracted from cells, then separated and quantitated by HPLC as previously described [28]. Each analog triphosphate separated completely from GTP, dGTP and other endogenous nucleotides. Analog triphosphates were analyzed in duplicate at each time point, and each experiment was performed once or twice.

2.4. pSP189 plasmid shuttle vector mutation assay

HCT116 and U251 cells were transfected overnight with the pSP189 plasmid [29] using SuperFect transfection reagent (Qiagen). Medium was replaced and drug was added for 24 h (one cell doubling time), followed by 24 h in drug-free medium to allow completion of DNA replication. pSP189 plasmid was then harvested and isolated using a Qiagen Miniprep

kit, incubated with *DpnI* (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 μ L of TE containing plasmid DNA and 20 μ L of electrocompetent MBM7070 *E. coli*. The transformation mixtures were plated onto agar plates containing 100 μ g/mL ampicillin (Roche), 50mg/mL isopropyl- β -thio-B-D-galactopyranoside (Invitrogen), and 20mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Roche). After 24 h incubation, 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).

2.5. Cell cycle progression and DNA synthesis

Flow cytometric analysis was performed as previously described [30]. Briefly, at the conclusion of the drug incubation, cells were pulse labeled with 30 μ M BrdUrd for 15 min, treated to bind antibody to BrdUrd in DNA and then stained with propidium iodide. Cell cycle analysis was performed using a Coulter EPICS Elite ESP flow cytometer (Coulter, Hialeah, FL, USA). Percent DNA synthesis was determined by the change in the mean fluorescence intensity of BrdUrd-incorporating cells.

3. Results

3.1. GCV-induced mutations

In order to measure the ability of GCV to induce mutations, a well characterized plasmid-based shuttle vector assay was employed [29]. 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 the indicator *E. coli* MB7070 strain allows blue/white screening for supF mutations in bacterial colonies stained with X-gal. When U251tk cells were incubated for 24 h (one cell doubling time) with a broad range of GCV concentrations (IC_{10} to $\geq IC_{90}$), a dose dependent increase in plasmid mutation frequency was observed (Fig. 1A). At concentrations of GCV ≥ 0.1 μ M (IC_{75}), the increase in mutation frequency was significantly different from control, achieving nearly a 4-fold increase at a concentration of 1 μ M.

Analysis of the nature of the resulting mutations revealed that GCV induced predominantly GC $\rightarrow$ TA transversions (Fig. 1B). Interestingly, at 0.03 and 0.05 μ M GCV there was no significant increase in mutation frequency, yet GC $\rightarrow$ TA transversions accounted for 56–72% of the total mutations compared to only 33% in control cells. At higher concentrations of GCV, up to 81% of the mutations were GC $\rightarrow$ TA transversions. The total increase in mutation frequency can be accounted for by the increase in GC $\rightarrow$ TA mutations, and the majority of these were C $\rightarrow$ A mutations.

Further analysis of the mutations revealed two sites in the supF tRNA sequence where the majority of GCV-induced mutations occurred (Fig. 2A). Following GCV exposure, the most frequent mutation was C $\rightarrow$ A at position 118 (C118A), accounting for 15–53% of total mutations. The prevalence of this mutation increased at higher GCV concentrations. The second most common mutation following treatment with GCV was C $\rightarrow$ A at position 146 (C146A), which accounted for up to 20% of the mutations. Although mutations at these sites were observed in control cells, they accounted for <5% of the total number of mutations. In order to ensure that each C118A and C146A mutation in supF tRNA represented separate

mutagenic events, we evaluated the 8 base pair signature sequence in pSP189 which provides over 65,000 unique signature sequences within the plasmid population [29]. This analysis demonstrated that each plasmid carrying a mutation had a unique signature sequence, and thus the predominance of the C→A mutations was not due to overrepresentation of a single plasmid.

3.2. Effect of mismatch repair on GCV-induced mutations

Previously we have determined that the absence of a functional MMR pathway enhanced cytotoxicity at high concentrations ($>IC_{90}$) of GCV [24]. 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 HCT116 colon carcinoma cells expressing HSV-TK that are either deficient (0–1tk) or proficient (1–2tk) in MMR. In addition, hydroxyurea (HU) was used to produce an imbalance in dNTP pools (via inhibition of ribonucleotide reductase) which induces mismatches in DNA and thereby activates MMR. 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 (Table 1). The addition of HU at 1 or 3mM decreased the IC_{50} for GCV in the MMR-deficient 0–1tk, and the combination was additive (determined by isobologram analysis; data not shown). In contrast, the combination of HU and GCV was antagonistic, as it increased the IC_{50} for GCV by ≥ 2 -fold in the MMR-proficient 1–2tk cells.

In order to determine if the differences in cytotoxicity with GCV+HU in the HCT116 cells reflected MMR-mediated correction of mismatched nucleotides in DNA, the mutation frequency was measured following treatment with GCV±HU for one cell doubling time in both cell lines. Although the mutation frequency of untreated cells was higher in the MMR-deficient compared to the MMR-proficient cells ($0.11\pm 0.01\%$ and $0.06\pm 0.01\%$, respectively, $p = 0.03$), $1\ \mu\text{M}$ GCV produced similar mutation frequencies in both the MMR-deficient 0–1tk and MMR-proficient 1–2tk cells ($0.17\pm 0.02\%$ and $0.14\pm 0.02\%$, respectively, $p = 0.37$) (Fig. 3A). Treatment with 2mM HU resulted in a significant increase in mutation frequency in the MMR-deficient 0–1tk cells ($0.28\pm 0.03\%$, $p = 0.005$), but not in the MMR-proficient 1–2tk cells ($0.03\pm 0.01\%$) compared to untreated controls, as expected. The combined treatment with GCV and HU produced an additive increase in mutation frequency in the MMR-deficient 0–1tk cells ($0.39\pm 0.06\%$). However, in the MMR-proficient 1–2tk cells, the combination of GCV and HU resulted in a significant decrease in the mutation frequency ($0.06\pm 0.01\%$) compared to cells treated with GCV alone.

Analysis of the nature of the resulting mutations again revealed that GCV induced a predominance of GC→TA transversions in both the HCT116 MMR-deficient 0–1tk and MMR-proficient 1–2tk cells (49% and 88%, respectively, Fig. 3B and C) whereas this specific type of mutation accounted for $<30\%$ of the total mutations in control cells from each cell line (Fig. 3B and C). Furthermore, HU induced single base substitutions in which no single type of mutation accounted for more than 35% of the total mutations. The combined treatment with GCV and HU in the MMR-deficient 0–1tk cells resulted in a pattern of mutations more closely resembling those induced by HU than GCV alone, consistent with the finding that HU produced more mutations than GCV. Although in the MMR-proficient HCT116 1–2tk cells the drug combination resulted in a decrease in the overall mutation frequency, GC→TA mutations still predominated (64%). The C118A mutation predominated in the MMR-proficient HCT116 1–2tk cells (55%) (Fig. 2C) but represented only 7% of total mutations in the MMR-deficient cells (Fig. 2B). The addition of HU resulted in fewer of the C118A mutations in both cell lines; however they still represented 31% of total mutations in the HCT116 MMR-proficient 1–2tk cells, whereas they were absent in the MMR-deficient 0–1tk cells.

3.3. Effect of alteration of the carbohydrate structure on mutation induction

To evaluate the importance of the acyclic moiety of GCV on nucleotide misincorporation into DNA, we compared mutations induced by GCV to those induced by two structurally related deoxyguanosine analogs. PCV is similar to GCV except that the ether oxygen is replaced by methylene in the acyclic moiety. CdG has the same replacement of methylene for the ether oxygen as PCV, but also contains another methylene to produce a carbocyclic analog (Fig. 4A), which resembles the natural 2'-deoxynucleosides and has a reduced flexibility compared to the other two molecules. GCV and CdG are similarly toxic in U251tk, whereas PCV was 10–100-fold less cytotoxic at equimolar concentrations (Fig. 4B). These concentrations of analogs were not toxic to non-HSV-TK-expressing cells, as reported previously [31,32]. The difference in analog cytotoxicity in the U251tk cells was not due to higher concentrations or better retention of GCV or CdG triphosphates. HPLC analysis of cell extracts at 4 and 24 h after drug addition demonstrated that 10–20 times more PCV triphosphate accumulated in cells from 10 μ M PCV compared to the amounts of analog triphosphate that accumulated from 1 μ M GCV or CdG. Furthermore, PCV triphosphate was better retained after drug washout, as 30.8% of the 24 h value remained in cells at 24 h after drug washout, whereas only 18.9% and 1.2% of the triphosphates of GCV and CdG, respectively, was retained at 24 h post-drug washout. Neither 1 μ M CdG (IC₉₉) nor 10 μ M PCV (IC₉₃) produced a significant increase in mutation frequencies compared to control (no drug treatment) cells (Fig. 4C). In addition, there was not a predominance of any specific mutation following exposure to these drugs (Fig. 4D), and neither the C118A nor C146A mutation occurred with either of these two drugs (data not shown).

3.4. Effect of carbohydrate structure on DNA synthesis and cell cycle progression

Because the structural alterations 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 caused cytotoxicity. Previously we have demonstrated a unique pattern of cell cycle progression and delayed cell death after treatment with GCV, compared to other nucleoside analogs [5], and we wished to determine whether CdG and/or PCV elicited similar effects. Consistent with a previous report examining DNA content alone [5], dual parameter flow cytometry evaluating DNA content and rate of DNA synthesis demonstrated that cells treated with GCV were slowed in S-phase during a 24 h incubation (Table 2). After drug washout, cells began to progress through the cell cycle, as indicated by the increase in cells from early to mid to late S. The increase in G₂/M cells at 12 h after drug washout, followed by increases in G₁ and early S-phase cells, plus a 2-fold increase in cell number by 24 h post washout demonstrated that these cells divided and then entered G₁. Measurement of DNA synthesis by mean BrdUrd fluorescence demonstrated that there was moderate inhibition during drug incubation, and DNA synthesis increased to control levels as cells progressed through the cell cycle. However, the capacity to synthesize DNA decreased dramatically after mitosis (24 h and later times after drug washout) despite the fact that more than half of the cells were in S-phase. Cells appeared permanently blocked early in the second S-phase, as DNA synthesis and cell number decreased through 72 h post drug washout.

Similar to GCV-treated cells, following exposure to CdG cells were able to continue through the cell cycle and divide, as indicated by an approximate 2-fold increase in cell number by 36 h after drug washout (Table 2). However, they were slowed as they progressed through G₂/M, as this cell cycle fraction remained elevated during drug incubation and after drug washout. As cell number decreased during the second cell cycle, a more pronounced block in S and G₂/M was apparent. CdG produced a moderate block in DNA synthesis during and after drug incubation.

PCV-treated cells exhibited a pattern of cell cycle progression distinct from either GCV or CdG. Within 12 h after drug addition, there was a clearing of cells from G1 and G2/M while the majority of cells accumulated in early S and remained there until at least 12 h after drug washout. The prolonged early S-phase block reflected the strong inhibition of DNA synthesis during drug incubation. By 24 h after drug washout, cells began to progress from early to mid and late S-phase. However, in contrast to the results with GCV and CdG, the majority of PCV-treated cells did not divide as indicated by the continuous decrease in cell number during and after PCV exposure. While the increase in G2/M cells at 72 h may indicate that some cells were able to progress out of S-phase, the increase in G1 cells while cell number declined indicates that many cells died in S leaving a higher proportion of surviving cells in G1 and G2/M.

4. Discussion

The majority of cytotoxic nucleoside analogs produce cell death following their intracellular phosphorylation to a 5'-triphosphate and subsequent incorporation into DNA in place of the corresponding deoxynucleotide. Studies have demonstrated a strong correlation between DNA incorporation and cytotoxicity for nucleoside analogs, including the commonly used anticancer drugs cytosine arabinoside and gemcitabine [3,9]. We have demonstrated previously that these drugs differ substantially with respect to their effects on DNA replication fidelity, with gemcitabine much more capable of inducing misincorporations into DNA compared to cytosine arabinoside [16]. These drugs both share the same cytosine base, but differ in their carbohydrate with substitution of the 2' hydrogens for fluorine atoms in gemcitabine, whereas for cytosine arabinoside a 2' hydrogen is replaced with a hydroxyl in the "up" configuration. We were then interested in evaluating the effect of other carbohydrate substitutions on DNA replication fidelity. The structural similarities of GCV, CdG and PCV allowed an interesting comparison of an acyclic carbohydrate with either an ether oxygen or methylene group (GCV and PCV, respectively) compared to the carbocyclic moiety on CdG. The results demonstrated that these structural alterations significantly impacted both DNA replication fidelity, as well as the mechanism and extent of cell death.

Results presented here demonstrated that, of the three drugs tested, only GCV induced mutations in the pSP189 plasmid grown in U251tk cells. Thus, mutation induction was not simply due to an acyclic moiety, since the acyclic PCV did not produce mutations. However, both PCV and CdG have the ether oxygen replaced by a methylene group, implicating the ether oxygen of GCV in mutation induction.

GCV induced a dose-dependent increase in mutation frequency, which contributed to but likely was not solely responsible for GCV-induced cytotoxicity. There was a significant increase (compared to controls) in the specific GC→TA (primarily C→A) mutations at all drug concentrations. It has been reported that this transversion occurs in approximately 31% of spontaneous mutations with the supF plasmid [33], similar to the percentage in the controls presented here but distinctly lower than the range of GC→TA mutations (49–88%) observed with GCV alone. Thus, this appears to be a unique mutation specifically induced by GCV.

This unique mutation is likely due to incorporation of GCV 5'-monophosphate (GCVMP) into DNA, since GCV does not induce an imbalance in dNTPs [5]. Previous studies with oligodeoxynucleotide duplexes synthesized with GCVMP demonstrated that the acyclic moiety decreased stability of DNA [34]. Determination of the solution structure of GCVMP-containing duplexes revealed that the most significant distortions occurred at the site of GCVMP incorporation with increased base stacking, and a distinct kink in the sugar-phosphate backbone that extended two bases after GCVMP [35]. The authors hypothesized

that this distortion would result in the pausing of DNA polymerases. Recognizing that there are significant structural differences between a deoxynucleotide decamer and replication sites within the more complex plasmid DNA or chromosomal DNA, particularly with respect to tertiary structure, nevertheless it is intriguing to speculate that polymerase pausing due to the presence of GCVMP would promote insertion of incorrect nucleotides. The C118A and C146A mutations occurred just two nucleotides following a G site. Thus, incorporation of GCV instead of G may have promoted the C→A mutations. Corresponding solution structures with CdG and PCV have not been reported, and thus whether the lack of mutations with these analogs is due to an inability to disrupt the DNA conformation is not known.

The specificity of GCV to induce the C118A and C146A mutations is supported by the lack of these mutations in any of the controls, indicating that these sites are not prone to misincorporation. In addition, the C118 site has not been reported by others to be more sensitive to mutation in the supF plasmid mutation assay [27,33]. Although the C146 site was reported as a significant hot spot for mutations [33], the majority were C→G with fewer C→A mutations. The highest number of spontaneous C→A mutations was reported at C139. While we observed C→A mutations at this site, they occurred at a similar frequency in control and GCV-treated cells. It is noteworthy that the C118A and C146A mutations occurred at higher frequencies in response to GCV in three different cell lines, independent of MMR capacity. In addition, induction of MMR with HU decreased mutation frequency in the HCT116 1–2tk cells, but it was unable to correct the majority of the C→A mutations. In our previous studies evaluating mutation induction by other nucleoside analogs using the pSP189 plasmid assay, we have not observed specific mutations at these sites [16]. Taken together, the data demonstrate that the C118A and C146A mutations are induced specifically by GCV exposure. Since the conditions used allowed only one complete replication of the plasmid within each cell, the mutations observed were the result of GCVMP in the nascent strand and not the template DNA. 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. [34]. Due to the small coding sequence of the supF cDNA, it was not possible to evaluate sequence-specific effects in this study, but may be of interest in future studies.

As we have observed previously [24], HU increased cytotoxicity with GCV in the MMR-deficient cells, and here we have demonstrated that this was associated with an increase in mutations in DNA. In contrast, in the MMR-proficient cells activation of MMR with HU decreased mutations, which resulted in decreased cytotoxicity. The antagonistic cytotoxicity observed with GCV and HU in MMR-proficient cells could not be explained by decreased incorporation of GCVMP into DNA (data not shown). Taken together, these data demonstrate that mutations in DNA contribute to cell death with GCV.

In addition to the differences in mutation frequency elicited by GCV, PCV and CdG, the cell cycle data demonstrated distinct differences between the three deoxyguanosine analogs. Compared to PCV, the more moderate effect of both GCV and CdG on DNA synthesis inhibition and cell cycle progression indicates that, as triphosphates or after incorporation into DNA, GCV and CdG interact with polymerases more similarly to deoxyguanosine than does PCV. Consistent with our findings, studies using purified polymerases with synthetic primer templates demonstrated that the 5'-triphosphates of GCV and PCV (GCVTP and PCVTP) could be incorporated into synthetic primer templates by polymerases α , δ or ϵ , although further elongation was slow [6,36]. GCVTP and PCVTP were poor inhibitors of polymerases α and ϵ , however GCVTP exhibited a low K_i (2 μ M) for inhibition of pol δ . In a separate study, the 5'-triphosphate of CdG (CdGTP) exhibited a K_i of 1 μ M and was

incorporated into DNA with DNA polymerase α although elongation was slowed [37]. While these findings may have predicted greater inhibition of DNA synthesis by GCVTP and CdGTP, the moderate inhibition presented here is likely due to the ability of polymerase switching or additional polymerases to assist in elongating DNA after incorporation of a fraudulent nucleotide in the intact cell [14].

The differences in cell cycle progression between GCV, CdG and PCV were due to the effects of the corresponding nucleotide analog on DNA replication and not the induction of mutations. Both GCV and CdG elicited a block in the cell cycle following drug exposure, yet only GCV produced mismatches in DNA. In addition, while neither CdG nor PCV induced mutations, only PCV produced a strong S-phase block during drug exposure. The second S-phase block observed with GCV compared to a more prominent second G2/M block with CdG suggests that GCVMP serves as a poorer substrate than CdG 5'-monophosphate (CdGMP) in template DNA. However, in the nascent DNA strand both analogs were superior substrates for elongation compared to PCV 5'-monophosphate. This result is consistent with the finding that GCVMP is incorporated into DNA to a higher degree than PCV monophosphate in intact cells and in a primer-template assay [6,21]. It is possible that the block of DNA synthesis by phosphorylated PCV induces DNA repair proteins that prevent incorporation or retention of this analog into DNA, thereby decreasing cytotoxicity. Alternatively, PCV monophosphate in DNA may be a poor substrate for elongation, thus slowing DNA synthesis and antagonizing its own activity. It is interesting to note that the effects of the acyclic GCV on DNA replication and cell cycle progression were more similar to the carbocyclic CdG than the acyclic PCV. Although PCV is an excellent antiviral, the replacement of the ether oxygen by amethylene group produced an inferior antitumor compound in these studies.

Our data suggest that GCVMP and CdGMP are more disruptive to DNA synthesis when present in the template versus the nascent DNA strand since the cells divided after drug treatment but halted in the next S and/or G2/M phases with subsequent cell death. This is a novel mechanism for nucleoside analogs, since most will produce a strong block in DNA synthesis during drug incubation but surviving cells then go on to divide well with template-incorporated analog [5,30]. GCVMP and CdGMP in template DNA may interfere with DNA synthesis by serving as poor substrates for nascent strand synthesis. The presence of these analogs 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. A similar scenario could occur if the lesion were on the lagging strand, with inhibition of the polymerase α /DNA primase complex. Alternatively, template-incorporated GCVMP and CdGMP may be recognized as fraudulent nucleotides but the lack of a repair system to repair these template lesions permanently arrests cells, as demonstrated for the futile attempted repair of cisplatin crosslinking in template DNA [38]. That GCVMP in DNA initiates a repair response is suggested by previous data demonstrating greater sensitivity to high concentrations of GCV in MMR-deficient cells [24]. In addition, inhibition of DNA polymerase β , a gap-filling DNA repair polymerase, sensitized cells to GCV [39]. However, we have demonstrated previously that GCVMP is well retained in DNA throughout several cell divisions after drug washout, demonstrating the lack of a repair system for this lesion when present in the DNA template [5]. Taken together, these data suggest that the inability to replicate DNA with GCVMP or CdGMP in the template produces a more efficient cytotoxic effect than nucleoside analogs that primarily disrupt DNA synthesis when present in the nascent strand.

Previous reports have demonstrated that GCV is more genotoxic than ACV or PCV. ACV induced SCEs and chromosomal aberrations immediately after drug exposure, but only at high, non-physiologic concentrations. PCV produced an increase in SCEs and chromosomal

aberrations at least one cell cycle time following drug exposure, but this occurred only at highly cytotoxic concentrations ($>IC_{75}$) and resulted in only a modest increase in SCEs (≤ 2 -fold greater than controls) with few chromosomal breaks or translocations [20]. In contrast, GCV induced more SCEs (2–12-fold compared to controls) with many chromosomal breaks and translocations at non-cytotoxic concentrations. Interestingly, these events occurred during the second cell cycle after drug exposure [19,20], suggesting that GCVMP in the template strand initiated these aberrations, consistent with our findings here. Because SCEs occur as a result of homologous recombination [40] this pathway may be activated in response to GCV-induced DNA damage. Indeed, results from a yeast deletion assay suggested greater GCV sensitivity in yeast deficient in homologous recombination genes [24]. Further evaluation of the role of homologous recombination in the cytotoxic activity of GCV is warranted.

While nucleoside analogs elicit their effects by incorporating into DNA, they have vastly different biologic effects which cannot be explained simply by the level of this incorporation. Here we have investigated the consequences of DNA incorporation on replication fidelity, cell cycle progression and cytotoxicity for three structurally related deoxyguanosine analogs. The results demonstrated a distinct pattern of effects on DNA replication fidelity and profoundly different effects on cell cycle progression, resulting in differential cell killing. Importantly, the novel ability of GCVMP and CdGMP in the DNA template to arrest cell cycle progression produced superior cytotoxicity in human tumor cells compared to the strong inhibition of DNA synthesis by PCV monophosphate in the nascent strand which resulted in significantly less cell killing. These results predict that additional nucleoside analogs based on the acyclic moiety of GCV or the carbocyclic structure of CdG could be promising antitumor drugs.

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Fig. 1.

GCV induces a dose dependent increase in mutation frequency with a predominance of GC→TA transversions. U251tk cells were transfected with the pSP189 plasmid overnight and incubated with GCV for 24 h. At 24 h after drug washout, DNA from replicated plasmids was harvested and electroporated into MB7070 *E. coli* indicator strain. (A) Mutation frequency was calculated as the number of white (mutation) colonies/total number of colonies (blue + white) counted. *Columns*, average of at least three separate experiments; *bars*, SE; *asterisks*, significantly greater than the corresponding non-drug treated control ($p < 0.05$). (B) Each mutant colony was submitted for DNA sequencing to determine the exact mutations in the supF sequence that occurred during replication of pSP189 plasmids in U251tk cells in the absence or presence of GCV. At least 59 mutant plasmids were analyzed for each condition.

(A) U251tk cells

Control																	
T T T G C T

Fig. 2. Sites of single base substitutions in the supF cDNA from pSP189 plasmid replicated in human tumor cells. Cells were transfected overnight and incubated with no drug (control),

GCV and/or 2mM HU for 24 h as indicated. DNA from replicated plasmids was extracted 24 h after drug washout and electroporated into an indicator strain of *E. coli*. Plasmid DNA from mutant colonies was extracted and submitted for DNA sequencing. The supF coding sequence is shown with the mutations at the individual sites. The asterisks denote frequently mutated positions, and the percentage of total base substitutions that occur at these positions is indicated. (A) U251tk cells; (B), HCT116 0–1tk (MMR-deficient) cells; (C), HCT116 1–2tk (MMR-proficient) cells.

Fig. 3.

pSP189 plasmid mutation frequency in MMR-deficient and proficient HCT116tk cell lines following exposure to GCV and/or HU. HCT116 cells were transfected with the pSP189 plasmid overnight and incubated with 1 μ M GCV and/or 2mM HU for 24 h. DNA from replicated plasmids was extracted 24 h after drug washout and mutation frequency and specific mutations were determined. (A) 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 (* $p < 0.02$; ** $p < 0.01$). Specific mutations in the supF sequence in pSP189 plasmids replicated in (B), MMR-deficient HCT116 0–1tk cells and (C) MMR-proficient HCT116 1–2tk cells. At least 24 mutant plasmids were sequenced for control cells, and ≥ 42 mutant colonies were sequenced in drug-treated cells.

Fig. 4.

(A) Structures of GCV, CdG, and PCV. (B) Sensitivity of U251tk cells to GCV, CdG, and PCV. Exponentially growing U251tk cells were exposed to the indicated concentrations of drug for 24 h. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. *Points*, mean of triplicate samples; *bars*, SE. (C) pSP189 plasmid mutation frequency in U251tk cells following exposure to 1 μM CdG or 10 μM PCV for 24 h. Plasmids were extracted 24 h after drug washout; mutation frequency and specific mutations were determined as described in Section 2. 18–135 plasmids were analyzed for each condition. *Columns*, average of at least three separate experiments; *bars*,

SE. (D) Specific mutations in the supF sequence in pSP189 plasmids replicated in U251tk cells.

Table 1

The addition of HU 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.

Cell line	mM HU	% Survival with HU alone	IC ₅₀ GCV (μM)
MMR-deficient HCT116 0–1tk	0	100	0.57 ± 0.04
	1	24	0.41 ± 0.06
	3	16	0.34 ± 0.01
	0	100	0.39 ± 0.09
MMR-proficient HCT116 1–2tk	1	49	0.77 ± 0.19
	3	30	>1.0 ^a

Exponentially growing HCT116 cells were treated with GCV and/or HU for 24 h. Clonogenic cell survival was determined and expressed as a fraction of plating efficiency for untreated cells. Values represent mean±SE.

^aSurvival = 59.2±8.6% control at 1μM GCV.

Table 2

Effect of GCV, CdG and PCV on the cell cycle distribution of U251tk cells.

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

U251tk cells were incubated with 1 μ M GCV, 1 μ M CdG, or 50 μ M PCV for 24 h. Drug containing medium was removed following the 24 h incubation (time = 0h) and replaced with fresh drug-free medium. Cells were analyzed at the indicated time points during drug incubation (-12 h) and after drug washout. Cells were then prepared for dual parameter flow cytometry to determine DNA synthesis and DNA content as described in Section 2. Control represents a 24 h period of cell growth without drug addition. Results of a single experiment repeated twice are shown.