5-Azacytidine Can Induce Human Immunodeficiency Virus Type 1 Lethal Mutagenesis

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Abstract

Ribonucleosides inhibit human immunodeficiency virus type 1 (HIV-1) replication by mechanisms that have not been fully elucidated. Here we report the antiviral mechanism for the ribonucleoside analog 5-azacytidine (5-AZC). We hypothesized that the anti-HIV-1 activity of 5-AZC was due to an increase in HIV-1 mutation rate following its incorporation into viral RNA during transcription. However, we demonstrate that 5-AZC’s primary antiviral activity is attributed to its effect on the early phase of HIV-1 replication. Furthermore, the antiviral activity was associated with an increase in viral mutant frequency, suggesting that 5-AZC’s primary target is reverse transcription. Sequencing analysis showed an enrichment in G-to-C transversion mutations and further supports that reverse transcription is an antiviral target of 5-AZC. These results indicate that 5-AZC is incorporated into viral DNA following reduction to 5-aza-2’-deoxycytidine (5-aza-dC). Incorporation into viral DNA leads to an increase in mutant frequency that is consistent with lethal mutagenesis during reverse transcription as the primary antiviral mechanism of 5-AZC. Antiviral activity and increased mutation frequency was also associated with the late phase of HIV-1 replication, however 5-AZC’s effect on the late phase was less robust. These results reveal that the primary antiviral mechanism of 5-AZC is attributed to its ability to increase the HIV-1 mutation frequency through viral DNA incorporation during reverse transcription. Our observations indicate that 5-AZC can affect two steps in HIV-1 replication (i.e., transcription and reverse transcription) but that its primary antiviral activity is due to incorporation during reverse transcription.
Introduction

Significant progress has been made in the clinical management of HIV-1 infection using antiretroviral drugs (11). Nonetheless, the emergence of drug resistance limits the efficacy of current HIV-1 drugs and drives the need for novel therapeutics (44). Although current therapy relies on combination drug therapy, low levels of viral replication coupled with the high HIV-1 mutation rate makes drug resistance difficult to prevent (41, 43). The development of novel anti-HIV-1 drugs that have a high barrier to the emergence of drug resistance would offer new treatment options as the current drugs become ineffective from the emergence and transmission of drug resistance.

The HIV-1 mutation rate represents a novel drug target that may offer a higher genetic barrier to the emergence of drug resistance relative to current anti-HIV-1 drugs. The high mutation rate of HIV-1, as well as other RNA viruses, enables virus survival in the face of a rapidly changing host environment (e.g., host immune response to viral infection) (15). However, because most mutations are detrimental, the high mutation rate leads to a large proportion of non-infectious virions (7). Thus, HIV-1 may have evolved a mutation rate that carefully balances the need for viral adaptation with the need to replicate with enough fidelity to remain viable. Based upon this high mutation rate, a novel therapeutic strategy is to tip this balance in favor of a higher mutation rate such that the virus is unable to replicate with enough fidelity to remain infectious. This strategy, termed lethal mutagenesis, is based on the concept that only a modest increase in the viral mutation rate is needed to render the virus non-viable (14, 27, 38).

The validity of lethal mutagenesis as an antiviral strategy is supported by experimental evidence showing an inverse correlation between mutation rate and infectivity of several RNA viruses (e.g., polio virus, foot and mouth disease virus, and hantaan virus) as well as retroviruses (e.g., spleen necrosis virus and HIV-1) (1, 5, 22, 24, 47). Nucleoside analogs have been shown
to effectively increase viral mutation rates (9, 24, 26, 38). However, there has yet to be a nucleoside analog of acceptable efficacy and safety to be clinically relevant for the purpose of lethal mutagenesis. One of the concerns regarding the use of mutagenic nucleoside analogs is the potential for toxicity and carcinogenicity since these compounds can be directly incorporated into the host genome.

Ribonucleoside analogs with mutagenic potential have also been explored for their antiviral activity against riboviruses (10, 23, 50). The use of ribonucleoside analogs for blocking retroviral replication have been used, but the mechanism of action is not clear. There are three models that could explain the mechanism by which ribonucleoside analogs inhibit retrovirus replication. First, ribonucleoside analogs could be incorporated into HIV-1 RNA during transcription of the genomic length RNA (21, 38). Alternatively, the ribonucleoside analog could be incorporated into viral DNA by reverse transcriptase (RT) following its reduction to the 2'-deoxynucleotide form. Finally, ribonucleoside analogs may exert antiviral activity through incorporation into both DNA and RNA. To date, a detailed understanding of how ribonucleosides manifest an antiretroviral effect is not well established, including those ribonucleosides with mutagenic potential.

5-azacytidine (5-AZC) is a ribonucleoside analog that is used clinically to treat myelodysplastic syndromes (29, 54). 5-AZC has previously been shown to inhibit HIV-1 infectivity (3). Based on its structure and its effect on the poliovirus mutation rate, 5-AZC was proposed to exert its antiviral activity by increasing the HIV-1 mutation rate although no data has been published to support lethal mutagenesis as its mechanism of action. As a ribonucleoside, 5-AZC was proposed to increase the HIV-1 mutation rate through direct incorporation into viral RNA. However, 5-AZC could also be incorporated into viral DNA after reduction by cellular ribonucleotide reductase. In this study, we examined the mechanism by which 5-AZC inhibits HIV-1. Our data reveal that 5-AZC exerts its antiviral activity through incorporation into both viral
RNA and DNA. Interestingly, the most potent antiviral activity was attributed to incorporation of 5-AZC into viral DNA following its reduction to the 2'-deoxy form. Further investigation into the mechanism of 5-AZC revealed that incorporation of 5-AZC into viral DNA during reverse transcription leads to lethal mutagenesis which is characterized by a significant increase in transition mutations within the provirus.
Materials and Methods

Plasmids, cell lines and reagents – The HIV-1 based vector, HIG, was constructed by cloning the IRES-eGFP sequence from pIRES2-eGFP (Clontech, Mountain View, CA) in the Xho I site of pNL4-3.HSA.R+.E- (the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH, Germantown, MD) (8, 25). The resulting vector, pHIG, has a vector cassette with the murine heat stable antigen (HSA) gene, IRES and GFP. The Env expression vector pIIINL4env was a kind gift from Eric Freed (NCI Drug Resistance Program, NCI, Frederick, MD). U373-MAGI\textsubscript{CXCR4} and CEM-GFP cell lines were also obtained from the AIDS Reagent Program (via M. Emerman and J. Corbeil, respectively) (19, 53). The PE-conjugated antibody to mouse HSA was obtained from BD Pharmingen (San Diego, CA). The CellTiter-Glo and CellTiter 96 cell proliferation kits were obtained from Promega (Madison, WI). 5-azacytidine was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in DMSO at 1M stock concentrations and stored at -20 °C. The high Pure PCR Template Preparation Kit was from Roche Applied Science (Indianapolis, IN) and the pCR2.1 TOPO cloning vector was from Invitrogen (Carlsbad, CA).

Transfections, infections, drug treatments, and mutant frequency - To produce the HIG vector as a virus, a calcium phosphate transfection protocol was used. Briefly, 1.5 x 10\(^6\) HEK 293T cells were transfected with 10\(\mu\)g pNL4-3.HIG.E- and 1\(\mu\)g pIIINL4env. Cells were washed the next day with PBS and resuspended in culture media (DMEM + 10% fetal calf serum). Cell culture supernatants were harvested 48 h post-transfection and passed through a 0.2 \(\mu\)m filter. For drug treatment during virus particle production, 5-AZC or 5-aza-dC was added for 12 h prior to the harvesting of cell culture supernatants. The amount of drug that could potentially be carried over while in the producer cell supernatant was calculated to be negligible after ensuing dilutions. U373-MAGI\textsubscript{CXCR4} cells were used as permissive target cells for infections and were maintained in DMEM supplemented with 10% fetal calf serum and in the presence of 0.2 mg/mL neomycin, 0.1 mg/mL hygromycin B, and 1.0 \(\mu\)g/mL puromycin. Cells were infected at an MOI of 0.3 in the
absence of selectable media. For treatment of target cells, 5-AZC was added 2 hours prior to infection and maintained for 24 hours post-infection. Mutant frequency was determined by the percentage of target cells expressing one of the reporter genes relative to that of the entire infected population (i.e. [HSA+/GFP-] + [HSA-/GFP+] relative to total percent of infected cells). Mutant frequencies were normalized to virus replication in the absence of drug treatment.

**Virus particle capsid protein (p24) assay** - ELISA plates (96 well) were incubated overnight with 1:1000 rabbit p24 antiserum (AIDS Reagent Program Cat. #4250). Plates were then washed with PBST (PBS/0.5% Tween 20) and blocked for 1 h with 3% milk in PBST. Samples and standards were prepared by adding 1:1 volume of PBS/0.1% Empigen and incubating at 56 °C for 30 minutes. Samples were then added to the plate and incubated at 37 °C for 1 h, wells washed with PBST, and then incubated with mouse anti-p24 in PBST/1% milk at 22 °C for 1 h. Incubation was then done with a secondary anti-mouse HRP in PBST/1% milk for 30 minutes, and wells washed with PBST and PBS before the addition of the TMB substrate. Reactions were stopped by the addition of 1M sulfuric acid and the absorbance was determined at 450nm.

**Cellular toxicity analysis** – Both 293T and U373-MAGI\_CXCR4 were diluted out on 96-well plates to determine the linear range for cell number in each assay. 5,000 293T cells and 6,000 U373-MAGI\_CXCR4 were plated and the CellTiter-Glo and Cell Titer96 assays were performed as described by manufacturer’s protocol. Briefly, the CellTiter-Glo assay generates a luminescent signal, which is proportional to ATP levels while the CellTiter-96 assay measures the amount of a colorimetric product produced after a substrate is reduced by mitochondrial reductase.

**Flow cytometry analysis** - Infected target cells were prepared for flow cytometry by harvesting cells with trypsin-EDTA. Cells were then centrifuged at 200 x g for 5 minutes, and cell pellets were then resuspended at a final concentration of 5 x 10^6 cells/mL in PBS/2% fetal calf serum. Cells (2.5 x 10^5) were incubated for 20 minutes on ice with 1:250 anti-HSA PE (BD Pharmingen).
Cells were then washed with PBS/2% fetal calf serum, centrifuged at 200 x g for 5 min, and then resuspended in PBS containing 1% FC3 and 1% paraformaldehyde.

Cells were analyzed using a FACScan (BD Biosciences) with CellQuest software. Cells were gated for morphology and a minimum of 10,000 cells counted. Excitation was done at 488nm; FL1 detected emission at 507nm and FL2 detected emission at 578nm. Compensation was set at FL2 - 99% FL1.

**Cell sorting and proviral DNA sequence analysis** - Target cells were collected and single cells sorted using a FACSAria (BD Biosciences) 48 h post-infection. Approximately ten thousand cells from the PE-HSA+/GFP- quadrant were collected. Total genomic DNA was purified from these cells (Roche High Pure PCR Template Preparation Kit) and used as a template for nested PCR to amplify the GFP gene. Outer primer pair: 5'-CTGAAGGATGCCCAGAAGG-3' and 5'-TGCTTCTAGCCAGGCACAAGC-3'; inner primer pair: 5'-TTACATGTGTTTAGTCGAGG-3' and 5'-GCTACTTGTGATTGCTCCATA-3'. The resulting PCR products were purified, ligated to pCR 2.1 (Invitrogen), and transformed into the DH5α strain of E. coli. Plasmid DNA was purified from cells (Invitrogen Quick Plasmid Miniprep Kit) and used for DNA sequencing analysis. Sequence alignment was performed using SeqMan program of the Lasergene 7 software package (DNASTAR, Madison, WI).

**Analysis of 5-AZC with replication competent HIV-1** - CEM-GFP cells (1.5 x 10^6) were infected with the HIV-1 NL4-3 molecular clone at an MOI of 0.05. 5-AZC or DMSO alone was added to each culture at a 1:1000 dilution. Cultures were monitored every two days by flow cytometry to determine the percentage of infected cells.

**Statistical analysis**

All statistical analyses, graphical representation and curve fitting were done using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).
Results

5-AZC inhibits HIV-1 replication and increases viral mutant frequency. To elucidate the stage of viral replication that 5-AZC inhibits, we used a single round vector assay that enables differentiation of early and late stages of viral replication (Figure 1). Specifically, cells cotransfected with envelope-deficient HIV-1 plasmid and a plasmid encoding the HIV-1 envelope support the late stages of viral replication by enabling viral transcription, translation, and particle production. In contrast, virus-infected target cells support the early stages of viral replication by enabling viral entry, reverse transcription, and integration. The stage at which 5-AZC exerts its antiviral activity can be determined by specifically treating either the virus-producing cells, to examine late stages of replication, or the target cells to examine the early stages of viral replication. From previous studies, it was expected that ribonucleosides exert their antiviral activity in the late phase of the HIV replication cycle (39, 49). In support of this, 5-AZC led to a concentration-dependent decrease in HIV-1 infectivity with a half maximal inhibitory concentration (IC_{50}) of 112 µM (Figure 2A). Importantly, there was no concentration-dependent effect of the deoxynucleoside 5-aza-dC during the late phase of viral replication suggesting that the antiviral activity is dependent on 5-AZC’s influence on viral RNA. Additionally, this decrease in infectivity was not due to changes in virus production (Figure S1). Unexpectedly, 5-AZC led to a more potent inhibition of HIV-1 infectivity in the early phase of the HIV-1 replication cycle with an IC_{50} of 57 µM (Figure 2C).

Since 5-AZC could cause mutations through its incorporation into viral RNA during transcription, we next examined whether 5-AZC increased HIV-1 mutant frequency. To do this, the single cycle assay described in Figure 1 was used to determine HIV-1 mutant frequency when either virus-producing cells or target cells were treated with 5-AZC. The single cycle assay enables the determination of mutant frequency through the use of two reporter genes, HSA and GFP. HSA serves as a reporter for infectivity, while the other reporter gene, GFP, is used as a
mutation target. Specifically, cells that express one target gene, but not the other, represent cells that are infected, but harbor a mutation that abrogates expression of one of the target genes. Since 5-AZC is a ribonucleoside analog, it was expected that an increase in mutant frequency would be observed in the late phase of the HIV-1 replication cycle. As expected, 5-AZC increased the HIV-1 mutant frequency by 2-fold when virus-producing cells were treated at the respective [IC\textsubscript{50}] (Figure 2B). This indicates that 5-AZC increases mutant frequency during the late phase of HIV-1 replication. Figure 2D shows that 5-AZC also increased the mutant frequency by 3-fold when target cells were treated also with the respective [IC\textsubscript{50}]. These data suggest that 5-AZC may inhibit HIV-1 infectivity by increasing the mutant frequency at two distinct stages of the HIV-1 replication cycle.

Although the loss of infectivity coincided with an increase in mutant frequency, it is possible that the loss of infectivity could be attributed to compound toxicity. To investigate this, the toxicity of 5-AZC was analyzed for two different endpoints including relative cellular ATP levels as well as mitochondrial reductase activity (data not shown) when either virus producing cells or permissive target cells were treated with 5-AZC. Figure 3 shows that loss of viral infectivity was not correlated with cellular toxicity. Similar results were observed when mitochondrial reductase activity was used as an endpoint for toxicity (data not shown).

\textit{Analysis of HIV-1 mutation spectra during viral replication in the presence of 5-AZC.} Based on the data shown in Figure 2, we hypothesized that 5-AZC increased mutation frequency by two different mechanisms. First, 5-AZCTP could induce mutations through its direct incorporation into viral RNA. Second, 5-AZC could be incorporated into viral DNA after its 2'-OH reduction by ribonucleotide reductase. Since incorporation during either RNA or DNA synthesis would result in mutations in the provirus, we examined the mutation spectra of the proviral GFP gene after treating either virus producing cells or target cells with 5-AZC. To do this, cells infected by
mutant HIV were isolated by cell sorting. In this case, cells expressing HSA but not GFP (HSA+/GFP-) were sorted and the mutant GFP gene was sequenced.

Figure 4 shows the location and mutation type of over 100 individual GFP gene sequences recovered from target cells. The mutation spectra revealed a dramatic enrichment in G-to-C transversion mutations from virus exposed to 5-AZC in the early phase of replication compared to untreated virus producing cells (Fig 4A, 4B). Besides a dramatic change in the mutation spectra, 5-AZC increased the average number of mutations per nucleotide sequenced by 2.3-fold compared to virus obtained from untreated cells (Table 1). This indicates that 5-AZC not only changes mutation type but also increases the mutation frequency. The finding that treatment of permissive target cells dramatically altered the HIV-1 mutation spectra suggests that 5-AZC was likely incorporated into viral DNA during reverse transcription after its reduction by cellular ribonucleotide reductase since a similar mutation spectra has been reported in cells exposed to 5-aza-dC (30, 47).

Similar G-to-C transversion mutations were observed when virus-producing cells were treated with 5-AZC (Fig 4C). However, in addition to an increase in G-to-C mutations, there was also an increase in C-to-G mutations consistent with previous observations that 5-AZC could alter mutation spectra of spleen necrosis virus (47). Additionally, 5-AZC treatment increased the average number of mutations per nucleotide sequenced by 1.5-fold when compared to untreated virus-producing cells (Table 1).

Analysis of the average number of mutations per GFP gene sequenced reveals a general trend towards increased mutational load when comparing 5-AZC early and late phase treatments with that of the no drug treatment (Fig 4D). The GFP proviral sequences analyzed had mutational loads ranging from 0 to over 10 mutations per GFP target gene. The high mutation load is unlikely to
produce infectious virus and suggests that 5-AZC's ability to increase the HIV-1 mutation rate leads to a significant decrease in viral replication capacity.

**Susceptibility of replication-competent HIV-1 to 5-AZC.** Although the results so far suggest that 5-AZC inhibits HIV-1 infectivity by increasing the mutation rate, we wanted to confirm that these results would extend to replication-competent HIV-1. Additionally, we hypothesized that 5-AZC would inhibit replication-competent virus at lower concentrations compared to that used in the single cycle assay. This hypothesis is based on two factors. First, unlike the experiments described thus far, both phases of the replication cycle will be exposed to 5AZC during treatment of cells infected with replication-competent HIV-1. Second, if the antiviral activity of 5-AZC is due to an increase in mutation frequency, then multiple rounds of replication would allow for the accumulation of mutations resulting in a greater proportion of lethally mutagenized viruses. The results in Figure 5 show that significantly lower concentrations were required to inhibit replication-competent HIV-1 compared to the concentrations required to inhibit HIV-1 in the single cycle assay.
Discussion

The emergence and transmission of drug resistance as well as the halt of the Merck vaccine trial emphasizes the continual need for the development of novel HIV-1 drugs as well as a return to fundamental aspects of vaccine development (20, 34). As an antiviral strategy, lethal mutagenesis offers a novel drug target (i.e., the viral mutation rate) and is likely to have a high barrier to drug resistance. However, little progress has been made in identifying compounds with enough therapeutic potential to be used clinically to promote HIV-1 lethal mutagenesis. The ribonucleoside analog, ribavirin, is the only clinically approved ribonucleoside identified so far that may act as a lethal mutagen to inhibit viral replication - specifically, hepatitis C virus (10). While ribavirin is not effective against HIV-1, other ribonucleoside analogs such as 5-AZC have been shown to have anti-HIV activity in cell culture (3). However, little is known about its mechanism of action.

In this study, we examined the antiviral activity, mechanism, and toxicity of 5-AZC. Since 5-AZC is a ribonucleoside analog, it was hypothesized that its antiviral activity would primarily be attributed to its incorporation into viral RNA and subsequent increase in HIV-1 mutation frequency. In support of this, several previous studies have shown that 5-AZC can be incorporated into RNA (6, 16, 36, 46). One study demonstrated that 5-AZC was weak competitive inhibitor having a 20-fold lower affinity than CTP for RNA polymerase II (35). However, our results show that the most potent antiviral activity of 5-AZC is actually attributed to its effect on the early phase of HIV-1 replication, which includes reverse transcription. In fact, while 5-AZC increased HIV-1 mutation frequency in both the late and early phases of HIV-1 replication, it had a greater effect on the early phase of replication. These data suggest that 5-AZC exerts its antiviral activity at both phases of replication through an increase in mutation frequency. Although 5-AZC led to a modest increase in mutant frequency, similar increases in mutation rates have been shown sufficient to lethally mutagenize other RNA viruses (24, 26, 33,
In fact, the theory of lethal mutagenesis suggests that small increases in viral mutation rates should lead to a disproportionately larger decrease in viral infectivity (13, 17).

Both phosphorylation and 2’-OH reduction are prerequisites for 5-AZC’s clinical use as a DNA hypomethylating agent (reviewed therein (29, 54)). Similarly, our results suggest that 5-AZC is likely to be phosphorylated and reduced prior to its incorporation into viral DNA by RT. Phosphorylation of 5-AZC is likely performed by uridine-cytidine kinase as this enzyme is responsible for the phosphorylation of CMP, UMP, dCMP, as well as many pyrimidine analogs used for cancer and antiviral therapy (28). Additionally, 5-AZC was shown to be a suitable substrate for uridine-cytidine kinase (37).

After phosphorylation, it is inferred that 5-aza-CDP is reduced by cellular ribonucleotide reductase before incorporation into viral DNA. It has been shown that 10-20% of 5-AZC is incorporated into cellular DNA, suggesting a metabolic pathway through ribonucleotide reductase (36). To further support a role for ribonucleotide reductase in 5-AZC metabolism, the ribonucleotide reductase inhibitor hydroxyurea, was found to block the epigenetic hypomethylation activity of 5-AZC in vivo (12). Furthermore, there is our mutational data to support the conversion 5-AZC and its incorporation of into DNA. Specifically, our data show a significant increase in G-to-C transversion mutations in proviral DNA after target cells were treated with 5-AZC (Figure 4 B and Table 1) and a similar increase in G-to-C mutations was reported for cellular DNA exposed to 5-aza-dC (30). To our knowledge, there have been no biochemical studies which have looked at the interaction between 5-AZCTP (or 5-aza-dCTP) and purified RT; however, it is well established that the DNA polymerase activity of RT is specific for dNTPs by preferentially excluding ribonucleoside-triphosphates (rNTPs) from entering the polymerase active site (18). Moreover, previous reports have shown that DNA polymerase α have similar affinities and rate of incorporation for 5-aza-dCTP and dCTP (2).
Based on the data presented here, we propose a model that accounts for 5-AZC's antiviral effect on the early phase of the HIV-1 replication cycle (Figure 6). In this model, 5-AZC is first reduced to 5-aza-dCDP by ribonucleotide reductase. Next, 5-aza-dCTP is incorporated into viral DNA during reverse transcription. Once incorporated into DNA, the 5-aza-cytosine triazine ring (i.e. the base), can undergo a ring-opening step which would enable it to base pair with cytosine (30). Thus, as shown in Figure 6, cytosine would be incorporated into the plus strand DNA opposite of 5-aza-dC. Finally, our model shows that integration is a key step in repairing the 5-AZC-induced mutations. The model proposes that 5-aza-dC is excised by host DNA repair machinery during integration. 5-aza-dC would be replaced with guanosine since it can base pair with the cytosine located in the plus strand DNA opposite from the abasic site. When transcribed, the minus strand DNA then results in viral progeny carrying G-to-C mutations. In contrast, 5-aza-dC could be incorporated into the positive strand viral DNA across from guanosines present in the minus strand. However, during integration, the DNA repair machinery would likely excise the 5-aza-dC and replace it with a cytosine which would not lead to a mutation.

The use of ribonucleoside analogs as lethal mutagens offer the benefit of being able to predict the type of mutations that give rise to virus lethality (39, 49). The increase in G-to-C mutations caused by 5-AZC could be predicted based on the base pairing properties of 5-AZC and 5-aza-dC. Thus, it is possible that ribonucleoside analogs could be designed to specifically target certain nucleotides for mutation. Ribonucleoside analogs may be superior to current HIV-1 drugs in their ability to delay the emergence of drug resistance. For high-level resistance to emerge against ribonucleoside analogs that function like 5-AZC, it is likely that mutations would have to be acquired in both RT and RNA Pol II. Although RT is likely to accumulate mutations, there is little pressure on RT to select for mutations that would exclude mutagenic nucleoside analogs since these drugs do not appear to prevent RT-mediated polymerization. This is in contrast to nucleoside analogs such as azidothymidine (AZT) which prevent replication by chain
termination, and therefore efficiently select for any mutations in RT that restore viral DNA synthesis.

A limitation to the development of ribonucleosides as potential antiretroviral agents are the relatively high concentrations needed to observe an antiviral effect (Figure 2 A,C). However, the high concentrations of 5-AZC shown here may be attributed in part by the cell lines used in this study. A previous meta-analysis study documented that cell lines have up to a 5-fold higher concentration of rNTPs than primary cells and that cellular rNTPs are 10-100 fold more abundant than dNTPs (51). This suggests that during late phase replication, 5-AZCTP must compete with an intracellular concentration of cytidine triphosphate (CTP) of 109 – 455µM. Similarly, dividing cells were found to have intracellular dCTP concentrations ranging from 27 – 50µM (51).

Because the current study investigates treatment with the ribonucleoside 5-AZC, which has the potential to undergo a number of nucleos(t)ide metabolic pathways, it is difficult to predict the intracellular concentration of the rNTP and dNTP forms of 5-AZC. Nonetheless, these particular cell lines were chosen because of technical reasons during the virological assays as well as requirements for the sheer number of cells needed for the sequencing experiments.

Differences in dNTP pools in the cell types used here may also account for the discrepancy in IC50 values when comparing the single-cycle assay (Figure 2) to the multiple round assay (Figure 5) although differences in transport pathways and metabolism could also play a role. Future studies will be needed to precisely measure intracellular 5-AZCTP and 5-aza-dCTP levels when exposing cells to ribonucleosides. It may also be of interest to measure endogenous dNTP pool levels when treating cells with nucleoside analogs because this has been shown to cause alterations in natural dNTP pool levels (52) and thus may contribute to an increase in retroviral mutation rate (4, 31-33, 40, 42). However, the presence of the specific G-to-C transversion in the presence of 5-AZC seems to argue against this notion (Figure 4 B and Table 1).
Since our data support a model in which 5-AZC is converted to the corresponding deoxyribonucleoside triphosphate, it is possible that it could be incorporated into the host genome. This raises concerns over the possible genotoxicity of potential mutagenic ribonucleosides. However, toxicity was not significant at the concentrations required to inhibit viral replication. Additionally, a nucleoside analog currently in development, KP1212/KP1461, induces mutations in viral DNA, but does not appear to do so in the host cell genome (24, 45). It is likely that the host DNA repair machinery is sufficiently effective to eliminate any of these analogs that are incorporated into genomic DNA. However, the novelty of these drugs warrants further investigation into potential long-term effects.

Previous studies have demonstrated the anti-HIV activity of 5-AZC (3). Based on the structure of 5-AZC, it was speculated that its antiviral activity was due to its ability to be incorporated into viral RNA during transcription. Here, our data show that while 5-AZC does demonstrate antiviral activity by this mechanism, its more potent anti-HIV activity can be attributed to its reduction to 5-aza-dCTP followed by incorporation into viral DNA during reverse transcription. Thus, 5-AZC inhibits HIV infectivity through its incorporation into both viral RNA and DNA. This incorporation significantly increases the HIV-1 mutation frequency to a point consistent with lethal mutagenesis. Compounds with a similar mechanism of action could represent an important new class of anti-HIV compounds to explore for clinical viability.
References


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Figure Legends

Figure 1. Human immunodeficiency virus type 1 (HIV-1) vector for monitoring viral infectivity and mutant frequency in the presence of antiretroviral drugs. An envelope and Nef-deficient HIV-1 vector was constructed with a gene cassette containing the mouse heat stable antigen (HSA), an internal ribosomal entry site (IRES) element and the GFP gene. This vector was cotransfected into 293T cells along with a HIV envelope expression plasmid for producing vector virus. Forty-eight hours posttransfection, cell culture supernatants were collected, filtered, and used to infect permissive U373-MAGI_{CXCR4} target cells at a multiplicity of infection of 0.3. Forty-eight hours postinfection, cells were harvested, stained with a PE-conjugated HSA antibody and analyzed by flow cytometry. Virus-producing cells or permissive target cells were pretreated with drug or DMSO for 12 hrs after either transfection or 2 hrs prior to infection and treatment continued until cell culture supernatant or cells were collected, respectively.

Figure 2. Concentration-dependent effects of 5-azacytidine (5-AZC) on HIV-1 infectivity and mutant frequency. 293T or U373-MAGI_{CXCR4} cells were treated with the indicated concentrations of compound prior to transfection (late phase replication) or infection (early phase replication), respectively. Infected cells (MOI of 0.3) were analyzed by flow cytometry. Only the ribonucleoside 5-AZC had a concentration-dependent effect on viral infectivity during treatment at late phase replication (A) and this coincided with an increase in viral mutant frequency (B). A more potent antiviral effect was observed with 5-AZC treatment during early phase replication (C). This antiviral activity coincided with a much more dramatic increase in viral mutant frequency (D). Data shown is mean ± SD of 5 independent experiments.

Figure 3. Cytotoxicity of 5-AZC. 293T (A) or U373-MAGI_{CXCR4} (B) cells were treated at the indicated concentrations of 5-AZC and were analyzed for cell viability by measuring cellular ATP levels. Data represent the mean ± SD of 4 independent experiments.
Figure 4. Mutation spectra of GFP gene sequences from vector proviral DNAs. The HSA+, GFP- cell population from infected U373-MAGI cells was sorted from cells treated with 5-AZC (Fig. 1). The sequence results from at least 100 mutants are shown from (A) no drug, (B) 5-AZC treatment of permissive target cells and (C) 5-AZC treatment of virus-producing cells. Each GFP gene sequence analyzed is represented by a thin gray horizontal line. The location of a mutation in the GFP gene sequence is indicated by a colored box perpendicular to the line relative to the 5’-end of the 720 bp ORF. Transition mutations are represented by either black (purine) or yellow (pyrimidine) rectangular boxes. G-to-C and C-to-G transversion mutations are indicated by red and green rectangular boxes, respectively. Transversion mutations (other than G-to-C and C-to-G) are indicated by blue rectangular boxes. (D) Mutational load in GFP gene from proviral DNAs. The summary of the mutational load (all mutation types) in the GFP gene from either no drug, 5-AZC early phase and 5-AZC late phase is shown, with each diamond symbol representing a proviral GFP sequence. The calculation of the average number of mutations per GFP target gene sequence indicated.

Figure 5. 5-AZC inhibits replication-competent HIV-1. The HIV-1 NL4-3 molecular clone was transfected into 293T cells to produce an infectious virus stock that was used to infect CEM-GFP cells. Cells were treated with 5-AZC at the indicated concentrations. Cells were split every 2 days and fresh media and 5-AZC was added. Flow cytometry was used to determine the percentage of infected cells every 2 days. Data represent the mean ± SE of parallel experiments done in triplicate from 8 days post treatment and are representative of 3 independent experiments.

Figure 6. Model of 5-AZC mutagenesis during minus-strand DNA synthesis during HIV-1 reverse transcription. Ribonucleotide reductase converts 5-AZCDP to 5-aza-dCDP. After the incorporation of 5-aza-dC (dZ) triphosphate into minus-strand viral DNA, a spontaneous cytosine ring opening to a Dimroth intermediate occurs, which allows the base to pair with deoxycytidine. During integration, 5-aza-dC is excised by DNA repair machinery and replaced with a guanosine since it base pairs with the
cytosine present opposite of the abasic site in the plus strand DNA. When transcribed, the guanosine in the minus strand codes for a cytosine thereby leading to an overall G-to-C mutation.
Figure 1
Dapp et. al.
Figure 2
Dapp et al.

A. Mutant Frequency of HIG

Normalized % Mutant Frequency

5-aza-2'-deoxycytidine

IC₅₀ = 112 µM

B. 5-azacytidine

Normalized % Infection

IC₅₀ = 57 µM

C. Late Phase Replication

Normalized % Infection

IC₅₀ = 57 µM

D. Late Phase Replication

Normalized % Mutant Frequency

IC₅₀ = 57 µM

Early Phase Replication

Late Phase Replication

Early Phase Replication

Late Phase Replication
Figure 3
Dapp et. al.

A. U373-MAGI (Target Cells) Normalized Toxicity Curve

B. HEK 293T (Producer Cells) Normalized Toxicity Curve
Figure 4
Dapp et. al.

Mutant GFP sequence

100 200 300 400

eGFP

500 600 700 nt

Transition (purine)
Transition (pyrimidine)
Transversion
G → C
C → G

No Drug
Figure 4
Dapp et. al.

B. 5-AZC early phase

Mutant GFP sequence

100 200 300 400 500 600 700 nt

eGFP
Figure 4
Dapp et. al.

Mutant GFP sequence

100 200 300 400

eGFP

500 600 700 nt

5-AZC late phase

C.

Mutant GFP sequence

100 200 300 400 500 600 700 nt

eGFP
Mutational Load in GFP Mutation Target
(mutations per GFP sequence)

Average # of mutations per GFP sequence:

- No Drug: 1.45
- 5-AZC early phase: 3.40
- 5-AZC late phase: 2.17

Figure 4
Dapp et al.
Figure 5
Dapp et. al.

![Graph showing normalized % infection with different [5-AZC] concentrations.](http://jvi.asm.org/)

Normalized % Infection

- No Drug
- 500 mM
- 1 mM
- 10 mM
- 50 mM
Figure 6
Dapp et. al.

viral
+RNA

G U A G C

REVERSE TRANSCRIPTION

-DNA

dZ A T dZ G

+RNA

G U A G C

-DNA

dZ A T dZ G

+DNA

C T A C C

INTEGRATION

G \rightarrow C

5-Azacytidine (5-AZC)

2'-OH reduction of 5-AZCDP by Ribonucleotide Reductase

Dimroth intermediate of 5-aza-dC
Table 1. Mutation spectra of eGFP reporter gene from background and 5-azacytidine treatments displayed as a percentage of the total number mutations.

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<th>G</th>
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. *** p<0.001