5-Azacytidine Enhances the Mutagenesis of HIV-1 by Reduction to 5-Aza-2'-Deoxycytidine

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5-Azacytidine Enhances the Mutagenesis of HIV-1 by Reduction to 5-Aza-2′-Deoxycytidine

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5-Azacytidine (5-aza-C) is a ribonucleoside analog that induces the lethal mutagenesis of human immunodeficiency virus type 1 (HIV-1) by causing predominantly G-to-C transversions during reverse transcription. 5-Aza-C could potentially act primarily as a ribonucleotide (5-aza-CTP) or as a deoxyribonucleotide (5-aza-2′-deoxycytidine triphosphate [5-aza-dCTP]) during reverse transcription. In order to determine the primary form of 5-aza-C that is active against HIV-1, Illumina sequencing was performed using proviral DNA from cells treated with 5-aza-C or 5-aza-dC. 5-Aza-C and 5-aza-dC were found to induce highly similar patterns of mutation in HIV-1 in terms of the types of mutations observed, the magnitudes of effects, and the distributions of mutations at individual sequence positions. Further, 5-aza-dCTP was detected by liquid chromatography–tandem mass spectrometry in cells treated with 5-aza-C, demonstrating that 5-aza-C was a substrate for ribonucleotide reductase. Notably, levels of 5-aza-dCTP were similar in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC. Lastly, HIV-1 reverse transcriptase was found to incorporate 5-aza-CTP in vitro at least 10,000-fold less efficiently than 5-aza-dCTP. Taken together, these data support the model that 5-aza-C enhances the mutagenesis of HIV-1 primarily after reduction to 5-aza-dC, which can then be incorporated during reverse transcription and lead to G-to-C hypermutation. These findings may have important implications for the design of new ribonucleoside analogs directed against retroviruses.

RNA viruses exhibit high mutation rates and have been postulated to replicate near the error threshold—the maximal mutation rate compatible with the maintenance of genetic information (1, 2). Thus, these viruses may be particularly sensitive to small molecules that promote viral mutations, an antiviral strategy called lethal mutagenesis (3). Lethal mutagenesis has been pursued as a potential antiviral approach for many different RNA viruses (4). Most small-molecule candidates for lethal mutagenesis identified thus far have been nucleoside analogs with altered base-pairing properties. These nucleoside analogs base pair promiscuously due to ionization, structural rearrangement, tautomeration, or conformational flexibility (5). Alternatively, small molecules can be used to promote viral mutagenesis by leveraging host nucleic acid-editing enzymes that are part of the innate immune response. For example, molecules have been identified that block the degradation of APOBEC3 enzymes by the human immunodeficiency virus type 1 (HIV-1) accessory protein Vif (6–9). These molecules ultimately promote the incorporation of APOBEC3 proteins into virions, resulting in lethal G-to-A hypermutation during the next cycle of replication.

The ribonucleoside analog 5-azacytidine (5-aza-C) reduces the infectivity of HIV-1 by inducing lethal mutagenesis (10). 5-Aza-C is active during both the early and late phases of viral replication, reflecting incorporation during both reverse transcription and the transcription of viral genomic RNA, respectively. When added during the late phase of viral replication, 5-aza-C induces primarily C-to-G transversions in HIV-1. In contrast, during the early phase of replication, 5-aza-C induces primarily G-to-C transversions in the virus. These G-to-C transversions are thought to be caused by the incorporation of 5-aza-C into minus-strand viral DNA, followed by the hydrolysis of 5-aza-C and its deformylation into ring-opened remnants (10). These ring-opened remnants can then mispair with deoxycytidine during plus-strand synthesis, leading to the fixation of G-to-C transversions in proviral DNA. However, it remains possible that 5-aza-C hydrolysis products are directly incorporated by HIV-1 reverse transcriptase (RT) as well. Notably, 5-aza-C is closely related to 5-aza-2′-deoxycytidine (5-aza-dC), another nucleoside analog that has been explored for the lethal mutagenesis of HIV-1 (11, 12); the primary difference is that 5-aza-dC is much more potent than 5-aza-C and likely is incorporated only into viral DNA.

Two different mechanisms could account for the antiviral activity of 5-aza-C during the early phase of replication: First, 5-aza-C could be incorporated during reverse transcription primarily as a deoxyribonucleotide (i.e., as 5-aza-2′-deoxycytidine 5′-triphosphate [5-aza-dCTP]). For this to occur, the cellular enzyme ribonucleotide reductase (RNR) would have to first convert 5-aza-C (in its diphosphate form) to 5-aza-dC, which could then be phosphorylated to form 5-aza-dCTP. Notably, one previous study has demonstrated that ~10 to 20% of 5-aza-C is reduced to 5-aza-dC by RNR (13), suggesting that 5-aza-dCTP would likely

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be available for incorporation during reverse transcription. However, the reduction of 5-aza-C to 5-aza-dC has not yet been demonstrated in cell types for which antiviral activity has been reported. In further support of this possibility, HIV-1 RT has been shown to selectively exclude ribonucleotides by using a residue (Y115) that acts as a steric gate (14–16). Alternatively, 5-aza-C might be incorporated directly as a ribonucleotide (i.e., as 5-aza-dCTP) during reverse transcription. Notably, HIV-1 RT has been found to incorporate significant levels of endogenous ribonucleotides when the levels of deoxyribonucleotides are very low (resulting in high nucleoside triphosphate [NTP]/deoxynucleoside triphosphate [dNTP] ratios), as they are in macrophages (17, 18). Previous studies have found that high concentrations of 5-aza-C are required to elicit antiviral activity in cell culture (10), potentially skewing the NTP/dNTP ratio enough to allow for significant 5-aza-CTP incorporation. However, these findings could also reflect the inefficient reduction of 5-aza-C to 5-aza-dC.

In order to determine the primary form of 5-aza-C that is active during HIV-1 reverse transcription, Illumina high-throughput sequencing was performed to compare viral mutagenesis in the presence of 5-aza-C with that in the presence of 5-aza-dC. 5-Aza-C and 5-aza-dC caused similar levels of G-to-C and C-to-G transversions in HIV-1. Further, G-to-C and C-to-G transversions at individual sequence positions were distributed in highly similar patterns for 5-aza-C and 5-aza-dC. In addition, 5-aza-dCTP was detected in cells treated with 5-aza-C, indicating that 5-aza-C was reduced to 5-aza-dC by RNR. Importantly, 5-aza-dCTP levels were similar in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC. Lastly, 5-aza-CTP was incorporated in vitro by HIV-1 RT, but much less efficiently than 5-aza-dCTP. Overall, the data support the conclusion that 5-aza-C enhances HIV-1 mutagenesis and diminishes HIV-1 infectivity primarily by acting as 5-aza-dCTP, which may have important implications for the design of antiretroviral ribonucleoside analogs.

### MATERIALS AND METHODS

**Plasmids, cell lines, and reagents.** For single-cycle infections, viral stocks were produced by cotransfecting pNL4-3 MIG with 1 μg of pHCMV-G per 10-cm plate of 293T cells. Transfections were carried out using the polyethylenimine (PEI) method, as described previously (19). Viral supernatants were collected 48 h posttransfection, treated with 10 U/ml of DNase I for 2 h at 37°C to reduce plasmid carryover from transfections, and frozen in aliquots at –80°C. The titers of viral stocks were determined by infecting U373-MAGI cells (31,250 cells/well in 24-well plates) with volumes of virus ranging from 1.25 to 40 μl (2-fold series). Viral infectivity was determined by performing flow cytometry on cells collected 72 h postinfection. Viral titers were determined by plotting the volume of virus against the percentage of infected cells (i.e., cells expressing mCherry and/or EGFP).

**Determination of viral mutation frequencies by Illumina sequencing.** Samples were prepared for Illumina amplicon sequencing as described previously (22), but with several minor modifications. Briefly, dimethyl sulfoxide (DMSO) (for the no-drug control), 5-aza-C (75% effective concentration [EC75], 260 μM), or 5-aza-dC (EC75, 3.75 μM) was added to U373-MAGI cells (1 million cells/sample) 2 h before infection. The cells were then infected at a multiplicity of infection (MOI) of 1.0 by adding the appropriate volume of NL4-3 MIG-VSVG. The medium was replaced 24 h postinfection, and cells were collected 72 h postinfection. Genomic DNA was purified from cell pellets, and plasmid carryover (from transfections) was then assessed by quantitative PCR (qPCR). Levels of plasmid carryover were determined by dividing the starting quantity of the ampicillin resistance gene by the starting quantity of HIV-1 vif, using previously published primers (22). The average level of plasmid carryover was found to be ~0.4% (range, 0.1 to 0.7%). Five small (~160- to 170-bp) amplicons (Gag, Pol, Vif, Env, Nef) were then prepared from each sample by PCR, using the primers listed in Table 1. PCRs were also performed with the purified plasmid to determine the level of background error due to PCR and Illumina sequencing. For each sample, all amplicons were gel purified, quantified, and pooled in an equimolar fashion to normalize coverage between amplicons. Sequencing libraries were prepared from each sample using the TruSeq Nano DNA kit, pooled in an equimolar fashion, and subjected to 2 × 250 paired-end sequencing on the Illumina MiSeq system. Sequencing reads were analyzed as described previously (22) but were demultiplexed based on library indices instead of internal bar codes. As before, background error hot spots (mostly G-to-T and C-to-A transversions) were identified using the plasmid controls and were masked prior to mutational analysis of the biological samples. The final sequencing data were also used in separate analyses that were focused on drug interactions between 5-aza-C and RNR inhibitors (J. M. O. Rawson, M. E. Roth, J. Xie, M. B. Daly, C. L. Clouser, S. R. Landman, C. S. Reilly, L. Bonnac, B. Kim, S. E. Patterson, L. M. Mansky, submitted for publication).

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Gag</td>
<td>NNGAACGGAGCTGACACAGGGAAA</td>
<td>NNTTCTGCGGTAAAGGCTTCTCT</td>
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<tr>
<td>Pol</td>
<td>NNAGCAGGGCTGAGGCTGAGGG</td>
<td>NNACAGGGGCTTAACTGCTTA</td>
</tr>
<tr>
<td>Vif</td>
<td>NNNACCTGAGCTAACACTGAACTAA</td>
<td>NNGCTCTGAGTCAGAAGTCTT</td>
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<tr>
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<td>NNNACGCTGAGACATTCGTAG</td>
<td>NNNAGTGGCATTCATTTGGTCC</td>
</tr>
<tr>
<td>Nef</td>
<td>NNNCCGAGCTGAGTATCTGAGCCA</td>
<td>NNTTACGGATATCGACCC</td>
</tr>
</tbody>
</table>

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**TABLE 1 Primer sequences for Illumina amplicon sequencing of HIV-1**

- **Gag**
- **Pol**
- **Vif**
- **Env**
- **Nef**

*Note: Illumina primers were designed to prepare five different amplicons (~160 to 170 bp) from different viral genes. Primers were designed with two 5’-terminal degenerate bases (NN) to promote similar efficiencies of ligation to Illumina adapters. The Gag and Vif primers are the same as those utilized in a previous study (22) but lack bar codes, since samples were demultiplexed based on the library index rather than the internal bar code for this study.*
Determination of 5-aza-CTP and 5-aza-dCTP levels by LC–MS-MS. Samples were prepared for liquid chromatography–tandem mass spectrometry (LC–MS-MS) by splitting U373-MAGI cells onto 10-cm plates (1.3 million cells/plate), using two plates per treatment group. The medium was replaced 24 h later, and 5-aza-C, 5-aza-dC, or DMSO (for the no-drug control) was added. 5-Aza-C and 5-aza-dC were added at equivalent effective concentrations (i.e., EC_{25}, EC_{50}, or EC_{75}). Cells were collected 4 h after 5-aza-C or 5-aza-dC addition, corresponding to the time of early reverse transcription (data not shown). Cell pellets were then resuspended in 750 μl of 60% methanol (stored at −20 °C) and were incubated at −20 °C for 18 h. The samples were vortexed, heated at 95°C for 3 min, and centrifuged at 16,000 g for 5 min. The supernatants were transferred to new microcentrifuge tubes, dried using a Savant SPD1010 SpeedVac concentrator (Thermo Fisher Scientific, Inc.) at maximum pressure, and frozen at −80°C until the time of analysis.

For LC–MS-MS, dried extracts were dissolved in 200 μl of water containing 10 μM 5-iodo-dCTP, an internal standard. The samples were centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were analyzed by LC–MS-MS. 5-Aza-C and 5-aza-dC samples were reconstituted immediately prior to LC–MS injection in order to minimize their degradation during the procedure. LC–MS-MS was used to determine the levels of 5-aza-CTP, 5-aza-dCTP, riboguanylurea 5′-triphosphate (RGU-TP), the final hydrolysis product of 5-aza-CTP, and 2′-deoxyriboguanylurea 5′-triphosphate (dRGU-TP). Cells were centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were transferred to new microcentrifuge tubes, dried using a Savant SPD1010 SpeedVac concentrator (Thermo Fisher Scientific, Inc.) at maximum pressure, and frozen at −80°C until the time of analysis.

RESULTS

5-Azacytidine and 5-aza-2′-deoxycytidine induce similar levels of G-to-C and C-to-G transversions in HIV-1. In order to determine the primary form of 5-aza-C that is active against HIV-1, the effects of 5-aza-C and 5-aza-dC on HIV-1 mutagenesis were compared using Illumina amplicon sequencing. The direct incorporation of 5-aza-C as a ribonucleotide (5-aza-CTP) was predicted to lead to a mutational pattern distinct from that of 5-aza-dCTP, because incorporated ribonucleotides can (i) be replaced (correctly or incorrectly) by RNase H2-mediated repair, (ii) cause mutations if left unrepaired, particularly short (2–to-5 bp) deletions, and (iii) elevate the HIV-1 RT mismatch extension frequency on the opposite strand (26–28).

To prepare samples for sequencing, U373-MAGI cells were treated with DMSO (i.e., no drug), 5-aza-C (EC_{75} ~ 260 μM) or 5-aza-dC (EC_{75} ~ 3.8 μM). Cells were then infected 2 h after drug addition at an MOI of 1.0 with NL4-3 MIG-VSVG. Genomic DNA was purified from cells collected 72 h postinfection, and PCR was performed to prepare multiple amplicons (Gag, Pol, Vif, Env, Nef) from proviral DNA. Plasmid control amplifications were included to measure the level of background error from PCR and sequencing. The amplicons were pooled, used to prepare libraries, and subjected to 2 × 250 paired-end sequencing on the Illumina MiSeq system. Illumina sequencing generated ~ 5.5 million read pairs after all bioinformatics processing steps, containing ~570,000 mutations and ~630 million reference bases. 5-Aza-C and 5-aza-dC were found to significantly increase the frequencies of G-to-C and C-to-G transversions compared to the no-drug control in all amplicons examined (P < 0.0001 for all comparisons), although C-to-G transversions were ~5- to 6-fold less prevalent than G-to-C transversions (Fig. 1). In the presence of 5-aza-C, ~67% of all mutations were G-to-C transversions and ~12% were C-to-G transversions (compared with 64% and 12% for 5-aza-dC). C-to-G transversions were not observed previously when 5-aza-C was added during the early phase of viral replication (10), likely due to the lower sequencing depth of earlier studies (see Discussion). Notably, 5-aza-C and 5-aza-dC increased the levels of G-to-C transversions (Fig. 1A) and C-to-G
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transversions (Fig. 1B) to similar extents in all five amplicons, and the resulting mutation frequencies did not differ significantly ($P > 0.05$ for all comparisons). Depending on the amplicon, 5-aza-C and 5-aza-dC raised G-to-C transversion frequencies by ~45- to 75-fold and C-to-G frequencies by ~25- to 60-fold. Further, we did not observe short (2- to 5-bp) deletions that are characteristic of ribonucleoside incorporation in the 5-aza-C-treated sample (data not shown). These data demonstrate that 5-aza-C and 5-aza-dC cause very similar changes in viral mutation frequencies and spectra, arguing that 5-aza-C acts primarily as 5-aza-dCTP during reverse transcription.

5-Azacytidine and 5-aza-2’-deoxycytidine cause highly similar HIV-1 mutational patterns. 5-Aza-dC has been demonstrated previously to cause highly variable levels of G-to-C and C-to-G transversions depending on the specific sequence position (12). If 5-aza-C acts against HIV-1 after reduction to 5-aza-dC, the susceptibilities of individual bases to 5-aza-C and 5-aza-dC should closely resemble one another. To address this, mutation frequencies were determined at individual sequence positions in the five amplicons. G-to-C mutation frequencies were determined at every guanine position (124 in total), while C-to-G mutation frequencies were determined at every cytosine position (116 in total). The extent of correlation between 5-aza-C- and 5-aza-dC-mediated mutation frequencies was then examined. For both 5-aza-C and 5-aza-dC, significant variability (as much as ~20-fold) was observed between G-to-C and C-to-G frequencies at individual sequence positions (Fig. 2). 5-Aza-C- and 5-aza-dC-mediated mutation frequencies were found to be strongly positively correlated, both for G-to-C transversions (Pearson’s $r$, 0.93 [95% confidence interval (CI), 0.90 to 0.95]; $P < 0.0001$) and for C-to-G transversions (Pearson’s $r$, 0.96 [95% CI, 0.94 to 0.97]; $P < 0.0001$). This correlation was able to explain most of the variability in the observed data ($R^2$, 0.87 [G-to-C transversions] or 0.91 [C-to-G transversions]). The slopes of the best-fit linear regression lines were also determined, since they would equal 1.0 if the 5-aza-C- and 5-aza-dC-induced mutation frequencies matched perfectly. The regression lines exhibited slopes of 0.71 for G-to-C transversions (95% CI, 0.66 to 0.76) and 0.88 for C-to-G transversions (95% CI, 0.83 to 0.93), indicating that the frequencies of 5-aza-C-mediated mutations were slightly higher than those of 5-aza-dC-mediated mutations. These findings indicate that the patterns of viral mutagenesis in the presence of 5-aza-C and 5-aza-dC closely resembled one another, further indicating...
that 5-aza-C likely reduces HIV-1 infectivity after conversion to 5-aza-dC.

Levels of 5-aza-dCTP are similar in cells treated with 5-aza-cytidine or 5-aza-2'-deoxyctydine. While a previous report indicated that ~10 to 20% of 5-aza-C is reduced to 5-aza-dC in one particular cell line (13), the conversion of 5-aza-C to 5-aza-dC has not yet been assessed in cell lines for which antiviral activity has been demonstrated. To address this, cells were treated with multiple concentrations of either 5-aza-C or 5-aza-dC for 4 h (corresponding to a time point during which reverse transcription should be actively occurring); nucleotides were then extracted, and LC–MS-MS was performed to determine the relative quantities of 5-aza-CTP and 5-aza-dCTP (see Materials and Methods). The relative levels of RGU-TP and dRGU-TP, which are the final hydrolysis products of 5-aza-CTP and 5-aza-dCTP, respectively, and may be relevant to antiviral activity, were also determined. Cells were treated with equivalent effective concentrations (EC25, EC50, or EC75) of 5-aza-C or 5-aza-dC. If 5-aza-C acts primarily as 5-aza-dCTP against HIV-1, cells treated with 5-aza-C or 5-aza-dC should contain approximately equal levels of 5-aza-dCTP at equivalent effective concentrations. 5-Aza-CTP and RGU-TP were detected only in cells treated with 5-aza-C (data not shown), as expected considering the lack of cellular pathways for the conversion of deoxyribonucleotides to ribonucleotides. In contrast, 5-aza-dCTP was detected in cells treated with either 5-aza-C or 5-aza-dC (Fig. 3A), indicating that 5-aza-C (in its diphosphate form) was reduced to 5-aza-dC by RNR. Further, the levels of 5-aza-dCTP either did not differ significantly between cells treated with 5-aza-C and cells treated with 5-aza-dC (EC25 and EC75) or were higher in cells treated with 5-aza-C (at the EC50) (P < 0.05). Similar trends were observed for dRGU-TP, the final hydrolysis product of 5-aza-dCTP (Fig. 3B). These findings further indicate that 5-aza-C reduces the infectivity of HIV-1 primarily after reduction to 5-aza-dC, since HIV-1 RT should incorporate 5-aza-dCTP much more readily than 5-aza-CTP.

HIV-1 reverse transcriptase incorporates 5-aza-CTP in vitro much less efficiently than 5-aza-dCTP. Although similar levels of 5-aza-dCTP were observed in cells treated with 5-aza-C or 5-aza-dC, the extents to which 5-aza-CTP and 5-aza-dCTP are incorporated by HIV-1 RT had not yet been determined. 5-Aza-CTP was hypothesized to be incorporated only weakly, while 5-aza-dCTP was hypothesized to be incorporated relatively efficiently, since 5-aza-dCTP can be efficiently incorporated by cellular DNA polymerases (29, 30). To address this, the incorporation of 5-aza-CTP and that of 5-aza-dCTP were compared using an HIV-1 RT in vitro single nucleotide extension assay, with dCTP and CTP included for comparative purposes. Additional control reactions were performed by incubation with all four standard dNTPs (50 μM) (positive control) or by the omission of RT (negative control). It was found that HIV-1 RT incorporated 5-aza-dCTP relatively efficiently (Fig. 4), resulting in total primer extension at 5 μM (lane 10) and some detectable extension at 5-aza-dCTP concentrations as low as 50 nM (lane 8). However, 5-aza-dCTP was incorporated somewhat less efficiently than dCTP (Fig. 4, compare lanes 7 to 9 with lanes 19 to 21). In contrast, HIV-1 RT incorporated 5-aza-CTP and CTP only at the highest concentration tested (500 μM) (Fig. 4, lanes 6 and 18). These data demonstrate that 5-aza-CTP can be directly incorporated by HIV-1 RT, but only at very high concentrations and in the absence of competing deoxyribonucleotides. 5-Aza-dCTP was incorporated at least 10,000-fold more efficiently than 5-aza-CTP (Fig. 4, compare lanes 6 and 8). These observations are consistent with previous findings that HIV-1 RT exhibits high selectivity for deoxyribo-
nucleotides, largely due to a steric gate (Y115) that blocks incoming ribonucleotides (14–16). Considering that similar levels of 5-aza-dCTP were observed in cells treated with equivalent effective concentrations of 5-aza-C or 5-aza-dC (Fig. 3), these findings strongly indicate that 5-aza-C exerts activity against HIV-1 primarily through its reduction to 5-aza-dC.

**DISCUSSION**

The ribonucleoside analog 5-aza-C could potentially act as a ribonucleotide or as a deoxyribonucleotide to reduce HIV-1 infectivity during the early phase of viral replication. To address this, Illumina sequencing was performed in order to compare the enhancement of HIV-1 mutagenesis by 5-aza-C and 5-aza-dC. 5-aza-C and 5-aza-dC were found to cause similar changes in viral mutation frequencies regardless of the amplicon examined (Fig. 1). Both drugs elicited primarily G-to-C transversions in HIV-1, but also significant levels of C-to-G transversions, which were not observed in a previous study of 5-aza-C (10). 5-aza-C was not found to induce other types of mutations that are considered indicative of ribonucleotide incorporation, such as short (2- to 5-bp) deletions (27). While the mechanism of C-to-G transversions remains unclear, they may result from the incorporation of 5-aza-dCTP into plus-strand viral DNA or, alternatively, from the direct incorporation of 5-aza-dCTP hydrolysis products (e.g., dRGU-TP) into minus-strand viral DNA. 5-Aza-C-mediated C-to-G transversions were not observed in a previous study (10), possibly due to the reduced depth of Sanger sequencing, the lower concentration of 5-aza-C used, or other differences in the mutational assay. Additionally, 5-aza-C- and 5-aza-dC-mediated mutational patterns correlated strongly with one another even at the level of individual sequence positions in HIV-1 (Fig. 2), further indicating that 5-aza-C exerts antiviral activity after reduction to 5-aza-dC.

5-Aza-dCTP was detected in cells treated with 5-aza-C, indicating that 5-aza-C was reduced to 5-aza-dC (Fig. 3), and, importantly, similar levels of 5-aza-dCTP were observed in cells treated with equivalent effective concentrations of 5-aza-C and 5-aza-dC. Notably, the absolute concentrations of 5-aza-C were ~70- to 300-fold higher than those of 5-aza-dC in these experiments, indicating that intracellular conversion of 5-aza-C to 5-aza-dCTP was much less efficient than the conversion of 5-aza-dC to 5-aza-dCTP. By use of an *in vitro* incorporation assay, very high concentrations of 5-aza-dCTP were found to be successfully incorporated by HIV-1 RT (Fig. 4). However, 5-aza-dCTP was incorporated at least 10,000-fold more efficiently than 5-aza-dCTP, further supporting the notion that 5-aza-dCTP is the principal form of 5-aza-C that is active against HIV-1.

Taken together, these findings clearly indicate that 5-aza-dCTP is the primary form of 5-aza-C that is active during the early phase of HIV-1 replication. Further, 5-aza-C has been shown to exert much more potent antiviral activity during the early phase of replication than during the late phase of replication (10), indicating that 5-aza-C likely acts primarily as 5-aza-dCTP during spreading infections as well. These observations lead to the following model for the antiviral activities of 5-aza-C and 5-aza-dC (Fig. 5). 5-Aza-C and 5-aza-dC enter the cell by facilitated diffusion through a transporter protein and are then phosphorylated by the appropriate cellular kinases. 5-Aza-CTP is incorporated into viral RNA during the late phase of replication, leading to the fixation of C-to-G transversions during the following round of reverse transcription. 5-Aza-C (in its diphosphate form) is also converted to 5-aza-dC by RNR, and the resulting 5-aza-CDP can then be phosphorylated to form 5-aza-dCTP. Lastly, 5-aza-dCTP is incorporated into viral DNA during reverse transcription, resulting in the fixation of G-to-C and (to a lesser extent) C-to-G transversions in HIV-1.

In the future, it would be of interest to investigate the antiviral activity of 5-aza-C in other cell types, such as primary CD4+ T cells and macrophages. 5-Aza-C would likely act primarily as 5-aza-dCTP in activated CD4+ T cells, since they divide rapidly and thus have high levels of RNR and dNTPs (17, 31). In contrast, 5-aza-C could potentially be directly incorporated at significant levels in macrophages, which have low levels of RNR, low dNTP pools, and much higher ratios of NTPs to dNTPs (17, 31). Indeed, previous work has shown that HIV-1 RT can incorporate significant levels of endogenous ribonucleotides in macrophages and *in vitro* under macrophage-like conditions (17, 18). Further, the ribonucleoside analog 3′-deoxyadenosine, which likely cannot be reduced, exhibits antiviral activity in macrophages but not in CD4+ T cells, indicating that ribonucleoside analog triphosphates can be directly incorporated in macrophages (17).

Notably, the findings in this study have important implications for the design of ribonucleoside analogs directed against retroviruses. Ribonucleoside analogs are of interest as antiretroviral agents because their synthesis is often less complicated, less time-intensive, and more affordable than that of their de-
oxyribonucleoside counterparts. Ribonucleoside analogs may also offer other advantages in certain instances, such as better activation by cellular enzymes or improved antiviral activity by the targeting of both viral RNA and DNA. However, the findings in this study suggest that it is important to consider the efficiency at which ribonucleoside analogs are reduced—at least for the targeting of viral replication in cell types with high dNTP pools, such as CD4+ T cells. In contrast, efficient reduction may not be necessary or even desired for the targeting of viral replication in cell types with low dNTP pools, such as macrophages. Ribonucleoside analogs that cannot be reduced (such as those lacking a 3’ hydroxyl group, e.g., 3’-deoxyadenosine) could be used to specifically target viral replication in macrophages, an important viral reservoir (17). In sum, these findings inform efforts to identify additional nucleoside analogs that target HIV-1 RT, which continue due to significant concerns regarding the development of drug resistance and off-target effects.

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