Effect of Cell Cycle Arrest on the Activity of Nucleoside Analogues against Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus (HIV) reverse transcription can be notably affected by cellular activation, differentiation, and division. We hypothesized that changes in the cell cycle could also affect HIV susceptibility to nucleoside analogues, which compete with natural nucleotides for incorporation into viral DNA and inhibit viral replication through premature termination of reverse transcription. Proliferating HeLa-derived indicator cells were arrested in the S/G2 phase with etoposide, a topoisomerase II inhibitor, or in the G1/S phase with aphidicolin, a polymerase ε inhibitor. Cell cycle arrest by both agents induced a remarkable decrease in HIV susceptibility to zidovudine (AZT). This decrease was seen both with a single-cycle infectivity assay and with a viral DNA quantitation assay, indicating that the effect of cell cycle arrest was exerted at the reverse transcription stage. The increase in the 50% inhibitory concentration (IC50) seen with arrested cells was strongest for AZT (23-fold) and stavudine (21-fold) but more modest for other drugs (lamivudine, 11-fold; dideoxynosine, 7-fold; and nevirapine, 3-fold). In drug-resistant reverse transcriptase mutants, the increase in AZT IC50 (relative to that in dividing cells) was most prominent with a Q151M mutant and was comparable to the wild type in other drug-resistant mutants. Quantitation of intracellular pools of dTTP and AZT 5’-triphosphate (AZTTP) showed that etoposide treatment induced a significant increase in intracellular dTTP and consequently a decrease in AZTTP/dTTP ratios, suggesting that the decrease in viral susceptibility to AZT was caused by reduced incorporation of the analogue into nascent viral DNA. These results emphasize the importance of cellular proliferation and deoxynucleoside triphosphate metabolism in HIV susceptibility to nucleoside analogues and underscore the need to study the activities of drugs of this class with natural target cells under physiological conditions of activation and proliferation.

Nucleoside analogues, a part of most combination therapy regimens prescribed for the treatment of human immunodeficiency virus (HIV) infection, are the most widely used class of antiretroviral drugs. These compounds become active after phosphorylation into their triphosphate derivatives (15) and compete with natural endogenous deoxynucleoside triphosphates (dNTPs) for incorporation into nascent viral DNA by reverse transcriptase (RT), where they block viral DNA synthesis through a chain termination mechanism (9, 23, 24). The triple phosphorylation of nucleoside analogues is performed by cellular kinases that also catalyze the phosphorylation of natural endogenous deoxynucleosides (7, 19, 27). Although it is well established that the expression and activity of these cellular kinases are regulated by the cell cycle and by the state of activation and division of the cells (13, 29), the extent to which these parameters can affect the antiviral activity of nucleoside analogues is not known. Changes in the metabolism of nucleosides and, in particular, changes in the phosphorylation of nucleosides by cellular kinases could affect the antiviral activity of nucleoside analogues by two principal mechanisms. First, changes in the intracellular concentrations of endogenous dNTPs could affect the rate of incorporation of competing nucleoside analogue triphosphates into viral DNA (3, 4). Second, changes in the phosphorylation of nucleoside analogues could directly and selectively affect the availability and antiviral activity of the active triphosphate derivatives of the analogues.

The impact of fluctuations in the metabolism of deoxynucleosides in relation to cell activation and division could have strong implications regarding the antiviral activity of nucleoside analogues in vivo, where HIV can enter and initiate its replicative cycle in cell types with variable levels of metabolic activation and of cell division activity (11, 22, 28, 30). Although the majority of the actively replicating virus populations in vivo are believed to be produced by activated and dividing CD4+ T lymphocytes, most potential HIV target cells in which nucleoside analogues need to exert their antiviral activity are either metabolically resting or nondividing. The precise impact of these conditions on the antiviral activity of nucleoside analogues, however, has been difficult to study with tissue culture using primary human T cells. In quiescent primary CD4+ T lymphocytes, HIV replication is indeed notoriously inefficient, in relation to low dNTP pools, low metabolic activity, and possibly other mechanisms restricting viral DNA synthesis (2). In this study, we have used tumor-derived HIV-susceptible cells as a model and examined the effects of two drugs that arrest the cell cycle, etoposide and aphidicolin, on the antiviral activity of nucleoside analogues. We observed that blocking the cell cycle in G1/S or in S/G2 induced a decrease in HIV susceptibility to nucleoside analogues, most notably zidovudine (AZT). Cells arrested in the cell cycle at these phases were found to contain significantly increased intracellular dTTP but no significant change in AZT 5’-triphosphate (AZTTP) content. These findings emphasize the potential impact of cell...
by MT-4 cells, was spinoculated (860 (AZT or nevirapine) for 12 h. DNase I-treated virus (100 ng of p24), produced

Amplification was performed with a 7000 sequence detection system (Applied /H9262 /H11003

phosphoramidite and TAMRA is 6-carboxytetramethylrhodamine). DNA was

aliquots were frozen. To produce viral stocks used for analyzing the effect of cell

m pore size) and stored in aliquots.

Infectivity of each supernatant was assessed using a colorimetric assay based on

viability fell below 80% was filtered (0.45-

cultures was monitored daily. Culture medium obtained the day before the

were fitted to a sigmoid dose-response curve with variable slope. The lower

tivity was assessed 48 h later by using a single-cycle colorimetric assay as de-

quantified using liquid chromatography coupled with tandem mass spectrometry,

as previously described for metabolites of stavudine (d4T), lamivudine (3TC),

dideoxyinosine (dld) (5) and for natural endogenous deoxy nucleotides (14).

Measurement of intracellular AZTTP and dTTP concentrations. For analyses of whole-cell extracts, 2 × 107 cells were washed twice in 140 mM NaCl and immediately lysed in a Tris buffer- methanol 30:70 (vol/vol) mixture. For analyses of nuclear and cytosolic extracts, cells were washed twice in 140 mM NaCl, treated with pronase (7 mg/ml in Dulbecco’s culture medium without serum, supplemented with 20 mM HEPEs) for 10 min, washed three times in 140 mM NaCl, and resuspended in 200 μl Tris-NaCl-EDTA buffer containing 100 μg/ml digitonin for 10 min at 4°C. The lysate was then centrifuged at 250 × g, yielding a nuclear pellet and cytosolic supernatant. These fractions were resuspended in Tris- methanol as described above and immediately frozen at ~80°C. Intracellular

triposphorylated metabolites of AZT (AZTTP and thymidine (dTTP) were

measured in triplicate, and results were analyzed using the Cell Quest soft-

ofluorometry (FACSCalibur; BD Biosciences). A total of 20,000 events were

with 75 μl of proteinase K and lysed. DNA was purified using a QIAquick 8 PCR puri-

for 6 h at 37°C. Following removal of culture medium, the cells were detached

293T cells were treated with D Nas (SIGMA-ALDRICH) for 30 min at room temperature. Cells were stained with

50 μg/ml G418.

Human 293T cells and HeLa P4

was expressed as fmol/106 cells.

were compared by using a previously described DNA-

base biochemical method (6) and the nucleotide concentration in the sample

RESULTS

Cell cycle arrest by etoposide and aphidicolin. Two drugs, etoposide and aphidicolin, were used to provoke an arrest in the cell cycle of HeLa-derived P4 HIV indicator cells. Etopo-

side, an inhibitor of topoisomerase II, blocks the cell cycle activity. They warn that, in vivo,

that the activity of nucleoside analogues in primary cells may be

quantified using liquid chromatography coupled with tandem mass spectrometry,

or aphidicolin for 72 h. Cells were collected in phosphate-buffered saline (PBS) containing 1 mM EDTA, resuspended in 70% ethanol for 10 min on ice, washed, and resuspended in PBS containing 180 μg/ml of R Na (Sigma-Aldrich) for 30 min at room temperature. Cells were stained with 75 μg/ml propidium iodide (Sigma-Aldrich) in PBS and analyzed by cyt-

lar triphosphorylated metabolites of AZT (AZTTP) and thymidine (dTTP) were

Intracellular triphosphate readings for each drug concentration were fitted to a sigmoid dose-response curve with variable slope. The lower

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were supplemented with 10% fetal calf serum, and 50 μg/ml streptomycin, and 50 U/ml penicillin G. HeLa P4 cells were cultured in the presence of 500 μg/ml G418.

To produce viral stocks for the analysis of resistance to RT inhibitors, 293T cells (1.5 × 106 cells/well in 25-cm2 flasks) were transfected with 8 μg of plasmid DNA by calcium phosphate precipitation. After culture for 12 h, cells were

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were fitted to a sigmoid dose-response curve.

The triplicate optical density readings for each drug concentration

The exception of analyses conducted with cytosolic fractions, the number of cells in the extracted sample was determined by using a previously described DNA-based biochemical method and the nucleotide concentration in the sample

was fitted to the background of the assay. The IC50 was determined from the dose-response curve.

Quantification of the effect of cell cycle arrest on HIV DNA synthesis. HeLa P4 cells (2 × 106 cells/well in 6-well plates) were treated with various concentrations of etoposide or aphidicolin for 18 h and serial dilutions of RT inhibitors for 4 h, infected with an equivalent of 5 ng of wild-type NL4.3 virus or isogenic RT mutant strains, and maintained in the presence of RT inhibitors and cell cycle inhibitors. Infect-

Intracellular triphosphate readings for each drug concentration were fitted to a sigmoid dose-response curve with variable slope. The lower

Assessing the concentrations that would induce a stable block in cell growth in the absence of significant cell death, P4 cells were treated for 48 h with increasing concentrations of aphidicolin or etoposide. As shown in Fig. 1, treatment of P4 cells with etoposide induced a strong reduction of cells in the G1 phase of the cell cycle, together with an accumulation of cells whose DNA contents were indicative of G0/G1 cell cycle arrest. The percentage of cells in G1 was 9% at 250 nM and 1% at 1 μM, with a percentage of cell death of <5% and <10%, respectively. With aphidicolin, a marked increase in the proportion of cells blocked at the S phase was observed. The percentage of cells in S phase was 33% at 250 nM and 44% at 1 μM, while the proportion of dead cells was <5% at 250 nM and <10% at 1 μM. By use of 1 μM aphidicolin, a minority of cells (26%) exhibited a DNA content characteristic of the G2 phase, in line with the fact that aphidicolin can either block the cell cycle during the course of the S phase or prevent cells from entering the S phase.

Effect of cell cycle arrest on HIV-1 susceptibility to AZT. To assay HIV-1 susceptibility to AZT in growth-arrested cells, P4

final volume, 50 μl) contained 1 × TaqMan universal PCR mixture (Applied Biosystems, Foster City, Calif.), 200 nM (each) primer, 100 nM TaqMan probe, and 10 μl of diluted DNA. Amplification was performed with a 7000 sequence detection system (Applied
cells were treated with 1 μM etoposide or aphidicolin and used as targets in a single-cycle AZT susceptibility assay, using wild-type pNL4-3-derived HIV-1 virions produced by transfection of 293T cells. The inhibition curves presented in Fig. 2A, which plot mean percent inhibition in viral infectivity as a function of AZT concentration from four independent experiments, reveal a marked decrease in the inhibitory activity of AZT in growth-arrested cells, most prominently in etoposide-treated cells. The mean increase in the IC50 of AZT was 30-fold for etoposide and 7-fold for aphidicolin. This increase in IC50 was found to be strongly dose dependent for both etoposide and aphidicolin (Fig. 2B). Again, the effect was most prominent with etoposide, for which an increase in the IC50 of AZT was clearly measurable at 0.1 μM, whereas the first perceptible effect of aphidicolin was seen at 1 μM. This effect was also strongly related to the proportion of growth-arrested cells in the treated cultures, with a linear relationship between the increase (n-fold) in AZT IC50 and the percentage of cells arrested at the S/G2 phase and the S phase for etoposide and aphidicolin, respectively (Fig. 2C). This indicates that the decrease in HIV-1 susceptibility to AZT produced by etoposide and aphidicolin was related to their effect on the cell cycle, rather than to a direct effect on HIV DNA synthesis. It remained possible, however, that the impact of aphidicolin and etoposide was related to changes in the induction of β-galactosidase by Tat, since the transactivating properties of Tat have been described to be influenced by the cell cycle (16, 25). To rule out this possibility, the effect of the cell cycle-blocking drugs on HIV susceptibility to AZT was examined using a direct quantitative assessment of HIV DNA synthesis by real-time PCR. P4 cells were pretreated with etoposide for 24 h, treated with serial dilutions of AZT for 12 h, and exposed by spinoculation to DNase-treated, wild-type pNL4-3 HIV-1 virions harvested from infected MT4 cell cultures. Six hours after inoculation, an intermediate reverse transcript corresponding to a segment of the env gene (nucleotides 6275 to 6380) was quantified and the effect of AZT was expressed as a percent reduction of viral DNA content relative to that of cells incubated in the absence of AZT. Figure 3A shows a marked shift in the AZT inhibition curve of HIV DNA synthesis for cells pretreated with etoposide, a shift amounting to a 22-fold increase in IC50. Etoposide induced no significant change in the inhibition curve of nevirapine, a nonnucleoside inhibitor of HIV-1 reverse transcriptase.

**Effect of cell cycle arrest on activity of nucleoside analogues against wild-type and resistant HIV-1.** To further evaluate the effect of cell cycle arrest on antiviral activity of antiretroviral drugs, we examined the etoposide-induced changes in the IC50 of three additional nucleoside analogues, d4T, ddI, and 3TC, and of a nonnucleoside RT inhibitor, nevirapine, with wild-type HIV-1. As shown in Fig. 4A, the increases in IC50 were different according to the drug. The effects were highest with AZT (23.5-fold, \( P = 0.008 \), Mann-Whitney test) and d4T.
More-modest changes were observed with ddI (7.4-fold, \( P = 0.110 \)) and with 3TC (11.2-fold, \( P = 0.100 \)). With nevirapine, the increase in IC\(_{50}\) produced by cell cycle arrest was always below 3.5-fold (mean of 2.5-fold, \( P = 0.030 \)).

The impact of etoposide on the antiviral activities of AZT, d4T, and 3TC was then measured with RT mutants representing the two principal mechanisms of HIV resistance to nucleoside analogues, mutant M41L/T215Y and mutant Q151M (Fig. 4B) (10). The increases in the IC\(_{50}\) of AZT, d4T, and 3TC induced by etoposide were not different for wild-type virus and for the M41L/T215Y virus. A significant difference, however, emerged regarding resistance to AZT when comparing mutant Q151M and the two other viruses (Q151M versus the wild type, \( P < 0.01 \); Q151M versus M41L+T215Y, \( P < 0.05 \); Kruskal-Wallis test). With d4T, an increase was also seen but was not found statistically significant. No differences between the various mutants were seen with 3TC. Mutation Q151M, which confers wide cross-resistance among nucleoside analogues, promotes resistance to AZT and d4T but not 3TC through an analogue discrimination mechanism that is distinct from the terminator excision mechanism produced by thymidine analogue mutations such as M41L and T215Y. Of note, no changes in the IC\(_{50}\) of 3TC could be measured with mutant M184V, which produced a level of 3TC resistance that was too high to allow accurate calculation of an IC\(_{50}\) value (data not shown).

Changes in intracellular nucleoside triphosphate concentration after cell cycle arrest. Since the effect of cell cycle arrest on HIV susceptibility to RT inhibitors appeared to be restricted to nucleoside analogues, we hypothesized that this effect was related to changes in the intracellular metabolism of endogenous nucleosides or the nucleoside analogues themselves. Thus, we examined the AZTTP and dTTP contents of P4 cells after treatment with etoposide, using combined mass spectrometry and high-performance liquid chromatography methods. To ascertain that the observed changes were relevant to reverse transcription, which is believed to occur in the cytoplasm of infected cells, AZTTP and dTTP contents were also measured in nuclear and cytosolic fractions, following disruption of the plasma membrane with digitonin and fractionation by centrifugation. As shown in Fig. 5, which summarizes the

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**FIG. 2.** HIV-1 susceptibility to AZT in growth-arrested cells. (A) HeLa-derived P4 cells were treated with 1 \( \mu \)M etoposide or aphidicolin for 18 h or were untreated. Serial dilutions of AZT were then added to the cultures 4 h before exposure to HIV-1 NL4-3. Measurement of AZT susceptibility was performed using a single-cycle, Tat-dependent, \( \beta \)-galactosidase colorimetric assay. The inhibition curves plot mean percent inhibition in viral infectivity as a function of AZT concentration from four independent experiments. (B) Effects of the concentrations of etoposide and aphidicolin on HIV-1 susceptibility to AZT. (C) Correlation between the increase \((n\text{-fold})\) in AZT IC\(_{50}\) relative to that of untreated cells, and the proportion (given in percentage) of S/G\(_{2}\)-arrested cells in the culture.
findings of at least three independent experiments, we observed a significant increase in dTTP content in cells treated by etoposide, relative to that of untreated controls. This increase was seen with whole cells ($P < 0.01$), cytosolic fractions ($P < 0.001$), and nuclear fractions ($P < 0.01$). With aphidicolin, an increase in dTTP content was also observed for whole-cell extracts, but this difference failed to reach statistical significance ($P = 0.06$; data not shown). Interestingly, there was no significant change in AZTTP content in cells treated by etoposide, whether in whole cells, nuclei, or cytosolic fractions ($P > 0.05$). Consequently, the AZTTP/dTTP ratios were also decreased in all fractions.

**DISCUSSION**

A large proportion of currently available antiretroviral drugs target HIV reverse transcription, a key step in the virus life cycle that occurs early after viral entry into the target cell. Unlike other steps of the retroviral replicative cycle, reverse transcription is subject to important variations according to cell activation and cell differentiation, in relation to fluctuations in the synthesis and metabolism of cellular nucleic acids (17, 21, 30). In vivo, while most virus particles are assumed to be produced by CD4$^+$ T cells that are proliferating and metabolically active, these particles mostly encounter resting, nonproliferating cells, which constitute the majority of the T-cell reservoir, or nondividing cells of the monocyte-macrophage lineage. Thus, most HIV reverse transcription events in an infected individual probably occur in cells that are not actively dividing (18, 20, 21). In addition, actively HIV-infected cells express the Vpr accessory protein, which is known to exert a block on the cell cycle through molecular mechanisms that are not fully elucidated (1, 12, 17). Thus, in cells subjected to multiple asynchronous infections, any reverse transcription events occurring following the expression of Vpr would take place under conditions where the cell cycle is arrested at the G2 phase.

We hypothesized here that the metabolic changes that occur during the cell cycle could impact the antiviral activity of antiretrovirals targeting HIV reverse transcription, particularly nucleoside analogues. Using inhibitors that arrest the cell cycle through a block of cellular DNA polymerases or topoisomerases, we observed a marked decrease in antiretroviral activity of nucleoside analogues in cells arrested in the S or G2 phase of the cell cycle. This decrease in susceptibility was most prominent with AZT and d4T, while other nucleoside analogues appeared to be less affected. Correspondingly, we found that the arrest in the cell cycle provoked by etoposide was accompanied by a significant increase in intracellular concentration of dTTP, while no significant change in AZTTP concentration was observed. Whether the increase in intracellular dTTP was...
The decrease in HIV susceptibility to nucleoside analogues in cell cycle-arrested target cells was found to be most prominent in a mutant bearing the Q151M mutation, a mutation that increases the capacity of RT to discriminate between natural nucleosides and their analogues (10), thereby promoting high levels of resistance to most of these drugs. This finding is consistent with our hypothesis that decreased susceptibility to nucleoside analogues by cell cycle arrest is mediated by a drop in the AZTTP/dTTP ratio. Intuitively, a higher discriminative capacity by RT should result in even higher resistance under conditions where the ratio between analogue and endogenous nucleoside is lower than under conditions where this ratio is high.

The enhancing effect of the Q151M mutation was found to be significant only with AZT ($P < 0.01$ versus the wild type), and $P$ of $<0.05$ versus M41L+T215Y) and was not observed with 3TC. This observation is consistent with the fact that the discriminating capacity of RT is most prominent with AZT, an analogue that differs markedly in size from its natural counterpart (9), compared to the other analogues tested (26), and which should therefore be more prone to discrimination by the Q151M mutation.

Our observations open the possibility that natural fluctuations in intracellular dNTP pools, whether related to cellular proliferation, activation, or differentiation, may have a notable impact on the selection for resistance to nucleoside analogues. In its initial stages in vivo, it is likely that selection for resistance is favored under conditions where the susceptibility of the wild-type virus is already significantly decreased. Although viruses with thymidine analogue mutations were found to be no more affected by cell cycle arrest and dNTP fluctuations than the wild-type virus, the increase in $IC_{50}$ seen with AZT and other drugs makes it more likely that HIV reverse transcription proceeds in spite of high concentrations of extracellular AZT and intracellular AZT $5'$-monophosphate, thereby favoring emergence of resistance. This increase in $IC_{50}$ also lowers the genetic barrier for resistance, since it allows reverse transcription and establishment of a productive infection by viruses with one or few resistance mutations.

Finally, our finding that higher dTTP content in HIV target cells results in higher observed levels of resistance to AZT may have important implications for the interpretation of phenotypic resistance values as measured by tissue culture-based assays. Most of these assays utilize indicator tumor cells as targets for HIV infection, and it is well established that such cells have markedly higher dNTP content than that usually found in the primary cells that are the natural targets of HIV in vivo. The association of increased intracellular dTTP content with increased apparent HIV resistance to AZT suggests that resistance to AZT, and possibly to other deoxynucleoside analogues, could be notably distorted with tumor cell-based phenotypic resistance assays and should caution against systematically translating resistance values, as observed with these assays, into clinically relevant resistance levels.

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**REFERENCES**
