0026-895X/93/010057-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:57-63

Effects of 3′-Azido-3′-deoxythymidine Metabolites on Simian Virus 40 Origin-Dependent Replication and Heteroduplex Repair in HeLa Cell Extracts

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Received June 30, 1992; Accepted October 1, 1992

SUMMARY

Although the capacity of 3′-azido-3′-deoxythymidine (AZT) triphosphate, an active metabolite of the antiviral agent zidovudine (AZT), to inhibit polymerization by a variety of purified DNA polymerases has been described, it is important to understand its effect on replication by the more complex protein assemblies responsible for DNA replication in human cells. In the present study, we have determined the effects of AZT metabolites on the efficiency of simian virus 40 origin-dependent bidirectional replication of double-stranded DNA in extracts of human HeLa cells. Replication was inhibited by AZT diphosphate and AZT triphosphate, but only at concentrations exceeding those thought to be present *in vivo*. However, replication was inhibited by AZT monophosphate at concentrations previously reported to accumulate in human cells cultured in the presence of AZT, suggesting that AZT monophosphate may contribute to cytotoxicity by inhibiting chromosomal replication. In an attempt to determine whether AZT treatment could have longer term mutagenic effects on cells, we also determined the effects of these AZT derivatives on replication fidelity and on the efficiency of repair of DNA substrates containing single-base mismatches. Despite the ability of a normal deoxynucleoside monophosphate to reduce the fidelity of DNA replication, presumably by reducing exonucleolytic proofreading of errors, neither the mono-, di-, nor triphosphate form of AZT reduced base substitution fidelity when present in replication reactions. Similarly, the efficiency of repair of DNA substrates containing single-base mismatches was unaffected by these compounds. However, replication fidelity was affected by perturbations in relative and absolute concentrations of deoxynucleoside triphosphate substrates similar to those reported to occur in AZT-treated cells. Thus, AZT treatment could potentially be mutagenic *in vivo* via reduced replication fidelity resulting from alterations in deoxynucleoside triphosphate pools.

AZT was the first clinically approved drug for use in the therapy of acquired immunodeficiency syndrome in humans. Inside the cell, AZT is phosphorylated, first by thymidine kinase to AZTMP, then by thymidylate kinase to AZTDP, and then by nucleoside diphosphate kinase to AZTTP (1). It is believed that the therapeutic action of AZT is through AZTTP. Studies have shown that AZTTP is a substrate for HIV reverse transcriptase, which incorporates the nucleotide analog into DNA almost as efficiently as TTP and much more readily than do cellular replicative DNA polymerases (2–6). Incorporation of AZTMP into DNA results in termination of viral replication due to formation of a primer terminus that cannot be extended because it lacks a 3′-OH group.

Unfortunately, AZT treatment of patients has severe side effects, including suppression of bone marrow proliferation, manifested as anemia and neutropenia (7). AZT treatment of cultured cells is cytotoxic (8). The biochemical mechanisms responsible for cytotoxicity are not well understood. Human cell lines treated with AZT accumulate AZTMP to concentrations about 100-fold higher than the concentrations of AZTDP and AZTTP (1, 9–12), consistent with the finding that AZTMP is an inhibitor of thymidylate kinase (1). AZT treatment has also been reported to cause transient but substantial changes in dNTP pools in cultured cells (10, 11), suggesting that toxicity may partly result from interference with normal nucleotide metabolism.

The toxic effect of AZT in human bone marrow cells has also been attributed to the inhibition of DNA replication resulting from AZTMP incorporation (12). Several AZTTP inhibition studies with purified cellular DNA polymerases have been described (4, 6, 13). However, whereas replication of the cellular genome requires the concerted action of a large number of host

This work was supported in part by the National Institutes of Health Intramural AIDS Targeted Antiviral Program.

ABBREVIATIONS: AZT, 3′-azido-3′-deoxythymidine; AZTMP, AZTDP, and AZTTP, 3′-azido-3′-deoxythymidine 5′-mono-, -di-, and -triphosphate, respectively; SV40, simian virus 40; HIV-1, human immunodeficiency virus type 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); dNTP, deoxynucleoside triphosphate; RF, replicative form.

cellular replication proteins, the ability of AZTTP to inhibit replication of double-stranded DNA with a human replication complex has not yet been reported. It is possible to examine this by using the SV40 origin-dependent replication system, currently the best available model for human chromosomal replication *in vitro* (14–16). Replication in cell extracts initiates at the unique SV40 origin and is bidirectional and semiconservative. With only one exception, the SV40 large tumor antigen, only host proteins are required, including two or more DNA polymerases. The system converts double-stranded circular DNA templates into double-stranded circular daughter molecules (17, 18). We, therefore, initiated the present study to determine whether AZTTP inhibits SV40 origin-dependent replication. Realizing that millimolar concentrations of AZTMP have been reported in AZT-treated cells (10) and that such concentrations of normal deoxynucleoside monophosphates inhibit purified replicative cellular DNA polymerases and replication in the SV40 system (19), we expanded the analysis to examine the effects of AZTMP, as well as AZTDP, on replication efficiency.

A long-standing interest in understanding the mechanisms used by cells to stably replicate and maintain genetic information has prompted us to consider a second issue of interest regarding AZT treatment of cells, i.e., its potential mutagenicity. We were interested in addressing three specific issues. The high fidelity of DNA replication results partly from exonucleolytic proofreading of replication errors, and addition of millimolar concentrations of normal nucleoside monophosphates can inhibit proofreading (20, 21) and reduce replication fidelity (19). Firstly, because AZTMP accumulates to millimolar concentrations in AZT-treated cells, we asked whether AZTMP inhibits replication fidelity in the SV40 replication system.

Secondly, replication fidelity depends on both the relative and the absolute concentrations of the four normal dNTP substrates. The relative dNTP concentrations affect the ratio of correct versus incorrect nucleotide insertion, whereas the absolute concentration of the next correct nucleotide to be incorporated after a misinsertion affects the probability of proofreading the error before it can be fixed into double-stranded DNA by further incorporation (20, 22). Because, as noted above, AZT treatment has been reported to cause transient but substantial changes in dNTP pools in cultured cells (10, 11), we established these changes during *in vitro* replication to determine their effects on fidelity.

Finally, low mutation rates *in vivo* result partly from repair of mismatched bases by a heteroduplex repair complex. We (23) and others (24) have recently described a general mismatch repair system operating in HeLa cell extracts comparable to the methyl-directed long patch repair system of *Escherichia coli* (for review see Ref. 25). This repair requires some of the same proteins used for replication (24, 23), including at least one DNA polymerase (either polymerase α, δ, or ϵ). We therefore asked whether AZTMP, AZTDP, or AZTTP inhibits mismatch repair in human cell extracts.

Materials and Methods

Bacterial strains, bacteriophage, and other materials. *E. coli* strains and bacteriophage M13 mp2 and its derivatives have been described (26, 27). RF DNA was prepared from M13-infected *E. coli* (NR9099) as described (26), followed by centrifugation in a CsCl/ethidium bromide density gradient. Double-stranded M13 mp2 DNA containing a G-G mispair at position 88 and a nick at the *Ava*II site at position −264 (position 1 is the first transcribed base of the *lacZ*α gene) was prepared as described previously (23, 28). Restriction enzymes were purchased from New England BioLabs and used as described (29). The HeLa cell cytoplasmic extract was prepared as described (30, 31). SV40 large T antigen was purchased from Molecular Biology Resources (Milwaukee, WI).

Preparation of AZTMP, AZTDP, and AZTTP. AZTTP was prepared according to the methods in Ref. 32. AZTDP was prepared according to the same procedure but using β-cyanoethyl phosphate instead of pyrophosphate. AZTMP was prepared according to the methods in Ref. 33. All nucleotides were purified by reverse phase high performance liquid chromatography.

Replication reactions. Reaction mixtures (25 μl) contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 200 mM each CTP, GTP, and UTP, all four dNTPs (at concentrations indicated in table or figure legends) and [α-^{32}P]dCTP (4000 cpm/pmol), 40 mM creatine phosphate, 2.5 mg of creatine phosphokinase, 15 mM sodium phosphate (pH 7.5), 40 ng of M13 mp2SVA89 RF DNA, 1 mg of T antigen, and 75 μg of protein from a HeLa cytoplasmic extract. After incubation for 2 hr at 37°, the reaction was stopped as described (34), 10 μl were removed and added to 0.5 ml of ice-cold 10% trichloroacetic acid, and the acid-insoluble products were collected on a glass fiber filter and analyzed for ^{32}P incorporation by liquid scintillation counting. The remaining DNA was processed as described (19). To examine the quality of DNA synthesis, aliquots of each replication reaction were analyzed by agarose gel electrophoresis as described (29). The remaining DNA was digested with *Dpn*I and used to transfect *E. coli* by electroporation with a Bio-Rad Gene Pulser. Plating and plaque color determinations were as described (27, 35).

Mismatch repair reactions and analysis of repair synthesis . Mismatch repair reactions (25 μl) were similar to replication reactions except that dNTPs were omitted from the reactions to enable high specific activity labeling of the repair products for analysis of repair synthesis (cytoplasmic extracts contain sufficient dNTPs to support mismatch repair) (23), T antigen was omitted, and 5 ng (1 fmol) of purified heteroduplex DNA were added in place of RF-I DNA. After incubation at 37° for 1 hr, the reactions were terminated and the products were extracted and purified as described (23). The purified repair products were used to transfect *E. coli* strain NR9162 by electroporation. Plating and scoring of plaque colors (27, 35) and calculation of repair efficiency have been described (23). For analysis of mismatch repair-associated synthesis, the purified repair products were digested by *Hin*fI restriction endonuclease and resolved on a 5% native polyacrylamide gel, which was then dried and exposed for autoradiography. Repair synthesis was calculated as described (23) after excision and counting of the radioactivity in the mismatch-containing fragment (529 bp) and a control background band (413 bp).

Results

Effects of AZT metabolites on replication in HeLa cell extracts . The effects of increasing concentrations of AZTMP, AZTDP, and AZTTP on SV40 origin-dependent replication of double-stranded circular M13 mp2 DNA in HeLa cell extracts are shown in Fig. 1. For AZTTP and AZTDP, replication was not inhibited until the concentration was increased to 100 μM. At 300 μM and 1 mM AZTTP, incorporation was inhibited by 30% and 70%, respectively. Similarly, 1 mM and 2 mM AZTDP inhibited incorporation by 50% and 60%, respectively. AZTMP had no inhibitory effect on dNTP incorporation up to a concentration of 1 mM, but synthesis was inhibited 20% and 50%, respectively, at 2 and 5 mM AZTMP. For comparison, 2 mM dGMP caused >50% inhibition (data not shown).

Because AZT analogs might have affected not just the quan-

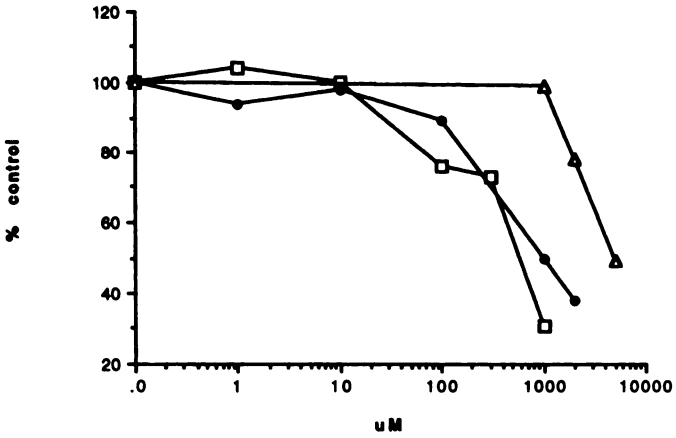

Fig. 1. Inhibition of replication by AZT metabolites. □, Synthesis in the presence of AZTTP; ●, synthesis in the presence of AZTDP; △, synthesis in the presence of AZTMP.

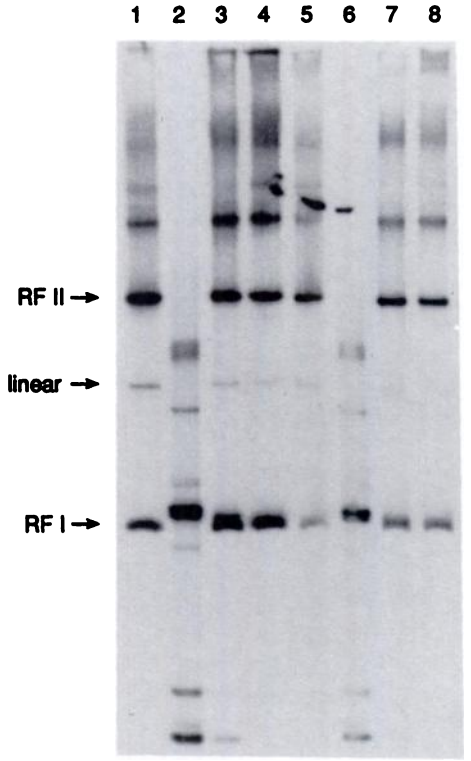

Fig. 2. Analysis of replication products by agarose gel electrophoresis. Aliquots of replication reaction mixtures (2 hr) with M13 mp2SV DNA were digested with appropriate restriction enzymes and analyzed by agarose gel electrophoresis followed by autoradiography of the dried gel. *Lanes 1-4*, replication products from reaction mixtures without monophosphate; *lanes 5-8*, replication products from reaction mixtures with 5 mM AZTMP. *Lanes 1* and *5*, reaction products treated with *Dpn*I; *lanes 2* and *6*, reaction products treated with *Sau*3AI; *lanes 3* and *7*, reaction products treated with *Mbo*I; *lanes 4* and *8*, uncut reaction products.

tity but also the quality of the products formed during replication, we analyzed these by diagnostic restriction enzyme digestion and agarose gel electrophoresis in the presence of ethidium bromide (Fig. 2). No radiolabeled DNA products were observed in reactions incubated in the absence of T antigen (data not shown). When the reaction was carried out in the presence of T antigen but without AZT analogs, the majority of the M13 mp2SV DNA products migrated coincident with RF-I (supercoiled) or RF-II (relaxed) monomer-length circular DNA (Fig. 2, *lane 4*). Electrophoresis in the absence of ethidium bromide (data not shown) indicated that the RF-I-type products are partially supercoiled monomer-length circles. The presence of higher molecular weight DNA products is consistent with similar observations in other laboratories (17, 18, 36) and is believed to represent replication intermediates (θ structures) or products of rolling circle replication. The ^{32}P-labeled product DNA was resistant to *Dpn*I digestion (Fig. 2, *lane 1*), as expected for the hemimethylated product of semiconservative DNA replication. The product DNA was completely cut by the methylation-insensitive *Dpn*I isoschizomer *Sau*3AI (Fig. 2, *lane 2*), demonstrating that the products retain cleavable sites. Digestion with *Mbo*I (Fig. 2, *lane 3*), which cuts only unmethylated GATC sequences, revealed that a portion of the monomer circles are the products of a single round of replication, i.e., resistant to *Mbo*I digestion. The presence of some *Mbo*I-sensitive monomers is consistent with previous studies of SV40 replication *in vitro* (18, 36, 37) and could reflect multiple rounds of replication in the extract or could result from a single round of replication of input template molecules that were not fully methylated. The products of replication in the presence of all concentrations of AZTMP, AZTDP, and AZTTP examined (Fig. 1) gave essentially the same pattern of product bands (e.g., Fig. 2, *lanes 5-8* for 5 mM AZTMP), although the bands were of lesser intensity as incorporation was inhibited at higher analog concentrations.

Effect of AZT metabolites on replication fidelity. Studies with purified DNA polymerases having associated 3′ to 5′ exonuclease activity demonstrate that the presence of a nucleoside monophosphate in a polymerization reaction decreases base substitution fidelity. This is believed to result from monophosphate binding to the exonuclease active site, thus inhibiting proofreading of primer-terminal mispairs (20, 38). Addition of dGMP to SV40 origin-dependent replication reactions also decreases replication fidelity (19), suggesting that proofreading activity associated with the replication complex has been inhibited (19). Given that AZT-treated cultured cells accumulate high levels of AZTMP (10, 11), we examined whether addition of AZTMP to a replication reaction reduces base substitution fidelity. Several reports indicate that AZT-treated cultured cells undergo changes of >20-fold in relative and absolute dNTP concentrations (1, 10, 11). Thus, we also measured replication error rates in reactions containing a 20-fold excess of one dNTP and in reactions in which the relative dNTP concentrations were held constant but the absolute concentrations were increased 10-fold.

To perform these fidelity measurements, we replicated an M13 mp2 DNA substrate containing an opal codon in the *lacZα* gene. Because this nonsense codon yields a colorless plaque phenotype, base substitution errors can be detected as blue M13 plaques on indicator plates after transfection of *E. coli* cells with *Dpn*I-resistant replication products. The reversion frequency, i.e., the proportion of blue to total plaques, reflects the base substitution error rate.

Replication reactions performed with equimolar concentrations of all four dNTPs yielded reversion frequencies (Table 1, line 2) <2-fold above the reversion frequency of unreplicated DNA (Table 1, line 1). Hence, replication is accurate under these conditions. However, reactions performed with a 20-fold excess of dCTP, which leads to increased misinsertion of dCTP

at the opal codon, yielded a small but reproducible increase in reversion frequency (Table 1, line 3).

The effect of increasing the absolute dNTP concentration, without a concomitant change in relative dNTP concentrations, can be seen by comparing Table 1, lines 3 and 4. Even reactions perfomed with a 20-fold excess of dCTP yielded a low reversion frequency when the concentration of the next correct nucleotide to be incorporated after misinsertion of dCTP remained low. However, a 5-fold increase in the concentration of all four dNTPs led to a reproducible increase in reversion frequency, i.e., lower replication fidelity. DNA sequence analysis of revertants from this reaction condition demonstrated that all were transition errors at the third position of the opal codon, consistent with misinsertion of dCMP opposite a template adenosine followed by correct incorporation of another dCMP opposite template guanosine. This is precisely the specificity predicted for a next-nucleotide effect on proofreading. These results, which are similar to earlier experiments in this system (19), demonstrate that changes in dNTP concentrations similar in magnitude to those reported in AZT-treated cells can reduce replication fidelity by reducing discrimination at two steps in a polymerization reaction.

Replication reactions were also performed with AZTMP and dGMP. The presence of 5 mM AZTMP did not increase the reversion frequency. In contrast, 2 mM dGMP substantially increased the reversion frequency, consistent with reduced proofreading efficiency. Similar results were obtained using excess dGTP (last three lines of Table 1), which promotes a different set of substitution errors, primarily T-dGTP mispairs. Similar to the results with AZTMP, addition of AZTTP or AZTDP also had no effect on the reversion frequency (data not shown).

Effect of AZT metabolites on mismatch repair and repair-associated DNA synthesis. To determine whether AZT metabolites affect the efficiency of mismatch repair in HeLa cell extracts, we used a recently developed heteroduplex repair assay (23). The assay is based on the analysis of plaque colors resulting from the transfection of a *mutS E. coli* strain (NR9162, which is defective in methyl-directed mismatch repair) with M13 mp2 heteroduplex DNAs that have been incubated in cell extracts. These DNAs contain base mispairs in the *lacZα* gene such that one strand encodes a blue plaque phenotype whereas the other encodes a colorless plaque phenotype. Upon transfection, unrepaired molecules yield characteristic proportions of mixed color and pure color plaques. When repair occurs, these proportions change such that the percentage of mixed bursts decreases and the percentage of one or both types of pure bursts increases depending on the extent of repair in each strand. If repair is directed primarily to one strand, the percentage of pure bursts representing the genotype of that strand decreases, whereas the other pure burst frequency increases. With proper plating conditions, the analysis of plaque phenotype ratios can be achieved with >95% accuracy (23).

The effect of AZTTP, AZTMP, and AZTDP on the efficiency of mismatch repair is shown in Table 2. The substrate contains a single G-G mismatch that is efficiently repaired under standard conditions in HeLa extracts (Table 2, experiments 1 and 2, compare lines 1 and 2). Concentrations of AZTTP up to 100 μM had no effect on repair efficiency, and repair was only slightly inhibited even at 1 mM AZTTP. Similarly, 1 mM AZTDP did not affect repair, nor did AZTMP at concentrations ranging from 1 to 10 mM (Table 2, experiment 2).

DNA synthesis associated with mismatch repair was also monitored. The AZT metabolites had essentially no effect on total incorporation (Table 2) except at the highest concentration of AZTTP examined. The pattern of incorporation into the mismatched substrate was similar for reactions containing the AZT metabolites, compared with the control reaction (Fig. 3). Comparison of the specific activity of the mismatch-containing restriction fragment (529 bp) with that of a control fragment (413 bp) gave the same result.

TABLE 1
Effect of AZTMP on opal codon reversion frequency

dCTP	dATP, dTTP, dGTP	AZTMP	dGMP	Reversion frequency ($\times 10^{-5}$)
μM	μM	mM	mM	
Unreplicated				1.3
100	100			2.4
1000	50			7.9
200	10			2.7
200	10	5		1.7
200	10		2	16.0
200[a]	10			3.1
200[a]	10	10		3.0
200[a]	10		2	25.0

[a] These experiments used excess dGTP rather than excess dCTP.

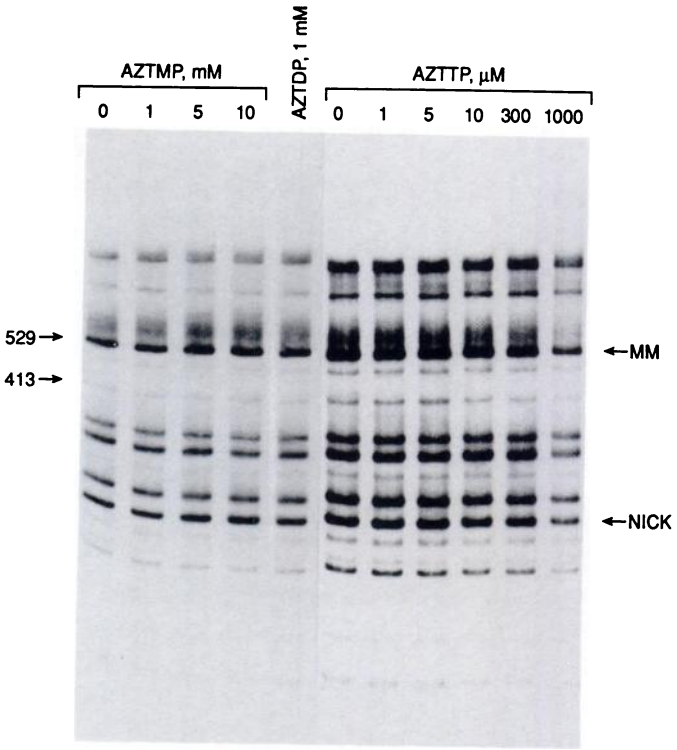

Fig. 3. Analysis of mismatch repair synthesis by polyacrylamide gel electrophoresis. Reaction conditions and preparation of the reaction products for gel electrophoresis (5%) are as stated in Materials and Methods. Concentrations of the metabolites are as indicated in the figure. The *HinfI* fragments used in calculating specific repair synthesis are indicated (529 and 413 bp). The 529-bp fragment contains the mismatch (*MM*); the nick-containing fragment (253 bp) is also indicated. The incorporation in other bands reflects mismatch repair-independent, nonspecific processing in extracts (see Ref. 23).

TABLE 2
Effect of AZT metabolites on repair of a G-G mispair in HeLa cell extracts

Addition	Plaque phenotype			Repair[a]	^{32}P incorporation[b]		Repair synthesis (529/413-bp bands)[c]
	(+)-Strand	(−)-Strand	Mixed				
	%			%	% of control	net cpm	
Experiment 1							
Untreated	13	49	38	0			
0	59	32	9	76	100	6469	2.6
1 μM AZTTP	60	32	8	79	104	6836	2.2
10 μM AZTTP	63	29	8	79	104	7000	2.0
100 μM AZTTP	64	30	6	84	110	6494	1.9
300 μM AZTTP	56	33	11	71	93	6671	2.6
1000 μM AZTTP	45	36	19	50	66	3858	2.3
Experiment 2							
Untreated	15	47	38	0			
No additions	57	32	11	71	100	6929	2.4
1 mM AZTMP	56	35	9	76	107	6450	2.8
5 mM AZTMP	60	32	8	79	111	6233	2.6
10 mM AZTMP	60	31	9	76	107	6689	2.3
1 mM AZTDP	53	37	10	74	104	5570	2.3

[a] Repair efficiency is expressed in percentage, as 100 × (1 − the ratio of the percentages of mixed bursts obtained from extract-treated and untreated samples).
[b] Net cpm represents the total incorporation of ^{32}P after subtraction of cpm obtained in an unincubated control reaction.
[c] Because nonspecific incorporation occurs throughout the DNA molecule (Fig. 3), an alternative assessment of mismatch repair-specific synthesis is achieved by excising and counting the mismatch-containing fragment and comparing incorporation with that in a fragment far removed from the site of the mispair (see Materials and Methods).

Discussion

The present study was undertaken to examine one possible explanation for the cytotoxic effects of AZT treatment, i.e., perturbation of cellular DNA replication. The data in Fig. 1 demonstrate that neither AZTTP nor AZTDP appreciably inhibits SV40 origin-dependent DNA replication in a human cell extract until the concentrations exceed 100 μM. For AZTTP, this result is consistent with studies of purified cellular polymerases, showing that the K_i value for AZTTP is above 1000 μM for polymerase α and above 200 and 300 μM, respectively, for polymerases δ and ε (4, 13). Due to the possible confounding influences of nucleotide-converting enzymes in the extract, the dose-response curves should not be considered as absolutely quantitative determinations. Nevertheless, these concentrations of AZTTP, as well as concentrations of AZTDP that are inhibitory to SV40 origin-dependent replication, are several orders of magnitude higher than the concentrations of these two AZT derivatives reported in cultured cells (1, 9, 12). A simple interpretation of these data is that the side effects of AZT treatment may not result from inhibition of chromosomal replication by AZTTP or AZTDP, at least for cell types that do not generate high intracellular concentrations of the di- and triphosphate forms. Nonetheless, it has been reported that AZT inhibits replication and growth of human bone marrow cells concomitant with AZTMP incorporation into DNA (12), although the mechanism of this incorporation has not been elucidated.

Interestingly, AZTMP at concentrations above 2 mM inhibits replication in extracts (Fig. 1). Given that millimolar concentrations of AZTMP have been reported to accumulate in cultured cells (10, 11), the inhibition of replication *in vitro* suggests that chromosomal replication could be affected in those cell types that generate high AZTMP concentrations. The mechanism of this inhibition is unknown.

Three experiments in the present study address a second possible effect of AZT treatment, i.e., its potential mutagenicity. The limited information available on this subject comes from bacterial studies and from studies with cultured human and rodent cells. AZT has been shown to induce the SOS response in *E. coli* and was shown to be nonmutagenic in *Salmonella typhimurium* (39). AZT has also been found to induce chromosomal aberrations in mouse bone marrow cells as determined by a micronuclei assay (40). Studies also indicate that AZT is mutagenic to cultured human cells and not to hamster cells (41). We were prompted to pursue this issue by the following four observations: (i) the presence of 1 mM dGMP reduces replication fidelity in HeLa cell extracts (19), consistent with nucleoside monophosphate inhibition of proofreading (42, 20); (ii) perturbations in dNTP concentrations affect DNA polymerization fidelity (43); (iii) AZTMP is present at high concentrations in AZT-treated cells; and (iv) several studies report substantial transient changes in dNTP pools in AZT-treated cells. We therefore determined to what extent imbalanced dNTP concentrations or AZTMP would alter the fidelity of replication *in vitro* with a human cell replication complex. If fidelity was reduced, AZT treatment could be mutagenic, which could have implications for the long term use of AZT.

The data in Table 1 indicate that the presence of even 10 mM AZTMP does not affect replication fidelity for base substitution errors. This is in contrast to the effect of dGMP. Normal deoxynucleoside monophosphates are known to bind to the active site for the 3′ to 5′ exonuclease of *E. coli* DNA polymerase I (38), to inhibit the 3′ to 5′ exonuclease activity of several proofreading-proficient DNA polymerases (42), and to reduce their fidelity (20). The data thus imply that 10 mM AZTMP does not inhibit proofreading during replication, perhaps because it does not bind effectively to the (yet to be identified) proofreading exonuclease.

Mismatch repair likely plays an important role in correcting replication errors in human cells just as it does in bacteria (23, 24). It is expected that inhibitors of this process will be mutagenic. Mismatch repair in HeLa cell extracts requires long patches of new DNA synthesis to repair just one mismatched base. This synthesis is already known to be sensitive to both

aphidicolin and butylphenyl-dGTP (23). We therefore tested the effect of AZT metabolites on repair of a single-base mismatch known to be efficiently repaired in HeLa cell extracts. None of the three AZT compounds tested affected repair efficiency or repair-dependent synthesis when present in reactions at concentrations similar to levels reported in cultured cells. This suggests that AZT metabolites are unlikely to be mutagenic by suppressing generalized mismatch repair *in vivo*.

Another parameter examined that is relevant to AZT effects was nucleotide pool variation. The 20-fold imbalance described in Table 1 is quantitatively similar to reports of dNTP fluctuations after AZT treatment of cultured cells (10). The reduced replication fidelity was predicted by the known effects of substrate variations on the fidelity of purified DNA polymerases (for review, see Refs. 43 and 44) and is similar to our earlier observations for replication. They illustrate that AZT-induced dNTP imbalances could be mutagenic by affecting two discrimination steps, i.e., nucleotide selectivity and exonucleolytic proofreading.

Because the fidelity of HIV-1 reverse transcriptase is strongly affected by variations in dNTP concentrations (45), imbalanced dNTP pools within AZT-treated cells infected with HIV-1 could also lead to higher mutation rates for the virus itself. This would provide additional variation upon which selection might act to more rapidly yield, for example, an AZT-resistant strain.

Acknowledgments

We thank Kenneth R. Tindall and Richard M. Philpot for critical evaluation of the manuscript.

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