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3''-AZIDO-3'-DEOXYTHYMIDINE AND 2',3'-DIDEOXYCYTIDINE DO NOT INHIBIT GENE-SPECIFIC DNA REPAIR IN HAMSTER CELLS

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SUMMARY: 3''-Azido-3'-deoxythymidine (AZT) was the first approved drug for the treatment of the AIDS; however, despite its usefulness, AZT often produces side effects that require cessation of therapy. 2',3'-Dideoxycytidine (ddC) is a related anti-retroviral agent in advanced stages of clinical testing. A previous report demonstrated that AZT decreased the repair of UV-induced DNA strand breaks in mammalian cells after ultraviolet (UV) irradiation. We studied the effect of AZT and ddC on DNA repair from the hamster DHFR gene of the major UV-induced DNA lesion, cyclobutane pyrimidine dimers (CPDs). We conclude that neither AZT nor ddC inhibited DNA replication or the gene-specific repair of CPDs in the hamster DHFR gene after 8 or 24 hrs of repair incubation at concentrations of 25 μM and 10 μM, respectively.

3'-Azido-3'-deoxythymidine (AZT) is widely used for the treatment of the Acquired Immune Deficiency Syndrome (AIDS) in humans. AZT is phosphorylated within the cell by thymidine kinase to AZT monophosphate (AZT-MP) and then cellular kinases convert AZT-MP to AZT-triphosphate (AZT-TP) (1). AZT-TP inhibits the viral reverse transcriptase with an IC_{50} which is 100-fold more than for its inhibition of the cellular DNA polymerase α (1). One of the main toxicities of AZT is bone marrow suppression that results in significant anemia and neutropenia (2). Biochemical mechanisms that cause this toxicity are not clearly defined, although alterations in nucleotide pools are believed to play a role. AZT-MP is also a potent inhibitor of

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thymidylate synthesis (3). Another possible explanation for the cytotoxic side effects of AZT is a proposed inhibition of DNA repair. AZT-MP has been demonstrated to be an inhibitor of exonucleolytic repair of AZT-terminated DNA in an *in vitro* testing system by a human 3'-exonuclease (4). Another research group reported that AZT at concentrations of 1-10 μM inhibited UV-induced strand break repair in lymphocytes (5). They proposed that dAZT might inhibit the gap-filling ligation step of DNA excision repair or block dTTP synthesis via the salvage pathway, thereby limiting supply of this nucleotide for excision nucleotide repair. The level of repair inhibition was modest and this phenomena has not been reported by other investigators (5). This finding, however, suggested to us that a more detailed examination of DNA repair at the level of individual genes might resolve the question as to whether DNA repair capacity was affected by these agents.

Several drugs or drug combinations have now been described which are able to inhibit gene-specific DNA repair: combinations of topoisomerase I and II inhibitors (6), caffeine and pentoxifylline (methylxanthines) (7), and the transcription blocking agent α-amanitin (8). In this study we tested whether AZT or ddC, potent inhibitors of some viral polymerases, could have such an effect. AZT was used in a concentration of 25 μM, because CEM cells exposed to this dose for 24 hours accumulate 900 μM of AZT-MP (9). *In vitro* studies have shown that ddC is about 10 fold more potent than AZT on a molar basis as an inhibitor of the viral polymerase (10). We studied the gene-specific repair rates in the DHFR gene in UV-irradiated CHO cells and evaluated overall genome replication by neutral CsCl gradient centrifugation. We found no measurable effects on either gene specific DNA repair or overall DNA replication after UV irradiation in our system.

MATERIALS AND METHODS

Isotopes, Enzymes, Hybridization Probes and Chemicals. [Methyl-^{3}H]Thymidine (>80 Ci/mmol) and [^{32}P]dCTP (3000 Ci/mmol) were purchased from Du Pont-New England Nuclear. Restriction endonuclease Kpn I was purchased from Boehringer Mannheim, New Haven, CT. The DNA probes for the DHFR gene (pMB5) have been described (11). The pMB5 probe was used to detect 14 kb Kpn I fragment containing the 5' half of the DHFR gene. AZT (Sigma Chemical Co.) and ddC (Pharmacia) were prepared as stock solutions in H_2O and used on the same day. AZT or ddC was added to repair media, in concentrations of 25 μM and 10 μM respectively, immediately after the cells were UV-irradiated.

Cell Culture. The Chinese hamster ovary cell line CHO-B11 which contains an amplified DHFR gene, was grown in Ham's F12 media without glycine, thymidine or hypoxanthine and supplemented with 10% fetal calf serum (dialyzed) from Gibco (BRL), Gaithersburg, MD (12). Cells were maintained in 500 nM methotrexate and grown in humidified 5% CO2, 95% air at 37 C. All experiments were done with cells growing in exponential phase.

Overall DNA Replication The complete procedure of the replication assay is described in detail elsewhere (13,14). Hamster cells were incubated for 3-4 days with 0.3 μCi/ml [3H]thymidine to label the DNA uniformly and then split into non-radioactive medium for one day. The cells were UV-irradiated at 20 J/m^2 with a germicidal lamp.

After UV-irradiation, the cells were incubated in repair media for 8 or 24 hrs with or without AZT or ddC (13). AZT or ddC were added to the media without preincubation. The DNA was phenol or salt extracted, treated with RNase and resuspended in Tris-EDTA. DNA was restricted to completion with endonuclease Kpn I. Samples were then centrifuged to equilibrium in neutral CsCl gradients, and the fractions were precipitated and assayed for radioactivity by liquid scintillation spectrometry for their ^{3}H-thymidine incorporation. The relative size of the density labeled fraction to the unlabeled fraction represented the relative amount of DNA replication that had occurred. The relative levels of genome replication were determined by comparing the ^{3}H radioactivity in the replicated fractions to the total amount of ^{3}H activity in all fractions from the neutral CsCl gradient.

Gene Specific DNA Repair. The complete procedure of the gene-specific DNA repair assay is described in detail elsewhere (13).The CsCl fractions containing parental density labeled DNA were pooled, dialyzed and concentrated by ethanol precipitation (from above). DNA aliquots (2 μg) were removed from each sample, treated with or without T4 endonuclease V and electrophoresed on 0.5%-0.7% alkaline agarose gels. After standard gel washes, the DNA was transferred to a nylon support membrane (Oncor Inc.). Pre-hybridizations, hybridizations and washes were performed as previously described. Hybridization was carried out at 45°C in 5 ml Hybrisol (Oncor Inc.) Random primed ^{32}P-labeled DNA probe (approx. $1x10^9$ cpm/μg) in the amount of 4×10^7 cpm was added to the hybridization. After standard washes of the support membrane, the final wash was in 0.1X SSPE, 0.1% SDS at 65°C. Support membranes were exposed to Kodak XAR5 film. Autoradiograms were scanned by a Hoefer Scientific GS300 densitometer. The CPDs frequencies were determined as previously described (13).

RESULTS

Effect of AZT and ddC on DNA replication in UV-irradiated hamster cells. Overall repair replication measurements were done to determine the effect of AZT and ddC on UV-irradiated CHO cells. Cells were incubated for 8 or 24 hours after irradiation with or without drug. The results are shown in Table 1. No significant differences were found in the amount of replicated DNA in UV-irradiated CHO cells incubated in the presence or absence of AZT or ddC. One biological experiment was done with each drug.

Effect of AZT and ddC on UV repair of the hamster DHFR gene. Repair analysis was performed in exponentially growing CHO cells that were exposed to 20 J/m^2 of UV light and incubated in repair media with or without AZT or ddC for up to 24 hrs after irradiation. The parental (non-replicated) DNA was isolated by neutral CsCl gradient

Table 1. **Effect of AZT and ddC on Overall Genome Replication in UV-Irradiated Hamster Cells**

Time (Hrs)	DRUG (μM)	%Replication
8	0	13
8	ddC (10)	12
8	AZT (25)	13
24	0	32
24	ddC (10)	42
24	AZT (25)	34

Values are expressed as percentage replication compared to unreplicated DNA peak isolated from UV-irradiated CHO cells incubated without AZT or ddC.

centrifugation. Repair of the transcriptionally active DHFR gene was analyzed in a 14 kb Kpn I fragment containing the 5' portion of the gene. A sample autoradiogram of a support membrane probed for this 5' region is shown in Fig. 1. CPDs repair was measured as the removal of T4 endonuclease V sensitive sites with time. Both experiments demonstrated normal repair patterns of CPDs from UV-irradiated CHO cells. One biological experiment was completed for each drug, and two sets of gel electrophoresis were done for each experiment. The results showed no inhibition of gene-specific DNA repair by 25 μM AZT (Table 2) or 10 μM ddC (Table 3).

DISCUSSION

The preferential DNA repair in mammalian cells of active genes compared to the overall genome is now well established (15). We have tested the hypothesis that AZT or ddC, another nucleoside analog, could effect DNA repair through an inhibition of active gene repair. The model we used for this study is the rodent DHFR gene, which has been shown be preferentially repaired compared to the inactive chromatin (9). We pursued this investigation because of suggestions by other investigators that AZT could inhibit DNA repair. Munch-Petersen demonstrated that phytohemagglutinin stimulated growth of human lymphocytes from healthy donors was significantly decreased at an AZT dose of 7 μM (5). Lymphocytes irradiated with 2 J/m^2 of UV light and incubated were studied for levels of single strand break (SSB) induction and persistence. AZT at concentrations between of 10-150 μM inhibited UV-induced SSB repair when measured by fluorometric analysis of DNA unwinding after lymphocytes were irradiated (5). Incubation of cells post-irradiation with 10 μM AZT resulted in 2.8 fold more unrepaired SSB compared to UV-irradiated lymphocytes incubated without drug. The magnitude of SSB inhibition measured varied significantly among the volunteers tested (5). These researchers suggested that the DNA polymerase(s) responsible for gap filling or ligation may be the target of the inhibitory effect. Another hypothesis was that AZT might inhibit the phosphorylation of thymidine and therefore

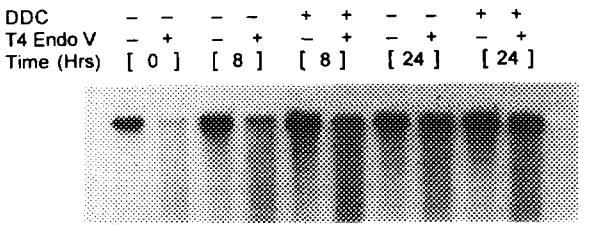

Figure 1. **Effect of ddC on Gene-specific Repair of CPDs from the Hamster DHFR gene.** DNA was isolated from hamster cells after irradiation with 20 J/m^2 UV light and cells were then allowed to repair for 8 or 24 hrs with or without ddC. DNA samples from cells treated with (+) or without (-) ddC were run in parallel (+) or (-) T4 endonuclease treatment as indicated. Support membrane probed for 14-kb region containing the 5' portion of the DHFR gene.

Table 2. **Effect of AZT on Gene-Specific Repair at 24 Hr in CHO-B11 Cells**

Time	AZT	CPDs in the DHFR gene			
Hrs	(μM)	Gel#1	Gel #2	Mean	%Repair
0	0	1.98	2.14	2.06	0
8	0	1.76	1.42	1.59	23
8	25	1.44	1.79	1.62	21
24	0	0.94	0.93	0.94	54
24	25	0.96	0.96	0.96	53

block the salvage pathway for dTTP formation. This would result in depletion of nucleotide pools required for repair synthesis of UV-induced CPDs. Differences in the availability of nucleotide salvage pools between human lymphocytes and CHO cells might explain why we did not observe any DNA repair inhibition in our experiments. Another possibility is that the inhibition is only present at low UV doses, such as the 2 J/m^2 used by Munch-Petersen (5).

Another study has demonstrated that AZT can induce chromosomal aberrations in murine bone marrow cells as shown by micronuclei assay (16). The mechanism of this effect is not defined, but among the possibilities are effects on DNA repair. Other investigators have evaluated the effect of AZT on HeLa cell extracts ability to affect SV40 replication and SV40 heteroduplexes repair (17). In these studies, the presence of AZT or its mono-, di-, or tri-phosphorylated derivatives at high concentrations had no effect on single base mismatch repair efficiency, although the replication fidelity was affected perhaps by perturbations in concentrations of deoxynucleotide triphosphate substrates. This may support our current findings, since this type of mismatch repair in HeLa cell extracts requires long patches of new DNA synthesis to repair the mismatched base, which may be analogous to cellular requirements for excision repair of CPDs. *In vitro* analysis of AZT-TP interactions with cellular DNA polymerases α, δ, ε and DNA primase has revealed that only DNA polymerase α was able to incorporate AZT-TP, but at a rate 2000-fold lower than natural dTTP (18). A 3'-exonuclease activity that can excise AZT-MP from the 3' terminus of DNA has been described (18) and AZT-MP can inhibit this 3'-exonuclease activity and possibly inhibit its own repair from DNA (4). At the AZT dose used in our present study (chosen to induce high levels of AZT-MP), we found no evidence that AZT-MP might be inhibiting repair patches with 3'-AZT-MP incorporation. The reasons likely reflect the very inefficient nature of this inappropriate incorporation relative to natural dTTP. No similar

Table 3. **Effect of ddC on Gene-Specific Repair at 24 Hr in CHO-B11 Cells**

Time	ddC	CPDs in the DHFR gene			
Hrs	(μM)	Gel#1	Gel #2	Mean	%Repair
0	0	1.94	1.89	1.92	0
8	0	1.39	1.22	1.30	32
8	10	1.50	1.15	1.32	31
24	0	0.52	0.58	0.55	71
24	10	0.65	0.71	0.68	65

data on ddC inhibition of DNA repair has been published. However, we included this agent since ddC, like AZT, has toxicities in patients for which biochemical mechanisms have not been defined. An important difference that might again explain our inability to detect repair inhibition by AZT or ddC is that our current study used hamster cells and not human cells. Significant differences may exist between the human and hamster DNA polymerases with regard to dideoxynucleosides.

In summary, we found no evidence of significant effects on gene-specific DNA repair inhibition or on overall genome replication in UV-irradiated CHO by AZT or ddC at the concentrations studied. These studies on the repair of UV-induced photoproduct damage do not of course exclude effects that AZT might have on other types of DNA damage (e.g., cisplatin) or in other cells types (e.g., bone marrow).

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