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Human Recombinant Interferon Alpha-2a Plus 3′-Azido-3′-deoxythymidine

SYNERGISTIC GROWTH INHIBITION WITH EVIDENCE OF IMPAIRED DNA REPAIR IN HUMAN COLON ADENOCARCINOMA CELLS

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ABSTRACT. We reported that 3′-azidothymidine-3′-deoxythymidine (AZT) plus 5-fluorouracil or methotrexate produces additive cytotoxicity in HCT-8 cells: a reflection of increased AZT metabolism when *de novo* thymidylate (dTMP) synthesis was inhibited. We now report that AZT plus human recombinant interferon alpha-2a (rIFN-α2a) produces synergistic growth inhibition in these cells. Evaluation of the effect of rIFN-α2a on dTMP metabolism revealed that exposure to rIFN-α2a (± AZT) did not affect dTMP synthase activity significantly but increased thymidine (dThd) kinase activity significantly. Consequently, AZT nucleotide production and incorporation into DNA were increased by coexposure to rIFN-α2a. This alone, however, cannot explain the observed synergism. Therefore, the effect of these agents on DNA excision/repair processes was assessed. Isotope clearance studies demonstrated that rIFN-α2a did not alter the rate of [^{3}H]AZT excision from DNA. In contrast, filter-elution studies revealed that rIFN-α2a (± AZT) produced more DNA damage and delayed repair compared with the effects produced by AZT alone. Since DNA polymerases alpha and beta are directly involved in gap-filling repair synthesis, experiments next assessed the effect of rIFN-α2a and/or 3′-azido-3′-deoxythymidine-5′-triphosphate (AZTTP) on their activities. Polymerase alpha was inhibited slightly by AZTTP but not by rIFN-α2a. Polymerase beta activity, however, was inhibited dramatically by rIFN-α2a + AZTTP. Finally, western analysis revealed that a 24-hr exposure to 5000 IU/mL rIFN-α2a (± 20 μM AZT) significantly reduced wild-type p53 expression compared with AZT-exposed cells. We conclude that rIFN-α2a enhances AZT-induced tumor cell growth inhibition by (i) increasing AZT metabolism, and (ii) inhibiting DNA repair and p53-mediated cell cycle control processes. BIOCHEM PHARMACOL 53;4:571–580, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. DNA polymerase beta; p53 protein; thymidine kinase; DNA excision/repair; human recombinant interferon alpha-2a; AZT

AZT[§] is a dThd analogue used to treat AIDS and AIDS-related complex [1, 2]. AZT also has been evaluated as an antineoplastic agent where studies revealed that it possessed activity when used with agents that inhibit *de novo* dTMP synthesis such as FUra [3, 4], MTX [5] and ICI D1694 [6]. Biochemical evaluation of these combinations demonstrated that, when *de novo* dTMP synthesis was inhibited, AZT salvage and incorporation into cancer cell DNA were enhanced. This resulted in tumor-cell-specific increases in AZT-induced DNA damage and cytotoxicity [3–6]. These findings helped launch a clinical analysis of AZT combined with FUra and LV [7–12]. Phase I studies in which bolus FUra (400 mg/m^2) + oral LV was administered weekly and followed by a 2-hr infusion of high-dose AZT (1–10 g/m^2) revealed this combination to be well tolerated and to produce predicted biochemical effects [8, 9]. Results from initial Phase II evaluation of this FUra + LV regimen followed by a 2-hr infusion of AZT (7 g/m^2) in patients with metastatic colon cancer indicated that a significant response rate could be achieved with this regimen [12].

The interferons were first described in the 1950s, and three classes, alpha, beta and gamma, were identified based on their diverse physical and biological properties [13–15]. With regard to cancer therapy, the antiproliferative activity of IFN-α has been studied extensively and appears related to its localization to the nucleus, where it directly affects nucleic acid and protein synthesis by disrupting the activities of 2′, 5′-oligoriboadenylate synthase, protein kinases,

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§ *Abbreviations:* AZT, 3′-azido-3′-deoxythymidine; IFN-α, interferon-alpha; rIFN-α2a, human recombinant interferon alpha-2a; FUra, 5-fluorouracil; MTX, methotrexate; dThd, thymidine; dUrd, 3′-deoxyuridine; LV, leucovorin; AZTMP, 3′-azido-3′-deoxythymidine-5′-monophosphate; AZTDP, 3′-azido-3′-deoxythymidine-5′-diphosphate; AZTTP, 3′-azido-3′-deoxythymidine-5′-triphosphate; DTT, dithiothreitol; FBS, fetal bovine serum; PCA, perchloric acid; TCA, trichloroacetic acid; and AIDS, acquired immunodeficiency syndrome.

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phosphodiesterases and specific transcriptional and translational factors [16–20]. Studies also have suggested that IFN-α can disrupt DNA synthesis by direct inhibition of DNA polymerases alpha and beta [21, 22].

Clinically, IFN-α alone is active against hairy cell leukemia [13, 17]. Preclinically, combinations of IFN-α with ionizing radiation or selected chemotherapeutic agents like doxorubicin and vinblastine produce significant cytotoxicity [17, 23, 24]. In this regard, one of the more extensively studied combinations has been IFN-α ($\leq 10^7$ IU/m^2) with FUra (±LV). Responses of 25–60% have been reported in patients with advanced colon cancer receiving these agents [25, 26]. Several mechanisms have been proposed to explain the activity of this combination, such as IFN-α-induced alterations in dThd transport and dThd kinase (EC 2.7.2.21; ATP: thymidine 5′-phosphotransferase) activity [27, 28]. In addition, a study by Houghton *et al.* [29] revealed that, in human colon tumor cells, exposure to rIFN-α2a (≤5000 IU/mL) + FUra/LV resulted in an increase in DNA damage and suggested that these agents interacted at the level of DNA excision/repair.

The above findings led us to predict that the combination of rIFN-α2a plus AZT would exert significant cytotoxicity in colon tumor cells. We now report that these agents combine to produce synergistic growth inhibition in the human colon tumor cell line HCT-8 which reflects the ability of (i) rIFN-α2a to selectively increase AZT metabolism and incorporation into DNA, and (ii) rIFN-α2a and AZT to disrupt selected aspects of DNA repair. These results are discussed in relation to the role of DNA repair processes in chemotherapeutic sensitivity and the design of future clinical regimens containing these agents. Preliminary aspects of this study have been reported [30, 31].

MATERIALS AND METHODS

Reagents

AZT was the gift of the Burroughs Wellcome Co. (Research Triangle Park, NC) and rIFN-α2a (Roferon-A) was purchased from Hoffmann-LaRoche Inc. (Nutley, NJ). [^{3}H]AZT (56 Ci/mmol), [5-*methyl*-^{3}H]dThd (61 Ci/mmol), [5-^{3}H]dUrd (20 Ci/mmol), and non-radiolabeled AZTTP were obtained from Moravek Biochemicals (Brea, CA), and [5-^{3}H]dCTP (18 Ci/mmol) was purchased from the New England Nuclear Corp. (Boston, MA). Purified human DNA polymerases alpha and beta were purchased from Molecular Biology Resources (Milwaukee, WI). Activated calf thymus DNA was from Worthington Biochemicals (Freehold, NJ). Wild-type p53 antibody for immunoprecipitation (Ab-5) and for western blotting (DO-1) were purchased from Oncogene Sciences (Uniondale, NY) and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. Protein A Sepharose was obtained from Pharmacia LKB (Piscataway, NJ). HPLC grade solvents and disposable tissue culture supplies were obtained from Fisher Scientific (Medford, MA). Reagents for SDS–PAGE were purchased from Bio-Rad Laboratories (Richmond, CA). RPMI 1640 medium, trypsin, and FBS were purchased from Gibco (Grand Island, NY). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell Line

Continuous cultures of HCT-8 human colon adenocarcinoma cells, obtained from the American Type Culture Collection (Bethesda, MD), were used in these studies [32]. Cells were cultured in plastic cell culture flasks as monolayers in RPMI 1640 medium supplemented with 10% FBS and passaged twice weekly. Cell cultures were maintained in a humidified incubator at 37° in an atmosphere of 5% CO_2. Under these conditions, their doubling time was ~22 hr, and cells in logarithmic growth were used in all studies.

Evaluation of Cell Growth Inhibition

Cells (5×10^4) were added to 10 mL of RPMI 1640 medium containing 10% FBS in 25 cm^2 culture flasks. AZT and/or rIFN-α2a, previously dissolved in medium, were added at concentrations of ≤100 μM (AZT) and ≤20,000 IU/mL (rIFN-α2a). Control cultures received the same amount of medium without drug. After 5 days, cells were harvested, and growth inhibition was determined as described previously [3, 5, 33]. Each experiment was performed in duplicate and repeated a minimum of three times.

Enzyme Assays

Thymidylate synthase (EC 2.1.1.45; 5,10-methylenetetrahydrofolate:deoxyuridine-5′-monophosphate C-methyltransferase) activity was measured in intact cells after a 1-day incubation in medium containing AZT (20 or 50 μM), rIFN-α2a (5000 or 10,000 IU/mL), or AZT (20 μM) plus rIFN-α2a (5000 IU/mL) by quantitation of ^3H_2O released from [5-^{3}H]dUrd, as previously described in detail [3, 34]. The effect of these drug exposures on cellular dThd kinase activity was assessed by TLC methods that we have reported elsewhere [35].

Quantitation of Intracellular Pools of dTTP and AZT Nucleotides

The effect of AZT (20 or 50 μM), rIFN-α2a (5000 or 10,000 IU/mL), or 20 μM AZT plus 5000 IU/mL rIFN-α2a on dTTP pools was quantitated by the incubation of $1–2 \times 10^7$ cells in 50 mL of medium containing selected drug combinations. After 24 hr, cells were harvested and homogenized in 0.5 mL of 0.2 N PCA. The acid-soluble material was removed, neutralized with KOH, processed, and analyzed by HPLC methods exactly as we have reported previously [5, 33].

To quantitate the effect of rIFN-α2a on the generation of [^{3}H]AZT nucleotides $\sim 2 \times 10^6$ cells were incubated in 15 mL medium containing 20 or 50 μM [^{3}H]AZT alone (100 or 40 mCi/mmol, respectively) or 20 μM [^{3}H]AZT + 5000

IU/mL rIFN-α2a. After 1 day, the cells were washed, harvested, and homogenized in 1 mL of 0.2 N PCA. The PCA-insoluble material was saved to quantitate [^{3}H]AZT incorporation into DNA as described below. The PCA-soluble material was analyzed to quantitate [^{3}H]AZT nucleotides by previously reported HPLC methods [5, 33].

Quantitation of [^{3}H]AZT Incorporation into, and Clearance from, DNA

AZT incorporated into nuclear DNA after a 1-day exposure to 20 or 50 μM [^{3}H]AZT alone or 20 μM [^{3}H]AZT + 5000 IU/mL rIFN-α2a was quantitated in the PCA-insoluble material generated above as previously described [5, 33, 36].

The effect of rIFN-α2a (5000 IU/mL) on the clearance of AZT from cellular DNA was quantitated by incubating ~2 × 10^6 cells in 15 mL of medium containing 20 or 50 μM [^{3}H]AZT ± rIFN-α2a (5000 IU/mL). After 24 hr, the medium was removed, and the cells were washed two times with 35 mL saline and incubated in 15 mL of medium + 10% FBS. At various times thereafter (≤6 hr), the cells were harvested, and [^{3}H]AZT incorporation into DNA was quantitated as described above.

Assessment of DNA Fragmentation/Repair

To assess the effect of rIFN-α2a (5000 IU/mL) on the repair of AZT-induced DNA damage, a modification of previously reported filter elution methods was employed [37]. Specifically, ~2 × 10^6 cells were incubated in 15 mL of RPMI 1640 medium containing 10% FBS and 1 μCi/mL of [^{3}H]dThd. After 24 hr, AZT alone (20 or 50 μM), rIFN-α2a alone (5000 IU/mL), or 20 μM AZT plus 5000 IU/mL rIFN-α2a was added to the [^{3}H]dThd-containing medium. After an additional 24 hr, the medium was removed, and the cells were washed two times with 35 mL saline, and incubated in 25 mL of drug- and isotope-free RPMI 1640 medium containing 10% FBS. At various times thereafter (≤6 hr) the cells were harvested, and ~6 × 10^5 cells were loaded onto a 2 μm polycarbonate filter (Millipore, Bedford, MA). Cells were lysed with 5 mL of 10 mM EDTA + 0.5% Triton X-100 + 2 M NaCl (pH 10.0) and then washed with 7 mL of 10 mM EDTA (pH 10.0), all applied at a flow rate of 2.5 mL/hr. Elution was then performed at 4° in the dark with a solution consisting of 2 M NaCl + 0.5 M H_3BO_3 + 20 mM EDTA (pH 12.6) applied at a flow rate of 2.5 mL/hr. One-milliliter fractions were collected over a 6-hr period and added to 18 mL of Ultima-Flo AP (Packard, Meriden, CT); radioactivity in these fractions, representing fragmented DNA, was determined by liquid scintillation. In addition, the filters were removed and incubated in 0.4 mL of 1 N HCl for 1 hr at 65°. To each filter was then added 1.0 mL of 1 N NaOH and 18 mL of Ultima-Flo AP to determine tritium-related radioactivity associated with each filter. DNA damage was estimated by calculating the percentage of total recovered counts per minute not retained on the filters.

Assessment of Human DNA Polymerase Alpha and Beta Activities

The effect of rIFN-α2a alone, AZTTP alone, or their combination on the activity of human DNA polymerase alpha activity was assessed by a modification of reported methods [33]. Specifically, 60 mM Tris (pH 8.0 at 37°), 5.0 mM magnesium acetate, 30 μg BSA, 1.0 mM DTT, 0.1 mM spermine, 100 μM dATP, dTTP, and dGTP, 20 μM [^{3}H]dCTP (0.2 mCi/mL), 10 μg activated calf thymus DNA, and various concentrations of AZTTP and rIFN-α2a were combined in a final volume of 90 μL. The reaction was initiated by the addition of purified human DNA polymerase alpha contained in a volume of 10 μL. After 45 min, the reaction was terminated by the addition of 100 μL of ice-cold 15% TCA. Acid-insoluble material was collected on a Whatman GF/A glass microfiber filter that was washed subsequently with 30 mL of ice-cold 15% TCA. Total DNA synthesis, represented by counts per minute retained on the filters, was quantitated by liquid scintillation.

The effect of rIFN-α2a and/or AZTTP on DNA polymerase beta activity was assessed by modification of the above method in which 50 mM Tris (pH 8.7 at 37°), 10 mM $MgCl_2$, 100 mM KCl, 40 μg BSA, 1 mM DTT, 100 μM dATP, dTTP, and dGTP, 20 μM [^{3}H]dCTP (0.2 mCi/mL), 10 μg activated calf thymus DNA, and various concentrations of AZTTP and rIFN-α2a were contained in a final volume of 90 μl. The reaction was initiated by the addition of purified human DNA polymerase beta contained in a volume of 10 μL. After 45 min, the reaction was terminated and DNA synthesis was assessed as described above.

Western Blot Analysis of p53 Expression

Cells 2 × 10^6 were seeded into a 75-cm^2 tissue culture flask containing 25 mL of RPMI 1640 medium + 10% FBS. Twenty-four hours later 20 or 50 μM AZT alone, 5000 IU/mL rIFN-α2a alone, or 20 μM AZT + 5000 IU/mL rIFN-α2a was added. Twenty-four hours later, wild-type p53 protein specifically was purified from these cells by microbatch affinity chromatography on the monoclonal Ab-5 antibody covalently coupled to Sepharose beads [38]. Then the immune complexes were solubilized and resolved by 7.5% SDS–PAGE exactly as previously described [38]. The proteins were transferred to nitrocellulose and probed with monoclonal DO-1 antibody (0.1 μg/mL), washed, and exposed to pre-flashed Kodak XAR film; bound proteins were visualized by enhanced chemiluminescence. Steady-state levels of p53 protein from 5–6 independent experiments were analyzed by scanning densitometry (model GS300; Hoefer Scientific Instruments, San Francisco, CA).

RESULTS

To assess the interaction between rIFN-α2a and AZT, cell growth inhibition after a 5-day exposure to these agents,

alone or in combination, was quantitated. Under these conditions, the IC_{50} of rIFN-α2a or AZT was ~14,000 IU/mL or ~65 μM, respectively. When these agents were combined, isobologram analysis [39, 40] of the resultant activity revealed synergistic cell growth inhibition over a wide range of AZT and rIFN-α2a concentrations (Fig. 1).

Exposure to IFN-α reportedly increases dThd kinase activity, and interferon gamma has been reported to inhibit the induction of dTMP synthase following exposure to FUra [28, 41]. Since such alterations can affect AZT metabolism and cytotoxicity [5, 33], studies next determined if rIFN-α2a, AZT, or their combination, affected these activities in HCT-8 cells. Exposure to rIFN-α2a or AZT for 24 hr slightly reduced dTMP synthase activity in whole cells (Table 1). Combining AZT (20 μM) with rIFN-α2a (5000 IU/mL) produced an additive inhibitory effect (Table 1).

Analysis of dThd kinase activity indicated that a 24-hr exposure to AZT alone (20 or 50 μM) did not alter this activity significantly. In contrast, exposure to 5000 or 10,000 IU/mL rIFN-α2a significantly increased dThd kinase activity. Interestingly, exposing cells to the combination of AZT plus rIFN-α2a resulted in a greater than additive increase in dThd kinase activity when compared with effects produced by these agents alone (Table 1).

Since AZT is salvaged as a dThd analogue [42], these findings suggested that, in the presence of rIFN-α2a, AZT nucleotide production was increased. Our previous studies indicated that such an increase could result in a reduced intracellular dTTP/AZTTP ratio, increased AZT incorporation into DNA, and increased cytotoxicity [5, 33]. To determine if the synergistic inhibition of HCT-8 cell growth produced by these agents reflected similar alterations, intracellular pools of dTTP and [^{3}H]AZT nucleotides were quantitated in cells incubated for 24 hr in medium containing either 5000 or 10,000 IU/mL rIFN-α2a, 20 or 50 μM [^{3}H]AZT, or the combination of 20 μM [^{3}H]AZT plus 5000 IU/mL rIFN-α2a. This exposure to AZT alone did not affect dTTP pools significantly (Table 2); thus confirming our previous findings. Additionally, in spite of its ability to alter dThd kinase activity, rIFN-α2a also, either alone or combined with AZT, did not affect dTTP pools significantly (Table 2). Quantitation of intracellular AZT nucleotides revealed that increasing the medium concentration of AZT from 20 to 50 μM increased intracellular pools of all AZT nucleotides and increased AZTTP pools by 56% to 19.6 pmol/10^6 cells. As predicted by the effect of these agents on dThd kinase activity, incubating cells in 5000 IU/mL rIFN-α2a and 20 μM AZT resulted in a measurable increase in AZT nucleotides compared with incubation in medium containing 20 μM [^{3}H]AZT alone. Specifically, AZTMP, AZTDP, and AZTTP pools were increased by 75, 37 and 27%, respectively, in the presence of rIFN-α2a (Table 2).

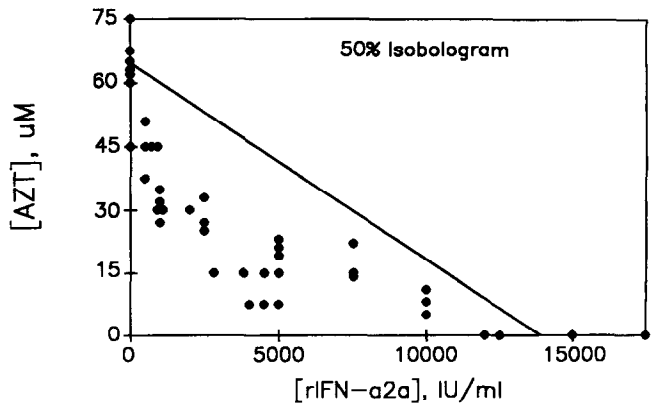

FIG. 1. Isobologram analysis of the growth inhibitory effects of the combination of rIFN-α2a and AZT in the human colon tumor cell line HCT-8. Cells (1 × 10^5) and various concentrations of rIFN-α2a and/or AZT were incubated at 37°. After 5 days, the cells were harvested and the cell number was determined as previously described [5]. Under these conditions, the IC_{50} of rIFN-α2a alone or AZT alone was 14,250 ± 1,460 IU/mL or 64.7 ± 3.2 μM, respectively. Each point represents the mean IC_{50} value of duplicate determinations.

This increase in AZT nucleotides reduced the intracellular dTTP/AZTTP ratio (Table 2) and suggested that, in the presence of rIFN-α2a, AZT incorporation into DNA increased. To determine if this was the case, HCT-8 cells were incubated in medium containing either 20 or 50 μM [^{3}H]AZT alone, or 20 μM [^{3}H]AZT plus 5000 IU/mL rIFN-α2a. After 24 hr, the cells were harvested, and AZT incorporation into DNA was quantitated. As predicted, in the presence of rIFN-α2a, AZT incorporation into DNA increased 2-fold to ~35 pmol incorporated/10^6 cells (Fig. 2).

Taken together, these findings suggested that the cell growth inhibitory effects produced by the combination of rIFN-α2a plus AZT resulted from a rIFN-α2a-induced increase in AZT metabolism and incorporation into DNA. However, AZT incorporation into DNA in the presence of 20 μM AZT + rIFN-α2a was not as great as that following exposure to 50 μM AZT alone while the cell growth inhibitory effects of the combination were significantly greater. Clearly, other factors were contributing to produce the observed synergism. Since, in this model, the cell growth inhibitory effects of AZT are directly related to its incorporation into and damage of DNA [33], these findings could suggest that rIFN-α2a also affected DNA excision/repair processes.

To evaluate this possibility, experiments first assessed the effect of rIFN-α2a on the rate of AZT excision from DNA. For these studies, cells were incubated in either 20 or 50 μM [^{3}H]AZT alone, or 20 μM [^{3}H]AZT plus 5000 IU/mL rIFN-α2a. After 24 hr, the cells were incubated in fresh medium without drug. At various times after drug removal (≤6 hr), the amount of AZT remaining in DNA was measured to assess its rate of clearance. AZT clearance from DNA in cells incubated in 20 or 50 μM AZT alone was parallel and appeared to be biphasic, with an apparent initial half-life of ~1 hr (Fig. 3). Incubating cells in both 20

TABLE 1. Effect of a 24-hr incubation in medium containing AZT alone, rIFN-α2a alone, or their combination on cellular dTMP synthase and dThd kinase activities in HCT-8 cells

Conditions	dTMP synthase (pmol/hr/10^6 cells)	dThd kinase (nmol/hr/mg protein)
No drug	10.6 ± 2.3	192.1 ± 11.2
AZT, 20 μM	8.4 ± 0.7	214.4 ± 16.9
AZT, 50 μM	8.3 ± 1.1	204.3 ± 6.4
rIFN-α2a, 5000 IU/mL	9.0 ± 1.6	241.3 ± 11.4*
rIFN-α2a, 10,000 IU/mL	9.3 ± 1.7	242.7 ± 12.0*
AZT, 20 μM + rIFN-α2a, 5000 IU/mL	7.4 ± 1.2	288.6 ± 14.7†

Cells (5×10^6) were incubated in 50 mL of RPMI 1640 medium containing 10% FBS and the noted concentrations of AZT, rIFN-α2a, or their combination for 1 day. To assess dTMP synthase activity cells were harvested and resuspended at 1×10^7 cells/mL in RPMI 1640 medium and the assay was initiated by the addition of 200 μL of cell suspension to 30 μL of 8.3 μM [5-^{3}H]dURd (30 μCi/mL) at 37°. Enzyme activity was quantitated by assessment of the formation of ^3H_2O as a function of time [3, 34]. To assess dThd kinase, cells were harvested, homogenized in 50 mM Tris (pH 7.4 at 37°), and centrifuged at 100,000 *g* for 1 hr. The enzyme reaction was initiated by incubating 10- to 30-μL aliquots of the resulting supernatant in a mixture of 200 μM [^{14}C]dThd (10 μCi/mL), 1 mM ATP, and 50 mM Tris (pH 7.4 at 37°) in a final volume of 100 μL for various times (≤10 min). Activity was determined by quantitation of the formation of [^{14}C]dThd nucleotides by TLC methods [35]. Each value represents the means ± SEM of 4 (dTMP synthase) or 8 (dThd kinase) determinations with *P* values determined by Student's *t* test.

* $P \leq 0.05$, compared with activity in non-treated controls.

† $P \leq 0.01$, compared with activity in non-treated controls.

μM AZT and 5000 IU/mL rIFN-α2a appeared not to effect the rate at which AZT was cleared from DNA (Fig. 3).

The effect of rIFN-α2a and/or AZT on DNA fragmentation and repair was assessed by monitoring DNA damage, using filter elution techniques, in cells exposed to these agents for 24 hr and then placed in medium without drug. The results indicated that the amount of DNA damage following exposure to 20 or 50 μM AZT was about equal (Fig. 4). Furthermore, the decrease in DNA fragmentation following removal of AZT from the medium, presumably a reflection of DNA repair, appeared to mirror AZT excision from DNA in that the degree of fragmentation was reduced dramatically within 1 hr after drug removal (Fig. 4). In contrast, exposure to rIFN-α2a alone or combined with 20 μM AZT resulted in greater DNA fragmentation than exposure to AZT alone. Also noteworthy, up to 3 hr after the removal of rIFN-α2a from the medium the amount of DNA fragmentation remained relatively unchanged. Indeed, over this interval the difference in DNA damage between rIFN-α2a (±AZT) exposed cells and cells exposed to AZT alone increased (Fig. 4). These findings suggested that, in rIFN-α2a exposed cells, the repair of DNA fragmentation was impaired.

DNA polymerases alpha and beta have been implicated in DNA repair [43–45], and it also has been reported that IFN-α can inhibit their activities [21, 22]. Therefore, the effect of rIFN-α2a, AZTTP, or their combination on these polymerase activities was assessed *in vitro*. The results revealed that while AZTTP alone slightly inhibited the activity of DNA polymerase alpha in a concentration-dependent manner, rIFN-α2a alone and the combination of AZTTP plus rIFN-α2a did not inhibit this polymerase

TABLE 2. Effect of a 24-hr incubation in medium containing AZT alone, rIFN-α2a alone, or their combination on intracellular pools of AZT nucleotides and dTTP

Condition	Nucleotides (pmol/10^6 cells) AZTMP	AZTDP	AZTTP	dTTP	TP ratio*
No drug				31.3 ± 1.5	
AZT, 20 μM	239.4 ± 18.1	12.9 ± 1.3	12.5 ± 1.4	30.2 ± 3.9	2.4:1
AZT, 50 μM	342.8 ± 39.8†	22.1 ± 4.2†	19.6 ± 3.3	34.6 ± 5.8	1.8:1
rIFN-α2a, 5000 IU/mL				31.7 ± 2.1	
rIFN-α2a, 10,000 IU/mL				34.7 ± 2.4	
AZT, 20 μM + rIFN-α2a 5000 IU/mL	418.5 ± 34.1†	17.7 ± 2.5	15.9 ± 1.9	30.8 ± 6.4	1.9:1

Cells ($5–20 \times 10^6$) were incubated in 10–50 mL of RPMI 1640 medium containing 10% FBS and the noted concentrations of [^{3}H]AZT alone (2.0 μCi/mL), rIFN-α2a alone, or their various combinations. After 1 day, the cells were harvested and processed to quantitate AZT nucleotides and dTTP by HPLC methods, as described in the text [5, 33]. Each value represents the mean ± SEM of 14–27 determinations with *P* values determined by Student's *t*-test.

* [dTTP]/[AZTTP].

† $P \leq 0.05$ vs that detected in cells exposed to 20 μM AZT.

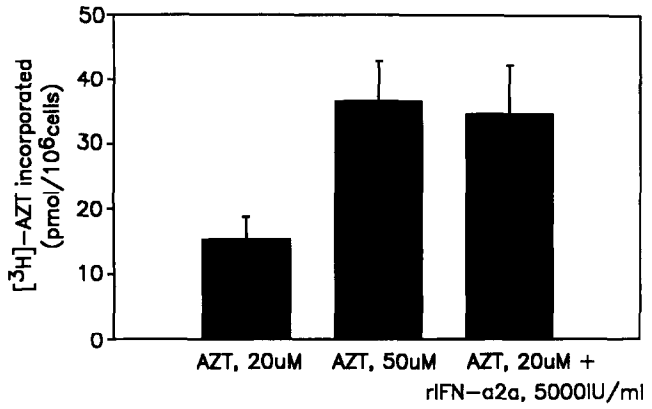

FIG. 2. **Effect of rIFN-α2a on the incorporation of AZT into the DNA fraction of HCT-8 cells. Approximately 2 × 10^6 HCT-8 cells were incubated in 10 mL of RPMI 1640 medium containing 10% FBS and the stated concentrations of [^{3}H]AZT ± rIFN-α2a (5000 IU/mL). After 24 hr, the cells were harvested and homogenized in 1 mL of 0.2 N PCA. The acid-insoluble material was assayed to quantitate [^{3}H]AZT content as previously described [3, 5, 33]. Each bar represents the mean ± SEM of 5–8 determinations.**

activity (Table 3). In contrast, rIFN-α2a alone or combined with AZTTP exerted a marked inhibitory effect on polymerase beta activity in spite of the fact that AZTTP at either 25 or 50 μM was non-inhibitory (Table 3). These findings suggested that the ability of rIFN-α2a and AZT to delay DNA fragment repair corresponded with the ability of these agents to inhibit DNA polymerase beta.

Exposure to various DNA damaging agents both increases the expression of, and activates, wild-type p53 [46]. This results in G_1 arrest and DNA repair, or can lead to

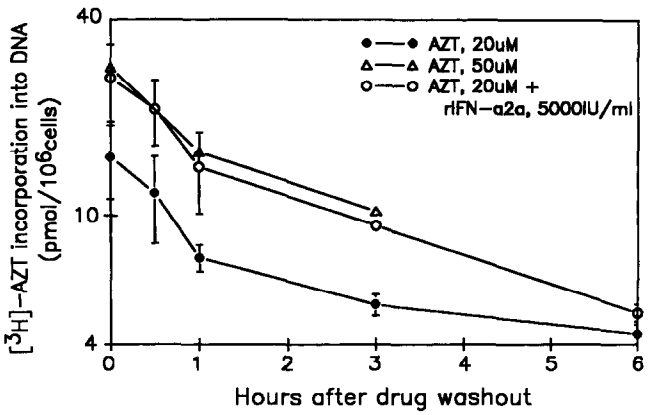

FIG. 3. **[^{3}H]AZT clearance from the DNA fraction of HCT-8 cells as a function of AZT concentration or co-exposure to rIFN-α2a. Approximately 2 × 10^6 HCT-8 cells were incubated in 10 mL of RPMI 1640 medium containing 10% FBS and the stated concentrations of [^{3}H]AZT ± rIFN-α2a. After 24 hr, the medium was removed, and the cells were washed with saline and then incubated in fresh medium without drug. At various times thereafter, the cells were harvested, and the [^{3}H]AZT content of cellular DNA was quantitated as described in Fig. 2. Each point represents the mean ± SEM of 4–6 determinations.**

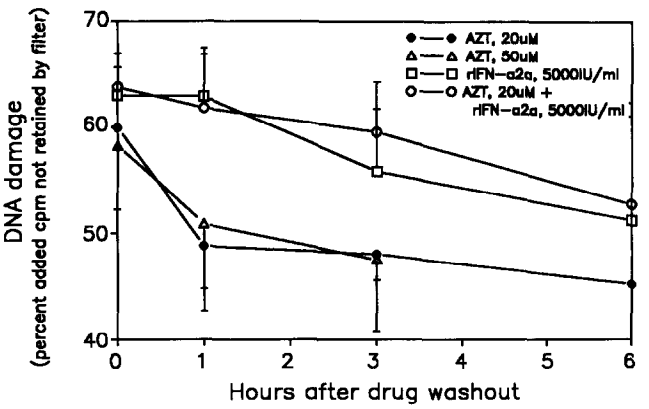

FIG. 4. **Assessment of DNA repair in cells exposed to AZT alone, rIFN-α2a alone, or the combination of AZT + rIFN-α2a. Approximately 2 × 10^6 cells were incubated in medium containing [^{3}H]dThd for 48 hr. For the final 24 hr cells were also exposed to either AZT alone (20 or 50 μM), rIFN-α2a alone (5000 IU/mL) or the combination of 20 μM AZT + 5000 IU/mL rIFN-α2a. Thereafter, the medium was removed, and the cells were washed with saline and then incubated in fresh medium without drug. At various times thereafter, the cells were harvested and DNA damage was assessed by filter elution techniques as described in the text. DNA damage is designated as the percentage of total counts per minute recovered not retained by the filter, and for the series of experiments summarized in this figure the total cpm recovered averaged 116,400. Each point represents the mean ± SEM of 4–6 determinations.**

apoptosis [47–49]. To determine if the ability of rIFN-α2a plus AZT to disrupt DNA repair also reflected a disruption of p53-mediated processes, studies next assessed the effect of these agents, alone or in combination, on wild-type p53 expression. As expected, following a 24-hr exposure to 20 μM AZT alone, wild-type p53 expression increased (Fig. 5A), and densitometric analysis revealed this increase to be significant ($P \leq 0.05$) (Fig. 5B). In contrast, exposure to rIFN-α2a (5000 IU/mL) alone significantly reduced ($P \leq 0.01$) wild-type p53 expression to a level below that detected in control (non-exposed) cells (Fig. 5B). When cells were exposed to 20 μM AZT + 5000 IU/mL rIFN-α2a, a condition that produced more DNA damage than exposure to 20 μM AZT alone, p53 expression was similar to that observed in control cells and was significantly lower ($P \leq 0.05$) than that in cells exposed to 20 μM AZT alone. Thus, in addition to inhibiting polymerase beta activity, rIFN-α2a significantly reduced the expression of wild-type p53.

DISCUSSION

The results indicate that AZT plus rIFN-α2a combine to produce synergistic inhibition of HCT-8 cell growth, which reflects (i) the ability of rIFN-α2a to increase dThd kinase activity and thus AZT metabolism and incorporation into DNA, and (ii) the combined inhibitory effects of rIFN-α2a

TABLE 3. Effect of AZTTP alone, rIFN-α2a alone, or their combination on the activities of human DNA polymerases alpha and beta

Conditions	Polymerase activity (% of control)	
	Alpha	Beta
No drug	100	100
Aphidicolin, 4 μg/mL	19.3 ± 10.3	ND*
ddCTP, 10 μM	ND	32.0 ± 7.6
rIFN-α2a, 15,000 IU/mL	124.0 ± 18.1	83.3 ± 11.9
rIFN-α2a, 30,000 IU/mL	126.9 ± 15.7	73.7 ± 10.8
AZTTP, 25 μM	89.3 ± 8.6	97.7 ± 1.5
AZTTP, 50 μM	76.8 ± 13.5	91.7 ± 7.3
rIFN-α2a, 15,000 IU/mL + AZTTP, 25 μM	106.3 (N = 2)	63.7 ± 8.9
rIFN-α2a, 15,000 IU/mL + AZTTP, 50 μM	105.5 (N = 2)	58.7 ± 8.1
rIFN-α2a, 30,000 IU/mL + AZTTP, 25 μM	109.1 (N = 2)	67.7 ± 3.7
rIFN-α2a, 30,000 IU/mL + AZTTP, 50 μM	113.3 ± 9.5	61.7 ± 6.7

Purified human DNA polymerases alpha and beta were obtained commercially. The effect of alterations in the concentration of rIFN-α2a and AZTTP on DNA synthesis mediated by these polymerases, using activated calf thymus DNA as a template and dTTP, dATP, [^{3}H]dCTP and dGTP as substrates, was carried out by methods described in the text, and DNA synthesis was quantiated by liquid scintillation techniques. Polymerase activity is presented as a percentage of cpm retained on the filters in the absence of inhibitors, and control cpm values were 1400 ± 130 for polymerase alpha and 16,600 ± 1,310 for polymerase beta. Values are the means ± SEM of 3 determinations unless otherwise noted.

* ND = not determined.

and AZT on DNA polymerase beta mediated gap-filling repair synthesis. This effect of rIFN-α2a on cellular dThd kinase activity has been observed by others using a variety of model systems [27, 28, 50]. The positive correlation between increased dThd kinase activity and increased AZT-associated cell growth inhibition is consistent with our previous observations that alterations in dThd or AZT metabolism, which result in an increased intracellular AZTTP/dTTP ratio, lead to increased AZT incorporation into DNA and cytotoxicity [5, 33].

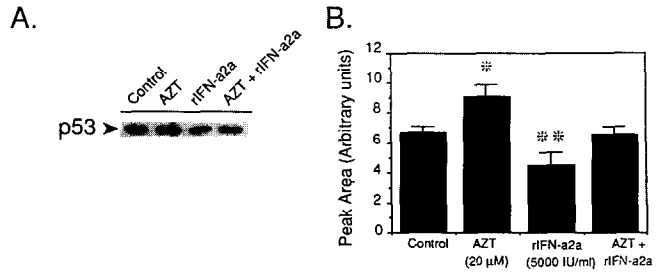

FIG. 5. (A) Results of a representative western blot to detect wild-type p53 protein in HCT-8 cells exposed to AZT and/or rIFN-α2a. Cells (2 × 10^6) were incubated in medium containing 20 μM AZT alone, 5000 IU/mL rIFN-α2a alone, or the combination of AZT + rIFN-α2a. After 24 hr, the cells were harvested and p53 protein was isolated by microbatch affinity chromatography using the Ab-5 antibody. The immune complexes were resolved by 7.5% SDS–PAGE and transferred to nitrocellulose, which was probed with the monoclonal antibody to wild-type p53, DO-1. p53 was then visualized by enhanced chemiluminescence. (B) Densitometric analysis of wild-type p53 expression in HCT-8 cells under the exposure conditions described above. Each bar represents the mean ± SEM of 5–6 western blots. Key: (*) $P \leq 0.05$ vs control (non-treated) cells, and (**) $P \leq 0.01$ vs control cells.

Our findings that alterations in DNA repair can affect AZT cytotoxicity support those of Vazquez–Padua *et al.* [51] and Harrington and coworkers [52], which indicated that DNA repair capacity can impact upon cellular sensitivity to AZT. In the present study, the effect of rIFN-α2a on AZT metabolism and incorporation into DNA, albeit significant, was similar in degree only to that produced when AZT was combined with either FUra or MTX [3, 5], combinations that produce only additive growth inhibitory effects. Therefore, we conclude that it is the added ability of rIFN-α2a + AZTTP to disrupt DNA repair that results in the synergistic activity observed in this study.

Of interest, we did not observe major differences in the rate of AZT excision from DNA among the exposure conditions analyzed. This is surprising since AZT excision, mediated by 3′-exonuclease, is reportedly inhibited by elevated AZTMP [51, 52]. In the present study, AZTMP pools were ~2-fold higher in cells exposed to 20 μM AZT plus rIFN-α2a compared with those in cells exposed to 20 μM AZT alone. The apparent lack of effect of this higher AZTMP on AZT excision, however, supports the findings of Harrington *et al.* [52] that under physiological conditions for Mg^{2+} and Mn^{2+}, 1 mM AZTMP (a concentration greater than that generated in the present study) inhibited 3′-exonuclease activity by <50%. Thus, while the ability of AZTMP to inhibit AZT excision from DNA may be relevant under some exposure conditions, it did not contribute to the activity observed in the present study.

Although AZT excision from DNA was unaltered, we have shown that rIFN-α2a, alone or combined with AZTTP, directly inhibited polymerase beta activity in a cell-free system. Our analysis of the effect of these agents on DNA fragmentation/repair in whole cells suggested that this inhibitory effect correlated with an impaired ability to

repair DNA damage. This supports the findings of Wilson and colleagues [53–55], who reported that alterations in the expression of DNA polymerase beta are associated with changes in cellular sensitivity to selected DNA damaging agents. It is fortuitous that, in the present study, the nucleoside analogue AZT contributes to the ability of rIFN-α2a to inhibit polymerase beta and also acts as a DNA damaging agent; thus, these agents combine to both directly damage DNA and inhibit subsequent repair.

It has been demonstrated that exposing cells to selected DNA damaging agents increases the expression of wild-type p53 [46–49, 56]. This can result in an inhibition of both replicative DNA synthesis and progression through the cell cycle to allow more effective DNA repair. Since DNA damage was greater in cells exposed to rIFN-α2a alone or combined with AZT, one could predict that exposure to these agents would increase expression of wild-type p53. We were surprised to find that exposure to rIFN-α2a resulted in a decreased expression of wild-type p53 in both the presence and absence of AZT. Functionally, this could result in relatively unrestricted cell growth in spite of DNA damage, a situation that can contribute to the cytotoxicity of this combination. Interestingly, these data suggest that functional DNA polymerase beta may be necessary for the early identification of DNA damage and subsequent increase in p53 expression. Alternatively, these findings may suggest that rIFN-α2a directly reduces p53 expression by inhibiting p53 synthesis or increasing its turnover. Indeed, our recent finding that rIFN-α2a can restore drug sensitivity in a cisplatin-resistant human melanoma line supports this later contention [38]. Experiments in progress are directed to further assessing these possibilities.

The present findings suggest that a previously unappreciated mechanism by which IFN-α enhances the cytotoxicity of selected DNA damaging agents, such as cisplatin, vinblastine, and UV radiation, is its ability to disrupt DNA gap-filling repair. Since, in the present study, rIFN-α2a concentrations as low as 500 IU/mL, a concentration that is clinically achievable, enhanced the growth inhibitory effect of AZT (Fig. 1), our findings also suggest that DNA repair may be therapeutically manipulated with rIFN-α2a. In light of the above data, the suggestive therapeutic activity of FUra/LV + AZT, and the reported efficacy of FUra + LV + IFN-α, we believe evaluation of FUra/LV + AZT + rIFN-α2a in patients with advanced cancer is warranted.

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