Azidothymidine-induced Cytotoxicity and Incorporation into DNA in the Human Colon Tumor Cell Line HCT-8 Is Enhanced by Methotrexate in Vitro and in Vivo

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ABSTRACT

We have reported that 5-fluorouracil can increase the cytotoxic and antineoplastic activity of 3'-azido-3'-deoxythymidine (AZT). To further evaluate the antineoplastic utility of AZT we now have assessed its effect in combination with methotrexate (MTX) in the human colon tumor model HCT-8. Incubation of these cells for 5 days in AZT and MTX caused a reduction in the 50% inhibitory concentration of AZT and isobologram analysis revealed additive effects which were reversed by the addition of 50 µm thymidine to the incubation media. This enhanced cytotoxicity appeared not to be related to an effect of AZT on MTX activity; in whole-cell assays the ability of MTX to inhibit de novo dTMP synthesis and deplete intracellular pools of dTTP was not affected by AZT. In contrast, although MTX did not alter AZT triphosphate production, it did affect AZT triphosphate utilization in DNA synthesis. Incubation of cells for 24 h in [3H]AZT alone (5 µM, 3 µCi/ml) resulted in 6.6 pmol AZT incorporated into cellular DNA/10^6 cells. Coincubation of these cells in [3H]AZT (5 µM) plus 5 or 15 nm MTX increased AZT incorporation into DNA to 8.0 and 20.5 pmol/10^6 cells, respectively. Biochemically, this effect appeared to correlate with the concentration-dependent ability of 5 or 15 nm MTX to deplete intracellular dTTP pools, which were reduced by 25 and 49%, respectively. Further evidence of the relationship between intracellular dTTP pools and AZT cytotoxicity was that, in the presence of both MTX and 50 µM thymidine, cellular dTTP pools remained near pretreatment levels and the incorporation of 5 µM AZT into DNA was not enhanced. Therapeutically, studies conducted in athymic (nude) mice bearing HCT-8 xenografts that received six weekly cycles of MTX (87.5 mg/kg) and AZT (300 mg/kg) revealed that the two-drug regimen exerted superior antineoplastic activity in tumors resulting from the combination of FUra and MTX. In vivo, we have evaluated the combination of FUra and AZT in human colon tumor-bearing athymic (nu/nu) mice and found significant antineoplastic activity in tumors relatively insensitive to either FUra or AZT alone, with no increase in host toxicity. These studies have led to a phase I and II clinical and pharmacological evaluation of high-dose AZT in combination with FUra and leucovorin (10). Initial results from these trials indicate that high-dose AZT is very well tolerated in this combination and produces expected biochemical effects.

Studies to identify the biochemical mechanism(s) responsible for the cytotoxic activity of AZT are ongoing. Previous reports by Frick et al. (11) suggested that, in normal human fibroblasts and human leukemia cells, AZT cytotoxicity was related to its ability to generate large intracellular pools of AZTMP. More recent studies by Sommadossi et al. (12) demonstrated that in human bone marrow cells AZT-induced cytotoxicity was related to its incorporation into DNA. In contrast, the findings of Vazquez-Padua et al. (13) using the human leukemia cell line K562 suggested that AZT cytotoxicity did not closely correlate with its incorporation into DNA but rather with the inability of cells to repair AZT-induced DNA damage. Recent preliminary findings from our group indicated that AZT-related cytotoxicity in a panel of human colon tumor cell models did indeed appear to be closely related to the degree to which AZT was incorporated into cellular DNA (14, 15).

Implied in these findings is that factors which increase AZT utilization in DNA synthesis should induce enhanced cytotoxicity. Indeed, results from our continuing evaluation of the combination of AZT and FUra suggest that FUra, presumably by its ability to inhibit de novo dTMP synthesis, specifically increases both AZT utilization in DNA synthesis and cytotoxicity (14). The combination of MTX and AZT should, by virtue of the same biochemical principles, produce similar effects. We now report the results of an assessment of the biochemical, cytotoxic, and antineoplastic activity of the combination of AZT and MTX in a human colon tumor model. Preliminary aspects of this study have appeared (16, 17).

INTRODUCTION

AZT* is a dThd analogue which is clinically useful in the treatment of AIDS and AIDS-related complex (1, 2). Although AZT was originally developed as an anticancer agent (3), early assessment of its cytotoxic properties was unimpressive and further development along these lines was abandoned. Recently, however, its potential use as an antineoplastic agent has again been suggested (4–9). Scanlon et al. (4, 5) have reported that AZT can restore sensitivity to cisplatin and MTX in vitro in cells resistant to these agents. Our group and others (6–9) have reported that significant cytotoxic activity in vitro in human colon tumor cells can be achieved when AZT is combined with FUra. In vivo, we have evaluated the combination of FUra and AZT in human colon tumor-bearing athymic (nu/nu) mice and found significant antineoplastic activity in tumors relatively insensitive to either FUra or AZT alone, with no increase in host toxicity (6). These studies have led to a phase I and II clinical and pharmacological evaluation of high-dose AZT in combination with FUra and leucovorin (10). Initial results from these trials indicate that high-dose AZT is very well tolerated in this combination and produces expected biochemical effects.

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MATERIALS AND METHODS

Drugs. AZT was the generous gift of the Burroughs Wellcome Company (Research Triangle Park, NC). MTX was purchased from Lederle (Caroline, PR). [3H]AZT (56 Ci/mmol) and [3H]deoxyuridine (56 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). RPMI 1640, FBS, and dazacycl FBS (molecular weight cutoff, 12,000) were purchased from Gibco (Grand Island, NY). RNase-free DNase I was purchased from Boehringer-Mannheim (Indianapolis, IN). HPLC grade solvents and chemicals were obtained from Fisher Scientific (Medford, MA).

Cell Lines. Continuous cultures of HCT-8 human colon adenocarcinoma cells were used in all studies. The biochemical and histological characterization of this cell line has been reported (18). Cells were cultured in sterile plastic tissue culture flasks as monolayers in RPMI
1640 supplemented with 10% FBS and passed twice weekly. Cell cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Under these conditions their doubling time was ~24 h.

Animals. Six- to 8-week-old athymic (nu/nu) mice (obtained from the Animal Care and Breeding Facility, Roger Williams Medical Center) bearing xenografts of the human colon tumor cell line HCT-8 were used to assess the therapeutic effectiveness and toxicity of various MTX and AZT regimens. Tumors were induced by a single s.c. injection of 6 × 10⁶ HCT-8 cells in the lower left axillary region (6). Two to 3 weeks later mice with palpable tumors (~100 mg) were culled for evaluation, as described below.

In Vitro Evaluation of Cytotoxicity. HCT-8 cells (1 × 10⁵) were added to 10 ml of RPMI 1640 containing 10% dialyzed FBS in 25-cm² culture flasks to which dThd had been added to achieve a final concentration of 0.1 μM. AZT, MTX, and additional dThd, previously dissolved in medium, were added at concentrations of 0.1 to 30 μM (AZT), 0.1 to 50 nM (MTX), and 50 μM (dThd), either alone or in various noted combinations. Control cultures received the same amount of medium without drug. After 5 days, cells were harvested by trypsinization and counted electronically (Coulter Counter model ZM, Coulter Electronics). Growth inhibition was calculated as a percentage of control culture.

Enzyme Assays. TS activity was measured in intact cells after a 90-min incubation in serum-free RPMI 1640 containing AZT alone (5 μM), MTX alone (50 or 100 nM), or the combination of AZT and MTX, at these concentrations. Enzyme activity was then assessed by the quantitation of H₂O released from [⁵²]Deoxyuridine, as previously described in detail (22).

Quantitation of Intracellular AZT-Nucleotide and dTTP Pools. The effect of MTX and/or exogenous dThd on the generation of [⁵²]AZT nucleotides was quantitated by incubating 2–4 × 10⁶ cells in 10 ml medium containing 30 μCi [⁵²]AZT alone (5 or 20 μM, concentrations which inhibit cell growth by ~10 and ~50%, respectively), after 5 days, [⁵²]AZT (5 or 20 μM) plus 50 μM dThd, [⁵²]AZT (5 μM) plus MTX (5 or 15 nM), or [⁵²]AZT (5 μM) plus MTX (5 or 15 nM) plus 50 μM dThd. After 24 h the medium was removed, and the cells were washed 3 times with 30 ml saline, harvested, pelleted, and homogenized in 1 ml 0.2 M PCA. The PCA-insoluble material was removed and utilized to quantitate [⁵²]AZT incorporation into DNA, as described below. The PCA-soluble material was analyzed to quantitate [⁵²]AZT nucleotides by a modification of previously reported HPLC methods (23) in which 450 μl were injected onto a computer-controlled Rainin modular HPLC system equipped with a Whatman Partisil SAX10 analytical column (4.6 mm x 25.0 cm). The column was eluted at 1.0 ml/min with a 1-h linear gradient of 0.01 M Na₂HPO₄ (pH 3.5) to 0.5 M Na₂HPO₄ plus 0.5 M KCl (pH 3.5). Column eluent was monitored with both a Gilson fixed wavelength (254 nm) UV detector and a Radiomatic scintillation detector (Model A-100). Under these conditions the retention times of ribonucleotides, standards containing 1–25 μM dTTP ± 100 μM UTP were also processed with each set of cell extracts. The dTTP content of cell extracts and standard solutions was quantitated by HPLC, as described above, and the dTTP peak areas for all standards were compared. Under these conditions the lower level of detectability for dTTP was ~30 pmol and the retention times for dTTP and UTP were 42.4 and 41.0 min, respectively.

Incorporation of [⁵²]AZT into Nuclear DNA. The incorporation of [⁵²]AZT into nuclear DNA and the effect of MTX and/or exogenous dThd on this parameter was quantitated in the [⁵²]AZT-exposed PCA-insoluble material generated as described above. This material was washed 5 times with 1 ml 0.2 M PCA and once in 350 μl of 0.1 M sodium acetate-5 mM MgSO₄ (pH 5.0) and incubated for 1 h at 37°C in 650 μl of 0.1 mM sodium acetate-5 mM MgSO₄ containing 300 units of RNase-free DNase I. To ensure extensive DNase I-mediated hydrolysis, the supernatant was removed and the insoluble material was incubated for an additional 2 times by the same method. [⁵²]H-related cpm in the pooled supernatant fractions, representing total cellular DNA (25), was quantitated by liquid scintillation techniques.

In Vivo Evaluation of Toxicity and Therapeutic Effectiveness. To evaluate the therapeutic effectiveness and toxicity of MTX combined with AZT, athymic (nu/nu) mice bearing HCT-8 xenografts were distributed by tumor weight in groups of 5–9. Animals received 6 weekly courses of AZT (300 mg/kg) and MTX (87.5 mg/kg) either alone or in combination. By this regimen, AZT was administered 2 h after MTX. The drugs were dissolved in normal saline immediately prior to use and administered by i.p. bolus so that 0.1 ml of drug was injected/10 g body weight. Therapeutic effectiveness and toxicity were evaluated as previously described (6). Experiments were performed in triplicate and the pooled number of animals in each treatment group was 19–26.

Statistical Analysis. Student’s t-test was used; P ≤ 0.05 was considered significant.

RESULTS

In HCT-8 cells the IC₅₀ of AZT after a 5-day continuous exposure was found to be 21.5 μM. Under similar conditions, the IC₅₀ of MTX was detected to be 12.1 nm (Table 1). When cells were incubated in both MTX and AZT for 5 days, an additive cytotoxic effect was observed (Fig. 1). Further in vitro evaluation of the combination indicated that, over a wide range of AZT and MTX concentrations, MTX was able to increase the cytotoxicity of AZT, while AZT exerted little effect on the IC₅₀ of MTX. This is in contrast to previous studies which suggested that, in HCT-8 cells, the cytotoxic effects of this combination were a consequence of the ability of AZT to competitively inhibit dThd kinase and enhance MTX-related disruptions in thymine nucleotide biosynthesis (8, 9). While we also have observed that AZT competitively inhibited dThd kinase in these cells, this effect did not appear to be closely associated with AZT-induced cytotoxicity; increasing the medium concentration of dThd 500-fold, to 50 μM, did not significantly affect the IC₅₀ of AZT (Table 1). That dThd “salvage” was not significantly inhibited by AZT was further evidenced by the fact that, even in the presence of AZT, this concentration of dThd (50 μM) could partially reduce the cytotoxicity of the MTX plus AZT combination to a degree observed to be associated with AZT alone (Table 1).

Complimentary biochemical studies were conducted to evaluate the effect of MTX, AZT, exogenous dThd, or their combinations on de novo dTMP biosynthesis and dTTP pool size in

<table>
<thead>
<tr>
<th>Compound</th>
<th>0.1 μM dThd</th>
<th>50.0 μM dThd</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>21.5 ± 2.2 μM</td>
<td>27.5 ± 7.5 μM</td>
</tr>
<tr>
<td>MTX</td>
<td>12.1 ± 2.0 μM</td>
<td>21.0 ± 1.8 μM</td>
</tr>
<tr>
<td>MTX (3 nM) + AZT</td>
<td>15.4 ± 2.0 μM</td>
<td>30.7 ± 2.3 μM</td>
</tr>
</tbody>
</table>

Table 1 Effect of 0.1 or 30.0 μM extracellular dThd on the IC₅₀ of MTX alone, AZT alone, or AZT in the presence of 3 nM MTX in HCT-8 cells

Twenty-five-cm² tissue culture flasks containing 10 ml of RPMI 1640 plus 10% dialyzed FBS, 1 × 10⁶ cells, and various concentrations of AZT, MTX, and dThd were incubated at 37°C. After 5 days, the cells were harvested and cell number was determined as described in the text. The percentage of growth inhibition was quantitated using cells incubated without AZT or MTX as control. Each value represents the mean ± SE of 6 to 18 determinations.

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and MTX were incubated at 37°C. After 5 days, the cells were harvested and the IC₅₀ of MTX alone or AZT alone was 12.1 nM and 21.5 nM, respectively. The mean IC₅₀ value of 6 to 10 determinations.

Table 2 Effect of various concentrations of AZT alone, MTX alone, 50 μM dThd, or their various combinations on intracellular pools of AZT nucleotides and dTTP

<table>
<thead>
<tr>
<th>Drug and concentration</th>
<th>AZTMP (pmol/10⁶ cells)</th>
<th>AZTDP (pmol/10⁶ cells)</th>
<th>AZTTP (pmol/10⁶ cells)</th>
<th>dTTP (pmol/10⁶ cells)</th>
<th>Triphosphate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+ dThd, 50 μM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AZT, 5 μM</td>
<td>25.3 ± 1.6</td>
<td>3.3 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>48.6 ± 2.6</td>
<td>—</td>
</tr>
<tr>
<td>+ dThd, 50 μM</td>
<td>29.5 ± 3.1</td>
<td>4.0 ± 0.4</td>
<td>2.7 ± 0.4</td>
<td>51.6 ± 4.0</td>
<td>21:1</td>
</tr>
<tr>
<td>AZT, 20 μM</td>
<td>215.7 ± 18.6</td>
<td>10.4 ± 1.6</td>
<td>6.9 ± 1.0</td>
<td>48.2 ± 6.6</td>
<td>7:1</td>
</tr>
<tr>
<td>+ dThd, 50 μM</td>
<td>225.0 ± 11.6</td>
<td>11.6 ± 2.4</td>
<td>7.6 ± 1.1</td>
<td>54.0 ± 4.4</td>
<td>7:1</td>
</tr>
<tr>
<td>MTX, 5 μM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>+ dThd, 50 μM</td>
<td>—</td>
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<tr>
<td>AZT, 5 μM</td>
<td>31.2 ± 4.8</td>
<td>3.5 ± 0.4</td>
<td>2.6 ± 0.5</td>
<td>38.1 ± 6.3</td>
<td>15:1</td>
</tr>
<tr>
<td>+ MTX, 5 μM</td>
<td>27.9 ± 2.5</td>
<td>4.3 ± 0.5</td>
<td>2.7 ± 0.6</td>
<td>53.2 ± 3.7</td>
<td>20:1</td>
</tr>
<tr>
<td>MTX, 15 μM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+ dThd, 50 μM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AZT, 5 μM</td>
<td>50.3 ± 5.2</td>
<td>4.0 ± 0.7</td>
<td>3.4 ± 0.8</td>
<td>28.6 ± 3.7</td>
<td>8:1</td>
</tr>
<tr>
<td>+ dThd, 50 μM</td>
<td>56.8 ± 12.5</td>
<td>4.2 ± 0.7</td>
<td>3.5 ± 1.2</td>
<td>43.4 ± 3.5</td>
<td>12:1</td>
</tr>
</tbody>
</table>

* [dTTP]/[AZTTP].
* P ≤ 0.001 versus 5 μM AZT.
# P ≤ 0.01 versus 5 μM AZT.
4 P ≤ 0.1 versus no drug control.
5 P ≤ 0.01 versus no drug control.
6 not determined.

Results of previous studies in our laboratory (6, 10, 14, 15) and others (12) have suggested that AZT-induced cytotoxicity is associated with the degree to which AZTTP is incorporated into DNA. Indeed, in the present study we have observed that as the extracellular concentration of AZT was increased from 5 to 20 μM (10 and 50% inhibitory concentrations, respectively, after a 5-day exposure) there was an increase in both AZT incorporation into DNA [3-fold (Fig.2)] and the intracellular concentration of AZT nucleotides [3- to 9-fold (Table 2)]. Therefore, we next evaluated the effect of MTX on both AZT nucleotide generation and incorporation into DNA. In HCT-8 cells, incubation in [³H]AZT (5 μM) for 24 h resulted in [³H]-AZTMP, [³H]AZTDP, and [³H]AZTTP pools of 25.3, 3.3, and 2.5 pmol/10⁶ cells, respectively. Incubation of cells in 5 μM [³H]AZT plus either 5 or 15 nM MTX for 24 h did not significantly alter AZTDP and AZTTP pools; however, in the presence of 15 nM MTX there was a 2-fold increase in AZTTP pools when compared to those generated from incubation in 5 μM [³H]AZT alone (Table 2). The addition of dThd (50 μM) to medium did not appear to affect AZT nucleotide generation under any of the drug combination conditions investigated.

In contrast, analysis of the DNase I-hydrolyzable fraction obtained from cells incubated for 24 h in 5 μM [³H]AZT plus 5 or 15 nM MTX revealed a 21% or 3-fold increase, respectively, in [³H]AZT incorporation in DNA when compared to incubation in 5 μM [³H]AZT alone (Fig.2). Of interest, although it did not affect the incorporation of either 5 or 20 μM [³H]AZT alone, 50 μM dThd did reduce [³H]AZT (5 μM) incorporation into DNA in the presence of MTX to a level of approximately that observed to result from incubation in medium containing 5 μM [³H]AZT alone (Fig.2).

Enhanced AZT incorporation into DNA and cytotoxicity is of no clinical benefit unless it results in improved therapeutic efficacy. To assess this possibility, the combination of MTX and AZT was evaluated in nude mice bearing HCT-8 xenografts. In these studies, the dose and schedule of MTX were selected to be approximately the maximum tolerated dose over 6 courses of weekly therapy. Weekly bolus doses of AZT alone up to 1200 mg/kg (due to the limited solubility the maximum which could be administered by bolus) were nontoxic but a 300-mg/kg dose was chosen for administration so that
compared to control regimens. Just as important, in the same
correlation with our previously published reports could be
was considered significant.

Bars, the mean ± SE of 7 to 15 determinations; P < 0.05 (versus 5 MM AZT alone)

Fig. 2. Effect of MTX (5 or 15 nm) or 50 µM dThd on the incorporation of
3H]AZT into the DNA fraction of HCT-8 cells. Two-4 x 10^6 HCT-8 cells were
incubated in 10 ml of RPMI 1640 containing 10% dialyzed FBS, the stated
centration of dThd, and either [3H]AZT alone (1.5 µCi/ml, 5 or 20 µM) or
[3H]AZT (5 µM, 1.5 µCi/ml) plus MTX (5 or 15 nm). After 24 h, the cells were
harvested and homogenized in 1 ml 0.2 M sodium acetate-5 mM
MgSO₄ (pH 5.0) containing 300 units of RNase-free DNase I, as described in the
text. The 3H-content of the pooled DNase I-hydrolyzable material was deter

Fig. 3. Effect of 6 weekly courses of MTX alone (87.5 mg/kg), AZT alone (300
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thesis. Indeed, examination of both de novo dTMP biosynthesis
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the ability of MTX to disturb these parameters nor did AZT
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Biochemically, although AZTTP is not a substrate for human
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can be postulated that the addition of an agent which limits dThd transport should be able to further enhance the cytotoxicity and tissue specificity of this combination by limiting dThd, but not AZT, "salvage" in neoplastic cells (35). To that end we are presently evaluating the combination of MTX and AZT and the nucleoside transport inhibitor dipyriramole (23, 35, 36).

Our in vivo results clearly indicate that these biochemical actions translate into a highly effective combination. In our murine studies both MTX and AZT alone were essentially ineffective at inhibiting human tumor xenograft growth while their combination significantly inhibited tumor growth. Since no added toxicity was generated by the drug combination over MTX alone this represents a dramatic improvement in therapeutic index. As a result of these findings the combination of MTX and AZT is presently undergoing a phase I and II clinical evaluation in our center in patients with advanced colon and breast cancer. Also warranted is an assessment of the activity of this, and related, combinations in AIDS and AIDS-related complex. Indeed, results from preliminary murine studies suggest that a significant increase in the antiviral activity of AZT may be achieved by this approach (30).

ACKNOWLEDGMENTS

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REFERENCES


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