

Telomerase Inhibition Using Azidothymidine in the HT-29 Colon Cancer Cell Line

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Background: We investigated the effects of telomerase inhibition by using the reverse transcriptase inhibitor azidothymidine (AZT) in the human colorectal cancer cell line HT-29 in the presence and absence of 5-fluorouracil (5-FU).

Methods: HT-29 cells were cultured in the presence of AZT. Telomerase activity was measured by using the telomerase repeat amplification protocol. Telomere length was determined by Southern analysis. The colorimetric microtiter assay was performed to determine the cytotoxic effects of AZT, alone and in combination with 5-FU.

Results: The presence of 3'-azido-3'-deoxythymidine triphosphate (AZT-TP) effectively inhibited telomerase extracted from HT-29 cells. HT-29 cells cultured with 125 μ M of AZT underwent fewer total population doublings over 91 days. Southern analysis revealed that telomere attrition occurred within this period. Exposure to 125 μ M of AZT resulted in slightly reduced viability (10%) of HT-29 cells. However, the presence of AZT increased 5-FU cytotoxicity, suggesting that the effects of these two drugs are synergistic.

Conclusions: The data are consistent with telomerase inhibition having growth-inhibitory effects in addition to those predicted to accompany loss of telomere function. Further studies using specific small-molecule inhibitors will confirm whether the growth-inhibitory and 5-FU-sensitivity effects seen here are a direct result of telomerase inhibition.

Key Words: Telomerase—AZT—HT-29 colon cancer cell line—Telomere.

Telomeres are specialized structures at the ends of eukaryotic chromosomes that are essential for genome stability, acting to cap the end of the chromosome and protect it from degradation and fusion. Human telomeres are composed of tandem repeats of the simple DNA sequence 5'-TTAGG-3', which may extend for more than 10 kilobases (kb), and associated proteins. The cellular immortality required for tumorigenesis is tightly linked to the maintenance of telomeric DNA, which is usually achieved through telomerase activation.

Complete replication of chromosomal termini is hampered by the unidirectional nature and primer require-

ments of conventional DNA polymerases. Because of these features, a region of unreplicated DNA will remain on the parental DNA strand, acting as the template for lagging strand synthesis after removal of the most terminal primer. This has become known as the end-replication problem.¹ The telomeric repeat array is maintained in the germline by telomerase, a reverse transcriptase that uses an RNA moiety as a template for the addition of telomeric sequences onto the 3' end of an existing DNA molecule.² In this way, terminal sequence loss is balanced by de novo addition of telomeric repeats. Telomerase activity is repressed in most somatic tissues, and, as a consequence of the end-replication problem and end processing, telomeric sequences are lost with each cell division. When a telomere becomes critically shortened, a signal is generated that causes the cell to undergo replicative senescence or apoptosis. In contrast, telomere length is stable, and telomerase is active in most tumors (approximately 90%) and immortal cell lines. Furthermore, forced expression of telomerase by introduction of

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the human telomerase catalytic subunit (hTERT), with concomitant extension of telomeric arrays, is sufficient to confer an immortal phenotype in primary human cells such as fibroblasts.^{3,4} Similarly, inhibition of telomerase with dominant negative alleles results in telomere shortening and eventual growth inhibition of previously immortal cells.^{5,6} It is intriguing to note that several recent reports making use of telomerase null mouse models and cell lines derived from such mice have indicated that telomerase promotes cell and tumor growth in a manner separable from its role in telomere maintenance.^{7,8} Given the prevalence of telomerase in tumors and its essential role in permitting continued cellular proliferation, it is regarded as a useful target for the development of new chemotherapeutic drugs.

Colorectal cancer is the fourth most frequently diagnosed cancer in the United States and has the second highest cancer-related mortality.⁹ It has been well documented that colon cancer responds to conventional chemotherapy (5-fluorouracil [5-FU] and leucovorin), resulting in improved survival.¹⁰ We sought to investigate the effects of telomerase inhibition in conjunction with conventional chemotherapy.

A number of telomerase inhibitors have been used. One of the earliest tested was the reverse transcriptase inhibitor azidothymidine (AZT). AZT was found to inhibit telomerase in the B-cell line JY616 and the T-cell line Jurkat E6-1, resulting in gradual telomere erosion and growth inhibition.¹¹ Recently, compounds more specifically targeted against telomerase have been developed and tested. These compounds fall into several broad categories: those that target the catalytic subunit of telomerase, those that target the RNA template subunit of telomerase, and those that target G-quartet structures.¹² AZT is a readily available inhibitor, and trials investigating its cytotoxicity in humans have already been performed.^{13,14} These aspects made AZT an attractive compound for determining the effects of telomerase inhibition in the human colorectal cancer cell line HT-29 in the presence and absence of 5-FU.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The HT-29 human colorectal cancer cell line was graciously donated by J. D. Chapman at Fox Chase Cancer Center. HT-29 was the only colorectal cancer cell line used in these experiments. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 2 mM of L-glutamine. The cells were incubated at 37°C in the presence of 5%

CO₂. AZT was obtained from Aldrich Chemical Company (Milwaukee, WI) and diluted to final concentrations of .1, 1, 10, 25, and 125 μ M. Cells were counted and subcultured weekly, reseeding with 1×10^4 cells. Medium was changed twice weekly. Population doublings were calculated as the log of the final concentration/initial concentration over the log of 2. All cultures were grown in triplicate.

Protein Extracts and Telomerase Assays

Telomerase activity was assessed by using the telomerase repeat amplification protocol (TRAP) assay. Whole-cell protein extracts were prepared as described previously.¹⁵ Protein concentrations were determined by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). The TRAP assay was used to detect telomerase activity as described previously.^{16,17} All reactions were performed with .5 μ g of protein extract in duplicate with or without the inclusion of 20 ng of ribonuclease. Ribonuclease sensitivity was used to ensure that reaction products were due to telomerase. Quantitation of telomerase activity was performed after densitometric analysis with NIH Image software. Activity is shown as arbitrary units, with all activities normalized to that in control HT-29 cells.

Southern Analysis

Genomic DNA was extracted by following standard procedures. Restriction enzyme digestion of genomic DNA was performed with *Hin*FI and *Rsa*I, and the resulting fragments were resolved on .7% agarose gels and transferred to Hybond N membranes (Amersham Biosciences, Piscataway, NJ) as described previously.^{15,18} Telomeric restriction fragments were detected after hybridization with oligonucleotides complementary to the telomeric repeats, TTAGGG and AATCCC, as described previously.¹⁹ A total of 100 ng of each oligonucleotide was labeled at its 5' end by using T4 polynucleotide kinase and γ ³²P-adenosine triphosphate. Mean telomere length was determined after densitometric analysis by using software developed at Fox Chase Cancer Center (<http://bioinformatics.fccc.edu/software>).

Colorimetric Microtiter (MTT) Cytotoxicity Assay

HT-29 cells were seeded into a 96-well plate at 2×10^3 cells per well in 150 μ L of medium. Cells were allowed to recover overnight before treatment. Cells were treated with AZT (125 μ m), 5-FU (.3 μ g/mL), and a combination of both agents at the aforementioned concentrations. Results were compared with those of an untreated control group. Cells were treated for 4 hours, and then the agents were removed and cells were cul-

tured for an additional 72 hours. Cultures were then exposed to 1 mg/mL of MTT (40 μ L of 5 μ g/mL; Sigma, St. Louis, MO) for 2 hours. Lysis buffer (100 μ L; 50% [v/v] *N,N*-dimethylformamide, 20% (w/v) sodium dodecyl sulfate, .025 N of HCl, and .03% acetic acid) was then added to wells, and they were allowed to incubate overnight. The optical density of each well at 570 nm was determined by using a SpectraMax Plus multiplate reader (Molecular Devices, Sunnyvale, CA). The assay was completed in triplicate for two different HT-29 cell groups. Group A was grown in standard media as described previously, and group B was grown in the presence of 100 μ M of AZT 1 week before the MTT assay was completed.

Statistical Analysis

Fixed-effects analysis of variance with three factors—treatment (treated or untreated), AZT (125 μ M or no AZT), and 5-FU (.3 μ g/mL or no 5-FU)—crossed in a balanced design was used to model cell viability. A summary is presented in Table 1. A normal probability plot of residuals was used as diagnostic tool to check the underlying model assumptions. First- and second-order interactions were used to assess multidrug synergistic and antagonistic interactions.²⁰

Random coefficient models were used to model the cell growth and examine the dose effect on the growth.²¹ The cell growth in each experiment was modeled as a quadratic function over time. Contrasts were used to test specific hypotheses. The critical comparison-wise critical significant value was set to 5%. Fixed-effects one-way analysis of variance was used to compare cell frequency at 91 days. Tukey's Studentized range was used as an a posteriori test.

RESULTS

AZT-TP Inhibits In Vitro Telomerase Activity Extracted From HT-29 Cells

HT-29 cells exhibited robust telomerase activity, as expected (Fig. 1). To determine whether the active me-

tabolite of AZT, 3'-azido-3'-deoxythymidine triphosphate (AZT-TP), was capable of inhibiting this activity, we performed TRAP assays by using equivalent protein (.5 μ g) and increasing amounts of AZT-TP. We found that AZT-TP inhibited telomerase activity in a dose-dependent manner (Fig. 1A); activity was completely abolished at 200 μ M of AZT-TP. Quantitation of this activity was performed by using densitometric scanning of reaction products. The amount of product resulting from a reaction performed in the absence of AZT-TP was arbitrarily set to 100% (Fig. 1B). This analysis confirmed the inhibition of telomerase by AZT-TP in a dose-dependent manner.

HT-29 Cells Grow More Slowly in the Presence of AZT

To determine whether AZT inhibited the growth of HT-29 cells, we monitored the growth rate of cells cultured in various concentrations of AZT (0–125 μ M) over 3 months. The cells were counted every week and reseeded at a known density, allowing calculation of the number of population doublings. Cumulative population doublings attained were plotted against elapsed time to generate the data shown in Fig. 2. The growth of HT-29 cells was adversely affected by the presence of AZT, and the effect was more dramatic at higher doses of AZT. Overall, untreated cells had undergone an average of 70.7 population doublings at the end of the experiment (91 days), whereas cells cultured in the presence of the maximum dose of 125 μ M of AZT underwent an average of only 36.5 population doublings in the same time period ($P < .0001$). The decreased population doublings attained reflects a decreased rate of cellular proliferation when cells are cultured at the maximum dose of 125 μ M of AZT relative to the untreated control culture ($P < .0001$). The decrease in total number of population doublings is primarily due to extremely slow growth over the first month of culture in the presence of AZT. During this period, HT-29 cells cultured in the presence of 125 μ M AZT grew at a rate that was only on average 12% that of untreated cells. By the end of the experiment, the HT-29 cells cultured in the presence of 125 μ M AZT were growing at a rate that was on average 70% of that observed in untreated cells cultured in parallel. Culturing HT-29 cells in the presence of 25 μ M AZT also adversely affected growth, although not to the extent observed with the higher dose of drug. Again, fewer overall population doublings are primarily a result of a slower rate of growth—75% of that observed in the controls—over the first month of treatment. Exposure to lower doses of AZT covering a 100-fold range from .1 to 10 μ M had only slight effects on the growth rate of HT-29 cells.

TABLE 1. Summary of three-way ANOVA used to model cell viability

Factor	P value
Treatment	<.001
AZT	<.001
5-FU	<.001
AZT 5-FU	.009
Group B: 5-FU	.012
Group B: AZT	.046
Group B: AZT 5-FU	.172

ANOVA, analysis of variance; AZT, azidothymidine; 5-FU, 5-fluorouracil.

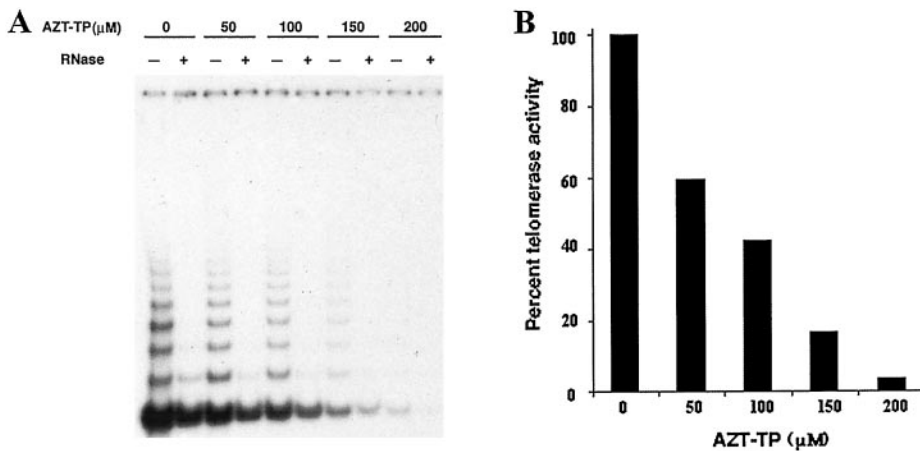


FIG. 1. Analysis of telomerase activity in the HT-29 colorectal cancer cell line. (A) Telomerase activity in HT-29 cells was measured by the telomerase repeat amplification protocol assay in the presence of the indicated concentrations of the active metabolite of azidothymidine (AZT-TP; 0, 50, 100, 150, or 200 μM). Each reaction was performed in the absence and presence of ribonuclease (RNase) A, which inhibits the formation of telomerase products by destroying the RNA template molecule. (B) Densitometric quantitation of the telomerase products.

Exposure to AZT Causes Telomere Erosion

The results obtained in Fig. 1 indicated that AZT-TP inhibited telomerase activity. A predicted consequence of telomere inhibition would be the gradual loss of DNA from chromosome ends. To confirm that AZT inhibited telomerase of HT-29 cells in vivo, we performed Southern analysis of telomeres isolated from HT-29 cells cultured in the presence or absence of 125 μM of AZT. There was no detectable telomere loss over the first month of culture (data not shown). However, telomeres isolated from HT-29 cells cultured for 3 months in the presence of AZT were much shorter than telomeres isolated from control cells (Fig. 3), with mean telomere lengths of 6.7 vs. 8.9 kb, respectively. These data confirm that AZT inhibits telomerase activity in vivo in HT-29 cells, resulting in gradual telomere attrition.

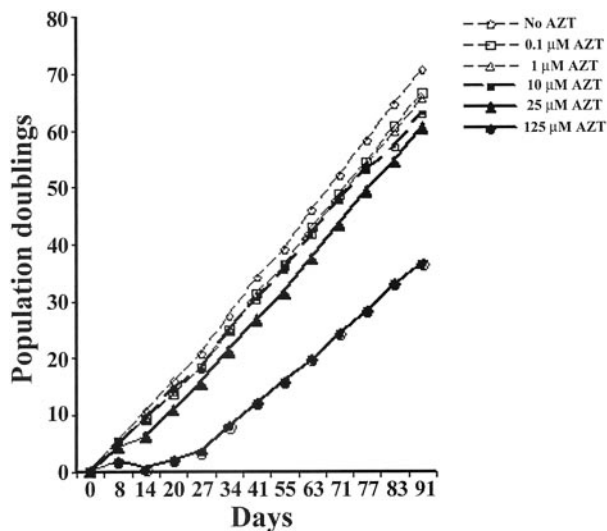


FIG. 2. Growth curves of HT-29 cells grown in the presence of the indicated concentrations of azidothymidine (AZT; 0, .1, 1, 10, 25, or 125 μM).

AZT Increases the Cytotoxicity of 5-FU

Telomerase has recently been shown to have growth-promoting activities that are separable from its required role in telomere maintenance. To determine whether

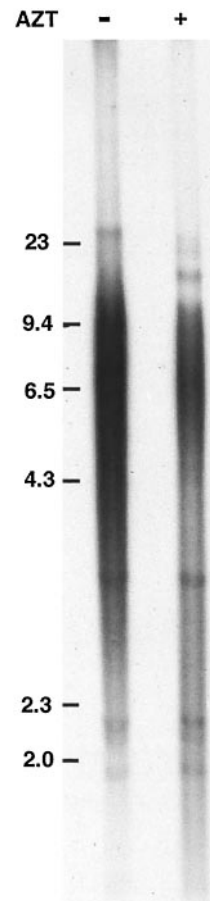


FIG. 3. Telomere length of HT-29 cells determined by Southern analysis cultured in the absence (-) or presence (+) of 125 μM of azidothymidine (AZT). Molecular weight markers (in kilobases) are indicated.

inhibition of telomerase with AZT increased the sensitivity of HT-29 cells to 5-FU, we determined cellular viability after treatment with AZT, 5-FU, or a combination of the two by using the MTT cytotoxicity assay. The assays were performed by using HT-29 cells cultured under two separate conditions. Group A cells were grown in standard culture media before assay treatment with AZT, 5-FU, or both. Group B cells were grown for 1 week in the presence of 100 μM of AZT before assay treatment with AZT, 5-FU, or both. As expected, in group A, we found that treatment with either AZT or 5-FU resulted in significantly reduced cell viability when compared with untreated controls (Fig. 4; Table 1; $P < .001$ for both treatments). Likewise, treating HT-29 cells with combined AZT and 5-FU significantly reduced cell viability ($P = .009$). Table 1 shows that all main effects and two-level interaction terms were statistically significant. Because statistical interaction is a valid approach for assessing multidrug synergistic and antagonistic interactions, we conclude that the combined effects of treatment/AZT, treatment/5-FU, and AZT/5-FU differ significantly from the pure additive effect that each factor has on its own.²⁰

Group B cells also demonstrated increased cytotoxicity when treated with either AZT, 5-FU, or the combination (Fig. 4; Table 1). However, for each treatment arm, the cytotoxicity was less than that seen in group A. This may indicate an acquired resistance arising from preexposure to AZT.

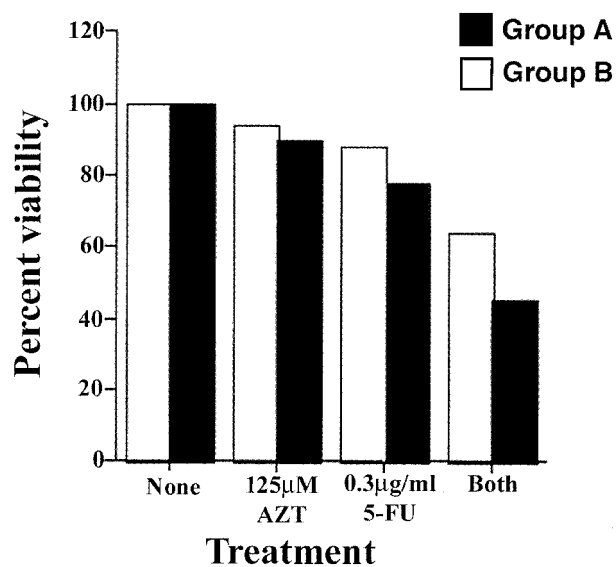


FIG. 4. Colorimetric microtiter cytotoxicity assay demonstrates increased sensitivity of HT-29 cells to .3 $\mu\text{g}/\text{mL}$ of 5-fluorouracil (5-FU) when treated in combination with 125 μM of azidothymidine (AZT). Group A cells were grown in standard media, and Group B cells were grown in the presence of AZT for 1 week before treatment.

DISCUSSION

Telomerase activation is thought to be a required step for tumor formation, stabilizing the telomeric array and conferring an unlimited proliferative potential on cells. However, recently it has been reported that telomerase has growth-promoting activities that are separable from its telomere replication function.^{7,8,22} In addition, reports suggest that inhibition of telomerase increases the sensitivity of cell lines to chemotherapeutic agents.^{23,24} However, the effect of telomerase inhibition on chemosensitivity seems to vary among different cell lines and agents, making it difficult to extrapolate between cell/tumor types and agents.^{24,25} Here we demonstrate that AZT inhibits telomerase in the HT-29 colorectal cancer cell line on the basis of the ability of its active metabolite AZT-TP to inhibit activity in vitro and telomere shortening accompanying long-term growth of HT-29 cells in the presence of AZT.

Our data demonstrate that AZT caused slower growth of HT-29 cells. This was primarily due to acute effects seen within the first month of culture. HT-29 cells seem to become acclimated to the presence of even high doses (125 μM) of AZT at later time points, resulting in a 12% comparative growth rate in the first month versus 70% in the final month. This could reflect selection of cells that are resistant to AZT within the population. Cellular acquired resistance to the presence of AZT has been described previously.²⁶ Additionally, the most significant growth delay was noted with 125 μM of AZT. Treatment with 25 μM of AZT resulted in an overall 25% reduction in population doubling. AZT is now prescribed as an anti-human immunodeficiency virus drug at 500 to 1500 mg/day, which corresponds to 20 to 60 μM of AZT.²⁷

The mean telomere length of HT-29 cells was reduced from 8.7 to 6.9 kb when cells were cultured in the presence of AZT. If one assumes an equivalent loss rate over the 3-month course of AZT treatment, this would correspond to approximately 60 bp of telomere attrition/population doubling. This rate of telomere loss is within that observed in somatic cells that do not contain telomerase activity and suggests that the telomerase inhibition achieved with AZT in these experiments was close to maximal. However, there was no detectable telomere shortening within the first month of culture, suggesting that the adverse growth effects of AZT on HT-29 cells are not a direct consequence of telomere shortening. This may relate to inhibition of telomere length-independent telomerase activity or other potential mechanisms of AZT activity, including inhibition of thymidine synthetase or DNA polymerase γ .^{28,29} Further studies now in progress with targeted telomerase inhibitors should provide insight into this question.

Finally, we found that AZT increased the sensitivity of HT-29 cells to the cytotoxic effects of 5-FU. Again, the cytotoxicity observed is unlikely to be a consequence of telomere shortening, because insufficient time elapsed during the cytotoxicity experiments for a critical amount of telomere attrition to occur. Early studies with AZT in combination with 5-FU were also promising for their synergistic effect. This effect was thought to be related to thymidine synthetase inhibition.^{13,14} However, the toxicity profile related to the combined treatment resulted in termination after phase II trials. What, then, is the clinical utility of the results if potentially toxic doses are required and the cells potentially acclimate to the AZT? This represents proof of principle, demonstrating that telomerase activity can be inhibited in this colorectal cancer cell line and that inhibition does result in growth delay, telomere loss consistent with somatic cell loss, and synergistic cytotoxicity when combined with conventional cytotoxic agents. Because of the significant toxicity associated with high doses of AZT and the broad inhibitory effects, we do not recommend it as a clinical tumoricidal agent.²⁷

The next step is to develop a targeted telomerase inhibitor. Targeted inhibitors should have a more favorable side-effect profile than the broad reverse transcriptase inhibitor AZT while maintaining the synergistic effects of telomerase inhibition. Present studies with targeted inhibitors indicate no inhibition of DNA and RNA polymerases at concentrations greatly exceeding the median inhibitory concentration for telomerase.³⁰ Studies addressing these questions are ongoing with similar inhibitors at Fox Chase Cancer Center.

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The acknowledgments are available online at www.annalsurgicaloncology.org.

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