Inhibition of Cell Growth and Telomerase Activity of Breast Cancer Cells in Vitro by 3'-Azido-3'-deoxythymidine

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ABSTRACT

The effect of zidovudine (3'-azido-3'-deoxythymidine; AZT) was investigated in four breast cancer cell lines, a T4 cell leukemia, and a normal breast cell line in vitro. AZT inhibited the growth of all tumoral cell lines, but it did so in a wide range of concentrations. The growth of a normal breast cell line was also inhibited, although it required a much higher concentration. Furthermore, AZT inhibited colony formation in soft agar and telomerase activity. These results indicated that AZT can be potentially used, alone or in combination, as an anti-breast cancer agent.

INTRODUCTION

Zidovudine, or AZT, is currently used in the treatment of AIDS. AZT is phosphorylated intracellularly to AZT-TP by thymidine kinase and is finally incorporated into viral DNA, blocking chain elongation by RT. AZT-TP can also be incorporated into eukaryotic DNA in place of thymidine, although it has low affinity for DNA polymerases α and β (1). Although it was developed as an antineoplastic agent, AZT was not considered as a potential antitumor agent because of its relatively high cytotoxicity in animals when administered in drinking water (2). However, it was found to have very low toxicity when administered as a bolus. Recently, AZT has been used in Phase I and II clinical trials alone or in combination with other drugs in gastrointestinal cancers. Some tumor regression in colorectal cancers has been reported (3–8).

Telomerase, a RNA-dependent DNA polymerase, maintains and elongates telomeres in human germ line and stem cells. Reduction of telomeres occurs during differentiation and normal aging. Telomerase activity has been strongly associated with immortalization of cells and cancer and is considered as a target for cancer treatment (9). Recently, the effect of AZT on Chinese hamster ovary cells that display telomerase activity was investigated. AZT was preferentially incorporated into telomeric DNA and Z-DNA-containing regions (2). AZT, alone or in combination with other antimetabolites, also inhibited the growth of human bladder cancer and colon cancer cell lines (10). Furthermore, AZT was shown to cause progressive telomere shortening in immortalized B and T human lymphocytic cell lines (11). Taken together, these results have stimulated further research on the effect of AZT on cancer cells. We have, therefore, investigated the effect of AZT on breast cancer cells that possess telomerase activity. The results indicated that AZT inhibits breast cancer cell growth, anchorage-independent growth, and telomerase activity.

MATERIALS AND METHODS

Cells. Four breast cancer lines [MCF-7 (12), T47D. (13) and two breast cancer cell lines, developed in our laboratory and designated ED and EK-2], a T4 lymphocytic leukemia cell line (Jurkat cells), and a normal breast cell line [MCF-10F (14)] were used.

MCF-7 and T47D cells (from American Type Culture Collection) were grown as monolayers in RPMI 1640 supplemented with 100 mg/ml streptomycin, 100 units/ml penicillin, 10% FCS, and 0.2 IU/ml of insulin. Jurkat cells (a gift from Dr. B. Hoyos) were grown in the same medium without insulin. MCF-10F cells (a gift from Dr. Mira y Lopez, Mount Sinai School of Medicine) were grown in 1:1 DMEM:Ham’s F-12 Nutrients medium supplemented with penicillin and streptomycin as above, 0.1 mg/ml choloroenterotoxin, 1 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2 ng/ml epidermal growth factor, 40 mM CaCl2, and 5% horse serum. ED and EK-2 cells were derived from heparinized pleural effusions from two patients with breast cancer following the procedure of Engel et al. (15) and maintained in MEGM medium (Clonetics) supplemented with streptomycin and penicillin, as described above, and 10% FCS. ED-R and ED-F are clones of ED. All cells were incubated at 37°C in the presence of 5% CO2. Doubling time was calculated as previously described (16).

Determination of IC50. Cells were grown in the presence of AZT concentrations ranging from 100 μM to 2 mM and counted at different times after exposure. The number of cells alive was determined by the trypan blue exclusion method.

Telomerase Assays. Cell extracts were prepared according to the conditions described by Kim and co-workers (17). The oligonucleotides TS (5’-AATCCGTCGAGCAGAGTT-3’) and CX (5’-CCCCGCTACCTAACCTACCTAA-3’) were purified by high-performance liquid chromatography and dissolved at 50 ng/ml. CX primer, 0–1 μl, was lyophilized in the reaction tube, and 15 μl of wax were added on top. The TS primer was 5’ end labeled with [γ-32P]ATP by the T4 polynucleotide kinase, purified through a Sephadex G-25 column, and diluted with non-

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3 The abbreviations used are: AZT, 3’-azido-3’-deoxythymidine; TP, triphosphate; MP, monophosphate; RT, reverse transcriptase.
Breast Cancer Cell Inhibition by AZT

TRAPeze telomerase detection kit (Oncor, Inc.) was also used. 15% cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. One The TRAP reaction mixture (17) was added, and the reaction Gels were exposed overnight at 4°C. In some experiments the radioactive TS to a specific activity of 5 × 10^6 cpm/μg (18). The TRAP reaction mixture (17) was added, and the reaction was allowed to proceed for 30 min at 23°C, followed by 27 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. One aliquot of the reaction (40 μl) was analyzed by electrophoresis on 15% polyacrylamide nondenaturing gel, with 0.045 M Tris-borate buffer with 0.001 M EDTA and run at 200 V for 12 h. Gels were exposed overnight at 4°C. In some experiments the TRAPeze telomerase detection kit (Oncor, Inc.) was also used.

Cell Growth in Soft Agar. Cells were resuspended in 0.45% agar (Difco Laboratories) in culture medium and seeded in 35 × 10-mm dishes containing a base of 0.6% agar in culture medium at the concentrations of 10^5, 10^4, and 10^3 cells/dish. The appropriate AZT IC_{50} for each cell line was incorporated in the medium except for controls. Cells were incubated for 3 weeks at 37°C and 5% CO₂, and then colonies were counted.

RESULTS

Evaluation of Cytotoxicity. The effect of AZT on cell growth was tested using different concentrations. AZT inhibited the growth of all of the cells tested. However, the IC_{50} varied from 0.25 to 1.35 mM as shown in Table 1 for the breast cancer cell lines, whereas the IC_{50} for the immortalized normal breast cell line was 1.75 mM. To evaluate whether rate of growth was related to the variation in IC_{50}, the doubling time of the cell lines was also determined. When the two parameters were compared by correlation analysis, no correlation between the rate of growth and IC_{50} was found (Table 1).

Cell Growth in Soft Agar. The effect of AZT on anchorage-independent growth was evaluated by measuring colony formation in agar. As shown in Table 2, at the IC_{50}, AZT inhibited colony formation of four breast cell lines to different degrees.

Table 1 Effect of AZT on human cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (hr)</th>
<th>AZT (IC_{50}, mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED-F_{p&lt;0.05}</td>
<td>40.0</td>
<td>0.25</td>
</tr>
<tr>
<td>ED-B_{p&lt;0.01}</td>
<td>17.5</td>
<td>0.50</td>
</tr>
<tr>
<td>T47D</td>
<td>29.0</td>
<td>0.75</td>
</tr>
<tr>
<td>ED_{p=0.25}</td>
<td>19.8</td>
<td>1.00</td>
</tr>
<tr>
<td>EK-2_{p&lt;0.05}</td>
<td>77.0</td>
<td>1.00</td>
</tr>
<tr>
<td>MCF-7</td>
<td>26.7</td>
<td>1.35</td>
</tr>
<tr>
<td>Jurkat</td>
<td>12.0</td>
<td>1.50</td>
</tr>
<tr>
<td>MCF-10F</td>
<td>67.3</td>
<td>1.75</td>
</tr>
</tbody>
</table>

“ p. passage number.

Table 2 Effect of AZT on cloning efficiency of human breast cancer cells

<table>
<thead>
<tr>
<th>Cloning efficiency (%)</th>
<th>Cell line</th>
<th>Control</th>
<th>AZT</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.70</td>
<td>MCF-7</td>
<td>1.06</td>
<td>77.5</td>
<td></td>
</tr>
<tr>
<td>1.60</td>
<td>T47D</td>
<td>0.27</td>
<td>83.0</td>
<td></td>
</tr>
<tr>
<td>1.12</td>
<td>ED-R</td>
<td>0.001</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>EK-2</td>
<td>0.001</td>
<td>99.9</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>MCF-10F</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Effect of AZT on Telomerase Activity. Since the presence of an endogenous inhibitor of telomerase activity has been reported, its presence was evaluated in MCF-7 cells using different concentrations of cell extracts (17). The results of telomerase activity in MCF-7 cells using the “TRAP reaction” are shown in Fig. 1. The best activity was detected using either 3 or 0.3 μg of protein of cell extract (Fig. 1, Lanes 4 and 5). At higher concentrations of cell extract (Fig. 1, Lanes 1–3), the activity was less, suggesting the presence of an inhibitor. The effect of AZT on telomerase activity was then assessed using 0.3 μg of protein of cell extract. The activity was partially decreased at 1 mM and completely inhibited at 2 mM (Fig. 2). Similar results were obtained with the other breast cancer cell lines. As previously reported, telomerase activity in MCF-10F cells was almost undetectable (Fig. 3), and therefore, AZT did not have any effect on it.

DISCUSSION

The results reported here indicate that AZT is able to inhibit the growth of breast cells in vitro. The IC_{50} varies from 0.25 to 1.35 mM for the cancer cell lines, whereas it is higher for the immortalized normal breast epithelial cell line MCF-10. No correlation of IC_{50} with doubling time is seen. Therefore, the IC_{50} is not related to the rate of cell growth. Variations in the IC_{50} may reflect differences in absorption, excretion, and/or phosphorylation of the compound between the cell lines.

At the respective IC_{50} for each cell line, AZT is able to inhibit colony formation in soft agar to different degrees (Table 2). Although the number of colonies decreased, the number of...
single cells did not decrease below the total cell and colony number of the untreated cultures, indicating that the drug was growth inhibitory but not cytocidal for breast cancer cells.

Finally, AZT inhibited telomerase activity of all of the breast cancer cells that were tested. As a result of telomerase inhibition, we have observed that MCF-7 cells cultured for five passages with AZT at the IC_{50} showed a marked reduction in the amount of telomeres.4

It was previously reported that when cell extract concentrations were high (17), inhibitor(s) of the reaction could be detected in the cell extract. Our results indicate that in the breast cancer cell lines tested, an endogenous inhibitor of the telomerase reaction was also present, consistent with the results of others (17). All experiments were conducted at the cell extract concentrations below the recognizable endogenous inhibitory effect.

The mechanism by which AZT inhibits growth of breast cancer cells in culture is complex. AZT is known to inhibit viral RT and integrase activities (19), as well as cellular enzymatic activities such as DNA polymerase γ (1, 20), thymidine kinase (21, 22), and telomerase (11) and has also been shown to be preferentially incorporated into telomeric DNA (9). In all cases, AZT-TP is the active molecule.

Breast cancer cell lines T47D, MCF-7, and ED displayed RT activity in the culture medium, whereas MCF-10F did not (Ref. 23).4 Furthermore, Patience et al. (24) reported that there is expression of endogenous retroviral sequences in T47D cells, whereas Wang et al. (25) showed the presence of mouse mammary tumor virus-env like gene sequences in 38% of human breast cancers and in some of the breast cancer cell lines, including ED, EK-2, and MCF-7. This would imply the involvement of a retroviral activity as a target for AZT. On the other hand, all of the breast cancer lines tested by us and the human breast tumors studied by Hiyama et al. (26) and Bednarek et al. (27) exhibited telomerase activity. Therefore, both enzymatic activities can be affected by AZT. But the effect of AZT on other enzymes, such as DNA polymerase γ and thymidine kinase, which are present in all cell types, cannot be ruled out. However, AZT has low affinity for these enzymes. In this context, it is remarkable that the growth of MCF-10F cells, which lack both RT and telomerase activities, is also inhibited by AZT, although at higher drug concentrations.

The active form of AZT is AZT-TP, which competes with the endogenous TTP. Conversion from the MP form (AZT-MP) to AZT-TP is mediated by thymidylate kinase and is very inefficient in most cells. It has been shown recently that the interaction between AZT-MP and the enzyme is structurally
unfavorable, thereby allowing accumulation of AZT-MP, which eventually inhibits the enzyme (28). This could explain the cytotoxicity of AZT. Designing a more favorable form of the analogue to interact efficiently with thymidylate kinase could improve AZT effects.

The results described here support the possibility of using AZT, alone or in combination with other agents that enhance AZT effects as a chemotherapeutic agent for breast cancer. They also support the recently proposed notion that telomerase activity could be useful as a novel marker for chemotherapy efficiency (29).

**Note Added in Proof**

After this paper was submitted for publication, a report describing the effects of AZT in one human breast cancer cell line appeared (30).

**REFERENCES**


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