Induction of Apoptosis by 5-Azacytidine: Drug Concentration-dependent Differences in Cell Cycle Specificity

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ABSTRACT

There are conflicting data in the literature as to whether cytotoxicity of the cytidine antimetabolite 5′-azacytidine (AZC) is a consequence of its incorporation into RNA, DNA, or both. Because apoptosis appears to be the predominant mode of tumor cell death after treatment with most anticancer drugs, and in the case of some drugs, the proclivity of the cell to undergo apoptosis varies depending on the cell cycle position, this study was aimed toward elucidating whether induction of apoptosis by AZC is cell cycle phase specific. Human promyelocytic leukemia HL-60 cells were treated with varying concentrations of AZC, and flow cytometric methods that identify apoptotic cells and provide information about the cell cycle distribution of the apoptotic and nonapoptotic cell populations were used. At 2–6 μM concentrations of AZC, the cells in the G1 phase preferentially underwent apoptosis, whereas the cells in G2-M were particularly resistant. Although incorporation of bromouridine into RNA was suppressed at that low AZC concentration, the rate of 5′-bromo-2-deoxyuridine incorporation into DNA was not significantly affected. At an AZC concentration of 8–40 μM, no cell cycle phase specificity in induction of apoptosis was apparent, but both the rate of 5′-bromo-2-deoxyuridine incorporation into DNA and bromouridine into RNA were reduced in proportion to drug concentration. The data suggest that the mechanism of cell killing by AZC may be different, depending on its concentration. Namely, whereas incorporation of AZC into RNA may play a predominant role in the induction of cytotoxicity of G1 cells at low drug concentrations, the perturbation of both RNA and DNA metabolism may be responsible for triggering cell death in the G1 and S phases, as is seen at higher concentrations of this antimetabolite.

INTRODUCTION

AZC is known to be an antileukemic agent and a useful tool to study the role of DNA methylation in cell differentiation and gene activation (1, 2). However, primary interest in AZC as a promising anticancer drug for solid tumors has diminished since clinical trials began nearly 20 years ago (3). The role of AZC in induction, intensification, maintenance, and relapse regimens targeting leukemias has not been defined clearly as yet (4), although significant activity has been demonstrated in some leukemia types (4–6). One reason for the disappointing results is insufficient information concerning the pharmacokinetic activity of AZC (clearance, elimination half-time, etc.), as noted by Glover et al. (4). There is also ambiguity regarding the mechanism of action of this antimetabolite in terms of its interference with RNA and DNA metabolism, as well as its cell cycle specificity. Such data are critical to the development of treatment protocols, especially those involving multidrug combinations.

AZC exerts its biological effects by different mechanisms. The substitution of the carbon at the 5 position in the pyrimidine ring with a nitrogen atom renders the ring unstable. Incorporation of the unstable base of AZC into RNA disrupts the synthesis and processing of various species of RNA and leads to inhibition of transcription (7–11). Incorporation of AZC into DNA affects the stability of the DNA double helix and its supercoiling, alters chromatin structure, and causes chromatid aberrations (12–14). Because the nitrogen atom in AZC cannot be methylated, DNA in which this antimetabolite is substituted for cytidine remains hypomethylated, which may be responsible for the maintenance of some genes in the active state and induction of cell differentiation (1, 2, 6).

There is a disagreement regarding the mechanism of induction of cytotoxicity by AZC (4–8, 15, 16). Some observations indicate that S-phase cells are more sensitive to AZC (15) and that the drug has an inhibitory effect on DNA synthesis (9, 12–14, 16). This would suggest that the effect of AZC on DNA replication and/or its incorporation into DNA may be the primary cause of cell death. On the other hand, there is extensive evidence that RNA metabolism is the primary target of this drug (8, 10, 11). Indeed, severe changes in synthesis, processing, and function of all RNA species occur in the presence of AZC, and some of these changes appear to be directly linked to drug cytotoxicity (4, 8, 10, 11).

The majority of anticancer agents are capable of inducing apoptosis (17–21), and AZC is not an exception (21, 22). Recently, flow cytometric methods that allow one to detect the cell cycle position of the cells undergoing apoptosis have been developed (21, 23–26). Preliminary observations suggested that AZC may preferentially trigger apoptosis of G1 cells (21). The aim of this study was to further investigate AZC cytotoxicity in relation to the cell cycle. Apoptosis of human promyelocytic HL-60 cells induced by different AZC concentrations was analyzed with respect to the cell cycle position at the time of exposure to the drug, and the cytotoxicity of AZC was correlated with its effect on DNA replication. The data indicate that the cell cycle-specific effects of AZC vary depending on drug concentration.

MATERIALS AND METHODS

Cell Culture. The HL-60 cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. The cells were split every third day and diluted to a concentration of 1–2 × 10⁶ cells/ml 1 day before each experiment. All experiments were performed on exponentially growing cell cultures. Cell densities did not exceed 4 × 10⁶ cells/ml in all experiments.

Drug Treatment. A stock solution of AZC (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving the drug at a concentration of 40 mM in distilled water. Final concentrations were obtained by diluting the stock solution directly into the tissue culture medium. Cells were incubated with varying concentrations of AZC for 1–24 h. In some experiments, cells were resuspended in conditioned, drug-free medium and cultured for up to 24 h following a 4-h (pulse) exposure to AZC. In all experiments, only the freshly made stock solutions of AZC, prepared up to 2 h before addition into the cultures, were used.

Cell Fixation. Two different methods of cell fixation were used, depending on which cytofluorometric assay would be used subsequently to identify apoptotic cells. In one set of experiments, cells were fixed in 70% ice-cold...
ethanol at −20°C for at least 14 h. After centrifugation, the cell pellets were resuspended in 40 μl of phosphate-citrate buffer consisting of 96 parts 0.2 M Na₂HPO₄ and 4 parts 0.1 M citric acid (pH 7.4) and shaken for 15–30 min. 

Incubation in this buffer results in extraction of the degraded, low molecular weight DNA from the ethanol-prefixed apoptotic cells (26). After centrifugation, the cell pellets were resuspended and stained for DNA content, and the supernatant was used for DNA gel electrophoresis as described previously (26). After such fixation and extraction, analysis of cellular DNA content revealed apoptotic cells as the cells represented by a "sub-G₁ peak" on DNA content frequency histograms (24, 26). Another method of cell fixation involved suspension of cells in 1% ethanol-free formaldehyde in PBS (pH 7.4) for 15 min on ice. After being washed in PBS, the cells were then resuspended in 70% ice-cold ethanol and stored at −20°C for 1–3 days. Unlike ethanol fixation, formaldehyde fixation prevents low molecular weight DNA fragments generated during apoptosis from being extracted during subsequent washings, thereby making it possible to measure the total DNA content of both nonapoptotic and apoptotic cells with the use of an assay that combines DNA staining and in situ strand break labeling (21, 23–25) as described below.

Detection of Apoptosis-Induced DNA Strand Breaks. In situ DNA strand break labeling to identify apoptotic cells was accomplished with the use of a commercial kit (Apoptag; kindly provided by ONCOR, Inc., Gaithersburg, MD) according to the protocol included with the kit by the vendor and as described previously (23, 25). In brief, after fixation in 1% formaldehyde, the cells were twice rinsed with PBS and suspended in equilibration buffer. The cells were then centrifuged; resuspended in reaction buffer containing terminal deoxynucleotidyl transferase, d-dUTP, and necessary ions; incubated for 1 h at 37°C; rinsed; and resuspended in a solution containing fluoresceinated anti-digoxigenin antibodies [F(ab)₂ fragments], Triton X-100, and the blocking reagent nonfat dry milk for an additional 30-min incubation.

Detection of BrdUrd Incorporation. To elucidate the effect of AZC on DNA synthesis, flow cytometric measurement of BrdUrd incorporation into cells was performed in some experiments according to our recently published technique, in which we use UV light to photolyse DNA at the site of BrdUrd incorporation (strand breaks induced by photolyse technique; Refs. 27 and 28). After AZC exposure for 1 h, cells were washed and incubated with 30 μM BrdUrd (Sigma) for 1 h. Cells were then washed, resuspended in cold PBS, and exposed to UV light by being placed in Petri dishes on the glass surface of a Fotodyne UV 300 analytic DNA transilluminator containing four 15-W bulbs emitting at a wavelength of 300 nm (Fotodyne, Inc., New Berlin, WI) for 5 min. The average intensity of UV light at the surface on which the cells were exposed, measured with the use of a UYV-25 sensor (UYV, Inc., Upland, CA), was 4.5 mW/cm². The cells were then fixed with 0.2% formaldehyde for 15 min, rinsed in PBS, suspended in 70% ethanol, and stored at −20°C overnight. The exposure of cells to UV induces DNA strand breaks preferentially at the sites of BrdUrd incorporation (29, 30). The UV-induced BrdUrd-associated breaks were then labeled with dUTP as described above.

BrdUrd Incorporation into RNA. Incorporation of BrUrd into RNA was detected according to the procedure of Jensen et al. (31) with minor modifications (32). In brief, after incubation with varying concentrations of AZC for 1 h, cells were incubated in the presence of 1 mM of BrUrd for 60 min at 37°C, then centrifuged, washed once with PBS, and fixed in 1% methanol-free formaldehyde for 15 min on ice. After fixation, the cells were washed in PBS and resuspended in 80% ethanol at −20°C. After removal of the ethanol, the cells were washed in PBS and incubated with an anti-BrdUrd antibody, which cross-reacts with BrUrd (generous gift of Dr. W. Gohde, PARTEC GmbH, Munster, Germany; dilution, 1:100) overnight at 4°C. As a control, a sample of cells was preincubated with 10 Kunitz units/ml of RNase A for 15 min at room temperature before incubation with anti-BrdUrd antibody. After cells were washed with 1% BSA in PBS, 100 μl of fluorescein-conjugated goat anti-mouse F(ab)₂ fragments (DAKO, Carpinteria, CA; dilution, 1:30) was added for 30 min at room temperature in the dark. Finally, 0.5 ml of a solution of PBS containing 10 μg/ml PI was added for 15 min.

DNA Staining and Flow Cytometry. Those apoptotic cells having DNA strand breaks that had been labeled with d-UdUTP and stained with fluoresceinated anti-d-UdUTP were treated with RNase A (Sigma) and stained with PI according to the protocol included in the ONCOR kit. Green (d-UdUTP) and red (DNA) fluorescence of individual cells was measured on a FACScan flow cytometer (Becton Dickinson). Detection of DNA-synthesizing cells was also measured on an FACScan flow cytometer; the green (FITC) fluorescence was proportional to d-dUTP incorporated into DNA breaks caused by UV-induced photolysis at the sites of BrdUrd incorporation, and red (PI) fluorescence represented DNA content. RNA synthesis was quantified based on a FITC-labeled (green fluorescence) mAb to BrdUrd, which cross-reacts with BrUrd, whereas DNA was stained with PI (32), again with the use of a FACScan flow cytometer.

The cell pellets removed from the phosphate-citrate buffer described above were resuspended in 1 ml of PBS. The cells were stained with 1 μg/ml diaminido-2-phenylindole as described previously (26, 33). The fluorescence of DNA-bound diaminido-2-phenylindole was measured with an ICP 22 flow cytometer (Ortho Diagnostics, Westwood, MA). The data were stored and analyzed with Acybyte software (Phoenix Flow Systems, San Diego, CA). The Multicycle program (Phoenix) was used for the analysis of cell cycle distributions, including the percentage of apoptotic cells that are observed as a "sub-G₁ peak" population.

DNA Gel Electrophoresis. The supernatant from AZC-treated and control cells, obtained by washing fixed cells in phosphate-citrate buffer (26), was concentrated by vacuum in a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, NY) for 15 min. A 3-μl aliquot of 0.25% Nonidet P-40 in distilled water was then added, followed by 3 μl of RNase A solution (1 mg/ml in distilled water). After 30-min incubation at 37°C, 3 μl of proteinase K solution (1 mg/ml; Boehringer-Mannheim, Indianapolis, IN) were added, and the extract was incubated for an additional 30 min at 37°C. Next, 12 μl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol FF, and 30% glycerol) were added, and the entire contents of the tube were transferred to the gel. Horizontal 0.8% agarose gel electrophoresis was carried out at 2 V/cm for 16 h. The DNA was visualized under UV light after staining with 5 μg/ml of ethidium bromide (Polysciences, Inc., Warrington, PA).

RESULTS

Several methods were used to investigate the mode and cell cycle specificity of death of HL-60 cells in cultures treated with different concentrations of AZC. Gel electrophoresis of DNA extracted from the AZC-treated cells revealed a distinct, discontinuous "ladder pattern," indicating preferential cleavage of DNA at the internucleosomal sections (Fig. 1), a feature characteristic of DNA degradation during apoptosis (34, 35). Such discrete cleavage of DNA, generating DNA fragments of a size of mononucleosomes and oligonucleosomes, was seen as early as 6 h after exposure to 6 μM AZC, and the bands on gels from cultures treated with 10 μM AZC were apparent after only 4 h of treatment (Fig. 1).

Apoptotic cells were also detected and quantified by flow cytometry with the use of two methods for their identification. The first method is based on extraction of the degraded DNA with high molarity phosphate-citrate buffer: after cell fixation in ethanol, the degraded DNA of low molecular weight is not stabilized within the cells and can be eluted with this buffer (26). DNA content analysis of so-treated cells reveals apoptotic cells as having a fractional DNA content, which, on DNA content frequency histograms, are represented by a "sub-G₁ peak" (24, 26). With the use of this methodology, no significant apoptosis was detected during the initial 3 h of cell incubation with up to 16 μM AZC (Fig. 2A). However, by 4 h, clear sub-G₁ peaks were present on DNA content frequency histograms of cells from cultures exposed to 6 μM and higher drug concentrations (Fig. 2). Accumulation of cells in early S phase among the nonapoptotic cell population (Fig. 2A, panels b and c, arrowheads) was also apparent at 10 μM AZC. With prolonged (12-h) exposure to AZC, the proportion of cells in the sub-G₁ peak at each drug concentration was increased (Fig. 2).

The cell cycle distribution of the nonapoptotic cells, i.e., the cells that retained normal DNA stainability, differed from that of the control (untreated) cells with increasing drug concentration and length

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INDUCTION OF APOPTOSIS BY AZACYCLOTHIDINE

Fig. 1. Electrophoretic pattern of DNA extracted from HL-60 cells treated with different concentrations of AZC for 4 (Lanes a) or 8 (Lanes b) h. The cells treated with 0, 2, 6, 10, 16, or 160 μM AZC for 4 or 8 h were fixed in ethanol, and the low molecular weight DNA extracted with phosphate-citrate buffer was subjected to electrophoresis directly as described previously (26). Note the bands of DNA observed at regular intervals in lanes beginning with extracts from cells treated with 6 μM AZC for 8 h. Each band represent multiples of DNA of 18(−2) Kbp in length.

of exposure. Namely, a 12-h exposure to 6 and 10 μM AZC resulted in a peak in the DNA distribution in mid to late S phase (Fig. 2A, panels e and f, arrowheads), as well as in an increase in the proportion of cells in G2-M. In the presence of 16 μM AZC, only 21% of cells retained normal DNA stainability after 12 h, and there was a predominance of cells in G1 (64%) and a diminished proportion of cells in S phase (23%) in treated cultures compared with untreated cultures (Fig. 2A, panel g).

The ability of AZC to induce apoptosis was both concentration and time dependent (Fig. 2B). The maximal increase in the frequency of apoptotic cells was observed between the third and fourth h of cell incubation with AZC, and after 4 h, the rate slowed for cells treated with 10–160 μM of AZC and reached a plateau after 8 h. However, in the case of cells treated with 6 μM AZC, the percentage of apoptotic cells increased continuously (Fig. 2B). Longer exposure times (>24 h) led to an additional increase in the percentage of apoptotic cells (data not shown).

The flow cytometric assay that provides information regarding cell cycle phase sensitivity to apoptosis is based on bivariate analysis of the cells with respect to the presence of DNA strand breaks versus their DNA content (Fig. 3). In this assay, the cells are fixed with the cross-linking agent formaldehyde, which, unlike fixation with ethanol, prevents extraction of DNA that is initially cleaved by the endonuclease in apoptotic cells (25). Therefore, unless DNA degradation is already very extensive or DNA loss occurred by previous shedding of apoptotic bodies (which appears to be a rather late event of apoptosis), DNA content of apoptotic cells provides information about their cell cycle position (21, 23-25). Analysis of DNA content of the cell population discriminated by the presence of DNA strand breaks (see
cells is very advanced, making it possible to estimate cell cycle position based on DNA content (23, 25). The populations of apoptotic (Ap) and nonapoptotic (N) cells were gated to cross-linking with ethanol (Figs. 1 and 2), cross-linking of DNA with formaldehyde prevents extraction of low molecular weight DNA, which, unless DNA degradation in apoptotic cells is very advanced, makes it possible to estimate cell cycle position based on DNA content (23, 25). The populations of apoptotic (Ap) and nonapoptotic (N) cells were gated to present their DNA content frequency histograms, respectively, as shown. Note that at 6 μM AZC, there is a predominance of G₁ cells in the apoptotic cell population in contrast with 16 μM AZC. The data in this figure and in Fig. 4 are represented by the 50% log-density contours of 10,000 cells measured per sample, where the innermost contour represents 50% of the peak height, the next contour represents 25% of the peak height, then 12.5, 6.25, 3.12, and 1% of the peak height.

As is evident from the raw data (Fig. 3), a 4-h exposure to 6 μM AZC cultures exposed to 2-6 μM AZC was preferential to G₁-phase cells. As is evident from the raw data (Fig. 3), a 4-h exposure to 6 μM AZC led to the appearance of a population of apoptotic cells consisting predominantly of cells with a G₁ DNA content. Analysis of the cell cycle distribution of the gated apoptotic and nonapoptotic populations, respectively, indicated that, whereas there were 40% G₁-phase and 40% S-phase cells in the control, untreated cultures, the proportion of G₁ cells was increased to 74% in the apoptotic population and was decreased to 33% among the nonapoptotic cells in the cultures after treatment with 6 μM AZC for 4 h (Table 1). In contrast, the proportion of S-phase cells was decreased from 40% to 23% among the apoptotic cells and was proportionally increased in the nonapoptotic cell population in the same cultures. At 2-4 μM concentrations of AZC, there was no evidence of apoptosis during the initial 4-h incubation with the drug. Apoptosis was apparent, however, when the cells were exposed for 4 h to these low concentrations of AZC but analyzed after an additional 4 h growth in the drug-free medium. In this case, as with 4-h exposure to 6 μM AZC, the proportion of G₁ cells was increased among apoptotic cells and decreased among non-apoptotic cells.

In contrast with the effects seen at low concentrations of AZC, the increase in the proportion of G₁ cells among the apoptotic cell population was less apparent when the cells were treated with 8-16 μM AZC (Fig. 3; Table 1). In particular, after exposure to 16 μM AZC, the cell cycle distributions of both apoptotic and nonapoptotic cell populations resembled the distributions of the untreated cells, except that there were always fewer G₂-M cells in the apoptotic cell population, regardless of the AZC concentration.

The rate of DNA replication as determined by incorporation of BrdUrd was decreased in the presence of AZC (Fig. 4; Table 2). The decrease, however, was apparent at AZC concentrations ≥6 μM. In contrast, the rate of RNA synthesis, determined as incorporation of BrdUrd into RNA under identical conditions of cell treatment with AZC, was already markedly diminished at a drug concentration of 4 μM, regardless of the phase of the cell cycle (Fig. 4; Table 2).

### Table 1 Cell cycle distribution of apoptotic and nonapoptotic cells after treatment with AZC

<table>
<thead>
<tr>
<th>AZC (μM)</th>
<th>Time of treatment (h)</th>
<th>Cell cycle distribution (%)</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G₁</td>
<td>S</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>4*</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>4*</td>
<td>35</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>33</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>29</td>
<td>47</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>45</td>
<td>40</td>
</tr>
</tbody>
</table>

*Apoptotic cells were identified after the incorporation of d-dUTP into DNA strand breaks by exogenous terminal deoxynucleotidyl transferase followed by staining of dUTP with fluorescein-antidigoxygenin (green) and DNA with PI (red) as in Fig. 3.

*4-h treatment with AZC followed by 4-h incubation in medium without AZC.

### DISCUSSION

Apoptosis is the mode of death of HL-60 cells exposed to pharmacological concentrations of AZC. This was evident because AZC triggered extensive endonucleolysis that was preferential to internucleosomal DNA sections. In addition, DNA degradation was manifested by the appearance of cells with fractional DNA content after extraction of low molecular weight DNA fragments and by the presence of numerous DNA strand breaks. The 3'-OH ends of the latter were labeled with fluoresceinated deoxynucleotides in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (23, 25). The morphology of the affected cells was typical of apoptosis (data not shown). Therefore, as do other antitumor drugs of different classes and mechanisms of action (21), AZC induces apoptosis of "primed" (36) hematopoietic tumor cells.

As mentioned above, there is a longstanding dispute regarding the mechanism of cytotoxicity induced by AZC. These results suggest that, although both DNA and RNA metabolism may be the drug target, there is a preference for RNA at low AZC concentration (2-8 μM). Namely, there was a predominance of G₁ cells, i.e., cells that did not replicate DNA, undergoing apoptosis at concentrations of 2-8 μM of AZC (Table 1). Because apoptosis of these cells was induced rapidly (4 h), it is unlikely that AZC could affect replication of their DNA during the preceding S phase. Furthermore, incorporation of BrdUrd into RNA was more affected at low AZC concentration compared with incorporation of BrdUrd into DNA (Fig. 4; Table 2). It appears, therefore, that incorporation of AZC into various types of
exposure to 16 μM AZC led to a decrease in the incorporation (No BrdUrd illustrates the background fluorescence of cells incubated in the absence of the precursor). In contrast, this mAb (31, 32). As can be noted from the left panels, BrdUrd incorporation was unaffected compared with that in untreated control (CTL) cells after exposure to 4 μM AZC, whereas exposure to 16 μM AZC led to a decrease in the incorporation (No BrdUrd illustrates the background fluorescence of cells incubated in the absence of the precursor). In contrast, incorporation of BrdUrd into RNA was suppressed at higher AZC concentrations (Table 1). This observation, together with the evidence that incorporation of BrdUrd into DNA was suppressed at higher AZC concentrations and of G1-phase and S-phase cells to higher drug concentrations of AZC and of G1-phase and S-phase cells to higher drug concentrations may bear implications in the clinic. One would expect, for example, that low doses of AZC (preferentially targeting G1 cells) may be more effective in combination with S-phase-specific drugs and in the treatment of tumors with a higher proportion of G1 cells. In contrast, AZC administered at high doses may be more effective when used alone or in situations in which drugs targeting S-phase cells are used. However, one cannot exclude the possibility that low concentrations of AZC may also be toxic to S-phase cells, but that cell death may occur at later times, manifesting as so-called “mitotic” or “reproductive” cell death. The apparent resistance of G2 cells to AZC suggests that its combination with antimitotic drugs or radiation may provide a wider spectrum of cell killing across the cell cycle.

RNA, by affecting the structure and stability of these RNAs and suppressing protein synthesis (7, 8), provides a trigger for apoptosis. The rate of RNA synthesis, as estimated by BrdUrd incorporation, appears to be highest during S phase (Fig. 4; see also Refs. 31 and 32). If the rate of RNA synthesis is considered to be a single factor sensitizing cells to AZC, one would expect S-phase cells to be more sensitive to this drug than are G1 cells. It is of interest, however, that the latter preferentially underwent apoptosis at low concentrations of this drug. This may be because perturbation of RNA and, subsequently, protein synthesis by AZC before the G1 restriction point (19), prevent cell entry to S phase. In such a case, induction of apoptosis in G1, especially of HL-60 cells that overexpress c-myc, resembles apoptosis that is triggered when cells are driven through the cell cycle by the overexpression of c-myc but are prevented from entering S phase, e.g., by deprivation of growth factors, as observed in other cell systems (37). The apparent resistance of the G2-M cells to AZC may be a reflection of the decreasing rate of RNA synthesis in these cells (known to occur before entrance to mitosis; see also Fig. 4), compared with that of cells in S phase.

There was an increase in the proportion of S-phase cells undergoing apoptosis in cultures treated with higher concentrations of AZC (Table 1). This observation, together with the evidence that incorporation of BrdUrd into DNA was suppressed at higher AZC concentrations, suggests that both DNA replication and RNA metabolism may be the targets of AZC under these conditions.

**Table 2. Effect of different concentrations of AZC on incorporation of BrdUrd into DNA and BrdUrd into RNA, respectively**

<table>
<thead>
<tr>
<th>Precursor incorporation</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdUrd</td>
<td>101</td>
<td>103 ± 8 (n = 4)</td>
<td>87</td>
<td>80</td>
<td>67 ± 14 (n = 4)</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>ND</td>
<td>75 ± 7 (n = 4)</td>
<td>61</td>
<td>ND</td>
<td>23 ± 13 (n = 4)</td>
</tr>
</tbody>
</table>

*As described in the legend to Fig. 4, cells were treated with varying concentrations of AZC for 1 h and then exposed to BrdUrd or BrdUrd for an additional hour. The mean fluorescence of all cells (G1, S, and G2-M) associated with either BrdUrd or BrdUrd incorporation was estimated for each AZC concentration by subtracting the background fluorescence (cells incubated in the absence of BrdUrd or treated with RNase, as shown in Fig. 4, respectively) from the total fluorescence of the cells stained with BrdUrd antibody. The data were normalized with respect to the fluorescence of the AZC untreated, control cells, which was considered 100% in each experiment. For AZC concentrations of 4 and 16 μg/ml, quadruplicate cultures were analyzed; means ± SE are presented. ND, not done.*

The strategy of antitumor drug use in the clinic, especially in combination with other drugs, is helped by knowledge of its cell cycle specificity. Although it is difficult to accurately estimate the drug concentration in the target tissue in vivo during chemotherapy, the observed preferential sensitivity of G1 cells to low concentrations of AZC and of G1-phase and S-phase cells to higher drug concentrations may bear implications in the clinic. One would expect, for example, that low doses of AZC (preferentially targeting G1 cells) may be more effective in combination with S-phase-specific drugs and in the treatment of tumors with a higher proportion of G1 cells. In contrast, AZC administered at high doses may be more effective when used alone or in situations in which drugs targeting S-phase cells are used. However, one cannot exclude the possibility that low concentrations of AZC may also be toxic to S-phase cells, but that cell death may occur at later times, manifesting as so-called “mitotic” or “reproductive” cell death. The apparent resistance of G2 cells to AZC suggests that its combination with antimitotic drugs or radiation may provide a wider spectrum of cell killing across the cell cycle.

HL-60 cells are unique in many respects, and in particular, they readily undergo apoptosis when treated with various cytotoxic agents (17). As such, they provide a useful model to study the responsiveness of cells to agents that induce by apoptosis. Many observations on HL-60 cells, especially pertaining to the mechanism of apoptosis, have been confirmed in other cell types, especially cells of the hematopoietic lineage. However, because of the exceptional propensity of HL-60 cells to respond by apoptosis, caution should be exercised in generalizing these observations to other cell systems (17).

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