Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture

L. H. Li, E. J. Olin, T. J. Fraser, and B. K. Bhuyan

Cancer Research, The Upjohn Company, Kalamazoo, Michigan 49001

SUMMARY

5-Azacytidine (1 to 5 μg/ml) significantly inhibited mitosis of L1210 cells in culture only after 2 hr of incubation. The inhibition of mitosis was correlated to the inhibition of DNA synthesis.

With asynchronous DON and L1210 cells, the cell-kill (inhibition of proliferative capacity) reached a saturation value at high doses of the drug. Any further increase in drug concentration did not result in a corresponding increase in percentage of cell-kill. This indicated that 5-azacytidine kills cells in certain phases of the cell cycle. With synchronous DON cells, it was shown that the drug was lethal predominantly in the S phase.

5-Azacytidine caused considerable chromosome damage when L1210 cells in culture were exposed to the drug at 5 μg/ml for 2 hr at 37°C.

INTRODUCTION

Some antitumor agents have selective biological and/or biochemical effects (1, 8, 9, 11–13, 16, 21) at certain phases of the cell cycle. Cytosar [cytarabine, (The Upjohn Co., Kalamazoo, Mich.) cytosine arabinoside, ara-C] and hydroxyurea inhibit cells primarily in the S phase, while vinblastine is effective only in the mitotic stage. Several agents, such as γ-radiation and nitrogen mustard, lack this phase specificity and kill cells in all phases of the cell cycle. Such information, for instance, was used by Skipper et al. (18) in designing effective dosage schedules for ara-C.

Since 5-azaCR inhibited DNA synthesis more than RNA synthesis (10), the action of the drug on asynchronous L1210 cells and asynchronous and synchronous DON cells was studied to determine whether the drug specifically inhibited a certain phase of the cell cycle. The effect of the drug on the chromosomal integrity and the inhibition of mitosis of L1210 cells is also reported.

MATERIALS AND METHODS

Tissue Culture Systems. L1210 cells were maintained in culture in RPMI 1634 medium (Grand Island Biological Company, Grand Island, N. Y.), developed by Moore et al. (14). The cells were used at 5 × 10⁶ cells/ml (in logarithmic phase of growth) in most cases. The incorporation of radioactive precursors, e.g., TdR-³H and uridine-³H, into respective macromolecules of intact L1210 cells was determined by the method described elsewhere (10).

DON cells, a Chinese hamster fibroblast line (American Type Culture Collection, No. CCL 16), were grown in McCoy's 5A medium modified by the addition of lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter) (Grand Island Biological Co.). The cell monolayer was detached from glass by treatment with a 0.1% trypsin solution. The cells were dispersed and grown in 8-oz. bottles planted with about 2 × 10⁶ cells in 25 ml medium. The cells were maintained in the logarithmic growth phase by subculturing every 2 days.

Chromosome Preparation. Chromosome preparations were made by the air-drying method described by Tijo and Whang (20). After exposure to 5-azaCR for a given period, the cells were centrifuged at 500 rpm for 2 min in a clinical centrifuge. The loosely packed cells were then suspended in 5 ml of hypotonic solution (1% sodium-citrate, pH 7) and allowed to stand for 10 min. The cells were centrifuged, and the loose pellet was resuspended in fixative (ethanol:glacial acetic acid, 3:1). After being fixed for 20 min, the cells were centrifuged, and the pellet was resuspended in a small amount of fresh fixative. A couple of drops of the cell suspension were placed on a clean glass slide and dried by an air stream. The cells were stained by an aceto-orcein stain (6). Two slides were prepared per sample for determination of chromosome damage and MI. Approximately 1000 cells per slide were counted to obtain the MI.

Determination of Cell Cycle of L1210 and DON Cells in Tissue Culture. Mean transit times through the cell cycle of both cell lines were determined from plots of labeled mitoses at various times after pulse labeling with TdR-³H (15).
RESULTS

Effect of 5-AzaCR on Macromolecule Synthesis. The effect of 5-azaCR on nucleotide synthesis in intact L1210 cells is shown in Chart 1 and indicates that DNA synthesis was inhibited to a greater extent than RNA synthesis at all doses used. Similar patterns of inhibition were also seen with DON cells (Chart 2).

Inhibition of Mitosis of L1210 Cells by 5-AzaCR. In order to accurately determine the MI, Colcemid was added to cells in logarithmic growth to accumulate cells in metaphase. Four experiments were done, and, since the results appeared to be quite reproducible, a representative example is shown in Chart 1. At 1 μg/ml of 5-azaCR, MI was not inhibited during the first 2 hr of incubation, and 30 to 50% inhibition was observed during the next 2 hr. Even at 5 μg/ml, MI was not significantly affected for the first 2 hr, but the passage of cells to mitosis was almost completely blocked after 2 hr of exposure to the drug. The inhibition of DNA synthesis, but not of RNA synthesis, parallels the inhibition of mitosis of L1210 cells, as seen in Chart 1.

Effect of 5-AzaCR on Proliferation Capacity of Asynchronous L1210 Cells. Chart 3 shows that the percentage of cell survival reaches a constant saturation value for each period of exposure. Cell survival was determined by injecting drug-treated and untreated cells into mice and comparing the respective median days of death.

Similar results were obtained when the number of cells surviving a 1-hr exposure to drug was determined by cloning, as seen in Chart 3.

Effect of 5-AzaCR on Proliferation Capacity of Asynchronous and Synchronous DON Cells. Chart 4 shows that when asynchronous DON cells were exposed to 5-azaCR the percentage of cell-kill reached a constant saturation value for each period of exposure, indicating that 5-azaCR kills cells in certain phases of the cell cycle. This possibility was con-
Chart 2. Inhibition of polynucleotide synthesis of DON cells in culture by 5-azaCR. Cells (circa 2 X 10⁶ cells/8-oz. prescription bottle) were incubated with 5-azaCR and labeled metabolite (Tdr-3H or Urd-3H, 6.4 μCi/4.6 μg/ml of reaction system) at 37° for 2 to 8 hr. After incubation, the radioactive medium was poured off, and cells (attached to the glass) were washed with fresh medium containing a high concentration (10 mg/ml) of either nonlabeled Tdr or uridine. After a quick rinse with 2 ml of trypsin (1 mg/ml), cells were detached from the glass by incubation with 0.5 ml of trypsin (1 mg/ml) for circa 5 min and then suspended in 10 ml of fresh medium. Acid-insoluble fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5 N perchloric acid (70°C) and used for the determination of radioactivity and nucleic acid content.

Chart 3. Survival of asynchronous L1210 cells after exposure to 5-azaCR. , in vivo (animal) experiment; - - - , in vitro experiment. For animal studies, cells were exposed to 5-azaCR for 2 or 4 hr. The cells were centrifuged and resuspended in fresh medium to give 10⁶ cells/ml, and 1 ml of cells were injected into mice (10 to 20 mice/group). The number of viable leukemic cells was determined by comparing the median days of death of mice injected with drug-treated cells to those injected with control (no drug) cells. Data presented in this chart are collective results of 4 experiments differing only in 5-azaCR concentration. For cloning experiments, to determine cell survival by the cloning technique, the following protocol was used. Cell suspensions were centrifuged after 1 hr of exposure to the drug, and the cells were resuspended in medium containing 5% calf serum to give a cell concentration of 10⁶/ml. Cells were further diluted in medium containing 20% serum. The cells were finally planted at 5 ml for each 10 X 1.50-cm tube in medium containing 20% calf serum and 0.2% agar. After 8 to 10 days incubation at 37° in a 5% CO₂ atmosphere, the colonies were visually counted. The plating efficiency was about 50%. The % coefficient of variation was about 25 to 30% of the mean value in the cloning experiment.

DISCUSSION

Fučík et al. (5) reported that 5-azaCR caused chromosomal mutation in the root meristem of Vicia faba and assumed the mutagenic activity of this compound was associated with its incorporation into DNA. The drug inhibited both DNA and RNA synthesis, was incorporated into the polynucleotides in L1210 cells (10), and caused sticky chromosomes, achromatic gaps, and chromosome fragmentation in these cells (Fig. 1).

Our results (Chart 1) showed that the inhibition of DNA synthesis (but not of RNA synthesis) parallels the inhibition of mitosis of L1210 cells; RNA synthesis is much less...
Chart 4. Survival of asynchronous DON cells after exposure to 5-azaCR. Cell monolayers were exposed to varying concentration of the drug for 3 to 5 hr. The drug was removed, and the cells from each sample were diluted and planted in 12 Petri plates. After 7 to 8 days incubation, the colonies were stained and counted. The percentage of cell survival was determined by comparing the percentage of surviving colonies in drug-treated samples to those in control samples. The cloning efficiency in the control (no drug) samples was about 78%; this represented 100% survival. The % coefficient of variation was about 20% of the mean value.

Chart 5. Survival of synchronous DON cells after exposure to drug. Mitotic cells were harvested from monolayers of DON cells, after pretreatment with Colcemid for 3 hr, and were used to start a synchronous culture. The cells were exposed to drug at different times after planting for 2 hr, and then the percentage of surviving cells was determined, as in Chart 4. The % coefficient of variation was about 20% of the mean value.

inhibited by this agent. Since the length of G1, S, G2, and M phases of L1210 cells in culture are, respectively, 1.2, 8.2, 1.2, and 0.6 hr (B. K. Bhuyan, unpublished data), these results indicated that cells previously in late S phase (2 hr prior to M phase) are blocked from proceeding to mitosis.

Bruce et al. (2, 3) have shown that with phase-specific agents, once the inhibitory concentration is reached, the percentage of cell-kill reached a constant saturation value for that period of exposure. With a longer period of exposure, more cells enter the sensitive phase and are killed, resulting in a higher saturation value for the percentage of cell-kill. Such data were obtained with presumably phase-specific agents such as Cytosar by Karon and Shirakawa (9) and with vinblastine, TdR-3H, and hydroxyurea by Bruce et al. (2, 3). With phase-nonspecific agents, however, an exponential cell-kill pattern is seen, and a saturation value for the percentage of cell-kill will not be obtained, as observed by Bruce et al. (2) with γ-radiation and nitrogen mustard.

Chart 3 illustrates the relationship between 5-azaCR dose and percentage of cell survival of asynchronous L1210 and DON cells, respectively. A saturation value of the percentage of cell-kill was obtained in both cell lines studied. In L1210 cells, a plateau of cell-kill was seen in both cloning and animal experiments when 5 μg/ml or higher concentrations of 5-azaCR were used. It must be realized, however, that results from the animal experiments are based on the assumptions that the drug-induced increase in host life-span results from the death of a high percentage of the L1210 cells and not from an increase in either the generation time or the lag phase of drug-treated L1210 cells. These possibilities have been appropriately discussed by Skipper et al. (17). In DON cells, the percentage of cell-kill becomes constant (about 90%) when the cells are incubated with 30 μg/ml 5-azaCR or more for 3 to 5 hr (37°). These results are in agreement with the previous findings, which suggest 5-azaCR acts more or less as a phase-specific agent, killing cells predominantly in a certain phase of the cell cycles of both cell lines studied.

The percentage of cell-kill of a synchronous DON cell population is minimal during M and G1 phases and increases as the cells progress into S phase. In the synchronous culture, there was a burst of mitosis about 8 hr after the mitotic cells were planted. When the synchronous culture was pulse-labeled with TdR-3H the percentage of labeled cells was 0, 72, 85, 88, 90, 62, and 44% at 1, 2, 3, 4, 6, 8, and 10 hr, respectively, after planting mitotic cells. This indicates that, while synchrony was maintained during G1 and early S phases, the cells in G2 phase are contaminated with a large percentage of S phase cells. Therefore, although it seems that percentage of cell survival decreases during G2 phase, it cannot be so stated. It is fairly obvious that 5-azaCR acts as an S phase-specific agent against DON cells, presumably through its greater inhibition of DNA synthesis (Chart 2). The shape of these curves (Chart 5) is similar to those obtained for Cytosar, a known S phase-specific agent (B. K. Bhuyan, unpublished data).

Work is in progress to determine the cell-cycle specificity of 5-azaCR upon a synchronous culture of L1210 cells. However, by carefully comparing the results of L1210 cells (Charts 1 and 3) with those obtained from DON cells (Charts 2, 4, and 5), it is reasonable to assume that 5-azaCR acts principally as an S phase-specific agent against leukemic L1210 cells in culture. Although this antimetabolite inhibits both DNA and RNA synthesis in leukemia L1210 systems, presumably via its incorporation into the respective molecules (10), the marked effect on DNA molecules

Chart 2. The percentage of cell-kill of a synchronous DON cell population is minimal during M and G1 phases and increases as the cells progress into S phase. In the synchronous culture, there was a burst of mitosis about 8 hr after the mitotic cells were planted. When the synchronous culture was pulse-labeled with TdR-3H the percentage of labeled cells was 0, 72, 85, 88, 90, 62, and 44% at 1, 2, 3, 4, 6, 8, and 10 hr, respectively, after planting mitotic cells. This indicates that, while synchrony was maintained during G1 and early S phases, the cells in G2 phase are contaminated with a large percentage of S phase cells. Therefore, although it seems that percentage of cell survival decreases during G2 phase, it cannot be so stated. It is fairly obvious that 5-azaCR acts as an S phase-specific agent against DON cells, presumably through its greater inhibition of DNA synthesis (Chart 2). The shape of these curves (Chart 5) is similar to those obtained for Cytosar, a known S phase-specific agent (B. K. Bhuyan, unpublished data).

Work is in progress to determine the cell-cycle specificity of 5-azaCR upon a synchronous culture of L1210 cells. However, by carefully comparing the results of L1210 cells (Charts 1 and 3) with those obtained from DON cells (Charts 2, 4, and 5), it is reasonable to assume that 5-azaCR acts principally as an S phase-specific agent against leukemic L1210 cells in culture. Although this antimetabolite inhibits both DNA and RNA synthesis in leukemia L1210 systems, presumably via its incorporation into the respective molecules (10), the marked effect on DNA molecules
appears to be one of the primary determinants of cytotoxicity.

ACKNOWLEDGMENTS

We thank Mr. B. E. Bowersox and Mrs. J. W. Culp for their skillful technical assistance and Mrs. Elizabeth L. Clark for her help in the preparation of this manuscript.

REFERENCES


Fig. 1. Chromosome aberrations in 5-azaCR-treated cells. L1210 cells were exposed to 5 μg/ml 5-azaCR for 2 to 3 hr. The cells were centrifuged and resuspended in warm medium containing Colcemid for 1 hr, and then slides were prepared. (A) Control (no drug) cells; (B) note achromatic gaps; (C) and (D) sticky and elongated chromosomes; and (E) fragmented chromosomes.
Phase Specificity of 5-azaCR
Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/30/11/2770