**In Vitro Cytotoxic and Biochemical Effects of 5-Aza-2'-deoxycytidine**

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**SUMMARY**

The in vitro effect of 5-aza-2'-deoxycytidine (5-aza-CdR) on cytotoxicity and macromolecular synthesis in A(T1)C1-3 hamster fibrosarcoma cells was investigated. The in vitro concentrations that produce 50% cell kill for 5-aza-CdR were about 1.0 and 0.01 μg/ml for a 2- and 24-hr exposure, respectively. 5-aza-CdR inhibited the growth of the fibrosarcoma cells by 40% at a concentration of 0.05 μg/ml. Deoxycytidine, but not cytidine, was a potent antagonist of the cytotoxicity produced by 5-aza-CdR. At cytotoxic concentrations 5-aza-CdR did not appear to inhibit DNA, RNA, or protein synthesis during a 1-hr incubation as measured by the incorporation of radioactive thymidine, uridine, or leucine into acid-insoluble material. At a concentration of 10 μg/ml, 5-aza-CdR stimulated the incorporation of radioactive thymidine into DNA by more than 50%. These results indicate that 5-aza-CdR is a very potent cytotoxic agent to tumor cells in vitro at concentrations that do not inhibit macromolecular synthesis.

**INTRODUCTION**

5-aza-CdR, a nucleoside analog, was first synthesized by Pliiml and Sorm (9) in 1964 and was shown by Sorm and Vesely (12) to be an active antileukemic agent in mice in 1968. Several lines of evidence suggest that 5-aza-CdR is an analog of deoxycytidine: (a) deoxycytidine can antagonize the antileukemic activity of 5-aza-CdR in mice (12); (b) 5-aza-CdR inhibits the incorporation of radioactive deoxycytidine into nucleic acids of leukemic cells (14); (c) leukemic cells resistant to 5-aza-CdR have been shown to be deficient in deoxycytidine kinase (13, 14). The phosphorylation and incorporation of 5-aza-CdR into nucleic acids occur primarily in rapidly proliferating tissue such as the thymus and spleen (11).

5-aza-CdR does not appear to inhibit RNA and DNA synthesis in mouse kidney cells in tissue culture except at high concentrations where only a weak inhibition is produced (11). The mechanism by which 5-aza-CdR produces its cytotoxic effects on mammalian cells is not known. In order to understand further the antineoplastic activity of 5-aza-CdR, we have investigated the cytotoxic, growth-inhibitory, and biochemical activity of this deoxycytidine analog on tumor cells in vitro.

**MATERIALS AND METHODS**

**Chemicals.** Radioactive nucleosides and leucine were obtained from New England Nuclear (Boston, Mass.). 5-aza-CdR was obtained from Chemapol (Prague, Czechoslovakia). Deoxycytidine and cytidine were obtained from Calbiochem (Los Angeles, Calif.).

**Cells and Media.** A(T1)C1-3 hamster fibrosarcoma cells (1) were grown in Minimal Essential Medium F-14 containing nonessential amino acids No. 114, 10 ml/liter (Grand Island Biological Co., Grand Island, N. Y.), and 10% fetal calf serum (Flow Laboratories, Rockville, Md.) as a suspension culture in a Vibro-Mixer E1 (Chemap, Inc., Hoboken, N. J.). The cell density was maintained at 1 x 10^4 to 5 x 10^5 cells/ml.

**Cytotoxicity and Growth Assay.** Cells from suspension culture were diluted with McCoy’s Medium 5A (Flow Laboratories) containing 5% serum to 60 cells/ml, and 5 ml (300 cells) were placed in 60- x 15-mm Falcon plastic Petri dishes (Scientific Products, Los Angeles, Calif.). The dishes were placed in the incubator for 4.5 days. The dishes were then rinsed twice with drug-free media. After the addition of 5 ml of McCoy’s Medium 5A containing 10% serum, the dishes were placed in the incubator for 4.5 days. The dishes were then rinsed rapidly with 0.9% NaCl solution, and the colonies were fixed with absolute methanol and stained with Giemsa. The colonies were counted electronically with an automatic colony counter (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). The plating efficiency was about 50%. Since the cells have a doubling time of about 10 hr, 1 viable cell would form a colony of 500 or more cells in 4.5 days.

For measurement of the effects of drugs on cell growth, 5 x 10^5 cells from the Vibro-Mixer were placed in 5 ml of medium in 17- x 100-mm Falcon plastic tubes. The tubes were gassed with air-5% CO₂, stoppered, and placed in a shaker bath at 37°. At different times 0.1 ml of the cell suspension was removed and counted with Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). The tubes were gassed with air-CO₂ each time after removal of a sample for cell count.
Biochemical Assay. Cells from the VibroMixer were centrifuged at 350 × g for 5 min at 20° and suspended in minimal essential medium containing 5% dialyzed serum to give a cell density of 1.5 × 10⁶ cells/ml. The cells (2 ml) were placed in plastic tubes containing 1.0 μCi of [methyl-³H]thymidine (20 Ci/mmmole), [6-³H]uridine (29.6 Ci/mmmole), or [4,5-³H]leucine (41.5 Ci/mmmole). The tubes were gassed with air-5% CO₂, stoppered, and incubated in a shaker bath at 37° for 1 hr. The cell suspensions were placed on 2.4-cm-diameter Whatman GF/C glass fiber filters (Scientific Products) that had been washed previously with sterile 0.9% NaCl solution. The filters were washed with 0.9% NaCl solution, cold 5% trichloroacetic acid, and ethanol and dried at 75° for 1 hr. The filters were placed in vials containing 10 ml of Omnifluor scintillation fluid (New England Nuclear) and counted in a liquid scintillation counter.

RESULTS

The cytotoxic effect of 2 or 24 hr exposure of A(T₁)C₁-3 hamster fibrosarcoma cells to different concentrations of 5-aza-CdR is shown in Chart 1. For a 2-hr exposure, a concentration of 1.0 μg/ml produced 50% cell kill. The 2-hr survival curve shows a shoulder at 5-aza-CdR concentrations between 0.4 and 10 μg/ml; between these concentrations 5-aza-CdR produced 44 to 60% cell kill. Only at very high concentrations (100 μg/ml) did 5-aza-CdR produce a 100% cell kill during a 2-hr exposure.

For a 24-hr exposure, 5-aza-CdR, 0.01 μg/ml, produced a 60% cell kill. For 100% cell kill with 5-aza-CdR during a 24-hr exposure, a drug concentration just greater than 0.04 μg/ml was required.

The cytotoxic effect of 0.01-, 0.1-, and 1.0-μg/ml concentrations of 5-aza-CdR for different exposure times on fibrosarcoma cells is shown in Chart 2. For a 6-hr exposure, 7, 60, and 88% cell kill were produced by 0.01-, 0.1-, and 1.0-μg/ml concentrations of 5-aza-CdR, respectively. For a 10-hr exposure, only the 5-aza-CdR concentration of 1.0 μg/ml produced close to 100% cell kill.

Forty, 50, and 53% inhibitions of the growth of the fibrosarcoma cells were produced by 0.05-, 0.5-, and 5.0-μg/ml concentrations of 5-aza-CdR during a 26-hr exposure (Chart 3). Cell division could occur in the presence of concentrations of 5-aza-CdR that were capable of producing 100% cell kill (compare Charts 1 and 3).

During a 2-hr exposure to 5-aza-CdR, 1.0 μg/ml, which produced 60% cell kill, the presence of deoxycytidine at concentrations of 1.0, 10, and 50 μg/ml blocked the cytotoxic action of 5-aza-CdR by 27, 85, and 100%, respectively (Table 1). Cytidine had only a weak antagonistic effect with respect to the 5-aza-CdR-induced cytotoxicity; cytidine, 50 μg/ml, blocked the cytotoxic action of 5-aza-CdR by only 13%.

The effect of 5-aza-CdR on DNA, RNA, and protein synthesis as measured by the incorporation of radioactive thymidine, uridine, and leucine into acid-insoluble material is shown in Table 2. 5-aza-CdR, 10 μg/ml, did not appear to inhibit DNA, RNA, or protein synthesis significantly in the fibrosarcoma cell during a 1-hr incubation. This concentration of 5-aza-CdR stimulated the incorporation of [³H]thymidine into DNA by 65%.
Effect of cytidine and deoxycytidine on the cytotoxicity produced by 5-aza-CdR on A(T1)C1-3 hamster fibrosarcoma cells

In our in vitro studies, we found that 5-aza-CdR is an active antileukemic agent in AKA mice. In our studies we found that 5-aza-CdR is also a very potent cytotoxic agent to tumor cells in vitro. The exposure time appears to play a major role in the cytotoxic activity of this nucleoside analog. In our in vitro studies, we found that the LD50 of 5-aza-CdR for a 24-hr exposure was 100-fold less than the LD50 for a 2-hr exposure of this agent (Chart 1).

There was a shoulder on the survival curve of the 2-hr exposure of 5-aza-CdR but not on the survival curve for the

DISCUSSION

In 1968 Šorm and Veselý (12) reported that 5-aza-CdR is an active antileukemic agent in AKR mice. In our studies we found that 5-aza-CdR is also a very potent cytotoxic agent to tumor cells in vitro. The exposure time appears to play a major role in the cytotoxic activity of this nucleoside analog. In our in vitro studies, we found that the LD50 of 5-aza-CdR for a 24-hr exposure was 100-fold less than the LD50 for a 2-hr exposure of this agent (Chart 1).

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Table 2

Effect of 5-aza-CdR on DNA, RNA, and protein synthesis in A(T1)C1-3 hamster fibrosarcoma cells

Three x 10^6 cells in logarithmic growth were placed in stoppered plastic tubes containing 1.0 µCi of [3H]thymidine (20 Ci/m mole), [3H]uridine (29.6 Ci/m mole), or [3H]leucine (41.5 Ci/m mole) as indicated and the indicated concentrations of 5-aza-CdR. After incubation for 60 min in a shaker bath, the amount of radioactivity incorporated in acid-insoluble material was measured as described under “Materials and Methods.”

<table>
<thead>
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<th>Concentration of 5-aza-CdR (µg/ml)</th>
<th>Incorporation (cpm)</th>
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<tr>
<td></td>
<td>[3H]Thymidine</td>
</tr>
<tr>
<td>0</td>
<td>20,921 ± 927*</td>
</tr>
<tr>
<td>0.1</td>
<td>22,351 ± 3,325</td>
</tr>
<tr>
<td>1.0</td>
<td>27,072 ± 4,926</td>
</tr>
<tr>
<td>10</td>
<td>33,966 ± 4,177</td>
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* Mean ± S.D.
produced a marked stimulation of the incorporation of radioactive thymidine into DNA. 5-aza-CdR also stimulated the incorporation of radioactive deoxyuridine into DNA (R. L. Momparler and J. Goodman, unpublished data). This apparent stimulation could be possibly due to the effect of 5-aza-CdR on the intracellular pool size of thymine nucleotides or the induction of thymidine kinase. Čihák et al. (2) reported that the administration of the riboside analog, 5-aza-CA, to rats resulted in an enhancement of thymidine incorporation into DNA of rhesus monkey kidney cells. There was no stimulation of the incorporation of thymidine into DNA by low concentrations of 5-aza-CdR by these investigators. The reason for the differences in the results obtained by this group and ours is not known.

At concentrations that are cytotoxic to tumor cells, 5-aza-CdR did not produce a marked inhibition of cellular proliferation (Chart 3). It appears that the cells are capable of undergoing a limited number of cell divisions after exposure to a lethal concentration of 5-aza-CdR, suggesting that the biochemical changes produced by this nucleoside analog do not produce an immediate effect on the molecular events that lead to mitosis.

It is interesting to compare the biological activity of 5-aza-CdR with that of the related riboside analog, 5-aza-CR. 5-aza-CR is a very potent cytotoxic agent (4, 6, 10) that inhibits DNA, RNA, and protein synthesis (4, 8, 9) and stimulates enzyme induction of specific enzymes (3). Lloyd et al. (6) and Li et al. (4) studied the in vitro cytotoxic and growth-inhibitory activity of 5-aza-CR on L1210 leukemic cells. Upon comparison of the 5-aza-CR data of these workers with our data on 5-aza-CdR, there appear to be 2 major differences between these 2 analogs with respect to their cytotoxic and growth-inhibitory effects: (a) the ratio of the in vitro LD_{50} for 2 hr to LD_{50} for 24 hr for 5-aza-CdR is approximately 100 (Chart 1), whereas the same ratio for 5-aza-CR is about 10; (b) during a 10- to 20-hr incubation 5-aza-CR is a more active inhibitor of cellular proliferation than is 5-aza-CdR (Chart 3). Although it is possible that these differences may be related to the different types of cells used, they are most probably correlated with the differences in the metabolic transformation and in the biochemical mode of action of these 2 5-aza-cytosine analogs. The comparative pharmacology of 5-aza-CdR and 5-aza-CR is under investigation in our laboratory.

REFERENCES

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