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Cell cycle effects and cellular pharmacology of 5-AZA-2′-deoxycytidine

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Summary. *The cytotoxic action of 5-aza-2′-deoxycytidine (5-AZA-CdR) in synchronized cells and logarithmic- and plateau-phase cultures of EMT_6 murine tumor cells was investigated. 5-AZA-CdR produced a greater cell kill of S phase cells than of cells in G_1 phase. Cells in the logarithmic phase of growth were more sensitive to the cytotoxic effects of 5-AZA-CdR than cells in the plateau phase of growth. These results indicate that 5-AZA-CdR produces a preferential kill of cells in the S phase of the cell cycle. 5-AZA-CdR did not block the cell cycle progression of cells into S phase. The survival curve of EMT_6 cells exposed to 5-AZA-CdR suggests that the cytotoxic action of this analogue is not self-limiting. The mutagenic activity of 5-AZA-CdR was investigated using induction of 6-thioguanine resistance in Chinese hamster ovary cells. 5-AZA-CdR was not a detectable mutagen in this assay system.*

Introduction

5-AZA-2′-deoxycitidine (5-AZA-CdR) is a very potent anti-leukemic agent in mice [8, 15, 19]. This analogue of deoxycytidine was first synthesized by Pliml and Sorm [11]. To become active 5-AZA-CdR must first be phosphorylated by deoxycytidine kinase [7]. Leukemic cells resistant to 5-AZA-CdR are deficient in this enzyme [20]. The lethal action produced by 5-AZA-CdR appears to be related to its incorporation into DNA [19], since agents which enhance its incorporation increase its cytotoxic action [9]. 5-AZA-CdR can also induce cells in vitro to differentiate, apparently by inhibition of DNA methylation [2]. The inhibition of DNA methylation appears to be related to the antileukemia action of 5-AZA-CdR [22].

A phase I study has been performed with 5-AZA-CdR in leukemia patients [12]. 5-AZA-CR is also related to a similar analogue, 5-azacytidine, an agent that is used for the clinical treatment of acute leukemia [1, 3, 21]. Since 5-azacytidine produces a preferential cell kill of S phase cells [5] and inhibits cell cycle progression [17], we have studied the cell cycle effects of 5-AZA-CdR. In addition, since 5-AZA-CdR is incorporated into DNA we have studied its potential mutagenic activity.

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Materials and methods

Chemicals. methyl-[H^3]thymidine and 5-AZA-CdR were obtained from New England Nuclear Corp. (Boston) and Chemapol (Prague), respectively. Cytosine arabinoside was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Ethyl methanesulfonate (EMS) was obtained from Fisher Scientific, Montreal.

Cells and media. EMT_6 murine mammary tumor cells [13] were grown as a monolayer culture in 25-cm^2 Falcon plastic flasks in minimal essential medium (F_{14}) containing nonessential amino acids (#114) 10 ml/l (Grand Island Biological Co.) and 10% fetal calf serum (Flow Laboratories, Rockville, Md). The doubling time of the EMT_6 cells was 11–12 h. Synchronized cells were obtained by mechanical detachment of mitotic cells from the monolayer [16]. Chinese hamster ovary (CHO) cells (proline auxotroph) were obtained from Dr Mark Meuth, Institut de Recherches Cliniques, Montreal.

Cytotoxicity assay. The cells were exposed to 5-AZA-CdR for the time intervals indicated in Tables 1–3 and Figs. 1 and 2. Cytotoxicity produced by 5-AZA-CdR was measured by placing 200 cells in a 60-mm Falcon plastic dish and counting the number of colonies formed 5 days after drug exposure. The dishes were rinsed once with drug-free medium at the end of drug exposure. A cell was considered to be viable if it gave rise to a colony of 50 or more cells. The plating efficiency of the EMT_6 cells was 60%. The experiments were repeated three times and gave similar results.

DNA synthesis assay. To measure DNA synthesis $1-4 \times 10^4$ cells were placed in 35-mm plastic dishes containing 2.0 ml medium with 10% dialyzed serum. At the times indicated in Tables 1–3 and Figs. 1 and 2, 1.0 μCi methyl-[H^3]thymidine, 20 Ci/mmol, was placed in the dish and the incubation continued for 30 min. The amount of radioactivity incorporated into the acid-insoluble fraction of the cells was determined as described previously [9]. Duplicate measurements were made for each experimental point.

Autoradiography techniques. The mitotic cells (20,000 cells/2 ml) were placed in a 35-mm Falcon petri dish and placed in a 5% CO_2 incubator at 37° C. At the time indicated (Table 3) 1 Ci methyl-[H^3]thymidine (20 Ci/mmol) was added to the dish and the incubation continued for 20 min. The medium was

then aspirated and the cells washed with 2 ml phosphate-buffered saline containing calcium. The cells were fixed for 5 min in 5% glutaraldehyde and washed with cold 5% TCA and H_2O. The photographic liquid emulsion (Kodak NTB) was added to the dry dish and the cells were exposed for 5 days. After photographic development the cells were stained with methylene blue and the fraction of labeled cells per dish was determined.

Assay for mutation induction. The mutagenic activity of the agents was determined by measurement of mutation induction at the hypoxynthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells utilizing resistance to 6-thioguanine [10]. Cells were plated at 5×10^5 cells/100-mm petri dish in 20 ml medium containing 5% serum. After a 24-h growth period, the cells were exposed to the indicated concentrations of the agents for 18 h (Table 3). The cells were then trypsinized and 10^6 cells plated in a 100-mm dish for expression of the mutation and 200 cells plated in a 60-mm dish to estimate cell survival. The cells in the 100-mm dish were subcultured every 48 h at the same cell density. For measurement of 6-thioguanine resistance 2×10^5 cells/100 mm dish were exposed to 10 μM of this agent in medium containing 5% dialyzed serum and 200 cells plated in the same medium without 6-thioguanine to determine the plating efficiency. After 7 days of incubation the colonies were fixed, stained, and counted. The mutation frequency was determined by dividing the total number of mutant colonies by the total number of cells plated corrected for the cloning efficiency.

Results

The cytotoxicity of 5-AZA-CdR on G_1 and S phase EMT_6 tumor cells is shown in Table 1. The cell cycle time of the EMT_6 cells was about 12 h with a G_1 phase time of about 4 h. 5-AZA-CdR at a concentration of 10 g/ml for a 2-h exposure produced only minimal cell kill of the G_1 phase EMT_6 cells. However, this same concentration and exposure time of 5-AZA-CdR produced about 60%–70% cell kill of early and late S phase cells.

The effect of 5-AZA-CdR on the progression of G_1 phase of EMT_6 tumor cells into S phase is shown in Fig. 1. Cell synchrony was obtained by detachment of mitotic cells from the monolayer. The cohort of G_1 phase cells was exposed continuously to 10 μg/ml 5-AZA-CdR starting 1.0 h after mitosis. This concentration of 5-AZA-CdR did not appear to inhibit the progression of the G_1 phase EMT_6 cells into S phase as measured by the incorporation of methyl-[H^3]thymidine into DNA (Fig. 1a) or by the percentage of cells labeled with methyl-[H^3]thymidine using autoradiography (Fig. 1b).

The effect of exposure time on the cytotoxic effect of different concentrations of 5-AZA-CdR on EMT_6 tumor cells is shown in Fig. 2. The cytotoxic activity of 5-AZA-CdR as measured by the colony-forming assay increased with the duration of the exposure time. 5-AZA-CdR at a concentration of 1.0 μg/ml produced 20%, 50%, and 80% cell kill for an exposure of 2, 4, and 8 h, respectively. There was a slight shoulder on the survival curve of 5-AZA-CdR at a concentration of 20 μg/ml for the exposure intervals of 2 h and 6 h.

Table 1. Cytotoxicity of 5-AZA-CdR in G_1 and S phase EMT_6 cells[a]

Phase of cell cycle[a]	Time interval 5-AZA-CdR exposure (h)	Cell kill by AZA-CdR (%)
G_1	2–4	10 ± 9[b]
Early S	6–8	72 ± 4
Late S	9–11	59 ± 8

[a] Mitotic cells were obtained by mechanical detachment from monolayers and placed in plastic dishes for cytotoxicity assay (120 cells) or DNA synthesis assay (2×10^4 cells). At the times indicated after mitosis the cells were exposed to 10 μg/ml 5-AZA-CdR for 2 h. Cells entered S phase 5 h after mitosis. Cytotoxicity was measured by counting the number of colonies formed 6 days after drug exposure

[b] Mean value and standard deviation

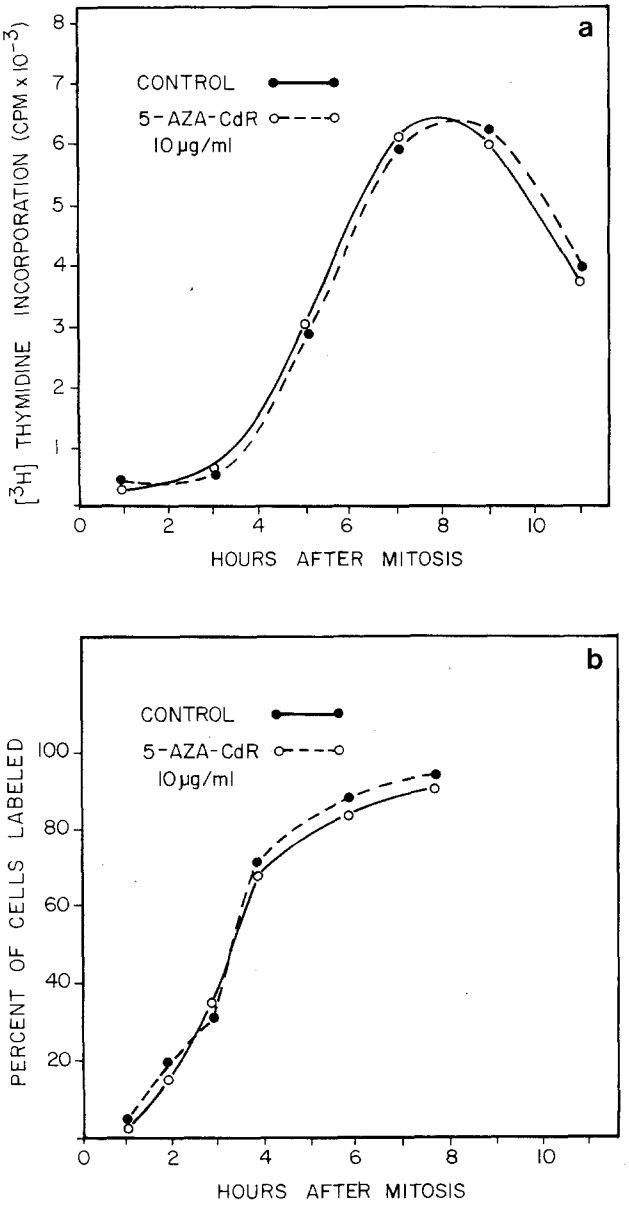

Fig. 1a, b. Effect of 5-AZA-CdR on progression of G_1 phase EMT_6 murine tumor cells into S phase. Mitotic cells were obtained by mechanical detachment from monolayers and placed in petri dish (20,000 cells) for DNA synthesis assay (*a*) or for labeling index using autoradiography (*b*). At the times indicated the cells were exposed to 1.0 μCi methyl-[H^3]thymidine (20 Ci/mmol) for 30 min

This concentration of 20 μg/ml 5-AZA-CdrR produced 60% and greater than 90% cell kill for the 2-h and 8-h exposures, respectively.

The cytotoxic effects of a 6-h exposure of 5-AZA-CdR on log-phase and plateau-phase EMT_6 cells are shown in Table 2. The log-phase cells were in a logarithmic phase of growth when exposed to 5-AZA-CdR, whereas the plateau-phase cells had

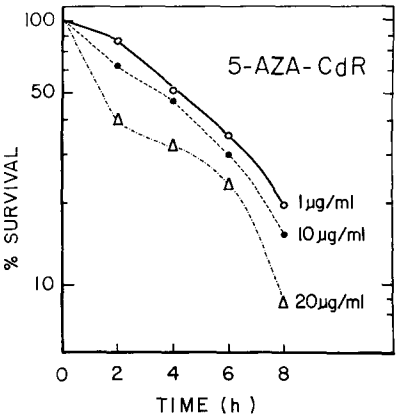

Fig. 2. Effect of exposure time on cytotoxicity of 5-AZA-CdR to EMT_6 tumor cells. Cells in logarithmic growth were placed in a 60-mm plastic petri dish for cytotoxicity assay (250 cells) and exposed to 1, 10, and 20 μg/ml 5-AZA-CdR for the times indicated. After drug exposure the number of colonies formed 5 days later was determined.

Table 2. Cytotoxicity of 5-AZA-CdR in log-phase and plateau-phase EMT_6 cells[a]

5-AZA-CdR concentration (μg/ml)	% Cell kill	
	Log phase	Plateau phase
2.0	32 ± 1[b]	< 5
5.0	31 ± 1	< 5
10.0	50 ± 4	< 5
20.0	51 ± 2	19 ± 5

[a] Monolayer log-phase cells (1.5×10^6 cells/25-cm^2 flask) or plateau-phase cells (5×10^6 cells/25-cm^2 flask) were exposed to the indicated concentrations of 5-AZA-CdR for 6 h. The cells were then trypsinized and 200 cells seded in a 60-mm petri dish to measure cell survival. The plating efficiency of the nontreated cells was 70%

[b] Mean value and standard deviation

Table 3. Induction of resistance to 6-thioguanine in CHO cells by 5-AZA-CdR or EMS[a]

Agent	Concentration (μg/ml)	Cytotoxicity (% kill)	6-Thioguanine resistance (mutation frequency)
None			2.1×10^{-5}
5-AZA-CdR	0.1	9 ± 2[b]	1.5×10^{-5}
5-AZA-CdR	1.0	53 ± 3	1.7×10^{-5}
5-AZA-CdR	10	84 ± 3	$< 1.0 \times 10^{-5}$
EMS	666	95	1.8×10^{-3}

[a] CHO cells were exposed to the indicated concentration of 5-AZA-CdR or EMS for 18 h. The cytotoxic effects and mutation frequency to 6-thioguanine produced by these agents were determined as described under *Methods*

[b] Mean value and standard deviation

reached a high cell density in which cell growth was markedly decreased [18]. At concentrations up to 10 μg/ml 5-AZA-CdR did not produce any significant cell kill in the plateau-phase cells, whereas this concentration produced 50% cell kill in the log-phase cells. Only at a concentration of 20 mg/ml did 5-AZA-CdR produce a significant cell kill (19%) in the plateau-phase cells.

The induction of resistance to 6-thioguanine in CHO cells by 5-AZA-CdR or EMS is shown in Table 3. The spontaneous mutation frequency in control cells not exposed to any agent was about 10^{-5}. EMS, a potent mutagen, at a concentration which killed 95% cells increased the mutation frequency to 6-thioguanine resistance in the surviving cells about 100-fold. 5-AZA-CdR at weakly and strongly cytotoxic concentrations was not detected to be a mutagen under these experimental conditions.

Discussion

For design of the optimal dose schedule for an antineoplastic agent, its pharmacological effects on normal and malignant cells should be investigated These studies should include experiments on the cytotoxic action of the agent on cells in different phases of the cell cycle and its effect on the progression of cells through the cell cycle. Agents whose cytotoxic action is specific for only one phase of the cell cycle usually show a marked schedule-dependency with respect to their in vivo antineoplastic activity. An example of such an agent is the deoxycytidine analogue, cytosine arabinoside [14]. 5-AZA-CdR is also a deoxycytidine analogue and a very potent experimental antileukemic agent [8, 15, 19]. In this report we have studied the cellular pharmacology of this interesting antileukemic agent.

Using synchronized EMT_6 cells we have observed that 5-AZA-CdR is markedly more cytotoxic to cells in S phase than in G_1 phase (Table 1). The small amount of cell kill produced by 5-AZA-CdR on G_1 phase cells may have been due to the contamination of this cohort of cells by cells from other phases of the cell cycle. The method of synchrony used in this study produces a yield of 90%–95% mitotic cells as determined by autoradiography. Support of the preferential cell kill of S phase cells by 5-AZA-CdR is also provided by our observations that this agent produces much greater cell kill of log-phase cells than of plateau-phase cells (Table 2). Twentyman et al. [18] reported that when EMT_6 cells enter the plateau phase of growth there is a marked reduction of the fraction of cells in S phase and a large increase in the fraction of cells in G_1 phase. These data suggest that 5-AZ-CdR is an S-phase-specific agent similar to cytosine arabinoside [23].

The preferential kill of cells in S phase by 5-AZA-CdR may be related to the incorporation of this analogue into DNA during chromosomal replication. It has been proposed that the lethal effects produced by 5-AZA-CdR are related to its incorporation into DNA [19], which is supported by the observation that agents which increase the incorporation of 5-AZA-CdR into DNA enhance its cytotoxic action [9].

5-AZA-CdR did not inhibit the progression of G_1 phase EMT_6 cells into S phase as determined by the incorporation of methyl-[H^3]thymidine into DNA (Fig. 1a) and by autoradiography using this radioactive nucleoside (Fig. 1b). These results suggest that the cytotoxic action of 5-AZA-CdR should not be self-limiting. A self-limiting agent blocks its own cytotoxic action by inhibiting the progression of cells into the phase of the cell cycle where the agent exerts its major

cytotoxic effects [14]. Analysis of the survival curve of EMT_6 cells exposed to 5-AZA-CdR (Fig. 2) supports the proposal that the cytotoxic action of this analogue is not self-limiting. The cell cycle of EMT_6 cells in logarithmic growth is 12 h, with 60% of the cells in S phase [18]. For an S-phase-specific agent such as 5-AZA-CdR a short exposure to a high concentration should produce 60% cell kill. As the exposure time was increased the fraction of cells killed exceeded that which was initially in S phase, indicating that the cytotoxic action of 5-AZA-CdR was not self-limiting.

Although the cytotoxic action of 5-AZA-CdR appears to be related to its incorporation into DNA, the precise mechanism of action of this analogue is unknown. Jones and Taylor [2] have shown that 5-AZA-CdR inhibits DNA methylation and induces cellular differentiation. It is not known whether these phenomena are responsible for the cytotoxic effects of 5-AZA-CdR or may due to the chemical decomposition of 5-aza-cytosine in DNA. 5-AZA-CdR does undergo slow chemical decomposition at neutral pH and 37° C [6]. If 5-AZA-CdrR produces its cytotoxic effect by erroneous base-pairing during DNA replication, it should be a potent mutagenic compound. To investigate this problem we studied the mutagenic activity of 5-AZA-CdR using the induction of 6-thioguanine resistance in Chinese hamster ovary cells (Table 3). Resistance to 6-thioguanine results from the deletion of HGPRT locus in these cells [10]. We observed that mutagenic activity of 5-AZA-CdR at different cytotoxic concentrations was not greater than the spontaneous rate of mutation in these cells under the experimental conditions used. Similar results have been reported by Landolph and Jones [4]. The molecular mechanism of action of this interesting antineoplastic agent still remains to be elucidated.

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