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Chemotherapy of L1210 and L1210/ARA-C leukemia with 5-aza-2'-deoxycytidine and 3-deazauridine*

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Summary. The in vitro and in vivo antineoplastic activity of 5-aza-2'-deoxycytidine (5-AZA-dCyd) and 3-deazauridine (3-DU) against L1210 and L1210/ARA-C (resistant to cytosine arabinoside) leukemic cells were investigated. L1210/ARA-C cells were more sensitive to the inhibitory effects of 3-DU than L1210 cells. Deoxycytidine completely reversed the in vitro cytotoxic effects produced by 3-DU on L1210 cells, but not those produced in L1210/ARA-C cells. L1210/ARA-C cells, which are deficient in deoxycytidine kinase, were completely resistant to the antileukemic effects of 5-AZA-dCyd, whereas this analogue produced a very potent antileukemic effect against L1210 cells. To study the in vivo interaction of 5-AZA-dCyd and 3-DU with respect to drug resistance, mice were simultaneously injected i.v. with 10^4 L1210 cells plus 10^2 L1210/ARA-C cells. A 9-h i.v. infusion of 5-AZA-dCyd (12.8 mg/kg) or 3-DU (186 mg/kg) produced an increase in life span of 56% and 26%, respectively. However, the sequential administration of 5-AZA-dCyd followed by 3-DU produced a 265% increase in life span and 7/10 longterm survivor, a very potent antileukemic effect. These results suggest that 3-DU is an excellent agent for use in combination chemotherapy to overcome drug resistance to the deoxycytidine analogue, 5-AZA-dCyd.

Introduction

5-Aza-2'-deoxycytidine (5-AZA-dCyd) has been demonstrated to be a very potent antineoplastic agent against murine leukemia [11, 14, 15]. The antileukemic effects produced by 5-AZA-dCyd result from its incorporation into DNA [2, 15], where it produces a potent inhibition of DNA methylation [2, 5] that can result in the induction of cellular differentiation [5, 10]. In clinical trials in patients with acute leukemia, 5-AZA-dCyd has been demonstrated to be an active agent [12, 13].

For the nucleoside analogue 5-AZA-dCyd to be an active inhibitor in the cell, it must first be phosphorylated by the enzyme deoxycytidine (dCyd) kinase [7]. Malignant cells that are deficient in dCyd kinase are resistant to 5-AZA-dCyd [9, 16]. Since drug resistance is a common problem in the chemotherapy of leukemia, a therapeutic regimen that uses 5-AZA-dCyd should include another drug that is active against leukemic cells resistant to this analogue.

A good candidate for use in combination with 5-AZA-dCyd 3-deazauridine (3-DU). In a previous report, we demonstrated that the antineoplastic action of the combination 5-AZA-dCyd plus 3-DU was synergistic against both L1210 leukemic and EMT_6 tumor cells [8]. After its conversion to its 5'-triphosphate, 3-DU is a potent competitive inhibitor of CTP synthetase [6]. In cells treated with 3-DU a reduction in the intracellular pool of CTP and dCTP occurs [3]. The reduced pool of dCTP produces less competition with the triphosphate of 5-AZA-dCyd for its incorporation into DNA [2] and also decreases the rate of phosphorylation of 5-AZA-dCyd by dCyd kinase, since dCTP is a potent feedback inhibitor of this enzyme [7].

L1210 leukemic cells that lack dCyd kinase (L1210/ARA-C) are resistant to cytosine arabinoside [4] and more sensitive to the inhibitory effects of 3-DU [3]. The wild-type L1210 leukemic cells are less sensitive to the inhibitory effects of 3-DU than L1210/ARA-C cells because they can use dCyd from the medium to maintain an adequate intracellular pool of dCTP.

In this study we investigated the antineoplastic activity of 5-AZA-dCyd and 3-DU against L1210 and L1210/ARA-C leukemic cells, showing that 3-DU is very effective against L1210/ARA-C cells and demonstrating that the sequential administration of 5-AZA-dCyd followed by 3-DU is very effective in mice with both L1210 and L1210/ARA-C leukemic cells.

Materials and methods

Chemicals. 5-AZA-dCyd and 3-DU were obtained from Chemapol (Prague, Czechoslovakia) and the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md), respectively. [5-^{3}H]-dCyd and [^{3}H]-5-AZA-dCyd were obtained from NEN Canada (Lachine, Québec) and Isotope Laboratory (Prague, Czechoslovakia), respectively. [^{3}H]-5-AZA-dCyd was purified by chromatography on a μBondapak/C_{18} column (Waters Scientific, Milford, Mass) by isocratic elution with H_2O. The chromatographic peak of [^{3}H]-5-AZA-dCyd was evaporated to 1/10 the original volume with a stream of nitrogen gas and stored at −70° C.

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Cytotoxicity assay. L1210 and L1210/ARA-C leukemic cells were maintained in culture at a cell density of 10^3-10^5/ml in minimal essential medium containing non-essential amino acids (Grand Island Biological Co., Grand Island, NY), 10 μ*M* 2-mercaptoethanol, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 5% fetal calf serum (Flow Laboratories, Rockville, Md). Both cell lines were obtained from Dr. T. Khwaja (Cancer Center, University of Southern California, Los Angeles). For cytotoxicity experiments, a 2-ml sample at 25,000 cells/ml were placed in plastic tubes and the indicated concentration of drugs was added. The tubes were gassed with 5% CO_2-air and placed in a shaker bath at 37° C for 9 h. The drugs were removed by centrifugation at 200 *g* for 5 min, the cells were counted, and 100 cells were placed in 2.0 ml medium containing 16% serum and 0.15% agar. The tubes were placed in an incubator containing 5% CO_2 at 37° C and the number of colonies were counted after 7 days, as previously described [8]. The cloning efficiency was about 60%.

Biochemical assay. L1210 or L1210/ARA-C cells ($2-4\times10^5$ cells/ml) were centrifuged at 200 *g* for 5 min; 2 ml was suspended in medium containing 5% dialyzed serum at a cell density of 1×10^5 cells/ml. After the addition of 1.0 μCi [5-^{3}H] dCyd (28.3 Ci/mmol) or 2 μCi [^{3}H]-5-AZA-dCyd (11 Ci/mmol) and the indicated concentration of drugs, the tubes were gassed with 5% CO_2-air and placed in a shaker bath at 37° C for 2.0 h. The amount of radioactivity incorporated into DNA was determined as previously described [8].

Chemotherapy in mice. The same L1210 or L1210/ARA-C cells used in tissue culture were used for in vivo studies; they were transplanted weekly from the ascitic fluid by i.p. injections of 10^4 cells in 0.1 ml medium (no serum or antibiotics) to male BALB/c × BDA/2F_1 (hereafter called CD2F_1) mice (Cumberland View Farms, Clinton, Tenn). For chemotherapy studies, the non-bloody ascitic fluid obtained 7 days after transplantation was diluted with medium to 10^5 cells/ml (L1210 leukemia) or 10^3 cells/ml (L1210/ARA-C leukemia) and 0.1 ml was injected i.v. into a lateral tail vein of 12- to 14-week-old male CD2F_1 mice (28–33 g). The 5-AZA-dCyd or 3-DU was dissolved in sterile 0.45% NaCl containing 10 m*M* potassium phosphate (pH 6.8). The drug solution was passed through a 0.22-μm filter placed in a 5-ml plastic syringe. The drug was infused into the tail vein at a rate of 0.22 ml/h using a Harvard syringe pump. The experiments were repeated at least three times and gave similar results.

Results

The in vitro cytotoxic effects of 3-DU, 5-AZA-dCyd, and/or dCyd on L1210 and L1210/ARA-C cells are shown in Table 1. 3-DU was more cytotoxic to L1210/ARA-C cells than to L1210 cells. dCyd (5 μg/ml) reduced the cell kill of L1210 cells produced by 0.5 μg/ml 3-DU from 71% to <2%; however, it did not significantly reduce the cell kill produced by 3-DU in L1210/ARA-C cells. dCyd also reduced the cytotoxic action of 5-AZA-dCyd on L1210 cells. At a concentration of 1.0 μg/ml, 5-AZA-dCyd did not produce any significant cell kill in L1210/ARA-C cells (data not shown).

Table 1. Effect of dCyd on the cytotoxic action of 3-DU or 5-AZA-dCyd on L1210 and L1210/ARA-C leukemic cells

Additions	Concentration (μg/ml)	Cell kill	
		L1210 (%)	L1210/ARA-C (%)
dCyd	5.0	<2	<2
3-DU	0.05	<2	24±5
3-DU	0.5	71±2[a]	98±2
3-DU+dCyd	0.5+5.0	<2	93±2
5-AZA-dCyd	0.01	52±2	<2
5-AZA-dCyd+dCyd	0.01+5.0	12±3	<3

L1210 or L1210/ARA-C cells (25,000 cells/ml) were exposed to the indicated concentration of drug for 9 h and cell survival was determined by colony-forming assay

[a] Mean±SD ($n=3$)

Table 2. Effect of 3-DU on the incorporation of dCyd or 5-AZA-dCyd into DNA of L1210 and L1210/ARA-C leukemic cells

Cells	3-DU concentration (μg/ml)	Incorporation (cpm)	
		[5-^{3}H]dCyd	[^{3}H]-5-AZA-dCyd
L1210	0	2,450± 420[a]	2,640± 190
L1210	0.1	2,180± 440	2,270± 250
L1210	1.0	8,310± 150	7,630± 610
L1210	10.0	10,790±1,220	11,060±1,170
L1210/ARA-C	0	290± 110	470± 180
L1210/ARA-C	1.0	220± 80	330± 110

L1210 or L1210/ARA-C cells (2×10^5/2 ml) were incubated in the presence of 1.0 μCi [5-^{3}H]dCyd (28.3 Ci/mmol) or 2.0 μCi [^{3}H]-5-AZA-CdR (11 Ci/mmol) at the indicated concentrations of 3-DU for 2.0 h and the incorporation into DNA was determined

[a] Mean±SD ($n=3$)

In Table 2, the data on the effect of 3-DU on the incorporation of radioactive dCyd and 5-AZA-dCyd into DNA of L1210 and L1210/ARA-C cells are presented. 3-DU stimulated the incorporation of both dCyd and 5-AZA-dCyd into the DNA of L1210 cells. The incorporation of both of these radioactive nucleosides into the DNA of L1210/ARA-C cells was close to the background level, and 3-DU did not produce a significant effect.

The effect of a 9-h i.v. infusion of 5-AZA-dCyd or 3-DU on the survival of CD2F_1 mice with either L1210 or L1210/ARA-C leukemia is shown in Table 3. The mice received an i.v. injection of 10^4 L1210 or 10^2 L1210/ARA-C cells 1 day before the start of chemotherapy. 5-AZA-dCyd given at a dose of 3.2 and 12.4 mg/kg produced an increase in life span (ILS) of 107% and 245%, respectively, in mice with L1210 leukemia. At a dose of 12.9 mg/kg, 5-AZA-dCyd was not effective against L1210/ARA-C leukemia. 3-DU was much more effective in mice with L1210/ARA-C leukemia than in animals with L1210 leukemia. At a dose of 177 mg/kg, 3-DU produced only a 17% ILS in mice with L1210 leukemia but resulted in 5/5 long-term survivors in mice with L1210/ARA-C leukemia.

The effect of a 9-h individual or sequential infusion of 5-AZA-dCyd and/or 3-DU on the survival of CD2F_1 mice with L1210 and L1210/ARA-C leukemia is shown in Table

Table 3. Effect of i.v. infusion of 5-AZA-dCyd or 3-DU on the survival of $CD2F_1$ mice with L1210 or L1210/ARA-C leukemia

Cells injected	Chemotherapy	Dose (mg/kg)	Survival (days)	ILS (%)	60-day survivors	% wt. change, day 7
10^4	None	0	7.5 ± 0.3[a]	0	0/4	
L1210	5-AZA-dCyd	3.2	15.5 ± 2.3	107	0/5	+8
	5-AZA-dCyd	12.4	25.9 ± 5.3	245	1/5	+1
	3-DU	177	8.8 ± 0.3	17	0/5	+6
10^2	None	0	10.5 ± 0.2	0	0/2	+3
L1210/ARA-C	5-AZA-dCyd	12.9	10.5 ± 0.2	0	0/5	+1
	3-DU	46.5	13.0 ± 0.8	24	0/5	+1
	3-DU	92	14.0 ± 0.6	33	1/5	+4
	3-DU	176	>60		5/5	+2

$CD2F_1$ mice were given an i.v. injection of 10^4 L1210 or 10^2 L1210/ARA-C cells as indicated on day 0. On day 1 the mice were given a 9-h i.v. infusion of 5-AZA-dCyd or 3-DU at the indicated dose

[a] Mean ± SD

Table 4. Effect of individual or sequential i.v. infusion of 5-AZA-dCyd and 3-DU on the survival of $CD2F_1$ mice with L1210 and L1210/ARA-C leukemia

Chemotherapy	Dose (mg/kg)	Survival (days)	ILS (%)	60-day survivors	% wt. change, (day 7)
None	0	7.7 ± 0.4[a]	0	0/6	
5-AZA-dCyd	12.8	12.1 ± 1.8	57	0/10	−3
3-DU	186	9.8 ± 1.4	27	0/10	+1
5-AZA-dCyd → 3-DU	12.1 → 182	28.2 ± 14.3	266	7/10	−16

$CD2F_1$ mice were given an i.v. injection of 10^4 L1210 and 10^2 L1210/ARA-C cells on day 0. On day 1, the mice were given a 9-h i.v. infusion of 5-AZA-dCyd or 3-DU at the indicated dose or a sequential infusion of 5-AZA-CdR (9 h) followed by 3-DU (9 h)

[a] Mean ± SD

4. At 1 day before therapy, the mice received a simultaneous i.v. injection of 10^4 L1210 and 10^2 L1210/ARA-C leukemic cells. 5-AZA-dCyd given at a dose of 12.8 mg/kg produced only a 57% ILS in these mice. At a dose of 186 mg/kg, 3-DU produced a 27% ILS. However, when a 9-h infusion of 5-AZA-dCyd was followed immediately by a 9-h infusion of 3-DU, this drug combination produced a 266% ILS and 7/10 long-term survivors. The drug combination produced greater host toxicity, as indicated by the 16% weight loss compared with no weight loss in mice receiving 3-DU alone and only 3% weight loss in animals given 5-AZA-dCyd alone.

Discussion

5-AZA-dCyd is a very active antileukemic agent in animal models [11, 14, 15]. In addition, this analogue has been shown to have clinical activity in patients with acute leukemia [12, 13]. However, single-agent chemotherapy of acute leukemia has only limited effectiveness due to the problem of drug resistance. One of the major mechanisms by which malignant cells become resistant to dCyd analogues is through the deletion of dCyd kinase activity [4, 9, 16], the enzyme that catalyzes the phosphorylation of these compounds. 3-DU is a very interesting antineoplastic agent, because malignant cells that are deficient in dCyd kinase and drug-resistant dCyd analogues are more sensitive to the cytotoxic action of 3-DU than are wild-type cells [3].

3-DU may be effective when given in combination with 5-AZA-dCyd for the following reasons. First, the antileukemic activity of 5-AZA-dCyd and 3-DU in combination is synergistic [8]. Second, 3-DU is more inhibitory to 5-AZA-dCyd-resistant cells (L1210/ARA-C) than to wild-type L1210 leukemic cells ([2], Tables 1, 3). Third, 3-DU is not very toxic to normal cells (weight loss) as compared with L1210/ARA-C cells (Tables 3, 4). The L1210/ARA-C leukemic cells used in this study were deficient in dCyd kinase, as indicated by the very low incorporation of [5-^{3}H]-dCyd and [^{3}H]-5-AZA-dCyd into DNA (Table 2).

To evaluate the interaction of 5-AZA-dCyd and 3-DU, the mechanism of action of 3-DU should be understood. After its conversion to its triphosphate form, 3-DU is a potent inhibitor of CTP synthetase [6]. The inhibition of this enzyme produces a reduction in the intracellular pool of CTP and, subsequently, a reduction in the dCTP pool [3]. The ability of dCyd to reverse the cytotoxic effects of 3-DU on L1210 leukemic cells (Table 1) suggests that the reduction in the dCTP pool plays an important role in the lethal action of this analogue. The greater sensitivity of L1210/ARA-C cells vs L1210 cells to 3-DU (Tables 1, 3) supports this hypothesis.

3-DU produced a very potent antineoplastic effect in mice with L1210/ARA-C leukemia, but not in animals with L1210 leukemia (Table 3). In the mice injected i.v. with 10^2 L1210/ARA-C cells, a dose of 176 mg/kg 3-DU given as a 9-h infusion produced 5/5 long-term survivors. There was no weight loss in these mice, indicating that very little observable host toxicity was produced by 3-DU. On the other hand, the same dose schedule produced only 17% ILS in mice with L1210 leukemia. This high therapeutic index of 3-DU against L1210/ARA-C leukemia in mice

makes it an interesting agent for use in combination with 5-AZA-dCyd.

Since drug resistance is a major problem in the treatment of acute leukemia, it is important that good animal models are available, in which new drug combinations can be evaluated to determine their efficacy against wild-type and drug-resistant cells. In the present study we simultaneously injected i.v. 10^4 L1210 cells plus 10^2 L1210/ARA-C cells, and gave drug therapy by i.v. infusion 1 day later. In this animal model in which 1% of the leukemic cells were resistant to 5-AZA-dCyd, we could determine whether the interaction of 5-AZA-dCyd and 3-DU was synergistic, additive, antagonistic, or curative by comparing the results obtained with the individual effects of 5-AZA-dCyd or 3-DU on L1210 or L1210/ARA-C leukemia (Table 3).

A dose of 12.8 mg/kg 5-AZA-dCyd given as a 9-h infusion produced a mean survival of 12.1 ± 1.8 days in mice with L1210 plus L1210/ARA-C leukemia (Table 4), as compared with that of 10.5 ± 0.2 days in animals with L1210/ARA-C leukemia (Table 3). The similarity in the survival of these two groups of mice suggests that 5-AZA-dCyd killed most of the L1210 cells but only a few, if any, of the L1210/ARA-C cells. This treatment with 5-AZA-dCyd was very effective against L1210 leukemia, producing a 245% ILS with 1/5 long-term survivors (Table 3).

In general, there are three possible schedules that can be used to give 5-AZA-dCyd and 3-DU as a therapeutic regimen for leukemia: the simultaneous administration of these two agents, the sequential administration of 5-AZA-dCyd followed by 3-DU, and the sequential administration of 3-DU followed by 5-AZA-dCyd. To produce long-term survivors in mice with L1210 leukemia by the simultaneous administration of 5-AZA-dCyd and 3-DU, it was necessary to use an infusion time of >12 h, which produced too much toxicity (R. L. Momparler, unpublished data); therefore, this schedule was not investigated further. Since 3-DU may block the cell-cycle progression of some G_1-phase cells into the S-phase [1] and thus possibly antagonize the cytotoxicity of 5-AZA-dCyd, an S-phase-specific agent, an infusion of 3-DU followed by 5-AZA-dCyd was also not studied. We chose to evaluate the sequential administration of 5-AZA-dCyd followed by 3-DU for the following reasons.

Since 5-AZA-dCyd is a very potent antileukemic agent [11, 14, 15], it should kill most of the L1210 cells that are sensitive to this agent (Table 3). Some L1210 cells may escape the lethal action of the 9-h infusion of 5-AZA-dCyd because (a) not enough 5-AZA-dCyd is incorporated into these cells, (b) the cells did not enter the S-phase during the treatment, or (c) the cells were resistant to this analogue (L1210/ARA-C cells). The 3-DU infusion should kill the L1210/ARA-C cells (Table 3) as well as some of the L1210 cells that took >9 h to enter the S-phase. In addition, 3-DU should stimulate the incorporation of 5-AZA-dCyd (remaining in the body fluids at the end of the infusion) into the DNA of L1210 cells (Table 2), producing a synergistic cytotoxic effect [8]. Since 3-DU is much less toxic than 5-AZA-dCyd (R. L. Momparler, unpublished results), a sequential infusion using these analogues should not produce unacceptable toxicity.

In testing the sequential administration of 5-AZA-dCyd followed by 3-DU in mice with L1210 plus L1210/ARA-C leukemia, we observed a very potent antileukemic effect, as shown by a 266% ILS and 7/10 long-term survivors (Table 4), and tolerable host toxicity. Further investigations should be carried out on this interesting drug combination. The combination of cytosine arabinoside and 3-DU used sequentially has been evaluated in patients with acute leukemia and showed some interesting clinical activity [1].

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