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Comparison of the *in Vitro* Cytotoxicity (L1210) of 5-Aza-2′-deoxycytidine with its Therapeutic and Toxic Effects in Mice*

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Abstract—*The* in vitro *and* in vivo *cytotoxic effects of 5-aza-2′-deoxycytidine (DAC) on L1210 leukemia are reported and related to the pharmacokinetics of DAC in CDF$_1$ mice. L1210-bearing mice* (1×10^4 cells, *i.v.*) *given DAC i.v.* (6.5–225 mg/kg) *on day 3 showed a 50–212% increase in lifespan (ILS), with an estimated 3–6 log cell kill of L1210. Optimal effects with late treatment were obtained when DAC was given either on a multiple-dose regimen* (10 mg/kg *i.v., q* 3 hr × 4, *day 3 or 5) or by a constant s.c. infusion* (2.0 mg/kg/hr × 12 hr, *day 3), ILS 328–414%. Following* 10 *or* 100 mg/kg *i.v., plasma DAC declined in a triexponential manner with an intermediate elimination* $t_{1/2}$ *of* 31 min. *Urinary excretion accounted for* 28.5% *of DAC plasma clearance. When L1210 cells were exposed to DAC* in vitro (0.5–100 μg/ml *for* 24–120 hr) *a maximum 3–4 log cell kill was obtained. Both* in vivo *and* in vitro *response to DAC demonstrated the importance of exposure time as a determinant of cell kill. DAC is estimated to be more cytotoxic* in vivo *than* in vitro. *The critical cytotoxic concentration of DAC appears to be between* 0.5 *and* 1.0 μg/ml.

INTRODUCTION

5-AZA-2′-DEOXYCYTIDINE (DAC) is a pyrimidine nucleoside analog with demonstrated antineoplastic activity *in vitro* [1, 2] and *in vivo* [2–4]. It has been shown to be effective against murine tumors AKR [3, 4], P388 [4] and L1210 [2]. It has also been used in preliminary trials in Canada in childhood leukemia [5]. DAC is phosphorylated [6] and incorporated into the DNA of rapidly proliferating tissues [4]. The cytotoxicity of DAC has been attributed to its inhibitory effect on DNA methylation [7, 8], but the chemical instability of the triazine ring [9] may also play a role in its cell killing properties. Since DAC is an S-phase-specific agent [10], its rational use should involve maximum prolongation of exposure times to ensure that all cycling cells synthesize DNA in the presence of the drug [11]. Momparler and coworkers have reported that i.v. infusions of DAC for 8–15 hr at rates producing plasma levels near 1 μg/ml give a potent antileukemic effect in mice [2]. We have extended these studies by investigating the *in vitro* activity of DAC on L1210 over a wide range of concentrations (0.001–100 μg/ml) and for exposure times up to 120 hr. We compare these *in vitro* results to DAC therapy and toxicity studies in L1210 bearing mice, performed according to a number of dosing protocols including prolonged s.c. infusion. Using the results of pharmacokinetic experiments in mice and an understanding of the spontaneous decomposition of DAC in aqueous solution, the *in vivo* and *in vitro* effects of DAC are related by the concept of total exposure (concentration and time) as a determinant of cytotoxicity.

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MATERIALS AND METHODS

Drugs and chemicals

DAC was provided by Dr D. deVos, Pharmachemie B.V. (Haarlem, Holland), and by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Silver Spring, MD), which also

provided 5-azacytidine. [6-^{3}H]-DAC was purchased from Moravek Biochemicals (Brea, CA). DAC is unstable in aqueous solution and decomposes in a temperature- and pH-dependent manner to form several non-cytotoxic products [9, 12]. In RPMI 1630 medium at 37°C DAC decomposed in a first-order fashion with a $t_{1/2}$ of 17.5 hr. To minimize the effects of drug decomposition, DAC was always dissolved in sterile phosphate buffered saline immediately prior to use.

In vitro *and media*

Mouse L1210 leukemia cells were grown at 37°C in suspension culture in Roswell Park Memorial Institute 1630 medium (RPMI 1630, HEM Research Inc.) supplemented with 20% heat-inactivated horse serum (GIBCO, Grand Island, NY), 0.84 mM L-glutamine, penicillin (82 units/ml), streptomycin (82 μg/ml) (all from the Media Unit, NIH) and 50 μM mercaptoethanol (SIGMA chemical Co, St. Louis, MO). Stock cultures were maintained in exponential growth at a density between 0.5×10^5 and 1×10^6 cells/ml. Periodic testing (FLOW Laboratories, McLean, VA) confirmed the absence of mycoplasma in the cell cultures.

In vitro *L1210—cytotoxicity assay*

The effect of DAC on L1210 viability was examined by determining the soft-agar colony-forming ability of treated cells. DAC was dissolved and diluted in PBS (pH 7.4) and added to cell suspensions (0.3×10^5–7.0×10^5 cells/ml) to give a final drug concentration between 0.001 and 100 μg/ml. After 1–120 hr incubation at 37°C the cells were washed three times by centrifugation and resuspended in fresh media. Cell density was determined using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, FL). Aliquots containing 10^2–10^5 cells were placed into plastic tubes (FALCON Plastics No. 2058, Oxnard, CA) containing RPMI 1630 medium plus 0.1% agar. Following incubation at 37°C for 12–19 days, the number of colonies was determined in each tube and expressed as a percentage of control viability (cloning efficiency).

Calculation of area under in vitro *DAC exposure curves*

Because of the instability of DAC, drug concentrations during cell incubations at 37°C were corrected and total area under the concentration × time ($C \times t$) exposure curves was calculated as:

$$\int_{t_0}^{t_i} C\,dt = \frac{-C_0 e^{-\lambda t}}{\lambda} \Bigg|_{t_0}^{t_i} \qquad (1)$$

where C_0 is the initial DAC concentration, λ is the first-order rate constant for DAC decomposition (0.0396/hr), $t_0 = 0$, and t_i = time of exposure.

Studies in mice

Normal (BALB/c × DBA/2)F_1 (hereinafter called CDF_1) mice weighing 20–25 g were used in these experiments. DAC was dissolved in PBS and injected via a lateral tail vein (0.005 ml/g) to yield desired doses in mg/kg. In some experiments [6-^{3}H]-DAC was added to give a specific activity of 300 μCi/mg (10 mg/kg dose) or 30 μCi/mg (100 mg/kg dose). For pharmacokinetic studies mice were decapitated at various times and blood collected into heparinized tubes. Mice were anesthetized with pentobarbital (80 mg/kg) 5–10 min before termination. Blood was centrifuged (2000 *g*, 10 min, 4°C) and the plasma collected.

Animals from which urine was collected were housed in glass funnels following DAC administration and were fitted with fecal cups. Total urine excreted and urine remaining in the bladder were collected. For infusion experiments Alzet osmotic minipumps (Model 2001, ALZA Corp, Palo Alto, CA) which had been pre-equilibrated without drug were used. With a mean output of 1.0 μl/hr at 37°C, the minipumps delivered DAC at an initial rate of 0.89–2.7 mg/kg/hr.

L1210 mouse leukemia was obtained from MASON Research Institute (Worcester, MA) and maintained by weekly i.p. passage for a maximum of 10 passages in CDF_1 mice. For *in vivo* therapy experiments tumor was transplanted i.p. or i.v. into CDF_1 male mice using second to tenth passage cells recovered from the peritoneal cavity of donor mice. Trypan blue exclusion was used to determine the number of viable cells injected. An untreated control group of mice-injected with the same number of L1210 cells and by the same route was always run simultaneously in each experiment. Each time a new batch of L1210 cells was received from MASON a log dilution titration assay was conducted. This assay was used to estimate log cell kill of L1210 followed drug therapy as proposed by Schabel *et al.* [13].

Mice were selected for experimental use 1 week in advance, housed six per cage, observed daily and weighed every 2 days. After drug treatment mice were observed daily for deaths and weighed every 2 days. All dead mice were autopsied and observed for the presence of tumors in lymph nodes and other organs. Tumor deaths were verified by spleen weights greater than 150 mg and liver weights greater than 1.6 g coupled with no excessive body weight loss (greater than 5% of starting body weight) at time of death. Toxic deaths were verified by spleen weights less than

80 mg and liver weights less than 1.0 g, coupled with excessive body weight loss.

Preparation of plasma and urine for HPLC analysis

Two methods were used to prepare plasma for HPLC analysis. In the first, plasma containing DAC was treated according to the method of Somogyi [14] for the precipitation of proteins. All manipulations were done on ice. Prior to treatment, 5-azacytidine was added to each sample as an internal standard at a concentration of 100 μg/ml. Aliquots (500 μl) of plasma were placed into plastic centrifuge tubes (Corning No. 25319), to which 4000 μl of deionized water was then added. After vortexing the diluted plasma, 250 μl of 10% $ZnSO_4$ and 250 μl of 0.5 N NaOH were added in rapid succession with additional vortexing. The mixture was centrifuged for 10 min at 4°C (1300 *g*), the supernatant collected and either immediately analyzed by HPLC or stored at -20°C. Recovery of DAC was determined by adding known amounts of standard to blank plasma and carrying out the above procedure. When the appropriate dilution (×10) was considered, DAC recovery was 80-95%. In later experiments a more efficient method was used by preparing ultrafiltrates of plasma with the Centrifree micropartition system (AMICON Corp, Danvers, MA). Plasma (0.2-0.5 ml) was placed in the Centrifree unit and centrifuged at 2000 *g* for 30 min. The resultant ultrafiltrate was frozen at -20°C until analysis. Recovery of DAC added to control plasma or urine was essentially quantitative, so an internal standard was not used. Urine samples were filtered (Millex GS, 0.22 μm, MILLIPORE Corp., Bedford, MA) and analyzed directly by HPLC.

HPLC analysis of DAC

The procedure for HLPC determination of DAC was adapted from that of Lin *et al.* [9]. Isocratic elution was with 0.01 M KH_2HPO_4/Na_2HPO_4 buffer, pH 6.8, at a flow rate of 1 ml/min. Reversed-phase columns used were an Altex Ultrasphere-ODS (4.6 mm i.d. × 25 cm, BECKMAN Instruments) or a μ Bondpak C18 (WATERS Instruments, Milford, MA). DAC was quantitated by u.v. absorbance at 240 nm and the peak area was integrated. DAC peak area was linear with injected concentration between 0.1 and 100 μg/ml. The sensitivity of the assay was at least 0.5 μg/ml in the Somogyi supernatants, which represents a concentration of 5 μg/ml in the original plasma. For plasma ultrafiltrates, DAC could be detected to 0.3 μg/ml. When samples contained [6-^{3}H]-DAC and the ^{3}H associated with the DAC peak was collected and quantitated using the experimentally determined specific activity of the injected stock solution, DAC was detectable to 0.01 μg/ml. In all analyses the spontaneous degradation of DAC was accounted for by maintaining standards and experimental samples under identical storage conditions.

Pharmacokinetic calculations

Plasma concentration vs time data after a single i.v. dose was fitted by computer [15] to the triexponential equation:

$$C = Ae^{-\alpha t} + Be^{-\beta t} + Ge^{-\gamma t}. \quad (2)$$

A weighting factor of $1/C^2$ was used in each case. Best-fit estimates for the first-order rate-constants α, β and γ were obtained. Half-lives ($t_{1/2}$), area under the $c \times t$ curve (AUC), plasma clearance (CL_p), renal clearance (CL_r) and volume of central compartment (V_c) were determined by standard pharmacokinetic methods [16]. Appropriate initial infusion rates (K_{in}) for the experiments utilizing Alzet minipumps were calculated according to the relation:

$$K_{in} = C_{ss} \cdot CL_p, \quad (3)$$

where C_{ss} is the steady-state concentration to be achieved and CL_p was determined from single bolus data. A true steady state was never obtained, since K_{in} constantly decreased as DAC decomposed within the minipump. This was accounted for in the pharmacokinetic calculations as follows:

$$K_{in_t} = K_{in}e^{-\lambda t}, \quad (4)$$

where K_{in_t} is the actual infusion rate at time t and $\lambda = 0.0396$/hr.

RESULTS

Effects of DAC on L1210 viability in vitro

Figure 1 illustrates that L1210 viability decreases as DAC concentration increases between 0.01 and 0.5 μg/ml. Little additional cell kill occurs above 0.5 μg/ml. Cytotoxicity is also dependent on duration of drug exposure. A 3 log cell kill is achieved at 24 hr, but when exposure was increased up to 120 hr there was only a moderate (0.5-1.0 log) increase in cytotoxicity. Since DAC is unstable in aqueous solution [9], it was postulated that degradation of the drug during the incubation period limited the cell kill and was responsible for the time dependent plateau. To investigate this possibility, *in vitro* drug exposure as function of area ($c \times t$) was calculated using (1) for each concentration and exposure time studied. It became clear that at each

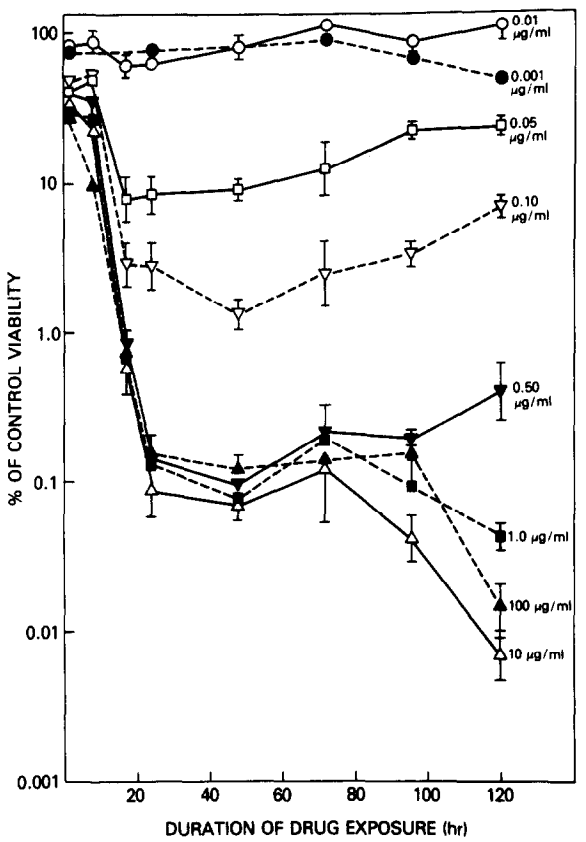

Fig. 1. DAC cytotoxicity as a function of exposure time in vitro. *L1210 cells were exposed to DAC at the indicated concentrations for* 1–120 hr. *Cells were then washed, diluted and resuspended in tubes containing fresh medium plus* 0.1% *agar. After* 13–17 days *incubation at* 37°C, *colonies in each tube were counted and L1210 viability expressed as a percentage of control cloning efficiency.*

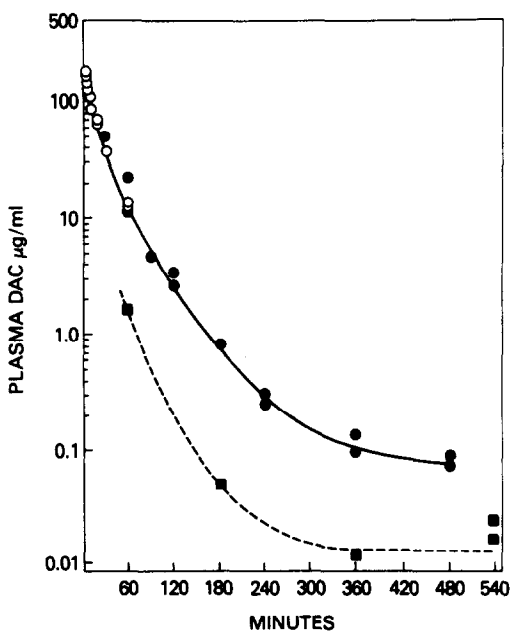

Fig. 2. Plasma concentrations of DAC in CDF$_1$ mice following i.v. bolus injection of 100 mg/kg (○, ●) *or* 10 mg/kg (■) [6-^{3}H]-*DAC (dark symbols) or unlabelled DAC (light symbols). Non-linear regression fit of the* 100 mg/kg *data (solid line) to a triexponential equation gave half-lives of* 11, 32 *and* 365 min.

concentration the relative increase in area from 1 to 24 hr is substantially greater than the relative increase from 24 to 120 hr. For example, at 1.0 μg/ml the area increases from 0.98 to 15.4 μg·hr/ml over 1 to 24 hr (15-fold) whereas the area increase is from 15.4 to 25.0 μg·hr/ml over 24 to 120 hr (2-fold)

Pharmacokinetics of DAC in CDF mice

Following an i.v. bolus of DAC (100 mg/kg or 300 mg/m^2), plasma concentration achieved a peak value near 130 μg/ml and then declined in a triexponential manner (Fig. 2). A non-linear regression fit of the data to (2) yielded an initial $t_{1/2}$ of 11.4 min, followed by intermediate and terminal phases of 31.5 min and 365 min respectively. At 4 hr plasma levels of DAC were already below the optimal concentration (0.5 μg/ml; Fig. 1) for maximal *in vitro* cytotoxicity. When the dose was reduced to 10 mg/kg, a similar triexponential plasma decay was observed which was proportional to the 100 mg/kg data. These results suggest that within this range of doses, DAC does not display saturation kinetics.

Pharmacokinetic parameters derived from the 100 mg/kg data are summarized in Table 1. Urinary clearance of intact drug (7.27 ml/min/kg) accounts for 28.5% of plasma clearance, which was 25.5 ml/min/kg or 76 ml/min/m^2. Excretion of DAC and its degradation products is rapid, with maximal cumulative urinary recovery generally achieved within 1 hr after administration. Degraded products represent about 55% of the total radioactivity recovered. Initial experiments indicate that urinary recovery of DAC is lower by an order of magnitude following 10 mg/kg as compared to the higher dose, which suggests saturation of a renal reabsorption process at higher DAC concentrations.

The *in vitro* cytotoxicity data indicate DAC is optimally effective at concentrations of 0.5–1.0 g/ml for 24 hr (Fig. 1), and suggests administration of the drug *in vivo* by infusion. Appropriate infusion rates (0.89–2.7 mg/kg/hr) were calculated and experiments were done to achieve plasma concentrations in the range 0.5–1.0 μg/ml in mice (Fig. 3). The solid line represents theoretical values at the highest

*Table 1. Pharmacokinetic parameters for DAC in mice**

$t_{1/2}^{\alpha} = 11.4$ min	$V_c = 594$ ml/kg
$t_{1/2}^{\beta} = 31.5$ min	$CL_p = 25.5$ ml/min/kg
$t_{1/2}^{\gamma} = 365$ min	$CL_r = 7.27$ ml/min/kg
AUC_{∞} 65 μg·hr/ml	$(CL_r/CL_p) \times 100 = 28.5\%$

*Determined following 100 mg/kg i.v.

Table 2. Dose response of DAC with single-dose treatment of advanced L1210 tumor in mice

Treatment and dose*	Toxic day of death	% Body weight (+, gain; -, loss)	Tumor day of death†	Median day of death Toxic	Median day of death Tumor	Survivors at 60 days	Log cell Δ
Control							
$L1210^{i.v.}$ 1 × 10^4 cells	-	-0, +11	8^6	-	8	0	+5
$DAC^{i.v.}_{225}$ d3q × 1	-	-7, +7	20,24,25,27 34,51	-	25	0	-6
$DAC^{i.v.}_{150}$ d3q × 1	-	-5, +3	20^2,22,23 24,26	-	22	0	-6
$DAC^{i.v.}_{100}$ d3q × 1	-	-0, +18	20^4,21,22	-	20	0	-6
$DAC^{i.v.}_{50}$ d3q × 1	-	-0, +13	20^4,22,35	-	20	0	-
$DAC^{i.v.}_{25}$ d3q × 1	-	-5, +8	18^2,20,24 28,31	-	20	0	-6
$DAC^{i.v.}_{12.5}$ d3q × 1	-	-0, +8	14,15^3,16,17	-	15	0	-5
$DAC^{i.v.}_{6.25}$ d3q × 1	-	-0, +6	12^6	-	12	0	-3
Control							
$L1210^{i.v.}$ 1 × 10^4 cells	-	-0, +20	7^5,8	-	7	0	+5
$DAC^{i.v.}_{50}$ d5q × 1	-	-7, +4	19^4,20,21	-	19	0	-7
$DAC^{i.v.}_{10}$ d5q × 1	-	-9, +0	11,12^2,13^2,14	-	12	0	-3
$DAC^{i.v.}_{1}$ d5q × 1	-	-0, +3	9^5,10	-	9	0	-1

*$DAC^{route}_{dose\ (mg/kg)}$ $dn_1q \times n_2$, where dn_1 is day of treatment following tumor administration on day 0 and n_2 is the number of times the dose was given.

†Superscript refers to number of mice.

infusion rate (2.7 mg/kg/hr) and the broken line represents the lower limit of expected values (0.89 mg/kg/hr). The slight decline of plasma DAC from 2 to 24 hr is due to the spontaneous degradation of DAC in the Alzet minipump.

Chemotherapeutic studies in L1210 bearing mice

To compare the *in vitro* cytotoxic effects of DAC with its *in vivo* therapeutic activity over a range of concentrations and exposure times, we

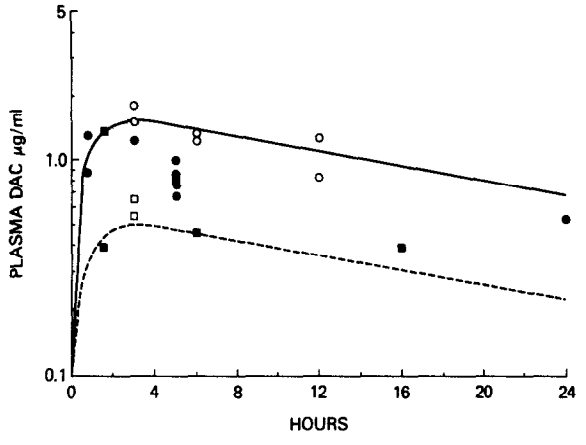

Fig. 3. Plasma concentrations of DAC in CDF_1 mice during continuous s.c. infusion by implanted Alzet minipumps. Initial infusion rates (mg/kg/hr): 2.7 (○); 1.7 (●); 1.4 (■); 0.89 (□). *Predicted plasma levels (C_{ss}) for* 2.7 mg/kg/hr *(solid line) and* 0.89 mg/kg/hr *(dashed line) were calculated according to the equation C_{ss} = infusion rate/plasma clearance with corrections made to account for the spontaneous degradation of DAC within the minipump.*

conducted single-dose, multiple-dose and infusion experiments in L1210 tumor-bearing mice. The results of DAC therapy over a wide range of single i.v. doses is presented in Table 2 for two degrees of staging of the tumor (treatment 3 and 5 days after innoculation). The maximum estimated cell kill (7 logs) is observed with day 5 treatment at the highest dose tested (50 mg/kg). When the dose is reduced to 10 mg/kg (day 5 treatment) cell kill is equivalent to that obtained *in vitro* (3 logs), and the *in vivo* AUC (6.5 µg·hr/ml) is about equal to the minimum *in vitro* AUC (7.7 µg·hr/ml) required to produce this degree of cytotoxicity (Table 3). These data may at first suggest a proportionality between AUC and cell kill which is similar in the *in vitro* and *in vivo* systems. However, when the relationship between AUC and cytotoxicity is examined over a range of *in vitro* concentrations and exposure times and *in vivo* doses and routes of administration (Table 3), the concept of proportionality between area (AUC) and cytotoxicity does not hold. If a comparison is made within the *in vivo* experiments, it is evident that infusion (2 mg/kg/hr) for 8 hr is considerably more cytotoxic than a single i.v. dose (10 mg/kg), although they produce a similar AUC. Increasing the i.v. dose to 50 mg/kg produces a cell kill equivalent to that of the infusion but represents a much larger AUC. Much of this area is a result of irrelevantly high concentrations. These results suggest that duration of exposure around a concentration of

Table 3. Area relationships for in vivo *and* in vitro *DAC cytotoxicity*

		DAC exposure as AUC (μg·hr/ml)	Estimated log cell kill
*In vivo**	50 mg/kg i.v. × 1	33	7
	10 mg/kg i.v. × 1	6.5	3
	10 mg/kg i.v. q3h × 4	26	7
	2 mg/kg/hr s.c. × 8 hr	7	6
In vitro†	1.0 μg/ml × 8 hr	6.8	1/2
	0.5 μg/ml × 24 hr	7.7	3
	1.0 μg/ml × 24 hr	15.4	3
	10 μg/ml × 120 hr	250	4
	100 μg/ml × 120 hr	2500	4

*AUC calculated from plasma $c \times t$ curve.
†AUC values corrected for degradation of DAC at 37°C.

0.5-1.0 μg/ml is an important determinant of DAC cytotoxicity *in vivo*. It also appears that the maximum cytotoxic effect *in vivo* (7 logs) is superior to that achievable *in vitro* (4 logs).

It is apparent from Fig. 1 that an exposure time of 24 hr or longer to 0.5 μg/ml DAC would be most desirable for maximum cell kill of L1210 cells. The *in vivo* toxicity studies in Table 4 indicate why such exposures are not reasonable *in vivo*. The maximal tolerable exposure time for mice at approximately 1 μg/ml is between 12 and 15 hr. Longer times of exposure near this concentration are lethal to host mice and concentrations above 1.0 μg/ml are lethal at exposure times of 12 hr or less.

When DAC is given *in vivo* on a multiple dose schedule it is again evident that exposure time is important in determining cytotoxicity to L1210. These results are summarized in Table 5 for experiments in which treatment was initiated 1 day after tumor inoculation. Note that a single dose of DAC at 10 mg/kg gave a median life span of 13 days and an estimated cell kill of 3 logs. Repeating this dose every 3 hr increased the cytotoxic effect of L1210 *in vivo* as indicated by increases in median life span, cell kill and long-term survivors. Our pharmacokinetic studies indicate that a single dose of 10 mg/kg would result in a plasma DAC concentration declining from 10 to 0.05 μg/ml in 3 hr. Yet the *in vitro* data (Fig. 1) indicate that concentrations as high as 100 μg/ml for 3 hr result in less than 1 log of cell kill. There is thus a greater cell kill estimated *in vivo* than would have been expected from the *in vitro* results. The multiple dose experiments (approximately 9-12 hr of exposure to median concentrations of 1 μg/ml) also suggest that the *in vivo* estimated cell kill (4.5 logs) is greater than *in*

Table 4. Toxicity resulting from infusion of 5-aza-2′-deoxycytidine in normal mice

Time of infusion (hr)	Dose (mg/kg/hr)	Est. C_{ss} (μg/ml)	Effect
48	0.5	0.4	LD_{30}
24	1.0	0.7	LD_{90}
16	2.4	1.6	LD_{30}
15	1.4	1.0	LD_0*
12	2.8	1.9	LD_{80}†
12	2.4	1.6	LD_{10}
12	1.4	1.0	LD_0
8	5.8	4.1	LD_{40}‡

*Data from [8].
†Data from Momparler and Firth *Drug Chem Toxicol* 1981, 4, 373-381).
‡Data from [2].

Table 5. Effects of exposure time to DAC on L1210 tumor (early staging) in mice

Treatment and dose*	Toxic day of death	% Body weight (+, gain; -, loss)	Tumor day of death	Median day of death Toxic	Median day of death Tumor	Survivors at 60 days	Log cell Δ	Comments
Control								
$L1210^{i.v.}$ 1 × 10^4 cells	-	-3, +15	8[5],14	-	8	0/6	+5	
$DAC_{10}^{i.v.}$ dlq × 1	-	-3, +9	13[5],20	-	13	0/6	-3	
$DAC_{10}^{i.v.}$ dlq3hr × 2	-	-9, +5	16,20[2],22,27	-	20	1/6	-4.5	
$DAC_{10}^{i.v.}$ dlq3hr × 3	-	-16, +20	44[2]	-	44	4/6	-4.5	note weight loss
$DAC_{10}^{i.v.}$ dlq3hr × 4	8[2]	-21, +30	34	8	34	3/6	-4.5	toxic deaths

*See footnote to Table 2; q3hr × n indicates the dose was given n times every 3 hr.

vitro cell kill (Fig. 1) at similar concentrations and exposure times.

Table 6 tabulates the results of multiple dose therapy with a more advanced tumor (treatment 5 days after tumor inoculation). Again, prolongation of exposure time to DAC increases the estimated cell kill, although no 'cures' of this advanced tumor were seen. The 7 log cell kill obtained in these experiments with treatment on day 5 indicates that the *in vivo* estimate of cytotoxicity upon day 1 treatment (maximum 4.5 log cell kill) (Table 5) was limited by the total tumor burden estimated to be present at that time.

In order to produce sustained plasma levels of DAC in mice, chemotherapy experiments were performed using Alzet minipumps. The results of this therapy are presented in Table 7, together with single- and multiple-dose data for comparison. Infusion of DAC at 2 mg/kg/hr for 8 hr ($C_{ss} \approx 1\ \mu$/ml) 3 days following tumor implant yields a median survival time (23 days) about equivalent to the optimal single dose of 225 mg/kg (25 days). If the infusion is extended to 12 hr, the median time of death is 36 days, the longest survival seen with late (day 3–5) treatment. Host toxicity becomes apparent as the infusion is extended, with marked weight loss and decreased median survival time after a 16 hr infusion.

DISCUSSION

In this paper we have confirmed the potent *in vitro* and *in vivo* cytotoxic effects of DAC on L1210 and have extended these findings by relating concepts of exposure time and concentration *in vitro* and the pharmacokinetics of DAC in mice to its cytotoxicity. *In vitro*, DAC produced a maximum of 3–4 logs cell kill which was concentration- and time-dependent.

Following i.v. administration of DAC to mice, the drug is cleared from plasma in a triexponential manner with an intermediate elimination half-life of 31.5 min. This value is in reasonable agreement with a half-life of 41 min reported in

Table 6. Effects of exposure time to DAC on L1210 tumor (advanced staging) in mice

Treatment and dose*	Toxic day of death	% Body weight (+, gain; -, loss)	Tumor day of death	Median day of death Toxic	Median day of death Tumor	Survivors at 60 days	Log cell Δ	Comments
Control								
$L1210^{i.v.}$ 1 × 10^4 cells	-	-0, +6	7^2,8^4	-	8	0	+5	
$DAC_{10}^{i.v.}$ d5q × 1	-	-9, +0	11,12^2,13^2,14	-	12	0	-3	weight loss; no gain
$DAC_{10}^{i.v.}$ d5q3hr × 2	-	-7, +4	20^2,21,23^3	-	21	0	-7	
$DAC_{10}^{i.v.}$ d5q3hr × 3	-	-0, +6	20,21^4	-	21	0	-7	
$DAC_{10}^{i.v.}$ d5q3hr × 4	-	-13, +8	21,22^3,23^2	-	22	0	-7	weight loss

*See footnote to Table 5.

Table 7. Effects of a variety of doses and schedules on day 3 staged L1210 tumor in mice

Treatment and dose*	Toxic day of death	% Body weight (+, gain; -, loss)	Tumor day of death	Median day of death Toxic	Median day of death Tumor	Survivors at 60 days	Log cell Δ	Comments
Control								
$L1210^{i.v.}$ 1 × 10^4 cells	-	-0, +5	7^3,8	-	7	0	+5	
$DAC_{10}^{i.v.}$ d3q3hr × 3	-	-5, +2	23,24,25,36 37,45	-	25	0	-6	
$DAC_{10}^{i.v.}$ d3q3hr × 4	59	-11, +5	26,27,30^2,52	59	30	0	-6	
$DAC_{Alza}^{s.c.}$ d3 2 mg/kg/hr × 8 hr	-	-4, +5	20^2,23,24,27 30	-	23	0	-6	
$DAC_{Alza}^{s.c.}$ d3 2 mg/kg/hr × 12 hr	-	-19, +6	27, 28, 36^2,50 59	-	36	0	-6	large weight loss
$DAC_{Alza}^{s.c.}$ d3 2 mg/kg/hr × 16 hr	13^2	-24, +6	24,25,26,43 30	13	25	0	-6	toxic
$DAC_{50}^{i.v.}$ d3q × 1	-	-0, +7	18^2,19^3,24	-	19	0	-6	
$DAC_{150}^{i.v.}$ d3q × 1	-	-5, +3	20^2,22,23 24,26	-	22	0	-6	
$DAC_{225}^{i.v.}$ d3q × 1	-	-7, +4	20,24,25,27 34,51	-	25	0	-6	

*See footnote to Table 5.

mice by Momparler and Gonzales [2] using a bioassay instead of HPLC. Plasma concentrations during s.c. infusion agreed well with values predicted using pharmacokinetic parameters obtained from an i.v. bolus dose. Mice rapidly degrade DAC, but some is excreted intact in the urine. There appears to be reabsorption of DAC by the kidneys which may be saturable at high doses.

An observation attracting our attention is the contrast between the *in vitro* and *in vivo* effects of DAC on L1210 viability. The maximal cell kill estimated *in vivo* (7 logs) was much greater than that estimated *in vitro* (4 logs). An 8-hr infusion *in vivo* that resulted in approximately 1 μg/ml plasma concentration gave an estimated 6 log cell kill, while a similar exposure *in vitro* produced less than a 1 log cell kill. It is possible that these observations result from the method used to estimate *in vivo* cytotoxicity. Our cell kill values are derived from median day of death extrapolations using log cell titration assays according to the method of Schabel *et al.* [13]. Overestimates of cell kill will occur if cells surviving drug treatment are delayed in their growth or grow more slowly than the control population. Clonogenic studies with DAC have shown that this drug does not markedly delay or slow down the growth of surviving L1210 cells *in vitro* [12]. An alternative explanation for the greater *in vivo* effect of DAC may be its effects on the immune system of host mice leading to augmentation of tumor cell kill. This explanation may have some validity because of the reported effects that DAC has on cell differentiation [17].

Through the use of pharmacokinetic data we were able to achieve *in vivo* the optimal DAC concentrations (0.5-1.0 μg/ml) observed to produce maximum L1210 cell kill *in vitro*. The optimal exposure time suggested from *in vitro* studies was not possible *in vivo* because of host toxicity limitations. Although *in vivo* treatment results in many cures (>60-day survivors) when DAC was given early after tumor implant, no cures were seen if treatment was delayed.

In these experiments the initial tumor quantity administered intravenously was 1×10^4 L1210 cells. With treatment given 1 day after tumor administration the estimated tumor burden would be 4×10^4 cells at the time of treatment. This relatively low tumor burden is the reason for the many long-term survivors, 'cures', seen in Table 5. With later treatment the estimated tumor burden would be higher and the chance for more extensive metastasis greater at the time of treatment. For example, if treatment is delayed, estimated tumor burden is 1×10^6 cells on day 3 and 3×10^7 cells on day 5. Even though cell kills as high as 7 logs were estimated with these experimental conditions, no long-term survivors were obtained (Tables 6 and 7). These findings indicate that there is a population of L1210 cells refractory to DAC. There are several potential determinants of this refractoriness: biochemical resistance (altered allosteric control of deoxycytidine kinase), resistant mutants (lack of deoxycytidine kinase), slowly growing tumor cells or cells residing in pharmacological sanctuaries. Experiments using either high-dose thymidine or the *de novo* pyrimidine synthesis inhibitor 3-deazauridine in combination with DAC demonstrated no additional therapeutic benefit, which tends to discount the first two determinants of refractoriness [18]. The observation that early treatment but not late treatment produces cures supports the idea of a pharmacological sanctuary, since a delay in treatment may allow time for tumor cells to metastasize to such sites. Of course a delay may also allow time for resistant mutants to arise. We are currently investigating the growth kinetics of cells treated *in vivo* to determine what role slow-growing cells may play in the refractory nature of advanced L1210 to DAC therapy.

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