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Adozelesin, a potent new alkylating agent: cell-killing kinetics and cell-cycle effects

Bijoy K. Bhuyan[1], Kathy S. Smith[1], Earl G. Adams[1], Tanya L. Wallace[1], Daniel D. Von Hoff[2], and Li H. Li[1]

[1] Cancer and Infectious Diseases Research, The Upjohn Company, Kalamazoo, MI 49001, USA
[2] Division of Oncology, University of Texas, Health Science Center, San Antonio, TX 78284, USA

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Summary. Adozelesin (U-73975) was highly cytotoxic to V79 cells in culture and was more cytotoxic than several clinically active antitumor drugs as determined in a human tumor-cloning assay. Phase-specificity studies showed that cells in the M + early G1 phase were most resistant to adozelesin and those in the late G1 + early S phase were most sensitive. Adozelesin transiently slowed cell progression through the S phase and then blocked cells in G2. Some cells escaped the G2 block and either divided or commenced a second round of DNA synthesis (without undergoing cytokinesis) to become tetraploid. Adozelesin inhibited DNA synthesis more than it did RNA or protein synthesis. However, the dose needed for inhibition of DNA synthesis was 10-fold that required for inhibition of L1210 cell growth. The observation that cell growth was inhibited at doses that did not cause significant inhibition of DNA synthesis and that cells were ultimately capable of completing two rounds of DNA synthesis in the presence of the drug suggests that adozelesin did not exert its cytotoxicity by significant inhibition of DNA synthesis. It is likely that adozelesin alkylates DNA at specific sites, which leads to transient inhibition of DNA synthesis and subsequent G2 blockade followed by a succession of events (polyploidy and unbalanced growth) that result in cell death.

Introduction

Adozelesin (U-73975) is a synthetically derived analog of the highly potent, alkylating, antitumor antibiotic CC-1065 [6]. CC-1065 binds nonintercalatively in the minor groove of double-stranded DNA at A-T-rich regions and subsequently undergoes covalent bonding with N-3 of adenine in preferred base sequences [9, 19]. Although CC-1065 has shown moderate antitumor activity in vivo, it has not been evaluated clinically due to the delayed deaths it caused in mice at therapeutic doses [16]. Since CC-1065's receptor (the A-T-rich region in the minor groove of DNA) has been identified and the alkylating cyclopropapyrroloindole (CPI) moiety has been synthesized, it has been speculated that the attachment of ligands of appropriate shape and length to the CPI moiety would optimize hydrophobic interactions with the DNA minor groove and yield potent analogs [10, 22, 23]. Adozelesin is one of these analogs that shows excellent broad-spectrum antitumor activity in vivo without causing delayed deaths [14]. On the basis of its superior solubility and stability in aqueous formulations, its in vivo antitumor activity, and its unique mechanism of action, adozelesin has been taken to phase I clinical trials. We report herein the in vitro cell-killing kinetics of adozelesin, its cytotoxicity for human tumor biopsies, and its cell-cycle effects. Parts of this report have been presented elsewhere as an abstract [17].

Materials and methods

Drug. Adozelesin (U-73975, mol. wt. 502.2) was synthesized at The Upjohn Company (Kalamazoo, Mich. USA) [10]. Stock solutions (1 mg/ml in dimethylacetamide) stored in a dark environment in a freezer remained chemically stable for at least 1 year. The drug was diluted in medium immediately prior to its addition to cell cultures. Based on cytotoxicity measurements, adozelesin remained stable in medium for at least 24 h at 37° C.

Cell culture. The following sources supplied the indicated cell lines: V79 (Chinese hamster lung fibroblast), Dr. R. Durand (British Columbia Cancer Research Center, Vancouver, Canada); CHO/WBL (clone of CHO-K1), Mr. R. Yu (The Upjohn Company); B16 (mouse melanoma), Dr. I. J. Fidler (M. D. Anderson Hospital and Tumor Institute, Houston, Tex.); and A2780 (human ovarian carcinoma), Dr. R. Ozols (National Cancer Institute, Md.). V79 cells were grown in Eagle's minimum essential medium (MEM) supplemented with essential MEM vitamins (10 ml of 100×), MEM nonessential amino acids (10 ml of 100×), 2 mM L-glutamine, and 15% fetal bovine serum. L1210 and A2780 cells were maintained in RPMI 1634 medium supplemented with fetal calf serum (5%), $NaHCO_3$ (0.75 mg/ml), penicillin (0.1 mg/ml), and streptomycin (0.05 mg/ml). The media used for growing CHO and B16 cells have been

Offprint requests to: B. K. Bhuyan, Cancer and Infectious Diseases Research, The Upjohn Company, Kalamazoo, MI 49001, USA

Table 1. Cytotoxicity of adozelesin and several clinically active drugs

Compounds	LD_{90} nm (ng/ml)[a]								
	Chinese hamster				Mouse				Human
	V79		CHO		L1210		B16		A2780
	Continuous[b]	2 h[b]	Continuous	2 h	Continuous	2 h	Continuous	2 h	2 h
U-73975	0.032 (0.016)	0.46 (0.23)	0.09	0.48	0.006	0.06	0.03	0.3	0.048
Adriamycin	50	258							
Actinomycin D	≅5								
Vinblastine	3.4								
Cisplatin	510	22,700							

[a] LD_{90} (90% cell-kill dose) values were estimated from dose-survival curves in which survival was determined by a cloning assay. Values shown within parentheses are expressed in ng/ml

[b] Cells were exposed to drug either for 2 h or continuously during colony formation

described elsewhere [4]. Adherent cultures (V79, CHO, B16, A2780) were harvested with 0.1% trypsin (type III; Sigma, St. Louis, Mo.) plus 0.02% ethylenediaminetetraacetic acid (EDTA; Eastman Kodak Co., Rochester, NY) in 0.9% NaCl solution and were maintained in exponential growth by subculturing prior to confluency.

Cell survival after drug exposure. B16, V79, CHO, and A2780 cells were planted as monolayers and L1210 cells, as suspension cultures at 24–48 h before an experiment to ensure that the cells were in exponential growth during drug exposure. For each drug concentration tested, two separate cultures were used. After drug exposure, cell survival was determined by colony-forming assay as previously described [2, 4]. The cloning efficiency of untreated (control) cells was normalized to 100%, and that of the treated cells was expressed as a percentage of control survival. The coefficient of variation (standard deviation expressed as a percentage of the mean) in determining cell survival was about 15% within each experiment. All experiments were repeated at least once.

Human tumor-cloning assay. The drug sensitivity of cells obtained from fresh human tumor biopsies was determined by a colony-forming assay [4].

Preparation of synchronous V79 cultures. Synchronous cultures were started from mitotic cells selectively harvested after a 2-h treatment with 0.033 μg colcemid/ml [5]. For determinations of phase-specific toxicity, mitotic cells were planted; when they had reached different phases of the cell cycle, they were exposed to drug for 1 h at 37° C, and cell survival was determined.

Growth-inhibition assay. V79 cell monolayers (about 10^5 cells/75 cm^2) were exposed for varying periods to the drug and the cells were then harvested and counted. The percentage of growth inhibition was calculated as:

$$100 - 100 \times \left(\frac{\text{number of cells in treated flask at 72 h} - \text{cells inoculated}}{\text{number of cells in control flask at 72 h} - \text{cells inoculated}} \right).$$

Macromolecule-synthesis inhibition. Logarithmically growing L1210 cells ($3-4 \times 10^5$ cells/ml) were incubated with drug for 1–5 h, following which they were centrifuged, washed twice, and resuspended in warm medium. For determinations of the subsequent effect on cell growth, cells were planted at 5×10^3/ml and counted after 3 days. In experiments conducted to determine the effect on macromolecule synthesis, cells were incubated for 45 min with [methyl-^{3}H]-thymidine, [5-^{3}H]-uridine, or D,L-[1-^{14}C]-leucine, after which the incorporation of radioactivity into DNA, RNA, or protein was determined [13].

DNA staining and flow cytometry. Cells fixed in ethanol and stained with mithramycin (MMC) [1] were analyzed for DNA content on a Becton Dickinson FACStarPLUS fluorescence-activated cell sorter [FACS; Becton Dickinson Immunocytometry Systems (BDIS), San Jose, Calif.]. Cells were illuminated by the 456.9-nm line of an argon ion laser at 150 mW. Illumination-beam geometry was elliptical (20×100 μm). A 515-nm long-pass Schott glass filter collected MMC fluorescence, and 456.9-nm 3C band-pass filters collected narrow-forward-angle and wide-angle light scatter. Data from the FACStarPLUS were acquired in the list mode on a VAXstation II/GPX computer using CONSORT/VAX software (BDIS). The following data were collected for about 4×10^4 cells: forward-scatter pulse height, side-scatter pulse height, mithramycin-fluorescence pulse area, and mithramycin-fluorescence pulse width.

Flow-cytometry list-mode data were analyzed using CONSORT/VAX software to eliminate subcellular debris and cell clumps. Subcellular debris was eliminated by gating raw list-mode data based on an operator-drawn region in forward-versus wide-angle light scatter. Cell clumps were eliminated by gating scatter-gated list-mode data based on an operator-drawn region in mithramycin-fluorescence pulse area versus pulse width. Single-parameter DNA histograms produced from the list-mode data were analyzed for cell-cycle-phase distribution using the program MODFIT (Verity Software House, Inc., Topsham) on an IBM PC/AT computer.

Results

Cytotoxicity of adozelesin for cell lines and human tumor biopsies

Table 1 shows the much higher cytotoxic potency of adozelesin as compared with several clinically active agents. The adozelesin dose that was lethal to 90% of the V79 cells population (LD_{90}) undergoing continuous drug exposure was 0.032 nM (0.016 ng/ml), and the potency of adozelesin was about 100-, 1,700-, and 17,000-fold that of vinblastine, Adriamycin, and cisplatin, respectively. The LD_{90} value for adozelesin in V79 cells exposed for 2 h was 0.46 nM (0.23 ng/ml) as compared with 258 and 22,700 nM for Adriamycin and cisplatin. Table 1 also compares the lethality of adozelesin for several rodent cell lines (V79, CHO, L1210) with its cytotoxicity to the human ovarian carcinoma A2780 cell line. Adozelesin was much more potent against human A2780 than against the rodent cell lines.

The lethality of adozelesin in the human tumor-cloning assay was compared with that of several clinically active

Table 2. Lethality for human tumors of adozelesin and several clinically active drugs

Compound	Tumor type	Number of sensitive tumors[a] / Number of tumors tested			
		Continuous[b]		1 h[b]	
		0.1	0.5	1	5
Adozelesin	Bladder	1/2	1/2	0/2	1/2
	Breast	1/11	4/11	2/13	7/13
	Colon	2/7	6/7	3/8	6/8
	Corpus uteri	0/1	0/1	0/1	0/1
	Head-neck	0/1	1/1	0/1	1/1
	Kidney	1/7	4/7	1/7	4/7
	Liver	0/1	0/1	0/1	1/1
	Lymphoma			1/1	1/1
	Melanoma	4/6	4/6	3/7	5/7
	Non-small-cell lung	3/11	4/11	2/11	7/11
	Ovary	1/3	2/3	2/4	3/4
	Stomach	2/2	2/2	1/2	2/2
Totals		15/52	28/52	15/58	38/58
Isophosphamide		9/23[c]			
Vinblastine				6/35[c]	
Adriamycin				2/31	
Mitomycin C				3/28	
VP-16				4/17	
Bleomycin				1/8	
Methotrexate				0/8	
5-Fluorouracil				2/22	

[a] Tumors were considered to be sensitive if the percentage of survival was <50%

[b] Continuous exposure to 0.1 or 0.5 ng/ml adozelesin. For 1 h exposure, 1 or 5 ng/ml was used

[c] Exposure to isophosphamide was continuous at 1 mg/ml. Cells were exposed to the other agents for 1 h (in ng/ml): vinblastine, 50; Adriamycin, 40; mitomycin C, 100; VP-16, 3,000; bleomycin, 200; methotrexate, 300; 5-fluorouracil, 6,000

antitumor drugs (Table 2). When a drug decreases the survival of tumor colony-forming units (T-cFU) to ≤50%, this assay predicts for clinical activity [21]. Drug concentrations selected for testing in this assay are usually 1/10 of the maximal plasma level in humans. In the absence of human data for adozelesin, we extrapolated from mouse data. The highest plasma level obtained in mice injected with the maximum tolerated dose (100 μg/kg) was about 450 ng/ml (Hamilton and Li, unpublished results). If the human level is even 1/10 of this value (i.e., 45 ng/ml), then it is acceptable to test 5 ng/ml in assay. Continuous exposure to an adozelesin concentration of 0.5 ng/ml was cytotoxic to 28 of 52 tumors tested; 1 h exposure to 5 ng/ml was cytotoxic to 38 of 58 tumors. The sensitivity of the same tumor samples to several clinically active antitumor drugs was as follows (sensitive tumors/number of tumors tested); isophosphamide, 9/23; vinblastine, 6/35; doxorubicin, 2/31; mitomycin C, 3/28; VP-16, 4/17; bleomycin, 1/8; 5-fluorouracil, 2/22; and methotrexate, 0/8 (Table 2). Following both continuous and 1 h incubation, higher percentages of the tumors tested were sensitive to adozelesin as compared with the other agents; significant antitumor activity was observed (≈25% of the tumors were sensitive),

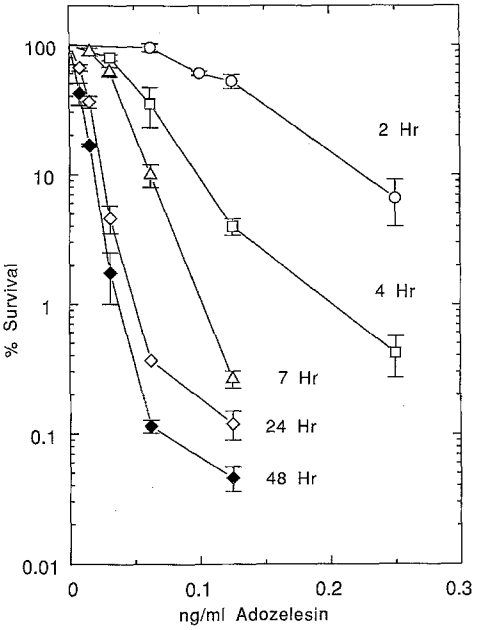

Fig. 1. Dose-survival curves generated for cells exposed to adozelesin for periods ranging from 2 to 48 h. Survival was determined by a colony-forming assay

even following exposure to a lower concentration of 1 ng/ml for 1 h or after continuous exposure to 0.1 ng/ml.

Time course of inhibition of V79 cell survival

Figure 1 shows the dose-survival response of cells exposed to adozelesin for 2, 4, 7, 24, or 48 h. In all cases, sigmoidal dose-survival curves were obtained that were characterized by an initial shoulder followed by an exponential (log-linear) decline in survival with increasing dose. As exposure duration increased, the initial shoulder (supposedly due to repair of sublethal damage) decreased and the dose-survival curves became steeper. LD_{50} values (2 h, 0.13 ng/ml; 4 h, 0.05 ng/ml; 7 h, 0.033 ng/ml; 24 h, 0.01 ng/ml; 48 h, 0.005 ng/ml) estimated from dose-response curves were used to calculate the concentration × time (cxt) factor needed to kill 50% of the cells. The cxt values (0.24 ± 0.02 ng ml^{-1} h) remained essentially constant for up to 48 h.

Phase-specific toxicity

The lethality of adozelesin for V79 cells in different phases of the cell cycle is shown in Fig. 2. The position of cells in the cell cycle at the time of drug exposure was determined either by flow cytometry or according to the lethality of cytosine arabinoside (Ara-C) or high-specific-activity (20 Ci/mmol) [^{3}H]-thymidine. Ara-C and [^{3}H]-thymidine kill only S-phase cells and thus demarcate the G1 and G2 phases from the S phase. Figure 2A shows minimal lethality for Ara-C over 3 h which suggests that cells progressed from mitosis into the G1 phase, in which they remained until about 3 h. At between 4 and 5 h, 90% of the cells

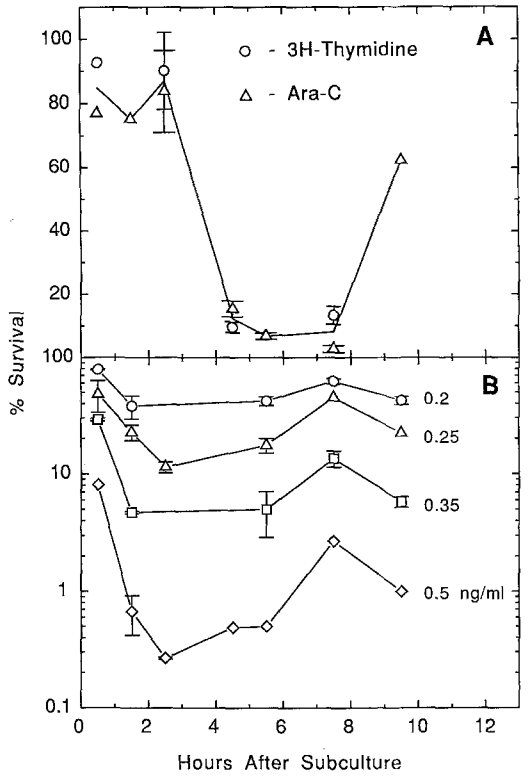

Fig. 2 A, B. Cytotoxicity of adozelesin, cytosine arabinoside (*Ara-C*), and high-specific-activity [^{3}H]-thymidine for cells in different cell-cycle phases. Selectively harvested mitotic cells were planted, and cells progressed into the G1 and S phases at different times after planting. When the cells had reached different points of the G1 and S phases, they were exposed to drug for 1 h. The time points shown lie halfway between the exposure periods; e.g., survival of cells following exposure to drug for 0–1 h is indicated at 0.5 h. **A** Cells exposed to Ara-C (100 μg/ml) and [^{3}H]-thymidine (10 μCi/ml). **B** Cells exposed to adozelesin at the concentrations indicated

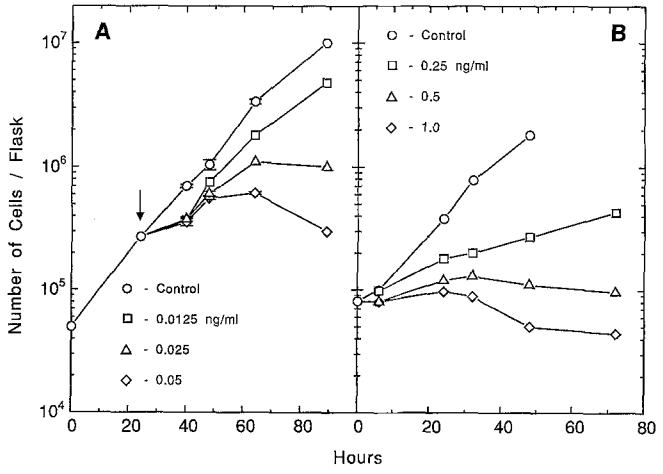

Fig. 3 A, B. Growth curve generated for cells exposed to adozelesin (**A**) continuously or (**B**) for 2 h. After 2 h exposure, monolayer cultures were washed and then incubated in fresh medium. The *arrow* indicates the addition of adozelesin to cell cultures

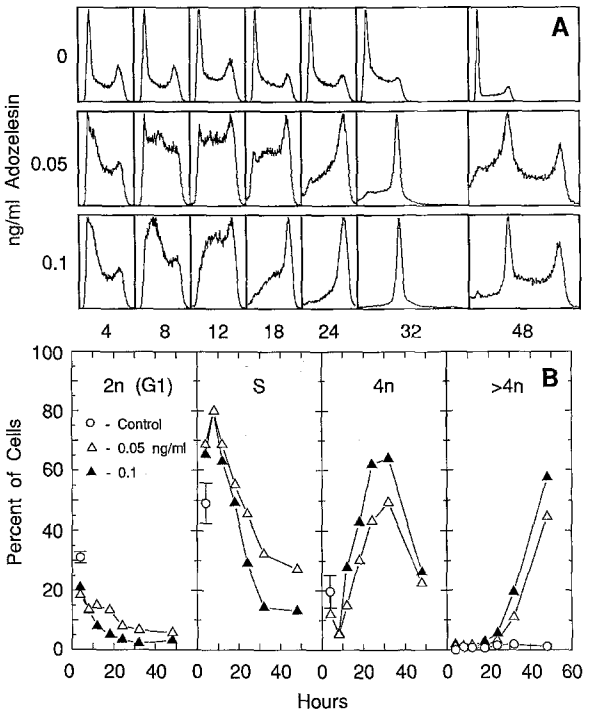

Fig. 4 A, B. Cell progression during continuous exposure to adozelesin. **A** DNA histograms of cells samples at different times during continuous exposure to the drug at 0.05 and 0.1 mg/ml. **B** Quantitative analysis of the DNA histograms shown in **A**. Diploid cells in the G1, S and G2-M phases contain 2n, 2n–4n, and 4n DNA, respectively; polyploid cells contain >4n DNA

entered the S phase and were killed on their exposure to Ara-C or [^{3}H]-thymidine. Cells stayed in the S phase until about 7.5 h and then progressed into G2 and M such that by 9.5 h, 68% of the cells had reached the G2-M phase. The results obtained from DNA histograms (not shown) agreed with those obtained after Ara-C or [^{3}H]-thymidine exposure. Figure 2B shows the sensitivity of cells in different phases to different concentrations of adozelesin. The results clearly demonstrate that cells in the M + early G1 phase (0–1 h) were most resistant to adozelesin whereas those in the late G1 (2–3 h) and the mid-S (5–6 h) phases were most sensitive and those in the late S phase displayed intermediate sensitivity.

Time course of growth inhibition

The inhibition of cell growth during continuous exposure to adozelesin is shown in Fig. 3 A. Cell growth was inhibited at all three doses tested, but the cultures treated with different doses grew at about the same rate for the first 24 h of drug exposure, after which growth inhibition was dose-dependent. Even at the highest dose (0.05 ng/ml), growth was not completely inhibited until 40 h (i.e., 64 h in Fig. 3 A) of drug exposure. The estimated 50% and 90% growth-inhibitory concentrations were 0.012 and 0.024 ng/ml, respectively, which corresponds well with the LD_{50} and LD_{90} values obtained in survival experiments. Figure 3B shows the growth-inhibition pattern obtained after 2 h drug exposure. At 0.25 ng/ml, the cells grew

continuously, albeit at a reduced rate, resulting in 89% growth inhibition. At 1 ng/ml, almost complete inhibition of growth was observed, and the number of cells declined after 32 h. The estimated 50% and 90% growth-inhibitory concentrations were 0.055 and 0.125 ng/ml, respectively. Only a 10-min exposure to adozelesin caused subsequent growth to be inhibited by 40%, which suggests that the drug binds to cells rapidly. However, in spite of this rapid binding, the maximal growth-inhibitory effect of the drug was not expressed until several hours later as seen from the above-mentioned experiments.

Inhibition of V79 cell progression

Cell progression was measured during continuous exposure to 0.1 and 0.05 ng/ml, which inhibited growth by 97% and 91%, respectively (Fig. 3A). Figure 4A shows the DNA histograms of cells obtained at different times after drug exposure, and the quantitative analysis of these histograms to determine the percentage of cells in the G1 (2n DNA), S (2n–4n), and G2-M (4n) phases is illustrated in Fig. 4B. Polyploid S and G2-M cells are represented as containing >4n DNA.

Adozelesin had 3 different effects on cell progression: (1) cell progression through S was slowed for about 8 h, (2) cells were blocked in the G2-M phase for about 32 h and (3) some cells escaped the G2-M block and entered a second round of DNA synthesis (without undergoing cytokinesis) to become polyploid. These effects are detailed below. At 0.1 ng/ml, the percentage of G1 cells gradually decreased to reach a nadir of 2.7% at 32 h as compared with a control value of 31%. This occurred because cells were blocked in G2-M and could not divide and feed the pool of G1 cells. The G1 pool was gradually depleted as G1 cells moved into the S phase. The failure of cells to accumulate in the G1 phase suggests that adozelesin apparently does not affect the progression of G1 cells to S-phase.

Adozelesin slowed cell progression through the S phase, thereby increasing the percentage of S-phase cells. Thus, after 4 and 8 h exposure to 0.1 ng/ml, the percentage of S-phase cells increased to 66.5% and 81%, respectively, as compared with the control value of about 48%. This inhibition was transient; after 8 h, cells started leaving the S phase to enter G2-M, thereby decreasing the percentage of S-phase cells to a nadir of 17.9% at 32 h.

The percentage of G2-M cells initially decreased for 8 h and then continuously increased until 32 h as cells were blocked in the G2-M phase. For an agent that induces G2-M blockade, we would expect the percentage of G2-M cells to increase continuously during drug exposure. Therefore, the initial decrease was surprising and was likely attributable to inhibition of cell progression through the S phase, resulting in a slower rate of entry of S-phase cells into the G2-M phase. After 8 h, the onset of cell progression from the S phase into G2-M (as shown by the decrease in the percentage of S-phase cells) combined with the G2-M block resulted in a rapid increase in the percentage of G2-M cells. After 32 h, by which time >60% of cells had reached the G2-M phase, <2% of cells were undergoing mitosis as determined microscopically (data not shown). Therefore, the cells were arrested in G2 rather than in M.

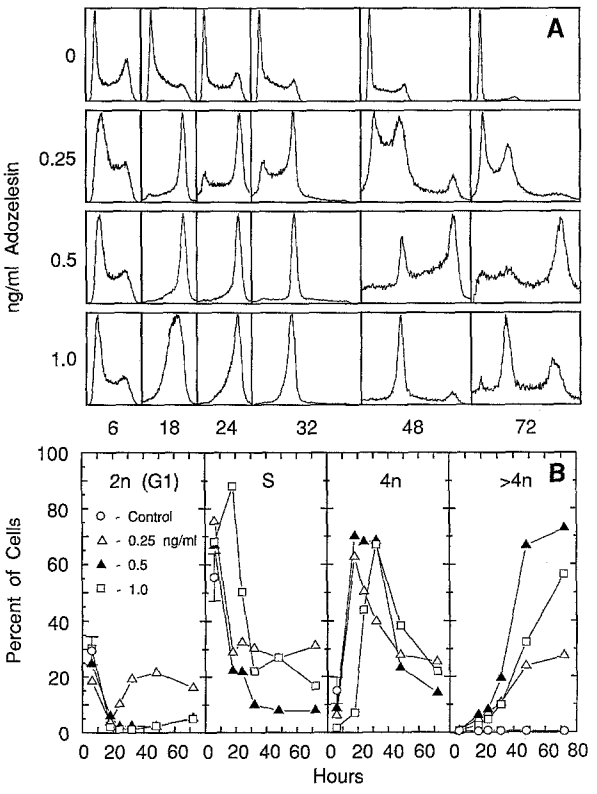

Fig. 5A, B. Cell progression after 2 h exposure to adozelesin. **A** DNA histograms of cells sampled as described in Fig. 4A. Quantitative analysis of the DNA histograms

By 32 h, cells had reached the early tetraploid S phase (>4n DNA), and by 48 h, 60% of the population had become tetraploid (>4n DNA). These results show that most cells escaped the G2 block without undergoing cytokinesis and entered a second round of DNA synthesis to become tetraploid. The high background activity visible in the 48-h histograms (Fig. 4A) also suggests possible abnormal cell division, cell death, and fragmentation. Therefore, the increased percentage of diploid G1 and S cells at 48 h is probably not a reliable indicator of normal cell division.

For cells exposed to 0.05 ng/ml, a similar pattern of inhibition occurring to a lesser degree may explain the changes seen in the DNA histograms. Cells exposed to 0.0225 ng/ml (which caused 50% growth inhibition) showed changes in cell progression similar to those observed at higher doses. Cells accumulated transiently in the S phase, and the percentage of cells in G2-M increased until 24 h; however, unlike the cells exposed to higher doses, these cells did not progress to polyploidy but divided and entered the diploid G1 phase.

V79 cell progression after 1 h drug exposure

The growth of cells under these exposure conditions is shown in Fig. 3B. Figure 5 illustrates that the cell-cycle effects (i.e., depletion of the G1 pool, slower progression

Table 3. Time-course study of the cytotoxic effect and inhibition of macromolecule synthesis by adozelesin

Incubation time[b] (h)	IC_{50} (ng/ml)[a]			
	Cell growth	DNA synthesis	RNA synthesis	Protein synthesis
1	0.06	0.67	1.4–2.4	>5
3	0.04	0.36–0.7	1.4–1.8	1.2
5	0.03	0.5–0.9	0.9–1.6	1.2

[a] IC_{50} represents the drug concentration required for 50% inhibition of the growth or macromolecule synthesis of L1210 leukemia cells. IC_{50} values were obtained by plotting the percentage of inhibition against the log concentrations of the agent. For cell-growth inhibition, cells were washed after 1, 3, or 5 h drug exposure and then incubated for 3 days, after which cells were counted. Macromolecule synthesis was measured immediately after drug exposure

[b] Incubated at 37° C

through the S phase, and blockade of cells in G2-M) of pulsed exposure were similar to those of continuous exposure. The inhibitory effects were dose-dependent. At 1 ng/ml, during the first 18 h the G1 pool was depleted; up to 90% of cells accumulated in the S phase; and due to slow progression through the S phase the percentage of G2-M cells declined. Thereafter, as S-phase cells moved into G2-M, this value increased to about 70% by 32 h. By 48 h, cells had escaped the G2-M block, with >30% of the cells becoming tetraploid and 3%, octaploid.

At 0.25 ng/ml, the inhibition was less intense and of shorter duration such that cells accumulated in the S phase for 6 h (vs 18 h at 1 ng/ml) and in the G2-M phase for 18 h (vs 32 h for 1 ng/ml). Since cells started escaping the G2-M block at 18 h, there was a corresponding earlier increase in the percentage of G1 cells. An interesting dose-response pattern was observed in the progression of cells from G2-M into G1 (2n DNA) or to polyploid (>4n DNA) at 48 and 72 h. At 0.25 ng/ml, the majority of cells remained in the diploid cycle (2n–4n) and only about 30% of them became polyploid. At 0.5 ng/ml, the majority of cells did not divide but up to 75% of them became polyploid. At 1 ng/ml, cytokinesis was almost completely inhibited and progression into polyploidy was partially inhibited such that a lower proportion of cells (55% vs 75% at 0.5 ng/ml) became polyploid.

Growth and macromolecule-synthesis inhibition in L1210 cells

Table 3 shows that DNA synthesis was more drug-sensitive than was RNA or protein synthesis. Thus, the dose needed for 50% inhibition of DNA synthesis was about 2–4 times lower than that required for 50% inhibition of RNA or protein synthesis. However, the 50% growth-inhibitory dose was about 10 times lower than the dose required for 50% inhibition of DNA synthesis, and cell growth was inhibited at doses that caused little inhibition of DNA synthesis.

Discussion

Our results clearly show that adozelesin is highly cytotoxic to cells in culture and to human tumor cells in the cloning assay. Thus, the LD_{90} after continuous exposure of V79 cells was 0.03 nM for adozelesin as compared with 50 nM for Adriamycin. Similarly, against human tumor cells, adozelesin was significantly active at concentrations as low as 0.5 ng/ml as compared with the 200-ng/ml dose used to test cisplatin and the 40-ng/ml concentration used for Adriamycin.

Although adozelesin alkylates DNA, our data suggest that the drug does not kill cells via significant inhibition of DNA synthesis. First, the ID_{50} (50% inhibitory dose) value for DNA-synthesis inhibition was about 10-fold that for growth inhibition. Therefore, very little inhibition of DNA synthesis occurred at growth-inhibitory doses. Furthermore, one would expect similarity between the ID_{50} needed for inhibition of a target macromolecule and that required for inhibition of cell growth in order to suggest a causal relationship between these effects. Obviously, we did not obtain such a correlation for adozelesin (Table 3), but we have previously obtained excellent correlations for other agents (e.g., nogalamycin inhibition of RNA synthesis and growth [12]) to establish causal relationship. Second, several agents (such as cytosine arabinoside or hydroxyurea) that cause significant inhibition of DNA synthesis block G1 cells from entering the S phase or prevent S-phase cells from progressing through S during drug exposure [20]. Neither of these effects was observed in cells treated with adozelesin; rather, the accumulation of cells in the S phase was transient, and cells ultimately completed DNA synthesis to enter the G2 phase. Finally, cells arrested in G2 ultimately escaped the G2 block to enter a second round of DNA synthesis and form tetraploid cells. Therefore, we would suggest that the interaction of adozelesin with DNA occurs at a few specific sites and thus does not result in significant inhibition of DNA synthesis but manifests as a G2 block and inhibition of cytokinesis.

Phase-specific toxicity studies showed that adozelesin is much less cytotoxic to M + early G1 cells as compared with late G1 + early S-phase cells. In this respect, it differs from its parent compound CC-1065 [5]. Like several other alkylating agents, CC-1065 is much more cytotoxic to mitotic cells than to interphase cells [3]. We are currently studying whether the phase-specific toxicity pattern of adozelesin can be explained by differences in cellular or nuclear drug uptake by M-early G1 cells as opposed to S-phase cells. Differences in the rate of DNA repair or in DNA binding sites are other possibilities that need to be investigated.

Adozelesin arrested cells in G2 and prevented them from making the G2-to-M transition. G2 arrest is elicited in cells exposed to a variety of DNA-damaging agents such as DNA alkylators and topoisomerase II inhibitors. Cells require an active p34 cdc2 kinase in association with cyclin to achieve an effective G2-to-M transition [7]. Lock and Ross [15] have shown that etoposide-induced G2 arrest results from the inhibition of p34 cdc2 kinase. We are interested in determining whether such an inhibition might

account for G2 arrest of adozelesin-treated cells. We have previously reported that V79 and CHO cells escape the G2 block to form polyploid cells, whereas B16 (mouse melanoma) and A2780 (human ovarian carcinoma) cells remain blocked in G2 [17]. Kung et al. [11] have reported species-specific differences between the response of human and rodent cell lines to mitotic blockade by colchicine and nocodazole. Human cells lines remained blocked in the M phase, whereas rodent cells escaped without undergoing cytokinesis and became polyploid. We did not see this clear-cut species-specific difference in the response of cells to adozelesin. However, it should be borne in mind that the mechanism of action of adozelesin is different from those tested by Kung et al. [11].

Adozelesin-treated cells undergo a progression of events, starting with a specific DNA lesion and leading to a long-term G2 block. Cell death leading to cell fragmentation and lysis occurs many hours later, as indicated by the high background activity observed in the DNA histograms at 48 h (Figs. 4, 5). Several authors have shown that a variety of drugs (e.g., the alkylator cisplatin and the antimetabolite cytosine arabinoside) induce damaged cells to enter programmed cell death or apoptosis [8, 18]. This process is characterized by induction of an endonuclease that cleaves chromatin into nucleosomal and polynucleosomal fragments. Although we have not yet found any evidence of such a process, the time course of the events observed in adozelesin-treated cells suggests that an apoptotic process might be operative.

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