Lethality, DNA Alkylation, and Cell Cycle Effects of Adozelesin (U-73975) on Rodent and Human Cells


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ABSTRACT

Adozelesin (U-73975) is an extremely potent cytotoxic agent which causes 90% lethality, after 2 h exposure in vitro, of Chinese hamster ovary and lung (CHO and V79), mouse melanoma (B16), and human ovarian carcinoma (A2780) cells at 0.33, 0.19, 0.2, and 0.025 ng/ml, respectively. Under similar conditions, Adriamycin and cisplatin had 90% lethality values in CHO cells of 150 ng/ml (=249 mi) and 6800 ng/ml (=2266 mi), respectively. The relative drug sensitivity of the cell lines (A2780 > V79, B16, CHO) was correlated to the relative amounts of [3H]adozelesin alkylated to DNA. The greater sensitivity of A2780 was due to (a) greater DNA alkylation at different drug doses and (b) greater intrinsic sensitivity of A2780 which resulted in greater cell kill at comparable DNA alkylation.

Phase specific toxicity studies show that adozelesin was least lethal to CHO cells in mitosis and very early G1. Lethality increased as cells progressed through G1 and was maximal in late G1 and early S. Mitotic cells had lower drug uptake and correspondingly less drug binding to DNA than G1 or early S phase cells. However, based on the amount of drug alkylated per µg of DNA, cells in M, G1, and S were equally sensitive. Therefore, the lower sensitivity of M-phase cells was due to lower drug uptake.

Adozelesin had three different effects on progression of CHO, V79, B16, and A2780 through the cell cycle: (a) slowed progression through S which resulted in significantly increasing the percentage of S-phase cells. This effect was transient; (b) cell progression was blocked in G2 for a long time period; (c) the response of the cell lines to the G2 block differed. CHO and V79 cells escaped G2 block by dividing and entered the diploid DNA cycle or did not undergo cytokinesis and became tetraploid. On the contrary, B16 and A2780 cells remained blocked in G2 and did not become tetraploid. Cell progression was inhibited in a similar manner when a synchronized population of M, G1, or S-phase cells were exposed to adozelesin.

INTRODUCTION

Adozelesin (U-73975) is a synthetically derived analogue of the highly potent, alkylating, antitumor antibiotic CC-1065 (1). CC-1065 binds intercalatively in the minor groove of double-stranded DNA at A-T rich regions followed by covalent bonding with N-3 of adenine in preferred base sequences (2, 3). Although CC-1065 showed moderate antitumor activity in vivo, it was not evaluated clinically because it caused delayed deaths in mice at therapeutic doses (4). Since the receptor of CC-1065 (the A-T rich minor groove of DNA) was identified and the alkylating cyclopropapyrroloindole moiety had been synthesized, it was speculated that attaching ligands of appropriate shape and length to the cyclopropapyrroloindole moiety would optimize hydrophobic interactions with the DNA minor groove and yield potent analogues (5–7). Adozelesin is one of those analogues which shows excellent broad spectrum anti-

MATERIALS AND METHODS

Drug. Adozelesin (U-73975, M, 502.2) was synthesized at The Upjohn Company, Kalamazoo, MI (6). Stock solutions (1 mg/ml in dimethylacetamide) stored in the dark in a freezer were chemically stable for at least 1 year. The drug was diluted in medium immediately prior to adding to cells. Based on cytotoxicity measurements, adozelesin was stable in medium for at least 24 h at 37°C.

[3H]Adozelesin (9.2 Ci/mm) was prepared by Chemsyn Science Labs (Lenexa, KA). DMA2 was obtained from Aldrich Chemical Company (Milwaukee, WI). Ready-Safe was purchased from Beckman Company (Fullerton, CA). EDTA, p-aminosalicylic acid, and PBS were purchased from Sigma Chemical Company (St. Louis, MO). SDS was purchased from Bio-Rad Laboratories (Richmond, CA). Redistilled phenol was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). High performance liquid chromatography grade chloroform (CHCl3) was purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). All other reagent grade compounds were obtained from Mallinckrodt Chemical Company (Paris, KY).

Cell Culture. The cell lines were obtained from sources listed previously (10). CHO cells were maintained in Ham’s F-10 medium supplemented with 10% fetal calf serum. B16 (clone F-10) cells grew in MEM supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, essential MEM vitamins (10 ml of 100X concentration/liter), MEM nonessential amino acids (10 ml of 100X concentration/liter), penicillin (60 µg/ml), and streptomycin (10 µg/ml). V79 cells grew in the same medium as B16 except pyruvate was not added and 15% fetal calf serum was used. A2780 cells were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and insulin (0.25 µg/ml). Monolayer cultures of all cell lines were harvested with 0.05% trypsin plus 0.02% EDTA in 0.9% NaCl solution (Irvine Scientific, Santa Ana, CA). CHO, B16, and A2780 and V79 cells grew exponentially with doubling times of about 14, 12, and 16 and 10 h, respectively, and were maintained in exponential growth by subculturing prior to confluency.

Drug Exposure and Cell Survival Determination. B16, V79, A2780, and CHO cells were plated as monolayer cultures 24–48 h before an experiment in order to assure exponential growth during drug exposure. Cells were exposed to drug at 37°C in their respective growth medium. For each drug concentration tested, two separate cultures were used.

After drug exposure, cell monolayers were washed with medium and harvested, and cell survival determined by a colony forming assay as previously described (11, 12). Cloning efficiency of exponentially growing CHO, V79, and B16 cells ranged between 60 and 80%, and that of A2780 was between 30 and 50%. The cloning efficiency of the untreated (control) cells was normalized to 100%, and the cloning efficiency of treated cells was expressed as a percentage of control survival. The coefficient of variation (SD expressed as a percentage of the mean) in determining cell survival was about 15% within each experiment. All experiments were repeated at least once.
Preparation of Synchronous CHO Cultures. Synchronous cultures were started from mitotic cells selectively harvested either after a 2-h treatment with 0.05 μg/ml of Colcemid or without any colcemid pretreatment (13). Mitotic cells were planted and cells entered G1, S, or G2-M at different times after planting. To determine phase specific toxicity, cells in different phases of the cell cycle were exposed to drug for 1 h and then cell survival was determined.

Growth Inhibition Assay. Cell monolayers (about 10^5 cells/75 cm^2) were exposed for varying periods to the drug and then cells were harvested and counted with a Coulter counter. The percentage of growth inhibition was calculated as

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\text{Growth Inhibition} = \frac{\text{cell no. in control at 72 h} - \text{cells inoculated}}{\text{cell no. in control at 72 h} - \text{cells inoculated}} \times 100
\]

DNA Staining and Flow Cytometry. Cells were fixed in ethanol and stained for DNA with mithramycin and then cells were analyzed for DNA content on a Becton Dickinson FACStarPLUS (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (10, 14). The following data were collected for about 4 × 10^5 cells of each sample: 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 on forward versus wide angle light scatter. Cell clumps were eliminated by gating scatter-gated list mode data on mithramycin fluorescence pulse area versus pulse width. Single parameter DNA histograms from gated list mode data were analyzed for cell cycle phase distribution using the program MODFIT (Verity Software House, Topsham) running on a Compaq 386/33 computer.

Uptake Studies. Cells were seeded in 25- or 75-cm² flasks 24 to 48 h before addition of drugs. [3H]Adozelesin was dissolved and diluted in DNA such that the final concentration of DNA added to the medium was 1%. At the indicated times, the medium containing the radiolabeled drug was aspirated off and the cells were washed twice with ice-cold complete medium and twice with ice-cold PBS. Cells were then harvested with trypsin and dispersed in 5 ml complete medium, and cells were transferred to a centrifuge tube. Cells remaining in culture flask were recovered by one wash with complete medium and added to the centrifuge tube. After centrifugation at 500 × g for 5 min, cells were resuspended in cold PBS and aliquots were taken to determine cell number, cellular uptake of labeled drug, and DNA isolation.

To determine cellular uptake, aliquots of the cell suspension were centrifuged at 500 x g for 5 min, cells were resuspended in cold PBS and aliquots were taken to determine cell number, cellular uptake of labeled drug, and DNA isolation.

To isolate DNA, cells were mixed with an equal volume of 15% trichloroacetic acid and then 10% SDS was added. Samples were mixed with 10 ml Ready-Safe and radioactivity was determined by liquid scintillation counting in a model 4640 Packard Tri-Carb (Packard Instrument Co., Inc., Downers Grove, IL). Drug uptake was calculated from the initial specific activity of labeled adozelesin. Uptake is expressed as fmol [3H]-drug equivalents bound/10^6 isolated cells.

To isolate DNA, cells were mixed with an equal volume of 15% p-aminosaliclyc acid and then 10% SDS was added to give a final concentration of 0.1% (final volume, 0.5 ml). An equal volume of phenol:CHCl₃:isoamyl alcohol (50:50:1, v/v/v) was added and samples vortexed. Samples were centrifuged in an Eppendorf Model 5414 centrifuge (Brinkmann Instrument Company) and the aqueous layer was transferred to a clean tube. One-tenth volume of 3 M NaCl was added to the sample and nucleic acid was precipitated by adding 2 volumes of absolute ethanol. Samples were maintained at -70°C for 1 h or -20°C overnight, and nucleic acids were pelleted by centrifugation. The pellets were washed once with cold 70% ethanol. DNA was dissolved in 0.15 ml of 10 mM Tris-1 mM EDTA, 0.1 M NaCl, pH 7.4. Aliquots (0.1 ml) were removed for scintillation counting, and appropriate aliquots were taken for DNA determination. DNA concentrations were determined by a Hoechst 33258 dye binding procedure (15) using a Model TKO-100 DNA Fluorometer (Hoefer Scientific Instrument Company, San Francisco, CA), as described by the manufacturer. DNA alkylation by adozelesin is expressed as [3H]-drug equivalents bound/µg DNA.

RESULTS

Cell Growth Inhibition. Dose-dependent growth inhibition was observed during continuous exposure to adozelesin (data not shown). These results were used to calculate the 50 and 90% growth inhibitory doses respectively: CHO, 0.024 and 0.08 ng/ml; V79, 0.02 and 0.04 ng/ml; B16, 0.006 and 0.02 ng/ml; and A2780, 0.0014 and 0.0047 ng/ml. B16, CHO, and V79 were about equally sensitive to the drug whereas A2780 was about 10-fold more sensitive.

Time Course of Inhibition of Cell Survival. Fig. 1 shows the dose-survival curves for CHO and A2780 cells exposed to adozelesin for 1, 2, 4, and 24 h and B16 and V79 exposed for 2 h. In all cases, sigmoidal dose-survival curves were obtained which were characterized by an initial shoulder followed by an exponential (log-linear) decline in survival as the dose increased. With increasing periods of exposure, the initial shoulder (the presumed region of repair of sublethal damage) decreased and the dose-survival curves became steeper. The LD₉₀ values, estimated from the dose-response curves show that after 2 h drug exposure, B16, CHO, and V79 cells were almost equally sensitive (LD₉₀ = 0.2, 0.33, and 0.19 ng/ml, respectively) to adozelesin, whereas A2780 (LD₉₀ = 0.025 ng/ml) was about 10-fold more sensitive. As a comparison, the LD₉₀ values for Adriamycin and cisplatin, after 2 h exposure of CHO cells, were 150 and 6800 ng/ml, respectively.

Since adozelesin binds covalently to DNA, we correlated the level of DNA alkylation in different cell lines to their drug sensitivity. The level of drug bound covalently to DNA after 2 h exposure of cells to different concentrations of [3H]adozelesin is shown in Fig. 2. A linear dose-response was obtained with different cell lines. For the same drug concentration in medium, the amount of drug alkylated per µg of DNA was A2780 > V79 > B16, CHO. For example, at 0.1 ng/ml drug the fmol of drug bound per µg of DNA were 14.6, 10, 4, and 4 for A2780, V79, B16, and CHO, respectively. Fig. 3 shows the cell kill obtained at different levels of DNA alkylation. The LD₉₀ values of the cells were approximately (fmol/µg DNA): A2780, 2.5; B16, 7.5 (extrapolated); CHO, 12 (extrapolated); and V79, 27. Therefore, the greater sensitivity of A2780 was due to (a) greater DNA alkylation at lower drug doses and (b) greater...
exposure to different concentrations of adozelesin. Bars, SD.

The intrinsic sensitivity of A2780 which resulted in greater cell kill samples were used to determine the percentage of cells killed by drug exposure.

adozelesin or unlabeled adozelesin. The former samples were processed to determine the level of [3H]adozelesin bound covalently to DNA (Fig. 2). The latter compound CC-1065) are more toxic to cells in M + very early G1 than to cells in other phases. Because of these concerns, we decided to repeat this experiment using mitotic CHO cells which were selectively harvested after 2 h of Colcemid pretreatment or without any Colcemid treatment. Mitotic cells were planted and as the cells passed through different phases of the cell cycle they were exposed to drug for 1 h.

The position of cells in the cell cycle at different times after planting mitotic cells was determined either by flow cytometry (data not shown) or toxicity of ara-C. ara-C kills only S-phase cells and thus demarcates G1 and G2 phases from S phase. The results showed that mitotic cells harvested without Colcemid progressed from mitosis into G1 and stayed in G1 until about 5 h (since there was no cell kill by ara-C at 5 h). At least 70–80% of cells had progressed into S by 7.5 h. Mitotic cells harvested with or without Colcemid pretreatment progressed through the cell cycle at similar rates. Flow cytometry results corroborated ara-C results.

The survival of cells exposed in different phases of the cell cycle for 1 h to adozelesin is shown in Fig. 4. Similar patterns of survival are obtained for synchronous cultures started from mitotic cells harvested with or without Colcemid pretreatment (Fig. 4). It is quite clear that cells in mitosis and very early G1 (0–1 h exposure) are much more resistant to the drug than cells in G1 and S.

To further analyze the phase related toxicity of the drug, we determined the intracellular uptake of [3H]adozelesin and the amount of drug covalently bound to DNA in different phases of the cell cycle. Fig. 5A shows dose dependent increase in drug uptake in M, G1, and S-phase cells and that intracellular drug concentration in M-phase cells was lower than in G1 and S cells. There was correspondingly less drug bound to DNA in M than in G1 or S cells (Fig. 5B). Fig. 6 shows that the percentage of cell kill observed for cells in M, G1, or S phase is proportional to the fmol adozelesin bound per μg DNA. Therefore, when cell survival is normalized for the amount of DNA bound drug, cells in different phases are equally sensitive. Thus lower uptake and resulting lower amount of drug bound to DNA in M-phase cells causes the lower drug sensitivity of these cells.

Cell Progression during Continuous Exposure to Drug. We previously reported (10) that adozelesin affected V79 cell progression at 3 different sites: (a) cell progression through S was

intrinsic sensitivity of A2780 which resulted in greater cell kill (than rodent cell lines) at comparable DNA alkylation.

Phase Specific Toxicity. We reported previously that adozelesin was least lethal to V79 cells in M + very early G1 and that drug toxicity increased significantly as cells progressed out of that phase (10). The V79 experiment used a synchronized culture started from mitotic cells harvested after Colcemid treatment. Because of our concern about Colcemid effects, we attempted to repeat the experiment by harvesting V79 mitotic cells without Colcemid pretreatment but were unsuccessful. The phase specificity pattern obtained with V79 cells was also surprising because we had expected that mitotic cells, since they do not have a nuclear membrane, would be more susceptible to this DNA binding drug. Furthermore, literature reports (16) show that many alkylating agents (e.g., melphalan and the parent compound CC-1065) are more toxic to cells in M + very early G1 than to cells in other phases. Because of these concerns, we decided to repeat this experiment using mitotic CHO cells which were selectively harvested either after 2 h of Colcemid exposure and then planted. Cells progressed through M to G1, S and G2-M phases of the cell cycle at different times after planting. Cells are in M + early G1 for 0–1 h exposure and in G1 at 3 and 5 h. Cells start entering S after 5 h such that 50 and 90% of cells are in S at 6 and 8 h, respectively. Cells in different phases were exposed for 1 h to different concentrations of adozelesin and survival was determined by a colony formation assay. The time points shown are halfway between actual exposure periods; e.g., survival from exposure at 0–1 h is shown at 0.5 h. Open and closed symbols refer to two separate experiments. Bars, SD.
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Fig. 5. A, intracellular uptake of \( ^{3}H \)adozelesin by cells in different phases; B, level of \( ^{3}H \)adozelesin bound covalently to cellular DNA of cells in different phases. Cells synchronized in different phases (see Fig. 4) were exposed to \( ^{3}H \)adozelesin for 1 h following which intracellular uptake and binding to DNA was measured.

Fig. 6. The percentage of cells killed was proportional to the amount of covalently bound \( ^{3}H \)adozelesin. The data for this figure were obtained by determining the percentage of survival and the DNA alkylation (Fig. 4 and Fig. 5) of M, G1, and S-phase cells exposed to different concentrations of \( ^{3}H \)adozelesin.

Inhibited temporarily, resulting in transient accumulation of cells in S. These cells subsequently progressed into G2; (b) cells were blocked in G2 for long periods of time; (c) some cells escaped the G2 block and divided whereas a large proportion of cells did not undergo cytokinesis but entered tetraploid S phase.

Fig. 7 shows progression of A2780, B16, CHO, and V79 cells during continuous exposure to approximately equally growth inhibitory (≥90% inhibition) doses of adozelesin. In all cell lines, adozelesin slowed cell progression through S so that cells accumulated in S by 12 h. This block was transient since the cells then progressed into G2-M, as seen by the decrease in S-phase population and the accumulation in G2-M at 24 h. Since the population at 24 h contained a very low percentage of mitotic cells, the G2-M peak at 24 h consists mostly of G2 cells. The response of the hamster (CHO and V79) cells to the G2 block differed from that of mouse B16 cells or human A2780 cells. Some of the CHO and V79 cells blocked in G2 progressed through M and divided to enter the diploid DNA cycle. This is seen in the increase in the G1 (2n) and S populations at 48 h, much more so in CHO than in V79 cells. Another fraction of the cells blocked in G2 did not undergo cytokinesis but entered the tetraploid DNA cycle. This is seen in the increase in tetraploid S (4n-8n) and G2 (8n) populations at 48 h, much more so in V79 than in CHO. In contrast, A2780 and B16 cells did not become tetraploid. B16 cells remained blocked in G2 (26- and 48-h samples) whereas A2780 remained blocked in G2 until 48 h and then some cells divided and entered G1 (76 h). Progression of CHO cells after 1 h exposure to drug showed the same pattern as seen after continuous drug exposure (data not shown).

Progression of Synchronized Mitotic, G1, or S-Phase Cells after Adozelesin Exposure. Since cells in different phases had different sensitivities to adozelesin (see Fig. 4), we wanted to determine if there were similar differences in cell progression if cells were exposed to drug in different parts of the cell cycle. Mitotic cells were selectively harvested and planted at 0 h, and cell progression after 1 h adozelesin exposure of cells in mitosis (0 h), mid-G1 (2 h), and S (8 h) was determined. DNA histograms and quantitative analysis of these histograms are shown in Figs. 8 and 9, respectively. The untreated (control) population progressed synchronously through the cell cycle as shown by the high (90%) G1 at 3 h, rapid decrease in G1 from 3 to 9 h (10% G1 at 9 h), and corresponding rapid increase in S (see Fig. 9). The maximum percentage of cells were in S at 9 h (89%) after which the cells progressed out of S into G2-M and

Fig. 7. Progression of CHO, V79, B16, and A2780 cell lines during continuous exposure to adozelesin. Several cell lines were exposed to varying concentrations of adozelesin sufficient to cause ≥90% growth inhibition. Cells were harvested at different times and cell distribution was determined by flow cytometry. Note the development of polyploidy in CHO and V79 cells at 48 h but not in B16 and A2780 cells.
phase CHO cells had been exposed for 1 h to adozelesin. Mitotic cells were harvested after a 2-h Colcemid exposure and planted at 0 h. Cells were exposed for 1 h to adozelesin (1 ng/ml) when they were in M (0-1 h treatment), mid-G1, or S phase cells. The DNA histograms of Fig. 8 were quantitatively analyzed to give the results shown here. The results for cells treated in small (i, (2-3 h treatment) are not shown because they were similar to results for cells treated in this G1. A, G1; B, S; C, G2-M.

Fig. 8. DNA histograms at different times after synchronized M, G1, or S phase cells. CHO cells had been exposed for 1 h to adozelesin. Mitotic cells were harvested after a 2-h Colcemid exposure and planted at 0 h. Cells were exposed for 1 h to adozelesin (1 ng/ml) when they were in M (0-1 h treatment), mid-G1 (2-3 h treatment) and in S (8-9 h treatment).

The time course of progression suggests that the cycling time for this synchronized population was about 12–13 h.

Fig. 9 compares the progression of the untreated (control) population to that of adozelesin treated cells. Since the progression of cells treated in M (0–1 h treatment) and in mid-G1 (treated at 2–3 h) were essentially the same, results with mid-G1 are not shown. The cells treated in M progressed normally into G1, as shown by the similar percentage of cells in G1 at 3 h in the treated and control populations. Cells treated in M or in mid-G1 progressed normally through G1 to S (similar percentage of S cells at 9 h) indicating that adozelesin does not affect progression through G1 or entry into S. Cells treated in M, G1, or S were significantly delayed in S. Thus 50% of the control population exited S at about 11.5 h compared to 22 h for cells treated in M or G1 and 17 h for cells treated in S. These results also show that drug binding to the different conformations of DNA (i.e., condensed DNA of mitotic cells, nonreplicating DNA in G1 cells and replicating DNA in S-phase cells) had similar effect.

By 49 h, a significant proportion of the cell population had become tetraploid with DNA content distribution representative of cycling cells distributed randomly through diploid and tetraploid G1, S, and G2-M phases. The distribution of cellular DNA in the region associated with diploid DNA content was abnormal and not analyzable by traditional means. These cells were still in the normal size range of diploid cells, as determined by forward versus side light scatter; therefore these events do not represent subcellular debris from lysed cells. This suggests that the abnormality in DNA content distribution probably results from drug toxicity leading to abnormal cellular and/or nuclear division, apoptosis, and cell death. Previous studies with cytotoxic compounds have shown that cells released from a temporary G2-M block will often divide abnormally to form daughter cells with severely abnormal DNA content distributions (13, 14).

DISCUSSION

Adozelesin is clearly a very potent antitumor agent (8) which, unlike many other alkylating agents (e.g., cisplatin), does not bind to RNA or protein. This DNA specific binding might account for its very high potency as compared to cisplatin and Adriamycin. For example, the LD50 values for 2 h exposure to adozelesin of A2780 and V79 cells were 50 and 380 pm, respectively, as compared to 4.7 μM (A2780) and 23 μM (V79) for cisplatin, respectively (16). Adozelesin was about 10-fold more cytotoxic to human ovarian carcinoma (A2780 LD50 = 50 pm) than to hamster or mouse cells (LD50 ~ 380–600 pm). The LD50 of adozelesin for 6 human carcinoma cell lines reported by Lee and Gibson (17) ranged from 20 to 60 pm which is similar to our results with human ovarian A2780 (LD50 = 50 pm). Therefore, in general, human cells may be more sensitive to adozelesin than rodent cells.

The difference in sensitivity between A2780 and the rodent cell lines cannot be explained by the cell distribution (% G1, S, G2-M) pattern of the cell lines. Thus, although A2780 was 10-fold more sensitive, its percentage of S-phase (39%) cells was within the range (30–55%) of S-phase cells in rodent cell lines. Qualitatively the difference in sensitivity in A2780 as compared to the rodent cells could be explained by the greater amount of drug alkylated to DNA for the same exposure. However, the amount of drug alkylated at 90% cell kill dose was very different for each cell line. Thus, at LD50 in A2780, B16, CHO, and V79 cells, the fmol adozelesin alkylated per μg DNA were 2.5, 7.5, 12, and 27.5, respectively. Since adozelesin alkylates DNA, we expected to see a correlation between amount of drug alkylated to DNA and drug cytotoxicity, i.e., the same amount of alkylation should cause the same cytotoxicity. Therefore, the lack of correlation suggests that: (a) DNA alkylation at specific sites, rather than total DNA alkylation, may be the factor controlling cytotoxicity; or (b) different cell lines have different abilities to repair alkylated DNA.

In recent years the role of p34 cdc2 kinase, cyclin B, and related proteins in regulating cell progression from G1 through mitosis has been identified (18). As cells proceed through S into G2, p34 cdc2 is phosphorylated and forms a complex with cyclin B. Dephosphorylation of this complex at prophase-metaphase boundary activates cdc2 kinase and allows cells to enter mitosis. Cells exit mitosis when cyclin B degrades and the p34 cdc2 kinase activity is lost. These regulating events may subsequently into G1. The time course of progression suggests that the cycling time for this synchronized population was about 12–13 h.
explain G₂ arrest by adozelesin and the subsequent escape from G₂ into the next cell cycle and polyploidy.

Lock and Ross (19) showed that etoposide treatment caused G₂ arrest and also resulted in rapid inhibition of p34 cdc2 kinase activity. Since etoposide did not directly inhibit the enzyme itself, these authors postulated that DNA damage by etoposide elicits a cellular response which causes inhibition of p34 cdc2 kinase activity and blocks cells from progressing into mitosis, i.e., G₂ arrest. As the G₂ arrested cells gradually recovered their kinase activity they were able to proceed into mitosis. It is likely that alkylation of cellular DNA by adozelesin results in a similar sequence of events which leads to G₂ arrest.

Various cell lines respond differently to G₂ arrest. Kung et al. (20) reported species specific differences in the response of cell lines to prolonged arrest of cells in mitosis by agents (e.g., Colcemid or nocodazole) that depolymerize microtubules. Human cell lines remained permanently blocked in M whereas rodent cell lines progressed into subsequent cell cycles in the absence of mitosis to generate polyploid cells. The response of mouse cells was related to the degree of transformation since fully transformed cultures became polyploid, whereas untransformed mouse embryo cultures remained blocked in M. This difference between cell lines was related to differences in regulation of cyclin B and cdc2 kinase. Human cell lines exposed to Colcemid maintained an elevated level of cyclin B and p34 cdc2 kinase and thereby remained arrested in M. In rodent cell lines cyclin B and cdc2 kinase were elevated only transiently and cells escaped M when cyclin B and cdc2 kinase levels decreased. Our results with adozelesin-treated cells also show differences between human and rodent cell lines. Human (A2780) cells remained blocked in G₂ whereas hamster (CHO and V79) cells escaped G₂ block into the next cell cycle and became polyploid. However, the malignant (i.e., transformed) mouse B16 cell line remained blocked in G₂ which is different than that expected from Kung’s studies (17). We do not know whether these differences can be related to differences in regulation of cdc2 kinase activity.

Although CC-1065 analogues interact with DNA (2, 3), Li et al. (8) have reported that inhibition of DNA synthesis alone did not account for the cytotoxicity of these analogues. We also observed that the dose required to inhibit growth was 10 times higher than that needed for inhibition of DNA synthesis; i.e., growth inhibition occurred at drug doses that marginally inhibited DNA synthesis (10). Lee and Gibson (17) were unable to detect any DNA damage (single strand breaks, DNA interstrand cross-linking, or DNA protein cross-links) at concentrations that produced up to a 3-log cell kill. These results suggest that adozelesin exerts its cytotoxicity, not by causing gross DNA damage but rather by acting at a limited number of specific vulnerable sites.

Azocelesin treated cells undergo a progression of events, possibly starting with a specific DNA lesion, leading to a long-term G₂ block. Cell death and subsequent cell fragmentation and lysis follow many hours later, as indicated by the high background observed in the DNA histograms at 48 h (Figs. 7 and 8). A variety of drugs (e.g., the alkylator cisplatin, and the antime-tabolite, ara-C) induce damaged cells to enter programmed cell death or apoptosis (21, 22). This process is characterized by induction of an endonuclease that cleaves chromatin into nucleosomal and polynucleosomal fragments. The time course of events in adozelesin treated cells suggests that an apoptotic process may be operative and studies to determine this are now in progress.

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