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In vitro evaluation of a novel chemotherapeutic agent, Adozelesin, in gynecologic-cancer cell lines*

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Summary. Adozelesin is a derivative of an extremely cytotoxic compound, CC1065. This entirely new class of drug binds preferentially to DNA and facilitates alkylation reaction. In the present study, we used the adenosine triphosphate (ATP) chemosensitivity assay to compare the cytotoxic potency of Adozelesin with that of common chemotherapeutic agents in ten gynecologic-cancer cell lines. Flow cytometry was also used to study its effects on cell-cycle kinetics. The mean drug concentrations required to produce a 50% reduction in ATP levels as compared with controls [IC_{50}] were: Adriamycin, 0.17 ± 0.06 μM; 4OH-Cytoxan, 18 ± 3 μM; cisplatin, 17 ± 7 μM; 5-fluorouracil, 183 ± 116 μM; and Adozelesin, 11.0 ± 5.4 pM. Thus, Adozelesin was 10^4–10^7 times more potent than Adriamycin, cisplatin, 5-fluorouracil, and Cytoxan. Cell kinetics studies revealed significant S and G2 blocks such as those previously reported for other alkylating agents.

Introduction

Adozelesin is a new synthetic derivative of CC-1065. The parent compound was discovered in 1978 and quickly became one of the most potent cytotoxic agents known to date [14, 19–21]. The development of CC-1065 as a potential chemotherapeutic agent was tainted by the delayed deaths observed in mice [22]. After numerous advances had been made in chemical synthesis and molecular biology, it was determined that the potency of this compound came from its alkylating function and from the spiral structure that facilitates its preferential binding to DNA [27, 35]. It has been shown that CC1065 binds to the minor groove of the DNA double helix, especially in adenine/thymidine-rich areas. Many derivatives have been synthesized and studied by Upjohn scientists [6, 34]. Adozelesin was chosen for further development due to its enhanced cytotoxicity and its lack of lethal hepatotoxicity [10, 33]. Preliminary in vitro studies showed that it was 10^5–10^6 times as potent as Adriamycin [2]. This drug is currently undergoing phase I clinical trials at various cancer centers in the United States. Because of its novel mechanism of action and its extreme sensitivity, this compound has the potential to overcome drug-resistance problems. In the present study, the performance of Adozelesin was compared with that of other commonly used agents in ten human gynecologic-cancer cell lines. The effects of this new drug on cell-cycle kinetics was also studied.

Materials and methods

Cell lines. Ten human gynecologic-cancer cell lines were used. Endometrial cell lines AE7 and ECC1 were obtained from Dr. P. G. Satyaswaroop (Hershey, Pa.); both of these lines were cultured from primary and untreated well-differentiated adenocarcinomas of the endometrium. HEC1A, HEC1B, AN3, and SKUT1B were obtained from American Type Culture Collection (ATCC). Both HEC1A and HEC1B were derived from an untreated moderately differentiated adenocarcinoma of the endometrium at the 122th and 128th passages, respectively. Cell line AN3 was derived from a metastatic lymph node arising from a poorly differentiated endometrial carcinoma obtained from a patient who had previously been treated with hormones. SKUT1B was cultivated from a patient with poorly differentiated leiomyosarcoma who had previously undergone irradiation. Ovarian-cancer cell lines CAOV3 and SKOV3 were obtained from ATCC at the 24th and 25th passages; both of these lines were derived from patients who had previously been treated with combinations of doxorubicin/cyclophosphamide and 5-fluorouracil/thiotepa, respectively [7, 11]. BG1, which was generated from an untreated ovarian-cancer patient, was kindly provided by Dr. J. Johnson of Bowman Gray University [13]. Cervical-cancer cell line ME180 was purchased from ATCC. All cell lines were grown in Eagle's modified essential medium (EMEM) supplemented with 10% fetal bovine serum, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 2.5 μg amphotericin B/ml. Cells were incubated at 37° C in an atmosphere containing 5% CO_2 and 95% humidity. The medium was replaced every 3 days, and cells

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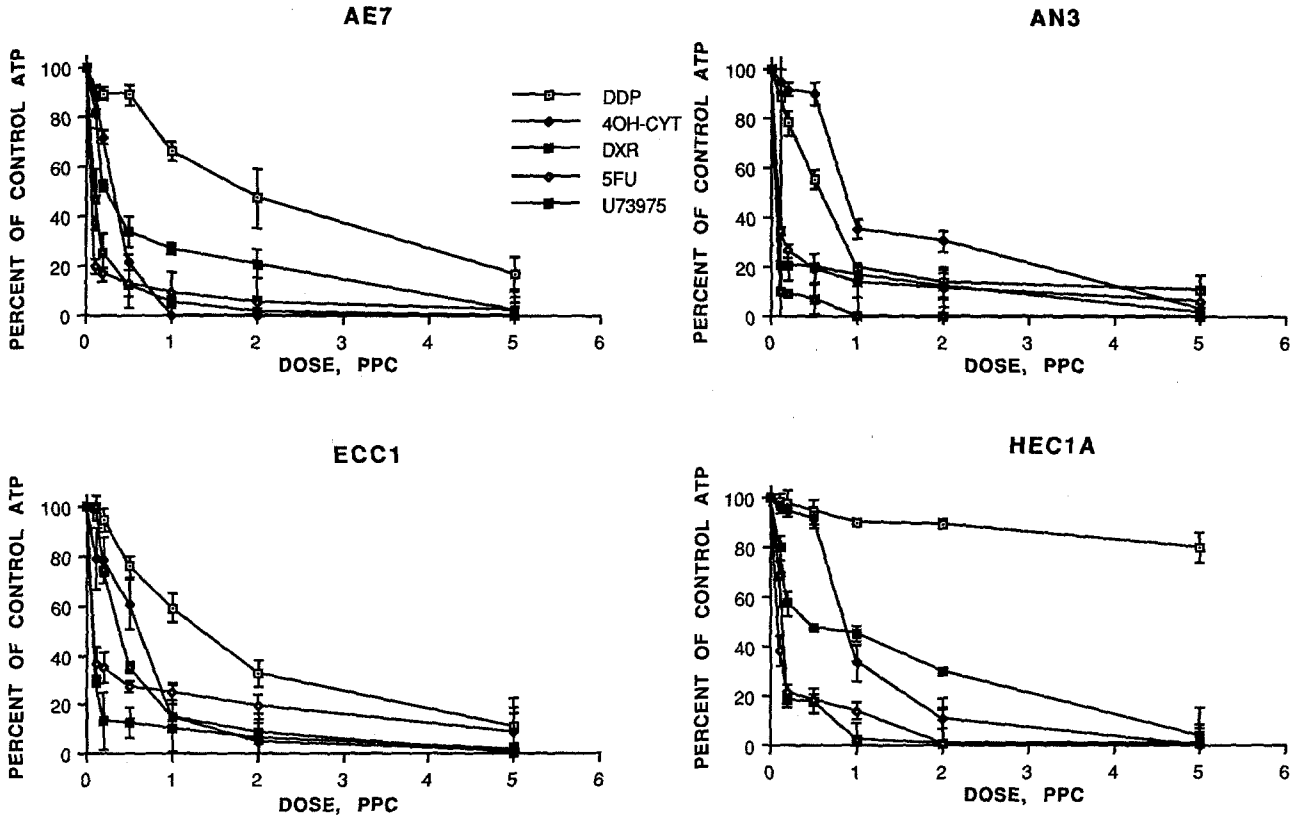

Fig. 1. Dose-response curves generated for cell lines AE7, AN3, ECC1, and HEC1A following their treatment with DDP, 4OH-CYT, DXR, 5FU, and U73975. Doses are indicated in peak plasma concentrations (*PPC*) for ease of comparison

were subcultured weekly following detachment with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA).

Drugs. The following drugs were used at the indicated peak plasma concentrations (PPC) as reference values [1]: cisplatin (DDP), 2.5 μg/ml or 8.3 μM, obtained from Bristol Myers; 5-fluorouracil (5FU), 50 μg/ml or 0.38 mM, obtained from Lymphomed; Adriamycin (DXR), 0.5 μg/ml or 0.92 μM, obtained from Adria Laboratory. Because Cytoxan remained inactive in the absence of hepatic enzyme conversion, we used its active metabolite, 4-hydroperoxy-cyclophosphamide (4OH-CYT), which was kindly provided by Dr. M. Colvin of John Hopkins Oncology Center. The level of this active metabolite in blood has been reported to be 20% that of cyclophosphamide, which reaches a PPC of 30 μg/ml [1, 8]; hence, a PPC of μg/ml was chosen for 4OH-CYT. Adozelesin was generously donated by the Upjohn Company Cancer Research Unit; a reference concentration of 10 pg/ml or 0.02 nM was used on the basis of preliminary in vitro and in vivo data and according to the manufacturer's recommendations. Adozelesin was maintained as a 1-mg/ml stock solution in dimethyl acetamide and was kept in a dark environment under refrigeration. All agents were dissolved in distilled water to the appropriate doses immediately before their use.

ATP chemosensitivity assay. In this assay, ATP activity is measured by determining the amount of light generated when an ATP molecule interacts with the luciferin-luciferase complex. Since the amount of ATP corresponds to cell number and cell mass, the ATP bioluminescence assay has been used to study cellular responses to various drug treatments [17–22]. Surviving fractions can then be determined by calculating the ratio of the ATP level in treated cells to that in control cells.

Briefly, a suspension of 20,000 cells/ml was used to plate 24-well tissue-culture flasks in triplicate. Except in the control wells, each drug was used at concentrations of 0.1, 0.2, 0.5, 1, 2, and 5 X, with X being equal to the PPC. At 24 h after the plating procedure, cells were exposed to the drugs for 90 min. Dose-response curves were obtained on day 7 by extracting the ATP from the cells in situ with an equal volume of 2% trichloroacetic acid. ATP bioluminescence was determined as previously described [30].

Flow cytometry. Dual-parameter flow cytometry was performed as previously described [16]. Briefly, a suspension of a million CAOV3 cells was plated into 25-cm^2 flasks. Drug exposure was done at near confluency at concentrations of 0, 0.1, 0.2, and 0.5 X. Samples of controls and treated cells were harvested by trypsinization at 0, 24, 48, 72, 96, and 168 h after treatment. Viable cell counts were performed with a hemocytometer using the trypan blue exclusion technique. A suspension of 300,000–500,000 cells/ml was centrifuged at 600 *g* for 5 min. The cell pellet was vortexed for 1 min with 1 ml nuclear isolating buffer consisting of 0.66% Trisma-HCl, 0.1% Trisma base, 0.27% sodium chloride, 0.037% EDTA, and 5% NP40. Bicarbonate buffer (0.9 ml) was added to the above preparation, and this mixture was subsequently stained with 0.1 ml fluoroisothiocyanate (FITC, 3 μg/ml) and 1 ml propidium iodide (PI, 0.07 μg/ml) and then vortexed for 30 s. Cell samples were kept refrigerated overnight and subjected to flow cytometry. Normal human lymphocytes were routinely used as internal controls. Three-dimensional graphs were generated by software programs (Coulter Electronics, Hialeah, Fla.) on an Easy-88 computer as previously described [16].

Data analysis. The drug concentration required to produce a 50% reduction in ATP levels as compared with the control value (IC_{50}) was calculated using the median-effect plot of log (fa/fu) vs log *c*, where *fu* represents the fraction unaffected, or the surviving fraction; *fa* indicates the fraction affected (=1 – *fu*); and *c* represents the drug concentration [9]. Nonparametric Wilcoxon matched-pair tests were used to determine the statistical significance of Adozelesin-induced cell-cycle kinetics.

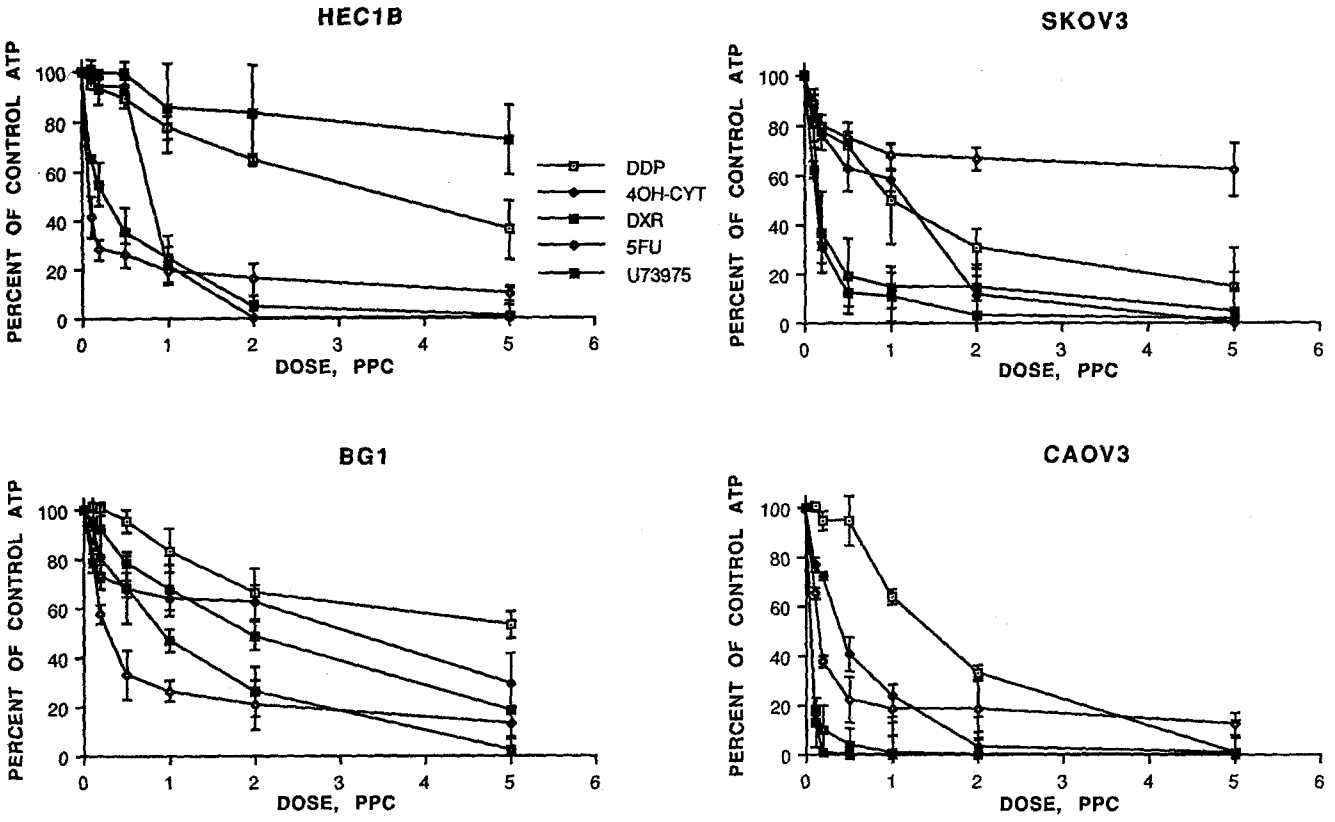

Fig. 2. Dose-response curves generated for cell lines HEC1B, BG1, CAOV3, and SKOV3 following their treatment with DDP, 4OH-CYT, DXR, 5FU, and U73975. Doses are indicated in peak plasma concentrations (*PPC*) for ease of comparison

Results

Following exposure to each drug, all cell lines exhibited a dose-response relationship; however, the response pattern of each individual cell line varied considerably. For descriptive purposes, a cell line showing a reduction in ATP levels amounting to $\geq 50\%$ of the control value at a drug concentration of 0.5 X was arbitrarily considered to be sensitive, whereas a reduction in ATP activity amounting to <50% of the control level was considered to indicate resistance [26]. As shown in Fig. 1, endometrial-cancer cell line AE7 was sensitive to all drugs except DDP. Endometrial cell lines AN3 and ECC1 produced similar chemosensitivity profiles; they were sensitive to DXR, 5FU, and Adozelesin but resistant to DDP and 4OH-CYT. HEC1A was a more resistant endometrial cell line; it was sensitive only to 5FU and Adozelesin. Figure 2 shows the dose-response curves generated for HEC1B, BG1, CAOV3, and SKOV3. Endometrial cell line HEC1B was sensitive to DXR and 5FU but was resistant to the alkylating agents Adozelesin, DDP, and 4OH-CYT. Ovarian-cancer cell line BG1 was sensitive only to 5FU. The previously treated ovarian cell line SKOV3 was sensitive to Adozelesin and DXR and resistant to 5FU, DDP, and 4OH-CYT. Of the three ovarian cell lines, CAOV3 was the most sensitive; it was sensitive to all of the drugs tested except DDP. As shown in Fig. 3, leiomyosarcoma cell line SKUT1B was sensitive to DXR, 5FU, and Adozelesin but was resistant to DDP and 4OH-CYT. Cervical-cancer cell line ME180 was sensitive to all of the drugs tested.

IC_{50} values were calculated in molar PPCs for purposes of comparison. Coefficients of variation (CVs) based on triplicate samples ranged from 1.0% to 37% of the mean fractional ATP value at each drug concentration. Higher CVs were usually observed at doses causing a large proportion of cell deaths. Mean IC_{50} values were calculated from data obtained in repeated experiments as follows: cisplatin, 17.0 ± 7.0 μM; 4OH-CYT, 18 ± 3 μM; 5FU, 183 ± 116 μM; Adriamycin, 0.17 ± 0.06 μM; and Adozelesin, 11.0 ± 5.4 pM. According to our strict criteria for drug sensitivity, only ME180 was sensitive to cisplatin. Three cell lines were sensitive to 4OH-CYT, and the other seven were resistant to this drug. 5FU, Adriamycin, and Adozelesin were cytotoxic to the majority of cell lines tested. Nine cell lines were sensitive to 5FU and DXR. All cell lines except HEC1B and BG1 were sensitive to Adozelesin. To identify a pattern of relative drug resistance in Table 1, we underlined the highest IC_{50} values obtained for each drug. We found that cell lines BG1 and HEC1B exhibited a pattern of relative resistance to several cytotoxic agents.

The effects of Adozelesin on cell-cycle kinetics are shown in Fig. 4. These cell-cycle effects exhibited a dose-response relationship and could be observed as late as at 168 h after drug exposure. Adozelesin caused significant S and G2 blocks in the CAOV3 cell line. The initial S-phase block was associated with a reduction in G2 phase at 24 h for doses 0.2 and 0.5 X. After this initial reduction, the G2 fraction started to rise and showed a maximal G2 block at 48 and 72 h. The mean S-phase fractions were

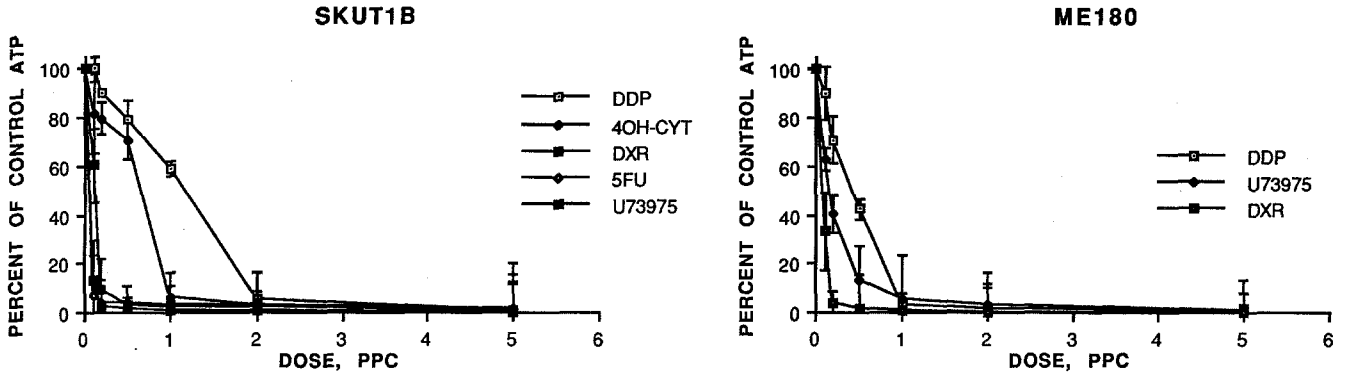

Fig. 3. Dose-response curves generated for cell lines SKUT1B and ME180 following their treatment with DDP, 4OH-CYT, DXR, 5FU, and U73975. Doses are indicated in peak plasma concentrations (*PPC*) for ease of comparison

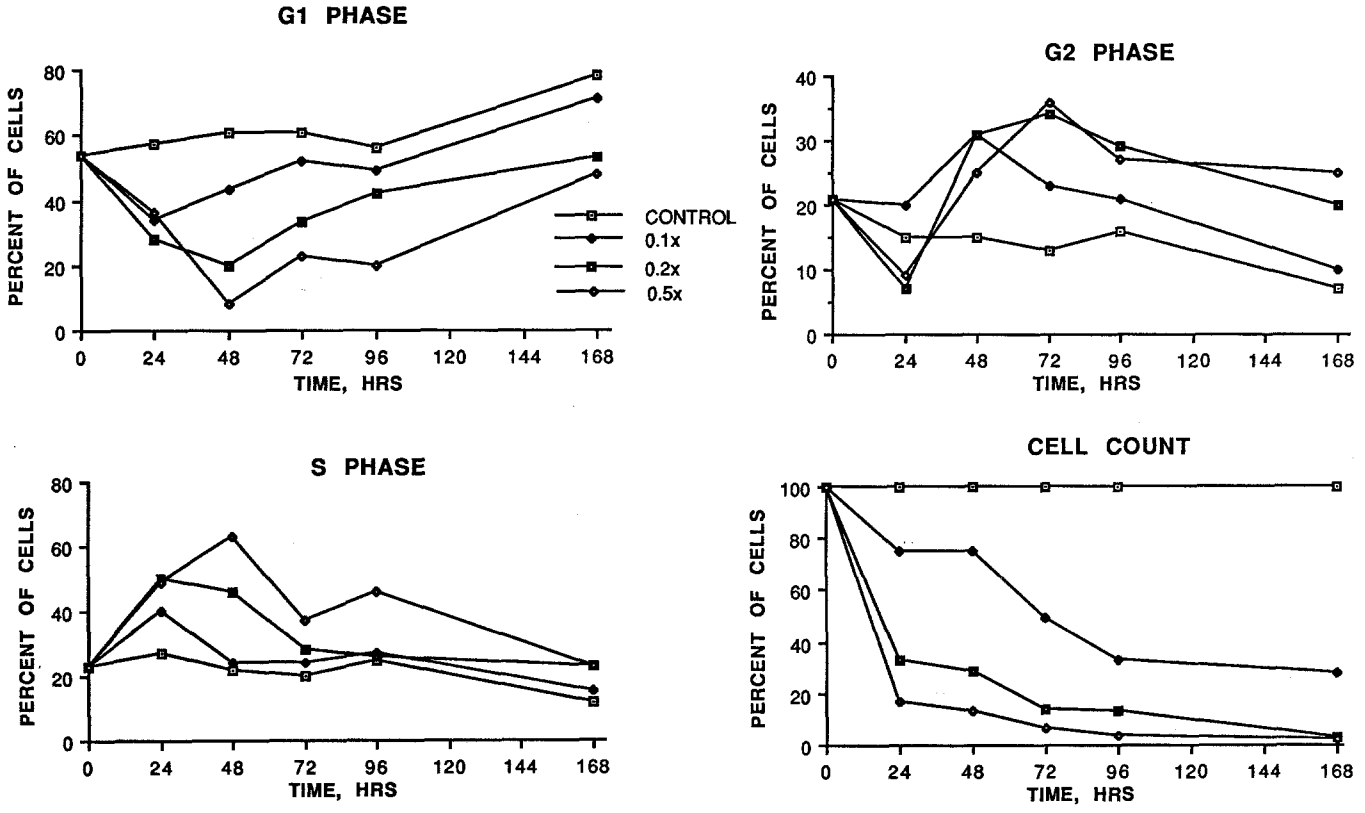

Fig. 4. Cell-cycle kinetics of the CAOV3 cell line following its treatment with U73975. Drug exposure was carried out at 0.1, 0.2, and 0.5 X, with X being equal to the peak plasma concentration

Table 1. IC_{50} values obtained for 10 gynecologic-cancer cell lines following their exposure to different cytotoxic agents

	Cell lines									
	AN3	AE7	ECC	HECA	HECB	BG1	CAOV	SKOV[a]	ME	SKUT
10^{-8} M DXR	2.8	8.6	39	8.3	21	63	2.9	20	3.2	2.9
10^{-5} M CYT	1.9	1.9	0.5	1.9	2.0	2.1	3.7	0.6	1.4	NA
10^{-5} M DDP	0.7	0.5	1.3	1.0	2.6	2.8	7.1	0.6	0.7	0.15
10^{-5} M 5FU	1.5	5.3	3.4	8.0	3.4	6.8	15	11	110	NA
10^{-12} M U73	1.6	6.9	0.8	9.3	51	33	0.6	2.6	2.8	1.6

Underlined data represent the two highest values for the above cytotoxic agents among 10 gynecologic-cancer cell lines, which were abbreviated as follows: CAOV for CAOV3, SKOV for SKOV3, SKUT for SKUT1B, ME for ME180, ECC for ECC1, HECA for HEC1A, and HECB for HEC1B

[a] SKOV3 was derived from an ovarian-cancer patient who had previously been treated with 5FU

21.5%±2.1%, 25.5%±3.3%, 32.7%±4.9%, and 40.2%±6.4% for controls and doses of 0.1, 0.2, and 0.5 X, respectively. At all doses, the S-phase blocks induced by Adozelesin were significant (P <0.02). The mean G2-phase fractions were 14.5%±1.8%, 21.0%±2.7%, 23.7%±4.0%, and 23.8%±3.6% for controls and doses of 0.1, 0.2, and 0.5 X, respectively. The Adozelesin-induced G2 blocks were also significant at all doses (P <0.04). The accumulation of cells in G2 and S blocks led to a depletion of cells in the G1 compartment. Viable cell counts appeared to parallel the changes observed in the G1 phase and reflected the cytotoxicity of Adozelesin.

Discussion

Adozelesin, a derivative of CC-1065, represents a new class of cytotoxic agents. The parent compound is one of the most potent cytotoxic agents ever discovered [14, 19–21]. Several important insights have been gained by studying the mechanism underlying the cytotoxicity of CC-1065. Its spiral structure was found to promote preferential and selective binding to specific DNA sequences [27, 31, 33–35]. The adjacent cyclopropyl group can then initiate alkylation reactions and lead to covalent bonding to cellular DNA. Because of these special characteristics, CC-1065 derivatives are very effective at low doses; the amount of drug required often lies in the range of picograms instead of the usual microgram doses for commonly used agents [14, 19–21]. The derivative Adozelesin has all of the advantages of the parent compound except the delayed death in mice previously reported for CC-1065 [10, 22, 33]. From the data shown in Table 1, it can be seen that this drug elicited a sensitivity response in 80% of the cell lines tested; its sensitivity profile was comparable with those of Adriamycin and 5FU. However, in comparative evaluations using molar concentrations, Adozelesin was 10^4 times more potent than Adriamycin and about 10^7 times more potent than 5FU, cisplatin, or 4OH-CYT. Because of its selective and preferential DNA binding, Adozelesin remained effective at picomolar doses (mean IC_{50}, 11.0±5.4 pM).

In Table 1, underlined the two highest IC_{50} values obtained for each drug. A certain degree of relative resistance to the above drugs became evident in the BG1 and HEC1B cell lines. Because of their broad pattern of resistance, both BG1 and HEC1B can be used as potential models to study the mechanisms of drug resistance. The SKOV3 cell line, which was previously treated with 5FU, exhibited the highest resistance to this drug. This observation supports the need to use different chemotherapeutic agents as second-line therapy following initial treatment failure.

Because Adozelesin alkylates and binds to DNA, its effects on the cell cycle were expected to be similar to those of other alkylating agents. Alkylation of DNA could interfere with cellular transcription and replication events [8]. Adozelesin caused significant S and G2 blocks in the CAOV3 cell line. These cell-cycle perturbations exhibited a dose-response relationship that correlated with cytotoxicity as determined using viable cell counts and the ATP chemosensitivity assay. However, the G2 block demonstrated a heterogeneous pattern of response; whereas the 0.1 X concentration caused a G2 accumulation at 24 h, the 0.2 and 0.5 X doses produced an initial depression of G2 cell fractions. The S-phase block caused by the 0.1 X dose was not as severe as those produced by doses of 0.2 and 0.5 X. The initial G2 reduction at doses of 0.2 and 0.5 X probably resulted from a significant S block that delayed the entry of sufficient cells into the G2 phase. Flow cytometric data showed that up to 50% of the cells were delayed in the S phase and prevented from entering the G2 phase. The initial G2 reduction at 24 h might also have been due to preferential killing of cells in the G2 and G2-M phases as previously reported for CC-1065 [5]. At 48 h, more cells began to enter the G2 phase and showed up as G2 blocks. The G2 and S blocks were maintained until 168 h posttreatment. In summary, the overall cell-kinetic effects of Adozelesin involved S and G2 blocks as well as G1 depletion. The S and G2 blocks were statistically significant as compared with control values (P <0.04). The severity of these blocks appeared to correlate with cytotoxicity as demonstrated by the ATP chemosensitivity assay and viable cell counts.

The above panel of gynecologic-cancer cell lines represent tumors from the ovary, uterus, and cervix. These cell lines appeared to be quite resistant to cisplatin. According to our criteria for drug sensitivity, the cell lines ME180 and AN3 (dose response, 20%) were considered to be sensitive to cisplatin. This low rate of response to cisplatin might have been due to a bias in cell-line selection or to the experimental conditions used and may not reflect the actual clinical activity of the drug.

Traditionally, colony-forming assays have been the gold standard in studies of drug response. However, these assays have several disadvantages that make them impractical for routine clinical use [4, 29]. One major drawback of the colony-forming assay is its low rate of clinical applicability (40%–50%) [4, 29, 32], which is partly attributable to poor tumor growth and to the large number of cells required for the assay. Since the assay depends on the ability of stem cells to form colonies, it could be argued that the low drug response was representative of stem cells but not of the entire tumor population [4, 29]. For several years, ATP bioluminescence assays have been used to study drug response in the United States, Japan, and Europe [12, 15, 17, 24, 25]. The ATP chemosensitivity test has several advantages. It is a highly quantitative assay that measures drug response according to cellular ATP activity instead of counting colonies. The assay is reproducible and reliable and its rate of clinical applicability is 95% [30]. Moreover, it is extremely sensitive, as its detection limit is 50 cells [23]. As a result, the ATP assay can be successfully performed even when cell harvest yields a small number of cells. In similar studies in Japan and Europe, ATP assays have been shown to correlate excellently with other assay systems [3, 18, 28].

In conclusion, Adozelesin is an interesting compound belonging to a new class of compounds with selective and preferential DNA-binding affinity. As an alkylating agent, it proved to be more potent than other alkylators such as cisplatin or Cytoxan. Adozelesin is currently undergoing phase I clinical trials at various cancer centers in the United

States; its toxicity to humans will not be known until the completion of these studies. Despite its potency. Adozelesin is not expected to distinguish between benign and malignant cells; thus, it would be expected to produce various organ toxicities, even, severe side effects, in patients. However, because of its extraordinary potency and its demonstrated efficacy against gynecologic cancer cells, Adozelesin has the potential to become an important chemotherapeutic agent.

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