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V79 Chinese hamster lung cells resistant to the bis-alkylator bizelesin are multidrug-resistant

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Abstract. Bizelesin (U-77779) is a highly potent bis-alkylating antitumor agent that is effective against several tumor systems in vitro and in vivo. V79 cells that were 125- to 250-fold resistant to bizelesin developed after constant exposure to gradually increasing concentrations of the drug. Resistant cells exhibited a multidrug-resistant phenotype and genotype as indicated by cross-resistance to several structurally and functionally unrelated drugs, e. g., colchicine, actinomycin D, and Adriamycin, and overexpression of mdr mRNA. Very low levels of cross-resistance to the alkylating agents cisplatin and melphalan were seen. Multidrug-resistant mouse leukemia (P388/Adriamycin-resistant) and human (KB/vinblastine-resistant) cells were also resistant to bizelesin. Bizelesin resistance was unstable and decreased when cells were grown in the absence of the drug. Resistant and sensitive cell lines had similar levels of glutathione, and bizelesin cytotoxicity for resistant cells was not markedly affected by treatment with buthionine sulfoximine. Cross-resistance between bizelesin and several of its analogs is reported.

Introduction

Bizelesin (U-77779) is a synthetically derived analog of the highly potent, alkylating, antitumor antibiotic CC-1065 [20]. CC-1065 binds nonintercalatively in the minor groove of double-stranded DNA at AT-rich regions followed by covalent bonding with N-3 of adenine in preferred base sequences [13, 29]. Although CC-1065 showed moderate antitumor activity in vivo, it was not evaluated clinically because it caused delayed deaths in mice at therapeutic doses [21]. Bizelesin is one of many CC-1065 analogs [22] that exert excellent broad-spectrum antitumor activity in vivo without causing delayed deaths [9, 10].

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Bizelesin is a dimeric molecule containing two of the DNA-alkylating cyclopropapyrroloindole (CPI) subunits of CC-1065 joined by a rigid bis(indolocarboxylic) linker [22]. Bizelesin, like its monomeric analogs, forms monoalkylation adducts with N-3 of adenine, but also forms DNA-DNA cross-links at AT-rich sequences [28]. On the basis of its in vivo antitumor activity and its unique mechanism of action, bizelesin is being considered for clinical trials.

Development of tumor cells resistant to antineoplastic agents is obviously clinically significant. When resistance to one drug was associated with cross-resistance to several structurally unrelated drugs, it was termed multidrug resistance (MDR) [14–16, 26]. This resistance phenotype was characterized by cross-resistance to structurally unrelated agents [15], reversal of resistance with verapamil [17], decreased intracellular drug concentration due to decreased drug uptake or increased efflux [18], and amplification or overexpression of one or more *mdr*-associated genes [25]. These characteristics pertain to P-glycoprotein-mediated MDR. MDR usually develops in response to large, hydrophobic, natural-product molecules, whereas cells resistant to known alkylating agents are usually not multidrug-resistant [30].

Materials and methods

Cell culture. V79 cells (Chinese hamster lung fibroblast, obtained from Dr. Ralph E. Durand, British Columbia Research Center, Canada) were grown as monolayer cultures as described previously [4]. Bizelesin-resistant V79 cells (V79/R-C6 or V79/R-D6) were grown in medium containing 0.5–1 ng of the drug/ml.

To maintain exponential growth, cells were subcultured prior to reaching a density of 5×10^6 cells/75-cm^2 flask. Monolayers were harvested by a washing step with phosphate-buffered saline followed by incubation at 37° C with a trypsin solution (0.05% trypsin and 0.02% ethylenediaminetetraacetic acid, EDTA). Since V79 cells clump readily, cells were dispersed while suspended in trypsin solution so as to obtain a single-cell suspension. Medium was added and the cells were redispersed and counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Cells were seeded 48 h prior to the experiments.

KB-3-1 (parent) and KB-V1 (vinblastine-resistant human epidermoid carcinoma) cell lines were obtained from Dr. M. Gottesman

(National Cancer Institute, NIH, Bethesda, Md.) and grown as monolayers in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% fetal calf serum [27]. KB-V1 was maintained in 1 μg vinblastine/ml. Adriamycin-resistant (P388/Adr) and sensitive (P388/O) mouse leukemia cells (Southern Research Institute, Birmingham, Ala.) were grown in suspension in RPMI 1640 medium supplemented with 5% fetal calf serum.

Cell survival and growth inhibition assay. Cell survival after drug exposure was determined by a colony-formation assay [3]. To determine cell survival after continuous exposure to drug, cells ($200-10^4$ cells) and drug were added to six-well Linbro plates. Plates were incubated for colony formation and the colonies were stained and counted [3]. The cloning efficiencies of V79/S and bizelesin-resistant cells were approximately 70% and 30%–40%, respectively. The cloning efficiency of the untreated (control) cells was normalized to 100%, and the cloning efficiency of the treated cells was expressed as a percentage of control survival. For each drug concentration, duplicate cultures were used. Vehicle or solvent controls were included in each experiment.

Growth inhibition of V79/S and V79/R cells was determined by exposing duplicate cultures to bizelesin. The variation between experiments was generally less than 10% for a given mean value. The percentage of growth inhibition was calculated as:

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

Growth inhibition of P388 cells was measured by plating cells (5 ml of 2×10^4 cells/ml) in 15-ml tubes, which were gassed with a 6% CO_2-air mixture. Drugs were added and the cells were incubated at 37° C. Growth inhibition of P388/O and P388/Adr by bizelesin versus Adriamycin was compared after 3 days of exposure to drug.

RNA isolation and Northern analysis. Total RNA was isolated from almost confluent cell cultures of V79/S and resistant clone V79/R-D6 by lysis with guanidine thiocyanate followed by ultracentrifugation over a CsCl cushion [7, 19]. Next, 10 μg RNA was glyoxylated and run on a 1.5% agarose gel in 10 m*M* NaH_2PO_4 buffer (pH 6.8). RNA was transferred to a GeneScreen filter membrane (NEN) by electroblotting in TAE (40 m*M* TRIS-acetate, 1 m*M* EDTA). After complete transfer, the damp blot was wrapped in Saran Wrap and the RNA was cross-linked to the membrane under short-wave UV light at 1200 μW/cm^2 for 2.5 min, for a dose of 1.6 kJ/m^2 [8].

A 3.4-kb *Eco*RI fragment containing a human *mdr* 1 coding sequence [24] was used to probe for MDR sequences. Hybridization with cyclophilin A was used as a standard to quantitate the amount of mRNA per lane. These cDNA probes were radiolabeled by random primer extension [11] to a specific activity above 1×10^9 cpm/μg. Filters were hybridized and washed as described elsewhere [1].

Source of materials. Bizelesin (U-77779, The Upjohn Company) was dissolved at 100 μg/ml and stored frozen in dimethylacetamide. Adriamycin (Sigma) was dissolved in 0.01 *M* glucuronic acid. Cisplatin (clinical sample containing 1 mg cisplatin, 9 mg NaCl, and 10 mg mannitol) was dissolved in 10 ml sterile H_2O. Colchicine (Sigma) was also dissolved in sterile H_2O. Vinblastine (Eli Lilly and Company, Indianapolis, Ind.) and actinomycin D (Calbiochem-Behring Corporation, La Jolla, Calif.) were dissolved in 100% ethanol. Melphalan (National Cancer Institute, Bethesda, Md.) was dissolved in a mixture of 0.1 *N* HCl plus ethanol (1 : 1, v/v). Drugs were diluted in medium immediately prior to their addition to the cells. All medium components (e. g., MEM, trypsin-EDTA, fetal calf serum) were obtained from Irvine Scientific (Irvine, Calif.). Verapamil hydrochloride and buthionine sulfoximine (BuSO) were obtained from Sigma (St. Louis, Mo.)

CPI analogs tested for cross-resistance to U-73975. The CPI analogs CC-1065, U-75559, and adozelesin were obtained from Dr. R. C. Kelly (The Upjohn Company). The drugs were dissolved in dimethyl-sulfoxide at 100 μg/ml and stored frozen. The structures of these analogs are shown in Fig. 1. The general structural features of the CPI analogs are as follows. CC-1065 (U-56314) is the parent compound and is comprised of three pyrroloindole subunits (A, B, and C) linked by amide bonds. The left-hand subunit (A) contains the reactive cyclopropyl ring, which forms an adduct with the N-3 of adenine in DNA. All of the analogs are based on the A-ring either in the form of spiro cyclopropyl cyclohexadienone (CC-1065 and adozelesin) or in its HCl opened form (bizelesin). The B- and C-ring systems vary significantly between the analogs. In adozelesin the tricyclic pyrroloindole B- and C-ring systems of CC-1065 have been converted to indole (B) and benzofuran (C). U-75559 and bizelesin contain two CPI subunits that are linked in U-75559 by a flexible tether (C-8 alkane dicarboxylic acid) and in bizelesin by a urea-linked pair of indole dicarboxylic acids.

Estimation of partition coefficients of CPI analogs. The partition coefficients were determined by methods described previously [5].

DNA staining and flow cytometry. Cells were fixed in ethanol and stained for DNA with mithramycin and were then analyzed for DNA content on a Becton-Dickinson FACStarPLUS (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) as described previously [4]. The following data were collected for about 4×10^4 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, Topshan) running on a Compaq 386/33 computer.

Glutathione determination. The cellular glutathione (GSH) level was determined by the method of Nakashima et al. [23] as described previously [2].

Results

Development of V79 cells resistant to bizelesin

Resistant cells developed after continuous exposure of parent V79/S cells to increasing levels of bizelesin starting at 0.0125 ng/ml (Fig. 2). Cells were maintained at each drug concentration for at least two passages. The drug dose was escalated in small increments until growth stopped and the cells appeared damaged (swollen and vacuolated). Cells were then allowed to recover in fresh drug-free medium. The procedure was repeated at a higher dose. After about 3 months, cells grew in the presence of 0.5–1 ng bizelesin/ml. Cells were cloned, and two distinct colonies were subcultured as resistant cell lines V79/R-C6 (or C6) and V79/R-D6 (or D6). It is clear from Fig. 2 that resistant cells lost viability after continuous exposure to more than 1 ng bizelesin/ml in spite of their being allowed to recover several times. Therefore, resistant cell lines were maintained in 1 ng bizelesin/ml.

Growth inhibition observed after continuous exposure to bizelesin of V79/S and resistant clones V79/R-C6 and V79/R-D6 is shown in Fig. 3. Bizelesin caused 50% growth inhibition (ID_{50}) in V79/S at 0.004 ng/ml as compared with 0.7–0.9 ng/ml for C6 and 0.9–1.2 ng/ml for

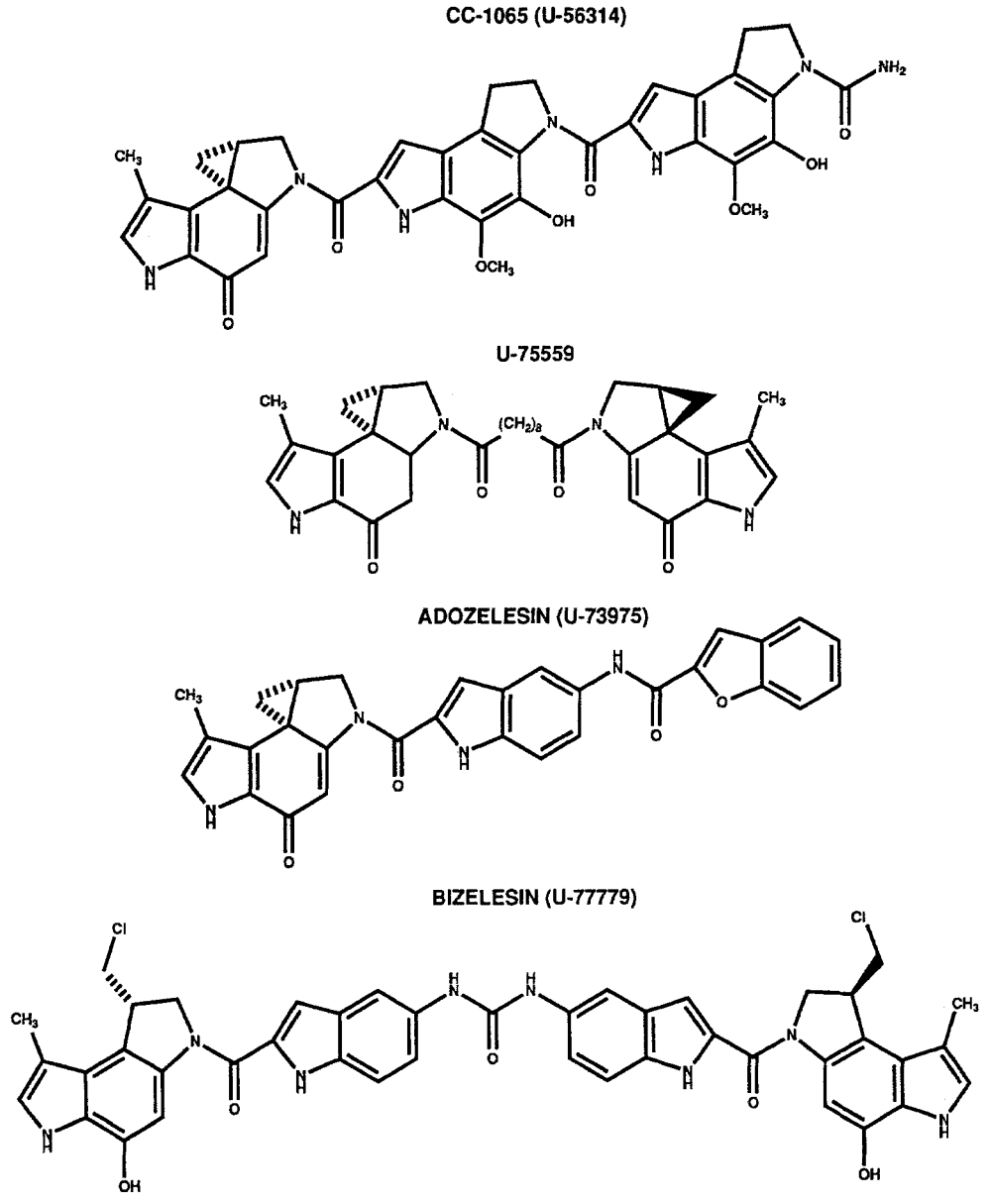

Fig. 1 Structures of bizelesin and CPI analogs

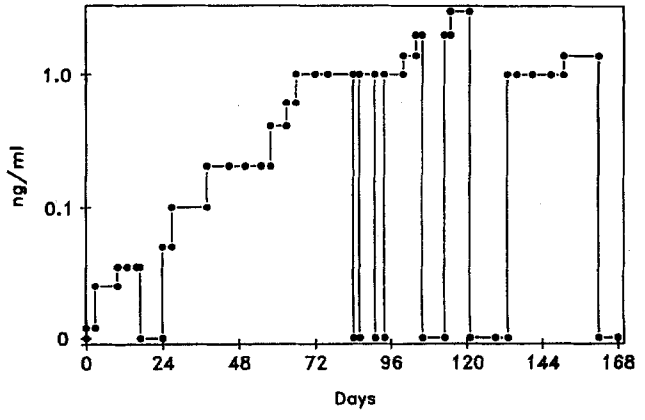

Fig. 2. Genesis of bizelesin-resistant V79 cells. V79/S cells were exposed to increasing concentrations of bizelesin starting at 0.0125 ng/ml. Cells were carried at each drug concentration for at least 2 passages before the drug concentration was increased. When the cells looked damaged, the drug was removed and the cells were allowed to recover before continuation of the procedure

D6, respectively. After 2 h of drug exposure, the ID_{50} for D6 cells was 13.1 ng/ml as compared with 0.05 ng/ml for V79/S.

The survival of V79/S, C6, and D6 cells after continuous exposure to bizelesin showed (Fig. 3B) that the 50% lethal dose (LD_{50}) for D6 and C6 cells was 0.67 and 0.75 ng/ml, respectively, as compared with 0.006 ng/ml for V79/S cells. These results show that bizelesin-resistant D6 cells were 250 (growth inhibition assay) or 125 times (survival assay) more resistant than the sensitive parent (V79/S) cells. D6 cells grown for 2 weeks without bizelesin (i.e., $D6^-$ cells in Fig. 3A) lost half of their resistance.

Figure 4 shows the time course of growth inhibition for V79/S and D6 cells exposed to bizelesin. The growth of V79/S cells exposed to 0.04 ng/ml stopped completely after 30 h of exposure to bizelesin, whereas D6 grew continuously, albeit at a slower rate than the control, even when exposed to 5 ng/ml.

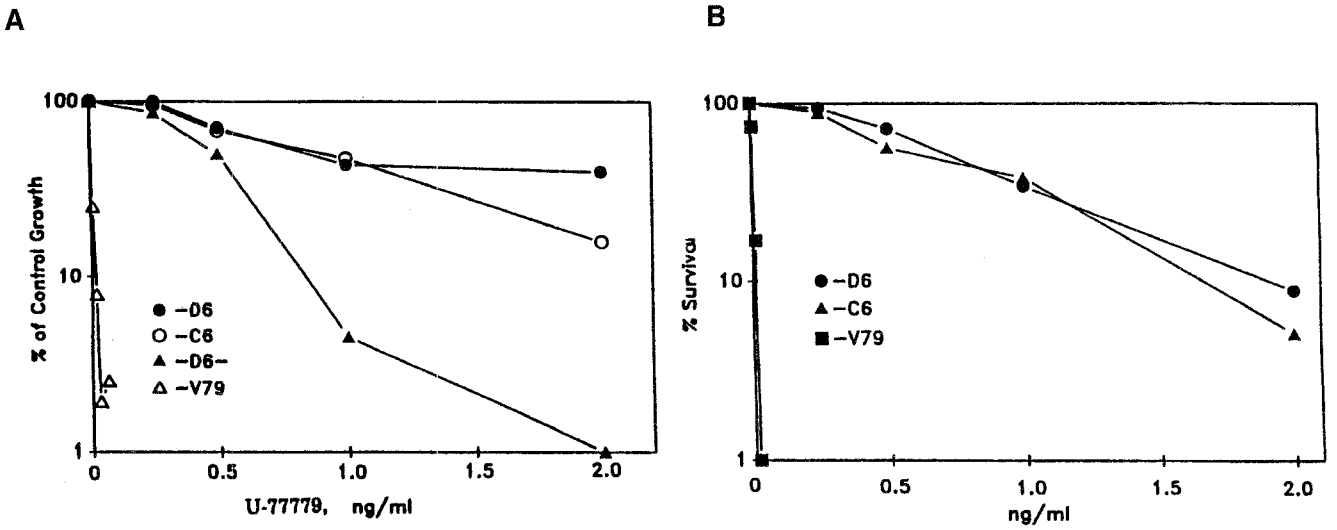

Fig. 3.A Growth inhibition after continuous (4-day) exposure to bizelesin. D6, C6, and V79/S cells were exposed to bizelesin for 4 days and growth inhibition was measured at the end of this period. C6 and D6 are bizelesin-resistant clones derived from the sensitive V79/S parent line. D6⁻ indicates D6 cells maintained without bizelesin for 2 weeks. Error bars are within the limits of the symbols (○, △, □) in the figure for both **A** and **B. B** Cell survival of D6, C6, and V79/S cells after continuous exposure to bizelesin. Cells were planted for colony formation in the presence of drug for 6–10 days

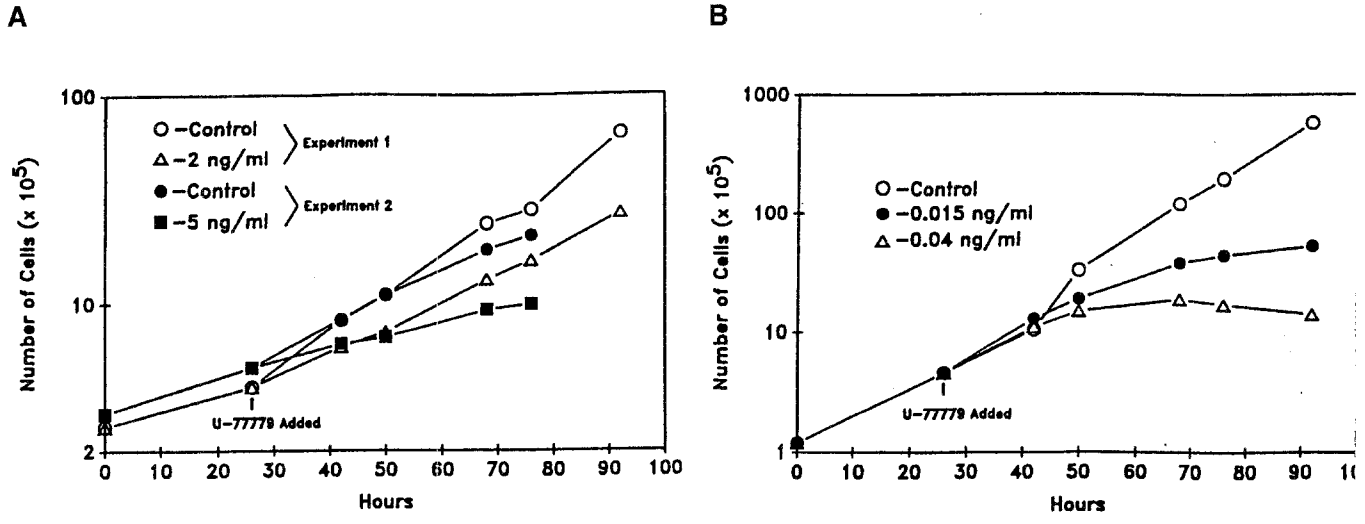

Fig. 4A, B. Time course of growth inhibition of V79/S (**B**) or D6 (**A**) cells by bizelesin. Error bars are within the limits of the symbols (○, △, □) in the figure

Cell-cycle progression of V79/S and D6 cells exposed to bizelesin

DNA histograms of V79/S and D6 cells are shown in Fig. 5. The 0-h samples represent control histograms and show essentially normal DNA distribution in resistant cells. V79/S cells exposed to 0.015 and 0.04 ng/ml for 16 h accumulated primarily in the G_2-M phase in a dose-dependent manner. After 50 h of exposure at 0.015 ng/ml, a significant proportion of G_2-M cells had escaped the G_2-M block to progress into the G_1 and S phases and a minor proportion of cells became polyploid. After 50 h of exposure at 0.04 ng/ml, a significant proportion of the V79/S population became polyploid. D6 cells exposed to 2 or 5 ng U-77779/ml accumulated in the G_2-M phase at 24 h. By 66 h, cells exposed to 2 ng/ml had escaped the G_2-M block and assumed a normal phase distribution. D6 cells exposed to 2 or 5 ng/ml showed a very small percentage of polyploid cells. Thus, these results show a significant difference in the dose-dependent cell-cycle response of sensitive and resistant cells.

MDR phenotype and genotype of bizelesin-resistant cells

When cells become resistant, particularly to a hydrophobic drug, they often become multidrug-resistant, i. e., become cross-resistant to a variety of structurally and functionally unrelated drugs. Therefore, the resistance of D6 cells to several drugs such as colchicine, actinomycin D, Adriamycin, vinblastine, cisplatin, and melphalan was measured. Colchicine and vinblastine affect the microtubules, acti-

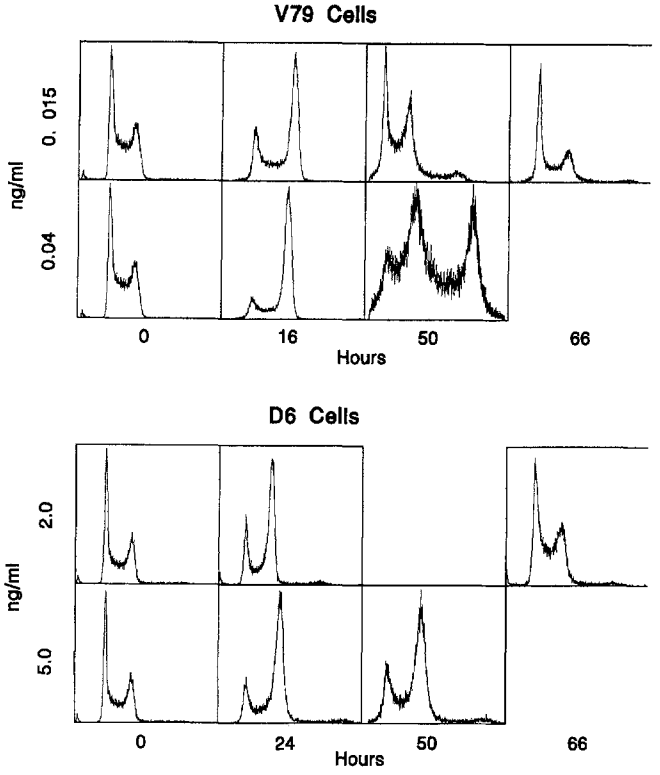

Fig. 5. Cell progression during continuous exposure to bizelesin. The DNA histograms show G_1 cells in the *left-hand peak*, G_2-M cells in the *right-hand peak*, and S cells in the *middle*. Note the much higher drug concentrations used for D6 as compared with V79/S cells

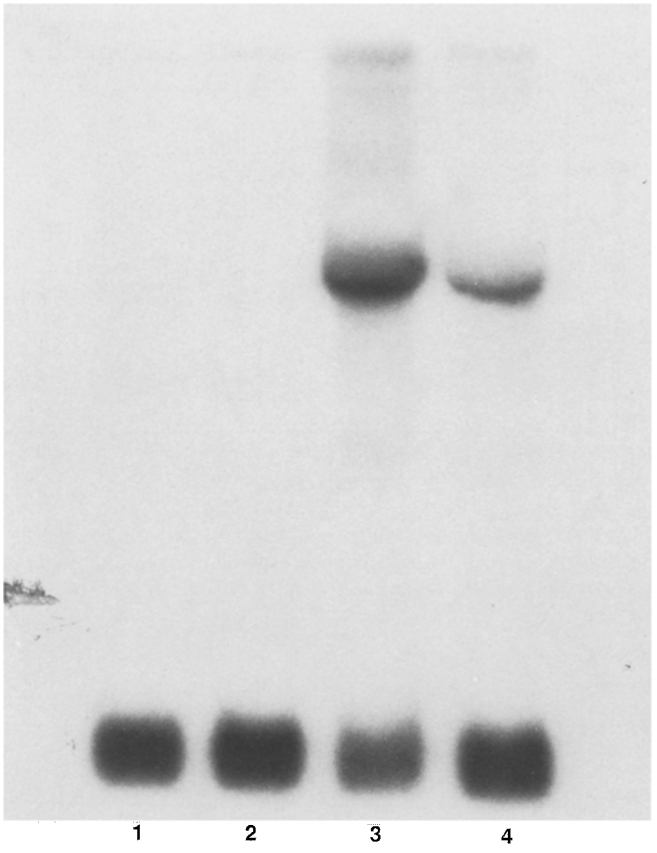

Fig. 6. Levels of mdr1-mRNA in a Northern blot of V79/S, D6, and D6$^-$ cells. The *upper band* indicates mdr-1 levels. *Lanes 1, 2*, V79/S cells; *lane 3*, D6 cells; *lane 4*, D6$^-$ cells (D6 cells grown in the absence of bizelesin for 2 weeks). Cyclophilin A (*lower band*) was also probed and used as a standard to determine relative amounts of mRNA loaded into each well

nomycin D and Adriamycin interact with DNA, and cisplatin and melphalan are alkylating agents. The drug concentrations required for 50% inhibition of growth (ID_{50}) or survival (LD_{50}) are presented in Table 1. D6 was 112 (based on the LD_{50}) or 256 times (based on the ID_{50}) more resistant to bizelesin than was V79/S. D6 was highly cross-resistant to colchicine, actinomycin D, Adriamycin, and vinblastine. There was a very low level of cross-resistance to cisplatin (1.7-fold) and melphalan (1.4-fold). These results show that U-77779-resistant (D6) cells were strongly cross-resistant to colchicine, actinomycin D, Adriamycin, and vinblastine and were very weakly cross-resistant to the alkylating agents.

The MDR human carcinoma cell line KB/V1 was also cross-resistant to bizelesin. KB/V1 was about 200 times more resistant to vinblastine and 100 times more resistant to bizelesin ($ID_{50} > 0.1$ ng/ml) than was KB 3.1. Similarly, the MDR mouse leukemia P388/Adr cell line was cross-resistant to bizelesin. P388/Adr was about 900 times more resistant to Adr (ID_{50} of Adr, 45 μg/ml) and 52 times more resistant to bizelesin (ID_{50}, 0.21 ng/ml) than was the parent line (P388/O). These results show that other MDR cell lines are cross-resistant to bizelesin.

MDR usually correlates with amplification and overexpression of the *mdr* gene(s). Figure 6 shows that the *mdr* transcript was barely detectable in RNA from V79/S but gave a strong signal in the bizelesin-resistant clone V79/R-

Table 1. MDR phenotype of D6 cells

	Bizelesin[a]		Colchicine[b]		Actinomycin D[b]		Adriamycin[b] LD_{50}	Vinblastine[b] LD_{50}	Cisplatin[b]		Melphalan[b] LD_{50}
	ID_{50}	LD_{50}	ID_{50}	LD_{50}	ID_{50}	LD_{50}			ID_{50}	LD_{50}	
V79/S	0.004	0.006	0.0023	0.004	0.0031	0.003	0.014	0.002	0.219	0.25	0.336
D6	1.06	0.67	0.169	0.58	0.181	0.22	≅6	> 0.1	0.375	0.44	0.475
Fold increase in resistance[c]	256	112	73.5	145	58.4	73	≅429	>50	1.72	1.8	1.41

ID_{50}, 50% growth inhibitory dose; LD_{50}, 50% cell-kill dose

[a] For bizelesin, ID_{50} and LD_{90} values are expressed in ng/ml

[b] For drugs other than bizelesin, ID_{50} and LD_{50} values are expressed in μg/ml

[c] Fold increase in resistance is the ratio of the ID_{50} or LD_{50} for D6 over that for V79/S

Table 2. Cross-resistance of bizelesin-resistant cells to other CPI analogs[a]

Compound	LD_{90} (ng/ml)[b]		Ratio[c] V79/R : V79/S	Log P[d]
	V79/S	V79/R		
Bizelesin	0.0045	0.8	178	2.75±0.35
Adozelesin	0.02	0.96	48	2.5 ±0.31
U-75559	0.0175	4.3	245	2.39±0.36
CC-1065	0.2	1.0	5	1.43±0.4

[a] The structures of these compounds are shown in Fig. 1

[b] LD_{90} (90% lethal dose by a cell-survival colony-counting assay) values are for continuous exposure of V79/S and V79/R cells to the drugs

[c] Ratio = LD_{90} for V79/R:LD_{90} for V79/S. This shows the increase in resistance to a test compound in V79/R as compared with V79/S cells. A high ratio indicates a high degree of cross-resistance between the test compound and bizelesin

[d] Log P values were obtained from Bhuyan et al. [5]. P (partition coefficient) increases with increased hydrophobicity of the compound

D6. $D6^-$ cells (grown for 2 weeks without bizelesin) lost half of their drug resistance (Fig. 3 A). This correlated with their lower level of expression of *mdr*-mRNA. Densitometric measurements showed that the hybridization of $D6^-$ mRNA to the *mdr* probe was 18.6% of that of D6.

Reversal of resistance by verapamil

Verapamil had a dual effect on resistant cells. It was more toxic to resistant D6 cells (verapamil LD_{90}, 0.5–1 μg/ml) than to V79/S cells (LD_{90}, >10 μg/ml). Verapamil also rendered D6 cells more sensitive to bizelesin. Thus, after normalizing for the toxicity of verapamil, we found that exposure to 0.25 ng bizelesin/ml allowed 50% of the resistant D6 cells to survive as compared with the 0.2% survival noted after bizelesin plus verapamil exposure.

Resistant cells do not contain increased cellular levels of GSH

Since cells often become resistant to alkylating agents by increasing the level of GSH-related metabolites [31], we compared GSH levels in V79/S and bizelesin-resistant C6 and D6 cells. The sensitive and resistant cells contained similar amounts of GSH (nanomoles/10^6 cells): V79/S, 7±0.35; C6, 6.9±0.4; and D6, 6.6±0.5. We also determined the effect of treatment with BuSO on bizelesin toxicity. BuSO lowers cellular GSH levels and thereby increases the sensitivity of resistant cells to alkylating agents [12]. Bizelesin-resistant cells were exposed to BuSO (30 μ*M*) for 24 h prior to the addition of bizelesin. Exposure to both compounds continued until colonies formed. Under these conditions, the LD_{90} of bizelesin was 0.45 ng/ml as compared with 0.31 ng/ml for bizelesin plus BuSO.

Cross-resistance of bizelesin-resistant cells to other CPI analogs

Table 2 presents the cross-resistance of V79/R-D6 to several CPI analogs. Table 2 shows that these resistant cells have a high degree of cross-resistance to adozelesin and U-75559 and a much lower level of cross-resistance to CC-1065 (U-56314). The correlation between the hydrophobicity of several analogs and their level of resistance is shown in Table 2. In general, the two compounds (U-75559 and adozelesin) with a high partition coefficient (log P > 2) showed a high degree of cross-resistance to bizelesin. CC-1065, with a low partition coefficient (log P = 1.43), had a low level of cross-resistance.

Discussion

The bizelesin-resistant cell lines were 125–250 times more resistant than the parent (V79/S) cell line, irrespective of whether the level of resistance was measured following long-term (8-day) or 2-h exposure to the drug or whether growth inhibition and cell survival were the measured end points.

The bizelesin-resistant cell line had the expected phenotypic and genotypic characteristics [15, 18] of P-glycoprotein-mediated MDR: (1) cells were cross-resistant to several structurally and functionally unrelated drugs, (2) there was significant overexpression of *mdr*-mRNA, and (3) verapamil reversed drug resistance. Two other MDR cell lines, KB-V1 and P388/Adr, were also resistant to bizelesin.

Since cells resistant to alkylating agents are usually not multidrug-resistant, the MDR phenotype of this cell line was unexpected. However, unlike most alkylating agents, bizelesin is a large hydrophobic molecule that probably meets the structural requirement for interacting with P-glycoprotein.

The greater toxicity of verapamil for the bizelesin-resistant cell line (LD_{90}, 0.5–1 μg/ml) as compared with V79/S (LD_{50}, >10 μg/ml) has been observed with other MDR cell lines. Cano-Gauci and Riordan [6] reported that the parent CHO cells tolerated 80 μ*M* verapamil, whereas 2 μ*M* verapamil was cytotoxic to MDR clones.

Our results agree with other studies showing that cells resistant to one alkylating agent are not cross-resistant to all other alkylating agents [30]. Bizelesin-resistant cells showed a very low level of cross-resistance to cisplatin and melphalan. BuSO did not greatly increase the toxicity of bizelesin for resistant cells. BuSO has been used to reduce cellular GSH levels and thereby modulate resistance to alkylating agents [12]. Also, the cellular level of GSH did not change with resistance development. Therefore, our results suggest that the GSH/glutathione-S-transferase pathway is not likely to be involved in bizelesin resistance.

We observed that CPI analogs (adozelesin, bizelesin, U-75559) with a high partition coefficient had a high level of resistance, whereas CC-1065, with a low partition coefficient, showed a low level of resistance. Zsido et al. [31] also reported that as compared with the sensitive parent the MDR cell line CH^R-C5 had a much lower level

of resistance to CC-1065 than to adozelesin and bizelesin. We also noticed a difference in cross-resistance among the three compounds (bizelesin, adozelesin, and U-75559) with similar log P values. U-75559 had a high level of cross-resistance and, like bizelesin, was a bis-alkylating agent. In contrast, adozelesin, a monofunctional agent, had a lower level of cross-resistance. We do not know whether this difference relates to the difference in alkylating functionality between adozelesin and bizelesin or to their cellular pharmacology.

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