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RESISTANCE OF CHO CELLS EXPRESSING P-GLYCOPROTEIN TO CYCLOPROPYLPYRROLOINDOLE (CPI) ALKYLATING AGENTS

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Abstract—Several new antitumor agents belonging to the class of minor groove binders that are able to form covalent bonds with DNA via a cyclopropylpyrroloindole (CPI) group are susceptible to a multidrug resistance (MDR) phenotype in Chinese hamster ovary (CHO) cells. The multidrug resistant CCH^R-C5 cell line was 16-, 23- and 13-fold more resistant to the analogs U-73,975, U-77,779 and U-80,244, respectively, although its cytotoxic response to the parent compound CC-1065 was similar to the response of the drug-sensitive wild-type cells (AuxB1). For a sequence of MDR cell lines showing increasing expression of P-glycoprotein (Pgp) there were corresponding increments in the level of resistance to U-73,975, arguing that Pgp is the key determinant in resistance of the MDR cells to CPI agents. MDR cells treated with U-73,975 showed diminished generation of covalent adducts on DNA as well as increased resistance to cytotoxicity.

A new class of antitumor agents which we refer to here as cyclopropylpyrroloindole (CPI†) compounds have the common property of binding to the DNA minor groove and forming covalent bonds with the DNA through a CPI moiety [1, 2]. CC-1065 (Fig. 1), the parent drug, was found to be very potent against a broad spectrum of human tumor cell lines in cloning assays and against *in vivo* murine tumor systems [3, 4], but preclinical studies revealed a delayed toxicity [5]. Recently, analogs still possessing extreme potency yet lacking delayed toxicities have been synthesized [6, 7]. Three analogs (Fig. 1), U-73,975, U-77,779 and U-80,244, are currently being developed for phase I clinical trials [8–10].

Studies in cell-free systems have suggested that the antiproliferative properties of these drugs are related to their potential to form covalent lesions on DNA, since analogs lacking the ability to form adducts with isolated DNA are significantly less cytotoxic [11, 12]. Moreover, we have shown that analogous covalent adducts are formed on the DNA of whole cells by CC-1065, consistent with a key role for these lesions in the cytotoxic action of this class of compounds [13].

To date, only one study has addressed resistance to CPI compounds. Moy *et al.* [14] isolated a B16 melanoma line which is 60- to 100-fold resistant to the selecting drug U-71,184 and exhibits cross-resistance to a number of CPI agents. Although the mechanism(s) responsible for the resistance has not been fully characterized, the diminished sensitivity could be partially accounted for by decreased uptake of the compound. This resistance phenotype was reported not to involve overexpression of P-glycoprotein (Pgp), the 170 kDa membrane protein which is the determining factor conferring "classic" multidrug resistance (MDR) [15].

We have examined the cytotoxicities of several CPI agents in well characterized Chinese hamster ovary (CHO) cell mutants that do exhibit Pgp-mediated MDR. These cells were selected for drug-resistance to colchicine (CCH) but were found to be cross-resistant to a variety of unrelated agents [16, 17] owing to overexpression of the membrane constituent Pgp [15, 18]. Using this model system we found that some CPI agents belong to the growing list of clinically relevant compounds recognized to be susceptible to Pgp-mediated MDR. Furthermore, the toxicities of two compounds for CHO cells expressing different levels of MDR corresponded to production of covalent adducts on the cellular DNA.

MATERIALS AND METHODS

Drugs. CC-1065 was isolated [19–20] and its analogs were synthesized at the Upjohn Co. (Kalamazoo, MI) [21, 22]. All CPI compounds (CC-1065, U-73,975 (adozelesin), U-77,779 and U-80,244) were dissolved in dimethylacetamide at 2 mg/mL and stored at −20°. The drugs were diluted in phosphate-buffered saline (PBS) prior to addition to cell monolayers.

Cell lines and culture. AuxB1 is a subclone of the CHO cell line [16]. CCH^R-A3, CCH^R-B3 and CCH^R-

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† Abbreviations: CCH, colchicine; CHO, Chinese hamster ovary; CPI, cyclopropylypyrroloindole; D_{10}, drug concentration resulting in 10% relative plating efficiency in clonogenic assays; MDR, multidrug resistance; PBS, phosphate-buffered saline (0.14 M NaCl, 2 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2); Pgp, P-glycoprotein; and TdR, thymidine.

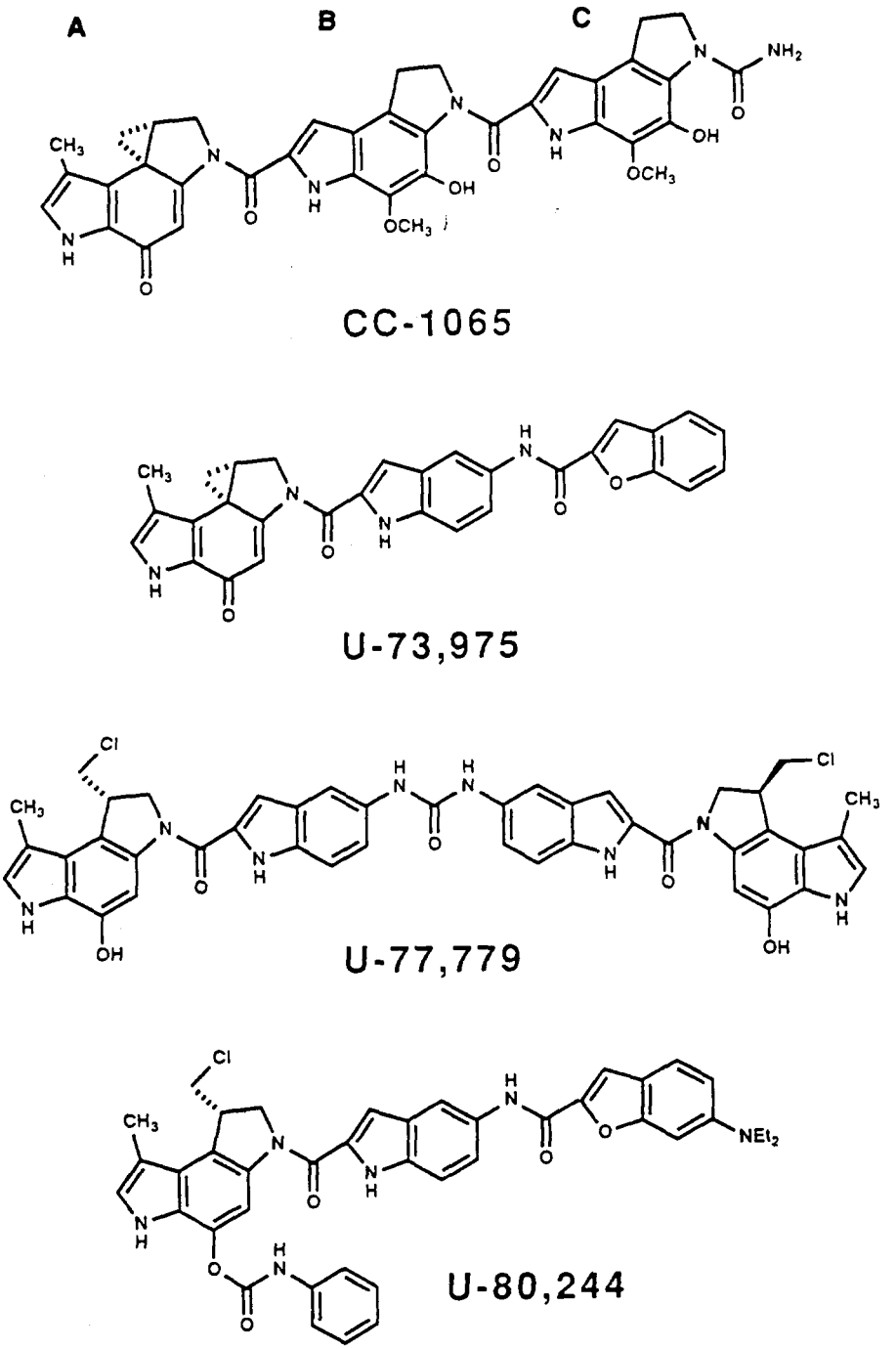

Fig. 1. Structures of CC-1065 and its analogs U-73,975, U-77,779 and U-80,244.

C5 were derived by Ling and colleagues from AuxB1 by serial selection with colchicine [16].

AuxB1 and its derivatives were grown in monolayer culture in α-modified minimum essential medium with nucleosides, 5% fetal bovine serum, and 1% Gentamycin (all from GIBCO, Grand Island, NY) at 37° in 5% CO_2.

Determination of relative plating efficiencies. Tissue culture dishes [multiwell (24 × 1-cm^2) plates or 60-mm petri dishes] were inoculated with 10^2–10^5 cells and the desired concentration of freshly diluted drug. Dishes were incubated at 37°, 5% CO_2 for 7–9 days (AuxB1, CCH^R-A3) or 10–13 days for the slower growing CCH^R-B3 and CCH^R-C5. Colonies were stained with methylene blue and counted, and plating efficiencies of drug-treated cells, relative to non-treated cells, were calculated. Cell sensitivity to drug cytotoxicity is expressed in terms of the

concentration that reduced relative plating efficiency to 10%, defined to be the D_{10}.

In some cases, to assess cytotoxicity under the same conditions as DNA damage (below), cultures were seeded at 4×10^5 cells/100-mm dish and incubated for 48 hr. Each dish was refed with fresh medium and treated with drug for 2 hr. Following drug treatment, the monolayers were washed twice with PBS, trypsinized and plated in triplicate at 2×10^2, 10^3, and 10^4 cells/60-mm dish and 10^5 cells/100-mm dish for incubation and staining as described above.

Sucrose gradient analysis for detection of heat-labile sites. Full experimental details have been described in Ref. 13. Briefly, [^{14}C]thymidine ([^{14}C]-TdR)-labeled cell monolayers were treated with drug for 2 hr at 37°. The cells were suspended in PBS (4°) followed by a 15-min ethanol extraction to reduce the amount of free and non-covalently bound drug. The suspensions were mixed 1:1 with untreated [^{3}H]TdR-labeled cells, lysed in gradient buffer (0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA, pH 8.6) containing 1% sarkosyl (v/v) and 2.5% sucrose (w/v) and concurrently heated at 90° for 15 min. (^{3}H-Labeled cells serve as an internal control, detecting damage that might occur during the post-treatment period by non-covalently bound drug.) Aliquots were loaded on alkaline sucrose gradients (5–30% sucrose in gradient buffer, underlaid with 0.5 mL of 60% sucrose in gradient buffer) and centrifuged at 16,000 rpm for 18 hr at 20° in an SW41 rotor. Gradients were fractionated, sedimentation profiles were calculated as percent recovered radioactivity, and frequencies of breaks were determined as described [13].

RESULTS

Cytotoxicities of CPI agents for CHO lines expressing P-glycoprotein-mediated multidrug resistance. To determine if CC-1065 or related analogs are susceptible to Pgp-mediated MDR, the well characterized MDR cell line CCH^R-C5 and its drug-sensitive counterpart CHO-AuxB1 were exposed to four different compounds of the CPI class. Table 1 summarizes the results of cytotoxicity measurements. The three CPI analogs U-73,975, U-77,779 and U-80,244 indeed showed considerable susceptibility to the MDR phentoype. The degrees of resistance exhibited by CCH^R-C5 are indicated by the ratios of D_{10} values for the MDR line relative to the sensitive line, which range from 13- to 23-fold for the three analogs. The result for CC-1065 was different, however; it was not affected much by MDR.

To further test whether resistance to the CPI compounds correlates with cellular levels of Pgp, relative resistance to U-73,975 was assayed in a series of CHO lines which exhibit graded increases in Pgp expression and MDR phenotype [15]. The CCH^R-A3, CCH^R-B3, and CCH^R-C5 lines are serial clonal isolates selected by Ling and Thompson for increasing degrees of resistance to colchicine [16]. CCH^R-A3 was derived from AuxB1 and is 7-fold resistant, while CCH^R-B3 was derived from CCH^R-A3 and is 31-fold more resistant than AuxB1. CCH^R-C5, selected from CCH^R-B3, is 160-fold more resistant to CCH than AuxB1. Each increment in CCH resistance is accompanied by increased Pgp expression [18].

Table 1. Relative cytotoxicities of CPI drugs to multidrug-resistant CHO cells

CPI drug	D_{10} concentration of drug (nM)		Ratio of D_{10} values CCH^R-C5:AuxB1	
	AuxB1 cells (sensitive)	CCH^R-C5 (multidrug-resistant)	Avg.	(range)
CC-1065	0.18	0.36	2.0	(2.0–2.0)
U-73,975	0.060	0.96	16	(15–17)
U-77,779	0.0070	0.16	23	(22–24)
U-80,244	0.17	2.2	13	(8.0–18)

Each D_{10} value represents an average from two independent experiments in which relative plating efficiency as a function of drug concentrations was assayed concurrently for the AuxB1 and CCH^R-C5 cell lines as described in Materials and Methods. In the different experiments, significant variability in drug potencies was observed (up to 4-fold for U-73,975), but relative cytotoxicities for the cell lines were reproducible as indicated by the ranges of the D_{10} ratios shown.

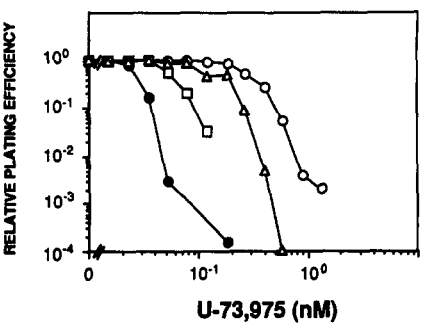

Fig. 2. Survival of CHO wild type (AuxB1) and derivative MDR clones exposed to U-73,975. Relative plating efficiency was measured by the colony-forming ability of a cell population in the presence of the drug compared with the colony-forming ability of the same population in the absence of drug. Cell lines assayed were: (●) AuxB1; (□) CCH^R-A3; (△) CCH^R-B3; and (○) CCH^R-C5.

Figure 2 shows the response curves describing U-73,975 cytotoxicity for each CHO line. A3, B3, and C5, respectively, showed 2.5-, 7.3- and 14.1-fold resistance to U-73,975 relative to the "wild type" AuxB1. The pattern of U-73,975 resistance corresponded to the graded expression of Pgp by the clones and implies that the decreased sensitivity to the CPI class of compounds is Pgp mediated. These studies focused on U-73,975 since the relative degree of drug-resistance of the MDR cells is representative of the responses of U-77,779 and U-80,244 yet U-73,975 retains the A subunit cyclopropyl group important for CC-1065 reactions with DNA.

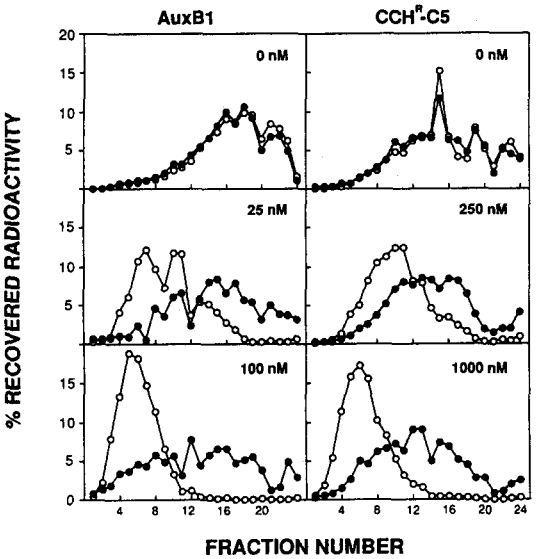

Fig. 3. Alkaline sucrose gradient profiles of U-73,975-induced damage to cellular DNA. Panels on the left are sedimentation profiles of AuxB1 genomic DNA while those on the right are of CCH^R-C5 DNA. Open symbols represent [^{14}C]TdR-labeled cells treated with U-73,975 at the concentrations indicated. Closed symbols represent non-treated [^{3}H]TdR-labeled cells. Drug-treated cells were extracted with ethanol and then mixed with [^{3}H]TdR-labeled non-treated cells prior to heating and lysis.

CPI-induced damage to genomic DNA. We examined whether the diminished cytotoxic potency in the Pgp-expressing cell lines might be correlated with alterations in cellular DNA damage following drug treatment. Thermal treatment generates strand breaks at sites of covalent adducts formed between CC-1065 and cellular DNA [23] detectable by alkaline sucrose gradient sedimentation [13]. We found that analogous damage was caused by the U-73,975 compound (Fig. 3).

Figure 3 illustrates the resistance of CCH^R-C5 cells to U-73,975-induction of heat-labile sites as evidenced by the drug concentrations required to degrade the DNA of the MDR cells to the same extent as the DNA of AuxB1 cells. The decreased frequency of DNA damage observed in the CCH^R-C5 cell line reflected susceptibility of U-73,975 to MDR. The ratio of the U-73,975 concentrations needed to generate 100 breaks/10^6 base pairs (calculations described in Ref. 13) indicates an approximately 10-fold degree of resistance for the CCH^R-C5 line relative to the AuxB1 line. For CC-1065 by comparison, sucrose sedimentation analysis of induced DNA damage showed an approximately 2-fold difference in sensitivities of the control and MDR cell lines (Table 2).

Table 2 shows the relationship between cytotoxicity and formation of covalent adducts for the sensitive (parental) and drug-resistant cell lines. To evaluate relative cytotoxic resistance under the same treatment conditions used to measure DNA damage, AuxB1 and CCH^R-C5 cultures at 8×10^4 cells/cm^2 were challenged with U-73,975 and CC-1065 for 2 hr and then plated in drug-free medium to determine survival, in terms of colony-forming efficiency. (The cytotoxicity assays referred to in Table 1 employed different drug-treatment conditions.) The parallels between relative levels of cytotoxicity and DNA damage for the cell lines provide new evidence suggesting that adduct formation by CC-1065 and U-73,975 may indeed be an important mechanism of CPI drug cytotoxicity.

DISCUSSION

We have shown that three CPI agents have diminished potency in multidrug resistant CHO cells. Resistance to U-73,975 was shown to increase with increasing levels of Pgp expression in CHO cell lines (using the same series of mutants that initially allowed correlation of Pgp with the MDR phenotype [15]).* These findings strongly imply that Pgp-mediated MDR is one mode of resistance to some important CPI compounds, as it is improbable that parallel increments in another mechanism could similarly account for the graded CPI responses of the cell lines tested. Recently we have also observed increased resistance to CPI agents correlated with Pgp level in a sequence of human MDR lines (unpublished data).

Subtle changes in the structures of other cytotoxic agents have been shown to elicit broad ranges of sensitivities when MDR mutants are exposed to a series of congeners [15, 17]. The general structural features of the four CPI agents used in the present study are as follows. CC-1065 (Fig. 1) is comprised of three benzodipyrrole subunits, A, B and C, linked together by amide bonds [2]. The left-hand subunit (A) contains the reactive cyclopropyl ring which is responsible for adduct formation with the N3 of DNA adenine groups. U-73,975 differs from the parent compound in that it has modified B and C subunits. U-80,244 is similar to U-73,975 in terms of its B and C subunits, but the A subunit is considerably altered. U-77,779 is a dimer that contains two reactive groups giving it the potential to form crosslink adducts on DNA.

The similarities in relative resistance (12- to 23-fold) of CCH^R-C5 to the three CPI analogs, compared to the relatively small degree of resistance to CC-1065, imply that a structural characteristic(s) that distinguishes the analogs from CC-1065 could explain their reduced potencies in the mutant cell lines. For example, a methylene group that bridges the pyrrolo ring within the B and C subunits is present in CC-1065 but not in these analogs. In fact it has been suggested that this moiety may be responsible for the delayed death toxicity caused by CC-1065 but not by the analogs [26, 27]. Whatever the case, it is curious that the three analogs which

* One might inquire whether agents such as verapamil and cyclosporin A that abate Pgp-mediated MDR in some cases also diminish the CPI resistance. The question could not be simply resolved with respect to specific reversal of Pgp-mediated MDR in this CHO cell system. Verapamil is differentially toxic for the mutant C5 cells ([24], data not shown), while both verapamil and cyclosporin A can sensitize AuxB1 as well as resistant cells [24, 25].

Table 2. DNA damage induced by CC-1065 and U-73,975 in sensitive and MDR cells, in comparison to cytotoxicities

Drug	Cell line	Drug concentrations required: Cytotoxicity (D_{10})	Drug concentrations required: DNA damage*
CC-1065			
	AuxB1	2.7 nM	80 nM
	CCH^R-C5	5.6 nM	190 nM
	Ratio		
	CCH^R-C5:AuxB1	2.1	2.4
U-73,975			
	AuxB1	3.1 nM	130 nM
	CCH^R-C5	19 nM	125 nM
	Ratio		
	CCH^R-C5:AuxB1	6.1	9.6

* Values expressed for DNA damage are the drug concentrations to give 100 breaks/10^6 base pairs. DNA lesion frequencies were calculated as described [13]. The D_{10} values for cytotoxicities are the drug concentrations that give 10% plating efficiencies relative to untreated controls, using the same conditions of drug treatment employed to measure DNA damage.

are susceptible to MDR all share the property of not causing delayed cytotoxicity.

The CPI-resistant B-16 variant isolated by Moy *et al.* [14] displays cross-resistance to a number of CPI analogs including CC-1065 and U-73,975 (12.5- and 9.7-fold resistance, respectively) unlike the Pgp-mediated resistance of the CHO mutants which distinguishes between CC-1065 and the tested analogs. Clearly the drug characteristics that influence the cytotoxic responses of the CPI-resistant B-16 clone are different from those that govern susceptibility to Pgp-mediated MDR.

The data presented here are the first direct demonstration of DNA damage by the CPI analog U-73,975 in whole cells. Our examination of responses to CC-1065 and U-73,975 in cell lines demonstrates an approximately 1:1 relationship between cytotoxicity and the potential of these drugs to generate covalent adducts, arguing that DNA lesions generated by these compounds or their metabolites do play a major role in cell killing.

Note added in proof. Consistent with our results described in this paper, Bhuyan *et al.* [28] have recently reported that a V79 hamster cell line selected for resistance to U-73,975 displays a Pgp-associated MDR phenotype, while an MDR vinblastine-selected human KB line shows cross-resistance to U-73,975.

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