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Cross-resistance to Antitumor Diarylsulfonylureas and Collateral Sensitivity to Mitochondrial Toxins in a Human Cell Line Selected for Resistance to the Antitumor Agent *N*-(5-Indanylsulfonyl)-*N'*-(4-chlorophenyl)urea

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SUMMARY

Diarylsulfonylurea (DSU) antitumor agents represent a new class of oncolytic compounds with an unknown, potentially novel, mechanism of action. At high concentrations of several of these agents, cytotoxicity appears to be a consequence of uncoupling of mitochondria. However, the mechanism of action at pharmacologically achievable concentrations is unknown. To further study these agents a subline of human colon carcinoma, GC_3/c1, was selected for resistance to *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (ISCU) (Sulofenur). This clone (designated LYC5) was stably resistant for 2 years in the absence of selection pressure and was characterized for cross-resistance to other antitumor DSU and therapeutically used oncolytic agents. LYC5 was cross-resistant to six of seven DSU analogues examined when cells were exposed to drugs for 7 days. However, the degree of resistance was inversely related to the potency of the individual DSU against the parental GC_3/c1 clone. Consequently, against LYC5 cells there was a relatively narrow range for concentrations inhibiting colony formation by 50% (4-fold), compared with that in GC_3/c1 cells (12-fold range). With a single exception, each DSU examined caused uncoupling of oxidative phosphorylation in isolated mitochondria at 50 μM, and data suggest that cytotoxicity in LYC5 cells may be a consequence of mitochondrial impairment. In contrast, LYC5 cells were collaterally sensitive to the mitochondrial toxins rotenone, antimycin, and oligomycin, by 11.4-, 7.2-, and 36.9-fold respectively. LYC5 cells were also collaterally sensitive to vincristine (7.7-fold), Actinomycin D (5.9-fold), and rhodamine-123 (10.5-fold), agents associated with P-glycoprotein (Pgp)-mediated multidrug resistance (MDR). LYC5 cells were slightly more sensitive to Melphalan and doxorubicin (2.8- and 2.3-fold, respectively) but not to cisplatin or dideazatetrahydrofolic acid. Collateral sensitivity to vincristine and Actinomycin D was consistent with decreased Pgp levels in LYC5 cells. Immunohistochemical staining and Western blotting with anti-Pgp antibodies indicated an 8-fold reduction in Pgp levels in LYC5 cells, relative to expression in parental GC_3/c1 cells. Consequently, association of mitochondrial toxins with resistance in MDR KB8-5 cells was examined in the presence or absence of the MDR-reversing agent verapamil. KB8-5 cells had equal or greater sensitivity, compared with parental KB3-1 cells, to rotenone, antimycin, and oligomycin and also to each DSU analogue examined. In addition, verapamil tended to have a protective effect against these mitochondrial toxins. It is thus concluded that these toxins are probably not part of the Pgp-mediated MDR phenotype and that collateral sensitivity of LYC5 cells to mitochondrial toxins is not a consequence of decreased Pgp expression. In contrast to results obtained with KB cells, verapamil significantly potentiated the toxicity of each mitochondrial toxin in GC_3/c1 cells by approximately 5-fold, such that in the presence of verapamil GC_3/c1 and LYC5 cells had similar sensitivities to rotenone and antimycin. Verapamil had significantly less effect in potentiating the cytotoxicity of mitochondrial toxins in LYC5 cells (<2-fold). Results suggested that resistance to DSU and collateral sensitivity to mitochondrial toxins may be linked and may be related to the mechanism of action of DSU at pharmacologically achievable concentrations. To test this, hybrids between GC_3/c1 and LYC5 cells were made and tested for resistance to ISCU and mitochondrial toxins. Results demonstrated that resistance to ISCU was a recessive trait, as was collateral sensitivity to rotenone, antimycin, and oligomycin.

DSU are a new class of antitumor agents with marked activity against chemorefractory solid tumors but little activity *in vivo* against rodent leukemia models (1–5). Although the mechanisms by which DSU exert cytotoxicity are unknown, these agents do not inhibit nucleic acid or protein synthesis except at very high concentrations (3, 6). Work from this and other laboratories has shown that MPCU (LY181984) and its 5-indanyl analogue ISCU (LY186641) enter cells, probably by passive diffusion, and accumulate approximately 4-fold (7).

This work is dedicated to the memory of Dr. Gerald B. Grindey, mentor, collaborator, and friend.

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ABBREVIATIONS: DSU, diarylsulfonylurea(s); ISCU, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (Sulofenur); MPCU, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; L-PAM, L-phenylalanine mustard (Melphalan); FUra, 5-fluorouracil; DDATHF, dideazatetrafolic acid; FBS, fetal bovine serum; Pgp, P-glycoprotein; MDR, multidrug resistance or resistant; DEPC, diethylpyrocarbonate; IO, iodoacetamide; PEG, polyethylene glycol.

Accumulation is inhibited by azide and ionophores that collapse the pH gradient across the mitochondrial inner membrane (7–9). Both toxicity and accumulation are dependent upon the extracellular pH, increasing as the extracellular pH is decreased, consistent with increased diffusion of the uncharged species (9–10). Unlike rhodamine-123, which accumulates in mitochondria as a consequence of the charge differential across the mitochondrial inner membrane, there is no cross-resistance to DSU in cells exhibiting the Pgp-mediated MDR phenotype (5).

At high concentrations for short periods of exposure, cytotoxicity is independent of cellular proliferation (6). Under these conditions, DSU cause a rapid loss of cellular ATP, consistent with the uncoupling activity of these compounds (11, 12). On the other hand, at therapeutic concentrations of free drug that can be achieved *in vivo* (≈0.5 μM), cytotoxicity is proliferation dependent and associated with formation of DNA nucleosomal ladders (13). However, despite considerable effort, no structure-activity relationship for antitumor DSU has been established (2). In general, the most efficacious DSU appear to be the most hydrophobic and also bind with high affinity to serum albumin (14).

Data derived using LYC5 human colon adenocarcinoma cells selected for resistance to ISCU have suggested that the mechanisms causing cytotoxicity at high concentrations (uncoupling) and at pharmacologically achievable concentrations may be distinct (11, 15). These cells were resistant to ISCU when exposed continuously to pharmacologically relevant concentrations of drug but were not resistant to short exposures to very high concentrations of ISCU known to uncouple oxidative phosphorylation (13). Here we report the profile of cross-resistance and collateral sensitivity of LYC5 cells. Data suggest that, although LYC5 cells are cross-resistant to other DSU, these cells are markedly more sensitive to mitochondrial toxins and also, to a lesser extent, to natural product anticancer agents associated with Pgp-mediated MDR. However, this collateral sensitivity to mitochondrial toxins appears not to be a consequence of reduced Pgp function.

Materials and Methods

Cell lines and reagents. A cloned human adenocarcinoma line, GC_3/c1 (16), was routinely grown in antibiotic-free RPMI 1640 medium (Whittaker, Walkersville, MD) supplemented with 2 mM glutamine and 10% FBS (Hyclone, Logan, UT). KB3-1 and MDR KB8-5 cells were maintained as described previously (17). DSU analogues reported here were supplied by Dr. G. B. Grindey, Eli Lilly and Co. (Indianapolis, IN). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or through Fisher Scientific (Springfield, NY).

Selection of GC_3/LYC5 clone. GC_3/c1 cells were mutagenized with *N*-methyl-*N'*-nitrosoguanidine and ethyl methanesulfonate, and surviving cells were exposed to 300 μM ISCU for 24 hr in the presence of 10% FBS. Four surviving colonies were isolated using cloning rings. One of these clones, designated LYC5, was exposed to increasing concentrations of ISCU. After 10 weeks it was capable of growing in medium containing 120 μM ISCU. Compared with parental GC_3/c1 cells, LYC5 was 4–6-fold resistant to ISCU (determined by colony formation assay after 7-day exposure to ISCU). For the experiments reported, LYC5 cells had been maintained in drug-free medium for >2 years. Resistance was stable over this period (13).

Colony-forming assays. GC_3/c1 and LYC5 cells were plated in triplicate at a density of 6000 cells/well, and KB cells were plated at 3000/well in Falcon no. 3046, six-well, flat-bottomed, tissue culture plates (Becton Dickinson Co., Lincoln Park, NJ). After 24 hr of incubation, medium was replaced with 3 ml of fresh medium containing drugs, and cells were incubated at 37° for an additional 7 days. The medium was aspirated and cells were washed once with 2 ml of 0.9% saline solution and dried overnight. Colonies were stained with 1 ml of 0.1% crystal violet solution, followed by washing twice with distilled water, and were counted using an automated ARTEK model 880 colony counter. The IC_{50} values were determined from concentration-percentage of cell survival curves and were defined as the concentrations of drugs required for 50% reduction in colonies, compared with controls. All experiments were repeated at least one time.

Effect of verapamil on *in vitro* cytotoxicity. Cells were treated with graded concentrations of cytotoxic agents in the absence or presence of a nontoxic concentration of verapamil (20 μM). The plates were then transferred to a CO_2 incubator and, after further incubation for 7 days at 37°, colonies were enumerated as described (17).

Isolation of mitochondria and measurement of oxidative phosphorylation. Mitochondria from mouse liver were prepared by the modified procedure of Thakar (18), as applied to liver tissue (19). Livers from three or four mice were pooled and homogenized at 4° in medium containing 0.1 M sucrose, 0.01 M EDTA, 0.046 M KCl, 0.1 M Tris, pH 7.4, and 0.5% bovine serum albumin. The homogenate was centrifuged at 478 × *g* for 10 min to remove nuclei, and the resulting supernatant was further centrifuged at 12,000 × *g* for 10 min to harvest the mitochondrial pellet. This pellet was washed once by resuspension in the homogenization medium and centrifugation. The final pellet was suspended in medium containing 223 mM mannitol, 70 mM sucrose, 0.02 mM EDTA, 5.0 mM KH_2PO_4, and 20 mM Tris, pH 7.4, which also was the medium used for measuring oxidative phosphorylation. The protein concentration was determined (Bio-Rad) and adjusted to 15–20 mg/ml. The oxygen consumption by mitochondria was measured using a YSI model 5300 biological oxygen-monitoring system equipped with a Clark electrode, in a water-jacketed chamber at 30°. The respiratory control ratio and ADP/oxygen ratios were calculated according to the method of Chance and Williams (20).

Selection of GC_3/c1/LYC5 cell hybrids. Hybrids were isolated by a selective system using irreversible biochemical inhibitors, as described by Wright (21), with several modifications. Briefly, confluent GC_3/c1 or LYC5 cells were trypsinized and replated at 1×10^7 cells/T-162 flask. After 24 hr, monolayers were washed in bicarbonate-free Hanks' solution, trypsinized, and pipetted to give a single-cell suspension. Cells were centrifuged and resuspended in 30 ml of bicarbonate-free Hanks' solution containing either 0.006% DEPC or 5 mM IO. Cells were incubated at 4° for 30 min, with mixing by inversion of the tube several times at 10-min intervals. Cells were centrifuged and resuspended in 20 ml of bicarbonate-free Hanks' solution without drug. An aliquot of these suspensions (0.5 ml from DEPC-treated or 0.5 ml from IO-treated populations, containing 2.5×10^6 cells) was plated to test for viability. To produce hybrids, the remaining suspensions were mixed and cells were pelleted. The supernatant was aspirated, and the pellet was loosened by gentle tapping of the tube. Instead of using Sendai virus-mediated fusion (21), we have used PEG. Briefly, over 1 min, 1 ml of a warm (37°) 50% PEG solution was added dropwise to the cells, with shaking after each addition and then with shaking for an additional 1 min. Cells and PEG were maintained at 37°. One milliliter of serum-free RPMI 1640 medium was then added dropwise over 1 min with shaking, cells were shaken for 1 min, and 5 ml of serum-free RPMI 1640 medium were added over 3 min. After 1 min, 15 ml of RPMI 1640 medium containing 10% FBS and 2 mM glutamine (complete medium) were added, and cells were incubated (37°, 95% air/5% CO_2) for 4–5 hr. Cells were pelleted by centrifugation and gently resuspended in 10 ml of complete medium. Two milliliters of this suspension were added to 35 ml of complete medium, and cells were incubated at 37° for 3 days, without the flasks being disturbed.

Separation of viable cells and culture conditions. After 3 days the cell suspension was collected after gentle shaking of the culture flask and was centrifuged. Cells were resuspended in 6 ml of phosphate-buffered saline, gently overlaid on 3 ml of lymphocyte separation medium (density, 1.080 g/ml; Organa Teknika), and centrifuged (200 × *g*, 5 min). Viable cells were collected from the interface and mixed with 10 ml of medium before centrifugation. The pellet was resuspended in 1 ml of complete medium (containing 25% FBS) and plated in one well of a 24-well tissue culture plate. Medium was changed every 3–4

TABLE 1
Derivation of GC_3/c1/LYC5 and GC_3/c1/GC_3/c1 hybrids

Hybrid	Treatment before fusion	
	DEPC	IO
1	LYC5	GC_3/c1
2	GC_3/c1	LYC5
3	GC_3/c1	GC_3/c1
4	LYC5	GC_3/c1

days. The derivation of four populations of hybrids that were subsequently characterized for sensitivity to ISCU and mitochondrial toxins is shown in Table 1. No growth was observed in GC_3/c1 or LYC5 cells treated with DEPC or IO but not fused or in hybrids formed between populations receiving the same treatment (e.g., DEPC/DEPC or IO/IO).

Detection and quantitation of Pgp. Membranes from GC_3/c1 and LYC5 cells were prepared by nitrogen cavitation (22), fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Phast System; Pharmacia), and transferred to nitrocellulose. Pgp was detected using C219 as the primary antibody, with sheep anti-mouse immunoglobulin peroxidase-linked second antibody. Bands were visualized by enhanced chemiluminescence (ECL, Amersham), according to the manufacturer's instructions. Membranes from KBV-1 cells were used as a control, and the signal was quantitated by scanning densitometry.

Results

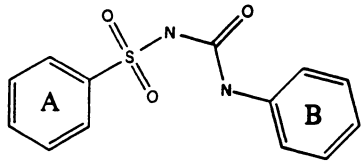
Cross-resistance to DSU analogues. LYC5, a clone resistant to ISCU, was selected from the GC_3/c1 human colon adenocarcinoma cell line. Numerous attempts to select for higher levels of resistance were unsuccessful, suggesting, perhaps, that at high concentrations a second mechanism of DSU was lethal and could not be circumvented. The LYC5 clone was maintained under continuous selective pressure (120 μM ISCU) or in drug-free medium for >2 years (13). For studies reported here, LYC5 cells that were grown without drug selection for >1.5 years were used. Sensitivity of GC_3/c1 and LYC5 cells to ISCU and six analogues is presented in Table 2. With the exception of LY195392, LYC5 cells were cross-resistant to each of the other DSU, to varying degrees. Of note was the finding that LY195392 was the least potent against GC_3/c1 cells, whereas the greatest degree of cross-resistance was found for the most potent compounds, LY195779 and LY186391. This inverse relationship is shown for each compound in Fig. 1.

Uncoupling activity of DSU. These data indicated that the product of IC_{50} × fold resistance was relatively constant for each DSU and may relate to the uncoupling activity of DSU. To examine this, the uncoupling activity of DSU on isolated mouse liver mitochondria was examined (Fig. 2). At 50 μM, six of seven analogues effectively uncoupled mitochondria, as determined by state 4 O_2 consumption, respiratory control ratio, and ADP/oxygen determinations. Their potencies as uncoupling agents did not correlate with their cytotoxic potencies against GC_3/c1 cells (proposed site 1). Of note, however, was the finding that the most effective uncoupling agent, LY195392, had similar potencies against GC_3/c1 and LYC5 cells. The other analogues were less potent uncoupling agents and also less potent cytotoxic agents against LYC5 cells. Thus, it is likely that, at the concentrations causing 50% inhibition of LYC5 colony formation (62–250 μM) (Table 1), DSU analogues act through uncoupling of mitochondria.

Sensitivity to mitochondrial toxins. These data and previous studies (6–8, 11) suggested that at high concentrations [either in LYC5 cells, as described above, or in GC_3/c1 cells exposed for short periods to DSU (13)] cytotoxicity is a consequence of uncoupling of oxidative phosphorylation. This would suggest that in LYC5 cells the mechanism of resistance relates to the primary site of action causing DSU cytotoxicity. At pharmacologically relevant concentrations (≈1 μM) DSU accumulate in mitochondria, but it is unknown whether this is the site of drug action. To examine this, the sensitivity of LYC5 cells to the mitochondrial toxins rotenone, antimycin, and oligomycin was examined (Table 3). LYC5 cells were 11.4-, 7.2-, and 36.9-fold more sensitive to rotenone, antimycin, and oligomycin, respectively, than were the parental GC_3/c1 cells.

Sensitivity to oncolytic agents. Collateral sensitivity to mitochondrial toxins could indicate specific changes in LYC5 cells or, alternatively, might reveal that LYC5 cells were generally more susceptible to toxic insult (i.e., more fragile). To determine whether LYC5 cells were generally more sensitive, we examined the cytotoxic potency of different oncolytic agents, including alkylating agents, antimetabolites, and drugs associated with Pgp-mediated MDR (Actinomycin D, doxorub-

TABLE 2
Cross-resistance of LYC5 to DSU



Compound	A	B	IC_{50}[a]		Fold resistance
			GC_3/c1	LYC5	
			μM		
LY181984	4-CH_3	4-Cl	18.8 ± 4	222	11.8
LY186391	H	3,4-DiCl	5.5 ± 1	249	45.3
LY186641	5-Indanyl	4-Cl	37.9 ± 3	208	5.5
LY188458	4-CH_3	4-CH_3	28.6 ± 12	215	7.5
LY195392	4-CH_3	2,4-DiCl	59.9 ± 6	62.9	1.05
LY195779	4-CH_3	4-NO_2	4.5 ± 2	235	52.4
LY219703	4-N_3	4-Cl	26.7 ± 6	147	5.5

[a] Values were determined after 7-day exposure to agents in RPMI 1640 medium plus 10% FBS.

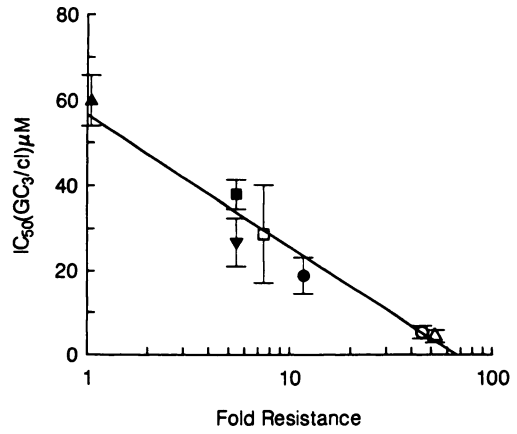


Fig. 1. Relationship between DSU potency against parental GC_3/c1 cells and degree of cross-resistance in LYC5 cells. IC_{50} values (mean ± standard deviation) were determined after exposure of cells to DSU analogues for 7 days. The relationship between potency against GC_3/c1 cells (*ordinate*) and fold resistance (*abscissa*) in LYC5 cells is shown for seven analogues. ▲, LY195392; ■, LY186641 (ISCU); ▼, LY219703; □, LY188458; ●, LY181984 (MPCU); ○, LY186391; △, LY195779.

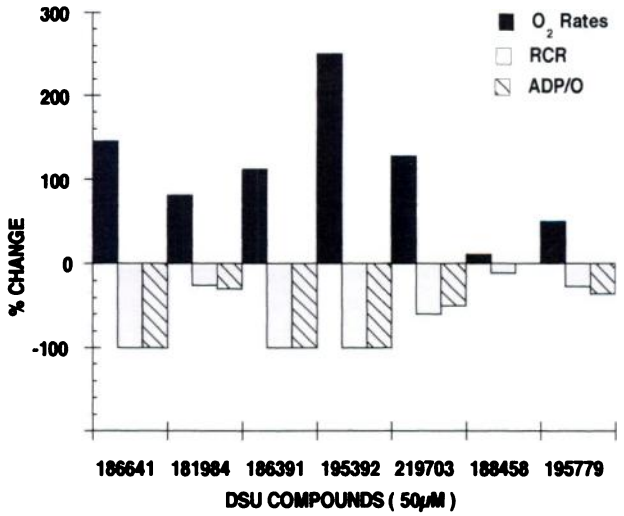


Fig. 2. Uncoupling activity of DSU analogues in mitochondria isolated from mouse liver. Mitochondria were incubated in the presence of 50 μM DSU. For each analogue, the effects on state 4 O_2 rate, respiratory control ratio (*RCR*), and ADP/oxygen (*ADP/O*) ratio were determined. Results are expressed as percentage change.

icin, and *Vinca* alkaloids). As shown in Fig. 3 and Table 3, the two cell lines were equally sensitive to *cis*-dichlorodiammine platinum(II) and antimetabolites (FUra and DDATHF). LYC5 cells were slightly more sensitive to L-PAM and doxorubicin but significantly more sensitive to Actinomycin D (5.9-fold), vincristine (7.7-fold), and rhodamine-123 (10.5-fold).

Cross-resistance to DSU and mitochondrial toxins in MDR cells. We previously showed that ISCU was equally active in MDR KB8-5 cells overexpressing Pgp and in parental KB3-1 cells (5). Similarly, there was no cross-resistance to any of the DSU analogues tested (Table 4). However, because Pgp-mediated MDR is associated with resistance to hydrophobic heterocyclic compounds (although usually basic substances) and LYC5 cells were collaterally sensitive to actinomycin D and vincristine, it seemed possible that the phenotype of LYC5 cells (i.e., collateral sensitivity to drugs) could be a consequence of decreased Pgp function. Immunohistochemical staining of GC_3/c1 and LYC5 cells with C219, a monoclonal antibody

TABLE 3

Sensitivity of GC_3/c1 and LYC5 cells to mitochondrial toxins and oncolytic agents

Agent	IC_{50}		Fold resistance
	GC_3/c1	LYC5	
	nM		
Rotenone	80 ± 21	7.0 ± 4.6	11.4
Antimycin	133 ± 33	18.4 ± 9.7	7.2
Oligomycin[a]	5.9 ± 0.9	0.16 ± 0.11	36.8
L-PAM	3,800	1,350	2.8
CDDP[b]	1,132	900	1.3
DDATHF	>25	>25	1.0
FUra	>10,000	>10,000	1.0
Vincristine	12.3 ± 3.2	1.6 ± 1.1	7.7
Vinblastine	5.2	1.4	3.7
Actinomycin D	1.0	0.17	5.9
Doxorubicin	23	10.2	2.3
Rhodamine-123	12,800	1,200	10.5

[a] ng/ml.
[b] CDDP, *cis*-dichlorodiammine platinum(II).

against an intracellular epitope of Pgp, showed a decreased intensity of staining in LYC5 cells. To quantitate Pgp, plasma membranes were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using C219. Results showed an 8-fold decrease in Pgp in LYC5 cells, relative to GC_3/c1 cells, which expressed 4.5% of the level detected in KBV-1 membranes (data not shown). The sensitivity of parental KB3-1 and MDR KB8-5 cells to mitochondrial toxins was next examined. As shown in Fig. 4, KB8-5 cells either had similar sensitivity or were more sensitive than KB3-1 cells to these agents. These data indicate that collateral sensitivity of LYC5 cells to mitochondrial toxins is not a consequence of decreased Pgp.

Effect of verapamil on sensitivity of cells to mitochondrial toxins. Verapamil has been shown to reverse Pgp-mediated MDR in many studies (23) and to sensitize KB8-5 and GC_3/c1 cells to *Vinca* alkaloids (22). Thus, it was of interest to use this modulator to determine whether there was similar Pgp function in each cell line and to determine whether verapamil modulated the activity of mitochondrial toxins. Verapamil, at a concentration that reduced cloning efficiency by <10%, significantly potentiated the effect of *Vinca* alkaloids in both GC_3/c1 and LYC5 cells (Table 5) but had little effect in sensitizing KB3-1 or KB8-5 cells to the action of rotenone, antimycin, or oligomycin (Fig. 4). The effect of verapamil on modulating toxicity of mitochondrial toxins in GC_3/c1 and LYC5 cells is shown in Fig. 5. In contrast to results obtained in the KB cell lines, a nontoxic concentration of verapamil (20 μM) significantly sensitized GC_3/c1 cells to each of the mitochondrial toxins, such that in the presence of verapamil these cells had similar sensitivities to rotenone and antimycin but remained about 4-fold resistant to oligomycin, compared with the DSU-resistant LYC5 cells. Verapamil caused a >5-, 6.0-, and >5.4-fold sensitization to antimycin, rotenone, and oligomycin, respectively. Against LYC5 cells, verapamil had a more modest effect, causing 1.9- and 2.2-fold sensitization to rotenone and antimycin, respectively, but did not modulate oligomycin toxicity (Fig. 5; Table 5).

Sensitivity of GC_3/c1/LYC5 hybrids to ISCU and mitochondrial toxins. Parental cells, resistant cells, and each of four hybrids (Table 1) were tested for sensitivity to ISCU and three mitochondrial toxins. Results are shown in Table 6. Each hybrid retained sensitivity to ISCU, suggesting that re-

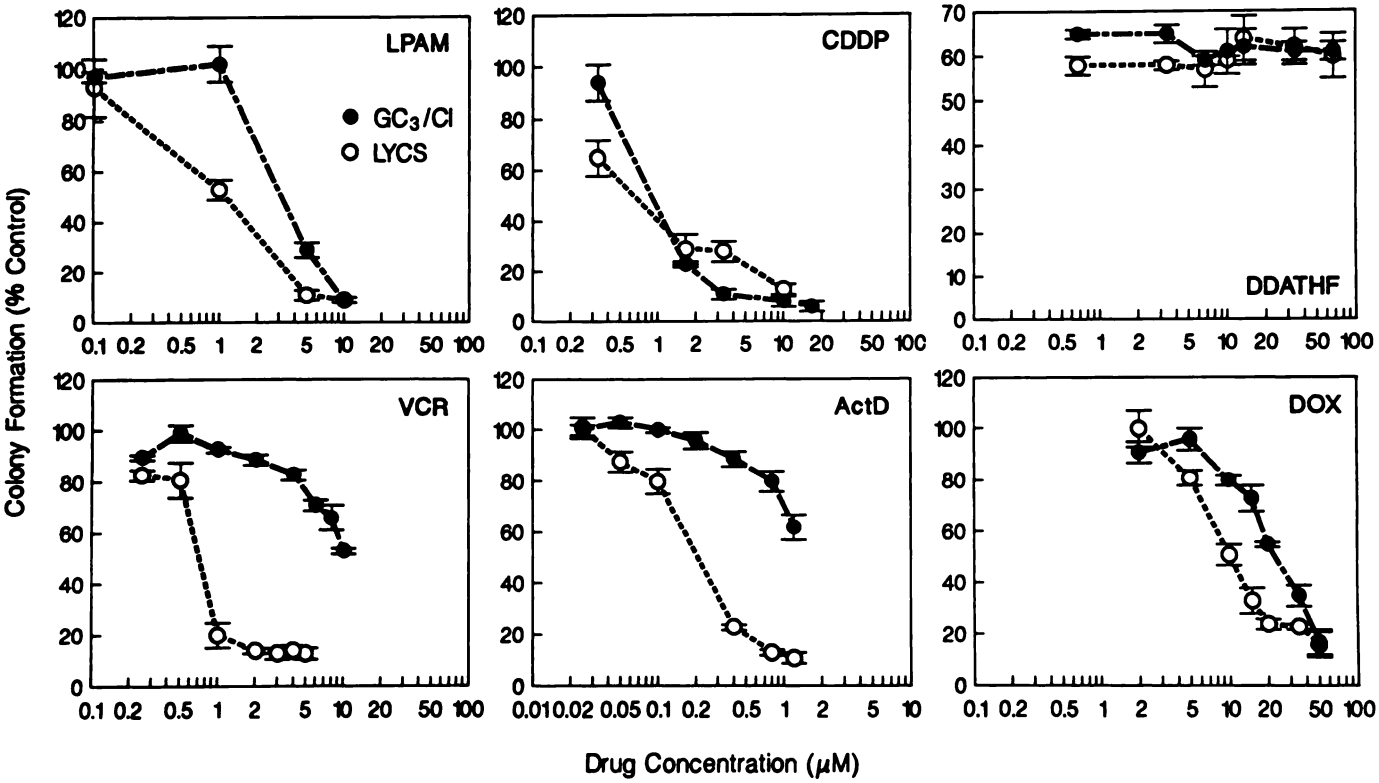


Fig. 3. Sensitivity of GC_3/c1 (●) and LYC5 (○) cells to therapeutic anticancer agents. Cells were exposed to each agent for 7 days and clonogenic survival was determined. Each *point* is the mean ± standard deviation for three determinations, and each experiment shown is representative of at least two replicate experiments. *CDDP*, *cis*-dichlorodiammine platinum(II); *VCR*, vincristine; *ActD*, Actinomycin D; *DOX*, doxorubicin.

TABLE 4
Sensitivity of KB3-1 and KB8-5 cells to DSU

Compound	IC_{50}[a]	
	KB3-1	KB8-5
LY181984	55.7	27.9
LY186391	26.3	7.2
LY186641	67.6	57.1
LY188458	62.4	59.1
LY195392	84.2	73.3
LY195779	12.2	8.7
LY219703	70	51.9

[a] Calculated IC_{50} values from a single experiment.

sistance in LYC5 cells is a recessive characteristic. Each hybrid was quite resistant to each mitochondrial toxin, having sensitivity similar to that of the parental GC_3/c1 cells. These data indicate that hypersensitivity or collateral sensitivity of LYC5 cells to these agents is also a recessive trait.

Discussion

DSU represent a new class of antitumor agents with considerable activity against a wide spectrum of human and rodent solid-tumor models. The mechanisms of action have not been elucidated, although at high concentrations cytotoxicity may be a consequence of uncoupling of oxidative phosphorylation (11, 12). The mechanism causing cytotoxicity at pharmacologically achievable plasma concentrations (<1 μM free drug) remains obscure. To further study the action of DSU, we have selected a resistant mutant, LYC5, subsequent to mutagenesis and growth in the presence of ISCU. This clone is 5.5-fold resistant to 7-day continuous exposure to relatively low concentrations of ISCU (proposed "site 1") but is not resistant to short exposures to very high concentrations of drug (proposed "site 2"). Although the mechanism by which LYC5 cells are resistant to ISCU is unknown, resistance is not a consequence of decreased accumulation of drug (13). Similar results were obtained with radiolabeled LY181984 and LY219703, for which accumulation at steady state was not significantly different between GC_3/c1 and LYC5 cells.[1] Resistance was stable in the absence of selective pressure, suggesting a genetic basis. Using the LYC5 clone, we have examined cross-resistance to other DSU and therapeutically used anticancer agents.

The resistant clone LYC5 was cross-resistant to each DSU analogue examined except LY195392 [*N*-(4-methylphenylsulfonyl)-*N'*-(2,4-dichlorophenyl)urea]. Of interest, this analogue was the least potent against parental GC_3/c1 cells, whereas the greatest degree of resistance was observed with the most potent compounds, LY195779 [*N*-(4-methylphenylsulfonyl)-*N'*-(4-nitrophenyl)urea] and LY186391 [*N*-(phenylsulfonyl)-*N'*-(3,4-dichlorophenyl)urea], to which LYC5 cells were 50- and 45-fold resistant, respectively. These data indicate that the concentration of DSU reducing colony formation by 50% in LYC5 cells may be a relatively constant value for each analogue examined. Indeed, the range of IC_{50} values against LYC5 cells was found to be relatively narrow, i.e., 4-fold including all analogues and <2-fold excluding LY195392. In contrast, there was greater variation in IC_{50} values for the parental GC_3/c1 cells (12-fold). At 50 μM six of seven analogues effectively uncoupled oxidative phosphorylation in isolated mitochondria; their potency as uncoupling agents did not correlate with

[1] J. Sosinski and P. Houghton, unpublished obeservations.

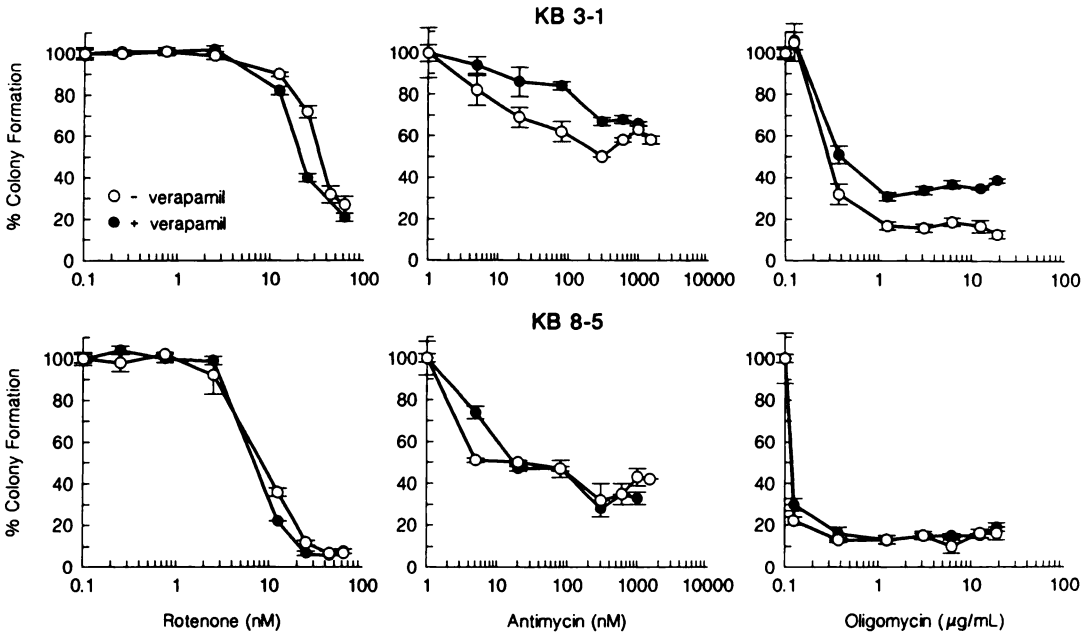


Fig. 4. Modulation of mitochondrial toxin potency by verapamil in KB cells. The sensitivity of parental KB3-1 cells and MDR KB8-5 cells to mitochondrial toxins was determined in the presence (●) or absence (○) of a nontoxic concentration of verapamil. Colony formation was determined after 7 days of simultaneous exposure to verapamil (20 μM) together with increasing concentrations of mitochondrial toxins. Each *point* represents the mean ± standard deviation for three determinations. Experiments are representative of at least two replicate experiments.

TABLE 5

Effect of verapamil on the toxicity of vincristine and mitochondrial toxins in GC_3/c1 and LYC5 cells

Agent	IC_{50}	
	−Verapamil	+Verapamil
	nM	
GC_5/c1		
Vincristine	12.3 ± 3.2[a]	0.26 ± 0.2
Vinblastine	5.2[b]	1.0
Rotenone	≥100	23 ± 7
Antimycin	142 ± 38	23.5 ± 1.5
Oligomycin[c]	7.0	1.3
LYC5		
Vincristine	1.6 ± 1.1	0.21
Vinblastine	1.4	1.0
Rotenone	9.7 ± 5.4	5.0 ± 1
Antimycin	24.7 ± 7.6	11.4 ± 6.8
Oligomycin[c]	0.32	0.37

[a] Mean ± standard deviation (three experiments).
[b] Mean (two experiments).
[c] ng/ml.

cytotoxic potency in GC_3/c1 cells (site 1), but it is probable that the mechanism of cytotoxicity in LYC5 cells pertains to that mechanism (site 2).

These results suggested that in LYC5 cells the primary target (site 1) for cytotoxicity of DSU at pharmacologically achievable concentrations was altered, such that it no longer became growth limiting. Because DSU accumulate in mitochondria even in cells exposed to micromolar concentrations of drug (9), this seemed to be a reasonable starting point for further studies. To further elucidate the site 1 mechanism of action, agents known to affect mitochondrial function were examined in parental and DSU-resistant cells. Three mitochondrial toxins that inhibit mitochondrial ATP synthesis were examined. Rotenone and antimycin inhibit electron transfer in the respiratory chain, and oligomycin inhibits the mitochondrial F_1/F_o ATPase. Rotenone blocks electron transfer from flavoprotein to ubiquinone, and the antibiotic antimycin inhibits transfer of electrons from cytochrome *b* to cytochrome c_1 (24, 25). Rather surprisingly, LYC5 cells were collaterally sensitive to these inhibitors, being 11-, 7-, and 37-fold more sensitive than wild-type GC_3/c1 cells to rotenone, antimycin, and oligomycin, respectively. These results seem to indicate that LYC5 cells were highly dependent upon respiratory chain phosphorylation for generation of ATP. Alternatively, the data could have indicated a general sensitivity (fragility) of LYC5 cells to chemical insult. To examine the latter possibility, the sensitivity of both cell lines to a diverse group of cytotoxic agents, including antimetabolites, alkylating agents, antimitotic agents, and topoisomerase II inhibitors, was examined. Results show that LYC5 cells were not necessarily more sensitive than GC_3/c1 cells to all agents, having similar sensitivity to cisplatin, DDATHF, and 5-FUra. There was slightly increased sensitivity to L-PAM and doxorubicin but greater sensitivity to actinomycin D (5-fold) and vincristine (7-fold). Of interest was the finding that, of the anticancer agents examined, the greatest collateral sensitivity was found with two natural products associated with Pgp-associated MDR, although the level of collateral sensitivity was relatively less than that found with the mitochondrial toxins.

Rotenone (a steroidal insecticide), antimycin, and oligomycin (a macrolide antibiotic) have not been reported to be associated with the Pgp-mediated MDR phenotype. However, steroidal drugs such as progesterone (26) and the macrolide immunosuppressants FK506 and rapamycin (27, 28) may act as either substrates or inhibitors of Pgp. The results with actinomycin D and vincristine could have indicated that LYC5 cells had reduced Pgp function, resulting in the observed pattern of collateral sensitivity to both mitochondrial toxins and cytotoxic agents. We have examined this possibility in two ways, by determining the degree of cross-resistance to mitochondrial toxins in a well characterized cell line where MDR is a consequence of *mdr1* overexpression (29) and by testing the ability of an MDR-reversing agent to sensitize KB and GC_3 cell

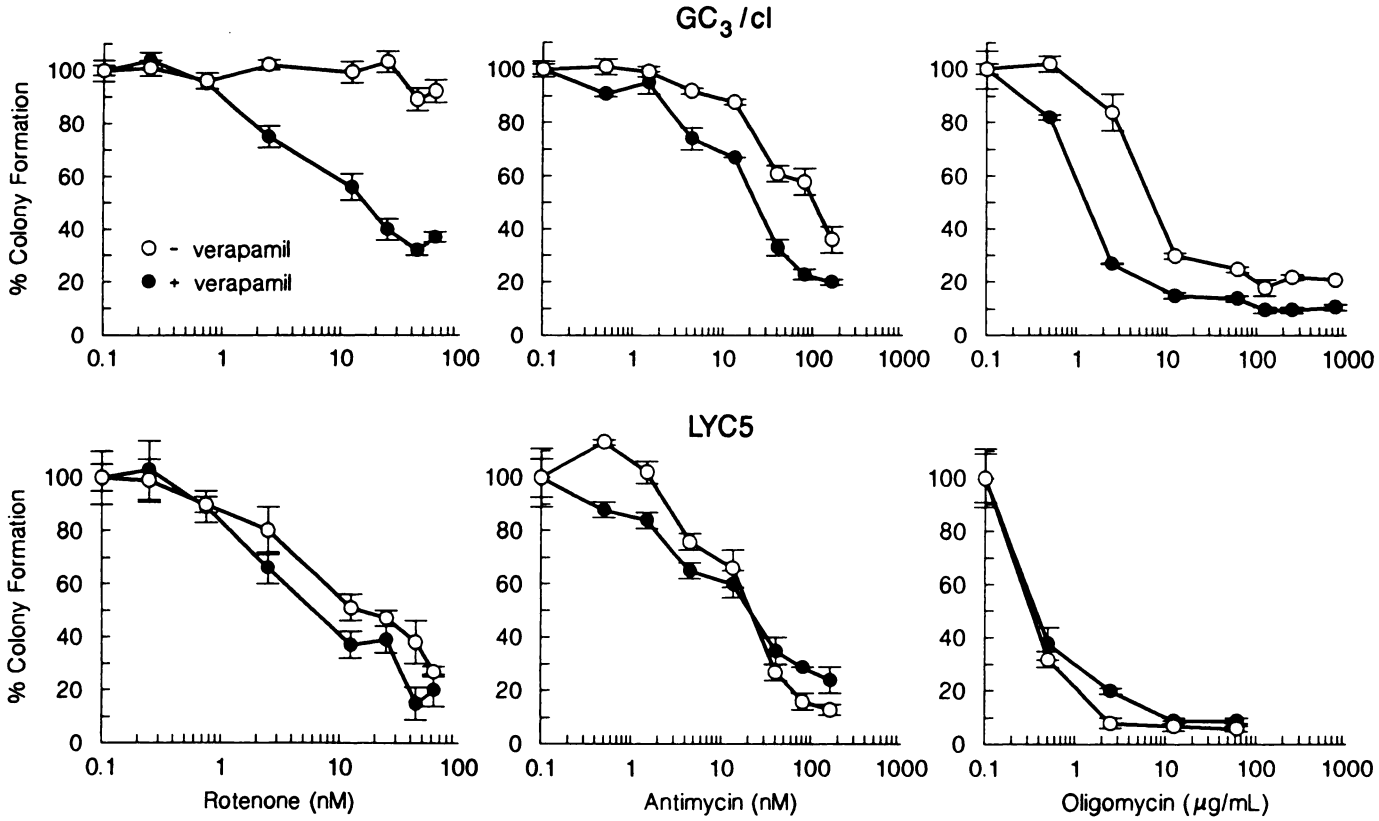


Fig. 5. Modulation of mitochondrial toxin potency by verapamil in GC_3/c1 and LYC5 cells. The sensitivity of parental GC_3/c1 cells and DSU-resistant LYC5 cells to mitochondrial toxins was determined in the presence (●) or absence (○) of 20 μM verapamil, as described for Fig. 4. Each *point* represents the mean ± standard deviation for three determinations. Experiments are representative of at least two replicate studies.

TABLE 6

Sensitivity of parental clones and hybrids to ISCU and mitochondrial toxins

Clone	IC_{50}			
	ISCU	Rotenone	Antimycin	Oligomycin
	μ	μM	μM	μg/ml
GC_3/c1	28	>125	90	18
LYC5	165	2.0	5.5	0.65
Hybrid 1	26	>125	160	18
Hybrid 2	30	>125	160	12
Hybrid 3	30	85	80	33
Hybrid 4	29	125	65	26

Cells were exposed to each agent for 7 days and colonies were enumerated.

variants to these agents. MDR KB8-5 cells were not resistant, relative to the parental KB3-1 cells, to mitochondrial toxins or DSU analogues. Verapamil at 20 μM, a concentration that reverses *Vinca* alkaloid resistance (22), failed to sensitize either KB line but, rather, had a slight protective effect against rotenone cytotoxicity in KB8-5 cells. These data argue that rotenone, antimycin, and oligomycin are not associated with the Pgp-mediated MDR phenotype.

Verapamil significantly sensitized GC_3/c1 colon adenocarcinoma cells to *Vinca* alkaloids, as reported previously (22), as well as to actinomycin D (data not shown). There was a lesser effect against the DSU-resistant variant LYC5. In addition, immunostaining and Western blotting analysis indicated a 8-fold reduction in expression of Pgp in LYC5 cells. How, or whether, reduced Pgp is linked to DSU resistance is obscure. One possibility is that, under the conditions used to select the LYC5 clone, high concentrations of ISCU (300 μM) that uncoupled mitochondrial oxidative phosphorylation were used. Because cells expressing Pgp have been shown to have higher ATP utilization, and possibly this protein actively transports ATP from cells (30), it is possible that Pgp-positive cells have a selective disadvantage during uncoupling caused by ISCU.

Perhaps of greatest interest was the significant collateral sensitivity of LYC5 cells to mitochondrial toxins and the sensitization of parental GC_3/c1 cells to each mitochondrial toxin by verapamil. Whether the hypersensitivity of LYC5 cells resides specifically at the level of mitochondrial metabolism or at some other locus (e.g., plasma membrane) is currently under investigation. However, these data would suggest that LYC5 cells are hypersensitive to effects of agents that inhibit electron transfer in the respiratory chain or to consequences of ATP depletion, indicative of altered energy metabolism as a consequence of DSU resistance. Sensitization of GC_3/c1 cells by verapamil was not anticipated, and the mechanism for this synergy is unknown. Verapamil did not sensitize KB8-5 cells to mitochondrial toxins, suggesting that this effect is independent of Pgp. Furthermore, it is not known whether mitochondrial toxins are potentiated by verapamil or whether cellular effects of verapamil are potentiated by mitochondrial toxins. Pertinent to the study of DSU is the finding that in the presence of verapamil GC_3/c1 cells had approximately equal sensitivity to the mitochondrial toxins rotenone and antimycin as did LYC5 cells. Furthermore, verapamil had only modest activity in potentiating the toxicity of mitochondrial toxins in LYC5 cells. Thus, it is possible that during selection for ISCU resistance the putative "verapamil target" involved in this synergy was lost or altered. If the verapamil-sensitive locus provides protec-

tion from mitochondrial toxins, such an alteration or deletion in LYC5 cells could confer hypersensitivity to these agents. It is well established that calcium channel antagonists exert effects on mitochondria. The 1,4-dihydropyridine nitrendipine and other calcium channel inhibitors such as verapamil have been shown to bind receptors on mitochondria (31, 32). In mitochondria isolated from Ehrlich carcinoma cells, diltiazem, and to a lesser degree verapamil, inhibited Na^+-dependent Na^+/Ca^{2+} exchange (33). Verapamil has also been reported to protect mitochondria from effects of ischemia, possibly by maintaining Mg^{2+}/Ca^{2+} ratios in cells (34). The finding that DSU antitumor agents accumulate in mitochondria and at high concentrations cause rapid alterations in cellular Ca^{2+} levels (35) suggests that the hypersensitivity of LYC5 cells to mitochondrial toxins and sensitization of parental cells with verapamil are phenomena related to cytotoxic mechanisms of DSU action. Such an hypothesis is amenable to testing.

Direct linkage of ISCU resistance to mitochondrial toxin hypersensitivity will require isolation of cDNA clones capable of conferring this phenotype. However, our initial studies using hybrids formed between GC_3/c1 and LYC5 cells have been informative. Parental and ISCU-resistant cells were treated with either IO or DEPC and fused. Under these conditions no colonies formed if (a) cells were not fused, (b) DEPC-treated cells were fused to DEPC-treated cells, or (c) IO-treated cells were fused with IO-treated cells. The sensitivities of three GC_3/LYC5 hybrids and one GC_3/GC_3 hybrid to ISCU, rotenone, antimycin, and oligomycin were compared with those of the parental and LYC5 clones. Each hybrid was sensitive to ISCU, demonstrating that resistance to ISCU is a recessive trait. Similarly, the sensitivity of each hybrid to mitochondrial toxins was similar to that of GC_3/c1 cells, again demonstrating that collateral sensitivity to these agents in LYC5 cells was also recessive.

In summary, LYC5 cells display a complex phenotype, demonstrating resistance to DSU but collateral sensitivity to several natural product anticancer agents and three mitochondrial toxins. The data presented suggest that collateral sensitivity to mitochondrial toxins and sensitization of parental GC_3/c1 cells to these agents by verapamil may be independent of Pgp, as is resistance to DSU. Resistance to ISCU and collateral sensitivity to mitochondrial toxins are both recessive traits and hence may be linked. Current studies are therefore focused on identifying the mechanism of resistance in LYC5 cells as an approach to understanding the mechanism by which this novel class of drug exerts cytotoxic activity.

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