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Separation of Resistance to Antitumor Diarylsulfonylurea Agents from Collateral Sensitivity to Mitochondrial Toxins

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SUMMARY

Compared with parental GC_3/c1 human colon adenocarcinoma cells, which are diarylsulfonylurea (DSU)-sensitive cells, the DSU-resistant clone LYC5 demonstrates 4.2-, 12.8-, and 5.3-fold increase in sensitivity to the mitochondrial toxins rotenone, antimycin, and oligomycin, respectively. Studies with hybrids formed by fusion of parental GC_3/c1 cells with LYC5 cells have indicated that resistance to antitumor DSUs and collateral sensitivity to mitochondrial toxins are recessive and therefore potentially linked. To examine this, we transfected a cDNA library from GC_3/c1 cells, constructed in pcDNA3, into LYC5 cells. G418-resistant colonies were selected and further selected in a single step for resistance to rotenone (100 nM). Individual colonies (designated T5LR) were expanded and tested for sensitivity to mitochondrial toxins, antitumor DSU agents (LY195779 and LY186391) that demonstrate a 45–50-fold differential potency against GC_3/c1, LYC5 cells, and the antimitotic agent vincristine. Results demonstrate that resistance to mitochondrial toxins rotenone, antimycin, and oligomycin can be transferred without conferring a DSU-sensitive phenotype. Furthermore, in T5LR clones, resistance to mitochondrial toxins was not associated with increased resistance to vincristine or increased P-glycoprotein expression, supporting the contention that resistance to these agents is independent of P-glycoprotein. Southern blot analysis of T5LR clones demonstrated unique integration sites for the neomycin phosphotransferase gene into genomic DNA in clones 4 and 9, indicating independent derivation. Analysis of clones 4, 6, and 9 with the use of polymerase chain reaction demonstrated a cDNA insert of ~1.0 kilobase.

DSU antitumor agents represent a new class of oncolytic with a potentially novel mechanism of action. At pharmacologically achievable concentrations of free drug (~1 μM), these agents do not inhibit macromolecular synthesis (1, 2) or arrest cells in any specific phase of the cell cycle. At low, physiologically achievable concentrations, cytotoxicity is proliferation dependent (designated site 1), but as the concentration of DSU is increased, cytotoxic activity becomes equal in proliferating and nonproliferating cells (site 2; Ref. 3). At high concentrations, cell killing may be a consequence of uncoupling oxidative phosphorylation (4, 5). Accumulation of DSU within cells is inhibited by agents that uncouple mitochondria and ionophores that collapse the pH gradient across the mitochondrial inner membrane. These data have been interpreted to implicate mitochondria as the site of drug sequestration and accumulation (6, 7), and the uncoupling activity of antitumor DSU would support this. Studies with the photoaffinity probe [^{3}H]LY219703 [*N*-(4-azidophenylsulfonyl)-*N'*-(4-chlorophenyl)urea] also indicate accumulation in mitochondria and nonmitochondrial membranes (8).

Alternatively, several reports have suggested that DSU may alter intracellular calcium regulation (9–11) or the cytoskeletal network (12) and that introduction of activated H-*ras* may sensitize NRK-52E cells to these effects. Furthermore, the prototypical DSU sulofenur has been reported to rapidly induce the expression of c-*fos*, c-*jun*, and *hsp*70 (≤15 min; Refs. 13 and 14). Although such observations are of interest, their significance to the pharmacologically relevant site of action of antitumor DSU is questionable. In the studies cited above, concentrations were used of >125 μM sulofenur under serum-free conditions, and most studies reported effects at 500 μM. Under these conditions, it has been shown that sulofenur causes rapid equilibration of extracellular and intracellular pH, possibly through direct damage to the plasma membrane (15). In plasma, sulofenur is highly protein bound (>99.9%), yielding free-drug concentrations in the range of 0.5–1 μM under therapeutic conditions (16, 17). Consequently, biochemical alterations reported with concentrations of drug of >1000-fold of those achievable in plasma

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ABBREVIATIONS: DSU, diarylsulfonylurea; sulofenur, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY195779, *N*-(4-methylphenylsulfonyl)-*N'*-(4-nitrophenyl)urea; LY186391, *N*-(phenylsulfonyl)-*N'*-(3,4-dichlorophenyl)urea; P-gp, P-glycoprotein; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

probably do not relate to the site 1 target of DSU cytotoxicity that seems to be relevant *in vivo* (3, 18).

Recently, we reported that a clone selected for resistance to sulofenur was collaterally sensitive to mitochondrial toxins and to agents associated with P-gp-mediated multidrug resistance (19). Through the use of hybrids formed by fusion of parental DSU-sensitive cells and their DSU-resistant derivatives, it was demonstrated that resistance to sulofenur was recessive, as was collateral sensitivity to rotenone, antimycin, and oligomycin. Therefore, these two traits may be linked and may be useful in dissecting the mechanism of action of antitumor DSU. In the current study, we report that resistance to rotenone and other mitochondrial toxins can be transferred without conferring sensitivity to DSU. Resistance to mitochondrial toxins was not associated with increased resistance to vincristine, further emphasizing that resistance to mitochondrial toxins is independent of P-gp.

Materials and Methods

Chemicals and reagents. DSU analogues were generously supplied by Dr. John Toth (Eli Lilly and Co., Indianapolis, IN). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Springfield, NY), and restriction enzymes were purchased from Fisher Scientific. DNA purification kit (QIAGEN-tip 2500) was purchased from QIAGEN (Chatsworth, CA). Lipofectin reagent was purchased from Life Technologies (Gaithersburg, MD). Micro-TurboGen kit was obtained from Invitrogen Co. (San Diego, CA).

cDNA library and primers. The size-selective (>500 bp) cDNA library, which was constructed from mRNA extracted from the GC_3/c1 cell line, was purchased from Invitrogen. The expression vector pcDNA3 and host cells Top10F′ were used for the library. pcDNA3 is a 5.4-kb vector designed for high level stable expression in eukaryotic hosts. The cytomegalovirus promoter has demonstrated high level expression in a wide range of mammalian cells. The neomycin phosphotransferase (*neo*) resistance marker, which is expressed from the SV40 early promoter, was used to select stable transformants in the presence of G418. The cDNA fragments were ligated into *Bst*X1/*Eco*R1 sites in the polylinker with the corresponding adaptors. The number of primary recombinants was 1.05×10^6, and the average insert size of the clones analyzed was 0.95 kb. Primers of pcDNA839f (5′-CACTGCTTACTGGCTTATCG-3′), pcDNA1069r (5′-ACAGATGGCTGGCAACTAGA-3′), *neo*-f (5′-ATGATTGAACAAGATGGATT-3′), and *neo*-r (5′-TCAAGAAGGCGATAGAAGGC-3′) were synthesized at the Molecular Resource Center, St. Jude Children's Research Hospital (Memphis, TN).

Cell lines and culture conditions. The cell lines, GC_3/cl derived from a human colon adenocarcinoma and its subline LYC5, selected for resistance to sulofenur, and conditions of growth have been described previously (3). Both cell lines were routinely grown in antibiotic-free RPMI-1640 (Whittaker, Walkersville, MD) supplemented with 2 mM glutamine and 10% fetal calf serum (Hyclone, Logan, UT). The LYC5 clone was derived from cloned GC_3/c1 cells that were mutagenized with MNNG and EMS. Compared with parental GC_3/c1 cells, LYC5 was 4–6-fold resistant to sulofenur and 45–50-fold resistant to the analogues LY186391 and LY195779 (19). Other cell culture reagents were from GIBCO.

Colony forming assay. Colony formation was determined essentially as reported previously (3). Briefly, cells were plated at a density of 6000/35-mm culture dish (model 3046, Falcon) and allowed to attach for 24 hr, after which medium was replaced with fresh medium-containing drugs, and cells were incubated for an additional 7 days. Colonies were quantified after staining with 0.1% crystal violet (model 800, Artek). IC_{50} values were calculated from concentration-survival curves of triplicate determinations at each drug concentration. All experiments were repeated at least once.

Transfection. The Top10F′ bacteria containing the cDNA library or vector only (pcDNA3) were grown separately in 500 ml LB medium with 50 μg/ml ampicillin. The large-scale plasmid DNA isolation and purification were performed with QIAGEN-tip 2500 columns, and DNA was dissolved at 0.5 μg/μl in Tris/EDTA buffer.

For transfection, 1×10^6 LYC5 cells were seeded in T12.5 flasks and allowed to attach overnight. When cells were 30–50% confluent, they were washed twice with serum-free RPMI 1640. For each flask to be transfected, 6 μg of library DNA or vector pcDNA3 was diluted into 100 μl serum-free RPMI 1640 (solution A). Ten microliters of lipofectin were diluted into 100 μl serum-free RPMI 1640 (solution B). Solutions A and B were combined, mixed gently, and incubated at room temperature for 15 min. While the lipofectin reagent/DNA complexes were forming, 1.8 ml of serum-free medium was added, and the mixture was overlaid onto cells. DNA-containing medium was replaced with normal RPMI 1640 on the second day after transfection.

After 48 hr transfected cells were transferred to T75 flasks (1:6 dilution). G418 (50 μg/ml) was used for selection of transfected LYC5 cells. Untransfected and pcDNA-transfected LYC5 cells were used as controls. Selection continued for 14 days, and media were changed every 3 days. Under these conditions, there were no surviving colonies in nontransfected LYC5 cultures.

Selection for rotenone resistance. G418-resistant LYC5 cells that were transfected with library cDNA were pooled and seeded (2×10^5 cells) into T162 flasks. Cells were allowed to attach overnight, and then 100 nM rotenone was added. Medium containing rotenone was replaced every 3 days. pcDNA3-transfected, G418-resistant LYC5 cells were used as a control. After 3 weeks of selection, rotenone-resistant clones were isolated by ring cloning and expanded; these clones were designated as T5LR.

Southern blot hybridization and PCR. Genomic DNA of rotenone-resistant T5LR clones was extracted with a Micro-TurboGen kit. Ten micrograms of genomic DNA of four T5LR clones were digested with *Bam*HI, *Hin*dIII, and *Eco*RI separately. After agarose gel electrophoresis (0.8%), DNA was transferred to a nitrocellulose membrane. The *Bsm*I and *Sma*I fragment containing neomycin gene was cut from pcDNA3 vector and labeled as a probe for Southern blot hybridization. Hybridization was performed according to the method of Sambrook *et al.* (20).

The primers *neo*-f and *neo*-r were used for amplification of the integrated neomycin gene in the vector pcDNA3. Another pair of primers (pcDNA839f and pcDNA1069r) were used for the amplification of integrated inserts from vector pcDNA3. Genomic DNA (200 ng) was used as template for PCR. The conditions used for amplification of cDNA inserts from genomic DNA were 5 min at 95°, 30 cycles of 1 min at 94°, 2 min at 50°, and then 3 min at 74° (21).

Determination of P-gp. To determine the expression of P-gp in GC_3/c1, LYC5, and T5LR cells, 2×10^7 cells were extracted (22). Cell extracts (200 μg of protein) were electrophoresed (10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with standard procedures (23) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). P-gp was detected with C219 monoclonal antibody (Signet Laboratories, Dedham, MA) and anti-mouse IgG horseradish peroxidase conjugate and visualized by the use of enhanced chemiluminescence according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Results

Collateral sensitivity to mitochondrial toxins. We previously reported that LYC5 cells were resistant to antitumor DSU but collaterally sensitive to rotenone, antimycin, and oligomycin. Since this report, LYC5 cells have been passaged in the absence of selecting pressure (no sulofenur) and

have retained resistance to sulofenur. Consequently, we first retested the sensitivity of GC_3/c1 and LYC5 cells to these mitochondrial toxins to determine whether collateral sensitivity to these agents was a stable characteristic. The results (Fig. 1) demonstrate that sensitivity to mitochondrial toxins was stable for >12 months in the absence of drug selection. The IC_{50} concentrations for rotenone, antimycin, and oligomycin were 17.6, 11.8, and 1.64 nM for LYC5 and 73.4, 151, and 8.6 nM for GC_3/c1 cells, respectively.

Selection of rotenone-resistant transfectants. The above results confirmed the original findings and demonstrated that collateral sensitivity to mitochondrial toxins and resistance to sulofenur were stable characteristics of LYC5 cells. The studies with hybrids (19) indicated that both sulofenur resistance and collateral sensitivity to mitochondrial toxins were recessive characteristics. Consequently, we theorized that the loss of function in LYC5 cells could be complemented by transfection of cDNA derived from parental GC_3/c1 cells. A cDNA library in the expression vector pcDNA3 was transfected into LYC5 cells (see Materials and Methods), and G418-resistant clones were selected under conditions where there were no surviving LYC5 cells in mock-transfected cultures. G418-resistant clones were pooled and selected in 100 nM rotenone (~6-fold the IC_{50} value for LYC5 cells). Under these conditions, no rotenone-resistant cells were obtained from cultures of nontransfected LYC5 cells or from LYC5 cells transfected with pcDNA3 vector alone. Four of the clones that grew in the presence of rotenone were identified, ring cloned, expanded, and characterized (designated T5LR clones 4, 6, 7, and 9).

Characterization of T5LR clones. Integration of plasmid sequences into genomic DNA of each clone was confirmed by Southern blot analysis of DNA restricted with *Hin*dIII, *Eco*RI, and *Bam*HI with a probe to detect the *neo* gene of pcDNA3 (Fig. 2). The recombinant plasmid 4 was used as a positive control; it was selected from the original cDNA library and was confirmed to have an insert of ~800 bp. As shown in Fig. 2, all of the four T5LR clones gave a positive signal with the neomycin probe in each of three independent digests. Plasmid sequences were detected on similar-sized fragments for *Eco*R1 digests (~6 kb) in DNA extracted from each T5LR clone. Clones 4 and 6 also had similar integration patterns in *Hin*dIII and *Bam*HI digests. Clones 7 and 9 had integration patterns in which the *Bam*HI fragment was smaller in extracts from clone 7, and the *Hin*dIII integration site for clone 9 was detected on a ~5.2-kb fragment. These results indicate that the integration sites for T5LR clones 4 and 6 were similar and therefore may have originated from the same cell but were different than for clone 9, suggesting that clone 9 was derived independently of clones 4 and 6. With use of the primers *neo*-f and *neo*-r, which span the entire *neo* gene, the predicted 781-bp fragment was detected in all four T5LR clones, indicating that the integrated *neo* gene was not rearranged in these clones. With the use of primer sets pcDNA839f and pcDNA1069r (see Materials and Methods), an insert of ~1000 bp was detected in clones T5LR clones 4, 6, and 9 (Fig. 2). No insert was detected in clone 7, which was not further characterized.

Resistance of T5LR clones to mitochondrial toxins. We next examined the sensitivity of T5LR c9 to each mitochondrial toxin. As shown in Fig. 3, this clone was resistant to each agent relative to LYC5. The IC_{50} values for rotenone, antimycin, and oligomycin were 17.9, 12.8, and 1.6 nM for LYC5 and 127, >300, and 17.6 nM for T5LRc9, respectively. Thus, T5LRc9 was 7.1-, >23-, and 11-fold resistant to rotenone, antimycin, and oligomycin, respectively, compared with LYC5. Similar results were obtained with 3 other T5LR clones (data not shown). Thus, each of the T5LR clones was resistant to mitochondrial toxins and therefore demonstrated a phenotype similar to that of the GC_3/c1 DSU-sensitive cells.

Sensitivity of T5LR clones to DSU. We examined the sensitivity of T5LR to the sulofenur analogues LY186391 and LY195779. These analogues were chosen because there is a 45–50-fold differential in the IC_{50} values between GC_3/c1 and LYC5 cells, allowing more accurate detection of any reversion of clones to DSU sensitivity. In contrast, the differential sensitivity between GC_3/c1 and LYC5 cells to sulofenur is only ~5-fold. As shown in Fig. 4, T5LR clones were as resistant to both of these sulofenur analogues as was LYC5.

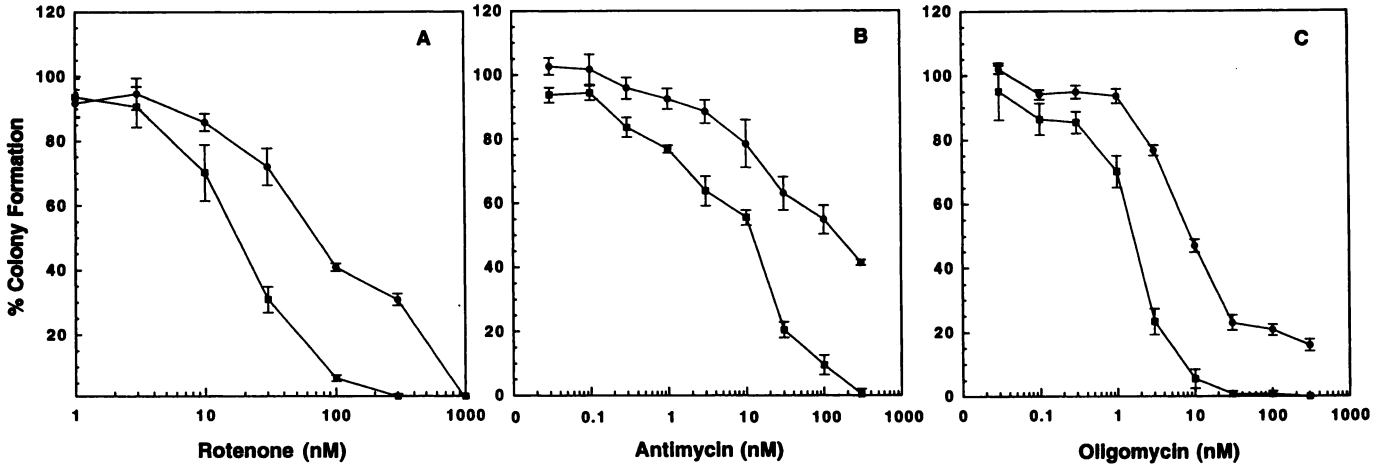

Fig. 1. Sensitivity of GC_3/c1 (●) and LYC5 (■) cells to mitochondrial toxins. GC_3/c1 and LYC5 cells were exposed to each agent for 7 days, and clonogenic survival was determined. Each value represents the mean of three determinations and is expressed as percentage of control colony number. A, For rotenone, calculated IC_{50} values were 73.4 and 17.6 nM for GC_3/c1 and LYC5 cells, respectively. B, For antimycin, IC_{50} values were 151 and 11.8 nM for GC_3/c1 and LYC5 cells, respectively. C, For oligomycin, IC_{50} values were 8.6 and 1.6 nM for GC_3/c1 and LYC5 cells, respectively. *Points*, mean ± standard deviation for a representative experiment.

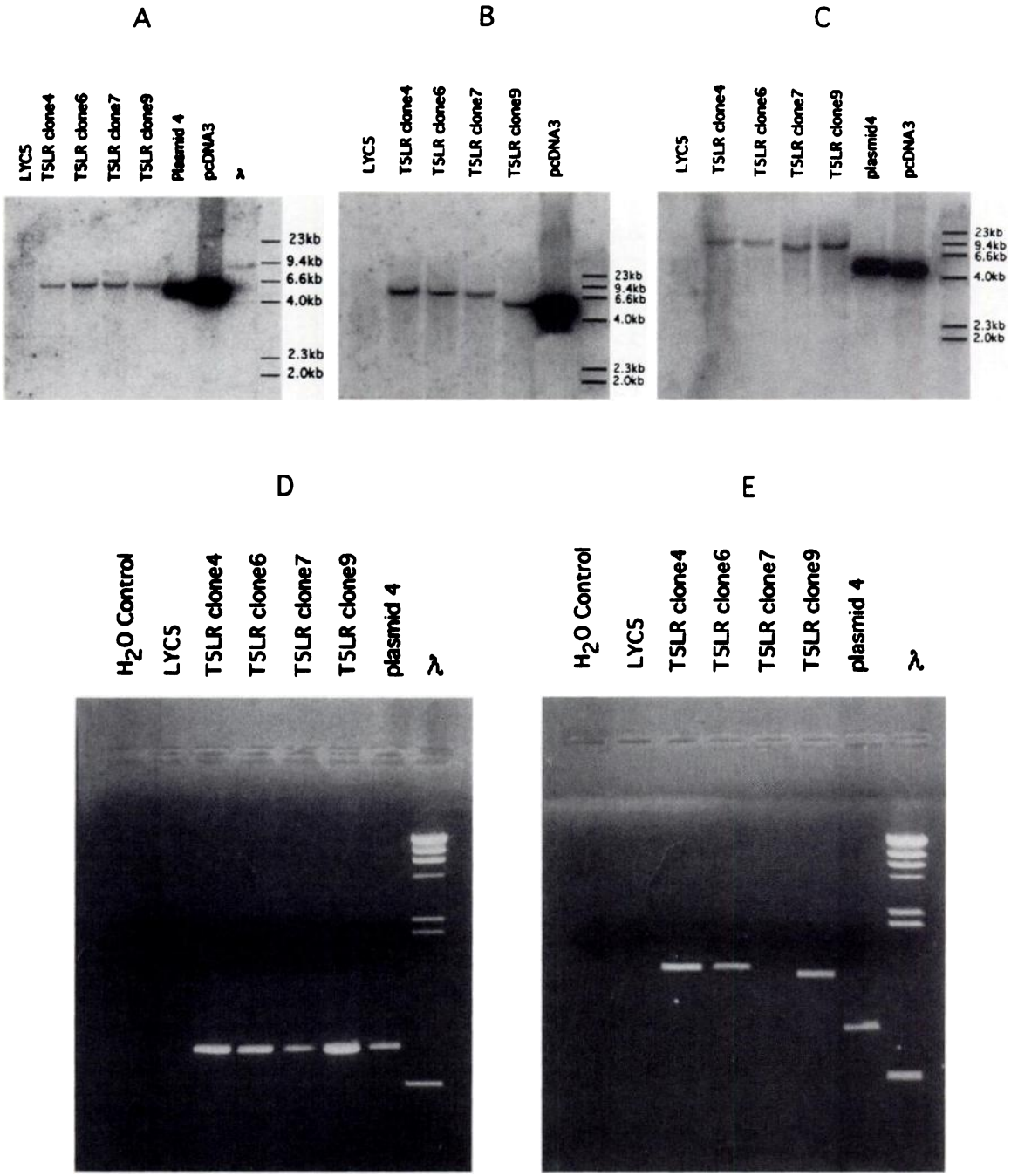

Fig. 2. Southern blot analysis of genomic DNA of T5LR clones and PCR showing cDNA inserts. Ten micrograms of genomic DNA were digested with *Eco*RI (A), *Hin*dIII (B), and *Bam*HI (C), respectively; electrophoresed through a 1% agarose gel; transferred to a hybridization transfer membrane (GeneScreen Plus, NEN Research Products, Boston, MA); and hybridized to a ^{32}P- labeled 1107-bp *Bsm*I and *Sma*I restriction fragment containing *neo* gene of pcDNA3. The genomic DNA of LYC5 cells was used as negative control, and pcDNA3 (5.4k kb) and plasmid 4, which has an 800-bp insert in pcDNA3, were used as positive controls for Southern blot analysis. D, DNA PCR analysis of T5LR cells. Primers specific to the 3′ and 5′ regions of *neo* gene were used to detect the integrated fragment of pcDNA. The predicted fragment (781 bp) was amplified from T5LR clones 4, 6, 7, and 9. Plasmid 4 was used as a positive control, and LYC5 DNA was used as the negative control. E, Primers specific to 3′and 5′ regions of polylinker in pcDNA3 were used to detect insert in pcDNA3 from T5LR cells. To confirm the sensitivity of PCR, 0.25 pg of DNA of plasmid 4 containing 800 bp insert was used as a positive control.

Resistance to mitochondrial toxins is not associated with vincristine resistance. In the previous study (19), it was shown that LYC5 cells were also collaterally sensitive to several agents associated with P-gp-mediated multidrug resistance (i.e., vincristine, rhodamine-123, actinomycin D), and this was associated with decreased expression of P-gp. To directly test whether conferring resistance to mitochondrial toxins was associated with a multidrug-resistant phenotype, the sensitivity of T5LRc9 to vincristine was examined. The results (Fig. 5) demonstrate that T5LRc9 cells were as sensitive to vincristine as were LYC5 cells. Analyses are given in Fig. 6 of P-gp expression by Western blot analysis in extracts from GC_3/c1, LYC5, and T5LR clones relative to standard lines, Rh30 (human rhabdomyosarcoma; P-gp-negative), and

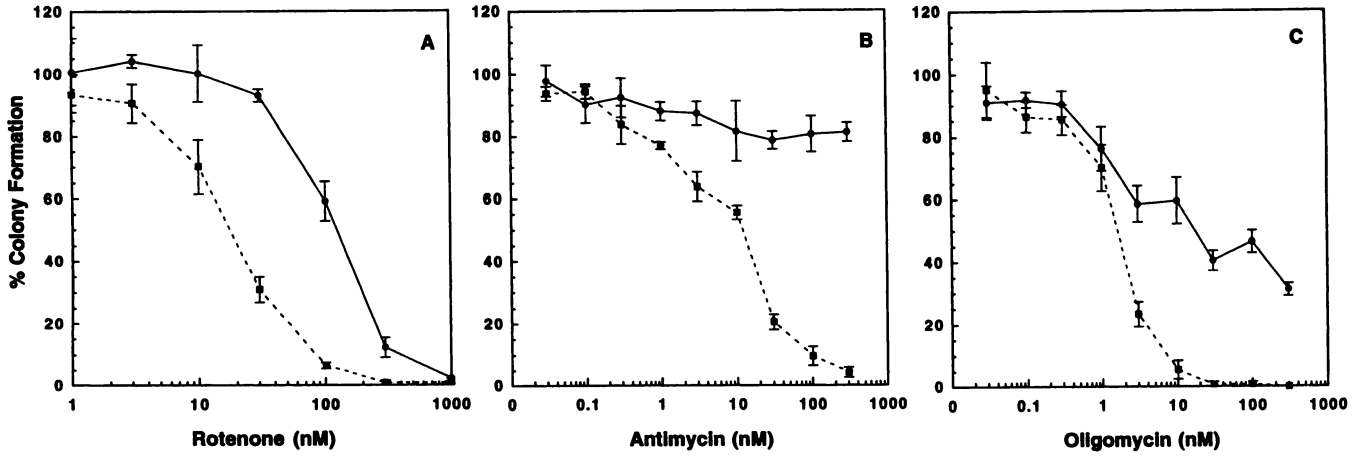

Fig. 3. Sensitivity of T5LRc9 (●) and LYC5 (■) cells to mitochondrial toxins. The sensitivity was determined as described in legend to Fig. 1. A, For rotenone, calculated IC_{50} values were 127.2 and 17.9 nM for T5LR and LYC5 cells, respectively. B, For antimycin, IC_{50} values were >300 and 12.8 nM for T5LR and LYC5 cells, respectively. C, For oligomycin, IC_{50} values were 17.6 and 1.6 nM for T5LR and LYC5 cells, respectively.

the multidrug-resistant variant CEM_{VLB100}, which overexpresses P-gp (24). These results show that P-gp expression was decreased below the level of detection in LYC5 cells, as previously reported, and was not increased in the T5LR clones selected for resistance to mitochondrial toxins subsequent to transfection of the cDNA library.

Discussion

Previous studies have suggested that antitumor DSU may have at least two potential mechanisms of action. At low pharmacologically achievable concentrations in humans (~0.5–1 μM free drug), the cytotoxic effects of sulofenur and other antitumor DSUs against cells in culture are proliferation dependent (3). Under these conditions, LYC5 cells were 5.5-fold resistant to sulofenur and 45–50-fold resistant to the analogues LY186391 and LY195779 (19). At concentrations required to kill cells during brief exposure (4–24 hr), cytotoxicity was independent of proliferation, and LYC5 cells were not resistant under these conditions (3). This second mechanism seems to correlate with DSU-induced uncoupling of mitochondria (4). Other data have implicated accumulation of DSU in mitochondria (3–5). At this time, neither the mechanism of action of DSU antitumor agents nor mechanisms that confer resistance are known. That LYC5 cells, resistant to antitumor DSU, were collaterally sensitive to mitochondrial toxins also seemed to implicate mitochondria in the action of this class of drug. Both resistance to antitumor DSU and collateral sensitivity to mitochondrial toxins segregated as recessive characteristics and therefore were potentially linked. Complicating this interpretation were that (i) LYC5 cells were collaterally sensitive to agents associated with P-gp-mediated multidrug resistance, and these cells demonstrated a significant decrease in detectable P-gp; and that (ii) the multidrug-resistance modulator, verapamil, markedly increased the sensitivity of parental GC_3/c1 cells to each mitochondrial toxin but had significantly less effect in potentiating the toxicity of these toxins in LYC5 cells. Thus, although from structural and charge considerations it seemed unlikely that agents such as rotenone, antimycin, and oligomycin were substrates for P-gp-mediated efflux, this potential did exist, and (iii) the selection of LYC5 used mutagens that may have resulted in several changes in addition to resistance to DSU.

Resistance to DSU in LYC5 cells is stable; consequently, it was not possible to check linkage between DSU resistance and mitochondrial toxin sensitivity in revertants. Thus, in the current study, a genetic approach was undertaken to transfer wild-type genes from parental GC_3/c1 cells that confer rotenone resistance to LYC5 cells, with the primary objective of determining whether this restored sensitivity to antitumor DSU. A secondary aim was to determine whether resistance to rotenone was associated with P-gp-mediated multidrug resistance. A size-selected cDNA library from parental GC_3/c1 cells was constructed in pcDNA3, a vector that carries the neomycin-selectable marker, and transfected into LYC5 cells. Clones selected for G418 resistance were pooled and placed under positive selection for rotenone resistance. Four colonies (designated T5LR) growing in 100 nM rotenone were expanded. Under identical conditions, no colonies were formed in control (pcDNA3 vector-transfected) or LYC5-untransfected cells. To determine whether these clones were independently derived, analysis of digested DNA with a probe to the *neo* gene was undertaken. Integration of plasmid DNA was confirmed in each clone by PCR and demonstrated that the integrated *neo* gene had not been rearranged. Southern blot analysis demonstrated similar integration sites in genomic DNA of clones 4 and 6 but distinct integration patterns for clone 9. This indicates that clones 4 and 6 may have a common derivation, which is independent of clone 9. No insert could be recovered from clone 7, possibly due to rearrangement or disruption during integration, and therefore clone 7 was not further studied. With the use of primers designed to allow recovery of cDNA inserts, a ~1.0-kb insert was identified in three of the T5LR clones. This cDNA is being cloned and characterized.

The sensitivity of T5LR clones to rotenone, antimycin, and oligomycin was next examined. Each clone demonstrated resistance to each of these toxins; therefore, T5LR clones were similar to parental GC_3/c1 cells. We next tested whether T5LR rotenone-resistant clones had reverted to DSU sensitivity with the use of two analogues of sulofenur. As reported previously (19), LYC5 cells were 45–50-fold resistant to LY186391 and LY195779 relative to GC_3/c1 cells,

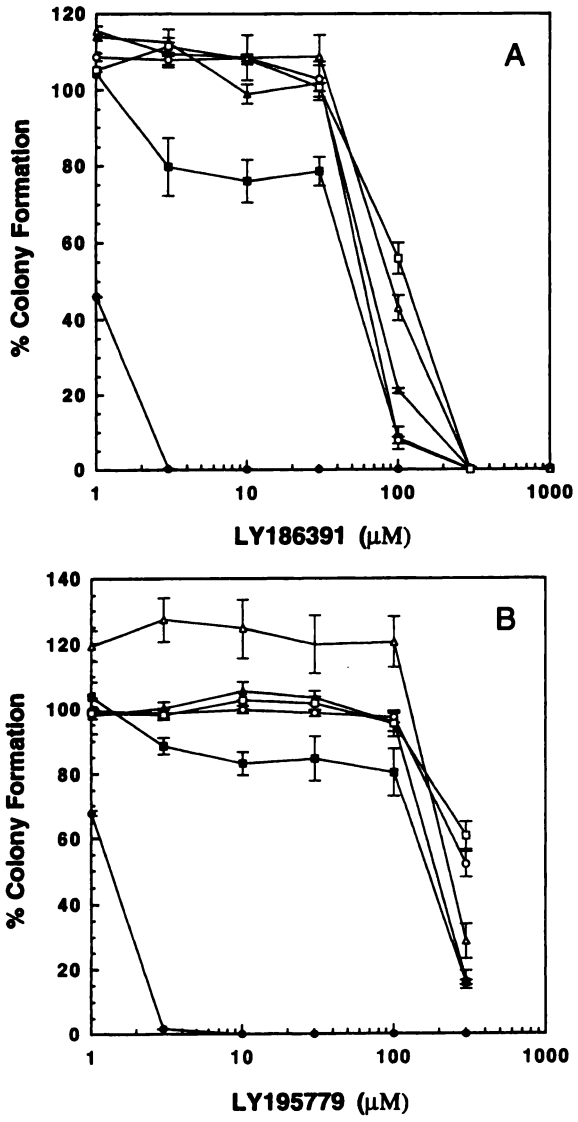

Fig. 4. Sensitivity of GC_3/c1 (●), LYC5 (■), and T5LR clones 4 (▲), 6(○), 7(□), and 9(△) to LY186391 (A) and LY195779 (B). The sensitivity was determined as described in legend to Fig. 1. *Points*, mean ± standard deviation for three determinations for a representative experiment.

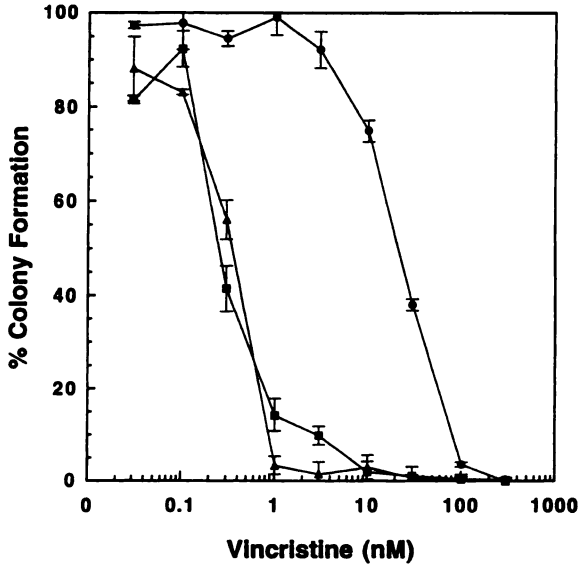

Fig. 5. Sensitivity of GC_3/c1 (●), LYC5 (■), and T5LR (▲) cells to vincristine. The sensitivity was determined as described in legend to Fig. 1. *Points*, mean ± standard deviation for three determinations for a representative experiment.

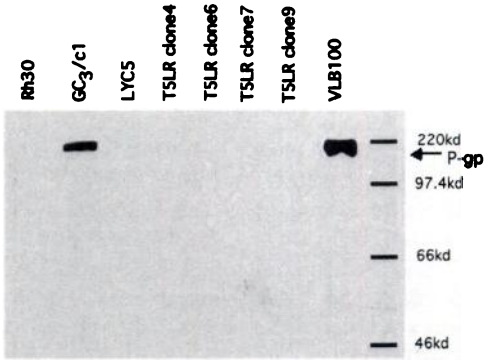

Fig. 6. Western blot for P-gp in cell lysates of GC_3/c1, LYC5, and T5LR clones 4, 6, 7, and 9. Rh30 (human rhabdomyosarcoma) and the multidrug-resistant human leukemic cell line CEM_{VLB100} cells were included as negative and positive controls, respectively. *Right*, position of molecular weight markers and P-gp.

whereas the level of resistance to sulofenur is ~5-fold. The reason for the greater differential toxicity of these analogues is their greater potency against GC_3/c1 cells, whereas each analogue inhibited growth of LYC5 cells at a similar concentration. Each of the rotenone-selected T5LR clones retained the same level of resistance to these DSU analogues as did LYC5. Consequently, we conclude that rotenone resistance and collateral sensitivity to antitumor DSU are not genetically linked. However, the potential exists that a single cDNA may confer resistance to three structurally different mitochondrial toxins that have independent loci of action in inhibiting respiration.

To examine whether T5LR clones, resistant to mitochondrial toxins, were cross-resistant to cytotoxic agents associated with P-gp-mediated multidrug resistance, we examined their sensitivity to vincristine. Parental GC_3/c1, LYC5, and T5LRc9 were exposed to vincristine for 7 days, and colony formation was determined. GC_3/c1 cells were ~40-fold resistant compared with LYC5 cells, whereas T5LRc9 cells had similar sensitivity as LYC5. Thus, conferring resistance to mitochondrial toxins was not associated with cross-resistance to vincristine. Immunoblotting also confirmed that LYC5 and T5LR have significantly less expression of P-gp than do GC_3/c1 cells. Thus, resistance to mitochondrial toxins can be conferred without an apparent increase in P-gp expression, suggesting that resistance to these agents is independent of this form of multidrug resistance.

In summary, resistance to the mitochondrial toxins rotenone, antimycin, and oligomycin has been transferred via a cDNA library derived from the toxin-resistant GC_3/c1 human colon adenocarcinoma cell line. Resistance was not associated with reversion to sensitivity to DSU antitumor agents, indicating that resistance to DSUs and collateral sensitivity to mitochondrial toxins are not genetically linked and probably arose as independent mutations during the selection of resistance in the LYC5 clone. Resistance to mitochondrial toxins was not associated with increased expression of P-gp or an increased resistance to vincristine.

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