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Collateral Sensitivity to Nitrosoureas in Multidrug-resistant Cells Selected with Verapamil[1]

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ABSTRACT

We have examined the effects of the nitrosoureas, streptozotocin (STZ) and 1,3-bis(chloroethyl)-1-nitrosourea (BCNU), on a human multiple myeloma cell line, RPMI 8226, and its drug-resistant variants. Cell lines selected for doxorubicin (DOX) resistance alone displayed a STZ and BCNU cytotoxicity profile similar to that of the parent cell line. In contrast, two of the drug-resistant variants selected with DOX plus verapamil, an agent which inhibits P-glycoprotein-mediated multidrug resistance, displayed a collateral sensitivity to STZ and BCNU. Verapamil was included in the selection protocol because it has been shown to inhibit the P-glycoprotein-mediated multidrug resistance phenotype and is now in clinical trials as a chemosensitizing agent. The collateral sensitivity to these nitrosoureas seen in the DOX plus verapamil-selected cell lines is due to the functional loss of a DNA repair molecule, O^6-Methylguanine DNA methyltransferase (MGMT). The functional loss of MGMT is secondary to the loss of MGMT gene expression. The loss of MGMT gene expression is not due to loss or gross rearrangement of the MGMT-coding region. If this selection pressure applied *in vitro* reflects the *in vivo* situation, then new chemotherapeutic strategies may be devised to exploit this phenomenon. These cell lines will serve as useful models for delineating mechanisms which govern MGMT expression.

INTRODUCTION

The development of multidrug drug resistance remains a primary obstacle to the success of chemotherapy of many human tumors. A variety of biochemical mechanisms are responsible for imparting multidrug resistance (1–3). One mechanism that confers multidrug resistance is the overproduction of P-glycoprotein, an integral membrane protein responsible for the active efflux of a broad spectrum of antitumor agents (3).

The overproduction of P-glycoprotein has been shown to be important in the acquired drug resistance of a variety of human tumor types. These tumor types include both hematological (*e.g.*, multiple myeloma, lymphoma) and solid tumors (*e.g.*, breast, childhood sarcoma) (4). The appearance of P-glycoprotein in the tumor has also been correlated with poor prognosis in neuroblastoma and childhood sarcoma (5, 6).

Because of the pervasiveness of P-glycoprotein-mediated drug resistance, strategies to pharmacologically neutralize P-glycoprotein function have been devised. While different approaches are currently under study, the first attempt to suppress P-glycoprotein function was through the use of verapamil (7). Verapamil is thought to bind P-glycoprotein and inhibit its drug efflux capacity. This results in greater intracellular drug accumulation, thereby increasing cell kill (8).

Verapamil can be used as a chemosensitizing agent in clinical situations in two different ways. One approach uses verapamil in chemotherapy following the emergence of the P-glycoprotein phenotype, in an effort to inhibit P-glycoprotein function (9). Although results are preliminary, the clinical studies suggest that using verapamil to reverse clinical multidrug resistance results in responses, but usually of short duration, and eventually the tumor becomes refractory to the sensitizing effects of verapamil (10–12). Another approach utilizes verapamil prior to the emergence of the phenotype (9). The theory behind this approach is that prophylactic use of verapamil would select against the population of cells that may express P-glycoprotein, thereby inhibiting its emergence as the primary mechanism of drug resistance.

We have created two different drug-resistant cell lines by using two different selection protocols that included verapamil.[3] The selection protocols were designed to imitate the clinical protocols described above. To mimic the use of verapamil as a prophylactic treatment, verapamil and DOX[4] were used concomitantly in selecting drug-resistant cells from the drug-sensitive human multiple myeloma cell line RPMI 8226 (8226/S). A multidrug-resistant variant emerged (8226/dox1V). This cell line is P-glycoprotein negative and has biochemical properties and a drug resistance profile consistent with multidrug resistance due to an altered topoisomerase II. To mimic the use of verapamil as a chemosensitizer, 8226/dox 40, a P-glycoprotein-positive variant of 8226/S, which was initially selected with DOX only, was grown in the continued presence of verapamil and DOX. A variant cell line emerged, 8226/mdr10V. 8226/mdr10V produces less P-glycoprotein than 8226/dox40, but has an increased drug resistance for many of the drugs tested.

In this report, we demonstrate that the drug-resistant cell lines selected in the presence of doxorubicin plus verapamil have become collaterally sensitive to the nitrosoureas, STZ and BCNU. This hypersensitivity is due to the functional loss of a DNA repair protein, MGMT, which is responsible for repairing a major mutagenic and cytotoxic lesion of the nitrosoureas, O^6-alkylguanine (13–15). The observed loss of activity is due to the inactivation of the MGMT gene.

MATERIALS AND METHODS

Cell Culture, Selection, Drugs, and *in Vitro* Cytotoxicity Assays. 8226/S was purchased from the American Type Culture Collection. 8226/S and drug resistant-variants were grown in RPMI 1640 supplemented with 5% fetal bovine serum (Tissue Culture Biologicals, Tulare, CA), penicillin (1 unit/ml), and glutamine (1%, v/v) (penicillin and glutamine from Grand Island Biological Co, Grand Island, NY). Cells were incubated at 37°C in 5% CO_2-95% air atmosphere and were passaged once every 6 days. Drug-resistant cells were cultured in the presence of selecting agents.

Drug-resistant cells were selected as follows. 8226/dox1V was selected from 8226/S by continuous culture in 10 μg/ml verapamil (2.2×10^{-5} M) with a stepwise increase in DOX concentration from 10^{-9} to 10^{-8} M over the course of 1 year. 8226/dox40 was selected from 8226/S

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[4] The abbreviations used are: DOX, doxorubicin; MGMT, O^6-methylguanine-DNA methyltransferase; STZ, streptozotocin; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; SDS, sodium dodecyl sulfate; cDNA, complementary DNA.

as previously described (3). 8226/mdr10V was selected from 8226/dox40 by continuous culture in 10 μg/ml verapamil with a stepwise increase in DOX concentration from 10^{-8} to 10^{-7} M over the course of 1 year. Verapamil alone was nontoxic to the cells.

In vitro cytotoxicity produced by STZ and BCNU was measured using a two-layer soft agar-cloning assay (16). Cells were exposed to STZ or BCNU for 1 h at 37°C, washed free of drug, and then plated in soft agar. Colonies were scored after 2 weeks. Data are expressed as the percentage of survival compared to untreated controls. *In vitro* cytotoxicity produced by DOX, mitoxantrone, vincristine, etoposide, and melphalan was measured using a tetrazolium-based semiautomated colorimetric assay, as previously described (17).

STZ was provided by Upjohn (Kalamazoo, MI), DOX was provided by Adria Labs, (Columbus, OH), verapamil was provided by Knoll Pharmaceutical (Whippany, NJ), mitoxantrone was provided by Lederle (Pearl River, NY), and BCNU and melphalan were obtained from the Drug Synthesis and Discovery Branch of the National Cancer Institute.

MGMT Activity. Measurement of cellular MGMT activity was performed essentially as described before (18). Briefly, 50 μg of total cellular protein was reacted with 0.1 pmol of an end-labeled, synthetic 18-mer containing a methyl group on the O^6 position of the 3'-guanine residue within a *Pvu*II restriction site, which blocks digestion by this restriction enzyme. The 18-mer, previously incubated with cellular protein, was subjected to *Pvu*II digestion. Relative levels of MGMT activity were determined by the conversion of the full-length 18-mer to a digested 8-mer.

Northern Blot Analysis. Total cellular RNA was purified by guanidium isothiocyanate cell lysis and CsCl gradient centrifugation (19). Northern blot analysis was performed as previously described (20). Briefly, prehybridization of the membrane was carried out in 50% formamide, 10% dextran sulfate 5× SSPE (1 × SSPE = 0.15 M NaCl, 0.015 M NaH_2PO_4, 0.002 M EDTA, pH 7.4), 1% SDS, 1× Denhardt's solution, and 250 μg/ml sheared, denatured, salmon sperm DNA at 42°C for at least 4 h. Hybridization was carried out in the same solution to which 10^6 cpm/ml denatured randomly primed ^{32}P-labeled probe was added. The probes used were a 738-base pair MGMT cDNA fragment (21) and a 2-kilobase chicken β-actin cDNA (22).

Following hybridization, the membrane was first washed in 2× standard sodium citrate (1 × SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate, pH 7.0), 0.5% SDS for 30 min at room temperature. The membrane was then washed twice in a large volume of 0.1× standard sodium citrate, 0.5% SDS for 30 min at 65°C.

Southern Blot Analysis. High molecular weight DNA was isolated, restriction enzyme digested, size fractionated on 0.8% agarose gels, and capillary transferred essentially as described previously (23). Hybridization and wash conditions were the same as those used in the Northern blot analysis. The 738-base pair MGMT cDNA was used as the hybridization probe.

RESULTS

Fig. 1 shows the results of colony formation assays in which 8226/S cells and multidrug-resistant variants were exposed to varying concentrations of drug. Fig. 1*A* shows results obtained using STZ. The data indicate that 8226/S and the multidrug-resistant variant 8226/dox40 are similarly resistant to the cytotoxic action of STZ. By contrast, the cells selected in the presence of DOX plus verapamil (8226/dox1V and 8226/mdr10V) are almost 2 logs of cell kill more sensitive to STZ than their parent cell lines. Fig. 1*B* shows the results obtained using BCNU. Again, the cells selected in the presence of DOX plus verapamil are more sensitive to the cytotoxic effects of nitrosoureas than their parent cell lines.

Table 1 shows the relative resistance of the salient cell lines to different classes of antitumor agents. All three multidrug-resistant variants are highly resistant to the anthracycline DOX and display moderate, but consistent, resistance to the nitrogen mustard, melphalan. Both mdr1 cell lines, 8226/dox40 and 8226/mdr10V, are >250-fold resistant to the *Vinca* alkaloid vincristine, while 8226/dox1V displays only 2-fold resistance to vincristine, a finding typical for resistance mediated by an altered topoisomerase II.

To determine the cause of enhanced sensitivity to STZ, we examined MGMT activity in various sensitive and drug-resistant myeloma cell lines. Fig. 2 shows the relative levels of functional MGMT in these cell lines and other drug-resistant cell lines derived from 8226/S. The end-labeled 18-mer, containing an O^6-methylguanine residue blocking a *Pvu*II restriction site, was incubated with 50 μg of total cellular protein from the various cell lines for 2 h at 37°C. Following organic solvent extraction and ethanol precipitation, the reacted probe was digested with *Pvu*II.

The ability of *Pvu*II to digest the end-labeled 18-mer to an 8-mer is dependent upon the removal of the methyl group from the O^6 position of the modified guanine and, thus, reflects the

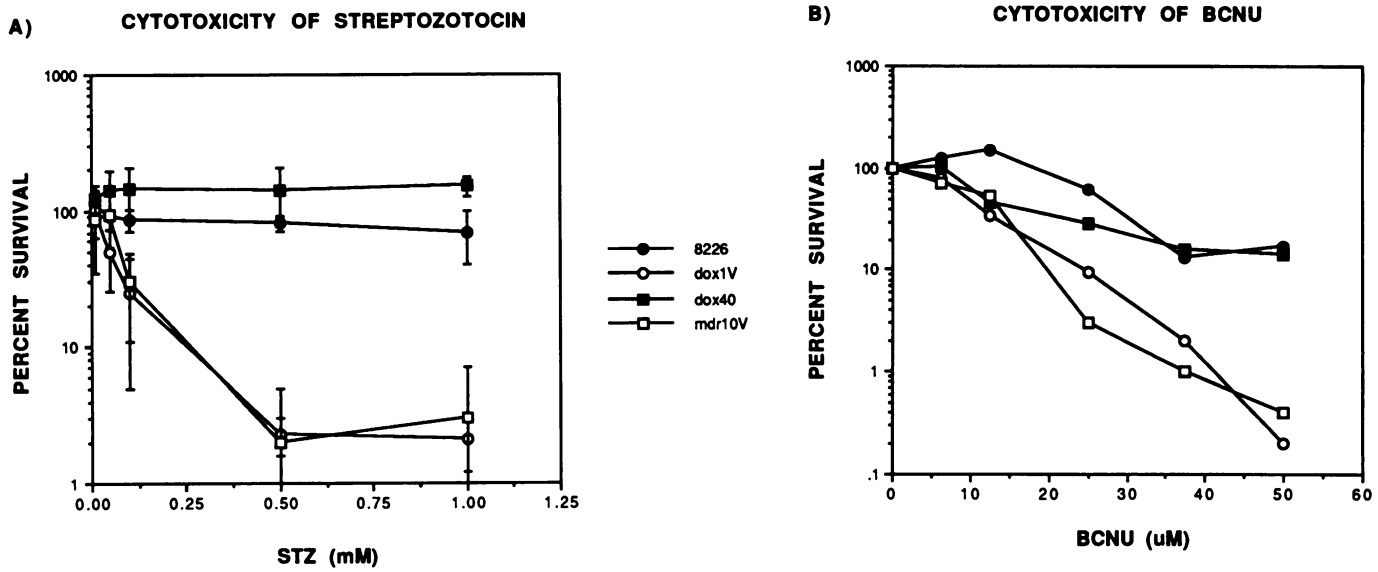

Fig. 1. Agar colony-forming ability of 8226/S, 8226/dox1v, 8226/dox40, and 8226/mdr10v exposed to varying concentrations of STZ (*A*) or BCNU (*B*) for 1 h at 37°C. Colonies were scored 12–14 days later. *Points*, mean survival of at least two independent experiments; *bars*, SD.

Table 1 *Degree of resistance of 8226 and drug-resistant cell lines to other antitumor agents*

Degree of resistance is relative to the nondrug-selected 8226 cell line and is calculated from the concentration which inhibited growth by 90%. Growth Inhibition was measured using a modified tetrazolium-based semiautomated colorimetric assay.

	8226	8226/dox1v	8226/dox40	8226/mdr10v
Doxorubicin	1	24	2800	3200
Etoposide	1	14	139	122
Mitoxantrone	1	10	78	6000
Vincristine	1	2	295	1400
Melphalan	1	8	11	6

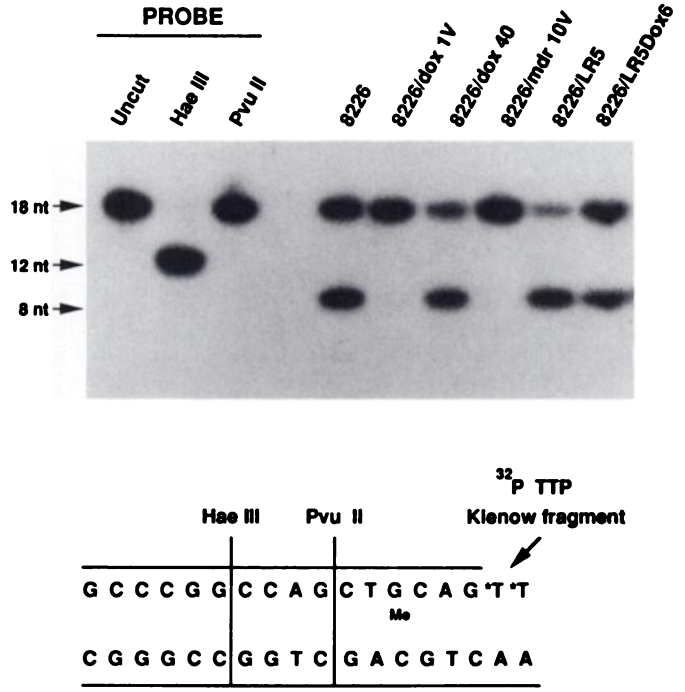

Fig. 2. MGMT activity assay in drug-sensitive (8226/S), DOX-selected (8226/dox40), DOX + verapamil-selected (8226/dox1V, 8226/mdr10V), melphalan-selected (8226/LR5), and melphalan + DOX-selected (8226/LR5dox6) cells. The end-labeled 18-mer, containing an O^6-methylguanine residue blocking a *Pvu*II restriction site, was reacted with 50 μg of total cellular protein from the various cell lines for 2 h at 37°C. The relative levels of MGMT are determined by the ability of *Pvu*II digestion to convert the ^{32}P end-labeled 18-mer to an 8-mer. Following *Pvu*II digestion, end-labeled products were fractionated on a 20% polyacrylamide, 7 M urea, 1 × buffer (0.089 M Tris, 0.089 M boric acid, 0.001M EDTA) gel. This autoradiograph is representative of those derived from at least three independent experiments. The three control lanes show the migration of unreacted, unrestricted 18-mer, the ability of the 18-mer to serve as a substrate for restriction enzyme digestion (*Hae*III), and the inability of *Pvu*II to digest the unreacted 18-mer. Bottom, a diagram of the 18-mer showing the salient restriction enzyme sites and the position of the O^6-methylguanine.

relative level of functional MGMT in the cell. It can be seen from Fig. 2 that the multidrug-resistant cell lines selected with verapamil, 8226/dox1V and 8226/mdr10V, are devoid of MGMT activity. In contrast, the parental cell lines, 8226/S and 8226/dox40, do have MGMT activity. In addition, no other drug-resistant variants of 8226/S tested have lost their MGMT activity. This includes cell lines selected for resistance to melphalan, melphalan plus doxorubicin (8226/LR5 and 8226/LR5Dox6, Fig. 2), and mitoxantrone (data not shown). Only cell lines chronically exposed to DOX plus verapamil are deficient in MGMT activity.

MGMT activity assays were done on 8226/S cells that received chronic or acute exposure to verapamil alone. Fig. 3 shows the results of this experiment. 8226/S cells were exposed to 10 μg/ml verapamil, the concentration used in the selection of 8226/dox1V and 8226/mdr10V, for 6 months. No difference in MGMT activity was detected (Fig. 3). Similarly, 24-h exposure of 8226/S cells to verapamil did not lessen their MGMT activity (Fig. 3).

Experiments were done to determine whether the observed ablation of MGMT activity was mediated at the level of gene expression. Total cellular RNA from the various cell lines (20 μg) was subjected to Northern blot analysis. Fig. 4 illustrates the results of this analysis. The parent cell lines, 8226/S and 8226/dox40, express the ~950-nucleotide MGMT mRNA, but the verapamil/DOX-selected variants do not express detectable levels of MGMT mRNA (Fig. 4). Fig. 4 also shows the same

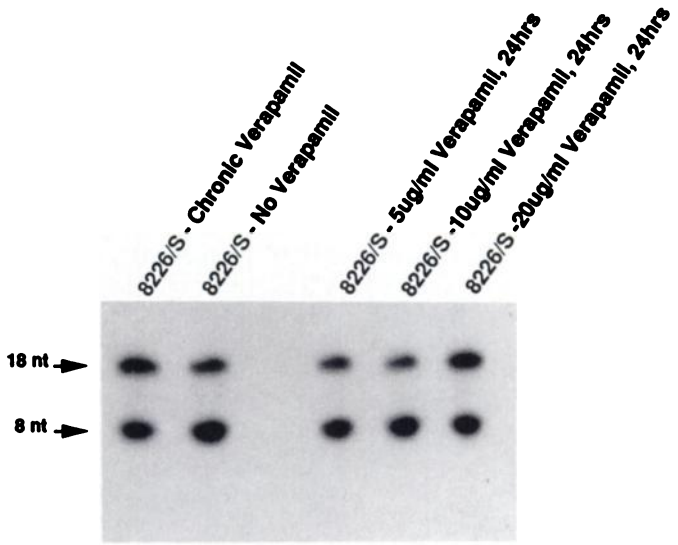

Fig. 3. MGMT activity assay on 8226/S cells that had received acute or chronic exposure to verapamil. Assay and analysis was performed as described for Fig. 2. nt, nucleotide.

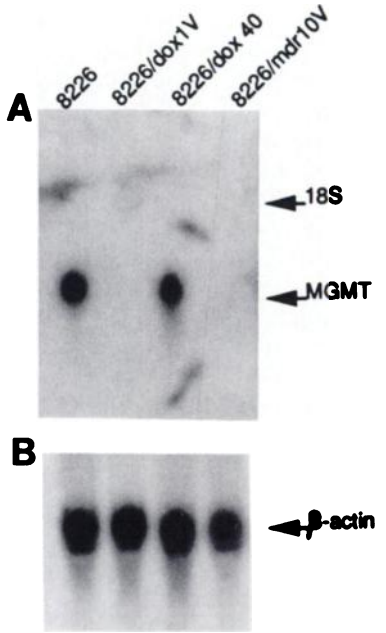

Fig. 4. *A*, Northern blot analysis of MGMT expression in 8226, 8226/dox1V, 8226/dox40, 8226/mdr10V. Total cellular RNA (20 μg) was fractionated on a 1.2% agarose gel, capillary transferred to a nylon filter, and hybridized with a human MGMT cDNA probe. The autoradiograph shown is representative of at least three independent experiments. *B*, the above blot stripped and rehybridized with a chicken β-actin cDNA probe.

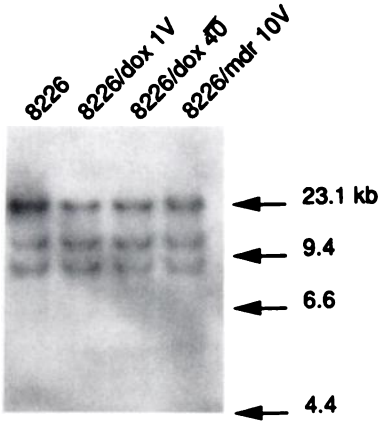

Fig. 5. Southern blot analysis of the MGMT gene in 8226, 8226/dox1V, 8226/dox40, and 8226/mdr10V cell lines. *Eco*RI-digested DNA (10 μg) was size fractionated on a 0.8% agarose gel, capillary transferred to a nylon filter, and hybridized with a full-length human MGMT cDNA probe. Molecular weight markers are derived from *Hind*III-digested λ. kb, kilobase.

blot reprobed with a probe specific for β-actin mRNA and served as a control for RNA loading and integrity.

Southern blot analysis was performed to determine the status of the MGMT gene. *Eco*RI-digested genomic DNA (10 μg) from the salient cell lines was probed with a full-length cDNA of the MGMT gene (Fig. 5). Results show that the gene is present, and the protein-coding region is not grossly rearranged. Thus, the loss of MGMT gene expression was not due to loss or gross rearrangement of the coding region of the MGMT gene.

DISCUSSION

In this report, we describe two mechanistically distinct multidrug-resistant cell lines that have become hypersensitive to the nitrosoureas, STZ and BCNU. These two cell lines, 8226/dox1V and 8226/mdr10V, were created from 8226/S and 8226/dox40, respectively, using verapamil and DOX as selecting agents. The two selection protocols were designed to mimic clinical protocols currently being used to either inhibit the emergence or suppress the function of P-glycoprotein-mediated multidrug resistance.

The hypersensitivity of these two cell lines to STZ is due to the loss of activity of the DNA repair molecule, MGMT. Collateral sensitivity to nitrosoureas has been observed previously in drug-resistant cells (24). Nitrogen mustard-resistant Walker 256 rat mammary carcinoma cells became collaterally sensitive to a chloroethylating agent, 5-[3-(2-chloroethyl)-1-triazenyl]-imidazo-4-carboxamide. This study demonstrated that the nitrosourea sensitivity was due to a loss of MGMT activity. Nucleic acid probes for MGMT were unavailable at this time and precluded a genetic analysis of these cells. In contrast to this rat cell line, the human myeloma cell line (8226/S) selected for resistance to a nitrogen mustard, melphalan, (8226/LR5, Ref. 25), retained MGMT activity.

The loss of MGMT activity in 8226/dox1V and 8226/mdr10V is due to loss of MGMT gene expression. The loss of gene expression is not due to the loss or gross rearrangement of the coding region of the MGMT gene. Although small deletions, point mutations, or frameshift mutations in the MGMT gene cannot be ruled out, DNA damage produced by DOX is usually associated with gross chromosomal damage, including inversions, deletions, and translocations (26). Additionally, we cannot rule out the possibility that genomic regions far removed from the protein-coding region of the MGMT gene may influence or regulate MGMT expression and that these regions have been disrupted. The cell lines, 8226/dox1V, 8226/mdr10V, and the parental cell lines, 8226/S and 8226/dox40, may serve as useful tools in elucidating mechanisms important in MGMT gene regulation.

Results presented here suggest that the inclusion of the nontoxic agent verapamil during DOX exposure results in the selection of a unique, stable phenotype that is devoid of MGMT activity. This is a viable possibility because (*a*) the loss of MGMT expression and activity has not been observed in any other 8226 drug-resistant variants. These drug-resistant variants include cell lines that have been selected with DOX alone, mitoxantrone, melphalan, or DOX plus melphalan; (*b*) the loss of MGMT activity also appears to be a stable event. 8226/mdr10V removed from selection pressure (DOX and verapamil) for at least 6 months has maintained the MGMT minus phenotype (data not shown); and (*c*) acute or chronic exposure to verapamil alone does not appear to have any effect on MGMT expression in 8226/S. This issue is not entirely resolved, however, because loss of MGMT expression may take a longer selection period than we have been able to test to date (*i.e.*, 6 months in nontoxic concentrations of verapamil). To test the generality of this finding, as well as to more clearly delineate the potential requirement of both DOX and verapamil in the selection of this phenotype, we are continuing to culture 8226/S cells in the presence of verapamil alone, and we are applying the selection pressure of verapamil alone and verapamil plus DOX to other human cell lines.

Taken together, the aforementioned results suggest that the selection pressure of DOX plus verapamil may be a unique selection pressure and results in decreased MGMT expression and collateral sensitivity to STZ. If this is the case *in vivo*, then patients being treated with chemotherapeutic regimens using these agents may become sensitive to chemotherapeutic nitrosoureas. This possibility is especially appealing because, rather than attempting to overcome an acquired drug resistance, this selection process ablates an innate resistance, a resistance mechanism which has severely limited the clinical usefulness of the nitrosoureas. Current studies are designed to determine whether this *in vitro* collateral sensitivity is also observed *in vivo*.

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