Mechanisms of Resistance to 1,3-Bis(2-chloroethyl)-1-nitrosourea in Human Medulloblastoma and Rhabdomyosarcoma[1]

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Abstract

Medulloblastoma (D-341 MED) and rhabdomyosarcoma (TE-671) cell lines, which are resistant to either 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or the combination of BCNU and O^6-benzylguanine (O^6-BG), were generated by serial escalation of BCNU. The activities of O^6-alkylguanine-DNA alkyltransferase (AGT), glutathione-*S*-transferase (GST), and total glutathione (GSH) of the parental, BCNU-resistant (BR), and BCNU + O^6-BG-resistant (OBR) cells were measured. No significant differences in GST activity or total GSH were seen between the parental, BR, and OBR cells of both TE-671 and D-341 MED. The AGT activities of D-341 MED (BR) and TE-671 (BR) were twice those of D-341 MED and TE-671, respectively, confirming the importance of this enzyme for BCNU resistance. The D-341 MED (OBR) cells did not exhibit any AGT activity, suggesting that another mechanism must play a role in the drug resistance. Fewer DNA interstrand cross-links (ICLs) were observed in D-341 MED (OBR) than in D-341 MED after 8 h BCNU (100–400 μM) treatment. However, the amounts of DNA ICLs observed in D-341 MED and D-341 MED (OBR) were stable after 24 h. Microarray analysis showed the increased expressions of several metallothionein genes and down-regulation of several proapoptotic genes. The AGT activity of TE-671 (OBR) was 223 fmol/mg when the cells were grown in 10 μM O^6-BG and decreased to about half this value when the O^6-BG concentration was increased 60 μM. The AGT cDNA of TE-671 (OBR) cells was cloned and found to contain a G-to-T transversion at codon 156, resulting in conversion of glycine to cysteine (G156C). *In vitro* mutagenesis has shown that the G156C AGT mutant is resistant to inactivation by O^6-BG. Thus, the selection of a mutant AGT with decreased sensitivity to O^6-BG is a significant contributing factor to BCNU + O^6-BG resistance.

Introduction

The alkylating drug BCNU,[3] which was first used in the 1960s, is one of the most commonly used drugs in treating intracranial tumors (1). The drug's active metabolite is the chloroethyl carbonium intermediate, which reacts with the O^6 position of guanine. This is followed by an intermolecular rearrangement forming an intramolecular N^1,O^6-ethanoguanine adduct. Ultimately, the exocyclic C—O bond is cleaved, and reaction with the N^3 position of cytosine in the complementary strand results in the N^3C-N^1G diadduct, which appears to be the cytotoxic molecular lesion (2–5). Unfortunately, the cytotoxicity of BCNU is frequently compromised by development of drug resistance leading to subsequent tumor growth. A major mechanism of resistance to alkylnitrosourea therapy is the DNA repair protein AGT (6). AGT removes the chloroethyl adduct from the O^6-position so that the DNA cross-link is not formed. Recent clinical trials have shown an inverse relationship between survival and AGT levels in patients with malignant glioma who receive BCNU therapy (7–10), thus providing a strong rationale for strategies designed to deplete tumor AGT levels before therapy with BCNU. Another possible mechanism of resistance is glutathione-mediated quenching of the alkylator (11–13). Once formed, the DNA ICL is not susceptible to either AGT removal or glutathione quenching (6, 12). Additional mechanisms of resistance must be operational, and enhanced repair of the DNA ICL must be considered (14). Indeed, several investigators have demonstrated repair of BCNU-induced DNA ICLs, although the biochemical pathway remains undefined (14–16).

O^6-BG is an AGT substrate rationally designed to produce suicide inactivation with restoration of sensitivity to chloroethylators or methylators (17–19). As noted above, the addition of O^6-BG to BCNU-containing regimens may not invariably restore or enhance BCNU sensitivity because other mechanisms of resistance may be operational in tumor cells (20).

We now report the generation of resistance to BCNU and BCNU plus O^6-BG in medulloblastoma (D-341 MED) and rhabdomyosarcoma (TE-671) cell lines and define the roles of AGT, GSH, and GST in these drug-resistant tumor cell

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[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; O^6-BG, O^6-benzylguanine; AGT, O^6-alkylguanine-DNA alkyltransferase; ICL, interstrand cross-link; GSH, glutathione; GST, glutathione-*S*-transferase; IGF, insulin-like growth factor.

lines. In addition, we are presenting a comparative analysis of DNA ICL formation in D-341 MED and D-341 MED (OBR) as well as a comparison of their gene expression profiles.

Materials and Methods

Drugs. BCNU was purchased from Sigma Chemical Co. (St. Louis, MO). O^6-BG was a generous gift from Dr. Robert Moschel of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD).

Generation of Drug-resistant Cells. To generate BCNU resistance, we began treating cells with ~10 μM BCNU for 1 h. The cells were maintained at this drug concentration until they showed markedly increased survival to drug treatment. After every three passages, the drug concentration was increased by 5–10 μM. OBR cell lines were generated the same way, except that: (*a*) the cells were exposed to 100 μM O^6-BG for 10 min before BCNU treatment; and (*b*) cells were maintained in medium with 10 μM O^6-BG.

Limiting Dilution Assay. The cytotoxicity of BCNU against D-341 MED, D-341 MED (BR), D-341 MED (OBR), TE-671, TE-671 (BR), and TE-671 (OBR) was determined using limiting dilution assay as described previously (21). OBR cells were initially exposed to 100 μM O^6-BG, treated with BCNU for 1 h and then plated in 10 μM O^6-BG.

AGT Assay. The activity of AGT was measured as described previously (18). The enzyme activity was defined as the fmol of O^6-[^{3}H]methylguanine removed from ^{3}H-methylated DNA per mg of extracted protein from sonicated cells after incubation at 37°C for 30 min. Briefly, the DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25 M and then hydrolyzed by incubating the precipitate with 500 ml of 0.1 M HCl at 70°C for 30 min. The modified bases were separated by reverse-phase high-performance liquid chromatography and quantified by scintillation counting. Protein concentration was determined using the method of Bradford (22) with BSA as the standard.

GST and GSH Assays. Total GSH was determined using the method of Tietze (23) and Griffith (24), with minor modifications described previously (25). The activity of GST was measured using the method of Habig (26).

Cloning and Sequencing the *AGT* Gene of TE-671 (OBR). The total cellular RNAs of the TE-671 and TE-671 (OBR) cells were prepared using Trizol reagent (Life Technologies, Inc.). The first-strand cDNA was synthesized from total RNA using Superscript RNase H$^-$ Reverse Transcriptase (Life Technologies, Inc.) and random hexamer primers (Life Technologies, Inc.). The AGT cDNAs were amplified using PCR Master Mix (Promega) along with the primers P1 (5′-GCGCGGATCCGGTACTTGGAAAAATGGACAAGGATTG-3′) and P2 (5′-GCGCGAATTCCAAACATCCATCCTACTGCACATAC-3′), which were designed based on the reported sequence of the human *AGT* gene (27). The PCR conditions were as follows: (*a*) initial denaturation at 95°C for 2 min; (*b*) 40 cycles of 95°C for 30 s, 62°C for 60 s, and 72°C for 60 s; and (*C*) final extension at 72°C for 7 min. The PCR products were extracted with chloroform, ethanol precipitated, redissolved in 15 μl of 10 mM Tris-Cl (pH 8.0), and 1 mM EDTA, and digested with *Eco*RI and *Bam*HI (New England Biolabs). The DNA products were electrophoresed on a 1.5% agarose gel (with 0.5 mg/ml ethidium bromide) and extracted from the gel using Qiaquick Gel Extraction kit (Qiagen). The extracted PCR fragment was ligated to *Eco*RI- and *Bam*HI-digested pTZ18u vector (Bio-Rad). After transformation of DH5α, plasmid DNAs were prepared from randomly picked white colonies using the Plasmid Mini kit (Qiagen). Sequencing of plasmids was carried out at the DNA Sequencing Facility of Duke University Medical Center (Durham, NC).

Determination of Gene-specific DNA ICLs. The medium of the exponentially growing D-341 MED or D-341 MED (OBR) cells was replaced with a fresh medium lacking FCS but containing 10 μM O^6-BG. After 1 h, BCNU was added to the medium to a final concentration of 0, 100, 200, 300, and 400 μM. Cells were harvested after 8 and 24 h of incubation with the drug. The cell pellets were washed with 1× PBS. Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen). Each DNA sample was digested with *Eco*RI for 12 h at 37°C. Ten μg of digested DNA was electrophoresed on 0.7%, 1× TAE agarose gel at 30 V for 16 h. Before electrophoresis, DNAs were denatured in 80% formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, and 1 mg/ml xylene xyanol by heating at 65°C for 5 min and then cooling on ice. In addition, a 10-μg DNA sample from untreated cells was loaded without denaturation. After the electrophoresis, the DNA bands were transferred to GeneScreen Plus membrane as described previously (28). The DNA bands were quantified by PhosphorImager, and the percentage of ICLs was calculated by dividing the amount of radioactivity in the double-stranded DNA band by the total radioactivity detected in double- and single-stranded DNA bands.

Gene Expression Analysis of D-341 MED and D-341 MED (OBR). Total RNA from D-341 MED and D-341 MED (OBR) cells was extracted using Trizol Reagent (Life Technologies, Inc.) and purified with RNeasy columns (Qiagen). Double-stranded cDNA was synthesized from 10 μg of total RNA using cDNA synthesis kit (Life Technologies, Inc.) and T7 $(dT)_{24}$ primer (GenSet). Using the BioArray High Yield RNA Transcription kit (with T7 RNA polymerase) from Enzo Diagnostics (New York, NY), the cDNA was transcribed *in vitro* to produce biotin-labeled cRNA. The cRNA was then hybridized to GeneChip Human Genome U95Av2 Array according to the Affymetrix protocol. Microarray data were analyzed using Microarray Suite 5.0 (Affymetrix Co.) A comparison analysis was made designating D-341 MED as baseline and D-341 MED (OBR) as experiment. This comparative analysis determined: (*a*) the change in *P*; (*b*) the associated change call; and (*c*) the signal log ratio.[4]

Results

Limiting Dilution Assays. BCNU was less cytotoxic to D-341 MED (BR) than the parental cell line D-341 MED (Fig. 1*a*). The survival of both cell lines was diminished considerably after exposure to O^6-BG prior to BCNU. However, O^6-BG improved the cytotoxicity of BCNU to D-341 MED

[4] A detailed explanation of the Microarray Protocol and change algorithm can be found at www.affymetrix.com.

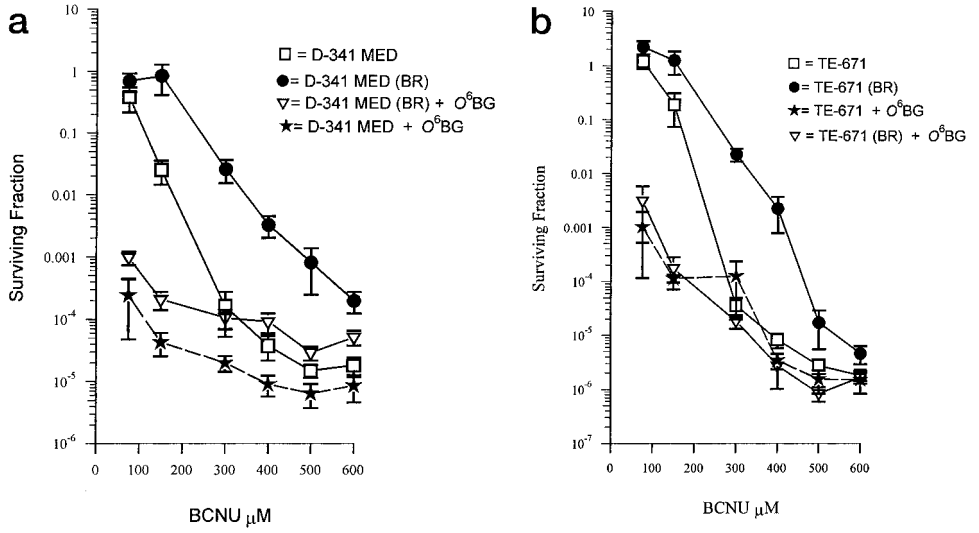

Fig. 1. Survival of D-341 MED and D-341 MED (BR) (*a*) and TE-671 and TE-671 (BR) (*b*) after exposure to various concentrations of BCNU ± O^6-BG. Each data point represents the average of at least three independent experiments; *bars,* SD.

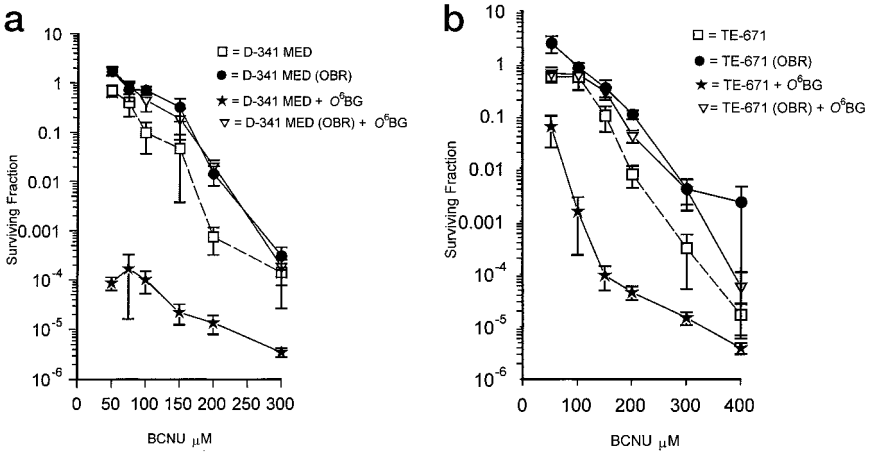

Fig. 2. Cytotoxicity of BCNU against D-341 MED (OBR) (*a*) and TE-671 (OBR) (*b*) in comparison to their corresponding parental cell lines with and without O^6-BG. Each data point represents the average of at least three independent experiments; *bars,* SD.

only at BCNU concentrations <300 μM. Similarly, TE-671 (BR) exhibited higher survival than TE-671 when treated with BCNU (Fig. 1*b*). With the addition of O^6-BG, the survival fraction of TE-671 (BR) decreased dramatically so that it was indistinguishable from TE-671.

The survival of D-341 MED after treatment with BCNU was somewhat less than D-341 MED (OBR) treated with BCNU plus O^6-BG (Fig. 2*a*). However, treatment of D-341 MED with BCNU plus O^6-BG produced substantially greater cytotoxicity. The survival of TE-671 after treatment with BCNU was less than TE-671 (OBR) treated with BCNU plus O^6-BG (Fig. 2*b*). Again, treatment of TE-671 with BCNU plus O^6-BG produced markedly increased cytotoxicity.

Total GSH and GST Activities. The total GSH for TE-671, TE-671 (BR), and TE-671 (OBR) were 35.4 ± 8.5, 40.8 ± 12.8, and 33.4 ± 9.4 nmol/mg protein extracts, respectively. The total GSH for D-341 MED, D-341 MED (BR), and D-341 (OBR) were 12.9 ± 4.0, 16.3 ± 8.0, and 13.5 ± 6.6 nmol/mg protein, respectively.

The total GST activities of TE-671, TE-671 (BR), and TE-671 (OBR) were determined to be 36.6 ± 5.5, 32.7 ± 9.8, and 28.0 ± 6.6 mmol/min/mg protein, respectively. D-341 MED, D-341 MED (BR), and D-341 MED (OBR) demonstrated specific GST activities of 61.0 ± 13.5, 88.4 ± 18.9, and 54.4 ± 13.1 nmol/min/mg protein, respectively.

AGT Activities. The parent cell line D-341 MED exhibited an AGT activity of 2232 ± 329 fmol/mg protein (Fig. 3*a*). The AGT activity of D-341 MED (BR) was significantly higher (4239 ± 170 fmol/mg), whereas that of D-341 MED (OBR) was essentially zero. The AGT activities of D-341 MED and D-341 MED (BR), grown in medium containing 10 μM O^6-BG, both declined to essentially zero.

TE-671 cells displayed an AGT activity of 3967 ± 341 fmol/mg protein compared with 6247 ± 348 for TE-671 (BR) (Fig. 3*b*). The AGT activities of both these cell lines were totally depleted when exposed to 10 μM BG. Unlike D-341 MED (OBR), TE-671 (OBR) cells clearly demonstrated residual AGT levels of 223 ± 36 fmol/mg. In a separate experiment, TE-671 (OBR) cells were treated with increasing O^6-BG concentrations (20, 40, and 60 μM) before AGT levels were quantitated. Even at elevated O^6-BG concentrations, TE-671 (OBR) cells continued to have active AGT, displaying

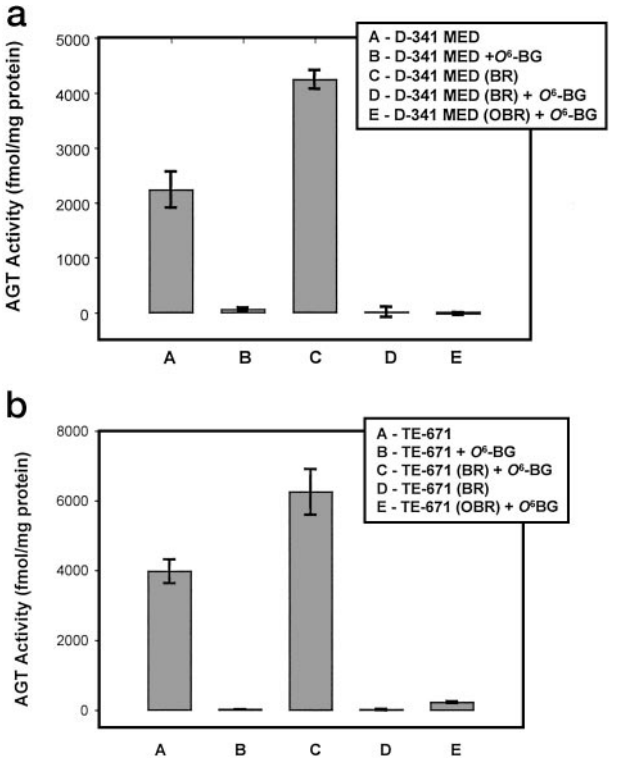

Fig. 3. AGT activities of D-341 MED, D-341 MED (BR), and D-341 MED (OBR) (*a*) and TE-671, TE-671 (BR), and TE-671 (OBR) (*b*) with and without exposure to 10 μM O^6-BG.

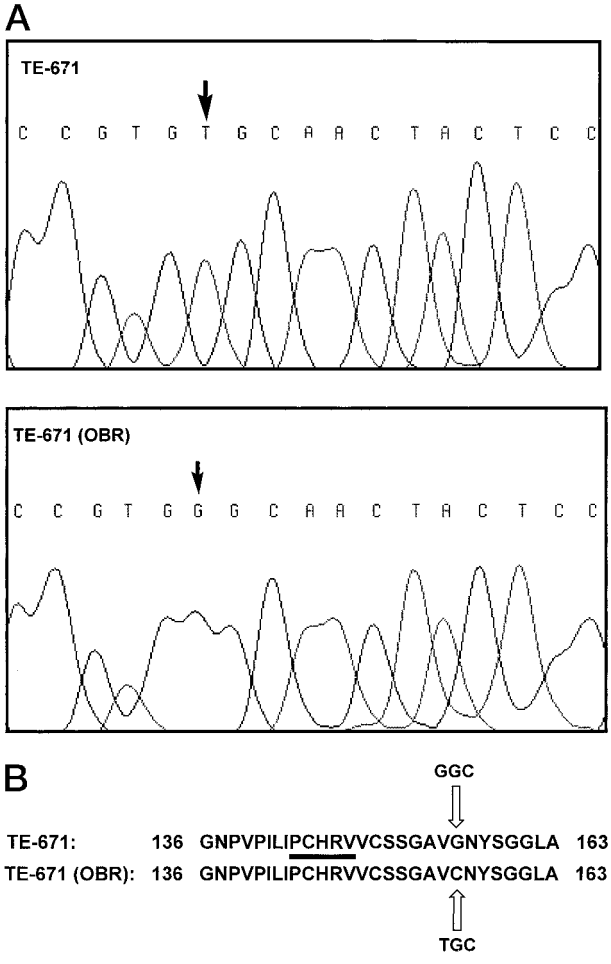

Fig. 4. *A,* comparison of TE-671 and TE-671 (OBR) AGT cDNAs. The TE-671 *AGT* gene has a G-to-T transversion relative to the parent cell line. *B,* a portion of the TE-671 (OBR) and TE-671 AGT primary structures. Mutation at codon 156 led to conversion of glycine to cysteine for TE-671 (OBR) AGT. The *underlined* stretch of amino acids is the known active site consensus sequence of the protein.

enzyme levels of 227 $\pm$ 13, 219 $\pm$ 35, and 109 $\pm$ 13 fmol/mg of 20, 40, and 60 μM O^6-BG, respectively. The observation that even 60 μM O^6-BG was not able to eliminate the AGT activity of TE-671 (OBR) indicated the possibility of a mutant AGT protein in this cell line.

Point Mutation in TE-671 (OBR) *AGT* Gene. We designed PCR primers for the amplification of the entire coding sequence for AGT. Sequencing of the TE-671 (OBR) *AGT* gene revealed a point mutation at codon 156. The triplet GGC (which codes for glycine in the wild-type gene) has become TGC (which codes for cysteine; Fig. 4). No other mutation was found in the entire 624 bases of the coding sequence. The *AGT* gene of TE-671 was also cloned and sequenced. No mutation was detected.

DNA ICL Formation. Because the BCNU resistance of D-341 MED (OBR) was not the result of quenching by elevated GSH or GST or the result of a mutant AGT, we investigated the formation of DNA ICLs in this cell line. Specifically, we measured DNA ICL formation within the c-*myc* gene by a DNA denaturation/renaturation assay in which cross-linked DNA is observed as double-stranded DNA after the DNA is denatured and separated by electrophoresis (29, 30). We treated D-341 MED and D-341 MED (OBR) at four BCNU concentrations (100–400 μM) for 8 h. At each BCNU concentration, the percentage of ICLs for D-341 MED was higher than that of D-341 MED (OBR) (Fig. 5). When the BCNU treatment was extended to 24 h, the amount of DNA ICL was almost unchanged from what was observed at 8 h for both the parent and drug-resistant cell lines (Fig. 5).

Gene Expression Analysis of D-341 MED and D-341 MED (OBR). The parent and resistant cell lines were compared for the expression of ~12,000 genes using microarray analysis. The expression ratio for a certain gene was defined as its signal for D-341 MED (OBR) divided by its signal for D-341 MED. Only genes with expression ratios of at least 3 (the up-regulated genes) and $\leq$0.3 (the down-regulated genes) are shown (Table 1). Each gene is grouped into the following categories: apoptosis-related, cell adhesion and extracellular matrix, cytoskeleton, DNA synthesis and repair, membrane structure and repair, metabolism, molecular transport, neuronal development, protein degradation, protein synthesis and processing, RNA processing, signal transduction, stress response, transcription, cancer-associated genes of unknown functions, and genes of unknown functions.

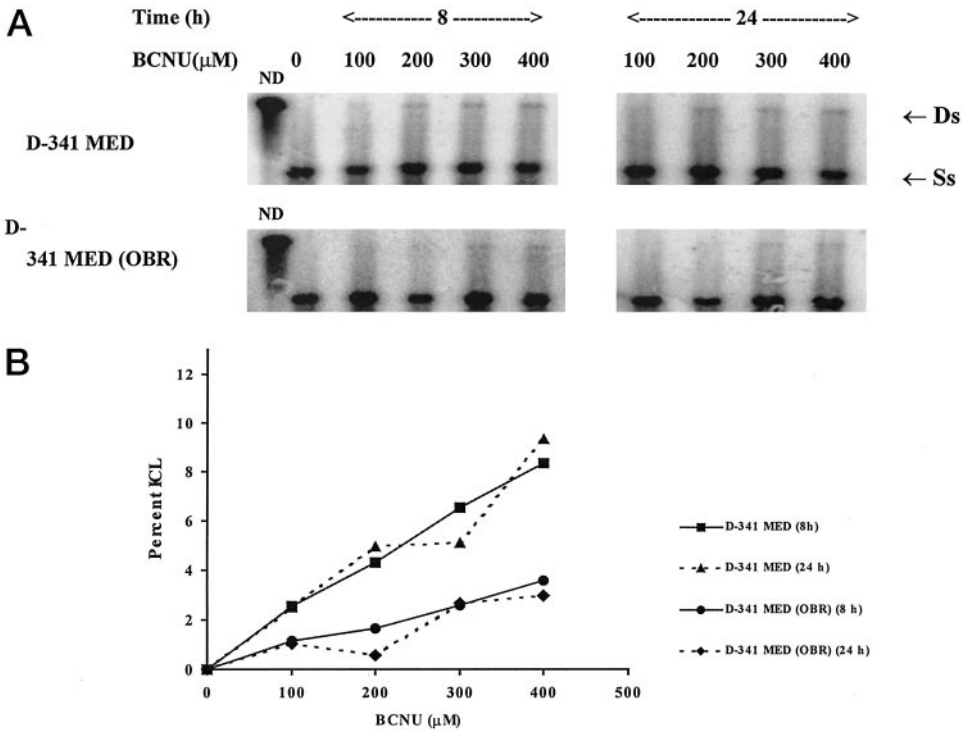

Fig. 5. *A,* denaturing/renaturing Southern blot analysis (using c-*myc* probe) of *Eco*RI-digested genomic DNA of D-341 MED and D-341 MED (OBR). The cells were treated at 0, 100, 200, 300, and 400 μM BCNU for 8 and 24 h. ND is nondenatured DNA. *Ds,* position of double-stranded DNA; *Ss,* position of single-stranded DNA. *B,* quantitative summary of the results shown in *A*.

Discussion

The effectiveness of alkylating agents in treating cancer cells is limited by a number of mechanisms that impart cellular resistance to the drugs. For example, a well-described mechanism of resistance to chloroethylnitrosoureas appears to be AGT-mediated monoadduct removal (6). Glutathione quenching of monoadduct formation also plays a role in reducing BCNU cytotoxicity (11–13). Chloroethylnitrosoureas also can be detoxified by either cytochrome P-450- or GST-mediated pathways (31, 32). Recent evidence suggests that p53 (33), the cell cycle regulator WAF1/Cip1 (34), the transforming growth factor β1 (35), and poly(ADP-ribose) polymerase (36) may play additional roles in generating BCNU resistance. However, the dealkylation at the O^6 position of guanine by AGT is now well recognized as the major mechanism of resistance to chloronitrosoureas as well as methylators (37–40). A number of laboratory and clinical studies have firmly established the correlation between BCNU resistance and elevated AGT activities in brain tumors (9, 41, 42).

O^6-BG was rationally developed to deplete AGT levels by binding to the enzyme with subsequent transfer of its benzyl group to the active site of the enzyme (19). Although the combination of BCNU and O^6-BG has been shown to be very promising *in vitro* as well as in xenograft studies (43–47), there is always a possibility that cellular resistance to this drug combination may modulate the effects of the drugs. The establishment of medulloblastoma and rhabdomyosarcoma sublines that are resistant to BCNU plus O^6-BG provides evidence that this possibility may occur in the clinic. Acquired resistance of breast cancer cells to BCNU and O^6-BG has also been reported (48).

Dramatic elevation in AGT activity was the primary reason for enhanced survival of D-341 MED (BR) and TE-671 (BR), after treatment with BCNU. The activity of AGT was still a significant factor in the survival of TE-671 (OBR) after treatment with BCNU plus O^6-BG. The glycine-to-cysteine AGT mutation at codon 156, which resulted in decreased effects of O^6-BG, was one of the mutant proteins shown previously to exhibit resistance to O^6-BG in the *in vitro* mutagenesis study by Xu-Welliver and Pegg (49). In their study, the region of residues between 150 and 173 was randomly mutated, and the resulting mutants with AGT activity and O^6-BG resistance were examined. G156C had an ED_{50} (for O^6-BG) of 50 μM compared with the 0.1 μM of the parent. Xu-Welliver and Pegg (49) found that glycine 156 and lysine 165 are two positions at which changes caused the largest increases in O^6-BG resistance. The mutant G156A, identified earlier for its O^6-BG resistance (50), has been studied extensively as a possible hematopoietic protectant during BCNU + O^6-BG chemotherapy (51–54). In the crystal structure model for AGT, the wing residues (amino acids 151 to 158) are proposed to play critical roles in stabilizing the position of O^6-BG in the AGT binding pocket (55). The conformational change brought about by conversion of glycine to cysteine may have been enough to disrupt the binding of O^6-BG by AGT.

The G156C mutant was caused by a GGC-to-TGC codon change. G-to-T transversion is the predominant mutation induced by BCNU in Chinese hamster ovary cells (56). Chinese hamster ovary cells transfected with AGT cDNA showed a decrease in BCNU-induced mutation (57). This inactivation of AGT by O^6-BG would contribute to increased mutational events by BCNU during the development of drug-resistant cell lines.

Table 1 Genes with marked expression change in D-341 MED (OBR) relative to D-341 MED

Shown here are genes with signal ratios (expression in D-341 MED (OBR): expression in D-341 MED) of at least 3.0 (up-regulated) and ≤0.3 (down-regulated).

Descriptions	Gene	GenBank or UniGene accession no.	Signal ratio [D-341 MED (OBR): D-341 MED]
Apoptosis-related			
Midkine (neurite growth-promoting factor 2)	*NEGF2*	X55110	13.93
Insulin-like growth factor 1 (somatomedin C)	*IGF1*	X57025	8.57
Programmed cell death 6	*PDCD6*	AF035606	0.31
Chemokine (C-X-C motif), receptor 4 (fusin)	*CXCR4*	L06797	0.14
Mitogen-activated protein kinase kinase 6	*MAP2K6*	U39064	0.13
Paternally expressed 3	*PEG3*	AB006625	0.09
Inhibin, β C	*INHBC*	Hs.199538	0.06
Cell-adhesion and extracellular matrix			
Epsillon sarcoglycan	*SGCE*	AJ000534	4.00
OB-cadherin (cadherin 11)	*CDH11*	D21255	4.00
Tetraspan 3	*TSPAN3*	M69023	0.33
Talin 2	*TLN2*	AB002318	0.33
Chondroitin sulfate proteoglycan 2 (versican)	*CSPG2*	D32039	0.19
Collagen, type IV, α 1	*COL4A1*	M26576	0.09
Chromatin structure			
H3 histone, family 3B	*H3F3B*	Hs.180877	0.18
Cytoskeleton			
Tropomyosin 1 (α)	*TPM1*	Z24727	3.03
Internexin neuronal intermediate filament protein, α	*INA*	S78296	0.31
γ-Tubulin ring complex protein (*76p* gene)	*76P*	W28255	0.13
Chaperonin containing TCP1, subunit 5 (epsilon)	*CCT5*	D43950	0.09
Tubulin, β-2	*TUBB2*	Hs.184582	0.05
DNA synthesis and repair			
Ligase IV, DNA, ATP-dependent	*LIG4*	X83441	0.14
Purine-rich element binding protein A	*PURA*	M96684	0.13
α-thalassemia/mental retardation syndrome X-linked RAD54 (*Saccharomyces cerevisiae*) homolog	*ATRX*	U72936	0.13
Membrane structure and repair			
Fer-1-like 3, myoferlin	*FER1L3*	AL096713	5.66
ADP-ribosylation factor 3	*ARF3*	M74491	0.33
Metabolism			
Arylsulfatase F	*ARSF*	X97868	6.06
Carbonic anhydrase 2	*CA2*	J03037	3.03
Fatty acid desaturase	*FADS1*	AF0097677	0.27
Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	*CAD*	Hs.154868	0.14
Citrate synthase	*CS*	AF047042	0.13
5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	*MTRR*	AF025794	0.07
Molecular transport			
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	*SLC25A6*	J03592	0.33
ATPase, Ca^{+2} transporting, plasma membrane 2	*ATP2B2*	L20977	0.23
Neuronal development			
Semaphorin 3A	*SEMA3A*	L26081	4.59
Protein degradation			
F box only protein 21	*FBXO21*	AB020682	6.50
Noncanonical ubiquitin conjugating enzyme 1	*NCUBE1*	AI557497	0.33
Protein synthesis and processing			
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	*KDELR2*	M88458	0.33
Ribosomal protein S20	*RPS20*	LO6498	0.16
Glutamyl-prolyl-tRNA synthetase	*EPRS*	X54326	0.10
Tyrosyl-tRNA synthetase	*YARS*	U89436	0.08
RNA processing			
RNA helicase-related protein	*RNAHP*	H68340	3.73
PRP4/STK/WD splicing factor	*HPRP4P*	AF016369	0.31
KH-type splicing regulatory protein (FUSE binding protein 2)	*KHSRP*	AA628946	0.12
Splicing factor, arginine/serine-rich 2	*SFRS2*	X75755	0.11
Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	*SFRS1*	M72709	0.10
Heterogeneous nuclear ribonucleoprotein H1 (H)	*HNRPH1*	W28483	0.09
Splicing factor proline/glutamine rich (polypyrimidine tract-binding protein-associated)	*SFPO*	W27611	0.07

Table 1 Continued

Shown here are genes with signal ratios (expression in D-341 MED (OBR): expression in D-341 MED) of at least 3.0 (up-regulated) and ≤0.3 (down-regulated).

Descriptions	Gene	GenBank or UniGene accession no.	Signal ratio [D-341 MED (OBR): D-341 MED]
Signal transduction			
Serine protease 11 (IGF binding)	*PRSS11*	D87258	9.85
Cysteine-rich, angiogenic inducer, 61	*CYR61*	Y11307	6.06
Serine protease 12 (neurotrypsin, motopsin)	*PRSS12*	AI810767	4.92
Teratocarcinoma-derived growth factor 1	*TDGF1*	M96956	3.73
Duffy antigen receptor for chemokines	*DARC*	X85785	3.73
Ectodermal-neural cortex (with BTB-like domain)	*ENC1*	AF059611	3.73
Protein phosphatase 2 regulatory subunit A, β isoform	*PPP2R1B*	M65254	3.03
O-Linked *N*-acetylglucosamine (GlcNAc) transferase (UDP-*N*-acetylglucosamine:polypeptide-*N*-acetylglucosaminyl transferase)	*OGT*	U77413	0.33
Pituitary tumor-transforming 1 interacting protein	*PTTG1IP*	Z50022	0.33
Interleukin-1 receptor-associated kinase 1	*IRAK1*	U52112	0.31
Serum constituent protein	*MSE55*	M88338	0.25
Acid sphingomyelinase-like phosphodiesterase	*ASM3A*	Y08136	0.25
FK506 binding protein 1A (12kD)	*FKBP1A*	V00599	0.18
Cyclin G1	*CCNG1*	X77794	0.13
Frizzled (*Drosophila*) homologue 7	*FZD7*	AB017365	0.08
Stress response			
Heat shock M_r 70,000 protein 6	*HSPA6*	X51757	12.13
Metallothionein 1L	*MT1L*	AA224832	7.46
Metallothionein 2A	*MT2A*	AI547258	6.06
Metallothionein 1A	*MT1A*	K01383	3.48
Transcription			
Inhibitor of DNA binding 2	*ID2*	D13891	4.59
Short stature homeobox 2	*SHOX2*	AF022654	3.25
LIM domain only 2 (rhombotin-like 1)	*LM02*	X61118	3.03
Suppressor of Ty (*Saccharomyces cerevisiae*) 6 homologue	*SUPT6H*	U46691	0.27
Zinc finger protein 161	*ZNF161*	D28118	0.27
Nescient helix loop helix 2	*NHLH2*	M96740	0.22
Zinc finger protein 124 (HZF-16)	*ZNF124*	S54641	0.20
Transcription elongation factor A (SII), 1	*TCEA1*	M81601	0.13
Tripartite motif-containing 28	*TRIM28*	X97548	0.09
T-box, brain, 1	*TBR1*	U49250	0.07
Cancer-associated genes of unknown functions			
P311 protein	*P311*	U30521	0.33
Genes of unknown functions			
Paternally expressed 10	*PEG10*	Hs.137476	32.00
KIAA0471 gene product	*KIAA0471*	AL049701	5.28
Hypothetical protein DKFZp564D0462	*DKFZP564D0462*	AL033377	3.48
Hypothetical protein FLJ10803	*FLJ10803*	AI765280	0.31
Homo sapiens clone 23664 and 23905 mRNA sequence	*N/A*	AF035315	0.29
Hypothetical protein FLB6421	*FLB6421*	AI133727	0.29
EGF-like-domain, multiple 5	*EGFL5*	AB011542	0.29
KIAA1111 protein	*KIAA1111*	AB029034	0.27
KIAA0794 protein	*KIAA0794*	AB018337	0.27
Human DNA sequence from cDNA 16pHQG	*N/A*	Z84717	0.22
HCGVIII-1 protein	*HCGVIII-1*	X92110	0.22
Leucine-rich PPR-motif containing	*LRPPRC*	M92439	0.09
SET domain, bifurcated 1	*SETDB1*	D31891	0.08

Alternatively, although treatment with O^6-BG eliminated the AGT content of D-341 MED (OBR), the cells still exhibited marked resistance to BCNU. Our results strongly support the presence of another mechanism of resistance that involves neither AGT, GSH, nor GST. We have investigated whether the drug-resistant cells have an increased ICL repair activity. If these cells have an increased repair capacity, it is most likely directed at the monoadducts because we observed no decrease in the DNA ICLs over time.

The results from the microarray experiment suggest several explanations for BCNU-resistance exhibited by D-341 MED (OBR). Among these possible resistance factors are the metallothionein isoforms 1-L, 2A, and 1-A (metallothioneins 1E and 1F increased by 2.6- and 2.5-fold, respectively, and were not included in the table). Although best associated with resistance of many cancer cells (including brain tumors) against platinum compounds (58, 59), these sulfur-rich peptides can also protect the

cells by covalent sequestration of electrophilic antineoplastic drugs (60–62).

The mitigation of apoptosis may also be a significant factor for BCNU resistance by D-341 MED (OBR). NEGF2 (which registered a 15-fold increase) has been shown to be antiapoptotic in Wilms' tumor cells (63) and cultured neurons (64) by inhibiting the activation of procaspase 3. IGF1 (increased 8.6-fold) is a well-known signaling protein for cell proliferation, and its elevation has been associated with several cancers (65). Interestingly, PRS11 (increased 10-fold) and CYR61 (increased 6-fold) are proteins with known IGF binding sites (66, 67). A stress-related protein, HSP70 protein 6 (HSPA6 or HSP70B′) also has increased expression in D-341 MED (OBR). Several studies have linked heat shock proteins to cancer drug resistance (68–70). Recently, heat shock proteins have been implicated in inhibition of procaspase 3 (71). In parallel with increased expression of antiapoptotic genes, several proapoptotic genes appeared down-regulated. PEG3 (decreased 11-fold) has been shown to induce p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria (72). PDCD6 (or ALG-2; which decreased 3.2-fold, is an essential component of Fas-mediated apoptosis (73). It is well known that the binding of HIV glycoprotein gp120 to the chemokine receptor CXCR4 (decreased 7-fold) leads to neuronal apoptosis (74). MAP2K6 (decreased 7.7-fold) was found necessary for c-Abl-induced apoptosis (75) but may also inhibit apoptosis in a p38MAPK (mitogen-activated protein kinase) manner (76). INHBC (which is the β chain of inhibin/activin, a member of the transforming growth factor β superfamily) decreased 17-fold. The proapoptotic action of inhibin/activin is well documented (77). Cells treated with inhibin have reduced *Bcl-2* (an apoptosis inhibitor) expression, which could also be a consequence of *p53* up-regulation. The effectiveness of many chemotherapeutic drugs has been correlated with their ability to induce apoptosis (78, 79). For example, glioblastoma cells overexpressing *Bcl-2* were less sensitive to BCNU (80). In another study, glioma cells with induced BCNU resistance were found to be resistant to apoptosis, and this correlated to altered nuclear localization of Bax protein (proapoptotic; Ref. 81).

Another interesting up-regulation is that of the gene for adhesion protein (osteoblast) OB-cadherin. A similar protein, E-cadherin, is believed to contribute to cancer drug resistance through contact-dependent growth inhibition (82). Semaphorin 3A (SEMA3A) is a member of a family of secreted or transmembrane neuronal proteins with a conserved 500-amino acid domain. A member of this family, SEMA3C (human semaphorin E), has been identified as a non-multidrug resistance factor in cisplatin-resistant ovarian cells (83).

The cellular resistance to BCNU and O^6-BG is a significant concern because trials of this regimen are now in the clinic (84, 85). We have now shown that brain tumor cells can be resistant to BCNU plus O^6-BG without the involvement of GST, GSH, AGT, or DNA ICL repair. However, the drug treatment regimen of BCNU plus O^6-BG has been shown to cause a mutant AGT unresponsive to O^6-BG inactivation in TE-671 (OBR) cells. Gene expression analysis suggests the roles of metallothioneins and apoptotic down-regulation in these cells. Further work will be required to sort through the list of genes with altered expression in D-341 MED (OBR) for their role in BCNU resistance.

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