In vitro Drug Response and Molecular Markers Associated with Drug Resistance in Malignant Gliomas

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Abstract

Purpose: Drug resistance in malignant gliomas contributes to poor clinical outcomes. We determined the *in vitro* drug response profiles for 478 biopsy specimens from patients with the following malignant glial histologies: astrocytoma (n = 71), anaplastic astrocytoma (n = 39), glioblastoma multiforme (n = 259), oligodendroglioma (n = 40), and glioma (n = 69).

Experimental Design: Samples were tested for drug resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), cisplatin, dacarbazine, paclitaxel, vincristine, and irinotecan. Biomarkers associated with drug resistance were detected by immunohistochemistry, including multidrug resistance gene-1, glutathione S-transferase π (GSTP1), O^6-methylguanine-DNA methyltransferase (MGMT), and mutant p53.

Results: *In vitro* drug resistance in malignant gliomas was independent of prior therapy. High-grade glioblastomas showed a lower level of extreme drug resistance than low-grade astrocytomas to cisplatin (11% versus 27%), temozolomide (14% versus 27%), irinotecan (33% versus 53%), and BCNU (29% versus 38%). A substantial percentage of brain tumors overexpressed biomarkers associated with drug resistance, including MGMT (67%), GSTP1 (49%), and mutant p53 (41%). MGMT and GSTP1 overexpression was independently associated with *in vitro* resistance to BCNU, whereas coexpression of these two markers was associated with the greatest degree of BCNU resistance.

Conclusions: Assessment of *in vitro* drug response and profiles of relevant tumor-associated biomarkers may assist the clinician in stratifying patient treatment regimens.

Gliomas account for 40% of new primary brain cancers diagnosed each year in the United States, with 5-year survival ranging from 22% for anaplastic astrocytomas to only 2% for glioblastomas (1, 2). Combined modality therapy, including image-based resections, radiotherapy, and chemotherapy, has produced only modest improvements in outcomes. Adjuvant chemotherapy with nitrosoureas has been the mainstay of treatment for the past three decades (3, 4). The recent introduction of temozolomide, CPT-11, and 13-*cis*-retinoic acid has broadened the armamentarium of agents with activity against gliomas without dramatically improving survival (5–7). Intrinsic and acquired drug resistance mechanisms are thought to be responsible for the poor response of glial tumors to chemotherapy (8).

In vitro drug response assays were developed to identify individual patterns of drug resistance to facilitate the selection of chemotherapy based on each patient's unique tumor biology. Various investigators have shown that *in vitro* tumor response to anticancer agents is significantly associated with clinical outcomes (9–13). Two recent clinical studies have shown a relationship between *in vitro* tumor response and survival in patients with breast and ovarian cancers (14, 15). More recently, Parker et al. carried out a prospective blinded study of the predictive value of extreme drug resistance (EDR) assays in patients receiving CPT-11 for recurrent glioma (16). This study showed that patient survival was significantly associated with *in vitro* drug resistance to CPT-11. Patients treated with agents showing EDR *in vitro* have consistently been found to be unresponsive to those agents, with shorter survival times. Most recently, Loizzi et al. have reported that patients with recurrent ovarian carcinoma who received therapy selected according to EDR assay results versus physician's choice had significantly longer survival (17). These findings support the notion that the EDR assay has been clinically validated and that it may provide a useful surrogate for clinical outcomes, making it possible to draw meaningful conclusions from studies that correlate specific mechanisms of resistance with *in vitro* drug response. We previously showed a relationship between increased multidrug resistance gene-1 (MDR1) expression in

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Received 8/23/05; revised 5/1/06; accepted 5/25/06.

Grant support: Oncotech, Inc., a commercial entity that markets the extreme drug resistance assay.

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Note: R. Parker, W. Huang, and J.P. Fruehauf were employees of Oncotech at the time this work was done. H. Brem, S. Brem, A. Sloan, and G. Barger have no financial interest in Oncotech.

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doi:10.1158/1078-0432.CCR-05-1830

freshly resected samples of ovarian and breast cancer and *in vitro* resistance to paclitaxel (18, 19). In this study, we evaluated the relationship between *in vitro* drug response of primary brain tumors and their expression of selected biomarkers putatively associated with drug resistance.

The cellular basis of drug resistance in malignant gliomas and other tumors seems to be associated with various mechanisms, including those that prevent drugs from reaching their target in active form, enhanced DNA repair, or disruption of the apoptotic response to DNA damage (20). A first line of cancer cell defense against chemotherapy stems from membrane-based efflux pumps, such as MDR1, which can reduce the intracellular levels of natural product-based agents (21–23). A second line of defense rests in the cytosol, where drug conjugation reactions, such as those mediated by the glutathione *S*-transferase family of enzymes, can inactivate drugs (24–29). O^6-methylguanine-DNA methyltransferase (MGMT) provides an important example of nuclear-based resistance mechanisms resulting from enhanced DNA repair capacity. MGMT carries out suicide repair of guanidine methyl residues formed after exposure to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or temozolomide (30–34). Another form of drug resistance is related to the regulation of apoptosis. Drug-induced DNA damage can activate the tumor suppressor protein p53, which can both induce apoptosis in the presence of irreparable damage and regulate several enzymes involved in DNA repair (35–38). These biomarkers can be semiquantitatively assayed by immunohistochemistry. Previous reports on biomarkers in gliomas have been relatively small, based on either cell lines derived from primary tumors or from limited series of cases. We therefore set out to determine if biomarker profiling of 478 primary brain tumors would reveal an association with *in vitro* drug resistance.

Materials and Methods

EDR assay. *In vitro* drug response was assessed by the Oncotech EDR assay. Surgical biopsies were obtained during debulking surgery and sent by overnight courier to Oncotech for analysis on a fee for service basis. Once received at Oncotech, specimens were accessioned and assigned a tracking number. Tumor histology and prior treatment history were reported by the referring institution. Pathology reports were obtained to provide the tissue diagnosis. Pathology review of all submitted specimens was done at Oncotech to confirm the malignant cell content. All tumor specimens had representative segments removed for formalin fixation and paraffin embedding. A board-certified pathologist (W.H.) reviewed stained sections to confirm the referring diagnosis by light microscopy.

Tissue culture was done as described previously in detail (10, 13). Tumor specimens were disaggregated mechanically and enzymatically into suspensions of small tumor clumps. To determine cell counts, small aliquots of the cell suspension were collected and mechanically disaggregated into a near single-cell suspension by repeated gentle pipetting followed by exposure to trypan blue and hemacytometer cell counting. Viable malignant cells were suspended in soft agar at ~30,000 per well in 24-well plates. Agar provides an anchorage-independent environment that selectively promotes transformed cell growth, whereas nontransformed cells undergo anoikis-related cell death or cell cycle arrest, preventing them from taking up radiolabeled thymidine (39–42). Huang et al. recently confirmed that the agar-based EDR assay promotes tumor cell proliferation while suppressing proliferation of normal tissue components in various types of solid tumors and therefore is an effective *in vitro* technique to analyze tumor-drug interactions (43). Single-agent chemotherapeutic drugs were added in duplicate at a concentration that approximated the *in vivo* peak plasma concentrations: BCNU (37 μmol/L), SN38 (0.01 μmol/L), cisplatin (1.67 μmol/L), dacarbazine (55 μmol/L), temozolomide (2 mmol/L), paclitaxel (2.45 μmol/L), vincristine (0.54 μmol/L), and etoposide (8.5 μmol/L; ref. 12). Treated tumor cell suspensions were continuously exposed to drug for 4 days. Tritiated thymidine (Amersham, Piscataway, NJ) was added to cells at 5 μCi/well during the final 48 hours of the assay to label proliferating cells. At the end of the exposure period, culture plates were incubated at 96°C to liquefy the agarose, well contents were harvested onto glass fiber filters, and the filter-trapped cells lysed with deionized water. The incorporated radioactivity in the filter-bound macromolecular DNA was measured by liquid scintillation as counts/min. Duplicate positive control wells (supralethal cisplatin exposure causing 100% cell death) and quadruplicate negative control (medium-exposed) cultures were done for each assay. Results were reported as percentage cell growth inhibition (PCI) for the individual drug compared with medium-exposed control cultures after subtraction of positive control counts/min. EDR assay performance characteristics, including the population median PCI and SD, were determined previously for each drug based on Oncotech's database of >60,000 independent cases evaluated using the same methods. Each suspension of brain tumor cells was tested against a panel of three to seven single agents per assay. The PCI calculated for the action of each drug on a given tumor was compared with the median and SD of the drugs in the database. Tumors with PCI values above the median for a given agent were classified as exhibiting low drug resistance (LDR) to that drug; tumors with PCI values between the median and −1 SD below the median were placed into the intermediate drug resistance category, whereas those tumors with PCI values ≥1 SD below the median were placed into the EDR category. Approximately 82% of submitted specimens contained adequate amounts of malignant cells and yielded an assay result. These categories have been described previously and validated clinically (10–17). The results reported were obtained on an unselected series of cases that yielded a successful EDR assay result. Biomarkers were then analyzed for these cases.

Immunohistochemistry. Immunohistochemical techniques to detect tumor cell-associated biomarkers MDR1, glutathione *S*-transferase π (GSTP1), MGMT, and mutant p53 (mp53), were done as described previously (19, 44–46). Immunohistochemical assays were done during the routine clinical testing of samples according to the physician's request as denoted on the requisition form. As a result, not all cases were tested for the markers studied. Sections of 3 to 4 μm thickness were cut and deparaffinized in histoclear and rehydrated in descending grades (100%-70%) of ethanol. Automated immunohistochemical procedures were done using the I-6000 robotics immunohistochemistry workstation (BioGenex, San Ramon, CA) for MDR1, whereas the NexEs automated workstation (Ventana, Phoenix, AZ) was employed for automated staining of all other biomarkers. Endogenous peroxide activity was blocked using a 10-minute treatment with 3% hydrogen peroxide in distilled water. Immunoperoxide staining was accomplished using the supersensitive streptavidin-biotin detection kit (BioGenex). Counterstaining was done using hematoxylin before coverslipping and viewing by light microscopy. For each tumor studied, negative controls using tissue sections from the same paraffin block were exposed to a nonspecific antibody. Biomarker positive and negative tissue controls were run in parallel. All antibody reagents were commercially available: MDR1 antibody JSB1 (BioGenex), GSTP1 (DAKO, Glostrup, Denmark), MGMT antibody MT3.1 (Chemicon, Inc., Temecula, CA), and mp53 antibody DO-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Titrations were done for all antibody reagents to insure minimal background staining and optimal antigen detection. Positive p53 immunostaining has been reported to be related to p53 mutations that increase p53 protein half-life, leading to intracellular accumulation (47, 48). We therefore refer to immunodetectable p53 as mp53 in this report. However, the sensitivity and specificity of immunohistochemistry for detection of mutant forms of

p53 is ~80%, indicating that all mutant forms cannot be detected and not all cases of detectable overexpression of p53 protein are related to mutations. Entire tissue sections were evaluated for intensity and percentage of tumor cells staining positively. Only the malignant component was scored for each case.

Tissue specimens that showed staining of >5% of malignant cells with ≥1+ intensity were considered positive for marker expression. All procedures were done by a pathologist who had no previous knowledge of the *in vitro* drug response for this series of cases.

Statistical analysis. We determined the degree of association between *in vitro* drug resistance and expression of MDR1, GSTP1, MGMT, and mp53. Biomarker histoscores were considered as dichotomous variables. Fisher's exact test, the unpaired *t* test, and the Mann-Whitney rank sum test were used to test for relationships between biomarker expression and drug resistance, where two-tailed *P*s < 0.05 were considered significant. Computations and proportionate graphics were done using InStat V3.0 and Prism 4 software (GraphPad Software, Inc., San Diego, CA, 1998).

Health Insurance Portability and Accountability Act compliance. All clinical specimens used for research were delinked from patient-specific identifiers and studied according to an institutional review board–approved protocol.

Results

The histologic profile of the 478 malignant gliomas evaluated in this study is shown in Table 1. Tumors were classified by pathologists at the referring institutions and underwent secondary review at Oncotech. The largest proportion of cases were classified as glioblastoma multiforme (n = 259; 54%) followed by astrocytomas (n = 71; 15%), gliomas (n = 69; 15%), oligodendrogliomas (n = 40; 8%), and anaplastic astrocytomas (n = 39; 8%). The incidence of the histologic subtypes of gliomas reported here does not mirror the clinical incidence of these cases. However, the series we report on was unselected and based on the collection of data for successful EDR assays. Overall *in vitro* drug response results determined for these cases are summarized in Table 2. The incidences of EDR for the agents tested, listed from least to most resistant, were 14% (n = 241) for cisplatin, 14% (n = 162) for etoposide, 16% (n = 99) for temozolomide, 19% (n = 172) for DTIC, 31% (n = 273) for BCNU, 35% (n = 113) for irinotecan (SN38), 48% (n = 224) for vincristine, and 59% (n = 121) for paclitaxel.

Astrocytic glioma and* in vitro *drug response. Astrocytic gliomas comprised 77% (n = 369) of the 478 specimens in our study set. Table 3 shows a comparison of the percentages of EDR for grade 1/2 astrocytomas (n = 71) versus the combined grade 3 anaplastic astrocytomas (n = 39) and grade 4 glioblastoma multiforme (n = 259). With the exception of vincristine, low-grade tumors were generally more resistant than the high-grade tumors to the drugs tested in the EDR assay. The following fold increases in EDR frequencies for the low-grade versus high-grade tumors were observed: 2.5-fold (cisplatin), 1.9-fold (temozolomide), 1.6-fold (SN38), 1.5-fold (dacarbazine), 1.3-fold (BCNU), and 1.3-fold (paclitaxel).

Table 1. Histologic profile of brain tumors studied

Histologic type*	*n* (%)
Astrocytoma	71 (15)
Anaplastic astrocytoma	39 (8)
Glioblastoma	259 (54)
Oligodendroglioma	40 (8)
Glioma	69 (15)
Total	478 (100)

*Tumor histology was determined by report from the physician referring the specimen to Oncotech and confirmed independently for the presence of malignant cells at Oncotech.

Oligodendrogliomas composed a smaller subset of cases (n = 40), limiting the extent of our analysis of their drug response. However, of the 26 cases examined for response to BCNU, only 15% showed EDR, whereas 62% fell into the LDR category. Their response to cisplatin (n = 24) was comparable, with only 8% showing EDR and 67% exhibiting LDR. These findings are consistent with the greater sensitivity these tumors exhibit clinically compared with glioblastoma.

To determine if prior therapy might contribute to acquired drug resistance, we compared EDR frequencies for specimens from untreated versus previously treated patients. The data depicted in Table 4 indicate that frequencies of EDR for tumors from previously treated individuals were generally comparable with the results for untreated patients, with the exception of etoposide, which exhibited a 1.7-fold higher rate of EDR among the treated cases.

Most patients who relapse and are treated with second-line agents have previously received an alkylating agent. We examined the rates of cross-resistance among patients found to exhibit EDR to BCNU. Cross-resistance was defined as those patients with tumors that showed EDR to BCNU that showed EDR to the second agent. Cross-resistance frequencies were 40% (vincristine), 18% (DTIC), 18% (cisplatin), and 15% (paclitaxel). These percentages were not significantly different than those seen in the overall group, suggesting that previous therapy with BCNU did not alter *in vitro* response to second line agents.

Frequency of biomarker expression in astrocytic tumors. The presence or overexpression of certain biomarkers may be associated with drug resistance and/or aggressive tumor behavior. Figure 1 shows examples of marker staining results: isotype control (Fig. 1A), GSTP1 (Fig. 1B), MGMT (Fig. 1C), and p53 (Fig. 1D). Table 5 shows the overall frequency of expression of the biomarkers studied, with an additional comparison of biomarker expression versus tumor grade. The DNA repair enzyme MGMT was present in 67% of cases, GSTP1 in 49%, mp53 in 41%, and MDR1 in only 7%. Although no significant differences in biomarker expression were noted between high-grade and low-grade cases, there was a trend toward slightly higher percentages of MGMT, GSTP1, and mp53 among high-grade tumors.

Coexpression of biomarkers in BCNU-resistant malignant glioma. To determine if additive effects of resistance markers might play a role in drug resistance, malignant gliomas were evaluated for coexpression of MGMT and GSTP1, GSTP1 and mp53, or mp53 and MGMT. Figure 2 shows the χ^2 analysis of biomarker coexpression. MGMT and GSTP1 were significantly coexpressed by 47% of the cases studied (P = 0.03). No significant coexpression was seen for the other comparisons. mp53 was coexpressed with GSTP1 in 20% of 206 tumors and with MGMT in 29% of 172 tumors. No significant differences in MGMT, GSTP1, or mp53 expression were detected between untreated and treated cases (Table 6).

Table 2. Overall drug response profile for all brain tumors (n = 478; 330 untreated and 148 previously treated)

		Response categories and % brain tumors showing *in vitro* drug resistance			
Drug name (abbreviation)	**Putative mechanism of action**	***n****	**%EDR**	**%IDR†**	**%LDR**
Carmustine (BCNU)	DNA alkylator	273	31	27	42
Dacarbazine (DTIC‡)	DNA alkylator	172	19	29	52
Temozolomide‡ (TEMO)	DNA alkylator, DTIC analogue	99	16	31	53
Cisplatin (CPLAT)	Platinum-DNA adducts and cross-links	241	14	16	70
Paclitaxel (TAX)	Blocks microtubule depolymerization	121	59	21	20
Vincristine (VCR)	Blocks microtubule polymerization	224	48	32	20
Irinotecan (SN38§)	Topoisomerase I inhibitor, blocks religation	113	35	25	40
Etoposide (VP16)	Topoisomerase II inhibitor, DNA intercalator	162	14	31	55

*Number of primary brain tumor cell cultures exposed for 96 hours to drugs at concentrations that approximate peak plasma levels (29).
†Intermediate drug resistance.
‡Temozolomide undergoes decomposition to a toxic species independent of hepatic enzymes, unlike DTIC, which is also thought to induce direct cytotoxicity *in vitro* via photodecomposition to the same toxic species as temozolomide (In: Chabner BA, Collins JM, editors. Cancer chemotherapy: principles and practices. Lippincott Co.; 1990. p. 314).
§SN38 (7-ethyl-10-hydroxycamptothecin) is the bioactive metabolite of irinotecan (CPT-11), respectively, used in the EDR assay.

To determine if drug response was influenced by biomarker coexpression, we compared the percentage of cell inhibition after BCNU exposure with the biomarker expression profile. As shown in Fig. 3, when compared with biomarker null cases (n = 21), independent expression of either MGMT (n = 14) or GSTP1 (n = 37) was associated with increased resistance to BCNU (P = 0.03), whereas tumors that coexpressed these two markers showed modestly additive resistance to BCNU (P = 0.002, two-tailed unpaired t test).

With respect to MGMT, GSTP1, mp53, and MDR1, no significant relationships were found between these markers and *in vitro* drug response to the other agents tested (data not shown).

Discussion

Treatment of primary central nervous system tumors includes surgery, radiation, and chemotherapy with single-agent temozolomide, BCNU, or 1-(2-chloroethyl)-3-cyclohexyl-L-nitrosourea combined with procarbazine and vincristine (4, 49). Unfortunately, combined modality therapy yields only 2% to 5% 5-year survival rates, and poor response to treatment is considered to be a function of intrinsic and/or acquired drug resistance (20). In this study, we set out to determine the rates of EDR for various agents employed in the treatment of gliomas using an assay shown to correlate with tumor response and patient survival (10–16). We examined an unselected series of 478 independent samples of gliomas to determine if their response profiles were associated with biomarkers linked previously to drug resistance. Our series was composed of astrocytomas (n = 71; 15%), anaplastic astrocytomas (n = 39; 8%), glioblastomas (n = 259; 54%), oligodendrogliomas (n = 40; 8%), and tumors generally classified as gliomas (n = 69; 15%). The relative distribution of these cases did not mirror the clinical incidence, particularly in anaplastic astrocytomas, which tend to occur at twice the frequency of

Table 3. Incidence of EDR for low-grade versus high-grade gliomas

	Low grade		High grade	
Drug	***n***	**%EDR**	***n***	**%EDR**
BCNU	55	38	218	29
DTIC	31	26	141	17
TEMO	11	27	90	14
CPLAT	44	27	195	11
TAX	25	76	95	58
SN38	15	53	182	49
VCR	42	53	100	33

NOTE: Low-grade tumors (n = 71) were grade 1 and 2 astrocytomas. High-grade tumors (n = 298) were grade 3 and 4 astrocytomas, including glioblastoma multiforme.

Table 4. Analysis of acquired resistance after prior therapy of malignant glioma

	Untreated patients		Treated patients	
Drug	***n****	**%EDR**	***n****	**%EDR**
Carmustine	145	31	76	36
Dacarbazine	101	16	46	20
Temozolomide	39	15	23	17
Cisplatin	135	13	62	16
Paclitaxel	75	60	30	57
Vincristine	121	44	61	47
Etoposide	82	12	44	20
SN38	56	45	32	34
Topotecan	55	24	21	24

NOTE: Tumor histology and prior treatment status were determined by report from the physician referring the specimen to our laboratory.
*n is the number of EDR-assayed gliomas.

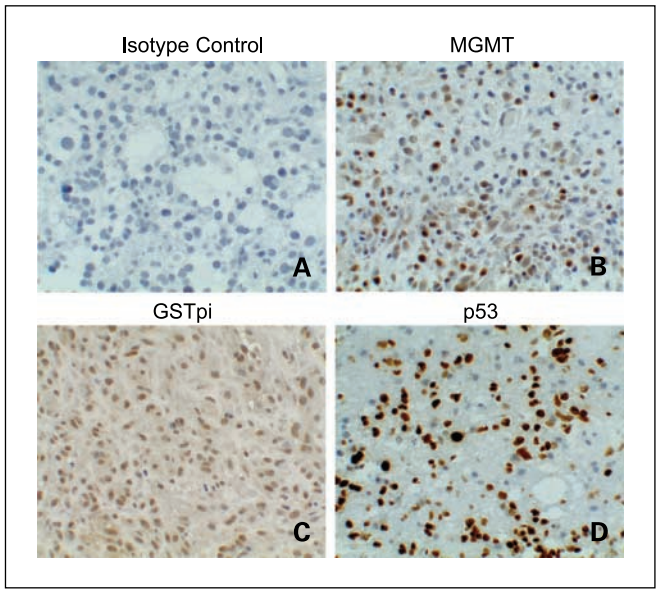

Fig. 1. Representative immunohistochemical staining patterns for MGMT (*B*), GSTP1 (*C*), and p53 (*D*) in gliomas. An isotype control is shown for comparison (*A*).

	GSTP1 +	GSTP1 -			P53 +	P53 -		MGMT +	MGMT -
MGMT +	64	37	+	GSTP1	42	62	p53+	46	26
MGMT -	14	21		GSTP1 -	37	82	p53-	73	47
	p=0.027				p=0.292				

Chi-square contingency table, 2-sided p values. N = 284 evaluable tumors.

Fig. 2. Associations between drug resistance biomarkers in malignant glioma. Paraffin-embedded tumor sections from 284 individual cases were assayed by immunohistochemistry to determine the presence of coexpressed tumor-associated biomarker proteins MGMT, GSTP1, and mp53.

astrocytomas. The ability of the testing laboratory to obtain EDR assay results for the subsets of these tumors did not differ, leading us to speculate that the selection bias relates to what tumors were accessible to the surgeon and which tumors they determined should be sent for assay.

We recently reported that poorer survival of patients treated for recurrent glioblastoma multiforme with CPT-11 was significantly associated with the *in vitro* resistance of these patient's tumors to CPT-11 (16). Although *in vitro* drug resistance has been found to correspond with poor outcomes clinically, it should be emphasized that LDR is not concordant with a successful outcome after chemotherapy (12, 13). Here, we sought to examine the potential relationships between known drug resistance biomarkers and *in vitro* drug response (18, 19). It has been shown previously that drugs falling into the EDR category have a very low probability of clinical activity, with response rates of <3%, whereas the intermediate drug resistance category response rates are ~50% of rates reported, and patients with tumors showing LDR *in vitro* have response rates ~2-fold higher than those reported in clinical trial data (10–17). The frequency of LDR for a given drug tends to parallel the clinical response rates for that agent (13). On the other hand, we noted a frequency of LDR that was generally higher than response rates seen clinically for these tumors. It should be noted that drug exposures in our study were generally 5- to 20-fold higher than those achieved clinically (12). These high exposures are employed to assure that the drug resistance end point is accurate as opposed to chemosensitivity assays that employ lower drug exposures. Drug resistance assays may therefore overestimate the frequency of responsive tumors. Further work to verify the relationship between *in vitro* drug response results for gliomas and clinical outcomes is needed to confirm the clinical utility of such tests for these patients and their relationship to the biomarkers studied.

We found that frequencies of LDR were least for cisplatin (70%) and VP16 (55%). These *in vitro* rates were greater than what would be expected in the clinic, where their activity may be diminished by the blood-brain barrier (50–52). Cisplatin and etoposide are both substrates for membrane-bound efflux pumps, such as MRP and MDR1, which prevent their entry into the extracellular space of the central nervous system (20, 52). The low levels of *in vitro* drug resistance noted for cisplatin and etoposide may be explained in part by the absence of such a barrier in our *in vitro* assay system.

Although the blood-brain barrier uses various pumps to protect the central nervous system, we found very low expression levels of MDR1 among the gliomas we analyzed. The role of MDR1 multidrug efflux pump in drug resistance in gliomas has been unclear (21–23). Only 7% of the 125 cases we studied showed detectable MDR1 expression, suggesting that it was not a major contributor to drug resistance for this

Table 5. Presence and frequency of tumor-associated biomarker expression in human brain tumors

Biomarker	Putative function	% Positive (*n* tested)			Fisher's exact *P*
		Overall	Low grade	High grade	
MGMT	Suicide repair of alkyl-DNA damage	67 (153)	59 (27)	69 (126)	0.37
GSTP1	Glutathione-dependent drug detoxification	49 (183)	43 (35)	50 (148)	0.46
MDR1	P-glycoprotein, natural drug pump	7 (135)	7 (27)	6 (108)	NS
p53	Cell cycle arrest, tumor suppressor	41 (232)	39 (36)	45 (196)	0.58

NOTE: A positive incidence of biomarker expression detected by immunohistochemistry included samples with at least 5% of tumor cells staining with a +1 intensity as described in Materials and Methods.

Table 6. Presence and frequency of tumor-associated biomarker expression in human brain tumors stratified by treatment status

Biomarker	% Positive*		Fisher's exact *P*
	Untreated (*n*)	Prior treatment (*n*)	
MGMT	75 (106)	63 (49)	0.21
GSTP1	51 (132)	44 (59)	0.44
p53	53 (233)	41 (69)	0.08

*A positive incidence of biomarker expression detected by immunohistochemistry included samples with at least 5% of tumor cells staining with a +1 intensity as described in Materials and Methods. ***n*** is the total number of cases studied.

series. Cordon-Cardo et al. and Tanaka et al. have reported finding strong MDR1 staining on glioma tumor vasculature without finding reactivity on glioma cells (53, 54). Toth et al. reported that 5 of 29 (17%) gliomas stained positive for P-glycoprotein (55). On the other hand, von Bossanyi et al. found that 31 of 50 (62%) cases showed MDR1 expression at a level of >5% of cells staining positive, with a significant trend toward higher expression levels with increasing grade (22). Although von Bossanyi et al.'s results differ from ours, we have carefully validated our P-glycoprotein immunohistochemical assay by correlating JSB1 staining intensity on control cells lines using both flow cytometry and image analysis (19). Furthermore, we have shown that the intensity of MDR1 staining obtained for breast or ovarian tumor samples was significantly associated with *in vitro* drug response (18, 19). On the other hand, the frequency of LDR that we noted for paclitaxel (20%) and vincristine (20%) was similar to the clinical response rates for these compounds (56, 57). These data suggest that although MDR1 expression by glial tumors may not be the dominant direct cellular process responsible for tumor resistance to natural products, other mechanisms are present that diminish their activity. The clinical mechanisms of natural product resistance may be a multifactorial function of endothelial expression of MDR1 at the blood-brain barrier in conjunction with glial tumor cell expression of alternative efflux pumps, such as MRP, altered tubulin with lower affinity binding sites, and/or protein kinase C signaling pathways that suppress apoptosis (8, 20, 52, 58, 59).

BCNU and temozolomide are preferred drugs for the treatment of central nervous system tumors based on their ability to bypass the blood-brain barrier (60, 61). Their *in vitro* activity should therefore better approximate their clinical activity than would agents that are restricted by the blood-brain barrier. The rate of LDR was 42% for BCNU and 53% for temozolomide, similar to their clinical response rates of ~30% to 50% (4, 62–67). Frequencies of resistance to these agents were not significantly associated with tumor grade or prior therapy, suggesting that intrinsic resistance mechanisms expressed by glial tumors at the time of diagnosis may be the major contributor to treatment failure (Tables 3 and 4). Glial tumor development may be accompanied early on by the acquisition of intrinsic drug resistance mechanisms expressed by both well-differentiated and poorly differentiated tumors. This model is consistent with our finding that a similar proportion of GSTP1 and MGMT expression was noted in both low-grade and high-grade gliomas (Table 5).

GSTP1 is the first major mechanism of resistance alkylator agents encounter after entering the cancer cell cytoplasm (8, 20, 29). GSTP1 acts to enzymatically conjugate glutathione to the reactive metabolites of BCNU (24–29). Strange et al. compared the GSTP1 isozyme profile and activity extracted from 21 brain tissue controls with 17 glioma samples (68). They noted that GSTP1 was the principle isozyme contributing to total glutathione *S*-transferase activity in both control tissue (70.9%) and tumors (82.3%), with significantly higher expression in tumors. We found GSTP1 expression in 49% of the gliomas in our series, a percentage similar to that reported by other groups (27, 28). It should be noted that detection methods and scoring systems vary between laboratories and this may contribute to the differences in the percentages of cases found to be positive among different reports. For example, immunohistochemistry will yield lower levels of expression than more sensitive techniques, such as gene expression measurements made using reverse transcription-PCR. What may be most important in evaluating the relationship between markers and phenotype is consistency in the techniques employed for the study set examined.

Winter et al. examined GSTP1 expression across a series of 22 glioblastoma cases using immunochemistry and found that 64% expressed >5% staining in 10 high-power fields (28). A higher percentage of expression was reported by Ali-Osman et al., who found that 43 of 61 (71%) cases showed moderate to high staining levels (27). Although Ali-Oman's group did not find a significant relationship between GSTP1 and tumor grade, they noted a trend toward higher staining in higher-grade

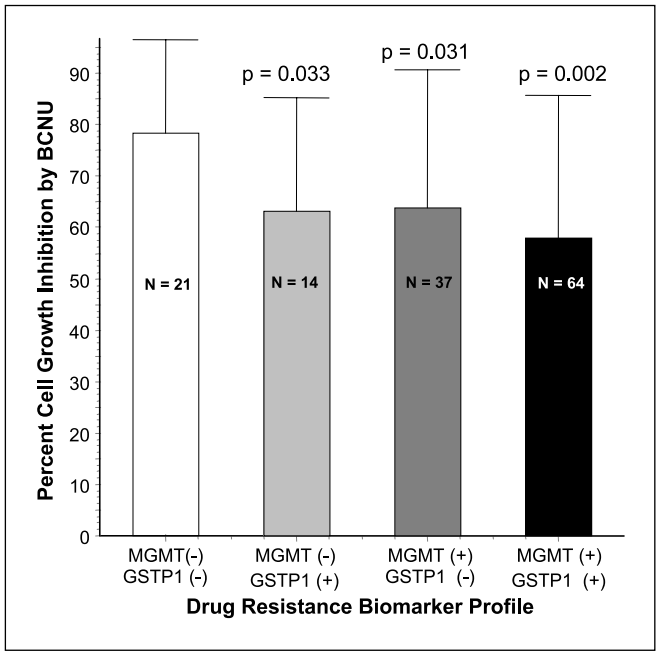

Fig. 3. Relationship between MGMT and GSTP1 coexpression and BCNU response in malignant glioma. Effect of biomarker coexpression on *in vitro* tumor response to BCNU in malignant glioma. *Bars,* 1 SD. The unpaired *t* test with two-tailed *P*s was done to compare the marker-negative group PCI after BCNU with the marker-positive groups.

tumors ($P = 0.16$, exact χ^2 test). Of note, their group found a significant reduction in the survival of patients with moderate or high staining levels compared with those cases that exhibited low levels (27). Although we also saw a trend toward increased staining by higher-grade tumors, we did not find a statistically significant difference in the percentage of positively staining tumors when comparing grade 1 and 2 cases (43%) with grade 3 and 4 cases (50%). Grant and Ironside reported that GSTP1 expression levels were increased with tumor grade in their study of 30 glioma cases (24). On the other hand, von Bossanyi et al. found that lower-grade gliomas had modestly higher expression levels of GSTP1, with 66% of the grade 1/2 cases showing high percentages of positive staining versus 54% of the grade 2/4 cases (22). Further, Matsumoto et al. reported that total glutathione *S*-transferase activity in glioblastomas was significantly less than in normal brain tissue (69). It is possible that intensity of staining varies with grade, but even low-grade tumors show significant levels of GSTP1, suggesting that this may be an intrinsic element of glial cell phenotype proffering protection against xenobiotics and that GSTP1 expression may be retained or increased during tumor progression.

The mechanisms by which GSTP1 may be up-regulated in gliomas are under investigation. Constitutive expression is thought to be influenced by the proximal promoter factor Sp1, whereas increased expression levels may result from stabilization of GSTP1 mRNA (70, 71). GSTP1 expression has been reported to be induced by drug exposure, indicating that it may play a role in acquired drug resistance (29). Further, nuclear localization has been reported to increase after treatment, potentially contributing to acquired drug resistance (27, 72). GSTP1 down-regulation in gliomas may be mediated, in part, by promoter hypermethylation (73, 74). Although we did not find an increased level of GSTP1 in previously treated patients (Table 6), we found that tumors expressing GSTP1 were significantly more resistant to BCNU than GSTP1-negative cases (Fig. 2). Results for BCNU action on the 21 GSTP1-negative cases fell into the LDR category, whereas the GSTP1-positive cases fell into the intermediate drug resistance category. These data suggest that GSTP1 contributes directly to glial tumor cell resistance to BCNU, a result consistent with other investigators (8, 25, 29). Another factor influencing the effect of GSTP1 on drug resistance is the availability of its cofactor, glutathione (29, 40). Glutathione content may vary among tumors, causing further variation in tumor response to BCNU (25). Strategies to deplete glutathione to augment BCNU action continue to be explored.

Although others have noted a relationship between GSTP1 expression and cisplatin resistance, we found no relationship between these two factors (75). Establishing this relationship has been somewhat tenuous, with one transfection study showing a 2- to 3-fold increase on the one hand (75), whereas another transfection study failed to show any change in cisplatin response (76). A possible explanation for these discrepancies may be related to the recent demonstration that GSTP1 is polymorphic, with at least four distinct alleles (26). Two of these alleles, GSTP1C and GSTP1B, have been shown to confer 5.6- and 2.5-fold resistance to cisplatin, respectively, whereas the other two alleles are not protective (77). Allelic variation among the samples examined in our study may have contributed to our failure to find an association between GSTP1 and *in vitro* response to cisplatin. The development of allele-specific monoclonal reagents would foster a more refined analysis of the relationship between GSTP1 polymorphism and cisplatin activity on gliomas.

For drugs that have evaded cytosolic mechanisms of drug resistance, the nucleus is equipped with the capacity to remove BCNU or temozolomide alkyl groups from the O^6-position of guanine via a reaction catalyzed by MGMT (30–34). Repair occurs before cross-link formation and involves an irreversible stoichiometric covalent transfer of the O^6-alkyl DNA adduct to a cysteine within the active site of MGMT, resulting in the inactivation and subsequent depletion of enzyme activity. MGMT-mediated repair is rapid, with a half-life of $\sim$35 hours. MGMT enzyme recovery occurs via *de novo* synthesis. In malignant glioma patients, MGMT overexpression has been associated with resistance to BCNU and similar alkylating agents and was an independent predictor of poor survival (33, 34). MGMT may be up-regulated by DNA-damaging agents or down-regulated by methylation of its promoter (78, 79). Differential transcriptional regulation is thought to be the major mechanism underlying the varying levels of MGMT between individual glial tumors (80).

We found that 62% of the 153 cases examined expressed immunodetectable levels of MGMT, with a nonsignificant trend toward a higher frequency of expression among high-grade lesions ($P = 0.37$). We also found that MGMT-positive cases were significantly more resistant to BCNU than MGMT-negative cases (Fig. 2). This is similar to the findings of the South West Oncology Group, who reported that survival of 64 glioma patients treated with BCNU was related to high versus low MGMT expression determined by a quantitative indirect immunofluorescence assay (34). Failure-free and overall survival for the 64% of cases that had high MGMT expression was significantly poorer, with a median survival of 8 months compared with 29 months for the low MGMT expressors ($P = 0.0002$). On the other hand, Silber at al., who employed a MGMT enzyme activity assay to compare MGMT content in 100 newly diagnosed gliomas versus 51 cases that recurred after surgery and radiotherapy versus 23 cases that recurred after surgery, radiotherapy, and alkylator therapy, failed to find a relationship between MGMT and survival (81). Of the 174 cases examined, Silber et al. group found detectable MGMT activity (Mer+) in 71%, with similar percentages for newly diagnosed cases (29%) and cases treated previously with surgery and radiation (24%). In contrast, a significantly higher percentage of MGMT-positive cases (95%) were found in recurrent specimens. No increase in MGMT activity was seen in cases where MGMT was measured before and after therapy. Although the data of Silber et al. are consistent with the notion that the subset of MGMT-positive cells may have had a selective advantage based on intrinsic resistance to BCNU, potentially becoming the dominant species at recurrence, the time to progression for the alkylator-treated patients in the Silber et al. series was not affected by MGMT status.

Recently, Esteller et al. correlated silencing of MGMT expression by promoter methylation with clinical response and survival of 47 newly diagnosed grade 3 or 4 glioma patients (82). MGMT promoter methylation was found in 40% of cases and was independent of tumor grade, patient age, and performance status. Methylation of the MGMT promoter was significantly associated in univariate analysis with increased response to cisplatin, whole-brain radiation, and BCNU

treatment. Median time to progression for patients with methylated MGMT promoters was 21 months compared with 8 months for unmethylated cases ($P < 0.001$), and overall survival was significantly longer for the methylated population ($P < 0.001$). Various factors may therefore influence alkylator resistance in gliomas. We found that both MGMT and GSTP1 were coexpressed in 47% of gliomas (Fig. 1). Cases that showed MGMT and GSTP1 coexpression were more resistant to BCNU than those tumors that expressed only one of these biomarkers, indicating that resistance mechanisms may produce additive effects (Fig. 2). Inactivation of MGMT to increase BCNU activity is currently being investigated in the clinic using the MGMT substrate O^6-benzylguanine (83, 84).

MGMT is also thought to contribute to temozolomide resistance, which we did not detect in our study. This may be related to the *in vitro* pharmacokinetic differences between BCNU and temozolomide (60, 61). The half-life of BCNU in serum is extremely short (15 minutes) due to rapid spontaneous hydrolysis and metabolic transformation of the parent species to the active intermediates, which are subsequently hydrolyzed to inactive metabolites. *In vitro* pharmacokinetics in the EDR assay are similar to *in vivo* kinetics, with the exception that no microsomal metabolism is present in the assay. Although the 10 μg/mL final concentration of BCNU in the EDR assay is 2-fold higher than the 5 μg/mL peak plasma concentration achieved clinically, both systems are subject to the rapid hydrolysis of BCNU. On the other hand, the *in vivo* half-life of temozolomide is 1.8 hours and is predominantly related to clearance. Thus, the *in vitro* exposure of temozolomide will be sustained in the EDR assay over several hours. As a result, MGMT may be depleted over the initial period of exposure, with a continued loss of function that could lead to decreased DNA repair capacity and increased drug efficacy (37, 67). Thus, the *in vitro* pharmacodynamics of temozolomide in the EDR assay may make it difficult to find an association between MGMT and temozolomide response.

In brain tumors, p53 mutation is one of the major genetic alterations that mark the change from a differentiated astrocyte to an astrocytoma (35). Although wild-type p53 has been reported to cause BCNU resistance in the U87MG glioma cell line, various studies suggest that mp53 is associated with drug resistance (36, 38, 85, 86). We failed to find a significant association between p53 status and *in vitro* drug resistance. This may be related to the notion that various mechanisms of drug resistance are at work simultaneously, making it difficult to discern the individual contributions of a given mechanism across the population of tumors studied.

Variations in area under the curve for the drugs studied may have contributed to our failure to find a relationship between some of the biomarkers and *in vitro* drug response. Because of the high drug exposures employed, for one individual marker to stand out as significantly associated with *in vitro* drug response to a given agent, it would have to make a very robust contribution to the net effect of all resistance mechanisms at work (58). Alternatively, the cut point we employed for calling a marker positive (>5%, 1+) may have been too low to show a relationship between a given marker and drug response, whereas a higher threshold or different scoring system may have yielded a different result. Furthermore, intra-assay and inter-assay variability may have introduced variance in the end points that obscured potential relationships between markers and *in vitro* drug response. The clinical value of the EDR assay is that it provides a net effect result for each agent by integrating the various and complex cellular mechanisms at work into a final drug response outcome (13).

The integration of biomarker status with other biological factors, including well-established variables, such as tumor grade and age, to predict clinical outcomes for individual patients with malignant glioma has become an area of active research. The results of our examination of *in vitro* drug resistance and resistance-related biomarkers in brain cancer lend support to the notion that intrinsic drug resistance to BCNU and other agents used to treat gliomas can be screened for using *in vitro* assays. However, results from such studies must be confirmed in clinical trials. Further, we found that BCNU resistance was related in part to tumor expression of GSTP1 and MGMT. Analysis of these factors before treatment may allow the clinician to stratify their treatment options and avoid unnecessarily toxic treatments that may have limited benefits. We are poised at the dawn of the genomic profiling era, and it is hoped that this approach will yield new insights into the classification of gliomas and their response to chemotherapy, potentially offering new tools to improve the lives of these patients (87–89).

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