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Biochemical changes associated with a multidrug-resistant phenotype of a human glioma cell line with temozolomide-acquired resistance

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Abstract

Temozolomide (TMZ) is a newly approved alkylating agent for the treatment of malignant gliomas. To investigate resistance mechanisms in a multidrug therapeutic approach, a TMZ-resistant human glioma cell line, SF188/TR, was established by stepwise exposure of human SF188 parental cells to TMZ for approximately 6 months. SF188/TR showed 6-fold resistance to TMZ and cross-resistance to a broad spectrum of other anticancer agents that included 3–5-fold resistance to melphalan (MEL), gemcitabine (GEM), paclitaxel (PAC), methotrexate (MTX), and doxorubicin (DOX), and 1.6–2-fold resistance to cisplatin (CDDP) and topotecan (TPT). Alkylguanine alkyltransferase (AGT) activity was increased significantly in the resistant cell line compared with the parental cell line ($P < 0.05$), whereas no significant differences occurred in the cellular uptake of TMZ and PAC between resistant and parental cells. Depletion of AGT by O^6-benzylguanine significantly increased the cytotoxicity of TMZ in both the sensitive and resistant cell lines, but did not influence the cytotoxicity of the other drugs tested. Treatment with TMZ caused SF188 cells to accumulate in S phase, whereas SF188/TR cells were unaffected. Expression of Bcl-2 family members in SF188/TR cells compared with SF188 cells indicated that the pro-apoptotic proteins (i.e. Bad, Bax, Bcl-X_S) were reduced 2–4-fold in the resistant cell line, whereas the anti-apoptotic proteins Bcl-2 and Bcl-X_L were expressed at similar levels in both cell lines. In conclusion, the mechanism of resistance of SF188/TR cells to TMZ involved increased activity of AGT, a primary resistance mechanism, whereas the broad cross-resistance pattern to other anticancer drugs was due to a common secondary resistance mechanism related to alterations in the relative expression of the pro-apoptotic and anti-apoptotic proteins.

Keywords: Pro-apoptotic proteins; Anti-apoptotic proteins; p53; Membrane transporters; Alkylguanine alkyltransferase

1. Introduction

Malignant gliomas remain an enormous therapeutic challenge despite advances in surgical, radiotherapeutic, and chemotherapeutic approaches. Although the diversity of drugs available for chemotherapy is increasing, alkylating agents remain the most commonly used in brain tumors, based on their inherent activities and their lipophilic nature, which enable appreciable blood–brain barrier transport. As with most malignant tumors, intrinsic or acquired drug resistance diminishes the response of the tumor. Numerous strategies based on the identification of the primary resistance pathway have been attempted to overcome drug resistance. Recently, apoptotic pathways have been implicated in the development of drug resistance [1–3]. This project investigated the basis of drug resistance to TMZ and of cross-resistance to a variety of other cytotoxic agents in glioma cell lines.

TMZ is an orally bioavailable imidazotetrazine compound that acts as a methylating agent and possesses clinical activity against melanomas, pancreatic cancer, and gliomas [4,5]. In biological media, TMZ undergoes pH-dependent degradation to its active metabolite, MTIC, which by further degradation to a DNA-alkylating species,

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Abbreviations: AGT, alkylguanine alkyltransferase; BCNU, *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea, carmustine; CDDP, *cis*-diamminedichloroplatinum(II), cisplatin; DOX, doxorubicin; GEM, gemcitabine; hMLH1, MutL homologue; hPMS2, postmeiotic segregation increased (*Saccharomyces cerevisiae*) 2; MEL, 4-[bis(2-chloroethyl)amino]-L-phenylalanine, melphalan; MRP, multidrug resistance-associated protein; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; MTX, methotrexate; O^6-BG, O^6-benzylguanine; O^6-MeG, O^6-methylguanine; PAC, paclitaxel; SRB, sulforhodamine B; TMZ, temozolomide; TPT, topotecan.

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is cytotoxic *via* the formation of O^6- and N^7-alkylguanine lesions in DNA. Resistance to alkylating agents is associated with increased AGT activity and repair of O^6-alkylguanine DNA adducts [6–10], increased detoxification by elevated levels of glutathione or glutathione-*S*-transferase [11–13], and excision or mismatch repair systems [14–17]. Among these factors, repair of DNA adducts by AGT represents the principal mechanism of cellular protection from DNA damage by alkylating agents. A recent study showed that inactivation of the *AGT* gene by methylating the AGT promoter region significantly correlated with the clinical outcome in glioma patients treated with the alkylating agent carmustine (BCNU) [18].

One mechanism of tumor cell death in response to various chemotherapeutic drugs including alkylating agents [19–21] is programmed cell death or apoptosis. It has been proposed that apoptotic-related proteins, such as the Bcl-2 family proteins and p53, play an important role in drug-induced apoptosis of tumor cells [1,2]. Bcl-2-related proteins either suppress (Bcl-2 and Bcl-X_L) or promote (Bad, Bax, and Bcl-X_S) apoptosis by interacting with and functionally antagonizing each other. These interactions either prevent or promote caspase activation, and concomitantly apoptosis. A recent report [22] discussed the important role of Bax in drug-induced apoptosis in human colorectal cancer cells, and illustrated that cancer cells with disrupted Bax function were partially resistant to the apoptotic effects of 5-fluorouracil. The present study determined the mechanism of resistance to TMZ in a human glioma cell line, and the mechanisms of cross-resistance to a wide array of anticancer drugs based on the supposition of the involvement of the Bcl-2 family members.

2. Materials and methods

2.1. Drugs and antibodies

TMZ and GEM were provided by the Schering-Plough Research Institute and Eli Lilly Pharmaceuticals, respectively. MTX was purchased from Lederle. All other chemicals and drugs, including PAC, CDDP, MEL, O^6-BG, and DOX were purchased from the Sigma. TPT was a gift from Smith Kline Beecham Pharmaceuticals. Bcl-2 monoclonal antibody was purchased from Oncogene Research Products. Bax, Bcl-x, and Bad polyclonal antibodies were purchased from Santa Cruz. hMHL1 and hPMS2 antibodies were purchased from Pharmingen. *N*-[^{3}H]Methyl-*N*-nitrosourea was obtained from Amersham.

2.2. Cell culture and development of a cell line with acquired resistance to TMZ

The human glioma cell line SF188 was provided by the Brain Tumor Research Center, University of California at San Francisco. It was grown as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°. To establish the TMZ resistant cell line, SF188 cells were exposed continuously to increasing concentrations of TMZ (from 50 to 300 μg/mL) over a 6-month period. The cytotoxicity of TMZ to the SF188 parental and the selected cells was tested frequently, and at 6 months the selected cells were about 6-fold resistant to TMZ. This cell line was denoted SF188/TR. TMZ was withdrawn from the SF188/TR cells, but the cells were challenged frequently by incubation with 300 μg/mL of TMZ.

2.3. Inhibition of cell growth

Sensitivity of SF188 and SF188/TR cells to TMZ, TPT, MEL, DOX, CDDP, PAC, MTX, and GEM was estimated by the SRB assay [23]. Briefly, exponentially growing cells were plated into 96-well plates (2000 cells/well). After 24 hr, serial dilutions of the drugs were added to the cells, which were incubated for 96 hr, subsequently fixed with 10% trichloroacetic acid (TCA), and then stained with 0.4% SRB in 1% acetic acid. After removal of the unbound SRB by washing, cell-bound SRB was dissolved in 150 μL of 10 mM Tris–HCl buffer. Absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories). Inhibition of cell growth was calculated by using the absorption ratio of drug-treated cells to untreated control cells.

2.4. Flow cytometric analysis and GSH assay

Cell cycle distribution was monitored following exposure of the cells (either SF188 or SF188/TR) to two concentrations of TMZ (100 or 300 μg/mL) for 0, 8, 24, and 48 hr. At each time point, one petri dish (60 mm) was withdrawn, and the cells were collected by trypsinization. Cells were fixed by ethanol and stained with propidium iodide (100 μg/mL) for 20 min in the dark at 4° after a 20-min incubation with 250 μg/mL of RNase A in 0.1% Triton X-100 at room temperature. DNA content was used to determine the cell cycle distribution according to a standard method [24].

The GSH concentration in the SF188 and SF188/TR cells was analyzed according to a published assay [25] by using a Bioxytech GSH-400 assay kit (OXIS International Inc.).

2.5. Intracellular uptake of TMZ and PAC

Time- and concentration-dependent uptake of PAC was compared in SF188 and SF188/TR cells. In the time-dependent studies, cell suspensions were prepared by trypsinization and then resuspended in serum-free medium at a concentration of 5×10^5 cells/mL and a PAC

concentration of 25 μg/mL. Cells were incubated for 30, 60, 120, and 240 min and then collected by centrifugation (1500 *g* for 5 min at 4°) and washed twice with cold PBS. The cell pellets were resuspended in 100 μL of water and sonicated for 20 s to release PAC. A 70-μL aliquot of the suspension was removed, and 3 vol. of cold methanol was added to precipitate proteins upon vortexing. Following centrifugation (15,000 *g* for 5 min at 4°), an aliquot of the supernatant was analyzed for PAC by HPLC. In the concentration-dependent studies, cells were incubated for 2 hr at PAC concentrations of 1, 2.5, 5, 10, 25, and 50 μg/mL, and then harvested and prepared for HPLC analysis as already outlined.

For the cellular uptake of TMZ, either SF188 or SF188/TR cells grown at 70–80% confluence were incubated with 500 μg/mL of TMZ for 2 hr. At the end of the incubation period, the medium containing TMZ was removed and the cells were washed twice in cold PBS. The cells were collected by scraping, then centrifuged (1500 *g* for 5 min at 4°), and the pellet was resuspended in 100 μL of water. Twenty microliters of 1 M HCl was added to the cell suspension. The suspension was then vortexed and centrifuged (15,000 *g* for 5 min at 4°), and 60 μL of the resultant supernatant was used for the HPLC analysis. The intracellular concentrations of PAC and TMZ were expressed as micrograms per milligram of cellular protein. In all of the drug uptake studies, small aliquots of the cell lysates were analyzed for total protein using a Bio-Rad assay [26].

2.6. Quantitation of TMZ and PAC by HPLC assay

TMZ and PAC were quantitated by HPLC. TMZ was separated on a CN column (Spherisorb, 5 μm, 4.6 × 150 mm, Alltech) with a mobile phase of 5% acetonitrile in 50 mM ammonium acetate (pH 6.8), and detected at a wavelength of 323 nm. The flow rate was 0.7 mL/min producing a retention time of TMZ of about 4.0 min [27]. PAC was isolated with an octadecylsilane column (Hypersil, 5 μm, 4.6 × 150 mm, Alltech) and a mobile phase of 45% (v/v) acetonitrile/water. PAC was detected at a wavelength of 230 nm.

2.7. Assay of AGT activity

O^6-MeG methyltransferase activity, commonly referred to as AGT activity, was determined in the SF188 and SF188/TR cell lines using an O^6-MeG DNA adduct repair assay [28]. Briefly, calf thymus DNA was reacted with *N*-[^{3}H]methyl-*N*-nitrosourea (1 μCi) for 30 min at 37° in 85 mM Tris buffer (pH 8.0). Methylated DNA was isolated, dissolved in 10 mM Tris buffer (pH 8.0), and stored at −80° until used. To determine AGT activity, extracts from SF188 and SF188/TR cells were prepared in buffer (1 mM EDTA, 70 mM HEPES, 5% glycerol, and 1 mM dithiothreitol, adjusted to pH 7.8) and ultrasonicated three times for 5 s each in ice water. Protein (200 μg) and ^{3}H-labeled methylguanine DNA (25 μg) were mixed in assay buffer (70 mM HEPES, pH 7.8, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, and 25 μM spermidine) in a total volume of 200 μL, and then incubated for 60 min at 37°. The reaction was stopped by adding 40 μL of 50% TCA at 4° for 30 min. Pellets obtained by centrifugation were hydrolyzed with 60 μL of 0.1 N HCl at 70° for 45 min, and the resultant hydrolysates were neutralized by the addition of 300 μL of 0.02 M Tris buffer (pH 10.6). Following centrifugation (10,000 *g* for 5 min at room temperature), supernatants were collected and combined with a water wash of the pellet residue. Aliquots of 100 μL were injected onto an HPLC system that separated N^7- and O^6-MeG using a mobile phase of 5% methanol in 100 mM sodium acetate (pH 5.2) solution containing 100 μL/L of triethylamine. Fractions of 1.5 mL of mobile phase eluent were collected, and radioactivity was assessed on a scintillation counter.

2.8. Western blot and protein quantitation

Western blot analyses were used to detect the expression of Bcl-2, Bcl-x, Bad, Bax, p53, p21, hPMS2, hMLH1, and MRP1. Total protein was extracted from the cells using lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl, 100 mM NaF, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1% phenylmethylsulfonyl fluoride, and 1% aprotinin). Protein concentrations were determined by a standard method [26], and then equal amounts of protein from SF188 and SF188/TR cells were subjected to SDS-PAGE on 4–15% Tris–glycine gels (Bio-Rad). Separated proteins on the gel were then transferred to nitrocellulose membranes, blotted with different antibodies, and detected by ECL (Amersham). Quantitation of the apoptotic protein bands was performed by densitometry.

2.9. p53 expression and induction

The tumor suppressor protein, p53, plays an important role in the induction of apoptosis in response to DNA damage caused by either radiation or chemotherapy. The DNA damaging agent DOX can induce p53 accumulation shortly after treatment of tumor cells in the presence of wild-type p53 but not in the presence of mutant p53 [29]. To evaluate the status of p53 and p21 in sensitive and resistant SF188 cells, both cell types were exposed to 0, 1, and 5 μM DOX for 4 hr. Cellular proteins were collected after treatment with the lysis buffer followed by western blot analyses.

2.10. Combination of O^6-BG and anticancer agents

A standard SRB assay was used to evaluate the ability of O^6-BG to modulate the cytotoxicity of TMZ, PAC, MTX,

Table 1
Characteristics of SF188 and SF188/TR cell lines

Cell lines	Doubling time[a] (hr)	Cell diameter[a] (μm)	GSH content[a] (nmol/mg protein)	Cell cycle[b] (%)		
				G1	S	G2–M
SF188	30.1 ± 1.4	11.60 ± 1.60	54.2 ± 4.6	52.8	27.1	20.1
SF188/TR	33.7 ± 0.2	12.44 ± 1.98	48.9 ± 3.9	49.5	32.1	18.3

[a] Values are means ± SD of three experiments.
[b] Data represent one of two identical experiments.

MEL, and GEM. Briefly, both SF188 and SF188/TR cells were plated onto 96-well plates, and allowed to attach overnight. Subsequently, 20 μM O^6-BG was added to each well for 6 hr, and then various concentrations of each anticancer drug was added to the wells and the plates were incubated for another 4 days. Cytotoxicity was evaluated as already outlined.

3. Results

3.1. Drug resistance profile of SF188/TR cells

The SF188/TR resistant cell line was developed by stepwise exposure to increasing TMZ concentrations for approximately 6 months. This cell line showed about 6-fold resistance to TMZ compared with the parental SF188 cells. After withdrawal of TMZ for 2 months and re-challenge with 300 μg/mL of TMZ for 1 week every month, the resistance factor to TMZ decreased to 4–5 and remained constant. SF188/TR cells developed resistance not only to TMZ, but also to other commonly used anticancer agents, such as PAC, GEM, MEL, CDDP, and MTX. The characteristics of the parental and resistant cell lines are shown in Table 1. There was no difference between the two cell lines in terms of diameter, GSH concentration, and cell cycle distribution. The doubling time of SF188/TR cells was 33.7 hr, whereas it was 30.1 hr for SF188 cells ($P < 0.05$). The IC_{50} values and resistance factors (R_f) of SF188/TR cells to different drugs are shown in Table 2.

3.2. Cell cycle distribution

There was no significant difference in the cell cycle distribution of SF188 and SF188/TR cells in the absence of TMZ (time 0). At a lower concentration of TMZ (100 μg/mL), the proportion of cells in S phase after 24 hr was increased in both cell lines. At 48 hr, SF188 cells were blocked significantly in the G_2–M phase, but the distribution of SF188/TR cells remained unchanged. Exposure of SF188 cells to a high concentration of TMZ (300 μg/mL) resulted in the accumulation of approximately 55 and 93% of the cells in S phase at 24 and 48 hr, respectively. The cell cycle distribution of SF188/TR cells was largely unchanged, with only a minor increase in the G_2–M phase (Fig. 1).

3.3. Intracellular accumulation of TMZ and PAC

TMZ achieved analogous intracellular concentrations in both SF188 and SF188/TR cells after a 2-hr incubation. The intracellular concentrations of TMZ in SF188 and SF188/TR cells were 26.4 ± 8.5 and 27.8 ± 2.7 ng/mg protein, respectively ($P > 0.05$). Similarly, there were no significant differences in the intracellular accumulation of PAC between the SF188 and SF188/TR cell lines as either a function of time or concentration (Fig. 2). These results support the notion that drug resistance to either TMZ or PAC in SF188/TR cells is unlikely to be associated with changes in membrane transport, and, specifically for PAC, are not due to MDR1 or MRP1.

3.4. AGT activity

AGT activity was increased significantly in the SF188/TR cells compared with the parental SF188 cells, being 52.0 ± 8.8 and 34.5 ± 7.7 fmol/mg protein, respectively ($P < 0.05$). Under the same substrate and protein concentration conditions, 44% of the O^6-MeG DNA adducts were repaired in the SF188 cells compared with 72% in the SF188/TR cells (Fig. 3, $P < 0.001$).

Table 2
Cytotoxicity (IC_{50}) and resistance factors (R_f) of TMZ and other anticancer drugs in SF188 and 188/TR glioma cells

Drugs	IC_{50}[a] (nM)		R_f	P values
	SF188	SF188/TR		
TMZ	426,300 ± 216,500	1,854,600 ± 60,000	4.4	<0.01
MEL	920.0 ± 300	3300 ± 500.0	3.6	<0.05
MTX	15.21 ± 5.6[b]	56.4 ± 27.9	3.7	<0.05
PAC	1.72 ± 0.84	5.86 ± 2.23	3.4	<0.05
GEM	1.87 ± 0.74	8.83 ± 1.51	4.7	<0.01
TPT	14.6 ± 3.9	24.6 ± 15.3	1.7	>0.05
DOX	20.5 ± 0.69	59.8 ± 1.72	2.9	<0.01
CDDP	683.7 ± 79.3	1115.01 ± 182.3	1.6	<0.05

[a] IC_{50} Values were statistically different ($P < 0.05$) between SF188 and SF188/TR cells for all drugs except TPT. Data are presented as the means ± SD of at least three individual experiments.
[b] IC_{20} Values are reported for MTX since SF188/TR cells can not reach the IC_{50} under the same conditions as the other drugs.

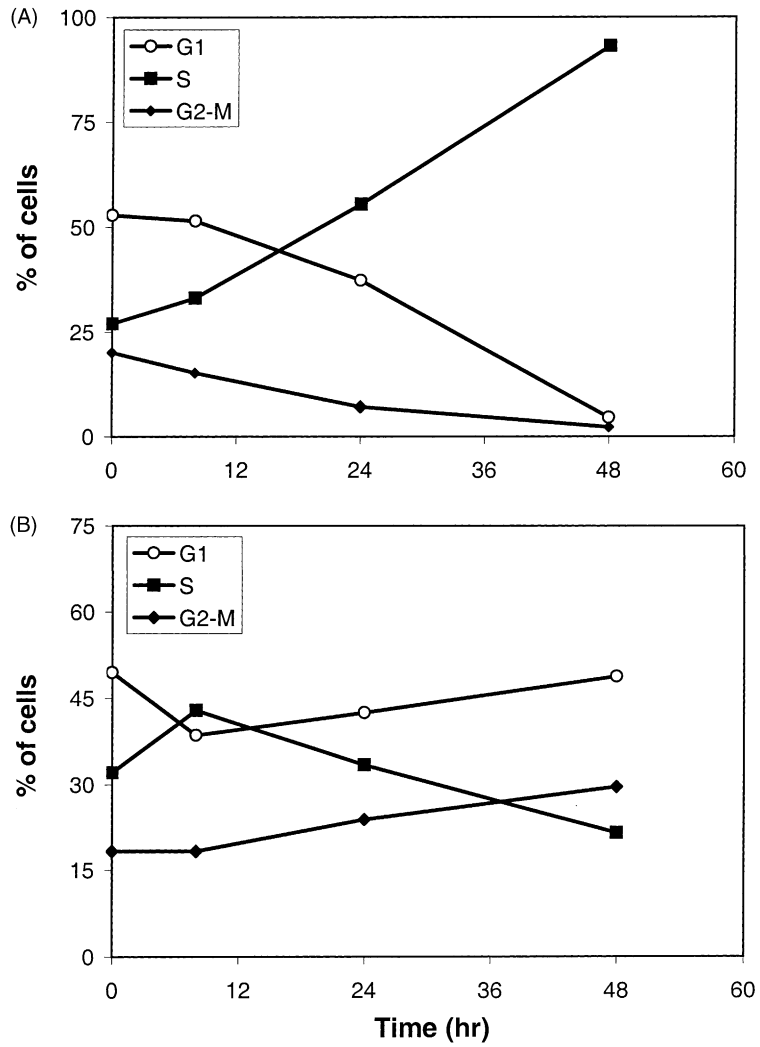

Fig. 1. Effects of 300 μg/mL of TMZ on SF188 (A) and SF188/TR (B) cell cycle distribution following different incubation times. Data represent one of two identical experiments.

3.5. Expression of mismatch repair protein hMLH1 and hPMS2 and induction of p53 and p21 by DOX

Resistance to methylating agents is frequently related to the alteration of mismatch repair protein expression [14–17]. Western blot analyses demonstrated that there were no changes in the expression of hMLH1 and hPMS2 between the sensitive and resistant cell lines (Fig. 4). This result indicates that the resistance of SF188/TR cells to TMZ is unlikely to be related to mismatch repair enzymes.

Analysis of the p53 status in the SF188 and SF188/TR cell lines revealed that both cell lines contain mutant p53, as indicated by immuno-reactivity with the mutant-specific conformational antibody pAb240 (Fig. 5). This observation was supported by a failure of the p53 protein in both cell lines to stabilize post-translationally in response to DNA damage, such as that caused by the anti-tumor drug DOX. Similarly, both cell lines failed to transcriptionally induce the p53-response gene $p21^{WAF1}$, in response to DNA damage (data not shown). This result indicated that the drug resistance in SF188/TR cells was not related to a loss of functional p53.

3.6. Expression of Bcl-2 family proteins

It was found that the steady-state expression levels of the pro-apoptotic proteins (Bad, Bax, and Bcl-X_S) were reduced 2–4-fold in the SF188/TR resistant cells compared with the parental cells. Expression of the anti-apoptotic proteins (Bcl-2 and Bcl-X_L) remained unchanged in both cell lines (Fig. 6). These results imply that a common apoptotic-related mechanism may have developed during the exposure to TMZ.

3.7. Modulation of TMZ resistance by O^6-BG

Based on the ability of O^6-BG to inactivate AGT, a number of clinical trials have been conducted in combination with alkylating agents [30,31]. Depletion of AGT by 20 μM O^6-BG did not influence the toxicities of MTX,

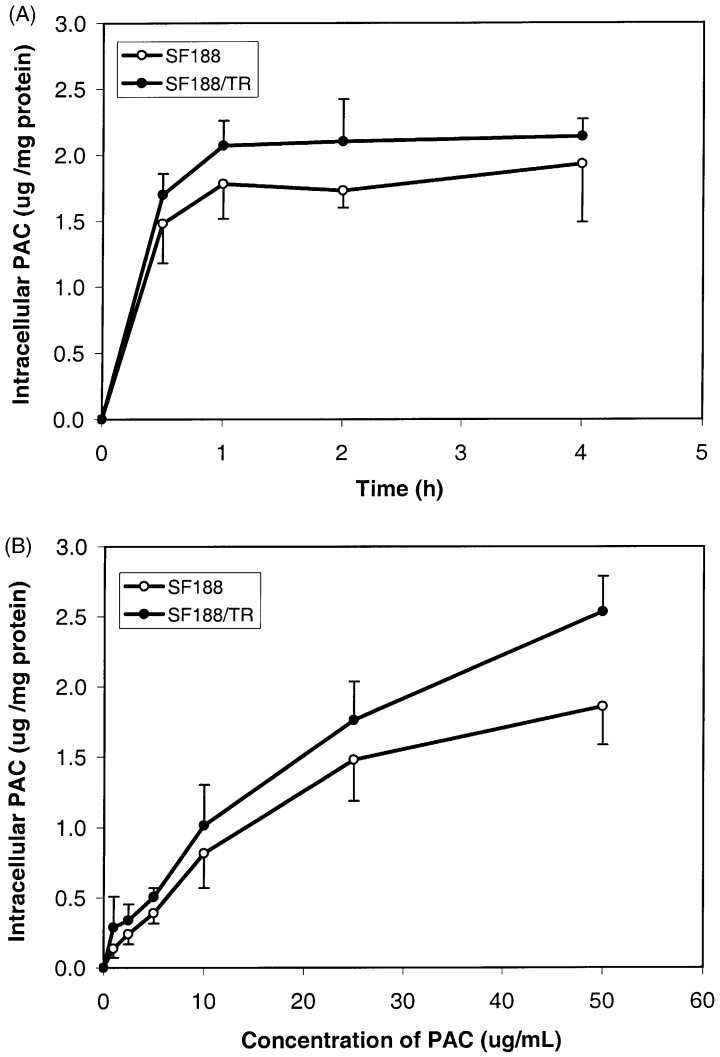

Fig. 2. Uptake of paclitaxel (PAC) in SF188 and SF188/TR cells. (A) Time-dependent uptake of PAC following incubation with 25 μg/mL of PAC; (B) concentration-dependent uptake of PAC following incubation for 2 hr. Data represent the means ± SD of at least three separate experiments.

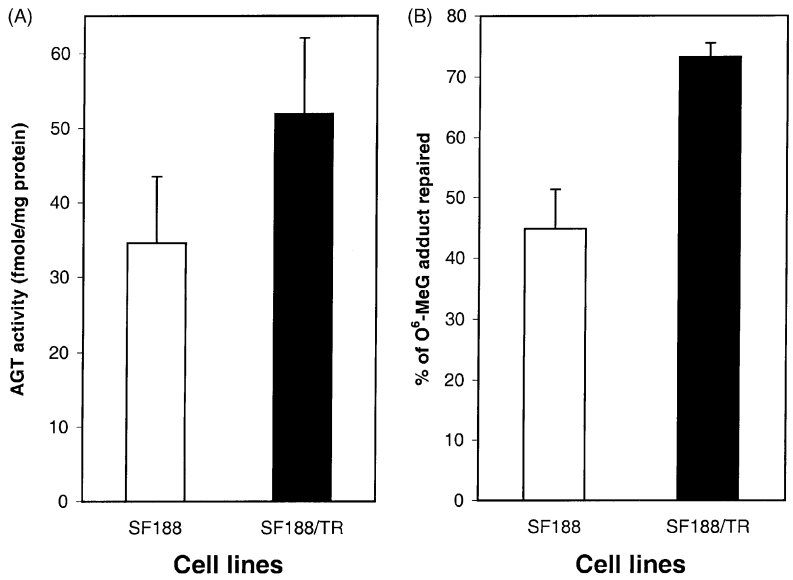

Fig. 3. Comparison of O^6-MeG AGT activity (A) and O^6-MeG DNA adduct repair (B) in SF188 and SF188/TR cells. In panel B, 200 μg of cellular protein was incubated with 16 μg of methylated DNA. Data represent the means ± SD of four experiments.

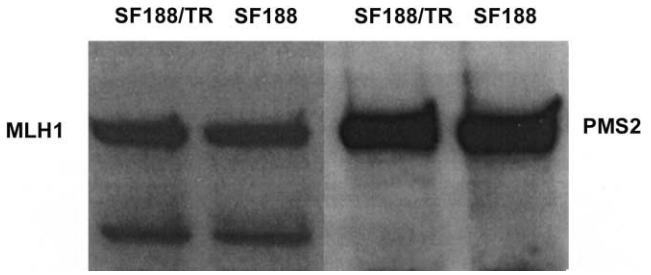

Fig. 4. Comparison of expression of mismatch repair proteins, hMLH1 and hPMS2, in SF188 and SF188/TR cells by western blot analysis.

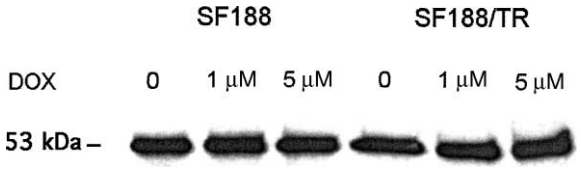

Fig. 5. Influence of DOX treatment on p53 expression in SF188 and SF188/TR cells. Cells were incubated with 1 and 5 μM DOX for 4 hr.

PAC, MEL, and GEM in either the SF188 or the SF188/TR cell line ($P > 0.05$), as expected. However, AGT depletion did increase TMZ activity significantly in both the SF188 and SF188/TR cell lines ($P < 0.01$), reducing the IC_{50} values by 20- and 40-fold, respectively (Fig. 7).

4. Discussion

TMZ produces mono-functional DNA adducts, either O^6-MeG or N^7-MeG adducts, with the former considered the lethal DNA lesion [10]. Tumor cell resistance to alkylating agents is generally mediated by DNA repair including AGT [6,7,10], excision repair [17], and mismatch repair system enzymes [14–16]. Although of less importance, other resistance pathways to nitrosoureas and nitrogen mustards may involve elevated levels of glutathione and/or glutathione-*S*-transferase [11–13]. AGT, a 16–28 kDa enzyme, is able to remove in a stoichiometric fashion O^6-MeG DNA adducts and transfer the methyl

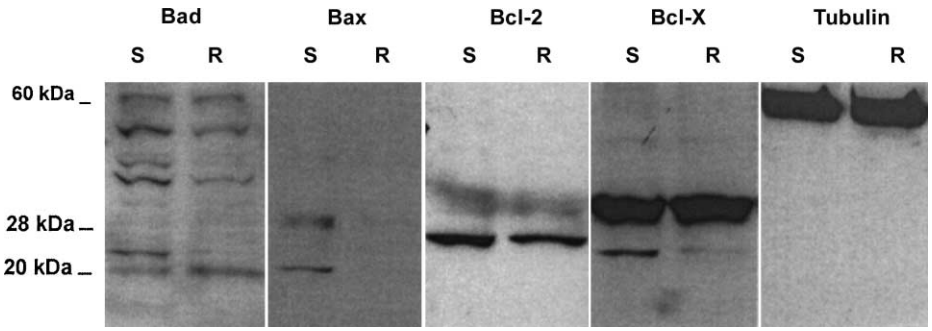

Fig. 6. Expression of Bcl-2 family proteins in SF188 (S) and SF188/TR (R) cells by western blot analysis. All proteins were detected on the same membrane by sequentially stripping and reprobing the blot. Each blot represents one of at least three identical experiments. Tubulin was used as a loading control.

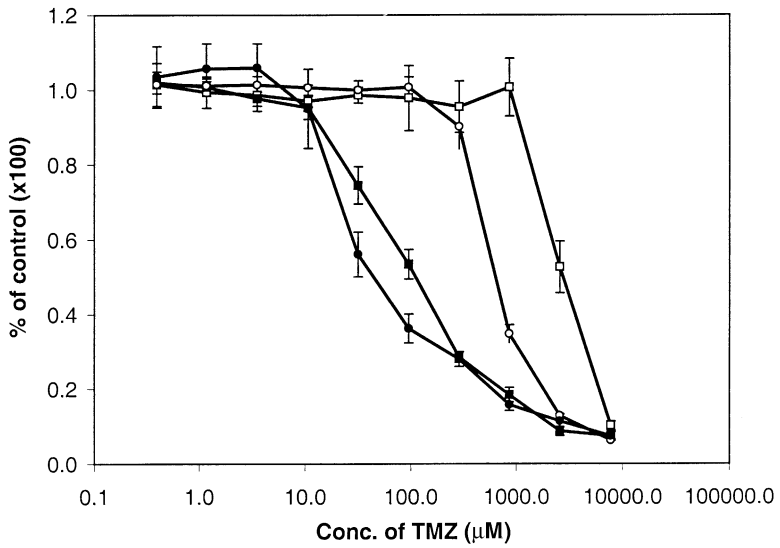

Fig. 7. Modulation of cytotoxicity of TMZ by O^6-BG. Cells were incubated with 20 μM O^6-BG for 4 hr prior to the addition of TMZ and then coincubated with TMZ for another 4 days. Cytotoxicity was evaluated by an SRB assay as described in Section 2. Data represent the means ± SD of at least three individual experiments, (□) SF188/TR without O^6-BG; (■) SF188/TR with O^6-BG; (○) SF188 without O^6-BG; and (●) SF188 with O^6-BG.

groups to cysteine residues. Tumor cells also may possess this protective action, rendering the cells resistant. In fact, tumor cell resistance to different types of alkylating agents, such as nitrosoureas, triazenes, and nitrogen mustards, has been correlated with cellular AGT levels, and many alkylation-resistant cell lines have shown increased concentrations of AGT or enhanced activity [6–9]. It was shown that compared with the TMZ-sensitive SF188 cell line, SF188/TR possessed elevated AGT activity. Consistent with the role of AGT in TMZ resistance, treatment of both SF188 and SF188/TR cells with O^6-BG sensitized the cells to TMZ but not to MEL, another alkylating agent that forms only the N^7 DNA adduct and subsequent DNA cross-links [32]. The resistance of SF188/TR cells to TMZ is caused apparently by increased AGT activity. The mismatch repair (MMR) system plays an important role in the control of genomic integrity in cells [15–17], and it has been reported [17] that cells with deficiencies in this system confer tolerance to methylating agents and other anticancer drugs, such as CDDP. CDDP-resistant cell lines show the loss of expression of MMR proteins, hPMS2 and hMLH1 [14]. The similar expression of hMLH1 and hPMS2 in SF188 and SF188/TR cells supports the existence of an intact MMR system and its unlikely role in TMZ resistance.

The ability of SF188/TR to exhibit cross-resistance to anticancer drugs with different mechanisms of action, including antimetabolites, an antimicrotubule agent, topoisomerase inhibitors, and other types of alkylating agents, suggested that there may be a common mechanism of resistance. Drug resistance can be related to alteration of drug transport, resulting in reduced intracellular drug accumulation. These membrane transport defects may be due to reduced uptake or enhanced drug efflux mediated by ATP-dependent efflux transporters, such as P-glycoprotein (MDR) or MRP [33,34]. The multidrug resistance associated with PAC and DOX is related to the overexpression of MDR1 or MRP1 transporters that limit their intracellular accumulation [35]. We found that PAC accumulation was similar in SF188 and SF188/TR cells, and expression levels of MRP1 were equivalent (data not shown), results that support the lack of involvement of membrane transport resistance pathways for PAC and DOX.

MTX is an S-phase cell cycle specific drug that may not reach maximum cytotoxicity within the 96-hr incubation period. Even though the distribution of cells in the different phases of the cell cycle was similar for both cell lines when treated (Table 1), there may have been a greater accumulation of sensitive than resistant cells in S phase after MTX treatment, which would parallel the results seem following treatment with TMZ (Fig. 1). The maximum cell kill attained with MTX was 60% in SF188 cells but only 25% in SF188/TR cells, which prevented calculation of the resistance factor based on the standard criteria of 50% cell kill. Calculation of the resistance factor of MTX at a 20% cell kill produced a resistance factor of 3.7, analogous to the values obtained for the other drugs.

To understand the mechanisms of resistance of SF188/TR cells to a broad range of anticancer drugs, we asked whether apoptosis could serve as a common cross-resistance mechanism. Apoptosis is a critical determinant of the control of cell number in physiological and pathological conditions [36–38], and is increasingly being studied in the context of drug resistance [3]. Anticancer drugs and radiotherapy can induce apoptosis of tumor cells through different signal transduction pathways that can be modulated by the Bcl-2 family of proteins or the p53 tumor suppressor protein [39,40]. The family of Bcl-2 proteins regulates the suppression (Bcl-2 and Bcl-X_L) and the promotion (Bax, Bad, and Bcl-X_S) of apoptosis by interacting with and functionally antagonizing each other. The pro-apoptotic proteins (Bad, Bax, and Bcl-X_S) can bind to either Bcl-2 or Bcl-X_L and are believed to form inactive heterodimer complexes [1,41,42] that inhibit caspase activation. The net balance in apoptotic-associated protein interactions could decide the fate of the cell and control the sensitivity of cells to anticancer drugs. SF188/TR cells showed a 2–4-fold reduced expression level of all the pro-apoptotic proteins, whereas the anti-apoptotic proteins were essentially unaltered. This shift in the balance or ratio of the pro- to anti-apoptotic factors could favor cell survival, and provide a common multidrug resistance mechanism for SF188/TR cells to escape the effects of diverse anticancer agents. Thus, it can be postulated that cells made resistant to a single drug undergo genotypic and phenotypic changes that produce both primary and secondary resistance pathways. In the case of SF188/TR cells, primary resistance to TMZ was due to overexpression of AGT, whereas secondary mechanisms of resistance were attributed to alterations in the pro- and anti-apoptotic expression profile.

In conclusion, a TMZ-resistant human glioma tumor cell line, SF188/TR, showed significant cross-resistance to a broad spectrum of anticancer agents. Under the assumption that acquired drug resistance produces secondary resistance pathways related to apoptotic protein expression, alternate drug treatment strategies may be clinically useful. Once primary drug resistance is established, possibly through multiple treatment cycles, institution of new cytotoxic drugs into the treatment plan may be beneficial. However, if these tumors have produced a secondary resistance through alteration of the apoptotic pathways, these agents will not be optimally effective. Thus, the addition of apoptotic protein modulators to the treatment regimen may increase the effectiveness of the cytotoxic drugs.

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