Sensitization of Pancreatic Tumor Xenografts to Carmustine and Temozolomide by Inactivation of Their O^6-Methylguanine-DNA Methyltransferase with O^6-Benzylguanine or O^6-Benzyl-2′-Deoxyguanosine[1]

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ABSTRACT

Adenocarcinoma of the pancreas is refractory to chemotherapeutic agents, including BCNU and streptozotocin. We have previously shown that drugs, which adduct the O^6-position of guanine, are ineffective against pancreatic tumor cell lines because of high expression of O^6-methylguanine-DNA methyltransferase (MGMT). The effect of MGMT inactivation on the resistance of pancreatic tumors to carmustine (BCNU) and to temozolomide (TMZ) was examined in five human pancreatic tumor xenografts in athymic mice. Tumor-bearing mice were treated: (*a*) with a single i.p. injection of BCNU or TMZ at the maximum-tolerated doses of 75 and 340 mg/m^2, respectively; and (*b*) with O^6-benzylguanine (BG) or O^6-benzyl-2′-deoxyguanosine (dBG) in combination with BCNU or TMZ. Pretreatment with the MGMT inactivators BG or dBG reduced the maximum-tolerated doses of BCNU and TMZ to 35 and 170 mg/m^2, respectively. MIA PaCa-2, CFPAC-1, PANC-1, CAPAN-2, and BxPC-3 having MGMT levels of 890, 1680, 680, 900, and 330 fmol/mg protein, respectively, were unresponsive to BCNU. MIA PaCa-2 and CFPAC-1 were also unresponsive to TMZ, whereas CAPAN-2 responded with a tumor delay of 32 days. BG or dBG sensitized all tumors to both BCNU and TMZ. BG plus BCNU treatment of MIA PaCa-2, CFPAC-1, PANC-1, CAPAN-2, and BxPC-3 induced tumor delays of 18, 16, 12, 14, and 16 days, respectively. In comparison, dBG plus BCNU at doses that were equitoxic to BCNU plus BG yielded tumor delays of 30, 19, 16, 21, and 22 days, respectively. The pancreatic tumors tested displayed functional mismatch repair that, however, may not be always sufficiently restrictive to prevent mutations under alkylation stress. Treatments with either BCNU or TMZ resulted in some degree of mutation in recurring tumors with the exception of CAPAN-2, the only wt-p53 xenograft. dBG, a weak MGMT inactivator *in vitro* as compared with BG, was markedly more effective than the latter in enhancing the efficacy of BCNU against pancreatic tumor xenografts. Both BG and dBG also enhanced the efficacy of TMZ against pancreatic tumors, possibly because of the repression of MGMT, which cannot be achieved with TMZ treatments alone. These results suggest that pancreatic tumors, which are resistant to DNA alkylating agents, may be sensitized to such agents when pretreated with MGMT inactivators.

INTRODUCTION

The incidence of the carcinoma of the pancreas has markedly increased over the past several decades and ranks as the fifth leading cause of cancer death in the United States. Despite the high mortality rate associated with pancreatic cancer, its etiology is poorly understood (1). Cancer of the exocrine pancreas is seldom curable and has an overall survival rate of <4% (2). Although tumors localized at the head of the pancreas have the highest potential to be cured, this stage of disease accounts for <20% of cases. For those patients with localized disease and cancers <2 cm with no lymph node metastases and no extension beyond the capsule of the pancreas, complete surgical resection can yield 5-year survival rates in 18–24% of such patients (3). Improvements in imaging technology, including spiral computed tomographic scans, magnetic resonance imaging scans, positron emission tomographic scans, endoscopic ultrasound examination, and laparoscopic staging, can aid in the diagnosis and the identification of patients with disease that is not amenable to resection (4, 5). For patients with advanced cancers, the overall survival rate of all stages is <1% at 5 years with most patients dying within 1 year (6–9). This abysmal prognosis underscores the need for developing improved effective chemotherapies based on our developing understanding of the mechanisms of resistance of the ductal adenocarcinoma of the pancreas. Nevertheless, the results obtained with cytotoxic chemotherapy have been disappointing for >40 Phase II studies of many new agents and combinations (10–14). The unresponsiveness of pancreatic tumors to several single agents (12, 13) may reflect the frequent gene modification in pancreatic tumors

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(15), including the activation of *K-ras* oncogene (90%), inactivation of the tumor suppressors *p16/Rb* (>90%), *p53* (75%), and *SMAD4/DPC4* (>50%). Of particular interest is the resistance of pancreatic tumors to 2-chloroethylating nitrosoureas (16) and to streprozotocin, a methylating agent (17). Resistance to methylating agents seen in some tumors, even in the presence of MGMT[3] inactivators (18), may be because of the enhanced capacity of pancreatic tumors to strand break repair by effective base excision, nucleotide excision, recombination repair, or most likely the presence of defective MMR, which could allow cells to replicate damaged DNA and undergo mitosis (19, 20).

Here, we investigate the effect of MGMT inactivators on the efficacy of BCNU and TMZ against pancreatic tumor xenografts in the athymic mouse model. The aims of the experiments presented here are: (*a*) to establish that DNA cross-linking drugs are effective against pancreatic tumors once their resistance to such drugs is quenched; (*b*) to compare the efficacy of BCNU *versus* TMZ, an agent that has been suggested to deplete MGMT levels in certain tumors, as well as cause cytotoxic damage leading to apoptotic death; and (*c*) to optimize the efficacy of BCNU and TMZ by using more effective MGMT inactivators than BG, which has undergone Phase I clinical trials (21, 22) and is now being tested with BCNU in patients with gliomas (23).

MATERIALS AND METHODS

Chemicals and Animals. BCNU was purchased from Bristol-Myers Squibb (Princeton, NJ), TMZ was generously donated by Schering Plough, Inc. (Madison, NJ), BG and dBG were synthesized as described previously (24, 25); 4-week-old male BALB/c nu/nu athymic mice were purchased from Harlan Labs (San Diego, CA).

Tumor Cell Lines. Five established pancreatic cancer cell lines (MIA PaCa-2, CFPAC-1, PANC-1, CAPAN-2, and BxPC-3) were obtained from American Type Culture Collection (Manassas, VA). Capan-2 is a p53 wt cell line. MIA PaCa-2, CFPAC-1, PANC-1, and BxPC-3 express mutant p53 (26). All cell lines were grown and maintained in DMEM with high glucose.

Tumor Implantation and Treatment. s.c. tumors grew upon injecting 3–4 million cells in the flank of the athymic mice. Treatment was administered when tumors were between 180 and 200 mm^3. Animals were treated i.p.: (*a*) with a high dose (75 mg/m^2) of BCNU in 15% ethanol in water; (*b*) with BG (160 mg/m^2) or dBG (235 or 260 mg/m^2) dissolved in 40% polyethylene glycol in PBS followed by BCNU (35 mg/m^2) in 15% ethanol in water 2 or 4 h after BG or dBG, respectively; (*c*) with a high dose (340 mg/m^2) of TMZ in DMSO; (*d*) with BG (160 mg/m^2) or dBG (260 mg/m^2) dissolved in 40% polyethylene glycol in PBS followed by TMZ (170 mg/m^2) in DMSO 2 or 4 h after BG or dBG, respectively. Tumors were allowed to grow until their volumes exceeded five times the volume of the tumor at treatment. Tumors were harvested and sampled for performing MIN assays and for histology. Toxicity of treatment was evaluated by determining the percent weight loss in treated mice. Regression was defined as the loss of at least 10% tumor volume after treatment. Growth delay was the difference between the median time to five times treatment volume in the treatment group minus the median time to five times treatment volume in the control group. All animals were killed either when their tumor size quintupled or at 90 days after treatment if tumors regressed and did not regrow.

MGMT Activity in Tumor Xenografts. MGMT was determined in tumor xenografts implanted s.c. in male athymic mice and grown to a size of 300–400 mm^3. The activity was determined in tumors of untreated animals and also at various time intervals after treatment with either TMZ, BG, or dBG, as indicated. The procedures for tissue handling and for MGMT determination in pancreatic tumors have been described previously (18).

MIN Analysis. The incidence of mutations in terms of MIN was determined by detecting length polymorphisms at different chromosomal loci in treated tumor DNA that are not present in untreated tumor DNA (27). The incidence of MIN was evaluated based on the extent of instability in the following loci: D2S123; D2S136; D3S1067; D5S107; D6S87; and D18S34. The sequences of MIN primers have been reported elsewhere (26–28). Briefly, the 5′ and 3′ primers of each locus were radiolabeled by the 5′-end labeling method, using [^{32}P]γ-ATP and T_4 polynucleotide kinase and amplified by PCR and electrophoresed in long-range urea gel (AT Biochem, Inc.). The radiolabeled PCR products were detected by autoradiography. Variations in terms of extra bands, shifts, or loss when compared with untreated DNA counterpart were designated as MIN for a particular chromosomal locus. A tumor was marked positive for MIN only when at least four of five xenografts tested (per treatment group) showed a positive pattern of MIN in at least one of the loci tested. For each analysis, xenografts were sampled in three different areas.

Colony-forming Assay. For clonogenic cell survival studies, two different cell concentrations in quadruplet sets were used for each thioguanine dose. Cell lines were left untreated or exposed to 1–20 μM thioguanine. After incubation for ≥10 days, each flask was stained with crystal violet, and the colonies containing >50 cells were counted. The surviving fraction was calculated as a ratio of the number of colonies formed to the product of the number of cells plated and the plating efficiency. The curve was plotted using X-Y log scatter (δ Graph 4.0; Ref. 28).

Statistical Analysis. Tumor growth data were analyzed using the Wilcoxon rank-sum test, comparing the time from treatment to five times treatment volume in the treatment group minus the median time to five times treatment volume in the control group. Groups were compared with the two-tailed Fisher's exact test.

RESULTS

BCNU at its maximum-tolerated dose in athymic mice had little effect on the growth of human pancreatic tumor xenografts

[3] The abbreviations used are: MGMT, O^6-methylguanine-DNA methyltransferase; CNS, central nervous system; BG, O^6-benzylguanine; dBG, O^6-benzyl-2′-deoxyguanosine; MMR, mismatch repair; BCNU, carmustine or *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; MIN, microsatellite instability; TMZ, temozolomide; wt, wild type.

Table 1 Enhancement of efficacy of BCNU in human pancreatic xenografts in athymic mice with the MGMT inhibitors BG and dBG

Mice no.	Treatment (dose)[a]	Weight loss	Regression[b] %[c]	T to 5× (days)[d]	T-C (days)[e]
PANC-1 (MGMT = 680 fmol/mg protein)					
7	None	0.0		11 (8–14)	
12	BCNU (75)	3.7	1/12	14 (11–24)	3
9	BCNU (35)[f] BG (160)	15.1	3/9	23 (20–32)	12
10	BCNU (35) dBG (235)	5.3	3/10	22 (14–38)	11
11	BCNU (35) dBG (260)	13.3	6/11	27 (19–48)	16
MIA PaCa-2 (MGMT = 890 fmol/mg protein)					
10	None	0.0		14 (11–25)	
10	BCNU (75)	3.7	1/10	17 (11–24)	3
10	BCNU (35) BG (160)	15.1	5/10	32 (26–45)	18
10	BCNU (35) dBG (235)	5.3	4/10	28 (22–23)	14
10	BCNU (35) dBG (260)	13.3	7/10	41 (31–49)	30
BxPC-3 (MGMT = 330 fmol/mg protein)					
10	None	0.0		9 (7–12)	
10	BCNU (75)	3.4	0/10	16 (9–22)	6
10	BCNU (35) BG (160)	16.4	3/10	25 (19–31)	16
10	BCNU (35) dBG (235)	6.7	2/10	24 (17–24)	15
10	BCNU (35) dBG (260)	16.3	4/10	31 (19–66)	22
CAPAN-2 (MGMT = 620 fmol/mg protein)					
10	None	0.0		17 (17–21)	
10	BCNU (75)	7.6	0/10	18 (15–19)	1
10	BCNU (35) BG (160)	18.2	3/10	31 (23–41)	14
10	BCNU (35) dBG (260)	16.4	3/10	38 (33–40)	21
CFPAC-1 (MGMT = 1680 fmol/mg protein)					
10	None	0.0		24 (18–26)	
10	BCNU (75)	4.6	0/10	20 (17–25)	−4
14	BCNU (35) BG (160)	16.9	4/14	40 (27–55)	16
10	BCNU (35) dBG (235)	4.7	2/10	30 (22–35)	6
10	BCNU (35) dBG (260)	16.4	6/10	43 (38–60)	19

[a] mg/m^2.
[b] Reduction of tumor volume by ≥10%.
[c] Average weight loss after treatment.
[d] Time for tumor to quintuple from its treatment size of 180–200 mm^3.
[e] Tumor delay.
[f] BCNU was administered 2 h after BG and 4 h after dBG.

(Table 1). This was probably because of high levels of MGMT activity present in these tumors. Depletion of MGMT with BG, 2 h before treatment with BCNU, resulted in a significant response of all tumor xenografts tested, with tumor growth delays ranging from 12 to 18 days. Tumor growth delays significantly increased ($P < 0.05$) in MIA PaCa-2, CAPAN-2, and BxPC-3 to 30, 21, and 22 days, respectively, when dBG was used instead of BG to suppress MGMT activity.

The response of pancreatic tumor xenografts to TMZ is shown in Table 2. TMZ alone had negligible activity against MIA PaCa-2 and CFPAC-1, which had the highest levels of MGMT among the tumors tested. However, it significantly slowed the growth of CAPAN-2 and PANC-1 inducing tumor growth delays of 32 and 13 days, respectively. Although this dose of TMZ reduced the MGMT activity in all of the pancreatic xenografts tested, it did not completely eliminate MGMT activity as effectively as BG or dBG (Fig. 1). A significant ($P < 0.05$) increase in the efficacy of TMZ by BG and especially by dBG against MIA PaCa-2, CFPAC-1, and CAPAN-2 xenografts with only marginal increase in its efficacy in PANC-1 tumors demonstrates the effectiveness of MGMT inactivation in reducing the resistance of tumors with high (>600 fmol/mg protein) to TMZ.

The effectiveness of TMZ to override MGMT resistance is because of partial inactivation of the tumor MGMT after treatment. A single treatment of animals bearing pancreatic tumor xenografts with 340 mg/m^2 TMZ resulted in loss of a fraction of the tumor MGMT. MGMT reduction varied from 300 to 500 fmol/mg protein, depending on the initial MGMT activity in the tumor (Fig. 1). The benefit of inactivating MGMT before treatment with TMZ is also shown in Fig. 1, in which BG and dBG not only caused complete suppression of the tumor MGMT, but also maintained its suppression for considerably longer time intervals. BG suppressed MGMT activity to below 10 fmol/mg protein 2 h after treatment, whereas comparable suppression was obtained with dBG at 4 h after treatment. Although both inactivators kept MGMT levels < 10 fmol/mg protein, MGMT recovered faster in BG than in dBG-treated pancreatic tumors.

MIN was examined in tumor xenografts that quintupled in volume after treatment with either BCNU (Table 3) or TMZ (Table 4) with or without MGMT inactivators and also in comparable volume control (untreated) xenografts. BCNU treatments induced alterations, resulting in the appearance of the unstable microsatellite phenotype in MIA PaCa-2 (one of six loci), CFPAC-1 (one of six loci), and BxPC-3 (five of six loci). A low incidence of MIN was observed in CFPAC-1 when this tumor was treated with dBG plus BCNU. A much greater incidence of instability (>80%) was found in BxPc3 tumors

Table 2 Enhancement of efficacy of TMZ in human pancreatic xenografts in athymic mice with the MGMT inhibitors BG and dBG

Mice no.	Treatment (dose)[a]	Weight loss	Regression[b] %[c]	T to 5× (days)[d]	T-C (days)[e]
PANC-1 (MGMT = 680 fmol/mg protein)					
10	None	0.0		11 (8–14)	
10	TMZ (340)[f]	8.6	3/10	24 (18–33)	13
10	TMZ (170), BG (160)	8.5	3/10	26 (21–27)	15
10	TMZ (170), dBG (260)	5.3	4/10	28 (24–36)	17
MIA PaCa-2 (MGMT = 890 fmol/mg protein)					
10	None	0.0		14 (11–25)	
10	TMZ (340)	10.9	4/10	18 (16–27)	4
10	TMZ (170), BG (160)	9.0	4/10	23 (20–33)	9
10	TMZ (170), dBG (260)	11.6	4/10	23 (20–31)	9
CAPAN-2 (MGMT = 620 fmol/mg protein)					
10	None	0.0		22 (16–26)	
10	TMZ (340)	12.3	9/10	54 (42–66)	32
10	TMZ (170), BG (160)	9.9	9/10	66 (32–88)	44
10	TMZ (170), dBG (260)	10.6	10/10	78 (66–94)	56
CFPAC-1 (MGMT = 1680 fmol/mg protein)					
10	None	0.0		19 (18–22)	
10	TMZ (340)	11.9	4/10	21 (19–28)	3
14	TMZ (170), BG (160)	9.9	7/14	32 (25–33)	13
10	TMZ (170), dBG (260)	10.6	5/10	36 (24–45)	17

[a] mg/m^2.
[b] Reduction of tumor volume by ≥10%.
[c] Average weight loss after treatment.
[d] Time for tumor to quintuple from its treatment size of 180–200 mm^3.
[e] Tumor delay.
[f] TMZ was administered 2 h after BG and 4 h after dBG.

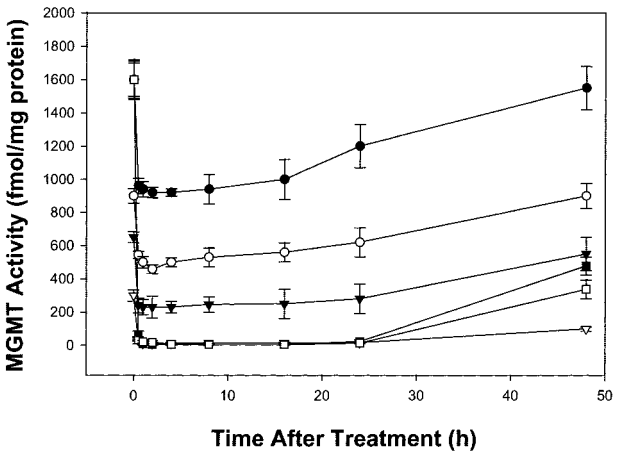

Fig. 1 Effect of TMZ, BG and dBG treatment on levels of MGMT activity at various time intervals after treatment. TMZ was administered i.p. at 340 mg/m^2 in athymic mice bearing CFPAC-1 (●), MIA PaCa-2 (○), PANC-1 (▲), and PxPC-3 (■) pancreatic tumor xenografts of approximate volume of 300–400 mm^3. BG (□) and dBG (△) were injected i.p. at 160 and 260 mg/m^2 in athymic mice bearing CFPAC-1 tumors. Animals (three/time point) were killed at the time intervals indicated, tumors were removed, and MGMT activity was determined. Each point represents the mean of three determinations ± SD.

treated with BCNU, but no detectable alterations were observed when MGMT was inactivated before BCNU treatment. No apparent changes in instability or loss of heterozygosity were observed in any of the loci tested in PANC-1 and CAPAN-2 as a result of BCNU treatment. TMZ induced MIN in MIA PaCa-2 (three of six loci) and PANC-1 (two of six loci) tumor xenografts under all of the treatment regimens tested. TMZ treatment with or without MGMT inhibition caused no changes in CFPAC-1 and CAPAN-2.

It is not clear whether the observed MIN in regrown tumor xenogbrafts treated with alkylating agents is attributable to lack of MMR in the implanted tumors. To ascertain this, we performed thioguanine clonogenic assays using pancreatic cancer cell lines. Cell lines of the most mutable tumor xenografts MIA PaCa-2 and PANC-1 have a significantly lower resistance to TG than the MMR-defective line LoVo (Fig. 2). However, resistance of the pancreatic cell lines was markedly greater than that of brain tumor neoplasms reported previously (29). These findings suggest that MIN observed in treated xenografts could arise from the selection of MMR-defective cell populations in the tumor by the treatment. However, additional investigation of the MMR response of pancreatic tumors is required to understand the acquisition of further mutations in tumors treated with TMZ.

DISCUSSION

Five pancreatic tumor xenografts were tested for resistance to the DNA cross-linking agent BCNU and to the methylating agent TMZ in the athymic mouse model. Levels of MGMT, a protein that repairs O^6-alkylguanine adducts that are essential for the cytotoxic action of these two drugs, ranged between 330 and 1680 fmol/mg of protein. MGMT activity > 600 fmol/mg protein completely inhibited the antitumor potential of the highest BCNU dose tolerable by athymic mice, but such inhibition was prevented by MGMT inactivation before BCNU treatment. Tumor response to combined BCNU/MGMT inactivator treatment varied depending on the inactivator and its dose, the time interval between inactivator and BCNU treatment, and the ge-

Table 3 Microsatellite instability in human pancreatic xenografts before and after treatment with BCNU alone and with BCNU after inactivation of MGMT with BG or dBG

Cell lines	D2S123 2	D2S136 (2p13-p14)	D3S1067 (3p14.3-p14)	D5S 107 (5q11.2-q13.3)	D6S87 (6q)	D18 S34 (18q12.2-q22.3)
PANC-1/control	−	−	−	−	−	−
BCNU	−	−	−	−	−	−
dBG/BCNU	−	−	−	−	−	−
BG/BCNU	−	−	−	−	−	−
CFPAC-1/control	−	−	−	−	−	−
BCNU	−	−	−	−	−	−
dBG/BCNU	−	−	+	−	−	−
BG/BCNU	−	−	−	−	−	−
MIA PaCa-2/control	−	−	−	−	−	−
BCNU	−	−	+	−	−	−
dBG/BCNU	−	−	−	−	−	−
BG/BCNU	−	−	+	−	−	−
BxPC-3/control	−	−	−	−	−	−
BCNU	+	+	+	−	+	+
dBG/BCNU	−	−	−	−	−	−
BG/BCNU	−	−	−	−	−	−
CAPAN-2/control	−	−	−	−	−	−
BCNU	−	−	−	−	−	−
BG/BCNU	−	−	−	−	−	−

Table 4 Microsatellite instability in human pancreatic tumor xenografts before and after treatment with TMZ alone and with TMZ after inactivation of MGMT with BG or dBG

	Chromosomal loci analyzed					
Cell lines	D2S123 2	D2S136 (2p13-p14)	D3S1067 (3p14.3-p14)	D5S107 (5q11.2-q13.3)	D6S87 (6q)	D18S34 (18q12.2-q12.3)
MIA PaCa-2						
Untreated	−	−	−	−	−	−
TMZ	+	−	−	−	−	−
dBG/TMZ	−	+	+	−	−	−
BG/TMZ	−	+	+	−	−	−
PANC-1						
Untreated	−	−	−	−	−	−
TMZ	−	−	+	−	−	+
dBG/TMZ	−	−	+	−	−	+
BG/TMZ	−	−	+	−	−	+
CFPAC-1						
Untreated	−	−	−	−	−	−
TMZ	−	−	−	−	−	−
dBG/TMZ	−	−	−	−	−	−

netic background of the tumor. The optimum treatment schedule was achieved when BG or dBG were administered 2 and 4 h, before BCNU, respectively. dBG was more effective than BG in enhancing the efficacy of BCNU. The optimal doses for combining dBG and BCNU were 260 and 35 mg/m^2, respectively. Escalation of either dose caused occasional deaths, whereas decreasing the dose of either drug resulted in a steep decline of the efficacy of the treatment. Equimolar doses of BG and dBG enhanced the efficacy of BCNU against pancreatic tumors similarly, but the combination of BG plus BCNU was more toxic than that of dBG plus BCNU. Because of the difference between BG and dBG in affecting BCNU toxicity, the dBG dose could be escalated and thus additionally increase the efficacy of BCNU beyond the level that could be achieved with BG. Therefore, at equitoxic responses, dBG was markedly more effective than BG in enhancing the antitumor efficacy of BCNU *in vivo*. Response to BCNU also varied depending on the tumor. Most responsive were MIA PaCa-2 and CFPAC-1, whereas PANC-1 was the most resistant. There was no correlation between p53/p21 or K-ras status of the tumor and the resistance to BCNU under MGMT-inactivating conditions.

Unlike BCNU, TMZ at its maximum-tolerated dose of 340 mg/m^2 had activity against PANC-1 and especially CAPAN-2 without the aid of MGMT inactivators. In addition, its activity was enhanced when animals were treated with either BG or dBG before treatment with a reduced dose of TMZ of 170 mg/m^2. An impressive response was seen after treatment of the p53-proficient tumor CAPAN-2 with dBG plus TMZ; the treatment resulted in a sustained regression in 9 of 10 xenografts and in the complete regression of three tumors for a period of at least 90

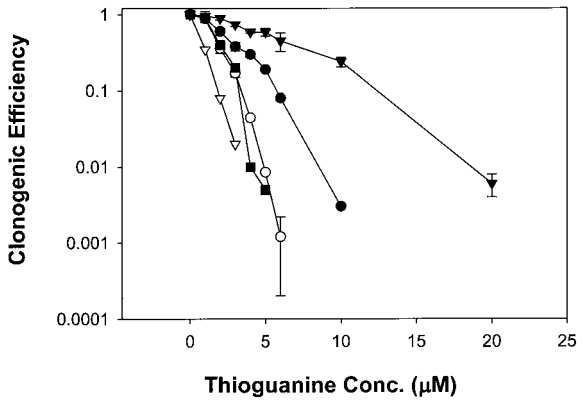

Fig. 2 Resistance of pancreatic tumor cell lines to thioguanine. The pancreatic cell lines MIA PaCa-2 (●), PANC-1 (○), CFPAC-1 (■), and CAPAN (▽) are compared with the MMR-defective line LoVo (▼, positive control). Cells were treated with various concentrations of thioguanine, and the surviving fraction was determined by a clonogenic assay.

days. On the other hand, MIAPaCa-2, a p53- and K-ras-mutated tumor, was the least responsive to TMZ. Unlike BCNU treatments in which a clear advantage of dBG over BG was demonstrated, BG was nearly as effective as dBG in enhancing the efficacy of TMZ. It is postulated that the cytotoxicity of the O^6-methylguanine adduct is manifested at much longer time than the 18 h needed for the completion of formation of the DNA cross-links by BCNU, which defines the cytotoxicity of this agent (30). Therefore, although dBG and BG differ in their efficacy to suppress MGMT for a time interval that is within the range of BCNU-induced DNA cross-link formation, their action in allowing persistence of O^6-methylguanine, which apparently leads to apoptosis, appears to be comparable.

Comparison between BCNU and TMZ shows that under MGMT inactivating conditions the two agents have nearly the same efficacy against PANC-1. BCNU is more effective than TMZ against MIA PaCa-2 and CFPAC-1, whereas TMZ is more effective against CAPAN-2. Because CAPAN-2 is the only tumor with a wt-p53, it is suggested that TMZ and perhaps other methylating agents could be more effective against tumors retaining a G_1-S cell cycle check point as it has been previously demonstrated with central nervous system tumors (29). This observation is of interest in that TMZ plus dBG could be combined with restoration of p53 function in a potentially effective treatment for pancreatic cancers. Restoration of p53 in tumors has been demonstrated with gene therapy technology (31, 32). More interestingly, pharmacological rescue of the p53 function can be accomplished either directly by stabilizing mutant p53 (33) or indirectly by activating a p53-dependent G_1 arrest via the stimulation of WAFp21 and of apoptotic pathways (34). Additional studies using isogenic pancreatic cell lines and xenografts, differing in their p53 status, are required to verify the significance of a functional p53 pathway in the resistance of pancreatic tumors to TMZ.

The limited efficacy of TMZ alone against pancreatic cancers may be attributable to the ability of this agent to yield high levels of methylation at the O^6 position of guanine that inactivate MGMT. Moderate levels of MGMT such as those found in primary brain tumors are expected to repair only a fraction of this cytotoxic adduct. However, in most pancreatic neoplasms, O^6-methylguanine formed by TMZ is expected to be effectively repaired by high levels of tumor MGMT. CFPAC-1, which has the highest level of MGMT of all tumors tested, retains more than half of its overall MGMT activity after a single treatment with 340 mg/m^2 TMZ. A greater fraction of MGMT is lost in MIA PaCa-2 and PANC-1 after TMZ treatment, but even then these tumors retain their capacity to repair O^6-alkylguanine adducts. It is suggested that TMZ may have an effect on such tumors by inactivating the MGMT activity in nonuniform manner within the tumor. For example, the tumor core may sustain less damage to a systemic agent such as TMZ, which is subsequently fully repaired by its MGMT reserves. In contrast, vascular regions may be exposed to high enough levels of TMZ to cause DNA damage that can completely saturate local MGMT.

It is interesting to note that the pancreatic tumors we examined showed evidence of genetic instability as determined by microsatellite assays after they were treated with either BCNU or TMZ. Although there was no evidence of gross genomic rearrangements or loss of heterozygosity in the loci examined, genomic instability could be detected in regrown tumors as a result of alkylation stress. The most responsive to the induction of an unstable phenotype was the line BxPC-3, which was affected by BCNU alone but not by the combination of BCNU plus MGMT inhibitors. This suggests that the tumor may have been mutated as a result of damage that is unrelated to the persistence of O^6-alkylguanine, because this adduct is expected to be effectively repaired by the high levels of MGMT in the tumor. MIA PaCa-2 was also altered to yield an unstable phenotype by the action of BCNU and BG/BCNU, however, such instability affected only one-third of the recurring tumors. A lower fraction of the tumor was altered in CFPAC-1 after treatment with dBG plus BCNU. No alterations were observed in PANC-1 or CAPAN-2. Overall, there was no correlation between induction of genetic instability and response to treatment, indicating that instability and cytotoxicity, even if induced by the same DNA modification by the alkylating agent, were mediated by pathways subject to control by the genetic characteristics of each tumor. Treatments with TMZ induced an unstable phenotype in PANC-1 and MIA PaCa-2, but no instability was observed in CFPAC-1 and CAPAN-2, which were responsive to TMZ in combination with MGMT inactivators.

The results presented here demonstrate that both BCNU and TMZ are effective against pancreatic tumors when they are combined with MGMT inactivators. Although the number of tumors is too small to draw a general conclusion, the studies suggest that pancreatic tumors with wt-p53 may be susceptible to TMZ once their MGMT is inactivated. Genomic instability is induced during treatment with alkylating agents, which may lead to a more aggressive genotype that would be refractive to additional treatment. However, such a response was not confined only to methylating agents such as TMZ as has been previously predicted (30) but also to BCNU treatment. The use of TMZ in the treatment of pancreatic cancers should not be restricted on the basis that as a methylating agent it presents a greater risk in inducing mutations than cross-linking alkylating agents. The mechanisms of BCNU- and TMZ-mediated cyto-

toxicity and mutagenicity in pancreatic cancers requires additional investigation.

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