Enhanced cytotoxicity of bioreductive antitumor agents with dimethyl fumarate in human glioblastoma cells

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We compared the cytotoxicity of the bioreductive antitumor agents mitomycin C (MMC) and streptonigrin (SN) with or without the DT-diaphorase (DTD) inducer dimethyl fumarate (DMF) in four human glioblastoma cell lines with the conventional chemotherapeutic agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). We also examined four other types of cancer cells to compare with glioblastoma cells. Cytotoxicity was measured with the sulforhodamine B (SRB) assay and was represented by 50% inhibition concentration (IC_{50}). Enzymatic activities of DTD, cytochrome b_5 reductase and glutathione-S-transferase (GST) in cells were measured spectrophotometrically. IC_{50} for BCNU was in a range of 28–300 μM in the glioblastoma cell lines. Glioblastoma cells were more sensitive to MMC or SN than to BCNU. Pretreatment with DMF significantly increased cytotoxicity of MMC and SN in glioblastoma cell lines and the NCI-H1299 lung cancer cell line, but had no effect on BCNU cytotoxicity. DMF significantly increased DTD and cytochrome b_5 reductase activity, and decreased GST in three of four glioblastoma cell lines. Addition of the DTD inhibitor, dicumarol, significantly inhibited cytotoxicity of MMC and SN, and reversed the increased cytotoxicity seen when DMF was combined with either MMC or SN in all glioblastoma cell lines. Combining inducers of DTD and cytochrome b_5 reductase with bioreductive agents may be a potential therapeutic strategy for glioblastoma. *Anti-Cancer Drugs* **16:167–174 © 2005 Lippincott Williams & Wilkins.**

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Introduction

Glioblastoma multiforme is the most common malignant neoplasm in the central nervous system (CNS). Primary brain tumors represent only *2%* of the estimated new cases of cancer occurring in adults annually in the US, but glioblastoma is among the most lethal and difficult to treat [1]. The most commonly used chemotherapy drugs have been alkylating agents, particularly the nitrosoureas. Although successfully used in some tumors to control growth or reduce tumor burden, their benefit in the treatment of malignant glioma is short-lived at best due to either *de novo* resistance or the rapid generation of acquired resistance of tumor cells [2,3]. Much of the ongoing research in CNS tumors is designed to find either a new chemotherapy strategy [4–6] or to improve delivery of currently available drugs to tumor cells [7,8].

A class of antitumor agents called bioreductive agents requires reductive activation by intracellular enzymes to function. These drugs are generally used to treat solid tumors as they are often more toxic under hypoxic conditions [9–13]. DT-diaphorase (DTD) named in 1960 from its reactivity with NADH and NADPH (at that time DPNH and TPNH) [16], also called NAD(P)H:quinone reductase (EC 1.6.99.2), is a highly inducible enzyme that activates bioreductive antitumor agents such as mitomycin C (MMC) and streptonigrin (SN) [14–16]. DTD can be induced in many tissues by a variety of chemicals, including quinones, isothiocyanates and diphenols, many of which are dietary components. Okamura *et al.* showed that glioblastoma cell lines expressed higher DTD activity than cell lines from other tumor types [17]. NADH:cytochrome b_5 reductase is potentially another important enzyme required for the reductive activation of MMC [18] and other bioreductive antitumor drugs [19].

Wang *et al.* showed that dimethyl maleate and dimethyl fumarate (DMF) were the most potent inducers of DTD in human tumor cells [20]. DMF has several favorable characteristics for use as a chemotherapy enhancer. It is a monofunctional rather than bifunctional inducer. This minimizes the potential complications of carcinogen activation through the induction of certain P-450 cytochromes. DMF has low toxicity and raises phase II enzyme levels in multiple tissues, thereby enhancing the potential for protecting a variety of organs [21]. Therefore, DMF as a potent non-toxic inducer of DTD may enhance the antitumor efficacy of bioreductive antitumor agents in glioblastoma. To develop a new chemotherapeutic strategy for glioblastoma, we compared the

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cytotoxicity of MMC and SN with or without the DTD inducer DMF in glioblastoma cell lines and four cell lines of other tumor types. Cell exposure to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a clinically employed conventional agent, was used as a basis of comparison for cytotoxic effect.

Materials and methods

Cell lines

Human glioblastoma cell lines T98G, U-87MG, U-138MG and A172, fibrosarcoma cell line HT-1080, colon adenocarcinoma cell line HCT-8, breast adenocarcinoma cell line MCF-7, and non-small cell lung cancer cell line NCI-H1299 were obtained from ATCC (Manassas, VA). T98G, U-138MG, U-87MG, MCF-7 and HT-1080 cells were cultured in MEM with 2 mM L-glutamine and 0.1 mM NEAA, and A172 cells were cultured in DMEM with 4 mM L-glutamine and 4.5 g/l glucose. HCT-8 was cultured in RPMI 1640 medium. NCI-H1299 was cultured in RPMI 1640 with 2 mM L-glutamine, 4.5 g/l glucose and 10 mM HEPES. All media were supplemented with 1.5 g/l sodium bicarbonate (except for HCT-8), 1.0 mM sodium pyruvate and 10% fetal bovine serum (FBS), and the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Drugs

MMC, SN, dicumarol and pHMB (*p*-hydroxymercuribenzoic acid, sodium salt) were obtained from Sigma (St Louis, MO). MMC was dissolved in DMSO:PBS (1:1), SN and dicumarol were dissolved in DMSO. DMF, obtained from Aldrich (Milwaukee, WI), was dissolved in methanol.

Cytotoxicity assay

Cytotoxicity to MMC or SN and other drugs was determined by the sulforhodamine B (SRB) cytotoxicity assay [22] carried out in 96-well microtiter plates. Cells were trypsinized, washed with PBS, resuspended in medium and plated in triplicate wells (1000–2000 cells/well). Cells were exposed to increasing concentrations of: (i) BCNU alone for 2 h; (ii) MMC or SN for 2 h, (iii) DMF alone for 48 h. The highest non-toxic concentration of DMF was identified for each cell type after the 48-h exposure as the highest concentration which did not affect cell survival. It was 30 μM for T98G, U-87MG, MCF-7 and HCT-8 cell lines, 20 μM for NCI-H1299, U-138MG and A172 cell lines, and 10 μM for HT-1080 cell line (Table 1).

MMC and SN were combined with DMF individually and cytotoxicity was assessed by SRB assay. Cells were exposed to the highest non-toxic concentration of DMF for 48 h followed by 2-h exposure to either MMC or SN. DMF was also combined with BCNU in glioblastoma cell lines as a control.

Dicumarol is a potent inhibitor of DTD [23]. It was combined with DMF plus MMC or SN to determine whether dicumarol could reverse the enhanced cytotoxicity achieved when DMF was added to the chemotherapeutic agents. Each cell line was exposed to: (i) DMF at the highest non-toxic concentration for 48 h followed by 50 μM dicumarol for 30 min and then MMC or SN for 2 h; (ii) dicumarol 50 μM for 30 min and then MMC or SN for 2 h. The SRB assay was then performed.

After drug exposure the cells were incubated with fresh drug-free medium for 3–5 days. Cells were fixed with 40% trichloroacetic acid solution for 1 h and 0.4% SRB was added to each well. After a 30-min incubation, the plates were washed with 1% acetic acid and read at 570 nm on a Biowhittaker microplate reader 2001. The wells with cells containing no drug and with medium plus drug but no cells were used as positive and negative controls, respectively. Dicumarol 50 μM alone for 2 h and DMF alone for 48 h had no effect on cell viability. IC_{50} was estimated from the dose–response curve.

Enzyme assay

DTD activity

The DTD activities were determined by measuring the rate of the NADPH-dependent menadiol-mediated reduction of MTT at 610 nm, as described by Prochaska and Santamaria [24]. Briefly, the cells were grown in duplicate 96-well microtiter plates for 24 h and then

Table 1 DMF cytotoxicity in human tumor cell lines

DMF (μM)	Tumor cell survival (%)							
	T98G	U-87MG	U-138MG	A172	HCT-8	MCF-7	HT-1080	H-1299
0.1	100 ± 5.1	100 ± 3.4	100 ± 3.5	100 ± 4.3	100 ± 3.5	100 ± 4.4	100 ± 4.5	100 ± 4.5
1	106 ± 3.4	98 ± 3.5	99 ± 4.1	99 ± 3.4	99 ± 3.5	100 ± 4.2	100 ± 4.3	100 ± 4.7
5	108 ± 4.6	106 ± 4.1	99 ± 3.6	99 ± 3.8	99 ± 5.1	101 ± 3.5	100 ± 3.8	101 ± 3.5
10	105 ± 4.3	105 ± 3.2	99 ± 3.7	99 ± 4.3	99 ± 4.5	99 ± 3.8	**99 ± 4.7**	100 ± 3.6
20	104 ± 5.2	103 ± 3.3	**98 ± 4.2**	**98 ± 4.6**	99 ± 4.2	99 ± 4.3	79 ± 5.3	**99 ± 3.8**
30	**101 ± 4.4**	**96 ± 3.9**	67 ± 3.8	50 ± 5.1	**99 ± 5.7**	**99 ± 4.1**	57 ± 4.2	77 ± 4.7
50	58 ± 3.9	63 ± 5.4	30 ± 5.1	28 ± 4.2	71 ± 5.6	73 ± 3.9	32 ± 3.5	66 ± 5.1
100	31 ± 4.2	38 ± 4.3	18 ± 4.3	11 ± 3.1	27 ± 4.3	30 ± 5.2	15 ± 2.1	45 ± 4.2

All cell lines were exposed to DMF for 48 h. Results are means ± SD of cell survival (%) for five separate experiments. The highest non-toxic concentration of DMF used in all subsequent experiments for each cell line is in bold.

treated for 48 h with DMF at highest non-toxic concentrations indicated in cytotoxicity assay. The assays were conducted at 25°C in 3.0-ml systems containing: 25 mM Tris–HCl (pH 7.4), 50 μM menadione in 10 μl of acetonitrile, 0.75 mM MTT, 30 μM NADP, 5 μM flavin-adenine dinucleotide, 1 mM glucose-6-phosphate, 5 U of yeast glucose-6-phosphate dehydrogenase, 0.07% bovine serum albumin and 0.01% Tween 20 [20]. The DTD activity was determined in cell lysates on one plate and related to the cell density determined on the second of each pair of plates stained with crystal violet. Protein concentrations were determined according to the method of Bradford [25]. DTD activity was expressed as nmol/min/mg protein.

GST activity

For measurement of total glutathione-S-transferase (GST) activity [17,26], trypsinized cells washed with PBS and resuspended in 500 μl of 1 mM Tris–HCl, pH 7.8, containing 0.2 M NaCl were sonicated and the solution was centrifuged at 10 000 *g* for 45 min at 4°C to collect the supernatant. The enzyme reaction was carried out in a 1-cm cuvette at 25°C. To give a final concentration of 1 mM, 80 mM 1-chloro-2,4-dinitrobenzene (CDNB) was diluted in 0.1 M potassium phosphate buffer, pH 6.5, after which sample and glutathione (GSH, 1.0 mM) were added. The optical density at 340 nm was monitored for at least 3 min at 30-s intervals. GST activity was calculated from the enzyme-dependent change in absorption. Protein concentrations were determined according to the method of Bradford [25]. GST activity was expressed as mU/mg protein. One unit of GST activity is defined as the amount of enzyme producing 1 μmol of CDNB–GSH conjugate/min under the conditions of the assay.

NADH:cytochrome b_5 reductase activity

NADH:cytochrome b_5 reductase activity was determined spectrophotometrically as the pHMB-inhibitable, NADH-dependent reduction of cytochrome *c* [27]. Briefly, trypsinized cells were washed with PBS and resuspended in 2 ml lysis buffer (10 mM HEPES–KOH, pH 7.4, 1.5 mM magnesium chloride, 10 mM potassium chloride, 0.05 mM DTT). The suspensions were then sonicated and centrifuged at 7800 *g* for 15 min at 4°C. The resulting lysates were removed and stored in liquid nitrogen until required. An assay mixture comprising 900 μM NADH and 70 μM cytochrome *c* in assay buffer (0.05 M phosphate buffer, pH6.8) was prepared immediately before use. To measure the cytochrome b_5 reductase activity of each lysate, paired samples were prepared which contained 1 ml of the assay mixture, 20 μl of lysate and 25 μl of either pHMB (final concentration 0.2 mM) or assay buffer. The change of absorbance at 550 nm was followed for 1 min. The pHMB-inhibitable activity was calculated as the difference between the activity of the two cuvettes and was expressed as nmol/min/mg protein. The protein concentration of the lysate was determined according to the method of Bradford [25].

Statistical analysis

All experiments were performed 5 times for each cell line. Results represent the mean of five experiments and the SDs. Statistical analysis was performed using the *t*-test.

Results

Cytotoxicity of BCNU, MMC, SN and DMF in human tumor cell lines

IC_{50} for BCNU was in a range of 28–320 μM in glioblastoma cell lines (Table 2). Glioblastoma cells were more sensitive to MMC or SN than to BCNU with IC_{50} ranging from 115 nM to 4.9 μM in T98G and U-87MG cells (Fig. 1). All cell lines were more sensitive to SN with $IC_{50} < 510$ nM than to MMC with $IC_{50} > 2.5$ μM. DMF alone had no effect on cell growth with concentrations less than 10 μM (Fig. 1D and Table 1).

Modifying effects of DMF and dicumarol on cytotoxicity of MMC and SN

Pretreatment with DMF significantly increased cytotoxicity of MMC in all glioblastoma cell lines tested and H1299 lung cancer cells (Fig. 2A). DMF also significantly increased cytotoxicity of SN in all cell lines except for HT-1080 (Fig. 2B and Table 3). In particular, the IC_{50} decreased from 40- to 140-fold for both drugs in the T98G and U-87MG glioblastoma cell lines. However, DMF had no effect on cytotoxicity of BCNU. The addition of the DTD inhibitor, dicumarol, significantly inhibited cytotoxicity of MMC in T98G and U-87MG cell lines, and also inhibited cytotoxicity of SN in all cell lines tested (Fig. 2D and E). In all glioblastoma cell lines tested, dicumarol reversed the increased cytotoxicity seen when DMF was added to MMC or SN (Fig. 2F and G).

Induction of DTD in human tumor cell lines by DMF

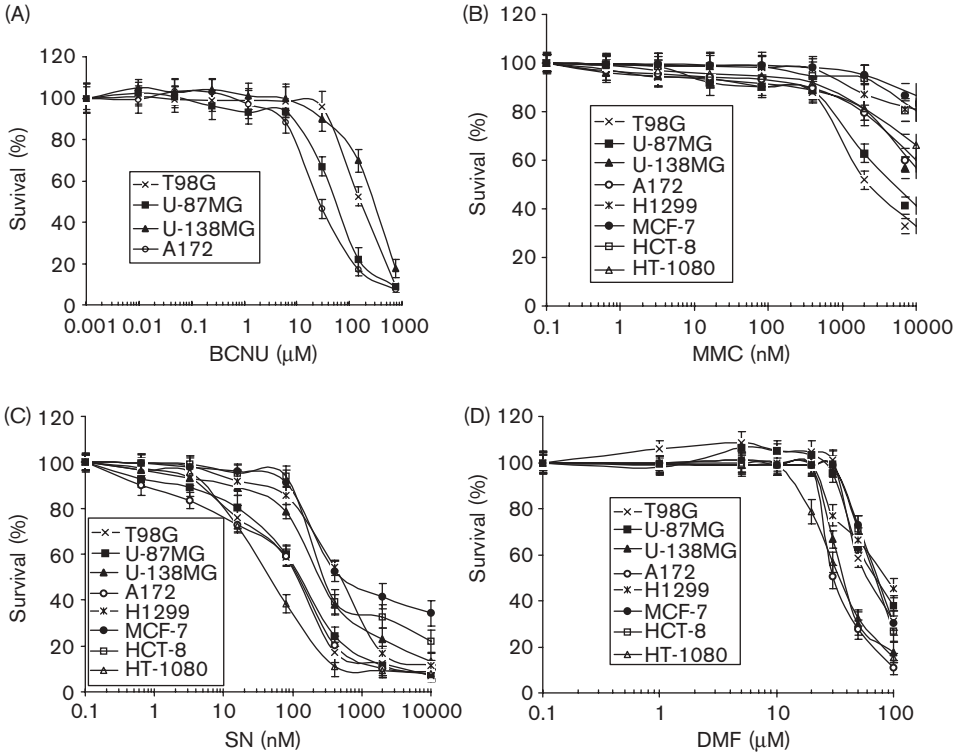
T98G cells had the highest baseline level of DTD activity and HT-1080 cells had the lowest DTD activity compared to other cell lines (Table 4). When the cells were incubated with DMF at highest non-toxic concentrations for 48 h, DTD was significantly increased in all

Table 2 IC_{50} **of the agents test in the cell lines listed below**

Cell lines	Tumor type	BCNU (μM)	MMC (μM)	SN (nM)
T98G	glioblastoma	161 ± 5	2.6 ± 0.5	115 ± 14
U-87MG	glioblastoma	58 ± 4	4.9 ± 0.8	125 ± 13
U-138MG	glioblastoma	300 ± 9	>10	242 ± 25
A172	glioblastoma	28 ± 3	>10	121 ± 14
HCT-8	colon	–	>10	257 ± 23
MCF-7	breast	–	>10	502 ± 34
HT-1080	fibrosarcoma	–	>10	58 ± 11
H-1299	lung	–	>10	512 ± 31

>10 μM means that IC_{50} values in five experiments are all greater than 10 μM. Results are means ± SD for five separate experiments.

Fig. 1



Sensitivity of eight human tumor cell lines to BCNU (A), mitomycin C (B), SN (C) and DMF (D). Data represent the means ± SD of five separate experiments.

glioblastoma cell lines except for T98G. DMF also significantly increased DTD activity in NCI-H1299 and MCF-7 cell lines, but not in HCT-8 and HT-1080 cell lines.

Induction of GST in human tumor cell lines by DMF

U-138MG and HCT-8 had higher levels of GST activity than the other cell lines (Table 5). When the cells were incubated with DMF at highest non-toxic concentrations for 48 h, GST was significantly decreased in three glioblastoma cell lines and HCT-8 cells ($p < 0.05$), but was significantly increased in HT-1080, NCI-H1299 and MCF-7 cell lines ($p < 0.05$).

Induction of NADH: cytochrome b_5 reductase by DMF

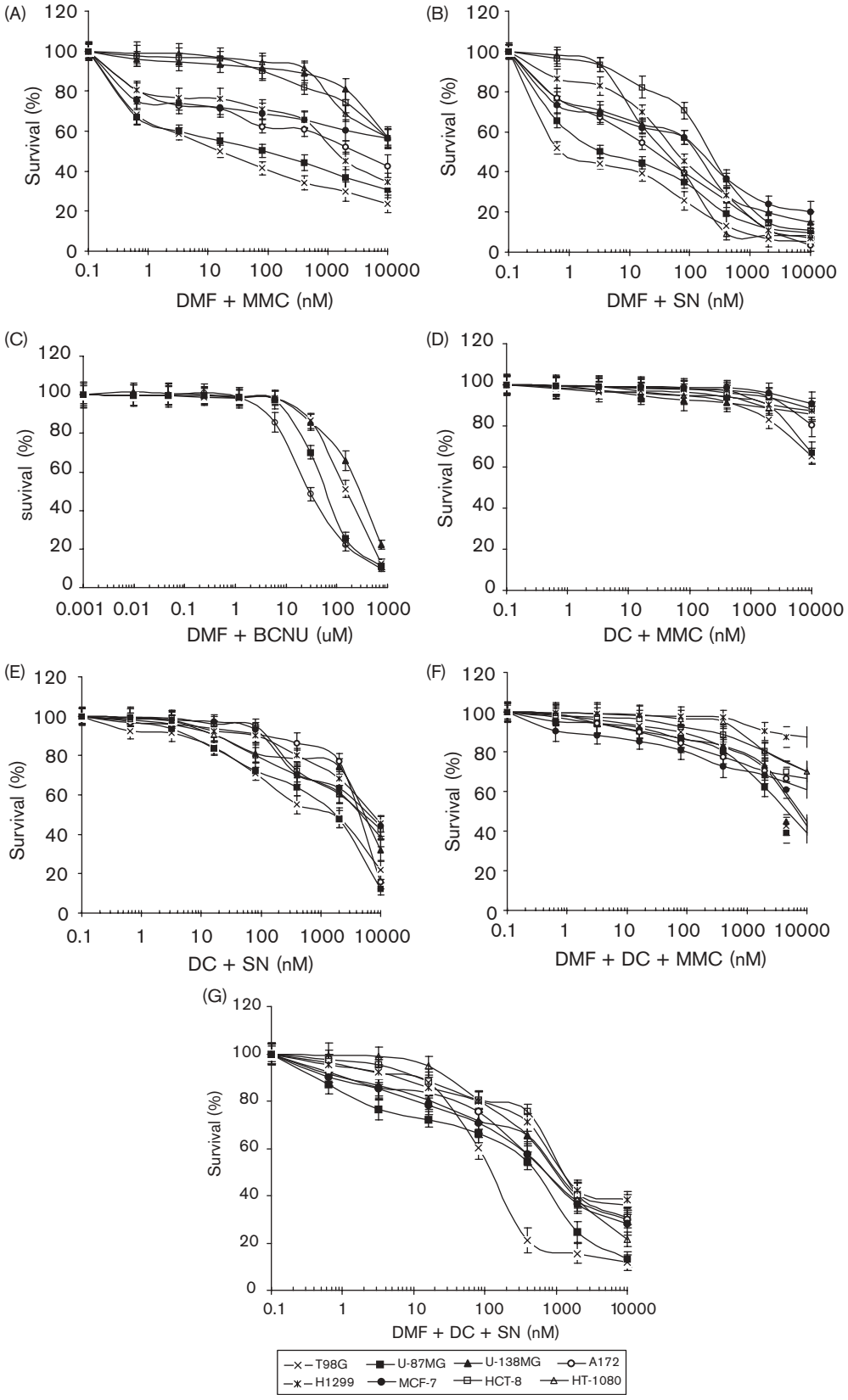
DMF significantly increased cytochrome b_5 reductase activity in three glioblastoma cell lines, T98G, U-87MG and U-138MG ($p < 0.01$), but not in other cell lines (Table 6).

Discussion

Glioblastoma multiforme is resistant to most chemotherapeutic agents. A clear understanding of the molecular basis of drug resistance is of key importance in developing new strategies for glioblastoma chemotherapy. Okamura *et al.* showed that human glioblastoma cells overexpressed a variety of genes related to drug resistance such as multidrug resistance 1 (MDR1), MDR-associated protein, NAPDH/cytochrome P-450 reductase, GST, γ-glutamyl cysteine synthetase and O^6-methylguanine DNA methyltransferase (MGMT), that contribute to the poor response of this tumor to various types of anticancer drugs [17]. Okamura *et al.* showed that the T98G cell line was highly resistant to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), docetaxel, irinotecan and cisplatin [17]. MGMT is an important determinant of BCNU and ACNU drug resistance as measured in experimental systems and clinically [28,29]. We also observed that all the glioblastoma cell lines tested were highly resistant to BCNU.

Significant cytotoxicity was observed in T98G and U-87MG cell lines when exposed to MMC and in all glioblastoma cell lines exposed to SN. DTD is a two-electron reductase that catalyzes MMC or SN to the active form, and has the potential to enhance cytotoxicity to these agents. Okamura *et al.* reported that three glioblastoma cell lines, T98G, U-373MG and U-251MG, had significantly higher levels of DTD activity than 10 cell lines derived from other human cancers [17]. In our

Fig. 2



Effects of DMF and dicumarol (DC) on cytotoxicity of MMC or SN in eight human tumor cell lines. Data represent the means ± SD of five separate experiments. (A) DMF + MMC, (B) DMF + SN, (C) DMF + BCNU, (D) DC + MMC, (E) DC + SN, (F) DMF + DC + MMC and (G) DMF + DC + SN.

Table 3 Modifying effects of DMF and DC on cytotoxicity of MMC or SN

Cell lines	DMF + BCNU	DMF + MMC	DMF + DC + MMC	DC + MMC	DMF + SN	DMF + DC + SN	DC + SN
T98G	163 ± 5 μM	18 ± 6 nM[a]	7.8 ± 1.1 μM[a]	> 10 μM[a]	1.0 ± 0.4 nM[a]	131 ± 12 nM[a]	2.0 ± 0.5 μM[a]
U-87MG	65 ± 4 μM	80 ± 18 nM[a]	6.1 ± 1.1 μM[a]	> 10 μM[a]	3.0 ± 0.8 nM[a]	666 ± 26 nM[a]	1.8 ± 0.3 μM[a]
U-138MG	320 ± 8 μM	389 ± 27 nM[a]	8.3 ± 1.2 μM[a]	> 10 μM	121 ± 13 nM[a]	1.1 ± 0.4 μM[a]	5.7 ± 1.1 μM[a]
A172	30 ± 3 μM	3.6 ± 0.9 μM[a]	> 10 μM[a]	> 10 μM	36 ± 9 nM[a]	823 ± 33 nM[a]	4.5 ± 0.8 μM[a]
HCT-8	–	> 10 μM	> 10 μM	> 10 μM	218 ± 18 nM[a]	1.4 ± 0.6 μM[a]	6.8 ± 1.2 μM[a]
MCF-7	–	> 10 μM	> 10 μM	> 10 μM	151 ± 12 nM[a]	811 ± 41 nM[a]	7.7 ± 1.9 μM[a]
HT-1080	–	> 10 μM	> 10 μM	> 10 μM	46 ± 9 nM	923 ± 45 nM[a]	5.9 ± 0.9 μM[a]
H-1299	–	1.5 ± 0.4 μM[a]	> 10 μM	> 10 μM	67 ± 14 nM[a]	1.3 ± 0.6 μM[a]	8.3 ± 1.4 μM[a]

[a]$p <$ 0.01 when (DMF + SN)-treated compared with SN-treated, (DMF + DC + SN)-treated compared with (DMF + SN)-treated, (DC + SN)-treated compared with SN (DMF + MMC)-treated compared with MMC-treated, (DMF + DC + MMC)-treated compared with (DMF + MMC)-treated, (DC + MMC)-treated compared with MMC. > 10 μM means that IC_{50} values in five experiments are all greater than 10 μM. Results are means ± SD of IC_{50} values for five separate experiments.

Table 4 DTD activity (nmol/min/mg protein)

Cell line	Control	DMF-treated	DMF + DC-treated
T98G	638 ± 34	664 ± 51	3.3 ± 2.4[b]
U-87MG	187 ± 14	399 ± 27[b]	4.1 ± 1.8[b]
U-138MG	106 ± 9	132 ± 12[b]	5.4 ± 1.1[b]
A172	115 ± 10	134 ± 12[a]	2.8 ± 0.5[b]
HCT-8	280 ± 27	311 ± 22	5.1 ± 1.5[b]
MCF-7	270 ± 21	319 ± 26[b]	6.2 ± 1.2[b]
NCI-H1299	184 ± 17	393 ± 30[b]	4.9 ± 0.3[b]
HT-1080	51 ± 8	61 ± 7	2.1 ± 0.5[b]

[a]$p < 0.05$ and [b]$p < 0.01$ when DMF-treated compared with control, (DMF + DC)-treated compared with DMF-treated. Results are means ± SD for five separate experiments.

Table 5 DMF effect on GST in cell lines listed

Cell lines	GST (mU/mg protein)	
	Control	DMF-treated
T98G	3037 ± 160	2032 ± 150[a]
U-87MG	3070 ± 185	1918 ± 142[a]
U-138MG	6973 ± 72	6714 ± 149[a]
A172	3583 ± 49	3404 ± 48
MCF-7	1391 ± 26	1603 ± 133[a]
HCT-8	5277 ± 158	4637 ± 140[a]
H1299	1252 ± 135	1782 ± 143[a]
HT-1080	1789 ± 130	2011 ± 139[a]

[a]$p < 0.05$ when DMF-treated compared with control. Results are means ± SD for five separate experiments.

Table 6 Cytochrome b_5 reductase activity (nmol/min/mg protein)

Cell line	Control	DMF-treated
T98G	94 ± 9	125 ± 7[a]
U-87MG	83 ± 7	118 ± 5[a]
U-138MG	179 ± 11	168 ± 13
A172	26 ± 4	62 ± 4[a]
HCT-8	74 ± 7	61 ± 9
MCF-7	91 ± 5	87 ± 7
H1299	60 ± 5	52 ± 6
HT-1080	55 ± 9	50 ± 6

[a]$p < 0.01$ when DMF-treated compared with control. Results are means ± SD for five separate experiments.

study, only T98G had a higher level of DTD compared to the other cell lines tested. We found a DTD level of 639 nmol/min/mg protein for T98G which is comparable to the levels identified by Okamura *et al.* for the glioblastoma cell lines he studied. Furthermore, the T98G cell line responded best to MMC compared to all other cell lines and had the greatest increase in cytotoxicity when DMF was added to the cell culture although all glioblastoma cell lines had a significant decrease in IC_{50} when DMF was added to MMC or SN. This was reversed with the addition of dicumarol, suggesting that activity of the DTD system can enhance the cytotoxicity of these agents. The addition of dicumarol to SN in the absence of DMF led to a marked increase in the IC_{50} for SN, as expected. Thus, inhibition of DTD with dicumarol protected the cells from SN cytotoxicity. This effect was less apparent with MMC. For T98G and U-87MG cell lines, the cytotoxicity of MMC decreased with the addition of dicumarol, but the U-138MG and A172 cell lines were so intrinsically resistant to MMC that no effect was observed.

DMF is a potent DTD inducer [20], and it increased the DTD activity in all glioblastoma cell lines except T98G. Wang *et al.* [20] found that another DTD inducer, 1,2-dithiole-3-thione, did not increase the DTD level or MMC cytotoxic activity in SK-MEL-28 human melanoma cells or AGS human gastric cancer cells that have high baseline levels of DTD activity of approximately 250 nmol/min/mg protein. T98G had the highest baseline level of DTD of all cell lines we tested, perhaps explaining the lack of enzyme induction by DMF administration. When MMC or SN was used in combination with DMF, enhanced cytotoxicity may be attributed to the increased activity of DTD in MCF-7, NCI-H1299 and all glioblastoma cell lines except for T98G. However, DMF increased the cytotoxicity of MMC or SN in T98G and HCT-8 even if it had no effect on DTD activity in these cell lines. Therefore, DMF must be having an effect in addition to enhancing DTD activity.

Cytochrome b_5 reductase is a FAD-containing flavoprotein [30] and potentially an important enzyme required for the reductive activation of bioreductive chemotherapeutic agents [18]. *In vitro* studies indicate that the purified cytochrome b_5 reductase is able to activate MMC [18] and the fused pyrazine mono-*N*-oxide bioreductive drug, RB90740 [19], and thus may enhance the

cytotoxicity of these compounds. Our data show that DMF is a potent inducer of cytochrome b_5 reductase in glioblastoma cell lines. It significantly increased cytochrome b_5 reductase activity in all glioblastoma cell lines except for U-138MG; this may contribute to the significantly enhanced cytotoxicity of MMC and SN by DMF. DMF induced cytochrome b_5 reductase in T98G even if it had no effect on the DTD level in this cell line. This may represent the mechanism whereby DMF increased MMC cytotoxicity in T98G cells. Very low activity of cytochrome b_5 reductase in A172 may explain why the cell line responded poorly to MMC alone. In A172 DMF increased activity of both DTD and cytochrome b_5 reductase, and concomitant increase in cytotoxicity was seen with MMC and SN.

GST-mediated inactivation is also a key determinant of MMC and SN cytotoxicity [31,32]. The GSTs are a family of phase II detoxifying enzymes that are coordinately induced with DTD in some tissues [21,33,34]. These enzymes may protect cells from the toxic and mutagenic effects of foreign chemicals [14] by direct inactivation. Therefore, GST may play an important role in tumor resistance to a variety of antitumor agents, including MMC, by reducing intracellular drug levels [35,36]. GST may have contributed to the poor response of all cell lines tested in this study to MMC or SN. However, DMF significantly inhibited GST in all glioblastoma cell lines except A172. Reduction of GST may have contributed to the enhanced cytotoxicity observed in glioblastoma cell lines (except A172) treated with DMF and MMC or SN. In A172, enhanced cytotoxicity was observed with the addition of DMF to MMC or SN even though GST was not significantly inhibited. Thus, induction of DTD and an increase in cytochrome b_5 reductase were likely sufficient to enhance cytotoxicity in this cell line

In summary, our study suggests that antitumor activity of MMC or SN is determined by the balance between intracellular activation of these agents by the DTD or cytochrome b_5 reductase systems and inactivation by GST. DMF enhanced the cytotoxicity of MMC and SN in all glioblastoma cell lines by increasing DTD or cytochrome b_5 reductase activity and decreasing GST activity. Manipulation of these enzyme pathways may guide use of bioreductive antitumor agents against individual tumors. MMC is not known to be an active agent against glioblastoma, perhaps in part because it does not penetrate an intact blood–brain barrier (BBB). SN can cross the BBB, but its clinical use in brain tumor treatment has been limited by myelotoxicity [37,38]. However, concurrent use of DMF with SN may allow administration of lower doses of the chemotherapeutic agent which will reduce systemic toxicity. This approach warrants further study.

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