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ORIGINAL ARTICLE

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Acquired resistance to O^6-benzylguanine plus chloroethylnitrosoureas in human breast cancer

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Abstract *Purpose*: O^6-benzylguanine (BG) is a pseudosubstrate inactivator of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) that has entered clinical trials as a potentiatier of the antitumor effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). This study was designed to evaluate potential mechanisms of BG + BCNU resistance in breast cancer cells. *Methods*: We treated MCF-7 breast cancer cells three times with cytotoxic concentrations of BG + BCNU to isolate a population of MCF-7 cells possessing BG + BCNU resistance (BBR). We measured drug resistance, AGT activity, cross-resistance to other agents and sensitivity of AGT to BG inactivation. *Results*: When compared with the parent line, MCF-7BBR cells were no longer sensitized to BCNU by BG, resulting in three-fold resistance to BG + BCNU and fourfold resistance to BG + 1-(2-chloroethyl)-1-nitrosourea (CNU). In contrast, MCF-7 and MCF-7BBR cells had similar sensitivity to BCNU, CNU, temozolomide (each in the absence of BG), cisplatin, and UV light. Acquired resistance to BG + chloroethylnitrosoureas (CENU) in MCF-7BBR cells was not accompanied by an increase in AGT or altered kinetics of BG inactivation of AGT. While glutathione-S transferase activity was increased modestly, its contribution to resistance in this setting remains unclear. *Conclusions*: This is the first report of acquired BG + CENU resistance in human tumor cells and the results indicate that selective pressure with BG + CENUs may result in nonresponsiveness to BG by one or more complex mechanisms. These results predict the development of acquired resistance to BG + BCNU in clinical trials.

Key words O^6-alkylguanine-DNA alkyltransferase · 1,3-bis(2-chloroethyl)-1-nitrosourea · O^6-benzylguanine · Drug resistance · Breast cancer

Introduction

Chloroethylnitrosoureas (CENUs) chloroethylate nucleophilic sites within macromolecules including at least 14 primary sites in DNA [1]. The cytotoxicity of these agents depends on chloroethylation of the O^6 position of guanine and conversion of that adduct to the N^3-cytosinyl-N^1-guanyl-ethane DNA interstrand crosslink [2–4]. Tumor cells resistant to CENUs typically express the O^6-alkylguanine-DNA alkyltransferase (AGT) DNA repair protein [5–7]. Human AGT encoded by the O^6-methylguanine-DNA methyltransferase (MGMT) gene irreversibly transfers alkyl groups from the O^6 position of guanine to an active-site cysteine residue, restoring the DNA to normal and preventing crosslink formation [8, 9]. In addition, AGT reacts with the cyclic intermediate N^1-O^6-ethanoguanine [10, 11] to prevent crosslink formation.

The discovery that O^6-benzylguanine (BG) is a potent pseudosubstrate (ED_{50} of 0.2 μM in a 30-min cell extract reaction) for human AGT has renewed interest in CENU therapy and focused efforts on reversing CENU resistance [12, 13]. AGT-expressing breast, central nervous system and colorectal cancer cell lines, among others, are sensitized to CENUs by BG and BG derivatives [14–17]. AGT-deficient cell lines and xenografts are not affected by AGT inhibitors [14, 17, 18]. The VACO8 colon cancer cell line sensitive to AGT and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) transduced with MGMT to express high AGT, is markedly resistant to BCNU [19]. Xenografts composed of cell mixtures of VACO8 and VACO8-MGMT cells (0.1% to 10% VACO8-MGMT) respond in vivo to treatment with

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BCNU, but regrow with delay periods that depend on the cell mixture. These regrowing tumors are always enriched in $MGMT^+$ cells [19]. Based on this evidence of very strong selection pressure in favor of MGMT-expressing cells, we hypothesized that cells expressing high amounts of MGMT could repopulate a tumor, resulting in acquired resistance to BCNU.

At the cellular level, acquired resistance to the combination of BG and BCNU could arise through improved processing of BCNU-induced DNA crosslinks, by increased nucleotide excision repair [20], or by increased topoisomerase 1 and 2 [21, 22]. Alternatively, 3-methyladenine glycosylase (MAG) may repair BCNU-induced DNA damage, although both increased MAG and MAG deficiency increase sensitivity to BCNU [23]. It is also possible that by increased conjugation of BCNU to glutathione (GSH) by the action of GST S transferase (GST) [24] may lead to drug resistance, although we have previously shown that levels of GSH and GST have a modest effect on BCNU resistance in MCF-7 cells [25].

Acquired resistance to the combination of BG and BCNU may also arise from mutations in AGT that reduce its reactivity with BG. Mutations at specific nonconserved sequences in both the bacterial and human AGT result in a protein that is either moderately or highly resistant to BG and thus to BCNU. These mutant AGTs retain the ability to repair O^6-methylguanine and O^6-chloroethylguanine adducts, making it likely that they could arise during drug selection by mutation [26–30].

Here, we report the isolation of MCF-7 breast cancer cells with resistance to BG + CENU after two in vitro exposures to BG + BCNU. Investigation of potential mechanisms indicates that AGT is unaltered and suggests increased processing of BCNU crosslinks.

Methods

Materials

BCNU and 1-(2-chlorethyl)-1-nitrosourea (CNU) were obtained from the Developmental Therapeutics Branch, National Cancer Institute (Bethesda, Md.). Cisplatin (CDDP) was obtained from Sigma (St. Louis, Mo.). BG was synthesized by Dr. Robert Moschel at the Frederick Cancer Research Institute (Fredericksburg, Md.). [^{3}H]*N*-Methylnitrosourea was obtained from Amersham (Arlington Heights, Ill.). The mT3.1 monoclonal antibody to the human AGT was kindly provided by Dr. D. Bigner at Duke University (Durham, N.C.).

Cell culture

MCF-7 and MCF-7BBR cells were grown as monolayers on plastic substrates in a humidified atmosphere at 37 °C. They were maintained in RPMI supplemented with 7% fetal bovine serum, 3% calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 2 m*M* glutamine for up to 15 passages. MCF-7BBR subclones were established via serial dilution in 96-well microtiter plates. Population doubling times were determined by plating 1×10^5 cells in 35 mm plates and monitoring their growth over several days. Growth curves were exponential in nature and population doubling times were derived from equations which fitted these curves.

Selection strategy

One semiconfluent 100-mm plate (5×10^6 cells) of MCF-7 cells was exposed to 10 μ*M* BG with 25, 50 or 100 μ*M* BCNU as described below. After 24 h, the treated cells were split 1:3 and replated to allow for colony formation. The colonies surviving treatment with 10 μ*M* BG + 50 μ*M* BCNU were pooled and expanded for a second selection. After two treatments with 10 μ*M* BG + 50 μ*M* BCNU, the surviving population of MCF-7 cells (MCF-7BBR) was resistant to BG + BCNU in colony formation assays.

Drug treatments

BG was dissolved in DMSO. BCNU and CNU were dissolved and serial diluted in 100% ethanol immediately prior to their use. CDDP was dissolved in 100% DMSO and diluted in medium. The maximum concentration of DMSO added was 0.2% and did not affect clonal growth. The cytotoxicity of all drug treatments was assessed in colony formation assays. Briefly, 3×10^3 cells were plated on 100 mm^3 plates in duplicate or triplicate. Drug treatments were initiated 24 h later by adding serum-free RPMI containing or lacking BG. After 1 h, BCNU, CNU or CDDP was added or the plates were exposed to UV light. Cells were washed twice 2 h later with serum-free RPMI and placed in serum-containing RPMI containing or lacking BG. After 15 days incubation, colonies were stained with methylene blue. Colonies containing ≥50 cells were counted. To evaluate repletion of AGT after its inactivation by BG, cells were exposed to 10 μ*M* BG for 1 h, washed twice in warm serum-containing medium and reincubated in medium without BG. Replicate dishes were harvested immediately after BG incubation and at various times up to 24 h later. Cells were then collected for AGT assay. The IC_{50} values represent the mean ± s.d. of a minimum of three independent experiments with two plates read per drug concentration.

Alkyltransferase assay

This assay has been described in detail elsewhere [31]. Briefly, substrate [^{3}H]methyl-DNA was prepared by reacting 70 mg calf thymus DNA with 10 mCi [^{3}H]*N*-methylnitrosourea. AGT activity in sonicated cell and tissue extracts was measured as the loss of [^{3}H]O^6-methylguanine, relative to [^{3}H]7-methylguanine (internal standard), from substrate DNA during a 1-h incubation at 37 °C in a Hepes assay buffer. The reaction mixture contained excess substrate DNA such that total alkyltransferase activity could be determined. Methylated purines were liberated from precipitated DNA by treatment with 0.1 *N* HCl at 80 °C for 1 h and separated by reverse-phase HPLC. [^{3}H]O^6-Methylguanine and [^{3}H]7-methylguanine (internal standard) were quantitated by liquid scintillation counting and AGT activity was expressed as femtomoles O^6-methylguanine removed per milligram protein or microgram DNA.

Western blots

Proteins were resolved by SDS-PAGE (10%) in a minigel apparatus at 130 V for 1 h and transferred onto polyvinyldifluoride (PVDF) membranes using a Trans-Blot minicell for 1 h at 100 V. PVDF membranes were blocked with 5% dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) and probed for 1 h with 7 μg/ml mT3.1 monoclonal antibody, which is specific for human AGT [32]. After three 5-min washes in TBS-T, membranes were incubated with horseradish peroxidase-linked antimouse sheep IgG for 1 h at 22 °C. After four additional washes, the band corresponding to human AGT was visualized

using chemiluminescence detection reagents and densitometric analysis.

Results

Generation of MCF-7 clones resistant to BG + BCNU

To investigate the ability of BG to reverse BCNU resistance in MCF-7 breast cancer cells that express high levels of AGT (35 fmol/μg DNA), we exposed MCF-7 cells to 0, 1, 10 or 25 μ*M* BG for 1 h prior to the addition of BCNU and throughout BCNU exposure and culture for colony formation. BG sensitized MCF-7 cells to BCNU in a concentration-dependent manner (Fig. 1A). The IC_{90} of BCNU alone (72 ± 16 μ*M*) was lowered to 24 ± 1, 19 ± 1 and 17 ± 1 μ*M* by the presence of 1, 10 and 25 μ*M* BG, respectively ($P < 0.05$). BG alone did not reduce colony formation at these concentrations.

Selection for BG + CENU-resistant human tumor cells

We treated 5×10^6 MCF-7 cells with 10 μ*M* BG followed by 25, 50 or 100 μ*M* BCNU. The surviving colonies were culture expanded and retreated with 10 μ*M* BG followed by 25, 50 or 100 μ*M* BCNU. After regrowth, 118 colonies formed in the 50 μ*M* BCNU plate. These were pooled and selected a third time in BG and BCNU. These cells were BG + BCNU-resistant (BBR), because confluent plates were observed 10 days after exposure to 10 μ*M* BG + 100 μ*M* BCNU. When compared to the parent line, MCF-7BBR cells had similar resistance to BCNU alone (IC_{90} of 65 ± 12 μ*M*; Fig. 1B), but were not sensitized to BCNU by BG. For example, the IC_{90} values for BCNU after treatment with 1, 10 and 25 μ*M* BG were 60 ± 11 μ*M*, 52 ± 5 μ*M* and 46 ± 12 μ*M*. Thus, MCF-7BBR cells were resistant to the BCNU-sensitizing effect of BG, compared to the parental cell line, even at high concentrations of BG.

To verify that differences in colony formation after BG + BCNU treatment were not due to differences in cell growth after exposure to BG alone, we exposed both MCF-7 and MCF-7BBR to various concentrations of BG alone and measured colony formation and growth rates. Up to 25 μ*M* BG was without effect on MCF-7 and MCF-7BBR colony formation (Fig. 1C). Higher concentrations of BG inhibited MCF-7 and MCF-7BBR colony formation equally (Fig. 1D) and increased cell doubling times to a similar extent (from 28 ± 3 h to 64 ± 9 h at 100 μ*M* in MCF-7 cells, and from 27 ± 3 h to 66 ± 3 h in MCF-7BBR cells). Cell cycle analysis using flow cytometry indicated that BG did no affect cell cycle progression at doses up to 10 μ*M* BG for 24 h (data not shown). Thus, BG had a similar cytostatic effect in both MCF-7 and MCF-7BBR cells, without selective action in any phase of the cell cycle.

Cross-resistance to other chemotherapeutic agents

Resistance of MCF-7BBR cells to the combination of CNU and 10 μ*M* BG was studied to determine whether cross-resistance extended to other CENUs (Fig. 2A). CNU, unlike BCNU, is a CENU which does not have carbamylating activity and thus relies more on formation of O^6-chloroethyl adducts to exert its toxicity. MCF-7 and MCF-7BBR cells were equally sensitive to CNU alone. BG sensitized MCF-7 cells to CNU, decreasing the CNU IC_{90} to 13 μ*M* (>13-fold sensitization), whereas in MCF-7BBR cells the BG plus CNU combination decreased the CNU IC_{90} to only 52 μ*M* (threefold sensitization, but more than fourfold more resistant than the parental cells).

To examine cross-resistance to DNA crosslinking agents repaired by nucleotide excision repair, MCF-7 and MCF-7BBR cells were exposed to CDDP and UV light, (Fig. 2B–D). MCF-7 and MCF-7BBR cells were equally sensitive to both agents alone and to BG + CDDP and BG + UV light. Thus, cellular resistance to CDDP and UV light was not affected by depletion of AGT activity or acquisition of BG + BCNU resistance.

Table 1 Drug resistance of MCF-7 and MCF-7BBR cells

Treatment	MCF-7 IC_{50}/IC_{90}	MCF-7BBR IC_{50}/IC_{90}	Fold resistance IC_{50}/IC_{90}
BCNU (μ*M*), *n* = 3	31 ± 11/72 ± 16	28 ± 10/65 ± 12	0.90/0.90
1 μ*M* BG + BCNU (μ*M*), *n* = 3	10 ± 1/24 ± 1	26 ± 6/60 ± 11	2.6/2.5
10 μ*M* BG + BCNU (μ*M*), *n* = 3	9 ± 2/19 ± 1	24 ± 3/52 ± 5	2.7/2.7
25 μ*M* BG + BCNU (μ*M*), *n* = 3	7 ± 1/17 ± 1	21 ± 5/46 ± 12	3.0/2.7
CNU (μ*M*), *n* = 3	51 ± 3/176 ± 18	49 ± 5/170 ± 19	0.96/0.97
10 μ*M* BG + CNU (μ*M*), *n* = 3	5 ± 1/13 ± 1	18 ± 1/52 ± 3	3.6/4.0
TMZ (μ*M*), *n* = 3	710 ± 160/>1000	710 ± 160/>1000	1.0/nd
10 μ*M* BG + TMZ (μ*M*), *n* = 4	50 ± 30/140 ± 30	90 ± 20/210 ± 30	1.8/1.5
CDDP (μ*M*), *n* = 2	2.7/9.3	2.9/9.0	1.1/0.97
10 μ*M* BG + CDDP (μ*M*), *n* = 2	2.9/9.5	3.2/9.6	1.1/1.0
UV light (J/m^2), *n* = 2	9.4/19	9.5/18	1.0/0.95
10 μ*M* BG + UV (J/m^2), *n* = 2	9.1/19	9.5/18	1.0/0.95

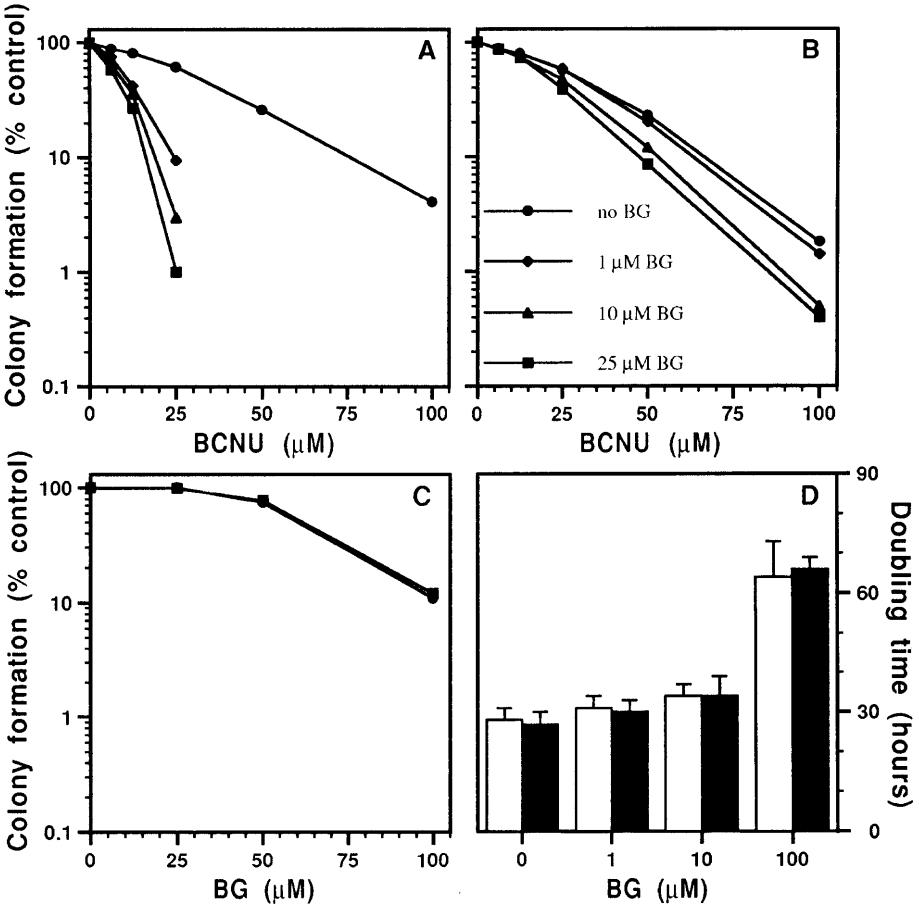

Fig. 1A–D Effect of BG and BCNU on MCF-7 and MCF-7BBR colony formation and population doubling times. **A**, **B** Colony formation by MCF-7 cells (**A**) and MCF-7BBR cells (**B**) after exposure to BCNU and 1 to 25 μ*M* BG + BCNU. BG was present 1 h before BCNU, during the 2-h BCNU exposure and until colony formation 15 days later. **C** Colony formation by MCF-7 cells (●) and MCF-7BBR cells (■) in the presence of BG only. **D** Population doubling times of MCF-7 cells (*white bars*) and MCF-7BBR cells (*black bars*) in the absence and presence of several concentrations BG. Values are the means from three independent experiments performed in duplicate or triplicate (*bars* in **D** represent the s.d.)

Finally, to examine cross-resistance to methylating agents, cells were exposed to temozolomide (TMZ) alone or to BG followed by TMZ. TMZ forms O^6-methylguanine DNA adducts, which should be repaired by AGT. BG should sensitize cells to TMZ unless either the AGT protein is altered or there is an acquired defect in mismatch repair which would result in TMZ resistance regardless of BG. Both MCF7 and MCF-7BBR cell lines were equally sensitive to the methylating agent TMZ, and both were sensitized to TMZ by BG (Fig. 2D). However, MCF-7BBR cells exhibited a slight increase in resistance to BG + TMZ ($P = 0.02$), based on a comparison of TMZ IC_{90} values in colony formation assays.

Analysis of BG and BCNU resistance subclones

To investigate heterogeneity in the MCF-7BBR population, we cloned six MCF-7BBR subclones. In each, resistance to the BG + BCNU combination was similar to that of MCF-7BBR cells (Fig. 3A). After treatment with 10 μ*M* BG + 25 μ*M* BCNU, survival in the subclones ranged from 29% to 43% (IC_{50} values of 16–21 μ*M* BCNU), compared to 2% with MCF-7 cells ($P > 0.05$) and 38% for the mass culture of MCF-7BBR cells. We then assessed AGT expression in these MCF-7BBR subclones using the activity assay and Western blotting. AGT activity in the MCF-7BBR population was 27 ± 6 fmol/μg DNA and ranged from 19 to 35 fmol/μg DNA in the subclones, a mean value slightly less than the 35 ± 3 fmol/μg DNA ($n = 5$) present in the MCF-7 cells. These differences did not correlate with resistance to BG + BCNU. Western blotting indicated that immunoreactive AGT was similar in the subclones and correlated with AGT activity (Fig. 3C). From this we conclude that the six clones were very similar and reflect a uniform population of drug-resistant cells.

AGT depletion on exposure to BG

The ability of BG to deplete AGT activity in MCF-7 and MCF-7BBR cells was also determined (Fig. 3D). Following a 1-h exposure to BG, a similar concentration-dependent decrease in AGT activity was noted in both MCF-7 and MCF-7BBR cells. For MCF-7 cells, the IC_{50} of BG was 0.5 ± 0.1 μ*M*, compared with 0.4 ± 0.1 μ*M* BG for MCF-7BBR cells. In addition, 10 μ*M* BG reduced AGT activity in MCF-7 and MCF-7BBR cells to < 1% of its pretreatment value. Thus, MCF-7BBR cells express slightly less AGT activity and BG + CENU resistance does not hinge on the expression of a BG-resistant AGT protein. To evaluate repletion of AGT after its inactivation by BG, cells were

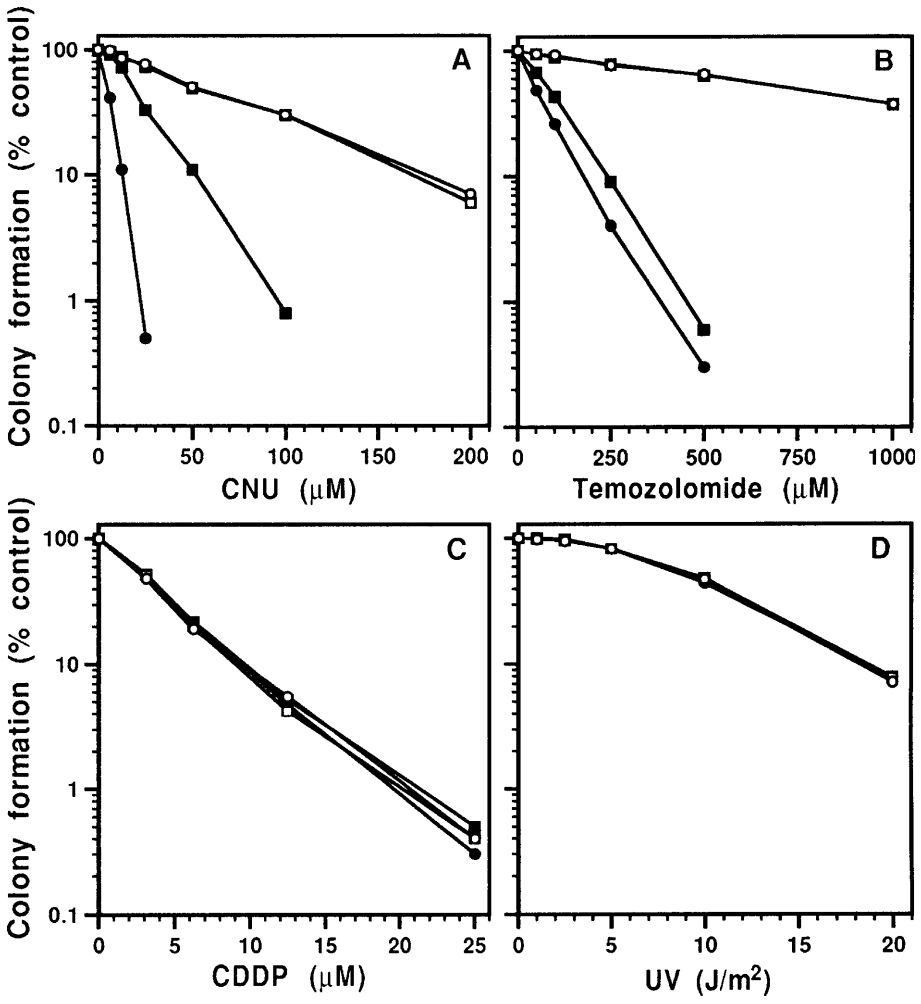

Fig. 2A–D Drug resistance of MCF-7 cells (*circles*) and MCF-7BBR cells (*squares*). **A** CNU resistance ($n = 3$); **B** Temazolomide resistance ($n = 4$); **C** CDDP resistance ($n = 2$); **D** UV light resistance ($n = 2$); ○, □ absence, ●, ■ presence of 10 μ*M* BG. Colony formation values are the means from two to four independent experiments performed in duplicate or triplicate

exposed to 10 μ*M* BG for 1 h, washed free of BG and incubated for up to 24 h. The repletion rate was identical in the two cell cultures, with 50% repletion in 12 h (Fig. 4). Thus, there did not appear to be differences in the rate or extent of repletion that could be correlated with drug resistance.

GSH metabolism in MCF-7BBR cells

Cell lines were then compared for compounds involved in BCNU intracellular detoxification through GSH conjugation. GST levels were higher in MCF-7BBR cells (3.2 ± 0.3 nmol/min per mg protein) than in MCF-7 cells (1.2 ± 0.2 nmol/min per mg protein; $P < 0.01$), although this was not associated with increased BCNU resistance. On the other hand, GSH levels were higher in MCF-7 cells (2.1 pmol/mg protein) than in MCF-7BBR cells (1.2 pmol/mg protein).

Discussion

This is the first report of the induction of resistance to BG + BCNU in MCF-7 cells, a cell line initially sensitive to these agents. The fact that drug-resistant cells emerged after two separate exposures to the drug combination, and that members of our laboratory have observed acquired resistance to this combination in three other cell lines [33], suggests that acquired resistance to BG + BCNU may be common, at least in vitro. BG + BCNU is in clinical trials [34] and it will be important to determine whether acquired resistance also is observed in the clinical setting, and if so whether it has similar characteristics to that observed in the MCF-7 cell line.

Our evaluation of drug resistance was performed in MCF-7BBR cells surviving high doses of the BCNU + BG combination. The results suggest that the selected cells were uniformly resistant to BG since six different clonal isolates had similar AGT activity, similar kinetics of AGT inactivation on exposure to BG, and similar resistance to BCNU + BG. Each of the subclones was completely impervious to BG sensitization to BCNU despite the ability of BG to inhibit AGT.

We were surprised to find that there was no alteration in AGT in the MCF-7BBR cells and that activity was regenerated after BG inactivation at the same rate in both cell lines. A number of mutant AGT proteins have been identified that are resistant to BG inactivation due

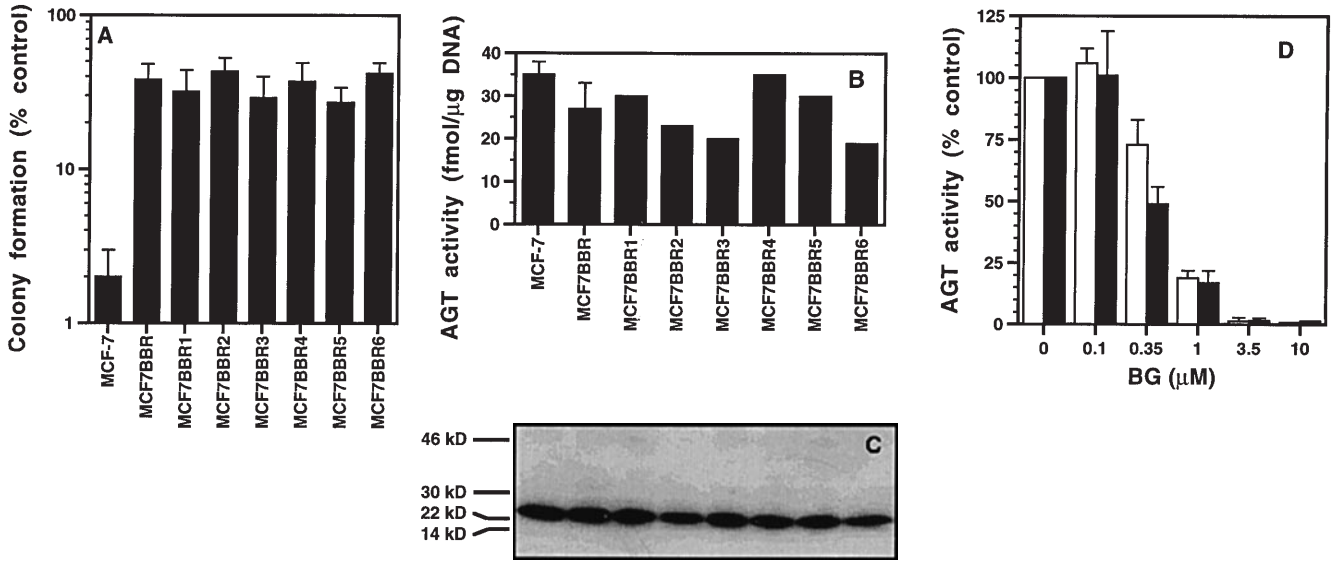

Fig. 3A–D BG + BCNU resistance, AGT expression and BG-sensitivity of AGT activity in MCF-7 cells, MCF-7BBR cells and MCF-7BBR subclones. **A** Resistance of MCF-7 cells, MCF-7BBR cells and six MCF-7BBR subclones to 10 μ*M* BG plus 25 μ*M* BCNU. **B**, **C** AGT expression based on AGT activity and immunoreactive protein. **D** BG-mediated inactivation of MCF-7 cells (*white bars*) and MCF-7BBR cells (*black bars*) AGT activity. When *bars* are present, the values are the means ± s.d. from three or more independent experiments. The AGT activity values shown in **B** are derived from two independent measurement for each subclone

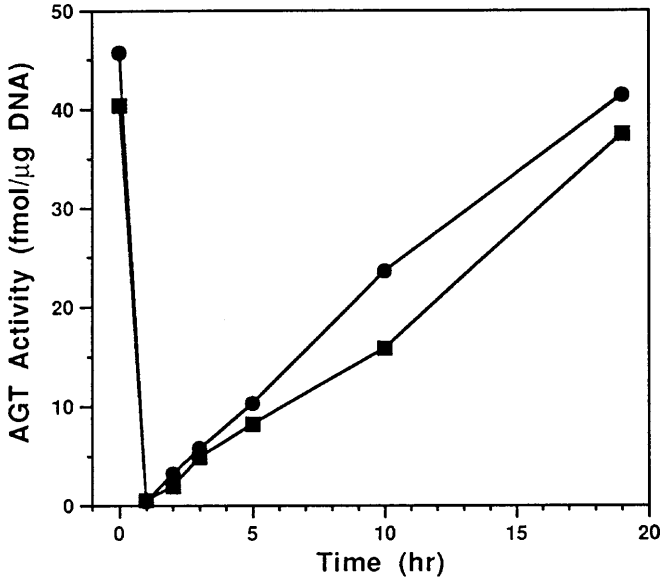

Fig. 4 Regeneration of AGT following BG depletion in MCF-7BBR and MCF-7BBR cells. Cells were exposed to 10 μ*M* BG for 1 h, washed extensively and cultured for up to 24 h in fresh medium. AGT was assayed in cells harvested at the time-points shown (● MCF-7, ■ MCF-7BBR)

to one or more amino acid mutations near the active site which alter the reactivity with BG on the basis of steric hindrance [26–28]. Since a single allelic mutation at one of a number of amino acids would render AGT BG-resistant, it is of note that an altered AGT was not uncovered during the selection process, particularly since BCNU is a potent mutagen [35]. Furthermore, there was only a slight increase in resistance to BG and TMZ, reinforcing the interpretation that resistance was not due to alterations in AGT. Rather, the mechanism of resistance appears to be AGT-independent and to become apparent only after BG inactivation of AGT, since there was no increase in resistance to BCNU or CNU alone.

Our results suggest that acquired resistance is not due to a defect in mismatch repair, nucleotide excision repair or apoptotic signalling. First, the cells were not resistant to BG + TMZ which we have found in all mismatch repair-defective cells [36]. We have also not observed cross-resistance between BG + TMZ and BG + BCNU in mismatch-resistant cells [36]. While it is possible that some cells with acquired mutations in mismatch repair would also be resistant to CENUs, that is not what is suggested by our results. Second, the MCF-7 and MCF-7BBR cells were equally sensitive to CDDP and UV, suggesting that the nucleotide excision repair pathway does not appear to be affected. Third, while there are recent studies identifying cells resistant to DNA damage-induced apoptosis due to the presence of decoy proteins which normally transmit the apoptotic signal [37], the cell sensitivity to TMZ, CDDP and UV indicates that both MCF-7 and MCF-7BBR cells respond appropriately to apoptotic signals following DNA damage. We measured bcl-2 and bax in these cell lines and found no difference (data not shown) which is further evidence that alterations in the apoptosis cascade are not present.

As shown in Fig. 1, BG doses of 10 and 25 μ*M*, well above the IC_{90} for AGT inactivation, resulted in increased BCNU cytotoxicity compared to 1 μ*M* BG. At first glance, this observation may seem to have no ready explanation, but we propose that it is due to two factors: (1) the higher affinity of AGT for DNA-bound substrates such as the chloroethyl adduct and (2) the ability

of the newly synthesized AGT to be DNA bound and preferentially repair DNA adducts rather than be inhibited by free BG. We have seen that high concentrations of BG sensitize cells to TMZ as well as BCNU, reinforcing the first point. In addition, since BCNU crosslinks form over 12 to 18 h, the pre-crosslink lesion is amenable to repair by the newly synthesized AGT over a prolonged period of time, supporting the second point. Thus, it is not surprising that high BG concentrations enhance BCNU and TMZ cytotoxicity.

While it was possible that MCF7BBR cells have altered uptake of BG, or sequester it in a cell compartment, this is highly unlikely given the fact that AGT inhibition by BG is unchanged and that TMZ cytotoxicity in the presence of BG is similar in MCF-7 and MCF-7BBR cells.

Although GST was increased in the MCF-7BBR cells this is not likely to explain the resistance to BCNU + BG. GSH, which was decreased in MCF-7BBR cells, binds BCNU in a conjugation reaction catalyzed by GST [21], reducing the amount of drug available for DNA damage. In other tumor cell lines, elevated GST is associated with BCNU resistance [38]. However, in MCF-7 cells, transfection of GST-mu does not result in BCNU resistance, L-buthionine-[S,R]-sul foxamine depletion of GSH modestly increases BCNU sensitivity, and inhibition of GST by ethacrynic acid also has only a modest effect on BCNU resistance [39, 40].

One of the remaining possible explanations for the observed resistance is enhanced repair of BCNU-induced crosslinks. Since repair kinetics of the cytotoxic N^3-cytosinyl-N^1-guanyl-ethane crosslink produced by CNUs remains ill-defined, it is difficult to precisely measure the rate of repair of this lesion in the MCF-7BBR cells. Previous investigators, including our own group, using alkaline elution or ethidium bromide incorporation to measure crosslinks, have shown increased crosslinks after inactivation of AGT (15). Whether BG alters crosslink formation in cells which are not sensitized by BG to killing with BCNU remains to be determined. However, the prediction would be that the increased capacity to repair crosslinks in these cells is due specifically to enhanced repair of the N^3-cytosinyl-N^1-guanyl-ethane crosslink. Studies quantifying repair of this crosslink in a defined nucleotide sequence are underway.

In summary, MCF-7 cells acquire BG + CENU resistance by a mechanism independent of AGT and cross-resistance to other crosslinking agents. Since we observed abrogation of the ability of BG to sensitize tumor cells to BCNU after only two discrete drug exposures, we predict that development of acquired resistance to BG + BCNU in clinical trials may be relatively common.

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