Chemotherapy-induced O6-Benzylguanine-resistant Alkyltransferase Mutations in Mismatch-deficient Colon Cancer

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ABSTRACT

The ability of O6-benzylguanine (BG) to inactivate alkyltransferase (AGT) to potentiate the antitumor efficacy of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is being tested in clinical trials. As of now, there are no examples of acquired resistance to BG+BCNU in the clinical setting. However, we hypothesized that genetically unstable tumors might develop resistance to the combination after repeated drug-exposures to achieve therapeutic efficacy. To evaluate this possibility, we treated three colon cancer cell lines that are either proficient in mismatch repair (MMR) [SW480 (MMR wild type)] or deficient in MMR [HCT116 (hMLH1 mutant) and HCT15 (hMSH6 mutant)] with three cycles of BG+BCNU. After drug-treatments, HCT116 and HCT15 were completely resistant to BG-potentiated cytotoxicity of BCNU. In these two cell lines, the acquired BG resistance resulted from two de novo and different mutations at amino acid 165 in AGT: 165-lysine (K) to glutamic acid (E) (K165E in HCT116), and 165-lysine to asparagine (N) (K165N in HCT115). Both K165-mutated AGTs had markedly decreased enzymatic activity because of unstable AGT protein but were remarkably resistant to BG inactivation. FISH analysis showed that only one copy of MGMT gene exists in HCT116 cells, and the status of promoter methylation of MGMT in HCT115 showed that one allele of the MGMT promoter has an aberrant methylation. Thus, the MGMT gene expressing AGT either from one copy (HCT116) or from unmethylated allele (HCT15) was mutated because of the exposure to BG+BCNU in these MMR-deficient cell lines. Conversely, MMR-proficient SW480 cells, treated with three cycles of BG+BCNU, maintained wt AGT and the sensitivity to BG-potentiated BCNU-cytotoxicity. To confirm that K165-mutated AGT proteins were responsible for resistance to BG+BCNU, we transfected K165E and K165N MGMT cDNAs into Chinese hamster ovary (CHO) cells. Transfected CHO cells had low AGT activity but increased IC50 for either BCNU or temozolomide (TMZ), compared with parental CHO cells. AGT did not potentiate the cytotoxicity of these two alkylating agents at concentrations up to 200 μM; in contrast, BG, at 25 μM, sensitized CHO-AGT (transfected with wt MGMT cDNA) cells to BCNU or TMZ-cytotoxicity by 3–4 fold. These results suggest that K165 AGT mutants arising in MMR-deficient tumor cells after treatment with chemotherapeutic agents are both resistant to BG-inactivation and are active in the repair of alkylated DNA adducts.

INTRODUCTION

AGT, encoded by MGMT, repairs adducts at the O6-position of guanine by a single reaction of transferring an alkyl group to an internal cysteine residue (1). A distinguishing property of AGT is that the same protein acts as a transferase and as the alkyl group acceptor, an irreversible process. This “suicidal” reaction mechanism means that AGT can function only once to accept a methyl or alkyl group linked to the O6-position of guanine (2, 3). DNA alkylation rapidly depletes active AGT molecules present in the cell until additional AGT is synthesized de novo. These unique properties make AGT an ideal target for biochemical modulation (4–7). BG, a direct substrate for AGT, has been demonstrated to be a potent inhibitor of human AGT (8). Compared with alkyl groups, benzyl groups react more readily to form S-benzyl-cysteine in the AGT active site because the electron charge stabilizes the benzyl group in the transition state (8). Therefore, BG causes a very rapid inactivation of AGT (ED50 ~0.2 μM) in cultured cells and xenografts. It has been consistently shown that BG renders AGT-competent tumor cells more sensitive to the cytotoxic effects of alkylating agents, including BCNU, chlorozotocin, clomosome, streptozocin, procarbazine, dacarbazine, and TMZ (9–17). The combination of BG and BCNU is currently undergoing clinical trials in brain tumors, colon tumors, melanoma, breast cancers, and other pediatric solid tumors (18). In our own clinical Phase I studies, we have shown that it is possible to completely inhibit AGT activity in patient tumors (19).

However, in clinical use, to achieve therapeutic efficacy, repeated administration of BG and BCNU is anticipated, raising the possibility that BG-resistant cells could develop. This is particularly an issue because many of the tumors targeted, such as gliomas and colon cancers, have genomic instability because of defects in MMR or mutations in p53 (20). Therefore, we mimicked the clinical application of BG combined with BCNU in colon cancer cell lines to evaluate whether BG-resistant tumor cells would develop and, if so, whether they carried mutations in AGT. AGT mutants have been constructed by site-specific or random mutagenesis, and some AGT mutants with remarkable degrees of BG resistance have been observed (20–23). Thus, the laboratory precedent for BG-resistant AGT exists.

Three human colon cancer cell lines with high AGT and different MMR and p53 status were chosen to select for BG+BCNU resistance. SW480 cells contain normal MMR activity and are mutant in p53, HCT116 cells are mutant in MLH1 and wild type for p53, and HCT15 cells are both hMSH6 and p53 mutant. We propose that the cell lines used in our studies represent intriguing models of acquired drug resistance because they have different genetic backgrounds, which would be expected to influence the type of resistance that develops.

MATERIALS AND METHODS

Chemicals and Reagents. BG, TMZ, and BCNU were obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutic Program, National Cancer Institute, NIH.

Selection for Acquired Resistance by BG+BCNU in Colon Cancer Cell Lines. On the basis of the observation that in colon cancer cell lines with either MMR proficiency or deficiency, resistance to BCNU was sensitized by BG (24), we selected these three colon cancer cell lines for acquired resistance to BG+BCNU. Cells were treated with 25 μM BG for 2 h prior to the 2-h treatment with IC50 BCNU concentration for each line. After this treatment, fewer than 10% of the cells survived. After growth to confluence, cells were exposed to the same treatment again. Three cycles of treatments with
BG+BCNU resulted in an estimated survival of $10^{-6}$. Surviving cells were named BBR cell lines and examined for drug resistance by colony-survival analysis.

Colony-Survival Assay. Two thousand cells/dish were plated, and pretreated with or without BG at concentrations of 25–200 μM for 2 h. BNU or TMZ was added for 2 h. After this, the medium was replaced with fresh medium. The cells were grown for an additional 7 days before staining with methylene blue for determination of colonies containing more than 50 cells (24).

AGT-Activity Assay. AGT activity was measured by the amount of [3H]methyl group removed from [3H]O6mG present in calf thymus that was methylated with N(2)-[3H]methyl-N-[3H]methyl-N-nitrosourea. The [3H]O6mG and [3H]methyl-N2-guanine bases were separated by HPLC and quantified by liquid scintillation (25).

Western Blot Analysis. Cell extracts were resolved by SDS-PAGE gels (12% polyacrylamide), and proteins were transferred onto polyvinylidene difluoride membranes. The blotted membranes were probed with a mouse monoclonal antibody, mT3.1 (Kamiya Biomedical Co., Seattle, WA), which is specific for human MGMT.

Northern Blot Analysis. Total RNA was prepared from 10 × 106 cells by lysing them in RNAzol according to the manufacturer’s protocol. RNA (10 μg) was size fractionated in 1% agarose gels containing 2.2 m formaldehyde and transferred to nylon membranes by capillary blotting. Membranes were probed with a 32P-labeled MGMT cDNA probe or with GAPDH cDNA (Clontech, Palo Alto, CA) to control for equal loading and transfer.

Sequencing of AGT. The PCR products were purified with a QIAEX II kit (Qiagen Inc., Valencia, CA) and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The sequence reactions were run on an Applied Biosystems 310 Genetic Analyzer. The data were collected and analyzed using Applied Biosystems sequencing analysis software, all according to the manufacturers’ protocols. Sequencing was performed in both directions using the primers used for PCR.

FISH Analysis. FISH was performed using two BACs (RPCI-11 109A6 and RPCI-11 218C11) containing at least a portion of the MGMT gene. BAC DNA was prepared using the Qiagen DNA prep kit following their protocol with the manufacturer’s recommended modification for BACs. The BAC probes were labeled with digoxigenin (Nick Translation kit 976-776; Roche; °C. The primer sets used for PCR were 5'-TCT AGG ATC CGT TTG CGA CTT GGT ACT TG-3' (upper primer) and 5'—GACTCTTCC-GAAAAGCAAACG-3' (lower primer). PCR products in parents were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Table 1 Relative resistance compared with parental cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μM) in parental lines</th>
<th>IC50 (μM) in BBR cell lines</th>
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<tr>
<td>SW480</td>
<td>16.2 ± 2.1</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>HCT116</td>
<td>30.0 ± 3.2</td>
<td>7.3 ± 1.7</td>
</tr>
<tr>
<td>HCT15</td>
<td>50.8 ± 1.9</td>
<td>14.8 ± 3.1</td>
</tr>
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</table>

Cloning 165-mutated MGMT by RT-PCR. Two independent total RNA preparations were made from 10 × 106 HCT116 BBR and HCT15 BBR cells by lysing them in RNAzol according to the manufacturer’s protocol. Each independent RNA preparation (1 μg) was reverse transcribed into cDNA with 200 units of superscript Reverse Transcription kit (Life Technologies, Inc., Carlsbad, CA) and 0.5 μg of oligo(dT) in a final volume of 20 μl of enzyme buffer for 60 min at 42°C. The primer sets used for PCR were 5’-TCT AGG ATC CGT TTG CGA CTT GGT ACT TG-3’ and 3’-CGG GAA ACT CTT CGA TAG CCT ACC CTA GGA TTC-5’. Alternatively, the primer of MFG vector used for PCR were 5’-TGG TAC TTC ACC CTT ACC GAG TC-3’ and 3’-CCG GGA ACT CCT CGA TAG CCT-5’.

Construction of the MFG-165-mutated MGMT. Two RT-PCR products, cDNA coding sequences of two AGT mutants (K165E or K165N), was inserted respectively into the unique EcoRI site in the pW derivatives. Six μg of plasmid DNA were transfected into 2 × 106 CHO cells with LipofectAMINE (Life Technologies, Inc.) following the protocol of the manufacturer. MFG-K165 cDNA was cotransfected with 0.6 μg of pSV2neo plasmid DNA as the selectable marker (28). Transfected cells are selected in G418 (0.1% w/v). The MFG-mutant AGT in CHO cells was confirmed by RT-PCR analysis.

RESULTS

Acquired Resistance to BG+BCNU in BBR Lines. We have shown previously that a single exposure to 10 μM BG sensitizes MMR-proficient and -deficient cell lines to BCNU (24). After BG+BCNU selection, SW480 BBR showed increased resistance to BCNU alone compared with parental SW480, but the cells were still sensitive to BG-enhanced BCNU-cytotoxicity, resulting in a 3-fold reduction in IC50 versus BCNU alone. In the two MMR-deficient cell lines, HCT116 BBR and HCT15 BBR, the IC50 for BCNU alone was similar to that in parental cell lines, but the cells were completely resistant to BG potentiation, resulting in a 3- to 4-fold higher DMF (DMF = IC50 of BCNU only ÷ IC50 of BG+BCNU) for the combination of BG with BCNU (Table 1).

AGT Activity, Protein, and mRNA in BBR Lines. To determine whether the loss of BG-sensitization in two MMR-deficient BBR lines was attributable to altered AGT, we measured the level of AGT activity, protein, and mRNA in parental and BBR cells. As shown in Table 2, in HCT15 BBR and HCT116 BBR cells, AGT activity...
Table 3 Inactivation of AGT by BG in parental and BBR cell lines*  

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Sublines</th>
<th>ED_{50} (µM)</th>
<th>Sublines</th>
<th>ED_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td></td>
<td>0.21 ± 0.05</td>
<td>SW480 BBR</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>HCT116</td>
<td></td>
<td>0.29 ± 0.03</td>
<td>HCT116 BBR</td>
<td>&gt;15</td>
</tr>
<tr>
<td>HCT15</td>
<td></td>
<td>0.31 ± 0.11</td>
<td>HCT15 BBR</td>
<td>&gt;15</td>
</tr>
</tbody>
</table>

* Results are the means ± SD of three independent experiments.

Fig. 1. A, the levels of AGT protein analyzed by Western blot in BBR lines and parental cells. Equivalent protein load confirmed by actin. B, comparison of the levels of RNA of MGMT gene between BBR lines and parental cells. Northern blot hybridization was used for MGMT mRNA analysis, and GAPDH was used as a loading control. P, parental cells.

markedly decreased compared with that in parental cells, from 22 to 2.5 fmol/µg DNA in HCT15 BBR and from 7.0 to 1.7 fmol/µg DNA in HCT116 BBR cells. AGT protein detected by Western blot analysis (Fig. 1A) was also reduced to 40% of that in parental lines for HCT116 BBR and to 10% of parental levels in HCT115 BBR cells. None of these changes were observed in SW480 BBR cells. Both AGT activity and AGT protein in SW480 BBR cells remained the same as in the parental cell line. To test whether the low level of protein expression and low enzymatic activity in the MMR-defective BBR cell lines resulted from decreased transcription, mRNA levels of MGMT were measured by Northern blot analysis (Fig. 1B). Interestingly, MGMT mRNA was expressed at a similar level in parental and BBR cell lines, which suggests that the low levels of AGT activity result from protein instability.

AGT Resistance to BG in BBR Lines. We have shown previously (24) that all three parental cell lines were very sensitive to BG, with 10 µM BG inhibiting AGT by >95%. However, both HCT116 BBR and HCT15 BBR cellular AGT was completely resistant to 15 µM BG. In contrast, SW480 BBR cellular AGT was not resistant to BG (ED_{50} of 0.2 µM). The data in Table 3 indicate that AGT in two MMR-deficient BBR lines was at least 50-fold resistant to inactivation by BG, compared with parental lines, despite low protein levels and reduced activity.

Mutations in Amino Acid 165 Position of AGT Were Identified in Two Colon Cancer Cell Lines with MMR Defect. We performed direct genomic DNA sequence of exon V, an active region of the MGMT gene and confirmed that the mutation occurred at position K165 of AGT. In HCT116 BBR cells, a mutation, AAG to GAG at amino acid 165, which substituted lysine to glutamic acid (K165E), was observed. A different mutation was observed in HCT115 BBR, AAG to AAT at 165 position, which substituted lysine to asparagine (K165N). The K165E mutation in HCT116 BBR appeared to be a homozygous mutation, whereas, K165N mutation in HCT15 BBR appeared to be heterozygous (Fig. 2). FISH analysis for chromosome 10, where human MGMT gene is located (10q26), in these two BBR cell lines and their parental cell lines showed that HCT116 parental cell line has only one copy of the MGMT gene (Fig. 3, A and B). It appeared that this single MGMT copy was mutated to K165E in HCT116 BBR cells after the selection with BG and BCNU. In contrast, HCT15 and HCT15 BBR showed two copies of the MGMT gene (Fig. 3, C and D). To determine whether aberrant methylation of the MGMT promoter might be present, resulting in transcriptional inactivation of one of the alleles of MGMT in HCT15 cells, we examined both HCT15 and HCT 15 BBR for the promoter-methylation status of the MGMT gene. Methylation of the promoter of the MGMT assay in HCT15 and HCT15 BBR cell lines revealed the presence of both unmethylated and methylated MGMT alleles in the parental line and its BBR line. This clearly indicated that, in HCT15 cell line, one allele of MGMT was inactivated and K165N was emanated from the allele that functionally expressed AGT (Fig. 4).

Expression of K165-mutant MGMT in CHO Cells. To further examine the hypothesis that the K165 AGT mutants were responsible for the acquired resistance to BG+BCNU in both of the BBR cell lines, we cloned K165E and K165N MGMT genes by RT-PCR from two BBR cell lines. These cDNAs were respectively inserted into the MFG retroviral vector and transfected into CHO cells that have undetectable AGT and are very sensitive to BCNU. RT-PCR analysis showed expression of K165E and K165N MGMT in CHO cells (Fig. 5A). Activity of these two mutant AGTs was extremely low, about
4–5 fmol/mg protein compared with 3100 fmol/mg protein for wt AGT derived from the same construction and expressed in CHO cells. However, Western blot analysis revealed that the amount of K165E AGT protein was higher than K165N AGT protein (Fig. 5B). AGT protein levels are not correlated with AGT activity in K165E cells, which suggests that K165E AGT detected by Western assay includes partially nonfunctional proteins. The levels of K165N-AGT protein, notably, appeared to be very low and completely degraded at 3 h after cycloheximide (Fig. 5C), although it had enzymatic activities similar to those of K165E AGT. These results suggest that the AGT with substituted asparagine is less stable than the glutamic acid substituted at position 165 and that two K165-mutant AGT proteins may have an increased rate of resynthesis because ubiquitin-mediated degradation of AGT protein serves a feedback signal for the regeneration of active AGT (29). Thus, low activity in K165-mutant cells may just reflect the specific repair capacity at a certain time point.

Resistance to BG and Alkylating Agents by K165-mutated MGMT Expressed in CHO Cells. Despite the very low level of AGT activity of the K165 mutants in CHO, these cells were still drug resistant. As shown in Fig. 6, after the inhibition of AGT by 25 μM of BG, CHO-AGT wt cells were sensitized to BCNU with a reduction in the IC50 from 28 μM to 6 μM, which is similar to the intrinsic sensitivity of parental CHO cells to BCNU (4 μM). In contrast, CHO-K165E and CHO-K165N cells were more resistant to BCNU than CHO parental cells with IC50 values of 15 μM and 9 μM, respectively, and concentrations of BG up to 200 μM did not sensitize these cells to BCNU (Fig. 6, top panels). Thus, these two K165 AGT mutants, despite very low AGT activity, were completely resistant to BG inactivation. Similar results were observed when cells were treated with TMZ and BG (Fig. 6, bottom panels). BG sensitized CHO-AGT wt cells to TMZ by 5-fold because of the effect of BG-inhibited AGT activity. However, K165 AGT mutants were resistant to BG and conferred a 5- to 6-fold more resistance to TMZ than did CHO parental cells, which indicated that K165 AGTs were active in the repair of both O6-chloroethyl guanine and O6-methyl guanine adducts formed by alkylating agents.

Expression of K165-mutated MGMT in SW480 Cells. We were still concerned that the low AGT activity seen in the BBR cells might be caused by a dominant-negative function of the K165 AGT mutants, inactivating wt AGT activity. To test this, K165E-MFG was trans-
DISCUSSION

This is the first report to show that after treatment with BG and BCNU, MMR-deficient cells are more likely to develop BG-resistant AGT. Because the loss of MMR activity is a common finding in human tumors, this study suggests that secondary mutations in drug resistance genes may contribute to clinically observed failure of chemotherapy and to tumor progression in patients whose tumors are MMR defective.

The human MMR genes contribute to genomic stability (28, 30, 31). Loss of the function of MMR leads to loss of surveillance and repair of replication misincorporation errors and to exogenous DNA damage, which results in genetic hypermutability. Human cell lines with defects in MMR have greatly enhanced spontaneous mutation rates at the hypoxanthine guanine phosphoribosyl-transferase (HPRT) which are up to 1000-fold higher than that in normal human fibroblasts or MMR-competent colorectal carcinoma cell lines (32–34). An accumulation of multiple mutations was found in the APC and p53 genes in a hereditary nonpolyposis colorectal carcinoma (HNPCC) patient with an inactivated MSH2 gene (35). Moreover, frequent inactivation of the TGFβ receptor, resulting from frameshift mutations, was observed in colorectal carcinoma cells with microsatellite instability (36). Thus, defects of MMR result in genomic instability, which is associated with an increase in the mutation rate of genes that may impact on DNA repair and cell proliferation.

In this study, we found that, after selection for BG and BCNU resistance, two MMR-deficient cell lines developed BG-resistant AGT because of two different mutations occurring at amino acid K165 of AGT, to form K165E and K165N, respectively. It has been previously shown that the mutagenesis of BCNU is mediated by O6-alkylguanine lesions and that the predominant spectrum of mutations induced by BCNU in CHO cells is transversions, comprising 69% of the mutations, whereas transitions occur in only 11% of BCNU-induced mutations (37). Therefore, the mutations that we observed in the MGMT gene, A:T to G:C in K165E and G:C to T:A in K165N, may well have resulted directly from the lesions induced by BCNU.

Because the activity of the K165 AGT mutants was very low in BBR cells, which suggested that they may not be capable of eliciting BG resistance, we were concerned with the possibility of other factors contributing to BG and BCNU resistance. First, because the two cell lines with AGT mutations were MMR defective, they may acquire many mutations both spontaneously and directly as a result of DNA damage throughout the genome. Moreover, compared with the parental cells, two BBR cell lines had similar sensitivities to BCNU alone even under such low AGT activity, which suggested that secondary mutation may also occur in other DNA repair genes or genes that are responsible for the regulation of cell cycle checkpoint and apoptotic death. Second, it has been demonstrated that the MMR defect may have an impact on the resistance to various chemotherapeutic agents because of the failure to detect DNA damage by the MMR system and to trigger the apoptotic cell death pathway (38, 39), which results in an elevated survival rate for drug-treated cells. Thus, we transferred K165-mutated MGMT genes into CHO cells to determine whether the K165 AGT mutants were responsible for the BG resistance. This transfer of drug resistance would rule out the presence of other resistant factors in the MMR-deficient BBR cell lines.

Although it is possible that a mutation in AGT, coupled with another mechanism of BCNU resistance, may be present in these cells, our results suggest otherwise. The results that emerged from experiments with CHO cell lines transfected with the two K165-mutated MGMT genes confirmed that the two K165 AGT mutants conferred increased resistance not only to BG+BCNU or

A. PCR Detection of K165 MGMT Proviruses

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
<th>Agarose (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>CHO-AGTw</td>
<td>1%</td>
<td>250</td>
</tr>
<tr>
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<td>150</td>
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<tr>
<td>3</td>
<td>CHO-K165N</td>
<td>3%</td>
<td>100</td>
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| 4    | K165E-mutant MGMT in SW480 cells (named SW/K165E), using the primers that identify only transfected MGMT (Fig. 7A). In the transfecants, total AGT activity, measured as repair of O6-mG, was not significantly changed; however, a small amount of AGT activity was resistant to BG inactivation, particularly at higher concentrations (Fig. 7B) because of the contribution of K165E AGT (estimated at about 5% of total AGT). Moreover, although BG potentiated BCNU cytotoxicity by 4-fold in SW480 cells, it failed to sensitize SW/K165E cells to BCNU (Fig. 7C). Thus, K165E AGT did not appear to confer a dominant-negative phenotype (AGT inactivation) to cells expressing wt AGT but did confer resistance to BG.

B. K165 AGT Proteins and activities

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>AGT activity (fmol/mg protein)</th>
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<tr>
<td>CHO-AGTw</td>
<td>3100 ± 200</td>
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<tr>
<td>CHO-K165E</td>
<td>4.3 ± 2.7</td>
</tr>
<tr>
<td>CHO-K165N</td>
<td>5.1 ± 0.8</td>
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</table>

C. AGT Protein Stability in wt and K165 Mutants CHO cells

<table>
<thead>
<tr>
<th>Time (h) after Cycloheximide (50 µg/ml)</th>
<th>CHO-AGTw</th>
<th>CHO-K165E</th>
<th>CHO-K165N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
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<td>24</td>
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Fig. 5. A, PCR detection of K165 MGMT using primers of MGMT in CHO transfectants. Lane 1, AGT-wt; Lane 2, K165E expressed in cell mass; Lane 3, K165E clone; Lane 4, K165N clone; Lane 5, CHO parental cells (nontransfectants). B, expression of K165 AGT protein measured by Western blot, and AGT activities measured by HPLC method in CHO cells. Results are the mean ± SD of three independent experiments. C, comparison of the stability of wt AGT with K165-mutant AGT. Cells were incubated with cycloheximide (50 µg/ml) for 0–24 h. AGT protein was detected by Western blot.
BG + TMZ but also to each of these alkylating agents alone. It has been noted that CHO-wt AGT cells, compared with CHO-K165 AGT mutant-cells, have an ~700-fold greater total of AGT activity but only 2- to 3-fold greater survival resistance to BCNU or TMZ alone, which indicates that the resistance to one alkylator alone is not proportionally dependent on AGT expression. K165 AGT mutants may have greater reactivity with O^6-alkylguanine than do wt AGT; thus, despite such low activity, these two AGT mutants are sufficient for drug resistance.

The analysis of species difference in MGMT gene sequence (40–45) has shown that amino acids in certain regions of the MGMT gene affect AGT activity and sensitivity to BG (20–23, 38). It has been demonstrated that all of the AGT mutants created by direct mutagenesis in vitro, or random substitution and genetic selection in bacteria, to date retain at least some ability to repair O^6-alkylguanine, even though they are resistant to BG inactivation. The characteristics of the K165 AGT mutants described here are consistent with those observed in mutations at K165 created by site-direct mutagenic approaches (46, 47). Although the precise mechanisms of BG resistance in these mutants have not been delineated, it has been suggested that the Lys-165 residue confers resistance to BG, through backbone distortions affecting the active site, and may play a structural role in the protein (45–47). Therefore, the K165 mutants that we identified would likely change the conformation of the protein and cause AGT instability. Moreover, our results also suggest that BCNU is highly mutagenic at amino acid position 165 in MMR-defective cells. Whether this amino acid is a “hot spot” for BCNU attack and mutation or for the recognition of MMR remain to be elucidated.

Although not the focus of this study, SW480 BBR cells had an increase in resistance both to BG + BCNU and to BCNU alone. However, the resistant pattern is different from that observed in MMR-defective cells. SW480 BBR cells were still sensitive to BG-potentiated cytotoxicity of BCNU, and no acquired mutations were found in MGMT. Thus, additional studies will be required to identify

Fig. 6. Cytotoxicity of BCNU (top panels)/TMZ (bottom panels) with or without BG in CHO transfectants of K165 AGT mutants and wt AGT. Cells were treated with 0–50 μM BCNU for 2 h or plus 25 μM BG for wt AGT-CHO cells and 200 μM for two K165 mutant AGT-CHO cells for 2 h prior to 2-h exposure to BCNU/TMZ. A, CHO-AGT (wt); B, CHO-AGT (K165E) cells; C, CHO-AGT (K165N). (■), CHO parental cells or nontransfectants were treated with either BCNU or TMZ; (○), cells were treated with either BCNU or TMZ alone; (●), cells were treated with BG + BCNU. Results are the mean ± SD of at least three separate experiments.
AGT-independent mechanism in acquired drug resistance to BG and BCNU in these cells.

REFERENCES


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