Differential competitive resistance to methylating versus chloroethylating agents among five O^6-alkylguanine DNA alkyltransferases in human hematopoietic cells

Aparecida Maria Fontes,[1] Brian M. Davis,[1] Lance P. Encell,[2] Karen Lingas,[1] Dimas Tadeu Covas,[3] Marco Antonio Zago,[3] Lawrence A. Loeb,[4] Anthony E. Pegg,[5] and Stanton L. Gerson[1]

[1]Division of Hematology/Oncology and Comprehensive Cancer Center, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio; [2]Promega Corporation, Madison, Wisconsin; [3]Regional Blood Center and Faculdade de Medicina de Ribeirão Preto da Universidad de São Paulo, Centro de Terapia Celular, Ribeirão Preto, Brazil; [4]The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington School of Medicine, Seattle, Washington; and [5]Department of Cellular and Molecular Physiology, Penn State Medical Center, Hershey, Pennsylvania

Abstract

P140K-MGMT **and** ***G156A-MGMT*** **genes encode two O^6-benzylguanine–resistant O^6-alkylguanine DNA alkyltransferase proteins that confer a high degree of O^6-benzylguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or O^6-benzylguanine and temozolomide resistance to primary hematopoietic cells. In this study, we directly compared these and three other O^6-benzylguanine–resistant *MGMT* genes for their ability to protect the human erythroleukemia cell line, K562, using a direct competitive selection strategy to identify the mutation that conferred the greatest degree of protection from O^6-benzylguanine and either BCNU or temozolomide. MFG retroviral vector plasmids for each of these mutants [*G156A-MGMT* (ED_{50} for O^6-benzylguanine, 60 μmol/L); and *P140K-MGMT, MGMT-2* (S152H, A154G, Y158H, G160S, L162V), *MGMT-3* (C150Y, A154G, Y158F, L162P, K165R), and *MGMT-5* (N157T, Y158H, A170S; ED_{50} for benzylguanine, >1,000 μmol/L)] were mixed, and the virus produced from Phoenix cells was transduced into K562 cells. Stringent selection used high doses of O^6-benzylguanine (800 μmol/L) and temozolomide (1,000 μmol/L) or BCNU (20 μmol/L) administered twice, and following regrowth, surviving clones were isolated, and the *MGMT* transgene was sequenced. None of the mutants was lost during selection. Using temozolomide, the enrichment factor was greatest for *P140K-MGMT* (1.7-fold). Using BCNU selection, the greatest enrichment was observed with *MGMT-2* (1.5-fold). *G156A-MGMT*, which is the least O^6-benzylguanine–resistant *MGMT* gene of the mutants tested, was not lost during selection but was selected against. The optimal mutant *MGMT* useful as a drug resistance gene may depend on whether a methylating or chloroethylating agent is used for drug selection. [Mol Cancer Ther 2006;5(1):121–8]**

Introduction

O^6-benzylguanine, an inhibitor of the DNA repair protein, O^6-alkylguanine DNA alkyltransferase (AGT) plus 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) or O^6-benzylguanine plus temozolomide have increased therapeutic efficacy over the chemotherapeutic agents alone in animal tumor xenograft models (1–8). Thus, these combinations are currently being evaluated in clinical trials with cutaneous lymphomas, malignant melanoma, brain neoplasms, multiple myeloma, and gastrointestinal carcinomas (9–12). However, the damage caused to the bone marrow by these alkylating agents manifests in the short term as myelosuppression or pancytopenia, the dose-limiting toxicity in clinical trials, and has the potential to induce therapy-related myelodysplasia or acute leukemia (13, 14).

Mutations in human *MGMT* have been developed, which retain AGT activity but are O^6-benzylguanine resistant. Based on the crystal structure of the AGT active site pocket, it is apparent that these mutants affect the size, hydrophobicity, and flexion of the active site pocket, resulting in exclusion of the benzyl moiety from the active site or a reduction in its ability to undergo an alkyl transfer reaction with the active site cysteine. *G156A-MGMT*, located within the DNA-binding wing flanking the alkyl-binding pocket (15), is moderately O^6-benzylguanine resistant (EC_{50} = 60 μmol/L). It distorts the adjacent loop (residues 158–160) that constitutes one wall of the benzyl-binding pocket (16, 17). The *P140K-MGMT* mutant alters the floor of the alkyl-binding pocket, excluding the benzyl group and

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Note: A.M. Fontes is currently at the Regional Blood Center of Ribeirão Preto, Ribeirão Preto, SP, 14051-140 Brazil. B. Davis is currently at Virexis, Gaithersburg, Maryland. L. Encell is currently at Tanox, Inc., Department of Molecular Biology, 10301 Stella Link, Houston, TX 77025.

Requests for reprints: Stanton L. Gerson, Division of Hematology Oncology, BEB-3 Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4955. Phone: 216-368-1177; Fax: 216-368-1166. E-mail: slg5@po.cwru.edu

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resulting in a very high degree of O^6-benzylguanine resistance (EC_{50} > 1.2 mmol/L; refs. 18, 19). Molecular evolution with selection allowed the identification of multiple *MGMT* mutants highly resistant to O^6-benzylguanine, which retain the AGT ability to remove alkyl adducts from the O^6-guanine position in DNA (20). Three mutants (i.e., *MGMT-2, MGMT-3*, and *MGMT-5*), each containing three to five amino acid substitutions in the region of the active site, were the most resistant to O^6-benzylguanine and BCNU (EC_{50} > 2 mmol/L; ref. 20).

These mutants, O^6-benzylguanine–resistant *MGMT* genes, provide a potent selection strategy for drug resistance gene transfer. We have used this for hematopoietic stem cell selection because two chemotherapeutic agents, BCNU, and methylating agents, such as temozolomide, are stem cell toxins, resulting in cumulative stem cell loss and delayed myelosuppression. We first introduced the use of O^6-benzylguanine–resistant *G156A-MGMT* to selectively protect the human CD34 cells and suggested that this selective protection could occur while sensitizing the tumor to chemotherapy using O^6-benzylguanine (21). Selecting the best O^6-benzylguanine–resistant *MGMT* for gene therapy is an important therapeutic decision. These mutants vary in stability, level of O^6-benzylguanine resistance, and repair capacity for either O^6-methylguanine or O^6-chloroethylguanine. The aim of the present study was to compare the ability of five of the most potent *MGMT* mutants to protect human hematopoietic cells against toxicity from O^6-benzylguanine + BCNU or O^6-benzylguanine + temozolomide treatment using a direct competitive selection strategy.

Materials and Methods

Chemicals

BCNU and temozolomide were obtained from the Developmental Therapeutics Branch, National Cancer Institute (Bethesda, MD). The BCNU was solubilized in 100% ethanol before dilution in PBS and used within 10 minutes of reconstitution. Temozolomide was solubilized in DMSO (Sigma, St. Louis, MO) before dilution in PBS and was used within 10 minutes of reconstitution. O^6-benzylguanine was synthesized by R. Moschel at the Frederick Cancer Research Institute and was solubilized in DMSO. Complete medium for K562 (human chronic myelogenous leukemia cell line) consisted of Iscove's modified Dulbecco's medium (Life Technologies/Bethesda Research Laboratories, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% L-glutamine plus 1% penicillin/streptomycin (Life Technologies/Bethesda Research Laboratories); and for producer cell line Phoenix, consisted of DMEM (Life Technologies/Bethesda Research Laboratories) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 1% L-glutamine plus 1% penicillin/streptomycin. Human interleukin-3 and human granulocyte macrophage colony-stimulating factor were purchased from R&D Systems (Minneapolis, MN). The monoclonal antibody anti-*MGMT* (monoclonal antibody clone MT3.1) was purchased from Kamiya Biomedical Co. (Seattle, WA), and R-phycoerythrin–conjugated antibodies were purchased from Caltag Laboratories (Burlingame, CA). The mouse isotype IgG1 was purchased from Becton Dickinson (San Jose, CA).

Retroviral Vectors

Retroviral vector pMFG-G156A was constructed to express cDNA sequence for *G156A-MGMT* as previously described (15). Retroviral vector pMFG-P140K was constructed to express cDNA sequence for *P140K-MGMT* by Davis et al. (22) and was originally selected for dual resistance to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and O^6-benzylguanine from a library of AGT mutants containing a random sequence at positions 138 to 140 (18). Retroviral vectors *pMFG-MGMT-2, pMFG-MGMT-3*, and *pMFG-MGMT-5*, containing the following mutations of the *MGMT* gene, as previously described, *MGMT-2* (S152H, A154G, Y158H, G160S, L162V), *MGMT-3* (C150Y, A154G, Y158F, L162P, K165R), and *MGMT-5* (N157T, Y158H, A170S), were subcloned into MFG retroviral vector (20). These three *MGMT* mutant genes were originally selected for dual resistance to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and O^6-benzylguanine from a library of *MGMT* mutant genes containing a random sequence at positions 150 to 172 (19) and conferred the greatest degree of resistance to O^6-benzylguanine and BCNU in K562 cells (20).

Cell Transfections and Transductions

DNA from each retroviral vector plasmid was isolated using NucleoBond Plasmid Max kit from Clontech (Palo Alto, CA) and then pooled by mixing 2 μg of each construct. A total of 10 μg of retroviral plasmid was transfected into 5×10^6 Phoenix amphotropic packaging cells (a gift from G. Nolon, Stanford University, Stanford, CA), using a calcium phosphate coprecipitation protocol.[4] To transduce K562 cells, these amphotropic producer cells were grown to confluence, and the virus was harvested in DMEM supplemented with 10% heat-inactivated fetal bovine serum. To remove contaminating producer cells, supernatant was filtered through a 0.45-μm filter (Millipore, Bedford, MA). Polybrene was added at 5.5 μg/mL. K562 cells were infected twice at intervals of 24 hours. The efficiency of transfection and transduction was measured by flow cytometry.

Flow Cytometric Analysis

To determine the *MGMT* expression in transduced cells, 1×10^6 cells were washed in 2% bovine serum albumin (BSA)/PBS, stabilized for 30 minutes at 4°C using 1% paraformaldehyde (in PBX 1×), and permeabilized by incubating in 1% Tween 20 (in 2% BSA/PBS) for 30 minutes at 37°C. After permeabilization, cells were washed in 2% BSA/PBS, and nonspecific binding sites were blocked for 30 minutes at 22°C with 10% normal goat serum. AGT antibody mT3.1 (1 μg) was added, and cells were incubated at 4°C overnight. Cells were washed twice with

[4] http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html

2% BSA/PBS and incubated with secondary antibody (goat anti-mouse IgG-1γ phycoerytrin conjugated) for 1 hour at 4°C. After washing, cells were resuspended in 300 μL of 2% BSA/PBS for fluorescence-activated cell sorting analysis. Flow cytometry analysis of 10,000 events was done on a FACScan (Becton Dickinson) running CellQuest data acquisition and analysis software (Becton Dickinson). Light scatter was used for gating on permeabilized cells.

Stringent Selection Protocol

High-stringency O^6-benzylguanine and BCNU and O^6-benzylguanine and temozolomide conditions were used to select O^6-benzylguanine–resistant *MGMT* mutant expressing K562 cells. For O^6-benzylguanine and BCNU selection, transfected K562 cells (30×10^6) were treated with 800 μmol/L O^6-benzylguanine for 1 hour followed by treatment with 20 μmol/L BCNU for 2 hours. For O^6-benzylguanine and temozolomide selection, transfected K562 cells (30×10^6) were treated with 800 μmol/L O^6-benzylguanine for 1 hour followed to treatment with 1,000 μmol/L temozolomide for 2 hours. Temozolomide-treated cells were washed in PBS and exposed additionally to 800 μmol/L O^6-benzylguanine for 16 to 24 hours to provide the maximal toxicity to residual O^6-methylguanine lesions. Cells treated with both agents were washed with PBS to remove the drugs and left to grow for 2 weeks in the presence of 25 μmol/L O^6-benzylguanine. This provides ongoing AGT inhibition and is the maximal amount of O^6-benzylguanine that can be added without slowing cell cycle times (3). After recovery, a second identical treatment was done. Outgrowth before analysis was for 14 days.

Analysis of the Cytotoxic Effects of O^6-Benzylguanine and Temozolomide

Colony-Forming Unit Assay. The O^6-benzylguanine and BCNU and O^6-benzylguanine and temozolomide resistance conferred to K562 cells by these five O^6-benzylguanine–resistant MGMT mutants was determined by clonogenic assays. Transfected K562 cells were exposed to increasing doses of temozolomide or BCNU, in the presence or absence of 25 μmol/L O^6-benzylguanine. After treatment, cells were washed in PBS, and 1,000 cells/mL were plated in triplicate in complete methylcellulose medium (0.8% methylcellulose, 1% BSA, 2 mmol/L L-glutamine, 0.1 mmol/L 1-mercaptoethanol, 10 ng/mL human interleukin-3, and 85 ng/mL human granulocyte macrophage colony-stimulating factor in Iscove's modified Dulbecco's medium; Stem Cell technologies, Vancouver, British Columbia, Canada). One-milliliter samples of the mixture were plated into 35-mm dishes and grown for 10 days. Colonies containing >50 cells was enumerated and compared with parental K562 cells.

PCR Analyses. In four independent experiments, individual colonies picked from methylcellulose cultures were washed with PBS, centrifuged, and then resuspended in 25 to 30 μL of a buffer containing 50 mmol/L Tris-HCl (pH 8), 10 mmol/L EDTA, 100 mmol/L NaCl, and 1% Triton X-100, plus 1 mg/mL proteinase K. The digestion was carried out at 55°C for 2 hours. Proteinase K was inactivated by heating at 95°C for 8 to 10 minutes. Two to five microliters of the digested sample were used for PCR analysis. Amplification was done (after an initial 3 minutes at 95°C) for 35 cycles of 30 seconds at 94°C, 40 seconds at the indicated annealing temperature, and 40 seconds at 72°C. A final extension period of 10 minutes was done. Annealing temperature was 62°C for the *MGMT* gene and 60°C for the *dystrophin* gene. Primers for *MGMT* gene (5′-CTTCACCATCCCGTTTTCCAG-3′ and 5′-CTGCCAGACCTGAGCTCCCTC-3′) amplify a 316-bp product; dystrophin primers (5′-TCACTTGCTTGTGCGCAGG-3′ and 5′-GAAAAGTGTATATCAAGGCAGCGACGATAA-3′) amplify a 500-bp product. The PCR was done with 40 pmol of each primer, 0.2 mmol/L deoxynucleotide triphosphates, 2 units of Taq polymerase, in a total volume of 50 μL. The PCR products were separated on 1.3% agarose gel and visualized after ethidium bromide staining. The amplified DNA was purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) and sequenced to characterize the *MGMT* gene mutant. Sequence data were analyzed by the MacVector program, version 3.1. The wild-type human *MGMT* is from Genbank (accession no. NM_002412).

Characterization of the Transduced K562 Cell Population

Analysis of Individual K562 Clones. Individual colonies of transduced K562 cells were picked from methylcellulose and cultured in Iscove's modified Dulbecco's medium as above, and cells were harvested for PCR analysis to evaluate the efficiency of the integration of retrovirus DNA. PCR-positive clones were submitted to fluorescence-activated cell sorting analysis for AGT protein expression as above and to reverse transcription-PCR for mRNA *MGMT* expression level.

Reverse Transcription-PCR Assay. Total cellular RNA was extracted from 1×10^6 K562 cells with SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Reverse transcription of RNA to cDNA was done according to the protocol of the SuperScript First Strand Synthesis System for reverse transcription-PCR (Life Technologies Bethesda Research Laboratories). For detection of *MGMT* mRNA, each K562 clone, 10% of the reverse transcriptase reaction with or without reverse transcriptase enzyme, was amplified by PCR with the same thermal cycling profile as above, using 25 cycles. Each PCR was carried out in 25 μL and resolved on a 1.3% agarose gel.

Results

Engineering Erythroleukemia K562 Cells to Constitutively Produce Five Mutant O^6-Benzylguanine–Resistant Human Alkyltransferase Proteins

The five O^6-benzylguanine–resistant *MGMT* mutants used in this study (Table 1) have different amino acid substitutions. The three mutants containing three to five amino acid changes near the active site and *P140K-MGMT* have

Table 1. Mutations in O^6-benzylguanine resistant *MGMT*s

O^6-benzylguanine–resistant MGMTs	Mutations
G156A-MGMT	$Gly^{156} \rightarrow Ala$
P140K-MGMT	$Pro^{140} \rightarrow Lys$
MGMT-2	$Ser^{152} \rightarrow His$; $Ala^{154} \rightarrow Gly$; $Tyr^{158} \rightarrow His$; $Gly^{160} \rightarrow Ser$; $Leu^{162} \rightarrow Val$
MGMT-3	$Cys^{150} \rightarrow Tyr$; $Ala^{154} \rightarrow Gly$; $Tyr^{158} \rightarrow Phe$; $Leu^{162} \rightarrow Pro$; $Lys^{165} \rightarrow Arg$
MGMT-5	$Asn^{157} \rightarrow Thr$; $Tyr^{158} \rightarrow His$; $Ala^{170} \rightarrow Ser$

millimolar EC_{50} values for O^6-benzylguanine inactivation, compared with the wild-type value of 0.1 μmol/L O^6-benzylguanine (Table 2). There is variability among the mutants in protein stability: *G156A-MGMT* is the least stable.

For transduction into K562 cells, we generated an amphotropic producer cell line by transfecting Phoenix cells with a pool of the five plasmids (pMFG-G156A-*MGMT*, pMFG-P140K-*MGMT*, pMFG-*MGMT-2*, pMFG-*MGMT-3*, and pMFG-*MGMT-5*) mixed at equimolar ratios. Twenty-four hours after transfection, 24% of Phoenix$^+$ cells expressed these transgenes as determined by flow cytometry using an anti-*MGMT* antibody (data not shown). After two cycles of transduction, the percentage of K562 cells expressing AGT by flow cytometry was 11% in two independent experiments (data not shown).

MGMT Expression in Clones of Transduced K562 Cells

To understand the results of the competitive selection, we determined the proportion of clones expressing *MGMT*. K562 clones positive for *MGMT* by PCR were expanded and *MGMT* expression analyzed by flow cytometric and reverse transcription-PCR assay. Of 14 *MGMT*$^+$ colonies analyzed by this method, three expressed high levels of AGT protein (89–96.2%, Fig. 1C–E), one moderate level (30%, Fig. 1F), four low level (4–7%, Fig. 1G–J), and six clones expressed at very low levels or not at all (1–2%, Fig. 1K–P). This analysis was repeated thrice with a total of 48 isolates, and similar results were obtained. Overall, 22% of PCR-positive clones expressed high levels of AGT protein, about 28% expressed low levels, and 43% had undetectable levels of expression.

We next asked whether differences in AGT expression were due to transcriptional activity effects or post-transcriptional events. Reverse transcription-PCR analysis of mRNA showed that the highest level of *MGMT* mRNA was detected in the clones that express high levels of AGT protein (Fig. 2, *lanes C1, C37*, and *C39*). Much lower levels of *MGMT* mRNA (Fig. 2, *lanes C5, C18, C20*, and *C32*) were detected for the clones that express 7% to 30% of AGT protein (Fig. 1, *C5, C18, C20*, and *C32*), whereas the absence of *MGMT* mRNA (Fig. 2, *lanes C4, C6, C22, C24, C26, C27*, and *C31*) was observed in clones with low or absent AGT protein (Fig. 1, *C4, C6, C22, C24, C26, C27*, and *C31*). Therefore, the absence of AGT protein is due to silencing of transcriptional activity and not due to translational effects.

Competitive Selection of Drug-Resistant K562 Clones by Stringent Drug Treatment

MGMT-transduced K562 cells were treated with 800 μmol/L O^6-benzylguanine for 1 hour and either 1,000 μmol/L temozolomide or 20 μmol/L BCNU for 2 hours. Surviving cells were retreated with the same drugs. Two independent experiments were done, and the selection results were pooled. Survival after the first drug selection was about 3% of control, with greater survival after the second treatment. To determine the degree of drug resistance conferred to K562 cells by these mutants after high-stringency selection, we determined the BCNU and temozolomide IC_{90} by seeding cells into methylcellulose medium and monitored for colony formation. K562 cell cultures containing unselected AGT mutants were 2-fold more resistant to BCNU (IC_{90} of 22 μmol/L) compared with parental K562 cells (IC_{90} around 10 μmol/L). After high-stringency O^6-benzylguanine and BCNU selection, K562 cells were 8-fold more resistance to BCNU (BCNU IC_{90} nearly 80 μmol/L; Fig. 3). Cells treated with BCNU alone had a similar survival curve, presumably because all O^6-benzylguanine–resistant *MGMT*s conferred O^6-benzylguanine resistance to the cell cultures, and a difference between the mutants could not be identified in the survival curves. Cells exposed to temozolomide had increased drug resistance after selection (Fig. 4). Parental K562 cells had a temozolomide $IC_{90} < 100$ μmol/L, whereas transduced K562 cells had a temozolomide IC_{90} of 600 μmol/L. After high-stringency O^6-benzylguanine and temozolomide selection, the temozolomide IC_{90} increased to >2,000 μmol/L, 20-fold above untransduced cells. When pretreated with 25 μmol/L benzylguanine, there is no significant difference in the temozolomide IC_{90} value.

Table 2. Features of O^6-benzylguanine–resistant MGMT proteins

O^6-benzylguanine–resistant MGMT proteins	ED_{50} O^6-benzylguanine (μmol/L)*	% AGT remaining after 12 hours of CHX†	Reference
Wild type	0.1	92	(20)
G156A-MGMT	60	30	(15)
P140K-MGMT	>1,200	86	(18)
MGMT-2	>2,000	70	(19, 20)
MGMT-3	>2,000	97	(19, 20)
MGMT-5	>2,000	67	(19, 20)

Abbreviation: CHX, cycloheximide.
*ED_{50} was determined by clonogenic assay following transduction into K562 cells.
†Stability was determined by Western blot.

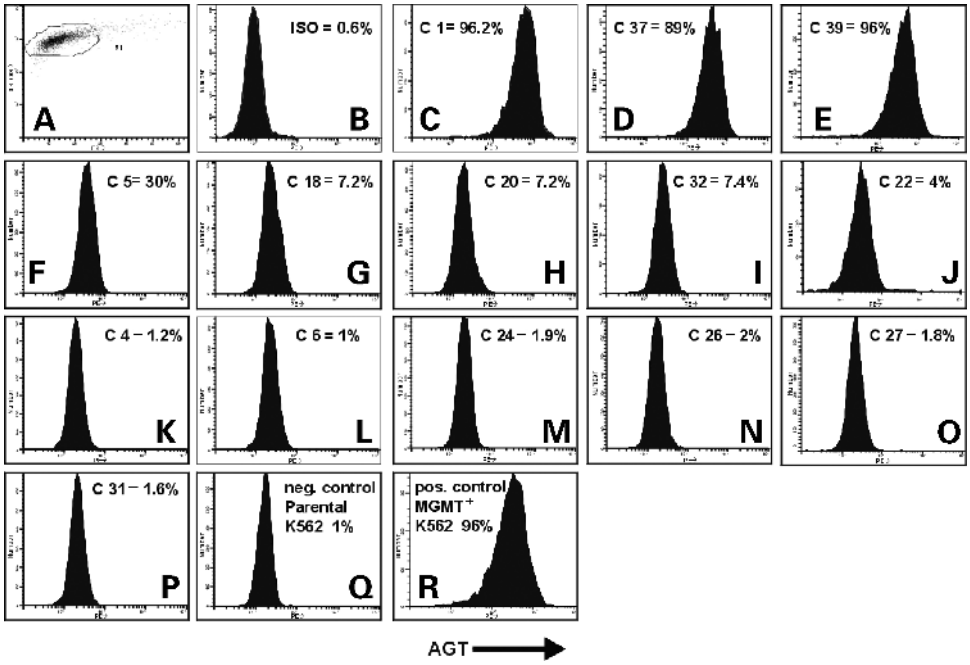

Figure 1. Alkyltransferase protein expression in *MGMT*$^+$ K562 clones. **A,** representative fluorescence-activated cell sorting dot blot light forward scatter/side scatter profile of K562 cells. **B,** representative histogram of K562 cells incubated with isotype-matched control antibody to establish profile setting. **C** to **P**, histograms showing AGT expression for each *MGMT*$^+$ K562 clone incubated with monoclonal antibody anti-MGMT (monoclonal antibody clone MT3.1). **Q** and **R**, histograms showing AGT expression from parental K562 (negative control) and *MGMT*$^+$/K562 cells known to express high level of this gene, respectively. Percentage of expression for each clone. *Top right,* % cells expressing AGT.

Enrichment of *P140K-MGMT*–, *MGMT-2*–, and *MGMT-5*–Transduced K562 Cell Clones after Stringent Selection with O^6-Benzylguanine *Temozolomide* or O^6-Benzylguanine *BCNU*

To identify whether there was competitive survival and expansion of K562 clones expressing a particular O^6-benzylguanine–resistant *MGMT* mutant and whether this depended on the drug used, we isolated K562 clones from methylcellulose and sequenced the mutant *MGMT* from sets of clones before and after drug treatment. As shown in Table 3, a total of 38 clones were sequenced from K562 clones without selection; 55 clones were sequenced after temozolomide selection and 55 clones were sequenced after BCNU selection. In the O^6-benzylguanine and temozolomide competitive selection studies, *P140K-MGMT*–transduced cells were enriched 1.7-fold and *MGMT-2* was enriched 1.2-fold ($P < 0.01$, χ^2 for the trend in distribution). In the O^6-benzylguanine and BCNU competitive selection studies, cells transduced with *MGMT-2* were enriched 1.5-fold, whereas *MGMT-5* was enriched 1.4-fold ($P = 0.18$, χ^2).

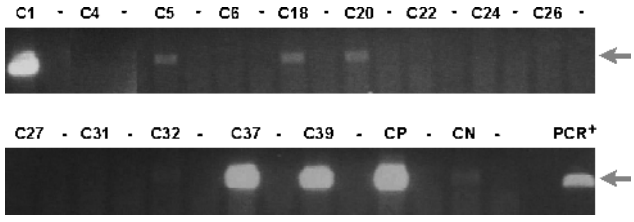

Figure 2. Expression *MGMT* mRNA in K562 *MGMT*$^+$ clones. Total RNA was prepared from each K562 clone and analyzed by reverse transcription-PCR for *MGMT* mRNA. Reactions were done without reverse transcriptase (–) to control for DNA contamination.

K562 clones transduced with the two other O^6-benzylguanine–resistant *MGMT* mutants (*G156A-MGMT* and *MGMT-3*) survived the drug treatment but were underrepresented among the clones isolated and sequenced. The rank order of O^6-benzylguanine–resistant *MGMT* mutants selected under these enrichment conditions differed after temozolomide compared with BCNU selection. In addition, the selection pressure for temozolomide seemed greater than with BCNU because the range of enrichment between

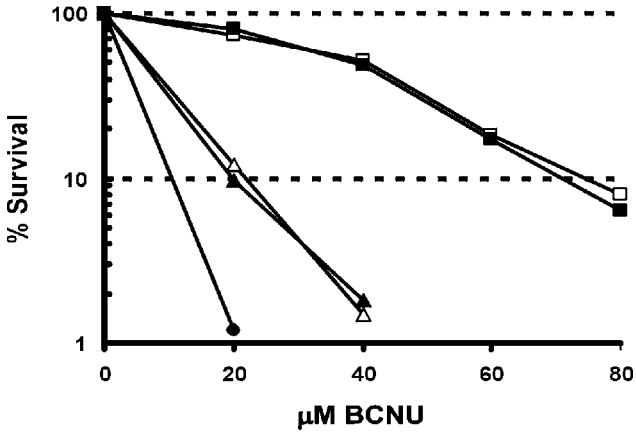

Figure 3. Comparison of the O^6-benzylguanine (*O^6-BG*) and BCNU resistance conferred to *MGMT*-transduced K562 cells before and after high-stringency selection. K562 cells were treated with BCNU alone (□, without selection; ■, after selection) or O^6-benzylguanine and BCNU (△, without selection; ▲, after selection). Cells were exposed to 25 μmol/L O^6-benzylguanine for 1 h followed with 0 to 80 μmol/L of BCNU. As a control, we measured the sensitivity of parental K562 cells to BCNU (●). K562 cells were plated at 1,000 per dish. After 10 to 12 d, colonies were enumerated.

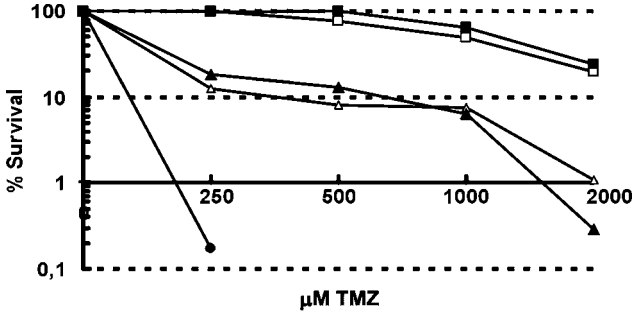

Figure 4. Comparison of the O^6-benzylguanine (*O^6-BG*) and temozolomide (*TMZ*) resistance conferred to MGMT-transduced K562 cells before and after high-stringency selection. K562 cells were treated with temozolomide alone (□, without selection; ■, after selection) or O^6-benzylguanine and temozolomide (△, without selection; ▲, after selection). Cells were exposed to O^6-benzylguanine (25 μmol/L) for 1 h before increasing doses of temozolomide (200 – 2,000 μmol/L). As a control, we measured the sensitivity of parental K562 cells (●) to temozolomide. K562 cells were plated at 1,000 per dish. After 10 to 12 d, colonies were enumerated.

the O^6-benzylguanine–resistant *MGMT* mutants was 4.9-fold with temozolomide but only 2.1-fold with BCNU. This may represent both intrinsic properties of drug selection and the dose intensity used.

Discussion

In this study, we compared the *in vitro* efficacy of five O^6-benzylguanine–resistant *MGMT* mutants in protecting K562 cells against cytotoxicity induced by temozolomide and BCNU. We found that competitive, high-stringency drug selection identified the most potent drug-resistant *MGMT* mutant, and that this O^6-benzylguanine–resistant *MGMT* mutant differed, depending on the drug used. The competitive *in vitro* selection and regrowth strategy is more discriminating than a simple survival assay and has been used to identify other drug resistance mutants, such as thymidine kinase (23), thymidylate synthase (24), dihydrofolate reductase (25), and glutathione *S*-transferase (26).

Our primary goal was to determine whether any of the five O^6-benzylguanine–resistant *MGMT* mutants would be lost during selection, and whether we could actually select from among these remarkably O^6-benzylguanine–resistant mutant proteins during high-dose O^6-benzylguanine exposure and drug treatment. We hypothesized that *G156A-MGMT* might be lost because it is less O^6-benzylguanine resistant, but it was not. Because all of the remaining four *MGMT* mutants are highly O^6-benzylguanine resistant, we might not have observed any selection. Instead, we found that after selection with O^6-benzylguanine and temozolomide, the most highly enriched mutant was *P140K-MGMT*, with an enrichment factor of 1.7 fold ($P = 0.01$). In contrast, resistance to O^6-benzylguanine and BCNU resulted in enrichment in favor of *MGMT-2* and *MGMT-5* by 1.5- and 1.4-fold, respectively. This selection only reached $P = 0.18$; thus, the selection was quite modest compared with O^6-benzylguanine and temozolomide. *MGMT-2* and *MGMT-5* also proved to be the most O^6-benzylguanine–resistant *MGMT* mutants in our previous study, where 11 different *MGMT* multiple mutants (20), not including *P140K-MGMT* and *G156A-MGMT*, were competed, and the enrichment factor for *MGMT*2 and *MGMT*5 was 10- and 6-fold, respectively. This study confirms those results.

One factor that might skew the results is to have one of the O^6-benzylguanine–resistant *MGMT* mutants transcribed or expressed at lower levels. This might overwhelm the ability to compete against another of the mutants. We analyzed this and found a similar distribution of gene expression and protein production among the mutants. On the other hand, the variation in viral production was not a factor because we analyzed the relative not the absolute enrichment after drug selection.

It is of interest that the *P140K-MGMT* mutant came in first with temozolomide and third during selection with O^6-benzylguanine and BCNU. Intrinsic properties of the various O^6-benzylguanine–resistant *MGMT* mutants likely contribute to the selection competition. The *MGMT-3* mutant is the most stable of the mutant AGTs (19, 20). Stability and expression levels are important for repair of

Table 3. Clonal O^6-benzylguanine–resistant *MGMT* enrichment in K562 cells after drug treatment

O^6-benzylguanine–resistant MGMT genes	No. selected	TMZ Selected	Fold enrichment	TMZ rank	BCNU selected	Fold enrichment	BCNU rank
Total isolates	38	55			55		
G156A-MGMT	6	3	0.35	5	7	0.8	3
P140K-MGMT	12	29	1.7	1	14	0.81	3
MGMT-2	6	10	1.16	2	13	1.5	1
MGMT-3	7	4	0.45	4	7	0.7	4
MGMT-5	7	9	1.0	3	14	1.38	2

NOTE: Clonal frequency is shown based on sequence of O^6-benzylguanine–resistant *MGMT* mutant recovered from K562 clones from unselected cultures and after either temozolomide or BCNU selection. Fold enrichment is the ratio of the proportion of recovered colonies in the selected versus unselected cohorts. The χ^2 for the distributions indicated $P < 0.01$ for the change in distribution after temozolomide and $P = 0.18$ for the change in distribution after BCNU. A total of 38 clones were analyzed from the unselected cultures and 55 each from the cultures selected with temozolomide and O^6-benzylguanine or BCNU and O^6-benzylguanine.
Abbreviation: TMZ, temozolomide.

O^6-methylguanine adducts generated by temozolomide because large numbers of adducts are formed, and removal of most of these adducts is required to protect the cell (27). In contrast, smaller numbers of BCNU-directed cross-links kill the cell; thus, a few highly O^6-benzylguanine-resistant molecules of AGT are sufficient, whereas stability and total expression level are less important. The fact that the *G156A-MGMT* mutant, whereas not the most potent, was not lost during either competitive drug selection suggests that the degree of O^6-benzylguanine resistance and protein stability are sufficient to repair DNA lesions in cells. This result is consistent with the ability of *G156A-MGMT* to protect CD34 cells *in vitro* (21), LTC-IC (28, 29), murine repopulating progenitor cells transplanted *in vivo* (30, 31), to increase the therapeutic efficacy of O^6-benzylguanine and BCNU when transduced into host marrow in tumor xenograft models (32) and in protecting hematopoietic cells against O^6-benzylguanine and temozolomide treatment *in vitro* (33). Although clinical concentrations of O^6-benzylguanine observed in phase I trials are up to 20 μmol/L (4, 10, 34), this approach was taken in an attempt to identify whether strong selection favor to select AGT with multiple mutations compared with single amino acid substitution, such as *G156A-MGMT*, which shows to be moderately O^6-benzylguanine resistant and the least stable AGT protein. In addition, among the other O^6-benzylguanine–resistant *MGMT* mutants, which showed to be highly O^6-benzylguanine resistant, we investigate whether some preferential amino acid substitutions would confer advantage to repair O^6-methylguanine or O^6-chloroethylguanine adducts on DNA. Surprisingly, *G156A-MGMT* was not lost during the competition study, and it shows the relative potency of *G156A-MGMT* in repair either O^6-methylguanine or O^6-chloroethylguanine lesions in DNA. Moreover, although *P140K-MGMT* was not preferred in the direct competition for O^6-benzylguanine and BCNU resistance, it is a stable AGT protein (22) and has shown efficacy as an O^6-benzylguanine and BCNU resistance gene protecting murine progenitor cells (35) *in vitro* and *in vivo* and in a dose-intensive tumor xenograft (36).

In summary, we have developed a direct competitive selection approach to analyze O^6-benzylguanine–resistant *MGMT* mutant enrichment after selection with either O^6-benzylguanine and temozolomide or O^6-benzylguanine and BCNU. This approach can detect differences not seen in comparative clonal survival curves. We find that *P140K-MGMT* is preferred during O^6-benzylguanine and temozolomide selection, whereas the multiple amino acid–substituted *MGMT-2* is slightly ($P = 0.18$) preferentially enriched after O^6-benzylguanine and BCNU. However, even the moderately O^6-benzylguanine–resistant mutant *G156A-MGMT* is not lost. This suggests that *MGMT-2*, in particular, should be added to the list of effective O^6-benzylguanine–resistant *MGMT* mutants that would be useful during drug resistance gene transfer into hematopoietic stem cells, and that the choice of O^6-benzylguanine–resistant *MGMT* mutant should depend on the drug selection anticipated.

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