Leukemia cells are sensitized to temozolomide, carmustine and melphalan by the inhibition of O^6-methylguanine-DNA methyltransferase

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Abstract. The cytotoxicity of the monofunctional alkylator, temozolomide (TMZ), is known to be mediated by mismatch repair (MMR) triggered by O^6-alkylguanine. By contrast, the cytotoxicity of bifunctional alkylators, including carmustine (BCNU) and melphalan (MEL), depends on interstrand cross-links formed through O^6-alkylguanine, which is repaired by nucleotide excision repair and recombination. O^6-alkylguanine is removed by O^6-methylguanine-DNA methyltransferase (MGMT). The aim of the present study was to evaluate the cytotoxicity of TMZ, BCNU and MEL in two different leukemic cell lines (HL-60 and MOLT-4) in the context of DNA repair. The transcript levels of MGMT, ERCC1, hMLH1 and hMSH2 were determined using reverse transcription-quantitative polymerase chain reaction. In addition, the proliferation was measured using the trypan blue exclusion assay. Drug sensitivity was found to vary between the two cell lines. Treatment of the cells with TMZ, BCNU or MEL in combination with O^6-benzylguanine, an MGMT inhibitor, was demonstrated to sensitize the two cell lines to these agents. However, the extent of sensitization was not found to be correlated with the expression levels of MGMT transcripts. Furthermore, the drug sensitivity was also not associated with the transcript levels of ERCC1, hMLH1 and hMSH2. Thus, leukemic cells were sensitized to alkylating agents by the inhibition of MGMT.

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Introduction

Alkylating agents comprise a major class of chemotherapeutic agents, widely used in various types of cancer, including leukemia (1,2). There are two types of alkylating agents: monofunctional and bifunctional agents. Bifunctional alkylating agents include cyclophosphamide, ifosfamide, melphalan (MEL) and carmustine (BCNU; also known as 1,3-bis(2-chloroethyl)-1-nitrosourea). Monofunctional agents include temozolomide [TMZ; also known as 3,4-dihydro-3-methyl-4-oxoimidazo (5,1-d)-as-tetrazine-8-carboxamide] and dacarbazine (1-3).

Alkylating agents form a variety of DNA adducts in cancer cells, including mono-adducts on N^1-alkylguanine, N^3-alkyladenine, N^7-alkylguanine or O^6-alkylguanine, and di-adducts between or within DNA strands (1-4). Bifunctional alkylating agents result in cytotoxicity due to the production of interstrand crosslinks, which are formed through the intermediate production of O^6-alkylguanine (5,6). These crosslinks are repaired through nucleotide excision repair (NER) and recombination. By contrast, monofunctional agents generate persistent O^6-methylguanine adducts that initiate futile cycling of the DNA mismatch repair (MMR) pathway, which causes DNA double-strand breaks (7-10). Intact MMR is required for the exertion of the cytotoxicity of monofunctional agents. The DNA repair enzyme, O^6-methylguanine-DNA methyltransferase (MGMT), repairs O^6-alkylguanine adducts (11,12) and reverses the cytotoxicity induced by the two types of alkylating agents.

The cytotoxic effects of alkylating agents are limited by a number of factors, including DNA repair (2,13,14). In the present study, the cytotoxic effects of the bifunctional BCNU and MEL agents, as well as the monofunctional TMZ agent, were evaluated in relation to DNA repair. The effects were compared in two cultured leukemia cell lines, HL-60 and MOLT-4. In addition, the sensitivity of the cells was manipulated by the addition of an MGMT inhibitor, O^6-benzylguanine (BG). The extent of the drug cytotoxicity was analyzed to determine its correlation with DNA repair, including any associations with MGMT, NER and MMR (15,16).

Our previous study demonstrated the important role of MMR in the exertion of the cytotoxicity of monofunctional agent temozolomide (17). Restored MMR sensitized the

cancer cells to temozolomide. Therefore, the aim of the present study was to evaluate the cytotoxicity of alkylating agents from the viewpoint of MGMT.

Materials and methods

Chemicals and reagents. BCNU, MEL and BG (all purchased from Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 99% ethanol immediately prior to use. TMZ (Schering-Plough KK, Osaka, Japan) was dissolved in 100% dimethyl sulfoxide immediately prior to use.

Cell culture. Human acute myeloid leukemia cell line, HL-60, and human acute T lymphoblastic leukemia cell line, MOLT-4, were used in this study (JCRB Cell Bank, Osaka, Japan). The cells were cultured in RPMI 1640 medium (Life Technologies Japan, Ltd., Tokyo, Japan) in a humidified atmosphere with 5% CO_2 at 37˚C.

Drug treatment and proliferation assay. To evaluate the growth-inhibitory effect of each agent on the two cell lines, the trypan blue exclusion assay was performed (17,18). Briefly, the cells were incubated with various concentrations of TMZ, BCNU or MEL (10 nM, 100 nM, 1 μM or 10 μM), alone or in combination with BG (10 μM), for 72 h. Subsequently, the samples were stained with trypan blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the viable cells, which exhibited negative staining, were counted. The 50% growth-inhibitory concentration (IC_{50}) was the concentration at which 50% of the growth of the untreated cells was inhibited. This value was extrapolated from the growth curve drawn for each drug treatment, with 100% considered to be the condition of untreated cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The transcript levels of MGMT, ERCC1, hMLH1 and hMSH2 were determined by RT-qPCR using the ABI Prism 7700 sequence detection system (Applied Biosystems Life Technologies, Foster City, CA, USA). RT-qPCR was performed according to the method of our previous study (17). ERCC1 is responsible for incision of the damaged DNA strand in the NER pathway, while hMLH1 and hMSH2 are two key factors in the MMR response. For MGMT, the sense primer sequence was 5'-TCCCGTTTTCCAGCAAGAGTC-3', and the antisense sequence was 5'-GGGCTGCTAATTGCTGGTAAGA-3'. The TaqMan probe DNA sequence was FAM-CCAGACA GGTGTTATGGAAGCTGCTGAAG-TAMRA (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). In addition, the primers for ERCC1, hMLH1 and hMSH2 were purchased from Mitsubishi Kagaku Bio-Clinical Laboratories. The absolute standard curve quantitation method was used for MGMT and ERCC1, and the relative standard curve quantitation method was used for hMLH1 and hMSH2. The values of HL-60 cells were set to 1 and relative values were determined for the MOLT-4 cells.

Statistical analyses. Graphs were generated using the GraphPad Prism software (version 5.0; GraphPad Software, Inc., San Diego, CA, USA). Spearman's rank correlation

Table I. Drug sensitivities of the two leukemia cell lines.

Drugs	IC_{50} (μM)	
	HL-60	MOLT-4
TMZ	49.0	191.5
BCNU	10.0	22.0
MEL	4.5	1.5
TMZ+BG	4.5	169.0
BCNU+BG	3.0	1.4
MEL+BG	0.3	1.4

Cells were incubated with various concentrations of TMZ, BCNU or MEL, with or without BG. The IC_{50} values were then determined using the trypan blue exclusion assay. TMZ, temozolomide; BCNU, carmustine; MEL, melphalan; BG, O^6-benzylguanine; IC_{50}, 50% growth-inhibitory concentration.

Table II. Sensitization by the addition of BG.

Drugs	Ratio of IC_{50}	
	HL-60	MOLT-4
TMZ / TMZ+BG	10.9	1.1
BCNU / BCNU+BG	3.3	15.7
MEL / MEL+BG	15.0	1.1

Ratios of IC_{50} values = (IC_{50} value of TMZ, BCNU or MEL treatment) / (IC_{50} value of TMZ, BCNU or MEL treatment in combination with BG). TMZ, temozolomide; BCNU, carmustine; MEL, melphalan; BG, O^6-benzylguanine; IC_{50}, 50% growth-inhibitory concentration.

was used for determination of any correlation between two parameters. All statistical analyses were performed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Growth-inhibitory effects of the alkylating agents. The growth-inhibitory effects of TMZ, BCNU and MEL were evaluated in the HL-60 and MOLT-4 cells. Overall, the sensitivity of the two cell lines to these agents varied; however, treatment with MEL appeared to be the most effective in inhibiting the cell growth (Table I).

Inhibition of MGMT by BG. The cytotoxic effect of alkylating agents is generally reduced by DNA repair in cancer cells (3-6). Upon treatment of the HL-60 and MOLT-4 cells with TMZ, BCNU or MEL in the presence of BG, an MGMT inhibitor, the two cell lines were apparently sensitized to all these agents (Table I). However, the extent of sensitization varied among the drugs in the two cell lines (Table II; Fig. 1). BG was not found to be cytotoxic to cells in the previous study (17).
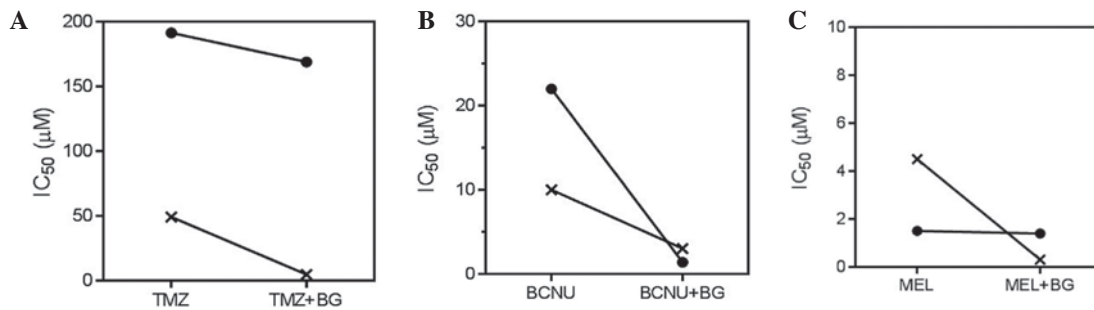
Figure 1. Changes in the IC$_{50}$ values following the addition of BG. The cellular sensitivity to (A) TMZ, (B) BCNU or (C) MEL were determined in the HL-60 and MOLT-4 cells in the presence or absence of BG using the trypan blue exclusion assay. Values are expressed as the mean of at least three independent experiments. TMZ, temozolomide; BCNU, carmustine; MEL, melphalan; BG, O^6-benzylguanine; IC$_{50}$, 50% growth-inhibitory concentration.
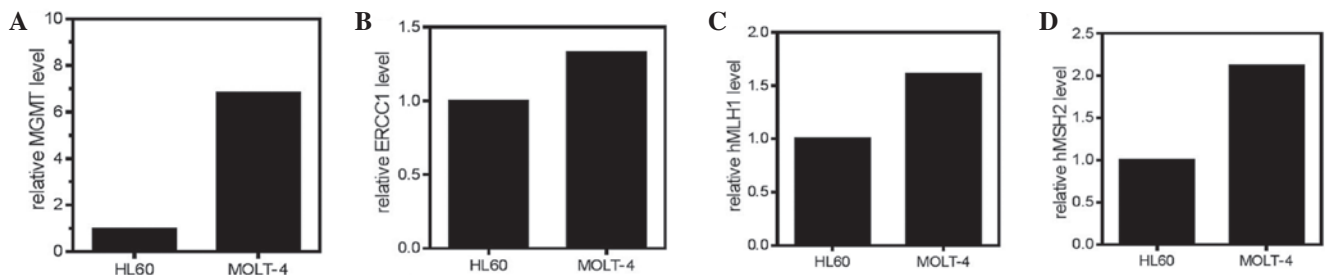

Figure 2. DNA repair-associated transcripts. Relative transcript levels of (A) MGMT, (B) ERCC1, (C) hMLH1 and (D) hMSH2 in the HL-60 and MOLT-4 cells, measured by using reverse transcription-quantitative polymerase chain reaction. The values of HL-60 cells were set to 1. MGMT, O^6-methylguanine-DNA methyltransferase.
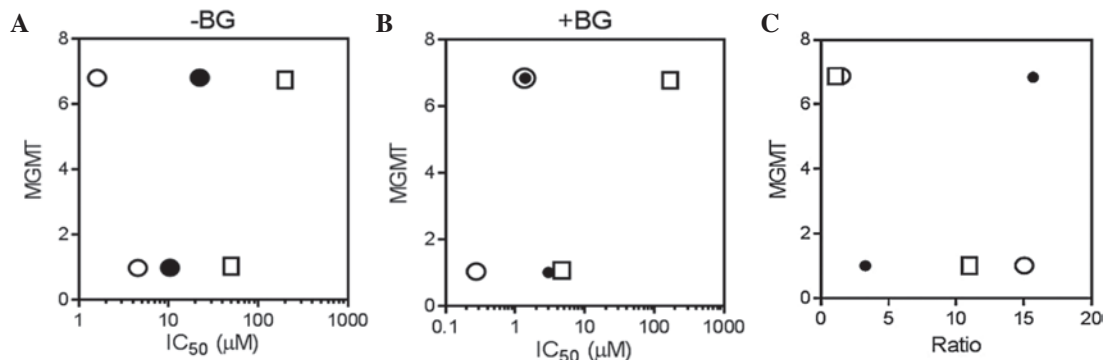

Figure 3. Correlation between MGMT transcript levels and drug sensitivity (in terms of IC$_{50}$ values) in HL-60 and MOLT-4 cells. Cells were incubated for 72 h with various concentrations of TMZ, BCNU or MEL, (A) with or (B) without a minimally toxic concentration of BG (10 μM). The growth inhibition effects were determined using the trypan blue exclusion assay. Values are expressed as the mean of at least three independent experiments. (C) The ratio between the IC$_{50}$ values of TMZ/BCNU/MEL to the values of TMZ/BCNU/MEL with BG was plotted against the MGMT transcript levels in each cell line. (A): P>0.99; (B): P>0.99 (Spearman's rank correlation). circle, MEL; filled circle, BCNU; square, TMZ. MGMT, O^6-methylguanine-DNA methyltransferase; TMZ, temozolomide; BCNU, carmustine; MEL, melphalan; BG, O^6-benzylguanine; IC$_{50}$, 50% growth-inhibitory concentration.

Transcript levels of DNA repair-associated genes. The transcript levels of MGMT, ERCC1, hMLH1 and hMSH2 were determined in the two cell lines (Fig. 2), and were found to be different between the two cell lines. Associations between these expression levels and the drug sensitivity were also investigated (Figs. 3 and 4). Alkylguanine is repaired primarily by MGMT (12,14,19). However, no apparent correlation was observed between the expression levels of MGMT and the drug sensitivity in the presence or absence of BG (Fig. 3A and B). Furthermore, the ratio of the IC$_{50}$ value of each agent over the IC$_{50}$ value of each agent + BG was calculated (Table II). These values were plotted against the MGMT

transcript levels (Fig. 3C). It was expected that the ratio may be higher in the cell line with a higher MGMT transcript level. However, the ratio was not correlated with the MGMT expression levels (Fig. 3C). This indicated that sensitization through the inhibition of MGMT could not be predicted based on the levels of MGMT transcript in these cell lines.

BCNU-mediated interstrand crosslinks are repaired by NER and recombination, while O^6-alkylguanine is repaired by NER (20). The ERCC1-XPF heterodimer is an endonuclease that is involved in the NER pathway (15). In the present study, the expression levels of ERCC1 were not found to be correlated to the drug sensitivity (Fig. 4A). No correlation was observed
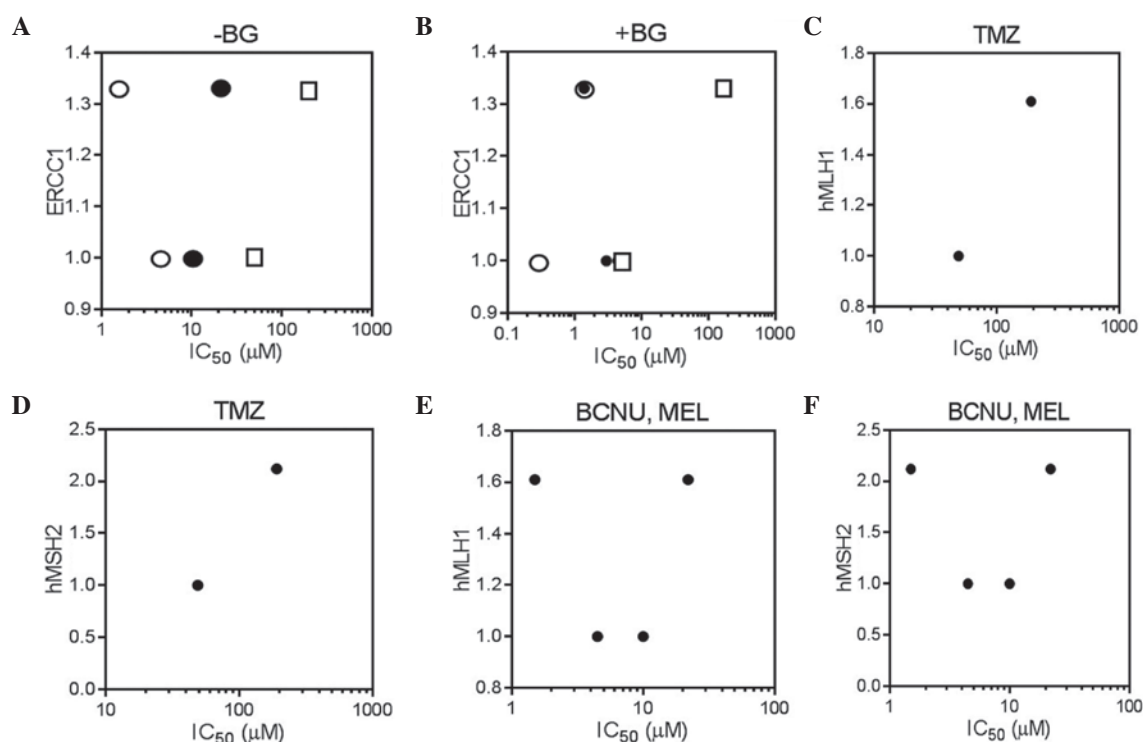
Figure 4. Correlation between ERCC1/hMLH1/hMSH2 transcript levels and IC_{50} values of BCNU/TMZ/MEL in HL-60 and MOLT-4 cells. The IC_{50} values of TMZ, BCNU and MEL, (A) with and (B) without a minimally toxic concentration of BG (10 μM) were plotted against ERCC1 transcript levels in the HL-60 and MOLT-4 cells (circle, MEL; filled circle, BCNU; square, TMZ). The IC_{50} values of TMZ were plotted against the (C) hMLH1 and (D) hMSH2 transcript levels of the HL-60 and MOLT-4 cells. The IC_{50} values of BCNU and MEL were plotted against the (E) hMLH1 and (F) hMSH2 transcript levels of the HL-60 and MOLT-4 cells. TMZ, temozolomide; BCNU, carmustine; MEL, melphalan; BG, O^6-benzylguanine; IC_{50}, 50% growth-inhibitory concentration.

using the IC_{50} values for the co-treatment with BG, which is an inhibitor of the activity of MGMT (Fig. 4B). Deficiency in MMR is known to result in cellular insensitivity to TMZ (10), while MMR is suggested to be involved in crosslink repair induced by BCNU (21). Based on these previous observations, a lower MMR function may be associated with lower sensitivity to a mono-functional TMZ, whereas a higher MMR function may confer the higher resistance to bifunctional BCNU and MEL. However, in the present study, no association was detected between the expression levels of the MMR-associated hMLH1/hMSH2 and the sensitivity to these two agents (Fig. 4C-F).

Discussion

The MMR response triggered by O^6-alkylguanine-mediated mismatch is indispensable to the exertion of the cytotoxicity of TMZ. Interstrand crosslinks are formed through the inter-mediate production of O^6-alkylguanine in the process of the cytotoxic action of BCNU and MEL (22). Therefore, MGMT is closely associated with the mechanisms of resistance to these agents. In the present study, two different leukemia cell lines were sensitized to monofunctional (TMZ) and bifunctional alkylating agents (BCNU and MEL) by an MGMT inhibitor, BG. The results indicated that O^6-alkylguanine was the major cytotoxic lesion generated by these alkylators. However, the sensitivity of the cells to these agents and the extent of the sensi-tization by BG were not found to be correlated with the MGMT transcript levels.

A previous study evaluated the role of BG in restoring TMZ sensitivity in patients with recurrent or progressive

TMZ-resistant malignant glioma in a phase II trial (23). Both TMZ and BG were administered on day 1 of a 28-day treat-ment cycle. Patients were administered a 1-h BG infusion at a dose of 120 mg/m^2, immediately followed by a 48-h infu-sion at a dose of 30 mg/m^2. TMZ was administered orally within 60 min after the end of the 1-h BG infusion at a dose of 472 mg/m^2. Out of the 66 patients treated with TMZ and BG, only six patients responded to the treatment, indicating that the efficacy of this combination was limited. In addition, the Children Oncology Group evaluated the combination treat-ment with BCNU and BG in pediatric patients with central nervous system tumors in a phase I study (24). The toxicity of this treatment was evaluated in 25 patients, and the maximum tolerated dose of BCNU administered with BG (120 mg/m^2) was 58 mg/m^2. Furthermore, the response to this treatment was evaluated in 24 patients, and only six patients were found to present stable disease, while one patient exhibited a minor response (24). A study by Hegi *et al* investigated the associa-tion between MGMT silencing and the survival of patients with glioblastoma, treated with radiotherapy alone or radiotherapy combined with TMZ (25). The MGMT promoter was methyl-ated in 45% of the 206 assessed cases and the methylation was an independent favorable prognostic factor (25). The authors concluded that patients with glioblastoma containing a methyl-ated MGMT promoter benefited from TMZ, whereas patients without a methylated MGMT promoter did not benefit from the treatment (25). Therefore, the critical role of MGMT was demonstrated in the therapeutic outcome of alkylator-based cancer treatment (25,26). However, the efficacy of combina-tion treatment with BG has not yet been confirmed clinically.

A number of proteins, including hMLH1 and hMSH2, participate in the process of MMR, which involves the mismatch recognition, excision of the DNA-containing error and resynthesis of the correct DNA (3,10). Intact MMR is required for the exertion of the cytotoxicity of TMZ, while interstrand crosslinks formed by bifunctional agents are, in part, repaired by MMR (21,27). Our previous study evaluated TMZ cytotoxicity in a BCNU-resistant variant leukemia cell line, in comparison with a BCNU-sensitive cell line (17). The study identified that the BCNU-resistant cells were more sensitive to TMZ compared with the BCNU-sensitive cells (17). In addition, the BCNU-resistant cells possessed increased hMLH1 and hMSH2 transcript levels (17). However, when the cells were transfected with shRNA against hMLH1, the sensitivity to TMZ was partially reversed. Therefore, the study suggested inverse roles of MMR on the cytotoxicity between TMZ and BCNU (17). The present study investigated only two leukemia cell lines and, therefore, it may be difficult to clarify the role of MMR in the sensitivity of leukemia cells to alkylating agents.

In conclusion, the present study evaluated the cytotoxic effects of monofunctional and bifunctional alkylating agents in relation to DNA repair in two different leukemic cell lines. The results revealed that the inhibition of MGMT appeared to sensitize the two leukemia cell lines to TMZ, BCNU and MEL. However, no correlation was identified between the drug sensitivity and MGMT transcript levels.

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