Inhibition of O\textsuperscript{6}-Methylguanine-DNA Methyltransferase by Glucose-Conjugated Inhibitors: Comparison with Nonconjugated Inhibitors and Effect on Fotemustine and Temozolomide-Induced Cell Death

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ABSTRACT

The DNA repair protein O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) is an important suicide enzyme involved in the defense against O\textsuperscript{6}-alkylating mutagens. It also plays a role in the resistance of tumors to anticancer drugs targeting the O\textsuperscript{6}-position of guanine, such as temozolomide and fotemustine. Several potent MGMT inhibitors have been developed sensitizing cells to O\textsuperscript{6}-alkylating agents. Aimed at targeting MGMT inhibitors to tumor cells, we synthesized MGMT inhibitory compounds conjugated with glucose to improve uptake in tumor cells. Here, we compared corresponding nonconjugated drugs as to MGMT inhibition pounds conjugated with glucose to improve uptake in tumor cells. All glucose conjugated inhibitors were 3- to 5-fold less effective than the corresponding nonconjugated drugs as to MGMT inhibition that was measured in cell extracts (in vitro) and cultivated HeLaS3 cells (in vivo). Except for O\textsuperscript{6}FPG, IC\textsubscript{50} values of the guanine derivatives applied in vitro and in vivo were correlated. A similar correlation was not obvious for the corresponding glucosides, indicating differences in cellular uptake. C8-\alpha-D-glucosides were less effective than \beta-glucosides. From the newly developed glucose-conjugated inhibitors tested, O\textsuperscript{6}-4-bromothenylguanine-C8-\beta-D-glucoside (O\textsuperscript{6}BTG-C8-\beta-Glu) was most potent in inhibiting MGMT both in vitro and in vivo. At a concentration of 0.1 \textmu M, it inhibited cellular MGMT to completion. It was not toxic, even when applied chronically to cells at high dose (up to 20 \textmu M). O\textsuperscript{6}BTG-C8-\beta-Glu strongly potentiated the killing effect of fotemustine and temozolomide, causing reversal from MGMT\textsuperscript{+} to MGMT\textsuperscript{-} phenotype. Therefore, O\textsuperscript{6}BTG-C8-\beta-Glu seems to be especially suitable for approaching MGMT inhibitor targeting in tumor therapy.

Tumor therapy with alkylating agents is often limited because of the development of tumor cell’s resistance and unwished systemic side effects. Attenuation of these limitations might be possible on the basis of our knowledge on the process by which antineoplastic agents exert their effects. For alkylating anticancer drugs belonging to the groups of methylating and chloroethylating agents, the mechanism of cytotoxicity has been studied in detail. It is well established that a critical site of attack of these drugs is the O\textsuperscript{6} position of guanine in the DNA, forming O\textsuperscript{6}-alkylguanine (Beranek, 1990). This lesion is considered to be a major cause of mutations and malignant transformation provoked by O\textsuperscript{6}-alkylating agents (for a recent review, see Margison and Santibanez-Koref, 2002). It also gives rise to genotoxicity and cell death via the induction of apoptosis (Kaina et al., 1997; Meikrantz et al., 1998), which is the underlying reason of the antineoplastic effect of O\textsuperscript{6}-alkylating agents such as procarbazine, dacarbazine, temozolomide, Carmustine, Lomustine, and fotemustine.

O\textsuperscript{6}-Alkylguanine is repaired by the DNA repair protein O\textsuperscript{6}-methylguanine-DNA methyltransferase (alkyltransferase; MGMT), which transfers the alkyl group to a cysteine in its own active center (for reviews, see Pegg et al., 1995; Gerson, 2004). Guanine in DNA is thereby restored, and MGMT gets inactivated. MGMT is unique among the re-
pair proteins because it acts only once (suicide enzyme). Therefore, the repair capacity of a cell correlates with the amount of preexisting molecules of MGMT and the rate of its resynthesis. MGMT has a strong protective effect on reproductive survival, apoptosis, and proliferation capacity of cells upon the treatment with \(O^6\)-alkylating agents, including antineoplastic drugs (Preuss et al., 1996b) and, therefore, it can be considered as a crucial marker of intrinsic and acquired tumor cell resistance to methylating and chloroethylating compounds (Kaina and Christmann, 2002).

MGMT is unique also from another point of view: it is highly variably expressed in normal and tumor tissues (Margison et al., 2003). For instance, in human lymphocytes it ranges in expression between 27 and 204 fmol/10⁶ cells (i.e., 7.6-fold). A given expression level in lymphocytes seems to reflect the personal situation (Jansen et al., 2001), although various environmental factors exert influence on MGMT expression (for review, see Margison et al., 2003). In tumors, MGMT is tumor type-specific, expressed with low expression in brain and malignant melanomas and high expression in ovarian and breast tumors (Preuss et al., 1996a; Hengstler et al., 1999; Margison et al., 2003). Different MGMT expression levels in tumors were related to MGMT promoter hypermethylation (Esteller et al., 2001). For brain tumors, data are available to show that the MGMT level correlates with the therapeutic response of patients treated with the \(O^6\)-alkylating agent carmustine \([N,N\text{'-bis}(2-chloroethyl)-N\text{-nitrosourea}\] (Belanich et al., 1999; Jaecle et al., 1998). MGMT was found to be inducible in rat and mouse tissues, and studies with human MGMT promoter revealed inducibility of the gene by glucocorticoids and genotoxic agents, including \(O^6\)-alkylating drugs and X-rays (Fritz et al., 1991; Grombacher et al., 1996).

Because MGMT is a most important determinant in alkylating drug resistance and because its expression may differ dramatically in individual tumors, determination of MGMT activity would be important as a predictive indicator. Even more important, however, would be to inactivate MGMT in tumors to sensitize the tumor to an antineoplastic agent. More important, however, would be to inactivate MGMT in tumors to sensitize the tumor to an antineoplastic agent.

Preparation of Cell Extracts and MGMT Assay.

Extracts were prepared from exponentially growing cells as described previously (Preuss et al., 1995). In brief, HeLa S3 cells expressing MGMT were harvested and homogenized by sonication in buffer containing 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM \(\beta\)-mercaptoethanol, 5% glycerol, and the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM). The sonication product was centrifuged at 10,000 rpm (10 min) in the cold to remove debris, and the supernatant was snap frozen in aliquots in liquid nitrogen and stored at \(-80\)°C until use. HeLa S3 cells express MGMT at a level of 588 ± 86 fmol/mg protein (mean of 13 determinations). In each assay, an extract of HeLa MR cells deficient in MGMT served as a negative background control.

Determination of MGMT Activity. MGMT activity in cell and tissue extracts was determined essentially as reported previously (Preuss et al., 1996a). The method is based on the radioactive assay in which the transfer of a tritium labeled methyl group from the \(O^6\) position of guanine in DNA to protein in the cell extract is measured. For the assays, at least 100 μg of cell extract protein was used. In each assay, a negative and positive MGMT sample was included of HeLa MR and HeLa S3 cell extract, respectively. The incubation of cell extract with \(\text{[H]methylthio} \)-labeled calf thymus DNA containing \(O^6\)-methylguanine (total 80,000 cpm/sample) occurred in buffer containing 70 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol, and 5 mM EDTA for 90 min. This was the optimal time span for the reaction to be completed (as demonstrated in background experiments; unpublished data). Data are expressed as femtomoles of radioactivity transferred from \(\text{[H]labeled DNA to protein per milligram of protein within the sample.}
Survival Experiments. Cells of the line AT17-C3, which is a CHO-9 derivative transfected with human MGMT cDNA expression vector expressing a high amount of MGMT (Kaina et al., 1991), were seeded at a density of 300 cells/5-cm dish in F12/Dulbecco's medium containing 10% fetal calf serum. Upon incubation at 37°C and 7% CO₂ for 4 h, cells were not pretreated or pretreated with the MGMT inhibitor for the indicated times. Fotemustine treatment occurred for 60 min. Thereafter, cells were rinsed with phosphate-buffered saline, and fresh medium was added. Alternatively, cells were seeded, and 12 h later fotemustine and the inhibitor were added. Cells were incubated until colonies formed (usually 7 days). They were fixed, stained, and colony formation efficiencies were determined in relation to the mock-treated control.

Results and Discussion

Inhibition of MGMT in Vitro and in Vivo by Various Inhibitors. First, we synthesized various guanine derivatives and checked their ability to inhibit MGMT in HeLaS3 cell extracts (in vitro) and HeLaS3 cells (in vivo). IC₅₀ values indicating the dose that inhibits MGMT activity by 50% were calculated from concentration-effect curves shown in Figs. 1 and 2 for in vitro and in vivo assays, respectively. IC₅₀ values are compiled in Table 2. Most efficient regarding MGMT inhibition both in vitro and in vivo was O₆BTG followed by O₆BG, O₆-benzylguanine, and O₆ITG. For these drugs, the efficiency of MGMT inhibition measured in cell extracts and in intact cells was correlated (Fig. 3A). An exception was provided by O₆FPG, which was less effective in inhibiting MGMT in vivo than in vitro, as revealed by comparison of IC₅₀ values (Fig. 3A). Although data on the uptake of the compounds are not available yet, it is supposed that O₆-methylguanine and the other guanine derivatives harboring larger groups at the O₆ position are able to enter the cell in a passive way. This might indeed be the case as suggested by the correlation of IC₅₀ values of MGMT inhibition determined in vitro and in vivo (Fig. 3A). The finding that O₆FPG is less active in MGMT inhibition in vivo, however, suggests that for some derivatives diffusion into the cell and/or into the nucleus is impaired or that an active influx or efflux mechanism is at least partially involved. The findings might also be attributed to the higher electronegativity of the fluorne atom in comparison with iodine and bromine, which can alter the lipophilicity and especially the electron density in

<table>
<thead>
<tr>
<th>R</th>
<th>IUPAC-name</th>
<th>Abbreviation</th>
<th>β-D-glucose</th>
<th>α-D-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-amino-6-(benzoyloxy)-9H-purine</td>
<td>O₆BG</td>
<td>2-amino-6-(benzoyloxy)-9-(octyl-β-D-glucosyl)-purine</td>
<td>-</td>
<td>O₆BG-C₈βGlu</td>
</tr>
<tr>
<td>2-amino-6-(3-iodo-benzoyloxy)-9H-purine</td>
<td>O₆IBG</td>
<td>2-amino-6-(3-iodo-benzoyloxy)-9-(octyl-β-D-glucosyl)-purine</td>
<td>-</td>
<td>O₆IBG-C₈βGlu</td>
</tr>
<tr>
<td>2-amino-6-(2-fluoropyridine-4-yl-methoxy)-9H-purine</td>
<td>O₆FPG</td>
<td>2-amino-6-(2-fluoropyridine-4-yl-methoxy)-9-(octyl-β-D-glucosyl)-purine</td>
<td>-</td>
<td>O₆FPG-C₈βGlu</td>
</tr>
<tr>
<td>2-amino-6-(4-bromothiophen-2-yl-methoxy)-9H-purine</td>
<td>O₆BTG</td>
<td>2-amino-6-(4-bromothiophen-2-yl-methoxy)-9-(octyl-β-D-glucosyl)-purine</td>
<td>2-amino-6-(4-bromothiophen-2-yl-methoxy)-9-(octyl-α-D-glucosyl)-purine</td>
<td>O₆BTG-C₈βGlu</td>
</tr>
<tr>
<td>2-amino-6-(5-iodothiophen-2-yl-methoxy)-9H-purine</td>
<td>O₆ITG</td>
<td>2-amino-6-(5-iodothiophen-2-yl-methoxy)-9-(octyl-β-D-glucosyl)-purine</td>
<td>2-amino-6-(5-iodothiophen-2-yl-methoxy)-9-(octyl-α-D-glucosyl)-purine</td>
<td>O₆ITG-C₈βGlu</td>
</tr>
</tbody>
</table>
the molecule. It also indicates that the in vitro inhibitory constant of a given nonconjugated inhibitor does not necessarily reflect the inhibitor capacity it exerts in intact cells. This at the same time underlines the need for in vivo testing of the compounds as to their MGMT inhibitor capacity.

Inhibition of MGMT by Glucose-Conjugated Inhibitors. To convert the guanine-derived MGMT inhibitors into drugs that are subject to active transport, various MGMT inhibitors were conjugated to D-glucose that cannot enter the cell by diffusion but is taken up actively. In previous exper-
TABLE 2
IC_{50} values determined in vitro (in HeLa S3 cell extracts) and in vivo (on HeLa S3 cells), and LD_{50} values as determined in colony-forming assays with Chinese hamster ovary cells

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Abbreviation</th>
<th>IC_{50} in Vitro</th>
<th>IC_{50} in Vivo</th>
<th>IC_{50} in Vitro/IC_{50} in Vivo</th>
<th>LD_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>O6-Benzylguanine</td>
<td>O6BG</td>
<td>0.2</td>
<td>0.035</td>
<td>8.6</td>
<td>51</td>
</tr>
<tr>
<td>O6-Benzylguanine-C8-glucose</td>
<td>O6BG-C8-Glu</td>
<td>1.1</td>
<td>1.2</td>
<td>0.91</td>
<td>0.8</td>
</tr>
<tr>
<td>O6-2-Fluoropyridinylmethyl-guanine</td>
<td>O6FPG</td>
<td>0.4</td>
<td>0.085</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td>O6-2-Fluoropyridinylmethyl-guanine-C8-Glu</td>
<td>O6FPG-C8-Glu</td>
<td>1.8</td>
<td>11</td>
<td>0.16</td>
<td>2.8</td>
</tr>
<tr>
<td>O6-m-Iodobenzylguanine</td>
<td>O6IBG</td>
<td>0.1</td>
<td>0.02</td>
<td>5.1</td>
<td>1.5</td>
</tr>
<tr>
<td>O6-m-Iodobenzylguanine-C8-Glu</td>
<td>O6IBG-C8-Glu</td>
<td>0.45</td>
<td>0.4</td>
<td>1.125</td>
<td>0.85</td>
</tr>
<tr>
<td>O6-Bromothenylguanine</td>
<td>O6BTG</td>
<td>0.009</td>
<td>0.004</td>
<td>2.25</td>
<td>0.73</td>
</tr>
<tr>
<td>O6-Bromothenylguanine-C8-alpha-Glucose</td>
<td>O6BTG-C8-Glu</td>
<td>0.45</td>
<td>0.08</td>
<td>5.625</td>
<td>1.5</td>
</tr>
<tr>
<td>O6-Bromothenylguanine-C8-beta-Glucose</td>
<td>O6BTG-C8-Glu</td>
<td>0.032</td>
<td>0.01</td>
<td>3.2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>O6-Iodothenylguanine</td>
<td>O6ITG</td>
<td>0.75</td>
<td>0.05</td>
<td>15</td>
<td>1.9</td>
</tr>
<tr>
<td>O6-Iodothenylguanine-C8-alpha-Glucose</td>
<td>O6ITG-C8-Glu</td>
<td>1.3</td>
<td>0.14</td>
<td>9.3</td>
<td>0.65</td>
</tr>
<tr>
<td>O6-Iodothenylguanine-C8-beta-Glucose</td>
<td>O6ITG-C8-Glu</td>
<td>0.8</td>
<td>0.36</td>
<td>2.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Therefore, we decided on conjugation with a C8 spacer. As shown in Figs. 1 and 2, and Table 2 for IC_{50} values, the glucose conjugates displayed reduced IC_{50} values compared with the parent compounds. This indicates that glucose conjugation reduces the inhibitor's ability to inhibit MGMT. This essentially confirms our previous data with a selected set of MGMT inhibitors (Reinhard et al., 2001b). Glucose conjugation (using a C8 spacer) reduced MGMT inhibition in the in vitro experiments shown here by a factor of 3 to 5.

It would be interesting to compare the inhibition of MGMT by the glucose-derivatives in vitro and in vivo. Most potent was O6BTG-C8-beta-Glu followed by O6IBG-C8-beta-Glu and O6BG-C8-beta-Glu. O6ITG-C8-beta-Glu was less efficient both in vitro and in vivo (Fig. 3B). Overall, in contrast to the nonconjugated inhibitors, the correlation between IC_{50} (in vitro) and IC_{50} (in vivo) of the glucose-conjugated inhibitors was not significant (Fig. 3B), which may be taken to indicate that cellular uptake and nuclear transport is different for the drugs. Again, the least efficient inhibitor was O6FPG-C8-beta-Glu, notably under in vivo conditions, which makes this compound not suitable for further studies.

Because of steric reasons one might assume that a beta-glucose linker is more advantageous than the alpha linker. Therefore, we compared two MGMT inhibitors linked to glucose by an alpha or beta linker. O6BTG-C8-alpha-glucose was clearly less efficient in MGMT inhibition both in vitro and in vivo than O6BTG-C8-beta-glucose. For O6ITG, the alpha-beta derivative was slightly less efficient in vitro, and, surprisingly, more efficient in vivo (Figs. 1 and 2; Table 2). In summary, the data revealed that 1) the glucose conjugates are 2- to 5-fold less effective than the nonconjugated compounds; 2) for inhibitors that were not coupled to glucose, the in vitro and in vivo MGMT inhibition constant was correlated (except for O6FPG); 3) for the glucose-conjugated inhibitors, the same correlation was lacking; and 4) the most efficient inhibitor, both as parent compound and glucoside in vitro and in vivo, was O6BTG. O6BTG and the corresponding derivative O6BTG-C8-beta-Glu inhibit MGMT nearly to completion already at a concentration of <0.1 \mu M (assayed upon incubation of cells with the inhibitor for 4 h). Because of the high potency of both O6BTG and O6BTG-C8-beta-Glu to inhibit MGMT, we decided to use O6BTG and the corresponding glucose derivative in further studies on cell killing.

**Cytotoxicity of MGMT Inhibitors.** The cytotoxicity of O6BTG and O6BTG-C8-beta-glucose as well as the other inhibitors synthesized in our laboratory was checked in colony-
forming assays, which is a very sensitive method for detection of killing effects on cellular level. LD$_{50}$ values calculated from dose-response curves (not shown) are compiled in Table 2. We should note that none of the inhibitors exerted immediate cytotoxic effects. In mass culture, cytotoxicity was not seen upon short-term treatment. Therefore, inhibition of MGMT observed in vivo cannot be due to unspecific cell killing. A reasonably good ratio between MGMT inhibition and cytotoxicity was observed for O$_6$BTG and the corresponding derivative. Thus, if CHO-9 cells were chronically exposed to the agents, O$_6$BTG and O$_6$BTG-C$_8$-βGlu were devoid of any toxicity up to a concentration of 0.5 μM (see Fig. 4 for representative survival curves). Interestingly, O$_6$BTG exerted cytotoxic effects upon chronic administration with a concentration >0.5 μM, whereas O$_6$BTG-C$_8$-βGlu was not toxic even with a 10-fold higher concentration (Fig. 4; data not shown). With a concentration of up to 100 μM O$_6$BTG-C$_8$-βGlu, colony formation gradually decreased to 60%. The LD$_{50}$ values were 0.73 and >100 μM for O$_6$BTG and O$_6$BTG-C$_8$-βGlu, respectively, which is far above the concentration inhibiting MGMT. If cells were treated for a short period of time (1 h), O$_6$BTG and O$_6$BTG-C$_8$-βGlu were even less toxic (Fig. 4; no killing effects were observed with a concentration of 5 and >100 μM, respectively (data not shown).

**Effect of MGMT Inhibitors on Fotemustine-Induced Cytotoxicity.** To check the efficiency of O$_6$BTG and O$_6$BTG-C$_8$-βGlu in increasing the killing effect of an O$_6$-alkylating agent on cells expressing human MGMT, we used CHO-9 cells stably transfected with human MGMT cDNA (AT17-C3 cells) as well as MGMT-expressing HeLa S3 cells. As a result of MGMT expression, these cells gained a high level of resistance to various O$_6$-alkylating agents (Kaina et al., 1991; Preuss et al., 1996b). As shown in Fig. 5, O$_6$BTG dramatically intensified the killing effect of the anticancer drug fotemustine (muphoran), which is an O$_6$-chloroethylylating agent that is in use in the treatment of malignant melanomas and glioblastomas (Boiardi et al., 2001; Tarhini and Agarwala, 2004). Whereas fotemustine at a concentration of 40 μg/ml (60-min treatment) was only very slightly toxic (80% colony formation), O$_6$BTG at a concentration of 0.1 μM given 60 min before the agent reduced cell survival by >99%. Interestingly, the glucose derivative O$_6$BTG-C$_8$-βGlu also intensified fotemustine-induced cell killing; however, higher concentrations of the inhibitor (up to 2 μM) were required to provoke a similar kill-intensifying effect (Fig. 5, bottom). This contrasts with MGMT inhibition experiments in which we showed that depletion of MGMT occurred already upon treatment of cells with 0.1 μM O$_6$BTG-C$_8$-βGlu. The discrepancy might be explained by different half-lives of O$_6$BTG and O$_6$BTG-C$_8$-βGlu within the cell and the kinetics of resynthesis of MGMT. If the intracellular half-life is short, the inactivated MGMT will be replaced by newly synthesized enzyme, which may still provoke resistance before O$_6$-chloroethylguanine lesions are...
converted into the corresponding interstrand cross-links, which are supposed to be the critical ultimate killing lesions (Erickson et al., 1980). If this is true, treatment with the inhibitor both before and after the administration of fotemustine should intensify fotemustine-induced cell kill. This was indeed the case. As shown in Fig. 6, the effect of O6BTG-C8-βGlu on fotemustine-induced cell inactivation was clearly enhanced if it was given both before and after the pulse treatment with the antineoplastic agent.

The intensifying effect of O6BTG and O6BTG-C8-βGlu on cell killing induced by increasing doses of fotemustine is shown in Fig. 7. Both the parent compound (Fig. 7A) and the corresponding C8-β-D-glucoside (Fig. 7B) dramatically sensitized MGMT-expressing AT17-C3 cells. Thereby, they responded to fotemustine in almost the same way as MGMT-nonexpressing cells. A similar sensitizing effect of O6BTG-C8-βGlu was found for HeLa S3 cells, which were reverted to the sensitivity of MGMT-deficient HeLa MR cells (Fig. 7C).

A representative of methylating agents used in tumor chemotherapy, notably in the therapy of glioblastomas and malignant melanomas, is temozolomide (Temodar), an imidazotetrazinone that undergoes spontaneous conversion in aqueous solution to an active methylating reagent (Tsang et al., 1991). The effect of O6BTG-C8-βGlu on temozolomide-induced cell death is shown in Fig. 8. Again, the glucose derivative was highly effective in sensitizing MGMT-expressing cells, which was shown for AT17-C3 (Fig. 8A) and HeLa S3 (Fig. 8B) cells.

**Fig. 7.** Survival of cells as a function of the concentration of fotemustine. A, AT17-C3 cells were seeded and treated with O6BTG as described in Fig. 5. B, AT17-C3 cells were seeded and treated with O6BTG-C8-βGlu 18 h later. One hour thereafter, fotemustine was given to the medium at the final concentrations indicated. Twenty-four hours thereafter, the medium was replaced by fresh medium, and cells were cultivated for 7 days until colonies formed. C, HeLa S3 cells were seeded and treated with O6BTG-C8-βGlu and fotemustine as described under B. MGMT lacking CHO-9 and HeLa MR cells treated with fotemustine only served as control.
chloroethylating and methylating fotemustine and temozolomide, which are representatives of cicient in inactivating MGMT in vitro and inside the cell. It

References

methyltransferase (MGMT) on the cytotoxic and recombinogenic activity of different antineoplastic drugs. *Int J Cancer* 65:506–512.


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