Role of Cytochrome P450 Isoenzymes in Metabolism of O^6^-Benzylguanine: Implications for Dacarbazine Activation

Lina Long and M. Eileen Dolan
Section of Hematology-Oncology, Department of Medicine and Cancer Research Center, Committee on Clinical Pharmacology, University of Chicago, Chicago, Illinois 60637

ABSTRACT

O^6^-Benzylguanine (BG) effectively inactivates the DNA repair protein O^6^-alkylguanine-DNA alkyltransferase and enhances the effectiveness of alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide, in vitro and in vivo. BG is presently in clinical trials with 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide. Preclinical data demonstrate that BG enhances the sensitivity of cells to 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide, the active intermediate of dacarbazine (DTIC), making the combination BG plus DTIC attractive for additional clinical development. DTIC requires metabolism by cytochrome P450 (CYP450) isoforms, 1A1, 1A2, and 2E1 to form two reactive N-demethylated metabolites, 5-[3-hydroxy-methyl-3-methyl-triazen-1-yl]-imidazole-4-carboxamide and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide, ultimately forming a methylating species responsible for its cytotoxicity. The objective of this study was to examine the role of CYP450 1A1 and 1A2 in the metabolism of BG and identify possible drug-drug interactions with DTIC. Our data show that CYP450 isoforms 1A1 and 1A2 are primarily responsible for both BG oxidation to O^6^-benzyl-8-oxoguanine (8-oxoBG) and additional debenzylation to 8-oxoguanine. The catalytic efficiency of BG oxidation is 16 times lower for CYP1A1 than CYP1A2; however, the catalytic efficiency of 8-oxoBG debenzylation is 11 times greater for CYP1A1 than CYP1A2. Furthermore, BG inhibits CYP1A1 and 1A2 catalyzed conversion of DTIC to active methylating species. 8-OxoBG also inhibited conversion of DTIC to active methylating species but to a much lesser extent. The concentrations of BG required to inhibit 50% of DTIC N-demethylation were 2.8, 0.13, and 3.8 μM in human liver microsomes.

INTRODUCTION

Alkylnitrosoureas [1,3-bis(2-chloroethyl)-1-nitrosourea and procarbazine] and alkyltriazenes (temozolomide and DTIC) are used to treat brain tumors, lymphomas, and malignant melanoma (1, 2). The repair of DNA damage by AGT is clearly one of the most important mechanisms of resistance to these agents (3). BG has been developed as a low molecular weight substrate that benzylates the cysteine residue within the active site of AGT, thereby inactivating the repair protein (4, 5). Inactivation of the AGT protein by nontoxic concentrations of BG renders a variety of human tumor cell lines more sensitive to the cytotoxic effects of alkylnitrosoureas and alkyltriazenes, including MTIC (active metabolite of DTIC; Refs. 3, 6, and 7). Phase I clinical trials of BG plus 1,3-bis(2-chloroethyl)-1-nitrosourea are now complete (2, 8–10). A series of Phase II studies with this combination and Phase I studies of BG plus temozolomide have been initiated.

The metabolism of BG involves oxidation to an equally potent derivative, 8-oxoBG in rats (11), mice (11), nonhuman primates (12), and humans (8, 13). In humans, BG oxidation is catalyzed by microsomal enzymes CYP1A2 and CYP3A4 and by cytosolic aldehyde oxidase (13). 8-OxoBG has been shown to be additionally metabolized to 8-oxoguanine in humans by the liver microsomal enzyme CYP1A2 (14).

DTIC, a methylating agent, has been in clinical use for many years to treat melanoma and lymphoma (15). A likely drug combination to emerge is BG plus DTIC because melanoma and lymphomas are known to express AGT, and AGT contributes to resistance of tumor cells to methylating agents, such as DTIC (16–18). DTIC requires metabolic activation by the CYP450 system through an N-demethylation reaction and subsequently undergoes spontaneous cleavage to yield a stable metabolite, AICA, and an active methylating species, diazomethane (1, 19). Recently, Reid et al. (19) demonstrated that the...
CYP450 isoforms responsible for N-demethylation of DTIC included CYP1A1, CYP1A2, and CYP2E1.

Because BG and DTIC are both metabolized CYP1A2 and the role of CYP1A1 in the metabolism of BG had not been studied, we reasoned there might be drug-drug interactions that would influence the amount of active methylating species generated by DTIC in the presence of BG. Therefore, the purpose of our work was as follows: (a) to determine the role of CYP1A1 in BG metabolism; and (b) to determine the effect of BG and 8-oxoBG on the extent of DTIC conversion to active methylating species.

MATERIALS AND METHODS

Materials. Sodium phosphate, phosphoric acid, HPLC-grade acetonitrile, and methanol were purchased from Fisher Scientific (Itasca, IL). Double-distilled deionized water was obtained by passing water through a Milli-Q reagent water system (Millipore, Bedford, MA). Phenacetin, 4-acetamidophenol, AICA, 6-methylthioguanine, 1-heptanesulfonic acid, and triethylamine were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO). DTIC was provided generously by the National Cancer Institute. BG and 8-oxoBG were provided generously by Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research & Development Center, Frederick, MD). Overexpressed human CYP450 isoforms (1A1, 1A2, and 2E1) expressed from cDNA on a baculovirus expression system were purchased from Gentest (Woburn, MA).

Preparation of Subcellular Fraction. Portions of human livers were obtained from National Disease Research Interchange (Philadelphia, PA) or the Cooperative Human Tissue Network Midwestern Division (Columbus, OH) and kept frozen at −70°C until use. Livers were homogenized in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M KCl and 1 mM EDTA (6.5 ml of buffer/gram liver) using a Potter-Elvehjem homogenizer and Teflon pestle (Biospec Products, Inc., Bertlesville, OK). Microsomes and cytosol were prepared by standard methods using differential centrifugation (20). The resulting pellets were resuspended in 0.1 M sodium phosphate buffer (pH 7.4) containing 5 mM MgCl₂ and 1 mM EDTA, followed by centrifugation at 105,000 × g for 1 h. The supernatant was stored as cytosolic fraction. The microsomal pellets were resuspended in the same buffer and stored at −70°C until use. Protein concentration was determined by the method of Bradford (21).

Incubation Conditions. All incubations with human liver microsomes or P450 isoforms were in a total volume of 1 ml containing 50 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl₂, and 1 mM NADPH at 37°C.

To evaluate enzymes responsible for BG oxidation, BG (0.25–60 μM) was incubated with 50 pmol of CYP1A1 or CYP1A2 for 15 min. After incubations, the reactions were terminated by the addition of 2 ml of chilled methanol. Aliquots of 10 μl of 0.25 mM 8-hydroxy-2′-deoxyguanosine (internal standard, made up in 0.1 M H₃PO₄) were added to the reaction vials.

To determine the effect of BG or 8-oxoBG on deethylation of phenacetin by CYP1A2, an incubation time of 30 min with 30 pmol of CYP1A2 and 100 μM phenacetin exhibited linear conditions and, therefore, was used in the experiments. BG (0.02–30 μM) or 8-oxoBG (2–50 μM) was added to reaction mixtures before incubation of phenacetin and CYP1A2. To determine kinetic parameters associated with inhibition of phenacetin deethylation by BG, phenacetin concentrations ranged from 0.01 to 1 mM, and BG concentrations ranged from 0 to 10 μM. After incubation, the reactions were terminated by the addition of 2 ml of chilled methanol. Aliquots of 10 μl of 1 mM 3-acetamidophenol (internal standard, made up in methanol) were added to the reaction vials.

To define optimal conditions for DTIC N-demethylation, DTIC (0–2 mM) was incubated with pooled human liver microsomes (0–2 mg/ml), CYP1A1 (0–50 pmol), and CYP1A2 (0–50 pmol) for various time periods for ≤1 h. Human microsomal protein (1 mg/ml), CYP1A1 (30 pmol), or CYP1A2 (30 pmol) incubated with DTIC (1 mM) for 15 min exhibited linear conditions and therefore was used in the following experiments, unless otherwise stated. To determine the effect of BG or 8-oxoBG on DTIC metabolism to AICA, BG (0.02–30 μM) or 8-oxoBG (2–50 μM) was added to reaction mixtures before incubation of DTIC with human liver microsomes, CYP1A1, or CYP1A2. To determine kinetic parameters associated with inhibition of DTIC metabolism by BG, DTIC concentrations ranged from 0.005 to 2 mM, and BG concentrations ranged from 0 to 2 μM for CYP1A1 and 0 to 10 μM for CYP1A2. After incubation, the reactions were terminated by the addition of 2 ml of chilled methanol. Aliquots of 10 μl of 1 mM 6-methylthioguanine (internal standard, made up in DMSO) were added to the reaction vials.

All reaction mixtures were vortexed for 15 s and centrifuged at 2,440 × g for 25 min to achieve precipitation of proteins. The supernatant was dried under a stream of nitrogen gas. The resulting residues were reconstituted in 200 μl of mobile phase. The reconstituted sample was centrifuged again for 10 min at 20,800 × g. Reaction mixtures measuring conversion of DTIC to AICA were kept at room temperature for >30 min to ensure spontaneous decomposition of 5-[3-hydroxy-methyl-3-methyl-triazen-1-yl]-imidazole-4-carboxamide and MTIC to AICA. Supernatant was taken, and aliquots of 150 μl for HPLC-UV system supernatant were injected onto the HPLC system.

HPLC Analysis. BG and 8-oxoBG were separated on a Beckman C18 reverse phase column (4.6 × 250 mm; Beckman Instruments, Inc., Fullerton, CA) at a flow rate of 1 ml/min using an isocratic mobile phase of 38% methanol in 10 mM potassium phosphate buffer (pH 7.5), and a Waters 996 Photodiode Array Detector (Waters Corp., Milford, MA) monitored at 280 nm. The retention times of O6-benzyl-8-hydroxymethylguanine (internal standard), 8-oxoBG, and BG were 9, 24, and 27 min, respectively.

An HPLC method with ED was used to quantify 8-
oxoguanine as described previously (22). Briefly, the chromatographic separation was achieved on a Tosohaas 5 μm, ODS-80T M column (Tosohaas, Montgomeryville, PA). A gradient mobile phase A consisted of 0.1 M sodium acetate adjusted to pH 5.2 with glacial acetic acid, and mobile phase B consisted of 100% acetonitrile. CoulArray potentials were set as follows: channel 1 at +280 mV, channel 2 at +300 mV, channel 3 at +320 mV, and channel 4 at +400 mV. The retention times of 8-oxoguanine, 8-hydroxyl-2'-deoxyguanosine (internal standard), and 8-oxoBG were 10, 18, and 24 min, respectively.

The condition used to analyze phenacetin and 4-acetamidophenol consisted of an acetonitrole gradient and 50 mM sodium phosphate buffer (pH 4.0) on a Beckman C18 reverse phase column (4.6 × 250 mm) at a flow rate of 1 ml/min. The gradient mobile phase started with 2% acetonitrile for 6 min and then increased linearly to 30% at 60 min. 4-acetamidophenol, 3-acetamidophenol (internal standard), and phenacetin were eluted at 21, 27, and 53 min, respectively, and monitored by UV at 244 nm using a Hitachi L-4500A Diode Array Detector (Tokyo, Japan).

DTIC and AICA were separated on a Beckman C18 reverse phase column (4.6 × 250 mm) using a gradient mobile phase of 10 mM heptane sulfonic acid, 0.1% triethylamine (pH 2.0) with phosphoric acid, starting with 5% methanol and increasing linearly to 40% methanol over 25 min, retaining at that level for an additional 20 min, and equilibrating back to 5% methanol over 15 min. The flow rate was 1 ml/min, and the column maintained at room temperature. AICA, DTIC, and 6-methylthioguanine were eluted at 15, 25, and 28 min, respectively, and monitored by UV at 265 nm using a Hitachi L-4500A Diode Array Detector and fluorescence detection at λexitation = 295 nm and λemission = 360 nm using a Hitachi F-7480 Fluorescence Detector. All compounds were identified by comparison of their retention time and UV spectrum with those of authentic standards. The limit of detection of AICA using UV detection was 163 ng/ml.

Statistical Analysis. IC50 values for inhibition of DTIC N-demethylation were calculated from the mean of six individual experiments using GraFit (Grafit version 4.0; Erithacus Software, Ltd., Middlesex, United Kingdom). In the determination of kinetic parameters, various concentrations of DTIC in the absence and presence of BG at concentrations between IC20 and IC80 and their corresponding rates were applied to estimate individual Km and Vmax values using GraFit software based on the single enzyme Michaelis-Menten kinetics. Ki values were determined by regression analysis of secondary plots (Km/Vmax ratio as a function of inhibitor concentration). The type of inhibition was estimated from observation of double reciprocal (Lineweaver-Burk) plots using various concentrations of BG.

RESULTS

Role of CYPIA1 in Metabolism of BG. Fig. 1A illustrates BG metabolism to 8-oxoBG and additional metabolism to
8-oxoguanine. Previous results indicated a role for CYP1A2, CYP3A4, and aldehyde oxidase in the oxidation of BG (13) and a role for CYP1A2 in the debenzylolation of 8-oxoBG using human liver microsomes and cytosol (14). CYP1A1, an extrahepatic P450 enzyme found in human lung, intestine, skin, lymphocytes, and placenta (23), was evaluated for its contribution to BG oxidation and 8-oxoBG debenzylolation. BG and 8-oxoBG are metabolized by CYP1A1 with a lower K_m value of 5 M and debenzylation of 8-oxoBG of 18.3 ± 1.3 μM compared with CYP1A2, 35.9 ± 12.8 μM (Table 1). The catalytic efficiency (V_max/K_m) for BG oxidation is 0.08 for CYP1A1 compared with 1.31 for CYP1A2 (13). CYP1A1 has a lower K_m and higher V_max value for 8-oxoBG debenzylation reaction compared with CYP1A2, resulting in 10 times higher catalytic efficiency (Table 1).

**Inhibition of DTIC N-demethylation by BG and 8-oxoBG.** Phenacetin, a probe substrate of CYP1A2, was used to evaluate the effect of BG on CYP1A2. Kinetic constants for phenacetin O-deethylation by CYP1A2 were K_m = 45.3 μM and V_max = 37.8 pmol/min/pmol. BG was a potent inhibitor of CYP1A2 catalyzed O-deethylation of phenacetin with an IC_50 value of 5 μM (Table 2). Fig. 1B illustrates the human metabolic profile of DTIC metabolism. DTIC is oxidized to form 5-[3-hydroxy-methyl-3-methyl-triazen-1-yl]-imidazole-4-carboxamide, which yields MTIC after elimination of formaldehyde (19). MTIC is then rapidly degraded to AICA and diazohydroxide with AICA being the only stable metabolite identified in rats, dogs, and man (19, 24–26). The effect of BG and 8-oxoBG on the extent of conversion to AICA using human liver microsomes, CYP1A1, or CYP1A2 is illustrated in Fig. 2. In the presence of 1 μM BG, the amount of AICA formed on incubation of DTIC with human liver microsomes, CYP1A1, or CYP1A2 was 88, 19, and 87% of that formed without the addition of BG, respectively. The concentrations of BG required to inhibit DTIC conversion to AICA by 50% in pooled human liver microsomes, CYP1A1, and CYP1A2 were 2.8 ± 0.7, 0.13 ± 0.02, and 3.8 ± 1.1 μM, respectively (Table 2). Maximal plasma concentrations of DTIC after IV bolus infusion for the treatment of malignant melanoma range between 55 and 165 μM (26). Therefore, we evaluated the effect of BG on DTIC conversion to active intermediate at a pharmacologically relevant concentration of 150 μM. The IC_50 for BG inhibition using human liver microsomes under these conditions was 1.5 ± 0.16 μM. 8-OxoBG also inhibited DTIC conversion to AICA, however, to a much lesser extent with no IC_50 >40% inhibition of 0.5 μM of modulator. There was no detectable AICA formed on incubation of DTIC (2 mM) with CYP2E1 (100 pmol) for 30 min (data not shown).

The representative Lineweaver-Burk plots for the inhibitory effect of BG on DTIC metabolism by CYP1A1 (A) and CYP1A2 (B) are shown in Fig. 3. The inhibitions were characterized by a concentration-dependent increase in the apparent

### Table 1: Kinetic parameters for metabolism of BG, 8-oxoBG and DTIC

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme</th>
<th>K_m (μM)</th>
<th>V_max (pmol/min/pmol)</th>
<th>V_max/K_m</th>
</tr>
</thead>
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<tr>
<td>BG</td>
<td>CYP1A1</td>
<td>6.4 ± 0.4</td>
<td>0.5 ± 0.09</td>
<td>0.08</td>
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<tr>
<td>BG</td>
<td>CYP1A2</td>
<td>1.3 ± 0.08</td>
<td>1.7 ± 0.04</td>
<td>1.31</td>
</tr>
<tr>
<td>BG</td>
<td>CYP3A4</td>
<td>52.2 ± 7.14</td>
<td>0.3 ± 0.01</td>
<td>0.0057</td>
</tr>
<tr>
<td>8-oxoBG</td>
<td>CYP1A1</td>
<td>18.3 ± 1.3</td>
<td>3.1 ± 0.07</td>
<td>0.17</td>
</tr>
<tr>
<td>8-oxoBG</td>
<td>CYP1A2</td>
<td>35.9 ± 12.8</td>
<td>0.6 ± 0.2</td>
<td>0.016</td>
</tr>
<tr>
<td>DTIC</td>
<td>CYP1A1</td>
<td>306 ± 24</td>
<td>477 ± 145</td>
<td>1.56</td>
</tr>
<tr>
<td>DTIC</td>
<td>CYP1A2</td>
<td>327 ± 16</td>
<td>165 ± 7</td>
<td>0.51</td>
</tr>
</tbody>
</table>

### Table 2: Kinetic parameters for BG inhibition of phenacetin O-deethylation and DTIC N-demethylation

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme</th>
<th>IC_50 (μM)</th>
<th>Apparent K_i (μM)</th>
<th>Type of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin</td>
<td>CYP1A2</td>
<td>5.0 ± 1.7</td>
<td>1.1 ± 0.1</td>
<td>Mixed</td>
</tr>
<tr>
<td>DTIC (1 mM)</td>
<td>Microsomes</td>
<td>2.8 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTIC (150 μM)</td>
<td>Microsomes</td>
<td>1.5 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTIC</td>
<td>CYP1A1</td>
<td>0.13 ± 0.02</td>
<td>0.19 ± 0.08</td>
<td>Mixed</td>
</tr>
<tr>
<td>DTIC</td>
<td>CYP1A2</td>
<td>3.8 ± 1.1</td>
<td>1.3 ± 0.2</td>
<td>Mixed</td>
</tr>
</tbody>
</table>
DISCUSSION

Previous studies demonstrated that BG oxidation to 8-oxoBG involves hepatic microsomal enzymes CYP1A2 and CYP3A4 and cytosolic aldehyde oxidase (13). CYP1A2 is also responsible, at least in part, for debenzylolation of 8-oxoBG to 8-oxoguanine (13). We report here that both BG and 8-oxoBG are substrates for CYP1A1, an extrahepatic enzyme. The catalytic efficiency for debenzylolation of 8-oxoBG by CYP1A1 is 10 times higher than for CYP1A2. BG is a potent inhibitor of CYP1A1 and CYP1A2, two enzymes responsible for conversion of DTIC to its active methylating species. The conversion of DTIC to its active methylating species is inhibited in the presence of pharmacological concentrations of BG and 8-oxoBG. Alternative AGT inactivators that are not substrates of CYP1A1 or 1A2 should be considered for combination studies with DTIC.

Recently Toft et al. (27) reported the inhibition of DTIC-induced apoptosis in the small intestine of mice by administration of BG. A normal apoptotic response to DTIC was restored by 24 h, suggesting that the effect by BG was reversible. Although the mechanism by which BG inhibited DTIC-induced apoptosis was not determined, BG inhibition of DTIC metabolism was proposed as a possible explanation. DTIC is metabolized to its active methylaing derivative by rat CYP1A enzymes (28) and human CYP1A1, 1A2, and 2E1 (19). We demonstrate a dose-dependent decrease in the amount of active methylating species generated upon incubation of DTIC with human liver microsomes in the presence of BG. Inhibition of CYP1A2-catalyzed conversion of DTIC by BG is primarily responsible for the observed effect in human liver microsomes because protein levels of CYP1A1 are low or undetectable in human liver (19, 23, 29). However, CYP1A1 is expressed in lung, gastrointestinal lumen, skin, lymphocytes, placenta, and some tumors and at relatively high levels in certain tumor cell lines, including ovarian, colon, and lung (19, 23, 29, 30). Our data suggest that in hepatic and extrahepatic tissue, BG inhibits DTIC conversion. Thus, the mechanism by which BG inhibited DTIC-induced apoptosis is likely a result of lack of conversion of DTIC to a methylating species capable of damaging DNA.

BG is presently in Phase II clinical trials. At the recommended Phase II dose (120 mg/m²), BG concentrations in plasma ranged from 0.5 to 16 μM (2). The concentration of BG required to inhibit 50% conversion of DTIC to its active methylating species in pooled human liver microsomes is 2.8 μM, well within the therapeutic range. Our data suggest that in hepatic and extrahepatic tissue, BG inhibits DTIC metabolism, making the combination unlikely to result in therapeutic benefit in humans. An alternative methylating agent is temozolomide. Temozolomide undergoes chemical degradation to MTIC in an aqueous environment (31), and therefore, BG would not be expected to interfere with the amount of active methylating species generated by this drug.

The pharmacokinetic profile of BG (t1/2 = 0.12 h) in humans shows rapid conversion to 8-oxoBG and slow elimination of 8-oxoBG (t1/2 = 5.4 h). Even during the 1-h infusion of BG, there are significantly higher concentrations of 8-oxoBG than BG. At 3-h postdrug administration, plasma concentrations of BG are undetectable, whereas 8-oxoBG reaches maximum concentrations (2, 8, 32). The extremely rapid conversion of BG to 8-oxoBG is most likely attributable to the high affinity of BG for CYP1A1 and CYP1A2 and moderate affinity for CYP3A4 and cytosolic aldehyde oxidase. The relatively long half-life of 8-oxoBG can be explained by its rapid formation and slower metabolism to 8-oxoguanine. The catalytic efficiency of BG oxidation by CYP1A2 is 82 times higher than that of 8-oxoBG debenzylolation. In addition, BG at pharmacological concentrations inhibits CYP1A1/2 and, therefore, may be preventing its further metabolism.

In conclusion, we have found that BG is a potent inhibitor of CYP1A1 and CYP1A2. BG and 8-oxoBG inhibit the conversion of DTIC to its active methylating agent. BG inhibition of DTIC conversion to an active methylating species suggests that the combination in human clinical trials should be approached with caution. BG combined with temozolomide would be a better choice because temozolomide does not require metabolic activation to produce the same active methylating species.
REFERENCES


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