Metabolism of O\textsuperscript{6}-Benzyguanine, an Inactivator of O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase\textsuperscript{1}

M. Eileen Dolan,\textsuperscript{2} Mi-Young Chae, Anthony E. Pegg, John H. Mullen, Henry S. Friedman, and Robert C. Moschel

Division of Hematology-Oncology, The University of Chicago, Chicago, Illinois 60637; \textsuperscript{2}Carcinogen-Modified Nucleic Acid Chemistry, ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Program, Frederick, Maryland 21702; \textsuperscript{3}Departments of Cellular and Molecular Physiology and of Pharmacology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033; \textsuperscript{4}Duke University Medical Center, Durham, North Carolina (H. S. F.)

ABSTRACT

O\textsuperscript{6}-Benzyguanine effectively inactivates the DNA repair protein, O\textsuperscript{6}-alkylguanine-DNA alkyltransferase, leading to an increase in the therapeutic index of 1,3-bis(2-chloroethyl)-1-nitrosourea in nude mouse xenograft studies. To investigate the fate of this inactivator in mammalian systems, we examined its biodistribution and metabolism following i.p. administration of 8-[\textsuperscript{3}H]-O\textsuperscript{6}-benzyguanine to male Sprague-Dawley rats and BALB/c mice. Following administration to rats, there were significantly higher levels of radioactivity in liver than in lungs, spleen, kidney, small intestine, and esophagus for up to 24 h. Major urinary metabolites were identified as O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine, N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzylguanine, and N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine. Debenzylated metabolites included guanine, 7,8-dihydro-8-oxoguanine, and N\textsuperscript{2}-acetylguanine. In contrast to rat metabolism, acetylated derivatives were not found in mouse urine. However, O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine was a major metabolite in the mouse. O\textsuperscript{6}-Benzyguanine-7,8-dihydro-8-oxoguanine was a very effective O\textsuperscript{6}-alkylguanine-DNA alkyltransferase inactivator and exhibited a 50% effective dose in HT29 cell extracts of 0.3 \mu M compared to 0.2 \mu M for O\textsuperscript{6}-benzyguanine. The O\textsuperscript{6}-alkylguanine-DNA alkyltransferase depleting activity of N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzylguanine and N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine were, respectively, 120- and 325-fold lower than O\textsuperscript{6}-benzyguanine in HT29 cell-free extracts.

INTRODUCTION

There is considerable evidence indicating that the presence in tumor cells of the DNA repair protein AGT\textsuperscript{3} imparts resistance to alkylating agents, the mechanism of action of which involves reaction with the O\textsuperscript{6}-position of guanine residues in DNA (1–3). Lack of AGT activity correlates with cellular sensitivity to the cytotoxic effects of chloroethyldating agents (e.g., 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and 1-(2-chloroethyl)-3-methylcyclohexyl-1-nitrosourea) and methylating agents (e.g., dacarbazine, procarbazine, and streptozotocin) (1, 4–7). We recently showed that several O\textsuperscript{6}-benzylated guanine derivatives are effective inactivators of AGT and that they can be used to enhance the cytotoxicity of chloroethylation and methylating antitumor drugs (8–10). Of the inactivators prepared thus far, O\textsuperscript{6}-benzyguanine is the most effective agent, requiring only \mu M concentrations for min to deplete AGT in HT29 cells and cell extracts (8–11). More importantly, significant growth inhibition has been observed in 1,3-bis(2-chloroethyl)-1-nitrosourea-resistant human brain and colon tumor xenografts after administration of O\textsuperscript{6}-benzyguanine (12–16).

Since metabolism of O\textsuperscript{6}-benzyguanine might compromise its potency as an AGT inactivator, we examined its metabolic fate in male Sprague-Dawley rats and BALB/c mice. For these experiments, we developed an HPLC assay for the determination of O\textsuperscript{6}-benzyguanine and its metabolites in biological fluids, we identified several of its metabolites in the urine and plasma of rats, and we evaluated the ability of the important metabolites to inactivate the AGT protein. The results of these investigations are presented below.

MATERIALS AND METHODS

Chemicals. O\textsuperscript{6}-Benzyguanine and N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzylguanine were synthesized as described (8, 17). 8-[\textsuperscript{3}H]-O\textsuperscript{6}-Benzyguanine was prepared by American Corp. (Arlington Heights, IL) by catalytic exchange using tritiated water and was purified by reverse phase HPLC under isocratic conditions with a mobile phase consisting of 50% methanol in 0.05 M ammonium formate at a flow rate of 1.0 ml/min at 35°C (retention time, 9 min). N\textsuperscript{2}-Acetylguanine and guanine were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Oxoguanine was purchased from Aldrich Chemical, Inc. (Milwaukee, WI). Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Animal Treatment. Male Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Athyamic BALB/c mice (nu/nu genotype, 6 weeks or older) were maintained as described previously (18). Animals were housed in an environmentally controlled room (12-h light/12-h dark) and were provided with food and water ad libitum. Rat procedures followed guidelines according to The University of ChicagoManual on Laboratory Animals prepared by the Animal Care Committee. During urine collection, rats were housed individually in metabolic cages.

Four rats (300 g) were each injected with 30 mg/kg 8-[\textsuperscript{3}H]-O\textsuperscript{6}-benzyguanine (134 \mu C/ml) in 10% cremophor/saline, and urine was collected at 5, 19, 29, 52, 72, 95, and 119 h. A second set of rats was injected with 30 mg/kg 8-[\textsuperscript{3}H]-O\textsuperscript{6}-benzyguanine (73 \mu Ci/ml), and rats were sacrificed at 1, 4, 14, and 24 h with a lethal dose of pentobarbital. Transmyocardial phlebectomy was performed to remove 3–10 ml of blood. Blood was centrifuged at 2500 rpm for 10 min, and plasma was removed. Liver, spleen, brain, small intestine, kidney, lung, and esophagus were excised and immediately frozen at −80°C until further analysis. Extract from tissues was prepared by the addition of 50 mm Tris, 0.1 mm EDTA, and 5 mm diithiothreitol buffer, pH 7.4 (2X v/w), and centrifugation was carried out for 35 s at 10,000 rpm. Total radioactivity was determined by mixing 100 \mu l extract with 10 ml of counting scintillant and counting on a Tri-Carb scintillation counter (Packard Instruments, Downers Grove, IL). Urine and plasma (10 ml each) were counted directly in scintillant.

Mice were injected with 30 mg/kg 8-[\textsuperscript{3}H]-O\textsuperscript{6}-benzyguanine (2.6 \mu Ci/20-g mouse) in 40% polyethylene glycol-400/60% saline, and urine was collected at 24, 36, and 48 h.

Sample Preparation. Methanol was added to the urine such that the final concentration was 35% (v/v) and was kept ice-cold for 2 h. After centrifugation at 14,000 × g for 10 min, the supernatant was further centrifuged through a 100,000 \mu M filter prior to HPLC injection. Plasma and liver extract were prepared for HPLC injection by the addition of 40 ml of acetonitrile:water (8:2 v/v) to 40 ml of acetonitrile:water (8:2 v/v) to the tissue. After centrifugation at 2200 rpm (1500 × g) for 10 min, samples were lyophilized and resuspended in a mobile phase for HPLC analysis (see below).

Received 2/23/94; accepted 8/29/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore hereby be marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work has been supported in part by the National Cancer Institute through Grants CA47226 (M. C. E.), CA57725 (A. E. P., M. E. D., H. S. F.), CA-18137 (A. E. P.), NCI Contract NOI-CO-74101 with ABL (M-Y. C., R. C. M.), and by NINDS through Grants NS30245 (H. S. F.) and NS20023 (H. S. F.) and AFs DHP-67E (H. S. F.).

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: AGT, O\textsuperscript{6}-alkylguanine-DNA alkyltransferase; O\textsuperscript{6}-benzyguanine, O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine; N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzylguanine, N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine; N\textsuperscript{2}-acetyl-O\textsuperscript{6}-benzyl-7,8-dihydro-8-oxoguanine; 8-oxoguanine, 7,8-dihydro-8-oxoguanine; HPLC, high pressure liquid chromatography; EI, electron impact; FAB, fast atom bombardment.
HPLC Analysis of Urinary Metabolites. The HPLC equipment consisted of an LKB controller with two pumps and a Rhodamine injector connected to an LKB variable wavelength detector (LKB, Piscataway, NJ) or a Hitachi diode array detector (Hitachi Instruments, San Jose, CA) scanning from 200 to 400 nm. Radioactivity was monitored by scintillation counting of fractions or by use of a radioactivity flow detector (Radiomatic Instruments, Tampa, FL) with a Flo-Scint II/mobile phase ratio of 4:5 (Packard Instruments). Reverse phase, strong cation exchange, and anion exchange chromatography systems were used for metabolite identification. For reverse phase chromatography, a Beckman ODS 5 μm C18 column (Beckman Instruments, Inc., San Ramon, CA) was eluted isocratically with 35% methanol in 0.05 M ammonium formate (pH 4.5) at 1 ml/min at room temperature. Strong cation exchange chromatography was carried out on a Whatman Partisil 10-SCX column eluted with 5 mM ammonium phosphate (pH 7.0) at a flow rate of 1 ml/min at room temperature. Anion exchange chromatography was carried out on a Rainin HP-PEI strong anion exchange column eluted with a mobile phase of 10 mM ammonium formate (pH 5.02) at a flow rate of 1 ml/min at room temperature.

Mass Spectral Analysis. El and positive ion FAB mass spectra were obtained on a reversed geometry VG-Micromass ZAB-2F spectrometer interfaced to a VG-2035 data system. Glycerol or a mixture of dithioerythritol and diithioerythritol (1:1) were used as a FAB matrix.

Synthesis of O'-Benzyl-8-oxoguanine. 2,5,6-Triamino-4-benzyloxypyrimidine (1.85 g, 8 mmol; Ref. 19) and 1,1'-carbonyldiimidazole (1.3g. 8 mmol) were dissolved in anhydrous N,N-dimethylformamide (5 ml) under argon. The solution was stirred at room temperature overnight and was mixed with water (200 ml) to precipitate a white solid. The solid was collected by filtration and dissolved in 250 ml of aqueous 2 N NaOH solution. Undissolved material was removed by filtration, and the filtrate was neutralized with glacial acetic acid to precipitate a white solid. The solid was collected by filtration, washed with water, and recrystallized from 50% aqueous ethanol to afford analytically pure white crystals. Yield, 1.63 g (79%); m.p. 256–257°C dec.; UV (pH 1) λmax 243 nm (ε = 0.717 × 10^4), 306 (1.499 × 10^4); (pH 6.9) 243 (0.915 × 10^4), 290 (1.108 × 10^4); (pH 13) 249 (0.443 × 10^4), 293 (1.368 × 10^4); 'H NMR δ 2.18 (s, 3 H, CH3), 2.57 (s, 3 H, CH3), 5.51 (s, 2 H, ArCH2), 7.30–7.57 (m, 5 H, ArH), 10.21 (s, 1 H, exchanges with D2O), 12.30 (s, 1 H, exchanges with D2O); MS (El) Calcd. m/z for C14H13N5O4: 285 (2.138 × 10^6); 'H NMR δ 2.18 (s, 3 H, CH3), 2.57 (s, 3 H, CH3), 5.51 (s, 2 H, ArCH2), 7.30–7.57 (m, 5 H, ArH), 10.41 (s, 1 H, exchanges with D2O), 11.04 (s, 1 H, exchanges with D2O); MS (EI) Calcd. m/z for C14H13N5O4: 285.0612. Found: 285.0610. Anal. C14H13N5O4. C, N, H.

Synthesis of N2-Acetyl-O'-benzyl-8-oxoguanine. Acetic anhydride (2 ml) was added to a suspension of O'-benzyl-8-oxoguanine (0.257 g, 1.0 mmol) in dry toluene (10 ml). The suspension was vigorously refluxed for 24 h and then cooled to room temperature. After storing at 4°C for 4 h, the resulting precipitate was collected by filtration and washed with benzene to give a diacetylated O'-benzyl-8-oxoguanine derivative. Yield, 0.287 g (84%); m.p. 272–274°C dec.; UV (100% MeOH) λmax 275 nm (ε = 1.313 × 10^4); (pH 1) 275 (1.143 × 10^4); (pH 6.9) 238 (0.995 × 10^4), 276 (1.115 × 10^4); (pH 13) 285 (2.138 × 10^4); 'H NMR δ 2.18 (s, 3 H, CH3), 2.57 (s, 3 H, CH3), 5.51 (s, 2 H, ArCH2), 7.30–7.57 (m, 5 H, ArH), 10.14 (s, 1 H, exchanges with D2O), 12.30 (s, 1 H, exchanges with D2O); MS (EI) Calcd. m/z for C16H16N5O4: 341.1123. Found: 341.1130. Anal. C16H16N5O4. C, N, H.

AGT Depletion Experiments. Crude extracts from HT29 cells were prepared as described (20) and were incubated for 30 min with concentrations between 0 and 200 μM O'-benzylguanine, N2-acetyl-O'-benzylguanine, O'-benzyl-8-oxoguanine, or N2-acetyl-O'-benzyl-8-oxoguanine in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM diithioerythritol for 30 min. For cell exposure experiments, cells in exponential growth were exposed to increasing concentrations of drug for 4 h as described (8). Alkyltransferase activity remaining was determined by measuring loss of O'-benzyl-8-oxoguanine from a [3H]-methylated DNA substrate (8, 20, 21). Protein was determined by the method of Bradford (22).

RESULTS

Biodistribution of 8-[3H]-O'-Benzylguanine. Treatment of rats with an i.p. injection of 8-[3H]-O'-benzylguanine (30 mg/kg; 73 μCi/mmoll in 10% cremophor/saline produced marked differences in the organ levels of radioactivity (Fig. 1). Significantly higher levels of radioactivity were observed in liver compared to lung, spleen, small intestine, kidney, or esophagus. Radioactivity levels decreased somewhat in the small intestine, lung, spleen, and esophagus over a 24-h period, while levels in the liver, kidney, and plasma remained fairly constant over the same time period.

Rat Urinary Metabolites. Metabolism of 8-[3H]-O'-benzylguanine (30 mg/kg; 134 μCi/mmoll) was studied by analyzing rat urine samples up to 119 h after i.p. administration of 5 μCi of the drug. Fig. 2 indicates the amount of radioactivity excreted in the urine of treated animals as a function of time. Highest levels were measured 19 h after drug administration. The total radioactivity excreted within 119 h was 20 ± 3.8% of the amount injected.

The separation of O'-benzylguanine and its metabolites was achieved using a reverse phase C18 column eluted with a mobile phase consisting of 35% methanol in 0.05 M ammonium formate (pH 4.5) at 1 ml/min at ambient temperature. Fig. 3 illustrates the radioactivity (Fig. 3A) and corresponding UV absorption profiles (Fig. 3B) of rat urine 19 h after an i.p. injection of 8-[3H]-O'-benzylguanine. The UV profile of urine from an untreated rat is illustrated in Fig. 3C. As indicated, there were six significant radioactive peaks that eluted at 3 min (peak 1), 4 min (peak 2, split), 6 min (peak 3), 35 min (peak 4), 40 min (peak 5), and 50 min (peak 6; Fig. 3A). The percentage of total radioactivity (± SD) associated with each peak at the time of urine collection is presented in Table 1. Nonradioactive, UV-absorbing peaks that were not found in untreated rat urine were observed at 32 min (peak 7) and 37 min (peak 8; Fig. 3B). To confirm that metabolism or degradation of the parent compound had not occurred after excretion prior to injection of urine at each time point, O'-benzylguanine (375 μm; 0.22 μCi/ml) was allowed to incubate in urine for 24 h at room temperature. Chromatography revealed that O'-benzylguanine was unchanged under these conditions (data not shown).

A combination of chromatography, UV, and mass spectroscopy was used to identify these various urinary metabolites. Peak 1 was determined to be composed of volatile tritiated components because 79% of the radioactivity associated with this peak was lost upon evaporation of urinary samples to dryness. Authentic samples of 3H2O exhibited the same retention time as peak 1 under these chromatographic conditions. Therefore, the major component associated with peak 1 was presumed to be 3H2O resulting from tritium exchange or oxidative release of tritium from the 8-position of 8-[3H]-O'-benzylguanine.

Peak 4 was identified as O'-benzylguanine by comparison of its retention time with that of an authentic sample. The UV spectrum of peak 4 was identical to that of the parent drug, O'-benzylguanine (Table 2; Fig. 4). Furthermore, peak 4 was isolated and subjected to El and FAB mass spectroscopy. The El spectrum showed a molecular ion at m/z = 241 and a base peak at m/z = 91 due to the benzyl group [C6H3]. The positive ion FAB mass spectrum showed a dominant ion at m/z = 242, corresponding to the molecular ion plus a proton (i.e., [M + H]+), and an intense ion at m/z = 91 for the benzyl group. Both spectra corresponded well with those of an authentic sample of O'-benzylguanine.

Metabolite 5 was isolated and subjected to El and FAB mass spectroscopy. The El spectrum for this metabolite showed a molecular ion at m/z = 283, indicating an acetylated derivative of O'-benzyl-
Fig. 1. Radioactivity in rat tissues after administration of 8-[3H]-O'-benzylguanine. Rats were injected i.p. with 30 mg/kg O'-benzylguanine (73 µCi/mmol) and sacrificed at 1, 4, 14, and 24 h. The radioactivity associated with plasma and extracts prepared from liver, intestine, lung, spleen, kidney, and esophagus was determined by scintillation counting. Results are expressed as cpm/g tissue or cpm/ml plasma.

Fig. 2. Urinary excretion of radioactivity after i.p. injection of 8-[3H]-O'-benzylguanine. Sprague-Dawley rats (300 g) were injected with 5 µCi 8-[3H]-O'-benzylguanine. For each urine collection, total radioactivity excreted was determined and expressed in percent total label excreted.

guanine. The spectrum also showed significant ions at m/z = 241, representing loss of CH2CO from the molecular ion and an ion at m/z = 91 for the benzyl group. The positive ion FAB mass spectrum showed major ions at m/z = 284 and m/z = 242 corresponding, respectively, to the molecular ion for an acetylated derivative of O'-benzylguanine plus a proton and loss of CH2CO from this material to produce protonated O'-benzylguanine. The chromatographic retention time as well as the UV spectroscopic properties of this metabolite were identical to those of an authentic sample of N2-acetyl-O'-benzylguanine (17), resulting from acetylation at the N2-position (Table 2; Fig. 4).

Metabolites 7 and 8 exhibited UV absorbance, but they contained no radioactivity. Metabolites 7 and 8, respectively, eluted about 3 min earlier than metabolites 4 and 5. These data suggested that metabolites 7 and 8 were related to O'-benzylguanine and N2-acetyl-O'-benzylguanine but were slightly more polar as a result of metabolic transformation at the 8-position. Since the UV absorption spectrum associated with peak 7 (Fig. 4) resembled the spectrum expected for an 8-hydroxy derivative of O'-benzylguanine (23) and since oxidation of the 8-position of purines is a well-known metabolic reaction (24), we presumed that metabolite 7 was O'-benzyl-8-oxoguanine. We confirmed this identification by synthesizing an authentic standard through reaction of 2,5,6-triamino-4-benzyloxypyrimidine with 1,1'-carbonyldiimidazole in N,N-dimethylformamide. The retention time and UV spectrum of the purified O'-benzyl-8-oxoguanine was identical to that associated with peak 7 (Table 2; Fig. 4). Upon the addition of authentic O'-benzyl-8-oxoguanine to rat urine collected 19 h after exposure to 8-[3H]-O'-benzylguanine, the peak height for peak 7 increased and remained symmetrical, indicating this peak was the identified metabolite (Table 3). To further confirm the identity of peaks 4 (O'-benzylguanine) and 7 (O'-benzyl-8-oxoguanine), the isolated materials were subjected to acid hydrolysis (1 N HCl; 30 min; 90°C). The retention time and UV spectrum of the liberated purine products were compared to those for authentic guanine and 8-oxoguanine samples (Table 2). The retention time (4 min) and spectra were in agreement.

The UV absorption spectrum associated with peak 8 resembled that of N2-acetyl-O'-benzylguanine more closely than the spectrum associated with peak 4 or 7 (Table 2; Fig. 4). Therefore, we presumed peak 8 was probably N2-acetyl-O'-benzyl-8-oxoguanine, resulting from
Table 1 Composition of rat urine following administration of 8-[3H]Oβ-benzylguanine
Urine was collected from rats injected with 30 mg/kg 8-[3H]Oβ-benzylguanine (5 μCi/300-g rat) and analyzed with a radioactivity detector after HPLC separation of peaks. Numbers represent the mean from 4 animals (± SD). At 5, 19, and 29 h, two additional minor peaks eluting at 10 and 13 min contained 8, 7, and 2% of total radioactivity, respectively.

<table>
<thead>
<tr>
<th>Radioactive metabolite peaks (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>29</td>
</tr>
<tr>
<td>43</td>
</tr>
<tr>
<td>52</td>
</tr>
</tbody>
</table>

Table 2 UV analysis of parent drug and metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>λ&lt;sub&gt;min&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N&lt;sup&gt;2&lt;/sup&gt;-acetylguanine</td>
<td>258.7</td>
<td>230.6</td>
</tr>
<tr>
<td>Metabolite 2</td>
<td>258.7</td>
<td>231.0</td>
</tr>
<tr>
<td>Metabolite 3</td>
<td>241.9, 280.5</td>
<td>229.4, 258.9</td>
</tr>
<tr>
<td>Oβ-Benzylguanine</td>
<td>240.0, 281.4</td>
<td>230.6, 257.4</td>
</tr>
<tr>
<td>Peak 4</td>
<td>240.4, 281.8</td>
<td>230.6, 258.2</td>
</tr>
<tr>
<td>N&lt;sup&gt;2&lt;/sup&gt;-Acetyl-Oβ-benzylguanine</td>
<td>266.0</td>
<td>235.5</td>
</tr>
<tr>
<td>Metabolite 5</td>
<td>266.0</td>
<td>235.9</td>
</tr>
<tr>
<td>Metabolite 6</td>
<td>275.9</td>
<td>244.7</td>
</tr>
<tr>
<td>Oβ-Benzyl-8-oxoguanine</td>
<td>243.2, 289.1</td>
<td>224.5, 263.9</td>
</tr>
<tr>
<td>Metabolite 7</td>
<td>243.2, 289.5</td>
<td>225.0, 263.9</td>
</tr>
<tr>
<td>Rat plasma metabolite</td>
<td>242.8, 289.5</td>
<td>225.8, 263.5</td>
</tr>
<tr>
<td>Rat liver metabolite</td>
<td>242.8, 287.9</td>
<td>227.0, 263.9</td>
</tr>
<tr>
<td>Mouse urine metabolite</td>
<td>242.8, 289.5</td>
<td>226.2, 264.3</td>
</tr>
<tr>
<td>N&lt;sup&gt;2&lt;/sup&gt;-Acetyl-Oβ-benzyl-8-oxoguanine</td>
<td>275.7</td>
<td>230.6</td>
</tr>
<tr>
<td>Metabolite 8</td>
<td>275.7</td>
<td>230.2</td>
</tr>
<tr>
<td>Guanine</td>
<td>246.5, 273.3</td>
<td>227.0, 262.7</td>
</tr>
<tr>
<td>Acid hydrolyzed peak 4</td>
<td>246.1, 273.7</td>
<td>230.6, 262.7</td>
</tr>
<tr>
<td>8-Oxoguanine</td>
<td>244.8, 289.9</td>
<td>226.6, 267.2</td>
</tr>
<tr>
<td>Acid hydrolyzed metabolite 7</td>
<td>244.8, 289.9</td>
<td>225.0, 267.6</td>
</tr>
<tr>
<td>Metabolite 9</td>
<td>244.0, 289.1</td>
<td>227.8, 267.6</td>
</tr>
</tbody>
</table>

Fig. 3. Reverse phase HPLC profile of rat urine after injection with 30 mg/kg 8-[3H]Oβ-benzylguanine. Rat urine collected at 19 h was prepared by the addition of methanol, centrifugation, and filtration. An aliquot was eluted with 35% methanol in 0.05 M ammonium formate (pH 4.5) from a C<sub>18</sub> reverse phase column. A, radioactivity as determined by scintillation counting of fractions. B, UV absorbance at 280 nm of rat urine collected at 19 h. C, UV absorbance at 280 nm of rat urine from an untreated rat.

Fig. 4. UV spectrum of parent drug and metabolites. The UV absorption spectrum was determined for metabolites 4, 5, 7, and 8 with a Hitachi diode array detector. The dotted line represents the spectrum of an authentic standard chromatographed in untreated rat urine which includes: Oβ-benzylguanine (Peak 4), N<sup>2</sup>-acetyl-Oβ-benzylguanine (Peak 5), Oβ-benzyl-8-oxoguanine (Peak 7), and N<sup>2</sup>-acetyl-Oβ-benzyl-8-oxoguanine (Peak 8). The solid line is the spectrum for the corresponding rat urinary metabolite.
and samples of O6-benzyl-8-oxoguanine or N2-acetyl-O6-benzyl-8-oxoguanine were added. The relative amounts of metabolites were based on peak heights at 280 nm and are corrected for absorbency differences of these metabolites at this wavelength. Urine Metabolite peak 8 resembles that for metabolite peak 8 and authentic N2-acetylguanine. The later peak and authentic N2-acetylguanine also coeluted with N2-acetylguanine at 17 min (data not shown). Guanine, 8-oxoguanine, and N2-acetylguanine presumably result from debenzylation of O6-benzylguanine, O6-benzyl-8-oxoguanine, and N2-acetyl-O6-benzylguanine, respectively. N2-Acetyl-8-oxoguanine resulting from debenzylation of N2-acetyl-O6-benzyl-8-oxoguanine has not been isolated.

Metabolite peak 3 and the minor metabolite 6 have not yet been identified. Their UV absorption properties are presented in Table 2. The UV spectrum for metabolite 6 resembles that for metabolite 8 and N2-acetyl-O6-benzyl-8-oxoguanine, while the spectrum for metabolite 3 resembles that of O6-benzylguanine.

Rat Liver and Plasma Metabolites. Chromatographic analysis of rat liver following exposure to 8-[3H]-O6-benzylguanine indicated that O6-benzyl-8-oxoguanine and O6-benzylguanine were both present at 1 and 4 h (Table 4; Fig. 5). Although the level of radioactivity remained fairly constant in the liver for up to 24 h, this radioactivity was made up of a volatile component (presumably 3H2O) and nonvolatile components, which included an early eluting peak (retention time, 5 min) and 8-[3H]-O6-benzylguanine (Fig. 5A). The percentage of this nonvolatile, early-eluting radioactive material was 43, 39, 94, and 100 at 1, 4, 14, and 24 h, respectively. The acetylated derivatives were not detected in rat liver (Fig. 5).

As we observed with liver samples, the UV spectrum of the nonradioactive, UV-absorbing peak, which elutes 3 min prior to parent drug in chromatograms from rat plasma (Fig. 5D), was identical to O6-benzyl-8-oxoguanine (Table 2). Only a small fraction of the parent drug was present as O6-benzyl-8-oxoguanine in rat liver and plasma compared to that found in the urine (Table 4). Although the radioactivity in plasma samples up to 24 h did not appear to change (Fig. 1), much was lost upon evaporation (30% was lost at 1 and 4 h, and 80–90% was lost at 14 and 24 h). Presumably, the radioactivity lost was 3H2O which might have formed as a result of oxidation at the C8 position and excretion of the metabolite. The
The effective dose required to inactivate 50% of AGT upon incubation of HT29 cells for 4 h with \( O^6 \)-benzylguanine, \( O^6 \)-benzyl-8-oxoguanine, \( N^2 \)-acetyl-\( O^6 \)-benzylguanine, and \( N^2 \)-acetyl-\( O^6 \)-benzyl-8-oxoguanine was 0.05, 0.15, 2, and 11 \( \mu M \), respectively.

**DISCUSSION**

This study was carried out to identify the major metabolites of \( O^6 \)-benzylguanine following its i.p. administration to rats and mice. Three benzylated derivatives were identified in rats. These were \( O^6 \)-benzyl-8-oxoguanine, \( N^2 \)-acetyl-\( O^6 \)-benzylguanine, and \( N^2 \)-acetyl-\( O^6 \)-benzyl-8-oxoguanine. Structures for these derivatives and their routes of formation are shown in Fig. 7.

Oxidation of \( O^6 \)-benzylguanine may have occurred initially since \( O^6 \)-benzyl-8-oxoguanine but not the acetylated metabolites were found in the liver and plasma. \( N^2 \)-Acetyl-\( O^6 \)-benzylguanine and \( N^2 \)-acetyl-\( O^6 \)-benzyl-8-oxoguanine were found only in the urine of rats. It is possible that acetylation is occurring in the kidney or the mucosa of the urinary bladder just prior to excretion. It has been reported that the major site of biological \( N \)-acetylation is the liver and gut mucosa, although this reaction can occur in other tissues (26). If acetylation of \( O^6 \)-benzylguanine and \( O^6 \)-benzyl-8-oxoguanine occurs in the liver, then the respective metabolites must get excreted rapidly; otherwise, this reaction is occurring elsewhere.

Since acetylation was not observed in mouse urine, then this must be a species-specific reaction. It is not known whether acetylation of \( O^6 \)-benzylguanine will occur in humans. Interestingly, the \( N \)-acetylating capacity of individuals is genetically determined and can vary remarkably from one person to another (24, 26). This implies that if \( N \)-acetylation of \( O^6 \)-benzylguanine occurs in humans, the extent to which these less active AGT-depleting derivatives are formed may differ considerably from one individual to another.

One of the major routes of metabolism of \( O^6 \)-benzylguanine in both rats and mice involves oxidation at the 8-position to form \( O^6 \)-benzyl-8-oxoguanine. The oxidative metabolism of drugs is confined mainly to the membrane-bound microsomal cytochrome P-450 system; however, oxidation may occur by nonspecific enzymes found in the soluble fraction of liver, such as xanthine oxidase or aldehyde oxidase (24). Although guanine itself is not a substrate for xanthine oxidase, several purine derivatives (e.g., 6-mercaptopurine, theophylline, and caffeine) are known to undergo 8-oxidation in vivo by this enzyme (27). Aldehyde oxidase is known to catalyse the oxidation of certain purines, peridine, and pyridimines in vivo (28). Studies to identify the enzyme(s) responsible for oxidation of \( O^6 \)-benzylguanine are currently under way.

Debenzylation of \( O^6 \)-benzylguanine, \( O^6 \)-benzyl-8-oxoguanine, and \( N^2 \)-acetyl-\( O^6 \)-benzylguanine occurred to produce guanine 8-oxoguanine, and \( N^2 \)-acetylguanine, respectively. There is a possibility that \( N^2 \)-acetyl-8-oxoguaine or other debenzylated products are also formed. \( N^2 \)-Acetyl-8-oxoguanine produced from \( 8 \)-[\( ^3 H \)]-\( O^6 \)-benzylguanine, \( O^6 \)-benzylguanine, and \( N^2 \)-acetylguanine, respectively. There is a possibility that hydroxylation of the benzene ring of an \( O^6 \)-benzylguanine derivative could produce a more labile compound which could spontaneously debenzylate.

\( O^6 \)-Benzyl-8-oxoguanine has AGT-inactivating activity similar to parent drug, indicating that conversion to this metabolite results in a slight loss of potency. However, a greater loss of AGT activity in vivo
can occur as a result of: (a) excretion of the parent drug or an active derivative such as O-benzyl-8-oxoguanine; (b) conversion to the less potent acetylated metabolites; or (c) debenzylation of the parent drug or metabolites.

ACKNOWLEDGMENTS

We thank Maricruz DeLeon for excellent technical assistance and Dr. Michael Sitrin for the use of his metabolic cages.

REFERENCES

METABOLISM OF O'-BENZYLGUANINE


Metabolism of $O^6$-Benzylguanine, an Inactivator of $O^6$ -Alkylguanine-DNA Alkyltransferase

M. Eileen Dolan, Mi-Young Chae, Anthony E. Pegg, et al.


Updated version
Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/54/19/5123

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.