Plasma and Cerebrospinal Fluid Pharmacokinetics of O6-Benzylguanine and Time Course of Peripheral Blood Mononuclear Cell O6-Methylguanine-DNA Methyltransferase Inhibition in the Nonhuman Primate

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ABSTRACT

O6-Benzylguanine (O6BG) enhances the cytotoxicity of the nitrosoureas by irreversibly binding and inhibiting the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT). The plasma and cerebrospinal fluid (CSF) pharmacokinetics of O6BG and its active metabolite, O6-benzyl-8-oxoguanine, were studied in a nonhuman primate model after 200 mg/m2 had been injected i.v. The parent drug and the metabolite were measured with a reverse-phase HPLC assay. A pharmacokinetic model incorporating separate compartments for O6BG and the O6-benzyl-8-oxoguanine metabolite, first-order conversion of O6BG to the metabolite, and additional first-order elimination rate constants for each compound, was simultaneously fitted to the parent drug and metabolite plasma concentration time data. Elimination of O6BG from plasma was rapid; it had a half-life of 1.6 h and a clearance of 68 μl/min/m2. On the basis of the pharmacokinetic model, essentially all of the O6BG was converted to O6-benzyl-8-oxoguanine. The plasma pharmacokinetic profile of the metabolite differed considerably from that of the parent drug. The half-life (14 h) was 10-fold longer and the area under the curve (2420 μl/h) was 11-fold higher than that of O6BG (212 μl/h). The clearance rate of O6-benzyl-8-oxoguanine was 6.4 ml/min/m2. The CSF/plasma ratio was 4.3% for O6BG and 36% for O6-benzyl-8-oxoguanine, and the metabolite area under the curve was 90-fold higher than that of O6BG in CSF. The excellent CSF penetration of the active metabolite provides a rationale for the use of O6BG as a chemosensitizing agent for brain tumors. We also studied the duration of MGMT inhibition in peripheral blood mononuclear cells. By 2 h after a 200 mg/m2 dose of O6BG, >98% of MGMT activity was suppressed, and >95% suppression of enzyme activity persisted at 18 and 48 h after the dose. By 2 weeks after the treatment, MGMT levels had returned to baseline. Persistent high concentrations of the active metabolite appear to provide a pharmacological explanation for the prolonged suppression of MGMT activity.

INTRODUCTION

Alkylating agents, such as the nitrosoureas, are chemically reactive compounds that bond covalently at the O6 position on guanine in DNA, leading to the formation of alkylguanine adducts and intra- and interstrand cross-links (1). Nitrosourea-resistant cell lines and human tumor specimens have the capacity to remove the alkyl group from guanine before the formation of DNA cross-links (2). The DNA repair enzyme MGMT (3) transfers the monooadduct to a cysteine in its active site, thereby restoring the guanosine in DNA and permanently inactivating the enzyme (3). Because MGMT is a single turnover enzyme, the number of DNA lesions that can be repaired is stoichiometrically proportional to the amount of MGMT in the cell (4); once all of the MGMT in the cell is consumed, additional DNA repair is dependent on de novo enzyme synthesis (5). Therefore, depletion of MGMT could increase the sensitivity of cells to nitrosourea-mediated DNA damage.

Therapeutic strategies for modulating intracellular levels of MGMT include the use of both agents that deplete MGMT by generating a large number of O6-methylguanosine DNA adducts, such as streptozotocin, and agents that directly inhibit MGMT, such as O6BG (6). O6BG, which currently is undergoing intensive evaluation, irreversibly inactivates MGMT by acting as a fraudulent substrate (3). O6BG has been shown to increase tumor cell sensitivity to nitrosoureas both in vitro and in vivo (6-10).

After i.p. administration to mice and rats, O6BG is oxidized to O6-benzyl-8-oxoguanine (Fig. 1) by cytochrome P450 enzymes CYP1A2 and CYP3A4 (11). This metabolite has MGMT-inactivating activity that is nearly identical with that of the parent drug; it has an ED50 of 0.3 μM in HT29 cell extracts compared with an ED50 of 0.2 μM for O6BG (12). Little is known, however, about O6BG metabolism in other species or about its penetration into the central nervous system.

To facilitate the design of rational dosing schedules of O6BG for human clinical trials, we investigated the pharmacokinetic behavior and CSF penetration of O6BG and O6-benzyl-8-oxoguanine in a nonhuman primate model. We also studied the duration of MGMT inhibition in peripheral blood mononuclear cells after a dose of O6BG.

MATERIALS AND METHODS

Animals. Six adult male Rhesus monkeys weighing 7.9-11.3 kg were used for this study. The animals were fed NIH open formula extruded nonhuman primate diet twice daily and were group housed in accordance with the Guide for the Care and Use of Laboratory Animals (13). Blood samples were drawn through a catheter placed in either the femoral or the saphenous vein contralateral to the site of drug infusion. CSF samples were drawn from a s.c. Ommaya reservoir attached to an indwelling Pudenz catheter, with its tip located in the fourth ventricle (14).

Reagents. O6BG and diluent (PEG 400; Union Carbide, sentry grade) were obtained from the Developmental Therapeutics Branch, National Cancer Institute (Bethesda, MD). The dose of O6BG was dissolved in PEG 400, diluted with 0.9% sodium chloride to a final concentration of 40% PEG 400, and sterilized by filtration through a 0.22-μm filter.

O6-Benzyloxanthine, N2-acetyl-O6-benzylguanine, and O6-benzyl-8-oxoguanine were provided by Dr. Robert C. Moschel (Frederick Cancer Research and Development Center, Frederick, MD).

Animal Experiments. A single 200 mg/m2 dose of O6BG was administered i.v. over 15 min to each animal. For the pharmacokinetic studies in three animals, blood samples were collected before the infusion, at the end of the infusion, and 5, 15, and 30 min and 1, 2, 3, 4, 6, 8, 10, 24, and 48 h after the end of the infusion. Plasma was separated immediately by centrifugation at 1500 rpm and frozen at -20°C until analysis. Ventricular CSF samples were collected before the infusion and 1, 2, 4, 6, 8, 10, 24, and 48 h after the end of the infusion. CSF was frozen at -20°C until analysis. Urine was collected from a different animal for 8 h after a 200 mg/m2 dose of O6BG and was frozen at -20°C until analysis.

For MGMT enzyme inhibition studies, heparinized blood samples were
collected from three animals before the infusion and at 2, 18, and 48 h and 2 weeks after the infusion. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation, and the resultant cell pellet was immediately frozen at −80°C until analysis.

**Plasma Protein Binding.** Fresh human and Rhesus monkey plasma was spiked with 100 μg O6-DG and incubated for 1 h at 37°C. The free drug was then separated by ultrafiltration on a Centricon Micropartition System (Amicon Division, W.R. Grace & Co., Danvers, MA) and centrifuged at 2000 × g for 20 min. The total drug concentration in plasma and the free drug concentration in the ultrafiltrate were measured. The percentage of protein-bound drug was calculated using the following formula:

\[
\frac{\text{O6-DG plasma} - \text{O6-DG ultrafiltrate}}{\text{O6-DG plasma}} \times 100\%
\]

**O6-DG Assay.** O6-DG concentration was measured using a modification of a reverse-phase HPLC method described previously (15). Plasma samples underwent solid-phase extraction using 3-ml Bond Elut C18 prep columns (Analytictech International, Harbor City, CA), which had been rinsed previously with 3 ml methanol and 3 ml 0.25 M ammonium acetate (pH 7.0). After 500 μl of plasma were loaded, the columns were washed with 3 ml 0.25 M ammonium acetate (pH 7.0), and eluted with 2 ml acetone. Eluates were evaporated to dryness under nitrogen at 37°C. Before injection onto the HPLC system, samples were reconstituted in the mobile phase and filtered through a 0.45-μm filter (Ultrafree-MC; Millipore Corporation, Bedford, MA). The recovery rate of O6-DG after solid-phase extraction from plasma was 86%. Urine and CSF were injected directly onto the HPLC system without solid-phase extraction.

Samples were injected onto a Beckman C18 5 μ 4.6 × 250 mm column (Beckman Instruments, Fullerton, CA) with a Brownlee ODS-GU C18 guard column (Applied Biosystems, San Jose, CA) and eluted with a mobile phase of 0.25 M ammonium acetate (pH 7.0)/methanol (50/50 v/v) at a flow rate of 1 ml/min. Peaks were monitored on a Waters Model 490 programmable multichannel detector at 280 nm or on a Waters Model 991 photodiode array detector (Waters Associates, Milford, MA). The retention time for O6-DG was approximately 11.5 min, and that for the O6-benzyl-8-oxoguanine metabolite was approximately 10.5 minutes. The coefficient of variation was <6%. A separate O6-DG standard curve was prepared with each set of samples. The concentration of O6-benzyl-8-oxoguanine in plasma was determined using a modification of O6-DG equivalents.

**MGMT Activity.** The activity of MGMT in peripheral blood mononuclear cells was measured as described previously (16). In brief, the mononuclear cell pellets were resuspended in 0.5 ml cell extract buffer (70 mM HEPES [pH 8.6], 1 mM EDTA, 5% glycerol, and 1 mM DTT). The cell suspension was sonicated three times for 10 s at 4°C to disrupt the cells and then centrifuged at 10,000 × g for 2 min to remove cellular debris. The protein and DNA content of the supernatant were measured, and then fixed amounts of protein from each sample were incubated with substrate DNA, which was prepared by incubating calf thymus DNA with [3H]MNU. The reaction mixture was incubated for 60 min at 37°C and then precipitated with 14% trichloroacetic acid at 4°C. MGMT activity in cell extracts was defined by the amount of the [3H]methyl group removed from O6-[3H]methylguanosine in the DNA. The alkylated O6-[3H]methylguanosine and N7-[3H]methylguanosine were liberated by acid hydrolysis and separated by HPLC, and radioactivity in the fractions of HPLC eluant was quantified by liquid scintillation. MGMT activity is expressed as fmol O6-[3H]methyl adduct removed/μg DNA.

**MGMT Protein Levels by Western Blot.** Cell extracts were resolved by SDS-PAGE. Samples were loaded onto 0.75-mm-thick gels (10% acrylamide) in a Bio-Rad minigel apparatus (Bio-Rad, Richmond, CA) and run at 150 V for 1 h. Proteins were transferred onto polyvinylidene difluoride membranes (Im mobilon-P; Millipore) using a Bio-Rad mini Trans-Blot cell for 1 h at 100 V. The blotted membranes were blocked with 5% dry milk in TBS [10 mM Tris-HCl (pH 7.4) with 0.9% NaCl] and then probed for 2 h with a mouse mAb specific for human cellular MGMT (MT 3.1, 5 μg/ml; gift of T. Brent, St. Jude Children’s Research Center, and D. Bigner, Duke University) in TBS containing 1% dry milk. After three 5-min washes with TBS containing 0.05% Tween 20, the blots were incubated with goat-antimouse horseradish peroxidase-conjugated anti-IgG for 1 h. Antibody binding was visualized by the Enhanced Chemiluminescence Kit (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions. For quantitative Western blotting, densitometric analysis was performed on a ScilScan 500 scanner (United States Biochemical Corp., Cleveland, OH) using Bioanalyzer software (Oberlin Scientific, Oberlin, OH).

**Pharmacokinetic Analysis.** The pharmacokinetic model depicted in Fig. 2 was fitted to the plasma O6-DG and O6-benzyl-8-oxoguanine concentration-time data using MLAB (18). The model incorporates separate compartments for O6-DG and the O6-benzyl-8-oxoguanine metabolite, first-order irreversible conversion of O6-DG to the metabolite, and additional first-order elimination rate constants for each compound. The differential equations describing the concentration of O6-DG and its metabolite are listed below:

\[
\frac{dC}{dt} = \frac{k_{0}C}{V_{d} - k_{dm}C - k_{m}C}
\]

\[
\frac{dM}{dt} = \frac{k_{d}CV_{d}}{V_{dm} - k_{m}M}
\]

where \(C\) is the concentration of O6-DG at time \(t\), \(M\) is the concentration of O6-benzyl-8-oxoguanine (in O6-DG equivalents) at time \(t\), \(k_{0}\) is the drug infusion rate (set to 0 at the end of the 15-min infusion), \(V_{d}\) is the volume of distribution of O6-DG, \(V_{dm}\) is the volume of distribution of the metabolite, \(k_{dm}\) is the rate constant describing the formation of metabolite from O6-DG, \(k_{del}\) is the elimination rate constant for O6-DG for all other routes of elimination, and \(k_{mel}\) is the elimination rate constant for the metabolite. The fits were weighted to 1/concentration². Initial model fits demonstrated that formation of metabolite accounted for virtually all of the elimination of O6-DG (\(k_{del}\) approached 0); therefore, \(k_{del}\) was set to 0, and this simpler model was fitted to the data. Clearance was calculated from the fitted model parameters (clearance = \(V_{d} \times k_{dm}\) for O6-DG, and apparent clearance = \(V_{dm} \times k_{mel}\) for the O6-benzyl-8-oxoguanine metabolite). A separate one-compartment model was fitted to the CSF concentration data to obtain the CSF elimination rate constants.

The degree of CSF penetration of O6-DG and O6-benzyl-8-oxoguanine was derived from the CSF:plasma AUC of both compounds as shown below:
The AUCs were calculated using the trapezoidal method (extrapolated to infinity).

RESULTS

The monkeys tolerated administration of \( O^6 \)-BG without clinical toxicity. Tables 1 and 2 list the pharmacokinetic parameters for \( O^6 \)-BG and \( O^6 \)-benzyl-8-oxoguanine in each animal. Disappearance of \( O^6 \)-BG from plasma was rapid; the mean (± SD) half-life was 1.6 ± 0.2 h and the mean (± SD) clearance was 68 ± 20 ml/min/m². The volume of distribution of \( O^6 \)-BG was 9.0 ± 1.4 liters/m² and the AUC was 210 ± 50 µM·h. \( O^6 \)-BG was 98 ± 0.7% bound in human plasma (single experiment).

\( O^6 \)-BG was detected in the CSF at the first time point measured (1 h after the infusion) and remained detectable for 7–9 h after the infusion. The mean (± SD) AUC of \( O^6 \)-BG in CSF was 9.1 ± 1.8 µM·h, and the disappearance of \( O^6 \)-BG from CSF paralleled that in plasma, having a half-life of 1.8 ± 0.2 h. The CSF:plasma ratio was low; it was only 4.3 ± 0.6% (Table 2).

\( O^6 \)-benzyl-8-oxoguanine was also identified in plasma and CSF of the nonhuman primates after administration of \( O^6 \)-BG. Identification was based on its retention time in the HPLC assay and the UV-visible absorption spectrum compared with an authentic standard. The retention time and the absorption spectrum of the metabolite in plasma were not compatible with those of \( O^6 \)-benzylxanthine or N²-acetyl-\( O^6 \)-benzylguanine.

\( O^6 \)-Benzyl-8-oxoguanine persisted in plasma for considerably longer than the parent drug, \( O^6 \)-BG. The mean (± SD) elimination half-life of the metabolite was 14 ± 5 h and the AUC (2420 µM·h) was 11-fold higher than that of \( O^6 \)-BG. The apparent clearance of \( O^6 \)-benzyl-8-oxoguanine was 6.4 ± 2.2 ml/min/m². On the basis of the model fit, all of the elimination of \( O^6 \)-BG can be accounted for by conversion to its \( O^6 \)-benzyl-8-oxoguanine metabolite. Although \( O^6 \)-benzyl-8-oxoguanine was quantified using the \( O^6 \)-BG standard curve, it is unlikely that the concentration of the metabolite was overestimated, because the recovery of \( O^6 \)-BG in our assay was high, and the UV absorption spectra and molar extinction coefficients of the parent drug and metabolite are very similar (12).

CSF concentrations of \( O^6 \)-benzyl-8-oxoguanine were also considerably higher than the concentrations of \( O^6 \)-BG (Table 2), and the elimination half-life was 6.5 ± 1.2 h. The CSF:plasma ratio was 36 ± 11%, nearly 10-fold higher than the ratio for \( O^6 \)-BG. At 48 h after \( O^6 \)-BG administration, \( O^6 \)-benzyl-8-oxoguanine still was measurable in the plasma of all animals and in the CSF of two of three animals. The plasma and CSF concentration-time profiles of \( O^6 \)-BG and \( O^6 \)-benzyl-8-oxoguanine are shown in Fig. 3.

Small amounts of \( O^6 \)-BG and \( O^6 \)-benzyl-8-oxoguanine were identified in the 8-h urine collection. Approximately 1% of the total dose of \( O^6 \)-BG was recovered in the urine as parent drug in this interval, and approximately 2% of the total dose was recovered as \( O^6 \)-benzyl-8-oxoguanine.

Baseline levels of MGMT activity in monkey peripheral blood mononuclear cells were 7.7 ± 1.9 fmol/µg DNA. Two h after a single dose of 200 mg/m² of \( O^6 \)-BG, >98% of MGMT activity was inhibited, and complete inactivation of the enzyme was observed at 18 h after the dose. Less than 3% of the baseline enzyme activity was regenerated by 48 h. At 2 weeks after \( O^6 \)-BG treatment, MGMT levels had returned to baseline (Fig. 4).

At 2 h after \( O^6 \)-BG administration, when MGMT activity was inhibited almost completely, the amount of immunoreactive protein measured by Western blot was unchanged from baseline (Fig. 5).

DISCUSSION

In primates, \( O^6 \)-BG was cleared rapidly from plasma; the half-life was 1.6 h. At a dose of 200 mg/m², the mean peak plasma concentration approached 90 µM, but by 10–12 h after administration, the plasma concentrations had fallen to approximately 1 µM.

The primary route of elimination of \( O^6 \)-BG was conversion to a metabolite that had a retention time on HPLC and a UV-visible absorption spectrum that were identical with those of an authentic standard of \( O^6 \)-benzyl-8-oxoguanine. The pharmacokinetic model, which was simultaneously fitted to the plasma concentration-time data of the parent drug and metabolite, predicted that the biotransformation of \( O^6 \)-BG to \( O^6 \)-benzyl-8-oxoguanine accounted for essentially all of the elimination of \( O^6 \)-BG from plasma. This finding is consistent with the urinary excretion of only 1% of the total dose as unchanged drug in the 8-h urine collection from a single animal. \( O^6 \)-Benzyl-8-oxoguanine was identified previously in the plasma and urine of rats treated with \( O^6 \)-BG, but the concentration of this metabolite in liver and plasma 4 h after administration was very low compared with that of \( O^6 \)-BG (12).

The rate of elimination of \( O^6 \)-benzyl-8-oxoguanine in primates was considerably slower than was that of \( O^6 \)-BG. The apparent clearance rate of the metabolite (6.4 ml/min/m²) was one tenth that of \( O^6 \)-BG (68

| Table 1 Pharmacokinetic parameters of \( O^6 \)-BG and its active metabolite, \( O^6 \)-benzyl-8-oxoguanine, in plasma |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Animal | \( k_{el} \) (h⁻¹) | \( V_d \) (liters/m²) | Clearance (ml/min/m²) | Half-life (h) | \( k_{el} \) (h⁻¹) | \( V_d \) (liters/m²) | Clearance (ml/min/m²) | Half-life (h) |
| 1 | 0.39 | 7.6 | 49 | 1.8 | 0.036 | 6.7 | 4.0 | 19 |
| 2 | 0.45 | 9.0 | 67 | 1.6 | 0.063 | 6.9 | 7.2 | 11 |
| 3 | 0.51 | 10.4 | 89 | 1.4 | 0.059 | 8.4 | 8.1 | 12 |
| Mean | 0.45 | 9.0 | 68 | 1.6 | 0.052 | 7.3 | 6.4 | 14 |
| SD | 0.06 | 1.4 | 20 | 0.2 | 0.015 | 0.9 | 2.2 | 5 |

| Table 2 Plasma and CSF exposure and CSF half-life of \( O^6 \)-BG and its active metabolite, \( O^6 \)-benzyl-8-oxoguanine |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Animal | \( AUC_{plasma} \) (µM·h) | \( AUC_{CSF} \) (µM·h) | Half-life CSF | CSF:plasma (h) | \( AUC_{plasma} \) (µM·h) | \( AUC_{CSF} \) (µM·h) | Half-life CSF | CSF:plasma (h) |
| 1 | 260 | 11.1 | 1.8 | 4.2 | 3410 | 860 | 5.8 | 25 |
| 2 | 210 | 8.1 | 2.0 | 3.8 | 2080 | 740 | 8.0 | 36 |
| 3 | 160 | 8.1 | 1.6 | 5.0 | 1780 | 860 | 5.8 | 48 |
| Mean | 210 | 9.1 | 1.8 | 4.3 | 2430 | 820 | 6.5 | 36 |
| SD | 50 | 1.8 | 0.2 | 0.6 | 870 | 70 | 1.2 | 11 |
ml/min/m²), and the half-life (14 h) was nearly 10 times longer. As a result, the total exposure to O⁶-benzyl-8-oxoguanine, as measured by the AUC, was 10-fold higher than was that of O⁶BG, and the O⁶-benzyl-8-oxoguanine concentration in plasma 48 h after the dose of O⁶BG still exceeded 10 μM (O⁶BG equivalents). Only 2% of the total O⁶BG dose was excreted in the urine as O⁶-benzyl-8-oxoguanine in the first 8 h, indicating that O⁶-benzyl-8-oxoguanine undergoes further metabolism as its primary route of elimination.

O⁶-Benzyl-8-oxoguanine is also a potent inhibitor of the target repair enzyme MGMT. In extracts from the HT29 cell line, a concentration of 0.3 μM for 30 min resulted in inactivation of 50% of the MGMT activity compared with an ED₅₀ of 0.2 μM for O⁶-BG (12). This study demonstrates that baseline MGMT activity in Rhesus monkey peripheral blood mononuclear cells is similar to that in human peripheral blood mononuclear cells (19), and that MGMT is inactivated for more than 48 h after a single 200 mg/m² dose of O⁶BG. Although the prolonged duration of MGMT inhibition may be accounted for, in part, by the time required for regeneration of the enzyme, it is likely that continued suppression by the persistent, high concentrations of O⁶-benzyl-8-oxoguanine played a major role. Furthermore, because Western blotting demonstrated the presence of MGMT protein at the 2-h time point (when enzyme activity was nearly absent), Western blotting cannot be used to distinguish between active and O⁶BG or metabolite-inactivated MGMT.

O⁶BG enhances the activity of alkylating agents that target the O⁶-position on guanosine as a preferred site of alkylation, such as the nitrosoureas and temozolomide. Because these alkylating agents are used primarily in the treatment of brain tumors, the ability of O⁶BG to cross the blood-brain barrier becomes a critical determinant of its ability to enhance the antitumor effects of these alkylating agents. The penetration of O⁶BG into CSF in this primate model, which has been highly predictive of the penetration of a wide range of drug in humans, was limited (4%). The mean CSF O⁶BG concentrations peaked at approximately 2.5 μM and fell below 1 μM within 4 h. In the plasma protein binding studies, O⁶BG was bound highly in both human and nonhuman primate plasma, and protein binding is an important determinant of the degree of CSF penetration (20). Although the CSF penetration of total drug was low, it appears likely that unbound drug diffuses freely into the CSF.

The O⁶-benzyl-8-oxoguanine penetrated into CSF to a much greater extent than did O⁶BG. The CSF:plasma ratio of the metabolite (37%) was approximately 8-fold higher than was that of O⁶BG, and the overall exposure to O⁶-benzyl-8-oxoguanine in CSF (AUCₜₚₛ) was 90-fold higher than was that of O⁶BG. At 24 h, the CSF O⁶-benzyl-8-oxoguanine concentration still exceeded 10 μM. The high degree of CSF penetration of the active metabolite suggests that O⁶BG would be potentially useful in enhancing the antitumor effects of the nitrosoureas in patients with brain tumors.

In summary, although O⁶BG was cleared rapidly from plasma and CSF, prolonged exposure to high concentrations of an active metabolite, O⁶-benzyl-8-oxoguanine, was achieved in plasma and CSF and provided a pharmacological explanation for the sustained suppression of MGMT activity in peripheral blood mononuclear cells after a single dose of 200 mg/m² of O⁶BG. These findings have important implications for the use of O⁶BG in clinical trials.

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Plasma and Cerebrospinal Fluid Pharmacokinetics of $O^6$-Benzylguanine and Time Course of Peripheral Blood Mononuclear Cell $O^6$-Methylguanine-DNA Methyltransferase Inhibition in the Nonhuman Primate


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