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HUMAN LIVER OXIDATIVE METABOLISM OF O^6-BENZYLGUANINE

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Abstract—The oxidation of O^6-benzylguanine, an inactivator of O^6-alkylguanine-DNA alkyltransferase, was examined using human liver cytosol, microsomes, and several P450 isoforms. Incubation of O^6-benzylguanine with human liver cytosol resulted in the formation of O^6-benzyl-8-oxoguanine, which was inhibited by menadione, a potent inhibitor of aldehyde oxidase. Inhibition by allopurinol, a xanthine oxidase inhibitor, was less dramatic. Oxidation of O^6-benzylguanine also occurred with pooled human liver microsomes and was inhibited by both furafylline and troleandomycin, selective inhibitors of CYP1A2 and CYP3A4, respectively. Human P450s CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4 expressed in Hep G2 hepatoma cells using vaccinia virus vectors were incubated with 10 or 200 μM O^6-benzylguanine. At 10 μM, O^6-benzylguanine was oxidized primarily by CYP1A2 and to a lesser extent by CYP3A4. However, an appreciable increase in CYP3A4 contribution was noted at 200 μM. CYP1A2 exhibited a more than 200-fold higher relative catalytic activity (V_{max}/K_m) compared with CYP3A4. Therefore, at therapeutically relevant concentrations of O^6-benzylguanine, CYP1A2 could be primarily involved in its oxidation since it shows a much lower K_m value (1.3 μM) than CYP3A4 (52.2 μM) and cytosol (81.5 μM). However, one would expect interindividual variation in the extent of oxidation of O^6-benzylguanine depending on the levels of aldehyde oxidase, CYP1A2, and CYP3A4.

Key words: O^6-benzylguanine; O^6-benzyl-8-oxoguanine; cytochrome P450; alkyltransferase; drug metabolism; aldehyde oxidase

O^6-Benzylguanine is presently one of the most effective inactivators of the DNA repair protein, AGT‖ [1]. Inactivation of this protein leads to an enhancement in the cytotoxic effect of chloroethylnitrosoureas (e.g. BCNU) and methylating agents (e.g. DTIC) [2]. More importantly, O^6-benzylguanine pretreatment results in a significant growth inhibition and an increase in the therapeutic index of BCNU in human brain [3–5] and colon tumor xenograft studies [6, 7]. Phase I human clinical trials of O^6-benzylguanine combined with BCNU have been initiated recently.

The metabolic fate of O^6-benzylguanine was examined in both rats and mice [8]. A major urinary and plasma-associated metabolite in both rats and mice was identified as O^6-benzyl-8-oxoguanine, which was found to be only slightly less potent as an inactivator of the alkyltransferase protein than O^6-benzylguanine [8]. The plasma half-life of O^6-benzyl-8-oxoguanine formed after O^6-benzylguanine administration was 3.3-fold longer than that of O^6-benzylguanine in rats¶ and 8.5-fold longer in nonhuman primates** [9].

The aim of the present study was to identify human enzymes that may be responsible for the conversion of O^6-benzylguanine to O^6-benzyl-8-oxoguanine. We investigated the oxidation of O^6-benzylguanine using human liver cytosol and microsomes as well as Hep G2 hepatoma cells expressing high levels of various human P450 isoforms. These studies have led to the identification of the cytosolic enzymes and P450s most likely to be involved in human metabolism of O^6-benzylguanine and provide information on the factors that may contribute to variations in O^6-benzylguanine metabolism among individuals.

MATERIALS AND METHODS

Chemicals

O^6-Benzylguanine and O^6-benzyl-8-oxoguanine were synthesized as described previously [1, 8]. Menadione, allopurinol, troleandomycin, sulfaphenazole, quinidine, diethyldithiocarbamate, coumarin, NADP, glucose-6-phosphate dehydrogenase, and buffers were purchased from the Sigma Chemical Co., St. Louis, MO. Furafylline was a gift from Dr. Kent Kunze (University of Washington, Seattle, WA). Pooled human liver microsomes and cytosols were obtained from Human Biologics, Inc., Phoenix, AZ.

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‖ Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; P450, cytochrome P450; O^6-benzyl-8-oxoguanine, O^6-benzyl-7,8-dihydro-8-oxoguanine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; and DTIC, 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide.

¶ Roy SK, Gupta E and Dolan ME, *Drug Metab Dispos*, in press.

** Berg SL, personal communication. Cited with permission.

Cytosolic oxidation of O^6-benzylguanine

O^6-Benzylguanine (100 μM) was incubated with 2.7 mg cytosolic protein at 37° in a buffer consisting of 100 mM potassium phosphate, pH 7.4, for 30 min. For inhibition studies, DMSO (final concentration < 0.2%, v/v), 200 μM menadione, a well-documented inhibitor of aldehyde oxidase [10], or 200 μM allopurinol, a xanthine oxidase inhibitor [10], was preincubated for 5 min prior to and for 0, 3, 10, 20, 30, and 60 min after the addition of O^6-benzylguanine to cytosolic extracts. Inhibitors were dissolved in DMSO. In a separate experiment to determine kinetic parameters of cytosol-mediated O^6-benzylguanine oxidation, 6.0 mg/mL cytosol was incubated with substrate (0.25 to 200 μM) for 20 min at 37°. All reactions (final volume = 500 μL) were terminated by the addition of 1.0 mL chilled methanol (100%). Samples were centrifuged, and the supernatant was lyophilized and reconstituted in mobile phase for HPLC injection (see below).

Microsomal oxidation of O^6-benzylguanine

Oxidation rates of O^6-benzylguanine in the presence or absence of inhibitor were compared using human liver microsomal protein. Inhibition of O^6-benzyl-8-oxoguanine formation by different isoform-selective P450 inhibitors, viz. troleandomycin (100 μM), furafylline (20 μM), coumarin (50 μM), sulfaphenazole (0.5 μM), diethyldithiocarbamate (100 μM), and quinidine (5 μM), was determined. Reaction mixtures containing sulfaphenazole and quinidine were preincubated at 37° for 5 min without the NADPH-generating system, and the oxidation reaction was initiated by simultaneous addition of the NADPH-generating system and O^6-benzylguanine. Reaction mixtures containing furafylline, diethyldithiocarbamate, and troleandomycin were preincubated at 37° for 10 min with the NADPH-generating system, and the reactions were initiated by adding O^6-benzylguanine. The oxidation rates of 10 and 200 μM O^6-benzylguanine in the presence or absence of inhibitors were compared using 1 and 0.1 mg human liver microsomal protein, respectively (final volume = 500 μL). Pooled liver microsomes used for experiments with 10 and 200 μM O^6-benzylguanine were from different sources. The reactions were terminated after 30 min as described above, and the oxidation rate was determined using the HPLC assay procedure described below. Results are expressed as the percentage of the rate of oxidation without addition of inhibitor.

Oxidation of O^6-benzylguanine by cDNA-expressed human P450s

The CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4 P450s were expressed using vaccinia virus vectors in human Hep G2 hepatoma cell lines as described previously [11]. The incubation mixtures included Hep G2 membrane fractions and 10 or 200 μM O^6-benzylguanine in 1 mL of 50 mM potassium phosphate buffer (pH 7.2). Cytochrome P450 contents (CYP1A2: 99 pmol, CYP2B6: 55 pmol, CYP2C8: 77 pmol, CYP2C9: 103 pmol, CYP2E1: 237 pmol, CYP3A4: 155 pmol) were determined by the method of Omura and Sato [12]. Substrate was added in 10 μL methanol, except for experiments with CYP2E1. Because methanol is an inhibitor of CYP2E1, it was removed by evaporation under nitrogen before subsequent addition of the CYP2E1 suspension to dissolve the substrate. To determine kinetic parameters for O^6-benzylguanine oxidation, 25 pmol CYP1A2 was incubated with substrate (0.25 to 10 μM) for 10 min or 96 pmol CYP3A4 was incubated with substrate (30 to 500 μM) for 30 min. All reactions were stopped by the addition of 2 mL of ice-cold methanol after 30-min incubations. Samples were centrifuged to remove the protein, and supernatants were lyophilized and reconstituted in the mobile phase for HPLC injection.

HPLC analysis

Samples resuspended in the mobile phase were injected onto a 25 cm × 4.6 mm Beckman ODS 5 μm C18 column (Beckman Instruments, Inc., San Ramon, CA) fitted with a Rainin ODS-GU 5 μm cartridge (Rainin Instrument Co., Woburn, MA). The column was eluted at a flow rate of 1 mL/min at room temperature using a mobile phase consisting of 43% methanol in 50 mM ammonium formate, pH 4.5, over 30 min. Column eluent was monitored continuously at 280 nm, and the identity of the reaction products was confirmed by comparison of their retention times and UV spectra with those of authentic standards using a Hitachi diode array detector (Hitachi Instruments, San Jose, CA). The elution time for O^6-benzyl-8-oxoguanine and O^6-benzylguanine was 16.6 and 18.3 min, respectively.

Data analysis

The kinetic constants and their standard errors were determined using the least squares regression analyses described by Cleland [13].

RESULTS

The amount of O^6-benzylguanine oxidized to O^6-benzyl-8-oxoguanine by human liver cytosol in 30 min was 10 nmol/mg protein. Figure 1 illustrates the effects of menadione and allopurinol on the formation of O^6-benzyl-8-oxoguanine catalyzed by human liver cytosol. Menadione, a potent aldehyde oxidase inhibitor, almost completely (90%) inhibited the reaction up to 60 min. In contrast, allopurinol, a xanthine oxidase inhibitor, reduced the formation of product at 30 min by only 24%. Kinetic constants, K_m and V_{max} values for cytosolic oxidation of O^6-benzylguanine, were determined from an Eadie–Hofstee plot to be 81.5 μM and 0.70 nmol/min/mg protein, respectively (Fig. 2). The values obtained for oxidation rates at substrate concentrations below 5 μM were lower than expected, which was probably due to nonspecific protein binding, and thus were not used in the analysis.

The amount of O^6-benzyl-8-oxoguanine formed by human liver microsomes in 30 min was 12 nmol/mg protein. Chemical inhibitors selective for particular P450s were used to probe their involvement in O^6-benzylguanine oxidation (Fig. 3). The results of previous *in vivo* and *in vitro* studies have established that furafylline, a methyl xanthine derivative, is a very potent and specific inhibitor of CYP1A2-catalyzed reactions [14, 15]. We therefore used furafylline to investigate the role of CYP1A2 in the oxidation of O^6-benzylguanine (10 μM) in pooled human liver microsomes. In the presence of 20 μM furafylline, the rate of oxidation decreased by 60%. However, at higher concentrations (200 μM), we observed 34% inhibition by furafylline and 46% inhibition by troleandomycin, a selective CYP3A inhibitor [16].

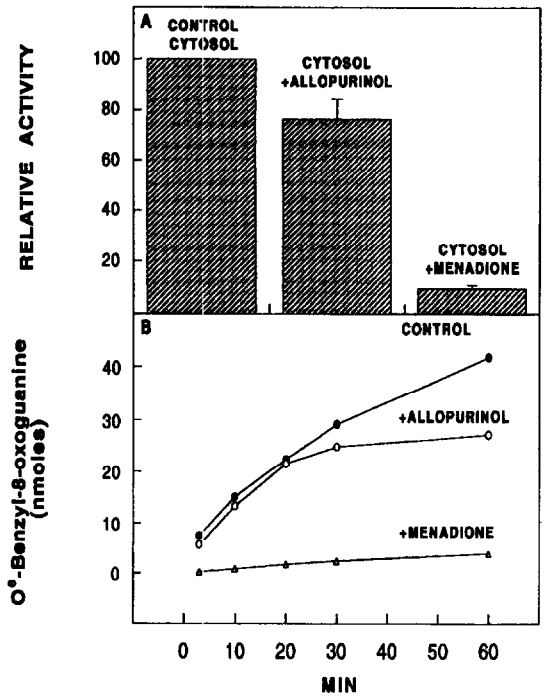

Fig. 1. Effect of allopurinol or menadione on the cytosolic conversion of O^6-benzylguanine to O^6-benzyl-8-oxoguanine. (A) Allopurinol (200 μM) and menadione (200 μM) were added 5 min prior to the addition of O^6-benzylguanine to cytosolic fractions. One hundred percent activity for oxidation of O^6-benzylguanine corresponds to the production of 10 ± 7 nmol/mg protein/30 min. The values represent means ± SD for three different human livers. (B) The time course for O^6-benzyl-8-oxoguanine formation in the presence and absence of inhibitors is representative of three pooled human liver cytosols. Incubation conditions are described in Materials and Methods.

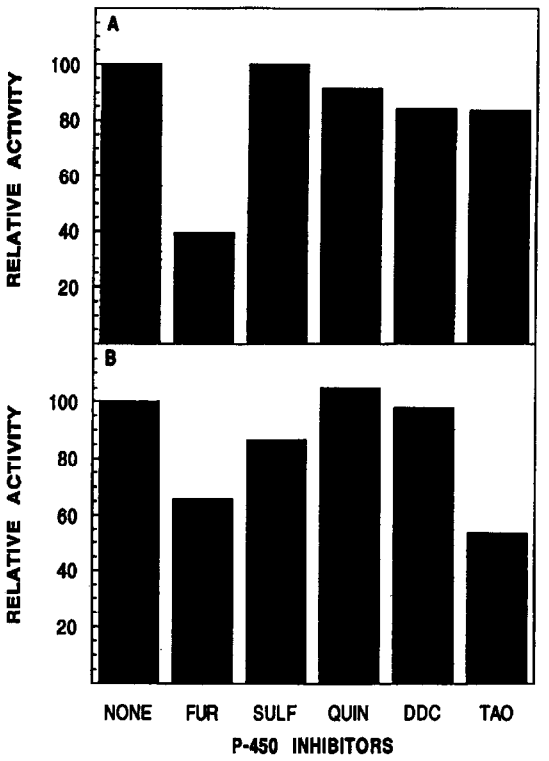

Fig. 3. Effect of P450 inhibitors on O^6-benzylguanine oxidation. Concentrations of (A) 10 μM and (B) 200 μM O^6-benzylguanine were incubated with microsomal protein in the presence or absence of inhibitors. Inhibitor concentrations and incubation conditions are described in Materials and Methods. One hundred percent activity for oxidation for O^6-benzylguanine corresponds to the production of 0.7 and 12 nmol/mg protein/30 min in the presence of 10 and 200 μM O^6-benzylguanine, respectively. Each data point is the mean of duplicate determinations. Abbreviations: FUR, furafylline; TAO, troleandomycin; SULF, sulfaphenazole; QUIN, quinidine; and DDC, diethyldithiocarbamate.

This indicates a predominance of CYP1A2-catalyzed oxidation at lower concentrations of O^6-benzylguanine and a contribution of CYP3A at higher concentrations. Other inhibitors selective for different isozymes were also tested. These included coumarin selective for CYP2A6, sulfaphenazole (CYP2C9/10), quinidine (CYP2D6) and diethyldithiocarbamate (CYP2E1). None of these selective inhibitors appreciably diminished the oxidation of O^6-benzylguanine. These results suggest that only CYP1A2 and CYP3A4 make significant contributions to the oxidation of O^6-benzylguanine.

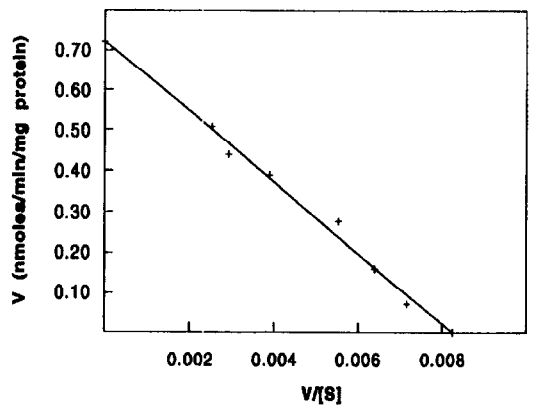

Fig. 2. Eadie–Hofstee plot for pooled human liver cytosol-mediated oxidation of O^6-benzylguanine. Substrate concentrations and incubation conditions are described in Materials and Methods. The K_m and V_{max} were determined as 81.5 ± 3.6 μM and 0.70 ± 0.01 nmol/min/mg protein (mean ± SEM, N = 8) using the least squares regression analysis described by Cleland [13].

Microsomes prepared from Hep G2 hepatoma cells expressing high levels of CYP2B6, CYP2C8, CYP2C9, CYP2E1, CYP3A4, and CYP1A2 were incubated with 10 μM (Fig. 4A) or 200 μM (Fig. 4B) O^6-benzylguanine. The amount of O^6-benzyl-8-oxoguanine formed within 30 min is shown for each P450 in Fig. 4. CYP1A2 microsomes oxidized O^6-benzylguanine to O^6-benzyl-8-oxoguanine to a greater extent than any other form at both the concentrations tested. The amount of product produced by CYP1A2 increased 1.6-fold when the substrate concentration was increased 20-fold. In contrast, when CYP3A4 microsomes were used, a 5.5-fold increase in the rate of product formation was observed at the higher substrate concentration.

To further assess the roles of CYP1A2 and CYP3A4 in O^6-benzylguanine oxidation, kinetic constants were determined for each P450 isoform. The K_m and V_{max} for the CYP1A2 or CYP3A4 oxidation of O^6-benzylguanine were determined from the Eadie–Hofstee plots. The K_m

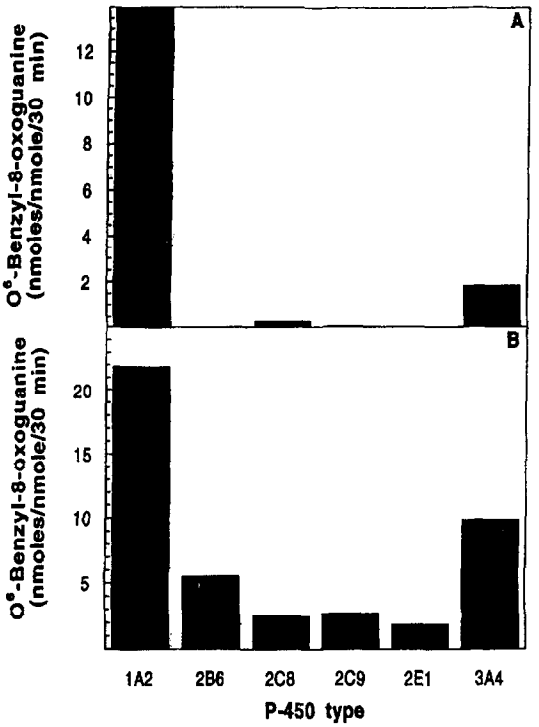

Fig. 4. Effect of P450 isoforms on the formation of O^6-benzyl-8-oxoguanine. CYP isoforms listed were incubated with (A) 10 μM and (B) 200 μM O^6-benzylguanine for 30 min at 37°. The amount of oxidized product was determined by HPLC analysis. Incubation conditions are described in Materials and Methods.

and V_{max} for CYP1A2 were 1.3 μM and 1.7 min^{-1}, respectively (Table 1). The K_m and V_{max} for CYP3A4 were 52.2 μM and 0.3 min^{-1}, respectively (Table 1). Thus, the relative catalytic activity (V_{max}/K_m) was more than 200-fold higher for CYP1A2 compared with CYP3A4.

DISCUSSION

Our studies indicate that the cytosolic enzymes, aldehyde oxidase and, to a lesser extent, xanthine oxidase, together with the microsomal enzymes CYP1A2 and CYP3A4, are principally responsible for the oxidation of O^6-benzylguanine to O^6-benzyl-8-oxoguanine in human liver. We observed inhibition of O^6-benzyl-8-oxoguanine formation upon addition of menadione (an inhibitor of aldehyde oxidase) and, to a lesser extent, allopurinol (an inhibitor of xanthine oxidase) to cytosolic preparations. The addition of furafylline (an inhibitor of CYP1A2) and troleandomycin (an inhibitor of CYP3A) to microsomal preparations containing O^6-benzylguanine also produced inhibition. Cytochrome P450s and aldehyde oxidase are coordinately involved in the oxidation of a number of agents [17, 18].

CYP1A2 oxidized O^6-benzylguanine more efficiently than CYP3A4. The former exhibited a more than 200-fold higher relative catalytic activity. However, the rate of oxidation of O^6-benzylguanine at the higher concentration (200 μM) by pooled human liver microsomes was inhibited to a greater extent (46%) in the presence of troleandomycin, a CYP3A inhibitor, than was observed in the presence of furafylline (34%), a CYP1A2 inhibitor. This can be explained by the high O^6-benzylguanine concentration and the predominance of CYP3A in the pooled microsomes. CYP3As are usually expressed at a higher level in human liver than CYP1A2 (28.8 ± 10.4 compared with 12.7 ± 6.7% of total human hepatic P450) [19]. However, a wide range of expression of CYP3A4 is possible in individual liver samples [20, 21]. Thus, CYP3A4 is expected to predominate in the metabolism of O^6-benzylguanine at higher cellular drug concentrations and when it is expressed at high levels. Glucocorticoids, macrolide antibiotics, or phenobarbital can elevate CYP3A4 levels [16, 22].

CYP1A2 is the predominant microsomal enzyme responsible for oxidation of O^6-benzylguanine at low concentrations. However, studies have shown that levels of CYP1A2 also vary significantly among samples as a result of both genetic and environmental factors including cigarette smoking [23–25]. Thus, there is a strong possibility that humans will vary considerably in their ability to oxidize O^6-benzylguanine.

O^6-Benzyl-8-oxoguanine is a very effective AGT inactivator and exhibited an EC_{50} in HT29 cell extracts of 0.3 μM compared with 0.2 μM for O^6-benzylguanine [8]. Since the plasma half-life of the metabolite is significantly longer than the half-life of parent drug in rats* and non-human primates† [9], it may be that the oxidized derivative would be the agent primarily responsible for AGT inactivation in certain species including humans. Therefore, the degree to which this product is formed in individuals may affect the extent and duration of AGT depletion. Interindividual differences in the ability to oxidize O^6-benzylguanine will depend primarily on the respective activities of their aldehyde oxidase, CYP1A2 and CYP3A4 liver enzymes.

Table 1. Kinetic parameters for the oxidation of O^6-benzylguanine by CYP1A2 and CYP3A4

Isoform	K_m (μM)	V_{max} (min^{-1})	V_{max}/K_m
CYP1A2	1.3 ± 0.08	1.7 ± 0.04	1.3
CYP3A4	52.2 ± 7.14	0.3 ± 0.01	0.0057

The kinetic constants and their standard errors (CYP1A2, n = 8; CYP3A4, n = 5) were determined using the least squares regression analyses described by Cleland [13].

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