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Debenzylation of O^6-benzyl-8-oxoguanine in human liver: implications for O^6-benzylguanine metabolism

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Abstract

O^6-Benzylguanine (BG) effectively inactivates the DNA repair protein O^6-alkylguanine-DNA alkyltransferase, and enhances the effectiveness of 1,3-bis(2-chloroethyl)-1-nitrosourea in cells in culture and tumor-bearing animals. BG is presently in phase II clinical trials. In humans, BG is converted to O^6-benzyl-8-oxoguanine (8-oxoBG), a longer-lived, yet equally potent inactivator. We have isolated and identified the debenzylated product, 8-oxoguanine, in plasma and urine of patients following administration of BG. The purpose of this work was to determine the human liver enzymes responsible for the debenzylation of 8-oxoBG. Therefore, 8-oxoBG was incubated with human liver microsomes and cytosol, and the concentration of 8-oxoguanine was determined. No appreciable product was formed in the cytosol; however, increasing amounts of 8-oxoguanine were formed with increasing concentrations of pooled human liver microsomes. The amount of 8-oxoguanine formed increased with time and substrate concentration. Co-incubation of human liver microsomes with 8-oxoBG and various cytochrome P450 isoform-selective inhibitors suggested the possible involvement of CYP1A2, 2E1, and/or 2A6 in this reaction. Incubation of 8-oxoBG with baculovirus cDNA-overexpressed CYP1A2, 2E1, 2A6, and 3A4 demonstrated that formation of 8-oxoguanine was due mainly to CYP1A2. Debenzylation of 8-oxoBG complied with Michaelis–Menten kinetics with K_m and V_{max} values of 35.9 μM and 0.59 pmol/min/pmol of CYP1A2, respectively. CYP1A2 appears to be mainly responsible for the debenzylation of 8-oxoBG in human liver.

Keywords: O^6-Benzyl-8-oxoguanine; O^6-Benzylguanine; 8-Oxoguanine; Metabolism; Alkyltransferase; CYP1A2

1. Introduction

Alkylnitrosourea effectiveness in chemotherapy is often compromised by resistance mechanisms present in tumor cells. The repair of alkylnitrosourea-induced DNA damage by AGT is clearly one of the most important mechanisms of nitrosourea resistance [1]. The mechanism of resistance by AGT involves the removal of lesions from the O^6-position of guanine by a cysteine residue within the active site of the protein [1]. Upon repair, the protein is inactivated, and guanine is restored in DNA. O^6-Chloroethylguanine, produced by chloroethylnitrosoureas, undergoes a slow intramolecular cyclization to produce an O^6,1-ethanoguanine intermediate that eventually reacts with the cytosine on the complementary strand of DNA to form an interstrand cross-link [2,3]. AGT can react with both O^6-chloroethylguanine and the ethanoguanine intermediate [4–6]. There is an inverse correlation between the level of this protein and the sensitivity of tumor cells grown in culture and as xenografts to the cytotoxic effect of the alkylnitrosoureas [1].

BG has been developed as a low molecular weight substrate for AGT that benzylates the cysteine residue in the active site of the protein [7,8]. Inactivation of the alkyltransferase protein by non-toxic doses of BG renders a variety of human tumor cell lines more sensitive to the cytotoxic effects of alkylating agents including BCNU, CCNU, chlorozotocin, clomesone, streptozotocin, and temozolomide [1,9,10]. BG in combination with BCNU just completed

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Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BG, O^6-benzylguanine; 8-oxoBG, O^6-benzyl-8-oxoguanine; 8OH2dG, 8-hydroxy-2′-deoxyguanosine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; and CYP or P450, cytochrome P450.

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phase I clinical trials [11–13], and a series of phase II studies have been initiated.

BG is oxidized to an equally potent derivative, 8-oxoBG, in rats [14], mice [14], non-human primates [15], and humans [11]. Debenzylation of BG and 8-oxoBG has been demonstrated in rats after administration of 8-[^{3}H]-BG [14]. We have identified 8-oxoguanine as a metabolite of BG in human plasma and urine [16,17]. However, there have been no reports to date with respect to the human enzymes involved. Only 3.4% of BG is accounted for in human urine as unchanged BG or 8-oxoBG [11], implying that there is extensive further metabolism of BG and/or 8-oxoBG. The aim of this work was to establish the hepatic enzyme(s) involved in the debenzylation of 8-oxoBG. In this report, we demonstrate that 8-oxoguanine most likely forms in liver from CYP1A2-mediated debenzylation of 8-oxoBG.

2. Materials and methods

2.1. Chemicals

Sodium acetate, glacial acetic acid, HPLC grade acetonitrile, and methanol were purchased from Fisher Scientific. Double-distilled deionized water was obtained by passing water through a Milli-Q reagent water system (Millipore). The following compounds were purchased from Sigma-Aldrich: 8-oxoguanine, 8OH2dG, 7,8-benzoflavone, 8-methoxypsoralen, 1,2-*trans*-dichloroethylene, diethyldithiocarbamate, β-phenylethyl isothiocyanate, aminobenzotriazole, quercetin, sulfaphenazole, troleandomycin, quinidine, coumarin, and allopurinol. Furafylline was purchased from Gentest. BG and 8-oxoBG were synthesized as described [7,14].

2.2. Preparation of subcellular fraction

Portions of human livers were obtained from the National Disease Research Interchange or the Cooperative Human Tissue Network Midwestern Division and kept frozen at −70° until used. Livers were homogenized in 0.1 M sodium phosphate buffer containing of 0.15 M potassium chloride and 1 mM EDTA (6.5 mL buffer/g liver), using a Potter–Elvehjem homogenizer and a Teflon pestle (Biospec Products Inc.). Microsomes and cytosol were prepared by standard methods using differential centrifugation [18]. The resulting pellets were resuspended in 0.1 M sodium phosphate buffer containing 5 mM $MgCl_2$ and 1 mM EDTA, followed by centrifugation at 105,000 *g* for 1 hr. The supernatant was stored as cytosolic fraction. The microsomal pellets were resuspended in the same buffer and stored at −70° until used. Protein concentration was determined by the method of Bradford [19].

2.3. 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 $MgCl_2$, and 1 mM NADPH at 37°. To define optimal conditions for incubation, 8-oxoBG (0–100 μM) was incubated with pooled human liver microsomes (0–3 mg/mL) for various time periods up to 4 hr. An incubation time of 30 min with human microsomal protein (2 mg/mL) and 8-oxoBG (50 μM) exhibited linear conditions and, therefore, was used in the subsequent experiments unless stated otherwise. Following incubation, the reactions were terminated by the addition of 2 mL of chilled methanol. Aliquots of 10 μL of 0.25 mM 8OH2dG (internal standard, made up in 0.1 M H_3PO_4) were added to the reaction vials. The mixture was vortexed for 15 sec and centrifuged at 10,500 *g* for 25 min to precipitate proteins. The supernatant was dried under a stream of nitrogen gas. The resulting residues were reconstituted in 200 μL of a solution containing 2% acetonitrile/0.1 M sodium acetate buffer (pH 5.2). The reconstituted samples were centrifuged again for 10 min at 20,800 *g*. The supernatants were taken, and 150-μL aliquots were injected onto the HPLC system.

2.4. Quantitation of 8-oxoguanine

An HPLC method with electrochemical detection (HPLC-ED) was utilized to quantitate 8-oxoguanine in human plasma and urine as described previously [16]. Briefly, the chromatographic separation was achieved on a TosoHaas 5 μm, ODS-80T$_M$ column (TosoHaas). Mobile phase A consisted of 0.1 M sodium acetate adjusted to pH 5.2 with glacial acetic acid; mobile phase B consisted of 100% acetonitrile (HPLC grade). The pump was set to deliver 2% of mobile phase B and 98% of mobile phase A at a flow rate of 1.0 mL/min for 10 min, then to increase linearly up to 50% B in 10 min. This condition was sustained for 5 min to allow washing out of additional peaks. CoulArray potentials were set as follows: channel 1 at +280 mV, channel 2 at +300 mV, channel 3 at +320 mV for detection of 8-oxoguanine and 8-OH2dG, and channel 4 at +400 mV for 8-oxoBG.

2.5. Chemical inhibition reactions

The concentrations of human P450 isoform-selective inhibitors and the conditions of reactions used to maximize selectivity were based on literature values [20–25]. The amount of 8-oxoguanine formed upon incubation of 50 μM 8-oxoBG in 50 mM potassium phosphate buffer (pH 7.4) with 2 mg/mL of human liver microsomes was evaluated in the absence (control) and the presence of various isoform-specific inhibitors used at a concentration of 100 μM. Inhibitors included: 7,8-benzoflavone for CYP1A2; furafylline for CYP1A2; 8-methoxypsoralen for CYP2A6, 1A2, and 3A4/5; 1,2-*trans*-dichloroethylene for CYP2E1, 1A2, and 2C6; diethyldithiocarbamate for CYP2A6 and 2E1; β-phenylethyl isothiocyanate for CYP1A2 and 2E1; aminobenzotriazole for all the P450 family; quercetin for

CYP3A4/5, 2D6, 2C8/9, 2B6, and 1A2; sulfaphenazole for CYP2C9; troleandomycin for CYP3A4/5; quinidine for CYP2D6; coumarin for CYP2A6; and allopurinol for xanthine oxidase. For inhibitors of CYP2A6, such as 8-methoxypsoralen and diethyldithiocarbamate, phosphate buffer was replaced with 50 mM Tris (pH 7.4). All solid chemical inhibitors were dissolved in either acetonitrile or ethanol according to known solubility of compounds, taking into account the sensitivity of isozyme to particular solvents [22]. Samples were dried under nitrogen before adding pooled human liver microsomes and NADPH to initiate the reaction. Reaction mixtures were incubated for 30 min at 37° in a final incubation volume of 1 mL.

2.6. cDNA-overexpressed human P450 isoforms

Human P450 isoforms overexpressed from cDNA cloned into a baculovirus expression system (Gentest) were incubated with 8-oxoBG (50 μM). For each reaction, 50 pmol of CYP1A2, 2E1, 2A6, and 3A4 were used, and all other conditions of incubation remained the same as those described above for experiments with human liver microsomes.

2.7. Kinetic analysis

Kinetic studies to evaluate K_m and V_{max} were performed using CYP1A2, which is expressed from human CYP1A2 cDNA using a baculovirus expression system. cDNA-expressed CYP1A2 was first incubated with 50 μM 8-oxoBG for 30 min; 8-oxoguanine formation was observed to be linear in CYP1A2 concentrations of 10–50 pmol. In determining kinetic parameters, the 8-oxoBG concentrations of 5, 12.5, 25, 50, 75, 100-ISO and 200 μM, respectively, were incubated for 0, 5, 15, 30, and 60 min with 30 pmol of cDNA-expressed CYP1A2. Linear regression lines were plotted for each substrate concentration. The slope of each plot was used as the rate of reaction and applied in the program GraFit (*GraFit version 4.0*, Erithacus Software Ltd.) to estimate apparent kinetic parameters according to Michaelis–Menten-type enzyme kinetics.

3. Results

3.1. Reaction of 8-oxoBG

Figure 1 illustrates the metabolic conversion of BG to 8-oxoBG and further conversion to 8-oxoguanine. In a phase I clinical trial [17], we observed that 21.1 ± 10.7% of BG administered is excreted as 8-oxoguanine (N = 8, range from 4.2 to 40.8%). The identity of 8-oxoguanine was confirmed by comparison of its chromatographic and spectroscopic properties with those of the authentic standard. Upon incubation of pooled human liver microsomes (2 mg protein/mL) with 8-oxoBG (50 μM) and NADPH, the 8-oxoguanine concentration increased over a 2-hr period (Fig. 2A). Similarly, as a function of human liver microsomal concentration, 8-oxoguanine concentration increased over 30 min of incubation (Fig. 2B). Additionally, there was an increase in 8-oxoguanine concentration, at a microsomal protein concentration of 2 mg/mL, with increasing substrate concentration over a 30-min period (Fig. 2C). There was little 8-oxoguanine formed upon incubation of 2 mg/mL of cytosolic protein with 8-oxoBG for up to 4 hr (Fig. 2A). Using the HPLC conditions described in this paper, no other metabolites were observed upon incubation of 8-oxoBG with human liver microsomes or CYP1A2.

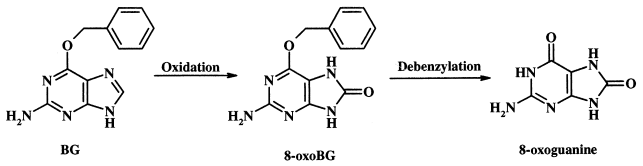

Fig. 1. Oxidation of BG and debenzylation of 8-oxoBG to 8-oxoguanine.

3.2. Chemical inhibition analysis

To identify the P450 isoform responsible for debenzylation, 8-oxoBG was incubated with human liver microsomes in the presence of P450 isoform-specific inhibitors (Fig. 3). Among the chemical inhibitors tested, a non-specific microsomal suicide inhibitor, 1-aminobenzotriazole [23], resulted in 54% inhibition of 8-oxoguanine formation compared with incubations in the absence of inhibitor. The amount of 8-oxoguanine formed was inhibited to the greatest extent by inhibitors of CYP1A2, 2A6, and 2E1. In the presence of furafylline, a mechanism-based CYP1A2 inhibitor [26], 8-oxoguanine formation was inhibited by 74%. 7,8-Benzoflavone, a competitive CYP1A2 inhibitor [26], inhibited the formation of product by 76%. Other selective CYP2E1 and/or CYP2A6 and/or CYP1A2 inhibitors, such as diethyldithiocarbamate [22], β-phenylethyl isothiocyanate [25, 27], and 8-methoxypsoralen [21,22] showed moderate inhibitory activity of 41, 48, and 68%, respectively. Overall, the information obtained from the chemical inhibition experiments suggested that CYP1A2, 2A6, and/or 2E1 are k;4most likely involved in the debenzylation reaction of 8-oxoBG.

3.3. cDNA-overexpressed human P450 isoforms

The extent of 8-oxoBG debenzylation was measured using selected cDNA-expressed P450 isoforms (Fig. 4). At increasing substrate concentrations of 10–60 μM, the concentration of 8-oxoguanine increased from 0.2 to 1.14 μM in the presence of CYP1A2. In contrast, the concentration of 8-oxoguanine in the presence of 50 μM 8-oxoBG and 50 pmol CYP2E1, 2A6, and 3A4 was 0.14, 0.17, and 0.09 μM,

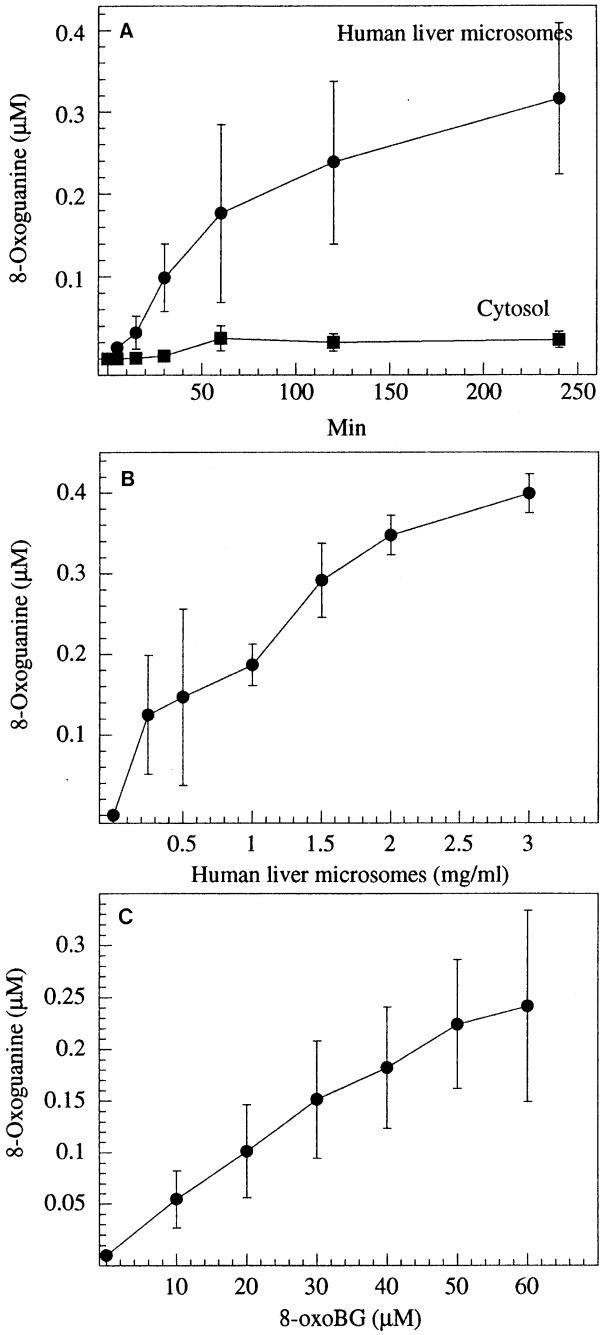

Fig. 2. Formation of 8-oxoguanine following incubation of 8-oxoBG with pooled human liver microsomes or cytosol. The concentration of 8-oxoguanine formed in the reaction mixture was measured by HPLC-ED. (A) 8-oxoBG (50 μM) was incubated with 2 mg/mL of pooled human liver microsomes (●) or cytosol (■) for 0–4 hr. (B) 8-oxoBG (50 μM) was incubated for 30 min with increasing concentrations of human liver microsomes. (C) Increasing concentrations of 8-oxoBG were incubated for 30 min with pooled human liver microsomes (2 mg/mL). Each value represents the mean ± SD of four separate incubations.

respectively. The rate of 8-oxoguanine formed in the presence of CYP1A2 was measured at 8-oxoBG concentrations between 5 and 200 μM. The K_m for the reaction was 35.9 ± 12.8 μM and the V_{max} was 0.59 ± 0.19 pmol/min/pmol of

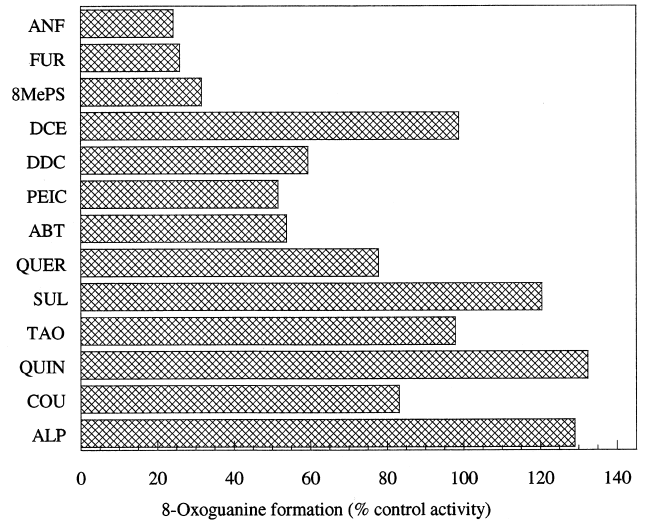

Fig. 3. Effect of CYP450 isoform-selective inhibitors on 8-oxoBG debenzylation by pooled human liver microsomes. Data are expressed as a percentage of 8-oxoguanine formation compared with control incubation (no inhibitor). 8-oxoBG (50 μM) was incubated with human liver microsomes (2 mg/mL) for 30 min, and the amount of 8-oxoguanine formed was measured. The amount of formation of 8-oxoguanine in the control sample was 0.26 μM. Each bar represents the average of two experiments. Abbreviations: ANF, 7,8-benzoflavone; FUR, furafylline; 8MePS, 8-methoxypsoralen; DCE, 1,2-*trans*-dichloroethylene; DDC, diethyldithiocarbamate; PEIC, β-phenylethyl isothiocyanate; ABT, aminobenzotriazole; QUER, quercetin; SUL, sulfaphenazole; TAO, troleandomycin; QUIN, quinidine, COU, coumarin; and ALP, allopurinol.

CYP1A2 (N = 4). The catalytic activity (V_{max}/K_m) was 0.016 for CYP1A2-catalyzed debenzylation of 8-oxoBG.

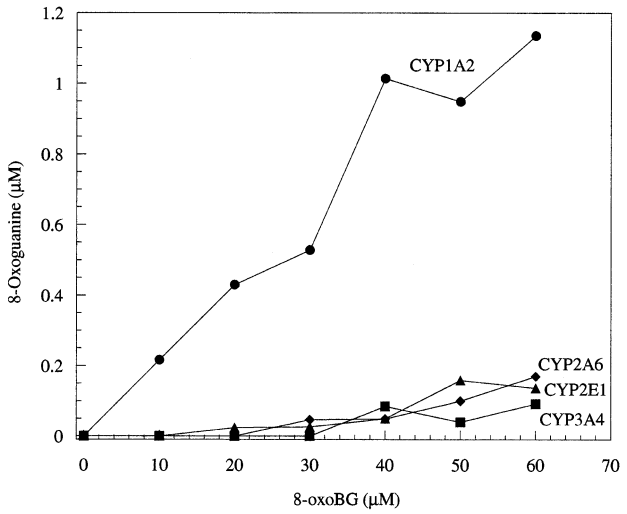

Fig. 4. Formation of 8-oxoguanine following incubation of 8-oxoBG with baculovirus-expressed human P450 isoforms. P450 isoforms (50 pmol) CYP1A2 (●), CYP2E1 (▲), CYP2A6 (♦), and CYP3A4 (■) were incubated with 8-oxoBG (0–60 μM) for 30 min, and the amount of 8-oxoguanine was measured. Each data point represents the average of duplicates in a single experiment. The experiment was done 2–3 times with similar results.

4. Discussion

Up to now, the metabolic fate of BG in humans has not been characterized fully. In fact, only 3.4% of total drug administered to patients is accounted for in the urine as BG and 8-oxoBG [11]. We recently identified 8-oxoguanine as a further metabolite of BG in human plasma and urine, accounting for 21% of BG administered [16,17]. We have now demonstrated that the conversion of 8-oxoBG to 8-oxoguanine is catalyzed in human liver by CYP1A2. Our studies have not ruled out the possibility of extra-hepatic conversion of 8-oxoBG to 8-oxoguanine. Debenzylation by CYP1A2 displayed an apparent K_m value of 35.9 μM and a V_{max} of 0.59 pmol/min/pmol of CYP1A2.

BG is oxidized to 8-oxoBG by CYP1A2, CYP3A4, and aldehyde oxidase [28]. The K_m and V_{max} for BG oxidation by CYP1A2 is 1.3 μM and 1.7 pmol/min/pmol of CYP1A2, respectively [28]. Catalytic activity (V_{max}/K_m) of CYP1A2 in the conversion of BG to 8-oxoBG is 81 times higher compared with conversion of 8-oxoBG to 8-oxoguanine. In humans, BG is rapidly oxidized to 8-oxoBG, and 8-oxoBG is slowly eliminated, resulting in a 28-fold higher AUC and a half-life 13 times longer than that of BG [11]. Our present data demonstrating a much lower catalytic efficiency of 8-oxoBG debenzylation are consistent with our proposed pharmacokinetic model in humans. Preliminary data from our laboratory indicate that BG acts as an inhibitor of CYP1A2, which may affect the rate of debenzylation of 8-oxoBG.

Human CYP1A2 metabolizes a large number of common drugs and engages in carcinogen metabolism and activation [26]. CYP1A2 is primarily responsible for the hepatic 3-demethylation of caffeine, the *N*-oxidation of carcinogenic arylamines, and the *O*-deethylation of phenacetin [29], and it catalyzes the oxidation of theophylline [30], estradiol, estrone [31], warfarin [32], and bufuralol [33]. CYP1A2 has also been reported to play a role in the *O*-deethylation of phenacetin [29]. Importantly for the clinical development of BG, the *N*-demethylation of dacarbazine is also catalyzed by CYP1A2 [34]. Competition of BG or 8-oxoBG with dacarbazine for CYP1A2 binding may result in a lower rate of conversion of dacarbazine to its active metabolite. Thus, the combination of BG and dacarbazine in humans should be approached with caution.

The proposed mechanism of 8-oxoBG debenzylation involves either oxidation of 8-oxoBG on the methylene carbon or on the benzene ring, which results in destabilization of the 8-oxoBG structure. Upon oxidation of the methylene carbon, 8-oxoBG could break down to benzaldehyde and 8-oxoguanine. Oxidation of the benzene ring could lead to the formation of hydroxymethylphenol(s) and 8-oxoguanine.

Preliminary data from patients indicate a 10-fold interindividual variability in the amount of BG accounted for as 8-oxoguanine in urine. CYP1A2 is known to be highly variable in humans as measured by mRNA [35] or protein [30] levels. Genetic polymorphism and environmental factors such as diet and/or concomitant drugs have been identified as contributing to this variability [36–38]. It was reported that smoking, oral contraceptives, and coffee consumption are independently related to changes in CYP1A2 activity, as cigarette smoking results in a marked induction of CYP1A2 [38] and caffeine acts as a nonspecific CYP1A2 substrate [39]. It is possible that there may be drug–drug interactions between BG and common medications, which may ultimately influence the rate and extent of 8-oxoBG debenzylation.

In conclusion, we have found that CYP1A2 is a major human liver P450 isoform contributing to further metabolism of BG to 8-oxoguanine through the *O*-debenzylation of 8-oxoBG. The mechanism of this reaction is currently under investigation.

Acknowledgments

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