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ORIGINAL ARTICLE

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Pharmacokinetics of oral O^6-benzylguanine and evidence of interaction with oral ketoconazole in the rat

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Abstract *Purpose*: O^6-Benzylguanine (BG) is a modulator of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT). BG is converted in mice, rats and humans to an equally active, yet longer-lived metabolite, O^6-benzyl-8-oxoguanine (8-oxo-BG) by CYP1A2, CYP3A4 and aldehyde oxidase. Since intravenous BG is expected to enter phase I development with orally administered anticancer agents such as temozolomide, procarbazine or SarCNU, we determined the bioavailability of orally administered BG, as well as the effect of ketoconazole, a potent intestinal and hepatic CYP3A4 inhibitor, on the disposition of BG. *Methods*: Following intravenous or oral administration of BG in PEG-400/saline (40:60) to Sprague-Dawley rats, the pharmacokinetics of BG and 8-oxo-BG were determined. To determine the effect of CYP3A inhibition on disposition, oral BG was coadministered with ketoconazole. *Results*: The peak plasma concentration (C_{max}), time to C_{max} (t_{max}), and bioavailability (F) of oral BG were: 2.3 ± 0.9 μg/ml, 2.3 ± 0.6 h, and 65.5% respectively. The AUCs of BG and 8-oxo-BG were 13.1 ± 4.6 μg · h/ml and 1.7 ± 0.4 μg · h/ml after oral administration of BG. Coadministration with ketoconazole resulted in an increase in mean absorption time from 2.0 ± 0.3 h to 6.0 ± 0.9 h, a shift in t_{max} to 5 ± 3.3 h, a decrease in C_{max} to 0.96 ± 0.8 μg/ml, and a decrease in $AUC_{0\text{-inf}}$ ratio of 8-oxo-BG:BG from about 0.12 to 0.04 ($P < 0.05$). The bioavailability of BG was not changed (65.5% vs 56.9%, $P = 0.78$). *Conclusions*: The oral bioavailability of BG is high, warranting consideration of an oral formulation for clinical development. Coadministration of ketoconazole and BG resulted in delayed oral absorption and inhibition of conversion of BG to 8-oxo-BG in the rat model.

Key words O^6-Benzylguanine · Bioavailability · Metabolism · Ketoconazole

Abbreviations *AGT* O^6-alkylguanine-DNA alkyltransferase · $AUC_{0\text{-inf}}$ area under the concentration-time curves from zero to infinite time · *AUMC* area under the moment curve from zero to infinite time · *BCNU* 1,3-bis(2-chloroethyl)-1-nitrosourea · *BG* O^6-benzylguanine · *CCNU* 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea · *DTIC* 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide · *MAT* mean absorption time · *MRT* mean residence time · *8-oxo-BG* O^6-benzyl-8-oxoguanine · *SarCNU* 2-chloroethyl-3-sarcosinamide-1-nitrosourea

Introduction

The DNA repair protein, O^6-alkylguanine-DNA alkyltransferase (AGT) plays an important role in the protection of cells from the cytotoxic effects of chloroethylnitrosoureas (BCNU, CCNU, SarCNU) and methylating agents (temozolomide, DTIC, streptozotocin, procarbazine) thereby limiting the clinical utility of these drugs in the presence of this protein [8, 21, 22]. The mechanism of resistance involves transfer of alkyl groups from the O^6 position of guanine to an internal cysteine residue within the protein [21]. In this process, the guanine is left intact within the DNA substrate and the protein is inactivated. The number of lesions that can be repaired is limited to the number of molecules of the AGT protein available. Furthermore, there is strong evidence that the absence or depletion of the AGT protein results in a higher number of interstrand cross-

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links following exposure to chloroethylnitrosoureas [4, 5, 11, 19].

The results of a number of preclinical studies in tumor cells in culture [1, 10] and animal xenograft models [27, 30, 32] suggest that AGT activity is inversely correlated with sensitivity to alkylating agents. In animal studies, the presence of a subpopulation of cells in a tumor with elevated AGT has been correlated with poor response to BCNU [23]. In addition, in both retrospective and prospective human studies, a correlation between AGT levels in brain tumor samples and clinical outcome following treatment with BCNU has been demonstrated [3, 18]. Following an evaluation of 226 high-grade astrocytoma patients receiving BCNU therapy, Belanich et al. [3] have found that a low AGT content in tumors correlates with better response to treatment and greater survival. Jaeckle et al. [18] have demonstrated that the AGT level in tumor tissue specimens may be a predictive marker of survival in patients with malignant astrocytoma that is independent of other previously described prognostic variables. The study was designed with adequate power to detect a tripling of survival hazards ratio for patients with high versus low AGT levels.

In an effort to increase the sensitivity of tumor cells to alkylnitrosoureas, specific potent inhibitors of the AGT protein such as O^6-benzylguanine (BG) have been developed [12]. Several studies have demonstrated that BG increases the sensitivity of tumor cells in vitro, and of xenografts to BCNU and other agents that produce a toxic lesion at the O^6 position of guanine [9, 17]. Detailed studies of the inactivation by BG using purified human alkyltransferase have revealed that this compound is accepted at the active site of the mammalian protein leading to the formation of S-benzylcysteine [9].

BG is currently in phase II human clinical trials. The pharmacokinetics of BG in humans is best described by a two-compartment model, and parent drug is rapidly biotransformed to an equally active metabolite, 8-oxo-BG [15]. The study also demonstrated that 8-oxo-BG is present in human plasma at a higher concentration and for a much longer duration than BG (the AUC of 8-oxo-BG is 12- to 20-fold higher than that of BG). Ex vivo metabolic studies using rat and human liver fractions have demonstrated that P450-mediated reactions (human 1A2 and 3A4) and cytosolic aldehyde oxidases are mainly responsible for the oxidation of BG to 8-oxo-BG [24, 25].

In ongoing clinical studies combining BG with anticancer agents including BCNU, the intravenous route is used for drug delivery. Undoubtedly, the premise for favoring this route for drug administration is that it potentially ensures delivery of adequate BG concentrations to target tissues, thereby resulting in the desired pharmacodynamic outcome(s). In contrast to the systemic route, one of the major problems with oral delivery as a mode of drug administration in cancer therapy is the poor oral bioavailability of some anticancer agents [7]. This phenomenon could result from factors including inadequate intestinal absorption. Presumably, if adequate plasma concentrations of BG can be achieved after oral administration, this more convenient route of drug administration should be considered. We investigated the bioavailability of orally administered BG in rats. Furthermore, CYP3A is expected to play a major role in the metabolism of BG since this isoform is the predominant liver and intestinal P-450 [28]. Since BG is being developed for use in patients with solid malignant tumors, especially brain tumors, it is reasonable to speculate, that some of these patients will also be on prescribed medications, such as glucocorticoids, macrolide antibiotics, or antifungal agents that are known modulators of CYP3A enzyme. Since CYP3A enzyme is present in the small intestine as well as the liver of rats [34], we hypothesized that coadministration of oral ketoconazole, an antifungal agent, and known potent inhibitor of intestinal and hepatic CYP3A in rats [26] would alter the disposition of BG.

Material and methods

Chemicals

BG and 8-oxo-BG were supplied by Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.). PEG-400 was obtained from Spectrum Chemicals (Gardena, Calif.). Ketoconazole (200 mg tablets, Janssen Pharmaceuticals, Titusville, N.J.) was obtained from the Pharmacy Department at the University of Chicago.

Animals

Male Sprague-Dawley rats (150–175 g, 6 weeks old) purchased from Charles River (Wilmington, Mass.) were used in all experiments. The rats were implanted with a catheter in the right jugular vein to allow for easy drawing of blood. Animals were housed in an environmentally controlled room (12-h light/dark cycle). Food and water were provided ad libitum, until approximately 12 h prior to drug administration, when only water was allowed. Experimental procedures followed the guidelines of The University of Chicago Manual on Laboratory Animals prepared by the Animal Care Committee.

Administration of BG

Rats were assigned to three different groups based on mode of drug administration (intravenous, oral, oral with ketoconazole). BG solution was prepared and administered (intravenously) as previously described by Roy et al. [24]. Briefly, BG (2 mg/ml) was prepared in PEG-400/saline (40:60). After administration of a total dose of 14 mg/kg over 2 min, the catheter was flushed with 0.9% saline to prevent contamination by residual dosing solution. Animal feeding needles (Popper and Sons, New Hyde Park, N.Y.) were used to administer the same dose of BG orally. Ketoconazole tablets (200 mg × 2) were dispersed in 2 ml sterile water to make a slurry, and an estimated dose of 100 mg/kg was administered orally in two doses to a predetermined set of rats. The first dose was administered at the onset of fasting, and the second dose approximately 1 h prior to oral BG administration. This dosing strategy was thought to be optimal in maximizing the effect of ketoconazole on CYP3A, following preliminary studies conducted in our laboratory. The rats were allowed food and water ad libitum 1 h after the administration of the BG solution. Blood samples (about

200 µl) were obtained at 0, 0.5, 1, 1.5, 2, 3, 5, 7, 9 and 12 h after BG administration. For each blood sample drawn, an equal volume of prewarmed 0.9% normal saline was infused through the catheter for blood volume replacement. Samples were immediately centrifuged at 14,000 *g* for 10 min to obtain plasma, which was then stored at −70 °C until analysis.

Quantitation of BG and 8-oxo-BG

The internal standard O^6-(*p*-fluorobenzyl)guanine in methanol was added to 100 µl rat plasma and the mixture was treated with 5 ml ethyl acetate, vortexed for 10 s, and centrifuged at 2500 rpm at room temperature for 20 min. The organic layer was transferred into a clean disposable culture tube, evaporated under a stream of nitrogen at 37 °C to dryness and reconstituted with 200 µl mobile phase (10 m*M* potassium phosphate, pH 7.5, in methanol, a ratio of 72:28). Reconstituted sample (100 µl) was injected onto a Waters Nova Pak Phenyl 125 Å 4-µm reverse-phase column (3.9 cm × 150 mm) at ambient temperature. The resolved peaks were monitored with UV detection ($\lambda = 280$ nm). BG and 8-oxo-BG concentrations in rat plasma were determined by ratios, in relation to a standard curve generated from known concentrations of the compounds spiked in rat plasma obtained from untreated animals. The observed coefficient of variability between days for the standard curves was less than 10%. The minimum level of detection for BG and 8-oxo-BG was 2.4 ng/ml and 2.5 ng/ml respectively.

Data analysis

The pharmacokinetics of BG were analyzed using noncompartmental methods with WinNonlin (PharSight Corporation, Apex, N.C.). The elimination half-life of BG ($t_{1/2}$) was estimated from the slope of the terminal phase of the log concentration-time curves after intravenous and oral administration of the drug, for each rat. The area under the concentration-time curves for the study period (AUC_{last}), and after intravenous (AUC_{iv}) and oral (AUC_{po}) administration were obtained by linear trapezoidal technique. The AUC (from zero time to infinity) was calculated by the relationship $AUC = AUC_{last} + C_{last}/\lambda_{term}$, where λ_{term} is the terminal elimination rate constant. The time points used to derive λ_{term} were chosen after visual inspection of the log concentration-time curves for each rat. AUC_{last} is the area under the concentration-time curves to the last sampling time and C_{last} is the concentration at the last sampling period. Pharmacokinetic parameters for each rat were estimated and the mean for a group ($n = 3$) was used as representative of that group. The ratio of AUC_{po} to AUC_{iv} was used to calculate oral bioavailability of BG. The mean residence time (MRT), defined as AUMC/AUC, was estimated for each set of animals with AUMC defined as area under the moment curve from zero to infinity. The mean absorption time (MAT), defined as $MRT_{po} - MRT_{iv}$, was then calculated. To assess delay in absorption, a first-order kinetic model with a lag-time was used to obtain the lag-time after oral administration.

The pharmacokinetics of 8-oxo-BG were also analyzed using a noncompartmental model and the AUC for 8-oxo-BG was estimated using the linear trapezoidal technique. The relative amount of metabolite formed from systemically available drug for all groups of rats was estimated as $AUC_{(8\text{-oxo-BG})}/AUC_{(BG)}$. Student's *t*-test was used to compare the pharmacokinetic parameters with or without ketoconazole. *P*-values < 0.05 were taken as statistically significant.

Results

The concentration-time curves for BG and 8-oxo-BG for rats given intravenous versus oral BG are shown in Fig. 1. The elimination half-life ($t_{1/2}$) for BG after intravenous administration was approximately 1.2 h. Previously, we have reported a terminal $t_{1/2}$ of 1.6 h for BG [24]. Other pharmacokinetic parameters are shown in Table 1. The lag-time for absorption (T_{lag}), maximum attainable BG concentration (C_{max}), and time to attain maximum concentration (T_{max}) after oral administration, were 0.5 ± 0.6 h, 2.3 ± 0.9 µg/ml and 2.3 ± 0.6 h, respectively. The AUCs for BG after intravenous and oral administration were 20 ± 5.4 µg · h/ml and 13.1 ± 4.6 µg · h/ml, respectively. Oral bioavailability of BG was estimated as 65.5%.

Administration of ketoconazole prior to oral BG resulted in delayed absorption with a C_{max} and time to attain maximum concentration (T_{max}) of 0.96 ± 0.8 µg/ml and 5 ± 3.3 h, respectively (Fig. 1, Table 1).

Interestingly, while there was no significant difference in the bioavailability of BG between the two oral groups (56.9% with and 65.5% without ketoconazole, $P = 0.78$), the amount of 8-oxo-BG ($AUC_{8\text{-oxo-BG}}$), formed from the systemically available parent drug, decreased significantly with coadministration of ketoconazole. There was a reduction in the ratio of plasma $AUC_{8\text{-oxo-BG}}$ to AUC_{BG} from approximately 0.1 to 0.04, a decrease of approximately 60% (Table 1). Also, coadministration of oral ketoconazole with BG resulted in an increase in mean absorption time of BG from 2.0 ± 0.3 h to 6.0 ± 0.9 h (Table 1).

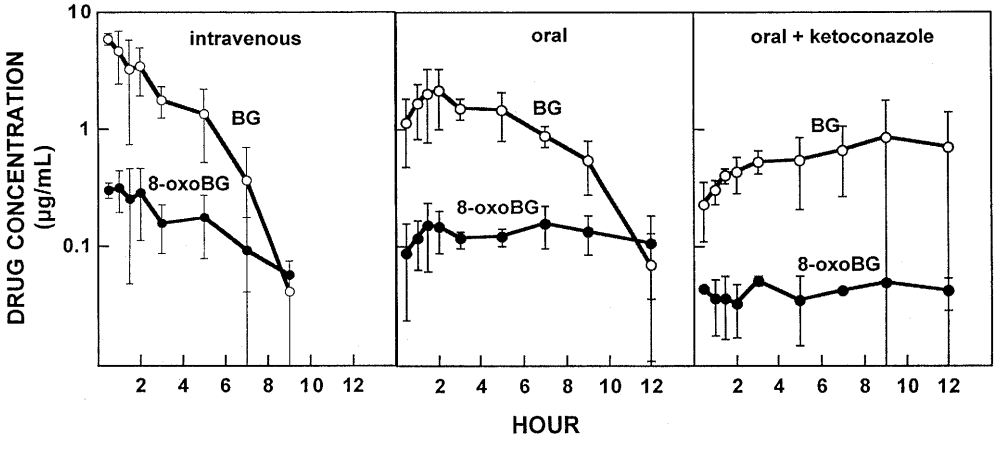

Fig. 1 Mean ± SD plasma concentrations-time profile of BG and 8-oxo-BG after intravenous ($n = 3$), oral ($n = 3$) and oral with ketoconazole ($n = 3$) administration in rats

Table 1 Pharmacokinetic parameters of O^6-benzylguanine and O^6-benzyl-8-oxoguanine (8-oxo-BG) in rat. All values except the AUC ratios and bioavailability are mean ± SD

Parameter	Mode of drug administration					
	Intravenous (n = 3)		Oral alone (n = 3)		Oral + ketoconazole (n = 3)	
	BG	8-oxo-BG	BG	8-oxo-BG	BG	8-oxo-BG
$AUC_{0\text{-inf}}$ (μg · h/ml)	20 ± 5.41	1.97 ± 1.2	13.1 ± 4.6	1.7 ± 0.4	11.4 ± 9.2	0.4 ± 0.1
$AUC_{0\text{-inf}}$ ratio (8-oxo-BG/BG)		0.09		0.12		0.04*
$T_{1/2}$ (h)	1.2 ± 0.7	2.6 ± 1.3	1.9 ± 0.9	4.5 ± 1.8	1.6 ± 0.3	2.8 ± 1.5
Bioavailability (F)			65.5%		56.9%**	
C_{max} (μg/ml)			2.3 ± 0.9		0.96 ± 0.8	
T_{max} (h)			2.3 ± 0.6		5 ± 3.3	
MRT (h)	2.1 ± 0.5		4.1 ± 0.08		8.1 ± 1.4	
MAT (h)			2.0 ± 0.3		6.0 ± 0.9	
T_{lag} (h)			0.5 ± 0.6		1.5 ± 2	

*P = 0.01, **P = 0.78, vs oral alone

Discussion

We have estimated the bioavailability for orally administered BG to be 65.5%. The maximum concentration of BG achieved was 2.3 ± 0.9 μg/ml after oral administration of the drug. Our data suggest a rapid absorption of BG from the gastrointestinal tract after oral administration. Administration of drugs orally is generally more convenient and less expensive to patients. There are compounds, including leucovorin, S-1 (an oral formulation of tegafur), and eniluracil (a modulator of 5-fluorouracil), that can be administered orally for the augmentation of antineoplastic efficacy [2, 31]. Our study shows that an adequate bioavailability of BG is possible in rats after its oral administration.

An important difference between humans and rats is the extent to which 8-oxo-BG is formed following intravenous administration of BG [15, 24]. The $AUC_{0\text{-inf}}$ ratio of 8-oxo-BG/BG in humans ranges from 12 to 20 for doses between 10 and 120 mg/m^2 (data not shown; [15]) compared to 0.09 for rats given 42 mg/m^2 (14 mg/kg). The difference is most likely due to a greater affinity of the enzymes responsible for oxidation of BG in humans [25]. It is known that human P450 isoforms CYP1A2 (Km 1.3 μM) and CYP3A4 (Km 52.2 μM) and cytosolic aldehyde oxidase (Km 81.5 μM) are responsible for oxidation in humans [25]. In rats, oxidation occurs in rat liver microsomes (apparent Km 19.6 μM) and cytosol (apparent Km 13.4 μM), although the CYP isoform has not been identified. Another difference is that acetylated metabolites including N^2-acetyl-BG and N^2-acetyl-8-oxo-BG are formed in rats [13] but not in humans following BG administration [15]. In spite of differences in the metabolic fate of BG and the extent of 8-oxo-BG formation, rats provide a reasonable model to evaluate oral bioavailability prior to human studies.

In the development of a BG formulation for oral administration in humans, an appropriate vehicle must be chosen that will not interfere with oral absorption. PEGs are polymers used in the formulation of various drugs. PEG 400 has been studied extensively in animals and has been shown to be non-toxic [14], and in humans it has been given orally [6] and is present in commercial drug formulations such as lorazepam [20]. Comparative solubility, metabolism, bioavailability and efficacy studies of BG as an adjuvant therapy with BCNU, using cremophor-EL and PEG 400 have been done in animals [14]. The authors demonstrated a more rapid distribution of BG to plasma, tumor, and other tissues when the drug is given in 40% PEG 400 than when given in cremophor-EL. In addition, there are limitations with the use of cremophor-EL formulations in humans and animal models, including hypersensitivity reactions [16, 33]. Thus, in this study, BG was dissolved in 40% PEG 400/saline for intravenous as well as oral administration.

We demonstrated in this study that while coadministration of ketoconazole did not significantly influence the oral bioavailability of BG, the MAT, T_{max} and C_{max} were affected. The MAT increased from 2.0 ± 0.3 to 6.0 ± 0.9, reflecting the effect of ketoconazole on the absorption of BG, T_{max} shifted from 2.3 ± 0.6 h to 5 ± 3.3 h suggesting delayed absorption of BG, and C_{max} decreased from 2.3 ± 0.9 μg/ml to 0.96 ± 0.8 μg/ml as a reflection of delayed absorption from the gastrointestinal tract. The decrease in the $AUC_{0\text{-inf}}$ ratio of 8-oxo-BG to BG when ketoconazole was coadministered with BG suggests a decrease in the amount of 8-oxo-BG formed from the systemically available parent drug. It is worth noting that in addition to being a CYP3A inhibitor in rats, ketoconazole has also been demonstrated to be an inhibitor of p-glycoprotein in the small intestine of rats [29]. However, studies in our laboratory have indicated that BG is not a substrate for p-glycoprotein (data not shown).

To the best of our knowledge, there are no data in the literature characterizing any interactions between ketoconazole and PEG 400. Thus, it is possible that such interaction might partly explain the delay in absorption and lack of increase in oral bioavailability of BG. Perhaps future studies using different delivery strategies for ketoconazole, for example the use of a pure powder formulation, or intraperitoneal administration of ketoconazole instead of administration as a slurry will

result in a disposition profile different from the one observed in this study. However, in the clinical setting it is more likely that ketoconazole will be administered as tablets, although the sequence of administration might vary.

In conclusion, we found that oral administration of BG in rats results in significant bioavailability. Coadministration of oral ketoconazole and BG resulted in delayed absorption and inhibition of P-450-mediated metabolism that did not result in a change in bioavailability. Further studies to determine the bioavailability of oral BG in humans are warranted.

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