Published in final edited form as:

Cancer Chemother Pharmacol. 2011 June ; 67(6): 1291–1297. doi:10.1007/s00280-010-1407-9.

Plasma and CNS pharmacokinetics of O⁴-benzylfolic acid (O⁴BF) and metabolite in a non-human primate model

Meredith K. Chuk,

Pediatric Oncology Branch, National Cancer Institute, 10 Center Drive, Building 10/Rm 1W-5750, Bethesda, MD 20892, USA

Diane E. Cole,

Pediatric Oncology Branch, National Cancer Institute, 10 Center Drive, Building 10/Rm 1W-5750, Bethesda, MD 20892, USA

Cynthia McCully,

Pediatric Oncology Branch, National Cancer Institute, 10 Center Drive, Building 10/Rm 1W-5750, Bethesda, MD 20892, USA

Natalia A. Laktionova,

Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, 500 University Drive, Hershey, PA 17033, USA

Anthony E. Pegg,

Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, 500 University Drive, Hershey, PA 17033, USA

Robert J. Parker,

Laboratory of Clinical Pharmacology, Center for Drug Evaluation and Research, US Food and Drug Administration, 10903 New Hampshire Ave., Silver Spring, MD 20993, USA

Gary Pauly,

Chemical Biology Laboratory, National Cancer Institute at Frederick, Frederick, MD 21702, USA

Brigitte C. Widemann,

Pediatric Oncology Branch, National Cancer Institute, 10 Center Drive, Building 10/Rm 1W-5750, Bethesda, MD 20892, USA

Frank M. Balis,

The Children's Hospital of Philadelphia, 3501 Civic Center Boulevard, CTRB 4016, Philadelphia, PA 19104, USA

Elizabeth Fox

chukmk@gmail.com.

The views presented in this manuscript do not necessarily represent the views of the National Institutes of Health or the Federal Government.

Electronic supplementary material The online version of this article (doi:10.1007/s00280-010-1407-9) contains supplementary material, which is available to authorized users.

The Children's Hospital of Philadelphia, 3501 Civic Center Boulevard, CTRB 4016, Philadelphia, PA 19104, USA

Abstract

Purpose—O⁶-alkylguanine-DNA alkyltransferase (AGT) repairs DNA damage from alkylating agents by transferring the alkyl adducts from the O⁶-position of guanine in DNA to AGT. The folate analog O⁴-benzylfolic acid (O⁴BF) is an inhibitor of AGT with reported selectivity of the alpha-folate receptor in tumors. We studied plasma and cerebrospinal fluid (CSF) pharmacokinetics and CSF penetration of O⁴BF in a non-human primate model.

Methods—Rhesus monkeys (*Macaca mulatta*) received O⁴BF (10–50 mg/kg) intravenously, and serial blood and CSF samples were obtained. Analyte concentrations in plasma were measured by HPLC/photo diode array, and an HPLC/MS/MS assay was used for CSF samples.

Results—A putative metabolite of O⁴BF was detected in plasma and CSF. O⁴BF and the metabolite inactivated purified AGT with ED₅₀ of 0.04 mM. The median clearance of O⁴BF was 8 ml/min/kg and half-life was 1.1 h. The metabolite had a substantially longer half-life (>20 h) and greater AUC than O⁴BF. The AUC of the metabolite increased disproportionately to the dose of O⁴BF, suggesting saturable elimination. CSF penetration of O⁴BF and its metabolite was < 1%. At the 50 mg/kg dose level, the C_{max} in CSF for O⁴BF was less than 0.09 mM and for the metabolite the C_{max} ranged from 0.02 to 0.04 mM (O⁴BF equivalents).

Conclusions—Concentrations of O⁴BF and the metabolite in CSF exceeded the ED₅₀ of AGT; however, recently reported lack of receptor specificity and pharmacokinetic data suggesting saturable elimination of both O⁴BF and its metabolite may limit dose-escalation and future clinical development of this agent.

Keywords

O⁶-alkylguanine-DNA alkyltransferase (AGT); O⁴-benzylfolic acid; Pharmacokinetics; CSF pharmacokinetics

Introduction

O⁶-alkylguanine-DNA alkyltransferase (AGT) repairs DNA damage from alkylating agents by transferring the alkyl group and other adducts from the O⁶-position of guanine in DNA to an internal cysteine residue in the AGT protein and rendering the protein inactive [1]. In tumors, AGT confers resistance to alkylating agents such as temozolomide and the chloroethylnitrosoureas.

O⁶-benzylguanine (O⁶-BG) depletes AGT by acting as a substrate and thereby enhances the activity of agents that alkylate the O⁶-position of guanine [2]. Continuous infusion of O⁶-BG for up to 48 h in patients depletes tumor AGT activity [3]. However, combining O⁶-BG with carmustine or temozolomide in adults and children with solid tumors enhanced the toxicity of the chemotherapy, requiring a reduction in the dose to less than half the standard dose [3–6].

O⁴-benzylfolic acid (O⁴BF) (Fig. 1) is a folate analogue and an inhibitor of AGT that is more water soluble and approximately 30-fold more potent than O⁶BG [7, 8]. Cellular uptake of O⁴BF appears to be mediated by the alpha-folate receptor [7], which has limited expression in normal tissues but is over-expressed in many primary and metastatic cancers, such as ovarian, uterine, endometrial, cervical and pancreatic carcinomas, ependymal brain tumors, mesothelioma and testicular choriocarcinoma, and to a lesser extent in breast, colon and renal cancers [9, 10]. Selective uptake of O⁴BF into tumor cells may lessen the severity of systemic toxicity that occurred when O⁶BG was combined with alkylating agents and allow for administration of higher doses of conventional chemotherapy in conjunction with O⁴BF.

The alkylating agents with which O⁴BF is likely to be combined are primarily used to treat brain tumors; therefore, the penetration of O⁴BF into the CNS may impact on its ability to enhance the anti-tumor effects of the alkylating agents. We studied the toxicity and the plasma and cerebral spinal fluid (CSF) pharmacokinetics of O⁴BF in a non-human primate model (NHP).

Materials and methods

Chemicals

O⁴-benzylfolic acid (O⁴BF, MW = 629 as disodium salt) and the internal standard O⁴-propylfolic acid (O⁴PF; MW = 527) were synthesized by and obtained from the Laboratory of Comparative Carcinogenesis at the National Cancer Institute, Frederick, MD [7]. Aldehyde oxidase (AO) was isolated as previously described from rabbit livers [11, 12].

Animals

Adult male rhesus monkeys (*Macaca mulatta*) weighing 8.1–14.8 kg with an indwelling Ommaya reservoir attached to a catheter in the fourth ventricle were utilized in this study (3 animals, 8 experiments) [13]. Animals were group housed in accordance with the Guide for the Care and Use of Laboratory Animals [14]. This study was approved by the NCI Animal Care and Use Committee.

Drug administration and PK sampling

O⁴BF (10–50 mg/kg) was diluted in normal saline to a final volume of 30 ml and infused intravenously (IV) over 30 min. Blood samples were drawn from a central or peripheral venous catheter contralateral to the site of drug infusion. CSF samples were drawn from the Ommaya reservoir. Paired CSF and blood samples were drawn prior to infusion, at end of infusion (EOI) and 30 min, 1, 2, 4, 6, 8, 10, 24, 48, and 72 h after infusion. Additional blood samples were drawn 5 and 15 min after the start of the infusion and 5 and 15 min after EOI. Blood samples were immediately centrifuged and plasma was separated. Plasma and CSF were stored at –70°C until assayed.

Sample processing, HPLC assay development and validation

The internal standard O⁴-propylfolic acid (40 µl of a 250 mM stock solution) was added to 0.5 ml of plasma, which was then deproteinated with 1 ml of acetonitrile (ACN). Samples

were vortexed, placed in a shaker for 10 min and centrifuged at 10,000 rpm for 10 min. Supernatant was collected, evaporated in a 37°C water bath under a stream of nitrogen and reconstituted in 200 µl of mobile phase. Analyte concentrations were measured by HPLC (Alliance 2695, Waters, Milford, CT) using a Waters Symmetry C8 (5 µm, 3.9 × 150 mm) column and Phenomenex security guard column (Torrance, CA) with a photo diode array (PDA) detector (Waters Model 996, Waters, Milford, CT). Mobile phase was 72% 0.01 M sodium phosphate (pH 6.9)/28% methanol at a flow rate of 1 ml/min. Injection volume was 50 µl. Empower Pro version 5.0 software was used for data analysis. The HPLC/PDA assay was linear from 0.2–20 µM in plasma. The inter- and intraday variability (CV%) was < 10%.

Analyte concentrations in CSF samples were undetectable using the HPLC/PDA method; therefore, an HPLC/MS/MS assay was developed and validated for CSF using a MDS Sciex API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with Shimadzu HPLC system (Columbia, MD). Chromatographic separation was performed with a Phenomenex Luna C8, 3 µm, 2 × 50 mm column (Torrance, CA), autosampler temperature 4°C and column heater 40°C. Mobile phase consisted of 0.1% formic acid and acetonitrile (80:20) at a flow rate of 0.25 ml/min. Source and compound-dependent MS parameters were optimal under the following conditions: collision gas 6 psi, curtain gas 20 psi and ion source 120 psi; ion spray voltage 5000 V, declustering potential 61 V, entrance potential 10 V and collision energy 25 V; ion source temperature 500°C. CSF injection volume was 20 µl. Quantification was performed using multiple reaction monitoring (MRM), and data analysis was performed with Analyst® software version 1.4.2. The HPLC/MS/MS assay for CSF was linear from 0.1 to 100 nM. The inter- and intraday variability (CV%) was 10%.

Plasma and aqueous assays were validated using FDA assay validation guidelines.

Metabolite evaluation

A putative metabolite of O⁴BF was detected in plasma, on the HPLC/PDA assay. Based on the previously described metabolism of methotrexate (MTX) to 7-OH-MTX by aldehyde oxidase, we hypothesized that the O⁴BF metabolite was similarly formed through hydroxylation at the 7-position. A 10 µM aqueous solution of O⁴BF was incubated with aldehyde oxidase (AO) for 30 min at room temperature. Protein was precipitated from solution with 1 ml ACN, centrifuged and resulting supernatant was analyzed by HPLC/PDA and mass spectrometry. Meteor (LHASA Limited, Leeds, UK) was used to predict possible metabolite structures.

The ability of the metabolite to inactivate purified AGT was assessed as previously described [15]. Briefly, purified hAGT protein (20 ng) was incubated with a range of concentrations of O⁴BF, and the metabolite formed by incubation of O⁴BF with AO in 0.5 ml of 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM dithiothreitol and 50 mg hemocyanin at 37°C for 30 min. The residual AGT activity was determined by incubation with [³H]methylated calf thymus DNA for 30 min and quantifying the resultant [³H]methylated protein formed. The results are expressed as the percentage of the residual AGT activity. The

ED₅₀ is defined as the concentration of O⁴BF or metabolite needed to reduce the AGT activity by 50% in a 30-min incubation period at 37°C.

Pharmacokinetics

The area under the curve (AUC) for O⁴BF and its metabolite in plasma and CSF was calculated using the linear trapezoidal method. Metabolite concentrations were reported as O⁴BF-equivalents. The half-life (T_{1/2}) for both compounds was calculated by dividing 0.693 by the slope of the terminal elimination phase of the plasma and CSF curves.

The CSF penetration of O⁴BF and metabolite was calculated from the AUC_{CSF}:AUC_{plasma} ratio.

Results

Metabolite identification and evaluation

The chromatograms from plasma showed a new peak that eluted after O⁴BF. This peak was not present in the preinfusion or 5-min sample (Fig. 2a) but was observed 30 min after the end of infusion (EOI) (Fig. 2b) and increased over time (Fig. 2c). UV spectra of O⁴BF (Fig. 3a) and this putative metabolite (Fig. 3b) are shown in Fig. 3. The UV and mass spectra of metabolite formed in vitro by incubation of O⁴BF with AO (Fig. 3c) and the metabolite peak in plasma (Fig. 3b) were identical. A positive ion scan of an aqueous solution of O⁴BF revealed a highly abundant analyte of 532.2 amu transitioning to product ion of 385.1 amu. A scan post-incubation of O⁴BF with AO found a second analyte of 549.0 amu which transitioned to a 364.8 amu production. This scan was repeated in CSF from NHP sample which contained metabolite and confirmed that enzymatic conversion of O⁴BF with aldehyde oxidase produced the metabolite seen in NHP.

AGT inhibition by O⁴BF and metabolite formed by incubation of O⁴BF with AO is shown in Fig. 4. The ED₅₀ of O⁴BF was 0.04 mcM, and the ED₅₀ of the metabolite was 0.04 mcM.

Pharmacokinetics

Plasma and CSF concentration–time profiles for parent drug and metabolite are shown in Fig. 5, and pharmacokinetic parameters of O⁴BF and its metabolite in plasma and CSF are shown in Table 1. O⁴BF was rapidly converted to metabolite. The median (range) clearance of O⁴BF was 8 (3–19) ml/min/kg and half-life was 1.1 (0.4–5)h. The metabolite had a substantially longer half-life and greater AUC than O⁴BF, and the AUC of the metabolite increased disproportionately to the dose of O⁴BF, suggesting there is a saturable elimination.

O⁴BF and metabolite were measurable in the CSF. However, CSF drug exposure of O⁴BF and its metabolite was < 1% of plasma drug exposure (Table 1). At the 50 mg/kg dose level, the C_{max} in CSF for O⁴BF was less than 0.09 mcM and for the metabolite the C_{max} ranged from 0.02 to 0.04 mcM (O⁴BF equivalents).

Toxicity

All animals tolerated O⁴BF well except for one animal that experienced emesis, transiently elevated serum transaminases, and a transient decrease in hemoglobin and platelets after receiving 50 mg/kg.

Discussion

AGT plays an important role in the mechanism of tumor resistance to agents, such as carmustine and temozolomide that produce their cytotoxic effect by alkylating the O⁶-position of guanine [16]. O⁶-benzylguanine (O⁶BG) is an AGT inhibitor that modulates resistance to these alkylating agents in preclinical models, and it has been tested clinically in combination with carmustine and temozolomide [5, 6]. However, combining O⁶BG with these alkylating agents also enhanced their toxicity and limited the dose of standard chemotherapy that can be safely administered. O⁴BF is a potent AGT inhibitor that was reported to have greater tumor selectivity because its uptake into cells was thought to be dependent on the alpha-folate receptor, which is more highly expressed in tumor cells than in normal tissues. Subsequent studies failed to confirm the proposed selective uptake of this modulating agent, which enhanced the myelotoxicity of carmustine in the CFU-GM clonogenic assay using human bone marrow cells [8, 17].

We studied the clinical pharmacology of O⁴BF in non-human primates. The pharmacokinetics of O⁴BF were characterized by a short half-life and rapid conversion to a metabolite with a substantially longer half-life and greater exposure than the parent compound. The metabolite, which is presumed to be 7-OH-O⁴BF, is an equipotent inhibitor of AGT.

The CSF penetration of O⁴BF and its metabolite is < 1% relative to plasma drug exposure. In comparison, O⁶BG has a CSF penetration of 4.3 (±0.6)% and its primary metabolite, O⁶-benzy-8-oxoguanine, has a CSF penetration of 36 (±11)% [17]. The ED₅₀ for AGT inhibition by O⁴BF is approximately 0.04 mM, and the C_{max} of O⁴BF in the CSF at doses above 40 mg/kg exceeds this level. However, the recently reported lack of receptor specificity [18] in conjunction with this pharmacokinetic data suggesting saturable elimination of both O⁴BF and its metabolite and GI toxicity and myelosuppression seen in one non-human primate treated with 50 mg/kg may limit dose escalation and future clinical development of this agent.

Acknowledgments

We would like to recognize Robert Moschel and John Strong for their contribution to this project and their enduring contributions to the advancement of pharmacology. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The work of NAL and AEP was supported in part by NIH grant R01 CA-071976.

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Fig. 1.
Chemical structure of O⁴-benzylfolic acid (O⁴BF)

Fig. 2. Chromatograms of O^4BF and its metabolite in the plasma of NHP 5 min after the start of the infusion (a) and 30 min (b) and 2 h (c) after the end of infusion. The peak representing the putative metabolite was not present prior to drug infusion and 5 min into the infusion but was present by 30 min into the infusion and exceeded O^4BF post-infusion

Fig. 3.

Ultraviolet absorption spectra of O^4BF (a), its metabolite in the plasma of NHP (b), and the incubation product after exposure of O^4BF to aldehyde oxidase (AO) (c). The spectrum of the putative metabolite in plasma was identical to the product produced by incubation of O^4BF with AO

Fig. 4. Inhibition of AGT activity by O⁴BF and its metabolite (presumed to be 7-OH-O⁴BF). The metabolite was produced by incubating O⁴BF with aldehyde oxidase. The metabolite concentration was assumed to be equimolar to the starting concentration of O⁴BF. Conversion was monitored by UV spectral absorbance and mass spectral scanning

Fig. 5. Concentration–time curves for O^4BF and its metabolite in plasma (a) and CSF (b) in one NHP after a 50 mg/kg dose of O^4BF . The metabolite concentrations are O^4BF equivalents (estimated from the O^4BF standard curve)

Table 1

Plasma and CSF pharmacokinetic parameters for O⁴BF and metabolite

Animal	Dose (mg/kg)	C _{max} (mM)		AUC (mM h) ^a		Half-life (h)		Clearance (ml/min/kg)	
Plasma pharmacokinetic parameters									
		O ⁴ BF	Metabolite	O ⁴ BF	Metabolite	O ⁴ BF	Metabolite	O ⁴ BF	Metabolite
RQ3588	10	25	9	14	103	0.4	18	19	
RQ3633	15	68	14	52	82	0.7	15	8	
15398	20	21	36	47	1,031	3.2	12	11	
RQ3588	20	105	42	65	1,110	0.8	>75	8	
15398	20	18	79	58	3,633	5	22	9	
RQ3633	40	489	183	347	6,058	1	16	3	
RQ3588	50	219	255	227	13,360	1.2	29	6	
RQ3633	50	301	281	333	12,570	1.3	32	5	
Animal	Dose (mg/kg)	C _{max} (mM)		AUC (mM h) ^a		CSF penetration (%)			
Cerebrospinal fluid pharmacokinetic parameters									
		O ⁴ BF	Metabolite	O ⁴ BF	Metabolite	O ⁴ BF	Metabolite	O ⁴ BF	Metabolite
RQ3588	10	0.02	0.0007	0.06	0.02	0.4	0.02	0.02	
RQ3633	15	0.02	0.0005	0.06	0.003 ^b	0.1	0.006	0.006	
15398	20	0.01	0.005	0.07	0.18	0.2	0.02	0.02	
RQ3588	20	0.03	0.002	0.17	0.08	0.3	0.01	0.01	
15398	20	0.01	0.01	0.11	0.7	0.2	0.02	0.02	
RQ3633	40	0.07	0.006	0.29	0.3	0.08	0.005	0.005	
RQ3588	50	0.07	0.02	0.4	0.8	0.2	0.01	0.01	
RQ3633	50	0.09	0.04	0.4	1.2	0.1	0.01	0.01	

^aFor O⁴BF plasma and CSF, AUC is extrapolated to infinity (∞) and AUC_{0-∞} is used to calculate the CSF penetration of O⁴BF; for the metabolite, the AUC is calculated is to the last measured time point and CSF penetration for the metabolite is the ratio of the AUC to the same time point in CSF and plasma

^bMeasured to 10 h