2017

Biotransformation and Pharmacokinetic Evaluation of PF-5190457, A Novel Drug Candidate for Alcoholism

Sravani Adusumalli
University of Rhode Island, sravani.adusumalli@gmail.com

BIOTRANSFORMATION AND PHARMACOKINETIC EVALUATION OF PF-5190457, A NOVEL DRUG CANDIDATE FOR ALCOHOLISM

BY

SRAVANI ADUSUMALLI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL AND PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2017

DOCTOR OF PHILOSOPHY DISSERTATION

OF

SRAVANI ADUSUMALLI

APPROVED:

Dissertation Committee:

Major Professor: Fatemeh Akhlaghi

Sara Rosenbaum

Ingrid Lofgren

Nasser H. Zawia
DEAN OF GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2017

Abstract

Alcohol use disorder is a chronic condition characterized by an inability to control alcohol intake. Recent research has shown that appetite-regulating hormones, ghrelin, and leptin, may play a role in alcohol craving. In addition, it is known that food and alcohol craving have overlapping receptors in the brain reward system. Ghrelin, a naturally occurring hormone in the human body, is produced mainly by ghrelinergic cells of the gastric mucosa. Once the hormone crosses the blood-brain barrier, it binds to growth hormone secretagogue receptor (ghrelin receptor) in the hypothalamus and stimulates the appetite and food intake. Leptin, a hormone produced from adipose tissue has an opposite effect to that of ghrelin and reduces hunger. Leptin and ghrelin work in tandem to maintain energy homeostasis in the body. It was demonstrated that antagonizing ghrelin receptor reduces alcohol craving thus making it a potential pharmacological target to treat alcohol use disorders.

PF-5190457 is a highly selective, orally bioavailable ghrelin receptor inverse agonist developed by Pfizer. PF-5190457 was made available for research by the National Center for Advancing Translational Sciences (NCATS) under the NIH New Therapeutic Uses Pilot Program. Initial studies by Pfizer had shown that the compound is safe and well tolerated in healthy volunteers after oral administration. To date, the compound has completed Phase 1b clinical trials in nontreatment-seeking patients with alcohol use disorder. The plasma samples obtained from the study was evaluated for the pharmacokinetics of PF-5190457 and its major hydroxy metabolite. In order to measure the concentration of the

drug and its metabolite, a liquid chromatography tandem mass spectroscopy method (LC-MS/MS) was developed and validated as described in Manuscript I (to be submitted to Journal of Chromatography B: Biomedical Sciences and Applications). The manuscript describes a new LC-MS/MS method developed for this purpose and the assay was fully validated according to the FDA guidance document for bioanalytical methods. The analytes of interest were extracted from plasma with methanol using a simple protein precipitation technique, and tacrine was used as the internal standard. All the bioanalysis method parameters, i.e., sensitivity, specificity, linearity, accuracy, precision, matrix effect, recovery, and stability were within the recommended FDA guidelines. Incurred sample reanalysis (10% of the patient plasma samples) was performed to evaluate the repeatability of the new method and was found to be within the acceptance criteria.

Manuscript II (to be submitted to *Drug Metabolism and Disposition*) describes the biotransformation of PF-5190457 (molecular weight: 513). Before this work in our lab, no information on the metabolism of the PF-5190457 in humans was available. Extensive LC-MS/MS profiling of plasma from Phase 1b study showed many circulating metabolites. Subsequent analysis revealed the presence of a significant hydroxy metabolite (*m/z* 529) along with minor glucuronide and hydroxy glucuronide metabolites. Further exploration of the fragmentation pattern of the major hydroxy metabolite (m/z 351 and m/z 225) suggested possible hydroxylation between the two nitrogen atoms of the drug. In parallel, *in vitro* experiments were conducted in human hepatic microsomes and

cytosolic fractions to characterize the drug metabolism enzymes responsible for the formation of metabolites. Preliminary data revealed the formation of hydroxy metabolite in the cytosol but not in the microsomes. The reaction was found to be independent of the presence of nicotinamide adenine dinucleotide phosphate (NADPH) in the incubation media. Addition of a non-selective CYP450 inhibitor (1-amino benzotriazole) inhibited the formation of all the minor metabolites but not the hydroxy metabolite. A comparative mass spectra analysis showed the presence of hydroxy metabolite in the human liver cytosol incubations which was previously found in human plasma. PF-5190457 was also incubated in hepatocytes leading to the formation of major and minor metabolites. Further addition of an aldehyde oxidase inhibitor (hydralazine) in hepatocytes completely inhibited the formation of the hydroxy metabolite indicating the involvement of aldehyde oxidase in the metabolism of the PF-5190457. The hydroxy metabolite was also found in incubations conducted with PF-5190457 and cytosol from different animal species. The aldehyde oxidase metabolite of PF-5190457 was biosynthesized, from female mouse liver cytosol and the structure of the metabolite was confirmed using NMR analysis indicating the hydroxylation position to be on the pyrimidine ring between the two nitrogen atoms. Literature shows both aldehyde and xanthine oxidase share substrate specificity. Specific inhibitors of both the enzymes were used to identify their involvement in the metabolism of the drug. Raloxifene and febuxostat were used as selective aldehyde oxidase and xanthine oxidase inhibitor, in human liver cytosol incubations with PF-5190457 respectively. The data from inhibition study

suggested a role for both the enzymes in the biotransformation of the drug with aldehyde oxidase enzyme being dominant over xanthine oxidase. The kinetic parameters Km and Vmax for hydroxy metabolite formation in human liver cytosol were 42 ± 4 µM and 0.12 ± 0.003 nmol/min/mg protein, respectively.

Aldehyde oxidase (AOX1) is gaining increased attention for its role in the metabolism of new drug candidates. A significant variability in the levels of the AOX1 protein expression in the human liver has been reported in published studies. We assessed the AOX1 levels in a novel repository of human liver (n=104) using mass spectroscopy as described in **Manuscript III** (to be submitted to *Drug Metabolism and Disposition*). The levels of AOX1 were estimated using traditional data-dependent acquisition and upcoming SWATH-MS method. A strong and significant correlation (Spearman r-0.72, *P<0.0001*) was observed between the two methods making a case for the use of SWATH based methods in the field of drug metabolism. Expression levels of AOX1 determined in our liver bank using MaxQuant (proteomics data analysis software) were found to be well within the literature reported range (2 - 49 pmol/mg total cytosolic protein). Further, we evaluated the effect of demographic factors (gender, age, ethnicity, drug usage, alcohol consumption, and smoking) and disease conditions (diabetes, non-alcoholic fatty liver disease) on the AOX1 expression. We found no significant association between demographic factors with the expression of the AOX1 protein. Similarly, disease conditions were not found to influence the expression of AOX1 indicating other unidentified factors influencing the enzyme expression.

Manuscript IV (to be submitted to *British Journal of Clinical Pharmacology*) outlines the pharmacokinetics data via non-compartmental analysis of PF-5190457 and its hydroxy metabolite. The concentrations of analytes from phase 1b trials were evaluated in N=12 patients at placebo, 50 and 100 mg doses using validated LC-MS/MS method as described in Manuscript I. The half-life of the metabolite (~13 h) was found to be longer than the parent drug (~6 h). Additionally, the rate of formation of hydroxy metabolite was studied in cytosol prepared from 10 human liver samples. The rate of formation of hydroxy metabolite was found to be highly variable with a 20.5-fold (0.02 – 0.41 nmol/min/mg protein) variation among the ten samples. Interestingly, the AOX1 protein expression for same samples showed only 2.5-fold variability (16.8 - 44.1 pmol/mg protein).

In summary, an understanding of the enzymes responsible for the metabolism of a drug is essential to avoid drug-drug interactions or the presence of polymorphic enzymes interfering with the therapeutic outcome of the drug. Collectively, we for the first time determined the clinical pharmacokinetics of PF-5190457 and its major metabolite (hydroxy metabolite) in alcoholic patients. Further studies suggested the involvement of aldehyde oxidase (major role) and xanthine oxidase (minor role) in the metabolism of the PF-5190457. Moreover, to examine the variability in the protein expression of the enzymes, we quantified AOX1 in a large liver databank. This work also makes a case for considering non-cytochrome-P450 enzymes in the early screening during drug discovery. Considering the longer half-life of metabolite than the parent, it will be warranted

to investigate the pharmacological properties of hydroxy metabolite in future studies.

ACKNOWLEDGEMENTS

I take this opportunity to extend my sincere thanks and gratitude to all those who made this Ph.D. dissertation possible. First and foremost, I would like to thank my major professor Dr. Fatemeh Akhlaghi for giving me the opportunity to work in her laboratory and having the confidence in me. I am extremely indebted to her for the help, advice, encouragement and continuous support throughout my Ph.D. journey. Without her guidance and persistent support this dissertation would not have been possible. For everything you have done for me, Dr. Akhlaghi, I sincerely thank you.

I thank our collaborator, Dr. R Scott Obach at Pfizer Inc for his valuable advice and suggestions. In addition, I would like to thank Dr. Lorenzo Leggio and his clinical team at the National Institute of Alcohol Abuse and Addiction for providing the clinical samples used in my dissertation I would like to extend sincere thanks to each of my doctoral committee members, Dr. Sara Rosenbaum, Dr. Ingrid Lofgren and Dr. Matthew Delmonico.

I thank my parents Prasad Adusumalli and Bhavani Adusumalli for their support, motivation and prayers and patience all through this time. Also, I cannot thank enough, my younger sister, Pavani, and her husband Anil Velaga who have been a continuous source of motivation and support throughout my life. I also would like to thank my in-laws for always being there.

I thank all my present and past lab members for all the team work and support. I would like to especially thank Shripad, Joyce, Ariel, Armin, Enoch and

Ben for always being there when needed. Special thanks to my lab mate, Rohitash for his support, candid feedback and the fun discussions we had during the lab work. Also, I would like to thank Kathy Hayes, Gerralyn Perry, Kim Andrews and Dr. Al Bach for their help throughout these years. In addition, I would like to extend my sincere thanks to College of Pharmacy at URI for giving me the opportunity to pursue my graduate studies here.

I owe a very big thanks to a very special person, my husband, Ramakrishna Parchuri for his continued support and understanding during my journey for Ph.D. degree. Thank you for being an amazing husband and you are the best. I really really really appreciate my little son Arjun for his patience especially during my dissertation writing. I fall short of words to say how grateful I am to both of you. I would also like to thank all my friends and family members who were very supportive during this journey.

PREFACE

This dissertation was prepared according to the University of Rhode Island Thesis/Dissertation Process: From Proposal to Defense standards for Manuscript format. This dissertation consists of four manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT-I: Analysis and Validation of an Assay for a Novel Ghrelin Receptor Inverse Agonist PF-5190457 and its Major Hydroxy Metabolite by LC-MS/MS in human plasma

This manuscript has been prepared for submission as a research article to Journal of Chromatography B: Biomedical Sciences and Applications.

MANUSCRIPT-II: The Role of Molybdenum-Containing Enzymes in the Biotransformation of PF-5190457 - a Novel Inverse Agonist of Ghrelin Receptor to Treat Alcoholism

This manuscript has been prepared for submission as a research article to Drug Metabolism and Disposition.

MANUSCRIPT-III: Assessment of AOX1 Levels Variability in Human Liver Cytosol with Label-Free Approach Using Mass Spectrometry

This manuscript has been prepared for submission as a research article to Drug Metabolism and Disposition.

MANUSCRIPT-IV: Clinical Pharmacokinetics of a Major Hydroxy Metabolite of PF-5190457, a Ghrelin Receptor Inverse Agonist- Evidence from In vivo and In vitro Studies

This manuscript has been prepared for submission as a research article to British Journal of Clinical Pharmacology for publication.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

MANUSCRIPT-I

This manuscript has been prepared for submission to "Journal of Chromatography B: Biomedical Sciences and Applications"

Analysis and Validation of an Assay for a Novel Ghrelin Receptor Inverse Agonist PF-5190457 and its Major Hydroxy Metabolite by LC-MS/MS in human plasma

Adusumalli S[1], Obach RS[2], Leggio L[3,4,5], Akhlaghi F[1]

1. Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island Kingston, RI 02881
2. Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Worldwide Research and Development, Pfizer, Inc., Groton, Connecticut
3. Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology, Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA
4. Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA
5. Center for Alcohol and Addiction Studies, Department of Behavioral and Social Sciences, Brown University, Providence, RI 02903, USA

Running title: LC-MS/MS Assay for PF-5190457 and its metabolite

Number of words in the text: 2600

Number of words in the abstract: 199

Number of tables: 4

Number of figures: 5

Name and address for correspondence:

Fatemeh Akhlaghi, PhD

Clinical Pharmacokinetics Research Laboratory

Biomedical and Pharmaceutical Sciences

University of Rhode Island

7 Greenhouse Rd, Kingston, RI 02881, USA Phone: (401) 874 9205/ Fax: (401) 874 5787 Email: fatemeh@uri.edu

Abstract

PF-5190457 is a selective and potent ghrelin receptor inverse agonist presently undergoing clinical trials to treat alcoholism. We have established a selective and sensitive liquid chromatography-tandem mass spectrometry method for determination of PF-5190457 and its hydroxy metabolite (PF-6870961) in human plasma and validated the assay according to FDA guidelines. Plasma samples were prepared by simple protein precipitation in methanol using tacrine as an internal standard (IS). Chromatographic separation was carried out on an Acquity UPLC BEH C18 (2.1mm×50mm) column with 1.7 µm particle size and 130 Å pore size column at a flow rate of 0.25 mL/min by a gradient method with a runtime of 11.30 min. Detection was performed using multiple reaction monitoring (MRM) at positive ion mode with two fragments for each analyte. Tacrine was found to be a suitable IS. The calibration curves were linear in the concentration range of 1-1000 ng/mL and 2-250 ng/mL for PF-5190457 and hydroxy metabolite respectively with a correlation coefficient ≥0.996. The inter-assay precision and accuracy results obtained were within the FDA recommended guidelines. The analytes were found to be stable under varied stability conditions. The validated method was utilized to measure plasma concentration of PF-5190457 and its hydroxy metabolite in clinical studies.

Keywords Alcoholism · Bioanalytical methods · Ghrelin · LC-MS/MS · PF-5190457 · Hydroxy metabolite · Pharmacokinetics

Abbreviations

CV Coefficient of variation

IS Internal standard

LLOQ Lower limit of quantification

ME Matrix effect

MeOH Methanol

MRM Multiple reaction monitoring

MS Mass spectrometry

Mw Molecular weight

PLs Phospholipids

QCs Quality controls

UPLC-MS/MS Ultra-performance liquid chromatography-tandem mass spectrometry

1. Introduction

PF-5190457 (Fig. 1) is a novel peripherally acting inverse agonist of ghrelin receptor discovered by Pfizer pharmaceuticals. It is a small molecule with a molecular weight (Mw) 512.67 belonging to a member of spiro-azetidino-piperidine series [1, 2]. Ghrelin, an endogenous peptide released from the stomach, is a ligand for growth hormone secretagogue receptor in the hypothalamus and regulates appetite and food intake in the body [3-5]. Antagonists of ghrelin could be potentially therapeutic to treat type 2 diabetes mellitus, eating disorders, obesity, and alcohol use disorder. They are believed to regulate these disorders by stimulating glucose-dependent insulin secretion, reduce food intake and craving for alcohol consumption respectively [6-12].

Clinical studies of PF-5190457 in healthy volunteers showed the drug is well-tolerated and exhibit linear pharmacokinetics [13]. Following oral administration, PF-5190457 is rapidly absorbed with a T_{max} of 0.3-3 hour and its elimination half-life ($t_{1/2}$) was ~8 h [13]. The metabolism pathway of PF-5190457 suggests cytosolic oxidative reaction involving molybdenum co-factor containing enzymes, aldehyde oxidase, and xanthine oxidase, leading to the formation of a hydroxy metabolite (Figure 1b) (Manuscript II). PF-5190457 is currently undergoing clinical trials for the treatment for alcohol use disorder and type 2 diabetes [8, 14].

Previously developed validated UPLC-MS/MS method in the laboratory did not include the hydroxy metabolite in the assay [14]. The current manuscript

demonstrates a simple and selective ultra performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) for simultaneous determination of PF-5190457 and its hydroxy metabolite (PF-6870961) in human plasma using tacrine as an IS. The present method is established with the aim of determining PF-5190457 and its hydroxy metabolite in plasma samples obtained in clinical trials of patients with alcohol use disorder.

2. Materials and methods

2.1. Chemicals and reagents

PF-5190457 and tacrine (Mw 198.26) were obtained from Sigma-Aldrich (St. Louis, MO, USA). PF-6870961 (hydroxy metabolite, Mw 528.23) was synthesized as described in the Manuscript II. LC-MS grade methanol and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Blank K_2EDTA human plasma from six subjects (three male, three female) was obtained from Bioreclamation (IVT Inc., Westbury, NY, USA).

2.2. LC–MS/MS instrumentation, conditions and data processing

The liquid chromatography separation was performed using an Acquity UPLC system comprising with a binary pump and a built-in column heater. A 10 µL sample loop was used to inject samples in partial loop injection mode. Chromatography separation of analytes and IS was accomplished within 11.30 min using gradient elution method using an Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm particle size, 130Å pore size) analytical column. An Acquity UPLC BEH C18 VanGuard pre-column (2.1 x 5 mm, 130Å) was used prior to the analytical column. Mobile phase consisted of water: methanol 95:5% (v/v) containing 0.1% formic acid (A) and 100% methanol containing 0.1% formic acid (B). The column and autosampler temperature were kept at 40 °C and 10 °C,

respectively. The mobile phase was delivered at 0.25 mL/min flow rate, and the injection volume was 5 µL. The initial conditions of mobile phase (98% A, 2% B) was maintained for 2 min, B was increased from 2% to 80% over 8.30 min, and held till 9.30 min and returned to initial conditions, followed by a 2 min equilibration for a total run time of 11.30 min.

Mass spectral analysis and quantification was carried out using multiple reaction monitoring (MRM) on a positive electrospray ionization mode with the following parameters: desolvation temperature of 350°C, desolvation gas flow 650 L/h, capillary voltage 3.50 kV and cone voltage 20 V. The fragmentation pattern for PF-5190457 and IS were similar to previously reported methods [14-16]. The optimized settings of mass spectrometer voltage and the retention time for analytes and internal standards with quantifier and qualifier fragments are summarized in Table 1.

Data acquisition and processing were performed using MassLynx™ software (V 4.1) and TargetLynx™ tool respectively. Calibration curves were constructed by calculating the analyte to IS peak area ratio against analyte concentrations.

2.3. Preparation of calibration standards, quality controls, and internal standard

Sub stock solutions of PF-5190457 and hydroxy metabolite were separately prepared in 50% methanol to obtain a final concentration of 10 µg/mL and were stored at 4°C. Working stock solutions for calibration and quality controls were

prepared from the stock solution by adequate dilution using diluent (methanol: water, 50:50, v/v). The calibration standards and quality control samples were prepared by spiking appropriate amount of the standard working solutions into pooled K_2EDTA human plasma with an organic solvent concentration less than 5% of the final volume. An eight-point calibration curve with concentrations ranging from 1-1000 ng/mL and 2-250 ng/mL for PF-5190457 and hydroxy metabolite were prepared respectively. Low, medium and high level QC samples were prepared for PF-5190457 (3, 200 and 750 ng/mL) and hydroxy metabolite (5, 25 and 200 ng/mL), respectively. A final working solution of 2.5 ng/mL tacrine (IS) in methanol was prepared.

2.4. Sample extraction procedure

Samples were prepared by a simple and direct protein precipitation. To 0.050 mL of sample was added 0.200 mL of precipitating solution containing IS in methanol. IS in methanol was added to all samples except blank. Control blank was extracted with IS whereas, the double blank was extracted in 100% methanol without any IS. The mixture was vortex mixed for 15 seconds, and then centrifuged at 10,000 rpm for 5 min. The clear supernatant (0.150 mL) was collected for further analysis.

2.5. Method validation

The validation of this assay was performed according to the requirements as outlined in guidelines established by the US Food and Drug Administration (US FDA) to evaluate the method with respect to selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, recovery and stability of analytes [17].

2.5.1. Specificity and Sensitivity

The method specificity was evaluated prior to the main validation batches for the presence of interference from endogenous compounds by screening blank plasma of six donors. The sensitivity of the assay was evaluated by analyzing blank plasma samples from six different donors and spiked plasma samples at the lower limit of quantification (LLOQ) levels of the analytes.

2.5.2. Linearity, accuracy, and precision

The linearity of the method was determined by analysis of standard plots associated with an eight-point calibration curve. Inter-assay precision and accuracy were calculated after the replicates in six different analytical runs. Peak area ratios of analytes to IS obtained from MRM were utilized for the construction of calibration curves, using least squares weighted (1/x) linear regression. Carry over test was assessed to demonstrate any carryover effect of analytes and IS.

2.5.3. Matrix effect and Recovery

The components present in the matrix (plasma) can interfere with ionization efficiency by coeluting with analytes of interest [18]. Matrix effect for ion suppression and enhancement was assessed using blank and spiked human plasma using post column infusion experiment as described previously [14, 19]. The chromatograms can be visually evaluated to verify there are no interfering peaks at the elution time of analytes and IS. Likewise, the interference of phospholipids was determined as they are highly abundant endogenous compounds in biological membranes and can interfere with elution of analytes [20, 21]. The major transitions of phospholipids were monitored with the precursor ion (Q1) at *m/z* 496, 522, 524, 758 and 782 and product ion (Q3) with *m/z* 184 in the method [22]. Recovery was determined at two concentration levels (LQC and HQC) by comparing the peak area of the analytes from the pre-extracted plasma standard with that obtained from post-extracted standard at the same concentration for the QC samples.

2.5.4. Stability

Stability of analytes were evaluated at two concentrations (LQC and HQC). The freeze–thaw stability was evaluated after undergoing three freeze thaw cycles, benchtop stability for 6 h and short-term stability was assessed after storage of the test samples at −80°C for 1 week. The autosampler storage stability was determined by storing the QC samples for ~48 h under autosampler condition (maintained at 10 C) before being analyzed.

2.5.5. Sample Reanalysis

The results from previously validated UPLC-MS/MS method for determination of PF-5190457 in patient plasma samples (n=45) by the laboratory was correlated with the current method to test the assay's ability to reproduce the data [14]. Plasma samples were obtained after oral administration of PF-5190457 from phase 1b clinical study (NCT02039349) in alcoholic patients conducted at the clinical research center (NIH clinical center at Bethesda, MD, USA). Incurred sample reanalysis (ISA) was performed to corroborate the results utilizing clinical samples. Samples (10% of the study size) around Cmax and terminal elimination phase were selected, reanalyzed and compared to the initial results obtained from clinical samples (described below). The results of repeated analysis were compared with original data, according to the following formula: (repeated sample − original sample) × 100/mean value. Two-thirds of the samples reanalyzed should be within ±20% between the two analyses [23].

3. Results and Discussion

3.1. LC-MS assay

The goal of this work was to develop and validate a simple and sensitive assay method for the simultaneous determination of PF-5190457 and its hydroxy metabolite in human plasma. The MS optimization was performed by direct infusion of solutions of PF-5190547, hydroxy metabolite and IS into the ESI source of the mass spectrometer. The best signal for both the analytes and IS was achieved using a positive ion mode. Protonated adducts for PF-5190547 (m/z 513) and hydroxy metabolite (m/z 529) and their respective high intensity fragments (m/z 209 and m/z 225) were selected respectively for quantification.

Several trials for chromatographic conditions were performed especially to select an optimal mobile phase and analytical C18 columns. These trials determined that the present method achieved an optimal degree of chromatographical separation for simultaneous separation of different hydroxy metabolites as well as the parent drug.

3.2. Sample preparation optimization:

A simple protein precipitation technique was utilized for the extraction of analytes and IS from the plasma samples. Acetonitrile, methanol, DMSO, 50% DMSO, 50% acetonitrile, 50% methanol mixture were investigated as organic solvents for stock solution preparation. It was noticed that the metabolite was

unstable in acetonitrile. Methanol was then chosen as the organic solvent for preparation of stock solution for the analytes and IS as well as only methanol was used in mobile phase.

3.3. Selection of internal standard

The stable isotopically labeled IS was not available for hydroxy metabolite and therefore we have demonstrated the suitability of readily available, cost-effective compounds with no evidence of cross-talk with the analytes of interest in the current assay. Various compounds with similar LogP and Pka to that of analytes like indoprofen, chloroxazane, naproxen, tacrine and rifampicin were tested for selection of suitable IS for the assay. Tacrine was deemed to be the most suitable IS for quantification of PF-5190457 and hydroxy metabolite as it ionizes in the same polarity along with the respective analytes.

3.4. Method Validation

3.4.1. Specificity and selectivity

The determination of PF-5190547, hydroxy metabolite and IS at their respective retention time were not affected by interference from co-extracted matrix components under the present LC–MS/MS conditions used in blank

plasma obtained from six different donors. Sensitivity was determined by comparing the baseline from blank plasma obtained from six different donors in comparison to that spiked with analytes at LLOQ. There was no interference observed from endogenous compounds. The representative extracted ion chromatograms of the analytes and the IS in human plasma are shown in Figure 2.

3.4.2. Linearity, Precision and Accuracy

Calibration was found to be linear over the concentration range of 1-1000 ng/mL for PF-5190547 and 2-250 ng/mL for hydroxy metabolite. The curve was fitted with linear regression using 1/x weighing function. The percentage deviation for PF-5190547 and hydroxy metabolite was less than 8.2% and 12.2% respectively (Table 2). The accuracy ranged from 96.2% to 113.2% for PF-5190547 and 88.0% to 106.6% for hydroxy metabolite. The correlation coefficients (r^2) for PF-5190547 and hydroxy metabolite were greater than 0.997 and 0.996 respectively for all curves. Precision and accuracy for this method was controlled by calculating the inter-batch variations of LLOQ and QC samples at four concentrations for PF-5190547 (1, 3, 200 and 750 ng/mL) and hydroxy metabolite (2, 5, 25 and 200 ng/mL) as shown in Table 3. The inter-day precision of 2.4–7.4% for PF-5190547 and 3.6–6% for hydroxy metabolite. This method demonstrated inter-day accuracy 88–104.2% for PF-5190547 and 97.2–109.7% for the hydroxy metabolite. There was no between-sample carryover detected following the double blank injections.

3.4.3. Matrix effect and Recovery

In the present study, the determination of PF-5190547 and hydroxy metabolite is not affected by co-extracted matrix components under the LC–MS/MS conditions used Figure 3a. There was no interference from the phospholipids on the elution of analytes Figure 3b. The percent mean recovery for PF-5190547 and hydroxy metabolite ranged from 95-103%.

3.4.4. Stability

The data representing the stability in plasma at two concentration levels (LQC and HQC) for PF-5190547 and hydroxy metabolite for bench top stability, three freeze and thaw cycles, autosampler and short-term stability are given in Table 4. These results indicate that the analytes are stable in human plasma as the mean of the results of the tested samples were within the acceptance criteria of ± 15% of the nominal concentrations. These findings suggest no stability-related problems would be expected during pharmacokinetic studies and analysis.

3.4.5. Sample Reanalysis

The patient samples (n=45) previously analyzed by UPLC-MS/MS method and subsequently analyzed by the current method were within ± 15.2% for PF-5190457. The data generated by this method is reproducible despite differing the LC-MS/MS conditions. Method reproducibility was also determined by ISA (n=45)

following the FDA Guidance for Industry for parent and metabolite. More than two-thirds of the sample were within ± 20%. The incurred sample analysis indicates that both parent and its metabolite are stable within human plasma from subjects. Bland- Altman plot is recommended over Pearson's or Spearman's correlation coefficient for correlation analysis for ISA as the range of data can influence the correlation [23]. The correlation between both the LC-MS/MS methods and ISA for PF-5190457 and hydroxy metabolite are depicted via Bland–Altman plots in Figure 4(a)-(c).

3.5. Method Application

The present validated method has been successfully applied to measure the concentration of PF-5190547 and its hydroxy metabolite after oral administration (50 mg and 100 mg) in human plasma obtained from phase 1b pharmacokinetics alcoholic patients admited to the clinical research center (NIH clinical center at Bethesda) for three visits (placebo, a dose of 50 mg and a dose of 100 mg). The study was approved by the University of Rhode Island Institutional Review Board HU# 123.172. The participants have filled out the informed consent form prior to the study. The plasma concentration ranges of PF-5190547 and hydroxy metabolite were measurable within their calibration curves. Figure 5 represents plasma concentration time profile after an oral dose of 50 mg dose in a study subject.

4. Conclusion

In this manuscript, we describe development of a sensitive LC–MS/MS for the accurate, precise and reliable measurement of PF-5190547 and its hydroxy metabolite in human plasma. This study demonstrates the suitability of tacrine as IS for accurate quantification of PF-5190547 and hydroxy metabolite. This method demonstrated adequate reliability and reproducibility of the results within the analytical range. We have shown that PF-5190547 and hydroxy metabolite are stable in human plasma under different storage conditions. This assay was successfully employed for determination concentration of PF-5190457 and the metabolite in a phase 1b clinical trial.

Conflict of interest

Dr. Obach is an employee of Pfizer Pharmaceuticals. Other authors declare no potential conflict of interest with any commercial entity whose products were used in the study.

Acknowledgements

The financial support of Grant #UH3TR000963 from National Institutes of Health is gratefully acknowledged.

References

1. Bhattacharya SK, Andrews K, Beveridge R, Cameron KO, Chen C, Dunn M, et al. Discovery of PF-5190457, a Potent, Selective, and Orally Bioavailable Ghrelin Receptor Inverse Agonist Clinical Candidate. ACS Med Chem Lett. 2014 May 8;5(5):474-9.
2. Orr ST, Beveridge R, Bhattacharya SK, Cameron KO, Coffey S, Fernando D, et al. Evaluation and synthesis of polar aryl- and heteroaryl spiroazetidine-piperidine acetamides as ghrelin inverse agonists. ACS Med Chem Lett. 2015 Feb 12;6(2):156-61.
3. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, et al. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology. 2001 Feb;120(2):337-45.
4. Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, et al. Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. FASEB J. 2004 Mar;18(3):439-56.
5. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999 Dec 9;402(6762):656-60.
6. Rudolph J, Esler WP, O'Connor S, Coish PD, Wickens PL, Brands M, et al. Quinazolinone derivatives as orally available ghrelin receptor antagonists for the treatment of diabetes and obesity. J Med Chem. 2007 Oct 18;50(21):5202-16.
7. Hanrahan P, Bell J, Bottomley G, Bradley S, Clarke P, Curtis E, et al. Substituted azaquinazolinones as modulators of GHSr-1a for the treatment of type II diabetes and obesity. Bioorg Med Chem Lett. 2012 Mar 15;22(6):2271-8.
8. Kong J, Chuddy J, Stock IA, Loria PM, Straub SV, Vage C, et al. Pharmacological characterization of the first in class clinical candidate PF-05190457: a selective ghrelin receptor competitive antagonist with inverse agonism that increases vagal afferent firing and glucose-dependent insulin secretion ex vivo. Br J Pharmacol. 2016 May;173(9):1452-64.
9. Kaur S, Ryabinin AE. Ghrelin receptor antagonism decreases alcohol consumption and activation of perioculomotor urocortin-containing neurons. Alcohol Clin Exp Res. 2010 Sep 01;34(9):1525-34.
10. Leggio L, Zywiak WH, Fricchione SR, Edwards SM, de la Monte SM, Swift RM, et al. Intravenous ghrelin administration increases alcohol craving in alcohol-dependent heavy drinkers: a preliminary investigation. Biol Psychiatry. 2014 Nov 1;76(9):734-41.
11. Yu M, Lizarzaburu M, Beckmann H, Connors R, Dai K, Haller K, et al. Identification of piperazine-bisamide GHSR antagonists for the treatment of obesity. Bioorg Med Chem Lett. 2010 Mar 01;20(5):1758-62.
12. Suchankova P, Steensland P, Fredriksson I, Engel JA, Jerlhag E. Ghrelin receptor (GHS-R1A) antagonism suppresses both alcohol consumption and the alcohol deprivation effect in rats following long-term voluntary alcohol consumption. PLoS One. 2013;8(8):e71284.

13. Denney WS, Sonnenberg GE, Carvajal-Gonzalez S, Tuthill T, Jackson VM. Pharmacokinetics and pharmacodynamics of PF-05190457: The first oral ghrelin receptor inverse agonist to be profiled in healthy subjects. Br J Clin Pharmacol. 2017 Feb;83(2):326-38.
14. Ghareeb M, Leggio L, El-Kattan A, Akhlaghi F. Development and validation of an UPLC-MS/MS assay for quantitative analysis of the ghrelin receptor inverse agonist PF-5190457 in human or rat plasma and rat brain. Anal Bioanal Chem. 2015 Jul;407(19):5603-13.
15. Camacho-Munoz D, Kasprzyk-Hordern B. Multi-residue enantiomeric analysis of human and veterinary pharmaceuticals and their metabolites in environmental samples by chiral liquid chromatography coupled with tandem mass spectrometry detection. Anal Bioanal Chem. 2015 Dec;407(30):9085-104.
16. Ponnayyan Sulochana S, Ravichandiran V, Mullangi R, Sukumaran SK. Highly Sensitive LC-MS-MS Method for the Determination of Tacrine in Rat Plasma: Application to Pharmacokinetic Studies in Rats. J Chromatogr Sci. 2016 Mar;54(3):397-404.
17. US FDA hwfgddggup. Bioanalytical method validation, Guidance for Industry,. 2013 [cited; Available from:
18. Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. Clin Biochem. 2005 Apr;38(4):328-34.
19. Bonfiglio R, King RC, Olah TV, Merkle K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun Mass Spectrom. 1999 Jun;13(12):1175-85.
20. Carmical J, Brown S. The impact of phospholipids and phospholipid removal on bioanalytical method performance. Biomed Chromatogr. 2016 May;30(5):710-20.
21. Little JL, Wempe MF, Buchanan CM. Liquid chromatography-mass spectrometry/mass spectrometry method development for drug metabolism studies: Examining lipid matrix ionization effects in plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2006 Apr 03;833(2):219-30.
22. Macwan JS, Ionita IA, Dostalek M, Akhlaghi F. Development and validation of a sensitive, simple, and rapid method for simultaneous quantitation of atorvastatin and its acid and lactone metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Anal Bioanal Chem. 2011 Apr;400(2):423-33.
23. Rocci ML, Jr., Devanarayan V, Haughey DB, Jardieu P. Confirmatory reanalysis of incurred bioanalytical samples. AAPS J. 2007 Oct 05;9(3):E336-43.

Tables Legend

Table 1. Mass spectrometry parameters for analytes and IS

Table 2. Summary of calibration standards from six validation runs

Table 3. Summary of QC samples from six validation runs

Table 4. Summary of stability studies

Figures Legend

Figure 1 (a) and (b). Structure of PF-5190457 and Hydroxy metabolite.

Figure 2. Representative chromatograms of (a) drug-free plasma sample (b) plasma sample extracted with IS and (c) plasma sample spiked at lower limit of quantification for PF-5190457 and hydroxy metabolite, and extracted with 2.5 ng/mL of tacrine respectively.

Figure 3(a). Chromatogram obtained with post column infusion shows no matrix effect at the retention times of analytes and IS. Arrows indicates region where the signal of compounds infused post-column is suppressed during the elution of endogenous matrix components **(b)** Chromatogram showing the peaks of analytes and corresponding IS along with key phospholipids.

Figure 4. Bland–Altman plots for **(a).** the correlation of PF-5190457 samples (n=45) analyzed by LC-MS/MS by method 1 vs. the current LC/MS/MS Dashed lines represent 95% limits of agreement (lower = −13.7%, upper = 13%) **(b) and (c)** ISA (Incurred sample reanalysis) showing the correlation for original vs.

repeat analysis for PF-5190457 (lower = −2.7%, upper = 22.7%) and hydroxy metabolite (lower = −22%, upper = 11%) respectively.

Figure 5. Representative graph showing plasma concentration time profiles for parent (PF-5190547) and hydroxy metabolite after the administration of 50 mg dose respectively in a study volunteer.

Table I-1.

Mass spectrometry parameters for analytes and IS

Compound	**Parent Ion (m/z)**	**Daughter ion (m/z)**	**Dwell (s)**	**Cone (V)**	**Collision energy (V)**	**Retention Time (min)**
PF-5190457	513	209, 335	0.025	30, 30	44, 16	4.4
Hydroxy metabolite	529	225, 351	0.025	34, 32	40, 20	3.8
Tacrine	199	170, 143	0.025	46, 42	30, 36	4.6

Table I-2.

Summary of calibration standards from six validation runs

PF-5190457	**Nominal Conc. (ng/mL)**	**Mean**	**SD**	**% Bias**	**CV**	**%Accuracy**
STD 1	1	1	0	0.9	3.9	99.1
STD 2	2	1.9	0.2	-4.8	8.2	104.8
STD 3	10	11.2	0.4	12	3.7	88
STD 4	100	108.8	3.8	8.8	3.5	91.2
STD 5	250	266.8	8.4	6.7	3.2	93.3
STD 6	500	521.2	10.2	4.2	2	95.8
STD 7	800	822.3	20.5	2.8	2.5	97.2
STD 8	1000	936.4	20.3	-6.4	2.2	106.4
r2		0.997			0.2	
Hydroxy metabolite	**Nominal Conc. (ng/mL)**	**Mean**	**SD**	**% Bias**	**CV**	**%Accuracy**
STD 1	2	2.1	0.1	3.4	5.8	96.6
STD 2	3.9	3.4	0.1	-12.9	2.5	112.9
STD 3	7.8	6.9	0.8	-11.7	12.2	111.7
STD 4	15.6	13.5	0.8	-13.2	5.8	111.6
STD 5	31.5	28.3	0.9	-10.1	3	110.1
STD 6	62.5	58.4	3.4	-6.6	5.7	106.6
STD 7	125	127.6	1.6	2.1	1.2	97.9
STD 8	250	256.7	2.4	2.7	0.9	97.3
r2		0.996			0.3	

n=6 (one replicate for each of the six validation runs), %Accuracy=100−[(mean-nominal)/nominal] *100, %CV calculated as (SD/mean) *100, %bias calculated as 100×(mean−nominal)/nominal

Table I-3.

Summary of QC samples from six validation runs

Analyte		LLOQ	QC1	QC2	QC3
PF-5190457	Nominal Conc (ng/mL)	1.0	3.0	200.0	750.0
	Inter-run Mean	1.0	2.9	224.0	776.6
	Inter-run SD	0.1	0.2	5.3	28.3
	Inter-run % Bias	4.9	-4.2	12.0	3.5
	Inter-run CV	11.4	7.6	2.4	3.6
	%Accuracy	95.1	104.2	88.0	96.5
		LLOQ	QC1	QC2	QC3
Hydroxy metabolite	Nominal Conc (ng/mL)	2.0	5.0	25.0	200.0
	Inter-run Mean	2.0	4.9	22.6	205.5
	Inter-run SD	0.1	0.2	0.8	9.8
	Inter-run % Bias	2.1	-2.6	-9.7	2.8
	Inter-run CV	6.0	4.7	3.6	4.8
	%Accuracy	97.9	102.6	109.7	97.2

n=18 (three replicates for each of the six validation runs), %Accuracy=100−[(mean-nominal)/nominal] *100, %CV calculated as (SD/mean) *100, %bias calculated as 100×(mean−nominal)/nominal

Table I-4.

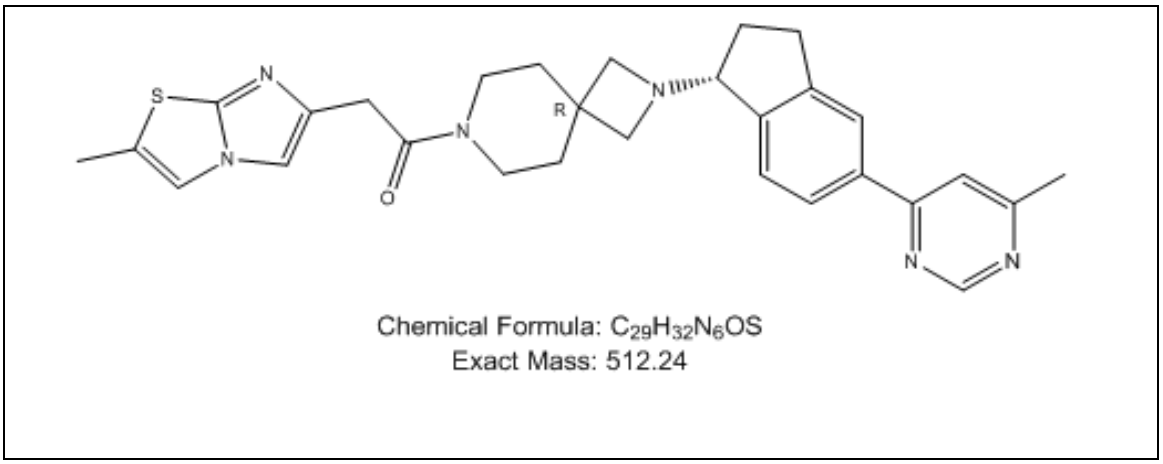
Summary of stability studies

		Bench top (6h)		**Freeze thaw (3 cycles)**		**Autosampler (36h)**		**Short-term (1 week)**	
		LQC	***HQC***	***LQC***	***HQC***	***LQC***	***HQC***	***LQC***	***HQC***
PF-5190457	Mean	3.0	795.0	2.7	782.2	2.8	759.1	3.0	759.3
	% Bias	0.3	6.0	-9.4	4.3	-5.3	1.2	-0.8	1.2
	% CV	9.6	3.7	6.4	4.2	2.2	3.1	2.1	3.1
Hydroxy Metabolite	Mean	5.0	215.2	4.8	222.3	-1.5	211.0	4.2	220.0
	% Bias	-3.9	7.6	-5.9	11.1	-3.3	5.5	1.0	10.0
	% CV	7.7	2.8	7.0	0.9	8.2	3.8	4.6	0.8

%CV calculated as (SD/mean) *100, %bias calculated as

100×(mean−nominal)/nominal

LQC and HQC for PF-5190547and hydroxy metabolite are 3 and 750 ng/mL, 5 and 200 ng/mL respectively



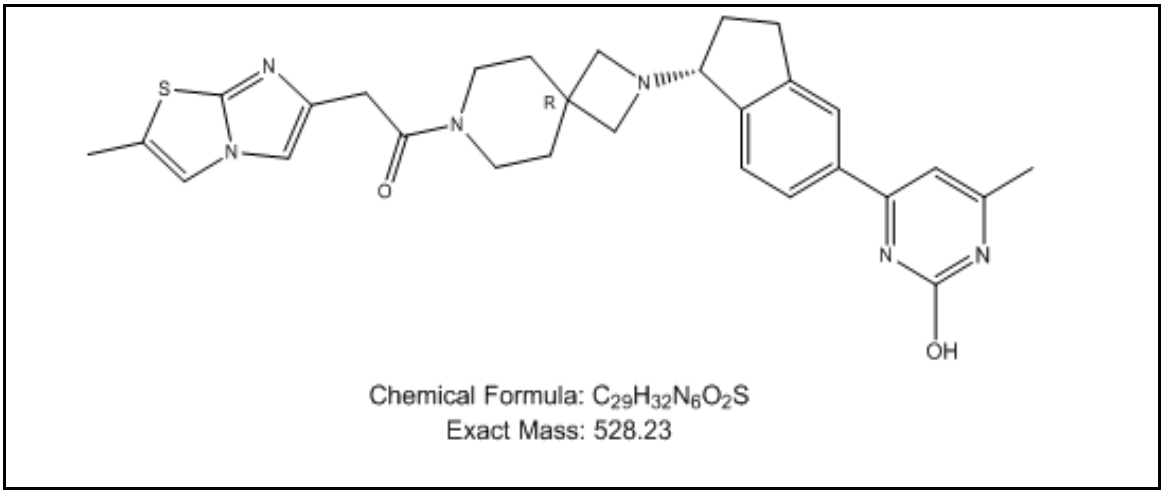
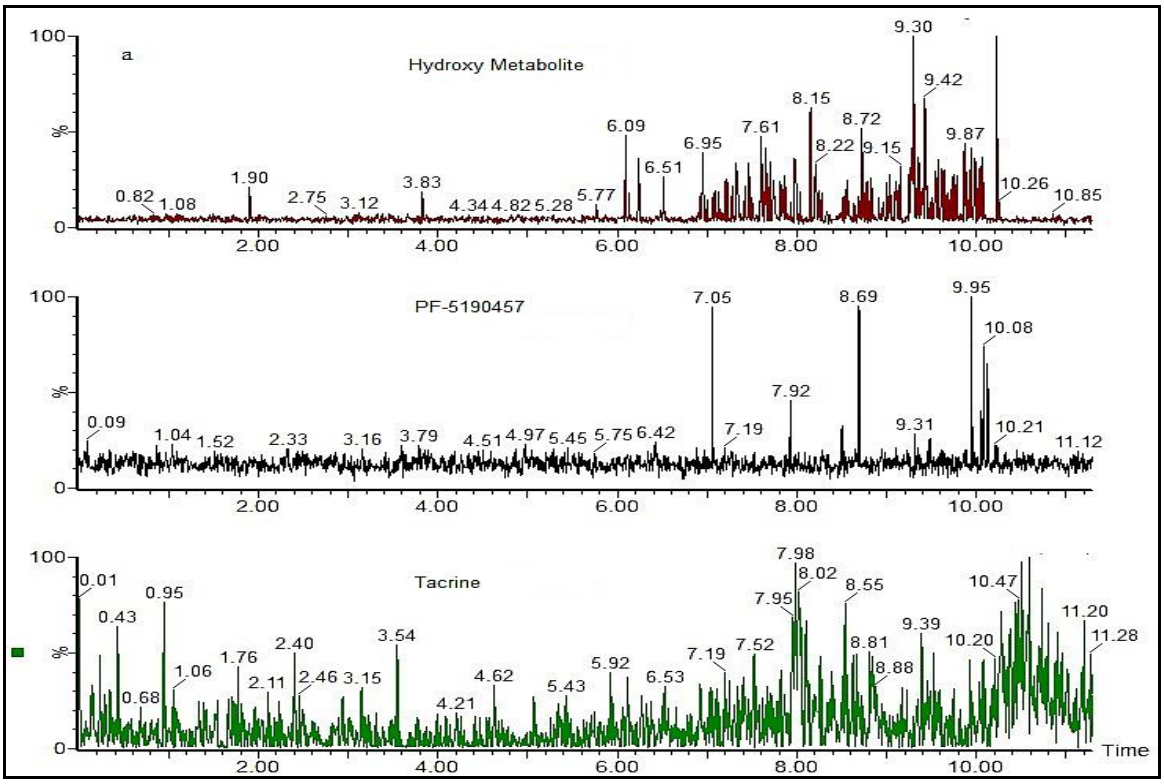
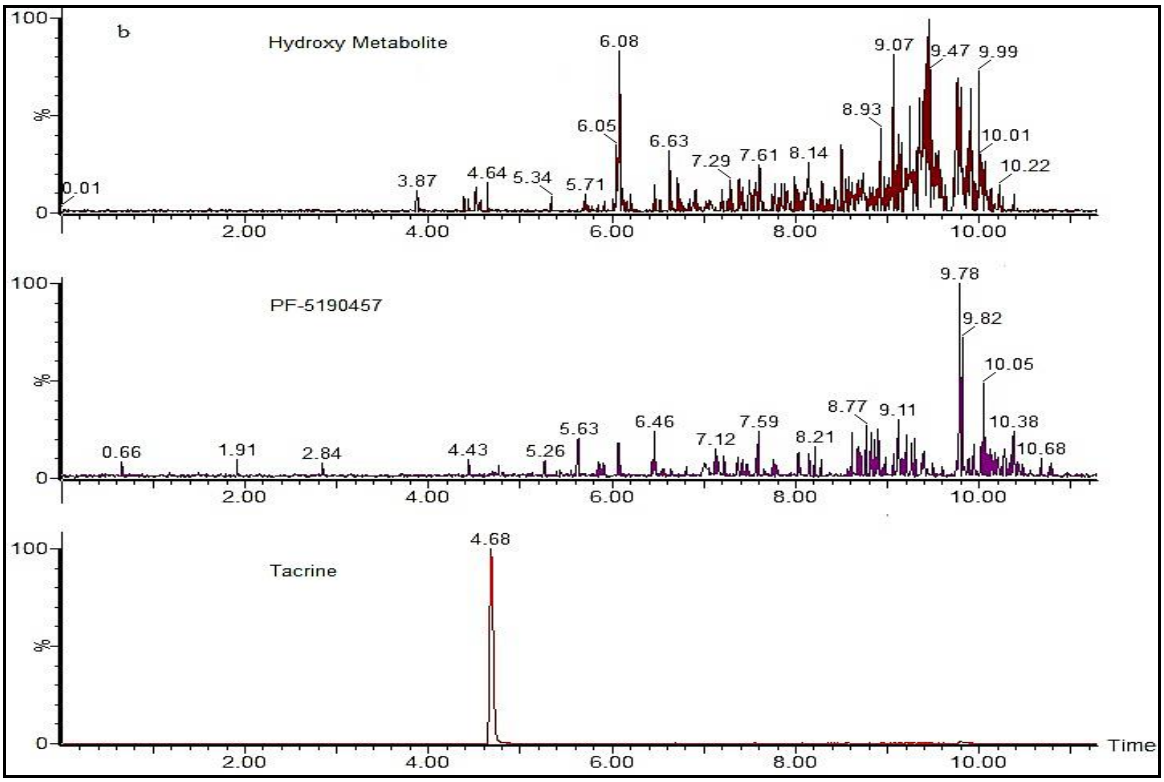


Figure I-1 (a) and (b). Structure of PF-5190457 and hydroxy metabolite


a
Hydroxy Metabolite
PF-5190457
Tacrine
%
100
0
Time
2.00
4.00
6.00
8.00
10.00
0.82
1.08
1.90
2.75
3.12
3.83
4.34
4.82
5.28
5.77
6.09
6.51
6.95
7.61
8.15
8.22
8.72
9.15
9.30
9.42
9.87
10.26
10.85
0.09
1.04
1.52
2.33
3.16
3.79
4.51
4.97
5.45
5.75
6.42
7.05
7.19
7.92
8.69
9.31
9.95
10.08
10.21
11.12
0.01
0.43
0.68
0.95
1.06
1.76
2.11
2.40
2.46
3.15
3.54
4.21
4.62
5.43
5.92
6.53
7.19
7.52
7.95
7.98
8.02
8.55
8.81
8.88
9.39
10.20
10.47
11.20
11.28


b
Hydroxy Metabolite
6.08
9.07
9.47
9.99
8.93
6.05
6.63
10.01
7.29
7.61
8.14
10.22
0.01
3.87
4.64
5.34
5.71
PF-5190457
9.78
9.82
10.05
5.63
6.46
7.59
8.77
9.11
10.38
0.66
1.91
2.84
4.43
5.26
7.12
8.21
10.68
Tacrine
4.68
Time
100
%
0
2.00
4.00
6.00
8.00
10.00

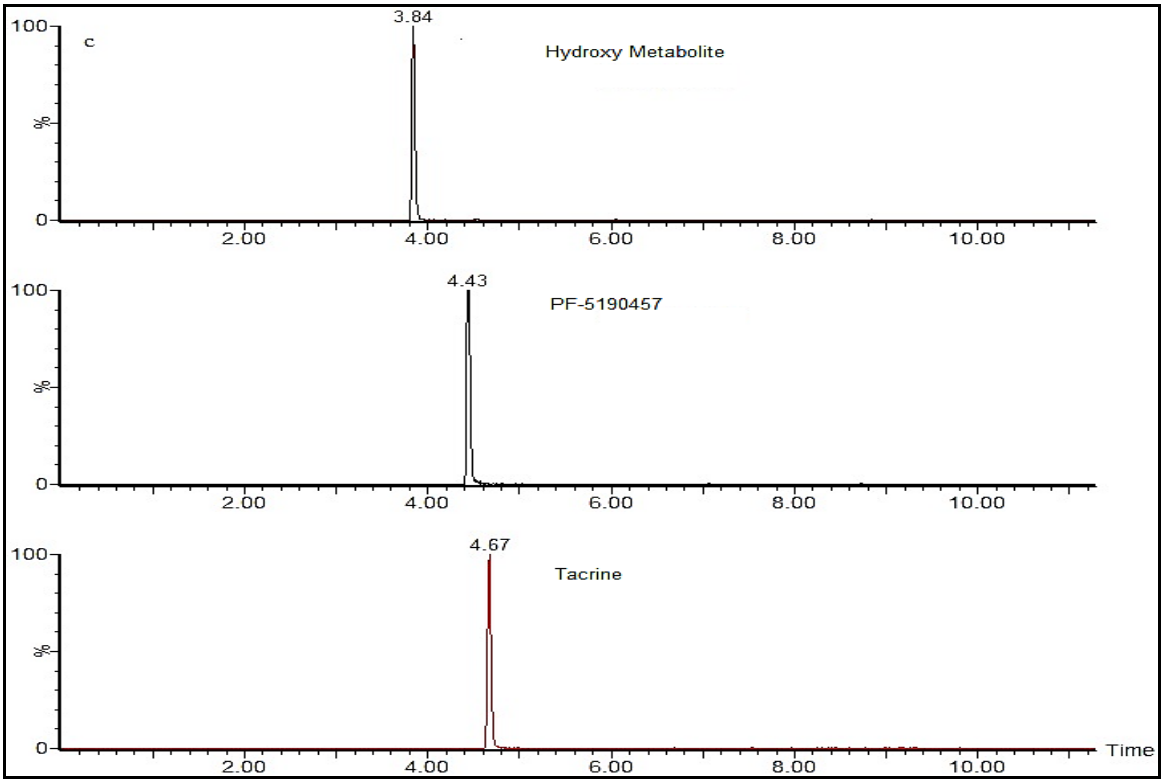
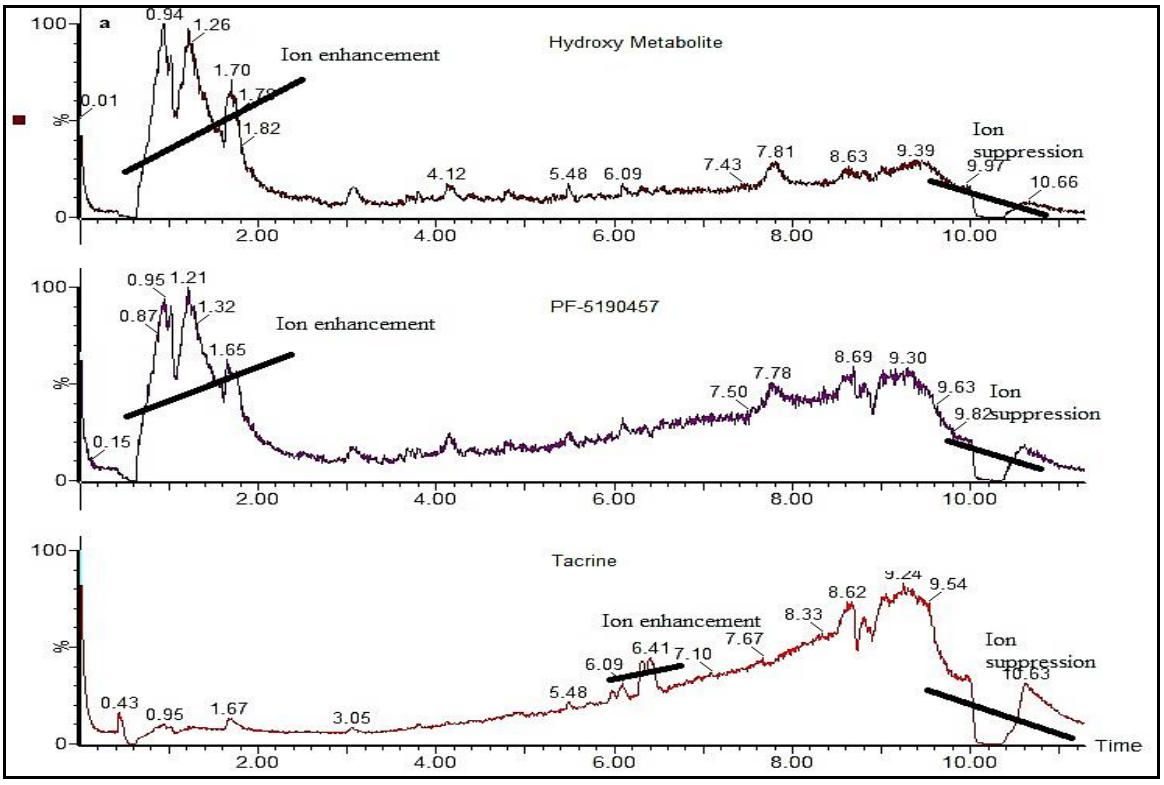

c
3.84
Hydroxy Metabolite
4.43
PF-5190457
4.67
Tacrine
Time
100
%
0
2.00
4.00
6.00
8.00
10.00

Figure I-2. Representative chromatograms of (a) drug-free plasma sample (b) plasma sample extracted with IS and (c) plasma sample spiked at lower limit of quantification for PF-5190457 and hydroxy metabolite, and extracted with 2.5 ng/mL of tacrine respectively.


a
Hydroxy Metabolite
Ion enhancement
Ion suppression
PF-5190457
Ion enhancement
Ion suppression
Tacrine
Ion enhancement
Ion suppression
Time

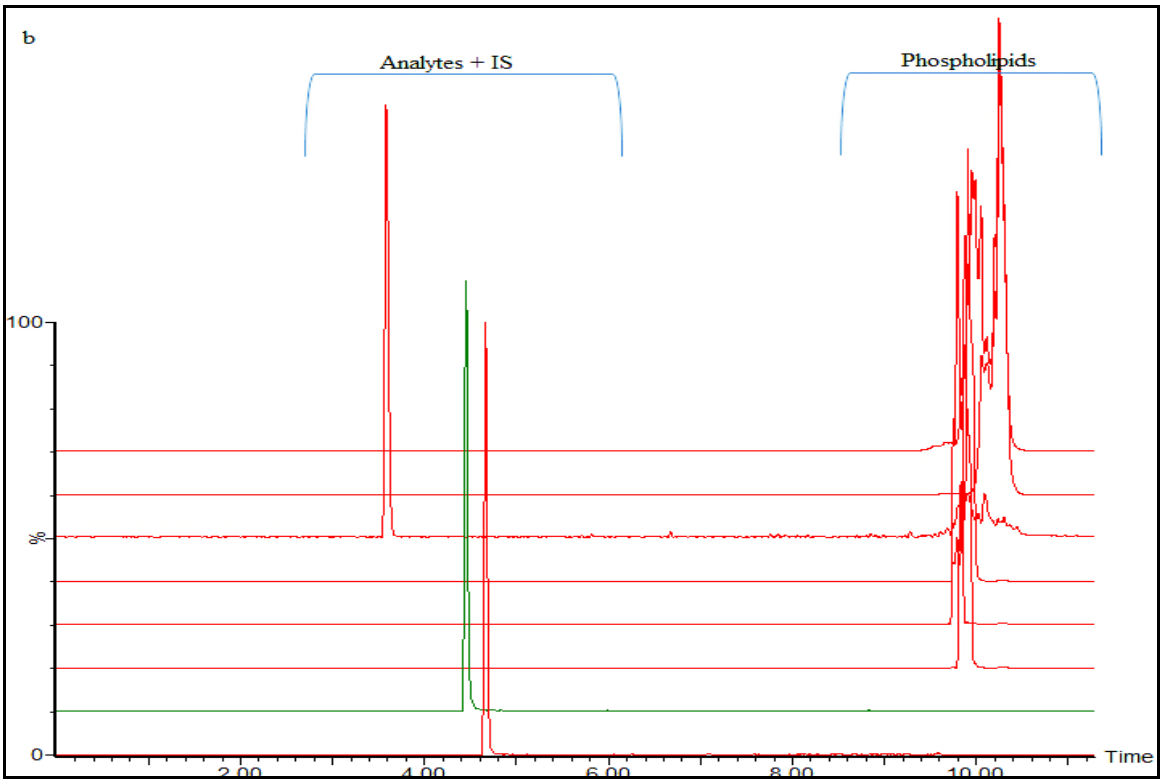
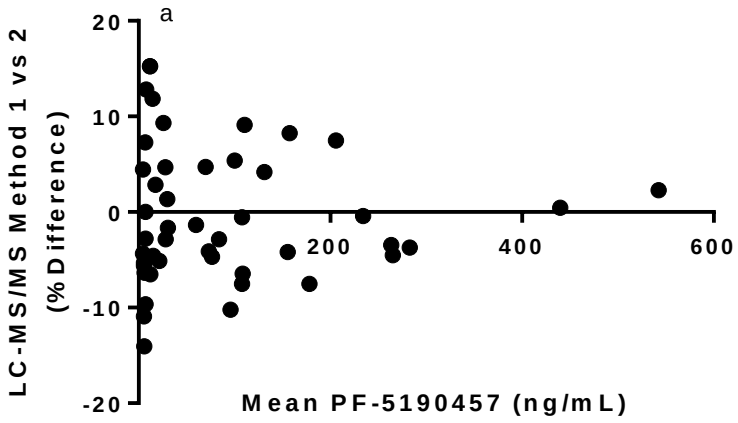
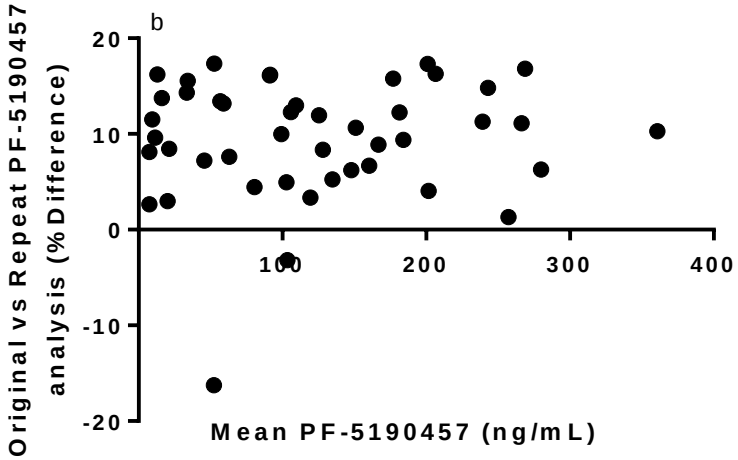

b
Analytes + IS
Phospholipids
Time

Figure I-3(a). Chromatogram obtained with post column infusion shows no matrix effect at the retention times of analytes and IS. Arrows indicates region where the signal of compounds infused post-column is suppressed during the elution of endogenous matrix components **(b)** Chromatogram showing the peaks of analytes and corresponding IS along with key phospholipids


a
LC-MS/MS Method 1 vs 2 (% Difference)
20
10
0
-10
-20
200
400
600
Mean PF-5190457 (ng/mL)


b
Original vs Repeat PF-5190457 analysis (% Difference)
20
10
0
-10
-20
100
200
300
400
Mean PF-5190457 (ng/mL)

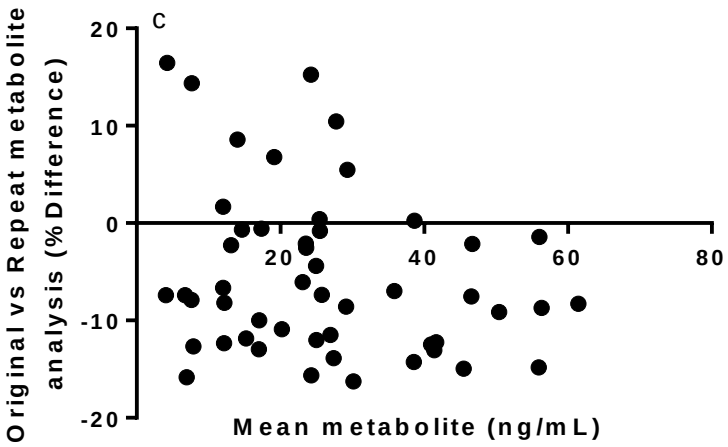


Figure I-4 Bland–Altman plots for **(a).** the correlation of PF-5190457 samples (n=45) analyzed by LC-MS/MS by method 1 vs. the current LC/MS/MS **(b) and (c)** ISA (Incurred sample reanalysis) showing the correlation for original vs. repeat analysis for PF-5190457 and hydroxy metabolite respectively. All the plots indicate the percentage difference between the concentrations of analytes measured were within ±20%.

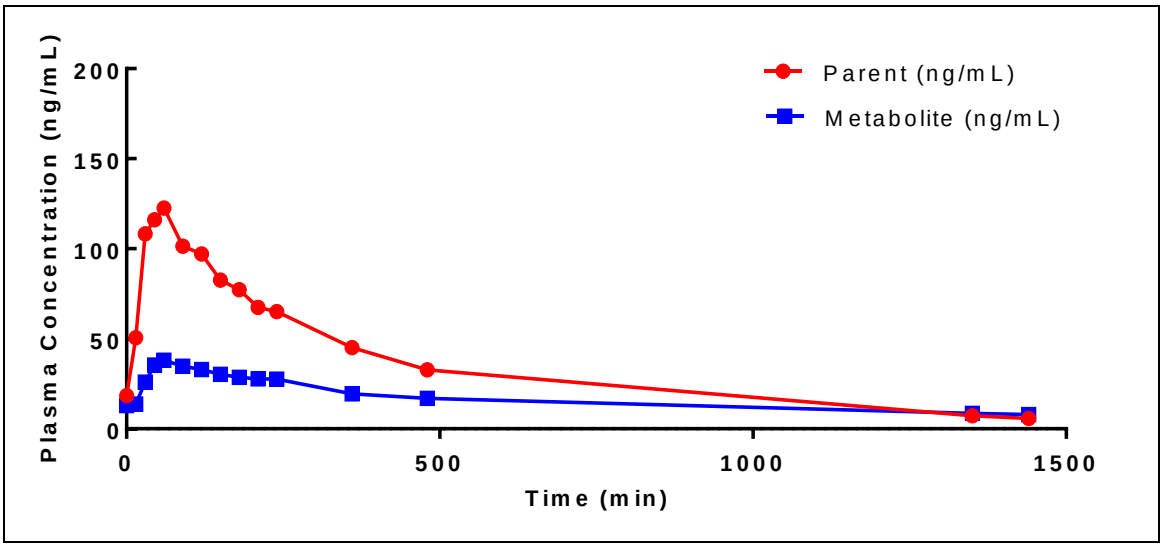


Figure I-5. Representative graph showing plasma concentration time profiles for parent (PF-5190547) and hydroxy metabolite after the administration of 50 mg dose respectively in a study volunteer.

MANUSCRIPT-II

This manuscript has been prepared for submission to “Drug Metabolism and Disposition”

The Role of Molybdenum-Containing Enzymes in the Biotransformation of PF-5190457 - a Novel Inverse Agonist of Ghrelin Receptor to Treat Alcoholism

Sravani Adusumalli, R. Scott Obach, Tim Ryder, Lorenzo Leggio, Fatemeh Akhlaghi

Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island Kingston, RI 02881 (A.S., A.F.), Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Worldwide Research and Development, Pfizer, Inc., Groton, Connecticut (O.R.,R.T.), Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology, Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA (L.L.), Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA (L.L.), Center for Alcohol and Addiction Studies, Department of Behavioral and Social Sciences, Brown University, Providence, RI 02903, USA (L.L.)

Running Title: Biotransformation of PF-5190457

Name and address for correspondence:

Fatemeh Akhlaghi, PhD

Clinical Pharmacokinetics Research Laboratory

Biomedical and Pharmaceutical Sciences

University of Rhode Island

7 Greenhouse Rd, Kingston, RI 02881, USA Phone: (401) 874 9205

Fax: (401) 874 5787 Email: fatemeh@uri.edu

Number of Text Pages: 45

Number of Tables: 1

Number of Figures: 10

Number of References: 58

Number of Words in Abstract: 170

Number of Words in Introduction: 545

Number of Words in Discussion: 1085

Number of supplemental figures: 3

Abbreviations: AO - Aldehyde Oxidase, IS - Internal standard, HLC - Human liver cytosol, HLM - Human liver microsomes, rAOX - Recombinant aldehyde oxidase, XO - Xanthine Oxidase, ABT- 1 amino benzotriazole

Abstract

PF-5190457 ((R)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)-1-(2-(5-(6-methylpyrimidin-4-yl)-2,3-dihydro-1H-inden-1-yl)-2,7-diazaspiro[3.5]nonan-7-yl) ethan-1-one), was identified as a potent and selective ghrelin receptor inverse agonist. The present study characterizes the routes of biotransformation of this compound *in vivo* and then further explores *in vitro* metabolism using subcellular fractions of human liver and primary hepatocytes. Following oral administration of PF-5190457, hydroxyl metabolites were observed, including one that had not been observed in human liver microsomal incubations. This major hydroxy metabolite was biosynthesized using liver cytosol and the site of hydroxylation was shown to be on the pyrimidine using nuclear magnetic resonance spectroscopy. The chemical inhibitors of AO (raloxifene) and XO (febuxostat) inhibited the formation of the major hydroxy metabolite in human liver cytosol. Both the enzymes were involved in the metabolism of the drug whereas, greater inhibition was observed to be achieved using AO inhibitor indicating AO to be a dominant enzyme. The Cl_{int} of the drug in human liver cytosol was 0.002 mL/min/mg protein. This study demonstrates the importance of considering the molybdenum-containing oxidases during the development of new drug entities.

Introduction

Ghrelin is a 28 amino acid peptide released from the stomach fundus and has a key role in growth and energy balance in the body (Korbonits et al., 2004). It is an endogenous ligand binding to the growth hormone secretagogue receptor in the hypothalamus resulting in increased food intake and decreased energy expenditure (Kojima et al., 1999; Asakawa et al., 2001; Inui et al., 2004). Leptin has an opposite effect to that of ghrelin and both the hormones together regulate energy homeostasis in the body (Yildiz et al., 2004). Ghrelin is used as an orexigenic agent to stimulate food intake as shown in clinical studies (Nagaya et al., 2004; Neary et al., 2004; Nagaya et al., 2005). Moreover, intravenous ghrelin administration suppresses glucose dependent insulin secretion in healthy subjects (Tong et al., 2010). It has been observed that neurotransmitters and peptides regulating food intake can also influence craving for alcohol in alcoholic individuals (Leggio et al., 2011; Volkow et al., 2013). In a human study, intravenous administration of ghrelin (3 mcg/kg in 45 subjects) increased the craving towards alcohol in alcohol dependent heavy drinkers (Leggio et al., 2014). Furthermore, in a rat study, it was demonstrated that antagonizing ghrelin receptor could prove as a potential target to treat alcohol addiction (Suchankova et al., 2013). Thus, inhibition of ghrelin and growth hormone secretagogue system can potentially lead to the discovery of novel pharmacological treatments for alcoholism.

PF-5190457 ((R)-2-(2-methylimidazo[2,1-b]thiazol-6-yl)-1-(2-(5-(6-methyl-

pyrimidin-4-yl)-2,3-dihydro-1H-inden-1-yl)-2,7-diazaspiro[3.5]nonan-7-yl)ethan-1-one; Fig. 1) is a member of spiro-azetidino-piperidine series identified as a potent ghrelin receptor inverse agonist (Bhattacharya et al., 2014). It is presently undergoing clinical trials at the National Institutes of Health for the treatment of alcohol use disorder (Ghareeb et al., 2015; Kong et al., 2016). In healthy volunteers, PF-5190457 was observed to be well tolerated (Denney et al., 2017) and it was found to be safe in a phase 1b study in subjects with alcohol use disorder (Lee et al, to be submitted for publication). The absorption of the drug after oral administration was rapid with a T_{max} of 0.3-3 h and the elimination half-life ($t_{1/2}$) was found to be ~8 h (Denney et al., 2017).

Limited information is available on the biotransformation of PF-5190457. The assessment of metabolic pathways of a new drug is important as it helps to understand the pharmacokinetics and pharmacodynamics of the compound in human. It is essential to identify the enzymes responsible for biotransformation, as this information contributes to an understanding of interindividual variability in pharmacokinetics that can be due to genetic polymorphism or drug-drug interactions. In addition, metabolites generated by these enzymes could be pharmacologically active or have off-target effects (Lin and Lu, 1997; Kumar and Surapaneni, 2001).

The objective of the present study was to characterize the biotransformation of PF-5190457 in vitro and to identify the major human circulating metabolites. Specifically, the objectives include 1) to characterize the

circulating metabolites *in vivo* in human plasma, 2) to determine of formation of metabolites *in vitro*, in sub-cellular fractions of human liver and human hepatocytes, 3) to confirm the structure of identified metabolites by mass spectrometry and NMR analyses 4) to assess the kinetic parameters of the metabolite formation, and 5) to identify the enzyme/s responsible for the formation of the major circulating metabolite using specific inhibitors for each enzyme.

Materials and Methods

Reagents.

PF-5190457, raloxifene hydrochloride, febuxostat and tacrine (internal standard) were purchased from Sigma Aldrich (St. Louise, MO, USA). Allopurinol was purchased from Toronto Research Chemicals (Toronto, Canada). PF-5190457 major hydroxy metabolite (also called PF-6870961) was biosynthesized as described below. Recombinant aldehyde oxidase (rAOX) was produced by Pfizer La Jolla, CA, USA). Dihydronicotinamide-adenine dinucleotide phosphate (NADPH) tetrasodium salt was obtained from Calbiochem (EMD Millipore, Billerica, MA). LC-MS grade methanol, acetonitrile and formic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). All other reagents and chemicals used in the study were of analytical grade and used without further purification.

In Vivo Metabolite Profiling.

Study Samples.

Plasma samples obtained after oral administration of PF-5190457 from phase 1b clinical study (NCT02039349) in alcoholic patients conducted at the NIH Clinical Center (Bethesda, MD, USA) were analyzed by high-resolution mass spectrometry (HRMS) to identify the circulating metabolites. The study was approved by the University of Rhode Island Institutional Review Board HU#

123.172. The participants have filled out the informed consent form prior to the study.

Sample Preparation.

Pooled plasma samples from representative patients were prepared for metabolite profiling experiments at various sampling times (predose, early and late post dose). Plasma proteins were precipitated by methanol followed by vortex mixing and centrifugation at 1,700 *g* for 5 min. The supernatant was then transferred into a (Genevac, Valley Cottage, NY), evaporated for 4h, and then reconstituted in 30 µL methanol followed by 120 µL water. The reconstituted samples were spun in a microfuge for 3 min to remove particulates, and 10 µL of aliquot of the reconstituted solution was injected onto HRMS system.

LC-TOF mass spectrometry

Chromatographic separation for metabolite profiling was achieved using a Waters HSS T3 C18 column (1.8 µm; 2.1x100 mm; Waters Corp) connected to Thermo Accela HPLC pump, CTC Analytics autoinjector, a diode array detector (PDA or DAD) and a Thermo Orbitrap Elite high resolution mass spectrometer (HRMS) (Thermo Fisher Scientific Inc, Waltham, MA, USA). The mobile phase consisted of 10 mM ammonium acetate (A) and methanol (B) with a flow rate of 0.35 mL/min. The gradient elution was started from 5% B, maintained for 0.5 min, increased to 50% B over 10 min, and then to 95% B over the next 2 min and finally decreased to 5% B to re-equilibrate the column with a total run time of 15

min. The MS detection was operated in positive ion electrospray (ES-positive) mode.

In Vitro Biotransformation of PF-5190457

Preparation of subcellular fractions

Human liver tissues (n=104) were obtained from Xenotech (Lenexa, KS, USA). Microsomal (HLM) and cytosolic (HLC) fractions were prepared by differential ultracentrifugation from human liver as described previously (Jamwal et al., 2017). Total protein concentration for the HLM and HLC were determined using Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) and the fractions were stored in -80°C until further analysis.

HLM and HLC Incubations

The *in vitro* metabolism of PF-5190457 was studied using HLM and HLC. PF-5190457 was incubated at relatively high concentrations (50-100 µM) with pooled HLM and HLC in a total volume of 50 µL of 100 mM potassium phosphate buffer (pH-7.4) such that the final concentration of methanol was less than 0.25%. Incubations were conducted at 37°C in an oscillating water bath. The total protein concentration used for both HLM and HLC ranged from 0.05 - 2 mg/mL. PF-5190457 was also incubated in only 100 mM potassium phosphate buffer (pH-7.4) without HLM or HLC as control. Microsomal incubations were conducted

with and without the addition of NADPH. The reaction was terminated by addition of 200 µL of ice-cold methanol at various incubation times (0-120 min). Subsequently, the samples were centrifuged for 5 min at 10,000 rpm to remove the precipitated protein.

LC-MS/MS analysis of HLM and HLC samples

The supernatant obtained from the samples was analyzed using an Acquity UPLC coupled with a Xevo-TQ mass spectrometer (Waters Corp, Milford, MA, USA). Samples (5 µL) were injected onto a C18 column (Acquity UPLC BEH 2.1 x 50 mm) with 1.7 µm particle size and 130 Å porosity. A pre-column (Acquity UPLC BEH C18, 2.1 x 5 mm) was connected prior to the analytical column. Mobile phase consisted of water: methanol 95:5% (v/v) containing 0.1% formic acid (A) and 100% methanol containing 0.1% formic acid (B). The column and autosampler temperature were kept at 40°C and 10°C, respectively. The mobile phase was delivered at 0.25 mL/min flow rate and the injection volume was 5 µL. Mobile phase initial condition of 98% solvent A, 2% solvent B was maintained for 2 min, but proportion of solvent B was increased to 50% over 8.30 min, and then to 95% solvent B over the next 2 min and returned to the initial condition for equilibration. The total run time was 12.30 min. A full scan from 100-1200 Da was carried out in a positive electrospray ionization mode with the following parameters: desolvation temperature of 600°C, desolvation gas flow of 1000 L/h, capillary voltage of 3.50 kV and cone voltage of 30 V. Similarly, single-ion monitoring for the $[M+H]^+$ ions of the anticipated

metabolites - hydroxy metabolite (513.2431+16), glucuronide (513.2431+176) and hydroxy glucuronide (513.2431+192) conjugates with daughter scan was performed.

Hepatocytes incubations

PF-05190457 was incubated in human hepatocytes with and without 1 amino benzotriazole (ABT) - a pan CYP450 inhibitor and hydralazine - a selective probe inactivator of aldehyde oxidase (AO) respectively (Ortiz de Montellano and Mathews, 1981; Strelevitz et al., 2012).

Incubations in liver cytosol of various species

PF-5190457 was incubated at 20 µM, with pooled mouse (male and female), rat and monkey (male and female) liver cytosol (2 mg/mL) in a total volume of 1 mL potassium phosphate buffer (100 mM; pH 7.5). Incubations were performed at 37°C for 75 min and then stopped by addition of acetonitrile (5 mL). Samples were centrifuged at 1700 g for 5 min and the supernatant was evaporated in vacuo, reconstituted in 1% formic acid (0.2 mL) for analysis by HPLC-UV-MS.

Biosynthesis and NMR Analysis

PF-5190457 (20 µM) was incubated with female mouse liver cytosol (20 mg/mL; Corning-Gentest, Woburn, MA) in 40 mL potassium phosphate buffer

(100 mM, pH 7.5) in a shaking water bath maintained at 37°C for 1.5 hr. The incubation was terminated with addition of CH_3CN (40 mL) and the precipitated material was removed by spinning in a centrifuge for 5 min at 1,700 g. The supernatant was partially evaporated in a Genevac vacuum centrifuge for 2 hr. To the remaining mixture was added 0.25 mL neat formic acid, 0.25 mL CH_3CN, and water to a final volume of 25 mL. This mixture was spun in a centrifuge at 40,000 g for 30 min. The clarified supernatant was applied to an HPLC column (Polaris C18, 4.6 x 250 mm; 5µ particle size) through a Jasco HPLC pump at a rate of 0.8 mL/min. After the entire sample was applied, an additional ~5 mL of mobile phase (0.1% formic acid containing 1% CH_3CN) was pumped through the system. The column was moved to a Thermo LTQ HPLC-MS system containing a photodiode array detector and a mobile phase gradient was applied to elute material of interest. The mobile phase was comprised of 0.1% formic acid in water and CH_3CN and was run at a flow rate of 0.8 mL/min. The gradient began at a composition of 2%B for 5 min followed by a linear gradient to 25%B at 70 min. The eluent passed through the photodiode array detector scanning from 200-400 nm and then to a splitter (ratio was approximately 15:1) with the larger portion going to a CTC Leaptec fraction collector (CTC, Cary, NC). Fractions were collected every 20 sec. The remainder was introduced into the mass spectrometer operated in the positive ion mode. The fractions proposed to contain the hydroxy metabolites of interest were analyzed for identity and purity on a Thermo Orbitrap Elite UHPLC-UV-MS system containing an Acquity column (HSS T3 C18, 2.1 x 100 mm, 1.7 µm

particle size) using the mobile phases described above at a flow rate of 0.4 mL/min and an injection volume of 5 µL. The composition began at 5% solvent B for 0.5 min followed by a gradient to 35% solvent B at 8 min, a second gradient to 95% solvent B at 10 min, and re-equilibration to initial conditions. Fractions containing single peaks by UV and the desired protonated molecular ions were combined and evaporated by vacuum centrifugation for analysis by NMR spectroscopy. Later, the fraction was reconstituted with 100 µL of DMSO-d6 prior to NMR analysis. NMR spectra were recorded on a Bruker Avance 600 MHz instrument (Bruker BioSpin Corporation, Billerica, MA). The ^{1}H, and 2D NMR (^{1}H COSY, HMBC, and HSQC) analyses were carried out in DMSO solutions of analyte.

Enzyme Kinetic Studies

The formation of major hydroxy metabolite in rAOX and HLC was studied to determine the enzyme kinetic parameters. Before the assessment of the enzyme kinetics, protein concentration and incubation time for the kinetic studies were evaluated to ensure the linearity of major hydroxy metabolite formation with respect to protein concentration and time. It was also seen that substrate utilization during the reaction was less than 10%.

rAOX incubations

PF-5190457 (0.5-125 µM) was incubated with rAOX (0.090 mg/mL) in 100 mM potassium phosphate buffer. The incubations were conducted at 37°C in

shaking water bath. The incubation mixture volume was 50 µL and all incubation samples were in triplicate. The reactions were terminated after 120 min using ice-cold methanol containing 2.5 ng/mL tacrine as IS.

Screening of allopurinol and oxypurinol in HLC.

The human livers utilized in this study were obtained from Sekisui XenoTech LLC which is typically retrieved from brain dead individuals for organ transplantation purpose but could not be transplanted. As such, the livers were perfused with the University of Wisconsin Cold Storage Solution (UW) containing allopurinol. However, it is known that allopurinol and its primary metabolite oxypurinol to be potent inhibitors of xanthine oxidase (XO) activity (Barr et al., 2014).

To detect the presence the XO inhibitors, HLC (n=104) were tested for the presence of allopurinol and oxypurinol using the same LC-MS/MS instrumentation and mobile phases as described earlier in HLC and HLM analysis. The flow rate was 0.3 mL/min, the column was equilibrated at initial conditions of 90% solvent A for 1.2 min and then to 10% at 2 min. Over the next minute, solvent A was increased to 90% and then kept constant for a re-equilibration time for 3 min. The total chromatographic run time was 3 min per sample. The multiple reaction monitoring (MRM) transitions used for allopurinol and oxypurinol detection were 137.1/110.1 and 153.1/136.1 respectively.

HLC incubations

Kinetic parameters for the formation of the major hydroxy metabolite from PF-5190457 was determined by incubating the drug in pooled, allopurinol and oxypurinol free, HLC. The optimized total protein concentration and incubation times in HLC was 0.1 mg/mL and 5 min, respectively. PF-5190457 (0.05–100 *μ*M) with HLC in a total volume of 50 µL of 100 mM potassium phosphate buffer (pH-7.4) were incubated at 37°C in duplicate. The final concentration of methanol in the incubations was less than 0.25%. The reaction was initiated by the addition of cytosol. Incubations (50 *μ*L) were terminated by protein precipitation with the addition of methanol (200 *μ*L) containing the 2.5 ng/mL IS.

LC-MS/MS analysis for kinetic studies

All the samples from rAOX and HLC were centrifuged for 5 min at 10,000 *rpm*. The supernatants were removed and analyzed. The parent, metabolite and IS were monitored using Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled to Xevo TQ-MS (Waters Corp., Milford, MA, USA). The analytes were separated using an aquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm particle size, 130Å pore size) analytical column with an Acquity UPLC BEH C18 VanGuard pre-column (2.1 x 5 mm, 130Å). Mobile phase solution A consisted of water: methanol 95/5 % (v/v) and mobile phase solution B consisted of methanol (100%). Formic acid (0.1%) was added to both the mobile phase solutions. The flow rate was 0.25 mL/min with a 10.30 min run time. The column temperature

was maintained at 40°C. The gradient consisted of 2% methanol for 2 min, increased to 35% for 2.0 - 7.30 min, further increased to 95% methanol till 8 min

and held until 8.30 min before returning to initial conditions to equilibrate with a total run time of 10.30 min. Mass spectral analysis and quantification was carried out using MRM on a positive electrospray ionization mode with the following parameters: desolvation temperature of 350°C, desolvation gas flow 650 L/h, capillary voltage 3.50 kV and cone voltage 20 V. The optimized settings of mass spectrometer voltage and the retention time (R_t) for the analytes and IS with quantifier and qualifier fragments are summarized in Table 1. Data acquisition and processing were performed using MassLynx™ software (V 4.1) and TargetLynx™ tool respectively. Retention times for hydroxy metabolite and internal standard were 4.4 and 5.1 min, respectively. Quantitation was done using a hydroxy metabolite standard curve ranging from 0.1-1 µM. The calibration standards were within ± 15% of nominal values.

Inhibition Studies

Incubation of PF-5190457 in HLC (with AO and XO inhibitors)

PF-5190457 (25 µM) was incubated with pooled allopurinol/oxypurinol free HLC (n=9) in a total volume of 50 µL of 100 mM potassium phosphate buffer (pH-7.4) in the presence and absence of inhibitors. Incubation conditions were similar to previous HLC kinetic experiments. To identify the enzymes involved in the

formation major hydroxy metabolite, HLC was incubated with and without AO inhibitor (raloxifene 0.005-50 µM), and XO inhibitor (febuxostat 0.001-50 µM). The reactions were terminated at 5 min using methanol containing 2.5 ng/mL tacrine as internal standard (IS). The samples were analyzed using LC-MS/MS as described under enzyme kinetic studies.

Data Analysis

Estimations of the maximum rate of major hydroxy metabolite formation (V_{max}) and the Michaelis-Menten constant (K_m) were performed using nonlinear regression analysis within GraphPad Prism (GraphPad Software Inc., La Jolla, CA) using the Michaelis-Menten equation given below.

$$V = (V_{max}*S)/(K_m+S)$$

The apparent *in vitro* intrinsic clearance (Cl_{int}) was calculated as V_{max}/K_m.

Results

Characterization of PF-5190457 Metabolites in Human Plasma

The circulating metabolites of PF-5190457 were determined in the plasma of human subjects who were administered PF-5190457 orally in a phase 1b clinical study (Fig. 2). Two peaks with protonated molecular ions of 16 mass units greater than PF-5190457 were observed (*m/z* 529.2382), indicating the addition of oxygen. Fragment ions of the hydroxyl metabolite that had a greater ion abundance included *m/z* 351.2179, 305.1430, and 225.1022 (Fig. 3) which is indicative of an oxidative biotransformation on the indenyl-pyrimidine portion of the molecule. Additional metabolites proposed as glucuronide and hydroxy glucuronide conjugates (*m/z* 689 and 705) were detected at apparent lower levels in the plasma.

In Vitro Biotransformation of PF-5190457

HLM and HLC

Preliminary experiments conducted in the subcellular fractions of the human liver generated the major hydroxyl metabolite in HLC without the addition of cofactors. The metabolite formed in HLC increased with incubation time, concentration of substrate, and concentration of cytosol. The hydroxy metabolite was detected in HLC as the protonated molecular ion $[M + H]^+$ at *m/z* 529.

Fragmentation of this ion produced fragments at *m/z* 225 and 351 (Fig. 4). This metabolite was not observed in HLM supplemented with NADPH.

Hepatocytes

The formation of hydroxyl metabolites of PF-5190457 was observed in human hepatocytes as shown in the chromatogram (Fig. 5). The traces were extracted ion chromatograms of m/z 529.2376 (5 ppm tolerance) representing the protonated molecular ion of a hydroxylated metabolite. The addition of ABT, a broad-spectrum P450 inactivator inhibited the formation of the apparent minor metabolites at R_t 4.12, 4.62, and 5.57 min and had no effect on the metabolite eluting at R_t 3.98 min suggesting that CYP mediated metabolism for the minor metabolites and not the major metabolite. It was also observed that addition of hydralazine inhibited formation of the metabolite at R_t 3.97 min indicating that AO could be responsible for the generation of this metabolite.

Incubations in liver cytosol from various species

The formation of hydroxy metabolite (513+13) was observed in cytosolic incubations of PF-5190457 in mouse (male and female), rat (male), monkey (female) indicating the metabolism to be independent of NADPH (Fig. 6). The hydroxy metabolite formed in cytosol of various species had similar retention time to that of the metabolite formed in HLC.

NMR Analysis

Characterization of biosynthesized hydroxy metabolite by NMR analysis demonstrated that the site of hydroxylation was on the carbon between the nitrogen on the 4-methylpyrimidine moiety (Fig. 7). ^{1}H COSY, HMQC, and HMBC spectra were obtained to support the hydroxylation of pyrimidine moiety (Supplemental data Fig. 2, 3 (a) and (b)).

Enzyme Kinetic Analysis

The HLC that were free (n=9) of allopurinol and oxypurinol were pooled together for further kinetic and inhibition experiments. The kinetics for the formation of major hydroxy metabolite from PF-5190457 was determined by incubating in rAOX and HLC. Representative Michaelis-Menten kinetic plots in rAOX and HLC are depicted in Fig. 8 and Fig. 9. Substrate inhibition kinetics at higher concentration of substrate was observed for rAOX incubations. The K_m and V_{max} values for the formation of hydroxy metabolite in rAOX and HLC were found to be 6.9 µM and 0.35 nmol/min/mg protein and 42 µM and 0.12 nmol/min/mg protein respectively. The Cl_{int} in rAOX and HLC were 0.05 and 0.002 mL/min/mg protein respectively.

Biotransformation of PF-5190457 is mediated by AO and XO

The effect of AO (raloxifene) and XO (febuxostat) inhibitors on the metabolism of PF-5190457 was investigated. There was considerable (>75%) but not complete inhibition of formation of the major hydroxy metabolite with 50 µM raloxifene (Fig. 10a). The extent of inhibition with febuxostat was greater than 50% at 1 µM (Fig. 10b) and further increases in inhibitor concentration did not inhibit the formation of metabolite.

Discussion

Characterization of circulating metabolites from patients orally administered with PF-5190457 revealed the biotransformation pathways for this ghrelin receptor inverse agonist. The primary route of metabolism observed in humans was hydroxylation yielding a major hydroxy metabolite of PF-5190457). In vitro experiments showed that this metabolite was generated in human liver cytosol. The metabolite profiling of major circulating metabolite (m/z 529a) in human plasma and the metabolite formed in HLC (m/z 529) showed similar MS/MS fragmentation pattern (m/z 225, m/z 351) suggesting hydroxylation on the pyrimidine ring yielding strong correlation between *in vivo* and *in vitro* data. Results from these experiments suggest that primary biotransformation process is non-NADPH-dependent metabolism in HLC leading to the formation of a hydoxy pyrimidine metabolite. The formation of the metabolite by NADPH independent process in HLC and the site of hydroxylation position on the PF-5190457 confirmed from NMR analysis suggested the involvement of molybdenum co-factor containing enzymes like AO and/or XO mediated biotransformation. The position of hydroxylation on the pyrimidine ring between the two nitrogen atoms suggests nucleophilic attack via AO and/or XO enzymes in the metabolism of the drug (Krenitsky et al., 1972; Beedham, 1985; Kitamura et al., 2006). In contrast to electrophillic oxidation by CYP450 enzymes, both AO and XO enzymes display nucleophilic attack on carbon atom which is electron deficient or carbon adjacent to N-heterocycle (eg. purines, pyrimidines and pteridines) resulting in the formation of distinct metabolites (Krenitsky et al.,

1972). Furthermore, the use of inhibitors indicated contribution of both AO and XO enzymes to the formation of hydoxy pyrimidine metabolite of PF-5190457. The data also suggest AO as a dominant enzyme in comparison to XO in the biotransformation of PF-5190457.

AO and XO enzymes belong to a family of molybdo-flavoenzymes and have a high level of similarity between their amino acid sequence homology (Beedham, 1987; Terao et al., 2000; Hille, 2005). XO can exists as two interconvertible forms as xanthine oxidase and xanthine dehydrogenase unlike AO (Della Corte et al., 1969). AO was found to be one of the highly abundant enzymes in HLC and the expression of AO was greater in comparison to XO in human liver (Nishimura and Naito, 2006; Sodhi et al., 2015). Physiological relevance of AO is still not known but XO plays an important role in catalyzing hypoxanthine to xanthine and later to uric acid (Beedham, 1985; Kitamura et al., 2006). Both the enzymes differ in substrate and inhibitors specificity. Increasing number of substrates in the past few decades have been identified to be catalyzed by the two enzymes especially involving AO which also has broader substrate specificity in comparison to XO (O'Connor et al., 2006; Pryde et al., 2010; Sanoh et al., 2015; Battelli et al., 2016). There are only few substrates which show biotransformation by both the enzymes like 6-deoxyclovir, 6-thioxanthine, and recently VU0409106 a lead compound for childhood developmental disorders (Krenitsky et al., 1984; Morrison et al., 2012; Choughule et al., 2014). Though some overlap exists between the substrates, there has been various panel of chemical inhibitors identified specifically for AO and XO

inhibitors including raloxifene (Obach, 2004), hydralazine (Strelevitz et al., 2012), allopurinol (Panoutsopoulos et al., 2004) and febuxostat (Weidert et al., 2014). Allopurinol has been widely used as a known XO inhibitor but recently it was identified that febuxostat is a more potent inhibitor of XO than allopurinol (Malik et al., 2011). There are not any important clinical drug-drug interactions associated with inhibition of AO but as growth in new chemical entities metabolized by AO is increasing, there could arise a need to characterize in drug-drug interaction potential at AO level (Obach, 2004; Pryde et al., 2010). It is a challenge to capture the drug interactions associated with these enzymes especially for AO enzyme in animal models as there exist species differences for expression and activity along with disparity with gender and strain (Itoh, 2009; Crouch et al., 2017; Mao et al., 2017).

Both potent XO inhibitors, allopurinol and febuxostat are clinically used for treatment of chronic gout and hyperuricemia. This condition is also present in disease states like metabolic syndrome, diabetes, chronic liver disease and cardiovascular disease (Hu and Tomlinson, 2008; George and Minter, 2017) some of which are target population for PF-5190457. There have been drug-drug interactions reported in man through the inhibition of XO substrates. For example, in one clinical study, allopurinol significantly inhibited 6-mercaptopurine which is a substrate for XO when administered orally and there was significant increase in peak plasma concentration and area under the curve of 6-mercaptopurine in patients with acute lymphoblastic leukemia (Zimm et al., 1983).

Inter-individual variability between donors has been observed in *in vitro* studies for hepatic AO and XO activity (Guerciolini et al., 1991; Al-Salmy, 2001; Hutzler et al., 2014). However, single nucleotide polymorphisms in the AO and XO genes has not been extensively studied in various populations (Levartovsky et al., 2000; Gok et al., 2003; Hutzler et al., 2014). Genetic polymorphism of AO gene was studied in an Italian study and the presence of both fast and poor metabolizers was observed (Hartmann et al., 2012). Moreover, two *in vitro* studies showed genetic variation in the XO gene could be responsible in individual variations in XO activity (Kudo et al., 2008; Kudo et al., 2010). A recent study in European population suggests that XO variants had influence on uric acid production since XO also catalyzes uric acid production (Scheepers et al., 2016).

Both *in vitro* and *in vivo* studies recognized the hydroxy pyrimidine metabolite as the major metabolite of PF-5190457. The findings presented here shows that both AO and XO contribute to the metabolism of PF-5190457 resulting in the formation of a major circulating metabolite. The study also emphasizes the importance of these non-CYP enzymes in the drug metabolism during the early stages of drug development. The kinetics of PF-5190457 in rAOX and HLC gave an impression on the sub saturation concentrations in the allopurinol free livers. The data and the experimental conditions could be used in the future to investigate the Cl_{int} to study the effect of various factors like age, gender, smoking, alcohol use or disease states to improve the clinical pharmacokinetics and efficacy of the drug in that respective population.

The pharmacokinetic profile of the PF-5190457 and its major hydroxy metabolite has been investigated in plasma samples obtained from human subjects during the phase 1b study which is described elsewhere (Manuscript IV). It is important to identify the other metabolic pathways and route of elimination of PF-5190457 in the context of a mass balance study.

Conflict of interest

Dr. Obach is an employee of Pfizer Pharmaceuticals. Other authors declare no potential conflict of interest with any commercial entity whose products were used in the study.

Acknowledgements

The financial support of Grant #UH3TR000963 from National Institutes of Health is gratefully acknowledged.

References

Al-Salmy HS (2001) Individual variation in hepatic aldehyde oxidase activity. *IUBMB Life* **51:**249-253.

Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, and Kasuga M (2001) Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* **120:**337-345.

Barr JT, Choughule KV, Nepal S, Wong T, Chaudhry AS, Joswig-Jones CA, Zientek M, Strom SC, Schuetz EG, Thummel KE, and Jones JP (2014) Why do most human liver cytosol preparations lack xanthine oxidase activity? *Drug Metab Dispos* **42:**695-699.

Battelli MG, Polito L, Bortolotti M, and Bolognesi A (2016) Xanthine Oxidoreductase in Drug Metabolism: Beyond a Role as a Detoxifying Enzyme. *Curr Med Chem* **23:**4027-4036.

Beedham C (1985) Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metab Rev* **16:**119-156.

Beedham C (1987) Molybdenum hydroxylases: biological distribution and substrate-inhibitor specificity. *Prog Med Chem* **24:**85-127.

Bhattacharya SK, Andrews K, Beveridge R, Cameron KO, Chen C, Dunn M, Fernando D, Gao H, Hepworth D, Jackson VM, Khot V, Kong J, Kosa RE, Lapham K, Loria PM, Londregan AT, McClure KF, Orr ST, Patel J, Rose C, Saenz J, Stock IA, Storer G, VanVolkenburg M, Vrieze D, Wang G, Xiao J, and Zhang Y (2014) Discovery of PF-5190457, a Potent, Selective, and Orally Bioavailable Ghrelin Receptor Inverse Agonist Clinical Candidate. *ACS Med Chem Lett* **5:**474-479.

Choughule KV, Barnaba C, Joswig-Jones CA, and Jones JP (2014) In vitro oxidative metabolism of 6-mercaptopurine in human liver: insights into the role of the molybdoflavoenzymes aldehyde oxidase, xanthine oxidase, and xanthine dehydrogenase. *Drug Metab Dispos* **42:**1334-1340.

Crouch RD, Blobaum AL, Felts AS, Conn PJ, and Lindsley CW (2017) Species-Specific Involvement of Aldehyde Oxidase and Xanthine Oxidase in the Metabolism of the Pyrimidine-Containing mGlu5-Negative Allosteric Modulator VU0424238 (Auglurant). *Drug Metab Dispos* **45:**1245-1259.

Della Corte E, Gozzetti G, Novello F, and Stirpe F (1969) Properties of the xanthine oxidase from human liver. *Biochim Biophys Acta* **191:**164-166.

Denney WS, Sonnenberg GE, Carvajal-Gonzalez S, Tuthill T, and Jackson VM (2017) Pharmacokinetics and pharmacodynamics of PF-05190457: The first oral ghrelin receptor inverse agonist to be profiled in healthy subjects. *Br J Clin Pharmacol* **83:**326-338.

George C and Minter DA (2017) Hyperuricemia, in: *StatPearls*, Treasure Island (FL).

Ghareeb M, Leggio L, El-Kattan A, and Akhlaghi F (2015) Development and validation of an UPLC-MS/MS assay for quantitative analysis of the ghrelin receptor inverse agonist PF-5190457 in human or rat plasma and rat brain. *Anal Bioanal Chem* **407:**5603-5613.

Gok F, Ichida K, and Topaloglu R (2003) Mutational analysis of the xanthine dehydrogenase gene in a Turkish family with autosomal recessive classical xanthinuria. *Nephrol Dial Transplant* **18:**2278-2283.
Guerciolini R, Szumlanski C, and Weinshilboum RM (1991) Human liver xanthine oxidase: nature and extent of individual variation. *Clin Pharmacol Ther* **50:**663-672.
Hartmann T, Terao M, Garattini E, Teutloff C, Alfaro JF, Jones JP, and Leimkuhler S (2012) The impact of single nucleotide polymorphisms on human aldehyde oxidase. *Drug Metab Dispos* **40:**856-864.
Hille R (2005) Molybdenum-containing hydroxylases. *Arch Biochem Biophys* **433:**107-116.
Hu M and Tomlinson B (2008) Febuxostat in the management of hyperuricemia and chronic gout: a review. *Ther Clin Risk Manag* **4:**1209-1220.
Hutzler JM, Yang YS, Brown C, Heyward S, and Moeller T (2014) Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. *Drug Metab Dispos* **42:**1090-1097.
Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, and Fujimiya M (2004) Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J* **18:**439-456.
Itoh K (2009) [Individual and strain differences of aldehyde oxidase in the rat]. *Yakugaku Zasshi* **129:**1487-1493.
Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, and Akhlaghi F (2017) Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res*.
Kitamura S, Sugihara K, and Ohta S (2006) Drug-metabolizing ability of molybdenum hydroxylases. *Drug Metab Pharmacokinet* **21:**83-98.
Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, and Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402:**656-660.
Kong J, Chuddy J, Stock IA, Loria PM, Straub SV, Vage C, Cameron KO, Bhattacharya SK, Lapham K, McClure KF, Zhang Y, and Jackson VM (2016) Pharmacological characterization of the first in class clinical candidate PF-05190457: a selective ghrelin receptor competitive antagonist with inverse agonism that increases vagal afferent firing and glucose-dependent insulin secretion ex vivo. *Br J Pharmacol* **173:**1452-1464.
Korbonits M, Goldstone AP, Gueorguiev M, and Grossman AB (2004) Ghrelin--a hormone with multiple functions. *Front Neuroendocrinol* **25:**27-68.
Krenitsky TA, Hall WW, de Miranda P, Beauchamp LM, Schaeffer HJ, and Whiteman PD (1984) 6-Deoxyacyclovir: a xanthine oxidase-activated prodrug of acyclovir. *Proc Natl Acad Sci U S A* **81:**3209-3213.
Krenitsky TA, Neil SM, Elion GB, and Hitchings GH (1972) A comparison of the specificities of xanthine oxidase and aldehyde oxidase. *Arch Biochem Biophys* **150:**585-599.

Kudo M, Moteki T, Sasaki T, Konno Y, Ujiie S, Onose A, Mizugaki M, Ishikawa M, and Hiratsuka M (2008) Functional characterization of human xanthine oxidase allelic variants. *Pharmacogenet Genomics* **18:**243-251.
Kudo M, Sasaki T, Ishikawa M, Hirasawa N, and Hiratsuka M (2010) Functional characterization of genetic polymorphisms identified in the promoter region of the xanthine oxidase gene. *Drug Metab Pharmacokinet* **25:**599-604.
Kumar GN and Surapaneni S (2001) Role of drug metabolism in drug discovery and development. *Med Res Rev* **21:**397-411.
Leggio L, Addolorato G, Cippitelli A, Jerlhag E, Kampov-Polevoy AB, and Swift RM (2011) Role of feeding-related pathways in alcohol dependence: A focus on sweet preference, NPY, and ghrelin. *Alcohol Clin Exp Res* **35:**194-202.
Leggio L, Zywiak WH, Fricchione SR, Edwards SM, de la Monte SM, Swift RM, and Kenna GA (2014) Intravenous ghrelin administration increases alcohol craving in alcohol-dependent heavy drinkers: a preliminary investigation. *Biol Psychiatry* **76:**734-741.
Levartovsky D, Lagziel A, Sperling O, Liberman U, Yaron M, Hosoya T, Ichida K, and Peretz H (2000) XDH gene mutation is the underlying cause of classical xanthinuria: a second report. *Kidney Int* **57:**2215-2220.
Lin JH and Lu AY (1997) Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol Rev* **49:**403-449.
Malik UZ, Hundley NJ, Romero G, Radi R, Freeman BA, Tarpey MM, and Kelley EE (2011) Febuxostat inhibition of endothelial-bound XO: implications for targeting vascular ROS production. *Free Radic Biol Med* **51:**179-184.
Mao Z, Wu Y, Li Q, Wang X, Liu Y, and Di X (2017) Aldehyde oxidase-dependent species difference in hepatic metabolism of fasudil to hydroxyfasudil. *Xenobiotica***:**1-8.
Morrison RD, Blobaum AL, Byers FW, Santomango TS, Bridges TM, Stec D, Brewer KA, Sanchez-Ponce R, Corlew MM, Rush R, Felts AS, Manka J, Bates BS, Venable DF, Rodriguez AL, Jones CK, Niswender CM, Conn PJ, Lindsley CW, Emmitte KA, and Daniels JS (2012) The role of aldehyde oxidase and xanthine oxidase in the biotransformation of a novel negative allosteric modulator of metabotropic glutamate receptor subtype 5. *Drug Metab Dispos* **40:**1834-1845.
Nagaya N, Itoh T, Murakami S, Oya H, Uematsu M, Miyatake K, and Kangawa K (2005) Treatment of cachexia with ghrelin in patients with COPD. *Chest* **128:**1187-1193.
Nagaya N, Moriya J, Yasumura Y, Uematsu M, Ono F, Shimizu W, Ueno K, Kitakaze M, Miyatake K, and Kangawa K (2004) Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure. *Circulation* **110:**3674-3679.
Neary NM, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, and Bloom SR (2004) Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J Clin Endocrinol Metab* **89:**2832-2836.

Nishimura M and Naito S (2006) Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. *Drug Metab Pharmacokinet* **21:**357-374.
O'Connor D, Jones P, Chambers MS, Maxey R, Szekeres HJ, Szeto N, Scott-Stevens P, Macleod AM, Braun M, and Cato B (2006) Aldehyde oxidase and its contribution to the metabolism of a structurally novel, functionally selective GABAA alpha5-subtype inverse agonist. *Xenobiotica* **36:**315-330.
Obach RS (2004) Potent inhibition of human liver aldehyde oxidase by raloxifene. *Drug Metab Dispos* **32:**89-97.
Ortiz de Montellano PR and Mathews JM (1981) Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. Isolation of an NN-bridged benzyne-protoporphyrin IX adduct. *Biochem J* **195:**761-764.
Panoutsopoulos GI, Kouretas D, and Beedham C (2004) Contribution of aldehyde oxidase, xanthine oxidase, and aldehyde dehydrogenase on the oxidation of aromatic aldehydes. *Chem Res Toxicol* **17:**1368-1376.
Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, and Tran TD (2010) Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *J Med Chem* **53:**8441-8460.
Sanoh S, Tayama Y, Sugihara K, Kitamura S, and Ohta S (2015) Significance of aldehyde oxidase during drug development: Effects on drug metabolism, pharmacokinetics, toxicity, and efficacy. *Drug Metab Pharmacokinet* **30:**52-63.
Scheepers LE, Wei FF, Stolarz-Skrzypek K, Malyutina S, Tikhonoff V, Thijs L, Salvi E, Barlassina C, Filipovsky J, Casiglia E, Nikitin Y, Kawecka-Jaszcz K, Manunta P, Cusi D, Boonen A, Staessen JA, and Arts IC (2016) Xanthine oxidase gene variants and their association with blood pressure and incident hypertension: a population study. *J Hypertens* **34:**2147-2154.
Sodhi JK, Wong S, Kirkpatrick DS, Liu L, Khojasteh SC, Hop CE, Barr JT, Jones JP, and Halladay JS (2015) A novel reaction mediated by human aldehyde oxidase: amide hydrolysis of GDC-0834. *Drug Metab Dispos* **43:**908-915.
Strelevitz TJ, Orozco CC, and Obach RS (2012) Hydralazine as a selective probe inactivator of aldehyde oxidase in human hepatocytes: estimation of the contribution of aldehyde oxidase to metabolic clearance. *Drug Metab Dispos* **40:**1441-1448.
Suchankova P, Steensland P, Fredriksson I, Engel JA, and Jerlhag E (2013) Ghrelin receptor (GHS-R1A) antagonism suppresses both alcohol consumption and the alcohol deprivation effect in rats following long-term voluntary alcohol consumption. *PLoS One* **8:**e71284.
Terao M, Kurosaki M, Saltini G, Demontis S, Marini M, Salmona M, and Garattini E (2000) Cloning of the cDNAs coding for two novel molybdo-flavoproteins showing high similarity with aldehyde oxidase and xanthine oxidoreductase. *J Biol Chem* **275:**30690-30700.
Tong J, Prigeon RL, Davis HW, Bidlingmaier M, Kahn SE, Cummings DE, Tschop MH, and D'Alessio D (2010) Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. *Diabetes* **59:**2145-2151.

Volkow ND, Wang GJ, Tomasi D, and Baler RD (2013) Obesity and addiction: neurobiological overlaps. *Obes Rev* **14:**2-18.
Weidert ER, Schoenborn SO, Cantu-Medellin N, Choughule KV, Jones JP, and Kelley EE (2014) Inhibition of xanthine oxidase by the aldehyde oxidase inhibitor raloxifene: implications for identifying molybdopterin nitrite reductases. *Nitric Oxide* **37:**41-45.
Yildiz BO, Suchard MA, Wong ML, McCann SM, and Licinio J (2004) Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A* **101:**10434-10439.
Zimm S, Collins JM, O'Neill D, Chabner BA, and Poplack DG (1983) Inhibition of first-pass metabolism in cancer chemotherapy: interaction of 6-mercaptopurine and allopurinol. *Clin Pharmacol Ther* **34:**810-817.

Legend for Tables

Table II-1. Mass spectrometry parameters for analytes and IS

Compound	Parent ion (m/z)	Daughter Ion (m/z)	Dwell (s)	Cone (V)	Collision energy (V)	Retention Time (min)
Parent drug (PF-5190457)	513	209, 335	0.025	30, 30	44, 16	5.6
Major hydroxy metabolite	529	225, 351	0.025	34, 32	40, 20	4.5
Internal standard (tacrine)	199	170, 143	0.025	46, 42	30, 36	5.9

Legend of Figures

Fig. 1. Structure of PF-5190457

Fig. 2. Metabolic profiles of pooled patient plasma samples at various sampling times (predose, early and late times) after administration of PF-5190457 analyzed by HPLC-UV and HPLC-MS/MS (representative m/z of the detected metabolites).

Fig. 3. Full scan and daughter ion scan of the major metabolite 529a detected at 7:30 min in the pooled human plasma samples.

Fig. 4. Representative mass spectra in HLC. (a) Experimental PF-5190457 incubation sample in HLC (b) Blank cytosol without the addition of substrate

Fig. 5. HPLC-MS traces for PF-5190457 incubated in pooled human hepatocytes

Fig. 6. HPLC-MS traces for PF-5190457 incubated in liver cytosol various species.

Fig. 7. ^{1}H Proton NMR Spectra of hydroxy metabolite of PF-5190457

Fig. 8. Representative kinetics for the metabolism of PF-5190457 to hydroxy metabolite in recombinant aldehyde oxidase incubations.

Fig. 9. Representative kinetics for the metabolism of PF-5190457 to hydroxy metabolite in human liver cytosolic incubations.

Fig. 10. Hydroxy metabolite formation in the presence of inhibitors (% of control) at various concentrations of, (a) raloxifene and (b) febuxostat with 25 µM PF-5190457 in human liver cytosolic incubations.

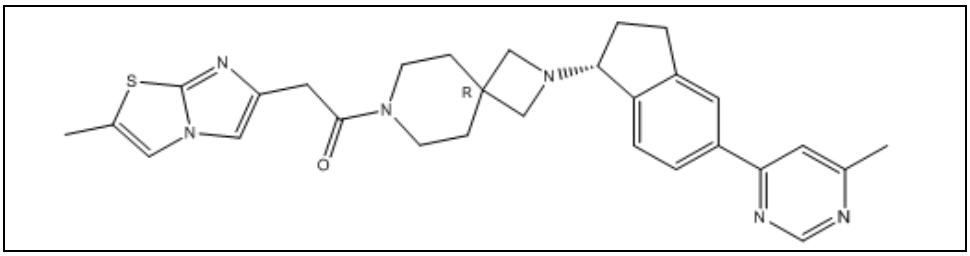


Fig. II-1. Structure of PF-5190457

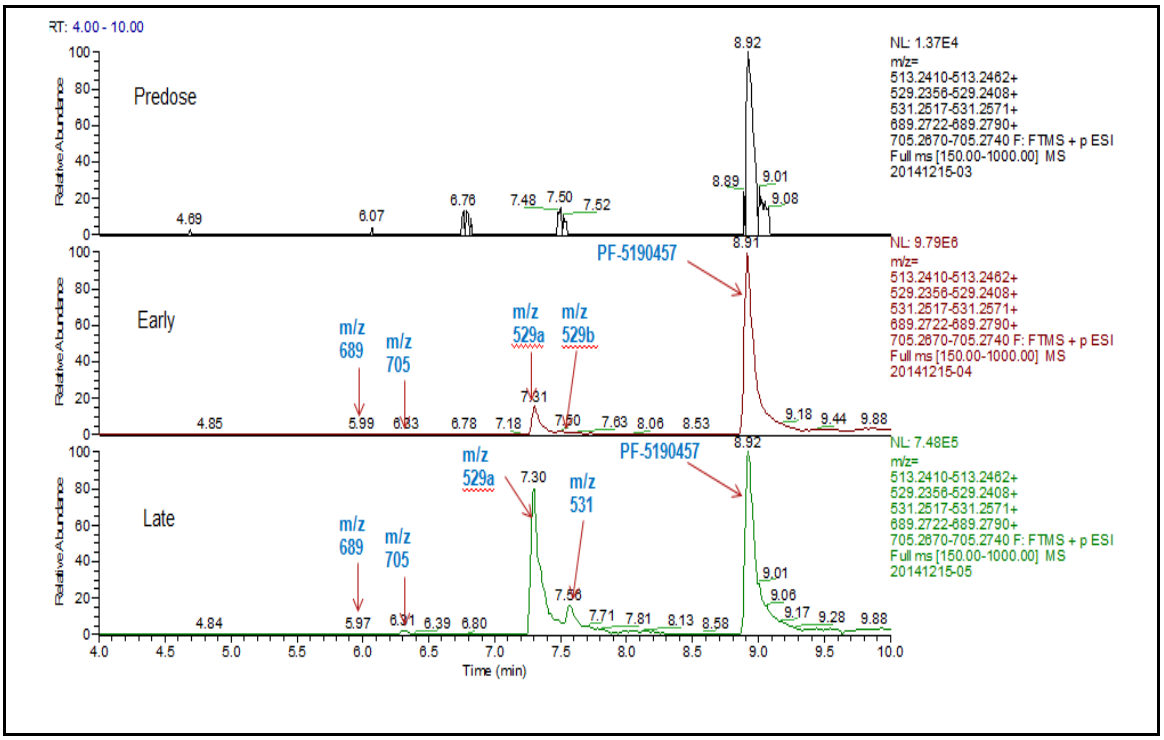


Fig. II-2. Metabolic profiles of pooled patient plasma samples at various sampling times (predose, early and late) after administration of PF-5190457 after HPLC-UV and HPLC-MS/MS analysis (representative m/z of the detected metabolites).

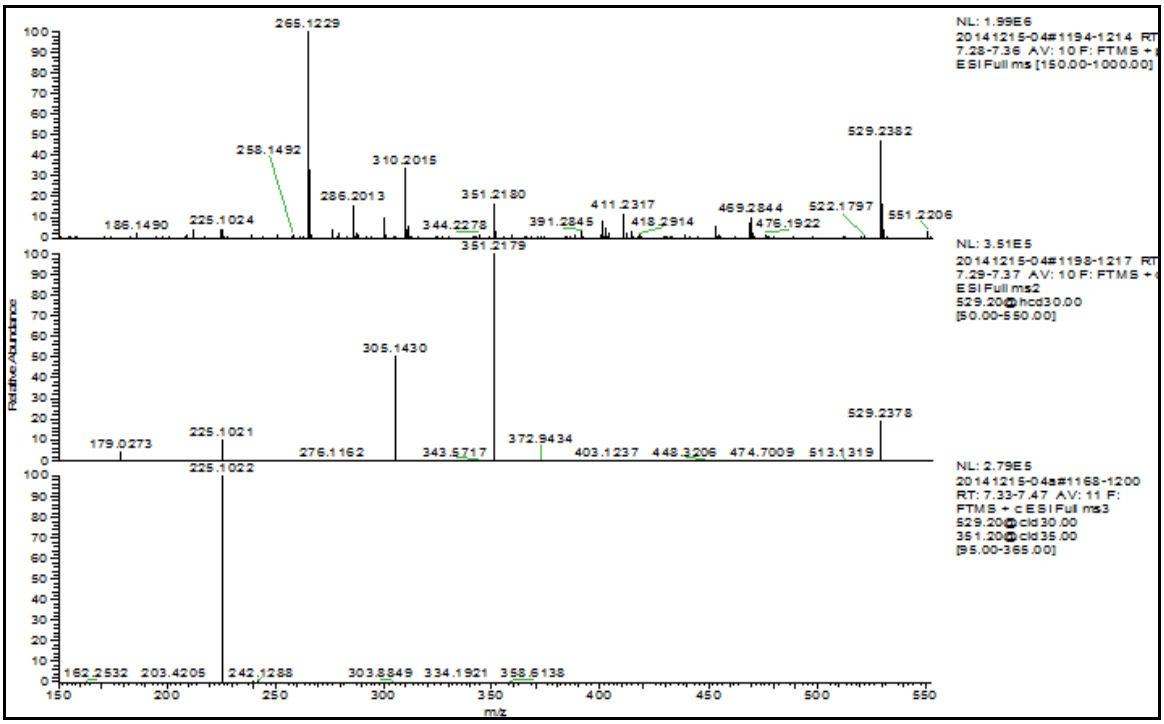


Fig. II-3. Full scan and daughter scan of the major metabolite 529a detected at 7:30 min in the pooled human plasma samples.

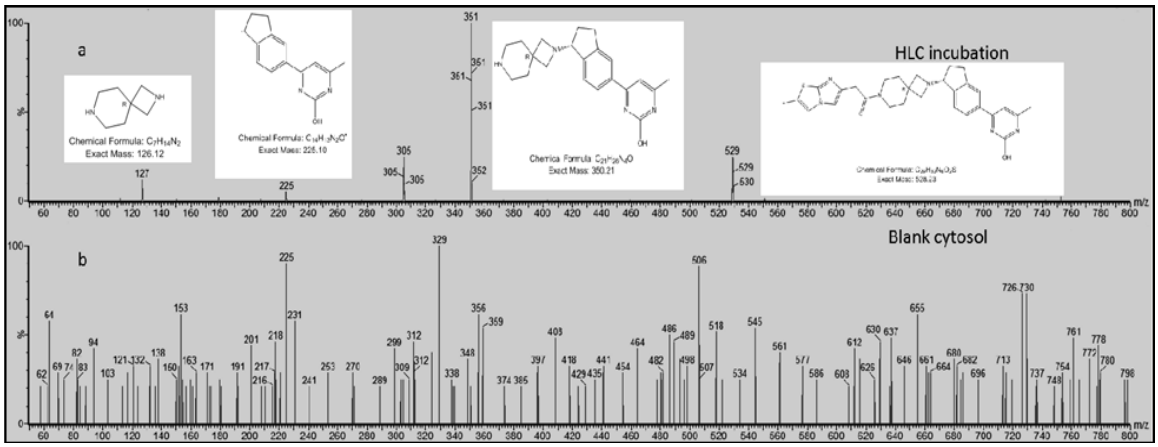


Fig. II-4. Representative mass spectra in HLC. (a) Experimental PF-5190457 incubation sample in HLC (b) Blank cytosol without the addition of substrate

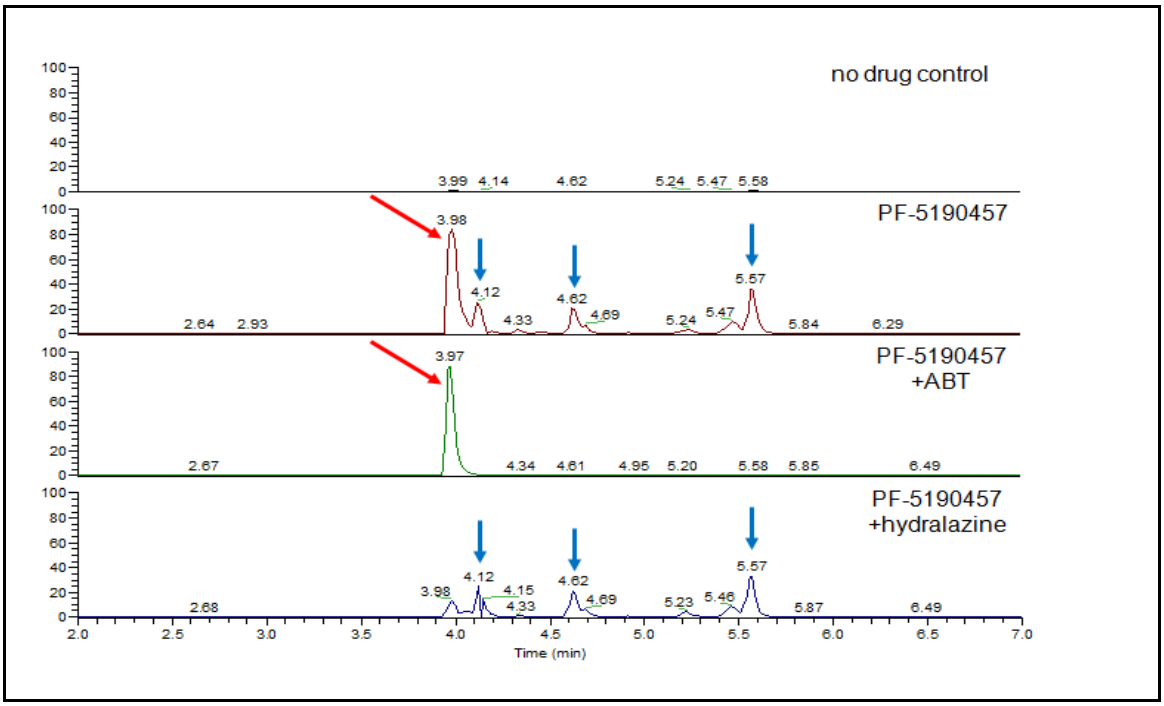


Fig. II-5. HPLC-MS Traces for PF-5190457 incubated in pooled human hepatocytes. Peaks at R_t of 4.12, 4.62, and 5.57 min (denoted with blue arrows) were sensitive to ABT. The peak eluting at 3.97 min (denoted with red arrows) was insensitive to ABT but was inhibited by hydralazine.

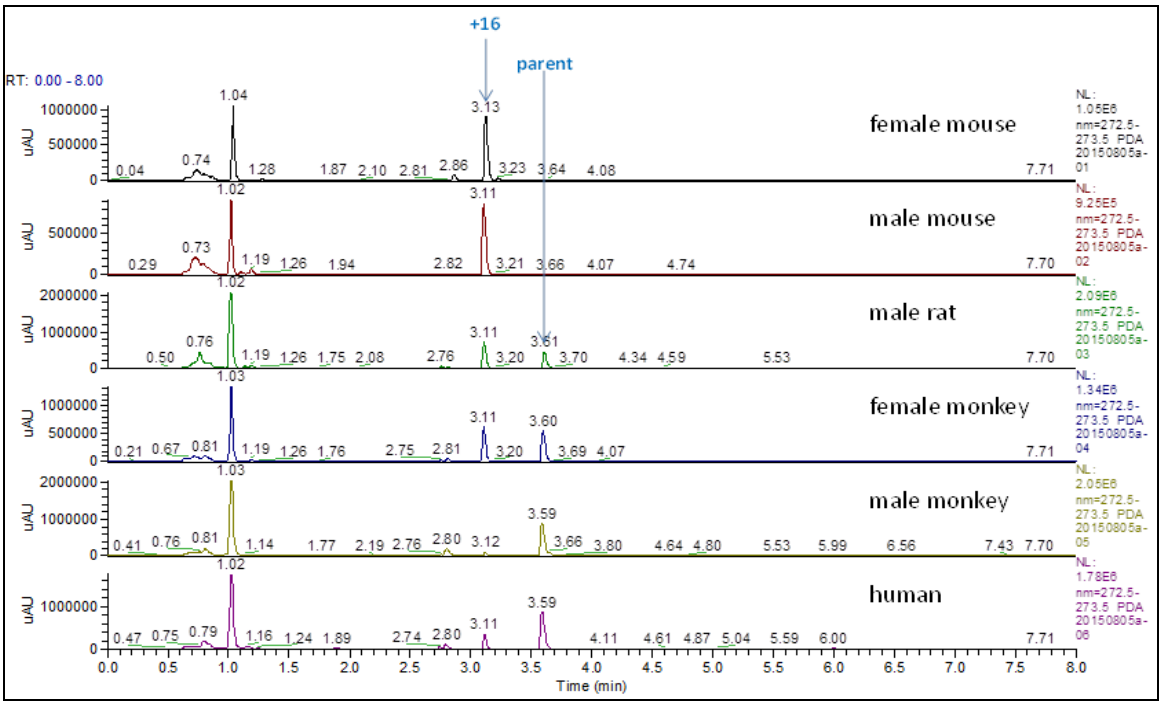


Fig. II-6. HPLC-MS Traces for PF-5190457 incubated in liver cytosol various species. Peaks at R_t of 3.12 and 3.61 min represents hydroxylated metabolite (513+16) and parent drug respectively.

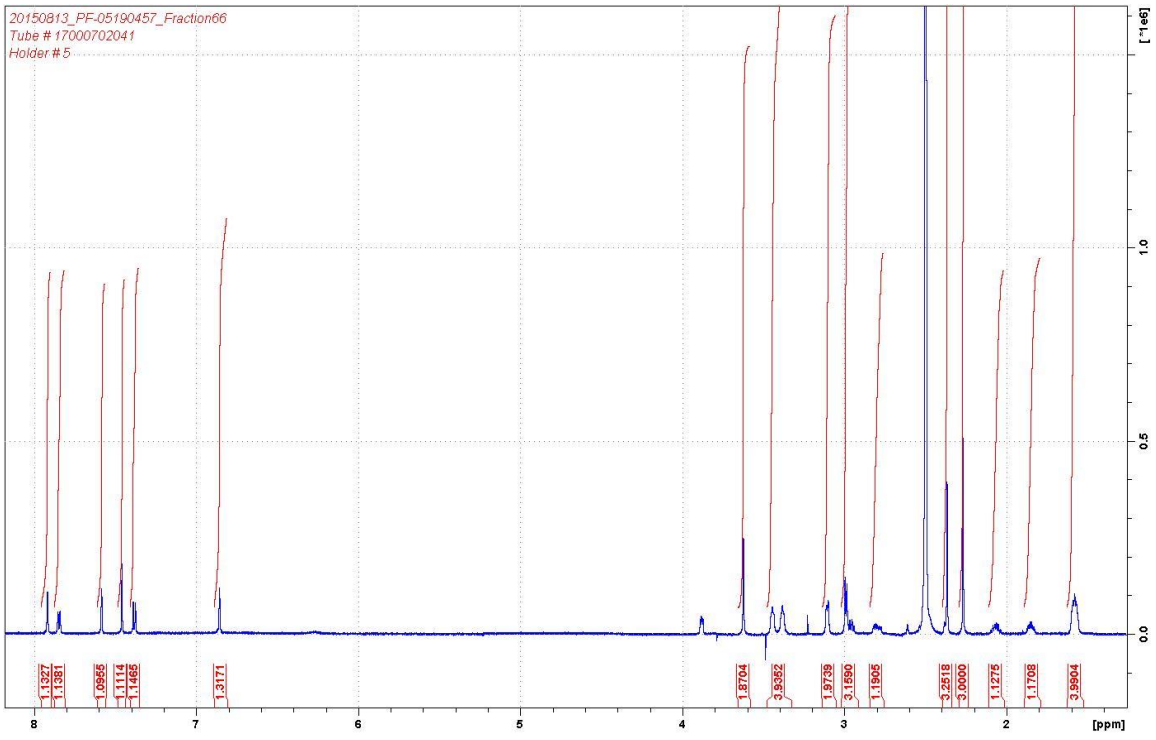


Fig.II-7. ^{1}H Proton NMR Spectra of hydroxy metabolite of PF-5190457

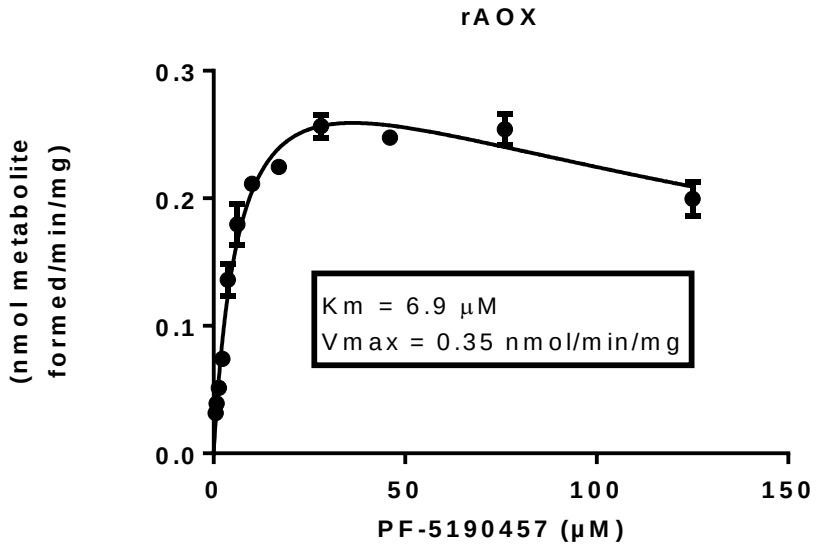


Fig. II-8. Representative kinetics for the metabolism of PF-5190457 to hydroxy metabolite formation in recombinant aldehyde oxidase incubations. Increasing concentrations of PF-5190457 (0.52 – 125 µM) were incubated with recombinant aldehyde oxidase (0.090 mg/mL) for 120 min at 37°C. Formation rates of metabolite (nmol/min/mg protein) versus PF-5190457 concentration (µM) were best-fit using nonlinear-regression analysis.

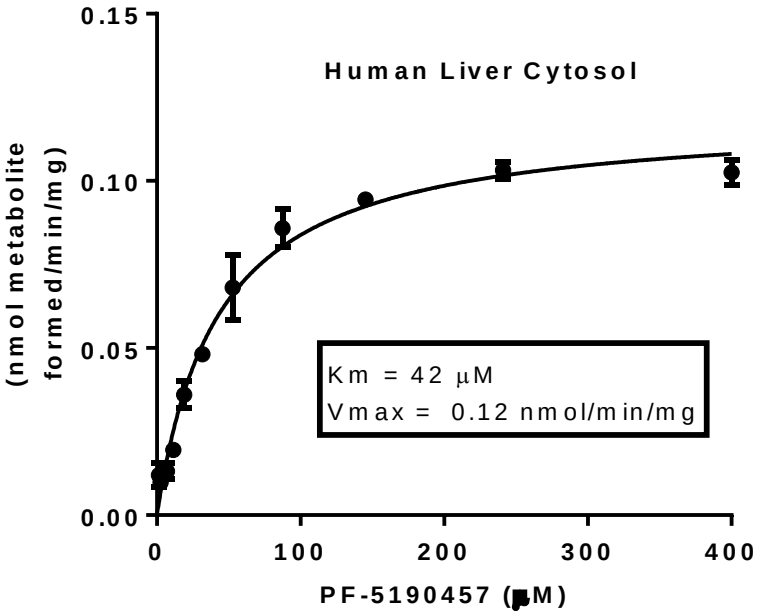


Fig. II-9. Representative kinetics for the metabolism of PF-5190457 to hydroxy metabolite in human liver cytosolic incubations. Increasing concentrations of PF-5190457 (1.5 – 400 µM) were incubated with human liver cytosolic samples (0.1 mg/mL) for 5 min at 37°C. Formation rates of metabolite (nmol/min/mg protein) versus PF-5190457 concentration (µM) were best-fit using non-linear regression analysis

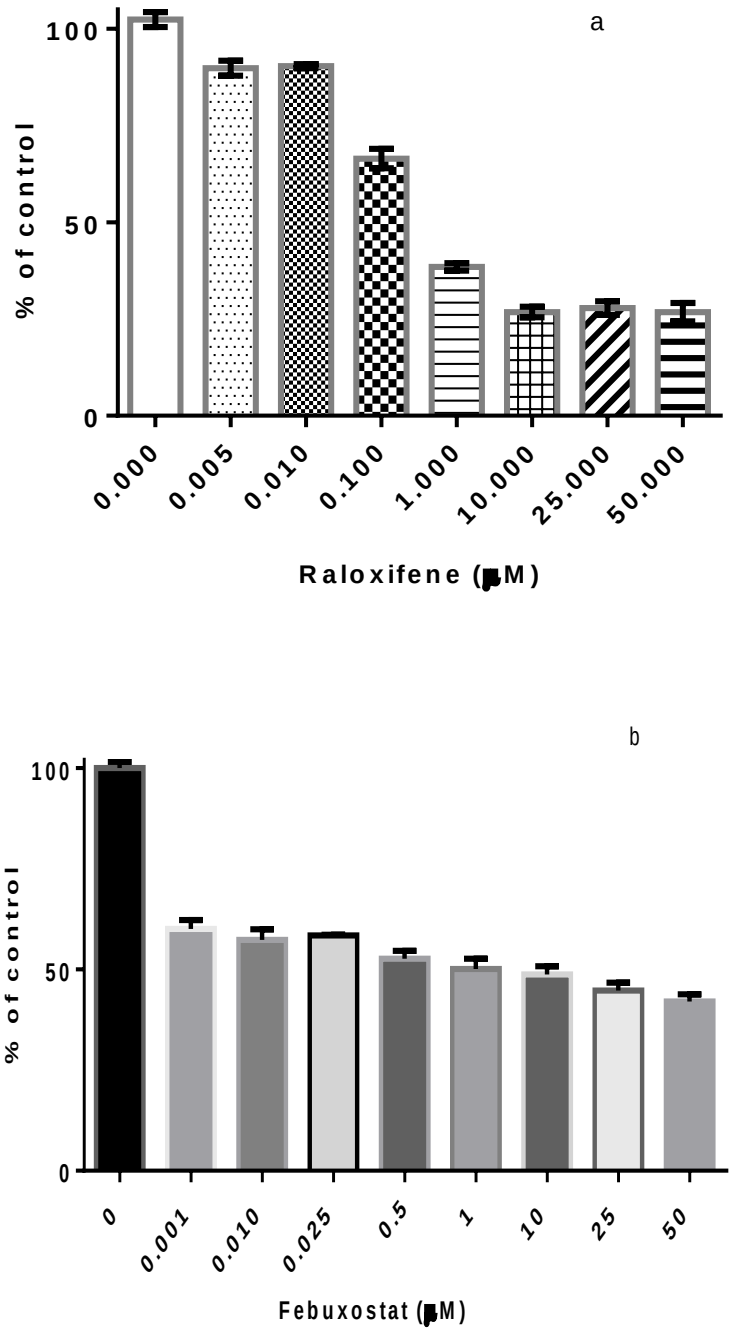
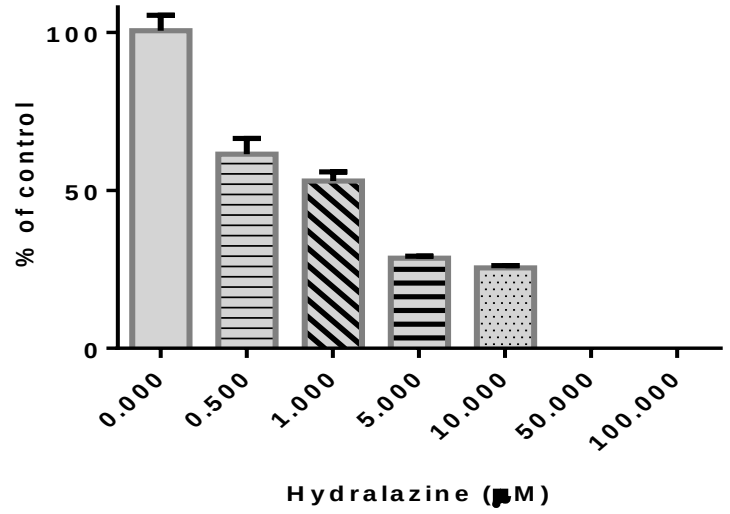
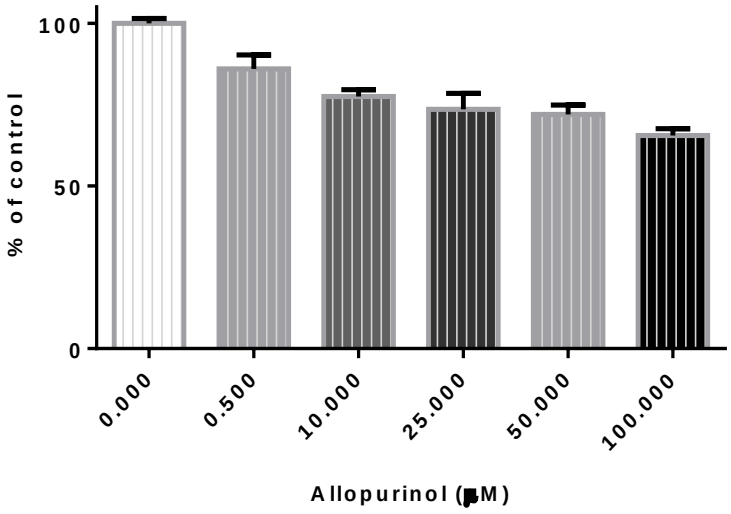


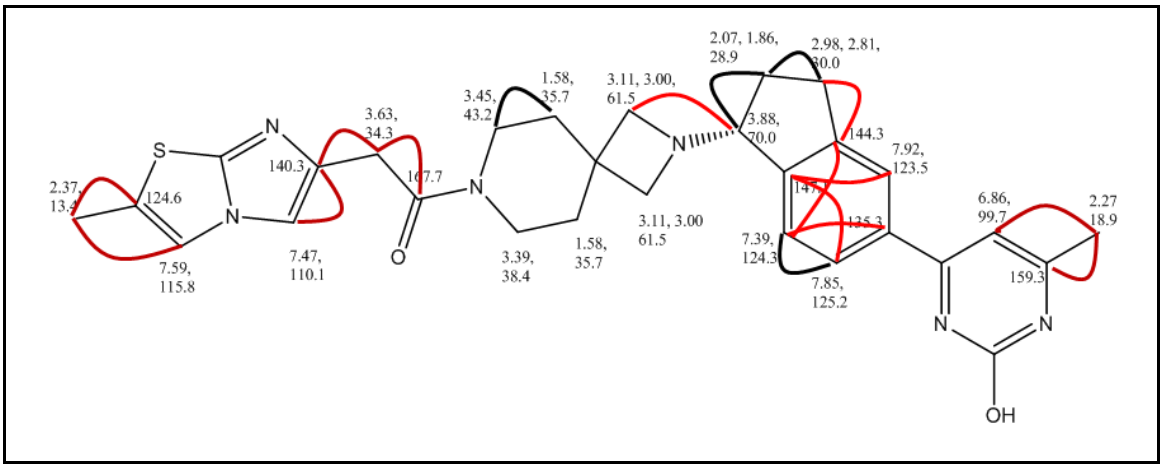
Fig. II-10. Hydroxy metabolite formation in the presence of inhibitors (% of control) at various concentrations of, (a) raloxifene and (b) febuxostat with 25 µM PF-5190457 in allopurinol free (n=9) pooled human liver cytosolic incubations.

Supplemental Data

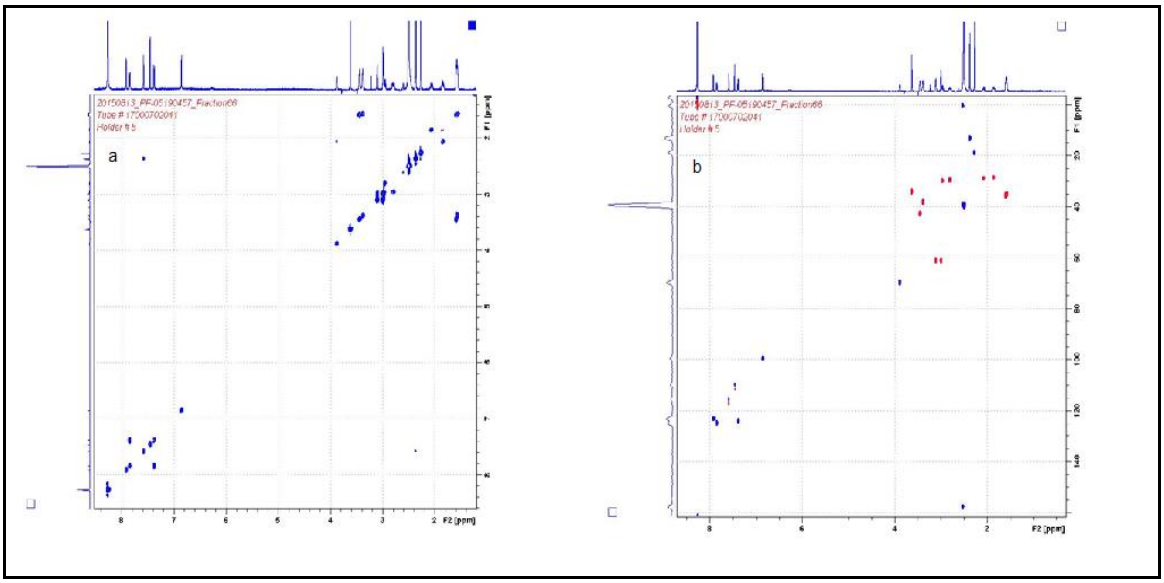




Supplemental Fig II-1 Shows percentage of PF-5190457 hydroxy metabolite formed in the presence and absence of inhibitors at various concentrations of (a) hydralazine, (b) allopurinol; the incubations contained 25 µM PF-5190457 in allopurinol free (n=9) pooled human liver cytosolic fraction



Supplemental Fig. II-2. COSY: Black Curves HMBC: Red Curves



Supplemental Fig II-3a and b. COSY NMR Spectra and HSQC NMR Spectra of hydroxy metabolite supporting oxidation at pyrimidine moiety

MANUSCRIPT-III

This manuscript has been prepared for submission to “Drug Metabolism and Disposition”

Assessment of AOX1 Levels Variability in Human Liver Cytosol with Label-Free Approach Using Mass Spectrometry

Sravani Adusumalli, Rohitash Jamwal, Ben Barlock, Scott R Obach, Fatemeh Akhlaghi

Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island Kingston, RI 02881 (A.S., A.F, J.R., B.B), Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Worldwide Research and Development, Pfizer, Inc., Groton, Connecticut (O.S.),

Running Title: Assessment of AOX1 levels variability in human liver cytosol with label free approach using mass spectrometry

Name and address for correspondence:

Fatemeh Akhlaghi, PhD
Clinical Pharmacokinetics Research Laboratory
Biomedical and Pharmaceutical Sciences
University of Rhode Island
7 Greenhouse Rd, Kingston, RI 02881, USA Phone: (401) 874 9205/
Fax: (401) 874 5787 Email: fatemeh@uri.edu

Number of Text Pages: 30

Number of Tables: 2

Number of Figures: 6

Number of References: 43

Number of Words in Abstract: 286

Number of Words in Introduction: 493

Number of Words in Discussion: 891

Abbreviations: – Aldehyde oxidase, AOX1 – Aldehyde oxidase protein, DIA – Data independent acquisition, DDA – Data dependent acquisition, SWATH-MS- Sequential windowed acquisition of all theoretical fragment ion mass spectra

Abstract

The present study describes the use of a mass spectrometry based label-free quantification approach to estimate the expression of aldehyde oxidase protein (AOX1) and also, to study the impact of various demographic factors on this enzyme in liver cytosolic fractions in a well-characterized bank of human liver (n = 104). Trypsin was used to digest cytosolic fractions, and a faster (90 min) digestion method was aided by a pressure cycling technology. Tryptically digested peptides were separated on an Acquity UHPLC Peptide BEH C18 column. The total run time was 60 min using a gradient method at a flow rate of 0.1 mL/min. The HLC samples were analyzed using two acquisition methods by data-dependent acquisition (DDA) and SWATH-MS (sequential windowed acquisition of all theoretical fragment ion mass spectra) mode on quadrupole-time-of-flight mass spectrometer (ESI-QTOFMS) operated in positive electrospray ionization mode. SWATH-MS data extracted using Skyline (targeted proteomics data extraction software) generated values for relative quantification while absolute protein of AOX1 was calculated by MaxQuant (identification and quantitative proteomics software) using total protein approach. We found that results from SWATH-MS based acquisition were comparable to traditional DDA method. The AOX1 levels quantified using MaxQuant was found to be in the range of 2 - 49 pmol/mg cytosolic protein (19.5 fold), which are well comparable to published reports on the expression of the enzyme. The effect of demographic factors such as age, gender, ethnicity, smoking, drug use, alcohol consumption and disease condition like diabetes and non alcoholic fatty liver disease (NAFLD)

on AOX1 protein in individual HLC were studied, but no significant association was found. The reasons for observed variability could be because of other underlying and unknown factors which show a need for further studies on the enzyme.

Abbreviations

AO aldehyde oxidase, AOX1 human aldehyde oxidase protein, DDA Data dependent acquisition, DIA Data independent acquisition, SWATH-MS Sequential windowed acquisition of all theoretical fragment ion mass spectra, XO Xanthine Oxidase

Introduction

Aldehyde oxidase (AO), along with xanthine oxidase (XO), belongs to the family of molybdo-flavoenzyme or molybdenum co factor containing enzymes and does not require NADPH as a cofactor for oxidation (Beedham, 1985; Hille, 2005). Unlike XO which catalyzes uric acid, the physiological function of AO in not known yet (Kitamura et al., 2006). In the recent year, AO is gaining significance in drug discovery programs because of a consorted effort to reduce the contribution of cytochrome P450 (CYP450) mediated biotransformation to the elimination of new drugs, (Pryde et al., 2010; Rashidi and Soltani, 2017). The new drug molecules are designed to have nitrogens in their rings like purines pyrimidines and pteridines to enable aldehyde oxidase metabolism (Lepri et al., 2017; Paragas et al., 2017). In contrast to electrophilic attack by CYP450 enzymes, aldehyde oxidase follows nucleophilic attack using the oxygen from the water (Beedham, 1985; Garattini et al., 2003). Various isoforms AOX1, AOX3, AOX3L1, and AOX4, have been identified with species differences. The functionally active aldehyde oxidase that is expressed in the human liver cytosol as AOX1 along with two other pseudogenes AOX3 and AOX3L1 (Kurosaki et al., 2013).

While a limited number of studies are published assessing the AOX1 levels in HLC, it is known that a significant degree of variability is associated with the expression of this enzyme (Barr et al., 2013; Fu et al., 2013). In one of the studies, the AOX1 levels reported in three batches of pooled HLC was found to

be between 2 - 40 pmol/mg (20 fold) (Barr et al., 2013). The other study assessed the levels in 20 individual HLC donors and the results observed were much tighter ranging from 0.74 – 2.30 pmol/mg (~3 fold) (Fu et al., 2013). The variation between both the studies was observed to be about 17 – 28-fold. Both the studies mentioned above used the absolute method of quantification (MRM) using mass spectroscopy.

The use of mass spectrometry is gaining popularity over traditional methods, such as Western blotting, enzyme-linked immunosorbent assays (ELISAs) and reverse-transcriptase polymerase chain reactions (RT-PCR) to monitor or quantify the expression of proteins (Han et al., 2008; Aebersold et al., 2013). Label-Free relative quantification approach for studying protein abundance of metabolizing enzymes in biological samples using mass spectrometry acquisition methods like data independent acquisition (DIA) and data dependent acquisition (DDA) (or information dependent acquisition) have been explored in the recent years (Hopfgartner et al., 2012). Our lab has recently published on using sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS-MS), which is a DIA method, to study relative quantification of enzymes using a label free approach (Jamwal et al., 2017).

In the present study, we used the DDA, and SWATH-MS to quantify the relative expression of AOX1 protein in the human liver cytosol. The main objective of the study was to quantify the AOX1 levels in a novel bank of human liver and to study the effect of various demographic factors on AO protein expression.

Materials and Methods

Chemical and Reagents. Trypsin digested β-galactosidase (E. coli), protein preparation kit, and L-1-*p*-Tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin were purchased from Sciex (Framingham, MA). Mass spectrometry grade acetonitrile and formic acid were from ThermoFisher Scientific (Waltham, MA). Acquity UHPLC Peptide BEH C18 analytical column and VanGuard precolumn were from Waters Corp. (Milford, MA).

Human liver bank.

Human liver tissue (N=104) was purchased from Sekisui XenoTech LLC (Kansas City, KS). The livers were well characterized for age, gender, ethnicity, the cause of death, cold ischemia time, liver and body weight, smoking, alcohol consumption, diabetes and NAFLD. The information was available for ≥95% of the samples.

HLC Preparation and Digestion.

Preparation of HLC and digestion of the samples was performed as described previously (Jamwal et al., 2017). Briefly, human liver samples were homogenized using a bead homogenizer (Bead Ruptor 24, Omni International, Kennesaw, GA). Ultracentrifugation was done to separate microsomes and cytosol. The cytosolic fraction thus obtained was stored in -80°c for future use. Cytosolic protein concentration was estimated using a bicinchoninic acid method (Pierce-Fisher, Rockford, IL) with bovine serum albumin as a standard. The

samples were diluted to 2.5 mg/mL in phosphate-buffered saline (pH 7.4) before protein digestion. The samples (150 µg of cytosolic protein) initially were denatured to disrupt the hydrogen and hydrophobic bonds of the proteins. Subsequent steps were to reduce the disulfide bonds of the proteins and addition of an alkylating agent to block the cysteine group in the samples. The digestion buffer was added to the cysteine blocked samples to maintain the optimum pH before digesting the samples. The proteins in the samples were digested with TPCK treated trypsin (1:20 enzyme/protein) in a Barocyler NEP2320− 45k (Pressure BioSciences Inc.) working on the principle of pressure aided digestion of proteins (pressure cycling technology-PCT). The optimized settings for temperature, cycles and pressure on the barocycler were 50 °C for 90 cycles, 50 s at 35 kpsi and 10 s at ambient pressure for every cycle. Two technical replicates for each HLC sample were digested and analyzed by mass spectrometry. XTreme 200 pool of HLC sample was used as digestion control to monitor the batch-to-batch variation of protein digestion carried out in six batches. Mostly, 12 cytosolic samples and one XTreme 200 pool of HLC as control sample were digested and run in every batch.

LC-QTOF/MS Analysis.

All the cytosolic digested samples were analyzed on a SCIEX 5600 TripleTOF mass spectrometer equipped with a DuoSpray ion source (SCIEX, Concord, Canada) coupled to Acquity UHPLC HClass system (Waters Corp., Milford, MA, USA) which has a with a binary pump and a built-in column heater.

Mass spectral analysis was carried out using a positive electrospray ionization mode. Chromatography separation of peptides was accomplished within 11.30 min using gradient elution method using an Acquity UHPLC Peptide BEH C18 (2.1 × 150 mm2, 300 Å, 1.7 µm) analytical column. An Acquity VanGuard precolumn (2.1 × 5 mm 2, 300 Å, 1.7 µm) was used prior to the analytical column. The analytical column and autosampler temperature consisting digested samples were kept at 40 °C and 10°C, respectively. Mobile phase A consisted of 98% water, 2% acetonitrile containing 0.1% formic acid and mobile phase B 98% acetonitrile, 2% water containing 0.1% formic acid. The mobile phase was delivered at 100 µL/min flow rate with a gradient method and total run time of 60 min. The initial conditions of mobile phase composition are as follows: (98% A) was maintained for 3 min, 60% to 90% A from 3 to 48 min, and 20% A held till 52 min and returned to initial conditions for equilibration. The amount of protein per injection on the column was 10 µg. In each batch, Trypsin-predigested beta-galactosidase peptides were injected (~30 pmol/injection) as quality control every 10 samples during the analysis to monitor mass calibration of the TOF detector and normalization of intensity during relative quantification of AOX1 protein (described below). The average intensity of the β-galactosidase peptide (APLDNDIGVSEATR) in a batch was used for data normalization of the respective batch of samples.

DDA and SWATH-MS Data Acquisition Settings.

Two acquisition methods, DDA and SWATH-MS method were used to

identify and quantify AOX1 protein. Analyst TF 1.7 was used to acquire data during the study (SCIEX, Framingham, MA). The detailed settings for source and compound parameters for both the acquisition methods are as described in previously published paper from our lab (Jamwal et al., 2017). The DDA experiments were performed in positive resolution mode over a mass range of m/z 350−950 with a charge state 2 to 4, and quadrupole resolution of 0.7 AMU were used for automated MS/MS analysis. Whereas, SWATH-MS-MS. based spectra were acquired for mass range m/z 400−900 Da with SWATH-MS window width of 25 m/z resulting in 20 overlapping mass windows per cycle.

Raw Data Processing.

Generation of spectral reference library

The data files of HLC samples obtained from the DDA acquisition were analyzed using the database search engine ProteinPilot 5.0 (SCIEX; Framingham, MA, USA) The data were searched against the UniProt protein database. The search was specified using the following parameters in the Paragon method: sample type - identification; cysteine alkylation - MMTS; digestion - Trypsin; instrument - TripleTOF 5600, species - *Homo sapiens*; search effort -thorough and false discovery analysis - none.

Method 1 Based on DIA-SWATH-MS MS and Skyline analysis.

The spectral library generated from DDA files was uploaded in Skyline to identify AOX1 protein, and SWATH-MS data files were processed using the full

scan MS/MS filtering at a resolution of 10000. Three unique AOX1 peptides were identified and curated for reproducible fragment ions. The total area of representative peptides for AOX1 protein was summed, and resulting intensity was normalized by total intensity of tryptic peptide of β-galactosidase. MultiQuant v 3.0 (SCIEX, Framingham, MA) was used to retrieve intensity for APLDNDIGVSEATR peptide and was subsequently used for normalization among different batches as described above (Nakamura et al., 2016).

*Method 2 Based on DDA acquisition and MaxQuant analysis***.**

The raw data files obtained from DDA data was analyzed for AOX1 using MaxQuant (Version 1.5) and was searched against UniProt human protein database at a false discovery rate (FDR) of 0.01. The absolute protein concentration was calculated from label-free quantification (LFQ) intensities using “Total Protein Approach”(Wisniewski and Rakus, 2014).

Statistical analysis.

Statistical analysis was performed with SPSS version 24 (IBM Analytics, Armonk, NY), and Prism® version 6 (GraphPad Software Inc., San Diego, CA) was used for graphs. Non-parametric Mann-Whitney U test and Kruskal-Wallis test (2-tailed) were used to study the effect of three or more groups. The correlation was studied using nonparametric Spearman correlation analysis. $P < 0.05$ was considered significant for all the statistical tests and correlation analysis.

Results

SWATH-MS based relative AOX1 levels

Three unique peptides for AOX1 protein DILADVVQK, MIQVVSR, GTSTETVPNANISGGSVVADLNGLAVK and three fragment ions per peptide for the protein were identified. AOX1 levels from SWATH-MS files are represented after normalization Selected peptides for the relative quantification of the AOX1 protein were correlated with each other and also, with sum of the three peptides to validate the selection of peptides (Fig 1a and b). The correlation between the surrogate peptides (Spearman, $r = 0.94$, $p<0.0001$) was significant. Likewise, the correlation between one of the selected peptides (DILADVVQK) of AOX1 and sum of the peptides was significant (Spearman, $r = 0.98$, $p<0.0001$). Two other peptides had similar significant correlation (data not shown) generating confidence in the selection of peptides.

DDA based absolute AOX1 levels

The AOX1 levels in the cytosolic samples determined via total protein approach ranged from 2.46 – 49.02 pmol/mg cytosolic membrane protein. There was variability with about 19-fold difference between the samples. Fig 3 shows the distribution of AOX1 levels across various cytosolic samples. AOX1 levels obtained via MaxQuant were used to determine the effect of various demographic factors on the AO enzyme.

Correlation between SWATH-MS and DDA based results

The correlation between the AOX1 levels measured via both the acquisition methods (DIA and DDA) was significant (Spearman, r = 0.72, p<0.0001) (Fig 3). The results indicate the reliability of either of the acquisition methods for determination of AOX1 levels.

Effect of demographic factors on AOX1 levels.

The effect of various factors like gender, age, weight, drug usage, ethnicity, smoking, alcohol consumption along with disease conditions like diabetes and NAFLD (non alcoholic fatty liver disease) was evaluated on AOX1 levels.

Cytosolic fatty acid synthase (FAS). FAS is observed to be a diagnostic marker for progression in NAFLD (Dorn et al., 2010). As the disease progresses, the FAS expression is expected to increase as seen in Fig 4. This enzyme was used as a positive control to determine the quality of data generated from mass spectroscopy.

Effect of Ethnicity.

The number of African American, Caucasian and Hispanic included in this study are as shown in Table 1**.** In general, African Americans exhibited marginally higher AOX1 levels in comparison to Caucasian and Hispanic donors. However, no significant association (*P>0.4*) was observed between Ethnicity and AOX1 levels (Fig 5) (Table. 2).

Effect of Alcohol.

Alcohol consumption in the liver donors were evaluated and scores were determined based on the drinking scale (None = 0, Safe = 1, Moderate = 2, Heavy = 3). The trend of AOX1 levels decreased with increase in alcohol consumption but not significant (*P>0.4*) (Fig 5) (Table.2)

Effect of other demographic factors.

Other demographic factors such as gender, age, weight, drug usage, smoking, along with disease conditions like diabetes and NAFLD (non alcoholic fatty liver disease) were not detected to have an impact on AOX1 levels based on our results. Table. 2 shows the effect of selected demographic factors on AOX1 with mean ±SE

Discussion

The AOX1 levels measured in HLC were found to be variable in the present study. Cytosolic AOX1 levels in human livers were quantified using two different acquisition methods in mass spectroscopy. Our lab had initially developed a DIA method using SWATH-MS method to determine the relative protein levels of various drug metabolizing enzymes which was published recently (Jamwal et al., 2017). DDA was used for identification of proteins in the earlier method (details in the method section). In due course, MaxQuant a quantitative proteomics software was utilized which allows both in identifying and quantifying the proteins of interest using the raw files generated from DDA (Cox et al., 2014). Both the acquisition methods (DIA and DDA) demonstrated good correlation with each other and reliability in using either method to quantify AOX1 protein. The cytosolic FAS protein (a marker for progression of NAFLD) assessment gave confidence in the reliability of the quantified protein levels using mass spectroscopy. The leverage of total protein approach in MaxQuant is that it gives accurate estimations of protein concentrations (Wisniewski et al., 2014). The absolute values of pmol of AOX1/mg of total cytosolic protein values generated from MaxQuant in the present study were comparable to other studies (Barr et al., 2013; Fu et al., 2013). The variation in our study was observed to be about 25-fold (2 - 49 pmol/mg) in a novel bank of human livers. The AOX1 levels quantified were much closer to the results reported by Barr et al. in pooled HLC (Barr et al., 2013).

The expression of drug metabolizing enzymes can be influenced by many intrinsic and extrinsic factors (Rogers et al., 2002; Wilkinson, 2005; Fisher et al., 2009; Merrell and Cherrington, 2011; Yang et al., 2013; He et al., 2015). Most importantly factors like genetic polymorphism (Meyer, 2004), epigenetic modifications like DNA methylation, histone protein modification or interaction with microRNAs (Chuang and Jones, 2007; Ingelman-Sundberg and Gomez, 2010), and non genetic factors like age (Kinirons and O'Mahony, 2004), gender (Beierle et al., 1999) or disease state (Elbekai et al., 2004) can influence the expression of metabolizing enzymes. Pharmacokinetic variability in individuals can be largely explained by the above mentioned factors influencing the metabolizing enzymes and the knowledge of these factors is important to monitor the drug response (Lin, 2007).

AOX1 is one of the highly abundant enzymes found in human liver (Beedham, 1987; Nishimura and Naito, 2006). Non alcoholic fatty liver disease (NAFLD) has demonstrated to influence the AOX1 levels. NAFLD is characterized by a spectrum of conditions ranging from simple steatosis, non-alcoholic steatohepatitis with fibrosis progressing to cirrhosis (Targher et al., 2008; Younossi et al., 2011). The rats fed with high-fat diet and primary hepatocytes loaded with palmitic acid showed an increase in AOX1 expression (Neumeier et al., 2006). It was observed that the primary hepatocytes for the study were isolated and cultured from tissue samples obtained were not from healthy subjects but from patients with liver tumors of colorectal cancer who were undergoing hepatectomy. The levels of AOX1 could also be influenced by

disease conditions like cancer. Certain phase I and II drug metabolizing enzymes, AOX1, CYP1B1, GSTM3 and GSTP1 gene and protein expression were found to be elevated in brain tumor specimens obtained from patients (n=77) undergoing primary resection (Stavrinou et al., 2015). Whereas, in the present study NAFLD did not have a significant impact on the AOX1 levels.

In an *in vitro* study (Fu et al., 2013) poor correlation was observed between AOX1 activity and expression levels in HLC. Several demographic factors along with protein mis-folding, polymorphism and co-factor deficiency were suspected to be the reasons for observed poor correlation, but no clear conclusions were drawn owing to their small sample size (n=20). In the same study, chronic alcohol consumption in liver donors (n=2) decreased the AO activity significantly but not the AOX1 levels. The AOX1 levels in the present study were not altered significantly with increase in alcohol consumption. In other *in vitro* studies, considerable inter-individual variability in the AO metabolism has been observed using different substrates (Al-Salmy, 2001; Hutzler et al., 2014). It would have been beneficial if the studies had AOX1 levels measured to correlate with the observed activity and if any other demographic factors or post-transitional modifications are influencing the activity of the protein.

AO contains molybedenum and iron sulfur centers along with flavin adenine dinucleotide (Beedham, 1985). Iron deficiency diet decreased the activity of AO in rats and increasing the iron reverted the activity to normal indicating iron to have a significant impact on AO enzyme (Mackler et al., 1978).

It is known that XO belongs to the same family as AO and is closely related enzyme to AO (Beedham, 1985). In a rat study, XO levels and activity were observed to be decreased with iron-depleted diet and increased when supplemented with iron (Ghio et al., 2002). Both these studies indicate iron could play an important role in modulating the AOX1 levels.

Many other unknown factors could be the reason for observed variability in the AOX1 levels in our study. Recognizing the sources of variability could be challenging as there may be other underlying unknown epigenetic factors, disease state or dietary constituents influencing the enzyme. To the best of our knowledge, for the first time, the effect of demographic factors on AOX1 levels in a large number of human livers was studied.

Conflict of interest

Dr. Obach is an employee of Pfizer Pharmaceuticals. Other authors declare no potential conflict of interest with any commercial entity whose products were used in the study.

Acknowledgements

The financial support of Grant #UH3TR000963 from National Institutes of Health is gratefully acknowledged.

References

Aebersold R, Burlingame AL, and Bradshaw RA (2013) Western blots versus selected reaction monitoring assays: time to turn the tables? *Mol Cell Proteomics* **12:**2381-2382.

Al-Salmy HS (2001) Individual variation in hepatic aldehyde oxidase activity. *IUBMB Life* **51:**249-253.

Barr JT, Jones JP, Joswig-Jones CA, and Rock DA (2013) Absolute quantification of aldehyde oxidase protein in human liver using liquid chromatography-tandem mass spectrometry. *Mol Pharm* **10:**3842-3849.

Beedham C (1985) Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metab Rev* **16:**119-156.

Beedham C (1987) Molybdenum hydroxylases: biological distribution and substrate-inhibitor specificity. *Prog Med Chem* **24:**85-127.

Beierle I, Meibohm B, and Derendorf H (1999) Gender differences in pharmacokinetics and pharmacodynamics. *Int J Clin Pharmacol Ther* **37:**529-547.

Chuang JC and Jones PA (2007) Epigenetics and microRNAs. *Pediatr Res* **61:**24R-29R.

Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, and Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* **13:**2513-2526.

Dorn C, Riener MO, Kirovski G, Saugspier M, Steib K, Weiss TS, Gabele E, Kristiansen G, Hartmann A, and Hellerbrand C (2010) Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol* **3:**505-514.

Elbekai RH, Korashy HM, and El-Kadi AO (2004) The effect of liver cirrhosis on the regulation and expression of drug metabolizing enzymes. *Curr Drug Metab* **5:**157-167.

Fisher CD, Lickteig AJ, Augustine LM, Ranger-Moore J, Jackson JP, Ferguson SS, and Cherrington NJ (2009) Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos* **37:**2087-2094.

Fu C, Di L, Han X, Soderstrom C, Snyder M, Troutman MD, Obach RS, and Zhang H (2013) Aldehyde oxidase 1 (AOX1) in human liver cytosols: quantitative characterization of AOX1 expression level and activity relationship. *Drug Metab Dispos* **41:**1797-1804.

Garattini E, Mendel R, Romao MJ, Wright R, and Terao M (2003) Mammalian molybdo-flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. *Biochem J* **372:**15-32.

Ghio AJ, Kennedy TP, Stonehuerner J, Carter JD, Skinner KA, Parks DA, and Hoidal JR (2002) Iron regulates xanthine oxidase activity in the lung. *Am J Physiol Lung Cell Mol Physiol* **283:**L563-572.

Han X, Aslanian A, and Yates JR, 3rd (2008) Mass spectrometry for proteomics. *Curr Opin Chem Biol* **12:**483-490.

He ZX, Chen XW, Zhou ZW, and Zhou SF (2015) Impact of physiological, pathological and environmental factors on the expression and activity of human

cytochrome P450 2D6 and implications in precision medicine. *Drug Metab Rev* **47:**470-519.
Hille R (2005) Molybdenum-containing hydroxylases. *Arch Biochem Biophys* **433:**107-116.
Hopfgartner G, Tonoli D, and Varesio E (2012) High-resolution mass spectrometry for integrated qualitative and quantitative analysis of pharmaceuticals in biological matrices. *Anal Bioanal Chem* **402:**2587-2596.
Hutzler JM, Yang YS, Brown C, Heyward S, and Moeller T (2014) Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. *Drug Metab Dispos* **42:**1090-1097.
Ingelman-Sundberg M and Gomez A (2010) The past, present and future of pharmacoepigenomics. *Pharmacogenomics* **11:**625-627.
Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, and Akhlaghi F (2017) Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS-MS. *J Proteome Res*.
Kinirons MT and O'Mahony MS (2004) Drug metabolism and ageing. *Br J Clin Pharmacol* **57:**540-544.
Kitamura S, Sugihara K, and Ohta S (2006) Drug-metabolizing ability of molybdenum hydroxylases. *Drug Metab Pharmacokinet* **21:**83-98.
Kurosaki M, Bolis M, Fratelli M, Barzago MM, Pattini L, Perretta G, Terao M, and Garattini E (2013) Structure and evolution of vertebrate aldehyde oxidases: from gene duplication to gene suppression. *Cell Mol Life Sci* **70:**1807-1830.
Lepri S, Ceccarelli M, Milani N, Tortorella S, Cucco A, Valeri A, Goracci L, Brink A, and Cruciani G (2017) Structure-metabolism relationships in human-AOX: Chemical insights from a large database of aza-aromatic and amide compounds. *Proc Natl Acad Sci U S A* **114:**E3178-E3187.
Lin JH (2007) Pharmacokinetic and pharmacodynamic variability: a daunting challenge in drug therapy. *Curr Drug Metab* **8:**109-136.
Mackler B, Person R, Miller LR, Inamdar AR, and Finch CA (1978) Iron deficiency in the rat: biochemical studies of brain metabolism. *Pediatr Res* **12:**217-220.
Merrell MD and Cherrington NJ (2011) Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug Metab Rev* **43:**317-334.
Meyer UA (2004) Pharmacogenetics - five decades of therapeutic lessons from genetic diversity. *Nat Rev Genet* **5:**669-676.
Nakamura K, Hirayama-Kurogi M, Ito S, Kuno T, Yoneyama T, Obuchi W, Terasaki T, and Ohtsuki S (2016) Large-scale multiplex absolute protein quantification of drug-metabolizing enzymes and transporters in human intestine, liver, and kidney microsomes by SWATH-MS-MS: Comparison with MRM/SRM and HR-MRM/PRM. *Proteomics* **16:**2106-2117.
Neumeier M, Weigert J, Schaffler A, Weiss TS, Schmidl C, Buttner R, Bollheimer C, Aslanidis C, Scholmerich J, and Buechler C (2006) Aldehyde oxidase 1 is highly abundant in hepatic steatosis and is downregulated by adiponectin and fenofibric acid in hepatocytes in vitro. *Biochem Biophys Res Commun* **350:**731-735.

Nishimura M and Naito S (2006) Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. *Drug Metab Pharmacokinet* **21:**357-374.
Paragas EM, Humphreys SC, Min J, Joswig-Jones CA, and Jones JP (2017) The two faces of aldehyde oxidase: Oxidative and reductive transformations of 5-nitroquinoline. *Biochem Pharmacol*.
Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, and Tran TD (2010) Aldehyde oxidase: an enzyme of emerging importance in drug discovery. *J Med Chem* **53:**8441-8460.
Rashidi MR and Soltani S (2017) An overview of aldehyde oxidase: an enzyme of emerging importance in novel drug discovery. *Expert Opin Drug Discov* **12:**305-316.
Rogers JF, Nafziger AN, and Bertino JS, Jr. (2002) Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs. *Am J Med* **113:**746-750.
Stavrinou P, Mavrogiorgou MC, Polyzoidis K, Kreft-Kerekes V, Timmer M, Marselos M, and Pappas P (2015) Expression Profile of Genes Related to Drug Metabolism in Human Brain Tumors. *PLoS One* **10:**e0143285.
Targher G, Marra F, and Marchesini G (2008) Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? *Diabetologia* **51:**1947-1953.
Wilkinson GR (2005) Drug metabolism and variability among patients in drug response. *N Engl J Med* **352:**2211-2221.
Wisniewski JR, Hein MY, Cox J, and Mann M (2014) A "proteomic ruler" for protein copy number and concentration estimation without spike-in standards. *Mol Cell Proteomics* **13:**3497-3506.
Wisniewski JR and Rakus D (2014) Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics* **109:**322-331.
Yang L, Price ET, Chang CW, Li Y, Huang Y, Guo LW, Guo Y, Kaput J, Shi L, and Ning B (2013) Gene expression variability in human hepatic drug metabolizing enzymes and transporters. *PLoS One* **8:**e60368.
Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, and Srishord M (2011) Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* **9:**524-530 e521; quiz e560.

Tables

Table. III-1. Detailed donor demographics

N (total)	104
Male, Female	49, 55
Ethnicity[#] (n) C, AA, H	89, 10, 5
Age[1] (years)	50.2 ± 1.1
Body-mass index[1] (kg/m^2)	31.4 ± 1.07
Diabetes mellitus (no, yes)	51, 53
Liver weight[1] (kg)	1.59 ± 0.58
Body weight[1] (kg)	88.7 ± 2.84
Smoking (no, yes)	46, 58
Drug Usage (no, yes)	83, 21
Alcohol consumption (Scale 0 -4) (no –heavy)	42, 21, 19, 10, 12
NAFLD (Scale 0 - 2) (none - disease progression)	41, 34, 29

[#]C-Caucasian, AA-African-American, H-Hispanic; [1]mean±SE.

Table. III-2. Effect of demographic factors on AOX1 levels

Factors	Groups	AOX1 (pmol/mg)[1]	Test	P value
Gender	Male (n=55)	26.7 ± 1.2	Mann-Whitney U	0.94
	Female (n=49)	26.9 ± 1.1		
Ethnicity[#]	AA (n=10)	31 ± 0.9	Kruskal Wallis	0.459
	CC (n=89)	26.4 ± 3		
	H (n=5)	25.2 ± 1		
Alcohol Consumption Scale	0 (n=42)	27 ± 1.1	Kruskal Wallis	0.466
	1 (n=21)	28.8 ± 2		
	2 (n=19)	28.2 ± 2.4		
	3 (n=10)	24.6 ± 1.8		
	4 (n=12)	22.4 ± 2.5		
Smoking	Yes (n=58)	26.7 ± 1.1	Mann-Whitney U	0.587
	NO (n=46)	26.6 ± 1.3		
Diabetes	Yes (n=53)	26.1 ± 1	Mann-Whitney U	0.797
	NO (n=51)	27.7 ± 1.3		
NAFLD (Scale 0-2) (none - progression of disease)	0 (n=41)	27.7 ± 1.3	Kruskal Wallis	0.254
	1 (n=34)	25.9 ± 1.2		
	2 (n=29)	26.5 ± 1.9		

[#]C-Caucasian, AA-African-American, H-Hispanic; [1]mean±SE

Legend of Figures

Fig. 1. Correlation plots

Fig. 2. Correlation plot between DIA (SWATH-MS-MS) and DDA for AOX1 levels

Fig. 3. Distribution of AOX1 levels across individual human liver cytosolic samples (n=104)

Fig. 4. Cytosolic fatty acid synthase in NAFLD.

Fig. 5. Effect of ethnicity on AOX1 levels

Fig. 6. Effect of alcohol on AOX1 levels

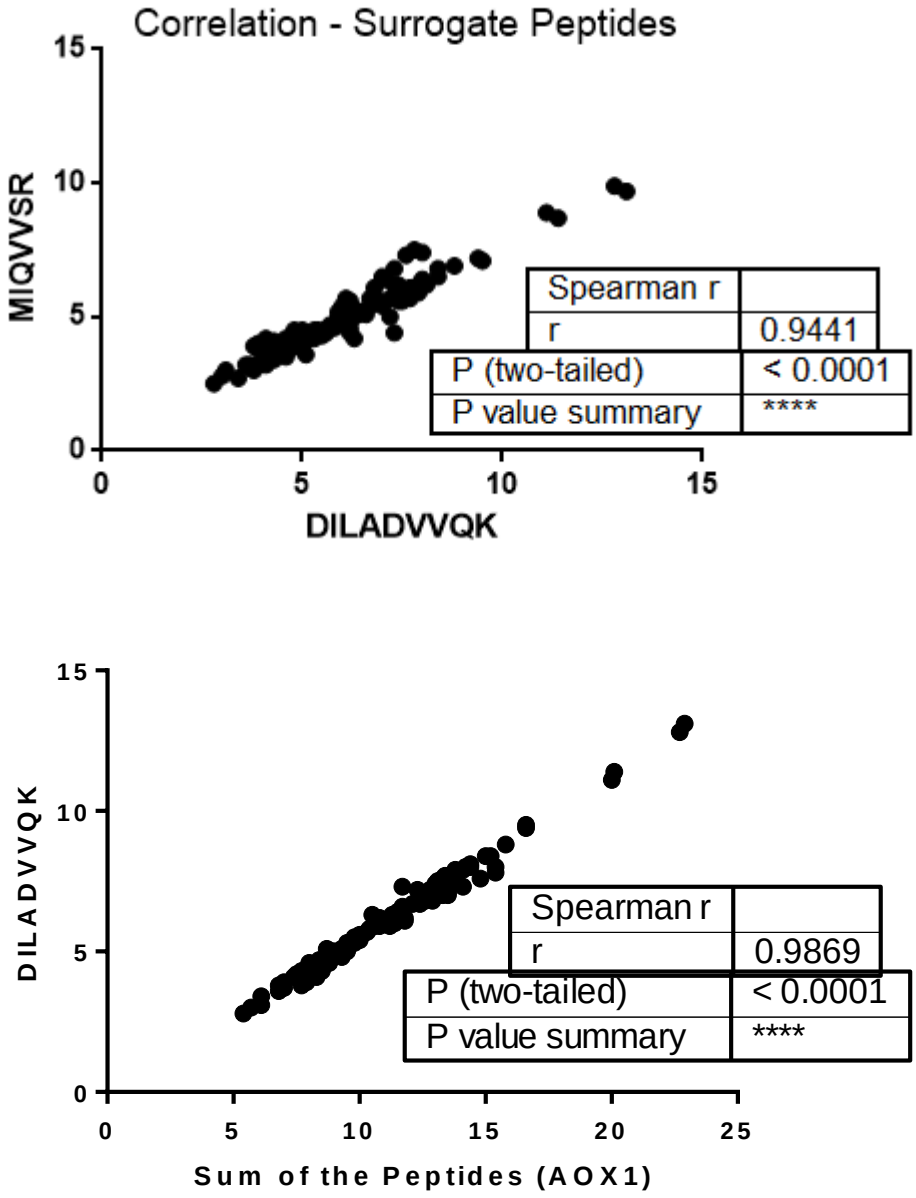


Fig. III-1. Correlation plots (a) correlation between surrogate peptides of AOX1 and (b) correlation between one of the unique peptide and sum of the peptides for AOX1 from SWATH-MS was considered significant ($p<0.05$) using correlation coefficient Spearman r.

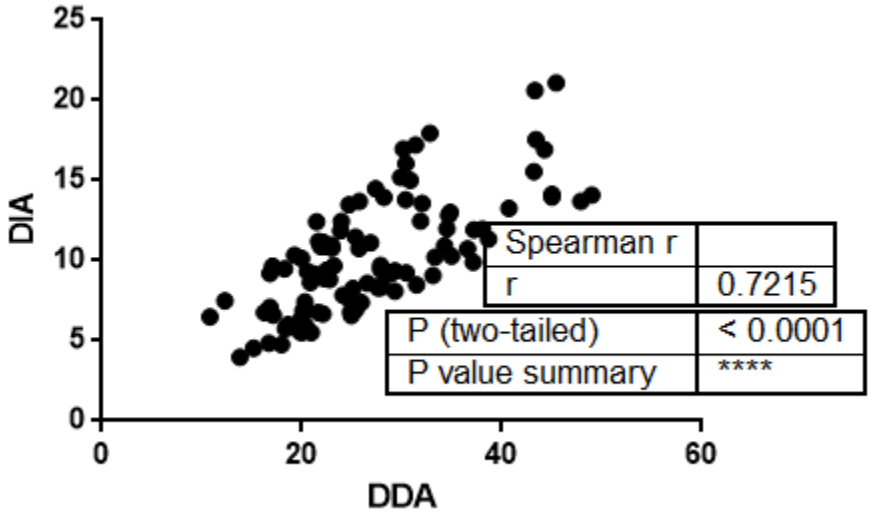


Fig. III- 2. Correlation plot between DIA (SWATH-MS) and DDA for AOX1 levels. Correlation between two acquisition methods SWATH-MS and traditional DDA was considered significant ($p<0.05$) using correlation coefficient Spearman r

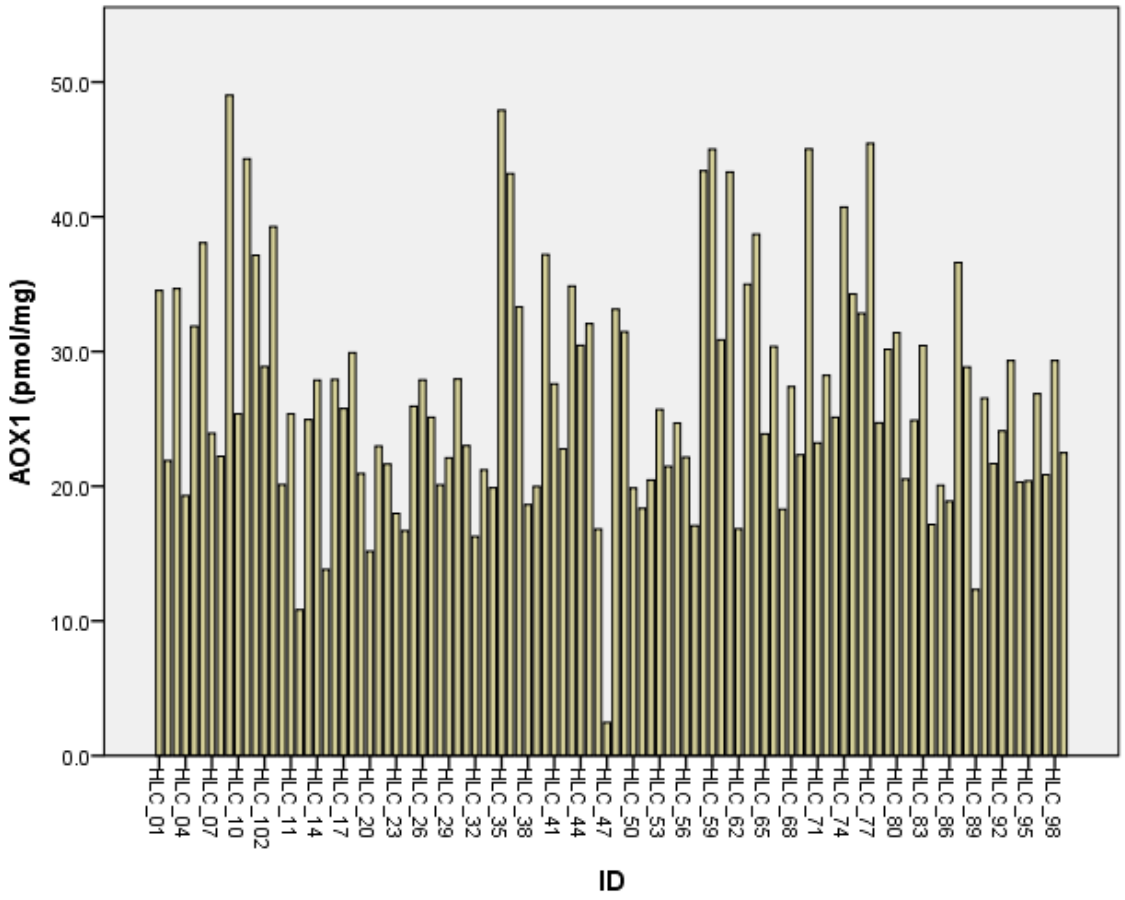


Fig. III-3. Distribution of AOX1 levels across individual human liver cytosolic samples (n=104). The AOX1 levels ranged from of 2-49 pmol/mg cytosolic protein (19.5 fold).

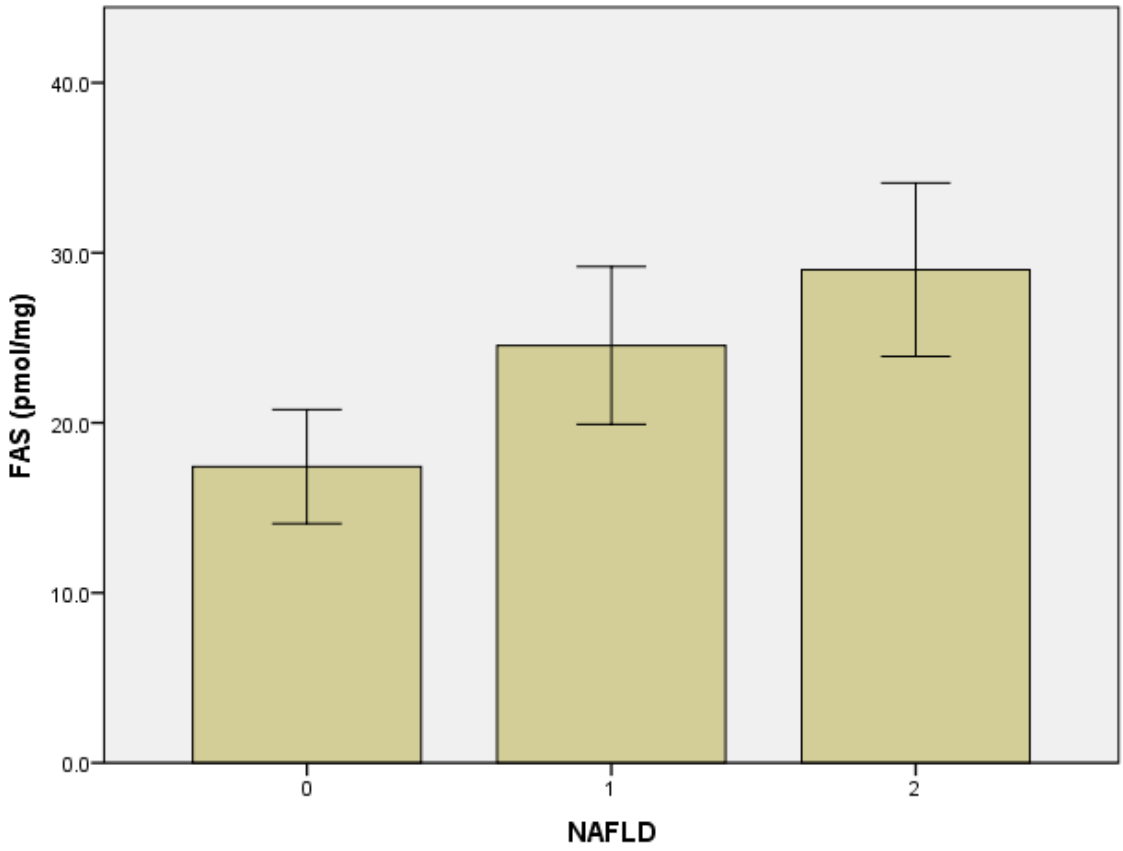


Fig. III-4. Cytosolic fatty acid synthase in non alcoholic fatty liver disease (NAFLD). Increase in cytosolic fatty acid synthase with progress in NAFLD was observed to be significant in HLC. NAFLD score (0 - 2) indicates none – 0, non alcoholic fatty liver – 1, progression to non-alcoholic steatohepatitis – 2. Column and error bars represent mean±SE. *P<0.05 reported from nonparametric Kruskal-Wallis test (2-sided).

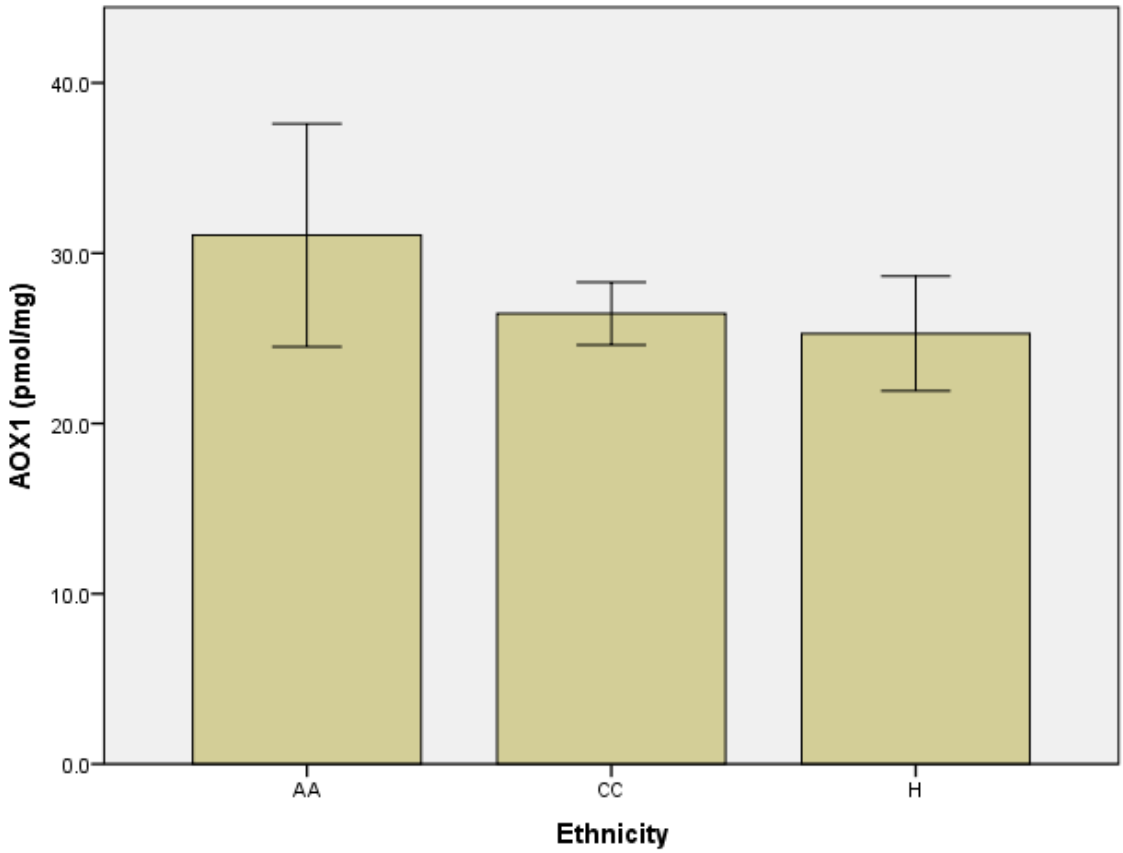


Fig. III-5. Effect of ethnicity on AOX1 levels. Effect of ethnicity (AA – African American, CC – Caucasian, H – Hispanic) on AOX1 was observed to be not significant across the groups in HLC Column and error bars represent mean±SE. *P* >0.4 reported from nonparametric Kruskal-Wallis test (2-sided).

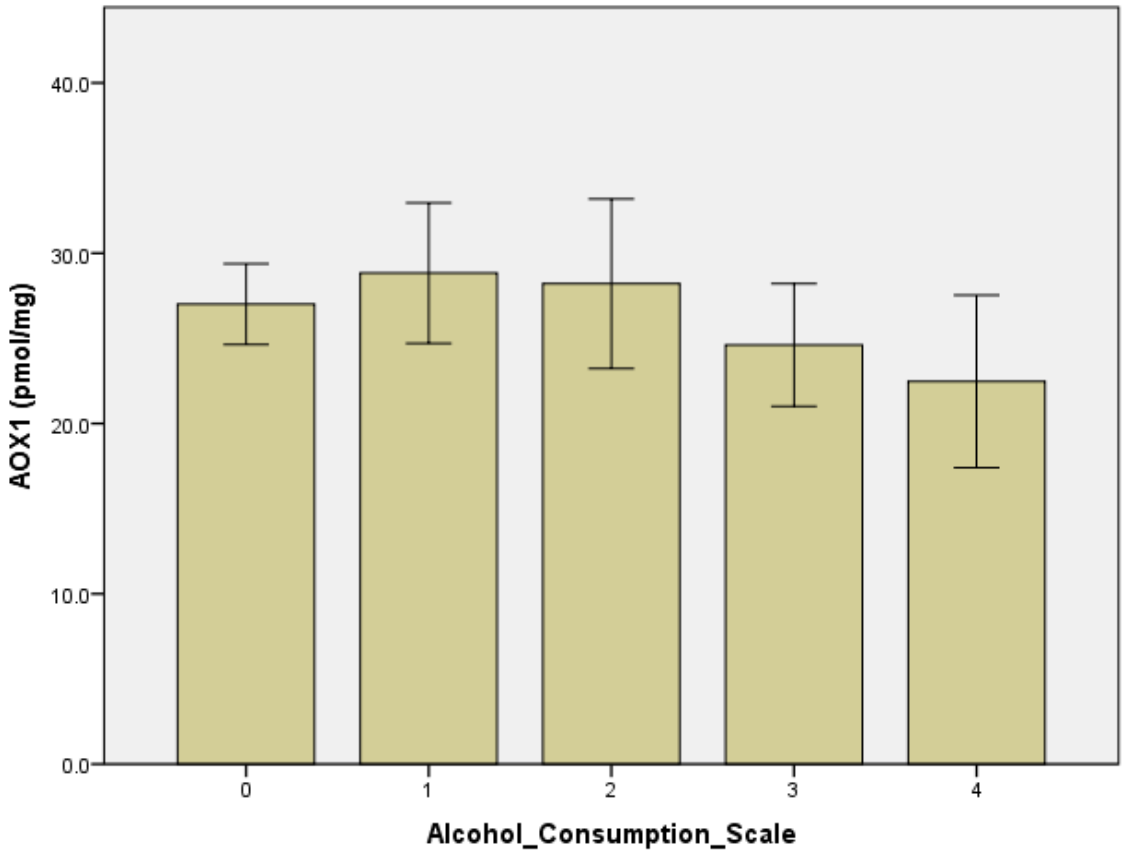


Fig. III-6. Effect of alcohol on AOX1 levels. Alcohol consumption had no significant effect on AOX1 levels in HLC. Alcohol consumption scale (0-4) indicated none to heavy drinking. (None = 0, Safe = 1, Moderate = 2, Heavy = 3). Column and error bars represent mean±SE. *P* >0.4 reported from nonparametric Kruskal-Wallis test (2-sided).

MANUSCRIPT-IV

This manuscript has been prepared for submission to British Journal of Clinical Pharmacology

Clinical Pharmacokinetics of a Major Hydroxy Metabolite of PF-5190457, a Ghrelin Receptor Inverse Agonist- Evidence from *In vivo* and *In vitro* Studies

Sravani Adusumalli[1], R. Scott Obach[2], Lorenzo Leggio[3,4,5], Rohitash Jamwal[1], Benjamin Barlock[1], Fatemeh Akhlaghi[1]

1 Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island Kingston, RI 02881

2. Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Worldwide Research and Development, Pfizer, Inc., Groton, Connecticut

3. Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology, Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA

4. Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA

5. Center for Alcohol and Addiction Studies, Department of Behavioral and Social Sciences, Brown University, Providence, RI 02903, USA

All authors have read and approved the content of this manuscript

Address for correspondence:

Fatemeh Akhlaghi, PhD

Clinical Pharmacokinetics Research Laboratory

Biomedical and Pharmaceutical Sciences

University of Rhode Island

7 Greenhouse Rd, Kingston, RI 02881, USA Phone: (401) 874 9205

Fax: (401) 874 5787 Email: fatemeh@uri.edu

Word count

Abstract: 249

Words in paper (without title page, abstract, references, tables, figures): 2847

Tables: 4

Figures: 6

Running title: Clinical Pharmacokinetics and *In Vitro* study of PF-5190457

Clinical Pharmacokinetics of a Major Hydroxy Metabolite of PF-5190457, a Ghrelin Receptor Inverse Agonist- Evidence from *In vivo* and *In vitro* Studies

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- PF-5190457 is a ghrelin receptor inverse agonist. It has been proposed to treat alcohol use disorder and type 2 diabetes. In phase 1 clinical studies, PF-5190457 was well tolerated in healthy volunteers and subjects in alcohol use disorder.

- PF-5190457 is majorly metabolized by aldehyde oxidase enzyme to (add the name of AO metabolite)

- Aldehyde oxidase is a cytosolic enzyme that displays high interindividual variability with different substrates

WHAT THIS PAPER ADDS

The manuscript, for the first time, reports the pharmacokinetics of PF-5190457 and its major hydroxy metabolite in alcoholic subjects. The half-life of the metabolite was higher in comparison to the parent drug. The metabolism of PF-5190457 was found to be variable across various human liver cytosolic fractions

AIM: To investigate the pharmacokinetics of PF-5190457 and its major hydroxy metabolite at 50 and 100 mg doses in alcoholic patients. Moreover, an *in vitro* study was conducted to examine the variability of PF-5190457 metabolite formation in individual human liver cytosol (HLC).

METHODS: The concentrations of PF-5190457 and its metabolite in patient plasma samples obtained through phase 1 b clinical trials were determined using a validated LC-MS/MS method. Previous studies from our group has demonstrated that PF-5190457 is metabolized by aldehyde oxidase (AOX1) to a major hydroxyl metabolite. To determine the variability of hydroxy metabolite formation, the substrate was incubated in n=10 individual HLCs. The AOX1 protein levels were obtained by mass spectroscopy using a label free quantitation approach and information dependent acquisition.

RESULTS: The half-life of parent and metabolite was found to be approximately 6 and 13 h respectively for both doses. The CL_{int} in HLC for PF-5190457 ranged from 0.4 – 7.1 µL/min/mg protein with 17-fold variability. The estimated AOX1 protein expression were much closer ranging from 16.8 - 44.1 pmol/mg (2.6-fold variability). The correlation between AOX1 protein expression and aldehyde oxidase (AO) activity was poor.

CONCLUSION: The pharmacokinetics of PF-5190457 and its major metabolite was evaluated after oral administration of 50 and 100 mg and importantly the half-life of the metabolite was found to be longer than the parent. Inter-individual variability in the hydroxy metabolite formation was observed in HLC indicating a

need to assess the influence of various demographic factors on a larger number of samples.

Keywords

Aldehyde oxidase, alcoholism, IDA Information dependent acquisition, HLC Human liver cytosol, PF-5190457, XO Xanthine Oxidase

Introduction

PF-5190457 is a ghrelin receptor inverse agonist and is currently undergoing clinical trials to treat alcoholism and type 2 diabetes mellitus [1-3]. This drug was originally developed by Pfizer and the tolerability of the compound in healthy volunteers was evaluated. The pharmacokinetics of the drug in healthy volunteers (n=35) was determined at single (2, 10, 40, 50, 100, 150, and 300 mg) and multiple (2, 10, 40 and 100 mg BID for two weeks). The absorption of the PF-5190457 was observed to be rapid and the elimination half-life was found to be ~9 h [4].

We have previously showed that PF-5190457 is metabolized to a hydroxy metabolite by the molybdenum co-factor containing enzymes AO and xanthine oxidase (XO). The contribution of AO in the metabolism of the drug was identified to more dominant (manuscript II). AO enzyme is gaining increasing importance in the metabolism and clearance of new drug entities in the recent years [5, 6]. There are various *in vitro* studies in S9 fraction, cytosol and hepatocytes demonstrating high interindividual variability in AO activity with use of various substrates [7-9]. For example, AO activity measured in cytosol from same donors using carbazeran, zoniporide and phthalazine as probe substrates varied 90, 42 and 17-fold respectively [10].

In the present paper, we determine the concentration of PF-5190457 and its hydroxy metabolite in alcoholic patients (N=12) enrolled in a phase 1b clinical study in subjects with alcohol use disorder. In an effort to address the variability

of PF-5190457 metabolism with respect to AO activity, the metabolite formation was measured in HLC from 10 individual donors. In addition, the measured activity was correlated with the expression of AO protein and the characteristics of HLC donors were examined to understand the impact of demographic variables such as age, gender, ethnicity, smoking, alcohol, medical history.

Materials and methods

Chemicals and reagents

PF-5190457 (Mw 512.67) and tacrine (Mw 198.26) were obtained from Sigma-Aldrich (St. Louis, MO, USA). PF-6870961 (Hydroxy metabolite Mw 528.23) was synthesized as described in manuscript II. LC-MS grade methanol and formic acid were obtained from Fisher Scientific (FairLawn, NJ, USA). Blank K2EDTA human plasma from six subjects (three male, three female) were obtained from Bioreclamation (IVT Inc., Westbury, NY, USA).

Pharmacokinetic study in Alcohol Dependent Subjects

Phase 1b clinical study of PF-5190457 was conducted in non treatment seeking alcohol dependent individuals at NIH clinical center at Bethesda. Men and women between 21 – 65 years were eligible. The study was approved by the University of Rhode Island Institutional Review Board HU# 123.172. The participants of the study have filled out the informed consent form prior to the study. Briefly, the key inclusion criteria were heavy drinking (on an average for

men – 21 drinks and women – 14 drinks a week) and good health which was confirmed by medical history, physical examination, ECG, blood/urine lab tests. Moreover, the female subjects must be of non-childbearing potential, and male subjects must follow contraception for a period of 28 days after dosing. Briefly the key exclusion criteria were interest in receiving treatment for alcohol use disorder, diagnosis of substance dependence (other than alcohol and/or nicotine), schizophrenia, bipolar disorder, or other psychoses, clinically significant medical abnormalities, heart rate >100 at screening on two separate measurements given potential of study medication to increase heart rate and BMI less than or equal to 18.5.

This phase 1b study was a within-subject design and a single blind dose-escalating placebo controlled inpatient study. Subjects (N=12) were admited to the clinical research center (NIH clinical center at Bethesda) for three visits (placebo, a dose of 50 mg and a dose of 100 mg). The subjects received PF-5190457 orally as solution every twelve hours for a total of 5 doses. Blood samples for pharmacokinetics analysis were collected in the morning of the third day of hospital stay after the administration of a fifth dose of PF-5190457 at various time points (0, 15, 30, 45, 60, 90, 120, 150,180, 210, 240, 360, 480, 1350 and 1440 min). The concentration of PF-5190457 and its hydroxy metabolite (PF-6870961) in plasma were measured using an LC-MS/MS assay specifically developed and validated for the purpose of this study as described in manuscript I. Briefly, the plasma extraction method involved simple precipitation in methanol and samples were analyzed using an Acquity UPLC system coupled with Xevo

TQ MS detector (Waters Corp, Milford, MA, USA). The pharmacokinetic parameters were determined using non-compartmental analysis. C_{max} (maximum observed drug concentration during the study), $AUC_{0–24}$ is area under the plasma concentration–time curve measured for 24 h, using the trapezoidal rule, $AUC_{last-\infty}$ is based on the last observed concentration extrapolated to infinity time (Cplast/k), k is rate constant, $AUC_{0–\infty}$ is area under the curve from time 0 extrapolated to infinite time ($AUC_{0–24}$ + $AUC_{last-\infty}$), T_{max} is time to observe maximum drug concentration, and $T_{1/2}$ is half-life determined by 0.693/*K*). The apparent volume of distribution and clearance were calculated using the relations V_z/F= Dose / ($AUC_{0–\infty}$* k) and Cl/F = Dose / $AUC_{0–\infty}$ respectively [11]. The pharmacokinetic parameters were evaluated for each subject at respective doses (50 and 100 mg).

Human Liver Selection for *In vitro* studies

The livers (n=10) for the *in vitro* study were selected from the existing novel human liver bank (n=104) in the lab purchased from Sekisui XenoTech LLC (Kansas City, KS). The primary criteria for selecting the livers in this study were based on ethnicity (African American - AA, Caucasian - CC). It is a common practice to perfuse livers with University of Wisconsin solution containing allopurinol (a well-known XO inhibitor) and its primary metabolite oxypurinol and they have shown to completely inhibit XO activity [12]. From our liver bank, we identified that only n=9 out of 104 were allopurinol free and none of the donors

were African American. For the purpose of uniformity, the livers selected for the present study were containing allopurinol/oxypurinol which was detected by mass spectrometry analysis as described in Manuscript II. The livers were well characterized and documented for demographic factors like age, gender, BMI, weight of body and liver, ethnicity, alcohol consumption, smoking, along with medical history for diabetes and non-alcoholic fatty liver disease. Table 1 shows demographic characteristics of donors.

Human liver cytosol Incubations

The procedure that was used to prepare liver cytosol has been described elsewhere [13]. The hydroxy metabolite formation was examined in the 10 individual HLC selected over the PF-5190547 concentration range of 0 - 400 µM and cytosolic protein concentration of 0.1 mg/ml. All experiments were performed in triplicates. The details for incubation conditions and subsequent LC-MS/MS analysis of cytosolic samples in Acquity UPLC coupled to Xevo TQ MS detector (Waters Corp, Milford, MA, USA) for determining the kinetic parameters are described in Manuscript II. The velocity of the reaction was calculated from Michealis Menton equation and CL_{int} (intrinsic clearance) was calculated as V_{max}/K_m where V_{max} is maximum rate of product formation and K_m is Michaelis-Menten constant (substrate concentration required for an enzyme to reach one-half of its maximum velocity).

Estimation of AOX1 protein expression in Human liver cytosol using LC-MS/MS

AOX1 levels (pmol/mg) in cytosolic fractions were determined using a label free approach on mass spectrometry as described in Manuscript III. Briefly, the digestion of proteins in cytosolic samples was performed using trypsin and the resulting peptides were analyzed using information dependent acquisition (IDA) mode on an AB Sciex TripleTOF 5600+ mass spectrometer. The subsequent data analysis was performed using MaxQuant (Version 1.5) proteomics software for protein (AOX1) identification by searching against UniProt human protein database (updated Oct 2016) and quantification. The maximum false peptide discovery rate was specified as 0.01. AOX1 protein was calculated based on the raw spectral protein intensity from label-free quantification (LFQ) intensities of the MaxQuant software output using the "Total Protein Approach" (TPA).

Statistical analysis

Statistical analysis was performed with SPSS version 24 (IBM Analytics, Armonk, NY), and Prism® version 6 (GraphPad Software Inc., San Diego, CA) was used for graphing and calculation of kinetic parameters (V_{max} and K_m). Since most of the datasets were not normally distributed, nonparametric methods were generally used for statistical analyses. Non-parametric Mann-Whitney U test (2-tailed) was used to compare the effect of ethnicity and other demographic factors on enzyme activity and AOX1 levels. The correlation was studied using nonparametric Spearman correlation analysis.

Results

Pharmacokinetics analysis

The validated LC-MS/MS method (Manuscript I) has been successfully applied to measure the concentrations of PF-5190547 and its hydroxy metabolite after oral administration of the drug at 50 mg and 100 mg in human plasma. The mean concentration versus time profiles of the analytes in human plasma are shown in Figure 1 a and b. The plasma concentration ranges of the analytes were measurable within their calibration curves. The concentrations of PF-5190547 and its hydroxy metabolite ranged from 2.8 to 213.3 ng/mL and 3.9 to 73.8 ng/mL for 50 mg dose and 3.5 to 813.7 ng/mL, and 6.6 to 124.8 ng/mL for 100 mg dose, respectively. Pharmacokinetic details for the area under the curve from initial time to infinity ($AUC_{0-\infty}$), C_{max}, T_{max}, V_z/F, Cl/F and $t_{1/2}$ are shown in Table 2 for both 50 and 100 mg doses. The half-life for the metabolite (14.7 ± 2.1 and 12.8 ± 2.2 h) at both the doses (50 and 100 mg) was observed to be longer in comparison to the parent (6.2 ± 1 and 5.8 ± 0.5 h).

Individual PF-5190457 metabolism in HLC

Formation of hydroxy metabolite of PF-5190457 in this study was mediated by AO as HLCs in the selected livers were devoid of XO activity. In general, individual variation in AO activity (Figure 2) was observed in all kinetic parameters for the hydroxy metabolite formation (Table 3). K_m and V_{max} values ranged from 8.1 to 68.3 µM and 0.02 to 0.41 nmol/min/mg protein respectively. CL_{int} was found to be between 0.4 – 7.1 µL/min/mg (~ 17 fold variability). African

American donors exhibited marginally lower AO activity in comparison to Caucasian donors. However, no significant association (*P>0.1,)* was observed between ethnicity and AO activity (Figure 3a). The effect of other demographic factors was examined and no significant relationship was observed. Nevertheless, the effect of smoking on AO activity was observed to be significant with $p<0.017$ (Figure 4a). The limitation of this study was small sample size as there were only n=3 out of 10 livers were obtained from non-smoker individuals.

Expression of AOX1 protein in HLC and correlation to *In vitro* AO activity

The AOX1 levels obtained from data analysis using MaxQuant TPA approach ranged from 16.8 – 44.2 pmol/mg with 2.6 fold difference (Figure 5) across the examined HLCs. There was no significant effect of ethnicity (Figure 3b) and smoking (Figure 4b) on AOX1 levels observed. Moreover, other demographic factors mentioned in **Table 1** did not significantly influence AOX1 levels. The AO activity characterized by rate of formation of hydroxy metabolite showed a weak correlation (Figure 6) (Spearman $r = 0.2$ and $p>0.5$) with AOX1 protein levels.

Discussion

The *in vivo* study evaluated pharmacokinetics of PF-5190457 and its hydroxy metabolite in alcohol dependent subjects. The pharmacodynamics of the drug in the same study population is evaluated by our lab and will be discussed elsewhere. Previously, published study determined PF-5190457 pharmacokinetics in healthy subjects [4] and the half-life of the parent was

observed to be higher in comparison to our study in alcoholic subjects (~9 vs ~6 hours) whereas, the other parameters (C_{max}, T_{max}) were comparable. It could be because of differences in blood sampling points or differences in the population (healthy vs alcoholic subjects).

The kinetics of major hydroxyl metabolite was not reported previously. The findings from the present study also indicate that the half-life of the hydroxy metabolite in heavy drinkers was higher in comparison to the parent drug. The higher exposure of the metabolite in the body could be of an advantage if the metabolite would have any pharmacological significance and further evaluations with respect to safety profile and byproducts of the hydroxy metabolite are necessary. The clinical study for PF-5190457 recruited n=12 subjects and the majority of the subjects where African American (n=11). The differences in ethnicity could affect the pharmacokinetics and subsequent pharmacodynamics of the drug [14, 15]. In addition, the variability of pharmacokinetic parameters is largely explained by genetic variation in metabolizing enzymes [16], disease state, physiological and environmental factors [17, 18]. The primary objective for the *in vitro* study in the present paper was to determine differences in metabolism of PF-5190457 with respect to ethnicity (African American vs. Caucasians) and inter-individual variability.

PF-5190457 is predominantly metabolized by AO enzyme (Manuscript II). Wide variability has been reported for substrates metabolized by AO and the metabolism has demonstrated to be highly substrate dependent with high donor

to donor variability [10, 19, 20]. An example, the AO activity was measured using different probe substrates N-[(2-dimethylamino) ethyl] acridine-4-carboxamide (DACA) and benzaldehyde in the same HLC donors (n=13). The CL_{int} with respect to DACA and benzaldehyde was 18-fold and 5-fold respectively across the individual donors [21]. In our study, the CL_{int} varied 17-fold for PF-5190457 across individual HLC. Further, weak correlation between the AOX1 protein levels and AO activity was observed which were in accordance with findings from another *in vitro* study in HLC [10]. Few studies published to determine correlation between AO protein and activity. The hydroxy metabolite formation was found to be reduced in AA but the non-significant change could be because of the small sample size of HLC. There is only one study so far, showing the influence of ethnicity on AO activity in cryopreserved hepatocytes using O6-benzylguanine as a probe substrate which showed no significant effect between AA (n=7) vs CC (n=50) on AO activity. The above-mentioned study too had several livers from AA donors. Further exploration of other demographic factors on PF-5190457 metabolism indicated smoking could lead to low AO activity but study in large sample size of donors would be essential for confident results. There is only limited information published on the impact of smoking on AO protein and activity. One study discussed no relationship between AO activity and smoking but it was based on 1 out of 13 HLC donors with tobacco use [21]. In one other study AO activity in large sample size (75 donors) was assessed using hepatocytes and the livers were characterized for tobacco use but the effect of smoking was not mentioned [22]. It is also known that nicotine is primarily

metabolized by CYP1A2 to an iminium ion and then the second step of metabolism primarily involves AO enzyme [23-25]. Fu et al. showed chronic alcohol use in (n=2) HLC donors could influence the AO activity but the small sample size was a limitation. The effect of both smoking and alcohol use could have a significant impact on AO activity and warrants further examination in a larger number of samples.

AO protein is a homodimer and disruption in homodimer formation can affect AO activity [26]. Other reasons for variation in AO activity could be protein misfolding or genetic polymorphism [27]. Genetic polymorphism of hAOX1 was studied in Italian population and the presence of functionally inactive hAOX1 allelic variants enzymes was determined [26]. To the best of our knowledge there are no studies conducted exclusively in AA population to assess the presence of allelic variants. One study determined the impact of common single nucleotide polymorphism on the AO activity in 75 donors in which there were only seven African American and 50 Caucasians and the rest were Hispanic. The study showed no statistical significance for polymorphism on AO activity [22].

Dietary constituents could also influence the AO activity [28]. AO enzyme contains iron along with molybdopterin cofactor and flavin adenine dinucleotide. It has been reported that iron deficiency could lead to lower AO activity as reported in a rat study. AO is the main enzyme for serotonin degradation and rats on iron deficient diet exhibited significantly increased concentrations of serotonin. The levels of serotonin reverted back to normal when the rats were supplemented with iron [29]. It is known that AO and XO enzyme share closely related

structures. The XO expression and activity in lung were reduced in rats fed on an iron-depleted diet and increased in rats supplemented with iron [30]. AO belongs to the same family iron supplementation could be one of the reasons for the variability observed in AO activity.

The findings from the present study could be important as variability in PF-5190457 was observed across individual HLC. Since multiple factors could be contributing to variability in AO activity, larger sample size is necessary to address the connections.

In conclusion, the manuscript describes the successful application of LC-MS/MS method to a pharmacokinetic study of PF-5190547 at 50 and 100 mg in healthy volunteers in phase 1b clinical trials to treat alcoholism. The *in vitro* study shows donor variability in hydroxylation of PF-5190457 in human liver cytosol.

Conflict of interest

Dr. Obach is an employee of Pfizer Pharmaceuticals. Other authors declare no potential conflict of interest with any commercial entity whose products were used in the study.

Acknowledgements

The financial support of Grant #UH3TR000963 from National Institutes of Health is gratefully acknowledged.

References

1. Bhattacharya SK, Andrews K, Beveridge R, Cameron KO, Chen C, Dunn M, et al. Discovery of PF-5190457, a Potent, Selective, and Orally Bioavailable Ghrelin Receptor Inverse Agonist Clinical Candidate. ACS Med Chem Lett. 2014 May 8;5(5):474-9.
2. Kong J, Chuddy J, Stock IA, Loria PM, Straub SV, Vage C, et al. Pharmacological characterization of the first in class clinical candidate PF-05190457: a selective ghrelin receptor competitive antagonist with inverse agonism that increases vagal afferent firing and glucose-dependent insulin secretion ex vivo. Br J Pharmacol. 2016 May;173(9):1452-64.
3. Ghareeb M, Leggio L, El-Kattan A, Akhlaghi F. Development and validation of an UPLC-MS/MS assay for quantitative analysis of the ghrelin receptor inverse agonist PF-5190457 in human or rat plasma and rat brain. Anal Bioanal Chem. 2015 Jul;407(19):5603-13.
4. Denney WS, Sonnenberg GE, Carvajal-Gonzalez S, Tuthill T, Jackson VM. Pharmacokinetics and pharmacodynamics of PF-05190457: The first oral ghrelin receptor inverse agonist to be profiled in healthy subjects. Br J Clin Pharmacol. 2017 Feb;83(2):326-38.
5. Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, Tran TD. Aldehyde oxidase: an enzyme of emerging importance in drug discovery. J Med Chem. 2010 Dec 23;53(24):8441-60.
6. Garattini E, Terao M. The role of aldehyde oxidase in drug metabolism. Expert Opin Drug Metab Toxicol. 2012 Apr;8(4):487-503.
7. Sahi J, Khan KK, Black CB. Aldehyde oxidase activity and inhibition in hepatocytes and cytosolic fractions from mouse, rat, monkey and human. Drug Metab Lett. 2008 Aug;2(3):176-83.
8. Kitamura S, Sugihara K, Nakatani K, Ohta S, Ohhara T, Ninomiya S, et al. Variation of hepatic methotrexate 7-hydroxylase activity in animals and humans. IUBMB Life. 1999 Dec;48(6):607-11.
9. Rodrigues AD, Ferrero JL, Amann MT, Rotert GA, Cepa SP, Surber BW, et al. The in vitro hepatic metabolism of ABT-418, a cholinergic channel activator, in rats, dogs, cynomolgus monkeys, and humans. Drug Metab Dispos. 1994 Sep-Oct;22(5):788-98.
10. Fu C, Di L, Han X, Soderstrom C, Snyder M, Troutman MD, et al. Aldehyde oxidase 1 (AOX1) in human liver cytosols: quantitative characterization of AOX1 expression level and activity relationship. Drug Metab Dispos. 2013 Oct;41(10):1797-804.
11. Rosenbaum SE. Basic Pharmacokinetics and Pharmacodynamics: An Integrated Textbook and Computer Simulations; May 2011.
12. Barr JT, Choughule KV, Nepal S, Wong T, Chaudhry AS, Joswig-Jones CA, et al. Why do most human liver cytosol preparations lack xanthine oxidase activity? Drug Metab Dispos. 2014 Apr;42(4):695-9.
13. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying

Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. J Proteome Res. 2017 Oct 10.
14. Donovan MD. Sex and racial differences in pharmacological response: effect of route of administration and drug delivery system on pharmacokinetics. J Womens Health (Larchmt). 2005 Jan-Feb;14(1):30-7.
15. Johnson JA. Influence of race or ethnicity on pharmacokinetics of drugs. J Pharm Sci. 1997 Dec;86(12):1328-33.
16. Rogers JF, Nafziger AN, Bertino JS, Jr. Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs. Am J Med. 2002 Dec 15;113(9):746-50.
17. Wilkinson GR. Drug metabolism and variability among patients in drug response. N Engl J Med. 2005 May 26;352(21):2211-21.
18. Merrell MD, Cherrington NJ. Drug metabolism alterations in nonalcoholic fatty liver disease. Drug Metab Rev. 2011 Aug;43(3):317-34.
19. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, et al. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology. 2001 Feb;120(2):337-45.
20. Sugihara K, Kitamura S, Tatsumi K, Asahara T, Dohi K. Differences in aldehyde oxidase activity in cytosolic preparations of human and monkey liver. Biochem Mol Biol Int. 1997 May;41(6):1153-60.
21. Al-Salmy HS. Individual variation in hepatic aldehyde oxidase activity. IUBMB Life. 2001 Apr;51(4):249-53.
22. Hutzler JM, Yang YS, Brown C, Heyward S, Moeller T. Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. Drug Metab Dispos. 2014 Jun;42(6):1090-7.
23. Brandange S, Lindblom L. The enzyme "aldehyde oxidase" is an iminium oxidase. Reaction with nicotine delta 1'(5') iminium ion. Biochem Biophys Res Commun. 1979 Dec 14;91(3):991-6.
24. Bielawski J, Brandange S, Rodriguez B. Kinetic resolution in the oxidation of iminium ion to lactam catalysed by aldehyde oxidase. Acta Chem Scand B. 1987 Mar;41(3):198-201.
25. Benowitz NL, Hukkanen J, Jacob P, 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. Handb Exp Pharmacol. 2009(192):29-60.
26. Hartmann T, Terao M, Garattini E, Teutloff C, Alfaro JF, Jones JP, et al. The impact of single nucleotide polymorphisms on human aldehyde oxidase. Drug Metab Dispos. 2012 May;40(5):856-64.
27. Itoh K, Maruyama H, Adachi M, Hoshino K, Watanabe N, Tanaka Y. Lack of dimer formation ability in rat strains with low aldehyde oxidase activity. Xenobiotica. 2007 Jul;37(7):709-16.
28. Barr JT, Jones JP, Oberlies NH, Paine MF. Inhibition of human aldehyde oxidase activity by diet-derived constituents: structural influence, enzyme-ligand interactions, and clinical relevance. Drug Metab Dispos. 2015 Jan;43(1):34-41.
29. Mackler B, Person R, Miller LR, Inamdar AR, Finch CA. Iron deficiency in the rat: biochemical studies of brain metabolism. Pediatr Res. 1978 Mar;12(3):217-20.

30. Ghio AJ, Kennedy TP, Stonehuerner J, Carter JD, Skinner KA, Parks DA, et al. Iron regulates xanthine oxidase activity in the lung. Am J Physiol Lung Cell Mol Physiol. 2002 Sep;283(3):L563-72.

FIGURE LEGENDS

Figure 1 (a) and (b) Plasma concentration time profiles for parent (PF-5190547) and hydroxy metabolite after the administration of 50 and 100 mg dose respectively. Data are shown as mean ± SD at each time point.

Figure 2 Bar chart demonstrating AO activity in individual human liver cytosol. AO activity was characterized by hydroxylation of PF-5190457 across n=10 human liver cytosol. The data was found to be non normally distributed with a 20 fold variability.

Figure 3 (a) Effect of ethnicity on AO activity and (b) AOX1 levels. AO activity in African American, AA (n=5) was observed to be lower in comparison to Caucasians, CC (n=5) but not significantly different ($p>0.1$). AOX1 proteins levels were observed to be not significantly different across both the groups

Figure 4 (a) Effect of smoking on AO activity and (b) AOX1 levels. AO activity in smokers (n=7) was observed to be significantly lower ($p<0.017$ Mann Whitney U test). in comparison to and non-smokers (n=3), CC (n=5). AOX1 proteins levels were observed to be not significantly different across both the groups.

Figure 5 Distribution of AOX1 protein levels in 10 individual human liver cytosol. The range of AOX1 levels was between 16 – 44 pmol/mg as determined by label free quantification approach using information dependent acquisition on mass spectroscopy.

Figure 6. Correlation between AO activity and AOX1 levels (pmol/mg). Open circles represent Caucasians and closed circles for African Americans. The equation of the line for the best fit was generated by least square linear regression analysis. Statistical significance of the correlations was determined by the Spearman correlation test. A weak correlation ($p>0.5$) was observed between AO activity (hydroxy metabolite of PF-5190457) formation and AOX1 levels was observed.

Table IV-1. Demographics and baseline characteristics of the human subjects

Number (*N*)	12
Females (*n*, %)	1 (8%)
Age (years)	
Range	23-58
M ± *SD*	40.5 ± 13.1
Median	40.0
Race/Ethnicity (%)	
African-Americans	11 (91.7%)
Caucasians	1 (8.3%)
Cigarette smoking Status (*n,* %)	
Smoker	9 (75.0%)
Non-Smoker	3 (25.0%)
DSM-IV diagnosis of alcohol dependence (*n*, %)	11 (91.7%)
BMI, Kg/m^2, (M ± SD)	27.1 ± 4.7
90-day baseline average drinks/drinking day, (M ± SD)	10.7 ± 4.2

Table IV-2. Demographic and donor data details

ID	Gender	Age	Ethnicity	Smoking	Drug_Use	Medical History
HLC_01	M	44	AA	Yes	Yes	Diabetes
HLC_02	F	49	AA	Yes	No	Diabetes
HLC_03	M	48	AA	Yes	Yes	No
HLC_04	M	23	AA	Yes	Yes	No
HLC_05	F	49	AA	Yes	No	Diabetes
HLC_06	F	78	CC	No	No	Diabetes
HLC_07	F	53	CC	No	No	Diabetes
HLC_08	M	59	CC	Yes	No	No
HLC_09	M	44	CC	No	Yes	No
HLC_10	M	53	CC	Yes	No	Diabetes

Table IV-3. Summary of pharmacokinetic parameters

a. 50 mg PF-5190457 dose (mean ± SD, n = 12)

Dose-50 mg	**Parent (PF-5190457)**	**Hydroxy Metabolite**
AUC $_{(0-\infty)}$ (ng.min)/mL	53184.8 ± 20694.8	33945.5 ± 9753.4
C_{max} (ng/mL)	134.3 ± 43.8	39.8 ± 14.1
T_{max} (min)	56.2 ± 24	56.2 ± 12.9
Half life (h)	6.2 ± 1	14.7 ± 2.1
CL/F (L/h)	60.7 ± 16.8	93.6 ± 28.8
Vz/F(L)	554.7 ± 156.7	2069.4 ± 781.2

b. 100 mg PF-5190457 dose (mean ± SD, n = 12)

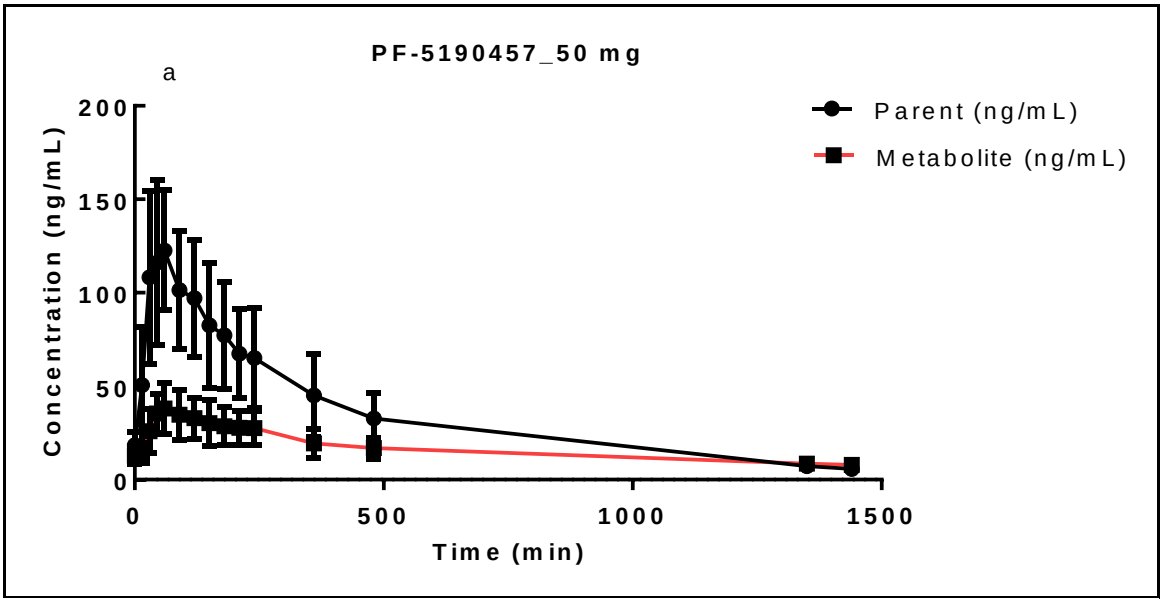
Dose-100 mg	**Parent (PF-5190457)**	**Hydroxy Metabolite**
AUC $_{(0-\infty)}$ (ng.min)/mL	108528.7 ± 64556.3	57497.6 ± 18588.6
C_{max} (ng/mL)	305.6 ± 189.4	76.2 ± 23.1
T_{max} (min)	50 ± 28.8	68.7 ± 48
Half life (h)	5.8 ± 0.5	12.8 ± 2.2
CL/F (L/h)	70 ± 40.2	111.6 ± 33.9
Vz/F(L)	604.7 ± 364.5	2162.7 ± 930.9

AUC = area under the plasma concentration time curve, C_{max} = maximum observed plasma concentration, T_{max} = time of Cmax, Cl/F = Apparent Clearance, Vz/F = Apparent volume of distribution

Table IV-4. Kinetic parameters for hydroxy metabolite formation of PF-5190457 in individual human liver Cytosol

ID	V_{max} (nmol/min/mg)	K_m (μM)	CL_{int} (μL/min/mg)	AOX1 (pmol/mg)
HLC_01	0.06	17.7	3.4	25.70
HLC_02	0.13	27.9	4.6	43.40
HLC_03	0.14	57.1	2.5	23.21
HLC_04	0.07	43.7	1.7	20.54
HLC_05	0.04	8.1	5.3	44.29
HLC_06	0.23	42.5	5.4	25.37
HLC_07	0.24	36.1	5.4	32.06
HLC_08	0.02	68.3	0.4	16.83
HLC_09	0.41	58.1	7.1	30.37
HLC_10	0.15	43.4	2.0	22.47

Figure IV-1 (a) and (b).



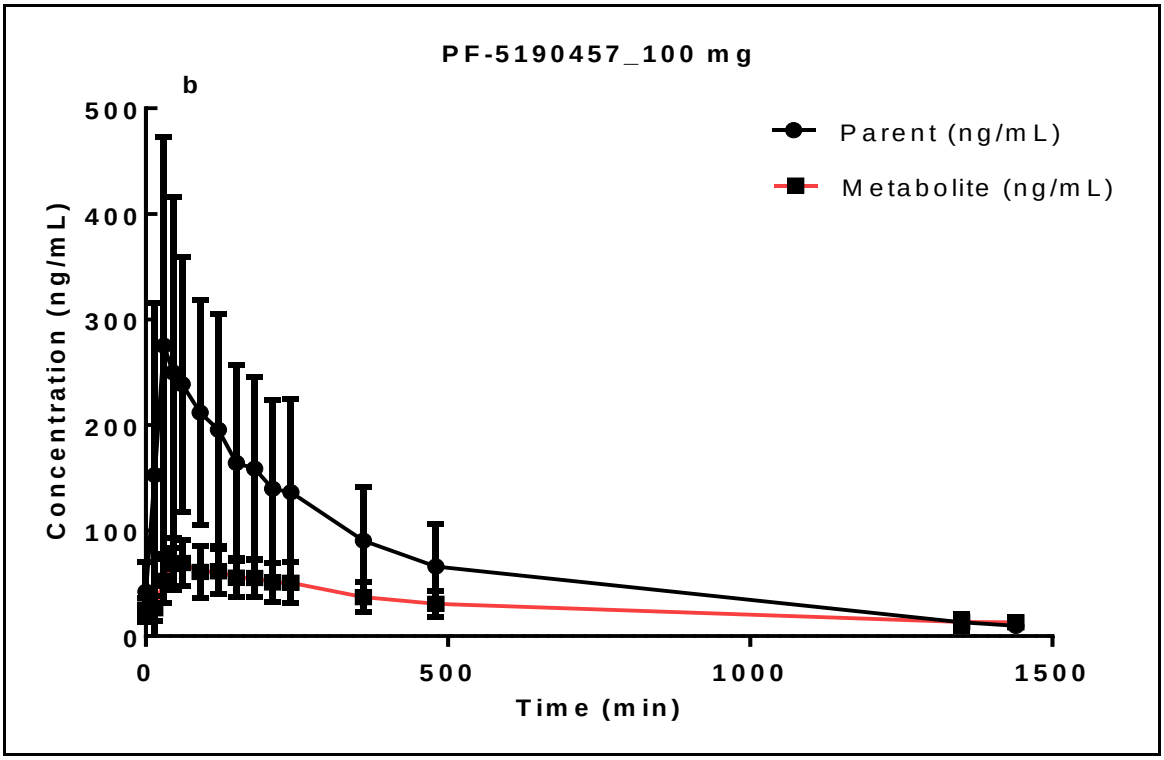


Figure IV-2.

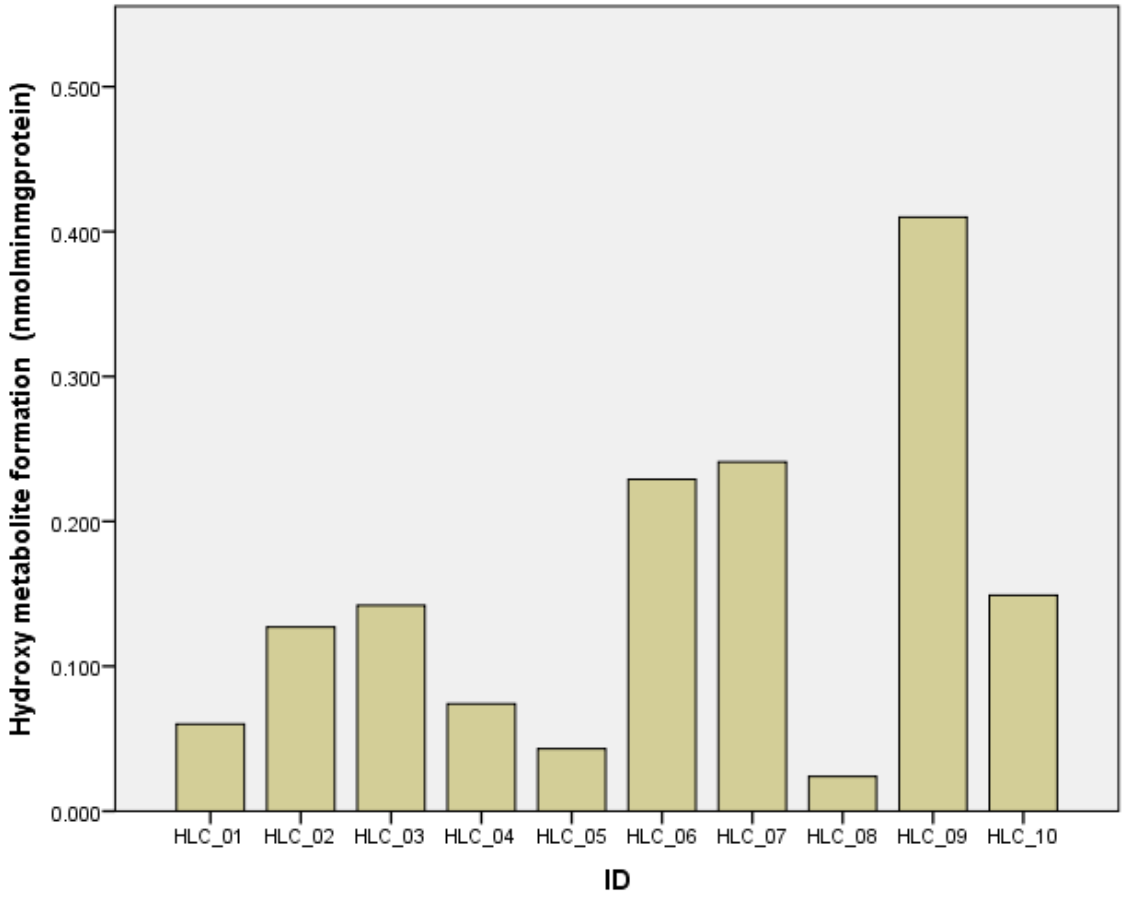

Hydroxy metabolite formation (nmolminmgprotein)
0.500
0.400
0.300
0.200
0.100
0.000
HLC_01
HLC_02
HLC_03
HLC_04
HLC_05
HLC_06
HLC_07
HLC_08
HLC_09
HLC_10
ID

Figure IV-3.

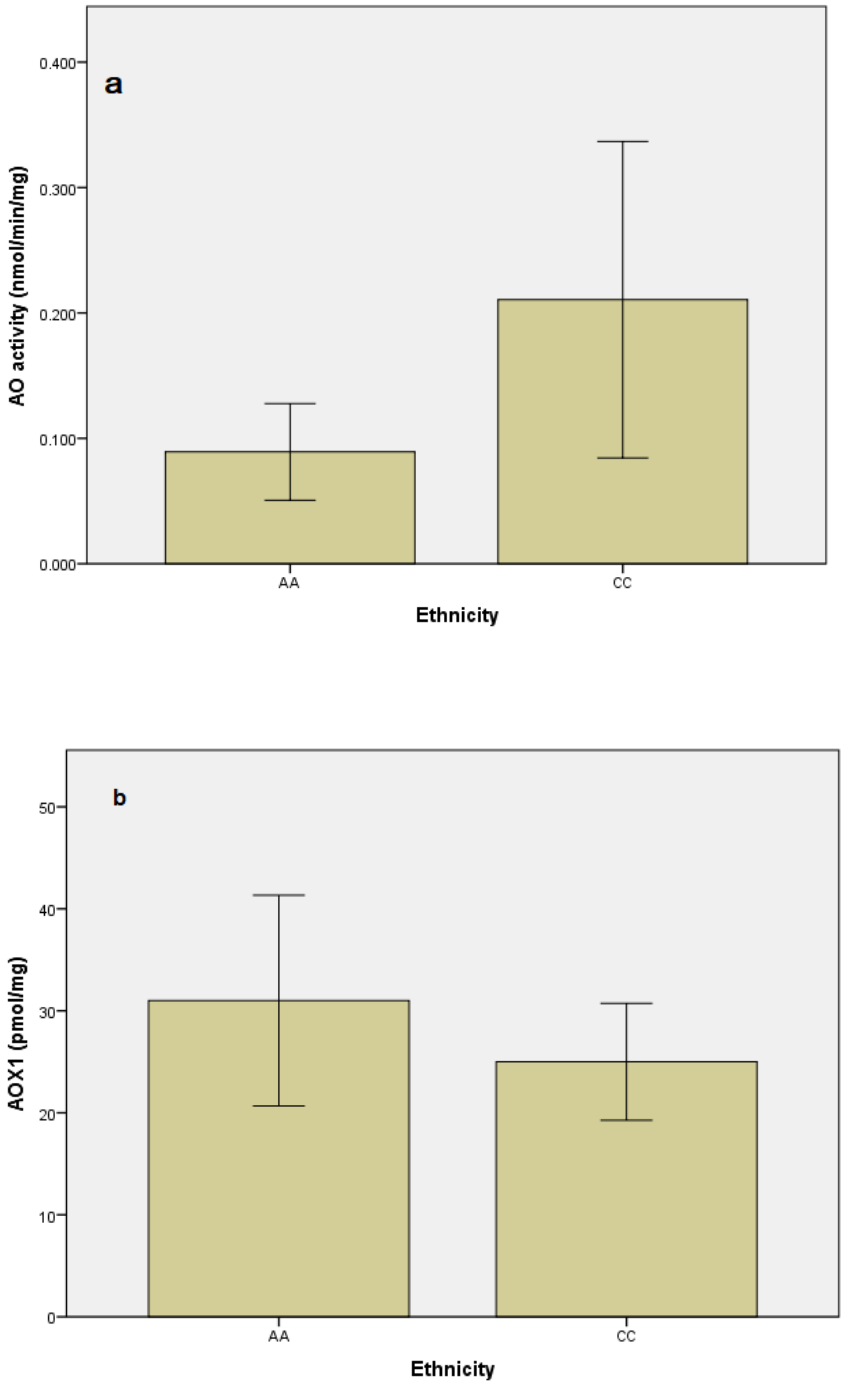
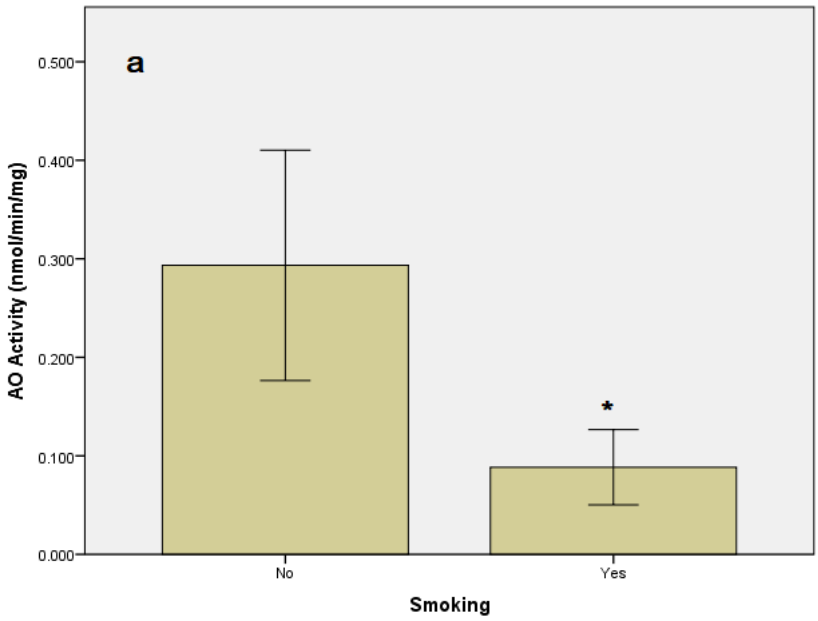

a
AO activity (nmol/min/mg)
0.400
0.300
0.200
0.100
0.000
AA
CC
Ethnicity
b
AOX1 (pmol/mg)
50
40
30
20
10
0
AA
CC
Ethnicity

Figure IV-4.


a
AO Activity (nmol/min/mg)
0.500
0.400
0.300
0.200
0.100
0.000
*
No
Yes
Smoking

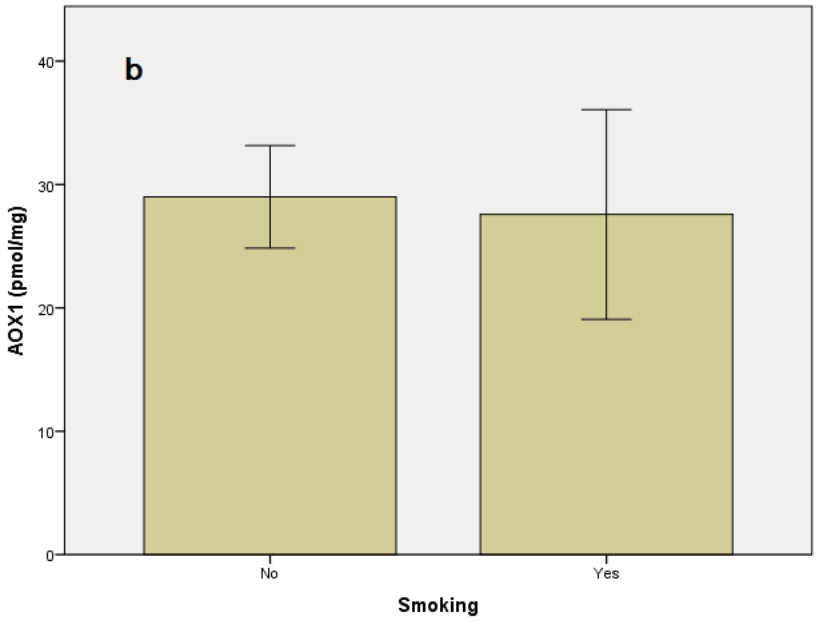

b
AOX1 (pmol/mg)
40
30
20
10
0
No
Yes
Smoking

Figure IV-5.

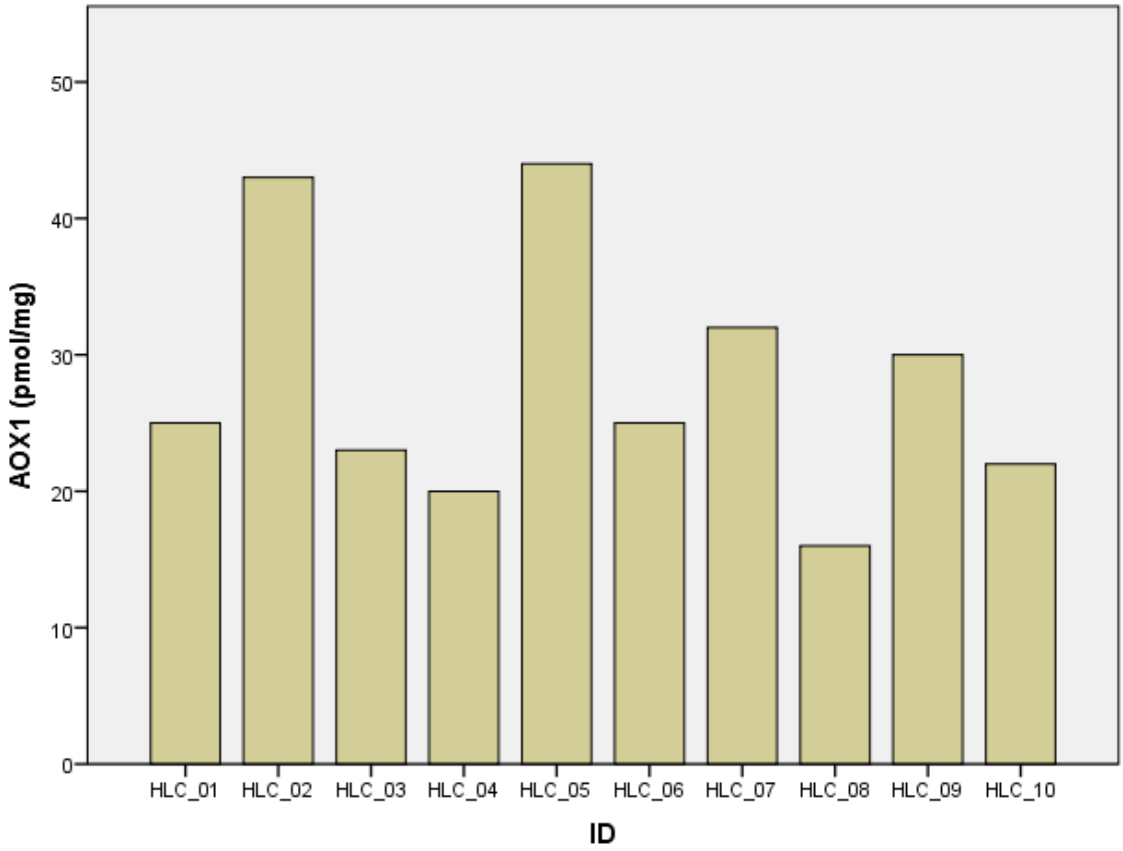
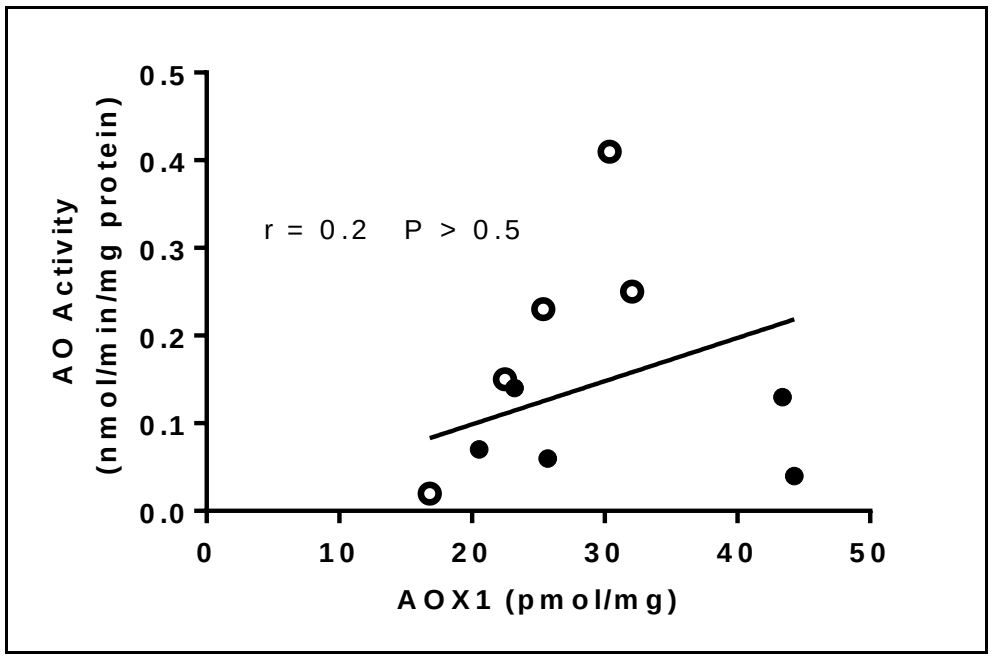

AOX1 (pmol/mg)
50
40
30
20
10
0
HLC_01
HLC_02
HLC_03
HLC_04
HLC_05
HLC_06
HLC_07
HLC_08
HLC_09
HLC_10
ID

Figure IV-6.


AO Activity
(nmol/min/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
r = 0.2 P > 0.5
0
10
20
30
40
50
AOX1 (pmol/mg)