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**Evaluation of Carbazeran 4-Oxidation and  $O^6$ -Benzylguanine 8-Oxidation as  
Catalytic Markers of Human Aldehyde Oxidase: Impact of Cytosolic  
Contamination of Liver Microsomes**

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## Running Title

AOX-1, Catalytic Markers, and Cytosol-Contaminated Microsomes

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**ABBREVIATIONS:** 1-ABT, 1-aminobenzotriazole; AOX, aldehyde oxidase; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide;  $k_{cat}$ , turnover number;  $K_m$ , Michaelis-Menten constant; MRM, multiple reaction monitoring; MS, mass spectrometry; UPLC, ultra-high performance liquid chromatography;  $V_{max}$ , maximum velocity.

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## **ABSTRACT**

The current study investigated the contribution of microsomal cytochrome P450 and cytosolic aldehyde oxidase-1 (AOX-1) to carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in human liver microsomal, cytosolic, and S9 fractions. Incubations containing carbazeran and human liver microsomes with or without exogenously added NADPH yielded comparable levels of 4-oxo-carbazeran. *O*<sup>6</sup>-Benzylguanine 8-oxidation occurred in microsomal incubations, and the extent was increased by NADPH. Human recombinant CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 did not catalyze carbazeran 4-oxidation, whereas CYP1A2 was highly active in *O*<sup>6</sup>-benzylguanine 8-oxidation. 1-Aminobenzotriazole, a pan-cytochrome P450 inhibitor, decreased *O*<sup>6</sup>-benzylguanine 8-oxidation, but not carbazeran 4-oxidation, in microsomal incubations, whereas 1-aminobenzotriazole and furafylline (a CYP1A2-selective inhibitor) did not inhibit carbazeran 4-oxidation or *O*<sup>6</sup>-benzylguanine 8-oxidation in human liver S9 fraction. Carbazeran 4-oxidation in incubations containing human liver microsomes (from multiple donors and commercial suppliers) was attributed to microsomal preparations contaminated with AOX-1, as suggested by liver microsomal experiments indicating a decrease in carbazeran 4-oxidation by an AOX-1 inhibitor (hydralazine), and detection of AOX-1 protein (at one-third the level of that in liver cytosol). Cytosolic contamination of liver microsomes was further demonstrated by the formation of dehydroepiandrosterone sulfate (catalyzed by cytosolic sulfotransferases) in liver microsomal incubations containing dehydroepiandrosterone. In conclusion, carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation are enzyme-selective catalytic markers of human AOX-1, as shown in human liver S9 fraction expressing cytochrome P450 and AOX-1. This study highlights the negative impact of cytosolic contamination of liver microsomes on the interpretation of reaction phenotyping data collected in an *in vitro* study performed in microsomal fractions.

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## Introduction

There has been an increase in efforts to improve the metabolic stability of new drug entities by reducing their susceptibility to cytochrome P450-mediated metabolism (Rashidi and Soltani, 2017). However, such strategies often lead to an increase in metabolic clearance by non-cytochrome P450 enzymes, such as aldehyde oxidase (AOX) (Argikar et al., 2016). AOX belongs to the family of molybdo-flavoenzymes that require flavin adenine dinucleotide and molybdo-pterin cofactors for their catalytic activities (Romao et al., 2017). The single AOX isoform in humans, AOX-1, is expressed in various tissues, but predominantly in the liver (Terao et al., 2016). AOX-1 catalyzes the oxidation of aldehydes, azaheterocycles, and iminium groups, and reduction of *N*-oxides, nitro groups, and sulfoxides (Garattini and Terao, 2013). Pharmacologically important substrates of AOX-1 include famciclovir (Rashidi et al., 1997), methotrexate (Chladek et al., 1997; Jordan et al., 1999), and idelalisib (Ramanathan et al., 2016). Chemical inhibitors of human AOX-1 have been identified, including hydralazine (Strelevitz et al., 2012), raloxifene (Obach, 2004), menadione (Johns, 1967), thioridazine (Obach et al., 2004), estradiol (Obach, 2004), amitriptyline (Obach et al., 2004), and tamoxifen (Obach, 2004).

Various substrate oxidation reactions are catalyzed by human AOX-1, including carbazeran 4-oxidation (Kaye et al., 1984; Kaye et al., 1985) and *O*<sup>6</sup>-benzylguanine 8-oxidation (Roy et al., 1995). Carbazeran 4-oxidation is used as a catalytic marker of this cytosolic enzyme (Zientek et al., 2010; Hutzler et al., 2012; Fu et al., 2013). However, it is not certain whether carbazeran 4-oxidation is an enzyme-selective catalytic marker of AOX-1 because of a study reporting that a general inhibitor of cytochrome P450, 1-aminobenzotriazole (1-ABT), decreased the *in vitro* intrinsic clearance of carbazeran in human liver microsomes, although the extent of the decrease was marginal and it was based on a single pool of human liver microsomes (Wilkinson et al., 2017). *O*<sup>6</sup>-Benzylguanine 8-oxidation is also used as a catalytic marker of AOX-1 (Zientek et al., 2010;

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Hutzler et al., 2012; Barr et al., 2015). However, experiments with a panel of individual human cytochrome P450 enzymes expressed in HepG2 cells and human liver microsomal experiments with cytochrome P450 enzyme-selective chemical inhibitors showed that several cytochrome P450 enzymes, especially CYP1A2, is active in *O*<sup>6</sup>-benzylguanine 8-oxidation (Roy et al., 1995). To date, it has yet to be evaluated systematically whether *O*<sup>6</sup>-benzylguanine 8-oxidation is a suitable enzyme-selective catalytic marker for AOX-1 in tissues or cells expressing both microsomal cytochrome P450 and cytosolic AOX-1.

The overall aim of the present study was to investigate systematically the appropriateness of utilizing carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation as enzyme-selective catalytic markers of AOX-1, as assessed in various *in vitro* human liver drug metabolism models (microsomes, cytosol, and S9 fraction). It is important to identify AOX-1 selective catalytic markers because they will facilitate functional studies on AOX-1 in cells or cellular fractions that contain a multitude of oxidative drug-metabolizing enzymes such as AOX-1 and cytochrome P450. The results of our detailed study indicate that carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation are suitable *in vitro* catalytic markers of AOX-1. They also highlight an important practical issue of cytosolic contamination of isolated microsomes in a reaction phenotyping study.

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## Materials and Methods

**Chemicals and Reagents.** Carbazeran, 4-oxo-carbazeran, *O*<sup>6</sup>-benzylguanine, and 8-oxo-*O*<sup>6</sup>-benzylguanine were purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Tolbutamide, hydralazine, 1-aminobenzotriazole (1-ABT),  $\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Pte. Ltd. (St. Louis, MO, USA). Dehydroepiandrosterone (DHEA) and DHEA sulfate were purchased from Steraloids, Inc. (Newport, RI, USA), and 3'-phosphoadenosine 5'-phosphosulfate solution (concentration of 9.9 mM in 25 mM Tris, 23% ethanol, pH 7.5; >90% purity) was from R&D Systems Inc. (Minneapolis, MN, USA). Methanol and acetonitrile, which were of high performance liquid chromatography-grade, were purchased from Tedia Company, Inc. (Fairfield, OH, USA), and formic acid was from VWR International Ltd. (Singapore). All other commercially available chemicals were of analytical grade. Rabbit anti-AOX-1 primary antibody (catalog #TA321295) and recombinant human AOX-1 (catalog #TP319221) for protein quantification were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (catalog #043-426) and Rabbit (12-180 kDa) Size Separation Master Kit (catalog #CBS-01-01) were bought from ProteinSimple (San Jose, CA, USA).

**Cytosols, Microsomes, S9 Fraction, and Recombinant Enzymes.** Human liver cytosol (mixed sex; pool of 150 donors; catalog #452115, lot #38290, Gentest<sup>®</sup> brand; 75 males and 75 females) and human liver S9 fraction (mixed sex; pool of 150 donors; catalog #452116, lot #38292, Gentest<sup>®</sup> brand; 75 males and 75 females) from different panels of donors were purchased from Corning, Inc. (Corning, NY, USA). Various lots of human liver microsomes (mixed sex; HLM) were purchased from Corning, Inc., Sekisui XenoTech, LLC (Kansas City, KS, USA), and Bioreclamation IVT (Westbury, NY, USA). HLM #1 was a pool of 50 donors (Corning, catalog

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#452156, lot #17358; 25 males and 25 females), HLM #2 was a pool of 150 donors (Corning, catalog #452117, lot #38291; 74 males and 76 females), HLM #3 was a pool of 50 donors (XenoTech, catalog #H0610, lot #1410013; 26 males and 24 females), HLM #4 was a pool of 200 donors (XenoTech, catalog #H2610, lot #1410230; 100 males and 100 females), and HLM #5 was a pool of 150 donors (Bioreclamation IVT, catalog #X008070, lot #SSP; 76 males and 74 females). Human recombinant AOX-1 enzyme (catalog #CYP150, lot #150011B) and control cytosol (isolated from *Escherichia coli* host cells; catalog #CYP099, lot #INT016E18C) were purchased from Cypex Ltd. (Dundee, Scotland, UK). Insect cell control (catalog #456201, lot #5274001), insect cell control with oxidoreductase and cytochrome b5 (catalog #456244, lot #3002971), and microsomes from baculovirus infected insect cells (SUPERSOMES™) expressing human CYP1A2 with oxidoreductase (catalog #456203, lot #5056003), CYP2C8 with oxidoreductase and cytochrome b5 (catalog #456252, lot #5057003), CYP2C9 with oxidoreductase and cytochrome b5 (catalog #456258, lot #5224006), CYP2C19 with oxidoreductase and cytochrome b5 (catalog #456259, lot #5229003), CYP2D6 with oxidoreductase (catalog #456217, lot #5236001), CYP3A4 with oxidoreductase and cytochrome b5 (catalog #456202, lot #4329002), CYP3A5 with oxidoreductase and cytochrome b5 (catalog #456256, lot #5258001) were purchased from Corning, Inc.

**Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation Assays.** The total volume of each incubation was 200  $\mu$ l (for assays containing human liver cytosol, human liver microsomes or S9 fraction) or 100  $\mu$ l (for assays containing human recombinant AOX-1 or cytochrome P450 enzymes). Unless indicated otherwise, each standard incubation mixture contained potassium phosphate buffer (100 mM, pH 7.4), carbazeran (2  $\mu$ M for cytosol, 3 or 7  $\mu$ M for microsomes, 5  $\mu$ M for S9 fraction, 10  $\mu$ M for cytochrome P450 enzymes, 15  $\mu$ M for recombinant AOX-1) or *O*<sup>6</sup>-benzylguanine (40  $\mu$ M for cytosol, 50  $\mu$ M for microsomes, 50  $\mu$ M for S9 fraction, 50  $\mu$ M for

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cytochrome P450 enzymes, 200  $\mu$ M for recombinant AOX-1), and various amounts of cytosol, microsomes, S9 fraction, or recombinant enzymes. In microsomal, S9 fraction, and recombinant cytochrome P450 enzyme assays, NADPH (1 mM; dissolved in assay buffer) or equivalent volume of assay buffer (in control groups) was added to the incubation mixture. The final concentration of vehicle was 0.5% v/v of DMSO, which was reported not to affect the cytosolic AOX-1 assay (Behera et al., 2014) or 0.5% v/v methanol, which was reported not to affect the microsomal and cytochrome P450 assay (Chauret et al., 1998). Each incubation mixture was pre-warmed for 3 min at 37°C in a low-speed shaking water bath. The enzymatic reaction was initiated by adding cytosol (20  $\mu$ g total protein; 1  $\mu$ l), microsomes (60-100  $\mu$ g total protein; 3-5  $\mu$ l), S9 fraction (60  $\mu$ g total protein; 3  $\mu$ l), recombinant cytochrome P450 enzyme (20 pmol enzyme; 20  $\mu$ l), or recombinant AOX-1 enzyme (30  $\mu$ g protein; 3  $\mu$ l), and the mixture was incubated for a specific duration (for carbazeren: 5 min for cytosol, 5 or 15 min for microsomes, 5 min for S9 fraction, 30 min for cytochrome P450 enzymes, or 45 min for recombinant AOX-1 enzyme; for *O*<sup>6</sup>-benzylguanine: 5 min for cytosol, 15 min for microsomes, 10 min for S9 fraction, 30 min for cytochrome P450 enzymes, or 45 min for recombinant AOX-1 enzyme), as specified in each figure legend. The reaction was terminated by adding an equal volume of ice-cold acetonitrile containing tolbutamide (25 nM final concentration in 400  $\mu$ l total volume of incubation mixture and acetonitrile; or 50 nM final concentration in 200  $\mu$ l total volume of acetonitrile alone; internal standard). Each sample was immediately mixed, placed on ice, and subsequently centrifuged at 16,000  $\times$  g for 15 min at 4°C. The supernatant was then transferred into a 96-well microplate for ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. Calibration standards were prepared by adding stock solutions (1-1000  $\mu$ M in DMSO) of 4-oxo-carbazeren or 8-oxo-*O*<sup>6</sup>-benzylguanine into the incubation mixture to give final concentrations of 1-1000 nM



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(equivalent to 0.2 – 200 pmol). The standard and quality control samples were subsequently processed in the same manner as that for unknown samples.

**Quantification of 4-Oxo-Carbazeran and 8-Oxo-*O*<sup>6</sup>-Benzylguanine by UPLC-MS/MS.**

The amount of 4-oxo-carbazeran and 8-oxo-*O*<sup>6</sup>-benzylguanine was quantified using UPLC-MS/MS. The UPLC-MS/MS system consisted of an Agilent 1290 Infinity LC System (Santa Clara, CA, USA) coupled to an AB SCIEX Triple Quad 3500 System (triple quadrupole mass spectrometer) (AB Sciex LLC, Framingham, MA, USA). Chromatographic separation was achieved using a Waters ACQUITY™ UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) coupled to Waters ACQUITY BEH C18 VanGuard Pre-column (2.1 × 5 mm, 1.7 μm), which were purchased from Waters Corporation (Milford, MA, USA). The column was maintained at 45°C for the carbazeran 4-oxidation assay and 40°C for the *O*<sup>6</sup>-benzylguanine 8-oxidation assay. The mobile phases were (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid for both assays. The flow rate was 0.5 ml/min and total run time was 4.5 min. In the carbazeran 4-oxidation assay, the gradient elution was 5% B from 0.0 to 1.0 min, linear increase to 95% B from 1.0 to 2.0 min, 95% B from 2.0 to 3.5 min, linear decrease to 5% B from 3.5 to 3.6 min, and 5% B from 3.6 to 4.5 min. In the *O*<sup>6</sup>-benzylguanine 8-oxidation assay, the gradient elution was 5% B from 0.0 to 0.4 min, linear increase to 95% B from 0.4 to 3.0 min, 95% B from 3.0 to 3.6 min, linear decrease to 5% B from 3.6 to 3.61 min, and 5% B from 3.61 to 4.5 min. The chromatographic effluent was introduced directly into the mass spectrometer only from 1.5 to 3.5 min. The autosampler sample compartment was kept at 4°C and the sample injection volume was 5 μl. Nitrogen gas was used as the collision gas and ion source gas. The analytes were determined in the positive electrospray ionization mode with multiple reaction monitoring (MRM). The MS source parameters, mass-to-charge (*m/z*) transitions, and other compound-dependent MS

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parameters for each analyte and internal standard are listed in Table 1. The amount of a metabolite was quantified using MultiQuant<sup>TM</sup> software (v3.0.1, AB Sciex). The calibration curves were plotted using the peak area ratios (analyte to internal standard) versus known amount of analyte, and weighted ( $1/x^2$ ) linear least-squares regression was performed to obtain the best-fit line.

**Rapid Equilibrium Dialysis.** Nonspecific binding of carbazeran and *O*<sup>6</sup>-benzylguanine to human liver microsomes, cytosol, and S9 fraction was determined using a Rapid Equilibrium Dialysis Kit (Thermo Fisher Scientific, Waltham, MA). Potassium phosphate buffer (100 mM, pH 7.4, 400  $\mu$ l) was added to the buffer chamber of the Rapid Equilibrium Dialysis plate. A mixture (total volume of 200  $\mu$ l) containing potassium phosphate buffer (100 mM, pH 7.4), a drug (2 or 10  $\mu$ M carbazeran in a final concentration of 0.5% v/v DMSO or 50 or 200  $\mu$ M *O*<sup>6</sup>-benzylguanine in a final concentration of 0.5% v/v DMSO), and a human liver tissue fraction (100  $\mu$ g total microsomal protein for carbazeran and 60  $\mu$ g total microsomal protein for *O*<sup>6</sup>-benzylguanine; 20  $\mu$ g total cytosolic protein; or 60  $\mu$ g total protein in the S9 fraction) was added to the sample chamber of the dialysis plate. The dialysis plate was incubated on an orbital plate shaker at 37°C for 4 h with a rotating speed of 300 rpm. At the end of the incubation period, a 25  $\mu$ l aliquot of the mixture from each of the buffer and the sample chamber was mixed with 475  $\mu$ l of ice-cold acetonitrile containing 50 nM tolbutamide (internal standard; final concentration of 47.5 nM in 500  $\mu$ l final volume). Control protein binding experiment was performed on human liver microsomes (100  $\mu$ g) with drugs known to be highly bound to microsomal or plasma proteins; i.e., gefitinib (1  $\mu$ M) (Burns et al., 2015) and abiraterone (1  $\mu$ M). (Marbury et al., 2014). Initial experiments with buffer (no enzymes) on both chambers were conducted to ensure that equilibrium of carbazeran, *O*<sup>6</sup>-benzylguanine, and controls (gefitinib and abiraterone) was achieved after 4 h. Each sample was mixed and centrifuged at 16,000  $\times$  g for 15 min at 4°C. The supernatant was then transferred

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into a 96-well microplate for UPLC-MS/MS analysis. Calibration standards were prepared by adding stock solutions of carbazeran (0.01-10 mM in DMSO) or *O*<sup>6</sup>-benzylguanine (0.3-300 mM in DMSO) into the incubation mixture to give final concentrations of 0.01-10 μM or 0.3-300 μM, respectively. The unbound fraction (*f*<sub>u</sub>) of a drug in the human liver microsomal, cytosolic, and S9 fraction was calculated as the ratio of drug concentration in buffer chamber and drug concentration in sample chamber (Obach, 1997).

**AOX-1 Protein Quantification by a Capillary Nano-proteomic Immunoassay.** AOX-1 protein level was quantified in a capillary nano-proteomic immunoassay (SimpleWestern System, ProteinSimple) (Chen et al., 2013). Standards were diluted from recombinant AOX-1 protein (OriGene Technologies). Each diluted standard and test sample (at 0.5 mg/ml) was mixed in 4 μl 5× reducing buffer with fluorescent molecular weight standards. After these samples were heated to 95°C for 5 min, 5 μl samples were loaded into each capillary tube. Proteins were passed and separated through stacking and separation matrices for 30 min at 250 V. Proteins were then immobilized to capillary walls using optimal photoactivated capture chemistry. Following proteins immobilization, capillaries were flushed with a wash buffer and incubated with a blocking reagent for 23 min. AOX-1 was probed with an anti-AOX-1 primary antibody (diluted in blocking buffer and incubated for 200 min) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody. A mixture of luminol and peroxide was added following manufacturer's protocol. The resulting chemiluminescent signal was captured by a charge-coupled device camera, and signal intensities were analyzed using Compass Software Version 3.1.7 (ProteinSimple).

**Enzyme Kinetic Analysis.** Carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation assays were conducted using varying concentrations of carbazeran (0.125 to 32 μM for human liver cytosol, 0.1 to 30 μM for microsomes, and 0.125 to 32 μM for S9 fraction) and *O*<sup>6</sup>-benzylguanine

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(1 to 400  $\mu\text{M}$  for cytosol, 2.5 to 100  $\mu\text{M}$  for microsomes, and 1 to 400  $\mu\text{M}$  for S9 fraction), respectively. The values of  $V_{\text{max}}$  and apparent  $K_m$  were determined by nonlinear least-squares regression analysis (SigmaPlot 12.5; Systat Software Inc., San Jose, CA, USA) of the velocity of metabolite formation ( $V$ ) and substrate concentration ( $S$ ) data using the equations for Michaelis-Menten, Hill, and substrate inhibition models. The goodness of fit for each model was evaluated by considering the Akaike information criterion,  $R^2$ , and visual inspection of the substrate-velocity data. Based on the above evaluation criteria, the data for carbazeran 4-oxidation and  $O^6$ -benzylguanine 8-oxidation were fitted using the Michaelis-Menten model:

$$V = \frac{V_{\text{max}} * S}{K_m + S}$$

where  $S$  represents the substrate concentration,  $V_{\text{max}}$  represents the maximum reaction velocity,  $K_m$  represents the substrate concentration at which the reaction rate is half of  $V_{\text{max}}$ . Turnover number ( $k_{\text{cat}}$ ) was calculated by dividing  $V_{\text{max}}$  by AOX-1 protein concentration (Hollaway et al., 1980). Corrected  $K_m$  was calculated by multiplying the unbound fraction ( $f_u$ ) by the apparent  $K_m$  (Obach, 1997). Unbound intrinsic clearance ( $Cl_{\text{int,u}}$ ) was calculated by dividing  $V_{\text{max}}$  by the corrected  $K_m$  (Giuliano et al., 2005).

**Enzyme Inhibition Experiments.** Enzyme inhibition was determined by conducting the carbazeran 4-oxidation and  $O^6$ -benzylguanine 8-oxidation assays in the presence of a known inhibitor. AOX-1 inhibition experiments were performed with hydralazine (5-40  $\mu\text{M}$ ), a selective inhibitor of AOX-1 (Johnson et al., 1985; Strelevitz et al., 2012). Cytochrome P450 inhibition experiments were conducted with 1-aminobenzotriazole (1-ABT, 100-3000  $\mu\text{M}$ ), an inactivator of multiple CYP enzymes (Linder et al., 2009; Zientek and Youdim, 2015), or with furafylline (25-75  $\mu\text{M}$ ), a CYP1A2-selective inhibitor (Sesardic et al., 1990; Kunze and Trager, 1993). For incubations containing 1-ABT or furafylline, human liver microsomes and S9 fractions were

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preincubated at 37°C in the presence of the inactivator and NADPH for 30 min prior to the addition of substrate.

**Testosterone 6 $\beta$ -Hydroxylation Assay.** The testosterone 6 $\beta$ -hydroxylation assay was conducted as described previously (Foo et al., 2015). Briefly, each standard 200  $\mu$ l incubation mixture contained potassium phosphate buffer (100 mM, pH 7.4), NADPH (1 mM), testosterone (40  $\mu$ M), and human liver microsomes (60  $\mu$ g protein). Each incubation mixture was pre-warmed for 3 min at 37°C and the enzyme reaction was initiated by adding NADPH. After a 10 min incubation period, the reaction was terminated by adding 200  $\mu$ l ice-cold acetonitrile containing prednisolone (1  $\mu$ M final concentration; internal standard). Each sample was processed for analysis of 6 $\beta$ -hydroxytestosterone and prednisolone by UPLC-MS-MS (Foo et al., 2015).

**7-Ethoxycoumarin *O*-Deethylation Assay.** The 7-ethoxycoumarin *O*-deethylation assay was adapted from a published method (Waxman and Chang, 2006). Each 200  $\mu$ l incubation mixture contained potassium phosphate buffer (100 mM, pH 7.4), 7-ethoxycoumarin (500  $\mu$ M), human recombinant cytochrome P450 enzyme (4 pmol; CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, or CYP3A5), and NADPH (1 mM), as specified in the figure legend. The final concentration of solvent in the incubation mixture was 0.5% v/v methanol, which was reported not to affect the catalytic activity of the cytochrome P450 enzymes listed above (Chauret et al., 1998). In the negative control groups, the recombinant enzyme was replaced with an equivalent protein amount (30  $\mu$ g) of insect cell microsomes or insect cell microsomes with oxidoreductase and cytochrome b<sub>5</sub>. Calibration standards were prepared by adding known amount of 7-hydroxycoumarin (100, 200, 400, 600, 800, or 1200 pmol in 0.1% v/v methanol) into the incubation mixture. Blank samples, which did not contain the metabolite or enzymes, were prepared in a similar manner as the samples. Each incubation mixture was pre-warmed for 3 min at 37°C in a

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low-speed shaking water bath. The enzymatic reaction was initiated by adding NADPH, and the mixture was incubated for 30 min. The reaction was terminated with ice-cold hydrochloric acid (2 M; 25  $\mu$ l). The enzymatic product was extracted with chloroform (450  $\mu$ l) and centrifuged (3000  $\times$  g for 5 min). The bottom organic layer (300  $\mu$ l) was back-extracted with 1 ml of 30 mM sodium borate (pH 9.2) and centrifuged (3000  $\times$  g for 5 min). The amount of 7-hydroxycoumarin in the top aqueous layer was quantified by fluorescence spectrophotometry, using an excitation wavelength of 370 nm and an emission wavelength of 450 nm (Waxman and Chang, 2006).

**Dehydroepiandrosterone Sulfonation Assay.** The dehydroepiandrosterone (DHEA) sulfonation assay was conducted as described previously (Yip et al., 2018). In brief, each incubation mixture (200  $\mu$ l) contained potassium phosphate buffer (100 mM, pH 7.4), magnesium chloride (2.5 mM), DHEA (1  $\mu$ M), and various amounts of microsomes (0, 100, 200, or 300  $\mu$ g protein). The enzyme reaction was initiated with 3'-phosphoadenosine 5'-phosphosulfate (5  $\mu$ M), and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 200  $\mu$ l ice-cold acetonitrile containing cortisol (0.05  $\mu$ M final concentration; internal standard). Each sample was processed for analysis of DHEA sulfate and cortisol by UPLC-MS-MS as detailed elsewhere (Bansal and Lau, 2016).

**Statistical Analysis.** Data were analyzed by one-way or two-way analysis of variance and, where appropriate, was followed by the Student-Newman-Keuls *post-hoc* test (SigmaPlot 12.5). The level of statistical significance was set *a priori* at  $p < 0.05$ .

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## Results

**Development and Validation of UPLC-MS/MS Methods for Quantification of 4-Oxo-Carbazeran and 8-oxo-*O*<sup>6</sup>-Benzylguanine.** UPLC-MS/MS methods were developed for the quantification of 4-oxo-carbazeran and 8-oxo-*O*<sup>6</sup>-benzylguanine, which are formed by carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation, respectively. Similar to previous reports (Sodhi et al., 2015), the precursor ion of 4-oxo-carbazeran  $[M + H]^+$  occurred at  $m/z$  of 377.2 and in the product ion scan, the most abundant product ion was at  $m/z$  of 288.0 (Supplemental Fig. S1A). Under optimized mass spectrometric conditions (Table 1), 4-oxo-carbazeran eluted as a single sharp peak and with a retention time of 2.81 min (Supplemental Fig. S1B). In the UPLC-MS/MS method for detecting 8-oxo-*O*<sup>6</sup>-benzylguanine, the  $m/z$  transition of 258.0 to 91.1 was selected (Supplemental Fig. S1C), which was consistent with the literature (Sodhi et al., 2015; Barr et al., 2015), and a single peak was obtained at a retention time of 2.02 min (Supplemental Fig. S1D). The blank samples did not show any peaks in the chromatograms (data not shown), indicating specificity of the methods. The calibration curve was linear from 0.2 to 600 pmol of 4-oxo-carbazeran and 0.2 to 200 pmol of 8-oxo-*O*<sup>6</sup>-benzylguanine. Using the criteria of signal-to-noise ratio of  $> 5:1$ , accuracy of  $\pm 20\%$  of the nominal concentration, and precision of  $< 20\%$  coefficient of variation, the lower limit of quantification (LLOQ) for 4-oxo-carbazeran and 8-oxo-*O*<sup>6</sup>-benzylguanine was 0.2 pmol or 0.5 nM (in the entire 400  $\mu$ l incubation mixture, of which 5  $\mu$ l was injected into the UPLC-MS). When quality control samples were analyzed at low (0.4 pmol), mid (4 pmol), and high (40 pmol) amounts, the intra-day and inter-day accuracy and precision were  $< 10.3\%$  for 4-oxo-carbazeran and  $< 12.7\%$  for 8-oxo-*O*<sup>6</sup>-benzylguanine (Supplemental Table S1). UPLC-MS/MS methods for quantifying the substrates (carbazeran and *O*<sup>6</sup>-benzylguanine) and internal standard (tolbutamide) were also developed and the conditions optimized (Table 1).

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**Optimization of Enzymatic Assay Conditions for Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation Catalyzed by Human Liver Microsomes, Cytosol, and S9 Fraction.** Carbazeran 4-oxidation catalyzed by human liver microsomes (in the absence or presence of NADPH) was linear up to 200 µg of microsomal protein (Supplemental Fig. S2A) and 5 min incubation time (Supplemental Fig. S2D). By comparison, in enzymatic incubations containing human liver cytosol, carbazeran 4-oxidation was linear up to 30 µg of cytosolic protein (Supplemental Fig. S2B) and 15 min incubation time (Supplemental Fig. S2E), whereas carbazeran 4-oxidation catalyzed by human S9 fraction (in the absence or presence of NADPH) was linear up to 150 µg of protein (Supplemental Fig. S2C) and 10 min incubation time (Supplemental Fig. S2F). In all subsequent experiments, the carbazeran 4-oxidation assay was conducted under linear conditions with respect to incubation time (5 min) and amount of protein (100, 20, and 60 µg protein in enzymatic incubations containing microsomes, cytosol, and S9 fraction, respectively). Under these incubation conditions, the level of carbazeran was decreased by 23% in liver S9 fraction and ≤ 10% in microsomes and cytosol (Supplemental Table S2), indicating that substrate concentration was not a rate-limiting factor in the assay.

*O*<sup>6</sup>-Benzylguanine 8-oxidation catalyzed by human liver microsomes (in the absence or presence of NADPH) was linear up to 200 µg of microsomal protein (Supplemental Fig. S3A) and 20 min (without NADPH) or 15 min (with NADPH) incubation time (Supplemental Fig. S3D). By comparison, in enzymatic incubations containing human liver cytosol, *O*<sup>6</sup>-benzylguanine 8-oxidation was linear up to 200 µg of cytosolic protein (Supplemental Fig. S3B) and 30 min incubation time (Supplemental Fig. S3E), whereas in enzymatic incubations containing human liver S9 fraction (in the absence or presence of NADPH), it was linear up to 150 µg of protein (Supplemental Fig. S3C) and 30 min incubation time (Supplemental Fig. S3F). In all subsequent experiments, the *O*<sup>6</sup>-benzylguanine 8-oxidation assay was conducted under linear conditions with



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respect to incubation time (15, 5, and 10 min in enzymatic incubations containing microsomes, cytosol, and S9 fraction, respectively) and amount of protein (60, 20, and 60  $\mu\text{g}$  protein in enzymatic incubations containing microsomes, cytosol, and S9 fraction, respectively). Under these incubation conditions, substrate depletion in the  $O^6$ -benzylguanine 8-oxidation assay was  $\leq 8\%$  (Supplemental Table S2), indicating that substrate concentration was not a rate-limiting factor in the assay.

**Detection of Carbazeran 4-Oxidation and  $O^6$ -Benzylguanine 8-Oxidation in Incubations Containing Human Liver Microsomes: Differential Effect of Exogenous NADPH.** Carbazeran 4-oxidation (Fig. 1A) and  $O^6$ -benzylguanine 8-oxidation (Fig. 1B) were detected in enzymatic incubations containing human liver microsomes. The extent of 4-oxo-carbazeran formation and 8-oxo- $O^6$ -benzylguanine formation among the different pools of microsomes varied by approximately 4-fold (Fig. 1A) and 1.6-fold (Fig. 1B), respectively. The inclusion of NADPH in the microsomal incubations did not alter the extent of carbazeran 4-oxidation (Fig. 1A). In contrast, it consistently increased the level of  $O^6$ -benzylguanine 8-oxidation in each of the microsomal preparations (Fig. 1B), suggesting the presence of AOX-1 in those samples.

**Comparison of AOX-1 Protein Expression Level in Human Liver Cytosol, Microsomes, and S9 Fraction.** To determine whether AOX-1 protein was present in our panel of human liver microsomal preparations, we conducted a capillary nano-proteomic immunoassay (also known as capillary electrophoresis immunoassay system) (Chen et al., 2015). Supplemental Fig. S4 shows that a single peak was obtained using recombinant AOX-1 protein, and chemiluminescent signal increased with increasing concentration of the AOX-1 protein standard, thereby achieving a good linearity in the calibration curve. AOX-1 protein was present in cytosol, S9 fraction, and in each of the five pools of microsomes used in the present study (Supplemental

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Fig. S4). The level of AOX-1 protein in the various microsomal preparations corresponded to 19-36% of that in the cytosol preparation and 23-43% of that in the S9 fraction (Table 2).

**Kinetic Comparison of Human Liver Microsomes, Cytosol, and S9 Fraction in the Catalysis of Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation.** Carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes (Fig. 2A and 2D), cytosol (Fig. 2B and 2E), and S9 fraction (Fig. 2C and 2F) increased with increasing substrate concentration, and the data were best fitted by the Michaelis-Menten model. The addition of NADPH to incubations containing microsomes (Fig. 2A) or S9 (Fig. 2C) did not modify the product formation *vs.* substrate concentration curves in the carbazeran 4-oxidation assay, consistent with the lack of an effect on the values of the various kinetic parameters, such as  $V_{max}$ , turnover number ( $k_{cat}$ ), apparent  $K_m$  (Table 3 and Supplemental Table S4). By comparison, the addition of NADPH to microsomal incubations increased *O*<sup>6</sup>-benzylguanine 8-oxidation with increasing substrate concentration (Fig. 2D), but this did not occur in S9 incubations supplemented with NADPH (Fig. 2F). Equilibrium dialysis was performed to investigate the extent of nonspecific binding of carbazeran and *O*<sup>6</sup>-benzylguanine to human liver microsomes, cytosol, and S9 fraction. As shown in Supplemental Table S3, the fraction unbound ( $f_u$ ) for each of these chemicals was comparable in each of these isolated fractions, as determined after a dialysis time of 4 h where there was still a substantive concentration of the substrate (which was far above the LLOQ of 0.5 nM for 4-oxo-carbazeran and 8-oxo-*O*<sup>6</sup>-benzylguanine). A kinetic comparison based on unbound drug showed comparable values of corrected  $K_m$  and unbound intrinsic clearance ( $Cl_{int,u}$ , when normalized to the amount of AOX-1) for carbazeran 4-oxidation in incubations containing human liver microsomes, cytosol, and S9 fraction (Table 3). The same trend was obtained for *O*<sup>6</sup>-benzylguanine 8-oxidation (Table 3).

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**Differential Catalysis of Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation by Human Recombinant Cytochrome P450 Enzymes.** As shown in Fig. 1A and 2A, 4-oxo-carbazeran was detected in enzymatic incubations containing carbazeran, human liver microsomes and NADPH. Cytochrome P450 enzymes are the major drug-metabolizing enzymes present in liver microsomes and require NADPH as a co-factor. Therefore, we determined whether a cytochrome P450 enzyme plays a role in carbazeran 4-oxidation by conducting experiments utilizing human recombinant cytochrome P450 enzymes. As a comparison, we also determined the catalysis of *O*<sup>6</sup>-benzylguanine 8-oxidation by the recombinant enzymes because 8-oxo-*O*<sup>6</sup>-benzylguanine was detected in liver microsomal incubations (Fig. 1B and 2D). As shown in Fig. 3A, little or no 4-oxo-carbazeran was formed by various human recombinant cytochrome P450 enzymes; i.e., CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, or CYP3A5. In contrast, *O*<sup>6</sup>-benzylguanine 8-oxidation was catalyzed by CYP1A2, and to a much lesser extent by CYP2C19, CYP2D6, CYP3A4, and CYP3A5 (Fig. 3B). Control experiments confirmed human recombinant AOX-1 enzyme as a catalyst of carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation (Fig. 3C). Multiple human recombinant cytochrome P450 enzymes are active catalysts of 7-ethoxcoumarin *O*-deethylation (Waxman et al., 1991). Therefore, the 7-ethoxcoumarin *O*-deethylation assay (Waxman and Chang, 2006) was conducted as a control experiment. As shown in Fig. 3D, each of the human recombinant cytochrome P450 enzymes investigated in the present study (Fig. 3A and 3B) was indeed catalytically activity. As expected, the insect control cell microsomes or *Escherichia coli* control cytosol did not yield any metabolite in enzymatic incubations containing a cytochrome P450 (Fig. 3A, 3B, and 3D) or AOX-1 (Fig. 3C).

**Differential Effect of a Cytochrome P450 Inhibitor on Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation in Enzymatic Incubations Containing Human Liver Microsomes.** To corroborate the results obtained from the recombinant enzyme experiments (Fig.

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3A and 3B), we performed chemical inhibition experiments with 1-ABT, known to inhibit multiple cytochrome P450 enzymes (Linder et al., 2009; Zientek and Youdim, 2015). 1-ABT did not decrease carbazeran 4-oxidation (Fig. 4A), whereas it decreased *O*<sup>6</sup>-benzylguanine 8-oxidation (Fig. 4B) in human liver microsomal incubations. A control experiment indicated that 1-ABT yielded the expected results in the microsomal CYP3A-catalyzed testosterone 6 $\beta$ -hydroxylation assay (Fig. 4C).

**Relative Contribution of Cytochrome P450 and AOX-1 Enzymes to Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation in Human Liver S9 Fraction.** Chemical inhibition experiments were performed to determine the relative contribution of cytochrome P450 and AOX-1 enzymes to carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation catalyzed by a tissue fraction (e.g., liver S9 fraction) containing both cytochrome P450 and AOX-1. Varying concentrations of 1-ABT, an inactivator of multiple CYP enzymes (Linder et al., 2009; Zientek and Youdim, 2015), and furafylline, a CYP1A2-selective inhibitor (Sesardic et al., 1990; Kunze and Trager, 1993), did not affect carbazeran 4-oxidation (Fig. 5A and 5B) or *O*<sup>6</sup>-benzylguanine 8-oxidation (Fig. 5D and 5E), whereas hydralazine, an enzyme-selective chemical inhibitor of AOX-1 (Johnson et al., 1985; Strelevitz et al., 2012), decreased substantively the extent of carbazeran 4-oxidation (Fig. 5C) and *O*<sup>6</sup>-benzylguanine 8-oxidation (Fig. 5F) in our panel of human liver S9 fraction. Overall, these results identify AOX-1 rather than cytochrome P450 as the contributing enzyme in the catalysis of carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in a tissue fraction such as human liver S9 that expresses both cytochrome P450 and AOX-1.

**Effect of an AOX-1 Inhibitor on Carbazeran 4-Oxidation and *O*<sup>6</sup>-Benzylguanine 8-Oxidation in Human Liver Microsomal Incubations.** 4-Oxo-carbazeran (Fig. 1A and 2A) and 8-oxo-*O*<sup>6</sup>-benzylguanine (Fig. 1B and 2D) were detected in incubations containing human liver

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microsomes without supplementation of NADPH. Therefore, we determined whether AOX-1 was present in the microsomal preparations by conducting an experiment with hydralazine, an enzyme-selective chemical inhibitor of AOX-1 (Johnson et al., 1985; Strelevitz et al., 2012). Hydralazine (40  $\mu$ M) almost completely decreased carbazeran 4-oxidation in five different pools of microsomes (Fig. 6A) and at various concentrations (5-40  $\mu$ M) of hydralazine (Fig. 6B), as determined in microsomal incubations without the addition of exogenous NADPH. As shown with an optimized enzyme assay (Supplemental Fig. S3A and S3D), hydralazine also decreased *O*<sup>6</sup>-benzylguanine 8-oxidation in multiple pools of microsomes (Fig. 6D) and by multiple concentrations (15 to 40  $\mu$ M) (Fig. 6E). Collectively, the results of these experiments suggested AOX-1 catalytic function in our panel of human liver microsomal preparations. A control experiment confirmed that hydralazine decreased human liver cytosolic carbazeran 4-oxidation (Fig. 6C) and *O*<sup>6</sup>-benzylguanine 8-oxidation (Fig. 6F).

**Evidence for the Presence of Another Functional Cytosolic Enzyme in Our Panel of Cytosol-Contaminated Human Liver Microsomal Preparations.** To determine whether our panel of human liver microsomes contained a functional cytosolic enzyme other than AOX-1, a DHEA sulfonation assay was conducted. DHEA sulfonation is a typical and well-characterized cytosolic enzyme reaction catalyzed by various sulfotransferases, namely, SULT2A1 (Falany et al., 1989), SULT2B1 (Meloche and Falany, 2001), and SULT1E1 (Falany et al., 1995). As shown in Supplemental Fig. S5, DHEA sulfonation increased with increasing amount of microsomal protein, as assessed in two pools of microsomes, indicating the presence of functional cytosolic enzymes other than AOX-1 (Table 2) in the cytosol-contaminated human liver microsomal preparations used in the present study.

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## Discussion

Previous *in vitro* and *in vivo* human studies have shown that carbazeran undergoes oxidation (Kaye et al., 1984) and glucuronidation (Kaye et al., 1984; Ballard et al., 2016). Experiments with enzyme-selective chemical inhibitors indicated that in human liver cytosol, AOX-1, but not xanthine oxidase, is a catalyst of carbazeran 4-oxidation (Kaye et al., 1985). The present study shows that in contrast to AOX-1, cytochrome P450 does not catalyze carbazeran 4-oxidation. This conclusion was based on our experimental evidence indicating that: 1) carbazeran 4-oxidation was inhibited by an AOX-1 inhibitor (hydralazine), regardless of whether the enzymatic incubation was conducted with human liver microsomes, cytosol, or S9 fraction; 2) lack of differences in the extent of carbazeran 4-oxidation among the multiple pools of microsomes with or without exogenous addition of NADPH; 3) after correcting for the extent of protein binding (to yield corrected  $K_m$ ) and normalizing  $V_{max}$  and  $Cl_{int,u}$  to the amount of AOX-1 protein, the enzyme kinetics of carbazeran 4-oxidation in incubations containing cytosol were comparable to those obtained in incubations containing microsomes or S9 fraction, either with or without exogenous addition of NADPH; 4) a well-established chemical inhibitor of cytochrome P450 (1-aminobenzotriazole) did not decrease carbazeran 4-oxidation; and 5) a panel of individual human recombinant cytochrome P450 enzymes (i.e., CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) did not catalyze carbazeran 4-oxidation to any appreciable extent. Consistent with our experimental results, it has been proposed that cytochrome P450 is not anticipated to catalyze carbazeran 4-oxidation based on differences in the substrate specificity of AOX-1 and cytochrome P450 (Crouch et al., 2016; Lepri et al., 2017). In a previous *in vivo* pharmacokinetic study, it was reported that carbazeran is metabolized extensively in humans, with a calculated total body clearance that was twice that of the literature value of human hepatic blood flow (Kaye et al. 1984). The clearance of carbazeran in humans (Kaye et al. 1984) is mainly the

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result of the catalysis of carbazeran glucuronidation by microsomal uridine 5'-diphosphoglucuronosyltransferase (Kaye et al., 1984; Ballard et al., 2016) and carbazeran 4-oxidation by the cytosolic AOX-1 (Kaye et al. 1985; present study) but not by the cytosolic xanthine oxidase (Kaye et al. 1985) or microsomal cytochrome P450 (present study). Collectively, the biochemical data, as summarized in Fig. 7, provide a mechanistic basis of the *in vivo* clearance of carbazeran in humans.

Enzyme-selective catalytic markers are important experimental tools in the functional studies of a drug-metabolizing enzyme (Zientek and Youdim, 2015). Carbazeran 4-oxidation is catalyzed by AOX-1 (Kaye et al., 1985), but not by xanthine oxidase (Kaye et al., 1985) or cytochromes P450 (present study). By comparison, *O*<sup>6</sup>-benzylguanine 8-oxidation is catalyzed by AOX-1 and to a lesser extent, by xanthine oxidase (Roy et al., 1995) and by cytochromes P450, especially CYP1A2 [(Roy et al., 1995) and present study]. However, a major conclusion from the present study is that AOX-1, but not cytochrome P450, is the primary catalyst of *O*<sup>6</sup>-benzylguanine 8-oxidation in a tissue fraction (e.g., S9 fraction) where both AOX-1 and cytochrome P450 enzymes are expressed. This conclusion is supported by the finding that an inhibitor of AOX-1 (hydralazine), but not inhibitors of cytochrome P450 (1-ABT and furafylline), decreased *O*<sup>6</sup>-benzylguanine 8-oxidation in human S9 fraction. Overall, based on the above findings and as summarized in Fig. 7, it is appropriate to use *O*<sup>6</sup>-benzylguanine 8-oxidation as catalytic markers of AOX-1 in a functional study performed in a cellular fraction (e.g., S9 fraction) or whole cell system (e.g., hepatocytes) where both the cytosol and endoplasmic reticulum are present.

The present investigation employing multiple complementary experimental approaches represents the first detailed quantitative analysis showing the presence of a substantial amount of AOX-1 protein in human liver microsomal preparations, accounting for up to 40% of that in human liver cytosol. Consistent with this finding, the extent of AOX-1-catalyzed carbazeran 4-oxidation

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in the microsomal preparations was also pronounced, as illustrated by an apparent  $V_{\max}$  obtained from microsomal incubations that was approximately 14% of that in cytosolic incubations. Other experimental evidence of cytosolic contamination of our microsomal preparations include: 1) the functional activity of AOX-1, as assessed by another AOX-1-catalyzed reaction,  $O^6$ -benzylguanine 8-oxidation, was detected in human liver microsomal incubations without the addition of NADPH, and this occurred in multiple pools of human liver microsomes obtained from multiple commercial suppliers; 2) an AOX-1 inhibitor (hydralazine) almost completely abolished the two AOX-1-mediated reactions (carbazeran 4-oxidation and  $O^6$ -benzylguanine 8-oxidation) in human liver microsomal incubations; and 3) another well-characterized cytosolic enzyme activity (DHEA sulfonation catalyzed by SULT2A1, SULT2B1, and SULT1E1) was also quantified in our pools of human liver microsomes. Previous studies detected AOX-1-catalyzed metabolites from carbazeran (Wilkinson et al., 2017), VU0409106 (Crouch et al., 2016), and SGX523 (Diamond et al., 2010) in human liver microsomes, but did not quantify microsomal AOX-1 protein content and postulated the metabolite formation was due to contamination of microsomes with cytosol (Crouch et al., 2016; Wilkinson et al., 2017). Overall, by using various experimental approaches to address the issue of cytosolic contamination of our panel of human liver microsomes, our results indicate that microsomal cytochrome P450 does not catalyze carbazeran 4-oxidation.

In conclusion, it is appropriate to utilize carbazeran 4-oxidation and  $O^6$ -benzylguanine 8-oxidation as enzyme-selective catalytic markers of AOX-1, including their use in *in vitro* and cell culture models known to contain both cytosolic and microsomal drug-metabolizing enzymes. Our expression and functional analyses demonstrated a substantial level of cytosolic AOX-1 protein and catalytic activity in multiple human liver microsomal preparations provided by multiple commercial suppliers. The use of cytosol-contaminated liver microsomal preparations in reaction phenotyping experiments will lead to an erroneous identification of the enzyme(s) responsible for



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the metabolism of a drug or new chemical entity under investigation. Therefore, it is important to quantify the extent of cytosolic contamination of each lot of human liver microsomes prior to performing reaction phenotyping experiments on chemicals or new drug entities. Protocols should include multiple washing and purification steps in the isolation of the microsomal fraction. The substrate oxidation assay protocols should also include a control group(s) where the microsomal incubation does not include NADPH, to gain insight into the possibility of cytosolic contamination of microsomal preparations and non-NADPH dependent drug metabolism. Although the present study did not use matched pairs of cytosol and microsomes, future studies should do so because it would provide a greater insight into lot to lot differences in the extent of cytosolic contamination of microsomes and help in the interpretation of cytosolic vs. microsomal drug metabolism data. Reaction phenotyping results obtained from experiment conducted in tissue microsomes should be verified in another drug metabolism model such as whole cells (e.g., hepatocytes) or an isolated cellular fraction containing cytosol and endoplasmic reticulum (e.g., S9 fraction).

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### **Authorship Contributions**

*Participated in research design:* Xie, Saburulla, Chen, Wong, and Lau.

*Conducted experiments:* Xie, Saburulla, Chen, Wong, Yap, Zhang, and Lau.

*Contributed new reagents or analytic tools:* N/A

*Performed data analysis:* Xie, Saburulla, Chen, Wong, Yap, Zhang, and Lau.

*Wrote or contributed to the writing of the manuscript:* Xie, Saburulla, and Lau.

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## Footnotes

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## Figure Legends

**Fig. 1.** Comparison of carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes. (A) Microsomes (100 µg protein; HLM Pools 1-5) was incubated with carbazeran (30 µM) at 37°C for 5 min in the absence or presence of NADPH (1 mM). (B) Microsomes (60 µg protein; Pools 1, 2, and 5) was incubated with *O*<sup>6</sup>-benzylguanine (50 µM) at 37°C for 15 min in the absence or presence of NADPH (1 mM). Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate or triplicate.

**Fig. 2.** Carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation as a function of substrate concentration in enzymatic incubations containing human liver microsomes, cytosol, or S9 fraction. (A) Microsomes (100 µg protein) was incubated with varying concentrations of carbazeran (0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 15, or 30 µM) at 37°C for 5 min in the absence or presence of NADPH (1 mM). (B) Cytosol (20 µg protein) was incubated with varying concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, or 32 µM) of carbazeran at 37°C for 5 min. (C) S9 fraction (60 µg protein) was incubated with carbazeran (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 µM) in the absence and presence of NADPH (1 mM) at 37°C for 5 min. (D) Microsomes (60 µg protein) was incubated with varying concentrations of *O*<sup>6</sup>-benzylguanine (2.5, 5, 10, 25, 50, 60, 70, 80, 90, or 100 µM) at 37°C for 15 min in the absence or presence of NADPH (1 mM). (E) Cytosol (20 µg protein) was incubated with varying concentrations (1, 2.5, 5, 10, 25, 50, 100, 200, 300, or 400 µM) of *O*<sup>6</sup>-benzylguanine at 37°C for 5 min. (F) S9 fraction (60 µg protein) was incubated with *O*<sup>6</sup>-benzylguanine (1, 2.5, 5, 10, 25, 50, 100, 200, 300, or 400 µM) in the absence and presence of NADPH (1 mM) at 37°C for 10 min. Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate or triplicate.



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**Fig. 3.** Carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in a panel of human recombinant cytochrome P450 and AOX-1 enzymes. (A, B) Carbazeran (10 μM) or *O*<sup>6</sup>-benzylguanine (50 μM) was incubated with NADPH (1 mM) and CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, or CYP3A5 (20 pmol) at 37°C for 30 min. (C) Carbazeran (15 μM) or *O*<sup>6</sup>-benzylguanine (200 μM) was incubated with human recombinant AOX-1 (30 μg protein) or *Escherichia coli* control cytosol (30 μg protein) at 37°C for 45 min. (D) 7-Ethoxycoumarin (500 μM) was incubated with NADPH (1 mM) and a cytochrome P450 enzyme listed above (4 pmol) at 37°C for 30 min. In the control incubations, the recombinant enzyme was replaced with insect cell microsomes (Control 1) or insect cell microsomes with oxidoreductase and cytochrome b5 (Control 2). Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate. \*, significantly different from the control groups (*p* < 0.05). LLOQ represents the lower limit of quantification.

**Fig. 4.** Effect of a cytochrome P450 enzyme inhibitor (1-ABT) on carbazeran 4-oxidation, *O*<sup>6</sup>-benzylguanine 8-oxidation, and testosterone 6β-hydroxylation in enzymatic incubations containing human liver microsomes. (A) Microsomes (100 μg protein) was incubated with NADPH (1 mM), carbazeran (7 μM), and 1-ABT (100, 300, 1000, 2000, or 3000 μM), or methanol (0.5% v/v; vehicle) at 37°C for 5 min. (B) Microsomes (60 μg protein) was incubated with NADPH (1 mM), *O*<sup>6</sup>-benzylguanine (50 μM), and 1-ABT (100, 300, or 1000 μM), or methanol (0.5% v/v; vehicle) at 37°C for 15 min. (C) As a control, microsomes (60 μg protein) was incubated with NADPH (1 mM), testosterone (40 μM), and 1-ABT (100, 300, 1000, 2000, or 3000 μM), or methanol (0.5% v/v; vehicle) at 37°C for 10 min. Data are expressed as mean ± S.E.M. of three independent

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experiments conducted in duplicate. \*, significantly different from the vehicle-treated control group ( $p < 0.05$ ). Carbazeran 4-oxidation, *O*<sup>6</sup>-benzylguanine 8-oxidation, and testosterone 6 $\beta$ -hydroxylation in the vehicle-treated control group were 62 pmol/min/mg protein (A), 42 pmol/min/mg protein (B), and 2861 pmol/min/mg protein (C), respectively.

**Fig. 5.** Effect of cytochrome P450 enzyme inhibitors (1-ABT and furafylline) and an AOX-1 inhibitor (hydralazine) on carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in enzymatic incubations containing human liver S9 fraction. (A-C) S9 fraction (60  $\mu$ g protein) was incubated with carbazeran (5  $\mu$ M), 1-ABT (100, 1000, or 3000  $\mu$ M) (A), furafylline (25, 50, or 75  $\mu$ M) (B), hydralazine (15, 25, or 40  $\mu$ M) (C), or DMSO (0.5% v/v; vehicle), and with 1 mM NADPH (A and B) or without NADPH (C) at 37°C for 5 min. (D-F) S9 fraction (60  $\mu$ g protein) was incubated with *O*<sup>6</sup>-benzylguanine (50  $\mu$ M), 1-ABT (100, 1000, or 3000  $\mu$ M) (D), furafylline (25, 50, or 75  $\mu$ M) (E), hydralazine (15, 25, or 40  $\mu$ M) (F), or DMSO (0.5% v/v; vehicle), and with 1 mM NADPH (D and E) or without NADPH (F) at 37°C for 10 min. Data are expressed as mean  $\pm$  S.E.M. of three independent experiments conducted in duplicate. \*, significantly different from the vehicle-treated control group ( $p < 0.05$ ). Carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in the vehicle-treated control group were 115-141 pmol/min/mg protein (A-C) and 96-144 pmol/min/mg protein (D-F), respectively.

**Fig. 6.** Effect of an AOX-1 inhibitor (hydralazine) on carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes (without exogenous supplementation of NADPH) or cytosol. (A) Microsomes (100  $\mu$ g protein; HLM Pools 1-5) was incubated with carbazeran (7  $\mu$ M) and hydralazine (40  $\mu$ M) or DMSO (0.5% v/v; vehicle)

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at 37°C for 5 min. (B) Microsomes (100 µg protein; Pool 1) was incubated with carbazeran (3 µM) and hydralazine (5, 10, 15, 25, or 40 µM) or DMSO (0.5% v/v; vehicle) at 37°C for 15 min. (C) Cytosol (20 µg protein) was incubated with carbazeran (2 µM) and hydralazine (5, 10, 15, 25, or 40 µM) or DMSO (0.5% v/v; vehicle) at 37°C for 5 min. (D) Microsomes (60 µg protein; HLM Pools 1, 2, and 5) was incubated with *O*<sup>6</sup>-benzylguanine (50 µM) and hydralazine (40 µM) or DMSO (0.5% v/v; vehicle) at 37°C for 15 min. (E) Microsomes (60 µg protein; Pool 2) was incubated with *O*<sup>6</sup>-benzylguanine (50 µM) and hydralazine (15, 25, or 40 µM) or DMSO (0.5% v/v; vehicle) at 37°C for 15 min. (F) Cytosol (20 µg protein) was incubated with *O*<sup>6</sup>-benzylguanine (40 µM) and hydralazine (15, 25, or 40 µM) or DMSO (0.5% v/v; vehicle) at 37°C for 5 min. Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate. \*, significantly different from the vehicle-treated control group ( $p < 0.05$ ). Carbazeran 4-oxidation in the vehicle-treated control group was 7.6 pmol/min/mg protein in human liver microsomes (B) and 477 pmol/min/mg protein in human liver cytosol (C). *O*<sup>6</sup>-Benzylguanine 8-oxidation in the vehicle-treated control group was 17.5 pmol/min/mg protein in human liver microsomes (E) and 396 pmol/min/mg protein in human liver cytosol (F).

**Fig. 7.** Differential role of AOX-1 and cytochrome P450 enzymes in (A) carbazeran 4-oxidation and (B) *O*<sup>6</sup>-benzylguanine 8-oxidation. This diagrammatic summary is based on the results of previous studies (Kaye et al., 1985; Roy et al., 1995) and the present study. AOX-1 appears to be a major catalyst of carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in human liver cytosol and S9 fraction. (\*) Cytochrome P450, mainly CYP1A2, catalyzes *O*<sup>6</sup>-benzylguanine 8-oxidation in liver microsomes, but with negligible contribution to *O*<sup>6</sup>-benzylguanine 8-oxidation in S9 fraction.

TABLE 1

Optimized mass spectrometric parameters for the analysis of 4-oxo-carbazeran, carbazeran, 8-oxo-O<sup>6</sup>-benzylguanine, O<sup>6</sup>-benzylguanine, and tolbutamide (internal standard) by UPLC-MS/MS.

Chemical	<i>m/z</i> transition ( <i>m/z</i> )	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell exit potential (V)	Dwell time (ms)	Curtain gas (psi)	Collision- activated dissociation gas (psi)	Ion spray voltage (V)	Ion source temperature (°C)	Ion source gas 1 (psi)	Ion source gas 2 (psi)
<i>Carbazeran 4-Oxidation Assay</i>												
4-Oxo-carbazeran	377.2→288.0	100	6.30	23.0	5.40	200	30	12	4000	550	50	50
Carbazeran	361.3→272.2	130	10.00	45.0	14.00	200	30	12	4000	550	50	50
Tolbutamide (internal standard)	271.1→91.1	67	6.00	45.0	6.50	200	30	12	4000	550	50	50

*O<sup>6</sup>-Benzylguanine 8-Oxidation Assay*

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8-Oxo-O <sup>6</sup> - benzylguanine	258.0→91.1	92.6	8.00	27.0	8.53	200	20	9	4000	400	40	40
O <sup>6</sup> - Benzylguanine	241.9→91.2	73.5	10.50	35.0	7.00	200	20	9	4000	400	40	40
Tolbutamide (internal standard)	271.1→172.1	76.9	8.19	17.0	5.50	200	20	9	4000	400	40	40

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TABLE 2

AOX-1 protein content in human liver cytosol, microsomes, and S9 fraction, as quantified in a capillary nano-proteomic immunoassay (SimpleWestern System, ProteinSimple). Data are expressed as mean  $\pm$  S.D. of three independent experiments.

Sample	AOX-1 Protein Content	
	(pmol/mg total protein) <sup>a</sup>	(percentage relative to cytosol)
Human Liver Cytosol	63.8 $\pm$ 7.8	100%
Human Liver Microsomes, Pool 1	12.3 $\pm$ 1.4	19%
Human Liver Microsomes, Pool 2	13.0 $\pm$ 1.4	20%
Human Liver Microsomes, Pool 3	23.1 $\pm$ 1.5	36%
Human Liver Microsomes, Pool 4	15.1 $\pm$ 1.1	24%
Human Liver Microsomes, Pool 5	16.1 $\pm$ 1.2	25%
Human Liver S9 Fraction	53.3 $\pm$ 3.5	83%

<sup>a</sup>,  $\mu$ g/mg total protein was converted to pmol/mg total protein, based on the molecular weight of 147,918 daltons for AOX-1 (UniProt Knowledgebase, Human AOX1, Entry #Q06278, <https://www.uniprot.org/uniprot/Q06278>)

TABLE 3

Enzyme kinetic analysis of carbazeran 4-oxidation and *O*<sup>6</sup>-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes, cytosol, or S9 fraction.  $V_{\max}$ , apparent  $K_m$ , fraction unbound ( $f_u$ ), corrected  $K_m$ , and unbound intrinsic clearance ( $Cl_{\text{int,u}}$ ) were calculated as described under Materials and Methods. Data are expressed as mean  $\pm$  S.E.M. of three independent experiments conducted in duplicate or triplicate.

Sample	$V_{\max}$ (pmol/min/mg protein)	$V_{\max}$ (pmol/min/pmol AOX-1)	Apparent $K_m$ ( $\mu\text{M}$ )	Fraction Unbound ( $f_u$ ) <sup>a</sup>	Corrected $K_m$ ( $\mu\text{M}$ )	$Cl_{\text{int,u}}$ ( $\mu\text{l/ min/mg}$ protein)	$Cl_{\text{int,u}}$ ( $\mu\text{l/ min/pmol}$ AOX-1)
<i>Carbazeran 4-Oxidation</i>							
Human Liver Microsomes (without NADPH) <sup>b</sup>	187 $\pm$ 17	14.5 $\pm$ 1.3	7.2 $\pm$ 1.0	0.87 $\pm$ 0.01	6.3 $\pm$ 0.9	30 $\pm$ 1	2.32 $\pm$ 0.11
Human Liver Microsomes (with NADPH) <sup>b</sup>	179 $\pm$ 14	13.8 $\pm$ 1.0	7.1 $\pm$ 0.5	0.87 $\pm$ 0.01	6.2 $\pm$ 0.4	29 $\pm$ 1	2.21 $\pm$ 0.04
Human Liver Cytosol	1290 $\pm$ 138	20.2 $\pm$ 2.2	6.3 $\pm$ 0.7	0.94 $\pm$ 0.02	5.9 $\pm$ 0.6	217 $\pm$ 5	3.41 $\pm$ 0.08

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Human Liver S9 Fraction (without NADPH)	671 ± 83	12.6 ± 1.6	4.9 ± 0.3	0.96 ± 0.01	4.7 ± 0.3	144 ± 19	2.61 ± 0.34
Human Liver S9 Fraction (with NADPH)	682 ± 83	12.8 ± 1.6	4.9 ± 0.3	0.96 ± 0.01	4.7 ± 0.3	144 ± 10	2.61 ± 0.17
<i>O<sup>6</sup>-Benzylguanine 8-Oxidation</i>							
Human Liver Microsomes (without NADPH) <sup>b</sup>	N/A <sup>c</sup>	N/A <sup>c</sup>	N/A <sup>c</sup>	0.99 ± 0.03	N/A <sup>c</sup>	N/A <sup>c</sup>	N/A <sup>c</sup>
Human Liver Microsomes (with NADPH) <sup>b</sup>	94 ± 11	7.2 ± 0.9	43 ± 4	0.99 ± 0.03	42 ± 4	2.3 ± 0.3	0.17 ± 0.03
Human Liver Cytosol	1254 ± 102	19.7 ± 1.6	70 ± 8	1.01 ± 0.02	71 ± 8	18 ± 2	0.28 ± 0.03
Human Liver S9 Fraction (without NADPH)	445 ± 10	8.35 ± 0.19	94 ± 3	0.96 ± 0.01	90 ± 3	4.9 ± 0.2	0.09 ± 0.003
Human Liver S9 Fraction (with NADPH)	454 ± 5	8.52 ± 0.09	85 ± 9	0.96 ± 0.01	81 ± 8	5.7 ± 0.6	0.10 ± 0.01

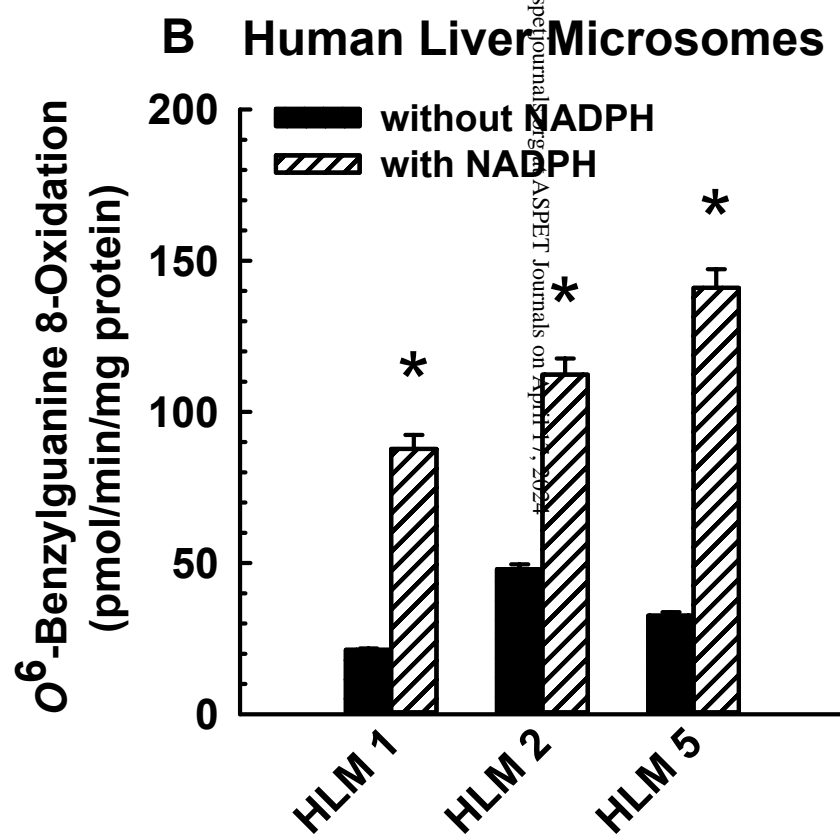
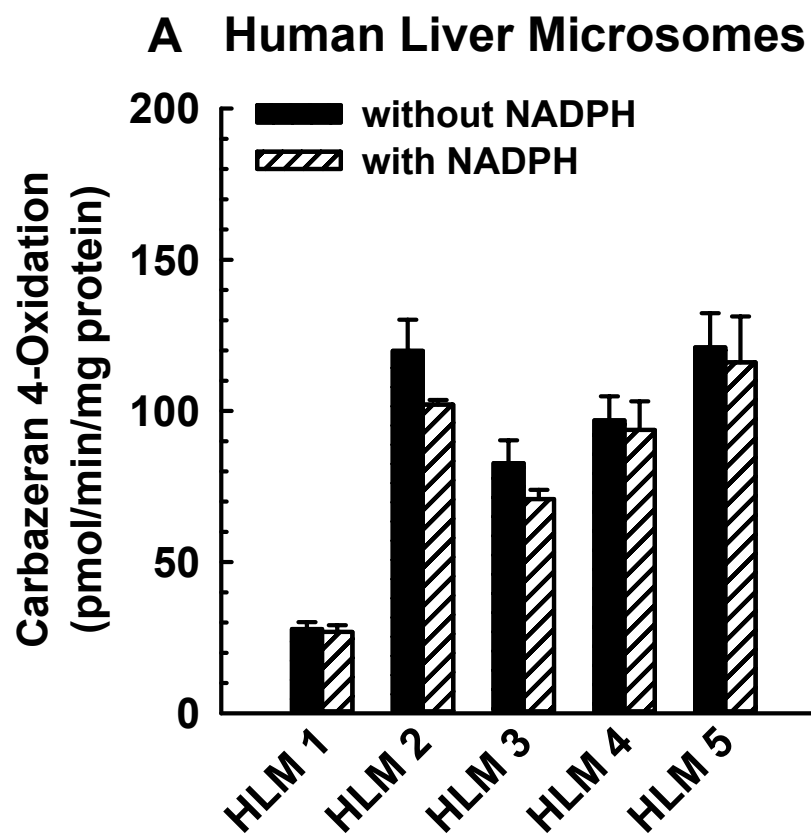


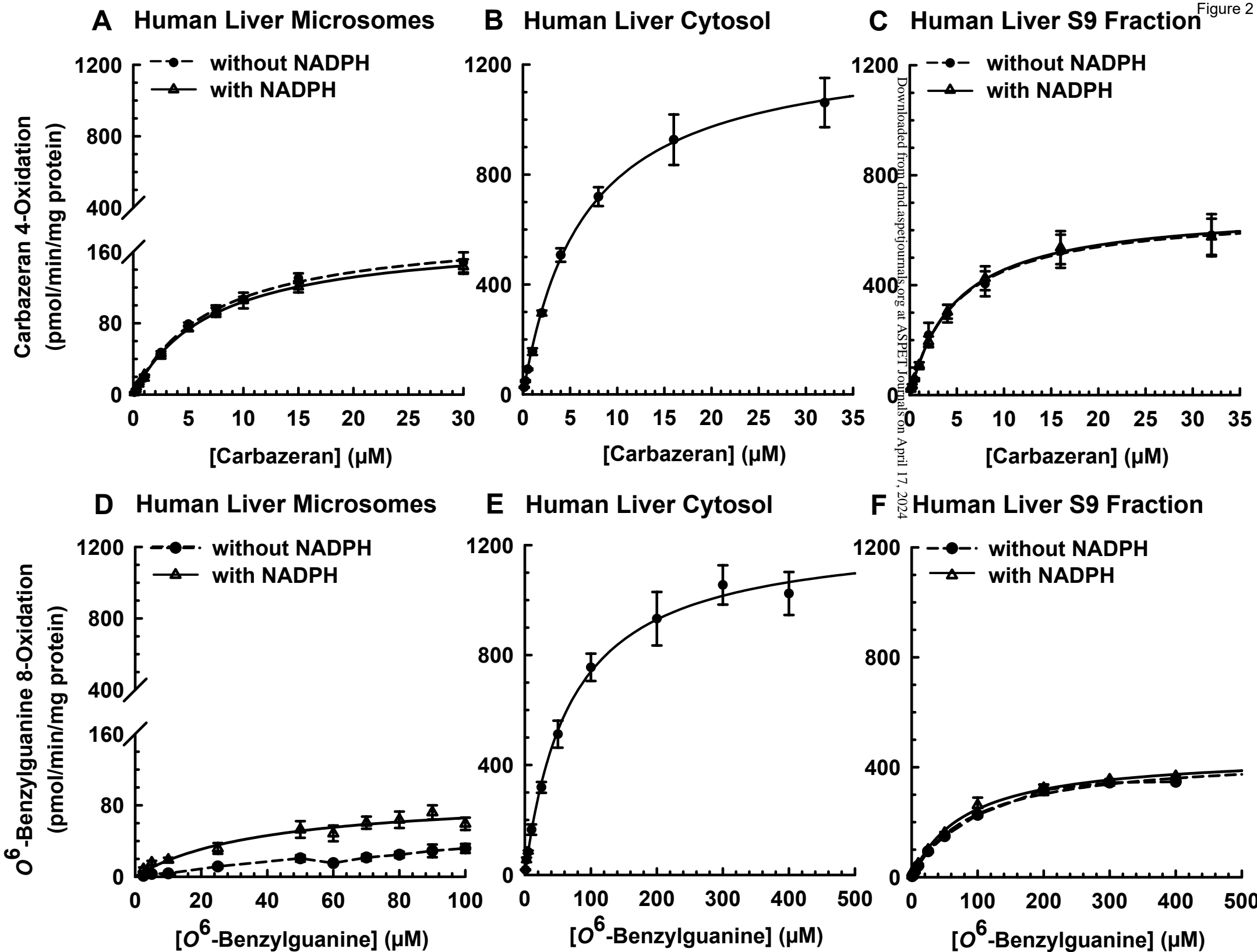
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<sup>a</sup>,  $f_u$  from 10  $\mu\text{M}$  carbazeran and 200  $\mu\text{M}$  *O*<sup>6</sup>-benzylguanine in the respective enzymes (Supplemental Table S3)

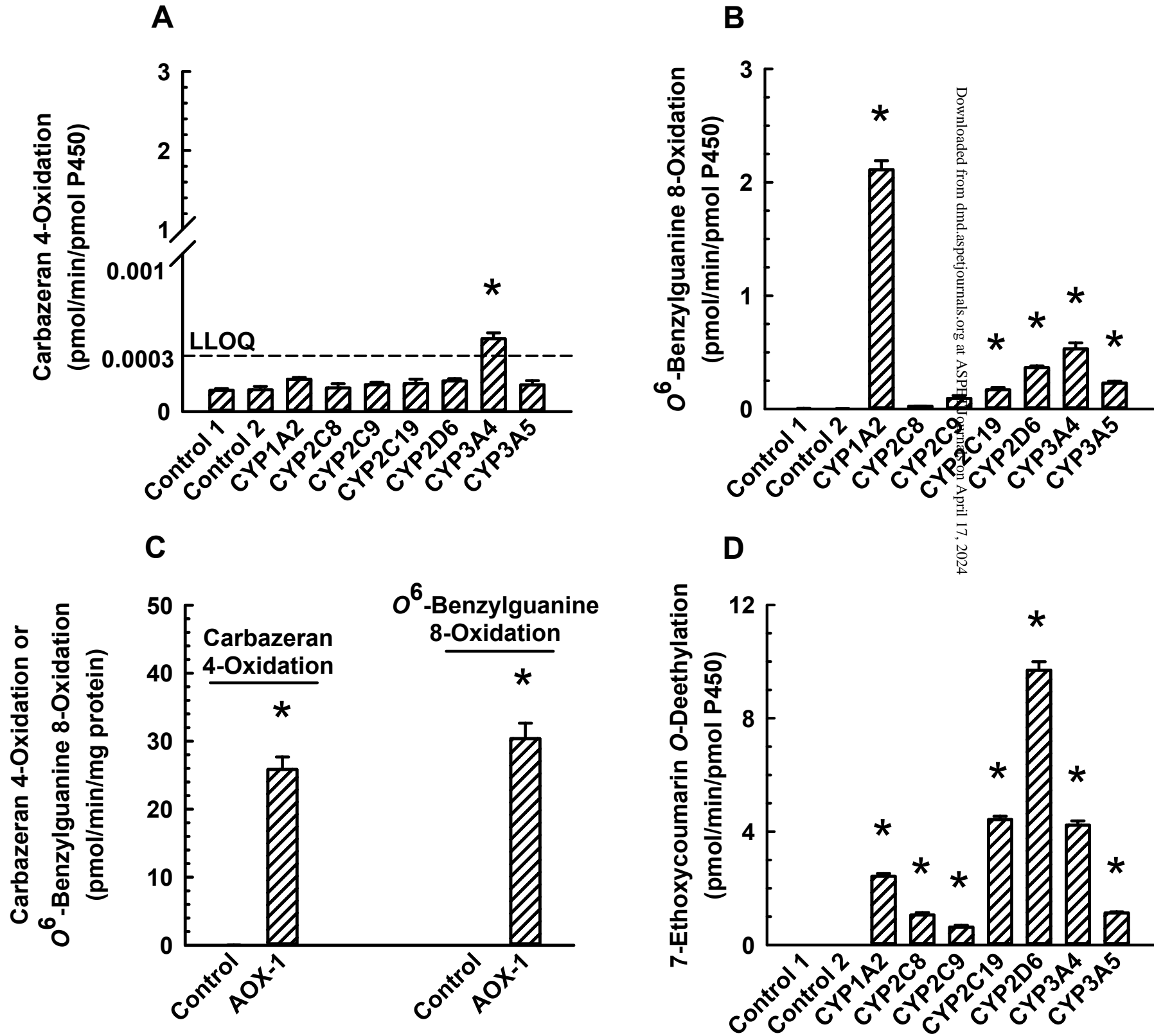
<sup>b</sup>, human liver microsomes from Pool #2

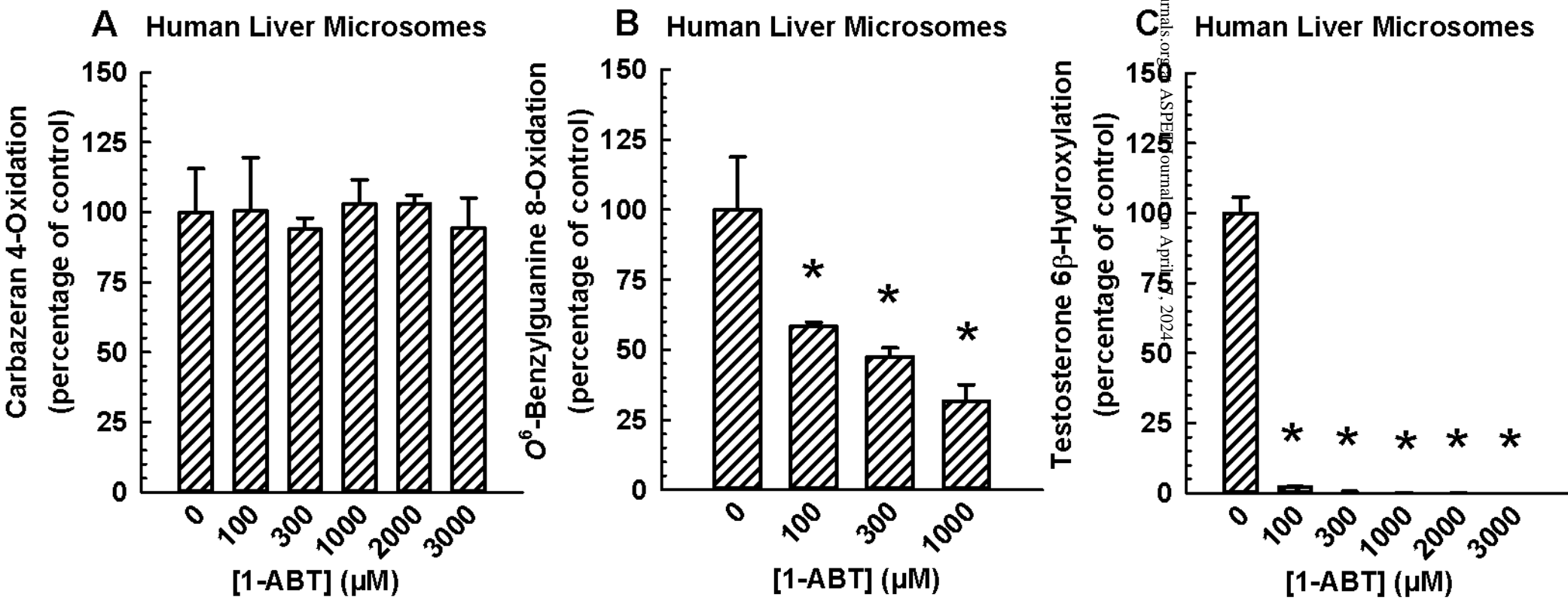
<sup>c</sup>, the  $V$  versus  $[S]$  plot did not reach plateau and kinetic values could not be determined. Due to solubility limit, the maximum substrate concentration was 100  $\mu\text{M}$ .

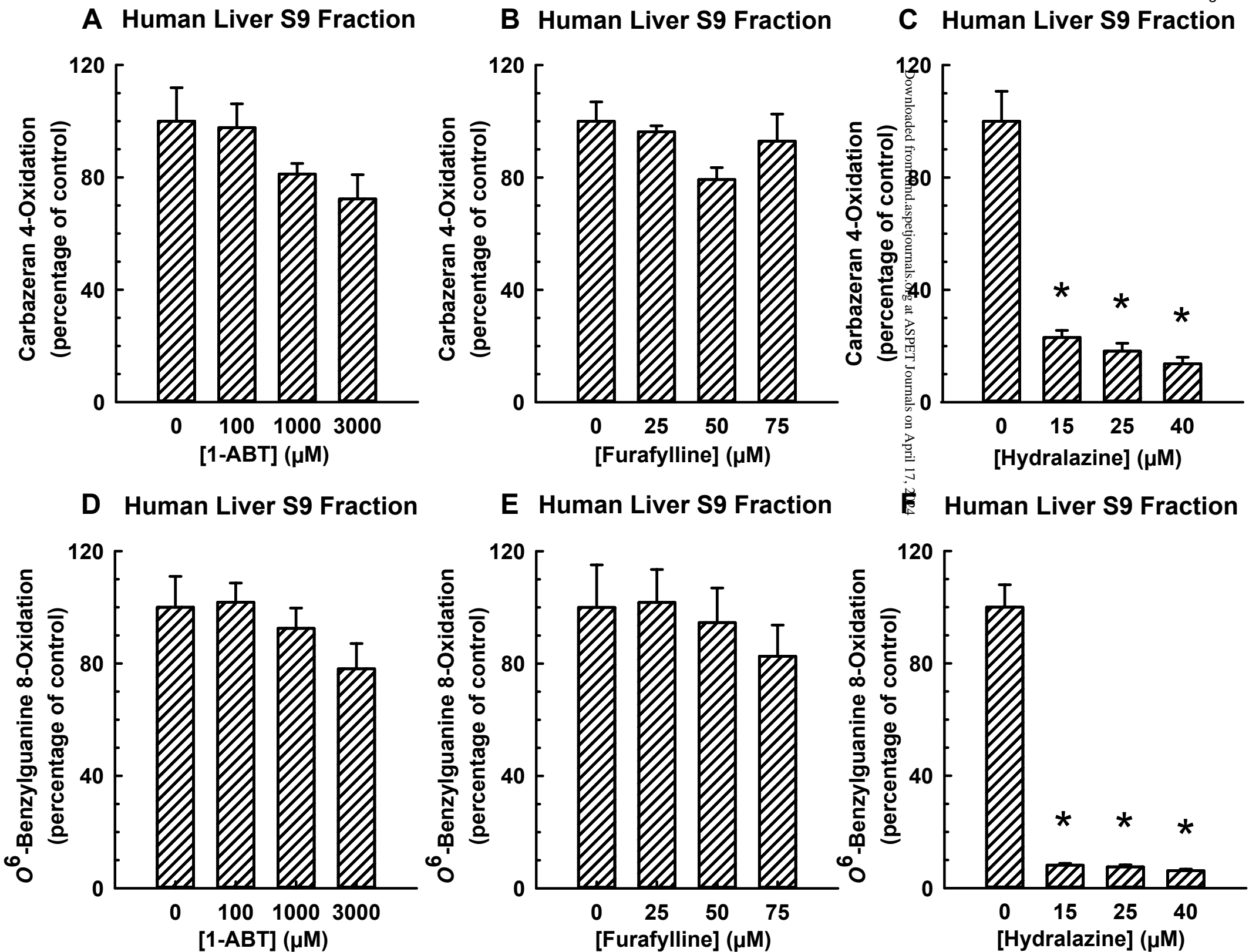


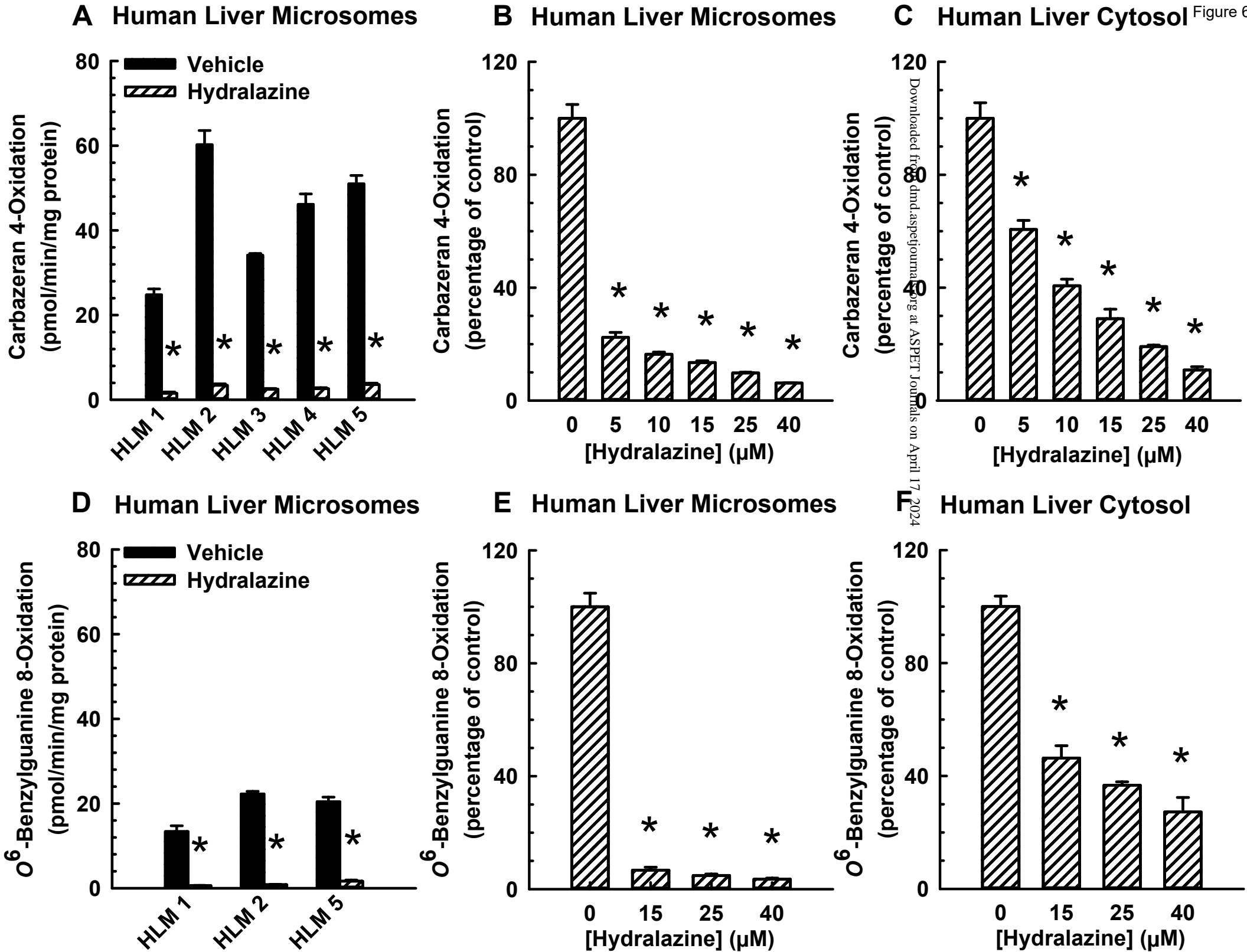


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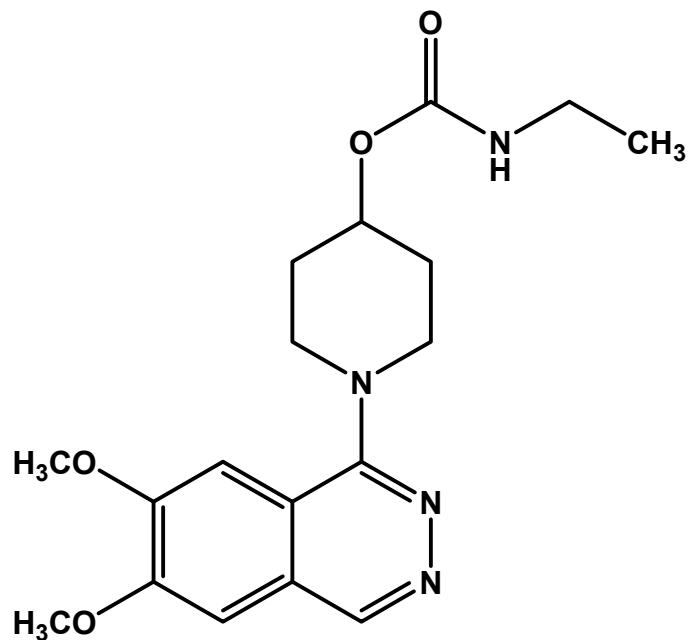






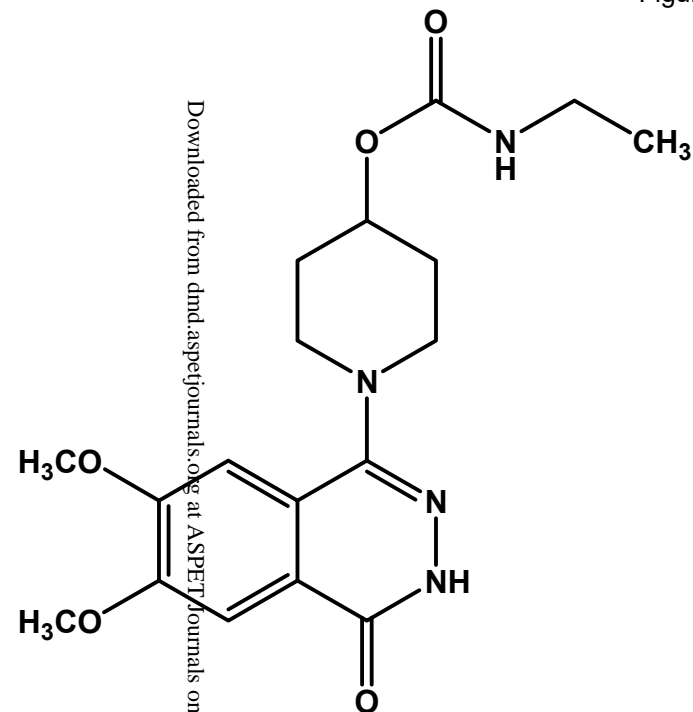


A



Carbazeran

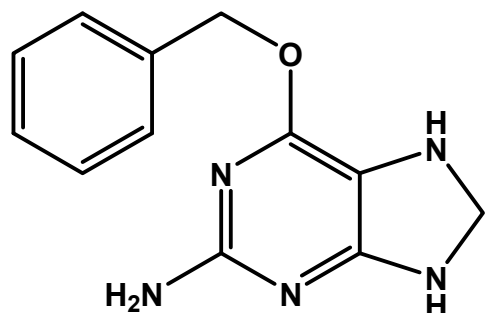
Catalyzed by  
AOX-1  
→  
Not catalyzed by  
cytochrome P450



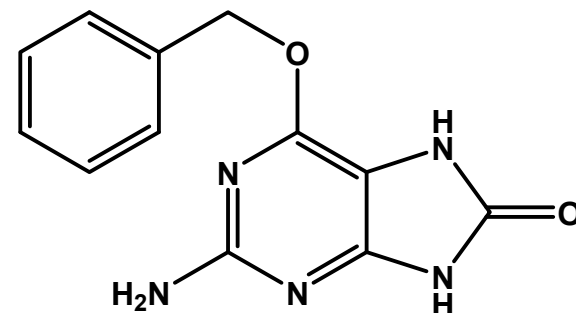
4-Oxo-Carbazeran

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B

O<sup>6</sup>-Benzylguanine

Catalyzed by  
AOX-1 and  
cytochrome P450 (mainly CYP1A2)\*  
→

8-Oxo-O<sup>6</sup>-Benzylguanine