Evaluation of Carbazeran 4-Oxidation and O⁶-Benzylguanine 8-Oxidation as Catalytic Markers of Human Aldehyde Oxidase: Impact of Cytosolic Contamination of Liver Microsomes

Jiarong Xie, Nur Fazilah Saburulla, Shiyan Chen, Siew Ying Wong, Ze Ping Yap, Linghua Harris Zhang, and Aik Jiang Lau

ABSTRACT

The present study investigated the contribution of microsomal cytochrome P450 and cytosolic aldehyde oxidase-1 (AOX-1) to carbazeran 4-oxidation and O⁶-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. O⁶-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 O⁶-benzylguanine 8-oxidation. 1-Aminobenzotriazole, a pan-cytochrome P450 inhibitor, decreased O⁶-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 O⁶-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 to 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 O⁶-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.

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 et al., 1997). 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, 1985) and O⁶-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 occurred within a single pool of human liver microsomes (Wilkinson et al., 2017). O⁶-Benzylguanine 8-oxidation is also used as a catalytic marker of AOX-1 (Zientek et al., 2010; Hutzler et al., 2012; Fu et al., 2013). However, experiments with a panel of individual human cytochrome P450 enzymes expressed in HepG2 cells and human liver microsomal experiments with cytochrome P450
enzymatic drug-metabolizing enzymes such as AOX-1 and cytochrome-selective catalytic markers because they will facilitate functional studies assessed in various in vitro human liver drug metabolism models. Guanine 8-oxidation as enzyme-selective catalytic markers of AOX-1, as an issue of cytosolic contamination of isolated microsomes in a reaction microsomal cytochrome P450 and cytosolic AOX-1. Selective catalytic marker for AOX-1 in tissues or cells expressing both oxidative drug-metabolizing enzymes such as AOX-1 and cytochrome P450. The results of our detailed study indicate that carbanzcer 4-oxidation and O6-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.

Materials and Methods

Chemicals and Reagents. Carbanzcer, 4-oxo-carbanzcer, O6-benzylguanine, and 8-oxo-O6-benzylguanine were purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Tolbutamide, hydralazine, 1-aminobenzotriazole, β-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Corp. (St. Louis, MO). Dehydroepiandrosterone hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Tolbutamide, hydralazine, 1-aminobenzotriazole, β-nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Corp. (St. Louis, MO). Dehydroepiandrosterone hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Corp. (St. Louis, MO). Dehydroepiandrosterone hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Corp. (St. Louis, MO). Dehydroepiandrosterone hydrate (NADPH), cortisol (hydrocortisone), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich Corp. (St. Louis, MO).

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(MMRM). The MS source parameters, mass-to-charge (m/z) transitions, and other compound-dependent MS parameters for each analyte and internal standard are listed in Table 1. The amount of metabolite was quantified using MultiQuant 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/x²) linear least-squares regression was performed to obtain the best-fit line.

**Rapid Equilibrium Dialysis.** Nonspecific binding of carbazeran and O⁶-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 μL) was added to the buffer chamber of the Rapid Equilibrium Dialysis plate. A mixture (total volume of 200 μL) containing potassium phosphate buffer (100 mM, pH 7.4), a drug (2 or 10 μM carbazeran in a final concentration of 0.5% v/v DMSO or 50 or 200 μM O⁶-benzylguanine in a final concentration of 0.5% v/v DMSO), and a human liver tissue fraction (100 μg total microsomal protein for carbazeran and 60 μg total microsomal protein for O⁶-benzylguanine; 20 μg total cytosolic protein; or 60 μg 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 hours with a rotating speed of 300 rpm. At the end of the incubation period, a 25-μL aliquot of the mixture from each of the buffer and the sample chamber was mixed with 475 μL of ice-cold acetone/ethyl acetate containing 50 mM tolbutamide (internal standard; final concentration of 47.5 mM) (Burns et al., 1980). Corrected unbound intrinsic clearance (Clint,α) of the velocity of metabolite formation (V) 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 (Simple Western system; ProteinSimple/BioTechnne, San Jose, CA) (Chen et al., 2013). Standards were prepared from recombinant AOX-1 protein (OriGene Technologies). Each diluted standard and test sample (at 0.01-10 μM) were mixed with 5 μL of 4 M reducing buffer with fluorescent molecular weight standards. After these samples were heated to 95°C for 5 minutes, 5 μL samples were loaded into each capillary tube. Proteins were passed and separated through stacking and separation matrices for 30 minutes at 250 V. Proteins were then immobilized to capillary walls using optimal photoactivated capture chemistry. Following protein immobilization, capillaries were flushed with a wash buffer and incubated with a blocking reagent for 23 minutes. AOX-1 was probed with an anti-AOX-1 primary antibody (diluted in blocking buffer and incubated for 200 minutes) 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 3.17 (ProteinSimple).

**Enzyme Kinetic Analysis.** Carbazeran 4-oxidation and O⁶-benzylguanine 8-oxidation assays were conducted using varying concentrations of carbazeran (0.125–32 μM for human liver cytosol, 0.1–30 μM for microsomes, and 0.125–32 μM for S9 fraction) and O⁶-benzylguanine (1–400 μM for cytosol, 2.5–100 μM for microsomes, and 1–400 μM for S9 fraction), respectively. The values of maximum velocity (Vmax) and apparent Michaelis-Menten constant (Km) were determined by nonlinear least-squares regression analysis (SigmaPlot 12.5; Systat Software Inc., San Jose, CA) 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, R², and visual inspection of the substrate-velocity data. On the basis of the above evaluation criteria, the data for carbazeran 4-oxidation and O⁶-benzylguanine 8-oxidation were fitted using the Michaelis-Menten model:

\[
V = \frac{V_{\text{max}} \times S}{K_m + S}
\]

where S represents the substrate concentration, Vmax represents the maximum velocity of the reaction, Km represents the substrate concentration at which the reaction rate is half of Vmax, Turnover number (kcat) was calculated by dividing Vmax by AOX-1 protein concentration (Hollaway et al., 1980). Corrected Km was calculated by multiplying the unbound fraction (fα) by the apparent Km (Obach, 1997). Unbound intrinsic clearance (Clint,α) was calculated by dividing Vmax by the corrected Km (Giuliano et al., 2005).

**Enzyme Inhibition Experiments.** Enzyme inhibition was determined by conducting the carbazeran 4-oxidation and O⁶-benzylguanine 8-oxidation assays in the presence of a known inhibitor. AOX-1 inhibition experiments were performed with hydralazine (5–40 μM), a selective inhibitor of AOX-1 (Johnson et al., 1983; Strelevitz et al., 2012). Cytochrome P450 inhibition experiments were conducted with 1-aminobenzotriazole (100–3000 μM), an inhibitor of multiple cytochrome P450 enzymes (Linder et al., 2009; Zientek and Youdim, 2015), or with furafylline (25–75 μM), a CYP1A2-selective inhibitor (Seresic et al., 1990; Kunze and Trager, 1993). For incubations containing 1-ABT or furafylline, human liver microsomes and S9 fractions were preincubated at 37°C in the presence of the inactivator and NADPH for 30 minutes prior to the addition of substrate.

### Table 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>m/z Transition</th>
<th>Untreated Potential</th>
<th>Entreated Potential</th>
<th>Collision Energy</th>
<th>Collision Cell Potential</th>
<th>Dwell Time</th>
<th>Curtain Gas</th>
<th>Collision-Activated Dissociation Gas</th>
<th>Ion Spray Voltage</th>
<th>Ion Source Temperature</th>
<th>Ion Source Gas 1</th>
<th>Ion Source Gas 2</th>
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<tbody>
<tr>
<td>Carbazeran 4-oxidation assay</td>
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<tr>
<td>4-Oxo-carbazeran</td>
<td>377.2→288.0</td>
<td>100</td>
<td>6.30</td>
<td>23.0</td>
<td>5.40</td>
<td>200</td>
<td>30</td>
<td>12</td>
<td>4000</td>
<td>550</td>
<td>50</td>
<td>50</td>
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<tr>
<td>Carbazeran</td>
<td>361.3→272.2</td>
<td>130</td>
<td>10.00</td>
<td>45.0</td>
<td>14.00</td>
<td>200</td>
<td>30</td>
<td>12</td>
<td>4000</td>
<td>550</td>
<td>50</td>
<td>50</td>
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<tr>
<td>Tolbutamide</td>
<td>271.1→911.1</td>
<td>67</td>
<td>6.00</td>
<td>45.0</td>
<td>6.50</td>
<td>200</td>
<td>30</td>
<td>12</td>
<td>4000</td>
<td>550</td>
<td>50</td>
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<td>O⁶-Benzylguanine 8-oxidation assay</td>
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<tr>
<td>8-OxooO⁶-Benzylguanine</td>
<td>258.0→911.1</td>
<td>92.6</td>
<td>8.00</td>
<td>27.0</td>
<td>8.53</td>
<td>200</td>
<td>20</td>
<td>9</td>
<td>4000</td>
<td>40</td>
<td>40</td>
<td>40</td>
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<tr>
<td>O⁶-Benzylguanine</td>
<td>240.1→912.1</td>
<td>73.5</td>
<td>10.50</td>
<td>35.0</td>
<td>7.00</td>
<td>200</td>
<td>20</td>
<td>9</td>
<td>4000</td>
<td>40</td>
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<td>40</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>271.1→172.1</td>
<td>76.9</td>
<td>8.19</td>
<td>17.0</td>
<td>5.50</td>
<td>200</td>
<td>20</td>
<td>9</td>
<td>4000</td>
<td>40</td>
<td>40</td>
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</table>
Testosterone 6β-Hydroxylation Assay. The testosterone 6β-hydroxylation assay was conducted as described previously (Foo et al., 2015). Briefly, each standard 200-μl incubation mixture contained potassium phosphate buffer (100 mM, pH 7.4), NADPH (1 mM), testosterone (40 μM), and human liver microsomes (60 μg protein). Each incubation mixture was prewarmed for 3 minutes at 37°C and the enzyme reaction was initiated by adding NADPH. After a 10-minute incubation period, the reaction was terminated by adding 200 μl of ice-cold acetonitrile containing prednisolone (1 μM final concentration; internal standard). Each sample was processed for analysis of 6β-hydroxytestosterone and prednisolone by UPLC-MS-MS (Foo et al., 2015).

7-Ethoxycoumarin O-Deethylation Assay. The 7-ethoxycoumarin O-deethylation assay was adopted from a published method (Waxman and Chang, 2006). Each 200-μl incubation mixture contained potassium phosphate buffer (100 mM, pH 7.4), 7-ethoxycoumarin (500 μM), 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 had been reported not to affect the catalytic activity of the cytochrome P450 enzymes listed above (Chauvet et al., 1998). In the negative control groups, the recombinant enzyme was replaced with an equivalent protein amount (30 μg) of insect cell microsomes or insect cell microsomes with oxidoreductase and cytochrome b5. Calibration standards were prepared by addition of a 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 prewarmed for 3 minutes at 37°C in a low-speed shaking water bath. The enzymatic reaction was initiated by addition of NADPH, and the mixture was incubated for 30 minutes. The reaction was terminated with ice-cold hydrochloric acid (2 M; 25 μl). The enzymatic product was extracted with chloroform (450 μl) and centrifuged (3000g for 5 minutes). 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 sulfonation assay was conducted as described previously (Yip et al., 2018). In brief, each incubation mixture (200 μl) contained potassium phosphate buffer (100 mM, pH 7.4), magnesium chloride (2.5 mM), DHEA (1 μM), and various amounts of microsomes (0, 100, 200, or 300 μg protein). The enzyme reaction was initiated with 3′-phosphoadenosine 5′-phosphosulfate (5 μM), and the mixture was incubated at 37°C for 30 minutes. The reaction was terminated by addition of 200 μl of ice-cold acetonitrile containing cortisol (0.05 μM 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, 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.

Results

Development and Validation of UPLC-MS/MS Methods for Quantification of 4-Oxo-Carbazeran and 8-Oxo-6-Benzylgua nine. UPLC-MS/MS methods were developed for the quantification of 4-oxo-carbazeran and 8-oxo-6-benzylgua nine, which are formed by carbazeran 4-oxidation and Oβ-benzylgua nine 8-oxidation, respectively. As in a previous report (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 minutes (Supplemental Fig. S1B). In the UPLC-MS/MS method for detecting 8-oxo-Oβ-benzylgua nine, the m/z transition of 258.0 to 91.1 was selected (Supplemental Fig. S1C), which was consistent with the literature (Barr et al., 2015; Sodhi et al., 2015), and a single peak was obtained at a retention time of 2.02 minutes (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 0.2–200 pmol of 8-oxo-6-benzylgua nine. Using the criteria of signal-to-noise ratio of >5:1, accuracy of ±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-6-benzylgua nine was 0.2 pmol or 0.5 nM (in the entire 400-μl incubation mixture, of which

<table>
<thead>
<tr>
<th>Sample</th>
<th>AOX-1 Protein Content</th>
<th>pmol/mg of total protein*</th>
<th>percentage relative to cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver cytosol</td>
<td>63.8 ± 7.8</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Human liver microsomes, Pool 1</td>
<td>12.5 ± 1.4</td>
<td>19%</td>
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</tr>
<tr>
<td>Human liver microsomes, Pool 2</td>
<td>13.0 ± 1.4</td>
<td>20%</td>
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</tr>
<tr>
<td>Human liver microsomes, Pool 3</td>
<td>23.1 ± 1.5</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>Human liver microsomes, Pool 4</td>
<td>15.1 ± 1.1</td>
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<tr>
<td>Human liver microsomes, Pool 5</td>
<td>16.1 ± 1.2</td>
<td>25%</td>
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<tr>
<td>Human liver S9 fraction</td>
<td>53.3 ± 3.5</td>
<td>83%</td>
<td></td>
</tr>
</tbody>
</table>

*Micrograms per milligram of total protein was converted to picomoles per milligram of total protein, on the basis of molecular weight of 147,918 Da for AOX-1 (UniProt Knowledgebase, Human AOX1, entry no. Q06278, https://www.uniprot.org/uniprot/Q06278).
5 μl was injected into the UPLC-MS/MS). When quality control samples were analyzed at low (0.4 pmol), mid (4 pmol), and high (40 pmol) amounts, the intraday and interday accuracy and precision were <10.3% for 4-oxo-carbazeran and <12.7% for 8-oxo-O⁶-benzylguanine (Supplemental Table S1). UPLC-MS/MS methods for quantifying the substrates (carbazeran and O⁶-benzylguanine) and internal standard (tolbutamide) were also developed and the conditions optimized (Table 1).

Optimization of Enzymatic Assay Conditions for Carbazeran 4-Oxidation and O⁶-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 micromosal protein (Supplemental Fig. S2A) and 5 minutes incubation time (Supplemental Fig. S2B). 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 minutes 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 minutes 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 minutes) and amount of protein (100, 20, 60, 200, 300, or 400 μg) of O⁶-benzylguanine at 37°C for 5 minutes. (D) Microsomes (60 μg protein) were incubated with various concentrations of O⁶-benzylguanine (2.5, 5, 10, 25, 50, 100, 200, 300, or 400 μM) at 37°C for 5 minutes. (E) Cytosol (20 μg protein) was incubated with various concentrations (1, 2.5, 5, 10, 25, 50, 100, 200, 300, or 400 μM) of O⁶-benzylguanine at 37°C for 5 minutes. (F) S9 fraction (60 μg protein) was incubated with O⁶-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 minutes. Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate or triplicate.
using recombinant AOX-1 protein, and the chemiluminescent signal (also known as capillary electrophoresis immunoassay system) (Chen preparations, we conducted a capillary nano-proteomic immunoassay Liver Cytosol, Microsomes, and S9 Fraction. absence of NADPH, suggesting the presence of AOX-1 in those samples. benzylguanine 8-oxidation were also detected in microsomes in the benzylguanine 8-oxidation assay was # factor in the assay. Under these incubation conditions, substrate depletion in the containing microsomes, cytosol, and S9 fraction, respectively). 8-Oxidation in Incubations Containing Human Liver Microsomes: 4-oxidation (Fig. 1A). In contrast, it consistently increased the level of (Fig. 1A) and 1.6-fold (Fig. 1B), respectively. The inclusion of NADPH among the different pools of microsomes varied by approximately 4-fold NADPH), it was linear up to 150 μg of protein (Supplemental Fig. S3). In all subsequent experiments, the O6-benzylguanine 8-oxidation assay was conducted under linear conditions with respect to incubation time (15, 5, and 10 minutes in enzymatic incubations containing microsomes, cytosol, and S9 fraction, respectively) and amount of protein (60, 20, and 60 μg protein in enzymatic incubations containing microsomes, cytosol, and S9 fraction, respectively). Under these incubation conditions, substrate depletion in the O6-benzylguanine 8-oxidation assay was ≤8% (Supplemental Table S2), indicating that substrate concentration was not a rate-limiting factor in the assay.

Detection of Carbazeneran 4-Oxidation and O6-Benzylguanine 8-Oxidation in Incubations Containing Human Liver Microsomes: Differential Effect of Exogenous NADPH. Carbazeneran 4-oxidation (Fig. 1A) and O6-benzylguanine 8-oxidation (Fig. 1B) were detected in enzymatic incubations containing human liver microsomes. The extent of 4-oxo-carbazeneran formation and 8-oxo-O6-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 carbazeneran 4-oxidation (Fig. 1A). In contrast, it consistently increased the level of O6-benzylguanine 8-oxidation in each of the microsomal preparations (Fig. 1B), suggesting NADPH-dependent microsomal metabolism of O6-benzylguanine. Interestingly, carbazeneran 4-oxidation and O6-benzylguanine 8-oxidation were also detected in microsomes in the absence of NADPH, 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 the chemiluminescent signal increased with increasing concentration of AOX-1 protein standard, thereby enabling 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 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 Carbazeneran 4-Oxidation and O6-Benzylguanine 8-Oxidation. Carbazeneran 4-oxidation and O6-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes (Fig. 2, A and D), cytosol (Fig. 2, B and E), and S9 fraction (Fig. 2, C and F) 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. 2, C and F) increased with increasing substrate concentration, and the data were best fitted by the Michaelis-Menten model. The addition of NADPH to microsomal incubations increased the extent of nonspecific binding of carbazeran and 6-benzylguanine (Fig. 2A) or S9 (Fig. 2D) did not modify the product formation-versus-substrate concentration curves in the carbazeneran 4-oxidation assay, consistent with the lack of an effect on the values of the various kinetic parameters, such as $V_{\text{max}}$, turnover number ($k_{\text{cat}}$, apparent $K_{\text{m}}$ (Supplemental Table S4; Table 3). By comparison, the addition of NADPH to microsomal incubations increased O6-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 carazeneran and O6-benzylguanine to human liver microsomes, cytosol, and S9 fraction. As shown in Supplemental Table S3, the fraction unbound ($I_0$) for each of these chemicals was comparable in each isolated fraction, as determined after a dialysis time of 4 hours, when there was still a substantive concentration of the substrate (which was far above the LLOQ of 0.5 nM for 4-oxo-carbazeneran and 8-oxo-O6-benzylguanine). A kinetic comparison with unbound drug as a basis showed comparable values of corrected $K_{\text{m}}$ and unbound intrinsic clearance (Clint_u, when normalized to the amount of AOX-1) for carazeneran 4-oxidation in incubations containing human liver microsomes, cytosol, and S9 fraction.

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$V_{\text{max}}$</th>
<th>$V_{\text{max}}$</th>
<th>Apparent $K_{\text{m}}$</th>
<th>Fraction Unbound$^c$</th>
<th>Corrected $K_{\text{m}}$</th>
<th>Clint_u</th>
<th>Clint_u</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbazeneran 4-oxidation</td>
<td></td>
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<tr>
<td>Human liver microsomes (without NADPH)$^a$</td>
<td>187 ± 17</td>
<td>14.5 ± 1.3</td>
<td>7.2 ± 1.0</td>
<td>0.87 ± 0.01</td>
<td>6.3 ± 0.9</td>
<td>30 ± 1</td>
<td>2.32 ± 0.11</td>
</tr>
<tr>
<td>Human liver microsomes (with NADPH)$^b$</td>
<td>179 ± 14</td>
<td>13.8 ± 1.0</td>
<td>7.1 ± 0.5</td>
<td>0.87 ± 0.01</td>
<td>6.2 ± 0.4</td>
<td>29 ± 1</td>
<td>2.21 ± 0.04</td>
</tr>
<tr>
<td>Human liver cytosol</td>
<td>1290 ± 138</td>
<td>20.2 ± 2.2</td>
<td>6.3 ± 0.7</td>
<td>0.94 ± 0.02</td>
<td>5.9 ± 0.6</td>
<td>217 ± 5</td>
<td>3.41 ± 0.08</td>
</tr>
<tr>
<td>Human liver S9 fraction (without NADPH)</td>
<td>671 ± 83</td>
<td>12.6 ± 1.6</td>
<td>4.9 ± 0.3</td>
<td>0.96 ± 0.01</td>
<td>4.7 ± 0.3</td>
<td>144 ± 19</td>
<td>2.61 ± 0.34</td>
</tr>
<tr>
<td>Human liver S9 fraction (with NADPH)</td>
<td>682 ± 83</td>
<td>12.8 ± 1.6</td>
<td>4.9 ± 0.3</td>
<td>0.96 ± 0.01</td>
<td>4.7 ± 0.3</td>
<td>144 ± 10</td>
<td>2.61 ± 0.17</td>
</tr>
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</table>

$^a$I0 from 10 μM carbazeran and 200 μM O6-benzylguanine in the respective enzymes (Supplemental Table S3).

$^b$Human liver microsomes from Pool 2.

$^c$The V vs. [S] plot did not reach plateau and kinetic values could not be determined. Owing to solubility limit, the maximum substrate concentration was 100 μM.
fraction (Table 3). The same trend was obtained for O₆-benzylguanine 8-oxidation (Table 3).

**Differential Catalysis of Carbazeran 4-Oxidation and O₆-Benzylguanine 8-Oxidation by Human Recombinant Cytochrome P450 Enzymes.** As shown in Fig. 1A and Fig. 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 cofactor. 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 O₆-benzylguanine 8-oxidation by the recombinant enzymes because 8-oxo-O₆-benzylguanine was detected in liver microsomal incubations (Fig. 1B; Fig. 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, O₆-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 O₆-benzylguanine 8-oxidation (Fig. 3C). Multiple human recombinant cytochrome P450 enzymes are active catalysts of 7-ethoxycoumarin O-deethylation (Waxman et al., 1991). Therefore, the 7-ethoxycoumarin 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. 3, A and B) was indeed catalytically active. As expected, the insect control cell microsomes or *E. coli* control cytosol did not yield any metabolite in enzymatic incubations containing a cytochrome P450 (Fig. 3, A, B, and D) or AOX-1 (Fig. 3C).

**Relative Contribution of Cytochrome P450 and AOX-1 Enzymes to Carbazeran 4-Oxidation and O₆-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 $O^6$-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 cytochrome P450 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. 5, A and B) or $O^6$-benzylguanine 8-oxidation (Fig. 5, D and E), whereas hydralazine, an enzyme-selective chemical inhibitor of AOX-1 (Johnson et al., 1985; Kunze and Trager, 1993), did not affect carbazeran 4-oxidation (Fig. 5, A and B) or $O^6$-benzylguanine 8-oxidation (Fig. 5, D and E) and hydralazine, an enzyme-selective chemical inhibitor of AOX-1. Varying concentrations of 1-ABT, an inactivator of multiple cytochrome P450 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. 5, A and B) or $O^6$-benzylguanine 8-oxidation (Fig. 5, D and E), 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 $O^6$-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 $O^6$-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 $O^6$-Benzylguanine 8-Oxidation in Human Liver Microsomal Incubations. 4-Oxo-carbazeran (Fig. 1A; Fig. 2A) and 8-oxo-$O^6$-benzylguanine (Fig. 1B; Fig. 2D) were detected in incubations containing human liver 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 (Joseph et al., 1985; Strelevitz et al., 2012). Hydralazine (40 mM) almost completely decreased carbazeran 4-oxidation in five different pools of microsomes (Fig. 6A) and at various concentrations (5–40 μM) of hydralazine (Fig. 6B), as determined in microsomal incubations without the addition of exogenous NADPH. As shown with an optimized enzyme assay (Supplemental Fig. S3, A and D), hydralazine also decreased $O^6$-benzylguanine 8-oxidation in multiple pools of microsomes (Fig. 6D) and by multiple concentrations (15–40 μM) (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 $O^6$-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 sulfortransferases, namely, SULT2A1 (Falany et al., 1989), SULT2B1 (Melcõ 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.

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. The basis of this conclusion was 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 correction for the extent of protein binding (to yield corrected $K_m$) and normalization of $V_{max}$ and $C_{IAPP}$ 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 when differences in the substrate specificity of AOX-1 and cytochrome P450 are the basis (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 result of the catalysis of carbazeran glucuronidation by microsomal uridine 5'-diphospho-glucuronosyltransferase (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 for 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, O6-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 O6-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 O6-benzylguanine 8-oxidation in human S9 fraction. Overall, on the basis of the above findings and as summarized in Fig. 7, it is appropriate to use O6-benzylguanine 8-oxidation as a catalytic marker of AOX-1 in a functional study.

**Fig. 5.** Effect of cytochrome P450 enzyme inhibitors (1-ABT and furafylline) and an AOX-1 inhibitor (hydralazine) on carbazeran 4-oxidation and O6-benzylguanine 8-oxidation in enzymatic incubations containing human liver S9 fraction. (A–C) S9 fraction (60 µg protein) was incubated with carbazeran (5 µM) and 1-ABT (100, 1000, or 3000 µM) (A), hydralazine (15, 25, or 40 µM) (B), or DMSO (0.5% v/v; vehicle), and with 1 mM NADPH (A and B) or without NADPH (C) at 37°C for 5 minutes. (D–F) S9 fraction (60 µg protein) was incubated with O6-benzylguanine (50 µM) and 1-ABT (100, 1000, or 3000 µM) (D), furafylline (25, 50, or 75 µM) (E), or DMSO (0.5% v/v; vehicle), and with 1 mM NADPH (D and E) or without NADPH (F) at 37°C for 10 minutes. 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 and O6-benzylguanine 8-oxidation in the vehicle-treated control group were 115–141 pmol/min per milligram protein (A–C) and 96–144 pmol/min per milligram protein (D–F), respectively.
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 in the microsomal preparations was also pronounced, as illustrated by an apparent $V_{\text{max}}$ 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 the result of 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.

![Fig. 6. Effect of an AOX-1 inhibitor (hydralazine) on carbazeran 4-oxidation and O$_6$-benzylguanine 8-oxidation in enzymatic incubations containing human liver microsomes (without exogenous supplementation of NADPH) or cytosol.](image-url)
Fig. 7. Differential role of AOX-1 and cytochrome P450 enzymes in (A) carbazeran 4-oxidation and (B) O^6^-benzylguanine 8-oxidation. The basis of this diagrammatic summary is 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^6^-benzylguanine 8-oxidation in human liver cytosol and S9 fraction. Cytochrome P450, mainly CYP1A2, catalyzes O^6^-benzylguanine 8-oxidation in liver microsomes, but with negligible contribution to O^6^-benzylguanine 8-oxidation in S9 fraction.

In conclusion, it is appropriate to use 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 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) whose 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-versus-microsomal drug metabolism data. Reaction phenotyping results obtained from experiments 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).

**Authorship Contributions**

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

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

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

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

**References**


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