Short Communication

Contrasting Influence of NADPH and a NADPH-Regenerating System on the Metabolism of Carbonyl-Containing Compounds in Hepatic Microsomes

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ABSTRACT:

Carbonyl containing xenobiotics may be susceptible to NADPH-dependent cytochrome P450 (P450) and carbonyl-reduction reactions. In vitro hepatic microsome assays are routinely supplied NADPH either by direct addition of NADPH or via an NADPH-regenerating system (NRS). In contrast to oxidative P450 transformations, which occur on the periphery of a microsome vesicle, intraluminal carbonyl reduction depends on transport of cofactors across the endoplasmic reticulum (ER) membrane into the lumen. Glucose 6-phosphate, a natural cofactor and component of the NRS matrix, is readily transported across the ER membrane and facilitates intraluminal NADPH production, whereas direct addition of NADPH has limited access to the lumen. In this study, we compared the effects of direct addition of NADPH and use of an NRS on the P450-mediated transformation of propiconazole and 11β-hydroxysteroid dehydrogenase type 1 (HSD1) carbonyl reduction of cortisone and the xenobiotic triadimefon in hepatic microsomes. Our results demonstrate that the use of NADPH rather than NRS can underestimate the kinetic rates of intraluminal carbonyl reduction, whereas P450-mediated transformations were unaffected. Therefore, in vitro depletion rates measured for a carbonyl-containing xenobiotic susceptible to both intraluminal carbonyl reduction and P450 processes may not be properly assessed with direct addition of NADPH. In addition, we used in silico predictions as follows: 1) to show that 11β-HSD1 carbonyl reduction was energetically more favorable than oxidative P450 transformation; and 2) to calculate chemical binding score and the distance between the carbonyl group and the hydride to be transferred by NADPH to identify other 11β-HSD1 substrates for which reaction kinetics may be underestimated by direct addition of NADPH.

In vitro assays using hepatic microsomes are a powerful tool for both drug discovery applications and human health risk assessment (Obach, 1997). Microsomes are the metabolically active subcellular tissue fraction of the endoplasmic reticulum (ER) and exist primarily as vesicles. Extensive research with hepatic microsomes has focused on the cytochrome P450 (P450) superfamily of enzymes, which are located on the periphery of the microsome vesicle (Cribb et al., 2005). In vivo, the cytosolic pentose pathway primarily supplies P450 enzymes with NADPH for oxidative transformation. In vitro microsomal assays, which are devoid of the cytosolic fraction, are routinely supplied NADPH by either direct addition of NADPH or use of an NADPH-regenerating system (NRS), consisting of β-NADP+, glucose-6-phosphate (G6P), and G6P dehydrogenase (G6PDH). In contrast to P450 enzymes, the susceptibility of a chemical to enzymatic processes inside the lumen (intraluminal) depends on the selective transport of cofactors across the ER membrane. Intraluminal carbonyl reduction is an important process for the normal function of various endogenous substrates, and many pharmaceutical agents and pesticides use carbonyl moieties in their mode of action. Although many cytosolic carbonyl-reducing enzymes have been identified, carbonyl reductase in hepatic microsomes is mainly attributed to 11β-hydroxysteroid dehydrogenase type 1 (HSD1), a short-chain dehydrogenase-reductase. 11β-HSD1 is an intraluminal NADPH-dependent enzyme that has been well characterized for the interconversion of the glucocorticoid hormone cortisone to cortisol and is considered the primary microsomal reductase for carbonyl-containing therapeutics and pesticides (Oppermann, 2007).

The majority of chemicals involved in intraluminal metabolism require protein-facilitated transport to cross the ER lipid bilayer. G6P, a natural cofactor and component of the NRS matrix, is readily transported across the ER membrane by a selective transporter protein (G6PT), whereas NADPH and NADP+ are relatively impermeable and have limited access to the lumen. G6P entering the lumen is used as a substrate by intraluminal hexose-6-phosphate dehydrogenase (H6PDH) for NADPH production from a separate NADP+ pool enclosed within the ER. The intraluminal production of NADPH is then directly coupled to 11β-HSD1 activity (Csala et al., 2006; Piccirella et al., 2006).

We recently reported on the 11β-HSD1-mediated carbonyl reduction of the broad spectrum 1,2,4-triazole fungicide triadimefon to triadenin (Kenneke et al., 2008). The current study focuses on the contrasting effects of NADPH and NRS on the kinetic transformation.

ABBREVIATIONS: ER, endoplasmic reticulum; P450, cytochrome P450; NRS, NADPH-regenerating system; G6P, glucose-6-phosphate; G6PDH, G6P dehydrogenase; HSD1, 11β-hydroxysteroid dehydrogenase type 1; G6PT, glucose-6-phosphate transport protein; H6PDH, hexose-6-phosphate dehydrogenase; PDB, Protein Data Bank; MOE, Molecular Operating Environment.
of carbonyl-containing xenobiotics in hepatic microsomes. We examined the P450-mediated transformation of propiconazole and the intraluminal carbonyl reduction of cortisone and the xenobiotic triadimefon with direct addition of NADPH and NRS. In addition, in silico predictions based on chemical binding score and carbonyl to hydride NADP distance were used to identify other 11β-HSD1 substrates that may yield discrepant reaction kinetics depending on the use of either NADPH or NRS.

Materials and Methods

Reagents. Triadimefon, triadimenol, and propiconazole were obtained from the U.S. Environmental Protection Agency (EPA) National Pesticide Standard Repository (Fort Meade, MD). NADP+, NADPH, G6P, G6PDH, alamethicin, magnesium chloride (MgCl₂), phosphate buffer (pH 7.4), cortisol, and cortisone were purchased from Sigma-Aldrich (St. Louis, MO).

Microsomal Incubation Procedure. Frozen hepatic microsomes from male Sprague-Dawley rats at a concentration of 20 mg of microsomal protein/ml were purchased from In Vitro Technologies (Baltimore, MD) and stored at −80°C until use. All microsomal incubations (0.3 mg of protein) were conducted using the same lot of microsomes and assayed as described previously (Kenneke et al., 2008). In brief, metabolism assays were initiated by the addition of either 250 μl of NRS or NADPH to the microsomal suspension. All alamethicin assays were conducted at 50 μg/ml microsomal protein and were incubated for 10 min before initiation of the reaction (Piccirella et al., 2006). Abiotic microsomal controls were conducted with addition of the parent compound and omission of either NADPH or G6P from the NRS.

Triadimefon Assays. All triadimefon and triadimenol standards were prepared in acetonitrile and stored in amber vials at 4°C. Substrate saturation kinetic profiles of triadimenol formation in the presence of either NADPH (1.8 mM) or NRS were conducted by varying the triadimefon stock solutions (0.3–170 μM) at a 1% final acetonitrile concentration. A saturation kinetic plot of triadimenol formation with respect to varying NADPH concentration (0–4 mM) was conducted at 170 μM triadimefon. Analysis of triadimenol formation while varying the cofactor composition was conducted in triplicate in the presence of triadimefon (170 μM). Varying cofactor additions were also conducted with the 1,2,4-triazole, propiconazole (180 μM), as a comparative control for P450-mediated transformation processes (Kenneke et al., 2008).

Cortisone Assays. Cortisone and cortisol standards were dissolved in 50% ethanol/water and stored in amber vials at 4°C. The final concentration of ethanol in metabolism assays was less than 1%. A saturation kinetic profile of cortisol formation with respect to varying NADPH concentration (0–4 mM) was conducted using cortisol (137 μM). Cortisol formation was analyzed in triplicate while varying the incubation matrix cofactor composition in the presence of cortisol (137 μM).

High-Performance Liquid Chromatography Analysis and Data Analysis. Analysis of triadimenol, triadimenol, cortisone, cortisol, and propiconazole metabolism samples was performed on an Agilent Series 1100 HPLC quaternary pump system (Agilent Technologies, Santa Clara, CA) as described previously (Kenneke et al., 2008). The Michaelis-Menten kinetic parameters Vmax and Km were determined based on nonlinear regression of product velocity formation at varying substrate concentrations (Sigma Plot; Systat Software, Inc., San Jose, CA).

Molecular Docking. Triadimefon was docked into two catalytic systems: 1) human 11β-HSD1 [Protein Data Bank (PDB) accession codes: 2IRW] and 2) CYP3A4 (PDB accession code 2V0M) to quantitatively determine the differences in binding energetics between the two enzyme systems. Additional carbonyl-containing substrates (Table 1) were built in MOE and optimized using the MMFFx2 (Halgren and Nachbar, 1996) force-field as implemented in MOE. All docking experiments for substrates listed in Table 1 were performed in MOE using MOE-Dock on the 2BEL crystal structure with energies being reported in kJ/mol (see supplemental information).

Results

Saturation Kinetics of Triadimenol and Cortisol Formation. The metabolism of triadimenol to triadimefon in rat hepatic microsomes displayed a near mass balance with varying substrate concentrations using both NADPH and NRS (Fig. 1). A linear rate of triadimenol formation was observed for each substrate concentration tested. Assays using either NADPH or NRS demonstrated saturation kinetics with increasing triadimefon concentrations. Michaelis-Menten data analysis applied to triadimenol formation in the NADPH assays resulted in a Vmax of 1917 pmol/min/mg protein and a Km of 77 μM. A Vmax of 9691 pmol/min/mg protein and a Km of 93 μM were measured for the NRS assays. To ensure the NADPH concentration was not limiting triadimefon metabolism, a saturation kinetic plot with respect to NADPH concentration was conducted at the highest triadimeno substrate concentration tested (170 μM) (see supplemental information). The natural cortisone reductase activity was measured on the basis of cortisol formation at saturating NADPH levels to ensure maximum enzyme activity was achieved at the selected substrate concentration (137 μM).

Influence of Cofactors on Triadimenol and Cortisol Formation. Triadimenol formation in assays with NRS displayed greater than a 4-fold increase in reaction velocity relative to NADPH; cortisol reduction to cortisol was enhanced over 2-fold (Fig. 2). Carbonyl reductase activity was also detected for both triadimenol and cortisol.

### Table 1

<table>
<thead>
<tr>
<th>Rank</th>
<th>Substrate</th>
<th>Substrate Class</th>
<th>11β-HSD1 Reactivity</th>
<th>Binding Score</th>
<th>Distance (Å)</th>
<th>O = C(HNADP)</th>
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<tbody>
<tr>
<td>1</td>
<td>15-Deoxy-Δ-12.4-PG12</td>
<td>Natural</td>
<td>Known</td>
<td>−54.3</td>
<td>3.3</td>
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<tr>
<td>2</td>
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<td>sunscreen</td>
<td>Possible</td>
<td>−54.2</td>
<td>3.1</td>
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<tr>
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<td>Known</td>
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<tr>
<td>5</td>
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<tr>
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<tr>
<td>7</td>
<td>7-Ketocholesterol</td>
<td>natural</td>
<td>Known</td>
<td>−52.3</td>
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<tr>
<td>8</td>
<td>Triadimefon</td>
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<td>9</td>
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<tr>
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<td>Known</td>
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during sole addition of G6P. Cortisol formation in the system containing G6P alone exhibited a faster rate of transformation compared with incubations amended with NADPH, whereas triadimenol formation with G6P displayed nearly half the NADPH rate. Increased triadimenol and cortisol formation was observed with addition of either NADP$^+$ or NADPH in conjunction with G6P (no G6PDH present). Triadimenol formation in microsomal assays containing G6P and NADPH displayed activity levels near the NRS, while cortisol formation in the same matrix displayed a faster rate than the NRS. The P450-mediated oxidative transformation of propiconazole using NRS and NADPH displayed equivalent activities of product formation, whereas the sole addition of G6P resulted in no biotransformation of propiconazole (see supplemental information).

Disruption of microsome ER membrane cofactor transport with the pore-forming alamethicin was conducted with NRS and NADPH using triadimefon and propiconazole. Triadimenol formation in assays containing NADPH with alamethicin displayed activity levels near the NRS, whereas alamethicin showed no adverse effect on P450-mediated propiconazole metabolism.

In Silico Prediction of Triadimefon Binding to Enzymatic Systems. Triadimefon metabolism was reported to proceed through both carbonyl reduction via 11β-HSD1 (Kenneke et al., 2008) and CYP3A-mediated oxidation of the tert-butyl group (Barton et al., 2006). Using a molecular docking approach, we compared the pre-equilibrium process of triadimefon binding to 11β-HSD1 (PDB ID: 2IRW) and CYP3A4 (PDB ID: 2V0M). A lower energy binding score and less sterically hindered distance between the minimum energy poses of triadimefon against the catalytic active site of each target system indicates a more favorable carbonyl-reduction pathway (see supplemental information). Other carbonyl-containing substrates that may yield discrepant reaction kinetics using either NADPH or NRS were calculated based on 11β-HSD1 binding score and carbonyl to hydride-NADP distance (Table 1).

Discussion

Carbonyl-containing xenobiotics, which may be susceptible to oxidative P450 pathway(s) positioned on the periphery of the ER, may serve as substrates for 11β-HSD1-reductase activity located within the ER lumen. Although both processes are NADPH-dependent, intraluminal carbonyl reduction relies on cofactor transport across the ER membrane (Csala et al., 2006). Our investigation of triadimefon biotransformation assessed with NADPH and NRS using hepatic microsomes resulted in contrasting kinetics (Fig. 1). Michaelis-Menten data analysis of triadimenol formation with the NRS matrix showed a nearly 5-fold increase in $V_{\text{max}}$ compared with the direct addition of NADPH while the $K_M$ values remained constant. Thus, calculation of hepatic clearance based on the ratio of the kinetic parameters $V_{\text{max}}/K_M$ would yield a 5-fold difference between NRS and NADPH (Obach, 1997). The implications of these findings are significant because in vitro kinetic measurements serve as a critical component to physiologically based models used in chemical risk assessment.

Assays conducted with the native 11β-HSD1 substrate, cortisone, in the presence of NRS and NADPH paralleled the triadimefon results in displaying a lower rate of carbonyl-reductase activity with NADPH (Fig. 2). In contrast, the P450-mediated oxidative transformation of propiconazole, which occurs on the periphery of the microsome vesicle, demonstrated the same activity levels with either NRS or NADPH (Fig. 3). These results indicate that direct addition of NADPH and use of an NRS may give differing results based on enzyme location with respect to the ER membrane.

To further assess the influence of NRS on carbonyl reduction, a
A significant finding was observed with cortisol and triadimenol formation occurring in incubation matrices containing only G6P, whereas no propiconazole transformation was observed. These results support the role of an intraluminal H6PDH that uses G6P to generate NADPH for direct use by 11β-HSD1 in the reduction of cortisone to cortisol. Intraluminal H6PDH activity is maintained by a specific transporter (G6PT) for G6P entrance into the ER, which contains a separate NADP+ pool for NADPH generation within the lumen of the microsome vesicle (Piccirella et al., 2006). The H6PDH production of NADPH remained compartmentalized and did not influence the extraluminal P450-mediated metabolism of propiconazole.

The pyridine nucleotides NADP+ and NADPH are considered relatively impermeable to the ER membrane, which is supported by the limited triadimefon and cortisone carbonyl-reductase activity observed with the sole addition of NADPH (Csala et al., 2006). The low activity observed with the direct addition of NADPH is likely the

![Triadimenol Formation](image)

![Cortisol Formation](image)

![Propiconazole Metabolite Formation](image)
Influence the intraluminal kinetics for carbonyl-containing xenobiotics. The implications of possible discrepancies among previously published results for both therapeutics and pesticides with carbonyl functional groups deserve further investigation. In addition, the physiological variability associated with intactness of microsomal preparations needs to be assessed to evaluate the degree of discrepancy posed by the use of NADPH and NRS for intraluminal processes.

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References