Short Communication

Covalent Modification and Time-Dependent Inhibition of Human CYP2E1 by the meta-Isomer of Acetaminophen

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ABSTRACT:
The hypothesis that N-acetyl-m-aminophenol (AMAP), the meta isomer of acetaminophen, will covalently bind to and inhibit human CYP2E1 in a time- and NADPH-dependent manner was investigated. Liquid chromatography/electrospray ionization-mass spectrometry analysis indicated that AMAP metabolites (i.e., AMAP*) selectively and covalently modified CYP2E1 apoprotein in a ratio of 1:4:1 (AMAP*/CYP2E1) in a reconstituted system. The deconvoluted spectra of CYP2E1 apoprotein from incubations containing NADPH and AMAP displayed mass shifts of 167.2 ± 7.1 and 334.4 ± 6.5 Da, suggesting the addition of one and two hydroxylated AMAP metabolites to CYP2E1, respectively. Mass shifts in cytochrome P450 reductase, cytochrome b5, and heme from these samples were not observed. CYP2E1 inhibition by AMAP increased with time in the presence of NADPH; a reversible inhibition component was also observed. The results support a bioactivation process that involves formation of a hydroquinone metabolite that undergoes further oxidation to a quinone, which reacts with CYP2E1 nucleophilic residues. The data are consistent with evidence from previous studies that identified hydroxylated AMAP glutathione conjugates collected from mice and indicate that cysteine residues are the most likely sites for adduct formation. This study reports the first direct evidence of AMAP-derived hydroquinone metabolites bound to human CYP2E1.

Introduction

The analgesic N-acetyl-p-aminophenol (APAP; acetaminophen) is safe at therapeutic doses but hepatotoxic at larger ones. APAP overdose is the most common cause of acute liver failure in the United States (Lee, 2004; Bower et al., 2007). An important step in APAP-induced toxicity is the formation of N-acetyl-p-benzoquinone imine (NAPQI) that reacts with liver proteins and glutathione and ultimately results in hepatocyte death (Hinson et al., 2010).

Metabolites of the meta isomer of acetaminophen, N-acetyl-m-aminophenol (AMAP) (Fig. 1A), bind to hepatic proteins in mice at levels comparable to that of APAP. However, unlike APAP, AMAP does not cause hepatotoxicity in mice or hamsters (Roberts and Jollow, 1978; Nelson, 1980; Tirmenstein and Nelson, 1989). One explanation derives from the identity of adducted proteins and their subcellular location. For example, AMAP displays a degree of selectivity for proteins in the cytosol and endoplasmic reticulum, whereas APAP tends to form adducts with cytosolic and mitochondrial proteins (Tirmenstein and Nelson, 1989; Rashed et al., 1990; Matthews et al., 1997; Qu et al., 2001). There is also evidence for the generation of a quasi-stable intermediate of NAPQI with glutathione (i.e., an ipso adduct), which permits distribution of NAPQI to sites more distant from the site of generation (Chen et al., 1999).

Cytochrome P450 2E1 is often studied for its involvement in toxic processes, in particular, the formation of reactive metabolites and reactive oxygen species (Caro and Cederbaum, 2004; Lieber, 2004). CYP2E1 has been implicated in benzene-mediated myelotoxicity, the activation of tobacco nitrosamines, and halothane hepatitis (Yamazaki et al., 1992; Valentine et al., 1996; Spracklin et al., 1997). CYP2E1 is an alcohol-inducible enzyme that catalyzes the formation of NAPQI from APAP. Compared with wild-type mice, Cyp2e1 knockout mice display decreased susceptibility to APAP-induced hepatotoxicity, requiring greater APAP doses to approach the mortality rates and liver transaminase elevations observed in wild-type mice (Lee et al., 1996).

CYP2E1 crystal structures reveal a relatively small but elastic active site. Rotation of Phe298 was identified as an important dynamic event for the accommodation of higher molecular weight substrates, such as fatty acid analogs (Porubsky et al., 2008, 2010). Although these studies have provided new structural insights, there remains a dearth of information with regard to the identification of key structural motifs that contribute to substrate recognition/orientation.

Another approach for elucidating protein structure-function relationships is to use a small molecule that is metabolized enzymatically to a reactive molecule that covalently adds to an amino acid on the enzyme peptide structure. When adduct formation results in enzyme inhibition, such molecules can be classified as time-dependent inhibitors (TDIs). If additional criteria are met, TDIs can be more strictly defined as mechanism-based inhibitors (Silverman, 1988). In addition to protein structure-function studies, TDIs are relevant to drug discovery. The early identification of new chemical entities that are TDIs of drug-metabolizing enzymes is an important step in the discovery of new drugs.

ABBREVIATIONS: APAP, N-acetyl-p-aminophenol; AMAP, N-acetyl-m-aminophenol; NAPQI, N-acetyl-p-benzoquinone imine; TDI, time-dependent inhibitor; P450, cytochrome P450; LC, liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; HPLC, high-performance liquid chromatography.
FIG. 1. A, constitutional isomers APAP and AMAP. B, HPLC trace for the separation of reconstituted proteins analyzed using UV absorption (λ = 280 nm) and electrospray ionization-mass spectrometry total ion current (m/z = 500-2000). Retention times were determined by comparison with protein standards. C, a representative deconvoluted mass spectrum of CYP2E1 isolated from reconstituted incubations containing AMAP and NADPH (top) or AMAP alone (bottom). D, a representative deconvoluted mass spectrum of CYP2E1 isolated from incubations containing AMAP and NADPH spiked with CYP2E1 standard.
process and for the prevention of drug-drug interactions (Grimm et al., 2009).

The interactions between AMAP and human CYP2E1 have not been well characterized. On the basis of the structural similarities between AMAP and APAP, the role of CYP2E1 in APAP bioactivation and evidence that AMAP forms an adduct with CYP2E1 in mice (Halmes et al., 1998), we hypothesized that CYP2E1 would catalyze the formation of an AMAP-derived reactive metabolite that could covalently bind to and inhibit human CYP2E1 in a time-dependent manner and serve as a probe for future investigations of CYP2E1 structure and function.

Materials and Methods

Materials. Purified human CYP2E1 and purified cytochrome $b_5$ were purchased from Invitrogen (Carlsbad, CA). Rat cytochrome P450 reductase was expressed and purified as reported previously (Shen et al., 1989). Supernatomes (human CYP2E1 with P450 reductase and cytochrome $b_5$) were obtained from BD Gentest (Woburn, MA). 1,6-Dilaurylphosphatidylcholine, AMAP, NADPH, glutathione, coumarin, 7-hydroxycoumarin, 1-aminoenobenzotriazole, and chlorozoxazone were purchased from Sigma-Aldrich (St. Louis, MO). 6-Hydroxycortroxazone was purchased from US Biologicals (Marblehead, MA). Slide-A-Lyzer dialysis cassettes were purchased from Thermo Fisher Scientific (Waltham, MA). POROS reverse-phase R2 resin was from Life Technologies (Carlsbad, CA).

Formation of CYP2E1 - AMAP Adducts. CYP2E1, P450 reductase, 1,6-Dilaurylphosphatidylcholine, and cytochrome $b_5$ were combined in a ratio of 1:1.6:0.1 and transferred to a Slide-A-Lyzer dialysis cassette (0.5-3 μl; 10,000 molecular weight cutoff). Each recombination component was added in the sequence listed and incubated for 5 min on ice before the addition of the next component. The mixture was dialyzed twice against 1 liter of 50 mM potassium phosphate (pH 7.4) for 2 h at 4°C to remove glycerol. Samples were preincubated for 3 min at 37°C after the addition of AMAP (5 mM). Incubations were initiated by the addition of NADPH (1 mM). Samples were incubated for 15 min at 37°C and then placed on ice until undergoing LC/ESI-MS analysis. The final concentration of CYP2E1 ranged from 2.3 to 2.8 μM. Incubation volumes varied slightly from experiment to experiment due to volume changes from dialysis but were approximately 200 μl with the goal to inject 100 to 200 pmol CYP2E1 on column for LC/ESI-MS analysis.

LC/ESI-MS Analysis of Reconstituted Incubations. CYP2E1, P450 reductase, cytochrome $b_5$, and heme were separated and analyzed by LC/ESI-MS. Samples (100 μl) were injected on a column packed with POROS reverse-phase R2 resin (20 μm, 2.1 mm × 100 mm) at a flow rate of 0.4 ml/min. The solvent system consisted of solvent A (0.05% trifluoroacetic acid) and solvent B (95% acetonitrile-0.05% trifluoroacetic acid). Incubation components were separated using a gradient. 50% solvent A-50% solvent B (t = 0 min to t = 15 min), 30% A (t = 20 min), 10% A (t = 25 min), and 65% A (t = 26 min). The Shimadzu HPLC system (Shimadzu, Kyoto, Japan) was interfaced with a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK). Samples were introduced using electrospray ionization, and spectra were obtained by scanning m/z = 500 to 2000 (3.5-s scan time) in the continuum mode with cone voltage set at 35 V. Spectra deconvolution was performed using MassLynx MaxEnt software Waters (Milford, MA). Experimental and control samples were compared with enzyme standards, which were analyzed using identical LC/ESI-MS conditions. Heme was detected as the potassium salt.

The mass of the AMAP-derived molecule covalently adducted to CYP2E1 apoprotein was evaluated by adding purified CYP2E1 standard to samples whose deconvoluted mass spectra exhibited detectable mass shifts for CYP2E1 apoprotein (i.e., samples containing both NADPH and AMAP). The mass difference between monoadducted CYP2E1 and CYP2E1 standard and the mass difference of diadducted CYP2E1 and monoadducted CYP2E1 were averaged to estimate the mass of the reactive metabolite, which is designated here as AMAP*: $\text{MW}_{\text{AMAP*}} = [(\text{MW}_{\text{CYP2E1} - \text{AMAP*}} - \text{MW}_{\text{unlabeled CYP2E1}}) + (\text{MW}_{\text{CYP2E1} - 2\text{AMAP*}} - \text{MW}_{\text{CYP2E1} - \text{AMAP*}})]/2$, where MW is molecular weight.

NADPH- and Time-Dependent Inhibition Experiments. CYP2E1 Supernatomes (0.30 mM) were preincubated in a total volume of 100 μl for 5 min at 37°C in incubation buffer (50 mM potassium phosphate, pH = 7.4). Select samples contained AMAP (2.5, 20, or 40 mM), NADPH (1 mM), and glutathione (5 mM), or a combination thereof. Incubations were initiated by the addition of NADPH and proceeded at 37°C for 72 min followed by dilution to 1000 μl with a solution of incubation buffer, chlorozoxazone (250 μM), and NADPH (1 mM) and subsequently incubated for an additional 10 min to assess remaining activity. A 100-μl aliquot of 7-hydroxycoumarin (42 μM) was added as an internal standard after termination with trichloroacetic acid (40 μl). Samples were agitated using a vortex mixer and centrifuged at 11,000g for 3 min. Supernatant (1000 μl) was transferred to a vial for HPLC analysis to quantify 6-hydroxycortroxazone as described below. Stock solutions of AMAP, NADPH, and glutathione solutions were prepared in incubation buffer. Chlorozoxazone stock solutions were prepared in dimethyl sulfoxide and diluted in incubation buffer before experiments. The final concentration of dimethyl sulfoxide in chlorozoxazone-containing incubations was 0.25% (v/v).

Results and Discussion

It was reported previously that a 50-kDa microsomal protein from mice treated with AMAP reacted with arylacetamide antiserum and also comigrated with a protein reacting with anti-CYP2E1 (Matthews et al., 1997). Moreover, AMAP inhibited the $\gamma$-nitrophenol hydroxylation activity of acetone-induced mouse liver microsomes (Halmes et al., 1998). The goal of the work reported here was to test whether human CYP2E1 catalyzes the generation of a reactive metabolite from AMAP (i.e., AMAP*) that covalently binds to and inhibits the enzyme and to provide information regarding the structure of the reactive metabolite to characterize the mechanism of inhibition.

Overall, the data presented here support a multistep process for the CYP2E1-mediated activation of AMAP and ultimately adduct formation. On the basis of the measured masses of CYP2E1 - AMAP*, reactive metabolite formation probably involves the formation of a hydroquinone metabolite that undergoes further oxidation to a quinone, which reacts with CYP2E1 nucleophilic residues (Fig. 2B).

Mass Spectral Characterization of Incubation Components and CYP2E1 - AMAP* Adducts. The HPLC separation and UV/mass spectral analysis of CYP2E1, P450 reductase, cytochrome $b_5$, and heme are shown in Fig. 1B. The average masses for individual
protein components and heme under the various incubation conditions (i.e., with AMAP, NADPH, or the combination of both AMAP and NADPH) are displayed in Table 1. The deconvoluted spectra of P450 reductase and cytochrome b5 from all samples displayed a single major peak with masses identical to the predominant peak in the deconvoluted spectra of standards (Supplemental Figs. 1–3). Adduct formation is selective for CYP2E1 apoprotein and is NADPH-dependent in the reconstituted system. That is, a single major peak was observed in the deconvoluted spectra of CYP2E1 apoprotein for most samples, except for those that contained both NADPH and AMAP; the spectra from these samples exhibited two major peaks (Fig. 1C). On the basis of the peak height of deconvoluted peaks, the ratio of monoadduct (CYP2E1·AMAP*) to diadduct (CYP2E1·2AMAP*) was 1.5 ± 0.5 and the ratio of AMAP* to CYP2E1 was 1.4 ± 0.1. The deconvoluted spectra from samples spiked with CYP2E1 standard provided a means to estimate the mass of the metabolite covalently bound to CYP2E1 apoprotein. When CYP2E1 standard was added to samples containing both NADPH and AMAP, the deconvoluted spectrum exhibited three major peaks corresponding to the mass of CYP2E1 apoprotein plus mass shifts of 167.2 ± 7.1 and 334.4 ± 6.5 Da (n = 3) (Fig. 1D; Table 1), suggesting additions of one and two AMAP metabolites, respectively. On the basis of the difference in mass between AMAP and the experimental mass addition to CYP2E1 = 167.5 ± 7.1 Da), the reactive metabolites that react with CYP2E1 are quinones generated from AMAP. The mass spectra of heme from all samples were identical, including those containing both AMAP and NADPH, and displayed a base peak m/z = 657, indicative of the potassium salt (Supplemental Fig. 1).

Although CYP2E1·AMAP* adducts are sufficiently stable to undergo whole protein LC/ESI-MS analysis, CYP2E1·AMAP* adducts did not withstand conditions currently used in typical proteomic studies. Similar results were observed in a study investigating specific protein residues that may be susceptible to formation of APAP adducts (Stamper et al., 2011). One fragmentation spectrum indicated the presence of an AMAP-adducted peptide (Supplemental Fig. 6).

Further investigation is necessary to develop conditions under which CYP2E1·AMAP* adducts are sufficiently stable to confirm this preliminary result.

Characterization of CYP2E1 Inhibition by AMAP. Inhibition of CYP2E1-mediated chlorzoxazone hydroxylase activity by AMAP was both time- and NADPH-dependent (Fig. 2A). Inhibition also involved a competitive component but was greatest when samples were preincubated with both NADPH and AMAP. Controls contained CYP2E1 Supersomes preincubated in incubation buffer (Table 2; Fig. 2A) (a representative HPLC trace for 6-hydroxychlorzoxazone and a standard curve are available in Supplemental Figs. 4 and 5). The Ki and k_inact values were 9.5 mM and 0.008 min⁻¹, respectively. Samples preincubated for 72 min with 5 mM glutathione, NADPH, and 20

### Table 1

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<thead>
<tr>
<th>Component</th>
<th>Detected Masses</th>
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<tr>
<td>+NADPH, +AMAP</td>
<td>CYP2E1 54,622.4 ± 8.9, 54,791.3 ± 6.1</td>
<td>6</td>
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<tr>
<td></td>
<td>Reductase 77,712.2 ± 2.4</td>
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<td></td>
<td>Cytochrome b5 15,949.3 ± 0.6</td>
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<td></td>
<td>K⁺ Heme 657.3 ± 0.1</td>
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<tr>
<td>+AMAP</td>
<td>CYP2E1 54,458.7 ± 3.3</td>
<td>5</td>
</tr>
<tr>
<td>+NADPH</td>
<td>54,465.5 ± 2.0</td>
<td>2</td>
</tr>
<tr>
<td>CYP2E1 standard</td>
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<tr>
<td>P450 reductase standard</td>
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<td>Cytochrome b5 standard</td>
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N.A., not applicable.
mM AMAP exhibited chlorzoxazone hydroxylase activity 26.5% greater than that of samples preincubated with NADPH and 20 mM AMAP and equal to that of samples preincubated with 20 mM AMAP or NADPH (Table 2).

The data from the current study are consistent with previous investigations of AMAP conducted in mice in which 2-acetamidohydroquinone and 3-hydroxyacetanilide were identified as major oxidative metabolites (Streeter et al., 1984; Rashed and Nelson, 1990). That is, AMAP underwent aromatic oxidation at positions that are ortho and para to the N-acetyl group. Phase II metabolites included glutathione conjugates of these oxidative metabolites and subsequent cysteine and mercapturate-related conjugates. It was proposed that the ring-hydroxylated metabolites undergo a second round of oxidation to generate the ortho- and para-quinones that react with the cysteinylic thiol of glutathione via conjugate addition. The current results provide the first direct structural evidence that an oxidized metabolite of AMAP is bound to protein, specifically CYP2E1, and further supports the proposed two-step activation process (Fig. 2B). A multistep bioactivation process could explain the comparatively high K\text{inact} value because it would encompass multiple binding affinities (e.g., AMAP and AMAP hydroquinone). Likewise, the relatively low K\text{inact} value is consistent with a multiphasic process because inactivation would depend on multiple rate constants including the formation of AMAP hydroquinone and AMAP quinone and ultimately the reaction between CYP2E1 and AMAP quinone. Samples containing glutathione, plus NADPH and AMAP, exhibited chlorzoxazone hydroxylase activity equal to that of controls containing CYP2E1 and NADPH and almost equal to that of controls containing CYP2E1 alone. Therefore, the reactive metabolite covalently binds to CYP2E1 apoprotein only in the absence of an electrophilic scavenger.

AMAP* · CYP2E1 monoaucts and diadducts were formed predominantly in the experiments described here. In regard to the function of individual adducted residues, at least one residue is important for activity because inhibition increased with time and in the presence of NADPH. If CYP2E1 activity loss requires adduct formation with at least two residues, this could also contribute to the lag period observed in the time-dependent inhibition curves. There is evidence suggesting that cysteine residues are the likely major adduct site(s). That is, glutathione protected CYP2E1 from inactivation, and previous studies indicated that glutathione conjugates of OH-AMAP were major routes of AMAP metabolism, evidence that the reactive metabolite readily reacts with thiols. Moreover, peaks in the deconvoluted mass spectra were consistent with a conjugate addition mechanism, which supports bond formation with soft nucleophiles (i.e., thiols). There are eight cysteines in human CYP2E1, and one (Cys480) is directly adjacent to substrate recognition site 6 as defined previously (Gotoh, 1992). Considering the dynamic nature of P450 enzymes (Scott et al., 2003), it is possible that other cysteine residues could be exposed to AMAP* even though they are not part of the defined substrate recognition sites.

In conclusion, the results from this study support the hypothesis that human CYP2E1 catalyzes the generation of a reactive metabolite from AMAP that covalently binds to and inhibits CYP2E1. The data support a multistep process that involves aromatic hydroxylation followed by a second oxidative event to form reactive quinones. The results are consistent with evidence from previous studies conducted in mice that identified AMAP glutathione conjugates. Moreover, adduct formation is specific for CYP2E1 apoprotein in the reconstituted system.

Acknowledgments

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Authorship Contributions

Participated in research design: Harrelson, Stamper, Goodlett, and Nelson.
Conducted experiments: Harrelson, Stamper, and Chapman.
Performed data analysis: Harrelson, Stamper, Chapman, Goodlett, and Nelson.
Wrote or contributed to the writing of the manuscript: Harrelson, Stamper, Chapman, Goodlett, and Nelson.

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Insights into the binding of inhibitors and both small molecular weight and fatty acid substrates. *J Biol Chem* **283**:33698–33707.


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