IN VITRO BIOTRANSFORMATION OF SILDENAFIL (VIAGRA) IN THE MALE RAT: THE ROLE OF CYP2C11

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ABSTRACT:

To assess the suitability of the male rat model for human studies on sildenafil metabolism, we examined the biotransformation of sildenafil in male rat liver microsomes and identified the role of specific cytochrome P450s (P450) using inhibitory antibodies and cDNA-expressed P450s. Rates of formation of the major circulating metabolite of sildenafil, UK-103,320, were 11-fold greater in the male rat than in human liver microsomes at 36 μM sildenafil, whereas substrate concentration corresponding to 50% \( V_{\text{max}} \) (\( K_{\text{m}} \) values) were 2.9-fold lower in the male rat. Although sildenafil is largely broken down by CYP3A isoforms in humans, coinubcation of rat liver microsomes with immunoinhibitory antibodies (CYP1A1/2, 2B1/2, 2C11, 2E1, and 3A1/2) revealed that metabolite formation was inhibited only by an antirat CYP2C11 antibody. Incubation of sildenafil with a cDNA-expressed CYP2C11 produced 10-fold higher levels of UK-103,320 than other P450s (CYP1A1, 1A2, 2B1, 2C6, 2C12, 2C13, 2E1, 3A1, and 3A2). Thus CYP2C11 contributes in a major way to the metabolism of sildenafil in the male rat. P450 isoforms mediating sildenafil biotransformation differ substantially between humans and the male rat, thereby limiting the applicability of this species as a model for sildenafil metabolism and drug interactions in humans.

Sildenafil (Viagra) is a cGMP phosphodiesterase type 5 inhibitor used in the treatment of erectile dysfunction. Pharmacokinetic studies of sildenafil demonstrate similarities between the rat and human in metabolite formation in vivo (Walker et al., 1999). Both species produce five principal metabolites and form UK-103,320 as the primary circulating metabolite. However, plasma clearance in the male rat is 8 times greater than that observed in male volunteers. Reflecting the high clearance of the parent drug, the male rat has a greater relative exposure to the pharmacologically active metabolite, UK-103,320, than to sildenafil. In humans, however, sildenafil is the principal pharmacologically active compound (Walker et al., 1999). Factors that contribute to differences in the clearance between the rat and human have not yet been identified.

A recent in vitro study using human liver microsomes demonstrates that 79% of sildenafil biotransformation to UK-103,320 is attributable to CYP3A (Warrington et al., 2000). A small percentage of metabolite formation is due to CYP2C9 (20%), CYP2D6 and CYP2C19 (collectively less than 2%) activity. These data are consistent with the findings of Hyland et al. (2001).

To determine whether differences in clearance between the rat and human are due to differences in metabolic pathways, we sought to identify which P450 isoforms mediate UK-103,320 formation in male rat liver microsomes.

Materials and Methods

Microsomes were prepared from the livers of 10 male Fischer 344 rats (ages 3–4 months) by differential centrifugation, as previously described (Warrington et al., 2000). Hepatic microsomes from 10 individual rat preparations were incubated with increasing concentrations of sildenafil (0–63 μM) in the presence of an NADPH-regenerating cofactor system. Samples were incubated for 20 min at 37°C and terminated with the addition of acetonitrile and exposure to ice. Buspirone (4 μM) was added as an internal standard and samples were centrifuged at 14,000 rpm for 10 min to remove the protein. The supernatants were transferred to autosampling vials for high-performance liquid chromatography (HPLC) analysis.

Samples were analyzed by HPLC, as previously described (Warrington et al., 2000). These conditions were found to be within the linear range with respect to time and protein for rat hepatic microsomes (data not shown). Negative controls without protein and cofactor demonstrated no metabolite formation (data not shown). Since reference samples of UK-103,320 were unavailable, identification of UK-103,320 was based on chromatographic retention times, as previously described (Warrington et al., 2000).

Formation of UK-103,320 in the male rat was consistent with a single enzyme Michaelis-Menten kinetic model. The following equation was fitted to UK-103,320 data points by nonlinear regression:

\[ V = \frac{V_{\text{max}} \cdot S}{K_{\text{m}} + S} \]

in which \( V \) is the rate of UK-103,320 formation and \( S \) represents the concentration of the substrate, sildenafil (Venkatakrishnan et al., 2001). Since pure samples of UK-103,320 were not available, absolute values of \( V_{\text{max}} \) could not

Abbreviations used are: P450, cytochrome P450; HPLC, high-performance liquid chromatography.
substrate concentration corresponding to 50% rat than in the human. In addition, reactions in the rat exhibited lower the appropriate serum control.

Fig. 1. Comparison of sildenafil biotransformation with its major circulating metabolite, UK-103,320, in human and rat liver microsomes.

Sildenafil biotransformation in 10 rat livers (mean ± S.E.) is indicated by dashed lines and square symbols. Denoted by solid lines and circle symbols, sildenafil biotransformation in a representative human liver is provided for comparison (Warrington et al., 2000).

be calculated. Therefore, the amount of UK-103,320 formed was expressed in arbitrary units reflecting the ratio of the chromatographic peak height of UK-103,320 relative to that of the internal standard.

Using five specific immunoinhibitory antibodies, we assessed the involvement of CYP1A1/2, CYP2B1/2, CYP2C11, CYP2E1, and CYP3A1/2 in the biotransformation of sildenafil in male rat hepatic microsomes. Hepatic microsomes from 10 rats were pooled, and samples of pooled microsomes were incubated in triplicate. At 37°C, sildenafil (36 μM) was incubated for 30 min with 10 μg of microsomes and either 50 mM potassium phosphate buffer, 100 μg of control serum (goat or rabbit), or 100 μg of each polyclonal antirat P450 antibody (Gentest, Woburn, MA). A NADPH-regenerating cofactor system was then added, and samples were incubated for an additional 20 min, as described above. The samples were then subjected to HPLC analysis, as previously described (Warrington et al., 2000). The degree of inhibition was determined by the peak height ratio of the antibody-containing sample divided by the peak height ratio of the appropriate serum control.

The role of specific P450s in sildenafil biotransformation was further examined using a screen of rat cDNA-expressed P450s. Sildenafil (15 and 132 μM) was incubated with either a rat cDNA-expressed P450 (CYP1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, or 3A2, Gentest) or a vector control (from an AHH-1 TK/H11001/H11002 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, or 3A2, Gentest) or a vector control (from an AHH-1 TK/H11001/H11002 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, or 3A2, Gentest) or a vector control (from an AHH-1 TK/H11001/H11002 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, or 3A2, Gentest).

The differences in clearance between species may be attributable, in part, to differences in the affinities of the enzymes controlling the metabolic pathways. While UK-103,320 formation is largely mediated by CYP3A in humans, it appears to be largely dependent on CYP2C11 in the male rat.

Many CYP3A substrates in humans are likely to be largely CYP3A substrates in rats such as cyclosporine (Smith, 1991). However, several CYP3A-specific reactions in humans such as nifedipine oxidation and lidocaine N-deethylation demonstrate overlapping substrate specificity with CYP2C in the rat (Smith, 1991). It has been suggested samples from the rat were compared to previously published human studies (Fig. 1; Table 1), comparable results were obtained when rat and human experiments were performed in parallel (data not shown). These in vitro experiments correlate well with in vivo human and rat studies (Walker et al., 1999). Studies with both inhibitory antibodies and cDNA-expressed enzymes identified the role of CYP2C11 in UK-103,320 formation in the rat liver (Figs. 2 and 3). As shown in Fig. 2, the antirat CYP2C11 antibody inhibited UK-103,320 formation by 87%, while the remaining antibodies (antirat CYP1A1/2, 2B1/2, 2E1, and 3A1/2) did not cause inhibition. When two concentrations of sildenafil (15 and 132 μM) were incubated with rat cDNA-expressed P450s (CYP1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, and 3A2), only CYP2C11 resulted in substantial UK-103,320 formation (Fig. 3). Incubation with CYP3A1 and 3A2 resulted in 6 and 10% of the metabolite formation observed with CYP2C11, respectively.

Results/Discussion

Rat liver microsomes formed UK-103,320 at higher rates than human liver microsomes under the same conditions (Fig. 1). At 36 μM sildenafil, UK-103,320 formation rate was 11-fold greater in the rat than in the human. In addition, reactions in the rat exhibited lower substrate concentration corresponding to 50% Vmax (Km values), reflecting a higher affinity of the rat enzyme for sildenafil biotransformation via the UK-103,320 metabolite pathway (Table 1). Although

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Km values</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Human</td>
<td>mean ± S.E.; n = 4 livers 14.4 ± 2.0 μM</td>
<td>Warrington et al., 2000</td>
</tr>
<tr>
<td></td>
<td>mean ± S.D.; n = 3 livers 6 ± 3 μM for Km1 a</td>
<td>Hyland et al., 2001</td>
</tr>
<tr>
<td></td>
<td>22 ± 9 μM for Km2.</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>mean ± S.E.; n = 4 livers 5.0 ± 0.2 μM</td>
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a Km1 refers to the high-affinity component. 
b Km2 describes the low-affinity component.

![Fig. 1. Comparison of sildenafil biotransformation with its major circulating metabolite, UK-103,320, in human and rat liver microsomes.](image1)

Fig. 1. Comparison of sildenafil biotransformation with its major circulating metabolite, UK-103,320, in human and rat liver microsomes.

**Fig. 2. Immunoinhibition of sildenafil biotransformation in male rat hepatic microsomes using a screen of antirat P450 antibodies.**

Sildenafil (36 μM) was incubated with either antirat CYP1A1/2 (3A), CYP2E1 (2E), CYP1A1/2 (1A), CYP2B1/2 (2B), CYP2C11 (2C), 50 mM potassium phosphate buffer (B), or goat or rabbit serum controls. Histograms represent UK-103,320 formation rate relative to serum controls (mean ± S.E.) for microsomal samples pooled from 10 rats. Samples were performed in triplicate. *p < 0.05 and **p < 0.001, as evaluated by Student’s t test.
that, for CYP3A substrates in humans, differences in clearance between the human and rat serve as a good predictor of CYP2C involvement in the rat (Smith, 1991). Nonetheless, while overlap between CYP3A- and CYP2C-mediated reactions has been previously documented, the role of parallel CYP2C isoforms has remained largely uncharacterized for these and other reactions.

Unlike many substrates, testosterone biotransformation has been well studied in the rat and specific isoforms that mediate the formation of several metabolites have been identified (Waxman et al., 1983; Waxman, 1984). However, there is significant overlap in the enzymes that mediate these reactions. For example, while 2α- and 16α-hydroxytestosterone formation have been commonly used as markers of CYP2C activity in rats (Shaw et al., 2002), 2α-hydroxytestosterone formation in the rat is mediated by CYP2C6 and 2C11 (Waxman, 1984; Jansson et al., 1985), and 16α-testosterone formation is shared by CYP2B1, 2C6, 2C11 and 2C13 (Cheng and Schenken, 1982; Waxman and Walsh, 1983; Waxman, 1984; Jansson et al., 1985). Since sildenafil appears to be predominantly metabolized by CYP2C11, its N-demethylation to UK-103,320 may serve as a useful index for analysis of CYP2C11 activity. As CYP2C11 represents one of the most abundantly expressed P450s in the rat liver (Guengerich et al., 1982), a specific index reaction may be particularly important.

The involvement of CYP2C11 in UK-103,320 formation is likely to explain differences in clearance found between the male and female rat. Plasma clearance in the female rat is comparable to that found in the human and, hence, is significantly lower than clearance in the male rat (Walker et al., 1999). While CYP2C12 is expressed in females, CYP2C11 and CYP2C13 are male-specific P450s (Imaoka et al., 1991). Thus, if CYP2C11 is the primary enzyme involved in sildenafil biotransformation in male rat liver microsomes and this enzyme is largely absent in the female, the metabolic pathways in the female rat are likely to be different.

These findings have two important implications. First, it is possible that sildenafil may be a useful CYP2C11 index substrate in the male rat. Second, this study, along with several others (Ring et al., 1994; Eagling et al., 1998; Perloff et al., 2000), emphasizes that caution is required in extrapolating rat metabolic data to humans.

References


Fig. 3. Sildenafil biotransformation in rat cDNA-expressed cytochromes.

Sildenafil at 15 μM (A) and 132 μM (B) was incubated with either a rat cDNA-expressed P450 (CYP1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2E1, 3A1, or 3A2; Gentest), a vector control (from a human lymphoblastoid (L. vector) or a Hi5 cell line) or human (HL) or rat liver (RLM) microsomes. Samples in which either protein, cofactor, or sildenafil were omitted were incubated in parallel and listed as no protein, no cofactor, and no drug. Metabolite formation is expressed in relative units.