IN VITRO BIOTRANSFORMATION OF SILDENAFIL (VIAGRA): IDENTIFICATION OF HUMAN CYTOCHROMES AND POTENTIAL DRUG INTERACTIONS

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(Received August 20, 1999; accepted December 17, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

The in vitro biotransformation of sildenafil to its major circulating metabolite, UK-103,320, was studied in human liver microsomes and in microsomes containing heterologously expressed human cytochromes. In human liver microsomes, the mean $K_m$ (± S.E.) was 14.4 ± 2.0 μM. A screen of the chemical inhibitors omeprazole (10 μM), quinidine (10 μM), sulfaphenazole (10 μM), and ketoconazole (2.5 μM) only revealed detectable inhibition with ketoconazole. Sildenafil biotransformation (36 μM) was inhibited by increasing concentrations of ketoconazole and ritonavir (IC$_{50}$ values less than 0.02 μM), which are established cytochrome P450 (CYP) 3A4 inhibitors. Using microsomes containing cDNA-expressed cytochromes, UK-103,320 formation was found to be mediated by four cytochromes: CYP3A4, -2C9, -2C19, and -2D6. Estimated relative contributions to net intrinsic clearance were 79% for CYP3A4 and 20% for CYP2C9; for CYP2C19 and -2D6, estimated contributions were less than 2%. These results demonstrate that CYP3A4 is the primary cytochrome mediating UK-103,320 formation and that drugs that inhibit CYP3A4 are likely to impair sildenafil biotransformation.

Sildenafil (Viagra), a selective inhibitor of cGMP phosphodiesterase type 5, is an orally active treatment for erectile dysfunction (Chuang et al., 1998). As many as 30 million men suffer from some degree of erectile dysfunction in the United States (Goldenberg, 1998; Kaplan et al., 1999). According to a report in August 1998, sildenafil had been the fastest selling drug in pharmaceutical history since its release in April 1998 (Nachtsheim, 1998) and, according to 1998 statistics, sildenafil was among the top 100 most commonly prescribed drugs in the United States. Recent clinical trials have also examined the use of sildenafil for women (Kaplan et al., 1999). One report also identified illicit use of sildenafil in British nightclubs (Aldridge and Measham, 1999). Given the widespread use of sildenafil, it can be expected that sildenafil will be coadministered with agents that either directly cause sexual dysfunction or are used to treat diseases associated with sexual dysfunction. These include antihypertensives, antidepressants, viral protease inhibitors, and azole antifungal agents. Several case reports have already recommended the use of sildenafil in pharmacologically induced impotence (Ashton and Bennett, 1999; Nurnberg et al., 1999; Rosenberg, 1999; Schaller and Behar, 1999), a leading cause of sexual dysfunction (Korenman, 1998). Thus, an understanding of the metabolism of sildenafil is imperative to anticipate and avoid important drug interactions.

Clinical pharmacokinetic data has identified the formation of a principal circulating metabolite, UK-103,320, which is a product of $N$-desmethylation (Muirhead et al., 1996) and, according to reports by the manufacturers of the drug, may contribute to approximately 20% of the net pharmacological effect of sildenafil. UK-103,320 has been identified as a metabolite of sildenafil in mouse, rat, dog, and humans (Muirhead et al., 1996; Walker et al., 1999).

Product labeling information has suggested the role of cytochrome P450 (CYP) 3A4 and CYP2C9 in formation of UK-103,320 in humans. Additional reports from the manufacturers of the drug have identified a minor role for CYP2D6 as well as an in vivo interaction between sildenafil and potent CYP3A4 inhibitors such as ketoconazole and ritonavir. However, aside from a recent study on the interaction of sildenafil and viral protease inhibitors (Merry et al., 1999), the details of these studies are not generally available in the medical literature.

The present study has examined the in vitro biotransformation of sildenafil using human liver microsomes as well as the identification of the role of specific cytochromes using chemical inhibitors and microsomes containing individual human cytochromes expressed by cDNA-transfected human lymphoblastoid cells.

Experimental Procedures

Materials. Liver samples from four human donors with no known liver disease were obtained from the Liver Tissue Procurement and Distribution System, University of Minnesota, Minneapolis, MN. Samples were chosen that demonstrated high CYP3A activity. Microsomes were prepared by differential centrifugation as described previously (von Moltke et al., 1993; Charpentier et al., 1997). Microsomal tissue was resuspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80°C until use. Microsomes containing individual human cytochromes expressed by cDNA-transfected...
human lymphoblastoid cells and control vector (Gentest, Woburn, MA) were also stored at −80°C until use (Crespi, 1995).

Because a pure sample of sildenafil was not available, sildenafil was extracted from a commercially available 50-mg tablet and dissolved in methanol to yield a final concentration of 250 μM, as described previously for other substrates (von Moltke et al., 1998). Other chemical reagents and materials were purchased from commercial sources or kindly provided by their manufacturers. Reference standards of UK-103,320 were not available.

**Incubations Using Human Liver Microsomes.** Varying quantities of sildenafil ranging from 0 to 250 μM were added to the incubation tubes. The methanol was evaporated to dryness in a 45°C vacuum oven. To achieve substrate concentrations greater than 250 μM, serial evaporations were conducted to yield substrate concentrations ranging from 0 to 750 μM. Incubation samples contained 50 mM phosphate buffer, 5 mM MgCl₂, 0.5 mM NADP⁺, and an isocitrate/isocitric dehydrogenase regenerating system. Samples were preincubated at 37°C for 5 to 10 min. Microsomal protein (0.05 mg/ml) was added to each reaction mixture and samples were incubated for 20 min at 37°C. Reactions were terminated with 100 μl of acetonitrile. Twenty microliters of buspirone (50 μM) were added as an internal standard. Samples were vortexed and centrifuged for 10 min. Supernatants were transferred to autosampling vials for HPLC analysis. All samples were incubated in duplicate.

Solubility studies showed that sildenafil is incompletely soluble in the microsomal incubation reaction mixture. Accordingly, the sildenafil concentrations used for kinetic analyses were adjusted to represent the actual sildenafil concentrations (range: 0–293 μM) present in the reaction mixture.

Samples were analyzed using a mobile phase of 30% acetonitrile/70% potassium phosphate buffer (27.75 mM, pH 4.5) at a flow rate of 1.4 ml/min, as described previously (Cooper et al., 1997). The analytical column was a stainless steel 30 cm × 3.9 mm reverse-phase NovaPak C₁₈ column (Waters Associates, Milford, MA) and column effluent was analyzed using UV detection at a wavelength of 230 nm.

Identification of UK-103,320 as the principal metabolite of sildenafil in incubation mixtures was based on its appearance as the quantitatively major metabolite product, as well as the similarity of relative retention times to previously published reports in which reference standards of UK-103,320 were available (Cooper et al., 1997). Formation of the UK-103,320 was found to be linear with respect to time up to 60 min and with respect to protein up to 0.1 mg/ml.

**Incubations Using Microsomes Containing cDNA-Expressed Cytochromes.** An initial screen with microsomes containing either one of eight cDNA-expressed cytochromes (CYP1A2, -2A6, -2B6, -2C9, -2C19, -2D6, -2E1, or -3A4), or a vector control, was conducted at two sildenafil concentrations (15 or 132 μM). Incubations were performed as described for human liver microsomes except that microsomes containing cDNA-expressed enzymes (1 mg/ml) were added in place of human liver microsomes. Full kinetic curves with increasing sildenafil concentrations were generated with microsomes containing one of four cytochromes (CYP2C9, -2C19, -2D6, or -3A4). Solubility studies with microsomes containing a cDNA-expressed vector control demonstrated that the actual sildenafil concentration present in the reaction mixture ranged from 0 to 588 μM.

**Chemical Inhibition of Sildenafil Biotransformation.** An initial screen was conducted using chemical inhibitors relatively specific to CYP2C19 (omeprazole), -2C9 (sulfaphenazole), -2D6 (quinidine), and -3A4 (ketoconazole). Inhibitor concentrations were chosen that exhibit maximal inhibition of
index substrates with relative specificity (Newton et al., 1995; Ko et al., 1997). Sildenafil (36 μM) was added to incubation tubes along with either sulfaphenazole (0 or 10 μM), omeprazole (0 or 10 μM), quinidine (0 or 10 μM), or ketoconazole (0 or 2.5 μM). The methanol was evaporated and incubations were conducted using human liver microsomes, as described above. All incubations were conducted in duplicate.

Inhibition curves using human liver microsomes were generated for two drugs known to be potent inhibitors of CYP3A4. Sildenafil (36 μM) was coincubated with ketoconazole and ritonavir at concentrations ranging from 0 to 2.5 μM. Inhibitor concentrations were chosen based on their ability to inhibit CYP3A4 with relative specificity, as described previously (von Moltke et al., 1998).

Data Analysis. The formation of UK-103,320 by human liver microsomes was consistent with Michaelis-Menten kinetics with uncompetitive substrate inhibition. The following equation was fitted to UK-103,320 data points:

\[ V = \frac{V_{\text{max}} S}{K_m + S \left(1 + \frac{S}{K_i}\right)} \]

in which V represents the velocity of UK-103,320 formation and S is the concentration of the substrate, sildenafil. \(V_{\text{max}}\) (representing the maximum reaction velocity), \(K_m\) (representing the uncompetitive substrate inhibition constant), and \(K_i\) (indicating the substrate concentration that corresponds to 50% \(V_{\text{max}}\)) were calculated by nonlinear regression. Because pure samples of UK-103,320 were not available, absolute values of \(V_{\text{max}}\) could not be calculated.

Formation of UK-103,320 by cDNA-expressed enzymes was fit to either a single enzyme Michaelis-Menten model (for CYP2C9 and -2C19) or a single enzyme Michaelis-Menten model with uncompetitive substrate inhibition (for CYP3A4). Formation of UK-103,320 by microsomes containing CYP2D6 could not be fit to Michaelis-Menten kinetics. Contributions of each cytochrome to sildenafil biotransformation were normalized for mean values of the relative abundance of individual cytochromes in the liver. The relative abundance of CYP3A4 (28.8%), CYP2D6 (1.5%), and CYP2C isoforms (18.2%) were determined by immunoquantification, as reported by Shimada et al. (1994); the relative abundance of CYP2C isoforms was subdivided into relative amounts of CYP2C9 (14.7%) and CYP2C19 (3.5%) using relative activity factors (Venkatakrishnan et al., 1998). The intrinsic clearance attributable to CYP3A4, CYP2C9, and CYP2C19 was multiplied by the relative abundance of each cytochrome in the liver. Percentage ratios of the intrinsic clearance of an individual cytochrome relative to the overall contribution of three cytochromes (CYP2C9, -2C19, and -3A4) were calculated.

Inhibitor data was analyzed by nonlinear regression to determine the estimated maximal degree of inhibition (\(E_{\text{max}}\)) and the \(IC_{50}\), which represents the inhibitor concentration that produces a reduction in metabolite formation to 50% of the control value without inhibition, as described previously (von Moltke et al., 1998).

### Table 1: Kinetic parameters for UK-103,320 formation

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Metabolite Formation</th>
</tr>
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<tbody>
<tr>
<td>Human liver microsomes</td>
<td>Mean ± S.E. for n = 4 livers</td>
</tr>
<tr>
<td>(K_m) (μM)</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td>(K_i) (μM)</td>
<td>164.7 ± 15.1</td>
</tr>
<tr>
<td>Expressed human cytochromes, Fitted function values ± asymptotic S.E.</td>
<td></td>
</tr>
<tr>
<td>CYP2C9 (V_{\text{max}}) (μM)</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>(K_m) (μM)</td>
<td>23.1 ± 7.5</td>
</tr>
<tr>
<td>(V_{\text{max}}/K_m) ratio (% of total)</td>
<td>79.1%</td>
</tr>
<tr>
<td>CYP2C19 (V_{\text{max}}) (μM)</td>
<td>0.2 ± 0.006</td>
</tr>
<tr>
<td>(K_m) (μM)</td>
<td>9.6 ± 1.2</td>
</tr>
<tr>
<td>(V_{\text{max}}/K_m) ratio (% of total)</td>
<td>20.0%</td>
</tr>
<tr>
<td>CYP2D6* (V_{\text{max}}) (μM)</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>(K_m) (μM)</td>
<td>23.1 ± 13.0</td>
</tr>
<tr>
<td>(V_{\text{max}}/K_m) ratio (% of total)</td>
<td>0.83%</td>
</tr>
</tbody>
</table>

* Data points could not be fit to Michaelis-Menten kinetics. However, CYP2D6 does not appear to account for greater than 2% of sildenafil biotransformation.
Results

A representative chromatogram of in vitro sildenafil biotransformation is shown in Fig. 1. UK-103,320 formation in liver microsomes was consistent with single-enzyme Michaelis-Menten kinetics with uncompetitive substrate inhibition (Fig. 2). Estimated $K_m$ and $K_s$ values are shown in Table 1.

A screen using microsomes containing cDNA-expressed cytochromes identified the possible roles of CYP2C9, -2C19, -2D6, and -3A4 in UK-103,320 formation. Formation of UK-103,320 using microsomes containing either CYP2C9 or -2C19 was consistent with single-enzyme Michaelis-Menten kinetics, whereas metabolite formation using microsomes containing CYP3A4 was consistent with single-enzyme Michaelis-Menten kinetics with uncompetitive substrate inhibition (Fig. 3). Formation of UK-103,320 using microsomes containing CYP2D6 could not be fit to Michaelis-Menten kinetics. However, since CYP2D6 only accounts for a small percentage of the total cytochrome content in the liver (1.5%; Shimada et al., 1994), it is unlikely to play a major role in sildenafil biotransformation. Estimated $K_m$, $K_s$, relative $V_{max}$, and relative intrinsic clearance ($V_{max}/K_m$) values for CYP2C9, -2C19, and -3A4 are shown in Table 1. Contributions of these cytochromes were determined after normalization for the predicted relative abundance of each cytochrome (Table 1). Normalization for the relative abundance of cytochromes confirms the predominant role of CYP3A4. CYP2C9 partly contributes to UK-103,320 formation, but CYP2C19 and -2D6 play only minor roles.

An initial screen of chemical inhibitors (quinidine, omeprazole, ketoconazole, and sulfaphenazole) using human liver microsomes demonstrated that only ketoconazole produced detectable inhibition of UK-103,320 formation (Fig. 4). Both ritonavir and ketoconazole produced almost complete inhibition of UK-103,320 formation at 36 $\mu$M sildenafil. The mean IC$_{50}$ values were: 0.010 ± 0.006 $\mu$M for ritonavir, and 0.019 ± 0.004 $\mu$M for ketoconazole (Fig. 5).

Discussion

Using microsomes containing lymphoblast cDNA-expressed cytochromes, we demonstrated that UK-103,320 formation is mediated by at least four cytochromes: CYP3A4, -2C9, -2C19, and -2D6. However, sildenafil is primarily metabolized by CYP3A4. The major role of CYP3A4 is evident when contributions of each cytochrome are normalized for their relative abundance in the liver. The mean $K_m$ for UK-103,320 formation in liver microsomes (14.4 $\mu$M) was similar to the $K_m$ values for UK-103,320 formation by microsomes expressing...
CYP3A4 (23.1 μM). This further supports the role for CYP3A4 in UK-103,320 formation.

Chemical inhibitor studies also support the role for CYP3A4 in the formation of UK-103,320. Ketoconazole (a CYP3A4 inhibitor) potently inhibited UK-103,320 formation, while sulfaphenazole (a CYP2C9 inhibitor), omeprazole (a CYP2C19 inhibitor) and quinidine (a CYP2D6 inhibitor) do not produce detectable inhibition of metabolite formation. Assays using microsomes containing cDNA-expressed cytochromes indicate that CYP2C9 may participate in sildenafil metabolism. However, the contribution of CYP2C9 does not appear to be quantitatively large enough for sulfaphenazole to produce detectable inhibition in human liver microsomes. Neither CYP2C19...
nor -2D6 appear to play an important role in the in vitro formation of UK-103,320. Ritonavir, a viral protease inhibitor that is also a strong inhibitor of CYP3A, impaired sildenafil biotransformation with potency similar to ketoconazole. The mean IC\textsubscript{50} value found for ritonavir (0.01 \mu M) was consistent with that recently reported (Merry et al., 1999).

Due to the high frequency of erectile dysfunction in the United States (Nachtsheim, 1998; Rosen, 1998) as well as to the possible role of a variety of diseases or pharmacological agents in the pathogenesis of sexual dysfunction (Korenman, 1998; Martinez et al., 1999), sildenafil is likely to be coadministered with other drugs. Sildenafil has already been recommended to counteract pharmacologically induced erectile dysfunction, a leading cause of sexual dysfunction (Ashton and Bennett, 1999; Nurnberg et al., 1999; Rosenberg, 1999; Schaller and Behar, 1999). Therefore, it is important to understand the potential for drug interactions with sildenafil.

In addition, in HIV patients with impotence, sildenafil may have a therapeutic advantage over intracavernosal injections due to the reduced risk of exposure of partners to infected blood (Nandwani and Gourlay, 1999). This use of sildenafil may incur a possibly clinically important drug interaction with highly active antiretroviral therapy (HAART) due to the potent inhibition of CYP3A4 by protease inhibitors, such as ritonavir, and by the non-nucleoside reverse transcriptase inhibitors such as delavirdine and efavirenz. In the United States, the possibility of a revision of sildenafil labeling to include warnings about possible drug interactions with the protease inhibitor amprenavir has been discussed in the medical press. In Europe, the manufacturer of sildenafil has agreed to change the labeling to include a warning about use of sildenafil with ritonavir. In Europe, these warnings will be extended to include other protease inhibitors.

Coadministration of sildenafil and CYP3A4 inhibitors may lead to increased plasma concentrations of sildenafil. This may, in turn, lead to an increase in adverse effects commonly associated with sildenafil such as headache, flushing, dyspepsia, and visual changes (Montorsi et al., 1999). According to one double-blind, randomized, placebo-controlled, fixed-dose study of 514 men, adverse effects of any cause were relatively frequent at 72% in patients receiving a 100-mg dose versus 33% in patients receiving placebo (Montorsi et al., 1999). Nonetheless, 95% of the adverse effects were considered mild to moderate in nature. The occurrence of most of these reactions appears to be dose-related. The in vitro studies described herein are consistent with a clinical study indicating that the coadministration of sildenafil and protease inhibitors, that inhibit CYP3A4 to varying degrees, may lead to increased plasma levels of sildenafil (Merry et al., 1999).

References