

Identification of the cytochrome P450 enzymes involved in the *N*-demethylation of sildenafil

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Aims To characterize the cytochrome P450 (CYP) enzymes responsible for the *N*-demethylation of sildenafil to its main metabolite, UK-103 320, to investigate the potential inhibitory effects of sildenafil on CYP enzymes and to evaluate the potential of selected drugs to affect sildenafil metabolism.

Methods The metabolic pathways of sildenafil *N*-demethylation were studied using human liver microsomes, as well as microsomes expressing individual human CYP enzymes. Further studies to identify the individual enzymes were performed at 2.5 and 250 μM sildenafil, and employed a combination of chemical inhibition, correlation analysis, and metabolism by expressed recombinant CYP enzymes. In addition, the effect of sildenafil on the activity of the six major drug metabolizing enzymes was investigated.

Results Sildenafil conversion was found to be mediated by at least two CYP enzymes, for which the mean kinetic parameters were $K_{m1} = 6(\pm 3 \mu\text{M})$, $K_{m2} = 81(\pm 45 \mu\text{M})$, $V_{\max1} = 22(\pm 9 \text{ pmol})$ and $V_{\max2} = 138(\pm 77 \text{ pmol})$ UK-103 320 formed $\text{min}^{-1} \text{mg}^{-1}$. At 250 μM sildenafil, *N*-demethylation was primarily mediated through the low-affinity, high- K_m enzyme (approximately 83%), whilst at 2.5 μM there was a greater role for the high-affinity, low- K_m enzyme (approximately 61%). Ketoconazole strongly inhibited metabolism at both sildenafil concentrations and was the only significant inhibitor at 250 μM sildenafil. At the lower sildenafil concentration, sulphaphenazole and quinidine also inhibited formation of UK-103 320. Overall, 75% or more of the *N*-demethylation of sildenafil at any concentration is probably attributable to CYP3A4. These results were supported by experiments using expressed human CYP enzymes, in which only CYP3A4 and CYP2C9 exhibited substantial sildenafil *N*-demethylase activity (respective K_m values of 221 μM and 27 μM). Sildenafil metabolism was inhibited by potent CYP3A4 inhibitors which are used clinically, but was found to be only a weak inhibitor of drug metabolizing enzymes itself, the strongest inhibition occurring against CYP2C9 ($K_i = 80 \mu\text{M}$).

Conclusions Evidence is provided for CYP3A4 and to a lesser extent CYP2C9-mediated metabolism of sildenafil. There is the possibility that elevated plasma concentrations of sildenafil could occur with coadministration of known inhibitors of CYP2C9 or CYP3A4. Since peak plasma concentrations of clinical doses of sildenafil are only 200 ng ml^{-1} ($\sim 0.4 \mu\text{M}$) it is very unlikely that sildenafil will significantly alter the plasma concentration of other compounds metabolized by cytochrome P450 enzymes.

Keywords: cytochrome P450 enzymes, drug interactions, sildenafil, UK-103 320

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Introduction

Sildenafil citrate (Viagra[®], 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl) phenylsulphonyl]-4-methyl piperazine, Figure 1) is a novel inhibitor of the human cyclic guanosine

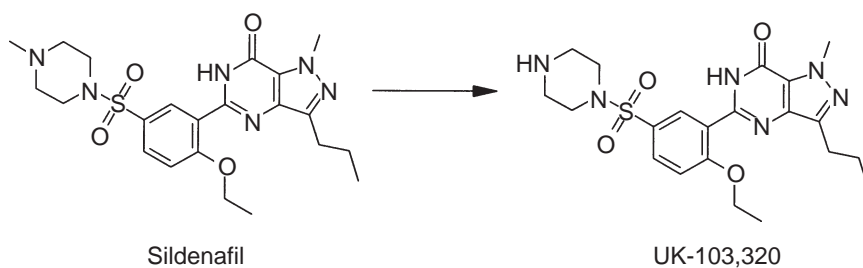


Figure 1 Structures of sildenafil and UK-103 320.

monophosphate (cGMP)-specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum and discovered through a rational drug design programme [1]. The compound is highly selective for inhibiting PDE5, with an IC_{50} of 4 nM, and has lower affinity for other PDE isozymes [2]. It has been shown to be effective as an oral treatment for male erectile dysfunction [3].

The major circulating metabolite of sildenafil results from piperazine *N*-demethylation (UK-103 320, Figure 1). Plasma concentrations of this metabolite are approximately 40% of those seen for sildenafil [4]. UK-103 320 has a PDE selectivity profile similar to that of sildenafil and a 2.5 fold lower *in vitro* potency for PDE5. This metabolite also has been identified in *in vitro* incubations with human liver microsomes. This paper describes *in vitro* studies carried out to characterize the human enzymes responsible for the *N*-demethylation of sildenafil to UK-103 320, to investigate the potential inhibitory effects of sildenafil on the CYP enzymes, and to evaluate the potential of selected drugs to affect sildenafil metabolism. Our work confirms and extends the results of a recently published study by Warrington *et al.* [21].

Methods

Drugs and chemicals

Sildenafil, authentic metabolite (UK-103 320), and an internal standard (UK-89 539) for high-performance liquid chromatography (h.p.l.c.) analysis were synthesized at Pfizer Central Research (Sandwich, UK). Furofylline (*S*)-mephentoin, 4-hydroxy mephentoin, bufuralol, 1'-hydroxybufuralol, 6-hydroxychlorzoxazone, hydroxy-terfenadine, and sulphaphenazole were obtained from Salford Ultrafine Chemicals and Research Ltd (Manchester, UK). Specific CYP cDNA-transfected human B-lymphoblastoid-derived microsomes were obtained from Gentest Corporation (Woburn, MA, USA). All other reagents were of at least Analar grade quality, obtained from Sigma Chemical Co (Poole, UK).

Preparation of liver microsomes

Transplant-quality human liver tissue was obtained from the International Institute for the Advancement of

Medicine (Exton, PA, USA). All tissues were procured with informed consent in accordance with the Uniform Anatomical Gift Act (UAGA) U.S.A. The donors ranged in age from 22 to 66 years and included nine males and five females. No donor had a known drug history, with the exception of one donor who had been treated with phenobarbitone, a known CYP3A4 inducer. No donor had a history of alcohol abuse.

Hepatic microsomes were prepared from individual human livers or a combination of four human livers by the process of differential centrifugation. Briefly, the liver tissue was homogenized in 0.05 M Tris HCl (pH 7.4) containing 0.25 M sucrose and then centrifuged at 9000 g for 20 min to remove the cell debris and nuclear fraction. The supernatant was removed and further centrifuged at 105 000 g for 60 min to pellet the microsomal fraction. This pellet was washed with 0.1 M Tris HCl (pH 7.4) and centrifuged at 105 000 g for 60 min to remove any contaminating haemoglobin. The final pellet was resuspended in 0.1 M potassium phosphate (pH 7.4) and stored at -80°C until use.

CYP content was determined using the method of Omuro & Sato [5] and the protein concentration was determined using the method of Lowry *et al.* [6], with bovine serum albumin as the protein standard.

Assay for sildenafil *N*-demethylation

The conversion of sildenafil to UK-103 320 by human liver microsomes or expressed recombinant CYP enzymes was determined according to the following method. Each incubation (final volume 1 ml) comprised of 50 mM Tris HCl (pH 7.4), 5 mM MgCl_2 , and 5 μM MnCl_2 . Reducing equivalents required for CYP metabolism were provided by NADPH, which was regenerated *in situ* by an isocitric acid/isocitric acid dehydrogenase system. The incubation mixture was preincubated for 5 min at 37°C in the presence of substrate prior to addition of NADPH.

At the end of the incubation (30 min) the reaction was terminated by the addition of 1 ml ice-cold 0.1 M Tris HCl (pH 9) followed by internal standard (UK-89 539 0.1 mg ml^{-1} , 10 μl). Samples were extracted using CH bond-elutes that had been activated with 1 ml methanol followed by 1 ml 0.1 M Tris HCl (pH 9). Samples were loaded onto SPE cartridges and slowly pushed through

(1 ml min⁻¹). The cartridges were washed with 1 ml 0.1 M Tris HCl (pH 9) and 1 ml 40% acetonitrile, and dried under vacuum. Samples were eluted with 1 ml methanol and reduced to dryness under nitrogen at 40°C. Samples were reconstituted in mobile phase (100 µl), and 80 µl was injected onto the h.p.l.c.

Samples were chromatographed on a 15 cm Spherisorb S5ODS2 column, with a mobile phase of 0.1 M *N,N,N',N'*-tetramethylethylene diamine, pH 5.1/methanol (50/50) delivered at a flow rate of 1 ml min⁻¹, and detection was by u.v. at 230 nm. The amount of UK-103 320 formed in the incubations was determined by interpolation from standard curves constructed in microsomes from 1 to 100 ng UK-103 320 (coefficient of variation was 7.9% and 4.5% for concentrations of 2.5 ng ml⁻¹ and 75 ng ml⁻¹). The rate of UK-103 320 formation was expressed as pmol UK-103 320 formed min⁻¹ mg⁻¹ microsomal protein.

Sildenafil kinetics in human liver microsomes

The rate of formation of UK-103 320 was determined in hepatic microsomal fraction prepared from three human liver samples. Initial studies were conducted to optimize the incubation time and protein concentration before the kinetic study, which was conducted over a sildenafil range of 1–750 µM. Rates of UK-103 320 formation were analysed to obtain values for K_m and V_{max} .

Chemical inhibition studies

The effect of specific inhibitors of CYP enzymes on sildenafil *N*-demethylation was investigated. The inhibitors were chosen on the basis of selective inhibition of a particular CYP enzyme, and the concentrations chosen were previously determined to cause significant inhibition of the corresponding CYP. The concentrations of inhibitor used, the CYP that they specifically inhibit, and the percentage inhibition of metabolism of a CYP specific substrate are shown in Table 1. For the mechanism-based inhibitor furafylline, a 15 min preincubation period was used. The inhibitors were coincubated with sildenafil at 2.5 and 250 µM, and their influence on the rate of UK-103 320 formation was investigated.

Correlation with probe substrate activities

A bank of 14 human livers was used to assess sildenafil metabolism. Microsomes from these livers had been previously characterized for CYP activities. A correlation was performed between sildenafil *N*-demethylation, at 2.5 and 250 µM sildenafil, and each CYP activity across the human liver bank. Probe substrates were caffeine, coumarin, phenytoin, (S)-mephenytoin, bufuralol, chlorzoxazone,

Table 1 Specific CYP inhibitors used to investigate inhibition of sildenafil *N*-demethylation by various CYP enzymes. The values are mean ± s.d. of triplicate determinations in microsomes prepared from a pool of four human livers.

<i>Isoform</i>	<i>Inhibitor</i>	<i>Concentration (µM)</i>	<i>% Effect on probe substrates</i>
CYP1A2	Furafylline	1	59 ± 6% inhibition
		10	95 ± 9% inhibition
CYP2C9	Sulphaphenazole	2.5	53 ± 4% inhibition
		25	85 ± 2% inhibition
CYP2D6	Quinidine	2.5	82 ± 3% inhibition
		25	95 ± 1% inhibition
CYP3A4	Ketoconazole	2.5	79 ± 1% inhibition
		25	88 ± 1% inhibition

and testosterone for CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, respectively. The specific analytical methods given below were used for each of these. To ensure that all the data were normally distributed, a log transformation was carried out prior to analysis to reduce the influence of very high or low activities.

H.p.l.c. analysis of probe substrates

Human liver caffeine *N*-demethylase activity was determined at 1000 µM caffeine and 1 mg ml⁻¹ microsomal protein for 60 min. After 60 min, the incubations were terminated by the addition of 7 ml dichloromethane/propan-2-ol (85/15 v/v) followed by 10 µl internal standard (β-hydroxyethyltheophylline, 100 µg ml⁻¹). After extraction, the organic layer was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 µl of h.p.l.c. mobile phase, and 80 µl was injected onto the h.p.l.c. The samples were chromatographed on a 25 cm Inertsil ODS2 column eluted at 1 ml min⁻¹ with an isocratic mixture of acetic acid (0.5%)/acetonitrile/methanol, 85/5/10 v/v/v. Detection was by u.v. absorbance (Shimadzu SPD-10 A) at 274 nm. Under these conditions, paraxanthine had a retention time of approximately 9 min, β-hydroxyethyltheophylline approximately 12 min, and caffeine approximately 17 min.

The remaining probe substrate activities were as described previously in the literature. Coumarin 7-hydroxylase activity was determined at a substrate concentration of 100 µM [7], phenytoin 4-hydroxylase activity at a substrate concentration of 500 µM [8], (S)-mephenytoin 4-hydroxylase activity at a substrate concentration of 500 µM [9], bufuralol 1'-hydroxylase at a substrate concentration of 10 µM [10], 4-nitrophenol 3-hydroxylase activity at a substrate concentration of 1000 µM [11],

and testosterone 6 β -hydroxylase activity at a substrate concentration of 250 μM [12].

Metabolism of sildenafil by expressed recombinant CYP enzymes

The formation of UK-103 320 from sildenafil (2.5 and 250 μM) was assessed in microsomes derived from specific CYP cDNA transfected human B-lymphoblastoid cells. The kinetics of UK-103 320 formation were determined in lymphoblastoid-derived microsomes expressing CYP3A4 and CYP2C9. Kinetic studies with CYP3A4 were performed at 1 mg ml^{-1} for 60 min over a substrate concentration range of 1–1000 μM , and at 0.5 mg ml^{-1} for 60 min at 1–500 μM sildenafil with CYP2C9.

Inhibition of sildenafil metabolism by CYP3A4 inhibitors

The effect of some potent CYP3A4 inhibitors which are used clinically (itraconazole, ketoconazole, indinavir, nelfinavir, saquinavir and ritonavir) on sildenafil *N*-demethylation was investigated in a pool of human liver microsomes. Incubations were performed at 2 μM sildenafil and 0.1 mg ml^{-1} protein for 15 min in the presence and absence of inhibitors (0.01–1000 μM).

Inhibition of CYP enzymes by sildenafil

The effect of sildenafil (0.01–1000 μM) on the metabolism of probe substrates for CYP enzymes was investigated in a pool of human liver microsomes. Drugs used as enzyme substrates were 10 μM phenacetin (CYP1A2), 20 μM phenytoin (CYP2C9), 130 μM (S)-mephenytoin (CYP2C19), 10 μM bufuralol (CYP2D6), 33 μM chlorzoxazone (CYP2E1), and 25 μM felodipine (CYP3A4). Specific assays were those previously described in the literature [9–13]. Samples were preincubated in the presence and absence of sildenafil (0.01–1000 μM) for 5 min at 37°C prior to the addition of NADPH. To further investigate the potential for interaction with CYP3A4, the effect of sildenafil on the metabolism of testosterone (150 μM) and terfenadine (25 μM) also was studied in the same way using specific assays [12–14].

Statistical analysis

All results are presented as mean \pm s.d. Determination of apparent K_m and V_{max} values were obtained by Grafit (version 3.01). Statistical analysis to investigate the effects of inhibitors was carried out in Microsoft Excel (version 5) using a two-sided *t*-test for independent samples. Correlations and multivariate analysis was also carried out in Microsoft Excel (version 5).

Results

Kinetics of sildenafil *N*-demethylation in human liver microsomes

The rate of formation of UK-103 320 in human liver microsomes was found to be linear with time up to 60 min and protein up to 0.1 mg ml^{-1} . Enzyme kinetics were therefore determined at a protein concentration of 0.1 mg ml^{-1} over 30 min. The apparent kinetic constants were estimated using sildenafil concentrations up to 750 μM . The conversion followed Michaelis–Menten kinetics, and examination of the Eadie–Hofstee transformation revealed a biphasic plot suggesting at least two

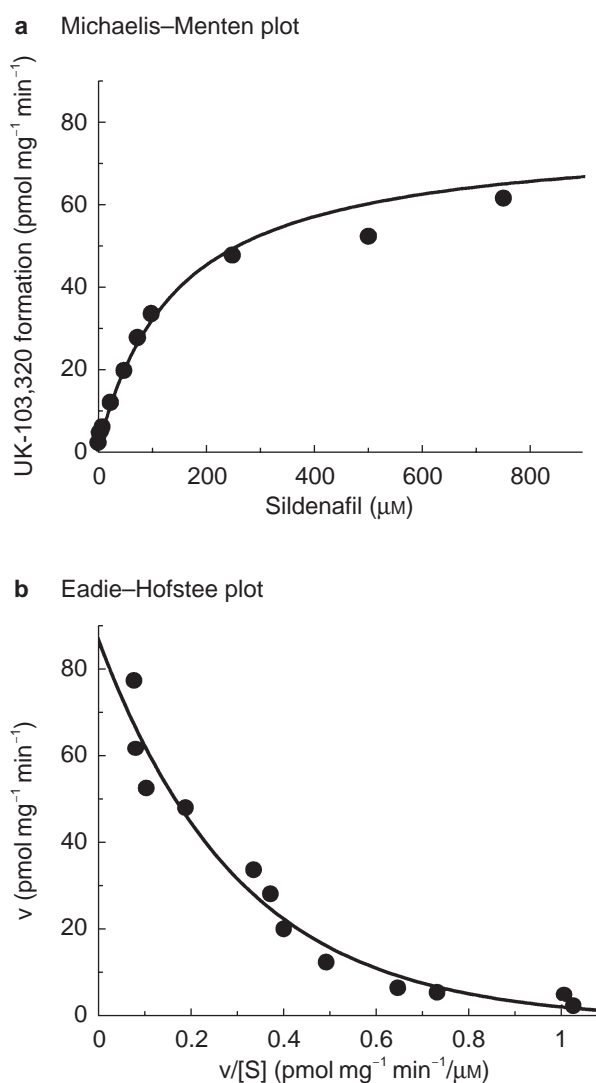


Figure 2 Representative Michaelis–Menten and Eadie–Hofstee plots for the conversion of sildenafil to UK-103 320 in human liver microsomes. Data illustrated from HM10. Each point represents the average of two determinations.

enzymes are involved in this reaction (Figure 2, for the liver designated HM10).

The apparent Michaelis–Menten kinetic parameters were estimated with the assumption that two enzymes were involved, by fitting the following equation to the data:

$$V = \frac{V_{\max 1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{\max 2} \cdot [S]}{K_{m2} + [S]}$$

where v is the velocity of formation of UK-103 320, S is the concentration of sildenafil in the incubation mixture, K_{m1} and K_{m2} are the Michaelis constants for the two enzymatic components, and $V_{\max 1}$ and $V_{\max 2}$ are the respective maximum velocities. The kinetic parameters obtained for the *N*-demethylation in the three human livers investigated are detailed in Table 2.

The mean apparent K_m values for the two components were $6 \mu\text{M}$ and $80 \mu\text{M}$, with V_{\max} values of $11\text{--}29 \text{ pmol mg}^{-1} \text{ min}^{-1}$ and $75\text{--}224 \text{ pmol mg}^{-1} \text{ min}^{-1}$, respectively. One of the livers showed autoinhibition

of the low-affinity enzyme characterized by a K_i of $655 \mu\text{M}$. No autoinhibition was seen in the other two livers.

Inhibition of sildenafil metabolism in human liver microsomes

The effects of specific CYP inhibitors on sildenafil metabolism at $2.5 \mu\text{M}$ and $250 \mu\text{M}$ were investigated using furafylline (CYP1A2), sulphaphenazole (CYP2C9), quinidine (CYP2D6), and ketoconazole (CYP3A4). These studies were carried out in microsomes produced from a combination of four human livers. The results of this investigation are illustrated in Figure 3.

Sildenafil metabolism was significantly inhibited by ketoconazole at both substrate concentrations ($P < 0.01$). At the low substrate concentration ($2.5 \mu\text{M}$), this was also true for the CYP2C9 inhibitor sulphaphenazole. There was also a significant inhibition of UK-103 320 formation at the low concentration in the presence of $25 \mu\text{M}$ quinidine but not with $2.5 \mu\text{M}$.

Table 2 Apparent kinetic parameters for the *N*-demethylation of sildenafil in three human livers.

Human liver	K_m (μM)	High affinity		Low affinity	
		K_m (μM)	V_{\max} ($\text{pmol mg}^{-1} \text{ min}^{-1}$)	K_m (μM)	V_{\max} ($\text{pmol mg}^{-1} \text{ min}^{-1}$)
HM6	3		29	75	224
HM10	7		11	129	75
HM13	8		27	39	115
Mean \pm s.d.	6 ± 3		22 ± 9	81 ± 45	138 ± 77

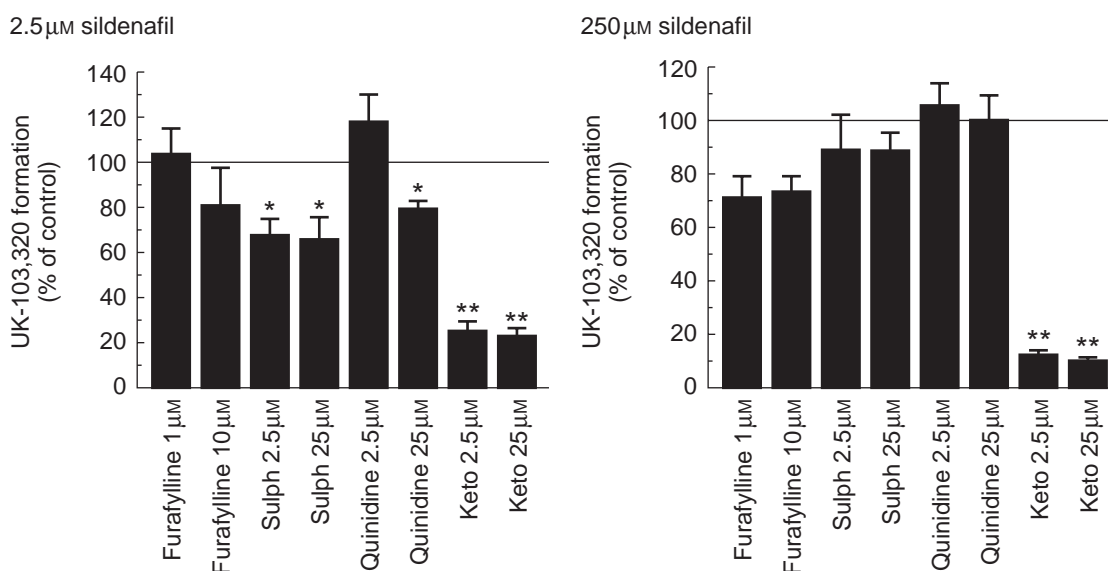


Figure 3 Effect of specific CYP inhibitors on the rate of *N*-demethylation of sildenafil ($2.5 \mu\text{M}$ and $250 \mu\text{M}$). The values are mean \pm s.d. of triplicate determinations in microsomes prepared from a pool of four human livers. * $P < 0.01$; ** $P < 0.001$ compared with the control value. sulph, sulphaphenazole; keto, ketoconazole.

Characterization of sildenafil N-demethylation in human liver bank

A bank of 14 human livers was used to assess the rate of UK-103 320 formation from sildenafil. The correlation data for sildenafil N-demethylation and the probe reactions for specific CYP enzymes are shown in Table 3. To avoid undue weighting by liver samples with very low or very high activities, all activities were correlated as their logarithm. At both concentrations of sildenafil there was a strong correlation with both CYP2C9 activity and CYP3A4 activity.

Multivariate analysis showed that the rate of UK-103 320 formation correlated strongly with both the rate of phenytoin 4-hydroxylation (CYP2C9) and testosterone 6 β -hydroxylation (CYP3A4) according to the following equations.

2.5 μM sildenafil

$$\log \text{UK-103 320 formation} = -1.7 + 0.74 \log \text{CYP2C9} + 0.49 \log \text{CYP3A4} \quad r=0.93$$

Table 3 Correlation of sildenafil N-demethylation and CYP activities (using log data for both).

Isoform (substrate)	Correlation coefficient (r)	
	2.5 μM sildenafil	250 μM sildenafil
CYP1A2 (caffeine)	0.49 ($n=10$)	0.53 ($n=10$)
CYP2A6 (coumarin)	0.24 ($n=12$)	0.44 ($n=12$)
CYP2C9 (phenytoin)	0.77* ($n=14$)	0.80** ($n=14$)
CYP2C19 (S-mephenytoin)	0.48 ($n=10$)	0.31 ($n=10$)
CYP2D6 (bufuralol)	0.34 ($n=14$)	0.33 ($n=14$)
CYP2E1 (chlorzoxazone)	0.36 ($n=14$)	0.49 ($n=14$)
CYP3A4 (testosterone)	0.87** ($n=14$)	0.84** ($n=14$)

* $P < 0.01$, ** $P < 0.001$. All other correlations were not statistically significant. n = number of human livers

250 μM sildenafil

$$\log \text{UK-103 320 formation} = 0.10 + 0.77 \log \text{CYP2C9} + 0.36 \log \text{CYP3A4} \quad r=0.91$$

The correlation equations are illustrated in Figure 4 as observed values against predicted values, calculated from the equations.

Metabolism of sildenafil in expressed recombinant CYP enzymes

The N-demethylation of sildenafil (2.5 and 250 μM) was investigated in microsomes derived from human B-lymphoblastoid cells expressing recombinant CYP1A2, CYP2C9, CYP2D6, CYP2E1, or CYP3A4 (Figure 5). At the low sildenafil concentration (2.5 μM) only CYP2C9 and CYP3A4 formed UK-103 320, whilst at the higher substrate concentration (250 μM) all the CYP enzymes formed UK-103 320 to some degree. However, CYP2C9 and CYP3A4 showed higher rates of formation. At 2.5 μM sildenafil, the rate of CYP3A4-mediated UK-103 320 formation was approximately six times greater than that of CYP2C9 determined on a pmol UK-103 320 $\text{mg}^{-1} \text{min}^{-1}$ basis. When the relative CYP activities in the expression systems were corrected for the average activity in human liver, then the rate of UK-103 320 formation was 20-fold greater for CYP3A4 compared with CYP2C9.

Kinetics of sildenafil N-demethylation by recombinant CYP3A4 and CYP2C9

The rate of formation of UK-103 320 in CYP3A4 microsomes was linear with time up to 60 min and protein up to 2 mg ml^{-1} , whilst in CYP2C9 microsomes the rate of formation was linear with time up to 60 min

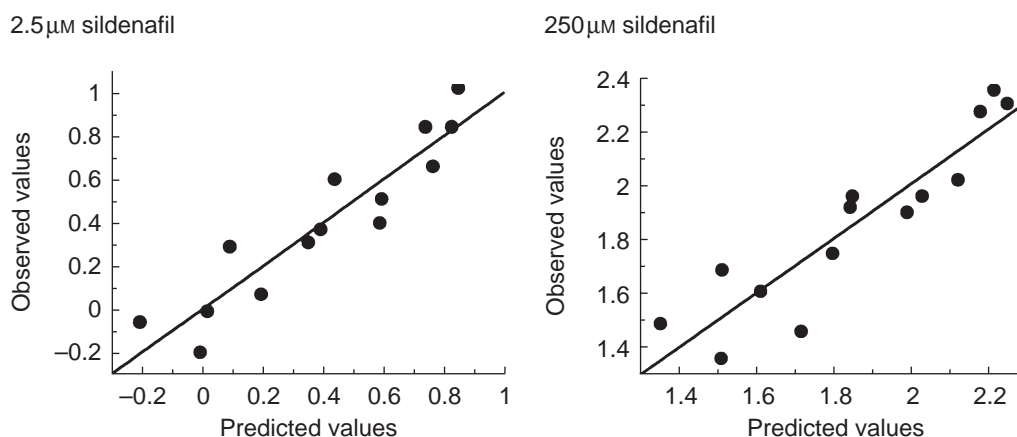


Figure 4 Plot of experimentally determined values (observed) for the rate of sildenafil conversion against values obtained using multivariate analysis to produce an equation relating the logarithm of the rate of sildenafil N-demethylation to the logarithm of the enzymatic activities of CYP2C9 and CYP3A4 in a panel of 14 human livers. Sildenafil concentrations were 2.5 μM and 250 μM and each point represents the mean \pm s.d. of triplicate determinations.

and protein up to 1 mg ml^{-1} . Data analysis indicated that the CYP3A4-mediated *N*-demethylation of sildenafil was characterized by a K_m of $221 \mu\text{M}$ and a V_{max} of $65 \text{ pmol min}^{-1} \text{ mg}^{-1}$ with autoinhibition at high concentrations (K_i of $288 \mu\text{M}$), whilst CYP2C9 mediated this transformation with a K_m of $27 \mu\text{M}$ and a V_{max} of $78 \text{ pmol min}^{-1} \text{ mg}^{-1}$ (Figure 6).

Inhibition of sildenafil metabolism by CYP3A4 inhibitors

Inhibition of sildenafil metabolism by the potent clinically used CYP3A4 inhibitors was investigated at concentrations up to $1000 \mu\text{M}$. Ritonavir, itraconazole and ketoconazole were the most potent inhibitors of sildenafil

N-demethylation, with IC_{50} values of $<0.05 \mu\text{M}$ (Table 4). The least potent inhibition was observed for saquinavir, with an IC_{50} value of approximately $5 \mu\text{M}$.

In vitro inhibition of CYP enzymes

Inhibition of CYP activity was investigated at concentrations up to $1000 \mu\text{M}$ sildenafil. Over this concentration range sildenafil was a weak inhibitor, with IC_{50} values of $>300 \mu\text{M}$ against all CYP probe substrates investigated, with the exception of CYP2C9 (Table 5). Further investigation of CYP2C9 inhibition showed it to be characterized by an approximate IC_{50} of $150 \mu\text{M}$, and a K_i of $80 \mu\text{M}$ (data not shown). Interactions with additional

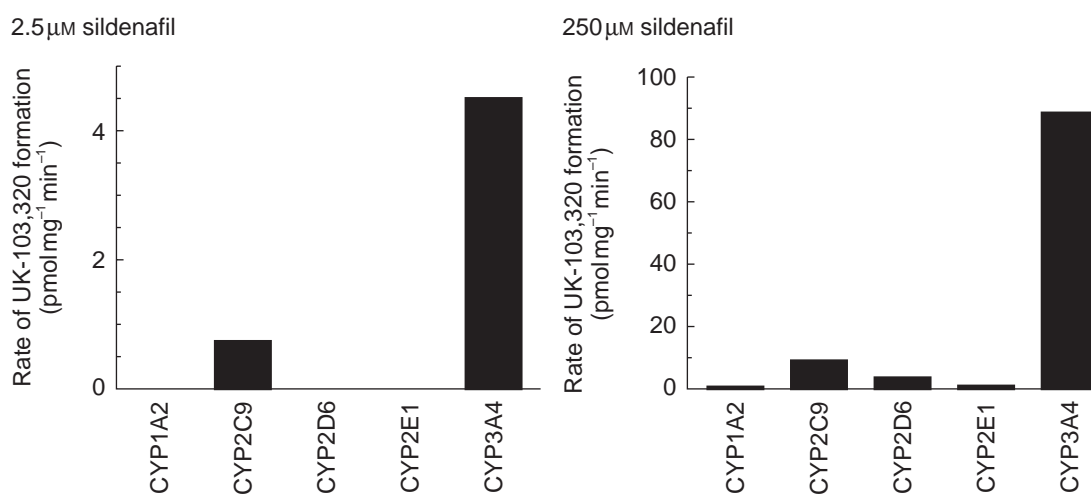


Figure 5 Rate of *N*-demethylation of sildenafil in a panel of microsomes derived from B-lymphoblastoid cells expressing CYP1A2, CYP2C9, CYP2D6, CYP2E1, or CYP3A4. Sildenafil concentrations were 2.5 and $250 \mu\text{M}$, and each assay was carried out in triplicate with results expressed as mean \pm s.d.

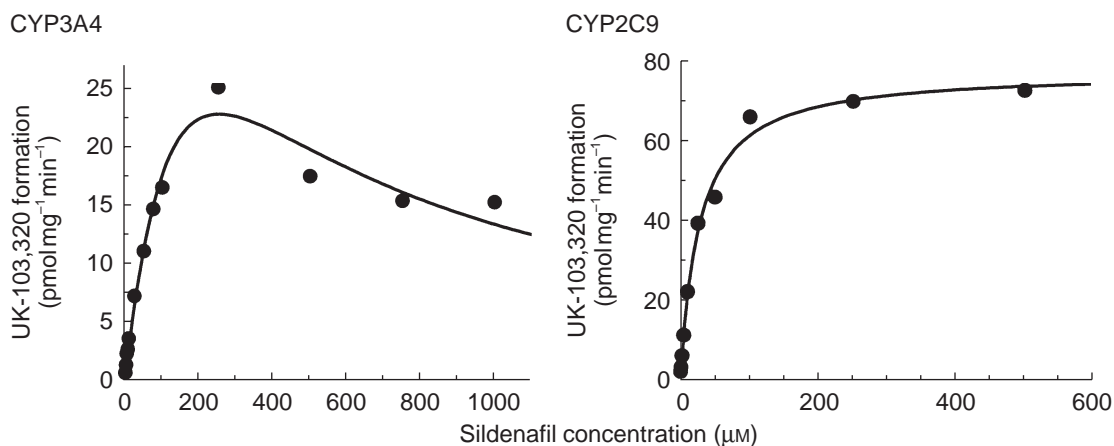


Figure 6 Michaelis–Menten plots for the conversion of sildenafil to UK-103 320 in microsomes prepared from B-lymphoblastoid cells expressing CYP3A4 and CYP2C9. Each data point represents an average of two determinations.

Table 4 *In vitro* inhibition of sildenafil metabolism by CYP3A4 inhibitors. The values are means \pm s.d. of triplicate determinations in microsomes prepared from a pool of four human livers.

Inhibitor	IC ₅₀ (μ M)
Itraconazole	0.010 \pm 0.001
Ketoconazole	0.043 \pm 0.008
Indinavir	0.254 \pm 0.073
Nelfinavir	0.611 \pm 0.113
Saquinavir	\sim 5
Ritonavir	0.003 \pm 0.0003

Table 5 *In vitro* inhibition of CYP activities by sildenafil. The values are mean \pm s.d. of triplicate determinations in microsomes prepared from a pool of four human livers.

Isozyme	Activity measured	IC ₅₀ (μ M)
CYP1A2	Phenacetin O-deethylase	> 300
CYP2C9	Phenytoin 4-hydroxylase	150 \pm 23
CYP2C19	(S)-mephenytoin 4-hydroxylase	> 300
CYP2D6	Bufuralol 1'-hydroxylase	> 300
CYP2E1	Chlorzoxazone 6-hydroxylase	> 300
CYP3A4	Felodipine oxidase	> 300
	Testosterone 6 β -hydroxylase	> 300
	Terfenadine hydroxylase	105 \pm 7

CYP3A4 substrates (testosterone and terfenadine) also indicated that sildenafil was a weak inhibitor of CYP activity, with IC₅₀ values of > 300 μ M and \sim 100 μ M, respectively.

Discussion

Sildenafil is *N*-demethylated in human liver microsomes to produce UK-103 320, which is the major circulating metabolite of sildenafil in man [4]. This pathway was found to be mediated by at least two CYP enzymes, evidenced by biphasic Eadie-Hofstee plots. The mean kinetic parameters for this reaction in microsomes from three human livers were $K_{m1} = 6 \pm 3$ μ M, $K_{m2} = 81 \pm 45$ μ M, $V_{max1} = 22 \pm 9$ and $V_{max2} = 138 \pm 77$ pmol UK-103 320 formed min⁻¹ mg⁻¹. Further studies to identify the individual enzymes were performed at 2.5 and 250 μ M sildenafil and employed a combination of chemical inhibition, correlation analysis in human liver microsomes and metabolism by expressed recombinant CYP enzymes. Using Equation 1, kinetic data indicated that at 250 μ M sildenafil, *N*-demethylation was primarily mediated through the low-affinity, high- K_m enzyme (approximately 83%), whilst at 2.5 μ M there was a greater role for the high-affinity, low- K_m enzyme (approximately

61%). However, the results of inhibition studies suggested a somewhat greater role for CYP3A4 at all concentrations.

Of the various specific CYP inhibitors examined, ketoconazole (2.5 and 25 μ M) strongly inhibited metabolism at both sildenafil concentrations and was the only statistically significant inhibitor at 250 μ M sildenafil. At the lower sildenafil concentration, sulphaphenazole (2.5 and 25 μ M) and quinidine (25 μ M) also inhibited formation of UK-103 320. Although high concentrations of ketoconazole inhibit reactions mediated by several CYP enzymes, lower concentrations specifically inhibit CYP3A4 [15–16]. These data therefore suggest a specific involvement of CYP3A4 as the low-affinity, high- K_m enzyme. The effect of ketoconazole at the low sildenafil concentration also can be attributed to the CYP3A4 contribution at this level, because it was not possible to use a concentration of sildenafil where the low-affinity enzyme contribution was negligible.

Inhibition at 2.5 μ M sildenafil also was observed with the CYP2C9 inhibitor sulphaphenazole [15], suggesting that CYP2C9 is the high-affinity enzyme. Inhibition was also observed with the higher concentration of quinidine, due to interaction with CYP3A4 rather than CYP2D6. Metabolism of quinidine itself is believed to be mediated by CYP3A4 [17], and quinidine also has been shown to be a weak inhibitor of testosterone 6 β -hydroxylase activity [16]. Several CYP3A4-mediated hydroxylations of benzodiazepines also are weakly inhibited by quinidine [18–20]. In addition, 2.5 μ M quinidine showed no inhibition, and this concentration is still very effective at inhibiting CYP2D6. The results of the inhibition studies indicate that overall, 75% or more of the *N*-demethylation of sildenafil at any concentration is attributable to CYP3A4.

In the experiment using expressed human CYP enzymes, only CYP3A4 and CYP2C9 exhibited substantial sildenafil *N*-demethylase activity. These two enzymes were characterized by K_m values of 221 μ M and 27 μ M for CYP3A4 and CYP2C9, respectively. These values are in keeping with CYP2C9 being the high-affinity enzyme and CYP3A4 the low-affinity enzyme in human liver. Hence, the metabolism of sildenafil in human liver is mediated by CYP2C9 and CYP3A4. These data are in agreement with Warrington *et al.* [21].

In addition, the effect of sildenafil itself on the activity of the six major drug metabolizing enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) was investigated. Sildenafil was found to be a weak inhibitor of all these enzymes; the most potent inhibition was against CYP2C9 activity, with a K_i of 80 μ M. Due to the major role of CYP3A4 in metabolism of sildenafil, interactions with additional CYP3A4 substrates were investigated. The inhibition of testosterone metabolism was in close agreement with that of felodipine (IC₅₀ > 300 μ M),

whilst the IC_{50} against terfenadine metabolism, although still weak, was threefold lower at 100 μM . However, marked inhibition of the metabolism of CYP2C9 substrates, or substrates for any other CYP enzymes, would not be predicted, as a 100-mg dose of sildenafil produces peak plasma concentrations of only 200 ng ml⁻¹ (approximately 0.5 μM) [4], which then decline relatively rapidly. The effects of coadministration of sildenafil with two CYP2C9 substrates, tolbutamide and warfarin, have specifically been investigated. Sildenafil did not affect either the pharmacokinetics of tolbutamide (250 mg), or the bleeding time or prothrombin time associated with a single 40 mg dose of warfarin [Data on file, Pfizer Inc].

Whilst drug–drug interactions would not be predicted on the basis of sildenafil inhibiting the metabolism of coadministered drugs, this does not preclude the possibility of sildenafil metabolism being inhibited by other drugs. As we have shown, sildenafil *N*-demethylation is mediated via CYP2C9 and CYP3A4, and any drugs that modulate their activities could affect sildenafil plasma concentrations. Based on the present data, the greatest effect is likely to be observed with inhibitors of CYP3A4, which is the major CYP enzyme present in human liver [22]. Further *in vitro* studies with the clinically used potent CYP3A4 inhibitors demonstrated a range of inhibitory potencies in close agreement with their rank order of potency against CYP3A4 [23, 24]. The net effect would be inhibition of sildenafil metabolism, leading to greater exposure to the parent drug. Clinical data support these *in vitro* findings. For example, in six HIV-positive men at steady state in treatment with indinavir, administration of 25 mg sildenafil resulted in plasma sildenafil concentrations 4.4 fold higher than data from historical controls [25]. Furthermore, treatment with ritonavir and saquinavir has recently been shown to increase plasma sildenafil concentrations by 11-fold and 4-fold, respectively [26]. Co-administration of CYP2C9 inhibitors, such as nonsteroidal anti-inflammatory agents, tolbutamide and warfarin, does not have any significant effect on the pharmacokinetics of sildenafil, whilst inhibitors of CYP3A4, including itraconazole and ketoconazole, do increase exposure to sildenafil.

This paper provides evidence for CYP2C9- and CYP3A4-mediated metabolism of sildenafil. Inhibitors of CYP enzymes, specifically the CYP3A4 inhibitor erythromycin, are known to produce significant increases in sildenafil C_{max} and AUC. Clinicians should therefore be aware of the possibility of drug–drug interactions when prescribing sildenafil concomitantly with known inhibitors of CYP3A4. Marked inhibition of the metabolism of CYP3A4 substrates, CYP2C9 substrates, or substrates for any other CYP enzymes, would not be predicted, as the clinical dose of sildenafil produces peak plasma concentrations of only 200 ng ml⁻¹.

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