Effect of tadalafil on cytochrome P450 3A4–mediated clearance: Studies in vitro and in vivo

Objectives: **Tadalafil was examined in vitro and in vivo for its ability to affect human cytochrome P450 (CYP) 3A–mediated metabolism.**

Methods: **Reversible and mechanism-based inhibition of CYP3A by tadalafil was examined in human liver microsomes. The ability of tadalafil to influence CYP3A activity was also examined in primary cultures of human hepatocytes. The effect of tadalafil on the pharmacokinetics of CYP3A probe substrates was evaluated in human volunteers before and after coadministration with either a single dose or multiple doses of tadalafil (10 or 20 mg).**

Results: **Negligible competitive inhibition of CYP3A was observed in vitro. Mechanism-based inhibition of CYP3A was detected, albeit with a low potency. In human hepatocytes, exposure to 1 μmol/L or greater of tadalafil resulted in increased CYP3A protein expression; however, as with a combined effect of induction and inhibition, a corresponding increase in CYP3A activity did not occur. The clinical pharmacokinetics of midazolam and lovastatin, probe substrates of CYP3A, were unaffected by up to 14 days of tadalafil administration (90% confidence intervals for the ratio of least squares means for the pharmacokinetic parameters of tadalafil were contained within the no-effect boundaries of 0.7 to 1.43).**

Conclusions: **In vitro results suggested that tadalafil would have little effect on the pharmacokinetics of drugs metabolized by CYP3A. Clinical studies demonstrated that the pharmacokinetics of 2 different CYP3A substrates, midazolam and lovastatin, were virtually unchanged after tadalafil coadministration. Thus therapeutic concentrations of tadalafil do not produce clinically significant changes in the clearance of drugs metabolized by CYP3A. (Clin Pharmacol Ther 2005;77:63-75.)**

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Tadalafil (Cialis) is a potent reversible phosphodiesterase-5 (PDE5) inhibitor used for the treatment of erectile dysfunction.[1-3] The recommended starting dose of tadalafil is generally 10 or 20 mg taken before anticipated sexual activity. The pharmacokinetic profile of tadalafil has been determined from dose-normalized data pooled across 13 single-dose studies of 10 or 20 mg tadalafil in healthy subjects. Peak concentrations of tadalafil are achieved over a range of 0.5 to 6 hours (median time to reach peak concentration, 2 hours). The half-life of tadalafil is 17.5 hours, with an apparent oral clearance (Cl/F) of 2.48 L/h.[4] Within 5 days of once-daily dosing, steady-state plasma concentrations are attained and exposure is approximately 1.6-fold greater than after a single dose.[5]

Physiologically, in response to sexual stimulation, nitric oxide is released into the smooth muscle of the corpus cavernosum of the penis, resulting in elevation of cyclic guanosine monophosphate (cGMP) levels and relaxation of the smooth muscle to produce an erection.[6,7] Because PDE5 inactivates cGMP, inhibition of PDE5 by tadalafil increases the intracellular levels of cGMP in the corpus cavernosum, facilitating the erectile response. Patients who are treated for erectile dysfunction are likely to take additional medications for pre-existing conditions such as cardiovascular disease

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and diabetes mellitus.[8] Therefore the potential for tadalafil to cause metabolism-based drug-drug interactions was assessed in vitro and in vivo.

The human cytochromes P450 (CYPs) are the major family of enzymes involved in the oxidative metabolism of drugs. In this family of enzymes, CYP3A is the dominant CYP in terms of both expression levels in the liver and the number of drugs metabolized.[9] As a result of the broad substrate specificity exhibited by CYP3A and the polypharmacy often used by patients, alterations in CYP3A activity by induction or inhibition can result in metabolically based drug-drug interactions. To evaluate the potential for tadalafil to cause drug-drug interactions, in vitro studies were performed examining the ability of tadalafil to alter metabolism mediated by CYP3A (and CYP1A2) in primary cultures of human hepatocytes or reversibly inhibit metabolism mediated by CYP3A (and CYP2D6, CYP2C9, CYP1A2, and CYP2C19) in human liver microsomes. In addition, tadalafil was examined for its ability to cause mechanism-based inhibition of CYP3A-mediated reactions in human liver microsomes. Mechanism-based inhibition occurs when the drug is converted to a metabolite that binds irreversibly to the enzyme active site, permanently inactivating the enzyme. The inactivated enzyme must be replaced by newly synthesized CYP to regain activity; thus recovery is slowed after mechanism-based inactivation as compared with reversible inhibition.[10] Tadalafil was examined as a mechanism-based inhibitor because of the presence of a methylenedioxyphenyl functional group in its structure.[11,12]

The in vitro results led to the conduct of clinical studies evaluating the effect of 2 dose levels of tadalafil on the pharmacokinetics of coadministered drugs that are metabolized by CYP3A. These in vivo studies were designed to examine the impact of a single dose (to investigate competitive inhibition) and multiple doses (to investigate mechanism-based inhibition and/or induction) of tadalafil on the pharmacokinetics of 2 well-recognized, sensitive probe substrates of CYP3A, midazolam and lovastatin.[13] In the clinical studies reported, 2 separate probes were tested to provide conclusive evidence concerning the effect of tadalafil on CYP3A-mediated clearance. This was thought to be especially valuable because the in vitro signals, which triggered the initial midazolam study, did not correlate with a clinical effect.

METHODS

Materials

Tadalafil and [$^{13}C^{2}H_3$]-tadalafil were obtained from Lilly ICOS LLC (Indianapolis, Ind, and Bothell, Wash). Midazolam for in vitro studies was obtained from Hoffmann-La Roche (Nutley, NJ). Bufuralol, 1′–hydroxy (OH)–bufuralol, 1′-OH-midazolam, *S*-mephenytoin, and 4′-OH-mephenytoin were obtained from Ultrafine Ltd (Manchester, United Kingdom). Midazolam for the clinical bioanalytic assay was obtained from Radian International (Austin, Tex). For in vitro bioanalytic studies, 1′-OH-[$^{13}C_5$]-midazolam was biologically derived from [$^{13}C_5$]-midazolam synthesized at Eli Lilly and Company, and for clinical bioanalytic assays, [$^{13}C_3$]-midazolam was purchased from Bridge Organics (Vicksburg, Mich). Diclofenac, phenacetin, NADPH, flunitrazepam, rifampin (INN, rifampicin), 3-methylcholanthrene, metoprolol, and salicylamide were purchased from Sigma Chemical Co (St Louis, Mo). 4′-OH-diclofenac was obtained from BD Gentest (Woburn, Mass). Acetaminophen was obtained from Kodak (Rochester, NY). Trolox and 4′-OH-phenytoin were obtained from Aldrich Chemical Co (Milwaukee, Wis). Resorufin and 7-ethoxyresorufin were obtained from Molecular Probes (Eugene, Ore). Lovastatin and simvastatin for bioanalytic assays were obtained from US Pharmacopeia (Rockville, Md). Hepatocyte Maintenance Medium (HMM) was purchased from BioWhittaker (Walkersville, Md). Mouse monoclonal antibody to CYP3A was a gift from P. Beaune at Universite de Liege (Liege, Belgium).[14] Horseradish peroxidase–conjugated secondary antibodies were purchased from Bio-Rad (Hercules, Calif). ECL+ detection reagents were purchased from Amersham (Arlington Heights, Ill).

Human liver samples designated HLB, HLH, HLM, and HLP were obtained from the liver transplant units at the Medical College of Wisconsin (Milwaukee, Wis) and Indiana University School of Medicine (Indianapolis, Ind) under protocols approved by the appropriate committees for the conduct of human research. Microsomes were prepared by differential centrifugation[15] and stored at −70°C. A mixture of equal protein concentrations of microsomes from HLB, HLH, HLM, and HLP was prepared and used in the in vitro microsomal studies. Microsomes prepared from a baculovirus-infected insect cell system containing complementary deoxyribonucleic acid–expressed CYP3A4, CYP reductase, and cytochrome b_5 (Supersomes) were obtained from BD Gentest (Woburn, Mass). Primary cultures of human hepatocytes were obtained from S. Strom at the University of Pittsburgh (Pittsburgh, Pa).[16]

In vitro reversible inhibition

Microsomal incubations were performed in duplicate with form-selective CYP substrates and 1-mmol/L

NADPH under linear rate conditions (CYP1A2 [acetaminophen (INN, paracetamol) formation], 0.5 mg/mL protein and 30-minute incubation; CYP2C9 [4′-OH-diclofenac formation], 0.25 mg/mL protein and 15-minute incubation; CYP2C19 [4′-OH-mephenytoin formation], 0.5 mg/mL protein and 30-minute incubation; CYP2D6 [1′-OH-bufuralol formation], 0.1 mg/mL protein and 30-minute incubation; and CYP3A [1′-OH-midazolam formation], 0.5 mg/mL protein and 1-minute incubation), with or without tadalafil. The samples were analyzed for the formation of the form-selective metabolite, and where warranted, an apparent K_i value (dissociation constant for the enzyme inhibitor complex) was generated by fit of the appropriate inhibition model to the data.[17-21] Concentrations of substrate and tadalafil for the various reactions were as follows: midazolam (CYP3A), 5, 10, 25, 50, or 100 μmol/L, and tadalafil, 1, 10, 25, or 50 μmol/L; bufuralol (CYP2D6), 5 μmol/L, and tadalafil, 0.5, 1, 10, 25, 50, or 100 μmol/L; diclofenac (CYP2C9), 2.5, 5, 10, 25, or 50 μmol/L, and tadalafil, 10, 25, 50, or 100 μmol/L; phenacetin (CYP1A2), 12.5, 25, 50, 75, or 100 μmol/L, and tadalafil, 0.1, 1, 10, or 25 μmol/L; and *S*-mephenytoin (CYP2C19), 5, 10, 25, 50, or 100 μmol/L, and tadalafil, 35, 50, 65, or 80 μmol/L. The potential for significant drug-drug interaction was evaluated by calculation of a ratio of inhibitor concentration (I) over K_i, where a ratio lower than 0.1 suggests low risk for drug-drug interactions, 0.1 to 1 suggests medium risk, and greater than 1 suggests high risk.[13]

In vitro mechanism-based inhibition (CYP3A)

CYP3A4 Supersomes (20 pmol CYP/mL) were preincubated in 100-mmol/L sodium phosphate buffer, pH 7.4, containing 1-mmol/L ethylenediaminetetraacetic acid, and tadalafil (0, 1, 2.5, 5, 10, or 20 μmol/L), erythromycin (0, 5, 10, 25, or 50 μmol/L), or diltiazem (0, 0.1, 0.5, 1, or 5 μmol/L) for 3 minutes at 37°C in duplicate. The mechanism-based inhibition reaction was initiated with the addition of NADPH (1 mmol/L). After incubations at various times, an aliquot of the mixture was withdrawn and diluted 20-fold into a prewarmed (37°C) CYP3A4 activity assay incubation system containing 1-mmol/L NADPH and midazolam (100 μmol/L). This activity assay mixture was allowed to incubate a further 2 minutes (linear rate conditions), and the supernatant was analyzed for 1′-OH-midazolam levels.[20]

To obtain the mechanism-based inhibition kinetic parameters of k_{inact} (the formation rate constant of the inactive complex with the enzyme) and K_I (the dissociation constant for the inactivator), equation 1[22] was fit to the observed rate of 1′-OH-midazolam formation by the samples after different times of incubation with tadalafil, erythromycin, or diltiazem by use of WinNonlin Professional software (Pharsight Corporation, Mountain View, Calif) as follows:

$$\text{Percent inhibition}_{(t)} = 100_{(t=0)} \cdot e^{(-\lambda t)} \qquad (1)$$

where λ, the pseudo first-order rate constant for enzyme inactivation, was defined by the following:

$$\lambda = (k_{inact} \cdot I)/(K_I + I) \qquad (2)$$

The mechanism-based inhibitory potency of the tested compounds was evaluated by calculating inactivation clearance (Cl_{inact}) (ratio of k_{inact}/K_I).[23]

In vitro human hepatocyte incubations

Hepatocyte monolayers in 6-well culture plates (approximately 1×10^6 cells/well) were incubated in triplicate with tadalafil (0.1, 1, 3, or 10 μmol/L), vehicle control (0.1% dimethylsulfoxide), or known inducers (1 μg/mL 3-methylcholanthrene or 10 μmol/L rifampin) in HMM for 48 hours. For the short-term experiment, cultures were treated for 0, 5, 15, 30, or 60 minutes with 0.1, 1, and 10 μmol/L tadalafil. After incubation with tadalafil, the medium was removed and cells were rinsed with HMM and incubated with midazolam (10 μmol/L) or 7-ethoxyresorufin (2 μmol/L) in HMM (containing 3 mmol/L salicylamide) for 30 minutes. Samples of the medium were analyzed by validated assays for the formation of the products 1′-OH-midazolam or resorufin.[20,24] Cells were harvested and protein content determined by the method of Lowry et al.[25] To determine which treatment groups were statistically different from controls, a variety of statistical evaluations were performed by use of JMP software (SAS Institute, Cary, NC) as described previously.[26]

After 48 hours of exposure to tadalafil, CYP3A4 immunoreactive protein content in the hepatocyte cultures was determined by Western blot analysis. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) followed by transfer to nitrocellulose paper. The blot was probed with a mouse monoclonal antibody to CYP3A4 followed by a goat antimouse horseradish peroxidase–conjugated antibody.[27] The blots were developed with ECL+ reagents according to the manufacturer′s instructions, visualized by use of a Storm 860 imager (Molecular Dynamics, Sunnyvale, Calif) and quantified by use of ImageQuant v 3.3 (Molecular Dynamics).

Clinical investigations

Open-label outpatient studies were conducted to examine the effect of coadministration of 10 or 20 mg tadalafil on the pharmacokinetics of 2 well-established probes of CYP3A activity, midazolam and lovastatin. Studies were conducted and patient written informed consent was obtained in conformity with the ethical principles of the Declaration of Helsinki (adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964, and revised at the World Medical Assembly, Tokyo, Japan, 1975; Venice, Italy, 1983; and Hong Kong, 1989) and the applicable European laws. Approval of the midazolam protocol and consent form was obtained by the Medeval Independent Ethics Committee, and the study was conducted by Medeval Ltd, Manchester, United Kingdom. Approval of the lovastatin protocol and consent form was obtained by the Independent Ethical Committee of the Phase I Clinical Trials Unit Ltd, Plymouth, United Kingdom, and the study was conducted by the Phase I Clinical Trials Unit Ltd. All subjects were overtly healthy as determined by medical history and physical examination. Alcohol use was not permitted for 48 hours preceding the days of pharmacokinetic blood sampling. At other times during both studies, alcohol use was permitted and smoking was permitted in the lovastatin interaction study, because neither of these habits affects CYP3A activity.[28]

Study subjects and experimental protocol for midazolam-tadalafil interaction study. For the midazolam-tadalafil interaction study, 12 male subjects (10 white, 1 biracial [black and white], and 1 black Caribbean subject) entered the study. All subjects were nonsmokers or former smokers who had stopped smoking more than 6 months before screening. Eleven subjects reported alcohol consumption ranging from 2 to 26 U (1 U of alcohol defined as 0.5 pt [284 mL] of beer or lager, 1 glass of wine, or 25 mL of spirits) per week before the study. Although alcohol use was discouraged, alcohol consumption of no more than 2 U/d was allowed during the study.

Tadalafil (10-mg tablets) was given once daily for 14 consecutive days (days 15 to 28). Midazolam (Dormicum; Hoffmann-La Roche) was administered as a single 15-mg tablet on 5 separate occasions (days 1, 8, 15, 28, and 42). Subjects were required to abstain from food and fluids, with the exception of water, starting at midnight before each dose. Water was not permitted from the beginning of dosing until 2 hours after tadalafil dosing. Food was consumed at least 2 hours after either midazolam or tadalafil dosing. Xanthine-containing drinks were restricted to 2 cups per day from 48 hours before the first dose of tadalafil until discharge, and consumption of grapefruit-containing products was not allowed from 48 hours before the first dose of midazolam until discharge.

Plasma concentrations of midazolam were measured on days 1 and 8 (to obtain baseline concentrations from midazolam administered alone on both days), 15 (first dose of tadalafil), 28 (last dose of tadalafil), and 42 (after a 2-week washout period after completion of tadalafil dosing). Samples for midazolam were collected before dosing and at 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 16, and 24 hours after dosing. Plasma concentrations of tadalafil were measured before dosing and 3 hours after dosing on days 15, 21, 28, and 42.

Midazolam concentrations were measured by use of a validated HPLC assay with tandem mass spectrometric detection (HPLC-MS/MS), with a lower limit of quantitation of 0.5 ng/mL. After addition of [$^{13}C_3$]-midazolam internal standard to the sample, the analytes were extracted with methyl *tert*-butyl ether and reconstituted in methanol for direct injection by the autosampler. Chromatography of the extracts was performed by use of a BetaBasic (100 × 2 mm, 5 μmol/L) C18 column (Keystone Scientific, Bellfonte, Pa) at 45°C and a mobile phase consisting of 2.5-mmol/L ammonium acetate buffer in 50:50 (vol/vol) methanol/water (mobile phase A) and methanol (mobile phase B). The gradient profile was as follows (in minutes per percent mobile phase B): 0/0, 2/40, 2.2/90, 2.7/90, and 3/0, with a column flow rate of 0.4 mL/min and a total run time of 6 minutes. The extracts were analyzed on a Finnegan TSQ-7000 equipped with a Finnegan APCI Interface (Finnegan Corporation, San Jose, Calif). Tandem mass spectrometry (positive ion mode) was used to monitor the transitions mass-to-charge ratio (*m/z*) 326.1 → 291.1 for midazolam and *m/z* 329.1 → 294.1 for internal standard. Standard curves and quality control samples were analyzed with the study samples, with the overall accuracy (percent relative error) of the method being lower than 17% and overall precision (percent coefficient of variation) being lower than 7%.

Tadalafil concentrations were measured by use of a validated HPLC-MS/MS assay with a lower limit of quantitation of 0.5 ng/mL. After addition of [$^{13}C^2H_3$]-tadalafil internal standard to the sample, the analytes were extracted by use of 3M Empore 3-mL/7-mm C2 disk extraction cartridges (3M Company, St Paul, Minn). Elution was achieved with 150 μL of methanol/water (90:10 [vol/vol]). Extracts were diluted 1:2 in water and underwent chromatography by use of a Luna (100 × 4.6 mm, 5 μm) phenyl-hexyl column (Phenomenex, Torrance, Calif), ambient column temperature, and a mobile phase consisting of methanol/water (90:10 [vol/vol]). The isocratic flow rate was 1.0 mL/min, with a total run time of 3 minutes. The extracts were analyzed on a PE Sciex API III Plus mass spectrometer equipped with a Sciex APCI Interface (Applied Biosystems/MDS Sciex, Foster City, Calif). Tandem mass spectrometry (positive ion mode) was used to monitor the transitions *m/z* 390 → 268 for tadalafil and *m/z* 394 → 272 for the internal standard. Standard curves and quality control samples were analyzed with the study samples, with the overall accuracy (percent relative error) of the method being lower than 10% and overall precision (percent coefficient of variation) being lower than 11%.

Study subjects and experimental protocol for lovastatin-tadalafil interaction study. For the lovastatin-tadalafil interaction study, 16 white subjects (10 women and 6 men) entered the study. Eight subjects smoked tobacco (ranging from 2 to 8 cigarettes per day) and 14 consumed alcohol (ranging from 1 to 20 U/wk) before screening. Female subjects of childbearing potential (not surgically sterilized between menarche and menopause) had a negative pregnancy test result at the time of enrollment and were using a reliable method of contraception (no subjects were taking oral contraceptives).

Tadalafil (two 10-mg tablets) was given once daily for 14 consecutive days (study days 8 to 21). Lovastatin (Mevacor; Merck Frosst Canada Ltd, Montreal, Quebec, Canada) was administered as a single 40-mg dose on 4 occasions (study days 1, 9, 21, and 35). Subjects were required to eat a standard breakfast 1 hour before tadalafil and/or lovastatin administration on each pharmacokinetic sampling day to maximize the absorption of lovastatin.[29] Water was not permitted for up to 2 hours after lovastatin dosing. Restrictions regarding xanthine-containing drinks, alcohol, and grapefruit products were similar to those for the midazolam study.

Plasma concentrations of lovastatin were measured on days 1 (baseline concentrations from lovastatin administered alone), 9 (second dose of tadalafil), 21 (last dose of tadalafil), and 35 (after a 2-week washout period after completion of tadalafil dosing). Samples for lovastatin were collected before dosing and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 9, 12, 16, and 24 hours after dosing. Plasma concentrations of tadalafil were measured before dosing and at 0.5, 1, 2, 3, 4, 6, 12, 16, and 24 hours after dosing on days 9 and 21.

Tadalafil concentrations were measured by use of a validated HPLC-MS/MS assay as outlined above. Lovastatin concentrations were measured by use of a validated HPLC-MS/MS assay, with a lower limit of quantitation of 0.25 ng/mL. After addition of simvastatin internal standard to the sample, the analytes were extracted by use of Isolute C18 solid-phase extraction cartridges (100 mg sorbent; Isolute International Sorbent Technology, Ystrad Mynach, United Kingdom). The cartridges were conditioned with methanol and 10-mmol/L ammonium acetate (pH 4.5) and washed with HPLC-grade water and 10:90 water/acetonitrile (vol/vol), and the analytes were eluted with acetonitrile. After evaporation, extracts were reconstituted in HPLC mobile phase for direct injection by the autosampler. Extracts underwent chromatography by use of a Phenomenex Luna C18(2) (50 × 2.1 mm, 5 μmol/L) column and a mobile phase consisting of 50% 4-mmol/L ammonium acetate buffer (pH 4.5)/acetonitrile (20:80 [vol/vol]) and 50% acetonitrile with a column flow rate of 0.3 mL/min and a total run time of 3.5 minutes. The extracts were analyzed on a PE Sciex API III Plus equipped with a Turbo Ion Spray interface (PE Sciex, Concord, Ontario, Canada). Tandem mass spectrometry (positive ion mode) was used to monitor the transitions *m/z* 405 → 285 for lovastatin and *m/z* 419 → 285 for internal standard. Standard curves and quality control samples were analyzed with the study samples, with the overall accuracy (percent relative error) of the method being lower than 14% and overall precision (percent coefficient of variation) being lower than 8%.

Pharmacokinetic and statistical analyses

Pharmacokinetic parameters were calculated by noncompartmental methods by use of WinNonlin Professional software. Statistical evaluation was conducted by use of a mixed effects model by ANOVA techniques on the log-transformed data.[30] The geometric least squares (LS) mean ratios and 90% confidence intervals (confidence intervals were predefined in the clinical protocols) for comparisons were calculated. An equivalence approach was used to compare area under the curve (AUC) and peak concentration (C_{max}) values for midazolam or lovastatin with and without coadministration of tadalafil. No clinically significant interactions were declared if the 90% confidence intervals for the ratio of geometric LS means of these parameters were contained within the equivalence limits of 0.70 to 1.43. The sample size of the studies was chosen to have a greater than 90% chance to rule out a clinically significant effect by use of these confidence intervals.

RESULTS

In vitro reversible inhibition

Tadalafil was examined for its ability to reversibly inhibit CYP form–selective catalytic activities. The K_i values for the inhibition by tadalafil of CYP3A-, CYP2C9-, CYP2C19-, and CYP1A2-mediated metabolism were 41 ± 5 μmol/L (noncompetitive), 66 ± 6

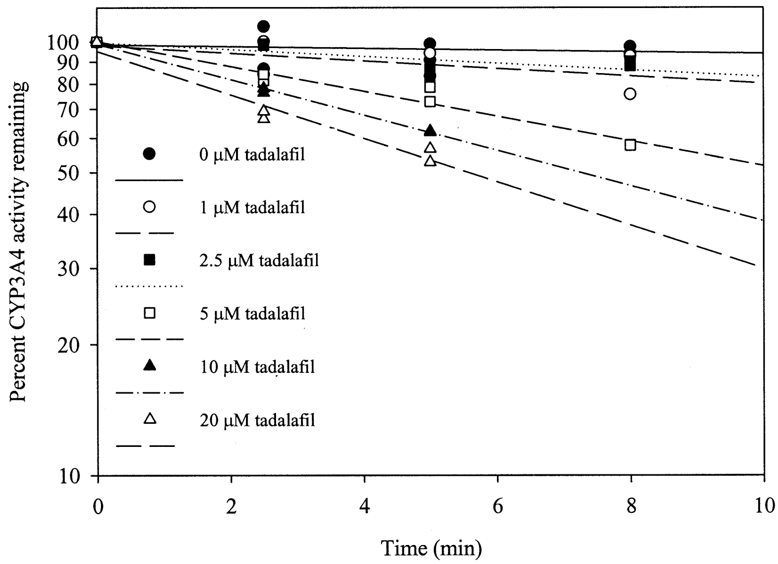

Fig 1. Time- and concentration-dependent loss of CYP3A4-mediated formation of 1′–hydroxy (OH)–midazolam by tadalafil after incubation with 100 μmol/L midazolam.

μmol/L (competitive), 73 ± 8 μmol/L (noncompetitive), and 14 ± 1 μmol/L (noncompetitive), respectively. By use of these K_i values, given an inhibitor concentration (I) of 2.02 μmol/L, which is the highest individual plasma tadalafil concentration observed for once-daily dosing of 20 mg, I/K_i ratios of 0.05, 0.03, 0.03, and 0.14 were obtained for CYP3A, CYP2C9, CYP2C19, and CYP1A2, respectively. Little inhibition (≤15%) of bufuralol 1′-hydroxylation by CYP2D6 occurred at a Michaelis-Menten constant concentration of bufuralol (5 μmol/L) with up to 100 μmol/L tadalafil.

In vitro mechanism-based inhibition

In these studies tadalafil (Fig 1), erythromycin (data not shown), or diltiazem (data not shown) inhibited 1′-OH-midazolam formation in a time- and concentration-dependent manner. Values for k_{inact} and K_I of 0.21 ± 0.04 min^{-1} and 12 ± 4 μmol/L, respectively, were determined by fit[19] of equation 1 to the data for tadalafil inhibition and used to calculate a Cl_{inact} of 17 $min^{-1} \cdot mmol/L^{-1}$. An erythromycin k_{inact} value of 0.30 ± 0.02 min^{-1} and K_I value of 5.1 ± 1.5 μmol/L and a diltiazem k_{inact} value of 0.17 ± 0.02 min^{-1} and K_I value of 0.52 ± 0.11 μmol/L were also determined. Calculated values of Cl_{inact} for erythromycin and diltiazem were 59 $min^{-1} \cdot mmol/L^{-1}$ and 327 $min^{-1} \cdot mmol/L^{-1}$, respectively, which were 3- to 19-fold greater than the Cl_{inact} value for tadalafil.

Hepatocyte incubations

Tadalafil (0.1 to 10 μmol/L) was examined for its ability to induce or inhibit CYP1A2 (7-ethoxyresorufin deethylation) and CYP3A (midazolam 1′-hydroxylation) after incubation for 48 hours in cultures of primary human hepatocytes. These activities were compared with vehicle-treated cultures and in cultures exposed to known inducers of CYP1A2 (3-methylcholanthrene) or CYP3A (rifampin). In hepatocyte cultures from 2 different donors, the positive control 3-methylcholanthrene induced CYP1A2 activity by 47- and 118-fold. In 1 culture a slight increase (1.7-fold) in CYP1A2 activity was observed after exposure to 10 μmol/L tadalafil, and in the second culture, no significant induction was observed (data not shown). Tadalafil was also examined for its ability to alter CYP3A activity (Fig 2). A significant induction of 1′-OH-midazolam formation (6.6- and 13.3-fold increase in activity) was observed with 10 μmol/L rifampin (positive control). In contrast, exposure to 0.1 μmol/L tadalafil did not affect either CYP3A activity or immunoreactive protein levels in these samples. Induction of CYP3A protein levels clearly occurred with exposure of the hepatocytes to 1 μmol/L tadalafil or greater. Increased CYP3A activity was observed with 1 μmol/L tadalafil, but this induction response was decreased after exposure to 10 μmol/L tadalafil (Fig 2).

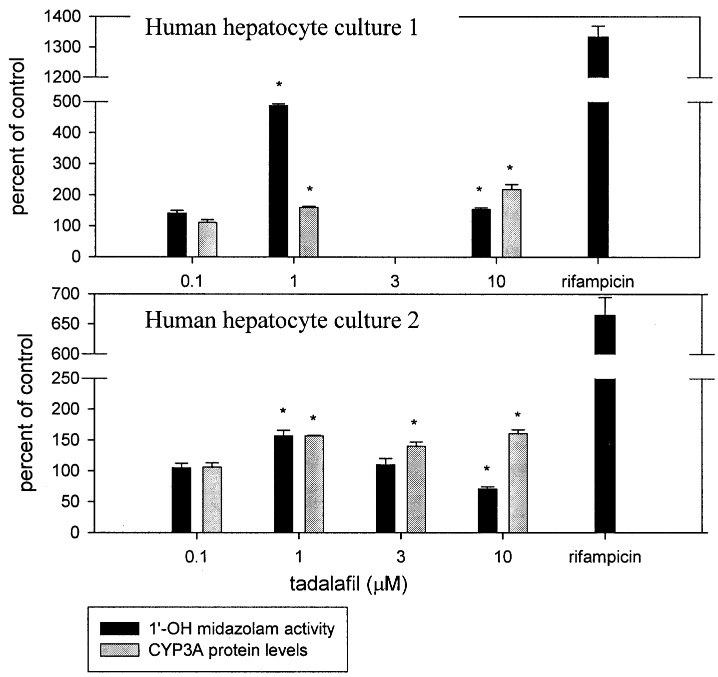

Fig 2. Effect of tadalafil treatment on midazolam 1′-hydroxylase activity (*black bars*) and CYP3A protein expression (*gray bars*) (triplicate determination) of 2 human hepatocyte preparations. Activities are expressed as percent of control with SEM. *Asterisk*, Significantly different from control (see Methods section).

Table I. Geometric mean (percent coefficient of variation) and statistical comparison of midazolam pharmacokinetic parameters after single oral dose (15 mg midazolam) on days 1, 8, 15, 28, and 42 and daily oral dose of tadalafil from days 15 to 28 in 10 healthy subjects

Parameter	*Day 1: Midazolam alone*	*Day 8: Midazolam alone*	*Day 15: Midazolam with day 1 tadalafil*	*Day 28: Midazolam with day 14 tadalafil*	*Day 42: Midazolam alone*
AUC (μg · h/L)	206 (37.8)	222 (50.2)	235 (51.7)	185 (48.0)	218 (54.0)
Ratio and 90% CI of geometric LS means*		1.08 (0.89-1.30)	1.10 (0.96-1.25)	0.87 (0.76-0.99)	1.02 (0.90-1.16)
C_{max} (μg/L)	110 (46.3)	115 (39.5)	114 (43.4)	117 (28.9)	127 (45.0)
Ratio and 90% CI of geometric LS means		1.04 (0.75-1.43)	1.02 (0.85-1.23)	1.05 (0.87-1.26)	1.13 (0.94-1.36)
Cl/F ($L \cdot h^{-1} \cdot kg^{-1}$)	0.357 (35.9)	0.334 (53.7)	0.319 (54.0)	0.404 (48.7)	0.342 (55.0)
Ratio and 90% CI of geometric LS means		0.93 (0.77-1.12)	0.91 (0.80-1.04)	1.15 (1.02-1.31)	0.98 (0.86-1.12)

AUC, Area under curve; CI, confidence interval; LS, least squares; C_{max}, peak concentration; Cl/F, apparent oral clearance.
*Ratio and 90% CI for day of treatment versus baseline (mean of days 1 and 8).

These results suggest both inductive and inhibitory effects on CYP3A.

For an evaluation of mechanism-based inhibition of CYP3A activity in hepatocyte cultures, an additional experiment was performed to investigate the effect of short-term exposure (0 to 60 minutes) to tadalafil. At 0.1 and 1 μmol/L tadalafil, a slight inhibitory effect (15% to 31%) on CYP3A-mediated midazolam 1′-

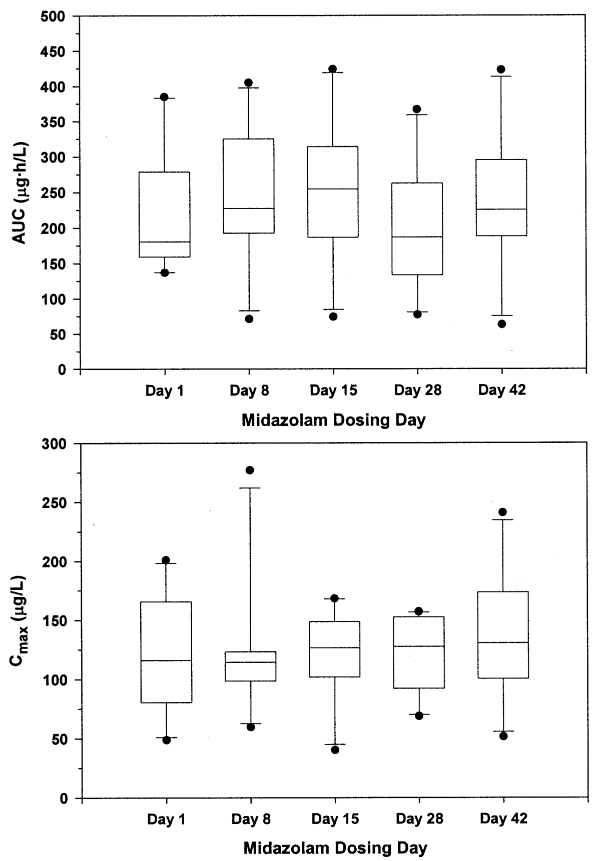

Fig 3. Midazolam pharmacokinetic parameters after administration of 15 mg midazolam at baseline (days 1 and 8), after first dose (day 15) and last dose (day 28) of daily dosing with 10 mg tadalafil, and after 2-week washout period (day 42). *Boxes* indicate 25th and 75th percentiles, and *whisker bars* indicate 10th and 90th percentiles. *Solid horizontal bars* within boxes indicate mean data. *Circles* represent values falling outside 10th and 90th percentiles. AUC, Area under curve; C_{max}, peak concentration.

hydroxylase activity was seen. With 10 μmol/L tadalafil, statistically significant time-dependent inhibition of 1′-OH-midazolam formation was observed, with inhibition ranging from 51% to 82% at 5 and 60 minutes of exposure, respectively.

Clinical investigations

Midazolam-tadalafil interaction study. Twelve healthy male subjects entered the midazolam-tadalafil interaction study. All subjects were aged between 24 and 58 years (mean, 33 ± 10.8 years), with body weights and heights ranging between 57.5 and 95.0 kg (mean, 73.8 ± 10.38 kg) and 169 and 186 cm (mean, 179 ± 5.6 cm), respectively. Ten subjects completed the study, whereas 2 discontinued because of adverse events. These 2 subjects received 3 of the 5 planned doses of midazolam (days 1, 8, and 15) and 11 of the 14 planned doses of tadalafil (days 15 to 25). Adverse events were similar to those reported previously[2,31] and included headache and myalgia.

Summary pharmacokinetic parameters for midazolam are shown in Table I. Summary statistics were representative of individual subject data (data not shown). Data are shown for the 10 subjects completing the study, although midazolam summary pharmacokinetic parameters were similar on days that data were available for all 12 subjects (data not shown). The distributions of midazolam AUC and C_{max} values suggested a negligible effect of tadalafil relative to the control treatments on days 1, 8, and 42 (Fig 3). A statistical comparison of the primary pharmacokinetic parameters of midazolam was conducted for baseline day 8 and compared with baseline day 1 to assess the variability in midazolam pharmacokinetics (Table I). The 90% confidence intervals for the ratio of geometric LS means for the pharmacokinetic parameters of midazolam were evaluated on days 15 (first dose of tadalafil), 28 (last dose of tadalafil), and 42 (after a 2-week washout period after completion of tadalafil dosing) versus mean baseline values (mean of days 1 and 8) to determine whether they were contained within the equivalence limits of 0.70 to 1.43 (Table I). Comparison of these geometric means showed no statistically significant differences relative to baseline for midazolam AUC, C_{max}, or Cl/F on tadalafil treatment days 1 or 14 (study days 15 or 29) or after a 2-week washout period after the last dose of tadalafil.

Plasma concentrations of tadalafil were measured to verify that subjects were exposed to the study drug. Tadalafil concentrations were measured before dosing and at 3 hours after dosing on days 15 (first dose of tadalafil), 21 (day 7 of tadalafil dosing), 28 (last dose of tadalafil), and 42 (after a 2-week washout period after completion of tadalafil dosing) (Fig 4). From the 3-hour data, it was determined that exposures after single and multiple tadalafil doses were within the ranges expected for a 10-mg tadalafil dose.[5]

Lovastatin-tadalafil interaction study. All 16 subjects completed the lovastatin-tadalafil interaction study. Their ages, body weights, and heights were between 22 and 47 years (mean, 38 ± 7.2 years), 57.8 and 99.1 kg (mean, 73.7 ± 12.91 kg), and 156 and 185 cm (mean, 170 ± 9.8 cm), respectively. Previously reported adverse events with tadalafil were also reported in this study as outlined here.

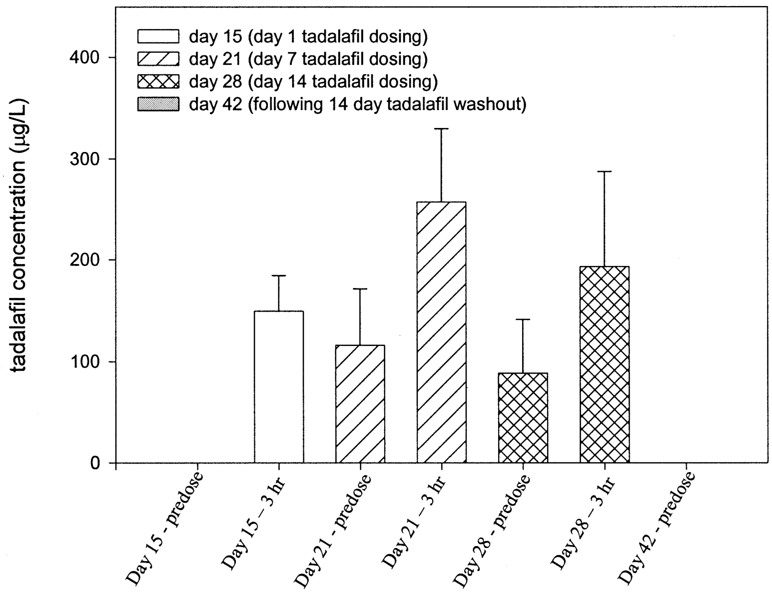

Fig 4. Arithmetic mean plasma concentrations of tadalafil in midazolam interaction study before dosing (0 hours) and at 3 hours on days 1, 7, and 14 (study days 15, 21, and 28, respectively) of once-daily multiple-dose administration of 10 mg tadalafil orally and after 14-day washout period (study day 42) in healthy subjects (n = 10).

The AUC was calculated for 9 to 10 subjects on each lovastatin dosing day. The distribution of AUC and C_{max} values for lovastatin is displayed in Fig 5, with considerable overlap of the interquartile ranges being noted between study days. As in the midazolam study, summary statistics were representative of individual subject data (data not shown).

A statistical comparison of the primary pharmacokinetic parameters of lovastatin on days 9 (second dose of tadalafil), 21 (last day of tadalafil treatment), and 35 (after a 2-week washout period after completion of tadalafil dosing) versus baseline values (day 1) was conducted (Table II). For both tadalafil treatment groups (days 9 and 21), as well as at 2 weeks after tadalafil treatment (day 35), lovastatin AUC and C_{max} were considered to be equivalent to day 1 values, because the 90% confidence intervals for the geometric LS mean ratios were contained within the limits of 0.70 to 1.43.

Exposure to tadalafil was confirmed on days 9 (second dose of tadalafil) and 21 (last day of tadalafil treatment), with concentrations determined over 10 time points up to 24 hours after dosing (Fig 6). The results exhibited tadalafil exposure consistent with 20-mg dosing, with the expected slight accumulation of tadalafil occurring on multiple dosing.

DISCUSSION

The first in vitro experiments indicated that coadministration of tadalafil had a low potential to cause any clinically significant, reversible inhibition of the metabolism of coadministered drugs cleared by CYP3A or the other CYPs tested. However, methylenedioxyphenyl functional groups have been implicated in mechanism-based inhibition of CYPs,[11] and tadalafil contains this group. With mechanism-based inhibition, the substrate is metabolized by a CYP to an intermediate that binds either irreversibly or essentially irreversibly to the catalytic site of the enzyme. Because CYP3A is the major CYP involved in drug metabolism[9] and tadalafil is metabolized by CYP3A,[32] tadalafil was evaluated in vitro for its ability to inactivate CYP3A metabolism. The inactivation parameters obtained with tadalafil were compared with those generated for known mechanism-based inhibitors that have exhibited moderate levels of in vivo inhibition, erythromycin[9] and diltiazem.[22,33] As a measure of inhibitory potency, Cl_{inact} values were calculated and exhibited the following rank order: diltiazem (327 $min^{-1} \cdot mmol/L^{-1}$) >> erythromycin (59 $min^{-1} \cdot mmol/L^{-1}$) > tadalafil (17.1 $min^{-1} \cdot mmol/L^{-1}$). These results indicate that tadalafil is a substantially less efficient

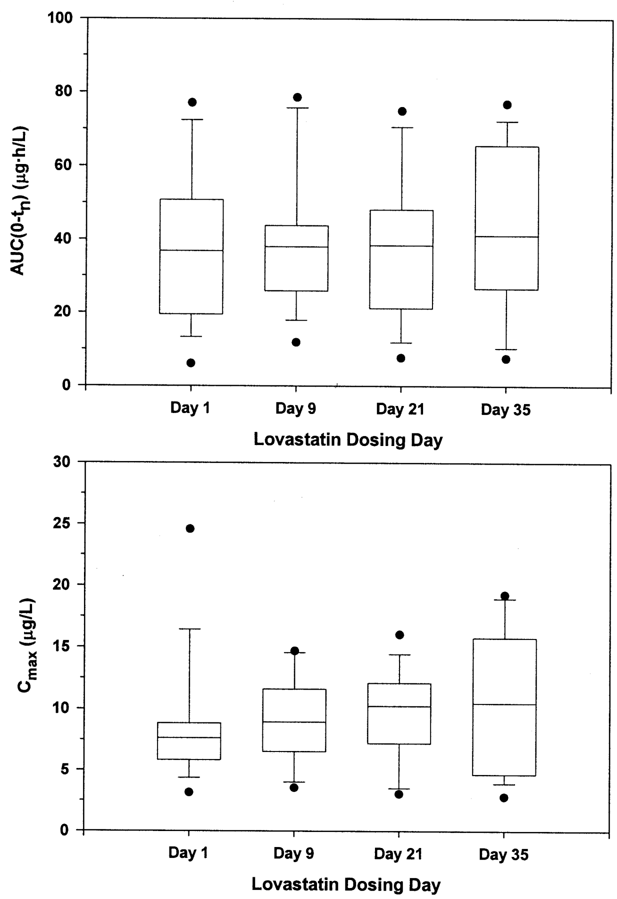

Fig 5. Lovastatin pharmacokinetic parameters after administration of 40 mg lovastatin at baseline (day 1), after second dose (day 9) and last dose (day 21) of daily dosing with 20 mg tadalafil, and after 2-week washout period (day 35). *Boxes* indicate 25th and 75th percentiles, and *whisker bars* indicate 10th and 90th percentiles. *Solid horizontal bars* within boxes indicate mean data. *Circles* represent values falling outside 10th and 90th percentiles.

mechanism-based inhibitor in vitro than either of the 2 positive controls.

Tadalafil was also evaluated for its ability to induce CYP3A and CYP1A2 activity in primary cultures of human hepatocytes. On the basis of the results of these studies, it does not appear that tadalafil would have a marked effect on in vivo CYP1A2 activity. Hepatocyte preparations exposed to 1 µmol/L tadalafil over a 2-day period demonstrated induction of CYP3A protein and activity. However, at higher doses of tadalafil, although CYP3A protein levels were induced, a reduction of CYP3A activity relative to protein levels was observed. These results suggest that tadalafil caused both inhibition and induction of CYP3A. Although there was a biphasic effect on activity dependent on concentration, measurement of CYP3A protein demonstrated that induction of CYP3A protein occurred with hepatocytes from all donors at nearly all tadalafil concentrations tested. A short-term exposure study of tadalafil with hepatocytes (0 to 60 minutes) confirmed that there was a time-dependent loss of CYP3A activity, further suggesting that mechanism-based inhibition occurs. This pattern of inhibition and induction by tadalafil suggests that mechanism-based inhibition of CYP3A is occurring in concert with CYP3A induction in hepatocytes. The net effect of these processes in vitro appears to be related to tadalafil concentration and results in essentially little change in CYP3A activity.

The clinical significance of the in vitro results depends on the ability of tadalafil to bind to the appropriate receptor for induction and reach the enzyme for inhibition. In vivo results may range from either inhibition or induction prevailing or no net effect. Interestingly, it has been reported that other methylenedioxyphenyl-containing compounds can not only cause mechanism-based inhibition but also induce enzymatic activity.[12] Therefore clinical studies with recognized CYP3A probe substrates were conducted to definitively evaluate possible inhibition or induction of CYP3A activity in vivo by tadalafil. Evaluation after 1 or 2 or multiple tadalafil doses would distinguish the potential effect of time of onset on the inhibition or induction of CYP3A by tadalafil. The progressive nature of mechanism-based inhibition is exemplified by erythromycin treatment, where erythromycin dosing for 1 day had a negligible effect on systemic clearance of alfentanil but treatment for 7 days decreased alfentanil clearance by 26%.[34] In another study accumulation of the CYP3A substrate terfenadine was observed after 7 days of co-administration with erythromycin.[35]

Because CYP3A probe substrates typically exhibit high between- and within-subject variability in pharmacokinetics,[36,37] the equivalence limits for the 90% confidence intervals of the geometric LS means were set at 0.73 and 1.43. These equivalence limits were selected on the basis of the knowledge that the 0.8 to 1.25 limits are unnecessarily rigorous for highly variable probes, which would lead to a false-positive claim of a statistically significant difference[38] even for the data obtained on the control days (days 1 and 8, before tadalafil dosing). Midazolam pharmacokinetics after the first and last dose during a 2-week daily-dose regimen of 10 mg tadalafil, as well as 2 weeks after completion of tadalafil dosing, was determined to be equivalent to the pharmacokinetics determined before

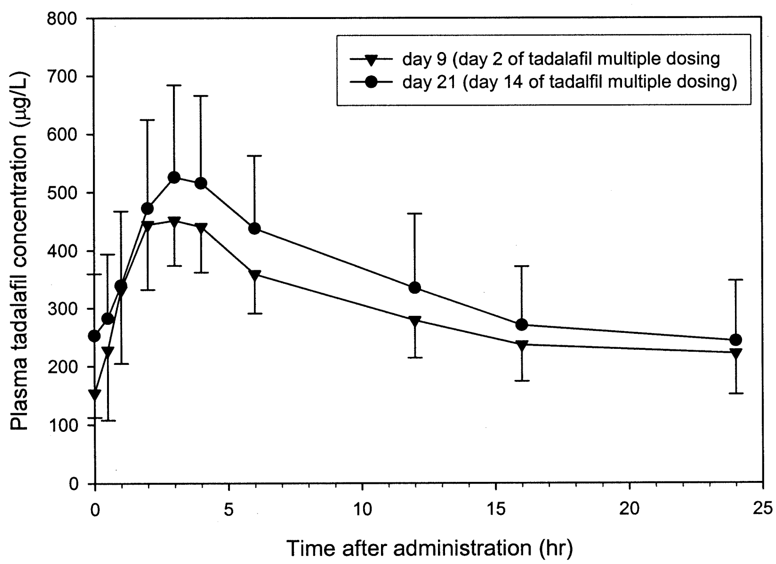

Fig 6. Arithmetic mean plasma concentration versus time profiles in lovastatin interaction study on days 2 and 14 (study days 9 and 21, respectively) of once-daily multiple-dose administration of 20 mg tadalafil orally in healthy subjects (N = 16).

Table II. Geometric mean with percent coefficient of variation and statistical comparison of pharmacokinetic parameters of lovastatin after single oral dose (40 mg lovastatin) on days 1, 9, 21, and 35 and daily oral dose of tadalafil from days 9 to 21

Parameter	*Day 1 (N = 16): Lovastatin alone*	*Day 9 (N = 16): Lovastatin with of day 2 tadalafil*	*Day 21 (N = 16): Lovastatin with of day 14 tadalafil*	*Day 35 (N = 16): Lovastatin alone*
AUC (μg · h/L)	30.8 (95.5)†	43.8 (66.6)†	34.4 (48.3)‡	44.1 (64.8)†
Ratio and 90% CI of geometric LS means*		1.15 (0.985-1.35)	1.03 (0.884-1.21)	1.16 (0.989-1.35)
C_{max} (μg/L)	7.62 (47.1)	8.38 (43.6)	8.82 (49.9)	8.93 (70.0)
Ratio and 90% CI of geometric LS means		1.10 (0.929-1.30)	1.16 (0.977-1.37)	1.17 (0.989-1.39)
Cl/F ($L \cdot h^{-1} \cdot kg^{-1}$)	17.3 (95.5)†	13.8 (75.1)†	15.9 (48.7)‡	12.3 (71.6)†
Ratio and 90% CI of geometric LS means		0.87 (0.74-1.015)	0.97 (0.826-1.13)	0.86 (0.74-1.011)

*Ratio and 90% CI for day of treatment versus baseline (mean of days 1 and 8)
†n = 10.
‡n = 9.

tadalafil administration. Thus these results indicate that there was no evidence that single or multiple doses of 10 mg tadalafil resulted in a change in CYP3A activity in vivo. These findings are in stark contrast with trials evaluating the interaction of midazolam with known potent CYP3A4 inhibitors, such as itraconazole, or known CYP3A4 inducers, such as rifampin. In such studies coadministration of itraconazole increased midazolam exposure by approximately 800% and coadministration of rifampin decreased midazolam exposure by 98%.[39]

An additional clinical study was conducted to confirm the lack of an effect on CYP3A4 activity when a higher dose of tadalafil (20 mg) was administered. Because no change was detected after 1 day of tadalafil exposure in the midazolam study, the pharmacokinetics of lovastatin was evaluated after the second tadalafil dose. Furthermore, lovastatin pharmacokinetics was evaluated after the second 20-mg tadalafil dose, rather than the first, to best detect a mechanism-based inhibitory effect because this may occur rapidly but may be subsequently disguised by offsetting induction. The

90% confidence intervals for ratios (lovastatin with tadalafil:lovastatin alone) of LS mean AUC and C_{max} values were completely contained in the prespecified no-effect boundary. Therefore it was concluded that 20 mg tadalafil administered daily had no significant effect on the pharmacokinetics of the CYP3A probe substrate lovastatin. These results are in contrast to the observed inhibition of CYP3A4 by itraconazole, which increased lovastatin AUC and C_{max} values by approximately 15-fold.[40]

The findings with midazolam and lovastatin provide strong evidence that short-term and long-term exposure to therapeutic concentrations of tadalafil do not alter the clearance of other drugs metabolized by CYP3A4. As further support of this conclusion, it is known that mechanism-based inhibitors often alter their own metabolic clearance in vivo. Thus exposure to a drug that may be a mechanism-based inhibitor would be predicted to be dose- and time-dependent. However, for tadalafil, over a dose range of 2.5 to 20 mg, exposure increased proportionally with dose, and single-dose pharmacokinetics (half-life, 17.5 hours) is predictive of observed plasma concentrations during multiple dosing.[5,41]

In conclusion, in vitro results suggest that tadalafil would not reversibly inhibit the metabolism of coadministered substrates of the major human CYPs but may have the potential to be a weak mechanism-based inhibitor and an inducer of CYP3A. Definitive clinical assessments demonstrated that, if induction and inhibition occurred, they were offset, because the pharmacokinetics of midazolam and lovastatin, CYP3A probe substrates, was virtually unchanged after coadministration with 10 and 20 mg tadalafil. Thus therapeutic concentrations of tadalafil do not produce clinically significantly changes in the clearance of drugs metabolized by CYP3A.

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All authors are employed by Eli Lilly and Company and own stock and/or stock options in the company.

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