Time- and NADPH-Dependent Inhibition of Cytochrome P450 3A4 by the Cyclopentapeptide Cilengitide: Significance of the Guanidine Group and Accompanying Spectral Changes

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ABSTRACT

Cilengitide is a stable cyclic pentapeptide containing an Arg-Gly-Asp motif responsible for selective binding to \( \alpha V \beta 3 \) and \( \alpha V \beta 5 \) integrins. The candidate drug showed unexpected inhibition of cytochrome P450 3A4 (P450 (3A4)) at high concentrations, that is, a 15-mM concentration caused attenuation of P450 3A4 activity (depending on the probe substrate): 15–19% direct inhibition, 10–23% time-dependent inhibition (30-minute preincubation), and 54–60% metabolism-dependent inhibition (30-minute preincubation). The inactivation efficiency determined with human liver microsomes was 0.003 ± 0.001 min\(^{-1} \) mM\(^{-1} \) and was 0.04 ± 0.01 min\(^{-1} \) mM\(^{-1} \) with baculovirus-based microsomes containing recombinant P450 3A4. Neither heme loss nor covalent binding to apoprotein could explain the observed reductions in residual activity. Slowly forming type II difference spectra were observed, with maximum spectral changes after 2 hours. Binding to both reduced and oxidized P450 3A4 was observed, with apparent \( K_d \) values of 0.66 \( \mu M \) and 6 \( \mu M \). The significance of the guanidine group in inhibition was demonstrated using ligand binding spectral changes and inactivation assays with guanidine analogs (debrisoquine, \( N \)-acetylariginine-O-methyl ester) and the acetylated ornithine derivative of cilengitide. The observed inhibition could be explained by direct inhibition, plus formation of stable complexes with both ferric and ferrous forms of heme iron and to some extent by the formation of reactive species capable to react to the protein or heme. Formation of the complex required time and NADPH and is attributed to the guanidine group. Thus, the NADPH-dependent inhibition is considered to be mainly due to the formation of a stable complex rather than the formation of reactive species.

Introduction

Cilengitide is a stable cyclic pentapeptide containing the Arg-Gly-Asp (RGD; Fig. 1) motif responsible for selective binding to \( \alpha V \beta 3 \) and \( \alpha V \beta 5 \) integrins (Dechantsreiter et al., 1999). The blocking of integrins prevents tumor angiogenesis, providing the prospect of a broader pharmacological action. Although unique in its mode of action and having favorable pharmacokinetic properties, cilengitide did not meet its primary end point of significantly increasing overall survival when added to the current standard chemoradiotherapy regimen for brain tumors (i.e., temozolomide and radiotherapy), in the most advanced phase III clinical trial (CENTRIC) (Soffietti et al., 2014).

During the course of the drug development, cilengitide showed unexpected inhibition of P450 3A4 at recommended therapeutic concentrations, associated with nonextractable radioactivity during in vivo experiments on human volunteers. Physiologically based pharmacokinetic modeling predicted a 1.3-fold increase of the area under the curve of the probe substrate midazolam after administration of clinically recommended dose of 2 g of cilengitide. Although potentially classified as a weak inhibitor, inactivation of P450 3A4 could contribute in relevant drug-drug interactions during multitreatment because P450 3A4 is involved in the metabolism of more than 50% of drugs (Williams et al., 2004; Guengerich, 2005a).

More than 100 peptide drugs are on the market (Craik et al., 2013), out of which only cyclosporine has shown clinically significant interactions (Amundsen et al., 2012). A predominant metabolic pathway for peptides is hydrolysis of peptide bonds (Bernkop-Schnurch and...
Schmitz, 2007). Based on the structural features of cilengitide (Fig. 1), potential metabolic reactions catalyzed by P450s could include aromatic hydroxylation of phenylalanine, aliphatic hydroxylation of valine, N-demethylation of the valine N-methyl group, and deamination and dealkylation of arginine (Guengerich, 2001).

Our study characterized the inhibition of P450 3A4 by cilengitide using human liver microsomes and recombinant P450 3A4, including the type and mechanism of inactivation, and we explain the observed nonextractable radioactivity observed in vivo.

Materials and Methods

**Chemicals and Enzymes.** Cilengitide, [14C]-guanidine]-labeled cilengitide, and the ornithine homolog of cilengitide were synthesized by Merck Serono GmbH (Darmstadt, Germany) (Jonczyk et al., 1999). Nifedipine was obtained from Sigma-Aldrich. The ornithine homolog of cilengitide were synthesized by Merck Serono and the ornithine derivative has an amine substituted for the guanidine group.

Fig. 1. Structure of cilengitide. The ornithine derivative has an amine substituted for the guanidine group.

Testosterone was obtained from Steraloids (Newport, RI). Baculosomes (microsomes from baculovirus-infected P450 3A4 recombinant insect cells) with human P450 3A4 or P450 3A5, or 2

**GmbH (Darmstadt, Germany) (Jonczyk et al., 1999). Nifedipine was obtained and the ornithine homolog of cilengitide were synthesized by Merck Serono with human liver microsomes and an NADPH-generating system for 0, 3, 6, 9, 15, and 30 minutes. After the preincubation, an aliquot of marker substrate was added, with the procedures of isolation and quantitation described earlier.

**Determination of Reversible and Quasi-irreversible Inhibition.** Cilengitide (final concentration 15 mM) was preincubated for 30 minutes with human liver microsomes (500 pmol P450) at 57 ± 1°C in 500 μl mixtures containing 50 mM potassium phosphate buffer (pH 7.4) and an NADPH-generating system. Preincubation and solvent controls were treated with potassium ferricyanide (final concentration 2 mM), after which samples and control were dialyzed for 24 hours against 50 mM phosphate buffer (pH 7.4). Three replicates of each incubation were used to measure residual enzyme activity with nifedipine and testosterone as substrates. When reversible or quasi-irreversible inhibition was observed with human liver microsomes, the results were confirmed using recombinant (baculovirus) P450 3A4.

**Pyridine Hemochrome Spectrophotometric Assay.** We added H2O (950 μl) to 250 μl of a P450 preincubation mixture (see above). After the addition of 0.20 ml of pyridine, the mixture was mixed using a vortex device, and 0.10 ml of 1 M NaOH was added, followed by more vortex mixing. The solution was split in two parts, reference and sample, and placed in cuvettes (capped) that were scanned from 500 to 600 nm to establish a baseline with an Aminco DW2a/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). A few crystals of solid sodium dithionate and 10 μl of H2O were added to the sample cuvette. Potassium ferricyanide (10 μl of a 1 M solution) was added to the reference cuvette. Samples were scanned within 1 minute after the addition of the alkaline solution because of the instability of the pyridine hemochromogen under basic conditions. The heme concentration was calculated based on an extinction coefficient of 20.7 mmol−1 cm−1 for the difference in absorption between peak at 557 nm and the trough at 541 nm (Flink and Watson, 1942; Paul et al., 1953).

**Radioactivity Assays.** The reconstituted P450 3A4 system containing 0.5 nmol P450 3A4, 1.0 nmol NADPH-P450 reductase, and 0.5 nmol cytochrome b5 was incubated with 7.5 mM 14C-labeled cilengitide (specific activity 8 Ci/mol). Two incubations, with and without (control) an NAPDH-generating system, were performed. The reactions were stopped by chilling the tubes on ice. Enzymes were separated by size-exclusion chromatography (Micro Biospin column P-6; Bio-Rad Laboratories, Hercules, CA), and cilengitide was recovered from the column by washing the column with a C2H5OH–H2O mixture (1:1, v/v, 4°C). Recovered enzyme samples were loaded on a gradient polyacrylamide electrophoresis gel (NuPAGE 4–12% Bis-Tris Gel; Life Technologies, Grand Island, NY). To test the specificity of binding, incubations included bovine serum albumin (4 μM) and ovalbumin (4 μM); proteins were separated on 7.5% (w/v) SDS-PAGE gels. After development, gels were stained with Colloidal Coomassie Blue (Kang et al., 2002).

For liquid scintillation counting, bands containing individual proteins were cut from the gel and were treated with 1.0 ml of 15% H2O2 (w/v) for 2 hours at 70°C in the dark. Scintillation fluid (10 ml of ScintiVerse II Cocktail; Fisher Scientific, Waltham, MA) was added, and the samples were left in the dark overnight to diminish luminescence, after which radioactivity was recorded on an LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Pasadena, CA). Percentages of covalently modified proteins were determined based on the difference in counts of incubations with and without NADPH.
One gel (loaded with proteins from the incubations with and without an NADPH-generating system) was treated with Autofluor (National Diagnostics, Atlanta, GA). Autofluor enables impregnation with phosphors, which convert radioactive β emission into more photons. After the treatment, the gel was dried, placed on an imaging screen (Kodak; Bio-Rad Laboratories), and left at −70°C. The imaging screen was visualized after 7 to 10 days on a Molecular Imager Pharos FX Plus Systems (Bio-Rad Laboratories).

**Spectrophotometry.** Spectral changes resulting from 2 μM ligand (cilengitide, cyclopeptide analogs of cilengitide, and guanidine derivatives) binding to 2.5 μM recombinant P450 3A4 were monitored for 8 hours. The binding affinity of
Cilengitide Inhibition of P450 3A4

Fig. 3. Heme assays. After incubation for 30 minutes with an NADPH-generating system in the presence of catalase, no statistically significant difference in pyridine hemochrome complex formation was observed in comparing a control incubation, a cilengitide incubation, and reconstituted P450 3A4 without incubation (enzymes mixture).

cilengitide to both reduced and oxidized CYP3A4 was estimated by monitoring the (absolute) spectral changes of 2.5 μM enzyme with each addition of cilengitide (every 10 minutes) in a total volume of 1.0 ml of 50 mM potassium phosphate buffer (pH 7.4) at 23°C. Binding to the ferrous form of enzyme was determined under anaerobic conditions (achieved under argon atmosphere and addition of protocatechuate-3,4-dioxygenase with 3,4-dihydoxybenzoic acid for removal of oxygen traces (Bull et al., 1981; Guengerich et al., 2004) after photoreduction (halogen lamp 500 W, 1 minute) in the presence of 1 mM 5-deazaflavin (Massey and Hemmerich, 1978).

Absorbance spectra were recorded from 350 to 500 nm using an Aminco DW2a/OLIS (for monitoring binding to oxidized P450 3A4) or a Cary 14/OLIS spectrophotometer (200–400 nm). Structure and purity of the product (55% yield) were confirmed by liquid chromatography with mass spectrometry and NMR (Supplemental Fig. 1).

Data Analysis. Data from assays were processed in GraphPad Prism software (GraphPad Software, San Diego, CA). As inhibition was monitored, a one-sided t-test (α = 0.05) was used for the assessment of statistical significance in the differences between samples and controls, based on measurements of residual activity. A nonlinear three parameter sigmoidal-logistic equation was used for IC₅₀ calculations: Residual activity = Bottom + (Top-Bottom)/(1 + 10exp((X - logIC₅₀))). The Michaelis-Menten equation was used for determination of inactivation constants (Kᵢ) and inactivation rate constants (kₙₐₜₐ) of inhibitor (I): kₜₜ = kₙₜₜ × [I]/(Kᵢ + [I]). A quadratic equation was used to determine dissociation constants (Kₒ) of ligands (L) with the enzyme (E): ΔA = Aₒ + (Bₒₒₐₜₐ/2)[E][Kₒ + [E] + [L]] − (Kₒ + [E] + [L])² − 4[E][L])½, Aₒ and Bₒₒₐₜₐ were constants in each analysis. DynaFit simulation software (Biokin, Pullman, WA) was used with a one-step enzyme-ligand binding model (Kuzmic, 1996), yielding similar results obtained from the quadratic nonlinear regression analyses in GraphPad Prism.

Results

Inhibition Assays and Kinetics. Cilengitide was tested with human liver microsomes for direct inhibition (no preincubation) and after preincubation with and without an NADPH-generating system. In both the nifedipine and testosterone assays, direct inhibition was observed when the highest concentrations of cilengitide were used. Cilengitide (15 mM) decreased enzyme activity by 15% (P = 0.034) in testosterone and nifedipine assays, respectively. When cilengitide was preincubated without the addition of NADPH, reductions of enzyme activity by 23% (testosterone assay, P = 0.031) and 10% (nifedipine assay, P = 0.008), respectively, were observed. Greater inhibition was observed when cilengitide was preincubated with NADPH: both nifedipine and testosterone assays showed statistically significant decreases in enzyme activity: 60% (P = 0.007) and 54% (P = 0.007), respectively.

Fig. 4. Binding of cilengitide to proteins. (A) An SDS-polyacrylamide gradient (4–12%, w/v) gel showed binding of cilengitide to NADPH-P450 reductase and P450 3A4 in a comparison of incubations of a reconstituted P450 3A4 system (P450 3A4, reductase, cytochrome b₅₆, phospholipids) with and without an NADPH-generating system. (B) To determine whether nonspecific covalent binding to other proteins occurred, bovine serum albumin (BSA) and ovalbumin (OVA) were added to the incubation mixtures and separated on an SDS-polyacrylamide (7.5%, w/v) gel. (B1) Autoradiography was performed after 10 days of exposure. (B2) The same gel after staining with Coomassie Brilliant Blue R-250. More intensive binding of [¹⁴C] cilengitide to bovine serum albumin was observed in incubations with the NADPH-generating system. B2: cytochrome b₅, b₅, NADPH-P450 reductase, NPR; P450 3A4: 3A4. (C) Nonspecific binding was observed when the incubation of cilengitide was performed with human liver microsomes (C1, autoradiography; C2, Coomassie Brilliant Blue R-250 staining). The percentages of binding to P450 3A4, NADPH-P450 reductase, and bovine serum albumin were determined after H₂O₂ digestion of gels by scintillation counting.
For the NADPH-dependent inhibition, inactivation kinetics were determined (Fig. 2). In the nifedipine assay, the inactivation constant $K_i$ was 6.3 ± 1.6 mM and the inactivation rate ($k_{\text{inact}}$) was 0.017 ± 0.002 min$^{-1}$, corresponding to an inactivation efficiency ($k_{\text{inact}}/K_i$) of 0.003 ± 0.001 min$^{-1}$ mM$^{-1}$. In the testosterone assay the apparent $K_i$ value was greater than the highest concentration analyzed ($K_i > 15$ mM).

In that nifedipine and testosterone are substrates for both P450 3A4 and P450 3A5, recombinant baculovirus-based microsomes were used to confirm the inhibitory effect of cilengitide. When 15 mM cilengitide was used, complete inhibition of P450 3A4 was observed in both assays. Preincubation of P450 3A5 with NADPH caused 9% ($P = 0.063$) and 23% ($P = 0.006$) decreases of activity in the nifedipine and testosterone assays, respectively.

The values of inactivation parameters determined for P450 3A4 were $K_i = 1.1 ± 0.2$ mM and $k_{\text{inact}} = 0.06 ± 0.03$ min$^{-1}$ in the testosterone assay and $K_i = 5.0 ± 1.1$ mM and $k_{\text{inact}} = 0.21 ± 0.03$ min$^{-1}$ in the nifedipine assay (Fig. 2). The inactivation efficiency ($k_{\text{inact}}/K_i$) in both assays with recombinant P450 3A4 was 0.04 ± 0.01 min$^{-1}$ mM$^{-1}$.

**Determination of Inactivation Mechanism.** The pyridine heme chromophore spectrophotometric assay was used to determine possible covalent modification of heme (Flink and Watson, 1942; Paul et al., 1953) after preincubation of 15 mM cilengitide and P450 3A4 with NADPH. When the assay was performed without the addition of an oxidizing agent (ferricyanide) to the reference cuvette, an apparent 72% ($P < 0.001$) decrease in heme concentration was observed. In the presence of ferricyanide (to reoxidize the P450 heme), the heme loss was reduced to 51% ($P < 0.001$). However, when preincubation was performed in the presence of catalase, no heme loss was observed (Fig. 3), in accordance with a lack of statistically significant changes in concentrations of (spectrally determined) P450 (P = 0.12) and cytochrome b$_5$ (P = 0.11), as well as residual activity (P = 0.070).

 Autoradiography of a reconstituted P450 3A4 system showed binding of $^{14}$C-labeled cilengitide to P450 3A4 and NADPH-P450 reductase (Fig. 4A). This result was confirmed with scintillation counting after digestion of gels with H$_2$O$_2$: 8% binding to P450 3A4 and 6% to the reductase. Binding was observed not only to P450 3A4 but also the reductase, and therefore incubations were performed with bovine serum albumin and ovalbumin as "traps" (Fig. 4, B1 and B2). Significant binding of cilengitide to bovine serum albumin was observed when compared with control (without NADPH) (4%).

In human liver microsomes, binding of $^{14}$C-labeled cilengitide was observed to proteins with higher molecular weights (Fig. 4, C1 and C2).

The reversibility of cilengitide binding was investigated by dialyzing incubations for 24 hours and testing residual activity. No reversibility was observed in the testosterone and nifedipine assays (Fig. 5). To determine possible quasi-irreversible inhibition (Silverman, 1995), preincubations were treated with potassium ferricyanide and dialyzed for 24 hours. Residual activity was assayed; no recovery of enzyme activity was observed in the nifedipine assay (Fig. 5). Recovery in the testosterone assay on human liver microsomes was observed (Fig. 5) but not confirmed when preincubations were performed with recombinant P450 3A4.

**Binding of Cilengitide to Oxidized and Reduced Forms of P450 3A4.** When monitoring an incubation of cilengitide with reconstituted P450 3A4 for 2 hours, slow changes in absorbance were observed at 426 nm (increase) and at 394 and 410 nm (decrease). To assess the binding of cilengitide to P450 3A4, spectral changes were monitored for 8 hours. Slow type II spectral changes were observed (Fig. 6, A and C). The maximal spectral change was achieved after 2 hours, indicating slow inhibitor-enzyme complex formation. This was further confirmed by testing residual activity after a 2-hour preincubation of cilengitide with human liver microsomes and without NADPH. Higher values of inhibition were observed in both the testosterone (46%, $P = 0.003$) and nifedipine (38%, $P = 0.001$) assays when compared with 30 minutes of preincubation (see *Inhibition Assays and Kinetics*).

A very low apparent second-order rate of cilengitide-P450 3A4 complex formation (130 M$^{-1}$ s$^{-1}$) was estimated on the basis of a one-ligand/two-state binding model, although it is not necessarily appropriate in that the binding of ligands to P450 3A4 involves multiple states/reactions after a diffusion-limited encounter (Isin and Guengerich, 2007).

A complex with the ferric form of P450 3A4 was formed at much lower concentrations ($K_d = 0.66$ μM; Fig. 6E) compared with the millimolar concentrations at which enzyme inhibition was observed. Opposite to the ferric system, the inhibitor-enzyme complex with ferrous
P450 3A4 formed quickly (Fig. 6, B and D). A $K_d$ for ferrous P450 3A4 (determined under anaerobic conditions) was also in the micromolar range ($K_d = 6 \mu M$; Fig. 6F), explaining the observed NADPH-dependent inhibition.

**Spectral Changes and Inhibition Assays of Guanidine Derivatives.** The guanidine group was hypothesized to be involved in cilengitide inhibition of P450 3A4. Debrisoquine and $N$-acetylariginine-$O$-methyl ester were tested for inhibitory effects and spectral changes. Binding constants were determined using a one-ligand/two-state binding model: $K_d = 1.0 \mu M$ for debrisoquine and $K_d = 0.14 \mu M$ for the arginine derivative (Fig. 7, A and B). The arginine derivative (final concentration 15 mM) caused statistically significant inhibition of P450 3A4 only in the nifedipine assay; the residual activity was 71 ± 4% (no preincubation), 68 ± 8% (preincubation without NADPH), 85 ± 6% (preincubation with NADPH) (Fig. 7D). Debrisoquine (final concentration 15 mM) caused significant reduction of P450 3A4 activity in both assays. A greater decrease was observed in the testosterone assay, with residual activity being 13 ± 5% (no preincubation), 24 ± 3% (15 minutes preincubation with NADPH).

**Fig. 6.** Binding of cilengitide to the ferric and ferrous forms of P450 3A4. (A) Type II spectral changes (maximum at 435 nm and minimum at 415 nm) when cilengitide was added to the oxidized (ferric) form of enzyme (A), and the appearance of a maximum at 417 nm and minimums at 403 and 447 nm were observed for binding to the ferrous (reduced) form of the enzyme (B). Binding to the oxidized (ferric) form was slow and reached a maximum after 2 to 3 hours (C), in contrast to the rapid binding to the reduced form of P450 3A4 (<2 minutes, D). Binding of cilengitide to both ferric (E) and ferrous (F) P450 3A4, fit to a one-ligand quadratic model: $K_{d,Fe^{3+}} = 0.66 \pm 0.09 \mu M$ and $K_{d,Fe^{2+}} = 6 \pm 3 \mu M$. 

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(preincubation without NADPH), and 19 ± 2% (preincubation with NADPH) (Fig. 7C). In the nifedipine assay, the residual activity was 46 ± 4% (no preincubation), 38 ± 4% (preincubation without NADPH), and 31 ± 1% (preincubation with NADPH) (Fig. 7D).

Spectral Changes and Inhibition Assays with Cyclopeptide Analogs. Spectral changes were monitored to determine the binding constants of cilengitide analogs. Based on the one-ligand/one-site binding model, the dissociation constants $K_d$ were 0.52 mM for the ornithine derivative and 0.44 μM for the acetylated ornithine derivative. Complete inhibition of P450 3A4 activity was observed with the ornithine derivative in the nifedipine assay whereas 17 ± 2% residual activity was observed in the testosterone assay (Fig. 8C). The acetylated derivative caused only a 6 ± 2% decrease of activity in the nifedipine assay ($P = 0.015$); a more potent decrease was observed in the testosterone assay (39 ± 6%, $P = 0.003$; Fig. 8C).

Discussion

In preliminary single-point inhibition assays, cilengitide (15 mM) caused direct (no preincubation), time-dependent (30 minute preincubation without NADPH-generating system), and also NADPH-dependent inhibition (30 minute preincubation with NADPH-generating system) of P450 3A4 in human liver microsomes, the most prominent being the latter (54–60% depending on the probe substrate used). Complete inactivation was confirmed with a baculosome-based recombinant P450 3A4 system for both substrate probes, and the maximal inhibition of P450 3A5 was 23% under the same conditions.

Inactivation kinetics for metabolism (NADPH)-dependent inhibition were studied with both human liver microsomes and recombinant baculosomes (Fig. 2). As the values of the inhibition constant are in the millimolar range and the maximal plasma concentrations in humans are in a low micromolar range, this would indicate low potential for drug-drug interactions.

In principle, a P450 inactivator can modify prosthetic heme, bind to apoprotein, or do both (Wienkers and Heath, 2005). The spectrophotometric assay (pyridine hemochrome) did not confirm any heme loss when incubations were performed in the presence of catalase to destroy any H$_2$O$_2$ generated (Fig. 3). This assay is based on determining heme iron in ferrous form as a complex with pyridine in basic media [versus a reference to which ferricyanide is added (Flink and Watson, 1942; Paul et al., 1953)]. During the optimization of assay, greater heme loss was observed when ferricyanide was not added (70 versus 27%), indicating the possible existence of a stable Fe$^{2+}$-cilengitide complex.
[14C] Cilengitide was bound not only to P450 3A4 but also to NADPH-P450 reductase (Fig. 4). Nonspecific binding was also confirmed when radioactive cilengitide was coincubated with bovine serum albumin and ovalbumin present. However, only 8% binding to P450 3A4 was observed (i.e., 0.08 nmol cilengitide label/nmol P450 3A4), which does not explain the complete inhibition of P450 3A4 observed with the baculovirus system under the same conditions.

In that covalent binding did not explain the observed inhibition, possible quasi-irreversible inhibition was analyzed. In this case, the heme iron would form a complex with cilengitide that appears irreversible but can be destabilized and enzyme activity recovered by subsequent incubation with ferricyanide and dialysis (Silverman, 1995). However, no recovery of enzyme activity was observed (Fig. 5). A disadvantage of this approach is the assumption that ligand binds to the ferric form (Ortiz de Montellano, 2005). However, cilengitide formed a more stable enzyme-ligand complex after one-electron reduction (Correia and De Voss, 2005; Sevrioukova and Poulos, 2013) and forms a more stable enzyme-ligand complex after one-electron reduction (Correia and Ortiz de Montellano, 2005). However, cilengitide formed a more stable complex with the oxidized form of P450 3A4 ($K_{d,Fe^3+} = 0.66 \mu M$ versus $K_{d,Fe^2+} = 6 \mu M$).

The quasi-irreversibility experiment suggested possible formation of a time-dependent cilengitide-P450 3A4 complex (i.e., slow tight-binding inhibition). This phenomenon has not been reported with P450s but has been reported for nonsteroid anti-inflammatory drug inhibition of cyclooxygenase (Prusakiewicz et al., 2004). Drug-enzyme complexes should eventually dissociate when dialyzed (Silverman, 1995; Szedlacsek and Duggleby, 1995), but no recovery of enzyme activity was observed after dialysis for 1 day (Fig. 5). Negative results for this experiment cannot eliminate the possibility of slow, tight binding, as has been shown for proteasome inhibitors (Manam et al., 2008).

Based on spectral changes, we postulated that the guanidine group of cilengitide was responsible for the observed inhibition. To test this hypothesis, we used guanidine-containing analogs (debrisoquine and N-acetyl-arginine-O-methyl ester) as well as the N-acetyl ornithine derivative of cilengitide (substitution for Arg). For both debrisoquine and N-acetyl-arginine-O-methyl ester, slow ligand-enzyme formation was observed, taking more than 1 and 6 hours to achieve the maximum type II spectral change. Inhibition assays also confirmed the low residual activity of P450 3A4 preincubated with debrisoquin in both the testosterone and nifedipine assays, while inhibition with the arginine derivative was only observed in the nifedipine assay (Fig. 7).

The ornithine derivative has an amino group on a residue chain and, as might be expected, caused type II spectral changes characteristic for N-acetyl ornithine derivative of cilengitide. Nonetheless, the inhibition assays showed a much lower reduction of catalytic activity compared with cilengitide and the ornithine derivative (Fig. 8).

Binding of ligands to P450 3A4 is not a one-step process and requires time (Isin and Guengerich, 2007; Isin and Guengerich, 2008). Recently, unusual type II spectral changes of bicalutamide (an antiandrogen for prostate cancer treatment) binding to P450 46A1 were reported and attributed to binding involving a water molecule as an intermediate (Mast et al., 2013). This phenomenon could provide an explanation for the observed changes, even with the protected amino derivative of cilengitide.

A guanidine group has not been reported to cause clinically significant interactions, at least not with P450s. Commonly used drugs containing a terminal guanidine group include famotidine (an antiulcer drug) and metformin (an oral hypoglycemic). Famotidine was developed as a histamine H2-receptor antagonist with low interaction potential compared with cimetidine and ranitidine (Humphries, 1987). Metformin exhibits interactions with organic cation transporters (the guanidine group is positively charged) (Somogyi et al., 1987).

To date, the drug-drug interaction package of P450 inhibition includes an assessment of the direct inhibition caused by the candidate drug. In addition, the indirect inhibition caused by the formation of a reactive...
metabolite or more potent metabolite is studied by preincubating the candidate drug with the test system (e.g., human liver microsomes) and NADPH- or NADP(H)-regenerating system. Our finding with cilengitide indicates that other relevant inhibition mechanisms such as time- but not NADPH-dependent (e.g., slow and tight binding) might also exist. Before including such a new evaluation into a drug-drug interaction package, it is important to learn how often this mechanism occurs in the more classic chemical space for drugs.

In conclusion, cilengitide causes time- and NADPH-dependent inhibition of CYP450 3A4. The observed inhibition can be explained by direct inhibition and by formation of stable complexes with both ferric and ferrous forms of heme iron. Formation of these stable complexes is time- and NADPH-dependent and is attributed to the guanidino group. Only a small part of the NADPH-dependent inhibition could be attributed to any reactive products capable of reacting with the protein or heme, explaining the nonextractable radioactivity observed in vivo. To date, the chemical mechanism of the adduct formation is not known.

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Authorship Contributions
Participated in research design: Guengerich, Barbero, Dolgos, Bojić. Conducted experiments: Bojić, Guengerich.

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Contributed new reagents or analytical tools: Barbero, Riva.

Performed data analysis: Bojić, Guengerich, Barbero, Dolgos, Gallemann.

Wrote or contributed to the writing of the manuscript: Bojić, Guengerich, Barbero, Dolgos, Gallemann, Freisleben.

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Authorship Contributions
Participated in research design: Guengerich, Barbero, Dolgos, Bojić. Conducted experiments: Bojić, Guengerich.

Contributed new reagents or analytical tools: Barbero, Riva.

Performed data analysis: Bojić, Guengerich, Barbero, Dolgos, Gallemann.

Wrote or contributed to the writing of the manuscript: Bojić, Guengerich, Barbero, Dolgos, Gallemann, Freisleben.
Data Supplement

Time- and NADPH-Dependent Inhibition of P450 3A4 by the Cyclopentapeptide Cilengitide: Significance of the Guanidine Group and Accompanying Spectral Changes

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Drug Metabolism and Disposition

Fig. S1. NMR and MS data of acetylated ornithine analogue of cilengitide. (A) NMR spectra were recorded on a Bruker AV400 (400 MHz) spectrometer (Bruker AXS Inc., Madison, WI). $^1$H NMR (D$_2$O, 400 MHz) showed a characteristic peak at δ 1.96 (s, 3H) that corresponds to the acetyl group, compared to the ornithine analogue of cilengitide (B). LC-MS analysis was performed on an Aquity UPLC (Waters, Milford, MA) coupled with an LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA). Separation was achieved on an Acquity UPLC BEH octadecylsilane (C$_{18}$) column (1.7 µm, 2.1 × 100 mm, Waters) with H$_2$O and CH$_3$OH as the mobile phase (flow: 0.3 ml/min, gradient: 0 min, 0% CH$_3$OH; 1 min, 0% CH$_3$OH; 4 min, 100% CH$_3$OH; 5.2 min, 100% CH$_3$OH; 5.3 min, 0% CH$_3$OH; 8 min, 0% CH$_3$OH). Mass spectrometry of the acetylated ornithine derivative in ESI positive mode (C) showed a peak ion at m/z 589 (M$^+$) that corresponds to the parent compound (calculated 589.2979, found 589.2980, Δ 0.2 ppm) vs. m/z 547 (M$^+$), which corresponds to the most intensive parent ion of the ornithine analogue (calculated 547.2875, found 547.2869, Δ 1.1 ppm).