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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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Running title: Cilengitide Inhibition of P450 3A4

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A list of nonstandard abbreviations used:
P450 cytochrome P450
ABSTRACT
Cilengitide is a stable cyclic pentapeptide containing an Arg-Gly-Asp motif responsible for selective binding to $\alpha\nu\beta^3$ and $\alpha\nu\beta^5$ integrins. The candidate drug showed unexpected inhibition of cytochrome P450 (P450) 3A4 at high concentrations, i.e. 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 min pre-incubation), and 54-60% metabolism-dependent inhibition (30 min pre-incubation). The inactivation efficiency determined with human liver microsomes was $0.003\pm0.001 \text{ min}^{-1} \text{mM}^{-1}$ and was $0.04\pm0.01 \text{ min}^{-1} \text{mM}^{-1}$ 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 h. 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 analogues (debrisoquine, N-acetylariginine-O-methyl ester) and the acetylated ornithine derivative of cilengitide. The observed inhibition could be explained by direct inhibition, plus by 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 complex required time and NADPH and is attributed to the guanidino 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 αvβ3 and αvβ5 integrins (Dechantsreiter et al., 1999). By blocking integrins, tumor angiogenesis is prevented, providing the prospect of a broader spectra of indications for cilengitide (e.g. newly diagnosed and recurrent glioblastoma, advanced solid tumors, pancreatic, prostate, non-small cell lung, and head and neck cancer) (Goodman and Picard, 2012). Cilengitide is not metabolized in vitro, neither in recombinant P450 microsomes, human liver microsomes, or human hepatocytes. About 70% of a 2 g dose administered intravenously in healthy volunteers undergoes renal excretion unchanged while the rest is eliminated mainly unchanged via biliary secretion in feces. Only very minor metabolites originating from cleavage have been observed. The maximal plasma concentration (C\text{max}) and total area-under-the-curve (AUC) increased in proportion to the dose, while no change in clearance, volume of distribution, or half-life was observed, indicating linear pharmacokinetics (Eskens et al., 2003). Cilengitide does not accumulate after repeated dosing, in that no significant changes were observed in pharmacokinetic parameters (Reardon et al., 2011). Although unique in its mode of action and having favorable pharmacokinetic properties, cilengitide did not meet its primary endpoint 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 non-extractable radioactivity during in vivo experiments on human volunteers. Physiologically-based pharmacokinetic modeling predicted a 1.3-fold increase of the AUC 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 multi-therapy because P450 3A4 is involved in the metabolism of more than 50% of drugs (Williams et al., 2004; Guengerich, 2005a).

More than one hundred 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, \textit{N}-demethylation of the valine \textit{N}-methyl group, and deamination and dealkylation of arginine (Guengerich, 2001).

The objective of this study was to characterize inhibition of P450 3A4 by cilengitide using human liver microsomes and recombinant P450 3A4, including the type and mechanism of inactivation, and to explain the observed non-extractable radioactivity observed \textit{in vivo}. 
Materials and Methods

Chemicals and Enzymes

Cilengitide, [14C-guanidine]-labeled cilengitide, and the ornithine homologue of cilengitide were synthetized by Merck Serono GmbH (Darmstadt, Germany) (Jonczyk et al., 1999). Nifedipine was obtained from Sigma-Aldrich (St. Louis, MO) and recrystallized from C2H5OH in amber glass (Guengerich et al., 1986). Testosterone was obtained from Steraloids (Newport, RI). Baculosomes (microsomes from baculovirus-infected P450 recombinant insect cells) with human P450 3A4 or P450 3A5, NADPH-P450 reductase, and cytochrome b5 were purchased from Life Technologies (Carlsbad, CA). Bovine serum albumin and ovalbumin were obtained from Sigma-Aldrich. The guanidine-based compounds debrisoquine and N-acetylarginine-O-methyl ester were purchased from Sigma-Aldrich and Bachem (Bubendorf, Switzerland), respectively. All other reagents and solvents were obtained from general commercial suppliers and were used without further purification.

Ten liver samples from a stock (Schadt et al, 2008) in our laboratory (Vanderbilt) were used to prepare human liver microsomes (4 males, 5 females, 1 unknown sex; median age 27 years). Recombinant P450 3A4 with a C-terminal (His)5 tag (Gillam et al., 1993; Hosea et al., 2000) was expressed in Escherichia coli and purified as described previously (Hosea et al., 2000). E. coli recombinant rat NADPH-P450 reductase (Hanna et al., 1998) and human liver cytochrome b5 (Guengerich, 2005b) were prepared as described elsewhere. All other proteins and enzymes were purchased from Sigma-Aldrich.

Incubations and Residual Activity
Incubations were conducted at 37°C in 100 µl incubation mixtures containing 50 mM potassium phosphate buffer (pH 7.4 for testosterone and pH 7.85 for nifedipine), an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mg/mL NADP+, and 2 µg/ml yeast glucose 6-phosphate dehydrogenase), and diagnostic substrate (testosterone 210 µM, nifedipine 20 µM). Depending on the P450 enzyme system used in incubations, final concentrations were 1 µM (P450) in human liver microsomes, 0.05 µM baculovirus recombinant P450 3A4 or 3A5, or 2 µM reconstituted recombinant P450 3A4. The reconstituted P450 3A4 system contained: P450 3A4 (2 µM), NADPH-P450 reductase (4 µM), cytochrome b₅ (2 µM), sodium cholate (0.5 mM), and a lipid mixture (L-α-1,2-dioleoyl-sn-glycero-3-phosphocholine, L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine, and bovine brain phosphatidylserine in a ratio of 1:1:1 (w/w/w), 40 µg/ml). Cilengitide solution in phosphate buffer was prepared ex tempore; the highest concentration used was 15 mM.

In general, pre-incubations were performed for 30 min with or without an NADPH-generating system. Residual activity was determined by co-incubating with a marker substrate for an additional 5 min (Sohl et al., 2009). Briefly, each reaction mixture was quenched by the addition of four volumes of CH₂Cl₂ and centrifuged at 1900 × g for 10 min. The organic layer was transferred and the solvent was evaporated under a stream of nitrogen; the dried sample was dissolved in CH₃OH for analysis. 6β-Hydroxytestosterone and oxidized nifedipine were determined using an LC-PDA Acquity system (Waters, Milford, MA) and an octadecylsilane (C₁₈) column (6.2 mm × 80 mm, 3 µm, Agilent Technologies, Santa Clara, CA) for the nifedipine assays and a similar octadecylsilane column (4.6 mm × 250 mm, 5 µm, Phenomenex, Torrance, CA) for the testosterone assays with CH₃OH–H₂O (64/36, v/v) for isocratic elution in both cases.
For determining inactivation kinetics with cilengitide, concentrations of zero, 0.75, 1, 3, 5, 7.5, and 15 mM were pre-incubated (in duplicate) with pooled human liver microsomes and an NADPH-generating system for zero, 3, 6, 9, 15, and 30 min. After the pre-incubation, an aliquot of marker substrate was added, with the procedures of isolation and quantitation described above.

**Determination of Reversible and Quasi-irreversible Inhibition**

Cilengitide (final concentration 15 mM) was pre-incubated for 30 min with human liver microsomes (500 pmol P450) at 37 ± 1°C in 500 μl mixtures containing 50 mM potassium phosphate buffer (pH 7.4) and an NADPH-generating system. Pre-incubation and solvent controls were treated with potassium ferricyanide (final concentration 2 mM), after which samples and control were dialyzed for 24 h 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**

H₂O (950 μl) was added to 250 μl of a P450 pre-incubation mixture (*vide supra*). 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, 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 dithionite 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 min following the addition of the alkaline solution, due to instability of the pyridine hemochromogen under basic conditions. The heme concentration was calculated based on an extinction coefficient of 20.7 mM⁻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 NADPH-generating system, were performed. The reactions were stopped by chilling the tubes on ice. Enzymes were separated by size exclusion chromatography (Micro Bios-Spin column P-6, Bio-Rad, 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% (w/v) 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 h at 70°C in the dark. Scintillation fluid (ScintiVerse II Cocktail, Fisher Scientific, Waltham, MA, 10 mL) was added and samples were left at 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). This 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), and left at -70 °C. The imagining screen was visualized after 7 to 10 days on a Molecular Imager Pharos FX Plus Systems (Bio-Rad).

**Spectrophotometry**

Spectral changes resulting from 2 μM ligand (cilengitide, cyclopeptide analogues of cilengitide, and guanidine derivatives) binding to 2.5 μM recombinant P450 3A4 were monitored for 8 h. The binding affinity of 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 min) 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-dihydroxybenzoic acid for removal of oxygen traces (Bull et al., 1981; Guengerich et al., 2004)) after photoreduction (halogen lamp 500 W, 1 min) in the presence of 1 μM 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 (On-Line Instrument Systems, for monitoring binding to reduced P450 3A4). Changes in the difference
spectra (at the wavelength maximum and minimum) were plotted against the cilengitide concentrations to estimate affinity.

**Synthesis of Acetylated Ornithine Derivative of Cilengitide**

The ornithine derivative of cilengitide (Merck Serono) was acetylated with acetic anhydride in pyridine at room temperature for 1 h (Karanewsky et al., 1988). The organic solvent was removed by co-evaporation with (C$_2$H$_5$)$_2$O *in vacuo*. Residual traces of solvents were removed *in vacuo* after addition of CH$_2$Cl$_2$ with (C$_2$H$_5$)$_3$N. A cation exchange column was used for purification, i.e. a Dowex AG 50W-X2 ion-exchange column (H$^+$ form) was equilibrated with acetic buffer (pH 4.5) before application of an aqueous solution of the residue (following evaporation). Fractions were monitored with an Aminco DW2a/OLIS spectrophotometer (200-400 nm). Structure and purity of the product (55% yield) were confirmed by LC-MS and NMR (Supplemental Figure 1).

**Data analysis.** Data from assays was processed in GraphPad Prism software (GraphPad Software, San Diego, CA). As inhibition was monitored, a one-sided *t*-test ($\alpha = 0.05$) was used for the assessment of significance in differences between samples and controls, based on measurements of residual activity. A non-linear three parameter sigmoidal-logistic equation was used for *IC*$_{50}$ calculations: Residual activity = Bottom + (Top-Bottom)/(1 + 10exp((X – log*IC*$_{50}$))). The Michaelis-Menten equation was used for determination of inactivation constants ($K_1$) and inactivation rate constants ($k_{inact}$) of inhibitor (I): $k_{obs} = k_{inact} \times [I] / (K_1 + [I])$. A quadratic equation was used to determine dissociation constants ($K_d$) of ligands (L) with the enzyme (E):

$$\Delta A = A_0 + (B_{max} / 2[E])([(K_d + [E] + [L]) - ((K_d + [E] + [L])^2 - 4[E][L])^{1/2}], A_0$$ and $B_{max}$ being
constants in each analysis. DynaFit simulation software (Biokin, Pullman, WA) simulation software 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 pre-incubation) and after pre-incubation 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% ($P = 0.033$) and 19% ($P = 0.034$) in testosterone and nifedipine assays, respectively. When cilengitide was pre-incubated without the addition of NADPH, reductions of enzyme activity by 23% (testosterone assay, $P = 0.031$) and 10% (nifedipine assay, $P = 0.008$), respectively, was observed. Greater inhibition was observed when cilengitide was pre-incubated with NADPH: both nifedipine and testosterone assays showed significant declines in enzyme activity, i.e. 60% ($P = 0.007$) and 54% ($P = 0.007$) respectively.

For the NADPH-dependent inhibition, inactivation kinetics were determined (Fig. 2). In the nifedipine assay, the inactivation constant $K_I$ was $6.3 \pm 1.6$ mM and the inactivation rate ($k_{\text{inact}}$) was $0.017 \pm 0.002$ min$^{-1}$, corresponding to an inactivation efficiency ($k_{\text{inact}}/K_I$) of $0.003 \pm 0.001$ 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. Pre-incubation 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 $K_I = 1.1 \pm 0.2$ mM and $k_{\text{inact}} = 0.06 \pm 0.03$ min$^{-1}$ in the testosterone assay and $K_I = 5.0 \pm 1.1$ mM and $k_{\text{inact}} = 0.21 \pm 0.03$ min$^{-1}$ in the nifedipine assay (Fig. 2). The inactivation efficiency ($k_{\text{inact}}/K_I$) in both assays with recombinant P450 3A4 was $0.04 \pm 0.01$ min$^{-1}$ mM$^{-1}$.

**Determination of Inactivation Mechanism**

The pyridine hemochrome spectrophotometric assay was used to determine possible covalent modification of heme (Flink and Watson, 1942; Paul et al., 1953) after pre-incubation 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 pre-incubation 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 as well, and
therefore incubations were performed with bovine serum albumin and ovalbumin as “traps” (Fig. 4B1, 4B2). Significant binding of cilengitide to bovine serum albumin was observed when compared to control (without NADPH) (4%). In human liver microsomes, binding of $^{14}$C-labeled cilengitide was observed to proteins with higher molecular weights (Fig. 4C1, 4C2).

The reversibility of cilengitide binding was investigated by dialyzing incubations for 24 h and testing residual activity. No reversibility was observed in the testosterone and nifedipine assays (Fig. 5). To determine possible quasi-irreversible inhibition (Silverman, 1995), pre-incubations were treated with potassium ferricyanide and dialyzed for 24 h. 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 pre-incubations 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 h, 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 h. Slow Type II spectral changes was observed (Fig. 6A, 6C). The maximal spectral change was achieved after 2 h, indicating slow inhibitor-enzyme complex formation. This was further confirmed by testing residual activity after a two hour pre-incubation 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 to 30 min pre-incubation (vide 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 binding of ligands to P450 3A4 involves multiple states/reactions following diffusion-limited encounter (Isin and Guengerich, 2007).

A complex with the ferric form of P450 3A4 was formed at much lower concentrations (\(K_d = 0.66 \mu\)M, Fig. 6E), compared to 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. 6B, 6D). 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. 7A, 7B). 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 pre-incubation), 68 ± 8% (pre-incubation without NADPH), 85 ± 6% (pre-incubation 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 pre-incubation), 24 ± 3% (pre-incubation without NADPH), and 19 ± 2% (pre-incubation with NADPH) (Fig. 7C). In the nifedipine assay the residual activity was 46 ±
4% (no pre-incubation), 38 ± 4% (pre-incubation without NADPH), and 31 ± 1% (pre-incubation with NADPH) (Fig. 7D).

**Spectral Changes and Inhibition Assays with Cyclopeptide Analogues**

Spectral changes were monitored to determine binding constants of cilengitide analogues. Based on one-ligand/two-site binding model, the dissociation constants $K_d$ were 0.52 μM 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, while 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 ($P = 0.015$), while 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 pre-incubation), time-dependent (30 min pre-incubation without NADPH-generating system), and also NADPH-dependent inhibition (30 min pre-incubation 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, while the maximal inhibition of P450 3A5 was 23% under the same conditions.

Inactivation kinetics for metabolism (NADPH)-dependent inhibition was studied with both human liver microsomes and recombinant baculosomes (Fig. 2). As values of inhibition
constant are in the millimolar range and 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 H₂O₂ generated (Fig. 3). This assay is based on determining heme iron in ferrous form as a complex with pyridine in basic media (vs. 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 vs. 27%), indicating possible existence of stable Fe²⁺-cilengitide complex.

[¹⁴C]-Cilengitide was bound not only to P450 3A4 but also to NADPH-P450 reductase (Fig. 4). Non-specific binding was also confirmed when radioactive cilengitide was co-incubated 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 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 ($K_{d,Fe^{3+}} = 0.66 \mu M$ vs. $K_{d,Fe^{2+}} = 6 \mu M$).

The quasi-irreversibility experiment suggested possible formation of 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 non-steroid 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 one day (Fig. 5). Negative results for this experiment cannot eliminate the possibility of slow, tight binding, e.g. as shown for proteasome inhibitors (Manam et al., 2008).

Based on spectral changes, it was postulated that the guanidine group of cilengitide was responsible for the observed inhibition. To test this hypothesis, guanidine-containing analogues (debrisoquine and $N$-acetyl-arginine-$O$-methyl ester) were used, 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 h to achieve the maximum Type II spectral change. Inhibition assays also confirmed the low residual activity of P450 3A4 pre-incubated with debrisoquine 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 inhibitors (Schenkman et al., 1967); strong inhibition was confirmed in inhibition assays with both testosterone and nifedipine as marker substrates. However, similar spectral changes were observed with the $N$-acetyl ornithine...
derivative of cilengitide. Nonetheless, inhibition assays showed much lower reduction of catalytic activity compared to 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 anti-androgen 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 guanidino group include famotidine (antiulcer drug) and metformin (oral hypoglycemic). Famotidine was developed as a histamine H2-receptor antagonist with low interaction potential compared to cimetidine and ranitidine (Humphries, 1987). Metformin exhibits interactions with organic cation transporters (the guanidino 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 reactive metabolite or more potent metabolite is studied by pre-incubating the candidate drug with the test system (e.g. human liver microsomes) and NADPH- or NADPH-re/generating 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. Prior to including such new evaluation into a drug-drug interaction package, it is important to learn how often this mechanism occurs in more classical chemical space for drugs.
In conclusion, cilengitide causes time- and NADPH-dependent inhibition of P450 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 non-extractable radioactivity observed in vivo. To date the chemical mechanism of the adduct formation is not known.
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Authorship Contributions

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References


Guengerich FP (2005b) Reduction of cytochrome *b*<sub>5</sub> by NADPH-cytochrome P450 reductase. *Arch Biochem Biophys* **440**:204-211.


Footnotes

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Figure Legends

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

Fig. 2. Inactivation kinetics determined with human liver microsomes and recombinant P450 3A4 systems using testosterone and nifedipine assays. (A) The cilengitide inactivation constant determined with human liver microsomes (testosterone assay), $K_{I,d}$ was >15 mM (highest concentration analyzed; if extrapolated, $K_I = 21 \pm 1$ mM and $k_{inact} = 0.07 \pm 0.01 \text{ min}^{-1}$). (B) For the nifedipine assay, $K_I = 6.3 \pm 1.6$ mM and inactivation rate $k_{inact} = 0.017 \pm 0.002 \text{ min}^{-1}$. For inactivation of recombinant P450 3A4 testosterone assay (C), $K_I = 1.1 \pm 0.2$ mM and $k_{inact} = 0.06 \pm 0.03 \text{ min}^{-1}$ and for the nifedipine assay (D), $K_I = 5.0 \pm 1.1$ mM and $k_{inact} = 0.21 \pm 0.03 \text{ min}^{-1}$. Thus with both testosterone and nifedipine assay, $k_{inact}/K_I \approx 0.003 \pm 0.001 \text{ min}^{-1} \text{ mM}^{-1}$ for recombinant P450 3A4.

Legend: concentrations of cilengitide $\bullet$ 0, $\blacksquare$ 0.75, $\blacktriangle$ 1, $\blacktriangledown$ 3, $\blacklozenge$ 5, $\circ$ 7.5, $\square$ 15 mM.

Fig. 3. Heme assays. After incubation for 30 min with an NADPH-generating system in the presence of catalase, no significant difference in pyridine hemochrome complex formation was observed between a control incubation, a cilengitide incubation, and reconstituted P450 3A4 without incubation (enzymes mixture).
Fig. 4. Binding of cilengitide to proteins. (A) An SDS-polyacrylamide gradient (4-12%, w/v) gel showed binding of cilengitide to NAPDH-P450 reductase and P450 3A4 when comparing incubations of a reconstituted P450 3A4 system (P450 3A4, reductase, cytochrome b₅, phospholipids) with and without an NADPH-generating system. (B) To determine if non-specific 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 done after 10 days of exposure. B2, 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 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.

Fig. 5. Reversibility of cilengitide binding to P450 3A4. To test the reversibility of cilengitide binding to P450 3A4, incubations with human liver microsomes were performed with the addition of an NAPDH-generating system for 30 min, after which the samples were dialyzed for 24 h at 4 °C. Negative controls contained no NADPH-generating system. Residual activity was tested with both the testosterone (left) and nifedipine assays (right). To test quasi-irreversibility, potassium ferricyanide (K₃Fe(CN)₆) was added after pre-incubation and before dialysis for 1 h.
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 minima 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 2-3 h (C), in contrast to the rapid binding to the reduced form of P450 3A4 (< 2 min, 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 $K_{d,Fe^{2+}} = 6 \pm 3 \mu M$.

Fig. 7. Spectral changes and inhibitory effects of guanidines. Spectral changes were recorded every 10 min, and difference spectra are shown (A and B). Type II spectral changes were observed with debrisoquine (A) and N-acetyl-arginine O-methyl ester (Ac-Arg-O-Me) (B). Residual activity was measured in testosterone and nifedipine assays with human liver microsomes, with or without pre-incubation and an NADPH-generating system. No statistically significant inhibition was observed with 15 mM Ac-Arg-O-Me (C). In the nifedipine assay, 15 mM debrisoquine caused a significant reduction of activity (up to 70%), most prominent being pre-incubation with NADPH-generating system (D). Ac-Arg-O-Me caused a 32% decrease of activity (D).

Fig. 8. Spectral changes and inhibitory effect of cyclopeptides. Spectral changes were recorded every 10 min, and difference spectra are shown (A and B). The ornithine derivative (cilengitide with arginine replaced with ornithine) caused Type II spectral changes that resemble cilengitide binding to P450 3A4 (A). Similar spectral changes were observed for binding of acetylated derivative to P450 3A4 (B). Residual activity was measured in testosterone and nifedipine
assays; pre-incubation was performed with human liver microsomes and an NADPH-generating system (C). The ornithine derivative caused complete inhibition of P450 3A4 in the nifedipine assay and a decrease of 82% in activity in the testosterone assay. Acetylation of the ornithine derivative reduced the inhibitory effect (testosterone assay: 39%, nifedipine assay: 6%).
Figure 1
Figure 2

Human liver microsomes

Testosterone assay

A

Inactivation Decay Plot

log(Residual activity / %)

0 10 20 30 40

t / min

Nifedipine assay

B

Inactivation Decay Plot

log(Residual activity / %)

0 10 20 30 40

t / min

Recombinant P450 3A4

Testosterone assay

C

Inactivation Decay Plot

log(Residual activity / %)

0 10 20 30 40

t / min

Nifedipine assay

D

Inactivation Decay Plot

log(Residual activity / %)

0 10 20 30 40

t / min

Inactivation kinetics

k_{obs} (min^{-1})

0 0.01 0.02 0.03

[cielengitide] (mM)

0 5 10 15 20

Inactivation Direct Plot

k_{obs} (min^{-1})

0 0.005 0.01 0.015

[cielengitide] (mM)

0 5 10 15 20
Figure 3

[Bar chart showing the [Heme] concentrations for different conditions: Control incubation, Cilengitide 15 mM, Enzymes mixture. The y-axis represents concentrations in μM, and the x-axis lists the different conditions.]
Figure 5
Figure 7

A and B: Absorbance measurements over wavelength (nm) showing changes at different wavelengths.

C and D: Bar graphs comparing residual activity (% 6BHT and % nifedipine oxidized) under different conditions:
- Control
- Debrisoquine
- Ac-Arg-O-Me

Conditions include:
- Pre-incubation with NADPH
- Pre-incubation without NADPH
- Direct inhibition

Significance indicators: *

** Alternate significance markers for specific comparisons.