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Mechanism-based Inhibition of CYP3A4 by Podophyllotoxin: Aging of an Intermediate is Important for in Vitro/in Vivo Correlations

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Abstract

An in vitro observation of time-dependent-inhibition of metabolic enzymes often results in removing a potential drug from the drug-pipeline. However, the accepted method for predicting TDIs of the important drug metabolizing cytochrome P450 enzymes often overestimates the drug interaction potential. Better models that take into account the complexities of the cytochrome P450 enzyme system will lead to better predictions. Herein we report the use of our previously described models for complex kinetics of podophyllotoxin. Spectral characterization of the kinetics indicates that an intermediate MI-complex is formed, which slowly progresses to an essentially irreversible MI-complex. The intermediate MI-complex can release free enzyme during the time-course of a typical 30 minute TDI experiment. This slow rate of MI-complex conversion results in an over-prediction of the k_{inact} value if this process is not included in the analysis of the activity versus time profile. In vitro kinetic experiments in rat liver microsomes predicted a lack of drug interaction between podophyllotoxin and midazolam. In vivo rat pharmacokinetic studies confirmed this lack of drug interaction.

Graphical abstract

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Supporting Information. Figures for binding of PPT to CYP3A4, and the structure of residuals after the subtraction of the contribution of the first 0, 1, 2 and 3 eigenvectors is supplied as Supporting Information.

Keywords

methylenedioxyphenyl compounds; time-dependent inhibition; carbene; podophyllotoxin; mechanism-based inhibition

INTRODUCTION

Avoiding drug-drug interactions (DDIs) is a critical effort during drug discovery and development. One of the most difficult DDIs to predict with preclinical data is the time-dependent inhibition (TDI) of the cytochrome P450 enzymes (CYPs). Since these TDIs result in a loss of enzyme over time,^{1–6} the in vivo impact of TDI is more difficult to assess than competitive inhibition. Whereas the DDI potential of a competitive inhibitor can be predicted from free drug concentration at the active site and a binding constant (at least in theory), the DDI potential of a TDI will depend on the affinity (K_I), inactivation rate (k_{inact}), and rate of enzyme regeneration (k_{deg}).^{5, 6} Given the complexity and uncertainty associated with DDI prediction, compounds that show TDI in vitro are most often removed from the development pipeline.

Current methods to determine TDI parameters have provided limited success in predicting clinical outcomes.^{7–10} A recent survey of 17 PhRMA member companies provides a number of reservations on the current use of TDI methodology.⁵ CYP kinetics are often complex, presumably due to the simultaneous interaction of multiple substrates with the active site, alternate site allosteric effects, and complex protein-protein interactions which modulate reactivity.^{11–16} Atypical kinetics are expected and observed for CYP-mediated TDI and we have reported a numeric method that can incorporate these and other observed mechanistic complexities.^{14, 17}

Another and probably more significant complexity in TDI kinetics is the mechanism of metabolic intermediate complex (MIC) formation. Many amines are known to form MICs via conversion to nitroso compounds and are classified as quasi-irreversible inhibitors.¹⁸ Methylenedioxyphenyl (MDP) compounds are also quasi-irreversible inhibitors via

activation to carbene MICs.^{2, 19} We have shown previously that the in vitro kinetics for MIC forming drugs is consistent with a reversible step that results in concave-upward PRA plots.^{14, 17} However, the rate constant for reversibility is not consistent with the persistence of in vivo inhibition for MIC-forming drugs.¹⁴ In order to resolve this discrepancy, it is imperative that we understand the detailed mechanism of MIC formation.

MIC formation from amines is complicated by the sequential oxidation steps required to generate the nitroso intermediate, which then binds to the heme. MIC formation from MDP compounds is more straightforward, since a single oxidation converts the methylenedioxy group to a carbene (Figure 1). MICs formed from MDP compounds have been studied with visible difference spectroscopy, since the porphyrinic adduct has two characteristic absorption peaks at 425–427 and 455 nm (“type III” spectrum).^{20–23} Elcombe and coworkers²⁴ observed type III interaction in rat hepatic microsomes treated with isosafrole, which in a further study they attributed to a stable ferrocyclochrome:carbene complex ($\text{Fe}^{2+}:\text{carbene}$).²⁵ The 425/455 nm absorbance is considered the defining characteristic of MIC formation.²⁶ An additional hypothesis reported by several authors is that the 425–427 nm peak is due to the carbene-induced displacement of the axial thiolate, leading to irreversible enzyme inactivation.^{2, 27} Moreover, an additional peak at 438 nm was identified and attributed to a supposed oxidized cytochrome P450-metabolite ($\text{Fe}^{3+}:\text{carbene}$).²⁵

The goal of the present study is to characterize the time-course of MIC formation using biophysical techniques and relate these rate constants to observed time-dependent inactivation kinetics in vivo. Here we report that MIC formation involves a moderately reversible intermediate ($\text{Fe}^{3+}:\text{carbene}$) that ages to a very slowly reversible intermediate ($\text{Fe}^{2+}:\text{carbene}$). The relative rate of this aging process is of fundamental importance in understanding if an in vitro TDI will result in an in vivo TDI. If aging is slow, as is the case for podophyllotoxin (PPT), longer pre-incubation times are required to determine k_{inact} and the in vivo inhibition due to MIC formation may be negligible.

MATERIALS AND METHODS

Materials

All reagents and solvents unless noted were obtained from Sigma-Aldrich/Fluka (St. Louis, MO). Midazolam (MDZ) and 1'-hydroxy-midazolam (1'-OH-MDZ) were purchased from Tocris Bioscience (Bristol, UK) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Podophyllotoxin (PPT) was purchased from Toronto Research Chemicals, Canada. 1-methyl-2-pyrrolidinone (NMP) was obtained from Avantor (Center Valley, PA, USA). CYP3A4-containing Baculosomes® (1 nmol/ml protein concentration) were purchased from Life Technologies (Carlsbad, CA, USA). Human liver microsomes pooled from 150 donors were purchased from Corning® Life Sciences (Tewksbury, MA, USA). Sprague-Dawley® rat liver microsomes from male donors were purchased from Invitrogen Co. (Carlsbad, CA, USA). Single jugular vein cannulated male Sprague Dawley rats were obtained from Charles River Laboratories (Malvern, PA, USA).

CYP3A4 Expression and Purification

CYP3A4 was expressed in *E. coli* and purified as described by Roberts.²⁸ For the purification step, the enzyme is first solubilized with 1% emulgen and loaded onto a Ni²⁺ column eluted with 100 mM Potassium phosphate (pH 7.4), 20% glycerol, 500 mM imidazole, and 0.05% cholate. The purified enzyme was then dialyzed overnight at 4 °C against 100 mM Potassium phosphate (pH 7.4) and 20% glycerol (v/v) and stored at –80 °C. The purity of CYP3A4 was >95% as determined by SDS–PAGE analysis and CO bound UV/Vis absorption. Protein concentration was determined by absorbance at 450 nm using an extinction coefficient of 91 mM^{–1}cm^{–1} for the CO bound protein.²⁹

Spectral Binding Titrations

Enzyme titrations were performed in split-beam mode with an Olis upgraded Aminco DW-2000 spectrophotometer (Olis, Inc., Bogart, GA) equipped with a Quantum NorthwestTLC50™ temperature-controlled cuvette holder (Quantum Northwest Inc., USA, Liberty Lake, WA). Experiments were performed at 37 °C, the wavelength range typically encompassed 350 to 500 nm, and readings averaged over 10 scans were taken in 1-nm steps. Binding affinities of PPT with CYP3A4 was determined by titration 0.5 μM of purified enzyme with the ligand, in a total volume of 1.0 ml of 100 mM Potassium phosphate buffer (pH 7.4). PPT was dissolved in 5% MeOH and the final concentration of solvent in the system was <2%. The reference cuvette contained buffer, and was titrated with an equal volume of ligand solution. UV-Vis spectra were recorded after each addition, and the absorbance differences were plotted against the added ligand concentration. Spectral dissociation constants (K_S) were estimated using OriginLab software (OriginLab Co., Northampton, MA, USA) with non-linear regression, using the hyperbolic equation $Abs = B_{max}[L]/(K_S + [L])$, where Abs is the absorbance difference, B_{max} is the maximum absorbance difference extrapolated to infinite ligand concentration, and $[L]$ is the ligand concentration.

Binding Kinetics of Ligand to CYP3A4

Stopped flow experiments were carried out using an Applied Photophysics RX2000 stopped-flow apparatus (Applied Photophysics LTD, Leatherhead, UK) connected to an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Changes in low-spin/high-spin heme spectra were monitored as a function of time. One drive syringe contained purified CYP3A4 diluted to 4 μM in 100 mM Potassium phosphate buffer (pH 7.4). The second drive syringe contained the ligand solution (200 μM PPT, dissolved in 5% MeOH). All stopped-flow measurements were carried out at room temperature (22 °C). After mixing equal amounts (200 μl) of each reagent from both syringes, UV-Vis spectra were collected at 10 scan/s for 30 s. Averages of six experiments were used in the subsequent data analysis. The difference in absorbance between high-spin and low-spin (390–417) was plotted against time, and the association rate (k_1) was obtained fitting a single and biexponential equations to the experimental data with non-linear regression using Mathematica 10.0 (Wolfram Research, Champagne, IL). Models were evaluated using AICc,³⁰ R^2 , and residual plots. The dissociation rate (k_2) was estimated

from $K_s = \frac{k_2}{k_1}$, where K_s is the spectral equilibrium constant obtained with UV-Vis titration of purified CYP3A4.

Spectral MIC Formation

Baculosomes® (1 nmol/ml protein concentration) were diluted to 0.1 nmol/ml in 100 mM Potassium phosphate buffer (pH 7.4). PPT (100 µM from a 10 mM stock solution in 10% MeOH) was added and after 3 min of incubation at 37 °C a baseline was taken. Reaction was started adding NADPH-generating system consisting of 100 µM NADP⁺, 5 mM glucose-6-phosphate and 1.0 UI/ml of glucose-6-phosphate dehydrogenase (final concentration); buffer was added in the reference cuvette. All spectra were determined in split-beam mode with an Olis upgraded Aminco DW-2000 spectrophotometer (Olis, Inc., Bogart, GA) equipped with a Quantum NorthwestTLC50™ temperature-controlled cuvette holder (Quantum Northwest Inc., USA, Liberty Lake, WA). The wavelength range typically encompassed 400 to 500 nm and readings averaged over 5 scans were taken in 1-nm steps. Experiments were performed for 30 min at 37 °C.

Cytochrome P450, P420 and P450:carbene Complex Quantification

P450 and P420 concentrations before and after reaction were determined by absorbance at 450 and 420 nm using an extinction coefficient (ϵ) of 91 mM⁻¹cm⁻¹ and 110 mM⁻¹cm⁻¹ respectively, for the carbon monoxide bound protein.²⁹ P450:carbene compound was determined using $\epsilon = 75 \text{ mM}^{-1} \text{ cm}^{-1}$ as reported by Chatterjee et al.³¹

Spectral Analysis and Singular Value Decomposition (SVD)

Spectra were collected and analyzed using Matlab (The Mathworks Inc., v.R2014a). Before SVD, Savitzky-Golay smoothing and peak-to-area normalization were performed on all the recorded spectra to increase signal-to-noise ratio. SVD analysis was then performed using the built-in Matlab function, obtaining the corresponding kinetic and spectral eigenvectors. Three components were considered as significantly above baseline, which account for > 70% of total variance in all the experiments.

In Vitro TDI Assays

In the inactivation preincubation, pooled HLM (containing 80 nM CYP3A4) were incubated at 37 °C with various concentration of PPT (0–20 µM) and NADPH (1 mM) in Potassium phosphate buffer (100 mM, pH 7.4). At seven time points (0–30 min), the activation mix containing the substrate (MDZ, 25 µM) and NADPH (1 mM) in Potassium phosphate buffer was added, and the reaction was carried on for 6 min at 37 °C. Both PPT and MDZ were dissolved in MeOH, and the final concentration of solvent in the system was 0.5%. Addition of activation solution causes an 8X dilution of the HLM (10 nM). Reaction with the substrate was quenched with 40 µl of 1 M formic acid in acetonitrile, containing a known concentration of 2-methyl-4(3H)-quinazolinone as internal standard, followed by centrifugation at 9,300 rcf for 10 min.

For rat liver microsomes (RLM), in the inactivation preincubation, pooled RLM (80 nM) were incubated at 37 °C with various concentration of PPT (0–50 µM) and NADPH (1 mM)

in Potassium phosphate buffer (100 mM, pH 7.4) containing TCEP 3 mM, and a reactive oxygen scavenging system consisting in superoxide dismutase and catalase, 250 U/ml each. The addition of superoxide dismutase and catalase was due to a considerable and biphasic decrease of enzymatic activity (up to 35%) observed at $[I] = 0$ (data not shown). At seven time points (0–30 min), the activation mix containing the substrate (MDZ, 100 μ M) and NADPH (1 mM) in Potassium phosphate buffer was added, and the reaction was carried on for 6 min at 37 °C. Both PPT and MDZ were dissolved in MeOH, and the final concentration of solvent in the system was 0.5%. Addition of activation solution causes an 8X dilution of the RLM (10 nM). Reaction with the substrate was quenched with a solution 1 M formic acid in acetonitrile, containing a known concentration of ^{13}C -1'-OH-MDZ as internal standard, followed by centrifugation at 9,300 ref for 10 min.

Rat Plasma Equilibrium Dialysis

Equilibrium dialysis was performed to determine the unbound fraction (f_u) of MDZ and PPT in rat plasma. Briefly, rat plasma was spiked with MDZ or PPT to a final concentration of 2 μ M. A 96 well equilibrium dialyzer™ (Harvard Apparatus, Holliston, MA, USA) was used with plasma sample on one side and blank phosphate buffer (pH 7.4) on other side with 5% CO_2 at 37 °C for 20 h. After equilibrium was achieved, samples on each side of the membrane were analyzed with LC-MS/MS.

In Vivo Rat DDI Studies

Male Sprague-Dawley single jugular cannulated rats weighing 250 to 350 g were maintained in the American Association for the Accreditation of Laboratory Animal Care–accredited University Laboratory Animal Resources of Temple University. Rats were housed individually under a 12 h light/dark cycle. Food and water were provided ad libitum. MDZ was dissolved in NMP and diluted with saline to 2.5 mg/ml. PPT was also dissolved in NMP and diluted with saline to 0.5 mg/ml. NMP percentage was 25% in both the formulations. In initial studies, animals were dosed PPT alone to determine the PK of PPT. Based on the observed half-life (37 min in rat), the following dosage regimen was designed for the DDI studies. Animals were divided in two groups, control and inhibitor treated group. In inhibitor treated group ($n = 5$) PPT (1 mg/kg, IV) was administered once every 2 h for a total of three doses. After 4 h of the last PPT dose, MDZ 5 mg/kg was administered through IV route. In control group ($n = 6$) blank vehicle was administered instead of PPT followed by MDZ. Heparin was used as an anticoagulant. Blood samples were collected at 0, 2, 5, 10, 15, 30 min and 1, 2, 3, 6, 9, 12 h in heparin dried tubes after MDZ administration. Plasma samples were analyzed by LC-MS/MS.

Liquid Chromatography-Mass Spectrometry

For the HLM in vitro assay, samples were analyzed using an 1100 series high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) and an API 4000 tandem mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster City, CA) using turbospray ESI operating in positive ion mode. Chromatography was performed on a Luna® reverse-phase column (50 $\times$ 2.0 mm, 5 μ m; Phenomenex, Torrance, CA). Mobile phase A consisted of 0.05% formic acid and 0.2% acetic acid in water, and mobile phase B comprised 90% acetonitrile, 9.9% water, and 0.1% formic acid. Using a

flow rate of 600 µl/min, the column was equilibrated at initial conditions of 95% mobile phase A for 0.5 min. Chromatographic separation was performed using a linear gradient over the next 1.5 min to 25% mobile phase A. Mobile phase A was then held constant at 25% over 0.5 min, followed by a linear gradient back to 95% A over 1.0 min. Finally, the column was re-equilibrated to the initial conditions over the last 2 min. The total chromatographic assay time was 5.0 min per sample, and the retention times for internal standard and metabolite were 2.4 and 2.8 min, respectively. The optimized mass spectrometer tune parameters were as follows: collision gas, 10; curtain gas, 15; ion source gas, 50; ion source gas, 25; ion spray voltage, 5000; desolvation temperature, 500; declustering potential, 85; entrance potential, 15; collision energy, 20; collision cell exit potential, 15. The analyte (1'-OH-MDZ)³² and the internal standard (2-methyl-4(3H)-quinazolinone) were detected using multiple reaction monitoring mode by monitoring the *m/z* transition from 342.2 to 203.2 and 161.0 to 120.0, respectively. Quantitation of product was achieved by extrapolating from a standard curve ranging from a 1 to 1000 nM concentration of authentic 1'-OH-MDZ.

For the RLM in vitro assay a different protocol was used since it was done in the different lab, an LC-20AD series high-performance liquid chromatography system (Shimadzu, Columbia, MD) fitted with a HTC PAL autosampler (LEAP Technologies, Carrboro, NC) was used to perform chromatography on a Luna® reverse-phase column (50 × 2.0 mm, 5 µm; Phenomenex, Torrance, CA). Using a flow rate of 500 µl/min, chromatographic separation was performed in isocratic mode, with 55% mobile phase A and 45% mobile phase B. The total chromatographic assay time was 5.0 min per sample, and the retention times for internal standard and metabolite were 1.1 min. The quantification of the metabolite was conducted using an API 4000 Q-Trap tandem mass spectrometry system manufactured by Applied Biosystems/MDS Sciex (Foster City, CA) using turbospray ESI operating in positive ion mode. The optimized mass spectrometer tune parameters were as follows: collision gas, 20; curtain gas, 20; ion source gas 1, 60; ion source gas 2, 40; ion spray voltage, 5500; desolvation temperature, 600; declustering potential, 70; entrance potential, 10; collision energy, 50; collision cell exit potential, 10. The analyte (1'-OH-MDZ) and the internal standard (¹³C-1'-OH-MDZ) were detected using multiple reaction monitoring mode by monitoring the *m/z* transition from 342.2 to 203.2 and 345.2 to 206.3, respectively. Quantitation of product was achieved by extrapolating from a standard curve ranging from a 1 to 1000 nM concentration of authentic 1'-OH-MDZ.

For chromatographic separation of MDZ and PPT in the in vivo rat plasma samples, a Phenomenex Luna-C18 (3 µm, 30 × 2 mm) analytical column coupled with a C18 guard column (4 × 2.0 mm) was used. Diltiazem and deoxypodophyllotoxin (DPT) were used as internal standards (IS) for MDZ and PPT respectively. AB Sciex API 4000 LC-MS/MS system was used for analyzing plasma samples in positive mode using following MRM transitions 327.050 to 292.160 *m/z* for MDZ, 415.500 to 178.400 *m/z* for diltiazem (IS), 415.126 to 397.100 *m/z* for PPT and 399.229 to 231.300 *m/z* for DPT. For MDZ and PPT gradient elution was used. Solvents used for MDZ and PPT LC-MS method consisted of aqueous mobile phase (A) and organic mobile phase (B) consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. For MDZ a gradient elution with 300 µl/min was programmed from 10% to 95% B in 0.5 min maintained at 95% until 1.5 min and returned to

initial condition at 2 min. The total run time was 8 min and the retention time was 3.7 min for MDZ. For PPT a gradient elution with 250 $\mu\text{l}/\text{min}$ was programmed from 10% to 95% B in 1.5 min maintained at 95% until 3 min and returned to initial conditions at 4.5 min. Total run time was 11 min and retention time was 5.37 min for PPT and 6.0 min for DPT. Calibration curves were linear for MDZ from 4 ng/ml to 10000 ng/ml, and 5ng/ml to 2000 ng/ml for PPT.

Kinetic Modeling and Simulations

For both CYP3A4 baculosomes and HLM, spectra were reconstructed using the three main components, and the corresponding concentration profiles were obtained. The extinction coefficients (ϵ) used for Fe^{2+} :carbene was $75 \text{ mM}^{-1} \text{ cm}^{-1}$,³¹ which was also assumed for the oxidized intermediate; the concentration of the third component over time was obtained using $\epsilon = 110 \text{ mM}^{-1} \text{ cm}^{-1}$, obtained from the P420 spectra (see Results section).³³ The differential equations to model the spectral complex formation kinetics were parameterized using the NonlinearModelFit function with 1/Y weighting in Mathematica 10.0 (Wolfram Research, Champagne, IL). When fitting parameters, the ParametricNDSolveValue function was used for numerical solutions of the differential equations with MaxSteps $\rightarrow 100,000$ and PrecisionGoal $\rightarrow \infty$. Models for CYP3A4 baculosomes and HLM were fit using the binding parameters obtained from stopped-flow experiments (binding constants k_1 and k_2). In HLM the P420:carbene component was not fit since its weighted spectral component was statistically close to background.

Fitting the PPT TDI Model

A kinetic model for TDI of CYP3A4 with PPT was parameterized using a numerical method described previously.^{14, 17} Briefly, the data were first evaluated using a standard replot of the log percent remaining activity versus preincubation time (PRA plot). The concave upward curvature is indicative of quasi-irreversible inactivation. The initial value of K_i was determined by fitting the competitive inhibition equation to the zero preincubation time data. The initial K_i estimate was significantly smaller than that estimated from the full TDI data suggesting that multiple binding constants are involved. Therefore, we evaluated both EII (two inhibitor molecules binding simultaneously) and ESI (simultaneous substrate and inhibitor binding). Enzyme loss (~20%) was observed with the zero inhibitor data and was added to the kinetic scheme from E, EI, ES, and ESI. In order to model the irreversible conversion of the Fe^{3+} :carbene to the Fe^{2+} :carbene, an additional rate constant was added (k_5 in Figure 3,) and compared to the model without k_5 . We will refer to the model without k_5 as the quasi-irreversible (Q) model, and the model with k_5 as the quasi-irreversible intermediate model (QI). Also, k_6 was optimum when set to zero. Model fitting was conducted with Mathematica 10.0.1.0 (Wolfram Research, Champagne, IL) using the NonlinearModelFit routine with PrecisionGoal = 12, MaxIterations = 10000, and 1/Y+ weighting. The WhenEvent function was used to simulate incubation dilution and substrate addition. As described previously,^{14, 17} association rate constants were fixed and dissociation rate constants were optimized to determine binding constants. For MDZ, the on-rate constant (k'_p) was fixed at $1 \mu\text{M s}^{-1}$ and the experimentally determined on-rate (k_p) of $270 \mu\text{M s}^{-1}$ was used for PPT. Models were evaluated using AICc,³⁰ R^2 , correlation

matrices, and residual plots. The results from this numerical modeling effort were compared to the standard replot method using all or only the first three (log linear) time points.

In vitro-in vivo extrapolation (IVIVE) of DDI between MDZ and PPT

In vitro parameters obtained with the standard replot method were used to predict the in vivo interaction between MDZ and PPT, by calculating the ratio of MDZ area under the plasma concentration-time curve in the presence and absence of PPT (AUC_i/AUC). This ratio was calculated with the standard static model⁴ assuming only hepatic metabolism via CYP3A:

$$\frac{AUC_i}{AUC} = \frac{1}{1 - f_m + \frac{f_m}{1 + \frac{[I]k_{inact}}{k_{deg}(K_I + [I])}}} \quad (\text{Eq. 1})$$

where f_m is the fraction of MDZ metabolized by CYP3A, $[I]$ is the in vivo PPT concentration, and k_{deg} is the resynthesis rate constant of the enzyme. The rat CYP3A k_{deg} range of 0.00018 – 0.00083 min⁻¹ was used.³⁴ A 2-compartmental model was fit to the PPT PK data in rats, to determine the total and unbound C_{max} to be used as $[I]$ in Eq. 1. AUC_i/AUC ratios were predicted with both k_{deg} values, and $[I]$ set as either total or unbound C_{max} .

The observed AUC_i/AUC was calculated with the in vivo rat data upon MDZ dosing with and without PPT. Standard 3-compartmental models were fit to the concentration-time curves of MDZ in the absence and presence of PPT, and AUCs were calculated with standard compartmental equations with Mathematica (version 10.2.0).

RESULTS

Spectral equilibrium and binding kinetics of PPT with purified CYP3A4

The affinity of PPT for CYP3A4 was estimated spectrophotometrically by monitoring the heme spectral changes upon the addition of ligand. PPT caused a Type I shift in the heme Soret band, reflecting the displacement of H₂O as the sixth ligand and a low to high spin conversion of the coordinated iron. The calculated spectral equilibrium constant (K_S) was 39.6 μM; however, the sigmoidal curve shape suggested cooperative binding to the enzyme (Supplemental Figure S1a). When a two-site binding equation was used, K_{S1} and K_{S2} values of 63.5 μM and 9.8 μM were obtained (data not shown).

To determine the association rate (k_I), the binding kinetics of PPT to CYP3A4 was investigated using absorbance measurements at 100 μM ligand concentration. The time-dependent spectra displayed also a Type I pattern; difference in absorbance at 390 and 417 nm were plotted against time, and apparent 2nd order constant (k_I) was obtained through nonlinear regression. The value ($4.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) is close to association kinetic rates of several others CYP3A4 substrates.³⁵ PPT binding kinetics was parameterized with a single exponential equation, and a biexponential equation. Unlike the equilibrium K_S measurement, the single exponential equation was selected for the following reason. Although the corrected AIC value was slightly lower for the biexponential equation (−261.3

vs -254.7), the kinetic parameters were statistically not well defined ($p > 0.1$). Thus, in order to calculate the dissociation constant (k_2), we used the K_S for one-site binding equilibrium (Supplemental Figure S1b).

MIC formation in baculosomes

CYP3A4 metabolism of MDP compounds results in the formation of a carbene intermediate which coordinates with the heme, resulting in a characteristic double Soret band (type III spectrum) at 425–427 and 455 nm.^{24, 36} Figure 2a shows the formation of the MIC in CYP3A4 baculosomes treated with PPT. The addition of an NADPH-generating system to the reaction mixture caused the appearance of the peak at 455 nm and a shoulder around 425 nm. We used SVD analysis to deconvolute the principal spectral components, a methodology previously used to characterize cytochrome P450 spectral kinetics.^{37, 38} SVD analysis resulted in three principal spectral components (Figure 2b), which cover >70% of total variance (Supplemental Figure S2). PC2 has a maximum at 437 nm, whereas PC1 and PC3 have a split Soret band at 425/455 nm and 421/455 nm, respectively. Considering the previous findings,²⁵ we attribute PC1 to the Fe^{2+} :carbene complex, whereas PC2 is the oxidized intermediate Fe^{3+} :carbene. Regarding PC3, Correia *et al.*² speculated that this corresponds to a P420:carbene complex, P420 being the enzymatically inactive displaced heme. However, given the difference in appearance kinetics it is more likely the third component is cytochrome P420 (without any ligand). The reason for such a concomitant degradation pathway is still unclear and deserves further investigation; nonetheless, the inactive P420 can be formed from cytochrome P450 in several conditions, including catalysis, as has already been reported.¹⁸ Plotting the eigenvector absorbance value against reaction time course, the disappearance of the oxidized intermediate and the consequent formation of the P420 species is followed by an increase of the Fe^{2+} :carbene complex, which does not reach a steady-state during the entire time course (Figure 2c). At the end of the kinetic experiment (~30 min), $52.0 \pm 5.8\%$ of total P450 was in the inactivated form (Fe^{2+} :carbene), whereas $17.5 \pm 11.0\%$ was converted into the P420 species and only $15.0 \pm 0.8\%$ was free P450.

MIC formation in HLM

Experiments performed in HLM are shown in Figure 2d. The spectral characterization was different from baculosomes, showing an intense peak at 425 nm, and only a shoulder at 455 nm. SVD analysis provided a closer insight of two main components, which accounted again for >70% of variance (Figure 2e); a third component is most likely attributable to background, as the associated dynamic component seemed to be not chemically related to PC1 and PC2. This is confirmed in that the subtraction of the first two principal components from the overall matrix resulted in noise (Supplemental Figure S3). Although less resolved, dynamic components are consistent with a Fe^{3+} :carbene intermediate which is reduced to the 455 nm absorbing complex. Similar to baculosomes, steady-state was not achieved during the time course for HLM (Figure 2f). The percentage of the different cytochrome fractions present at the end of the experiments were determined: only the $17.2 \pm 2.2\%$ of cytochrome P450 was in the Fe^{2+} :carbene form, whereas $40.7 \pm 7.3\%$ and $24.0 \pm 2.1\%$ were in the free and P420 form, respectively.

Kinetic modeling of the spectral components

Considering the previous findings, we propose a kinetic mechanism for CYP3A4-mediated PPT metabolism as shown in Figure 3. After inhibitor binding (EI), the reaction proceeds through the formation of the Fe^{3+} :carbene intermediate (k_3), which is reduced to the more stable Fe^{2+} :carbene complex (k_5). In order to account for the increase Fe^{2+} :carbene complex through the entire time-course of the experiment, a reversible pathway for enzyme regeneration needs to be included (k_4), which allows the release of carbene from the Fe^{3+} :carbene complex. The released carbene is unstable and would react with water to ultimately produce catechol.^{39, 40} An additional pathway leads to the formation of the inactive P420 (k_6) from the enzyme-inhibitor complex (EI), which degrades to an unknown product (k_7). The estimated parameters (with their respective standard deviations) are presented in Table 1 for both CYP3A4 baculosomes and HLM, whereas two representative fittings for CYP3A4 baculosomes and HLM are shown in Figures 4a and 4b, respectively. Non-linear regression on time-dependent curves resulted in R^2 values of 0.959 and 0.981, respectively. In both systems, the third electron transfer ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$), which results in the irreversible inactivation of the enzyme, is the rate limiting step. This results in the accumulation of the oxidized Fe^{3+} :carbene complex that can regenerate free enzyme much faster than the reduced Fe^{2+} :carbene complex. Interestingly, while P450 turnover rates were similar, enzyme regeneration though carbene release was about 3-fold faster in baculosomes than in HLM.

PPT TDI model

Three analyses of the PPT TDI data from HLM were conducted. Models were fit in the presence and absence of k_5 (QI and Q, respectively) and using the linear (first three) points with the replot method. The parameter estimates for these analyses are listed in Table 2, and the fits are shown in Figure 5. The two numerical method analyses gave similar values for K_i , but very different values for k_{inact} . The k_{inact} value for the QI model was calculated as the net rate constant that includes all rate constants associated with Fe^{3+} :carbene complex and Fe^{2+} :carbene complex:⁴¹

$$k_{inact} = \frac{1}{\frac{1}{k_5} + \frac{k_4 + k_5}{k_3 k_5}} \quad (\text{Eq. 2})$$

The k_{inact} value for the Q model is simply k_3 and is similar to that observed k_{inact} for the replot method. The K_i value for the replot method is $21.5 \pm 18.8 \mu\text{M}$. Given the non-linearity of the time-dependent inactivation the replot method is not a truly valid method for analyzing this data and is shown only for comparison since it is the excepted method for TDI evaluation.¹⁷ The PRA plot for the Q model (Figure 5b) exhibits an increase in activity at high $[I]$ and long pre-incubation times. This is because E^* is excluded from enzyme loss with this model. E^* effectively serves as a protected reservoir of active enzyme.

Similarly, the analysis of RLM data was performed using three models: a Michaelis-Menten (MM) quasi-irreversible inhibition scheme (Figure 6a), a EII quasi-irreversible model (Figure 6d) and the replot method (not shown), this last taking into account either the entire

kinetic, or the linear region of the PRA plot. The parameters estimated are listed in Table 3 and the numerical fits are shown in Figure 6. Both MM and EII models suggest that in RLM the inactivation is time-dependent during the first 10 min of reaction, and the aging is slower or absent ($k_{inact} = 0$) compared to HLM.

IVIVE of MDZ-PPT DDI

With equilibrium dialysis, the f_u of PPT in rat plasma was 0.044 ± 0.006 . The observed in vivo rat AUCi/AUC for PPT and MDZ was 1.2 ± 0.3 . The concentration-time profiles of MDZ with or without PPT are shown in Figure 7. Using $K_I = 2.2 \mu\text{M}$ and $k_{inact} = 0.058 \text{ min}^{-1}$ from Table 3, AUCi/AUC was predicted using the standard static model (Eq. 1). Since a wide range of k_{deg} values have been reported for rat CYP3A,³⁴ the high and low reported k_{deg} values were used, 0.00083 and 0.00018 min^{-1} , respectively. PK analysis with PPT alone in rats yielded a mean C_{max} of 3.94 μM , and a mean $C_{max,unbound}$ of 0.17 μM . For each k_{deg} value, PPT C_{max} or $C_{max,unbound}$ was used as [I] in Eq. 1. These results are listed in Table 4. Compared to the observed AUCi/AUC of 1.2, it is clear that the standard replot method greatly overpredicts the DDI potential for all k_{deg} and [I] values used. The numerical method yields a $k_{inact} = 0$ (see Table 3), suggesting that no DDI would be observed. In order to obtain the observed AUCi/AUC value, k_{inact} values of 0.0025 min^{-1} (for $k_{deg} = 0.00083 \text{ min}^{-1}$) and 0.0006 min^{-1} (for $k_{deg} = 0.00018 \text{ min}^{-1}$) would be required. Such low k_{inact} values would be difficult to obtain experimentally.

Although human clinical data is not available for PPT, DDI can be predicted from the in vitro HLM data (Table 2). DDIs were predicted using a theoretical $C_{max} = 1 \mu\text{M}$, $f_u = 0.05$, and human in vitro parameters from both the standard replot method and the numerical QI model. Human hepatic CYP3A4 k_{deg} values of 0.00013 or 0.0005 min^{-1} were used.⁴² As seen in Table 4, the standard replot method predicts a much greater AUCi/AUC compared to the numerical method. In fact, the numerical method predicts a minimal DDI using the unbound inhibitor concentration.

DISCUSSION

The ultimate value of any in vitro TDI method lies in the ability to predict clinical drug interactions. A recent perspective by Greenblatt⁴³ states “Despite numerous modifications and refinements of the core model over the following 40 years, we still have not achieved a predictive paradigm having accuracy sufficient to justify bypassing all, or even most, clinical DDI studies in the course of drug development.” Further, he notes, “overprediction is the rule”. The inability to accurately predict DDIs can result in progressing of flawed compounds into development or preventing good molecules from moving forward into development. One possible reason that DDIs due to TDI are not well predicted is that current methods have not been capable of incorporating known CYP complexities into in vitro and in vivo models. As described previously,^{14, 17} the numerical approach to TDI data analysis makes it possible to model complexities including multiple binding (ESI and EII), quasi-irreversibility (Q) and quasi-irreversible intermediate formation (QI). As can be seen in Figures 5 and 6, and Tables 2 and 3, the replot method cannot adequately model the data. The replot method is applicable only to Michaelis-Menten kinetics, and curvature in the

PRA plots cannot be modeled. The standard protocol is the use the initial log linear data points to calculate inactivation rates. This results in poor estimation of TDI parameters, and subsequent overprediction of DDIs. Although we have previously modeled and discussed quasi-irreversible TDI, those models did not consider the possibility of intermediate formation.

Carbene capture by cytochrome P450 and its putative role in mechanism-based inhibition of MDP compounds has been investigated in drugs and insecticides.^{3, 18, 22, 44} Herein, the mechanistic details of P450-mediated activation of MDP moiety to carbene have been related for the first time to the quasi-irreversible nature of the observed TDI. We relied on the optical absorbance of the intermediates in the Soret region of the visible spectrum, using SVD to deconvolute the different species which would otherwise not be resolved. The oxidized intermediate (Fe^{3+} :carbene), previously observed only by removal of the reducing agent,²⁴ is presumably reduced to the Fe^{2+} :carbene, which is energetically more stable.⁴⁵ The spectral nature of the reduced complex is consistent with a split Soret band with maxima at 427 and 455 nm, as reported.³ In addition, a third component was identified, corresponding to the inactive form P420, which spectrum partially overlapped with the Fe^{2+} :carbene complex (Figure 2). In baculosomes, up to 20% of the enzyme was converted to P420 after 30 min of reaction; however, the associated kinetic was not related to carbene activation (Figure 2c). As a matter of fact, enzyme loss was included in both Q and QI models (k_9), since time-dependent inactivation of CYP3A4 was observed even in the absence of the inhibitor (Figure 5c). The spectral analyses presented here led us to modify the Q model by adding an $\text{E}^* \rightarrow \text{E}^{**}$ pathway (QI model).

Using the binding data from the stopped-flow experiments the model in Figure 3 was parameterized with the spectral data in Figure 2. The resulting rate constant for Fe^{3+} :carbene formation was 1.1 min^{-1} for HLM (Table 1). This value is similar to the calculated rate constant of 0.49 min^{-1} for the in vitro HLM TDI assay (Table 2). The rate constants for Fe^{2+} :carbene formation (enzyme inactivation) can be calculated as a net rate constant and is 0.020 min^{-1} for the spectral MIC formation experiments in HLM (Table 1). This value is very close to k_{inact} for the HLM TDI experiments in HLM (0.013 min^{-1} , Table 2). K_I values were also comparable between experiments. Together, these data suggest that the reversible intermediate observed in the spectral studies is responsible for the quasi-irreversible in vitro TDI kinetics. In a previous report we noted that the observed in vitro reversibility is much faster than the rate of enzyme recovery in vivo, and hypothesized that an addition enzyme inactivation step may be involved. The data presented here provides experimental evidence that the conversion of the Fe^{3+} :carbene to the Fe^{2+} :carbene is the actual enzyme inactivation step.

The rate constants for Fe^{3+} :carbene formation were similar for the baculosomes and HLM (1.64 min^{-1} vs. 1.13 min^{-1} , respectively, Table 1). On the other hand, the formation rate constant (k_5) for the Fe^{2+} :carbene was greater in the baculosomes as compared to HLM (0.08 min^{-1} vs. 0.02 min^{-1}). This is likely due to faster rates of reduction because of higher levels of reductase in the baculosome system.¹⁶ Since this is the inactivation step, artificial expression systems may result in different DDI predictions.

The results for the Q and QI models for HLM and RLM data are shown in Tables 2 and 3 respectively. For HLM, the AICc for the QI model was lower ($AICc = 2.7$) indicating that this model was slightly preferred, although the main reason for choosing the more complex model is that it is consistent with the known kinetic mechanism based on the spectral data shown in Figure 2. The most profound and important result is that the k_{inact} value is almost 20 times lower when the intermediate is taken into account. This is a result of the branched pathway from the Fe^{3+} :carbene which can release the carbene to ultimately give free enzyme, and the catechol product (after the carbene reacts with water) instead of proceeding to the very slowly reversible Fe^{2+} :carbene complex. In essence, using the existing protocols overestimates k_{inact} because it does not take into account the branched pathway that rescues the enzyme. For RLM, the AICc values suggest that the EII Q model is appropriate (Table 3). The rate of intermediate conversion to inactive enzyme could not be determined for the RLM dataset ($k_{inact} = 0$). A low k_{inact} estimate is supported by in vivo studies in the rat.

The undertook a rat DDI study since the analysis using the model in Figure 3 predicts minimal DDIs whereas the currently accepted approach (using the initial rate of enzyme loss) would predict a large DDI. The rat in vivo DDI study showed a minimal DDI potential, with a mean AUCi/AUC of 1.2. Using the static model, low in vivo k_{inact} values (0.0025 or 0.0006 min^{-1}) were predicted for two standard rat CYP3A k_{deg} values (Table 4). It is noteworthy that k_{inact} values of this magnitude will be virtually impossible to determine experimentally. The numerical method with in vitro HLM data estimates a k_{inact} of $0.013 \pm 0.004 \text{ min}^{-1}$, but k_{inact} values of this magnitude are unlikely to predict a significant DDI.

As discussed by Greenblatt,⁴³ a recent publication from the FDA⁴⁶ using both publically available and confidential data shows that a vast majority of DDIs are overpredicted. The data herein strongly suggest that overprediction of DDIs for drugs that form MICs is due to models that do not capture the formation of a reversible intermediate. Formation of this intermediate results in effectively a biphasic inactivation process. Since the standard replot method assumes log linear inactivation, the first (log linear) phase of a PRA plot is typically used to estimate k_{inact} . However, the true rate of inactivation is determined by the slower second phase of a PRA plot (Figure 5D). Thus, the standard replot method systematically overpredicts k_{inact} for concave upward biphasic reactions.

The uncertainties in kinetic parameters, in particular the over prediction of k_{inact} for many drugs, may be one of the causes that the value used for the rate of CYP resynthesis (k_{deg}) does not match the observed rate in vivo. In vivo studies generally report a k_{deg} value of approximately 0.077 h^{-1} ($t_{1/2} = 90 \text{ h}$),⁴² but the best correlation between replot in vitro data and in vivo TDI data is obtained with a $t_{1/2} \sim 24\text{--}36 \text{ h}$. A majority of the TDI drugs used in these studies form MICs and over estimation of k_{inact} may have contributed to the selection of much faster k_{deg} values for in vivo DDI prediction.

The implications of overpredicting k_{inact} are substantial. As seen in Table 4, overestimating k_{inact} can erroneously predict a significant DDI when in fact no DDI may be observed in vivo. False positives due to inaccurate in vitro kinetic parameters can prevent good drug candidates from moving forward into development. It remains to be seen if we will be able to accurately predict small DDIs since the need to characterize the slow phase of enzyme

inactivation requires very accurate data at later time points. This effort may be complicated by other factors such as enzyme loss, e.g. due lipid peroxidation and reactive oxygen species formation.⁴⁶ However, there appears to be a clear need to alter our approach to nonlinear PRA plots. We should not assume that rapid initial loss of enzyme will result in significant irreversible loss of enzyme.

Conclusions

The MI-complex formed by podophyllotoxin first results in an oxidized Fe³⁺:carbene which can release the carbene to give the free enzyme or can age by reduction to the Fe²⁺:carbene which is only very slowly reversible. Since this aging process is very slow the TDI is overestimated if this process is not taken into account. Given that the general assumption is that free enzyme can only be formed by synthesis of new enzyme after MI-complex formation this newly described branched pathway explains why half-life for synthesis of new enzyme is often predicted to be shorter in TDI studies. Furthermore, when the aging process is very slow it is unlikely that the TDI will be of importance in vivo which may lead to the rescue of drugs during development that show in vitro TDI. Accurate models that provide concentration-time profiles for drug metabolizing enzymes can be used to help design dosing regimens that minimize DDIs and provide a sound basis for future DDI FDA guidances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Mechanism-based inhibition of cytochrome P450 by methylenedioxyphenyl compounds.

Figure 2.

Spectra of MI- complex formation upon metabolism of PPT by CYP3A4. (a) Raw spectra for metabolite complex formation in CYP3A4 baculosomes. (b) Principal spectral components (we attribute PC1 to the Fe^{2+} :carbene complex, PC2 to the oxidized intermediate Fe^{3+} :carbene and PC3 to cytochrome P420) and (c) dynamic components obtained through SVD analysis of raw spectra. (d) Raw spectra for metabolite complex formation in HLM. (e) Principal spectral components and (f) dynamic components obtained through SVD analysis of raw spectra.

Kinetic scheme for MIC formation

Figure 3.

Proposed kinetic scheme for MIC formation. The species depicted in the schemes are defined as follows: E, unbound active enzyme; S, substrate; ES, enzyme-substrate complex; Fe³⁺:carbene, ferricytochrome:carbene complex (absorbance maximum at 437 nm); Fe²⁺:carbene, ferrocyanochrome:carbene complex (absorbance maximum at 455 nm); P420, cytochrome P420 (absorbance maximum at 425 nm); Met, PPT metabolite derived from carbene release.

Figure 4. Numerical fitting of the spectral complexes for CYP3A4-mediated metabolism of PPT in (a) baculosomes and (b) HLM.

Figure 5.

Numerical fitting of the TDI in vitro data (HLM). ESI-QI scheme (**a**), standard replot for MM kinetics (**b**), ESI-Q concentration-time plot (**c**), ESI-Q PRA plot (**d**); ESI-QI concentration-time plot (**e**) ESI-QI PRA plot (**f**). $E^* = \text{Fe}^{3+}:\text{carbene}$; $E^{**} = \text{Fe}^{2+}:\text{carbene}$; enzyme loss (k_5) occurs from E, ES, EI, and ESI.

Figure 6.

Numerical fitting of the TDI in vitro data (RLM). MM-Q scheme (a), MM-Q concentration-time plot (b), MM-Q PRA plot (c); EII-Q scheme (d), EII-Q concentration time plot (e) and EII-Q PRA plot (f). The QI scheme is depicted but reduces to the Q-model since k_5 parameterized to zero.

Figure 7.
Concentration-time profile for MDZ in rats, with and without PPT.

Table 1

Kinetic parameters obtained from non-linear regression of time-dependent concentration profiles for CYP3A4 baculosomes and pooled human liver microsomes (HLM).

Rate Constant	System	
	<i>3A4 baculosomes</i>	<i>HLM</i>
$^a k_1 (\text{min}^{-1})$	1080	1080
$^a k_2 (\text{min}^{-1})$	$10.6 \times 10^{+3}$	$10.6 \times 10^{+3}$
$^b k_3 (\text{min}^{-1})$	1.8	1.134 ± 0.156
$k_4 (\text{min}^{-1})$	0.0780 ± 0.0186	0.0195 ± 0.000
$k_5 (\text{min}^{-1})$	0.0804 ± 0.0228	0.0954 ± 0.0024
$k_6 (\text{min}^{-1})$	0.1224 ± 0.0252	0.0506 ± 0.0401
$k_7 (\text{min}^{-1})$	0.0636 ± 0.0048	0
R^2	0.959	0.981
AICc	-238.0	-225.7

^a From binding kinetic experiment (see Materials and Methods)

^b Fixed value

Table 2

Parameter estimates for three methods of TDI data analysis in HLM. Data are expressed as estimates $\pm$ standard error.

Rate constant	QI model	Q model	Standard Replot
$k_3 (\text{min}^{-1})$	94.9 ± 1.6	95.3 ± 1.7	
$k_5 (\mu\text{M}^{-1} \text{min}^{-1})$	3371 ± 1567	2334 ± 977	
$k_7 (\text{min}^{-1})$	0.50 ± 0.23	0.23 ± 0.07	
$k_8 (\text{min}^{-1})$	0.15 ± 0.04	0.081 ± 0.012	
$k_9 (\text{min}^{-1})$	0.011 ± 0.001	–	
α	0.16 ± 0.07	0.21 ± 0.08	
$k_{10} (\text{min}^{-1})$	0.017 ± 0.006	0.011 ± 0.001	
$K_i (\mu\text{M})$	12.5 ± 5.8	8.6 ± 3.6	21.5 ± 18.8
$k_{inact} (\text{min}^{-1})$	0.013 ± 0.004	0.23 ± 0.07	0.29 ± 0.15
AICc	–182.8	–180.3	–

Table 3

Parameter estimates for three methods of TDI data analysis in RLM. Data are expressed as estimates $\pm$ standard error.

Rate constant	MM quasi-irreversible	EII quasi-irreversible	Standard Replot (all data)	Standard Replot (1 st 3 pts)
K_{11} (μM)	36.0 ± 14.2	3.82 ± 1.49	did not converge	2.2 ± 2.3
k_6 (min^{-1})	0.30 ± 0.10	0.071 ± 0.020		
k_7 (min^{-1})	0.12 ± 0.02	0.11 ± 0.02		
k_6/K_{12} ($\text{nM}^{-1}\text{min}^{-1}$)		3.66 ± 1.01		
k_{inact} (min^{-1})	0	0		0.058 ± 0.014
AICc	-243.4	-247.4	N.A	N.A

N.A.: not admitted

Table 4

IVIVE to predict DDI between PPT and MDZ in rats and humans

Rat, standard replot		
	$k_{deg} = 0.00083 \text{ min}^{-1}$	$k_{deg} = 0.00018 \text{ min}^{-1}$
AUCi/AUC when $[I] = C_{max}$	11.1	13.4
AUCi/AUC when $[I] = C_{max,unbound}$	4.5	9.3
k_{inact} required for AUCi/AUC = 1.2	0.0025 min^{-1}	0.0006 min^{-1}
Human, standard replot		
	$k_{deg} = 0.0005 \text{ min}^{-1}$	$k_{deg} = 0.00013 \text{ min}^{-1}$
AUCi/AUC when $[I] = C_{max}$	9.5	12.6
AUCi/AUC when $[I] = C_{max,unbound}$	2.1	4.5
Human, QI model		
	$k_{deg} = 0.0005 \text{ min}^{-1}$	$k_{deg} = 0.00013 \text{ min}^{-1}$
AUCi/AUC when $[I] = C_{max}$	2.6	5.5
AUCi/AUC when $[I] = C_{max,unbound}$	1.1	1.4