Simple, Direct, and Informative Method for the Assessment of CYP2C19 Enzyme Inactivation Kinetics

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ABSTRACT:

Many clinically relevant drug interactions involving cytochrome P450 (P450) inhibition are mediated by mechanism-based inactivation (MBI). Time-dependent inhibition is one of the major features distinguishing between reversible inhibition and MBI. It thus provides a useful screening approach for early drug interaction risk assessment. Accordingly, we developed an easy and informative fluorometric method for the assessment of CYP2C19 enzyme inactivation kinetics. Dibenzylfluorescein (DBF) is widely used as a profluorescent probe substrate for P450 activity and inhibition assays, but its use has been considered to be limited to traditional endpoint assays. We monitored CYP2C19-catalyzed metabolism of DBF using synthesized fluorescein benzyl ester and fluorescein benzyl ether along with commercially available fluorescein as intermediate standards. Furthermore, we demonstrated the use of DBF in a kinetic assay as a progress curve analysis for straightforward determination of whether a compound is a time-dependent inactivator of CYP2C19. The recombinant human CYP2C19 inactivation kinetics of isoniazid, ticlopidine, and tranylcypromine were evaluated, and their key kinetic parameters were measured from the same experiment. The known mechanism-based inactivators, isoniazid and ticlopidine, exhibited clear time-dependent inactivation with $k_{\text{inact}}$ values of $250.5 \pm 34 \mu M$ and $0.137 \pm 0.006 \text{min}^{-1}$ and $1.96 \pm 0.5 \mu M$ and $0.135 \pm 0.009 \text{min}^{-1}$, respectively. Tranylcypromine did not display any time-dependent inhibition, which is consistent with its reported mechanism of competitive inhibition. In summary, DBF is suitable for use in the progress curve analysis approach and can be used as an initial screen to identify compounds that require more detailed investigations in drug interaction optimization.

Introduction

The human cytochrome P450 (P450) enzymes play an important role in the metabolism of drugs and numerous other xenobiotics. Inhibition of P450 enzymes is a common mechanism that can lead to drug interactions. These can evoke severe adverse effects; they have resulted in early termination of drug development, refusal to obtain approval, prescribing restrictions, and even withdrawal of drugs from the market (Wienkers and Heath, 2005; Kalgutkar et al., 2007; Pelkonen et al., 2008).

P450 inhibition can be categorized as either reversible or irreversible. Irreversible inactivation is generally of greater concern than reversible inhibition because it can result in more profound and prolonged effects (Ghanbari et al., 2006; Kalgutkar et al., 2007). There is increasing awareness that many clinically relevant drug interactions involving P450 inhibition are mediated by irreversible mechanism-based inactivation (MBI) (Ghanbari et al., 2006; Grime et al., 2009). Today, potential in vivo effects of drug interactions caused by competitive inhibitors can be fairly well predicted from in vitro P450 kinetics. The current challenge is to detect time- and concentration-dependent effects of irreversible and quasi-irreversible inactivators among large numbers of early-phase compounds in the drug development pipeline (Wienkers and Heath, 2005; Fowler and Zhang, 2008). This information is especially important because failure to consider MBI in vitro can lead to serious underestimation of drug interaction magnitude in vivo, particularly when one is trying to predict drug interactions from in vitro data based on competitive models (Bjornsson et al., 2003; Polasek and Miners, 2007).

The updated regulatory guidances by the U.S. Food and Drug Administration (Guidance for industry: drug interaction studies—study design, data analysis, and implications for dosing and labeling, 2006, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) and European Medicines Agency (Guideline on the investigation of drug interactions, 2010, http://www.emea.europa.eu/htms/human/humanguidelines/efficacy.htm) for in vitro drug interaction studies include recommendations that drug candidates need to be tested for time-dependent and mechanism-based inactivator properties. Recently, a team of scientists from 16 pharmaceutical research organizations recommended the use of a tiered approach wherein abbreviated assays are first used to determine whether or not new chemical entities demonstrate time-dependent inhibition, followed by more thorough inactivation studies for those that do (Grimm et al., 2009).

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ABBREVIATIONS: P450, cytochrome P450; MBI, mechanism-based inactivation; DBF, dibenzylfluorescein; EtOAc, ethyl acetate; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
Current in vitro inactivation research methodologies have been criticized for being significantly influenced by the wide range in experimental conditions, complicating comparison across studies and jeopardizing clinical predictions. In addition, these complex experiments offer limited mechanistic insight (Ghanbari et al., 2006; Riley et al., 2007; Fowler and Zhang, 2008; Obach, 2009; Zhou and Zhou, 2009). Fairman et al. (2007) proposed an alternative in vitro approach for the investigation of pre-steady-state kinetics of CYP1A2 inactivation, referred to as progress curve analysis. Progress curve analysis uses an “all-in” approach in which the enzyme is exposed simultaneously to probe substrate and inactivator while enzyme activity is monitored throughout the inactivation. This type of analysis has long been an accepted tool for measuring pre-steady-state inhibition kinetics for a variety of physiological enzymes (Fairman et al., 2007; Obach, 2009; Zhou and Zhou, 2009).

Dibenzyllfluorescein (DBF) is widely used as a profluorescence probe substrate, in particular for CYP2C8, CYP2C9, CYP2C19, CYP3A4, and aromatase (CYP19), in high-throughput assays. CYP2C19 was chosen as the target enzyme in this study because it metabolizes several widely used drugs, such as proton pump inhibitors (Pelkonen et al., 2008). Recent data indicate that inhibition of CYP2C19 may lead to a reduction in clinical efficacy of the antithrombotic drug clopidogrel (Wallentin, 2009). The DBF assay is based on the general principles originally published by Crespi and coworkers (Crespi et al., 1997; Crespi and Streser, 2000). The utility of DBF for kinetic assays with continuous data acquisition has been questioned because the initial metabolite of DBF (fluorescein benzyl ester) requires very alkaline conditions for further hydrolysis to maximize the fluorescence intensity (Crespi and Streser, 2000; Miller et al., 2001).

The purpose of this study was to 1) characterize the properties of DBF as a probe substrate for CYP2C19 enzyme activity and inhibition assays, 2) assess whether DBF can be used as probe substrate in a real-time kinetic assay, and 3) demonstrate the use of the progress curve analysis approach for rapid identification of time-dependent P450 inactivators as well as the analysis of key inactivation kinetic parameters.

Materials and Methods

Materials. Isoniazid, trans-2-phenylcyclopropylamine hydrochloride (tryglypyromine), ticlopidine, and fluorescein were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Dibenzyllfluorescein (purity >99%) and cDNA-expressed human wild-type CYP2C19 (Supersomes) were purchased from BD Biosciences Discovery Labware (Bedford, MA). Fluorescein benzylic ester and fluorescein benzyl ester were synthesized with reagents of commercial high purity quality without further purification unless otherwise mentioned. Reactions were monitored by thin-layer chromatography using aluminum sheets coated with Silica Gel 60 F245 (0.24 mm) with suitable visualization. The microwave irradiation experiment was performed in a Biotage Initiator Microwave Reactor (Biotage, Uppsala, Sweden) in a pressure-rated glass tube. Purifications by flash chromatography were performed on Silica Gel 60 (0.063–0.200 mm mesh). 4H and 13C NMR spectra were recorded on a Bruker Avance AV 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 and 125.75 MHz, respectively, using tetramethylsilane as an internal standard. The products were also characterized by mass spectrometry with a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source. The purity was determined by elemental analysis (carbon, hydrogen, and nitrogen) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy). Synthesis of Fluorescein Benzyl Ester. Fluorescein (800 mg, 2.4 mmol), benzyl alcohol (10 ml), and concentrated sulfuric acid (1.2 g, 12 mmol) were irradiated with microwaves at 120°C for 1 h (Fig. 1). The reaction mixture was cooled to 37°C. The reactions were terminated by rapid cooling to 4°C after centrifugation, the supernatants were analyzed by LC-MS. The products were also characterized by mass spectrometry with a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source. The purity was determined by elemental analysis (carbon, hydrogen, and nitrogen) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy).
NaOH. All samples were analyzed with a Finnigan LTQ mass spectrometer using positive electrospray ionization and full scan or MS/MS measurements. The compounds were separated using a 1200 high-performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany) equipped with a 50 × 2 mm Gemini C18 column (Phenomenex, Torrance, CA) with a gradient starting from 10% acetonitrile, 0.1% formic acid and ending with 0% acetonitrile, 0.1% formic acid in 10 min at a flow rate of 200 µL/min and injection volume of 5 µL. The column temperature was 30°C. The compounds were identified on the basis of their retention times, molecular weights, and MS/MS spectra.

**Stability of Fluorescence Intensity.** Stability of the fluorescence intensity of DBF, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein (all 1 µM) was characterized at different pH and temperatures (room temperature or 37°C). The buffers were either 100 mM Tris-HCl (pH 7.0 and 7.4) or 100 mM KPO4 (pH 7.0 and 7.4). All samples were in a 150-µL total volume in duplicate in OptiPlate 96-well microplates, and the fluorescence intensity was measured with a Victor2 plate reader (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland) in a continuous mode at 1-min intervals for 90 min at excitation and emission wavelengths of 485 and 535 nm, respectively.

**Prerequisites for Kinetic Assay.** The initial prerequisites for a real-time kinetic assay were investigated using the endpoint assay procedure by determining whether enzyme activity could be detected without the use of 2 M NaOH. This was done by comparing signal/noise ratios between an enzyme-catalyzed sample and two blank (nonenzyme-catalyzed) samples. The experimental conditions were as described in Table 1. The reactions were initiated by addition of 50 µL of the NADPH-regenerating system after a 10-min preincubation at 37°C and were subsequently incubated for 30 min at 37°C in the dark. Blank I was similar to the control (enzyme-catalyzed) sample except that 110 µL of 2 M NaOH was added into the wells before addition of the NADPH-regenerating system. Blank II lacked the enzyme. After fluorescence was measured at the end of the 30-min incubation, 110 µL of 2 M NaOH was added into the wells containing control and blank II samples, and the fluorescence was measured again.

**Real-Time Kinetic Assay.** Real-time kinetic assays were conducted at 37°C in a Victor2 plate scanner using the experimental conditions in Table 1. Blank samples were treated similarly to the enzyme-catalyzed samples but in the absence of P450 enzyme. Reactions were initiated by addition of DBF (experiment A) or the NADPH-regenerating system (experiment B). Experiment C was conducted by measuring first the substrate in a Victor2 plate scanner for 15 min at 37°C, after which the prewarmed enzyme and the NADPH-regenerating system were added into the wells and immediately after that the actual reaction incubation, and fluorescence data acquisition was performed at 1-min intervals for 45 min. Each experiment was performed in duplicate. In addition, the linearity of the CYP2C19-catalyzed reaction with respect to enzyme concentration and incubation time was investigated by using varying amounts (0.125–2 pmol) of the enzyme.

**Determination of IC50.** To compare determination of IC50 values between endpoint and kinetic assays, the IC50 value of the known CYP2C19 inhibitor tranylcypromine was assessed using four modified procedures: (A) traditional endpoint assay, (B) endpoint assay combined with initial measurement of the substrate for 15 min before the reaction initiation, and kinetic assays without (C) and with (D) substrate premeasurement. The reactions were initiated by addition of the enzyme and were incubated for 30 min at 37°C. In the endpoint assays, the reactions were terminated by addition of 110 µL of 2 M NaOH. In the kinetic assays, linear regions of the progress curves were used for the reaction velocity calculations. All IC50 values were determined as a mean value from duplicate determinations. Percentages of remaining P450 activity were plotted as a function of the logarithm of the molar concentration of tranylcypromine, and the curves were fitted to a sigmoid dose-response equation with Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

**Progress Curve Analysis.** The procedure involving the 15-min initial measurement of substrate as shown in Fig. 3C was used in progress curve analysis. In these experiments, the enzyme is exposed simultaneously to the substrate and inactivator, and enzyme activity is monitored throughout the process (real-time kinetic assay). Progress curve experiments were performed by evaluating two known mechanism-based (time-dependent) inactivators, isoniazid and ticlopidine, and one known reversible (time-independent) inhibitor, tranylcypromine. Seven different concentrations of each test compound were used: 2.74 to 4000 M for isoniazid, 0.14 to 100 M for ticlopidine, and 0.14 to 100 µM for tranylcypromine. These concentrations were selected to ensure a wide range of inactivation across the 45-min time course. Isoniazid and ticlopidine were dissolved in water, and tranylcypromine was dissolved in acetonitrile and then further diluted with water. Consequently, the final solvent concentrations in the incubations did not exceed 2.2%. Controls were treated similarly but without the presence of inactivators. The fluorescence data obtained were analyzed to determine the key kinetic parameters and mechanistic information of the inactivation process.

Each progress curve was fitted by eq. 1 (Copeland, 2005), which contains terms for the initial and steady-state velocities (v0 and vs) and for the rate constant for onset of inhibition (kobs), i.e., conversion from the initial velocity phase to the steady-state velocity phase:

\[
\text{[Product]} = \text{v}_0 + \frac{\text{v}_0 - \text{v}_s}{k_{\text{obs}}} (1 - \exp(-k_{\text{obs}}t))
\]

**TABLE 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.4</td>
<td>100 mM</td>
</tr>
<tr>
<td>Substrate (DBF) concentration</td>
<td>1.0 µM (equals Km)</td>
</tr>
<tr>
<td>Enzyme (human recombinant CYP2C19)</td>
<td>1.5 pmol</td>
</tr>
<tr>
<td>NADPH-regenerating system</td>
<td>50 µL</td>
</tr>
<tr>
<td>Incubation time at 37°C</td>
<td>30–60 min</td>
</tr>
<tr>
<td>Excitation/emission wavelengths (nm)</td>
<td>485/535</td>
</tr>
</tbody>
</table>

**TABLE 2**

**LC/MS characteristics of DBF, fluorescein, fluorescein benzyl ester, and fluorescein benzyl ether**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>MH*</th>
<th>MS/MS Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibenzylfluorescein</td>
<td>9.84</td>
<td>513.4</td>
<td>485,421,345,333</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>7.25</td>
<td>333.2</td>
<td>305,287,271</td>
</tr>
<tr>
<td>Fluorescein benzyl ester</td>
<td>7.28</td>
<td>423.3</td>
<td>361,345,317</td>
</tr>
<tr>
<td>Fluorescein benzyl ether</td>
<td>9.75</td>
<td>423.3</td>
<td>395,377,345</td>
</tr>
</tbody>
</table>

\* Blank: I as enzyme-catalyzed sample but 2 M NaOH added into samples before initiation of the reactions; blank II: as enzyme-catalyzed sample but in the absence of enzyme.

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$K_{obs}$ values were then plotted against inactivator concentrations and fitted to eq. 2 (Fairman et al., 2007):

$$
\frac{k_{obs}}{H11005} \frac{1}{K_I} + \frac{1}{S/K_m} + \frac{1}{I}
$$

where $k_{obs}$ is the maximum rate of inactivation, $[I]$ is the concentration of inactivator, $K_I$ is the inactivator concentration required for half-maximal inactivation, $S$ is the substrate concentration, and $K_m$ is the Henri-Michaelis-Menten constant.

**Results**

**P450-Catalyzed Metabolism of DBF and Effect of Base.** The CYP2C19-catalyzed biotransformation of DBF and effect of base were investigated by LC-MS. The retention times, MH ions, and major MS/MS fragment ions for the standards were used for identification of the compounds (Table 2). Fluorescein benzyl ester was formed in the CYP2C19-catalyzed reaction as a major metabolite, and minor amounts of fluorescein benzyl ether were also detected. Fluorescein was not formed at the reaction pH 7.4. In the blank samples, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein were not formed from DBF. Addition of 2 M NaOH caused decomposition of DBF to fluorescein benzyl ether, whereas fluorescein and fluorescein benzyl ether remained unchanged in the presence of 2 M NaOH. The standard compounds remained unchanged in the absence of 2 M NaOH. The results are presented in Fig. 1.

**Stability of Fluorescence Intensity.** The fluorescence intensity of DBF decreased markedly during the first 10 min of the measurement until it reached a steady state (Fig. 2). The fluorescence intensity during the 90-min measurement was the same with the different buffers (Tris-HCl and KPO4), pH conditions (pH 7.0 and 7.4), and temperatures (room temperature and 37°C). A similar quenching of fluorescence was observed for fluorescein benzyl ester and fluorescein benzyl ether but not for fluorescein (data not shown). At steady state, when the amounts of the fluorescence intensity of DBF were compared with the intensities of fluorescein benzyl ether, fluorescein benzyl ester, and fluorescein, these were 20-, 120-, and 200-fold higher than that of DBF, respectively.

**Prerequisites for Kinetic Assay.** The optimal conditions for the kinetic assay were examined by comparing fluorescence intensities between enzyme-catalyzed sample and two kinds of blank samples and by evaluating the effects of NaOH on fluorescence intensity. The results are summarized in Table 3. An acceptable signal/noise ratio was achieved during the 30-min incubation without addition of 2 M NaOH to the samples, because the fluorescence intensity was 12-fold higher in the enzyme-catalyzed samples than in blank II (no enzyme) sample. In contrast, the fluorescence intensity was only approximately 2- to 3-fold higher in the enzyme-catalyzed samples than in blank I (NaOH added before initiation of the reaction). One important finding was that the signal/noise ratio was approximately 5 times greater between the enzyme sample and blank II than between enzyme sample and blank I. Addition of 2 M NaOH at the end of incubation resulted in only a 1.5-fold increase in fluorescence intensity. The result showed that it is feasible to detect enzyme activity without the use of 2 M NaOH and that blank II is a better choice for assessing background noise than blank I.
Real-Time Kinetic Assay. The progress of the P450-mediated catalytic reaction can be observed from the kinetic readouts as shown in Fig. 3. The fluorescence intensity of DBF declined to the steady-state value during the 15-min initial measurement, allowing for detection of the linear reaction kinetics from the very beginning of the reaction (Fig. 3C).

The time course of the CYP2C19-catalyzed reaction with varying amount of the enzyme is presented in Fig. 4. Linear kinetics with respect to enzyme amount and incubation time was evident from the plots. Linear regression analysis of the plot of enzyme amount versus incubation time indicated that the best linearity was obtained with 1 to 2 pmol of the enzyme.

Determination of IC₅₀. The IC₅₀ value of tranylcypromine was determined by the endpoint (experiments A and B) and the kinetic assays (experiments C and D). Inhibition of DBF metabolism by tranylcypromine is shown in Fig. 5.

Progress Curve Analysis. Progress curves for each of the inactivators are shown in Fig. 6. All reaction progress curves without inactivator displayed linear kinetics and confirmed that the measurements were made during the linear steady-state phase of the reaction. For isoniazid and ticlopidine, significant time- and concentration-dependent inactivation of CYP2C19 was observed, and their progress curves were fitted to eq. 1 to obtain estimates of $k_{\text{obs}}$, $v_i$, and $v_s$ at several concentrations. Except for tranylcypromine, best-fit lines of the inactivators yielded $v_s$ values of zero (Fig. 6).

The $K_{\text{obs}}$ values were replotted against inactivator concentrations and fitted to eq. 2 (Fig. 7). The $K_i$ and $k_{\text{inact}}$ values determined are presented in Table 4. Tranylcypromine showed concentration- but not time-dependent inhibition determined by its linear progress curves at each concentration. Thus, it was analyzed by linear fitting of each concentration because the relative reaction velocity can be determined from the slope of a linear fit, and the IC₅₀ value was determined via standard methodologies (4.72 ± 0.30 μM) and then converted to an absolute inhibition constant ($K_i$, 2.36 μM) for substrate affinity and concentration using the Cheng-Prusoff equation, where $K_i = IC_{50}/(1 + [S]/K_m)$. All results represent the mean of duplicate determinations.

Discussion

In the present study, we describe development of a simple, direct, and informative fluorometric method for the assessment of CYP2C19 enzyme inactivation kinetics. Well known inactivators were used to test the method. Isoniazid and ticlopidine were chosen because clinically relevant interactions between them and substrates of CYP2C19 have been reported, and they are known to act via MBI (Donahue et al., 1997; Tateishi et al., 1999; Nishimura et al., 2003; Richter et al., 2004; Kalugutkar et al., 2007; Venkatakrishnan and Obach, 2007). The selected competitive inhibitor, tranylcypromine, is commonly used as a positive control compound for CYP2C19 inhibition studies (BD Gentest, A high throughput method for measuring cytochrome P450 inhibition, 2000, http://www.bdj.co.jp/gentest/1f3pro0000osf5lj-att/P450-InhibitorScreeningTechBulletin-Ver4.2-2000-09.pdf) (Lin et al., 2007).

The data show that 2 M NaOH is not required for enzyme activity determinations using DBF as the probe substrate because the fluores-
cence intensity of the metabolite formed (fluorescein benzyl ester) is 120-fold higher than that of DBF. The transformation of fluorescein benzyl ester to fluorescein in the presence of 2 M NaOH is consistent with the reports of its use in the endpoint assays (Miller et al., 2001; Hong et al., 2008). However, it is not desirable that at the same time DBF is transformed to fluorescein benzyl ether in the presence of 2 M NaOH, which also increases the background fluorescence intensity and causes significant deterioration in the sensitivity, i.e., signal/noise ratio.

Therefore, CYP2C19 enzyme activity can be followed in real time using DBF as the substrate. The product formation is linear from the very beginning of the reaction, and the IC_{50} values calculated from the progress curves are comparable with the results obtained with the traditional endpoint assay and correlated well with literature data (a range of 1.9 to 9 μM) (BD Gentest, A high throughput method for measuring cytochrome P450 inhibition, 2000 (Diers et al., 2001; Lin et al., 2007). A kinetic assay provides the most reliable means of accurately determining reaction velocity from the slope of a plot of signal versus time, i.e., it is purely a result of the enzyme activity itself. Based on these experiments, the observed spontaneous quenching of DBF fluorescence is due to some fluorometric phenomenon instead of decomposition of the compound. The other related compounds possess higher fluorescence intensities than DBF.

The full progress curve of an enzymatic reaction contains an abundance of valuable kinetic information, allowing investigation of both reversible and irreversible components of the reaction mechanisms, and thus provides more information in one experiment. All irreversible enzyme inactivators display slow binding kinetics in initial binding equilibrium as reflected in the magnitude of reduction of the enzyme active site. The presence of substrate can hinder the enzyme reaction progress curve will be nonlinear and reflect two distinct velocities for the reaction (Copeland, 2005). This behavior was seen with the known time-dependent inactivators, isoniazid and ticlopidine, but not with the known time-independent inhibitor, tranylcypromine.

Thus, the enzyme reaction progress curve will be nonlinear and reflect two distinct velocities for the reaction (Copeland, 2005). This behavior was seen with the known time-dependent inactivators, isoniazid and ticlopidine, but not with the known time-independent inhibitor, tranylcypromine.

For time-dependent irreversible/quasi-irreversible inactivators, the value of $k_{\text{obs}}$ is generally expected to increase over a certain range of inactivator concentrations and then to undergo saturation at higher concentrations (Kalgutkar et al., 2007). The first step involves reversible binding of the inactivator to the enzyme, often under rapid equilibrium conditions. For mechanism-based inactivators, the second step involves some bioactivation/chemistry of the covalent bond formation or transformation into metabolic intermediate products that coordinate tightly to the heme iron atom of P450 enzyme. This behavior (saturation) was also seen with isoniazid and ticlopidine in this study, pointing to a two-step inactivation mechanism.

All mechanism-based inactivators are competitive with the normal substrate of the enzyme, because they rely on the catalytic mechanism of the enzyme active site. The presence of substrate can hinder the access of the inactivator to the enzyme, i.e., the substrate has to be at a relatively low concentration ($K_m$) so that it does not completely block the enzyme inactivation process (Ghanbari et al., 2006). For that reason, the probe substrate at the concentration corresponding to its measured apparent $K_m$ (data not shown) was used in the experiments, and this was taken into account when kinetic constants of the inactivation were determined. The inactivation kinetic constants ($K_i$ and $k_{\text{inact}}$) determined for isoniazid and ticlopidine correlated with published data (Wen et al., 2002; Nishimura et al., 2003; Polasek et al., 2006; Kalgutkar et al., 2007; Venkatakrishnan and Obach, 2007; Nishiya et al., 2009).

This progress curve analysis methodology allows estimates of the initial binding equilibrium as reflected in the magnitude of reduction of $v_i$ by the inactivator against respective control, as described by Fairman et al. (2007). In our study, $v_i$ was plotted against the initial

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>Determined</th>
<th>Published Data</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_i$ (μM)</td>
<td>$k_{\text{inact}}$ (min⁻¹)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>250.5 ± 34 0.137 ± 0.006</td>
<td>112 0.090</td>
</tr>
<tr>
<td></td>
<td></td>
<td>255 0.020</td>
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<td></td>
<td></td>
<td>79.3 0.039</td>
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<td></td>
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<td>1.65 0.192</td>
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<td></td>
<td></td>
<td>3.32 0.074</td>
</tr>
</tbody>
</table>

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This progress curve analysis methodology allows estimates of the initial binding equilibrium as reflected in the magnitude of reduction of $v_i$ by the inactivator against respective control, as described by Fairman et al. (2007). In our study, $v_i$ was plotted against the initial
inactivator concentration but no significant inhibition was seen across the concentration range (<40–50% inhibition at any concentration tested) so that no estimation of $K_i$ could be made.

In conclusion, time-dependent inhibition is one of the major distinguishing features between reversible and irreversible/quasi-irreversible inhibition. It thus provides a useful screening approach for identifying potential mechanism-based inactivators in early drug interaction studies for pharmaceutical agents under development. This finding is especially important because it has become widely recognized that detection and amelioration of time-dependent inactivation is a crucial aspect in drug interaction optimization for novel compounds (Polasek and Miners, 2007; Fowler and Zhang, 2008). DBF characterization, kinetic assay, and the progress curve analysis approach together offer a new response and improvement for the current challenge. The present work has shown that the widely used P450 substrate, DBF, is suitable for use in a progress curve analysis approach; i.e., this is a rapid and reliable method that can be used as an initial screen to help identify compounds that require more detailed investigations. However, this approach will require further evaluation with a broader set of P450 enzymes and inactivators before it is fully exploitable.

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Authorship Contributions

Participated in research design: Salminen, Venäläinen, and Raunio. Conducted experiments: Salminen and Auriola. Contributed new reagents or analytic tools: Leppänen. Performed data analysis: Salminen. Wrote or contributed to the writing of the manuscript: Salminen, Venäläinen, Pasanen, Auriola, Juvonen, and Raunio.

References


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