Progress Curve Analysis of CYP1A2 Inhibition: A More Informative Approach to the Assessment of Mechanism-Based Inactivation?

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ABSTRACT:
Mechanism-based cytochrome P450 inactivation is defined as a time- and NADPH-dependent inactivation that is not reversible upon extensive dialysis. Current methodologies use dilution approaches to estimate the rate of inactivation and offer limited mechanistic insight and are significantly influenced by experimental conditions. We investigated the potential of progress curve analysis because this experimental design allows investigation of both the reversible ($K_{\text{app}}$) and irreversible ($K_i$, $K_{\text{inact}}$) components of the reaction mechanism. The human liver microsomal CYP1A2 inactivation kinetics of resveratrol, oltipraz, furafylline, and dihydralazine (Fig. 2) were evaluated. The inactivation results for furafylline ($K_i$, 0.8 $\mu$M; $K_{\text{inact}}$, 0.16 min$^{-1}$) are within 2-fold to published data ($K_i$, 1.6 $\mu$M; $K_{\text{inact}}$, 0.19 min$^{-1}$). Resveratrol and dihydralazine results are within a 4.3-fold range of published data, which compares well with ranges of estimates of these parameters across publications (e.g., furafylline has estimates ranging of $K_i$ from 1.6 to 22.3 $\mu$M and $K_{\text{inact}}$ from 0.19 to 0.87 min$^{-1}$). This range of estimates highlights the potential caveats surrounding the existing methodologies that have been previously discussed in depth. In addition to these inactivation parameters, we have been able to demonstrate a variation in balance of reversible versus irreversible inhibition within these inactivators. Oltipraz and resveratrol have $K_{\text{inact}}$ values similar to their $K_i$, indicating a significant early onset reversible inhibition, whereas furafylline and dihydralazine are dominated by irreversible inactivation. This approach allows a more mechanistic investigation of an inactivator and in the future may improve the prediction of clinical drug-drug interactions.

Clinical drug-drug interactions (DDIs) can pose significant challenges to the development of novel chemical entities into marketable agents. Novel chemical entities may become victims to interactions that increase their maximal plasma concentration or plasma area under the curve and reduce their therapeutic index or alternatively cause increases in exposure and erode the therapeutic index of coadministered drugs. The most common DDIs are likely due to reversible inhibition of hepatic cytochrome P450 enzymes (P450s) (Lin and Lu, 1998). Alternatively, a more complex process can occur where drugs are metabolized into intermediates that can irreversibly inactivate P450s. This is known as mechanism-based inactivation (MBI) and results in a dose- and time-dependent loss of P450 activity (Silverman, 1995). Subsequent recovery of P450 activity is entirely due to de novo synthesis of P450 protein. This process leads to a significant delay between withdrawal of the inactivator and recovery of metabolism. An example of a mechanism-based clinical DDI involving CYP1A2 is the interaction between furafylline and caffeine, where coadministration increased the plasma area under the curve of caffeine 7- to 10-fold accompanied by significant incidence of unacceptable side effects (Tarrus et al., 1987).

Significant success has been achieved in the prediction of clinical DDI from in vitro analysis of reversible P450 inhibitors (Brown et al., 2005; Ito et al., 2005; Obach et al., 2006). These methodologies allow an accurate estimation of the likely magnitude of DDI using relatively simple in vitro techniques and scaling to humans using physiological parameters such as hepatic blood flow and plasma unbound fraction plus estimates of the fraction metabolized by the inhibited P450.

Due to the clinical significance of MBI, there continues to be a drive for improving DDI predictions in this area. The reaction scheme for mechanism-based inactivation is given in Fig. 1 (Walsh et al., 1978; Silverman, 1995). In brief, the scheme describes a rapid, reversible pre-equilibrium complex, which can then be activated to an intermediate form according to the rate constant $K_2$. This can then progress to either an irreversibly inactivated final complex, according to the rate constant $K_3$ or metabolism of the inactivator to a metabolite and the release of enzyme back into the active pool. The kinetic constants used to describe this process are the initial binding equilibrium, $K_i$, the maximum inactivation rate constant, $K_{\text{inact}}$, and the concentration of inactivator required to achieve the half-maximal rate of inactivation, $K_i$.

Conventional in vitro inactivation studies use a preincubation step followed by dilution with a probe substrate to estimate remaining P450 activity. These experiments have used widely different experimental conditions, including preincubation time, dilution factor, and activity measurement time, which make comparison across studies and clinical predictions difficult (Ghanbari et al., 2006; Yang et al., 2005). Recent publications have demonstrated that in vitro data can be used to predict the magnitude of these interactions using a standardized preincubation and dilution methodology (Obach et al., 2007).

ABBREVIATIONS: DDI, drug-drug interaction; P450, cytochrome P450; MBI, mechanism-based inactivation.
Although these methodologies are significantly improving the predictive power of in vitro studies, they are still complicated to perform because the design has to be tailored in terms of preincubation times and dilution factor. These can have significant effects because any inactivation occurring postdilution will lead to inaccurate estimations of the kinetic constants, especially if the initial velocities are not used (Ghanbari et al., 2006; Yang et al., 2005).

In this report, we outline an alternative in vitro approach for the investigation of presteady state kinetics of CYP1A2 inactivation. Progress curve analysis uses an “all-in” approach where the enzyme is exposed simultaneously to probe substrate and inactivator/inhibitor while enzyme activity is monitored throughout the inactivation. This type of analysis has long been an accepted tool for measuring pre-steady state kinetics for a variety of physiological enzymes (Orson and Tipton, 1979; Pope et al., 1998; Maurer et al., 2000; Dash et al., 2001). However, its use in the context of P450 inactivation is unprecedented. Our goals were to demonstrate that this alternative approach can yield comparable data with the conventional dilution approach, may be less prone to analytical caveats, and provide a more detailed interpretation of inactivation kinetics.

We investigated the time course of CYP1A2 inactivation with four preceded time-dependent inactivators (Fig. 2): furafylline, dihydralazine, oltipraz, and resveratrol, plus a time-independent inhibitor (paroxetine) (Von Molke et al., 1996). These data were analyzed to determine mechanistic information around the inactivation mechanism, the key kinetic parameters for comparison with previous studies, and, where appropriate, predictions of clinical DDI.

Materials and Methods

Materials. Furafylline, dihydralazine, oltipraz, resveratrol, paroxetine, and fluconazole were synthesized by Pfizer Global Research and Development (Sandwich, UK). Pooled human liver microsomes were supplied by BD-Gentest, Inc. (Woburn, MA). All other reagents were of the highest purity available and purchased from Sigma-Aldrich (Gillingham, UK).

Inactivator Time Course Studies. Individual incubation mixes (total, 1.7 ml) consisted of 50 mM phosphate buffer, pH 7.4, 5 mM MgCl₂, NADPH regeneration system (5 mM isocitric acid and isocitric acid dehydrogenase at 1 units/ml), human liver microsomes (0.1 mg/ml microsomal protein), and CYP1A2-specific probe substrate (5 μM tacrine) (Spaldin et al., 1995). The Kᵣ of tacrine in this batch of microsomes was previously determined to be 2 μM (data not shown). Reactions were carried out at 37°C and initiated by the addition of NADPH (1 mM). Initial studies with rich data sampling in the early phase (data not shown) demonstrated that the turnover of tacrine (as monitored by determination of OH-tacrine production) becomes linear approximately 5 min after the addition of NADPH. Therefore, all incubations were left for 8 min after the addition of NADPH before addition of inactivator or MeOH vehicle. Immediately after mixing, a 100-μl sample (representing t₀) was removed and quenched in ice-cold acetonitrile (0.05% formic acid) containing internal standard (250 ng/ml fluconazole). Additional samples were removed at 11 further time points postaddition of inactivator, where tacrine metabolism is linear (50 min).

Time course experiments were carried out to obtain inactivation progress curves with multiple concentrations (six to eight) of each inactivator (0.5–3.5 μM furafylline, 20–150 μM dihydralazine, 0.5–20 μM oltipraz, 4–80 μM resveratrol) on three to four occasions. These concentrations were chosen to ensure a wide range of inactivation across the 50-min time course. To account
for inactivator depletion in the incubation, a time-averaged concentration was determined by trapezoidal integration using eq. 1 in WinNonlin (version 4.0.1; Pharsight, Mountain view, CA) (Zhao et al., 2004).

\[
\int_{t=0}^{t=50 \text{ min}} [I] \cdot dt \quad \text{Time averaged } [I] = \frac{1}{50 \text{ min}} \int_{t=0}^{t=50 \text{ min}} [I] \cdot dt \tag{1}
\]

Progress curve analysis was then carried out to estimate the key constants defined in eqs. 2 to 4 (Silverman, 1995; Yang et al., 2005).

\[
K_i = \frac{(k_{-1} + k_2)}{k_1} \tag{2}
\]

\[
K_{\text{max}} = \frac{(k_2 \cdot k_3)}{(k_2 + k_1 + k_4)} \tag{3}
\]

\[
K_i = \frac{((k_{-1} + k_2) \cdot k_1) \cdot (k_3 + k_4) \cdot (k_2 + k_3 + k_4) \cdot (k_{-1} + k_2)}{k_1} \tag{4}
\]

For each experiment nonlinear regression of progress data were performed with eq. 5, using Grafit (version 5) for each inactivator concentration. This regression simultaneously estimates the apparent rate constant for the formation of the final steady state equilibrium \(K_{\text{obs}}\) plus the initial and steady state velocities of product formation \(V_0\) and \(V_s\) for each curve analyzed (Morrison and Walsh, 1988; Pope et al., 1998).

\[
[\text{Product}] = \frac{V_s \cdot t + (V_0 - V_s) \cdot (1 - e^{-k_{\text{obs}} t})}{K_{\text{obs}}} \tag{5}
\]

Using this approach a set of data can be generated for each of these parameters against inactivator concentration. This process was repeated for each experimental day to generate 3 to 4 sets of data for further analysis.

Plots of \(K_{\text{obs}}\) against inactivator concentration should yield hyperbolic profiles if inactivation is preceded by a saturable binding step and were analyzed by nonlinear regression (eq. 6 to estimate \(K_i\) and \(K_{\text{max}}\). I equals time averaged inactivator concentration as defined in eq. 1.

\[
K_{\text{obs}} = \frac{K_{\text{max}} \cdot [I]}{K_i \cdot (1 + S/K_{\text{obs}}) + [I]} \tag{6}
\]

FIG. 3. Plots of progress curves for CYP1A2 inactivation by 20 to 150 \(\mu\)M dihydralazine (A), 0.5 to 3.5 \(\mu\)M furafylline (B), 0.5 to 20 \(\mu\)M oltipraz (C), 4 to 80 \(\mu\)M resveratrol (D), and 1 to 12 \(\mu\)M paroxetine (E). Lines in A to D indicate best fit to eq. 4 and show increasing inactivation with time and concentration of inactivator. Paroxetine shows no time-dependent inhibition, and lines indicate linear best fits.
Where a linear relationship is observed it indicates that, within the operating limits of the assays, we were unable to establish conditions where a saturable inactivation was observed. In this case, a linear fit of the double reciprocal plot used to estimate $K_i$ and $K_{\text{inact}}$ (Kitz and Wilson, 1962). For comparison purposes, similar analyses were also undertaken for the compounds where hyperbolic profiles were obtained.

This methodology also allows estimates of the initial binding equilibrium as reflected in the magnitude of reduction of $V_0$ by the inactivator against respective vehicle control. Although the overall mode of inhibition with each of these inactivators is mixed, the contribution made by the inactivation process at this early time is likely to be minimal. Therefore, this inhibition is likely to reflect the initial rapid and reversible binding of the inactivator to the enzyme. As such, these data can be used to generate a value close to the initial rapid binding affinity that we have termed a $K_i$ apparent ($K_{\text{app}}$). Inhibition of $V_0$ was plotted against initial inactivator concentration and $IC_{50}$ values determined via standard methodologies, then adjusted for substrate affinity and concentration using the Cheng-Prussof equation, where $K_i = IC_{50}/(1 + [S]/K_m)$. Paroxetine is a non-time-dependent inhibitor and so was analyzed by determining average velocity across the time course by linear fitting of each concentration. $IC_{50}$ values were then determined, and $K_i$ was estimated using the Cheng-Prussof equation.

**Analytical Methods.** Quenched samples were centrifuged at 2000g (4°C) for 40 min to pellet out precipitated protein. The supernatant (20 µl) was analyzed for OH-tacrine and inactivator concentrations by liquid chromatography-mass spectrometry on an API4000 (MDS Sciex, Foster City, CA). Separation was achieved using an 8-min gradient reverse phase method with a 50 × 4.6-mm Luna Phenyl hexyl column (Phenomenex, Torrance, CA). The gradient was run from 100% MeOH:H2O (10:90) to 100% MeOH:H2O (90:10), with both containing 2 mM ammonium acetate and 0.027% formic acid and provided by Romil (Cambridge, UK).

**Results**

Linearity of probe substrate turnover was observed in vehicle incubations up to 50 min, indicating that no significant loss of CYP1A2 activity occurs over the time course. For all the inactivators tested, significant time- and concentration-dependent inactivation of CYP1A2 was observed. Paroxetine showed concentration- but not time-dependent inhibition consistent with its reported mechanism of competitive inhibition. Example progress curves for each of the inactivators are given in Fig. 3, with best fit lines to eq. 5. All inactivator depletion was monitored during the experiments, and time-averaged concentrations were calculated as described by eq. 1 (Table 1). Furafylline depletion was negligible (<2%), so initial concentration was used for all analyses.

$K_{\text{obs}}$ was then plotted against time-averaged concentrations for each inactivator (Fig. 4). The linear relationship for dihydralazine and furafylline showed that we were unable to demonstrate the presence of a saturable step before inactivation (Silverman, 1995). Double reciprocal analysis (Kits and Wilson, 1962) was therefore used to generate estimates of $K_i$ and $K_{\text{inact}}$ (Fig. 5; Table 2). Oltipraz and resveratrol show the predicted hyperbolic relationship, and fits to eq. 6 enabled estimation of $K_i$ and $K_{\text{inact}}$. To enable comparison of the analysis methods, double reciprocal analysis was also performed for these compounds.

Inhibition of $V_0$ was plotted against initial inactivator concentration assuming that at this stage, minimal inactivator was depleted. Oltipraz and resveratrol showed significant inhibition across the concentration range, thus enabling estimation of $K_{\text{app}}$ (Table 2; Fig. 6). Furafylline and dihydralazine were such weak inhibitors (<40% inhibition at any concentration tested) that no estimation of $K_{\text{app}}$ was possible.

**Table 1**

<table>
<thead>
<tr>
<th>Initial Inactivator Concentration</th>
<th>Time-Averaged Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>μM</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
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<tr>
<td>10</td>
<td>8.7</td>
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<tr>
<td>15</td>
<td>11</td>
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<tr>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>Oltipraz</td>
<td>μM</td>
</tr>
<tr>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>3.5</td>
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<tr>
<td>7.5</td>
<td>5.6</td>
</tr>
<tr>
<td>10</td>
<td>7.9</td>
</tr>
<tr>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Dihydralazine</td>
<td>μM</td>
</tr>
<tr>
<td>5</td>
<td>2.1</td>
</tr>
<tr>
<td>10</td>
<td>4.6</td>
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<td>15</td>
<td>5.4</td>
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<td>25</td>
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<td>75</td>
<td>49</td>
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<tr>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td>150</td>
<td>114</td>
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</table>

**Discussion**

In this investigation, we have been able to demonstrate the first use of progress curve analysis to investigate MBI of CYP1A2 inactivators. The furafylline data are within 2-fold of recently published dilution data generated within Pfizer laboratories with the same microsomal batch and near-identical assay conditions (Obach et al., 2007) (Table 2). In this publication, considerable attention has been spent on optimizing assay conditions for each inactivator, in terms of preincubation and minimizing postdilution inactivation, and therefore represents a relevant comparator.

Resveratrol and dihydralazine results are reasonably comparable with published data for $K_i$ and $K_{\text{inact}}$ (within 4.3-fold), especially given the published ranges for inactivation parameters across the literature. For example, furafylline has been cited as having a range of $K_i$ from 1.6 to 22.3 and $K_{\text{inact}}$ from 0.19 to 0.87 (Kunze and Trager, 1993; Obach et al., 2007). This range of estimate highlights the potential variations due to different microsomal batches and the caveats surrounding the existing analysis methodologies that have been discussed in depth and were the drivers for embarking on this investigation (Yang et al., 2005; Ghanbari et al., 2006). The results for oltipraz are not directly comparable because the literature values have been generated in a recombinant system (Chang et al., 2001).

Additionally, a clear differentiation between the balance of initial reversible and time-dependent inactivation in the compounds observed. Oltipraz and resveratrol show inhibition of $V_0$ that enabled estimation of the reversible $K_{\text{app}}$. When these are compared with $K_i$, estimated either via hyperbolic plots of $K_{\text{obs}}$ against I or double reciprocal plots, the values are similar, or $K_{\text{app}}$ is lower. This indicates that the rate of conversion of rapid, reversible pre-equilibrium complex to the activated intermediate complex ($K_2$) is likely to be the rate determining step in the overall inactivation process and that $K_i$ and $K_{\text{app}}$ are relatively fast. Conversely, the results for furafylline and dihydralazine show little inhibition of the initial velocity at any concentration, and so $K_i$ is significantly lower than $K_{\text{app}}$. This indicates that, compared with resveratrol and oltipraz, $K_i$, $K_{\text{app}}$, and $K_{\text{inact}}$ are relatively slow (Silverman, 1995). These data demonstrate that this analysis methodology can deliver a greater level of mechanistic insight than dilution methodologies. Reported studies have claimed the
inactivation kinetics of resveratrol to be comparable with furafylline (Thomas et al., 2001). Indeed, in this study, both inactivators exhibit moderate potency and inactivation rate; however, the two can be distinguished based upon a different balance of reversible ($K_{\text{app}}$) and irreversible ($K_{i}$ and $K_{\text{inact}}$) inactivation.

In recent years, it has been well established that in vitro-in vivo correlations in the field of MBI are limited. Although modeling recommendations have been made to improve clinical DDI predictions (Obach et al., 2007), the caveats associated with the dilution approach have not been fully surmounted (Yang et al., 2005; Ghanbari et al., 2006). Progress curve analysis is capable of assessing multiple mechanisms of inhibition and is not limited to MBI, although it is the focus of this study. Importantly, more confidence can be placed in the parameter estimates generated when we consider that the data are analyzed in a way that captures the inactivation process as it occurs as opposed to deriving the degree of inactivation by dilution.

In summary, this investigation has demonstrated that progress curve

![Graphs of $K_{\text{obs}}$ against time-averaged inactivator concentration for dihydralazine (A), furafylline (B), oltipraz (C), and resveratrol (D). A and B, linear fits; C and D, best fit according to eq. 5.](image)

![Graphs of double reciprocal plots and best fit lines of $K_{\text{obs}}$ against time-averaged inactivator concentration for dihydralazine (A), furafylline (B), oltipraz (C), and resveratrol (D).](image)
analysis can be successfully applied to investigate MBI of CYP1A2. Additionally, this analysis allows a more in-depth investigation of the balance of reversible and irreversible inactivation. Using this method, the same magnitude of the furafylline/caffeine interaction is predicted using precedent methods (Obach et al., 2007). Future studies will examine a wider range of inactivators and P450s to compare this methodology with the dilution approach. Particular focus will be paid to inactivators and P450s where a significantly greater sensitivity to $K_i$ and $K_{\text{inact}}$ estimates is predicted than the furafylline/caffeine interaction. In parallel, more powerful analysis and modeling techniques may be able to further use the added mechanistic insight offered.

Acknowledgments. We thank Barry Jones, Ruth Hyland, Kuresh Youdim, Neil Benson, and Maurice Dickins for support and advice throughout the investigation.

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>$[I]$</th>
<th>$v_0$</th>
<th>$K_{\text{app}}$</th>
<th>$K_i$</th>
<th>$K_{\text{inact}}$</th>
<th>$K_{\text{app}}/K_i$</th>
<th>$K_i$</th>
<th>$K_{\text{app}}$</th>
<th>$K_{\text{inact}}$</th>
<th>$K_{\text{app}}/K_{\text{inact}}$</th>
<th>$K_i$</th>
<th>Reference</th>
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<tr>
<td>Oltipraz</td>
<td>0.24</td>
<td>0.05</td>
<td>0.037</td>
<td>100</td>
<td>0.18</td>
<td>0.033</td>
<td>196</td>
<td>9$^a$</td>
<td>0.19$^a$</td>
<td>Langouet et al. (2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>3.3</td>
<td>0.4</td>
<td>0.41</td>
<td>(24)</td>
<td>3.3</td>
<td>0.09</td>
<td>31</td>
<td>8.5</td>
<td>0.28</td>
<td>Chang et al. (2001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furafylline</td>
<td>b</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.8</td>
<td>0.16</td>
<td>229</td>
<td>1.6</td>
<td>0.19</td>
<td>Obach et al. (2007)$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydralazine</td>
<td>&gt;45</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>11</td>
<td>0.13</td>
<td>18</td>
<td>42</td>
<td>0.03</td>
<td>Masubuchi and Horie (1999)</td>
<td></td>
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<tr>
<td>Paroxetine$^d$</td>
<td>1.4</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>(5)</td>
<td>(0.02)</td>
<td>(7)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined; N.A., not applicable.

$^a$ Recombinant CYP1A2.

$^b$ $K_i$ not determined since inhibition was <40% at highest concentration tested.

$^c$ Carried out within Pfizer laboratories with identical microsomal batch and similar incubation conditions.

$^d$ Non-time-dependent inhibitor.

References


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