Mechanism-Based Inhibition of CYP2C8: Differentiation by IC_{50} Shift Approach and Enzyme Inactivation Kinetics

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OVERVIEW
PURPOSE: To investigate the inhibitory pattern of reversible and mechanism-based inhibitors of CYP2C8 and estimate the enzyme inactivation kinetics of known mechanism-based inhibitors of CYP2C8.

METHODS: Determination of the IC_{50} values of known reversible and mechanism-based inhibitors of CYP2C8 using human liver microsomes with a 30-min pre-incubation in the presence and absence of NADPH. Estimation of the enzyme inactivation kinetics of known mechanism-based inhibitors of CYP2C8 by the pre-incubation in the presence of NADPH with multiple pre-incubation times and multiple inhibitor concentrations in human liver microsomes followed by the enzyme activity assay using amodiaquine as the probe substrate.

CONCLUSIONS: The IC_{50} shift approach can be used to differentiate reversible and mechanism-based inhibitors.

INTRODUCTION
Mechanism-based inhibition of cytochrome P450 (CYPs) reactions leads to irreversible or quasi-irreversible inactivation of CYPs, and has greater clinical impact than reversible inhibition. Determining whether a new chemical entity is a reversible or mechanism-based inhibitor of CYP-mediated reactions is critical in drug discovery and development. In this study, known reversible (quercetin and montelukast) and mechanism-based inhibitors (phenelzine, amiodarone, and gemfibrozil glucuronide) of human CYP2C8 were investigated with pooled human liver microsomes (HLMs) by an IC_{50} shift method [1]. In the IC_{50} shift approach, IC_{50} values are determined using a 30-min pre-incubation with chemical inhibitors in the presence and absence of NADPH prior to adding probe substrates. The inhibition type (reversible or mechanism-based) is estimated based on the relative IC_{50} values under the two incubation conditions.

METHODS
30-Minute Pre-Incubation in the presence and absence of NADPH
CYP inhibitors were pre-incubated at 37 °C for 30 min with HLMs (0.25 mg/mL) in the presence and absence of NADPH (1 mM). CYP reactions were initiated by adding probe substrates (final concentrations were at ~K_m) without and with NADPH. The HLMs and probe substrates were incubated at 37 °C for 20 min, and the reaction was terminated with ice-cold acetonitrile containing 0.1% formic acid. After centrifugation, the supernatant was injected for the quantitation of probe substrate metabolites by LC-MS/MS.

Co-Incubation and Pre-Incubation in the presence of NADPH [2]
For co-incubation, substrates and inhibitors were incubated at 37 °C for 20 min with HLMs (0.25 mg/mL) and NADPH (1 mM). For pre-incubation, inhibitors were pre-incubated in the presence of NADPH (1 mM) for 30 min with HLMs (0.25 mg/mL), and incubated for an additional 20 min after the addition of substrates. CYP reactions were terminated by adding ice-cold acetonitrile containing 0.1% formic acid. After centrifugation, the supernatant was injected for the quantitation of probe substrate metabolites by LC-MS/MS.

Estimation of Enzyme Inactivation Kinetics
Known mechanism-based inhibitors (phenelzine, amiodarone, and gemfibrozil glucuronide) of CYP2C8 at six concentration levels were pre-incubated with HLMs (1 mg/mL, in the final pre-incubation) containing phosphate buffer (0.1 M, pH 7.4), MgCl_2 (5 mM in the final pre-incubation), and NADPH (1 mM) in the final pre-incubation times (0, 5, 10, 15, 20, and 30 min). At each pre-incubation time, aliquots (10 μL) of the pre-incubated solutions were withdrawn and added into the CYP2C8 enzyme activity incubation solutions (190 μL) containing amiodarone (10 μM in the final incubation, at approximately 5 x K_m), phosphate buffer (0.1 M, pH 7.4), MgCl_2 (5 mM in the final incubation), and NADPH (1 mM in the final incubation) with a 20-fold dilution. CYP reactions were conducted by incubating at 37 °C for 20 minutes, and terminated with ice-cold acetonitrile containing 0.1% formic acid. After the removal of protein by centrifugation at 3,000 rpm for 10 min at 4°C, the supernatants were transferred into HPLC vials and the formation of desethylamodiaquine (metabolite of amodiaquine) was determined by LC-MS/MS. The inactivation parameters (K_i and K_{inact}) were estimated by fitting the experimental data to the following equation using a non-linear least-squares regression method by GraphPad Prism software:

\[ k_{obs} = \frac{k_{max} \times [I]}{K_i + [I]} \]

where, \( k_{obs} \) is the observed inactivation rate constant, \( K_i \) is the inhibitor concentration at half-maximal inactivation, \( k_{max} \) is the maximum inactivation rate constant, and \( I \) is the inhibitor concentration.

RESULTS

1. K_i and V_{max} of Amodiaquine for CYP2C8

![Figure 1](image1)

2. IC_{50} Values of Known Reversible and Mechanism-Based Inhibitors

![Figure 2](image2)

Table 1. IC_{50} shift values (n=2) of known reversible and mechanism-based inhibitors by pre-incubation and co-incubation approaches

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC_{50} (Pre-incubation: NADPH)</th>
<th>IC_{50} (Co-incubation: NADPH)</th>
<th>IC_{50} Shift (NADPH/NADPH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenelzine</td>
<td>150</td>
<td>100</td>
<td>0.18</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>250</td>
<td>150</td>
<td>0.02</td>
</tr>
<tr>
<td>Gemfibrozil Glucuronide</td>
<td>100</td>
<td>50</td>
<td>0.38</td>
</tr>
</tbody>
</table>

3. Enzyme Inactivation Kinetics of Known Mechanism-Based Inhibitors

![Figure 3](image3)

Table 2. K_i (inhibitor concentration at half-maximal inactivation) and K_{inact} (maximum inactivation rate constant) values (n=2) of known mechanism-based inhibitors for CYP2C8

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K_i (μM)</th>
<th>K_{inact} (min^-1)</th>
<th>K_{inact}/K_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenelzine</td>
<td>0.085</td>
<td>25</td>
<td>0.024</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>0.067</td>
<td>25</td>
<td>0.0082</td>
</tr>
<tr>
<td>Gemfibrozil Glucuronide</td>
<td>0.23</td>
<td>50</td>
<td>0.0044</td>
</tr>
</tbody>
</table>

CONCLUSION
Mechanism-based inhibitors demonstrated a significant leftward IC_{50} shift after pre-incubation in the presence of NADPH, in comparison with the IC_{50} after pre-incubation in the absence of NADPH. In contrast, reversible inhibitors produced a rightward IC_{50} shift or no change in IC_{50} after pre-incubation with NADPH. Similar IC_{50} alterations were observed by the co-incubation approach versus the pre-incubation approach in the presence of NADPH.

The IC_{50} shift estimated by either the pre-incubation or co-incubation approaches can be used to differentiate reversible and mechanism-based inhibitors of CYPs in vitro.

The enzyme inactivation kinetics of mechanism-based inhibitors can be assessed by pre-incubation in the presence of NADPH with multiple pre-incubation times (0, 5, 10, 15, 20, and 30 min) and multiple inhibitor concentrations using human liver microsomes (1 mg/mL) followed by the enzyme activity assay using a probe substrate at the concentration of approximately 5-fold K_i with a 20-fold dilution from the pre-incubated solutions.

References
1. Obach, et al., Drug Metab Dispos 2007, 35:246-255