Application of accurate mass spectrometry to mechanistic studies of metabolism-dependent inhibition of cytochrome P450 enzymes

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Overview

• Inhibition of drug metabolizing enzymes
  • Action
  • Implications
  • Cytochrome P450, CYP, enzyme inhibition
• Alkylamine CYP inhibition application
  • Elucidating mechanism of CYP3A4 inhibition by UPLC/QTOF-MS metabolite profiling with a novel reversibility sample prep approach
Reactive metabolites and enzyme inhibition

- Xenobiotic metabolism usually detoxifying, but sometimes chemically reactive metabolites form e.g. electrophiles, free radicals
- Metabolism-dependent inhibition can be a specific consequence of formation of some types of reactive metabolites
  - Metabolite binds to metabolizing enzyme and alters it chemically or functionally
  - Clinical implications e.g. drug-drug interactions, mortality
  - Compound development implications e.g. development halt, market withdrawal
- Mechanistic understanding can help direct compound design to minimize this behavior

Overview of cytochrome P450 (CYP) enzyme inhibition

- **CYP inhibition**
  - Parent drug-protein
  - Metabolite-protein
    - Direct inhibition (reversible)
    - Metabolism-dependent inhibition, MDI (irreversible)
    - More potent direct inhibition e.g. fluoxetine and norfluoxetine
    - Irreversible covalent inhibition e.g. mibebradil
    - Quasi-irreversible noncovalent inhibition e.g. troleandomycin (metabolite-intermediate complex, MIC)
MDI of CYP3A4 by Posicor (mibefradil), withdrawn 1998
- Measure effect of pre-incubation with the test article on the in vitro IC$_{50}$ of CYP3A4 marker substrate

![Graph showing MDI of CYP3A4 by Posicor](image)

- Validated GLP-compliant LC/MS/MS quantitation (Applied Bio API2000)

Metabolism-dependent CYP3A4 inhibition by troleandomycin

![Graph showing Metabolism-dependent CYP3A4 inhibition by troleandomycin](image)
Irreversible MDI of CYP3A4 by mibefradil

- Reversibility assay incorporating chemical oxidation and ultracentrifugation can distinguish irreversible and quasi-irreversible MDI
- CYP3A4 activity by formation of 6β-hydroxytestosterone (LC/MS/MS)

Reversibility assay yields no return of enzyme activity

Quasi-irreversible MDI of CYP3A4 by troleandomycin

Reversibility assay yields return of enzyme activity
2° and 3° alkylamines and MIC formation

- MIC formation has been attributed to interaction of a nitroso group introduced to the structure by CYP metabolism
- Only supported by indirect evidence
  - UV/vis detection of MIC
  - Formaldehyde assays
  - Structural modification & MIC
- Metabolite profiling and characterization by mass spectrometry can be used to find direct evidence of metabolites and intermediates


Alkylamine test articles

- Macrolide antibiotics

Troleandomycin  Erythromycin
Alkylamine test articles

• Diltiazem and metabolites

\[
\begin{align*}
\text{Diltiazem} & : & \text{H}_3\text{C} & - \text{O} & - \text{O} & - \text{N} - \text{CH}_3 \\
\text{N-Desmethyl diltiazem} & : & \text{H}_3\text{C} & - \text{O} & - \text{O} & - \text{N} & - \text{CH}_3 \\
\text{O-Desacetyl diltiazem} & : & \text{H}_3\text{C} & - \text{O} & - \text{O} & - \text{O} & - \text{OH} \\
\end{align*}
\]

Metabolism-dependent CYP3A4 inhibition summary

• All show evidence of metabolism-dependent inhibition of CYP3A4

<table>
<thead>
<tr>
<th>Test article</th>
<th>IC_{50} (\mu M)</th>
<th>No preincubation</th>
<th>30 min preincubation</th>
<th>Fold shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troleandomycin</td>
<td>9.5</td>
<td>0.8</td>
<td>~12</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>49.0</td>
<td>9.1</td>
<td>~5</td>
<td></td>
</tr>
<tr>
<td>Diltiazem</td>
<td>46.8</td>
<td>13.6</td>
<td>~3</td>
<td></td>
</tr>
<tr>
<td>N-Desmethyl diltiazem</td>
<td>8.2</td>
<td>1.9</td>
<td>~4</td>
<td></td>
</tr>
<tr>
<td>O-Desacetyl diltiazem</td>
<td>215.0</td>
<td>79.0</td>
<td>~3</td>
<td></td>
</tr>
</tbody>
</table>
Troleandomycin metabolite-intermediate complex (MIC) analysis

- MIC formation involving the ferrous heme iron can be detected spectrophotometrically

- HLM
- 100 μM TAO
- 1 mg/mL protein
- pH 7.4, 37°C
- 8 min incubation

- All test articles showed evidence of MIC involving ferrous heme iron

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Waters Synapt MS metabolite profiling analytical workflow

- Incubation of drug with *in vitro* test system
- Protein precipitation
- Acquity UPLC separation/Synapt (QTOF) detection using MS^E_
- MetaboLynx XS data processing for biotransformation assignment
- MS/MS acquisition if necessary
- Structural elucidation for biotransformation routes

- Compound-directed mass defect filtering & MS^E time alignment
- ± 50 mDa; C-heteroatom dealkylation MDF

- 100 μM drug; 0, 30 min; HLM; NADPH
- 10 min: 10-90% B; water & ACN (formic acid); Waters T3 column
- Positive ESI; V & centroid mode; MS^E and MS/MS (CE ramp)
- By spectral changes from known parent drug
Macrolide antibiotic biotransformation summary

- Biotransformation dominated by $N$-demethylation and ester hydrolysis
- No evidence of nitroso or intermediates

Diltiazem and metabolite biotransformation summary

- Biotransformation dominated by $N$-demethylation and ester hydrolysis
- No evidence of nitroso or intermediates
Sample preparation for metabolite profiling

- Incubation followed by protein precipitation

**Drug**
Buffer, pH 7.4
NADPH cofactor
Human liver microsomes, HLM

37°C

Aliquot at time-point
Add organic stop

Transfer supernatent

Controls: 0 minute, no cofactor

- Does inhibitory metabolite precipitate out bound to the protein?

Reversibility assay with metabolite profiling

Incubate 30 min

ICE & SPIN

Transfer

Met ID

Treat with FeK₃(CN)₆

Protein precipitation

SAMPLE should contain free inhibitory metabolite

CONTROL should not contain inhibitory metabolite

UPLC/accurate MS/MS

Sample should contain protein-bound inhibitory metabolite

No treatment

Protein precipitation

UPLC/accurate MS/MS
Normal diltiazem metabolic profile in human liver microsomes

Reversibility assay MDF chromatograms for diltiazem

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Low energy MS\textsuperscript{E} spectrum for diltiazem inhibitory metabolite

Parent diltiazem [M+H]\textsuperscript{+}

\[ m/z = 415.1707 \]
\[ \Delta_{413} = -2.0157 \text{ amu} \] (2H?)
\[ \Delta_{440} = +24.9960 \text{ amu} \]

MS/MS spectrum for diltiazem

NL 60
\[ \Delta_{m} = 0 \]

NL = Neutral loss
MS/MS spectrum for diltiazem inhibitory metabolite

Reversibility assay MDF chromatograms for N-desmethyl diltiazem

[Diagram showing MS/MS spectrum and chromatograms]

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Low energy MS\textsuperscript{E} spectrum for N-desmethyl diltiazem inhibitory metabolite

Identical to diltiazem inhibitory metabolite

MS/MS spectrum for N-desmethyl diltiazem inhibitory metabolite

\[ [M + H]^+ \]

\[ [X + H]^+ \]

\[ [X - HCN + H]^+ \]

\[ [X + Na]^+ \]

\[ [X + K]^+ \]
Reversibility assay MDF chromatograms for O-desacetyl diltiazem

Low energy MS² spectrum for O-desacetyl diltiazem inhibitory metabolite

-42 from diltiazem inhibitory metabolite

Y = X – acetyl

MS/MS data indicate modification of alkylamine
Inhibitory metabolite structure

- Inhibitory metabolite forms by modification of alkylamine
- Identical metabolite forms with mono and di methylamine

**Diltiazem metabolite**

**N-Desmethyl diltiazem metabolite**

- Mass error is high; unlikely structure
- Trace inhibitory metabolite in untreated sample: acetonitrile adduct?
Accurate mass elemental composition data for \( m/z \) 440.1645

- Compound specific element limits, 50 ppm tolerance, DBE -1.5-50

<table>
<thead>
<tr>
<th>Elemental composition</th>
<th>Theoretical ( m/z )</th>
<th>Mass error (ppm)</th>
<th>i-Fit score</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{C}<em>{23}\text{H}</em>{26}\text{N}<em>{3}\text{O}</em>{4}\text{S} )</td>
<td>440.1644</td>
<td>0.2</td>
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<tr>
<td>( \text{C}<em>{22}\text{H}</em>{25}\text{N}<em>{2}\text{O}</em>{7} )</td>
<td>440.1709</td>
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<td>7.5</td>
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<tr>
<td>( \text{C}<em>{24}\text{H}</em>{30}\text{NO}_{5}\text{S} )</td>
<td>440.1532</td>
<td>25.7</td>
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<tr>
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<tr>
<td>( \text{C}<em>{21}\text{H}</em>{30}\text{N}<em>{2}\text{O}</em>{7} )</td>
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<tr>
<td>( \text{C}<em>{22}\text{H}</em>{27}\text{N}<em>{2}O</em>{7} )</td>
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<tr>
<td>( \text{C}<em>{21}\text{H}</em>{29}\text{N}<em>{2}O</em>{9} )</td>
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<tr>
<td>( \text{C}<em>{20}\text{H}</em>{26}\text{N}<em>{2}O</em>{10} )</td>
<td>440.1557</td>
<td>28.0</td>
<td>11.4</td>
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<tr>
<td>( \text{C}<em>{19}\text{H}</em>{26}\text{N}<em>{3}O</em>{7} )</td>
<td>440.1491</td>
<td>35.0</td>
<td>12.3</td>
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<tr>
<td>( \text{C}<em>{20}\text{H}</em>{30}\text{N}<em>{3}O</em>{6} )</td>
<td>440.1855</td>
<td>-47.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Inhibitory metabolite structure

- \( \text{C}_{23}\text{H}_{26}\text{N}_{3}\text{O}_{4}\text{S} - \text{H}^+ - [\text{C}_{2}\text{H}_{3}\text{N}] = \text{C}_{21}\text{H}_{22}\text{N}_{2}\text{O}_{4}\text{S} \)
- Change from diltiazem: - \( \text{CH}_4 \)

Does this explain why in literature 1° amines do not form MIC?
Could blocking this group prevent metabolism-dependent inhibition?
Conclusions

- Metabolite profiling of this test group revealed no evidence of nitroso metabolite formation
- The reversibility assay with LC/MS/MS metabolite profiling can be used for identification of quasi-irreversible inhibitory metabolites
- Diltiazem metabolism-dependent inhibition of CYP3A4 may be due to formation of a methyleneamine metabolite
- Assess di-N-desmethyl diltiazem for evidence that methyl group is necessary for metabolism-dependent inhibition
- Revisit the macrolide antibiotics (stronger interaction?)

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End

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