Risk Assessment of Reactive Metabolites in Drug Discovery: A Focus on Acyl Glucuronides and Acyl-CoA Thioesters

The Delaware Valley Drug Metabolism Discussion Group
2017 Rozman Symposium
HUMAN BIOTRANSFORMATION: THROUGH THE MIST

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Preclinical Pharmacokinetics & Drug Metabolism Assays

**Target Selection & Validation**
- Discovery
- Screen
- Hit-to-Lead
- Lead Optimization

**Optimize Pharmacokinetics**
- Liver microsomal stability
- Membrane permeability
- Efflux/transport assays
- Plasma stability
- Rat PK
- Protein binding
- Blood to plasma ratio

**Early DDI Assessment**
- Enzyme induction PXR activation
- CYP inhibition

**Lead compound Selection**
- Characterize Preclinical Candidate
- Intrinsic clearance
- Excretion balance
- Definitive metabolite ID
- Higher species PK
- Human PK prediction

**Assess DDI Potential**
- Competitive CYP IC₅₀
- Time-dependent CYP inhibition
- Hepatocyte induction
- CYP reaction phenotyping

**Detection and Assessment of Chemically-Reactive Metabolites**
Toxicity is a frequent cause of drug withdrawal from the market.

Reactive drug metabolites may mediate liver injury via covalent modification of biological macromolecules.

Reactive metabolites of carboxylic acid-containing drugs, acyl glucuronides and acyl-CoA thioesters, might contribute to idiosyncratic drug toxicity.

Underlying mechanisms not clear.

Believed to be immune-mediated.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Category</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcofenac (anti-inflammatory)</td>
<td></td>
<td>Hepatits, rash</td>
</tr>
<tr>
<td>Alpidem (anxiolytic)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Amodiaquine (antimalarial)</td>
<td></td>
<td>Hepatits, agranulocytosis</td>
</tr>
<tr>
<td>Amineptine (antidepressant)</td>
<td></td>
<td>Hepatits, cutaneous ADRs</td>
</tr>
<tr>
<td>Benoxaprofen (anti-inflammatory)</td>
<td></td>
<td>Hepatits, cutaneous ADRs</td>
</tr>
<tr>
<td>Bromfenac (anti-inflammatory)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Carbutamide (antidiabetic)</td>
<td></td>
<td>Bone marrow toxicity</td>
</tr>
<tr>
<td>Ibufenac (anti-inflammatory)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Iproniazid (antidepressant)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Ibufenac (anti-inflammatory)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Iproniazid (antidepressant)</td>
<td></td>
<td>Hepatits (fatal), anemia</td>
</tr>
<tr>
<td>Metiamide (antiulcer)</td>
<td></td>
<td>Bone marrow toxicity</td>
</tr>
<tr>
<td>Nomifensine (antidepressant)</td>
<td></td>
<td>Hepatits (fatal), anemia</td>
</tr>
<tr>
<td>Practolol (antiarrhythmic)</td>
<td></td>
<td>Severe cutaneous ADRs</td>
</tr>
<tr>
<td>Remoxipride (antipsychotic)</td>
<td></td>
<td>Aplastic anaemia</td>
</tr>
<tr>
<td>Sudoxicam (anti-inflammatory)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Tienilic Acid (diuretic)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Tolrestat (antidiabetic)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Troglitazone (antidiabetic)</td>
<td></td>
<td>Hepatits (fatal)</td>
</tr>
<tr>
<td>Zomepirac (anti-inflammatory)</td>
<td></td>
<td>Hepatits, cutaneous ADRs</td>
</tr>
</tbody>
</table>

In many cases, metabolic activation reactive metabolites leading to covalent binding to protein demonstrated in vitro and/or in vivo.

Carboxylic acid drugs

Danger Hypothesis Illustration for Immune-mediated Idiosyncratic Hepatotoxicity

Assessing Formation of / Exposure to Reactive Drug Metabolites

- Formation of adducts with nucleophiles
  - *In vitro* “trapping” studies with glutathione
  - *In vivo* metabolic profiling studies (e.g., GSH-adducts in bile, NAC-adducts in urine)
- Covalent binding studies with radiolabeled drug
  - Definitive
  - Measures “total” burden of protein bound-drug residue
  - Helpful compliment to trapping studies

- Trapping studies with glutathione and covalent binding studies with radiolabel carboxylic acid-containing drugs to examine the importance of acyl glucuronides vs. acyl-CoA thioesters as reactive intermediates *in vitro* and *in vivo*.

Relationship between daily-dose of oral medications and idiosyncratic drug-induced liver injury (DILI)

- Drugs dosed at \( \leq 10 \) mg/day extremely rare to lead to idiosyncratic drug toxicity (whether or not these drugs are prone to metabolic activation).

<table>
<thead>
<tr>
<th>Dosage Groups</th>
<th>( \leq 10 ) mg/day</th>
<th>11-49 mg/day</th>
<th>( \geq 50 ) mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual agents</td>
<td>Cerivastatin (2)</td>
<td>Fialuridine (3)</td>
<td>Isoniazid (24)</td>
</tr>
<tr>
<td>(number of cases)</td>
<td>Lisinopril (1)</td>
<td>Propylthiouracil (13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Simvastatin (1)</td>
<td>Phenytoin (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pemoline (1)</td>
<td>Valproate (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zafirkulast (1)</td>
<td>Nitrofurantoin (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paroxetine (1)</td>
<td>Ketoconazole (6)</td>
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</tr>
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<td></td>
<td>Disulfiram (6)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Troglitazone (4)</td>
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<td></td>
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<tr>
<td></td>
<td>Sulfasalazine (3)</td>
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<td></td>
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<tr>
<td></td>
<td>Methyl dopa (3)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Nefazodone (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Labetalol (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amoxicillin/Clavulan (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromfenac (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ibuprofen (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydrocodone (1)</td>
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<td></td>
<td>6-Mercaptopurine</td>
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<td></td>
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<tr>
<td></td>
<td>Itraconazole (1)</td>
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<tr>
<td></td>
<td>Carbamazepine (1)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Trimethoprim/Sulfamethoxazole (1)</td>
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<tr>
<td></td>
<td>Bupropion (1)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Iron (1)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Naproxen (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total number: 2, 8, 101

*NOTE.* Excluded are inhalation agents (4), intravenous agents (3), herbal agents (7), *Amanita* mushrooms (9), and combination of agents (3).
Assessing the Potential for Risk of Idiosyncratic Drug Toxicity Caused by Candidate Drugs (AstraZeneca, 2012)

Drugs that demonstrated high covalent binding (CVB) burden (>1 mg/day, Zones 3 and 4) correlated with many of the toxic compounds.

<table>
<thead>
<tr>
<th>CVB Burden</th>
<th>Compound</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(#9)</td>
<td>Ibufenac</td>
<td>24</td>
</tr>
<tr>
<td>(#32)</td>
<td>Ibuprofen</td>
<td>5.2</td>
</tr>
<tr>
<td>(#20)</td>
<td>Diclofenac</td>
<td>1.8</td>
</tr>
<tr>
<td>(#29)</td>
<td>Caffeine</td>
<td>&lt;0.23</td>
</tr>
</tbody>
</table>

\[ f_{cvb} = \frac{(CVB \cdot 0.26 \text{ mg})}{(\text{turnover} \cdot 4000 \text{ pmol})} \]

CVB burden = \( f_{cvb} \cdot \text{dose} \)

Recommendation: <1 mg/day exposure (CVB burden) to reactive metabolites

Cross-species Liver Microsomes Metabolite Profile: LC/UV detection

Identification of GSH-adduct as Major Metabolite in HLM fortified with NADPH and GSH

- GSH-adduct major metabolite formed in HLM (1.5 % relative peak area to parent, ~50% of total metabolite peak area)
- Decision to perform reactive metabolite/toxicity risk assessment prior to advancement.

Unpublished observations
Covalent Binding (CVB) of Radiolabeled Test Drug and $[^{14}\text{C}]$Diclofenac to Protein in Human Hepatocytes in Suspension: Prediction of “CVB Burden” in Human

### Covalent Binding

<table>
<thead>
<tr>
<th>Compound</th>
<th>CVB (pmol/mg protein)</th>
<th>Incubation time (h)</th>
<th>Turnover (%)</th>
<th>$f_{cvb}$</th>
<th>Daily Dose (mg)</th>
<th>CVB Burden (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Compound</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0 ± 2.6</td>
<td>1</td>
<td>0 ± 3.0</td>
<td>0.174*</td>
<td>80†</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>34.8 ± 4.8</td>
<td>2</td>
<td>0 ± 2.9</td>
<td>0.252*</td>
<td></td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>49.8 ± 8.3</td>
<td>4</td>
<td>0 ± 4.3</td>
<td>0.361*</td>
<td></td>
<td>28.8</td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>78.9 ± 2.6</td>
<td>1</td>
<td>68.6 ± 2.8</td>
<td>0.008</td>
<td>200</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>109. ± 8.3</td>
<td>2</td>
<td>78.9 ± 1.1</td>
<td>0.010</td>
<td></td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>151.6 ± 7.5</td>
<td>4</td>
<td>87.6 ± 0.5</td>
<td>0.013</td>
<td></td>
<td>2.51</td>
</tr>
<tr>
<td>(Thompson, et al.)</td>
<td>140.8</td>
<td>2</td>
<td>100</td>
<td>0.0092</td>
<td>200</td>
<td>1.84</td>
</tr>
</tbody>
</table>

Substrate concentration (10 µM), incubation volume (2.5 mL); *no loss of drug detected (assume 1% turnover); †Preliminary prediction of drug dose in human based on rat PK/PD and simple allometric scaling (4-species) for human PK.
Metabolites that form chemically reactive intermediates can be difficult to detect and measure because of their short half-lives. However, they can form stable products (e.g., glutathione conjugates) that can be measured and, therefore, may eliminate the need for further evaluation. Phase II conjugation reactions generally render a compound more water soluble and pharmacologically inactive, thereby eliminating the need for further evaluation. However, if the conjugate forms a toxic compound such as acylglucuronide, additional safety assessment may be needed.\(^5\)

Partial list of carboxylic-acid containing drugs withdrawn from clinical use and/or also associated with allergic reactions

Withdrawn

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Allergic reactions</th>
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<tbody>
<tr>
<td>Alclofenac</td>
<td><img src="image1" alt="Alclofenac" /></td>
<td></td>
</tr>
<tr>
<td>Bromfenac</td>
<td><img src="image2" alt="Bromfenac" /></td>
<td></td>
</tr>
<tr>
<td>Ibufenac</td>
<td><img src="image3" alt="Ibufenac" /></td>
<td></td>
</tr>
<tr>
<td>Zomepirac</td>
<td><img src="image4" alt="Zomepirac" /></td>
<td></td>
</tr>
<tr>
<td>Indoprofen</td>
<td><img src="image5" alt="Indoprofen" /></td>
<td></td>
</tr>
<tr>
<td>Ibufenac</td>
<td><img src="image6" alt="Ibufenac" /></td>
<td></td>
</tr>
<tr>
<td>Flunoxaprofen</td>
<td><img src="image7" alt="Flunoxaprofen" /></td>
<td></td>
</tr>
<tr>
<td>Benoxaprofen</td>
<td><img src="image8" alt="Benoxaprofen" /></td>
<td></td>
</tr>
<tr>
<td>Tienilic Acid</td>
<td><img src="image9" alt="Tienilic Acid" /></td>
<td></td>
</tr>
<tr>
<td>Tolmetin</td>
<td><img src="image10" alt="Tolmetin" /></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td><img src="image11" alt="Diclofenac" /></td>
<td></td>
</tr>
<tr>
<td>Fenoprofen</td>
<td><img src="image12" alt="Fenoprofen" /></td>
<td></td>
</tr>
<tr>
<td>Diflunisal</td>
<td><img src="image13" alt="Diflunisal" /></td>
<td></td>
</tr>
<tr>
<td>Clofibric Acid</td>
<td><img src="image14" alt="Clofibric Acid" /></td>
<td></td>
</tr>
<tr>
<td>Probenecid</td>
<td><img src="image15" alt="Probenecid" /></td>
<td></td>
</tr>
<tr>
<td>Indoprofen</td>
<td><img src="image5" alt="Indoprofen" /></td>
<td></td>
</tr>
<tr>
<td>Valproic Acid</td>
<td><img src="image16" alt="Valproic Acid" /></td>
<td></td>
</tr>
</tbody>
</table>

Drug Acyl Glucuronide: Instability (Primarily by Acyl Migration)

- Incubation of 1-O-β-drug-acyl glucuronide in buffer (pH 7.4, 37 °C) to determine degradation rate/degradation half-life.


**Diagram**

**Time = 0 min**

pH 7.4, 37°C

1-O-β-isomer

**Time = 20 min**

pH 7.4, 37°C

1-O-β-isomer

*Acyl migration isomers

% Remaining

100
80
60
40
20
0

Incubation Time (min)

Retention Time (min)

Relative Intensity (%)
Mechanisms for A) the transacylation of protein nucleophiles by 1-β-O-acyl-linked glucuronides and B) for stable adduct formation with lysine-residues on proteins by reaction with open-chain acyl glucuronide migration isomers.
“Increasing the degree of substitution at the alpha-carboxy-carbon correlates with increasing stability (decreasing reactivity with protein) of the acyl glucuronide.


The data provide conclusive evidence for the covalent binding of tolmetin glucuronide to HSA by Schiff-base formation with lysine ε-amino groups. In all of the identified tolmetin-containing peptides, the glucuronic acid moiety was present.” Ding et al., Proc. Natl. Acad. Sci. 90 (1993).

“Our results establish that the binding of these reactive metabolites to nucleophilic sites of proteins occur via two different mechanisms: one involving imine (Schiff base) formation and the other involving nucleophilic displacement of glucuronic acid.” Ding et al., Drug Metab Dispos., 23 (1994).

Zomepirac

Benoxaprofen
Qiu et al. (1998) Drug Metab. Dispos. 26:246-56.

Diclofenac

Fevipiprant
Pearson et al. (April, 2017) DMD Fast Forward
The classification value of the degradation half-life which separated the safe drugs from the withdrawn drugs was calculated to be 3.6 h.

“The KPB system was considered to be the best for evaluating the stability of AGs, and the classification value of the half-life in KPB serves as a useful key predictor for the IDT risk.”

<table>
<thead>
<tr>
<th>No.</th>
<th>AG</th>
<th>KPB</th>
<th>HSA</th>
<th>Human Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flufenamic acid AG</td>
<td>7.2 ± 0.6</td>
<td>7.7 ± 1.1</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>Gemfibrozil AG</td>
<td>71.4 ± 7.1</td>
<td>7.6 ± 0.8</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>Levofloxacin AG</td>
<td>16.1 ± 2.3</td>
<td>16.3 ± 3.8</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>Meclofenamate AG</td>
<td>28.1 ± 1.6</td>
<td>16.6 ± 0.9</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>Montelukast AG</td>
<td>37.5 ± 5.9</td>
<td>21.8 ± 1.0</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>Repaglinide AG</td>
<td>11.5 ± 1.0</td>
<td>7.0 ± 0.2</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>Telmisartan AG</td>
<td>45.6 ± 5.5</td>
<td>111.8 ± 14.0</td>
<td>11.7 ± 1.2</td>
</tr>
<tr>
<td>8</td>
<td>Diclofenac AG</td>
<td>0.7 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>9</td>
<td>Furosemide AG</td>
<td>3.2 ± 0.0</td>
<td>7.2 ± 0.3</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>Ibuprofen AG</td>
<td>2.7 ± 0.1</td>
<td>1.3 ± 0.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>Indomethacin AG</td>
<td>1.7 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>12</td>
<td>Mefenamic acid AG</td>
<td>17.0 ± 0.5</td>
<td>51.3 ± 6.2</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>13</td>
<td>R-Naproxen AG</td>
<td>1.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>S-Naproxen AG</td>
<td>2.2 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>15</td>
<td>Probencid AG</td>
<td>0.3 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>16</td>
<td>Tolmetin AG</td>
<td>0.4 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>17</td>
<td>R-Benzoxaprofen AG</td>
<td>0.7 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>18</td>
<td>S-Benzoxaprofen AG</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>19</td>
<td>Fenclomenac AG</td>
<td>1.7 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td>Ibufenac AG</td>
<td>0.8 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>21</td>
<td>Zomepirac AG</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

Covalent Binding: Bioactivation of Valproic acid (VPA)

- Microvesicular steatosis proposed to be due to irreversible inhibition of fatty acid metabolism leading to a build-up of lipids.

- Covalent binding studies in rat hepatocytes showed that inhibition of P450 or Glucuronidation had no effect on covalent binding to protein, whereas inhibition of acyl-CoA formation led to a 90% decrease in covalent binding to protein.

- VPA-acyl-CoA implicated in CB to protein

Covalent binding studies in rat hepatocytes showed that inhibition of P450 or Glucuronidation had no effect on covalent binding to protein, whereas inhibition of acyl-CoA formation led to a 90% decrease in covalent binding to protein.

VPA-acyl-CoA implicated in CB to protein

**Proposed Metabolism Routes of Carboxylic Acid-Containing Drugs to Reactive Derivatives and Non-Toxic Products**

1. Acyl-CoA Formation
2. Acyl Glucuronidation
3. Acyl Migration
4. Conjugation Reactions
5. Ring-Chain Tautomerism
6. Schiff-Base Formation With Proteins

**Potential Toxicity**

**Conjugation Reactions:**
- Glycine Amides
- Taurine Amides
- Carnitine Esters
- Hybrid Triglycerides
- Choline Esters
- Fatty Acylation?

**Remains intracellular, not excreted, doesn’t circulate in plasma**

**Excretion in urine, bile, detection in plasma**

1. Acyl-CoA Thioester
2. Covalent Binding to Protein
3. 1-O-Acyl Glucuronide
4. 2-, 3-, 4-O-Acyl Glucuronides
5. Open-Chain Aldehydes
6. Potential Toxicity
Acyl-CoA Synthetase-mediated Formation of S-acyl-CoA Thioesters Leading to the Transacylation of GSH and Protein nucleophiles

Detoxification?

Adverse Drug Reactions?

Detoxification
Acyl-CoA Thioesters Shown to Acylate Proteins In Vitro (early reports)

**Salicyl-SCoA**


**Arachidonyl-SCoA**


**Palmitoyl-SCoA**


**Nafenopin-SCoA**


**Clofibryl-SCoA**

Enantioselective Covalent Binding Studies with a Model Profen, 2-Phenylpropionic Acid (2-PPA), in Rat Hepatocytes


*denotes ¹⁴C-label
Enantioselective Covalent Binding Studies with 2-Phenylpropionic Acid in vitro in Rat Hepatocytes

- Covalent binding to protein correlates closely with acyl-CoA formation but not acyl glucuronidation.
- Corresponding studies in vivo in rat showed CB to liver protein in favor of the acyl-CoA thioester.

Li et al., JPET, 305, 250-256 (2003)


Table 1. Effect of Trimethylacetic Acid (TMA) and (-)-Borneol on the Covalent Binding, Acyl-CoA Formation, and Acyl Glucuronidation of (R,S)-[1,2-14C2]-2-PPA in Incubations with Freshly Isolated Rat Hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>% of control</th>
<th>2-PPA-CoA</th>
<th>2-PPA glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>covalent binding</td>
<td>TMA-treated</td>
<td>47.4 ± 2.7</td>
<td>33.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>(-)-borneol-treated</td>
<td>81.3 ± 3.2</td>
<td>105.3 ± 2.5</td>
</tr>
</tbody>
</table>

A) Covalent Binding
B) 2-PPA-CoA Formation
C) 2-PPA-Acyl Glucuronidation
PAA-acyl-CoA formation in rat hepatocytes leads to the highly selective, but reversible, covalent binding to hepatocyte proteins, but not to the transacylation of glutathione.
Significantly Different Covalent Binding of Oxidative Metabolites, Acyl Glucuronides, and S-Acyl CoA Conjugates Formed from Xenobiotic Carboxylic Acids in Human Liver Microsomes

Malin Darnell,† Katarina Breitholtz,‡ Emre M. Isin,† Ulrik Jurva,† and Lars Weidolf‡,†

CVMD iMed DMPK, ‡Drug Safety & Metabolism, AstraZeneca R&D Malmö, 431 83 Malmö, Sweden

ABSTRACT: Xenobiotic carboxylic acids may be metabolized to oxidative metabolites, acyl glucuronides, and/or S-acyl-CoA thioesters (CoA conjugates) in vitro, e.g., in hepatocytes, and in vivo. These metabolites can potentially be reactive species and bind covalently to tissue proteins and are generally considered to mediate adverse drug reactions in humans. Acyl glucuronide metabolites have been the focus of reactive metabolite research for decades, whereas drug-CoA conjugates, which have been shown to be up to 40–70 times more reactive, have been given much less attention. In an attempt to dissect the contribution of different pathways to covalent binding, we utilized human liver microsomes supplemented with NADPH, uridine S′-diphosphoglucuronic acid (UDPGA), or CoA to evaluate the reactivity of each metabolite separately. Seven carboxylic acid drugs were included in this study. While ibuprofen and tolmetin are still on the market, ibufenac, fenclozic acid, tienilic acid, suprofen, and zomepirac were stopped before their launch or withdrawn. The reactivities of the CoA conjugates of ibuprofen, ibufenac, fenclozic acid, and tolmetin were higher

- Ibuprofen, Ibufenac, Zomepirac, Tolmetin, Suprofen, Tienilic acid, Fenclozic acid all formed acyl glucuronides, but did not lead to covalent binding to liver microsomal protein.
- Ibuprofen, Ibufenac, Fenclozic acid, and Tolmetin → Acyl-CoA-mediated covalent binding
- Suprofen and Tienilic acid → P450-mediated covalent binding

Metabolism of Nicotinic Acid

Flushing and hepatotoxicity are important adverse effects of nicotinic acid.

Liver Dysfunction
Cases of severe hepatic toxicity, including fulminant hepatic necrosis.

Dosing:
- Recommended dietary allowance = 12-16 mg/day.
- Tolerable upper intake level = 35 mg/day (to avoid flushing).
  - Hepatotoxicity observed at 500 mg/day and higher.

http://lpi.oregonstate.edu/mic/vitamins/niacin
**In Vitro Covalent Binding Studies with \([^{14}\text{C}]\text{Nicotinic Acid}\) and \([^{14}\text{C}]\text{Diclofenac}\) in Human Hepatocytes**

\[^{14}\text{C}\]-Nicotinic acid (10 \(\mu\text{M}\)) and \[^{14}\text{C}\]Diclofenac (10 \(\mu\text{M}\))

Time-dependent Covalent Binding to Protein in Incubations with Human Hepatocytes

(1 million viable cells/mL) in Suspension (Williams Media E)

### Covalent Binding to Protein

**Nicotinic Acid**
- CVB: 83 pmol/mg protein
- Turnover: 0.35
- \(f_{cvb} = 0.015\)
- Daily Dose: 500 mg
- CBV Burden: 7.7 mg

**Diclofenac**
- CVB: 114 pmol/mg protein
- Turnover: 0.95
- \(f_{cvb} = 0.008\)
- Daily Dose: 200 mg
- CBV Burden: 1.6 mg

\(*140*\) pmol/mg protein

\(*2*\) h

\(*1.0*\)

\(*0.0092*\)

Unpublished observations

Acyl-CoAs reported to be 20- to 100-fold more reactive toward GSH than the corresponding acyl glucuronides in buffer (pH 7.4, 37°C)

- Ibuprofen 20-fold
- Clofibric acid 40-fold
- 2-Phenylproprionic 70-fold
- Naproxen 100-fold

Grillo (2011) Current Drug Metab. 12:229-244.
Acyl-S-CoA Reactivity with Glutathione: Influenced by both Steric and Electronic Effects

- Increasing the degree of substitution at the alpha-carboxy-carbon correlates with increasing stability (decreasing reactivity with GSH) of the acyl-CoA.

Reactivity of S-Acyl-Glutathione Thioesters (0.1 mM) with N-Acetylcysteine (NAC, 1 mM) at 25°C and pH 7.4

Increasing the degree of substitution at the alpha-carboxy-carbon correlates with increasing stability (decreasing reactivity with NAC) of the acyl-SG.
Enantioselective Studies with (R)- and (S)-Ibuprofen/Hepatocytes

- Proposal that Ibuprofen-acyl-CoA thioester, and not ibuprofen acyl glucuronide, mediates the transacylation of GSH in rat hepatocytes.
Enantioselective Bioactivation of Ibuprofen by Acyl-CoA Formation Favoring the R-Ibuprofen Isomer in Rat Hepatocytes

**Acyl-CoA Formation**

- (R)-Ibuprofen
- (S)-Ibuprofen

**Acyl-SG Formation**

- (R)-Ibuprofen
- (S)-Ibuprofen

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>I-1-O-G (%)</th>
<th>I-CoA (%)</th>
<th>I-SG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-borneol (0.1 mM)</td>
<td>1.7 ± 0.5</td>
<td>n.d.</td>
<td>98 ± 9.0</td>
</tr>
<tr>
<td>pivalic acid (0.5 mM)</td>
<td>n.d.</td>
<td>53 ± 2.1</td>
<td>46 ± 5.1</td>
</tr>
<tr>
<td>valproic acid (0.5 mM)</td>
<td>n.d.</td>
<td>36 ± 4.7</td>
<td>34 ± 0.5</td>
</tr>
<tr>
<td>lauric acid (0.5 mM)</td>
<td>9.2 ± 1.3</td>
<td>7.3 ± 2.7</td>
<td>7.0 ± 2.5</td>
</tr>
</tbody>
</table>

Carboxylic Acids Bioactivated by Acyl-CoA Formation Leading to S-Acyl-Glutathione Adducts

Zomepirac

Ibuprofen

Mefenamic Acid

Tolmetin

Flunoxaprofen

Cholic Acid analogues

Diclofenac

Benoxaprofen

Grillo (2011) Current Drug Metab. 12:229-244.
In vivo (Advil, 800 mg)


(Amgen)
In Vivo Formation of Ibuprofen-N-Acyl-Cysteine in Rats dosed with Pseudoracemic Deuterated $D_3$-(R)- and $D_0$-(S)-Ibuprofen

LC-MS/MS Analysis of Rat Urine Extracts

(Pharmacia/Pfizer) Grillo and Hua (2008) Unpublished observations
Benoxaprofen: Mechanisms of Benoxaprofen-induced Cholestasis

- A number of cases of hepatotoxicity in elderly patients led to the withdrawal of the drug from the market.

- “...the history of this drug and its adverse effects is an important chapter in the annals of hepatotoxicity.”

- Hepatotoxicity observed mostly in elderly females.

- “Morphologic features consisted of severe hepatic cholestasis accompanied by slight to moderate necrosis. No evidence for immune-based hypersensitivity”.

- Bioactivation by P450 not observed.

- Metabolic idiosyncratic reaction proposed.

- Proposed to be due to insoluble metabolite (“acyl glucuronide”) excreted in bile leading to blockage of bile flow, cholestasis.

Benoxaprofen: Mechanisms of Benoxaprofen-induced Cholestasis

- Incubation of benoxaprofen-S-acyl-glutathione with bovine gamma-glutamyltransferase (Ƴ-GT) leads to precipitate formation in vitro.

- Identity of precipitate not yet known.

- Ƴ-GT is located in the bile canaliculus membrane.

- Species differences in hepatic Ƴ-GT levels.
  - Rat low, human high.

- Ƴ-GT levels increase with age.

- Proposal that insoluble precipitate formed from the Ƴ-GT-mediated breakdown of benoxaprofen-acyl-SG may play a role in hepatic cholestasis.

Role of Drug Metabolism in Drug-induced Liver Toxicity (Sid Nelson, University of Washington; Seattle, WA) Drug-induced liver injury is one of the most common reasons for failure of drugs in development, and for either removal of drugs from the market or "black-box" warnings on marketed drugs. This presentation will focus on the role of reactive metabolites as a major cause of drug-induced liver injury, on structure-toxicity relationships, and on methods to assess risk from reactive metabolites.

“The role of acyl glucuronides in carboxylic acid drug toxicity may have been oversold.”
Summary

- The chemical reactivity, covalent binding, and toxicity of acyl glucuronides has been widely-studied, although a clear link has not been established.

- Acyl-CoA thioesters are substantially more reactive than their corresponding acyl glucuronides, and increasing evidence demonstrates that acyl-CoA thioesters contribute more importantly than acyl glucuronides to the covalent modification of hepatic protein in vitro and in vivo.

- The importance of chemically-reactive acyl-CoA metabolites in drug-induced hepatotoxicity may be underestimated.
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