Applying NMR- and LC/MS-Based Metabolite Profiling to Predictive and Investigative Toxicology

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TOXICOLOGICAL SCIENCES

2011 SOT 50th Anniversary Featured Articles and Special March 2011 Supplement

Review Articles by Distinguished International Experts Will Focus on Major Milestones and Impactful Areas of Toxicology

The March 2011 Special Anniversary Supplement will represent areas that are, or have been, a major focus and impactful areas in toxicology, along with forward-looking topics, including reviews on carcinogenesis, toxicogenomics, nanotoxicology and synthetic biology. The articles provide historical perspective on the subject matter, major advances in the field, and thoughts on the future direction of research.

Important contributions to toxicology will be celebrated in each issue of Toxicological Sciences in 2011. The first article published in January 2011 will focus on the hallmarks and mechanisms of cell death, a subject that is fundamental to nearly all toxic responses. Future topics in the regular monthly issue will include genetic polymorphism, epigenetics, flame retardants, and the toxicology of climate change.
Determining Toxicant Effects at Different Levels of Biomolecular Organization

**Metabolomics: Phenotype**

- Altered gene expression (constitutive or induced)
- Environmental or dietary influence
  - Contribution of intestinal microbiota
- Biomarkers of toxicity or disease
  - Causally-related to toxicity or result of toxicity
Integrating Omics Data for Hazard Identification and Risk Assessment

HAZARD IDENTIFICATION
- Evaluating Toxicity
- Predicting Toxicity
- Developing Hypotheses
- Investigating Mechanisms

RISK ASSESSMENT
- Dose-response
- Exposure Analysis
- Cross-Species Analysis
- Mechanism(s)
## Application of NMR and MS Methods in Metabolomics

<table>
<thead>
<tr>
<th>Attribute</th>
<th>NMR</th>
<th>MS-based</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative</td>
<td>Yes</td>
<td>Requires standard</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Low (10^{-5} M)</td>
<td>Much more than NMR</td>
</tr>
<tr>
<td>Consistency</td>
<td>Yes: proton; Minimal sample prep</td>
<td>Ionization variable; extraction and chromatographic conditions</td>
</tr>
<tr>
<td>Overlapping peaks</td>
<td>Yes; Deconvolution required</td>
<td>Minimal; millions of ions</td>
</tr>
<tr>
<td>Annotation</td>
<td>Databases available (more needed)</td>
<td>Some available; varies with conditions</td>
</tr>
<tr>
<td>Status</td>
<td>Workhorse; mature Rapid, non-destructive</td>
<td>Increasing use; uHPLC, GC</td>
</tr>
</tbody>
</table>

Robertson et al. (Toxicol. Sci. 2011, in press)
Application of Metabolomics to Understand Phenotypic Changes

- **Genetic Basis**
  - Male and female mice distinguished by urinary excretion of trimethylamine and trimethylamine-oxide
  - FMO3 only expressed in females: TMAO $\gg$ TMA
    - Males: TMA $\gg$ $\gg$ TMAO
Characterization of Transporter Knockout Models: Oatps

Oatp1a1

- 521 bp
- 273 bp

Oatp1a4

- 476 bp
- 232 bp

Substrate Uptake (pmol/mg/min)

- E217G
- E3S
- TC
- Digoxin
- MPP+
- CSA

Wildtype

Oatp1a1-/-

Oatp1a4-/-
Urinary Metabolite Profiles in Oatp Null Mice

Serum LC-MS: Bile acids muricholic, deoxycholic

- Increased in nulls

Urinary NMR
A: Hippurate
- Increased in nulls
B, C: phenylpropionic acids
- Decreased in nulls
Reciprocal Relationship between Hippurate and Chlorogenic Acids

Wikoff W R et al. PNAS 2009;106:3698-3703

Distribution and quantity of gut anaerobes increased throughout entire gi tract
Is it possible to integrate omics data to define causal relationships?

- Time course of gene expression changes relative to effects on metabolites
- Integrating global metabonomic outcome with single organ transcriptional data
- Static and dynamic effects “merged”
  - Multiple time points for metabonomic samples and single time point for gene expression
  - Urine sample collected over 24 hr with gene expression determined only at the end of collection
Phenobarbital: Hepatic Transcriptional and Urinary Metabonomic Data in Rats

- Phenobarbital: widely used in transcriptional profiling studies
  - Cytochrome P450 enzymes
  - Phase II enzymes and xenobiotic transporters
  - Nuclear receptor activation and gene expression
  - Hepatic transcription factors
  - Cell cycle regulation and cell proliferation

Urinary NMR Metabolomic Profile after Phenobarbital Treatment

Ascorbic Acid

Gulonic Acid
Urinary Metabolomic Profile: Microsomal Enzyme Inducers

DMP 904; 1 Day
DMP 904; 5 Day
PB; 1 Day
PB; 5 Day
GA
AA
Hepatic Transcriptional Profiles Inform Metabolite Excretion

1: Glucuronate Reductase
2: Guloenolactonase
3: L-Guloenolactone Oxidase

After 5 days of dosing; (p < 0.001)
Transcriptional Effects on Hepatic Recycling of Ascorbic Acid

+ 3.5

+ 1.7

+ 1.6
Integrating Metabolomic and Transcriptional Profiling Data

• Metabolomic and transcriptomic data inform each other
  – Major urinary metabolomic changes reflected in transcriptional profiling data
    • not most affected genes
• Evidence that
  – Microsomal enzyme induction increases the demand for ascorbic acid
  – Ascorbate is required to support microsomal enzyme induction
• Urinary excretion of gulonic and ascorbic acid consistently increased by microsomal enzyme induction in rodents
Skeletal Muscle Fiber Type and Biochemistry

**Fiber selective toxicity**
- Statins: Fast twitch (Type II)
- PPARα agonists: slow twitch (Type I)

**Variable response to stress or insult**
- Cell death, atrophy, hypertrophy

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction Type</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td>Resistance to fatigue</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Force production</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Fuel source</td>
<td>Triglycerides</td>
<td>CP, glycogen</td>
</tr>
</tbody>
</table>
Preclinical and Clinical Biomarkers of Skeletal Muscle Toxicity

- **Creatine kinase**
  - Used widely clinically; limited utility in preclinical species

- **Non-specific markers**
  - AST, Aldolase, LDH

- **Muscle-specific proteins**
  - Troponins, myoglobin, myosin light chain 1 (Myl3), fatty acid binding protein 3 (Fabp3)
    - Tissue specificity: skeletal and cardiac muscle
      - Some expressed in other tissues (liver, kidney)
    - Short half-life can limit utility

- **Combinatorial measurements as best approach**
# Skeletal Muscle Biomarkers of Toxicity: Fast Twitch Troponin I and Myoglobin

<table>
<thead>
<tr>
<th></th>
<th>AST (U/L)</th>
<th>fsTnI (ng/ml)</th>
<th>uMB (ng/mg Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Cerivastatin-F</td>
<td>114 ± 6</td>
<td>1838 ± 772*</td>
<td>&lt; 9 ± 1</td>
</tr>
<tr>
<td>Cerivastatin-M</td>
<td>130 ± 21</td>
<td>151 ± 10</td>
<td>&lt; 7 ± 1</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>135 ± 19</td>
<td>157 ± 17</td>
<td>nd</td>
</tr>
</tbody>
</table>

Cerivastatin dosed for 14 d at 1 mg/kg  
Isoproterenol dosed to male rats at 0.5 mg/kg (single dose)


- **Cerivastatin:** severe skeletal muscle necrosis (Type II) in female rats  
  - FsTnI increased markedly  
  - AST highly variable

- **Isoproterenol:** cardiac (ventricular) necrosis
Carnosine and Methylhistidine Metabolism

Carnosine → \[ \beta \text{-alanine} + \text{histidine} \] → Anserine
1-Methylhistidinide

- Highly specific to skeletal muscle (anserine)
  - mM levels
  - Used to study muscle protein turnover
- Rat serum concentrations at µM levels
3-Methylhistidine

- Post-translational modification of actin and myosin
- Rat skeletal muscle concentrations $\geq 500 \mu$M
  - 3-MH > 1-MH in smooth muscle, heart
## Effect of Skeletal Muscle Toxicity on Methylhistididine Levels

### Serum

<table>
<thead>
<tr>
<th></th>
<th>1-MH (μM)</th>
<th>3-MH (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td><em>Cerivastatin-F</em></td>
<td>6.0 ± 0.9</td>
<td>69.7 ± 12.8*</td>
</tr>
<tr>
<td><em>Cerivastatin-M</em></td>
<td>15.8 ± 2.1</td>
<td>14.1 ± 0.8</td>
</tr>
</tbody>
</table>

### Urinary Excretion

<table>
<thead>
<tr>
<th></th>
<th>1-MH (nmol/mg Cr)</th>
<th>3-MH (nmol/mg Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td><em>Cerivastatin-F</em></td>
<td>494 ± 52</td>
<td>3142 ± 666*</td>
</tr>
<tr>
<td><em>Cerivastatin-M</em></td>
<td>452 ± 34</td>
<td>381 ± 45</td>
</tr>
</tbody>
</table>
Time Course of Biomarker Changes

3-Methylhistidine in Serum

Skeletal Troponin I

Myosin Light Chain 3

Control

day 9

day 15
The Paradox of Isoproterenol: Methylhistidines and Muscle Biology

- Cardiac necrosis
  - Myoglobin increased
- Dose-dependent effects on skeletal muscle
  - Low dose increases muscle growth
  - 1- and 3-MH decrease due to anabolic effect
- Methylhistidines may report hypertrophy, atrophy and necrosis

![Bar chart showing urinary MH (nmol/mg Cr) for 1-MH and 3-MH in control and Isoproterenol groups.](chart.png)

- Control
- Isoproterenol

0 100 200 300 400

Uriney MH (nmol/mg Cr)

1-MH 3-MH

* Statistical significance
Human Relevance of Methylhistidines

- All mammals except humans synthesize (much) anserine
  - 1-MH may only be applicable to preclinical species
- 3-MH is conserved across all species
- Meat consumption can affect constitutive levels
Integrating Omics Data for Hazard Identification and Risk Assessment

Toxicology will progress from predominantly individual chemical studies into a knowledge-based science in which computational and informatics tools will play a significant role in deriving new understanding of toxicant-related disease.
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