ENZYME MARKERS OF TOXICITY
Why do we need enzyme markers?

- *In vivo* monitoring
- Serial sampling
- Early detection of metabolic changes
- Detection of organ-specific effects
- Establishment of “NO EFFECT” level
- Determination of toxic mechanism
- Is required by regulatory agencies
ENZYMES: “highly specialized proteins that facilitate biochemical reactions that otherwise would proceed at a much lower rate”

- Are usually confined to a specific cellular (membrane, cytosol, mitochondria) and/or organ location
- Sensitive to membrane integrity, changes in metabolism, excretion, inactivation
- The magnitude of response often correlates with the severity of damage

Why “enzyme markers”? 
Where do enzyme markers fit?

**BIOMARKERS:**

molecular, biochemical, or cellular alterations that are measurable in biological samples (tissues, cells or fluids)

The “Ideal” Biomarker:

- Method of analysis is appropriate to species being evaluated (e.g., human/rodent insulin assays have no homology)
- Sensitive, specific, predictive, efficient
- Bridges animal and human applications
- Non-invasive sampling (e.g., survival blood collection)
- Assay easy and rapidly performed
- Assay is reliable
- Assay is “cost worthy”
Where do enzyme markers fit?

- **Markers of internal dose:** blood & urine levels, fat concentrations, exhaled breath, metabolites in urine
- **Markers of biologically active dose:** DNA & protein adducts (both in cells and in body fluids)
- **Markers of early biological effect:** genetic alterations in target and reporter genes, nuclear aberrations, **altered enzymatic activities**
- **Markers of altered structure/function:** enzyme markers, proliferation, cell differentiation, differential expression of genes, cellular/tissue changes

From: Kensler T.W.  SOT 1992 (AM#2)
Laboratory evaluation of organ-specific toxicity

IMPORTANT ISSUES TO REMEMBER:
- Cell types differ in susceptibility to toxic agents
- One organ – many cell types
- Cellular injury vs. organ function impairment
- Oxygen concentration gradients
- Metabolizing enzymes (e.g., Cyt. P450) concentration gradients
Localization of damage:

- **Centrilobular (zone 3):**
  - Most hepatotoxicants (CCl₄, APAP)
  - Less oxygen + high P450 concentration

- **Periportal (zone 1):**
  - Phosphorus, aflatoxin, allyl alcohol
  - High oxygen + highest dose at the site

- **Midzonal (zone 2):**
  - Beryllium

- **Massive necrosis:**
  - Iproniazid, MAO inhibitors

www.cvm.okstate.edu/instruction/mm_curr/histology
LIVER TOXICITY

- Cholestatic Injury
- Cytotoxic Injury
- Disturbances of hepatic function/clearance
LIVER TOXICITY

General properties that describe a useful biomarker of xenobiotic-induced hepatic toxicity

- **Availability:** present in biological fluids in detectable levels
- **Specificity:** is of liver origin (exclusively or predominantly), or its level is affected by a change in liver function
- **Prevalence:** can be applied across multiple species, including humans
- **Sensitivity:** can be reliably measured at sub-lethal doses of a xenobiotic
- **Persistence:** stable to allow studies within days or weeks after collection
- **Relevancy:** confirmed to be associated with histopathological or functional changes in the liver

Adapted from Amacher DE (2002)
Evaluation of liver toxicity *in vivo*

- Serum enzyme tests
- Hepatic excretory tests
- * Alterations in chemical constituents of the liver
- * Histological analysis of liver injury
**LIVER TOXICITY**

**Zimmerman classification of serum enzymes to monitor liver injury:**

1. **Cholestatic Injury (ALP, 5’-NT, GGT)**
2. **Cytotoxic Injury:**
   A. Somewhat non-specific enzymes (AST; LDH)
   B. Enzymes that are found **mainly** in liver (ALT)
   C. Enzymes that are found **only** in liver (OCT; SDH)
3. **Enzymes relatively insensitive to hepatic injury** (e.g., creatine phosphokinase)
4. **Enzymes that demonstrate reduced serum activity in liver disease** (cholinesterase)
Table 24.1
Effect of various hepatotoxic procedures on four liver function tests in mice$^a$

<table>
<thead>
<tr>
<th>Hepatotoxic Procedure$^b$</th>
<th>BSP Retention (mg/dl)</th>
<th>Alkaline Phosphatase (units)</th>
<th>Bilirubin Concentration (mg/dl)</th>
<th>ALT Activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>0.3 ± 0.3</td>
<td>3.0 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>ANIT (150 mg/kg po)</td>
<td>45.0 ± 23</td>
<td>5.6 ± 2.6</td>
<td>1.1 ± 0.4</td>
<td>282 ± 126</td>
</tr>
<tr>
<td>CCl$_4$ (1 mg/kg po)</td>
<td>13.0 ± 7</td>
<td>5.3 ± 1.3</td>
<td>0.4 ± 0.2</td>
<td>8510 ± 193</td>
</tr>
<tr>
<td>Bile duct ligation</td>
<td>26.0 ± 3</td>
<td>19.0 ± 10</td>
<td>3.8 ± 0.8</td>
<td>655 ± 132</td>
</tr>
</tbody>
</table>

Data obtained from Reference 246.

$^a$ Values are expressed as means ± SE; each group contained 10 mice.

$^b$ Hepatotoxic procedure was performed 24 h before assessing function.

from Hayes A.W. Principles and Methods of Toxicology, 4th Edition (Taylor & Francis, 2000)

ANIT  – α-naphthylisothiocyanate
BSP   – sulfobromophthalein
LIVER TOXICITY

I. Markers of cholestatic injury:
A. Enzymatic:

Alkaline Phosphatase [AP, ALP] (membrane)
Hydrolyzes phosphate esters (e.g. ATP) at pH>7.0
Normal circulating levels contributed by: intestine/bone (rat), intestine/bone/liver/placenta (humans)
Many isoforms: humans-3, rats-2
Affected by diet, age, pregnancy and other factors
Not a very reliable marker in rat studies (diet, strain)
LIVER TOXICITY

I. Markers of cholestatic injury:

A. Enzymatic:

5'-Nucleotidase [5'-NT] (membrane)
Hydrolyzes nucleoside 5'-monophosphates
Normally present in: kidney, intestinal mucosa, etc.
Many isoforms: humans-3, rats-2
Is made soluble from membranes by a detergent or bile acids – released during cholestasis
I. Markers of cholestatic injury:

A. Enzymatic:

\(\gamma\)-Glutamyl Transpeptidase [GGT] (membrane)

Participates in the transfer of amino acids across the cellular membrane and in glutathione metabolism.

High concentrations are found in the liver and kidney. GGT is measured in combination with other tests: ALP is increased in hepatobiliary disease and bone disease; GGT is elevated in hepatobiliary disease, but not in bone disease.
I. Markers of cholestatic injury:

B. Non-enzymatic markers:

Total Serum Bile Acids

Synthesized in the liver, important for digestion and absorption of lipids and lipid-soluble vitamins

Relatively sensitive, early marker of cholestasis

Could be affected by altered enterohepatic circulation and impaired hepatic function
I. Markers of cholestatic injury:

B. Non-enzymatic markers:

Plasma Bilirubin (Direct and Total)

Heme $\rightarrow$ biliverdin $\rightarrow$ bilirubin $\rightarrow$ conjugated bilirubin

Cholestasis: direct (conjugated) is $>50\%$ total bilirubin

Hemolysis: direct (conjugated) is $<50\%$ total bilirubin
II. Markers of hepatocellular injury:
A. Somewhat non-specific enzymes:

Aspartate aminotransferase [AST] (cytosol/mitochondria)
L-aspartate + 2-oxoglutarate $\leftrightarrow$ oxaloacetate + glutamate

*a.k.a.:* serum glutamate-oxaloacetate transaminase (SGOT)

Normally present in a wide variety of tissues (skeletal muscle, heart muscle, liver, etc.)

AST in serum is stable: RT - 3 days; frozen – 30 days

Red blood cells are loaded with AST: be careful (hemolysis)
II. Markers of hepatocellular injury:
A. Somewhat non-specific enzymes:

Lactate Dehydrogenase [LDH] (cytosol)
pyruvate $\leftrightarrow$ L-lactate

Normally present in a wide variety of tissues
Five isoenzymes, isoenzyme profile may help identify specific tissue origin (LDH-5 $\rightarrow$ liver; LDH-1,-2 $\rightarrow$ kidney)
II. Markers of hepatocellular injury:
B. Enzymes found mainly in liver:

**Alanine aminotransferase [ALT]** (cytosol)

L-alanine + \(\alpha\)-ketoglutarate \(\leftrightarrow\) pyruvate + glutamate

*a.k.a.*: serum glutamate-pyruvate transaminase (SGPT)

Greatest activity is found in the liver
Activity can be found in serum and CSF, but not in urine
Stable at RT, frozen and refrigerated
Hemolysis has a negligible effect on ALT activity
II. Markers of hepatocellular injury:

C. Enzymes almost exclusively located in liver:

**Ornithine carbamyl transferase [OCT]** (mitoch.)

Ornithine $\rightarrow$ citrulline

Is found in liver (>97%) and small intestine (<2%)

Activity increases in both acute and chronic liver disease

Diagnostically useful in all species

As sensitive as histopathological examination of the liver

Is elevated after acute obstruction of bile flow
II. Markers of hepatocellular injury:
C. Enzymes almost exclusively located in liver:

Sorbitol dehydrogenase [SDH] (cytosol)

D-sorbitol $\leftrightarrow$ D-fructose

Is found in liver and testes

Greatest activity is found in the liver

Diagnostically useful in all species

Sensitive enzyme marker for liver necrosis but shall be combined with measurements of ALT or other enzymes

Is elevated after acute obstruction of bile flow
III. Enzymes relatively insensitive to hepatic injury:

Creatine phosphokinase [CPK]

\[ \text{creatine + ATP} \leftrightarrow \text{creatine phosphate + ADP} \]

Greatest activity is found in skeletal muscle

Is used as a marker of muscle injury (clinical use – cardiac muscle injury)
IV. Enzymes that demonstrate reduced serum activity in liver disease:

Choline Esterase [ChE]

Acetylcholine esterase and butyrylcholine esterase

Inhibited by organophosphates and carbamates

Can not distinguish between decreased synthesis and decreased activity
Laboratory evaluation of hepatic clearance/function:

Decreased dye clearance -> loss of functional liver mass:

- **Sulfobromophthalein**
- **Indocyanine green**

- Serum (i.v. injection)
- Bile (concentrated)
- GI tract (excreted)
**Hepatocellular:** ALT > 2 times upper limit of normal (ULN) or ALT/alkaline phosphatase (AP) ratio is 5

**Cholestatic:** AP > 2 times ULN or ALT/AP ratio is 2

**Mixed:** ALT/AP ratio is 2 to 5; individual values are > 2 times ULN

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Table 1 Clinical chemistry variables that are considered useful in identifying liver toxicity.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hepatocellular</th>
<th>Hepatobiliary</th>
<th>Mitochondrial</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-nucleotidase (5-NT)</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>alanine aminotransferase (ALT)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alkaline phosphatase (ALP)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>aspartate aminotransferase (AST)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>gamma glutamyltransferase (GGT)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>glutamate dehydrogenase (GLDH)</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>lactate dehydrogenase (LDH)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>ornithine carbamyltransferase (OCT)</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>sorbitol dehydrogenase (SDH)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>total bile acids (TBA)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>total bilirubin (TBILI)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>unconjugated bilirubin (UBILI)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
Multi-strain profiling of APAP-induced liver injury:
- % liver necrosis (24h), reduced GSH (4h), ALT (24h), ALT (4h)

**Histopathology vs Clinical Chemistry**

C57BL/6J mice

300 mg/kg, gavage
KIDNEY TOXICITY

I. Serum indicators of renal injury:

Blood Urea Nitrogen (BUN)

Blood Creatinine

Are used as estimators of glomerular filtration rate

About 75% of nephrons should be nonfunctional before changes in serum concentrations can be detected

BUN could be affected by high protein diet, dehydration...

Creatinine is less affected by external factors
KIDNEY TOXICITY


DBCP: 1,2-dibromo-3-chloropropane


### TABLE 2

**DOSE–RESPONSE OF KIDNEY TOXICITY WITH DBCP AND PERDEUTERO-DBCP**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kidney/body wt ×10²</th>
<th>Creatinine (µmol/liter)</th>
<th>Urea (mmol/liter)</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>4+</th>
<th>Mean grade ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.84 ± 0.03</td>
<td>54 ± 8</td>
<td>7.6 ± 1.7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DBCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(42.5 µmol/kg)</td>
<td>0.84 ± 0.06</td>
<td>44 ± 3a</td>
<td>6.0 ± 0.5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>(85 µmol/kg)</td>
<td>0.90 ± 0.14</td>
<td>58 ± 20</td>
<td>7.0 ± 5.6</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.8 ± 0.8a</td>
</tr>
<tr>
<td>(170 µmol/kg)</td>
<td>1.08 ± 0.15a</td>
<td>150 ± 49a</td>
<td>28.3 ± 16.3a</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2.8 ± 0.4a</td>
</tr>
<tr>
<td>(340 µmol/kg)</td>
<td>1.05 ± 0.05a</td>
<td>168 ± 47a</td>
<td>36.6 ± 22.8a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>3.2 ± 0.4a</td>
</tr>
<tr>
<td>C1-C2-C3-D5-DBCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(42.5 µmol/kg)</td>
<td>0.89 ± 0.01</td>
<td>39 ± 3</td>
<td>4.6 ± 0.9</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>(85 µmol/kg)</td>
<td>0.90 ± 0.05</td>
<td>48 ± 7</td>
<td>5.6 ± 0.9</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1.2 ± 0.8a</td>
</tr>
<tr>
<td>(170 µmol/kg)</td>
<td>1.13 ± 0.12a</td>
<td>210 ± 117a</td>
<td>32.8 ± 20.9a</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2.8 ± 0.4a</td>
</tr>
<tr>
<td>(340 µmol/kg)</td>
<td>1.09 ± 0.07a</td>
<td>240 ± 154a</td>
<td>51.1 ± 34.8a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>3.0 ± 0.0a</td>
</tr>
</tbody>
</table>

*Note.* Five rats in each group were dosed with DBCP or D5-DBCP (42.5–340 µmol/kg) ip. All rats were killed 48 hr after administration of test compounds. Values are means ± SD.

*Significantly different from control values (p < 0.05).

*Significantly different from DBCP values (p < 0.05).*
II. Urine indicators of renal injury:

Physical Characteristics
Color/turbidity (RBC’s, bilirubin); volume; osmolality

Chemical Components
Urinary protein:	 tubular (low MW) or glomerular (high MW) function
Urinary glucose:	 no elevation of blood glucose but glucosuria (tubular)
Urinary brush border enzymes (ALP, AST, GGT):	 proximal tubule
KIDNEY TOXICITY: Example

Halogenated alkanes and alkenes

Glutathione conjugates

Cysteine conjugates

Concentrated in kidney by renal transport system

GSH S-transferase

Reactive electrophiles (thioacylating metabolites)

Protein alkylation

Cysteine S-conjugate β-lyase

liver

GGT
KIDNEY TOXICITY: Examples

METABOLIC ACTIVATION OF THE NEPHROTOXIC HALOALKENE 1,1,2-TRICHLORO-3,3,3-TRIFLUORO-1-PROPENE BY GLUTATHIONE CONJUGATION*

SPYRIDON VAMVAKAS, ELISABETH KREMLING and WOLFGANG DEKANT†


Table 1. Toxicity of 1,1,2-trichloro-3,3,3-trifluoro-1-propene (TCTFP) in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TCTFP (25 mg/kg)</th>
<th>TCTFP (50 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma urea (mg/%)</td>
<td>38 ± 4</td>
<td>85 ± 22**</td>
<td>143 ± 32**</td>
</tr>
<tr>
<td>Plasma ASAT (U/l)</td>
<td>68 ± 16</td>
<td>54 ± 17</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>Plasma ALAT (U/l)</td>
<td>16 ± 3</td>
<td>11 ± 4</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Urine glucose (mg/24 hr)</td>
<td>2 ± 0.6</td>
<td>85 ± 31**</td>
<td>125 ± 27**</td>
</tr>
<tr>
<td>Urine protein</td>
<td>5 ± 1</td>
<td>30 ± 13**</td>
<td>65 ± 14**</td>
</tr>
<tr>
<td>Urine GGT (U/24 hr)</td>
<td>3540 ± 783</td>
<td>22490 ± 1370**</td>
<td>39470 ± 2170**</td>
</tr>
</tbody>
</table>
Histopathological evidence of cisplatin- and gentamicin-induced proximal tubular toxicity.

Representative H&E-stained sections histological sections from kidney of (A) vehicle-treated (magnification 20x), (B) cisplatin-treated rats (5 mg/kg, 6 d) (magnification 20x), (C) vehicle-treated rats (magnification 200), (D) gentamicin-treated rats (80 mg/kg/day; 7 d) (magnification 200x).

Representative electron micrographs from (E) vehicle-treated rats (magnification 3,000x) and (F) gentamicin-treated rats (80 mg/kg/day, d. 7) (magnification 4,500x). Increased apoptosis and cellular infiltrates and myelin figures were observed in rats treated with gentamicin (80 mg/kg/day, d. 7).

Table 1. Changes in serum and urine chemistry and organ and body weights 7 days after cisplatin and gentamicin treatment and 21 days after puromycin treatment.

<table>
<thead>
<tr>
<th>Cisplatin dose (mg/kg)</th>
<th>Body weight (g)</th>
<th>Kwt (g)</th>
<th>Serum Cr (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>Urine Glucose/Cr</th>
<th>Prot/Cr</th>
<th>Ca/Cr</th>
<th>Protein excretion (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>279 ± 7</td>
<td>2.5 ± 0.1</td>
<td>0.30 ± 0.01</td>
<td>15 ± 1</td>
<td>0.23 ± 0.01</td>
<td>1.0 ± 0.1</td>
<td>0.07 ± 0.01</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>0.3</td>
<td>268 ± 8</td>
<td>2.7 ± 0.1</td>
<td>0.24 ± 0.02</td>
<td>14 ± 1</td>
<td>0.17 ± 0.06</td>
<td>1.1 ± 0.1</td>
<td>0.11 ± 0.02</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>1</td>
<td>269 ± 10</td>
<td>2.7 ± 0.1</td>
<td>0.22 ± 0.04</td>
<td>11 ± 1</td>
<td>0.15 ± 0.04</td>
<td>1.5 ± 0.2</td>
<td>0.12 ± 0.03</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>252 ± 3*</td>
<td>2.7 ± 0.3</td>
<td>1.4 ± 0.4*</td>
<td>63 ± 20*</td>
<td>6.1 ± 2.0*</td>
<td>1.8 ± 0.3</td>
<td>0.23 ± 0.08*</td>
<td>66 ± 10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gentamicin dose (mg/kg/day)</th>
<th>Body weight (g)</th>
<th>Kwt (g)</th>
<th>Serum Cr (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>Urine Glucose/Cr</th>
<th>Prot/Cr</th>
<th>Ca/Cr</th>
<th>Protein excretion (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>269 ± 7</td>
<td>2.0 ± 0.1</td>
<td>0.19 ± 0.02</td>
<td>16 ± 1</td>
<td>0.19 ± 0.02</td>
<td>0.5 ± 0.1</td>
<td>0.07 ± 0.01</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>278 ± 8</td>
<td>2.1 ± 0.1</td>
<td>0.15 ± 0.02</td>
<td>13 ± 1</td>
<td>0.16 ± 0.02</td>
<td>0.4 ± 0.1</td>
<td>0.06 ± 0.01</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>280 ± 6</td>
<td>2.1 ± 0.1</td>
<td>0.15 ± 0.03</td>
<td>12 ± 1</td>
<td>0.20 ± 0.02</td>
<td>0.5 ± 0.1</td>
<td>0.09 ± 0.02</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>80</td>
<td>264 ± 7</td>
<td>2.2 ± 0.2</td>
<td>0.16 ± 0.03</td>
<td>15 ± 2</td>
<td>0.23 ± 0.02</td>
<td>1.3 ± 0.1</td>
<td>0.14 ± 0.07*</td>
<td>14 ± 1*</td>
</tr>
<tr>
<td>240</td>
<td>252 ± 8</td>
<td>3.2 ± 0.2*</td>
<td>1.9 ± 0.1*</td>
<td>127 ± 41</td>
<td>0.49 ± 0.01*</td>
<td>4.9 ± 0.3</td>
<td>0.26 ± 0.07*</td>
<td>142 ± 31*</td>
</tr>
</tbody>
</table>

Figure 7. (A) Western blot confirms KIM-1 expression in rat kidney after cisplatin treatment (144 hr, 5 mg/kg) in vehicle control and cisplatin-treated animals. n = 5. (B) RT-PCR of KIM-1 mRNA, cyclophilin B (cyclo), cytB1, and GAPDH were used as controls. (C) Western blot of heme oxygenase. (D) Quantitation of heme oxygenase mRNA shows that increased mRNA expression at 4 hr precedes peak of protein expression (24 hr).

Figure 8. In situ hybridization confirms KIM-1 expression is very low in a kidney from a control animal (A) and highly expressed in a cisplatin-exposed kidney (B). Immunohistochemistry shows basal expression of vimentin expression in the kidney of a control rat (C) and induction in an animal treated with cisplatin (D).
Regulatory issues with novel biomarkers

菱 Only solid biomarkers may be used for regulatory decisions on higher level
  ▪ e.g. efficacy, safety, dose selection

菱 New biomarkers need to be validated, at least show significance in more than 1 experiment

- Assay valid?
- Animal model valid?
- Measure for efficacy/toxicity?
- Animal model predictive for human disease and clinical endpoints?
Lung

- **#1: Bronchialveolar Lavage Fluid Analysis**
  - Useful for markers of inflammation
  - Might be too invasive for main study animals; require satellite group

- **#2: Enhanced Histopathology**
  - Primary focus on novel stains, immunohistochemistry (e.g., Ki-67)
  - Might need extra animals depending on what else you plan to do with lungs

- **Other Markers With Promise**
  - Gene Expression Analysis: Probably can’t do all animals, but save (frozen) tissues for possible future analysis if lesions seen; as with other endpoints, might require additional animals depending on how much tissue you need or other uses
  - Imaging: Not quite ready for prime time, but has tremendous promise for the future
Heart

• #1: Troponins
  – Relatively inexpensive, non-invasive (blood), human correlates
  – Primarily an early event, may require early (days) interim sacrifice

• #2: B-type Natriuretic Protein (BNP)
  – More invasive (need RNA)
  – High negative predictivity, less so for a positive response

• #3: Ultrasound imaging
  – Non-invasive, good human correlates
  – Expensive, not high-throughput

• #4: α2-macroglobulin (rat only)
  – Analogous to human C-reactive protein
  – Requires early (48 hr) sample time?
Lipids/Carbohydrates

- **#1: Cholesterol/triglycerides**
  - Widely available, human correlates, most other labs already do this

- **#2: Insulin**
  - Applicable to human, but need rodent specific methods
  - Recommended for routine use, but cost relatively high

- **#3: Reduced Glutathione (GSH)**
  - Good indicator of oxidative stress, low cost, reliable
  - Not specific to lipid disorders

- **#4: Specialized Histopathology**
  - Micro vs. macrovesicular fatty acid change
  - Inexpensive and physiologically meaningful

- Several other endpoints possible, but not for routine use
  - Body composition, hepatic CHO/lipid levels, SREBP-1,2
General Observations

- Many endpoints are most predictive at early timepoints (2-7 days)
  - Will require adding animals for an interim sacrifice group

- Some endpoints are invasive or compromise integrity of other endpoints (e.g., brochialveolar lavage) requiring extra satellite animals

- A number of biomarkers have human analogs already in use in human diagnosis (troponins, insulin, ultrasound, etc.) and these seem like "low hanging fruit" that are worth pursuing

- Some endpoints seem very promising, but will require significant training time, capital investment, etc. (e.g., microimaging, ultrasound, storage facilities for frozen tissues)

- Need to develop a decision tree approach to all categories
  - e.g., for cardiac, routinely do troponin, α2-macroglobulin in the rat only, BNP in conjunction with ultrasound and markers of inflammation and necrosis. If a cardiotoxin suspected, add micro-CT.