BIOMARKERS OF TOXICITY

Why do we need biomarkers?

• *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
Why “enzyme markers”?

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

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
LIVER TOXICITY

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
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

<table>
<thead>
<tr>
<th>Hepatotoxic Procedure</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 ± 21</td>
<td>5.6 ± 2.6</td>
<td>1.1 ± 0.4</td>
<td>282 ± 126</td>
</tr>
<tr>
<td>CCl₄ (1 mg/kg po)</td>
<td>13.0 ± 7</td>
<td>5.3 ± 1.3</td>
<td>0.4 ± 0.2</td>
<td>8510 ± 190</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.
*Values are expressed as mean ± SE; each group contained 10 mice.

Hepatotoxic procedure was performed 36 h before assessing function.

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

ANIT = α-naphthylisothiocyanate
BSP = sulfobromophthalein

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**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

γ-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
LIVER TOXICITY

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

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**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
LIVER TOXICITY

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)

LIVER TOXICITY

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 + α-ketoglutarate $\rightarrow$ 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

C. Enzymes almost exclusively located in liver:

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

- ornithine $\rightarrow$ citrulline

- Is found in liver (>97%) and sm. 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
LIVER TOXICITY

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

LIVER TOXICITY

III. Enzymes relatively insensitive to hepatic injury:
Creatine phosphokinase [CPK]
creatine + ATP $\leftrightarrow$ creatine phosphate + ADP
Greatest activity is found in skeletal muscle
Is used as a marker of muscle injury (clinical use – cardiac muscle injury)
LIVER TOXICITY

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

LIVER TOXICITY

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

<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></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td></td>
<td>X</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>X</td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase (GLDH)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Lactase</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Ornithine carbamyltransferase (OCT)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Succinyl dehydrogenase (SDH)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Total bile acids (TBA)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Total bilirubin (TBIL)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Unconjugated bilirubin (UBIL)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

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

Histopathology vs Clinical Chemistry

![Histopathology vs Clinical Chemistry](image)
Properties which contribute to this protective role:

(a) -SH is a very reactive nucleophilic site

(b) tissue concn generally is relatively high - the normal concn of GSH in liver of various animals is 4-10 mM

(c) multiple forms of glutathione S-transferases which catalyze the reaction of various electrophilic compounds with GSH, thereby neutralizing their electrophilic sites and rendering the products more water-soluble in a (generally) overall detoxication process. 8 transferases can be chromatographically separated from rat liver.

Substrates of S-transferases:
Although each of the S-transferases has some preference for certain substrates, each of the transferases often have overlapping substrate specificities and not rigid specificities.

(d) GSH “can non-enzymatically reduce a number of substances, such as peroxides and free radicals” and a later example of carbonyl compounds with reactive double bonds
At least 2 separate cellular GSH pools:

Cytosol
Mitochondria

TABLE 10.2 Features of Cytosolic Glutathione That Are Important in Protection Against Chemical Toxicity.
- High intracellular concentration (1-8 mM)
- Rapid synthesis by cytosolic enzymes in response to chemical depletion
- GSH, glutathione conjugate, and GSSG transport out of most cell types via plasma membrane transporters
- Very dynamic glutathione redox cycle activity to maintain GSH/GSSG ratio of about 50-100
- Protein thiol/GSH ratio is about 2-4:1
- High concentration of glutathione transferases (about 0.1 mM)

TABLE 10.3 Features of Mitochondrial Glutathione That Are Important in Protection Against Chemical Toxicity.
- Separate physiological pool of glutathione from cytosolic glutathione
- High concentration in the matrix (estimated at 10-12 mM)
- Only extramitochondrial synthesis of glutathione
- GSH transport: lack of GSSG transport in and out mitochondrial inner membrane
- Active glutathione redox cycle that maintains a GSH/GSSG ratio of about 25-50
- Protein thiol/GSH ratio is about 10
- Glutathione transferase activity

INTRACELLULAR GLUTATHIONE HOMEOSTASIS

FIGURE 10.10 Modulation of glutathione and the glutathione redox cycle.
Mechanism-based inhibitor of P450s
Inhibition of heme synthesis
Induction of P450s

**Covalent Reaction of Acetaminophen with Liver Protein**

Glutathione not involved in this table.

**Basis for presenting this table:**
Covalent reaction correlates with tissue toxicity, and with metabolism by Cytchrome P450

**Data for Acetaminophen – Mouse Liver**

<table>
<thead>
<tr>
<th>Preincubation (of animals before acetaminophen)</th>
<th>Dosage of Acetaminophen (mg/kg)</th>
<th>Severity of Hepatic Necrosis after Acetaminophen</th>
<th>Covalently bound ['H]-Acetaminophen Liver (nanomoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>375</td>
<td>1-2+</td>
<td>0.3 (some covalent reaction without observable toxicity)</td>
</tr>
<tr>
<td>Piperoxyl butoxide</td>
<td>375</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>375</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>375</td>
<td>2-4+</td>
<td>1.6</td>
</tr>
<tr>
<td>None</td>
<td>750</td>
<td>3-4+</td>
<td>1.9</td>
</tr>
<tr>
<td>Piperoxyl butoxide</td>
<td>750</td>
<td>0-1+</td>
<td>0.8</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>750</td>
<td>0-1+</td>
<td>0.8</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>750</td>
<td>4-1+</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Correlation between severity of hepatotoxicity and the extent of covalent binding by the arylating metabolite of acetaminophen.

From the data in this Table, one can clearly establish a correlation – critically not necessarily a cause and effect – but a correlation between the extent of covalent reaction with protein and the cellular necrosis.

Two types of experiments:
At any dosage, 2-15% of acetaminophen administered to animals is transformed by the cytochrome P-450 dependent MFO to a chemically reactive metabolite. At low - or therapeutic – dosages, virtually all of the reactive metabolite is converted to a glutathione conjugate that is ultimately excreted as a mercapturic acid derivative. But at high dosages of the drug, the GSH in liver is decreased to such an extent that the reactive metabolite can no longer be completely inactivated by GSH and a portion of the metabolite becomes covalently bound to liver proteins.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic glutathione concentration (mg/g liver)</th>
<th>Serum alanine aminotransferase activity (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77 ± 0.11</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>Acetaminophen alone</td>
<td>0.61 ± 0.09</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>Acetaminophen + 200 mg/kg phenytoinamide</td>
<td>0.61 ± 0.09*</td>
<td>281 ± 225</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
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<td></td>
<td>2.77 ± 0.11</td>
<td>34 ± 4</td>
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<td>0.61 ± 0.09*</td>
<td>281 ± 225</td>
</tr>
</tbody>
</table>

Note: Phenytoinamide was administered 3 hr before acetaminophen. For each treatment, one group of mice (N = 8) was euthanized at 2 hr postacetaminophen for measurement of hepatic glutathione concentrations and a second group (N = 8) was euthanized at 26 hr for measurement of serum ALT activity. Results are expressed as mean ± SD. Values in parentheses are percentages of the mean hepatic glutathione concentration in saline-treated control.

* Significantly different from saline-treated control (p < 0.05).

+ James et al. Toxicol Appl Pharmacol. 1993 Feb;118(2):159-68
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

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**TABLE 2**

Influence of Hepatic Glutathione Depleters on Acetaminophen Hepatotoxicity

<table>
<thead>
<tr>
<th>Animal treatment regimen</th>
<th>Nonacetaminic glutathione depleters</th>
<th>N-acetylcysteine (222 mg/kg)</th>
<th>Dextromethorphan (125 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH depletor</td>
<td>40 ± 3</td>
<td>28 ± 3</td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>40 ± 3</td>
<td>40 ± 3</td>
<td></td>
</tr>
<tr>
<td>GSH depletor + APAP</td>
<td>328 ± 56*</td>
<td>839 ± 619*</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH depletor</td>
<td>53 ± 3</td>
<td>53 ± 4</td>
<td></td>
</tr>
<tr>
<td>APAP</td>
<td>80 ± 20</td>
<td>80 ± 20</td>
<td></td>
</tr>
<tr>
<td>GSH depletor + APAP</td>
<td>3125 ± 986*</td>
<td>6777 ± 2773*</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were administered the glutathione depletor alone, acetaminophen (APAP 400 mg/kg, ip) alone, or glutathione depletor. The prior to administration of acetaminophen. Serum ALT activities were measured 24 hr following the APAP dose and are expressed as the mean ± SEM, N = 6 ± 0.

** Control 34±4**

The serum ALT value was significantly different from that for mice not receiving glutathione depletor pretreatment. p < 0.05. In both experiments the results of the glutathione depletor groups were not significantly different from each other.

James et al. Toxicol Appl Pharmacol. 1993 Feb;118(2):159-68
**KIDNEY TOXICITY**

**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

---

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Kidney/body wt ( \times 10^2 )</th>
<th>Creatinine (( \mu \text{mol/liter} ))</th>
<th>Urea (( \text{mmol/liter} ))</th>
<th>Kidney necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54 ± 8</td>
<td>7.6 ± 1.7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>DBCP</td>
<td>0.84 ± 0.03</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(42.5 ( \mu \text{mol/kg} ))</td>
<td>64 ± 3</td>
<td>6.0 ± 0.5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(85 ( \mu \text{mol/kg} ))</td>
<td>0.90 ± 0.14</td>
<td>58 ± 20</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>(170 ( \mu \text{mol/kg} ))</td>
<td>1.08 ± 0.15</td>
<td>150 ± 49</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>(340 ( \mu \text{mol/kg} ))</td>
<td>1.05 ± 0.05</td>
<td>168 ± 47</td>
<td>3.3 ± 0.4*</td>
<td>0</td>
</tr>
<tr>
<td>( \text{C}<em>2 \text{-D}</em>{12}-\text{DBC} )</td>
<td>0.89 ± 0.01</td>
<td>39 ± 3</td>
<td>4.6 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>(42.5 ( \mu \text{mol/kg} ))</td>
<td>0.90 ± 0.05</td>
<td>48 ± 7</td>
<td>5.6 ± 0.9</td>
<td>1</td>
</tr>
<tr>
<td>(170 ( \mu \text{mol/kg} ))</td>
<td>1.13 ± 0.12*</td>
<td>210 ± 147*</td>
<td>32.8 ± 20.9*</td>
<td>0</td>
</tr>
<tr>
<td>(340 ( \mu \text{mol/kg} ))</td>
<td>1.69 ± 0.09*</td>
<td>240 ± 154*</td>
<td>51.1 ± 34.8*</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Five rats in each group were dosed with DBCP or \( \text{D}_2-\text{DBC} \) (42.5–340 \( \mu \text{mol/kg} \)). 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 (\( p < 0.05 \)).
KIDNEY TOXICITY: Examples

**Halogenated alkanes and alkenes**

- GSH S-transferase
- Glutathione conjugates
- Concentrated in kidney by renal transport system
- Cysteine conjugates
- Cysteine S-conjugate β-lyase
- Reactive electrophiles (thioacetylating metabolites)
- Kidney

**Glutathione conjugates**

- GGT
- Cysteine conjugates
- Liver

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**KIDNEY TOXICITY: Examples**


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

*Spyridon Vamvakas, Elisabeth Kremer and Wolfgang Duran*

| Table 1. Toxicity of 1,1,2-trichloro-3,3,3-trifluoro-1-propene (TCIFP) in rats |
|-----------------|-----------------|-----------------|
| Parameter       | Control         | TCIFP (25 mg/kg)| TCIFP (50 mg/kg) |
| Plasma urea (mg/dl) | 38 ± 4          | 85 ± 22**       | 143 ± 32**        |
| Plasma ASAT (U/l)| 68 ± 16         | 34 ± 17         | 70 ± 8            |
| Plasma ALT (U/l) | 16 ± 3          | 11 ± 4          | 10 ± 3            |
| Urine glucose (mg/24 hr) | 2 ± 0.6       | 85 ± 31**       | 125 ± 27**        |
| Urine protein (mg/24 hr) | 5 ± 1          | 30 ± 13**       | 65 ± 14**         |
| Urine GGT (U/24 hr) | 3540 ± 783     | 22490 ± 1370**  | 39470 ± 2170**    |
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 puroycin treatment. *

<table>
<thead>
<tr>
<th>Cisplatin Group</th>
<th>Body weight (g)</th>
<th>Serum Cr (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>Glucose/Cr</th>
<th>Protein excretion (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>270 ± 7</td>
<td>0.30 ± 0.01</td>
<td>15 ± 1</td>
<td>0.23 ± 0.01</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>289 ± 6</td>
<td>0.24 ± 0.02</td>
<td>14 ± 1</td>
<td>0.17 ± 0.06</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>299 ± 9</td>
<td>0.27 ± 0.04</td>
<td>13 ± 1</td>
<td>0.15 ± 0.04</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>254 ± 2</td>
<td>1.4 ± 0.4*</td>
<td>83 ± 20*</td>
<td>0.1 ± 0.5*</td>
<td>0.25 ± 0.5*</td>
</tr>
</tbody>
</table>

- Table 1. Changes in serum and urine chemistry and organ and body weights 7 days after cisplatin and gentamicin treatment and 21 days after puroycin treatment. *}

Figure 7. (A) Western blot confirms KIM-1 expression in rat kidney after cisplatin treatment (164 kDa). (B) Schematic representation of cisplatin and gentamicin treatment. Environ Health Perspect 112:465-479 (2004)
Regulatory issues with novel biomarkers

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

June 12, 2008
FDA, European Medicines Agency to Consider Additional Test Results When Assessing New Drug Safety

Collaborative effort by FDA and EMEA expected to yield additional safety data
In the first use of a framework allowing submission of a single application to the two agencies, the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) worked together to allow drug companies to submit the results of seven new tests that evaluate kidney damage during animal studies of new drugs. The tests measure the levels of seven key proteins or “biomarkers” found in urine that can provide additional information about drug-induced damage to kidney cells, also known as renal toxicity.

The new biomarkers are KIM-1, Albumin, Total Protein, β2-microglobulin, Cystatin C, Clusterin, and Trefoil Factor-3. For decades, both FDA and EMEA have required drug companies to submit the results of two blood tests, called blood urea nitrogen (BUN) and serum creatinine, to evaluate renal toxicity. In addition to those tests, the FDA and EMEA will now consider results from the seven new tests as part of their respective drug review processes. Although a decision by the sponsor to collect information using the new tests is voluntary, if collected, it must be submitted to FDA.

FDA scientists believe that the seven new tests may provide important advantages over the BUN and creatinine tests. For example, in experiments using rats, the two traditional tests can only detect kidney damage a week after it has begun to occur. The new tests, however, are more sensitive and can detect cellular damage within hours. And while BUN and serum creatinine show that damage has occurred somewhere in the kidneys, the new tests can pinpoint which parts of the kidney have been affected.

KIM-1
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., bronchoalveolar 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.