Mechanisms of Acute Toxicity

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The Dow Chemical Company
Acute Tox Workshop
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Acknowledgements

• Barun Bhhatarai
• Ed Carney
• Amanda Parks
• Paul Price
Pop quiz: Put these in order of lethality

- Which substance has **lowest** lethal dose? (i.e. the most ‘toxic’)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Toxicity ranking</th>
<th>LD50 (/kg bw)</th>
<th>GHS Cat</th>
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<tbody>
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</tr>
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<td>?</td>
<td>?</td>
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</tr>
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<td>Ethanol</td>
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<tr>
<td>Caffeine</td>
<td>4</td>
<td>130-320 mg</td>
<td>3</td>
</tr>
<tr>
<td>Arsenic</td>
<td>3</td>
<td>46 mg</td>
<td>2</td>
</tr>
<tr>
<td>Aspirin</td>
<td>5</td>
<td>1000 mg</td>
<td>4</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>6</td>
<td>3000 mg</td>
<td>5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7</td>
<td>14000 mg</td>
<td>5</td>
</tr>
<tr>
<td>Nicotine</td>
<td>2</td>
<td>1 mg</td>
<td>1</td>
</tr>
<tr>
<td>Botulism toxin</td>
<td>1</td>
<td>0.02 ng</td>
<td>1</td>
</tr>
</tbody>
</table>
# Acute classification categories

<table>
<thead>
<tr>
<th>Regulatory Agency (Authorizing Act)</th>
<th>Animals</th>
<th>Endpoint</th>
<th>Classification</th>
</tr>
</thead>
</table>
| **EPA (FIFRA)**                    | Use current EPA or OECD protocol | Death\(^1\) | I - $LD_{50} \leq 50 \text{ mg/kg}$  
II - $50 < LD_{50} \leq 500 \text{ mg/kg}$  
III - $500 < LD_{50} \leq 5000 \text{ mg/kg}$  
IV - $LD_{50} > 5000 \text{ mg/kg}$ |
| **CPSC (Federal Hazardous Substances Act)** | White rats, 200-300 g | Death\(^1\) within 14 days for $\geq$ half of a group of $\geq 10$ animals | Highly toxic - $LD_{50} \leq 50 \text{ mg/kg}$  
Toxic - $50 \text{ mg/kg} < LD_{50} < 5 \text{ g/kg}$ |
| **OSHA (Occupational Safety and Health Act)** | Albino rats, 200-300 g | Death\(^1\), duration not specified. | Highly toxic - $LD_{50} \leq 50 \text{ mg/kg}$  
Toxic - $50 < LD_{50} \leq 500 \text{ mg/kg}$ |
| **DOT (Federal Hazardous Material Transportation Act)** | Male and female young adult albino rats | Death\(^1\) within 14 days of half the animals tested. Number of animals tested must be sufficient for statistically valid results. | Packing Group 1 - $LD_{50} \leq 5 \text{ mg/kg}$  
Packing Group II - $5 < LD_{50} \leq 50 \text{ mg/kg}$  
Packing Group III - $LD_{50} < 500 \text{ mg/kg}$ (liquid)  
$LD_{50} < 200 \text{ mg/kg}$ (solid) |
| **OECD Guidance for Use of GHS (2001b)** | Protocols not specified | Not specified | I - $LD_{50} \leq 5 \text{ mg/kg}$  
II - $5 < LD_{50} \leq 50 \text{ mg/kg}$  
III - $50 < LD_{50} \leq 300 \text{ mg/kg}$  
IV - $300 < LD_{50} \leq 2000 \text{ mg/kg}$  
V - $2000 < LD_{50} \leq 5000 \text{ mg/kg}$  
Unclassified - $LD_{50} > 5000 \text{ mg/kg}$ |

Abbreviations:  
EPA=U.S. Environmental Protection Agency; OECD=Organisation for Economic Co-operation and Development; LD\(_{50}\)=Dose producing death in 50% of the animals tested; CPSC=U.S. Consumer Product Safety Commission; FIFRA=Federal Insecticide, Fungicide, and Rodenticide Act; OSHA=U.S. Occupational Safety and Health Administration; DOT=U.S. Department of Transportation; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
Requirement is to classify by LD/LC$_{50}$

- This can be done using guideline animal studies
- Do in vitro alternative methods offer a replacement?
- Do in silico alternative methods offer a replacement?
In silico approaches - QSAR

QSAR Tools
- TOPKAT
- ChemBench
- DEREK
- EPA-TEST
- OASIS-beta
- Terra-QSAR
- ACD/ToxSuite
- ADMET Predictor

Global QSAR models not as applicable for compounds acting via highly specific mechanisms
Why study acute mechanisms?

- In vitro and in silico approaches not yet a total replacement
- May direct in vitro HTS assays and enable building of QSARs
- The ‘future’ is mechanism- (AOP-) based
- Better enable read-across
- Focus on identify compounds of high inherent toxicity
- Important in poisoning cases
- Acute mechanism *in scope* for repeat-dose studies
- Understand if animal data relevant to humans
- Understanding *mechanisms* makes us better toxicologists and better able to interpret and troubleshoot studies
Challenges of identifying acute MOAs

- A workshop like *THIS* has never been held...
- Not a guideline study requirement
- Study doesn’t include organ weights, histopath or clin path
- DBs of LD/LC50 values don’t contain other mechanistic info
- Studies often conducted at CROs blinded to TM identity
- Specific mechanisms rarely examined
- Relationship of mechanistic effect to apical effect not clear
- Risk assessors didn’t consider acute toxicity ‘sexy’ – rare focus
- Mechanistic in vitro HTS assays may only look above cytotoxicity noise level yet the MOA may drive cytotoxicity
Facts about acute data

• Distribution of GHS classification not evenly distributed for oral route - most compounds are GHS 4-5

<table>
<thead>
<tr>
<th></th>
<th>GHS 1</th>
<th>GHS 2</th>
<th>GHS 3</th>
<th>GHS 4</th>
<th>GHS 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daphnia</td>
<td>498</td>
<td>456</td>
<td>970</td>
<td>484</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>640</td>
<td>565</td>
<td>830</td>
<td>580</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>311</td>
<td>828</td>
<td>1885</td>
<td>5189</td>
<td>3284</td>
</tr>
</tbody>
</table>

• Provides information on inherent toxicity

• IV Data
  – Compounds average 40x more toxic by iv than oral route
  – Sometimes it’s the only data you have, especially for highly insoluble compounds
  – Compounds that pass limit dose orally may cause lethality in seconds intravenously
  – Directly applicable for medical devices
Ways to identify potential mechanisms

• Determine whether ‘reactive’ or ‘pharmacologic’
• 3-D crystalline protein structure mapping
• HT Gene expression data-mining
• Identify protein targets using wet-lab binding interactions
• Examine pathology and clinical pathology data
• Consider *Time-to-death*
• Examine relationship of acute toxicity to HTS data results
• Similarity to compounds with known mechanisms
• Years of experience resulting in a logical ‘hunch’
• Use systems biology approach
• Focus on critical targets for high acute toxicity
Chemical reactivity

- Electrophilicity
- Hardness (HOMO/LUMO)
- Acylation
- Schiff base formation
- Michael addition reaction
- SN1 mechanism
- SN2 mechanism
- SNAr mechanism
- Polarizability

- Molecular wt
- Protein/DNA binding
- Substructures
- Solubility
- pKa
- Log KoW
Systems biology approach

Organism

Organs
Heart, Liver, Kidney, Nervous System...

Cells
Muscle, Cardiac, Hepatic, RBCs, Neurons, PMNs...

Organelles
Nucleus, Mitochondria, Peroxisome, Cytoplasm...

Biochemical Pathways
Gluconeogenesis, Pentose phosphate pathway, Kreb’s cycle, Electron transport, Fatty acid metabolism, Lipolysis, Lipogenesis, Pyrimidine/Purine biosynthesis, Urea cycle, Glycolysis, Vitamins...
Some mechanisms of acute toxicity

- Inhibit energy production
- Antimetabolites
- Anticoagulants
- Chelants
- Inhibit signal transduction
- Ion channel blockers
- Inhibit Na+/K+ ATPase
- Protein synthesis inhibitors
- Non-specific high chemical reactivity
- Physico-chemical properties
  - Acids, Bases
  - Surfactants
  - Accept protons and uncouple mitochondrial during diffusion
Metabolism - Bioavailability

- Physical
  - Mucous
  - Chewing
  - Mixing/churning
  - Acid
  - Emulsification
- Hormones
- Enzymes
Protein Digestion

• Stomach
  – HCl denatures
  – Pepsinogen $\rightarrow$ pepsin

• Small Intestine
  – Hormones
    • Cholecystokinin
    • Secretin
  – Pancreatic enzymes
    • Trypsin, peptidases, elastase

• Amino acids $\uparrow$ insulin, $\downarrow$ glucagon

• No storage form for protein
  – amino acids $\rightarrow$ protein; carbons $\rightarrow$ carbohydrate/lipid; amino “N” as urea
Carbohydrate Digestion

- Starch: glucose polymer $\alpha(1\rightarrow4)$ glycosidic bonds
  - Amylose
    - linear, 100’s glucose units
  - Amylopectin
    - branched, 1000’s units
    - linear $\alpha(1\rightarrow4)$
    - branch $\alpha(1\rightarrow6)$ each 24-30 units
  - Glycogen
    - branch each 8-12 units
- Pancreatic amylase breaks $\alpha$-1,4-bonds
Lipid Digestion

- **Stomach**
  - *Lingual and Gastric Lipase*

- **Small intestine**
  - Cystokinin → gallbladder
    - ↓ gastric motility
  - Secretin → pancreas
    - bicarbonate neutralizes pH
  - Emulsification → Bile saltss
  - *Pancreatic Lipase* → FA at C1 and C3
  - *Colipase* → stabilizes *Lipase*
  - *Cholesteryl ester hydrolase*
  - *Phospholipase A2* → FA at C2
  - *Lysophospholipase* → C2
Potentially labile subfragments

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Structures</th>
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<tr>
<td>Acyl Halides</td>
<td><img src="image1" alt="Structure" /></td>
<td>Aldoxime</td>
<td><img src="image2" alt="Structure" /></td>
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<tr>
<td>O-Alkylloxime</td>
<td><img src="image3" alt="Structure" /></td>
<td>Amides</td>
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<tr>
<td>Anhydride</td>
<td><img src="image5" alt="Structure" /></td>
<td>Carboximidate</td>
<td><img src="image6" alt="Structure" /></td>
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<tr>
<td>Ester</td>
<td><img src="image7" alt="Structure" /></td>
<td>Ether</td>
<td><img src="image8" alt="Structure" /></td>
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<tr>
<td>Hydrazone</td>
<td><img src="image9" alt="Structure" /></td>
<td>Imine-Hydrazone</td>
<td><img src="image10" alt="Structure" /></td>
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<tr>
<td>Imides</td>
<td><img src="image11" alt="Structure" /></td>
<td>Imine</td>
<td><img src="image12" alt="Structure" /></td>
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<tr>
<td>Ketoxime</td>
<td><img src="image13" alt="Structure" /></td>
<td>Sulphonamide</td>
<td><img src="image14" alt="Structure" /></td>
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<tr>
<td>Phosphoramide</td>
<td><img src="image15" alt="Structure" /></td>
<td>Organophosphates</td>
<td><img src="image16" alt="Structure" /></td>
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(thiophosphates, etc)
Modeling Systemic Bioavailability

Simulations Plus
Energy production

- **Adenosine Triphosphate (ATP)** used for most energy requiring reactions (e.g., active transport). Isn’t stored, consumption closely follows synthesis
- 1 kg created-recycled/hr. A cell uses 10 million ATP molecules/sec and recycles all of its ATP every 20-30 sec
- **Guanosine Triphosphate (GTP)** equivalent to ATP in energy content and preferentially used in some cellular reactions
- **Flavin Adenine Dinucleotide (FAD)** a cofactor reduced to FADH2, an energy-rich molecule
- **Nicotinamide Adenine Dinucleotide (NADH)**: a cofactor; reducing potential converted to ATP through the electron transport chain
- **Nicotinamide adenine dinucleotide phosphate (NADPH)** is used in fatty acid and nucleic acid synthesis
- **Phosphocreatine** is used to replenish ATP from creatine and ADP
Glycogen
Glucose 1-P
- Glucose 6-P
Fructose 6-F
Fructose 1,6-Bis P
Glyceraldehyde 3-P
- bisPhosphoglycerate
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate
Pyruvate
Acetyl CoA

Glycolysis
Pyruvate Dehydrogenase Complex

Thiamin coenzyme
Lipoic acid coenzyme
Coenzyme A-SH
Flavin coenzyme
NAD+

CO₂
Thiamin coenzyme
Lipoic acid coenzyme
Flavin coenzyme
NADH + H⁺

Arsenic inhibits

Pyruvate

Pyruvate Dehydrogenase Complex

Acetyl CoA

Mitochondria
Lipoic Acid: Cofactor for 2nd Enzyme

Hydroxyethyl derivative bound to reactive carbon of TPP

Lipoic acid

Arsenic inhibits

Coenzyme A-SH

Acetyl CoA

Reduced Lipoic acid

Dihydrolipoyl transacetylase
Niacin – Vitamin B3

Nicotinic acid

Nicotinamide

Tryptophan

Nicotinic acid

Nicotinamide

Tryptophan

NAD^+ NADP^+

NAD^+ NADH

: H- Hydride ion

NADH

R
Riboflavin – Vit B2

FAD (oxidized quinone form)  \[\xrightarrow{2H^+, 2e^-}\]  FADH\(_2\) (reduced hydroquinone form)

FMN and FAD tightly bound to enzymes that catalyze oxidation or reduction
Fish acute toxicity vs. ToxCast HTS

*627 of 1853 ToxCast II chemicals had fish acute tox data*
Mitochondrial Electron Transport

Intermembrane space
Matrix

ATP Synthase

H^+ H^+ H^+ H^+

ADP → ATP

H_2O → O_2

FADH2 → NADH

TCA Cycle

Succinate

IV III II I
Mitochondria membrane potential assay

Mitochondrial tox predicts upper boundary to Daphnia toxicity

AC$_{50}$ Inhibition of Mitochondrial Membrane Potential (uM)

Daphnia acute LC$_{50}$ (mg/l)
Mitochondrial toxicity predicts upper bound to acute intravenous toxicity
Mitochondrial toxicity doesn’t predict upper bound to oral rat toxicity
LD_{50} values adjusted downward by % bioavailable
Antimetabolites

Dihydrofolate reductase 2-steps requiring 2 NADPH

Purines

N^{10}-Formyl-THF  N^{5}N^{10}-Methenyl-THF  N^{5}N^{10}-Methylene-THF  N^{5}-Methyl-THF

Tetrahydrofolate (THF)

Methotrexate
LD50 135 mg/kg

Methionine

NADPH + H+

NADP+

NADH + H+

NAD+

Methotrexate
Purine synthesis

FOLIC ACID ANALOGS
Methotrexate etc inhibit reduction of dihydrofolate to THF; ↓ DNA replication in cancer and normal cells
Pyrimidine Biosynthesis

Base synthesized then added to preformed ribose
Anticoagulants

- Cofactor of enzyme that carboxylates γ-glutamyls in Prothrombin and Factors VII, IX and X
- Without carboxylation, don’t bind membrane phospholipids
- Deficiency in infants - hemorrhagic disease of the newborn
- Natural K vitamins free of toxic side effects

Warfarin is structural analog of Vit K
LD50 8.7 mg/kg
Chelators

- 104mg/kg rat iv
- 280 ug/kg rat iv
Signal transduction

Dopamine
Epinephrine
Norepinephrine
Serotonin
Histamine
Acetylcholine
Glutamic Acid
Aspartic Acid
GABA
Glycine
Tyrosine
Adenosine
Tetrodotoxin inhibits voltage-gated sodium channels. Oral LD50 is 334 μg/kg.
Cardiac glycosides (Inh Na+/K+ ATPase)

28.3 mg/kg rat LD50 oral

10.8 mg/kg rat LD50 iv
Michael acceptors

Acrolein
LD50 26 mg/kg
Acids

- Trifluoromethanesulfonic acid
- pH of 10% solution = 0.1
- Acute oral LD50: 1605.3 mg/kg bw
  - GHS Cat 4; H302: Harmful if swallowed
- Acute dermal LD50: > 50 mg/kg bw
  - test results inconclusive because of severe local effects on skin at 2000 mg/kg bw
- Acute inhalation LC50: ????
  - study scientifically unjustified

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\end{tikzpicture}}\]
Future mechanistic approaches...

- Phenotypic readouts
  - Cytotoxicity
  - Apoptosis: caspase 3/7, 8, 9
  - Membrane integrity: LDH, protease release
  - Mitochondrial toxicity (membrane potential)
  - Gene tox: p53, ELG1, DNA damage gene deficient lines (DT40 lines and mouse)

- Cell Signaling
  - Stress response: ARE, ESRE, HSP, Hypoxia, AP-1
  - Immune response: IL-8, TNFα, TTP
  - Other: AP-1, CRE, ERK, HRE, JNK3, NFκB, LDR

- Drug metabolism
  - CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4

- Target specific assays
  - Nuclear receptors: AR, AhR, ERα, FXR, GR, LXR, PPARα, PPARδ, PPARγ, PXR, RXR, TRβ, VDR, RORα, RORγ
  - hERG channel
  - Isolated molecular targets: 12hLO, 15hLO1, 15hLO2, ALDH1A1, HADH560, HPGD, HSD17b4, α-Glucosidase, α-Galactosidase, Glucocerebrosidase, APE1, TDP1, DNA polymerase III, RECQ1 helicase, RGS4, BRCA, IMPase, O-Glc NAc Transferase, Caspase-17, CBFB-RUNX1, PK, Tau, Cruzin, β-Lactamase, PRX, YjeE, NPS, Proteasome, SF1, SMN2, beta-globin splicing, Anthrax Lethal Factor, TSHR

- Genetic variation: 87 HapMap lines

New Robot Can Test 10,000 Chemicals
Questions?