

MoA-Based Selection of Reference Compounds

General Selection Criteria

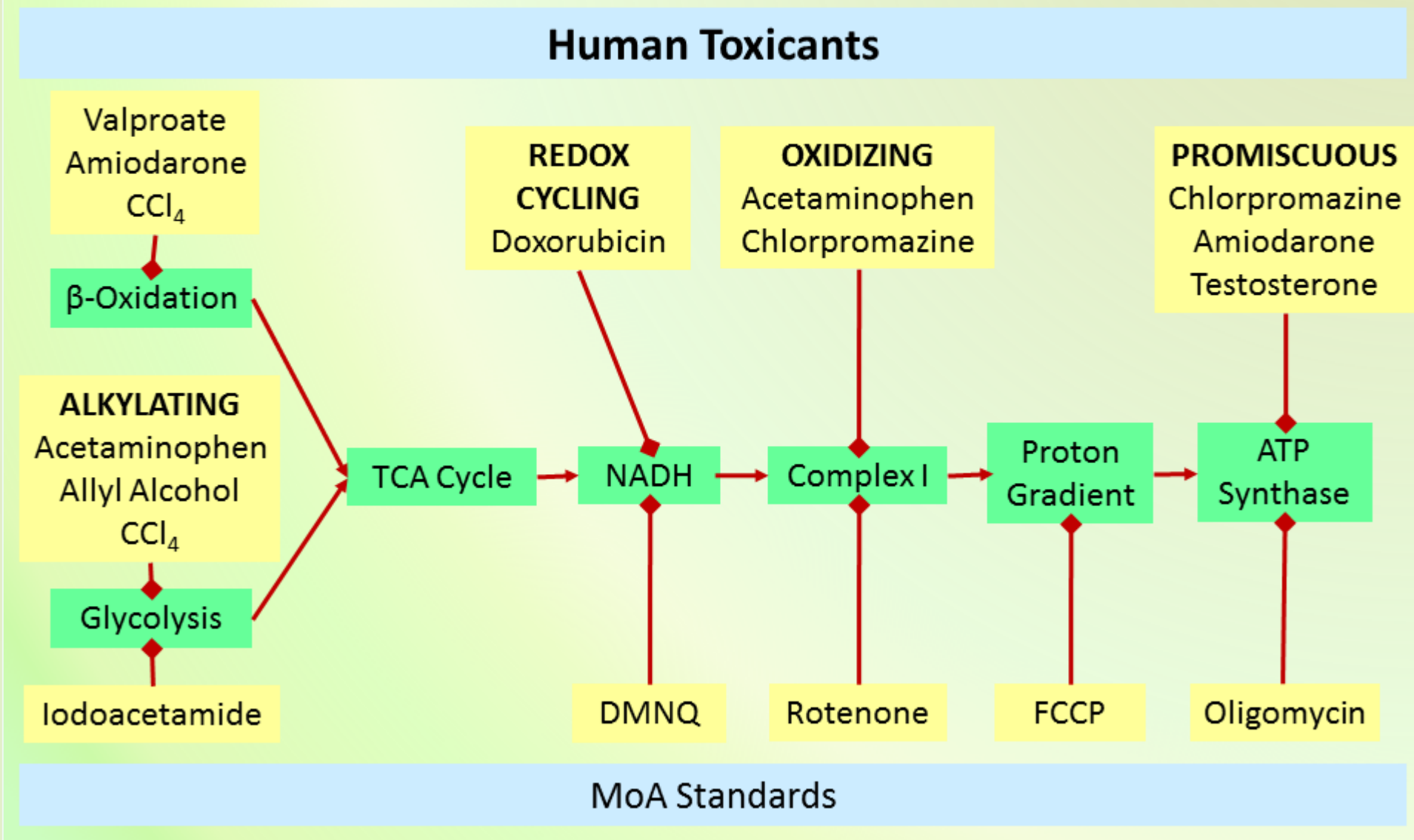
- SEURAT-1 goals [SEURAT-1 annual report, 2011]
 - Test systems for repeated dose systemic toxicity
 - Toxicological mode-of-action (MoA) framework
 - Applicable to any substance
- General and physical properties:
 - Defined, confirmed structure and isomeric form
 - Non-volatile and stable to storage, light, freeze-thaw
 - Soluble in buffer at 30 times the *in vitro* IC₅₀ for toxicity
 - Solubility in DMSO 100x buffer solubility, low binding to plasticware
 - Available commercially at >95% purity (>99% preferred)
 - Gene expression, proteomics, metabonomics/fluxomics, and/or epigenomics profiles known
 - Bioactivated (hepatotoxins - desirable, not required)

Reactive and Promiscuous Compounds

Prototypical Human Toxicant	MoA
Acetaminophen	Thiol alkylation, oxidation
Doxorubicin	Redox cycling
Allyl alcohol	Thiol alkylation
Carbon tetrachloride	Thiol alkylation, oxidation, protein adducts, DNA adducts
Aflatoxin B1	Lysine alkylation, DNA adducts
Chlorpromazine	Promiscuous, thiol alkylation, oxidation
Valproate	Promiscuous
Amiodarone	Promiscuous
Tamoxifen	Promiscuous

- Reactive compounds:
 - Alkylating agents (e.g. quinones, Michael acceptors)
 - Oxidizing agents (e.g. high potential quinones)
 - Redox cycling agents (e.g. low potential quinones)
 - Free radical carriers
- Promiscuous compounds: hydrophobic, non-selective interactions with multiple targets

Reactive and Promiscuous Molecules in Energy Metabolism



A superset of potential hepatotoxin standards were initially proposed based on their association with the adverse events of cytotoxicity, fibrosis, steatosis, cholestasis, and phospholipidosis. A subset of compounds was subsequently selected from the parent set based on the diversity and generality of their MoAs. These included compounds with alkylating, redox, and free radical reactivities and a group of compounds classified as promiscuous. Promiscuity is a concept derived from the characterization of high throughput screening hits and refers to a lack of selectivity, which is commonly associated with hydrophobic compounds. It is likely that disruption of membrane function contributes to the activities of this group.

In addition to the hepatotoxins, doxorubicin was selected as an archetypical reactive compound for developing assays of repeated dose cardiotoxicity.

Loss of reduction potential, disruption of mitochondrial membrane gradients, and inhibition of ATP formation are commonly cited as causes of cytotoxicity. This figure recasts these effects in terms of the energy metabolism pathway and illustrates the point that specific types of molecular reactivity can be associated with discrete points in this pathway. The thiol of glyceraldehyde phosphate dehydrogenase is highly reactive, for example, and production of ATP by glycolysis is therefore sensitive to alkylating agents. Similarly, DT diaphorase catalyzes the oxidation of NADH by redox cycling quinones and will deplete the cellular reduction potential, turning on redox-sensing receptors such as NRF2. Strongly oxidizing quinones such as NAPQI block the entry of reducing equivalents into the electron transfer chain at complex I and deplete the mitochondrial membrane potential. Finally ATP synthase is a common target of toxicants with promiscuous activity, presumably reflecting a sensitivity of this enzyme to membrane disruption.

Many toxicants have multiple potential MoAs, whereas an MoA-based approach to prediction of toxicity must rely on an understanding of discrete MoAs. Therefore we have identified additional compounds that are selective for each of the key points of interaction illustrated in the figure so that we can profile these more discrete MoAs. Comparison of these to profiles for less selective toxicants will help us understand which MoAs are dominant causes of toxicity for the more complex compounds – or perhaps reveal that the dominant MoA is not reflected in the pathways of energy metabolism.

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Lipid Accumulation Without Chemical Reactivity

Compound	Pharmacology	Toxicity Target	Toxicity
Bosentan	Endothelin antagonist	Bile salt export pump	Cholestasis
Dilrotapide	Intestinal microsomal phospholipid transfer inhibitor	Hepatic microsomal phospholipid transfer protein	Steatosis
Fluoxetine	Serotonin reuptake inhibitor	Phospholipid binding	Phospholipidosis

Since adverse events of lipid accumulation are commonly associated with reactive/promiscuous hepatotoxins, these events normally co-occur with other MoAs of cytotoxicity. It is not clear, therefore, to what extent these disorders are sources of toxicity or are in themselves benign. Therefore, a set of selective inhibitors of lipid transport is proposed as MoA standards that are devoid, to the best of our knowledge, of additional reactivities. These compounds may be employed to determine the effects of long-term lipid accumulation, in itself, on cellular function.

Summary of Compounds Selected

Compound	Organ	MoA	Adverse event
Reactive Compounds			
Acetaminophen	Liver	Thiol reagent, oxidizing agent	Necrosis
Doxorubicin	Heart	Redox cycling, DNA oxidation	Heart failure
Allyl alcohol	Liver	Thiol reagent	Fibrosis
Carbon tetrachloride	Liver	Free radical	Fibrosis, steatosis
Aflatoxin B1	Liver	Lysine reagent	Apoptosis
Chlorpromazine	Liver	Thiol reagent, oxidizing agent, free radical, lipid binding	Cholestasis, hepatitis
Iodoacetamide	Multiple	Thiol reagent	(MoA standard)
DMNQ	Multiple	Redox cycling	(MoA standard)
Promiscuous Ligands and Receptors			
Sodium valproate	Liver	Inhibition of multiple pathways, including β-oxidation	Steatosis, necrosis
Amiodarone	Liver	Phospholipid binding	Steatosis, necrosis, phospholipidosis
E 4031	Heart	hERG channel blocker	Arrhythmias
Tamoxifen (tentative)	Liver	Epigenetic modification (primary MoA of interest)	Steatosis
MoA Standards for Oxidative Phosphorylation			
Rotenone	Multiple	Complex I (electron transport)	(MoA standard)
Oligomycin	Multiple	ATP synthase inhibitor	(MoA standard)
FCCP	Multiple	Proton gradient uncoupler	(MoA standard)
MoA Standards for Lipid Metabolism			
Bosentan	Liver	BESP inhibition	Cholestasis
Dilrotapide	Liver	MTP inhibition	Steatosis
Fluoxetine	Liver	Phospholipid binding	Phospholipidosis
Non-MoA Based Selections			
Methotrexate	Multiple	Anti-metabolite	Hepatic fibrosis
Carbachol	Heart	Cholinergic agonist	(cell line characterization)
Isoproterenol	Heart	Adrenergic agonist	(cell line characterization)
Nifedipine	Heart	L-type Ca channel blocker	(cell line characterization)
Hygromycin B	Multiple	Protein synthesis inhibitor	(electron microscopy standard)

Nuclear Hormone Receptors

(Evaluation in Progress)

NHR Promiscuity: ToxCast Screen (2010)

Receptor	Hit Rate	Biological Pathways
PXR	76%	induction of metabolizing enzymes
AhR	17%	induction of metabolizing enzymes
CAR	1%	induction of metabolizing enzymes
NRF2	53%	response to ox. stress
HIF1α	8%	hypoxia and angiogenesis
HSE	7%	stress response, heat shock
PPARα	3%	lipid metabolism/glucose homeostasis
PPARγ	47%	lipid metabolism/glucose homeostasis
LXRα	7%	cholesterol homeostasis
LXRβ	7%	cholesterol homeostasis
FXR	<0.3%	bile acid homeostasis
RARα	16%	regulation of GSH
RXRβ	<0.3%	lipid/xenobiotic homeostasis/metabolism
ERα	29%	endocrine
AR	<0.3%	endocrine

*PPARγ and ERα activation linked to NRF2 activation

- Selection criteria:
 - Promiscuous receptor
 - Non-carcinogenic, non-mutagenic toxicity
 - Toxicity not due to increased reactive metabolites
- Candidate systems
 - Tamoxifen-ER (epigenetics)
 - AhR (chronic activation)
 - Conazoles-CAR/PXR (liver hypertrophy)
 - GW3965-LXR (steatosis)

In addition to promiscuous ligands, there are also promiscuous receptors that are relatively non-selective in ligand binding. The hERG ion channel is an archetypical example and is included as a target for cardiotoxicity standards. Of the major protein classes with cellular regulatory activity, the nuclear hormone receptors are considered relatively promiscuous as a class. This promiscuity is being assessed quantitatively in the ToxCast program of the EPA and representative results from this project are shown in the figure. Analysis of this protein class for inclusion as reference standards is currently underway, and the three systems that appear to be most relevant at this time are listed.