Selection Of Reference Chemicals To Validate *In Vitro* Cytotoxicity Assays For Estimating In Vivo Starting Doses For Acute Oral Toxicity

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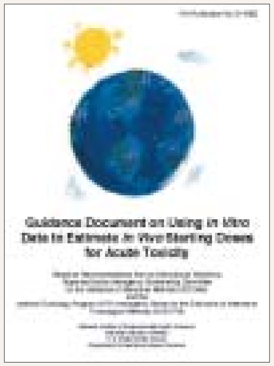
Introduction



Acute oral toxicity testing is one of the initial steps used to identify and characterize the potential hazards associated with a particular chemical. In October, 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods and approaches directed toward reducing and refining the use of laboratory animals for acute toxicity testing (ICCVAM, 2001a). One approach was the use of *in vitro* cytotoxicity assays to predict acute *in vivo* lethality (Spielmann et al., 1999). One of the workshop recommendations for reducing and refining the use of animals for lethality assays in the near-term was the publication of guidance for using *in vitro* cytotoxicity assays to estimate starting doses for acute oral lethality assays (ICCVAM, 2001b). The recommended publication, illustrated above, provides details and examples on how to implement such an approach. Another recommendation was that a validation study be conducted using this approach with at least two *in vitro* basal cytotoxicity test methods.

Study Objective:

- To evaluate the utility of two *in vitro* cytotoxicity tests for identifying the starting dose for *in vivo* acute lethality assays.

As the *Guidance Document* (ICCVAM, 2001b) describes, the approach is based on the linear regression analysis of rodent *in vivo* oral LD_{50}s and *in vitro* IC_{50}s for 347 chemicals in the Registry of Cytotoxicity (RC) (Halle, 1998):

$$\log LD_{50}\ (\text{mmol/kg}) = 0.435 \log IC_{50}\ (\text{mM}) + 0.625$$

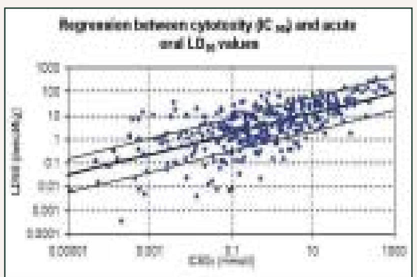


Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral LD_{50} values for 347 chemicals.
The heavy line shows the fit of the data to a linear regression model, $\log (LD_{50}) = 0.435 \times \log (IC_{50x}) + 0.625$; r=0.67. The thinner lines show the empirical $F_G = \log 5$ acceptance interval for the prediction model that is based on the anticipated precision of LD_{50} values from rodent studies Halle (1998).

This poster describes the selection of test chemicals for the study.

Methods

The following criteria, recommended by Workshop participants (ICCVAM, 2001a), were used to compile a database of 117 candidate chemicals by mining several publicly available databases:

1) Representative of all six categories of acute oral toxicity (OECD, 2001),
2) The types of chemicals regulated by the various U.S. regulatory agencies, and
3) Those with human toxicity data and/or human exposure potential.

Sources for Database of Candidate Chemicals

A database of 117 candidates was compiled with chemicals from the following sources, which were assumed to contain chemicals that met the criteria:

- Chemicals tested in the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC); all have significant human toxicity data that has been collected and analyzed by Ekwall et al. (1998).
- Chemicals recommended by U.S. EPA Office of Pesticide Programs and Office of Pollution Prevention and Toxics.
- Chemicals with the top five highest frequencies of human toxic exposures from the Toxic Exposure Surveillance System (Litovitz et al., 2000).
- Chemicals recommended by the *Guidance Document* (ICCVAM, 2001b) for qualifying cytotoxicity assays for this approach.
- Chemicals from those evaluated by the National Toxicology Program (NTP), and/or on the U.S. EPA High Production Volume list, and/or from the RC (Halle, 1998).

Selection of Chemicals for Testing

From the candidate database, 72 chemicals were selected, 12 from each of the six acute oral toxicity hazard classifications in the Globally Harmonised Scheme (GHS) (OECD, 2001).

Class	Oral LD_{50}
Class 1	< 5 mg/kg
Class 2	> 5 - < 50 mg/kg
Class 3	> 50 - < 300 mg/kg
Class 4	> 300 - < 2000 mg/kg
Class 5	> 2000 - < 5000 mg/kg
Unclassified	> 5000 mg/kg

Criteria for selecting 72 chemicals from the 117 candidates:

- Availability of human acute oral toxicity data (e.g., MEIC database)
- Availability of rodent acute oral toxicity data (e.g., RC, RTECS)
- Not highly volatile
- Not strictly controlled by U.S. Drug Enforcement Agency (DEA) (i.e, > Schedule II)
- Corrosivity. Corrosives were given a lower testing priority than noncorrosives since regulatory guidelines state that corrosive chemicals should not be tested in animals for acute toxicity. U.S. Department of Transportation (DOT) Packing Group (PG) designations were used. Chemicals in DOT PG I are most corrosive and lowest in testing priority.

Chemicals were selected so as to represent the range of toxicity in each GHS category, and/or so that the entire set of chemicals had proportionally no more "outliers" (i.e., chemicals outside the log 5 acceptance interval of the RC) than the RC database.

Results

Table 1 shows the selected chemicals and alternates (i.e., remainder of candidate chemicals that were not selected for testing).

Table 1. Selected and Alternate Chemicals

Selected Chemicals						Alternate Chemicals						
GHS[1] Category/Chemical	RC[2] No.	Rodent Oral LD50[3] (mg/kg)	Indication of Human Exposure Potential/Data[4]	Corrosivity[5]	Product/Use	GHS[1] Category/Chemical	RC[2] No.	Rodent Oral LD50[3] (mg/kg)	Indication of Human Exposure Potential/Data[4]	Corrosivity[5]	Notes[6]	Product/Use
LD50 < 5 mg/kg												
Mercury II chloride	29	1	MEIC, NTP,TESS	PGII	Pesticide							
Triethylenemelamine	143	1.0		PGII	Manufacturing							
Sodium selenate*10 H20		1.6	NTP,TESS	PGI	Feed additive							
Busulphan	177	1.9		PGIII	Pharmaceutical							
Cycloheximide	13	2.0			Pesticide							
Disulfoton	51	2.0	EPA	PGI	Insecticide							
Parathion	49	2.0	NTP, EPA	PGI	Insecticide							
Strychnine		2	MEIC, EPA,TESS	PGI	Pesticide							
Aminopterin	3	3.0		PGI	Medicinal							
Phenylthiourea	234	3.0	NTP	PGI	Pesticide							
Epinephrine bitartrate	169	4.0	NTP (HCl salt)		Pharmaceutical							
Aflatoxin B1	37	5.0		PGI	Food contaminant							
LD50 > 5 - < 50 mg/kg						***LD50 > 5 - < 50 mg/kg***						
Colchicine	6	6	MEIC,TESS		Pharmaceutical	t-Butylamine		30	NTP, HPV, EPA	PGII		Manufacturing
Potassium cyanide	252	10	MEIC,TESS	PGI	Electroplating	2,4-Dinitrophenol	68	30	NTP, HPV	PGI		Pesticide, manufacturing
Dichlorvos (DDVP)		17	NTP, HPV, EPA,TESS	PGI	Pesticide	Acrolein	179	46	NTP, HPV, EPA	PGI	Volatile (BP=52C)	Pesticide, manufacturing
Digoxin	22	18	MEIC,TESS	PGI	Pharmaceutical							
Fenpropathrin		18	EPA	PGII	Pesticide							
Endosulfan		18	EPA, NTP,TESS	PGI	Pesticide							
Arsenic III trioxide	153	20	MEIC, NTP,	PGII	Pesticide							
Thallium I sulfate	181	29	MEIC,TESS	PGII	Pesticide							
Sodium arsenite		41	TESS	PGII	Pesticide							
Triphenyltin hydroxide	132	44	NTP, HPV, EPA	PGII	Pesticide							
Sodium dichromate	144	50	NTP, EPA	PGII	Oxidizing agent							
Nicotine	103	50	MEIC, EPA,TESS	PGII	Pharmaceutical							
LD50 > 50 - < 300 mg/kg						***LD50 > 50 - < 300 mg/kg***						
Paraquat	235	58	MEIC, EPA,TESS	PGII	Pesticide	Pentachlorophenol	173	51	MEIC, NTP	PGII		Disinfectant
Hexachlorophene	157	61	MEIC, NTP,TESS	PGIII	Disinfectant	Amphetamine sulfate	262	55	MEIC, NTP,TESS	PGII	DEA	Pharmaceutical
Lindane	223	76	MEIC, NTP, EPA, TESS	PGIII	Pesticide	Rotenone	134	60	NTP, EPA,TESS			Pesticide
Cadmium II chloride	20	88	TESS		Veterinary pharmaceutical	Furfural		65	NTP, HPV	PGII		Solvent, food additive
Verapamil HCl	196	108	MEIC,TESS	PGIII	Pharmaceutical	p-Phenylenediamine	180	80	NTP	PGIII		Dyeing
Haloperidol		128	MEIC		Pharmaceutical	Chlorpyrifos		82	EPA,TESS	PGII		Pesticide
Sodium oxalate	227	155	MEIC, NTP,TESS		Paints, cleaner	Dextropropoxyphene HCl	229	83	MEIC,TESS			Pharmaceutical
Phenobarbital	118	163	MEIC,TESS		Pharmaceutical	Methadone		86	MEIC,TESS	PGII	DEA	Pharmaceutical
Sodium I fluoride	106	180	MEIC, NTP, EPA	PGIII	Electroplating, flouridation	Fipronil		92	EPA			Pesticide
Caffeine	112	192	MEIC, NTP, HPV, TESS	PGIII	Pharmaceutical, food additive	Pentobarbital		125	MEIC,TESS		DEA	Pharmaceutical
Diquat		231	MEIC,TESS		Pesticide	Bromoxynil (phenol)		190	EPA			Pesticide
Cupric sulfate * 5 H2O	81	300	MEIC, NTP, EPA	PGIII	Pesticide	Diphenylhydantoin	82	199	MEIC, NTP,TESS	PGIII		Pharmaceutical
						Carbaryl	73	230	EPA			Pesticide
						Metaldehyde		250	EPA,TESS			Pesticide
LD50 > 300 - < 2000 mg/kg						***LD50 > 300 - < 2000 mg/kg***						
Amitriptyline	183	319	MEIC,TESS		Pharmaceutical	Ferrous sulfate	261	319	MEIC			Food additive
Phenol	115	414	MEIC, NTP, HPV, EPA, TESS	PGII	Disinfectant	Warfarin	86	324	MEIC, EPA,TESS	PGI		Pharmaceutical, pesticide
Propranolol HCl	54	470	MEIC		Pharmaceutical	Disopyramide		333	MEIC			Pharmaceutical
Chloral hydrate	264	479	MEIC, NTP,TESS		Pharmaceutical	Barium II nitrate	246	355	MEIC,TESS	PGII		Pyrotechnic
Glutethimide	101	600	MEIC,TESS		Pharmaceutical	Thioridazine HCl	170	358	MEIC,TESS			Pharmaceutical
Atropine sulfate	70	623	MEIC,TESS		Pharmaceutical	Methyl phenidate		367	NTP		DEA	Pharmaceutical
Valproic acid		670	MEIC,TESS		Pharmaceutical	Molinate		369	EPA			Pesticide
Meprobamate		794	MEIC,TESS		Pharmaceutical	2,4-Dichlorophenoxyacetic acid	89	369	MEIC, NTP, HPV, EPA, TESS			Pesticide
Acetylsalicylic acid	107	1000	MEIC,TESS	PGIII	Pharmaceutical	Orphenadrine HCl	230	425	MEIC	PGIII		Pharmaceutical
Lithium I sulfate	327	1187	MEIC,TESS		Pharmaceutical	Trichlorfon	75	451	NTP, EPA	PGII		Pesticide
Procainamide		1950	MEIC		Pharmaceutical	Quinidine sulfate	53	456	MEIC			Pharmaceutical
Carbamazepine		1957	MEIC,TESS		Pharmaceutical	1,3-Dichloropropene		470	NTP, HPV, EPA			Pesticide
						Theophylline	105	600	MEIC, NTP,TESS			Pharmaceutical
						Isoniazid	123	650	MEIC,TESS			Pharmaceutical
						Diazepam	63	709	MEIC,NTP,TESS	PGIII	DEA	Pharmaceutical
						Maprotiline		760	MEIC,TESS			Pharmaceutical
						Methyl eugenol		810	NTP			Food additive
						Diphenhydramine HCl	71	855	MEIC, NTP,TESS			Pharmaceutical
						Malathion	67	885	MEIC, NTP, EPA, TESS	PGI		Pesticide
						Salicylic acid	272	891	HPV			Pharmaceutical
						Chloroform	308	908	MEIC, NTP, HPV	PGIII	Volatile (BP=61C)	Solvent
						Chloroquine diphosphate	31	970	MEIC			Pharmaceutical
						Ibuprofen	233	1009	TESS			Pharmaceutical
						Nalidixic acid	99	1349	NTP			Pharmaceutical
						Dichloromethane	328	1597	MEIC, NTP, HPV, TESS	PGIII	Volatile (BP=40C)	Solvent
						Antipyrene	300	1800				Pharmaceutical
LD50 > 2000 - < 5000 mg/kg						***LD50 > 2000 - < 5000 mg/kg***						
Acetaminophen	113	2404	MEIC, NTP,TESS		Pharmaceutical							
Potassium I chloride	346	2602	MEIC,TESS		Pharmaceutical, manufacturing							
Sodium chloride	344	2998	MEIC, EPA,TESS		Pharmaceutical, food additive							
Chloramphenicol	91	3393	MEIC, NTP		Pharmaceutical							
Boric aid		2660	NTP, EPA,TESS		Pesticide							
Lactic acid	341	3730	NTP, HPV		Food additive							
Citric Acid		3000	NTP, HPV, EPA		Food additive							
Dimethylformamide	351	2800	HPV		Solvent							
Xylene	301	4300	MEIC, NTP, HPV,TESS		Solvent							
Trichloroacetic acid	294	4999	NTP		Fixative							
Acetonitrile	358	3798	NTP, HPV		Solvent							
Carbon tetrachloride	125	2799	MEIC, NTP, HPV,TESS	PGII	Solvent							
LD50 > 5000 mg/kg						***LD50 > 5000 mg/kg***						
2-Propanol	128	5843	MEIC, HPV, EPA,TESS		Disinfectant							
Ethylene glycol	360	8567	MEIC, NTP, HPV,TESS		Antifreeze							
Ethanol	130	14008	MEIC, NTP, HPV, EPA,TESS		Solvent							
1,1,1-Trichloroethane	297	10298	MEIC, NTP, HPV	PGIII	Solvent							
Methanol	361	13012	MEIC, NTP, HPV,TESS	PGII	Solvent							
Propylparaben	209	6326	NTP		Food additive							
5-Aminosalicylic acid	120	7749	NTP		Pharmaceutical							
Sodium hypochlorite		8910	TESS		Disinfectant							
Dibutyl phthalate	88	11998	NTP, HPV		Plasticizer							
Glycerol	131	12691	NTP, HPV		Solvent							
Gibberellic acid	108	6305	NTP, EPA		Plant growth regulator							
Diethyl phthalate	122	6179	NTP, HPV		Plasticizer							
						Other Nonselected Candidates						
						Furan		5.2, ip	NTP, HPV		No air shipping, volatile (BP=31C)	Chemical intermediate

[1] GHS-Globally Harmonised System categories of acute oral toxicity (OECD, 2001).

[2] RC is Registry of Cytotoxicity, a database of chemical specific IC_{50}s and LD_{50}s. RC No. reflects numbers assigned/reported in Halle (1998).

[3] LD_{50} data are from Registry of Cytotoxicity, Registry of Toxic Effects of Chemical Substances (RTECS), or EPA Office of Pesticide Programs.

[4] The following items signify human toxicity/exposure data or potential for human exposure. MEIC is Multicentre Evaluation of In Vitro Cytotoxicity and indicates chemicals with monographs containing toxic and lethal human blood concentrations and analysis. NTP indicates chemicals, chosen by the likelihood of human exposure, evaluated by the National Toxicology Program. EPA indicates EPA registered pesticides (indicates human exposure potential). HPV indicates High Production Volume Chemicals that are imported or produced in amounts > 1,000,000 lbs/year. TESS indicates chemicals for which human poisonings are documented by the Toxic Exposure Surveillance System (Litovitz et al., 2000).

[5] Corrosivity. PGI-III refers to U.S. Department of Transportation 6.1 packing groups. PG1 denotes the most corrosive chemicals. PGIII is the least corrosive. Chemicals with no PG designation are expected to be noncorrosive.

[6] Notes. Only chemicals expected to be too volatile for the cytotoxicity assay system have "volatile" notations. BP = Boiling point. DEA (U.S. Drug Enforcement Agency) refers to Schedule II controlled substances. Chemicals with no "DEA" notation are expected to be under less strict control.

Table 2 shows the distribution, by GHS class, of candidate and selected chemicals used in MEIC and NTP studies and those tracked by the Toxic Exposure Surveillance System. Forty-two of the 72 selected chemicals are MEIC chemicals, 22 of the selected chemicals are NTP chemicals, and 43 of the selected chemicals have human poisoning incidences reported in the Toxic Exposure Surveillance System. "Other" refers to chemicals on the candidate list for which no rodent LD_{50} data could be located. Only intravenous or inhalation data were found.

Table 2. MEIC[1], NTP[2], and TESS[3] Chemical Distribution by GHS[4] Oral Toxicity Class

GHS Class	Selected Chemicals/ Candidate Chemicals	MEIC Chemicals/ Selected Chemicals	NTP Chemicals/ Selected Chemicals	TESS Chemicals/ Selected Chemicals
Class 1	12/12	2/12	5/12	3/12
Class 2	12/15	6/12	5/12	9/12
Class 3	12/26	11/12	4/12	10/12
Class 4	12/38	12/12	2/12	10/12
Class 5	12/12	6/12	6/12	6/12
Unclassified	12/12	5/12	6/12	5/12
Other	0/2	0/0	0/0	0/0
Total	72/117	42/72	22/72	43/72

[1] MEIC: Multicentre Evaluation of *In Vitro* Cytotoxicity

[2] NTP: National Toxicology Program

[3] TESS: Chemicals for which human exposures are tracked by the Toxic Exposure Surveillance System

Table 3 summarizes the number of Registry of Cytotoxicity "outliers" by GHS oral toxicity class as compiled in the list of 347 chemicals of the RC and as selected for testing. Although the percentage of "outliers" for the selected chemicals in each GHS category differs somewhat from the RC in several categories, the total percentage of "outliers" identified in the set of selected chemicals (i.e., 31%) is similar to the total percentage of outliers in the RC (i.e., 27%).

Table 3. Distribution of Registry of Cytotoxicity (RC) "Outliers"[1] by Chemical Class

Registry of Cytotoxicity		Candidate and Selected Chemicals		
GHS[2] Class	**"Outliers"/Total Chemicals**	**Candidate Chemicals**	**RC Chemicals/ Selected Chemicals**	**RC "Outliers"/Selected RC Chemicals**
Class 1	9/11 (82%)	12	10/12	9/12 (75%)
Class 2	15/26 (58%)	15	8/12	4/12 (33%)
Class 3	24/70 (34%)	26	10/12	4/12 (33%)
Class 4	14/139 (10%)	38	8/12	0/12 (0%)
Class 5	12/57 (21%)	12	10/12	0/12 (0%)
Unclassified	20/44 (45%)	12	11/12	5/12 (42%)
Other	0/0 (0%)	2	0/0	0/0 (0%)
Total	94/347 (27%)	117	57/72	22/72 (31%)

[1] Chemicals falling outside the empirical $F_G = \log 5$ acceptance interval for the RC prediction model Halle (1998).

[2] GHS: Globally Harmonised Scheme of acute oral toxicity hazard classifications (OECD, 2001)

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