SELECTION OF REFERENCE CHEMICALS FOR THE VALIDATION OF *IN VITRO* CYTOTOXICITY ASSAYS FOR PREDICTING *IN VIVO* ACUTE SYSTEMIC TOXICITY

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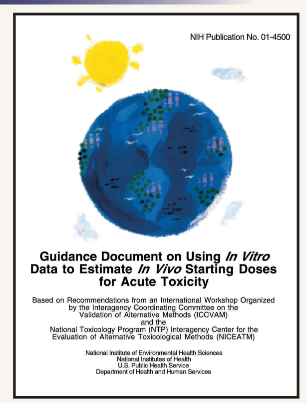
Abstract

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and NICEATM convened an international workshop in October 2000 to evaluate the validation status of *in vitro* methods for predicting acute systemic toxicity. Workshop participants recommended that *in vitro* basal cytotoxicity methods should be further evaluated. NICEATM and ECVAM subsequently designed a multi-laboratory validation study to evaluate the utility of two *in vitro* cytotoxicity tests for predicting acute oral toxicity in rodents and humans. A critical aspect of the study design was the selection of appropriate reference chemicals. Selection criteria included: 1) representation of chemicals across the full range of acute toxicity, 2) availability of high quality rodent acute toxicity test data, 3) availability of human toxicity data and/or exposure potential, and 4) representation of the types of regulated chemicals. A list of 116 candidates was compiled by mining several publicly available databases, including chemicals from the Multicentre Evaluation of *In Vitro* Cytotoxicity and the Registry of Cytotoxicity. Seventy-two chemicals were selected for testing: 12 chemicals for each of the five hazard classes of the Globally Harmonised Classification System and 12 chemicals classified as having no acute toxicity hazard. These reference chemicals and data will now be used to evaluate the predictive performance of the proposed *in vitro* test methods. Supported by NIEHS contract N01-ES-85424.

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. NICEATM and ECVAM subsequently designed a multi-laboratory validation study to evaluate the performance of two standardized *in vitro* cytotoxicity tests using this approach.[1]

This poster describes the selection rationale, which was based on workshop recommendations for selection of validation chemicals, for the 72 chemicals that will be tested during the validation study.

[1] See poster entitled "Validation Study Design to Evaluate *In Vitro* Cytotoxicity Assays for Predicting Rodent and Human Acute Systemic Toxicity" by Stokes et al. for more information on the study designed to implement this approach.

Prediction Model

As the Guidance Document (ICCVAM, 2001b) describes, the approach is based on the linear regression analysis of rodent *in vivo* oral LD50s and *in vitro* IC_{50}s for 347 chemicals in the Registry of Cytotoxicity (RC) (Halle, 1998), which resulted in the following prediction model:

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

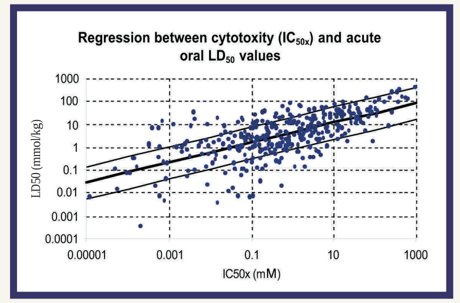


Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral LD_{50} values for 347 chemicals. The heavy line shows the fit of the data to a linear regression model, $\log (LD_{50}) = 0.435 \times \log (IC_{50x}) + 0.625$; r=0.67. The thinner lines show the empirical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

Methods

The following criteria, recommended by workshop participants (ICCVAM, 2001a), were used to compile a database of 116 candidate chemicals by mining several publicly available databases:

1) Representative of all five Globally Harmonised System (GHS) categories of acute oral toxicity as well as unclassified (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 116 candidates was compiled with chemicals from the following sources, which contained 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 Toxic Substances.
- Chemicals with the top five highest frequencies of human toxic exposures from the Toxic Exposure Surveillance System (TESS) (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 U.S. 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 five GHS acute oral toxicity hazard categories and 12 unclassified chemicals (OECD, 2001).

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

Criteria for selecting 72 chemicals from the 116 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. United Nations (U.N.) (also U.S. Department of Transportation) Packing Group (PG) designations were used. Chemicals in U.N. PG I are most corrosive and lowest in testing priority.

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

GHS[1] Category/Chemical	RC[2] No.	Rodent Oral LD50[3] (mg/kg)	Indication of Human Exposure Potential/Data[4]	Corrosivity[5]	Product/Use
LD50 < 5 mg/kg					
Mercury II chloride	29	1	MEIC, NTP,TESS	PGII	Pesticide
Triethylenemelamine	143	1.0		PGII	Manufacturing
Sodium selenate		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
Physostigmine		4.5			Pharmaceutical
LD50 > 5 - < 50 mg/kg					
Colchicine	6	6	MEIC,TESS		Pharmaceutical
Potassium cyanide	252	10	MEIC,TESS	PGI	Electroplating
Dichlorvos (DDVP)		17	NTP, HPV, EPA,TESS	PGI	Pesticide
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, TESS EPA	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					
Paraquat	235	58	MEIC, EPA,TESS	PGII	Pesticide
Hexachlorophene	157	61	MEIC, NTP,TESS	PGIII	Disinfectant
Lindane	223	76	MEIC, NTP, EPA	PGIII	Pesticide
Cadmium II chloride	20	88	TESS		Veterinary pharmaceutical
Verapamil HCl	196	108	MEIC,TESS	PGIII	Pharmaceutical
Haloperidol		128	MEIC, TESS		Pharmaceutical
Sodium oxalate	227	155	MEIC, NTP,TESS		Paints, cleaner
Phenobarbital	118	163	MEIC,TESS		Pharmaceutical
Sodium I fluoride	106	180	MEIC, NTP, EPA TESS	PGIII	Electroplating, flouridation
Caffeine	112	192	MEIC, NTP, HPV, TESS	PGIII	Pharmaceutical, food additive
Diquat		231	MEIC,TESS		Pesticide
Cupric sulfate * 5 H2O	81	300	MEIC, NTP, EPA TESS	PGIII	Pesticide
LD50 > 300 - < 2000 mg/kg					
Amitriptyline	183	319	MEIC,TESS		Pharmaceutical
Phenol	115	414	MEIC, NTP, HPV, EPA, TESS	PGII	Disinfectant
Propranolol HCl	54	470	MEIC, TESS		Pharmaceutical
Chloral hydrate	264	479	MEIC, NTP,TESS		Pharmaceutical
Glutethimide	101	600	MEIC,TESS		Pharmaceutical
Atropine sulfate	70	623	MEIC,TESS		Pharmaceutical
Valproic acid		670	MEIC,TESS		Pharmaceutical
Meprobamate		794	MEIC,TESS		Pharmaceutical
Acetylsalicylic acid	107	1000	MEIC,TESS	PGIII	Pharmaceutical
Lithium I sulfate	327	1187	MEIC,TESS		Pharmaceutical
Procainamide		1950	MEIC,TESS		Pharmaceutical
Carbamazepine		1957	MEIC,TESS		Pharmaceutical
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					
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

Alternate Chemicals

GHS[1] Category/Chemical	RC[2] No.	Rodent Oral LD50[3] (mg/kg)	Indication of Human Exposure Potential/Data[4]	Corrosivity[5]	Notes	Product/Use
LD50 < 5 mg/kg						
Aflatoxin B1	37	5.0		PGI		Food contaminant
LD50 > 5 - < 50 mg/kg						
t-Butylamine		30	NTP, HPV, EPA	PGII		Manufacturing
2,4-Dinitrophenol	68	30	NTP, HPV	PGI		Pesticide, manufacturing
Acrolein	179	46	NTP, HPV, EPA, TESS	PGI	Volatile (BP=52C)	Pesticide, manufacturing
LD50 > 50 - < 300 mg/kg						
Pentachlorophenol	173	51	MEIC, NTP	PGII		Disinfectant
Amphetamine sulfate	262	55	MEIC, NTP,TESS	PGII	DEA	Pharmaceutical
Rotenone	134	60	NTP, EPA,TESS			Pesticide
Furfural		65	NTP, HPV	PGII		Solvent, food additive
p-Phenylenediamine	180	80	NTP	PGIII		Dyeing
Chlorpyrifos		82	EPA,TESS	PGII		Pesticide
Dextropropoxyphene HCl	229	83	MEIC,TESS			Pharmaceutical
Methadone		86	MEIC,TESS	PGII	DEA	Pharmaceutical
Fipronil		92	EPA			Pesticide
Pentobarbital		125	MEIC,TESS		DEA	Pharmaceutical
Bromoxynil (phenol)		190	EPA			Pesticide
Diphenylhydantoin	82	199	MEIC, NTP,TESS	PGIII		Pharmaceutical
Carbaryl	73	230	EPA			Pesticide
Metaldehyde		250	EPA,TESS			Pesticide
LD50 > 300 - < 2000 mg/kg						
Ferrous sulfate	261	319	MEIC, TESS			Food additive
Warfarin	86	324	MEIC, EPA,TESS	PGI		Pharmaceutical, pesticide
Disopyramide		333	MEIC, TESS			Pharmaceutical
Barium II nitrate	246	355	MEIC,TESS	PGII		Pyrotechnic
Thioridazine HCl	170	358	MEIC,TESS			Pharmaceutical
Methyl phenidate		367	NTP		DEA	Pharmaceutical
Molinate		369	EPA			Pesticide
2,4-Dichlorophenoxyacetic acid	89	369	MEIC, NTP, HPV, EPA, TESS			Pesticide
Orphenadrine HCl	230	425	MEIC	PGIII		Pharmaceutical
Trichlorfon	75	451	NTP, EPA	PGII		Pesticide
Quinidine sulfate	53	456	MEIC			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, TESS			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 > 5000 mg/kg						

[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. EDIT is Evaluation-guided Development of New *In vitro* Tests and denotes the chemicals (C. Clemedson, Personal communication) chosen for a follow-on project to MEIC to develop a battery of *in vitro* tests to predict human toxicity. NTP indicates chemicals, chosen by the likelihood of human exposure, evaluated by the National Toxicology Program. U.S. EPA indicates U.S. 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.N. and 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 category, of candidate and selected chemicals used in MEIC, EDIT, and NTP studies and those tracked by TESS. Forty-two of the 72 selected chemicals are MEIC chemicals, 17 are EDIT chemicals, 37 are NTP chemicals, and 46 have human poisonings reported by TESS.

Table 2. MEIC[1], EDIT[2], NTP[3], TESS[4] Chemical Distribution by GHS[5] Oral Toxicity Category

GHS Category	Selected Chemicals/ Candidate Chemicals	Selected MEIC/ MEIC Candidates	Selected EDIT/ EDIT Candidates	Selected NTP/ NTP Candidates	Selected TESS/ TESS Candidates
Category 1	12/13	2/2	1/1	5/5	3/3
Category 2	12/15	6/6	5/5	5/8	9/10
Category 3	12/26	11/17	4/5	6/12	11/19
Category 4	12/38	12/29	3/5	2/14	12/27
Category 5	12/12	6/6	2/2	9/9	6/6
Unclassified	12/12	5/5	2/2	10/10	5/5
Total	72/116	42/65	17/20	37/58	46/70

[1]MEIC: Multicentre Evaluation of *In Vitro* Cytotoxicity (Ekwall et al., 1998)

[2]NTP: U.S. National Toxicology Program

[3]EDIT: Evaluation-guided Development of New *In Vitro* Cytotoxicity Tests (Ekwall et al., 1999)

[4]TESS: Chemicals for which human poisonings were reported by the Toxic Exposure Surveillance System (Litovitz et al., 2000)

[5]GHS: Globally Harmonised System of acute oral toxicity hazard classification (OECD, 2001)

Table 3 summarizes the number of RC chemicals in each GHS oral toxicity category, the number of RC chemicals considered as candidates for this study, the number of RC chemicals selected for testing, the number of "outliers" in the RC, and the number of RC "outliers" selected for testing. Although the percentage of "outliers" for the selected chemicals in most GHS categories is similar to the RC, the total percentage of RC "outliers" identified in the set of selected chemicals (i.e., 38%) is greater than the total percentage of outliers in the RC (i.e., 27%).

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

Registry of Cytotoxicity		Candidate and Selected Chemicals		
GHS[2] Category	"Outliers"/Total Chemicals	Candidate Chemicals	Selected RC Chemicals/ RC Candidates	Selected RC "Outliers"/ Selected RC Chemicals
Category 1	9/11 (82%)	13	9/10	8/9 (89%)
Category 2	15/26 (58%)	15	8/10	4/8 (50%)
Category 3	24/70 (34%)	26	10/17	4/10 (40%)
Category 4	14/139 (10%)	38	8/28	0/8 (0%)
Category 5	12/57 (21%)	12	10/10	0/10 (0%)
Unclassified	20/44 (45%)	12	11/11	5/11 (45%)
Total	94/347 (27%)	116	56/86	21/56 (38%)

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

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

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