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3.0 REFERENCE SUBSTANCES USED FOR VALIDATION OF THE 3T3 AND NHK NRU TEST METHODS

3.1 Rationale for the 72 Reference Substances Selected for Testing

This section describes the procedures used to select the 72 reference substances selected for testing in Phase Ia of the validation study.

3.1.1 Reference Substance Selection Criteria

The SMT (see **Appendix A**) selected reference substances for testing using a process based on general recommendations made by Workshop 2000 participants (ICCVAM 2001a). The following criteria were used:

- The toxicities of the reference substances should be evenly distributed across the expected range of rodent LD_{50} values, using the GHS classification for acute oral toxicity as a guide (UN 2005).
- The reference substances should cover a wide range of structural and use classes, and be relevant to the needs of the various user communities.
- Substances with human toxicity data and/or human exposure potential (i.e., substances of interest to society) should be included. Substances with human acute toxicity data were particularly important to ECVAM for determining the relationship of the NRU IC_{50} values to human blood/serum LC.

Table 3-1 shows the GHS scheme for classifying substances into six toxicity categories (five with measured LD_{50} ranges and an unclassified category with LD_{50} values greater than 5000 mg/kg) based on acute rodent oral LD_{50} values (UN 2005). The SMT used this scheme for the classification of candidate substances to assure that the reference substances selected for the validation study represented the full range of acute oral toxicity.

Table 3-1 GHS Classification Scheme for Acute Oral Toxicity

Category	LD_{50} (mg/kg)
1	$LD_{50} \leq 5$
2	$5 < LD_{50} \leq 50$
3	$50 < LD_{50} \leq 300$
4	$300 < LD_{50} \leq 2000$
5	$2000 < LD_{50} \leq 5000$
Unclassified	$LD_{50} > 5000$

Abbreviations: UN=United Nations; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
LD_{50}=Dose that produces lethality in 50% of the test animals.

For the purposes of the initial toxicity classification, the rodent oral LD_{50} values for the individual substances were obtained from readily available toxicological databases. These rodent oral LD_{50} values were re-evaluated in **Section 4** for the purpose of identifying the most appropriate reference LD_{50} values to use for the accuracy analyses (i.e., determine to

what extent there is agreement between a test method result and an accepted reference value [see **Section 6.3**]). Rat LD_{50} data were preferred because:

- The current acute oral toxicity test guidelines recommend using rats (OECD 2001a, c, d; EPA 2002a)
- The majority of LD_{50} data used in the RC millimole regression were from studies using rats (282 rat data points and 65 mouse data points) (Halle 1998, 2003)
- The great majority of acute oral systemic toxicity testing is performed with rats

Mouse oral LD_{50} values were used (10 substances) for the initial toxicity classification when rat data were unavailable, however, mouse data were not used in the regression analyses presented in **Section 6**. The toxicological databases, in order of preference, were:

- The RC, which contains LD_{50} values that came largely from the 1983/84 RTECS® (Halle 1998, 2003). The RC is a database of acute oral LD_{50} values for rats and mice obtained from RTECS® and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for chemicals with known molecular weights.
- The current RTECS® (MDL Information Systems 2001, 2002)
- The current Hazardous Substances Data Bank (HSDB; U.S. National Library of Medicine [NLM] 2001, 2002).

To insure that a wide range of structural and use classes were selected, reference substances of interest to the various U.S. regulatory agencies, as determined from substance lists received from the various agencies, were included. Substances with human toxicity data and/or human exposure potential were chosen by mining publicly available databases (e.g., the NTP test database, the MEIC database) for potential candidates.

3.1.2 Candidate Reference Substances

The process of identifying the 72 reference substances started with the compilation of a database of 116 candidates. The intent of the SMT was to compile a database with at least 12 substances in each GHS toxicity category that also met the other selection criteria, and then to prioritize the substances within each category to select the 72 to be tested. As recommended by Workshop 2000 (ICCVAM 2001a), the following publicly available databases and other sources were used to identify candidate substances:

- The MEIC program, which collected human toxicity data and *in vitro* toxicity data from 61 test methods for 50 substances (Ekwall et al. 1998)
- The EDIT program, which targeted development of *in vitro* test methods for endpoints other than basal cytotoxicity; includes 20 chemicals that are a subset of the MEIC chemicals
- The RC (Halle 1998, 2003), which contains *in vitro* cytotoxicity and *in vivo* rodent LD_{50} data for 347 substances
- The Toxic Exposure Surveillance System (TESS) (Litovitz et al. 2000), which compiles reports of toxic human exposures from poison control centers throughout the United States
- Pesticides recommended for consideration by the EPA Office of Pesticide Programs (OPP)

- The *Guidance Document* (ICCVAM 2001b), which reported *in vitro* NRU results for 11 RC substances using protocols similar to those to be used in the validation study
- The U.S. NTP test database, which contains information on the toxicity of substances relevant to human exposure (NTP 2002)
- The EPA High Production Volume (HPV) Challenge Program list of chemicals. The HPV is a voluntary testing program to provide the public with a complete set of baseline health and environmental effects data for each chemical that is manufactured within or imported into the United States at amounts >1 million pounds/year (EPA 2000a)

The candidate substances from the list of 116 that were not selected as reference substances to use in the validation study are listed in **Appendix F3**, grouped by GHS category, along with the rat or mouse oral LD_{50} value, the database(s) or other source(s) used to identify the substance as a potential candidate, and the type of product and/or use for the substance.

3.1.3 Selection of Reference Substances for Testing

Using the candidate substance database, 72 reference substances (12 GHS-unclassified substances and 12 substances from each of the five GHS acute oral toxicity hazard categories) were selected. This number of substances per GHS category was considered adequate by the ICCVAM Acute Toxicity Working Group (ATWG), ICCVAM, ECVAM, and the SMT to accurately evaluate the performance of these two *in vitro* NRU test methods for identifying the starting dose for rodent acute oral toxicity tests across the range of toxic levels that would be encountered during testing. The criteria used for prioritizing the candidate substances were:

- The availability of rodent acute oral toxicity data
- The availability of human acute oral toxicity data and/or relevance for human exposure
- The level of volatility (because the cells are exposed for 48 hours while incubated at 37 °C in 96-well plates, volatilization from wells containing a volatile reference substance would affect the accuracy of the IC_{50} calculation and potentially contaminate other wells)
- Not a controlled substance according to the U.S. Drug Enforcement Agency (DEA). Excluding substances that are listed in DEA Schedules I and II from consideration obviates the requirement for U.S. laboratories to obtain a DEA license and adhere to the DEA substance storage and control procedures
- Practical considerations such as cost and disposal

If more than 12 candidate substances in a GHS category met the above criteria, then selection was based on two further considerations. One consideration was the distribution of substance toxicities within each toxicity category so as to select substances that represented the entire range of toxicity within each category. Another consideration, which applied only to candidate substances selected from the RC database, was the fit of the toxicity to the RC millimole regression. Substances with the best fit to the RC millimole regression were preferentially selected to prevent the entire set of reference substances from having proportionally more "outlier" substances (i.e., greater than one-half log from the RC millimole regression) than the entire RC database.

The final list of selected reference substances is sorted by GHS acute oral toxicity category in **Table 3-2**.

3.2 Characteristics of the Selected Reference Substances

The physical/chemical and toxicological information in **Appendix F** may be useful for characterizing the performance of the *in vitro* NRU test methods for various chemical types (e.g., chemical class, toxic effect class). **Appendix F1** lists the reference substances in alphabetical order with information on the CASRN, purity, supplier, pH (of the highest concentration tested in NRU), and concentrations tested. **Appendix F2** provides the reference substances in alphabetical order, and information on physical/chemical characteristics such as molecular weight, chemical class, water solubility, acid/base dissociation constant (pK), boiling point, and octanol-water partition coefficient (log K_{ow}), a measure of lipid solubility. Although test substance concentration and toxicity may be heavily influenced by molecular charge and surface activity (ICCVAM 2006), these attributes were not characterized because this type of information is not readily available. **Appendix F2** also includes the major toxic effects attributed to each chemical, ability to pass the blood:brain barrier (BBB), metabolic activation/inactivation (whether or not it is metabolized, or the identification of the metabolites), and mechanism of lethality (where known) for each of the reference substances. The remainder of this section summarizes selected characteristics of the reference substances.

3.2.1 Source Databases Represented by the Selected Reference Substances

The primary sources of substances were well represented in the final list of reference substances. **Table 3-3** shows the distribution of reference substances by GHS category from each of the source lists. Forty-two (58%) of the 72 substances were MEIC chemicals (17 of the 42 MEIC chemicals [40%] were also EDIT chemicals), 46 (64%) were involved in human poisonings as reported by TESS, 51 (71%) have been evaluated by the NTP, and 18 (25%) are listed in the EPA's HPV Challenge Program. Some substances were present in more than one database.

The other major source of reference substances was the RC, which contributed 58 (81%) of the 72 chemicals, as shown in **Table 3-4**. Because the RC millimole regression was used to identify outlier substances (see **Section 6.2**), the fit of the RC substances to this regression was relevant (Halle 1998, 2003). Halle (1998, 2003) defined outliers as those chemicals with log IC_{50}-log LD_{50} points that were >0.699 (i.e., log 5) from the RC millimole regression. **Table 3-4** shows the number of RC outliers selected for testing and the corresponding number of outliers in the RC. Although the percentage of outliers in several GHS categories is similar to the percentage in the RC, the total percentage of RC outliers in the set of reference substances (i.e., 38% [22/58]) is greater than the percentage in the RC (i.e., 27% [95/347]). This occurred because the fit to the RC millimole regression was not the major deciding factor during selection of the 72 reference substances.

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

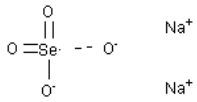
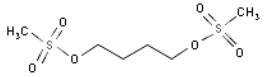
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
$LD_{50} \leq 5$ mg/kg							
Mercury II chloride	1	MEIC, EDIT, RC (outlier), TESS, NTP	Preservative; Manufacturing; Insecticide	271.50	0.22	Inorganic compound; Mercury compound; Chlorine compound	
Triethylenemelamine	1	RC (outlier), NTP	Manufacturing; Insect chemosterilant	204.23	–0.54	Organic compound; Heterocyclic compound	
Sodium selenate	2**	TESS, NTP	Feed additive	188.90	NA	Inorganic compound; Sodium compound; Selenium compound	
Busulfan	2	RC (outlier), NTP	Pharmaceutical (antineoplastic)	246.31	–0.52	Organic compound; Alcohol; Acyclic hydrocarbon; Sulfur compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

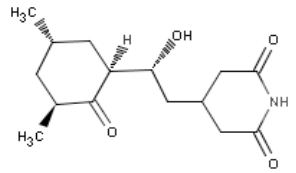
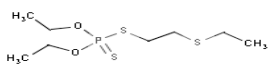
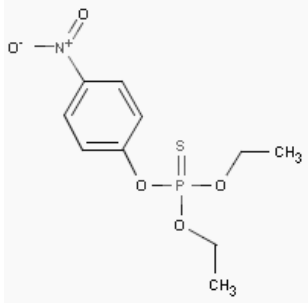
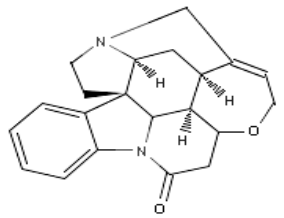
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Cycloheximide	2	RC (outlier), NTP	Antibiotic Fungicide	281.40	0.55	Organic compound; Heterocyclic compound	
Disulfoton	2	RC (outlier), EPA, NTP	Pesticide (insecticide)	274.42	4.02	Organic compound; Organophosphorous compound; Sulfur compound	
Parathion	2	RC (outlier), EPA, NTP	Pesticide (insecticide)	291.28	3.83	Organic compound; Organophosphorous compound; Sulfur compound	
Strychnine	2*	MEIC, TESS, EPA	Pesticide (rodenticide)	334.40	1.93	Organic compound; Heterocyclic compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

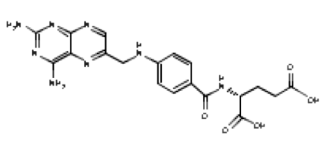
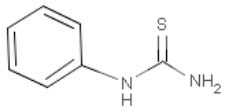
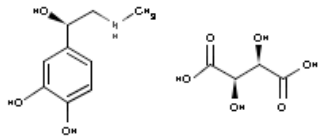
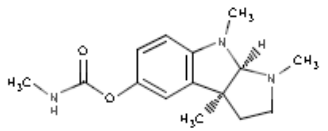
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Aminopterin	3**	RC	Pharmaceutical (antineoplastic); Pesticide (rodenticide)	476.45	NA	Organic compound; Heterocyclic compound	
Phenylthiourea	3	RC (outlier), NTP	Pesticide (rodenticide)	152.20	0.71	Organic compound; Sulfur compound; Urea	
Epinephrine bitartrate	4**	RC (outlier), NTP (HCl salt)	Pharmaceutical (adrenergic)	333.30	–1.52	Organic compound; Alcohol; Amine	
Physostigmine	5*	EHS	Pharmaceutical (anticholinesterase)	275.40	NA	Organic compound; Carboxylic acid; Heterocyclic compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

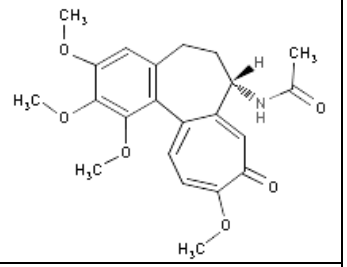
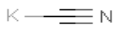
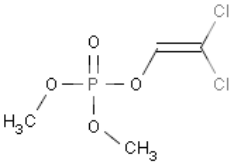
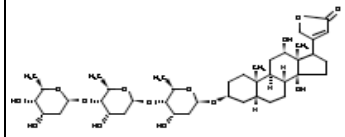
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
5 < LD_{50} ≤50 mg/kg							
Colchicine	6**	MEIC, RC, TESS, NTP	Pharmaceutical (gout suppressant)	399.45	1.03	Organic compound; Polycyclic compound	
Potassium cyanide	10	MEIC, EDIT, RC (outlier), TESS	Electroplating	65.12	NA	Inorganic compound; Potassium compound; Nitrogen compound	
Dichlorvos	17*	TESS, EPA, NTP, HPV	Pesticide (insecticide)	220.98	1.43, 1.45	Organic compound; Organophosphorous compound	
Digoxin	18**	MEIC, EDIT, RC (outlier), TESS	Pharmaceutical (antiarrhythmic)	780.90	1.26	Organic compound; Polycyclic compound; Carbohydrate	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

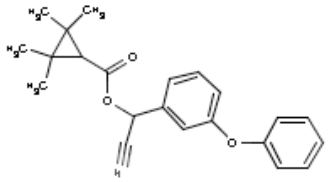
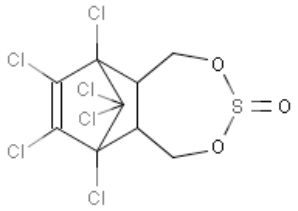
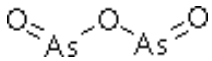
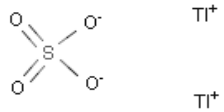
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Fenpropathrin	18*	EPA	Pesticide (insecticide)	349.43	6.0 @ 20° C	Organic compound; Nitrile; Ester; Ether	
Endosulfan	18*	TESS, EPA, NTP	Pesticide (insecticide)	406.91	3.83	Organic compound; Heterocyclic Compound; Sulfur compound	
Arsenic III trioxide	20	MEIC, EDIT, RC, TESS, EPA, NTP	Pesticide (insecticide)	197.80	NA	Inorganic compound; Arsenical	
Thallium I sulfate	29**	MEIC, EDIT, RC (outlier), TESS	Pesticide (rodenticide/insecticide)	504.80	NA	Inorganic compound; Metal; Sulfur compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

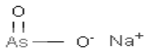
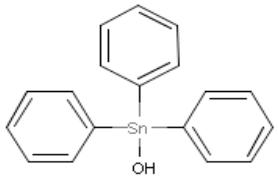
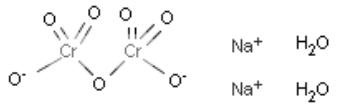
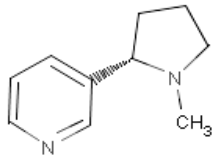
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Sodium arsenite	41*	TESS, NTP	Pesticide (herbicide, insecticide, fungicide)	129.90	NA	Inorganic compound; Arsenical; Sodium compound	
Triphenyltin hydroxide	44	RC, EPA, NTP, HPV	Pesticide (fungicide/insecticide)	367.02	NA	Organic compound; Organometallic compound	
Sodium dichromate dihydrate	50	RC, EPA, GD, NTP	Oxidizing agent	298.00	NA	Inorganic compound; Sodium compound; Chromium compound	
Nicotine	50	MEIC, EDIT, RC (outlier), TESS, EPA, NTP	Pharmaceutical (stimulant)	162.020	1.17	Organic compound; Heterocyclic compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

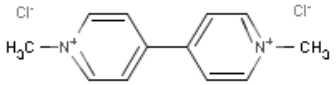
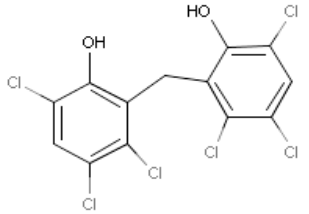
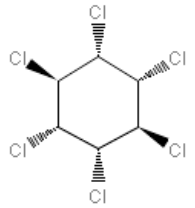
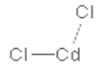
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
50 < LD_{50} ≤300 mg/kg							
Paraquat	58	MEIC, EDIT, RC (outlier), TESS, EPA	Pesticide (herbicide)	257.20	–4.22 @ pH 7.4	Organic compound; Heterocyclic compound	
Hexachlorophene	61	MEIC, RC, TESS, NTP	Disinfectant	406.91	6.91	Organic compound; Cyclic hydrocarbon; Phenol	
Lindane	76	MEIC, EDIT, RC (outlier), EPA, NTP	Pesticide (insecticide)	290.80	3.72	Organic compound; Halogenated hydrocarbon	
Cadmium II chloride	88	RC, TESS, GD, NTP	Consumer; Industrial products	183.31	NA	Inorganic compound; Cadmium compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

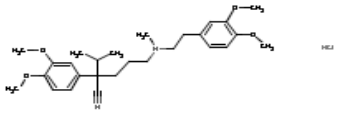
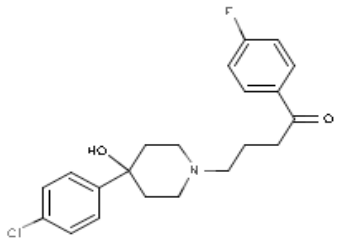
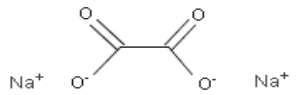
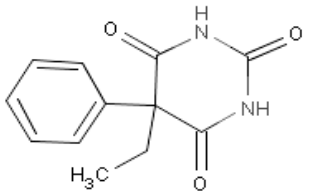
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Verapamil HCl	108	MEIC, EDIT, RC (outlier), TESS, NTP	Pharmaceutical (antiarrhythmic)	491.08	3.79	Organic compound; Amine	
Haloperidol	128*	MEIC, TESS	Pharmaceutical (antipsychotic)	375.90	3.36	Organic compound; Ketone	
Sodium oxalate	155	MEIC, EDIT, RC, TESS, NTP	Paints; Cleaners	134.00	NA	Organic compound; Carboxylic acid; Sodium compound	
Phenobarbital	163	MEIC, RC (outlier), TESS, NTP	Pharmaceutical (anticonvulsant)	232.23	1.47	Organic compound; Heterocyclic compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

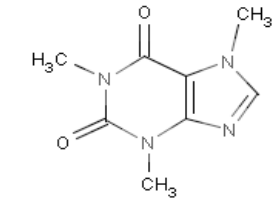
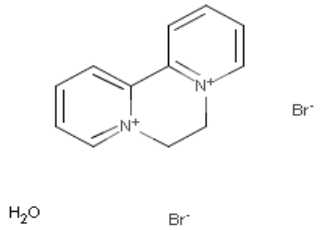
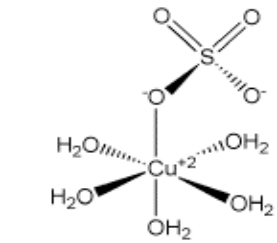
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Sodium I fluoride	180	MEIC, RC, TESS, EPA, NTP	Electroplating; Water fluoridation	41.99	NA	Inorganic compound; Sodium compound; Fluorine compound	
Caffeine	192	MEIC, RC (outlier), TESS, NTP, HPV	Pharmaceutical (stimulant); Food additive	194.20	–0.07	Organic compound; Heterocyclic compound	
Diquat dibromide	231	MEIC, RC, TESS	Pesticide (herbicide)	362.10	–3.05	Organic compound; Heterocyclic compound	
Cupric sulfate * 5 H2O	300	MEIC, RC, TESS, EPA, NTP	Pesticide (insecticide/fungicide)	249.70	NA	Inorganic compound; Sulfur compound; Metal	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

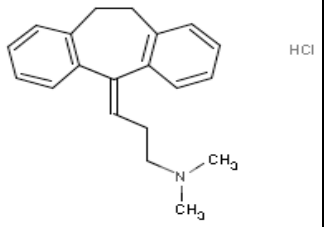
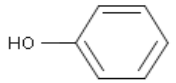
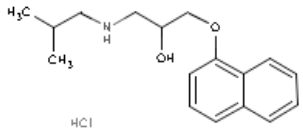
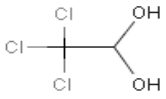
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
300 < LD_{50} ≤2000 mg/kg							
Amitriptyline HCl	319	MEIC, EDIT, RC, TESS	Pharmaceutical (antidepressant)	313.90	5.04	Organic compound; Polycyclic compound	
Phenol	414	MEIC, RC, TESS, EPA, NTP, HPV	Disinfectant	94.11	1.46	Organic compound; Phenol	
Propranolol HCl	470**	MEIC, RC, TESS, GD	Pharmaceutical (antiarrhythmic)	295.80	3.09	Organic compound; Alcohol; Amine; Polycyclic compound	
Chloral hydrate	479	MEIC, RC, TESS, NTP	Pharmaceutical (sedative)	165.40	0.99	Organic compound; Alcohol	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

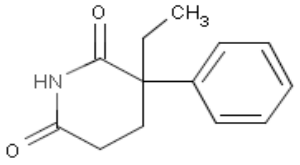
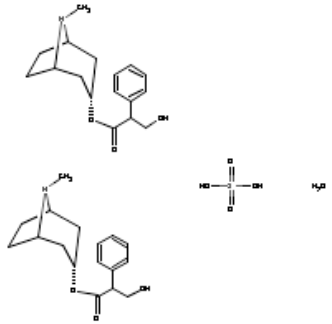
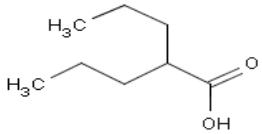
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Glutethimide	600	MEIC, RC, TESS	Pharmaceutical (sedative)	217.30	1.9	Organic compound; Heterocyclic compound	
Atropine sulfate	623	MEIC, EDIT, RC, TESS	Pharmaceutical (antimuscarinic)	694.80	1.83	Organic compound; Heterocyclic compound	
Valproic acid	1695 **	RC, MEIC, TESS, NTP	Pharmaceutical (anticonvulsant)	144.20	2.75	Organic compound; Carboxylic acid; Lipids	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

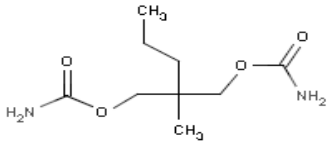
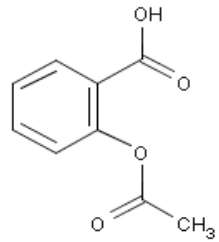
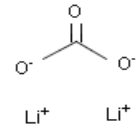
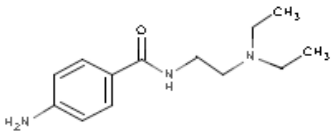
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Meprobamate	794*	MEIC, TESS	Pharmaceutical (antidepressant)	218.30	NA	Organic compound; Carboxylic acid	
Acetylsalicylic acid	1000	MEIC, EDIT, RC, TESS, NTP	Pharmaceutical (analgesic)	180.20	1.19	Organic compound; Carboxylic acid; Phenol	
Lithium I carbonate	1187[7]	MEIC, RC, TESS, NTP (Cl salt)	Pharmaceutical (mood stabilizer)	73.89	NA	Inorganic compound; Lithium compound; Alkylies; Carbon compound	
Procainamide	1950*	MEIC, TESS	Pharmaceutical (antiarrythmic)	271.79	NA	Organic compound; Carboxylic acid; Amide	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

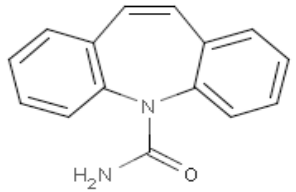
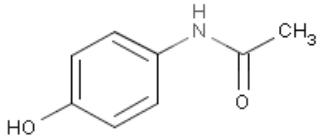
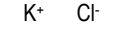
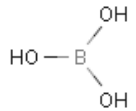
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Carbamazepine	1957*	MEIC, TESS	Pharmaceutical (antiepileptic)	236.30	2.45	Organic compound; Heterocyclic compound	
2000 < LD_{50} ≤5000 mg/kg							
Acetaminophen	2404	MEIC, EDIT, RC, TESS, NTP	Pharmaceutical (analgesic)	151.20	0.8	Organic compound; Amide	
Potassium I chloride	2602	MEIC, RC, TESS, NTP	Pharmaceutical (electrolyte); Manufacturing	74.55	NA	Inorganic compound; Potassium compound; Chlorine compound	
Boric aid	2660*	TESS, EPA, NTP	Pesticide (insecticide)	61.83	NA	Inorganic compound; Boron compound; Acids	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

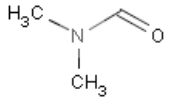
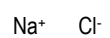
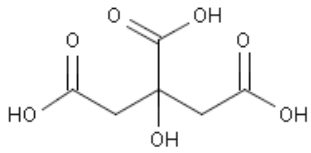
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Carbon tetrachloride	2799	MEIC, RC, TESS, NTP, HPV	Solvent	153.82	2.83	Organic compound; Halogenated hydrocarbon	
Dimethylformamide	2800	RC, GD, NTP, HPV	Solvent	73.10	–1.01	Organic compound; Amide; Carboxylic acid	
Sodium chloride	2998	MEIC, EDIT, RC, TESS, EPA, NTP	Pharmaceutical (electrolyte); Food additive	58.44	NA	Inorganic compound; Sodium compound; Chlorine compound	
Citric Acid	3000*	EPA, NTP, HPV	Food additive	192.10	–1.72	Organic compound; Carboxylic acid	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

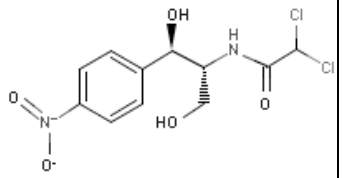
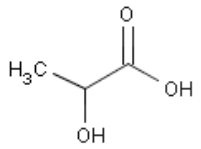
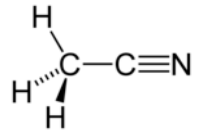
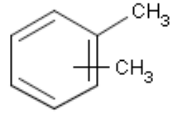
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Chloramphenicol	3393	MEIC, RC, NTP	Pharmaceutical (antibiotic)	323.14	1.14	Organic compound; Alcohol; Cyclic hydrocarbon; Nitro compound	
Lactic acid	3730	RC, NTP, HPV	Food additive	90.08	–0.72	Organic compound; Carboxylic acid	
Acetonitrile	3798	RC, NTP, HPV	Solvent	41.05	–0.34	Organic compound; Nitrile	
Xylene (mixed isomers)	4300	MEIC, RC, TESS, NTP, HPV	Solvent	106.17	3.12 – 3.2	Organic compound; Cyclic hydrocarbon	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

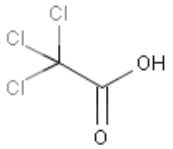
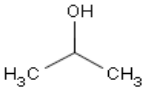
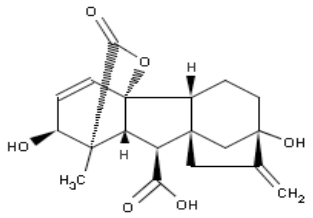
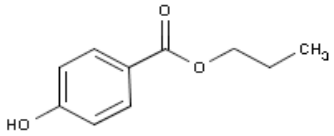
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Trichloroacetic acid	4999	RC, NTP	Fixative	163.40	1.33	Organic compound; Carboxylic acid	
LD_{50} >5000 mg/kg							
2-Propanol	5843	MEIC, RC, TESS, EPA, NTP, HPV	Disinfectant	60.10	0.05	Organic compound; Alcohol	
Gibberellic acid	6305	RC, EPA, NTP	Plant growth regulator	346.38	0.24	Organic compound; Polycyclic compound	
Propylparaben	6326**	RC (outlier), NTP	Food additive	180.20	3.04	Organic compound; Carboxylic acid; Phenol	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

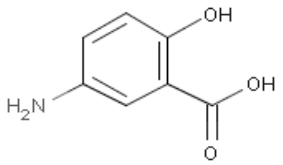
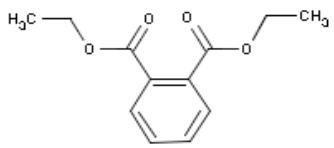
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
5-Aminosalicylic acid	7749**	RC (outlier), NTP	Pharmaceutical (antibiotic)	153.10	1.32	Organic compound; Carboxylic acid; Phenol	
Ethylene glycol	8567	MEIC, EDIT, RC, TESS, NTP, HPV	Antifreeze	62.07	–1.36	Organic compound; Alcohol	
Diethyl phthalate	8602	RC (outlier), NTP, HPV	Plasticizer	222.20	2.47	Organic compound; Carboxylic acid	
Sodium hypochlorite	8910[8]	TESS, NTP	Disinfectant	74.44	NA	Inorganic compound; Sodiumcompound; Oxygen compound; Chlorine compound	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

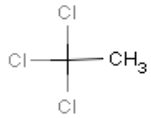
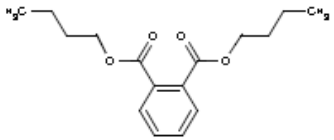
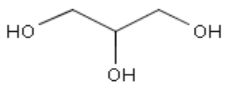
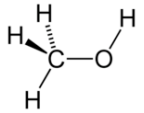
GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
1,1,1-Trichloroethane	10298	MEIC, RC, NTP, HPV	Solvent	133.41	2.49	Organic compound; Halogenated hydrocarbon	
Dibutyl phthalate	11998	RC (outlier), NTP, HPV	Plasticizer	278.30	4.9	Organic compound; Carboxylic acid	
Glycerol	12691	RC, GD, NTP, HPV	Solvent	92.09	-1.76	Organic compound; Alcohol	
Methanol	13012	MEIC, EDIT, RC, TESS, NTP, HPV	Solvent	32.04	–0.77	Organic compound; Alcohol	

Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity

GHS Category[1]/Substance	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	Molecular Weight (g/mol)	log Kow[5]	Chemical Class[6]	Molecular Structure
Ethanol	14008	MEIC, RC (outlier), TESS, EPA, NTP, HPV	Solvent	46.07	–0.31	Organic compound; Alcohol	

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); LD_{50}=Dose that produces lethality in 50% of the test animals; K_{ow}=Octanol:water partition coefficient; EDIT=Evaluation-guided Development of New *In vitro* Test Batteries (substances in EDIT program are a subset of the MEIC substance set); EPA=Pesticides registered with the Environmental Protection Agency; EHS=EPA's Extremely Hazardous Substance list; HPV=High Production Volume chemicals (i.e., those that are imported into or produced in the United States in amounts ≥1,000,000 lbs/year); GD=*Guidance Document* (ICCVAM 2001b); MEIC=Multicentre Evaluation of *In Vitro* Cytotoxicity; NA=Non applicable; NTP=National Toxicology Program; RC=Registry of Cytotoxicity with the chemicals classified as regression outliers shown in parentheses; TESS=Toxic Exposure Surveillance System (Litovitz et al. 2000); HSDB=Hazardous Substances Data Bank; RTECS®=Registry of Toxic Effects of Chemical Substances.

*From RTECS® (MDL Information Systems 2002).

**Mouse.

[1]GHS category designation for the substance (e.g., LD_{50} <5 mg/kg)

[2]LD_{50} data are from the Registry of Cytotoxicity (Halle 1998, 2003) and are for rats, unless otherwise noted. The LD_{50} values are rounded to the nearest whole number.

[3]Sources used to identify candidate chemicals.

[4]Product/use categories from HSDB (NLM 2002) or RTECS®(MDL Information Systems 2002). Pharmaceutical uses from Gilman et al. (1985) or Thomson PDR® (2004).

[5]From HSDB (NLM 2001, 2002) or Material Safety Data Sheets.

[6]Based on Medical Subject Heading [MeSH®] descriptors (NLM 2005).

[7]Mouse data for lithium sulfate (Halle 1998, 2003).

[8]From HSDB (NLM 2002).

Table 3-3 Distribution of Candidate Substances and Reference Substances by Source[1] and Toxicity Category

GHS Category (mg/kg)	Reference Substances/ Candidate Substances	MEIC Reference/ MEIC Candidates	EDIT Reference/ EDIT Candidates	TESS Reference/ TESS Candidates	NTP Reference/ NTP Candidates	HPV Reference/ HPV Candidates
$LD_{50} \leq 5$	12/13	2/2	1/1	3/3	5/9	0/0
$5 < LD_{50} \leq 50$	12/15	6/6	5/5	9/10	8/11	2/5
$50 < LD_{50} \leq 300$	12/26	11/17	4/5	11/19	9/18	1/3
$300 < LD_{50} \leq 2000$	12/38	12/29	3/5	12/27	5/23	1/5
$2000 < LD_{50} \leq 5000$	12/12	6/6	2/2	6/6	12/12	6/6
$LD_{50} > 5000$	12/12	5/5	2/2	5/5	12/12	8/8
Total	72/116	42/65	17/20	46/70	51/85	18/27

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); LD_{50}=Dose that produces lethality in 50% of the test animals; MEIC=Multicentre Evaluation of *In Vitro* Cytotoxicity**;** EDIT=Evaluation-Guided Development of *In vitro* Tests; TESS=Toxic Exposure Surveillance System; NTP=U.S. National Toxicology Program; HPV=U.S. Environmental Protection Agency (EPA) High Production Volume program.
[1]Substances may have been selected from more than one source (see **Table 3-2** and **Appendix F3**).

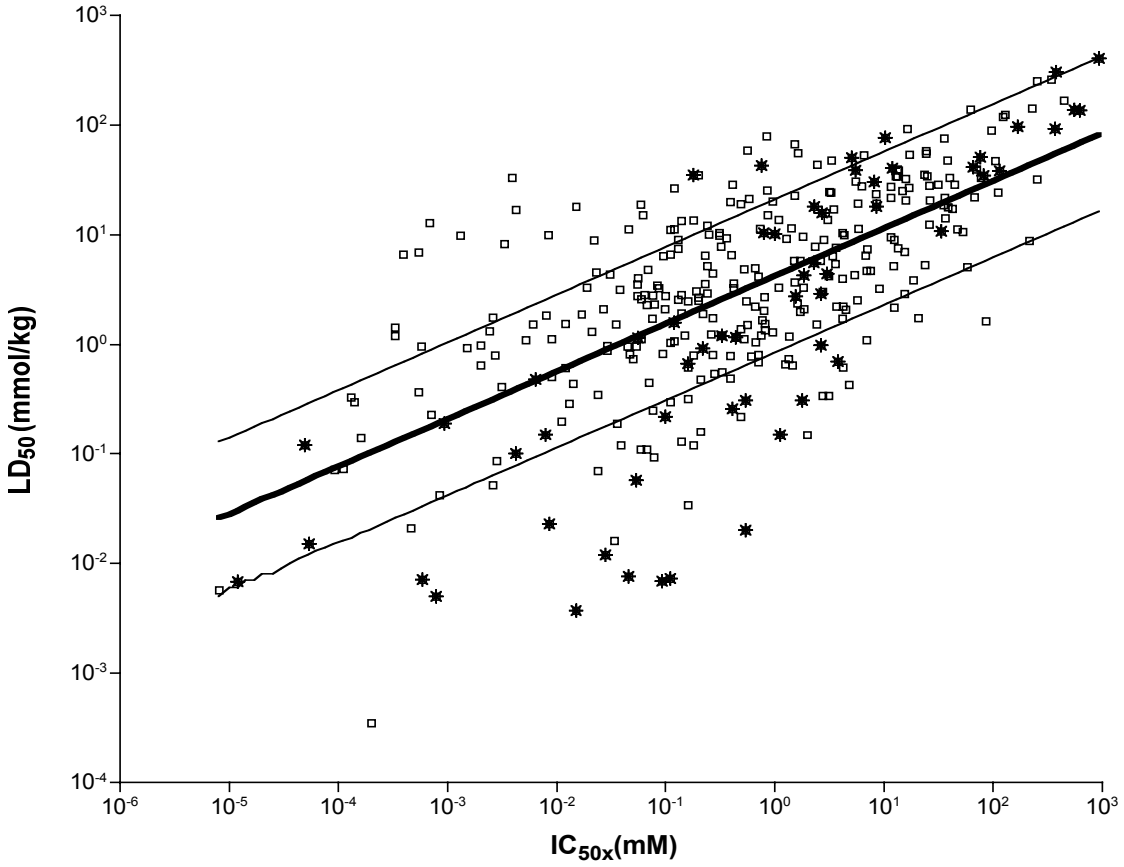
Table 3-4 Selected Substances: Distribution of RC Chemicals and RC Outliers[1] by Toxicity Category

GHS Category (mg/kg)	RC Outliers/ Total Chemicals	Candidate and Selected Substances		
		Candidate Substances	RC Reference / RC Candidates	RC Reference Outliers/ RC Reference Chemicals
$LD_{50} \leq 5$	10/11 (91%)	13	9/10	8/9 (89%)
$5 < LD_{50} \leq 50$	15/26 (58%)	15	8/10	4/8 (50%)
$50 < LD_{50} \leq 300$	24/70 (34%)	26	11/18	5/11 (45%)
$300 < LD_{50} \leq 2000$	14/139 (10%)	38	9/29	0/9 (0%)
$2000 < LD_{50} \leq 5000$	12/57 (21%)	12	10/10	0/10 (0%)
$LD_{50} > 5000$	20/44 (45%)	12	11/11	5/11 (45%)
Total	95/347 (27%)	116	58/88	22/58 (38%)

Abbreviations: RC=Registry of Cytotoxicity; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); LD_{50}=Dose that produces lethality in 50% of the test animals.
[1]Chemicals falling outside the log 5 (i.e., > ±0.699) prediction interval for the RC millimole regression (Halle 1998, 2003).

Among the 58 RC substances selected for use in the validation study, 22 (38%) were outliers for the RC millimole regression. Toxicity[1] was underpredicted for 17 (77%) of these outlier substances and overpredicted (i.e., predicted LD_{50} was lower than measured *in vivo* LD_{50}) for the remaining five (23%). For the 95 outlier substances in the RC, the number of substances for which toxicity was over- or under-predicted was approximately the same. Toxicity was underpredicted for 49 (52%) outliers and overpredicted for 46 (48%) outliers (Halle 1998, 2003). **Figure 3-1** shows the 58 RC chemicals selected for testing, in addition to the 289 RC chemicals that were not selected, and the RC millimole regression. In the figure, the outliers are those points outside the RC prediction interval. For the 58 RC substances selected for testing, the majority (17/22) of the outliers are below the RC millimole regression line.

Figure 3-1 The Fifty-Eight (58) Selected RC Reference Substances on the RC Millimole Regression



Abbreviations: RC=Registry of Cytotoxicity; LD_{50}=Dose that produces lethality in 50% of the test animals; IC_{50}=Test substance concentration that reduces cell viability by 50%.
The 58 RC chemicals tested in the NICEATM/ECVAM validation study are shown by *. The RC regression, $\log (LD_{50}) = 0.435 \times \log (IC_{50x}) + 0.625$, is shown by the bold line. The lighter lines show the ± log 5 (i.e., ±0.699) prediction interval (Halle 1998, 2003). The open boxes represent the 289 chemicals not included in the validation study.

[1] Toxicity is inversely proportional to LD_{50}. High LD_{50} values reflect low toxicity and low LD_{50} values reflect high toxicity

3.2.2 Chemical Classes Represented by the Selected Reference Substances

Medical subject heading (MeSH®) descriptors from the NLM were used to determine chemical class designations for the selected substances. Of the 72 reference substances, 57 (79%) were organic and 15 (21%) were inorganic. The number of substances in the organic (79) and inorganic (31) subclasses is greater than the number of substances in each class because some of the substances are classified in more than one subclass. The most commonly represented classes of organic compounds were heterocyclics (14/57, 25%), carboxylic acids (14/57, 25%), and alcohols (10/57, 18%). **Table 3-5** shows the distribution of the substances among the GHS toxicity categories. The 14 heterocyclics were evenly distributed among the first four GHS toxicity categories for LD_{50} ≤2000 mg/kg with the majority of the heterocyclics (11/14) in the categories for LD_{50} <300 mg/kg. The majority of the carboxylic acids (12/14) and alcohols (8/10) had an LD_{50} >300 mg/kg, while the majority of the inorganics (10/15) had an LD_{50} <300 mg/kg.

3.2.3 Product/Use Classes Represented by the Selected Reference Substances

Product and use information was obtained from HSDB (NLM 2002) or RTECS® (MDL Information Systems 2002). The number of assigned uses (77) is greater than the number of selected substances because some of the substances have more than one use. **Table 3-6** shows the distribution of products and uses of the selected substances according to their GHS categories. Pharmaceutical (27/77; 35%) and pesticide (17/77; 22%) uses were observed most frequently. The toxicity category of 300 < LD_{50} ≤2000 mg/kg had the highest number of pharmaceuticals. Every toxicity category except LD_{50} >5000 mg/kg had at least four substances with pharmaceutical uses. The majority of pesticides (16/17; 94%) had an LD_{50} <300 mg/kg. The next most frequent uses were as solvents (8/77; 10%) and food additives (5/77; 6%); LD_{50} >2000 mg/kg contained most of the substances with solvent (8/8; 100%) and food additive (4/5; 80%) uses.

3.2.4 Toxicological Characteristics of the Selected Reference Substances

3.2.4.1 *Corrosivity*

The intent of the SMT was to prioritize only those substances with low corrosivity because guidelines for acute systemic toxicity testing indicate that corrosive or severely irritating substances need not be tested (OECD 2001a, c, d). The UN and U.S. Department of Transportation Packing Group (DOT PG) classification system was used to classify the corrosivity hazard associated with the candidate substances. However, after substance selection was completed and testing had begun, the SMT learned that the PG classification system was also based on hazards other than corrosivity (e.g., dermal and inhalation toxicity, flammability, etc.). Therefore, the selected substances were not actually prioritized by corrosivity. Subsequent information on the corrosivity of the selected substances was obtained from HSDB (NLM 2004) and the Material Safety Data Sheets (MSDS) provided with the purchased substances. Seven substances that were not identified by the DOT PG classification system had corrosive notations. The MSDS notations for lactic acid, sodium hypochlorite, sodium oxalate, and trichloroacetic acid indicated that these substances should carry a corrosive label. Chloral hydrate, mercury II chloride, and potassium cyanide were noted by HSDB to be corrosive to eyes or skin.

Table 3-5 Distribution of Chemical Class for the 72 Reference Substances by Toxicity Category

Chemical Class[1]	GHS Acute Oral Toxicity Category (mg/kg)						Total
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2000$	$2000 < LD_{50} \leq 5000$	$LD_{50} > 5000$	
Organic							
Carboxylic acid	1	0	1	4	4	4	14
Heterocyclic compound	5	2	4	3	0	0	14
Alcohol	2	0	0	2	1	5	10
Phenol	0	0	1	2	0	2	5
Polycyclic compound	0	2	0	2	0	1	5
Sulfur compound	4	1	0	0	0	0	5
Amine	1	0	1	1	0	0	3
Cyclic hydrocarbon	0	0	1	0	1	1	3
Halogenated hydrocarbon	0	0	1	0	1	1	3
Organophosphorous compound	2	1	0	0	0	0	3
Amide	0	0	0	1	2	0	3
Nitrile	0	1	0	0	1	0	2
Acyclic hydrocarbon	1	0	0	0	0	0	1
Carbohydrate	0	1	0	0	0	0	1
Ester	0	1	0	0	0	0	1
Ether	0	1	0	0	0	0	1
Ketone	0	0	1	0	0	0	1
Lipid	0	0	0	1	0	0	1
Nitro compound	0	0	0	0	1	0	1
Organometallic compound	0	1	0	0	0	0	1
Sodium compound	0	0	1	0	0	0	1
Urea	1	0	0	0	0	0	1
Total Organics	17	11	11	16	11	14	79

Table 3-5 Distribution of Chemical Class for the 72 Reference Substances by Toxicity Category

Chemical Class[1]	GHS Acute Oral Toxicity Category (mg/kg)						Total
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2000$	$2000 < LD_{50} \leq 5000$	$LD_{50} > 5000$	
Inorganic							
Sodium compound	1	2	1	0	1	1	6
Chlorine compound	1	0	1	0	2	1	5
Arsenical	0	2	0	0	0	0	2
Metal	0	1	1	0	0	0	2
Potassium compound	0	1	0	0	1	0	2
Sulfur compound	0	1	1	0	0	0	2
Acid	0	0	0	0	1	0	1
Alkalies	0	0	1	0	0	0	1
Boron compound	0	0	0	0	1	0	1
Cadmium compound	0	0	1	0	0	0	1
Carbon compound	0	0	0	1	0	0	1
Chromium compound	0	1	0	0	0	0	1
Fluorine compound	0	0	1	0	0	0	1
Lithium compound	0	0	0	1	0	0	1
Mercury compound	1	0	0	0	0	0	1
Nitrogen compound	0	1	0	0	0	0	1
Oxygen compound	0	0	0	0	0	1	1
Selenium compound	1	0	0	0	0	0	1
Total Inorganic	4	9	7	2	6	3	31

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
[1]Based on the Medical Subject Heading [MeSH®] descriptor (NLM 2005). Some substances are counted more than once because they appear in more than one subclass under the organic or inorganic classes.

Table 3-6 Distribution of Product/Use[1] Class for the 72 Reference Substances by Toxicity Category

Product/Use Class[1]	GHS Acute Oral Toxicity Category (mg/kg)						Total
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2000$	$2000 < LD_{50} \leq 5000$	$LD_{50} > 5000$	
Antibiotic/fungicide	1	0	0	0	0	0	1
Antifreeze	0	0	0	0	0	1	1
Consumer/industrial products	0	0	1	0	0	0	1
Disinfectant	0	0	1	1	0	2	4
Electroplating	0	2	0	0	0	0	2
Fluoridation	0	0	1	0	0	0	1
Feed additive	1	0	0	0	0	0	1
Fixative	0	0	0	0	1	0	1
Food additive	0	0	1	0	3	1	5
Manufacturing	1	0	0	0	1	0	2
Oxidizing agent	0	1	0	0	0	0	1
Paints, cleaners	0	0	1	0	0	0	1
Pesticide	5	7	4	0	1	0	17
Pharmaceutical	4	3	4	11	4	1	27
Plant growth regulator	0	0	0	0	0	1	1
Plasticizer	0	0	0	0	0	2	2
Preservative	1	0	0	0	0	0	1
Solvent	0	0	0	0	4	4	8

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
[1]Product/use information from Hazardous Substances Data Bank (NLM 2002) or Registry of Toxic Effects of Chemical Substances ([RTECS®], MDL Information Systems 2002). Some substances are counted more than once because they appear in more than one use category.

3.2.4.2 *Toxicity Targets*

As shown in **Appendix F2**, the most common toxicological effects in humans or rodents were neurological (40 substances); 26 cause central nervous system (CNS) depression, seven produce CNS stimulation, four produce CNS affects such as encephalopathy, and three affect the peripheral nervous system. Other common target systems include the liver (17 substances), kidney (15 substances), and cardiovascular system (10 substances). No target organ information was available for gibberellic acid. Among the 72 reference substances, 27 had more than one toxicity target.

3.2.4.3 *Metabolism*

Table 3-7 shows the 22 reference substances that are known or expected to produce active/toxic metabolites *in vivo*. In contrast, dichlorvos, fenpropathrin, meprobamate, phenylthiourea, and sodium dichromate are rapidly metabolized to less toxic compounds. Because the NHK and 3T3 cells have little (Babich 1991) or no (INVITTOX 1991) metabolic capability, respectively, metabolites of these compounds would not be expected to be present *in vitro*. **Appendix F2** provides for more information on the metabolism (activation/inactivation) of the selected reference substances.

Table 3-7 Reference Substances Metabolized to Active Metabolites

Known to Have Active Metabolites				Active Metabolites Expected
Acetaminophen	Carbamazepine	Digoxin	Methanol	Carbon tetrachloride
Acetonitrile	Chloral hydrate	Disulfoton	Parathion	Triethylenemelamine
Acetylsalicylic acid	Cycloheximide	Ethanol	Procainamide HCl	Valproic acid
Amitriptyline HCl	Dibutyl phthalate	Ethylene glycol	Verapamil HCl	
Busulfan	Diethyl phthalate	Glutethimide		

3.2.5 Selection of Reference Substances for Testing in Phases Ib and II

Based on the *Guidance Document* (ICCVAM 2001b) recommendation that 10 to 20 substances be tested to qualify candidate *in vitro* cytotoxicity tests for determining starting doses for rodent acute oral toxicity assays, 12 reference substances were chosen from among the 72 reference substances for testing in Phases Ib and II (see **Table 3-8**). The criteria for choosing these reference substances, in order of importance, were:

- Two reference substances must be included from each of the five GHS toxicity categories and the unclassified category.
- The log LD_{50} (mmol/kg) must be within the prediction interval (±0.699) of the RC millimole regression. The *Guidance Document* (ICCVAM 2001b) recommends that reference substances for evaluating an *in vitro* basal cytotoxicity test to use with the RC millimole regression fit the regression as closely as possible.
- MEIC chemicals must be included. Cytotoxicity data from these phases (and Phase III of this study), and the available human toxicity information for the MEIC chemicals, could be used to build a prediction model for estimating

human LC values. The Phase Ib reference substances arsenic trioxide and ethylene glycol are also EDIT chemicals (subset of MEIC chemicals).

If more than two substances in a GHS category met the above criteria, reference substances were selected so that the LD_{50} was as close to the RC millimole regression as possible and/or to represent the full range of toxicity in each GHS category.

Table 3-8 Reference Substances Tested in Phases Ib and II

Reference Substances	CASRN	RC Reference No.	MEIC Reference No.	Rodent Oral LD_{50}[1] (mg/kg)	Observed – Predicted log LD_{50}[2]
LD_{50} ≤5 mg/kg					
Aminopterin	54-62-6	3	NA	3	-0.652
Sodium selenate	13410-01-0	NA	NA	1.6[3]	NA
5 < LD_{50} ≤50 mg/kg					
Colchicine	64-86-8	6	60	6[4]	-0.593
Arsenic III trioxide	1327-53-3	153	26	20	-0.591
50 < LD_{50} ≤300 mg/kg					
Cadmium II chloride	10108-64-2	81	NA	88	0.011
Sodium I fluoride	7681-49-4	106	14	180	-0.109
300 < LD_{50} ≤2000 mg/kg					
DL-Propranolol HCl	350-60-90	54	23	470[4]	-0.023
Lithium I carbonate	544-13-2	327[4]	20	1187[4,5]	-0.256[4]
2000 < LD_{50} ≤5000 mg/kg					
Potassium I chloride	7447-40-7	346	50	2602	0.085
Chloramphenicol	56-75-7	91	45	3393	0.441
LD_{50} >5000 mg/kg					
2-Propanol	67-63-0	128	10	5843	0.396
Ethylene glycol	107-21-1	360	7	8567	0.321

Abbreviations: CASRN=Chemical Abstracts Service Registry Number; RC=Registry of Cytotoxicity; MEIC=Multicentre Evaluation of *In Vitro* Cytotoxicity; NA=Not applicable *(*i.e., substances not included in the RC and/or MEIC studies); RTECS®=Registry of Toxic Effects of Chemical Substances.

[1]From the RC (Halle 1998, 2003) unless otherwise indicated. Data are for rats unless otherwise indicated.

[2]Available only for substances included in the RC. This figure characterizes the log LD_{50} deviation from the RC regression. Outliers are > ±0.699 from the regression line.

[3]RTECS® (MDL Information Systems 2002).

[4]Mouse data.

[5]For lithium sulfate.

Only nine of the 72 reference substances met all three criteria. In the most toxic category (i.e., LD_{50} ≤5 mg/kg), only one RC chemical, aminopterin, was within 0.699 of the RC millimole regression. Sodium selenate was selected as the second reference substance in this category even though its fit to the RC millimole regression was not known. Neither aminopterin nor sodium selenate were MEIC chemicals. For the 50 < LD_{50} ≤300 mg/kg category, cadmium chloride was selected over the MEIC chemicals cupric sulfate $5H_2O$, diquat dibromide, sodium oxalate, and hexachlorophene because it fit the RC millimole regression better than the four MEIC chemicals (the observed LD_{50} minus log predicted LD_{50} values were -0.534 to -0.337).

3.2.6 Unsuitable and Challenging Reference Substances

Several reference substances could not be adequately tested for cytotoxicity in 3T3 cells and/or NHKs in from one to all three of the laboratories. The following reference substances did not produce sufficient toxicity at soluble concentrations for calculation of an IC_{50} at the highest concentrations tested under the testing conditions used in the study (see also **Tables 5-2, 5-4,** and **5-5**):

- Carbon tetrachloride (no 3T3 or NHK NRU IC_{50} data from ECBC, FAL, or IIVS)
- Xylene (no 3T3 or NHK NRU IC_{50} data from ECBC or FAL)
- Methanol (no 3T3 NRU IC_{50} data from ECBC, FAL, or IIVS; no NHK NRU IC_{50} data from ECBC)
- Lithium carbonate (no 3T3 NRU IC_{50} data from FAL or IIVS)
- 1,1,1-Trichloroethane (no 3T3 NRU IC_{50} data from FAL or IIVS; no NHK NRU IC_{50} data from ECBC)
- Valproic acid (no 3T3 NRU IC_{50} data from ECBC or FAL; no NHK NRU IC_{50} data from ECBC, FAL, or IIVS)

Other reference substances were difficult to test because of volatility or lack of toxicity, but three acceptable tests could be obtained after a number of trials.

- Acetonitrile and 2-propanol were highly volatile and nontoxic, so that even with the use of film plate sealers, from one to seven tests failed the VC and data points test acceptance criteria at each laboratory.
- Disulfoton failed at least one test in both test methods at ECBC and FAL because of inadequate toxicity (i.e., an IC_{50} could not be detected) and insolubility. All laboratories reported precipitate in the test plates for 3T3 and NHK NRU tests. IIVS had no failed tests in either test method.
- Dibutyl phthalate failed one 3T3 NRU test at ECBC and one NHK NRU test at FAL because of inadequate toxicity and solubility.
- Lindane failed one 3T3 NRU test at FAL because of inadequate toxicity and solubility and one because of its volatility.
- Parathion failed one test because of inadequate toxicity and solubility in both test methods and one NHK NRU test because of volatility at FAL.
- Diethyl phthalate failed one NHK NRU test because of volatility at FAL.
- Digoxin (all laboratories), gibberellic acid (ECBC and FAL), and strychnine (ECBC and FAL) failed at least one 3T3 NRU test because of inadequate toxicity and solubility.

3.3 Reference Substance Procurement, Coding, and Distribution

BioReliance collected information from the suppliers of the reference substances on their analytical purity, composition, and stability (see **Appendix F1**), tested the reference substances for solubility, packaged them into 4 g aliquots for shipment to the testing laboratories, and archived two additional samples. All reference substances were given a random number code that was unique for each testing facility to conceal the identities from the testing laboratories. Approximately 100 g of the PC substance, SLS, was distributed, uncoded, to each laboratory and one additional sample was archived.

Reference substances were packaged so as to minimize damage during transit, and shipped under appropriate storage conditions and according to the appropriate regulatory transportation procedures. Testing facilities were notified upon shipment in order to prepare for receipt. With the exception of the PC substance which was shipped directly to the Study Directors, the reference substances were shipped to the test facility Safety Officers. Shipments were accompanied by a sealed information packet containing the appropriate health and safety procedures (i.e., MSDS or equivalent documentation with information regarding the proper protection for handling, procedures for dealing with accidental ingestion or contact with skin or eyes, and for containing and recovering spills), and a code disclosure key. Also provided was a data sheet giving a minimum of essential information needed by the testing laboratory for each reference substance, including color, odor, physical state, weight or volume of sample, specific density for liquid reference substances, and storage instructions. The shipment directed the Safety Officer to:

- Notify BioReliance and the SMT upon receipt of reference substances
- Retain the health and safety package and provide the coded reference substances and chemical data sheets with minimum essential information to the laboratory Study Director without revealing the identities of the test substances
- Notify the SMT if test facility personnel open the health and safety packet at any time, for any reason, during the study
- Return the unopened health and safety package to BioReliance after testing is completed

3.3.1 Exceptions

The Safety Officer for ECBC required the information on reference substance codes before the substances were shipped in order to satisfy the facility's environmental procedures and requirements. The reference substance codes were stored in a classified safe located in the Safety Office which was in a building separate from the cytotoxicity testing laboratory, and were to be opened only by the Safety Officer. The ECBC Safety Officer opened the sealed health and safety packets for lithium carbonate and ethanol upon receipt of those substances because the code information for these substances was not included in the list originally provided. ECBC cytotoxicity testing personnel did not have direct access to the reference substance codes.

3.4 Reference Substances Recommended by the *Guidance Document*

The *Guidance Document* specifically recommended testing the following 11 substances to validate candidate *in vitro* basal cytotoxicity assays: sodium dichromate dihydrate, cadmium chloride, *p*-phenylenediamine, DL-propranolol HCl, trichlorfon, ibuprofen, nalidixic acid, salicylic acid, antipyrene, dimethylformamide, and glycerol (ICCVAM 2001b). Of these 11 substances (see **Appendix F3** and **Section 3.1.2**), five (sodium dichromate dihydrate, cadmium chloride, DL-propranolol HCl, dimethylformamide, and glycerol) were chosen for testing after the candidate substances were prioritized as described in **Section 3.1.3**. The seven that were not selected did not satisfy the selection criteria (e.g., not MEIC chemicals, not identified as high exposure risk in TESS)

3.5 Summary

Seventy-two reference substances were selected for testing in the NICEATM/ECVAM validation study. These substances were selected to represent: (1) the complete range of *in vivo* acute oral LD_{50} values; (2) the types of substances regulated by the various regulatory authorities; and (3) those with human toxicity data and/or human exposure potential. To insure that the complete range of toxicity was covered, the GHS (UN 2005) was used to select 12 substances for each acute oral toxicity category and 12 unclassified substances. The set of selected reference substances had the following characteristics:

- Thirty-five percent (27/77 uses) were pharmaceuticals, 22% (17/77 uses) were pesticides, 10% (8/77 uses) were solvents, and 6% (5/77 uses) were food additives. The remaining substances were used for a variety of manufacturing and consumer products.
- In terms of relevance of the substances to human exposure, 58% (42/72) were included in the MEIC study (substances chosen because of availability of human lethality data), 24% (17/72) were included also in the EDIT program (EDIT substances are a subset of the MEIC substances), 64% (46/72) had human exposure data reported by TESS, 71% (51/72) had been evaluated by NTP, and 25% (18/72) were on the EPA HPV list.
- Eighty-one percent (58/72) of the substances were in the RC and 38% (22/58) of these were outliers with respect to the RC millimole regression. The RC millimole regression underpredicted the toxicity of 77% (17/22) of the outliers and overpredicted the toxicity of 23% (5/22). For the 95 outlier substances in the RC, however, the number of substances for which toxicity was over- or under-predicted was approximately the same (i.e., toxicity was underpredicted for 49 [52%] outliers and overpredicted for 46 [48%] outliers [Halle 1998, 2003]).
- Seventy-nine percent (57/72) were organic compounds and 21% (15/72) were inorganic. The most commonly represented classes of organic compounds were heterocyclics (25%, 14/57), carboxylic acids (25%, 14/57), and alcohols (18%, 10/57).
- Nineteen substances (26%, 19/72,) were known to have active metabolites and three others were expected to have active metabolites based on their chemical structures.
- Many of the substances produced toxicity in more than one organ system. The most common target systems were neurological (40 substances), liver (17 substances), kidney (15 substances), and cardiovascular (10 substances). No target organ information was available for one substance (gibberellic acid).

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4.0 RODENT ACUTE ORAL LD_{50} REFERENCE VALUES USED TO ASSESS THE ACCURACY OF THE 3T3 AND NHK NRU TEST METHODS

The procedures and analyses presented in this section were designed to identify the most accurate rodent acute oral LD_{50} values for the 72 reference substances used in the validation study. These values were needed to ensure that the reference substances were correctly placed within the different GHS toxicity categories and to provide a data set against which to compare the predicted LD_{50} values estimated using the IC_{50} data obtained from the 3T3 and NHK NRU test methods (see **Section 6**). The predicted LD_{50} values are used to determine the starting dose for rodent acute oral toxicity tests and the more accurate the prediction, the fewer the number of rodents that would be used in an acute oral toxicity test (see **Sections 1.0** and **1.2.2**).

4.1 Methods Used to Obtain Rodent Acute Oral LD_{50} Reference Values

4.1.1 Identification of Candidate Rodent Acute Oral LD_{50} Reference Data

No animal testing was performed to obtain the rodent oral acute LD_{50} reference data for this validation study. To identify reference data for the 72 substances, rat acute oral LD_{50} studies were located using literature searches, secondary references, and electronic database searches. Literature searches were conducted in PubMed (U.S. NLM) and the Institute of Scientific Information (ISI) Web of Science® (Thomson Scientific, Philadelphia, PA) using each chemical name and "lethal dose 50" as search terms. Secondary references included NTP technical reports, Toxicological Profiles from the Agency for Toxic Substances and Disease Registry (ATSDR), Cosmetic Ingredient Reviews by the Cosmetics Industry Council, pesticide handbooks, the Merck Index, and various other summary sources. **Table 4-1** lists the electronic databases searched to locate references for rat oral LD_{50} values. Rat LD_{50} data were preferred because:

- The current acute oral toxicity test guidelines recommend using rats (OECD 2001a, c, d; EPA 2002a)
- The majority of LD_{50} data used in the RC millimole regression were from studies using rats (282 rat data points and 65 mouse data points) (Halle 1998, 2003)
- The majority of acute oral systemic toxicity testing is performed with rats

Table 4-1 Internet-Accessible Databases Searched for LD_{50} Information

Database/Source[1]	Sponsor(s)
Agency for Toxic Substances and Disease Registry (ATSDR)	U.S. Department of Health and Human Services (DHHS)
Center for Drug Evaluation and Research (CDER)	U.S. Food and Drug Administration (FDA)
CHEMFINDER	CambridgeSoft Corporation
Chemical Carcinogenesis Research Information System (CCRIS); National Cancer Institute (NCI) Website	NCI; National Institutes of Health (NIH); DHHS
Chemical Evaluation Search and Retrieval System (CESARS)	Michigan Department of Natural Resources; Ontario Ministry of the Environment; Canadian Centre for Occupational Health and Safety (CCOHS) CHEMpendium™
Chemical Hazard Response (CHRIS)	U.S. Coast Guard

Table 4-1 Internet-Accessible Databases Searched for LD_{50} Information

Database/Source[1]	Sponsor(s)
Chemical Ingredients Database	U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP); California EPA Department of Pesticide Regulation
CHEMINDEX; CHEMINFO	(CCOHS) CHEMpendium™
ChemRTK High Production Volume (HPV) Challenge Program; OPPT Chemical Fact Sheets; Chemical Information Collection and Data Development	EPA Office of Pollution Prevention and Toxics (OPPT)
CIS Chemical Information	World Health Organization (WHO) International Programme on Chemical Safety (IPCS); CCOHS; International Labour Organisation (ILO) Occupational Safety and Health Information Centre (CIS)
Concise International Chemical Assessment Documents (CICADS)	WHO IPCS; CCOHS; ILO; United Nations Environment Programme (UNEP)
Consumer Product Safety Commission Website	U.S. Consumer Product Safety Commission (CPSC)
Deutsches Institut fur Medizinische Dokumentation und Information (DIMDI) [The German Institute for Medical Documentation and Information]; Registry of Cytotoxicity (RC)	Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Erganzungsmethoden zum Tierversuch (ZEBET) [German Centre for the Documentation and Validation of Alternative Methods]
Developmental and Reproductive Toxicology/Environmental Teratology Information Center (DART®/ETIC)	EPA; The National Library of Medicine (NLM); The National Institute of Environmental Health Sciences (NIEHS); National Center for Toxicological Research (NCTR)
Emergency Response Guidebook (ERG 2000)	Transport Canada; U.S. Department of Transportation (DOT); Secretariat of Communications and Transportation of Mexico
Environmental Health Criteria (EHC) monographs; Health and Safety Guides (HSG); International Agency for Research on Cancer (IARC)	WHO IPCS; CCOHS
European Centre for the Validation of Alternative Methods (ECVAM) Scientific Information Service (ECVAM SIS)	European Commission Joint Research Centre
HAZARDTEXT®; MEDITEXT®; INFOTEXT®; SARATEXT®; REPROTEXT®; REPROTOX®	TOMES Plus®, MICROMEDEX, Greenwood Village, CO
Integrated Risk Information System (IRIS)	EPA Office of Research and Development (ORD)
International Chemical Safety Cards (ICSC) IPCS/EC Evaluation of Antidotes Series	WHO IPCS; CCOHS; Commission of the European Union (EU)
International Uniform Chemical Information Database (IUCLID)	European Chemicals Bureau
Joint Expert Committee on Food Additives (JECFA); Joint Meeting on Pesticide Residues (JMPR); Pesticide Data Sheets (PDS)	WHO IPCS; CCOHS; Food and Agriculture Organization (FAO) of the United Nations
Material Safety Data Sheets (MSDS)	Interactive Learning Paradigms, Incorporated
Multicentre Evaluation of In Vitro Cytotoxicity (MEIC)	Scandinavian Society for Cell Toxicology
The National MSDS Repository	MSDSSEARCH, Inc.
National Toxicology Program (NTP) Chemical Health and Safety Database	NIEHS
National Transportation Library	DOT
New Jersey Hazardous Substance Fact Sheets	New Jersey Department of Health and Senior Services
Oil and Hazardous Materials/Technical Assistance	EPA Office of Waste and Water Management

Table 4-1 Internet-Accessible Databases Searched for LD_{50} Information

Database/Source[1]	Sponsor(s)
Data System (OHM/TADS)	
Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS)	IPCS; CCOHS; International Register of Potentially Toxic Chemicals (IRPTC); UNEP
Pesticide Action Network Pesticide Database	Pesticide Action Network North America
Pesticide Product Information System (PPIS)	EPA Office of Pesticide Programs (OPP)
Poisons Information Monographs (PIMs)	IPCS; CCOHS
Registry of Toxic Effects of Chemical Substances (RTECS®);NIOSH Pocket Guide to Chemical Hazards	National Institute for Occupational Safety and Health (NIOSH)
SCORECARD	Environmental Defense
The EXtension TOXicology NETwork (EXTOXNET)	University of California, Davis; Oregon State University; Michigan State University; Cornell University; University of Idaho
The Right-to-Know Network (RTK NET)	Office of Management and Budget Watch; Center for Public Data access
Toxic Chemical Release Inventory (TRI); GENE-TOX	The National Library of Medicine (NLM)
Toxic Substances Control Act Test Submissions (TSCATS)	EPA OPPT
TOXLINE®; Hazardous Substances Data Bank (HSDB); ChemIDplus	NLM (TOXNET)

Abbreviations: LD_{50}=Dose lethal to 50% of the animals tested
[1]Includes public and proprietary databases

A total of 195 references containing LD_{50} data retrieved through these searches were reviewed and evaluated. Information regarding the materials, animals, and methods used to derive the 491 LD_{50} values reported by these references were compiled and are provided in **Appendix H1**. **Appendix H2** provides a narrative characterization and evaluation of the LD_{50} values.

4.1.2 Criteria Used to Select Candidate Rodent Acute Oral Data for Determination of LD_{50} Reference Values

This effort was to designed to derive a set of high quality reference oral LD_{50} values from data that were collected using standardized protocols, accompanied by documentation showing that established testing procedures were followed in compliance with national and international GLP guidelines (OECD 1998; FDA 2003; EPA 2003a,b). After a review of the collected data, the SMT determined that a requirement for GLP compliance would eliminate 99% (452 of the 459 values remaining after exclusion of 30 duplicate values and two erroneous values) of the oral LD_{50} values.

The SMT then considered limiting the selection of LD_{50} values to those from studies that used the specifications for animals recommended by the current acute oral toxicity test guidelines. The current guidelines recommend using young adult rats, 8 to 12 weeks of age, of a common laboratory strain (e.g., Sprague-Dawley) and the most sensitive sex (OECD 2001a, c, d; EPA 2002a). Female animals are recommended if there is no information from which to determine the most sensitive sex. A limited number of LD_{50} values were available

from animals that fit this description; only 3% (14/459) of the oral LD_{50} values were determined using 8 to 12 week old female laboratory rats. An additional 15 LD_{50} values were obtained from female rats in an appropriate weight range (age not provided in the reference) for that age range (~ 176-250 g according to Charles River [http://www.criver.com], Harlan [http://www.harlan.com/us/index.htm], and Taconic Farms [http://www.taconic.com/anmodels/spragued.htm] websites). Thus, only 6% (29/459) of the acute oral LD_{50} values in the database, covering 21 of the 72 reference substances (29%), were from studies that used the strain, sex, and age of rats recommended by current test guidelines (OECD 2001a; EPA 2002a).

4.1.2.1 *Final Exclusion Criteria*

Because so few studies met the initial criteria (i.e., GLP compliance and use of animals recommended by current acute oral toxicity test guidelines), the database was reviewed and evaluated to derive alternative criteria for the development of reference LD_{50} values. For this evaluation, the SMT looked for commonalities among the data records that, when selected, provided a comparable data set for each chemical. Review of the available data indicated that the majority of acute oral toxicity tests were conducted by gavage to unanesthetized, young adult laboratory rats of both genders. Thus, the selection process was revised to exclude studies that reflected the following, less typical, materials, animals, and methods in order to compile a homogenous set of reference LD_{50} values for each chemical. The studies excluded were those with:

- Feral rats
- Rats <4 weeks of age
- Anesthetized rats
- Test chemical administered in food or capsule
- LD_{50} reported as a range or inequality

Data from feral rats were excluded because the health status and age of these animals was uncertain. All laboratory rat strains/stocks were deemed acceptable on the assumption that they were healthy and provided with adequate care and housing during testing. Data from neonates and weanlings were excluded because their sensitivity to chemical toxicity may differ from that of adults. Four weeks was considered the minimum acceptable age because rats are typically weaned at approximately three weeks of age (Barrow 2000). Data from feeding experiments or experiments that involved administration of the chemical in capsules were also excluded because gavage is the most common mode of administration for acute oral studies and the rate of gastrointestinal absorption for these other methods is likely to be different (Nebendahl 2000). Because LD_{50} point estimates are required for the prediction model, LD_{50} values reported as ranges or inequalities were unacceptable.

4.1.2.2 *Assumptions Regarding Materials, Animals, and Methods*

The level of detail for describing the materials, animals, and methods for the LD_{50} studies varied greatly. For example, some studies reported only that white rats were used, while others provided complete information on stock/strain, gender, and age of animals. Details on other protocol components such as the number of animals tested per dose group, method of administration, doses administered, clinical signs, and times of death varied as well. In order to use as much of the available data as possible, the following assumptions were made if a study report did not state otherwise:

- Rats were young adults of a common laboratory strain
- Rats were not anesthetized
- Oral route of administration was by gavage

4.1.2.3 *Calculation of Reference LD_{50} Values*

If a substance had multiple LD_{50} values after the application of the exclusion criteria, the outliers at the 99% level (Dixon and Massey 1981) were excluded. A geometric mean and 95% confidence limits were calculated from the remaining values, and used as the reference LD_{50}. A geometric mean was used because it is the antilog of the mean of the logarithm of the values and is less affected than the arithmetic mean by extreme values. The use of a geometric mean also corresponds with the approach used for the RC millimole regression to derive a single IC_{50} value from multiple IC_{50} values (Halle 1998, 2003), and with the approach used to derive the IC_{50} value for each chemical for the *in vitro* - *in vivo* regressions evaluated in the NICEATM/ECVAM validation study (see **Section 6**).

In addition to the statistical evaluation of outliers, an extreme value, which was not a statistical outlier but was based on biological plausibility, was identified for trichloroacetic acid. This chemical had five reported LD_{50} values ranging from 400-8900 mg/kg after applying the exclusionary criteria. The lowest value (400 mg/kg) was rejected as biologically implausible because up to 1000 mg/kg/day had been used in an oral chronic rodent carcinogenicity study with no, or only minimal, toxicity (EPA 1996).

4.1.2.4 *Use of Rat and Mouse Data*

If no rat oral LD_{50} values could be found for a reference substance, mouse acute oral LD_{50} values were evaluated using the same approach as was used for rat values. Because an IC_{50}-LD_{50} regression model using only rat data was preferable, the three reference substances (i.e., epinephrine bitartrate, colchicine, and propylparaben) for which mouse values only were available were not used for the evaluations of accuracy (**Section 6**) or animal reduction (**Section 10**).

4.2 Final Rodent Acute Oral LD_{50} Reference Values

After the application of the exclusionary criteria, there were 385 acceptable rodent acute oral LD_{50} values from which to calculate reference LD_{50} values. **Table 4-2** shows the reference LD_{50} value for each substance in descending order of toxicity, presented both as mg/kg and as mmol/kg. Data are presented as mmol/kg in order to be consistent with the RC approach. The RC millimole regression used units of mmol/kg for the LD_{50} and mM for the IC_{50} (see **Section 1.1.3**). Also shown for each substance are the 95% confidence limits around the geometric mean, the ratio of the maximum to the minimum acceptable value, the number of LD_{50} values used to calculate the reference value, the number of LD_{50} values available (not including duplicate values or erroneous values), and the LD_{50} value initially used for hazard classification of the reference substance (see **Table 3-2**).

Table 4-2 lists the reference substances grouped by GHS acute oral toxicity category (UN 2005) using the reference LD_{50} values that were derived as described above. The initial categorization for this study, which used the LD_{50} values in the far right column of **Table 4-2** (i.e., values reported in **Table 3-2**, which come from the RC unless otherwise specified), placed 12 substances in each toxicity category. **Table 4-3** compares the number of substances in each GHS toxicity category based on their reference LD_{50} values with the number in each

category based on the initial LD_{50} values. The initial and reference LD_{50} values placed 53 (74%) of the substances in the same GHS category. Nineteen substances (26%) were reclassified based on the reference LD_{50} values (this value is the sum of the numbers in the discordant cells in **Table 4-3)**. Compared with the initial LD_{50} value, the reference LD_{50} value was higher for 18 (25%) and lower for only one (1%) of the substances.

Of the 19 reference substances that were reclassified because of the reference LD_{50} values, five substances originally assigned to the most toxic, $LD_{50} \leq 5$ mg/kg, category (i.e., aminopterin, mercury chloride, busulfan, parathion, and strychnine) were moved to the next, less toxic, category ($5 < LD_{50} \leq 50$ mg/kg). In the $5 < LD_{50} \leq 50$ mg/kg category, four substances (dichlorvos, fenpropathrin, sodium dichromate dihydrate, and nicotine) moved to the less toxic $50 < LD_{50} \leq 300$ mg/kg category, and one (triphenyltin hydroxide) moved two categories to $300 < LD_{50} \leq 2000$ mg/kg. In the $50 < LD_{50} \leq 300$ category, four substances (haloperidol, caffeine, copper sulfate pentahydrate, and sodium oxalate) moved to a lower toxicity category ($300 < LD_{50} \leq 2000$ mg/kg). Only carbamazepine moved from the $300 < LD_{50} \leq 2000$ mg/kg category to the $2000 < LD_{50} \leq 5000$ mg/kg category. In the $2000 < LD_{50} \leq 5000$ mg/kg category, citric acid, trichloroacetic acid and dimethylformamide moved to the next lower toxicity category ($LD_{50} > 5000$ mg/kg). In the $LD_{50} > 5000$ mg/kg category, 5-aminosalicylic acid moved to the higher toxicity, $2000 < LD_{50} \leq 5000$ mg/kg category. This was the only substance that moved to a more toxic category

4.3 Relevant Toxicity Information for Humans

The relevance of rodent acute oral LD_{50} data to human LC values was assessed by the MEIC program (Ekwall et al. 1998b), which used mouse and rat oral LD_{50} data from RTECS® (Ekwall et al. 1998a). Mean lethal doses in humans were collected primarily from handbooks containing human clinical toxicity information (Ekwall et al. 1998a) supplemented, when necessary, by an in-house compendium from the Swedish Poisons Information Centre. Ekwall et al. (1998b) calculated least squares linear regressions for the prediction of the mean human LC values by rat and/or mouse oral LD_{50} data for the 50 MEIC substances using units of log mol/kg. They reported a correlation of $R^2 = 0.607$ for the rat oral LD_{50} prediction of mean human LC values and $R^2 = 0.653$ for the mouse oral LD_{50} prediction of mean human LC values. It is important for comparisons of MEIC data with rodent LD_{50} values to note that the MEIC human values are not lethal doses, and therefore not equivalent to LD_{50} values. Many of the values (if not the majority) are blood concentrations that were associated with morbidity or mortality, and usually do not reflect the actual dose consumed by the patient. These are not necessarily the peak blood concentrations, but only the concentrations at the time of ascertainment, which could have ranged from immediately after onset of medical treatment to post-mortem. The MEIC organizers readily admitted that they could not relate the blood concentrations to the administered dose.

The relevance of the NRU data collected in the NICEATM/ECVAM validation study to the prediction of human acute toxicity will be addressed elsewhere by ECVAM in a separate evaluation.

Table 4-2 Rodent Acute Oral Reference LD_{50} Values Listed by GHS Category[1]

GHS Category[1]/ Reference Substance	Reference Acute Oral LD_{50}[2,3] (mg/kg)	95% Confidence Interval[4] (mg/kg)	Reference Acute Oral LD_{50} Range[5] (mg/kg)	Reference Acute Oral LD_{50}[2] (mmol/kg)	95% Confidence Interval[4] (mmol/kg)	Maximum: Minimum Value[6]	N	Initial Rodent Acute Oral LD_{50}[3,7] (mg/kg)
LD_{50} ≤5 mg/kg (N =7)								
Cycloheximide	2	NC	1-2.5	0.00711	NC	2.5	3	2
Phenylthiourea	3	NC	3	0.0197	NC	NC	1	3
Sodium selenate	3	NC	1.6-5.98	0.0159	NC	3.7	2	2[8]
Epinephrine bitartrate	4 (mouse)	NC	4	0.0196	NC	NC	1	4 (mouse)
Triethylenemelamine	4	1-25	1-13	0.0120	0.0037-0.12	13.0	4	1
Physostigmine	5	NC	5	0.0182	NC	NC	1	5[8]
Disulfoton	5	2-10	2.3-12.6	0.0182	0.009-0.036	5.5	6	2
5 < LD_{50} ≤50 mg/kg (N =12)								
Parathion	6	3-12	1.8-30	0.0209	0.010-0.041	16.7	10	2
Strychnine	6	NC	2.35-16.2	0.0188	NC	6.9	3	2[8]
Aminopterin	7	NC	7	0.016	NC	NC	1	3 (mouse)
Potassium cyanide	7	5-10	5-10	0.111	0.077-0.15	2.0	7	10
Busulfan	12	NC	1.9-29	0.049	0.008-0.38	15.3	4	2
Colchicine	15 (mouse)	NC	5.886-29	0.0375	NC	4.9	3	6 (mouse)
Thallium I sulfate	25	NC	25	0.0495	NC	NC	1	29 (mouse)
Arsenic III trioxide	25	10-64	13-81.5	0.127	0.050-0.32	6.3	5	20
Endosulfan	28	NC	18-43	0.068	NC	2.4	2	18[8]
Digoxin	28	NC	28	0.0362	NC	NC	1	18 (mouse)
Mercury II chloride	40	27-60	12-92	0.148	0.010-0.22	7.7	10	1
Sodium arsenite	44	36-53	36-53	0.336	0.28-0.40	1.5	5	41[8]
50 < LD_{50} ≤300 mg/kg (N =12)								
Sodium dichromate dihydrate	51	44-58	34.17-64.5	0.193	0.17-0.22	1.9	11	50
Dichlorvos	59	40-88	17-97.5	0.266	0.18-0.40	5.7	9	17[8]
Nicotine	70	68-72	68-71	0.430	0.42-0.44	1.0	4	50
Fenpropathrin	76	57-100	48.5-164	0.217	0.16-0.29	3.4	9	18[8]
Hexachlorophene	82	68-98	56-215	0.202	0.17-0.24	3.8	19	61
Paraquat	93	65-132	57-115	0.498	0.35-0.71	2.0	5	58
Lindane	100	78-129	88-125	0.344	0.27-0.44	1.4	4	76
Verapamil HCl	111	NC	108-114	0.226	NC	1.1	2	108

Table 4-2 Rodent Acute Oral Reference LD_{50} Values Listed by GHS Category[1]

GHS Category[1]/ Reference Substance	Reference Acute Oral LD_{50}[2,3] (mg/kg)	95% Confidence Interval[4] (mg/kg)	Reference Acute Oral LD_{50} Range[5] (mg/kg)	Reference Acute Oral LD_{50}[2] (mmol/kg)	95% Confidence Interval[4] (mmol/kg)	Maximum: Minimum Value[6]	N	Initial Rodent Acute Oral LD_{50}[3,7] (mg/kg)
Sodium I fluoride	127	92-175	64-279	3.020	2.19-4.16	4.4	12	180
Cadmium II chloride	135	88-208	88-211	0.738	0.48-1.14	2.4	5	88
Diquat dibromide	160	NC	121-231	0.466	NC	1.9	3	231
Phenobarbital	224	NC	162-318	0.966	NC	2.0	3	163
300 < LD_{50} ≤2000 mg/kg (N =16)								
Caffeine	310	256-374	192-483	1.59	1.32-1.93	2.5	10	192
Triphenyltin hydroxide	329	208-520	46.4-1200	0.896	0.57-1.42	25.9	15	44
Haloperidol	330	NC	128-850	0.877	NC	6.6	2	128[8]
Amitriptyline HCl	348	NC	320-380	1.18	NC	1.2	2	319
Propranolol HCl	466	NC	466	1.575	NC	NC	1	470 (mouse)
Cupric sulfate • 5 H_2O	474	269-836	236.2-960	1.90	1.08-3.35	4.1	6	300
Phenol	548	434-692	317-1500	5.82	4.82-7.68	4.7	14	414
Lithium carbonate	590	479-728	525-710	7.98	6.5-9.9	1.4	4	1187 (mouse; sulfate salt)
Glutethimide	600	NC	600	2.76	NC	NC	1	600
Sodium oxalate	633	NC	558-707	4.724	NC	1.3	2[11]	155 (mouse)[9]
Chloral hydrate	638	391-1040	479-863	3.86	2.36-6.29	1.8	4	479
Atropine sulfate	819	641-1045	600-1136	1.21	0.95-1.54	1.9	7	623
Valproic acid	995	NC	670-1480	6.91	NC	2.2	2	1695 (mouse)
Meprobamate	1387	1291-1489	1286-1522	6.35	5.92-6.82	1.2	6	794[8]
Acetylsalicylic acid	1506	1224-1854	616-2840	8.36	6.8-10.3	4.6	14[11]	1000
Procainamide HCl	1950	NC	1950	8.286	NC	NC	1	1950[8]
2000 < LD_{50} ≤5000 mg/kg (N =11)								
Acetaminophen	2163	NC	1944-2404	14.3	NC	1.2	2	2404
Potassium I chloride	2799	NC	2600-3020	37.6	NC	1.2	2	2602
Carbamazepine	2805	NC	1957-4025	11.9	NC	2.1	2	1957[8]
Boric aid	3426	2617-4486	2660-5140	55.4	42.3-72.6	1.9	6	2660[8]
5-Aminosalicylic acid	3429	NC	2800-4200	22.4	NC	1.5	2	7749 (mouse)
Chloramphenicol	3491	NC	2500-5000	10.8	NC	2.0	3	3393
Acetonitrile	3598	2951-4375	1320-8120	87.6	71.9-107	6.2	26	3798
Lactic acid	3639	NC	3543-3730	40.3	NC	1.1	2	3730

Table 4-2 Rodent Acute Oral Reference LD_{50} Values Listed by GHS Category[1]

GHS Category[1]/ Reference Substance	Reference Acute Oral LD_{50}[2,3] (mg/kg)	95% Confidence Interval[4] (mg/kg)	Reference Acute Oral LD_{50} Range[5] (mg/kg)	Reference Acute Oral LD_{50}[2] (mmol/kg)	95% Confidence Interval[4] (mmol/kg)	Maximum: Minimum Value[6]	N	Initial Rodent Acute Oral LD_{50}[3,7] (mg/kg)
Carbon tetrachloride	3783	3024-4732	2350-10054	24.6	20-31	4.3	15	2799
Sodium chloride	4046	2917-5623	3000-6140	69.3	50-96	2.0	5	2998
Xylene	4667	1294-16827	1537-8620	43.9	12-158	5.6	4	4300
LD_{50} >5000 mg/kg (N =14)								
2-Propanol	5105	4624-5636	4500-5840	84.9	77-94	1.3	6	5843
Trichloroacetic acid	5229	2745-9961	3320-8900	32.0	16.8-61.0	2.7	4	4999
Dimethylformamide	5309	3548-7925	2800-7182	72.6	49-108	2.6	6	2800
Citric Acid	5929	NC	3000-11700	30.9	NC	3.9	2	3000[8]
Gibberellic acid	6040	NC	5780-6300	17.4	NC	1.1	2	6305
Propylparaben	6332 (mouse)	NC	6332	35.1	NC	NC	1	6326 (mouse)
Ethylene glycol	7161	6266-8204	4000-9900	115.4	101-132	2.5	16	8567
Methanol	8710	6223-12218	5628-12880	272	194-381	2.3	6	13012
Dibutyl phthalate	8892	6180-12794	7499-12436	31.9	22-46	1.7	4	11998
Diethyl phthalate	9311	NC	8600-10100	41.9	NC	1.2	2	8602
Sodium hypochlorite	10328	NC	8200-13000	62.8	NC	1.6	2	8910[10]
Ethanol	11324	8610-14894	7060-17775	245.7	187-323	2.5	8	14008
1,1,1-Trichloroethane	12078	10000-14588	9600-16000	90.5	75-109	1.7	6	10298
Glycerol	19770	10495-37154	12600-27650	215	114-403	2.2	4	12691

Abbreviations: LD_{50}=dose lethal to 50% of the animals tested; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); N=Number of acceptable values used for geometric mean; NC=Not calculated.

[1]Categorized using the reference oral LD_{50}.

[2]Based on a geometric mean of acceptable LD_{50} values from adult laboratory rats unless otherwise specified.

[3]Values rounded to the nearest whole number.

[4]For the geometric mean of the acceptable LD_{50} values, NC is used for substances with three acceptable values or less, which was considered too few for calculation of a valid confidence interval.

[5]Range of acceptable oral LD_{50} values.

[6]Ratio of minimum acceptable LD_{50} to maximum acceptable LD_{50}.

[7]Values rounded to the nearest whole number. Values are from the RC unless otherwise specified; rat data unless otherwise specified.

[8]RTECS® (MDL Information Systems 2002).

[9]RC reference for rat oral LD_{50} of 155 mg/kg is Shrivastava et al. (1992), which references Klinger and Kersten (1961). Klinger and Kersten (1961) indicate the value was determined by intraperitoneal administration to mice.

[10]HSDB (NLM 2002).

[11]An erroneous value obtained from the literature was not included.

Table 4-3 GHS Category Matches for the Rodent Acute Oral LD_{50} Initial and Reference Values

Initial LD_{50} (mg/kg[1])	Reference LD_{50} (mg/kg)						Total	Category Match	Reference LD_{50} Lower	Reference LD_{50} Higher
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2000$	$2000 < LD_{50} \leq 5000$	$LD_{50} > 5000$				
$LD_{50} \leq 5$	7	5	0	0	0	0	12	58%	0%	42% (5)
$5 < LD_{50} \leq 50$	0	7	4	1	0	0	12	58%	0%	42% (5)
$50 < LD_{50} \leq 300$	0	0	8	4	0	0	12	67%	0%	33% (4)
$300 < LD_{50} \leq 2000$	0	0	0	11	1	0	12	92%	0%	8% (1)
$2000 < LD_{50} \leq 5000$	0	0	0	0	9	3	12	75%	0%	25% (3)
$LD_{50} > 5000$	0	0	0	0	1	11	12	92%	8%	0% (0)
Total	7	12	12	16	11	14	72	74%	1%	25% (18)

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); LD_{50}=Dose lethal to 50% of animals tested.
Note: Shaded cells show the number of chemicals for which both LD_{50} categories agree.
[1]Initial LD_{50} values were used for reference substance selection and were obtained from the RC (Halle 1998, 2003), RTECS® (MDL Information Systems 2002), and HSDB (NLM 2002) (see **Table 3-2**).

4.4 Accuracy and Reliability of the Rodent Acute Oral LD_{50} Reference Values

Accuracy (concordance) is the closeness of agreement between a test method result and an accepted reference value (in this case to the rodent acute oral LD_{50} measurement) (ICCVAM 2003). Because there are insufficient data to permit a comparison between rodent and human lethal doses, the accuracy of rodent acute oral LD_{50} values for predicting the oral LD_{50} in humans cannot be determined. Acute toxicity testing in rodents leads to a relative ranking of the toxicity of chemicals for regulatory purposes, with the default assumption that the rodent values and ranking are predictive of the human values and ranking.

The among laboratory reproducibility of the reference LD_{50} values determined in this section may be judged by evaluating the range of acceptable LD_{50} values for each reference substance and by comparing the values (and their variability) with the variability of LD_{50} values derived from controlled acute oral toxicity studies.

4.4.1 Variability Among the Acceptable LD_{50} Values

The variability among the acceptable rodent acute oral LD_{50} values used to calculate the reference LD_{50} value for each reference substance was assessed by calculating the ratio of the maximum to the minimum value (see **Table 4-2**). For the 62 reference substances with more than one acceptable LD_{50} value, the maximum:minimum ratio ranged from 1.1 to 25.9, with a mean of 4.3 and a median of 2.2. The maximum:minimum ratios were greater than 10 for four substances: triethylenemelamine, parathion, busulfan, and triphenyltin hydroxide. The low LD_{50} values for triethylenemelamine, busulfan, and parathion may have contributed to the high maximum:minimum ratios. The four LD_{50} values for triethylenemelamine ranged from 1 to 13 mg/kg, the four values for busulfan ranged from 1.9 to 29 mg/kg, and the 10 values for parathion ranged from 1.8 to 30 mg/kg.

Table 4-4 shows the maximum:minimum LD_{50} ratios by toxicity category. The more toxic substances (i.e., $LD_{50} \leq 50$ mg/kg) tended to have higher maximum:minimum ratios than substances with lower toxicity (i.e., $LD_{50} > 50$ mg/kg). This is anticipated because small day-to-day, or laboratory-to-laboratory variations in weighing and dosing the lower concentrations would have a higher impact on the chemicals being administered in low doses than those being administered in the high dose range.

Table 4-4 Maximum:Minimum LD_{50} Ratios by GHS Toxicity Category

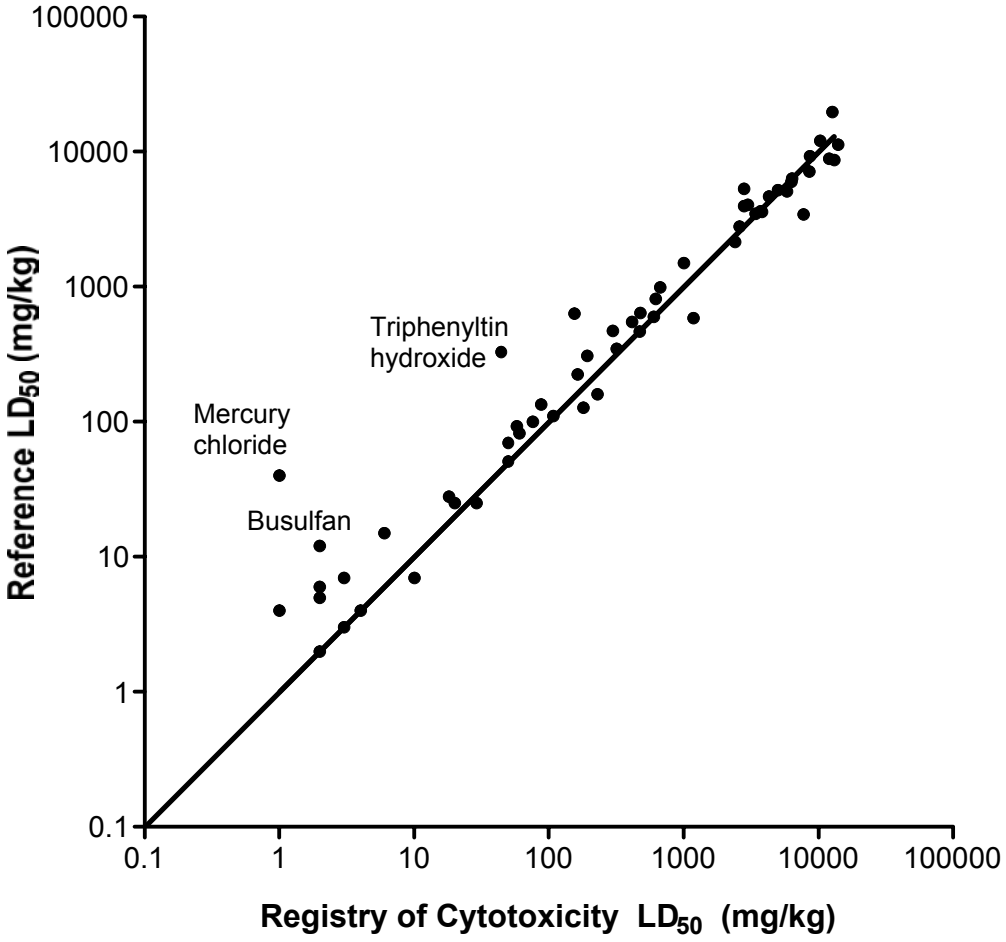
GHS Category (LD_{50} in mg/kg)	Mean Maximum:Minimum LD_{50} Ratio	Median Maximum:Minimum LD_{50} Ratio	Range of Maximum:Minimum LD_{50} Ratio	N
$LD_{50} \leq 5$	6.2	4.6	2.5 – 13.0	4
$5 < LD_{50} \leq 50$	7.1	6.3	2.0 - 16.7	9
$50 < LD_{50} \leq 300$	2.4	1.9	1.1 - 5.7	12
$300 < LD_{50} \leq 2000$	4.6	2.2	1.2 - 25.9	13
$2000 < LD_{50} \leq 5000$	2.6	2.0	1.2- 22.3	11
$LD_{50} > 5000$	2.3	2.3	1.1 - 3.9	13

Abbreviations: LD_{50}=Dose lethal to 50% of animals tested; GHS-Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); N=Number of chemicals with more than one acceptable LD_{50} value after application of the exclusion criteria described in **Section 4.1.2**.

4.4.2 Comparison of Rodent Acute Oral LD_{50} Reference Values with the Corresponding RC LD_{50} Values

The correspondence of the rodent acute oral LD_{50} reference values with the RC LD_{50} values for the 58 reference substances in common with the RC are shown on a log scale in **Figure 4-1**. Not surprisingly, a Spearman correlation analysis for the two sets of log transformed values yielded a significant correlation ($p < 0.0001$) with a correlation coefficient, r_s, of 0.97. **Figure 4-1** shows that the LD_{50} reference values tended to be higher than the RC LD_{50} values. One factor in this difference is that the majority of LD_{50} values used in the RC were from the 1983/84 RTECS®, which contains the lowest LD_{50} value found for a particular chemical without regard to the available methodological information, without consideration of whether it is an outlier with respect to the other available values, and without scientific review before publication. Thus, because the reference LD_{50} values are based on the geometric mean from multiple studies, it is not surprising that these values tended to be higher than the single values in the RC database.

Figure 4-1 Correlation of LD_{50} Values With the Reference LD_{50} Values for the 58 RC Chemicals



Abbreviations: LD_{50}=Dose lethal to 50% of animals tested; RC=Registry of Cytotoxicity.
The diagonal line shows the 1:1 relationship.

When comparing the reference LD_{50} values to the RC values, the substances with the largest differences were busulfan, triphenyltin hydroxide, and mercury chloride **(**see **Figure 4-1)**.

- The LD_{50} reference value for busulfan was six times that of the RC value (12 mg/kg vs. 1.9 mg/kg). The RC value (from 1983/84 RTECS®) was from a paper by Schmahl and Osswald (1970) in which they cited a rat oral LD_{50} of 1.86 mg/kg. The literature also contained rat oral LD_{50} values of 28 and 29 mg/kg for male and female Sprague-Dawley rats, respectively (Matsuno et al. 1971).
- The LD_{50} reference value for triphenyltin hydroxide was 7.5 times the RC LD_{50} (329 mg/kg vs. 44 mg/kg). The 15 LD_{50} values used to determine the reference value included the RC value, and had a wide range, 44-1200 mg/kg. Because of the large variation in the data, which was evenly distributed throughout the range neither the highest nor the lowest values were outliers.
- The LD_{50} reference value for mercury chloride was 40 mg/kg, while the RC value was 1 mg/kg. The RC value was from a summary document that reported the rat oral LD_{50} as a range of 1-5 mg/kg (Worthing and Walker 1991). Because it was reported as a range, it was excluded from the calculation of the reference value (see **Section 4.1.2.1**). The remaining 11 values ranged from 12 to 160 mg/kg. The highest value (160 mg/kg) was considered an outlier when compared to the other 10 values and therefore excluded from the reference value calculation.

4.4.3 Comparison of the Variability Among Acceptable LD_{50} Values to Those Obtained in Other Studies

The variation seen here for 62 reference substances is not atypical, considering the results of other studies that examined the variation among rodent acute oral LD_{50} values derived for the same substance. For example, Weil and Wright (1967) showed that LD_{50} values varied by as much as five-fold for the 10 substances tested in eight laboratories using exactly the same protocol. Another international study involving 65 participating laboratories in eight countries that did not control the LD_{50} protocols among laboratories, reported maximum:minimum ratios from 3.6 to 11.3 (with LD_{50} values ranging from 44 to 5420 mg/kg) for five substances (Hunter et al. 1979). The chemicals tested, and the LD_{50} ranges were:

- PCP[1] 44-523 mg/kg
- Sodium salicylate 800-4150 mg/kg
- Aniline 350-1280 mg/kg
- Acetanilide 805-5420 mg/kg
- Cadmium chloride 70-513 mg/kg

The results of a follow-on study in which the same substances were tested by 100 laboratories in 13 countries showed that adherence to a specific protocol reduced the range of maximum:minimum LD_{50} ratios from 3.6 to 11.3 to 2.4 to 8.4 (Zbinden and Flury-Roversi 1981).

[1] Compound undefined in the publication.

Although the LD_{50} data collected from the literature for the NICEATM/ECVAM validation study used various rat strains, sexes, observation durations, and calculation methods for estimating the LD_{50}, the variation in LD_{50} values for individual substances was similar to the data of the earlier cited studies. The current study found four of the 62 substances with multiple LD_{50} values had maximum:minimum LD_{50} values higher than that reported by Hunter et al. (1979) (i.e., >11.3), and three of those were in the highest toxicity category. Hunter et al. (1979) also observed that the largest variation was associated with the more highly toxic substances.

4.5 Summary

To enable the comparison of *in vitro* NRU data with rodent acute oral toxicity data, LD_{50} reference values for the 72 reference substances were calculated using data obtained from the literature, database searches, and secondary references. Rat acute oral LD_{50} values were preferred, but mouse acute oral LD_{50} values were collected for three substances with no available or acceptable rat data. The 491 LD_{50} values that were retrieved comprised 485 rat LD_{50} values and six mouse values. It was not possible to identify a high quality data set produced under GLP guidelines because only 3% of the data records were in GLP compliance. Instead, as described in **Section 4.1.2.1**, a homogenous set of LD_{50} values for each substance was identified by applying specific exclusion criteria related to the materials, animals, and methods used for each study.

After analysis of the acceptable values for outliers, the remaining 385 values were used to derive rodent acute oral LD_{50} reference values by calculation of a geometric mean of the values for each substance. As a result of this procedure, the LD_{50} reference values for 19 of the 72 reference substances were sufficiently different from the values that were used in the RC and other summary sources, so that they were reclassified into different GHS oral toxicity categories.

Because there is no reference standard against which to evaluate the accuracy of the rodent acute oral toxicity test, the reliability of the LD_{50} reference values was assessed by comparison to other evaluations of the performance of this test method. The maximum:minimum ratio of the acceptable values for the 62 reference substances that had more than one LD_{50} value ranged from 1.1 to 25.9, and the ratios for four of the substances were greater than one order of magnitude.

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5.0 3T3 AND NHK NRU TEST METHOD DATA AND RESULTS

This section summarizes the IC_{50} results generated by testing 72 coded reference substances (see **Section 3**) in the 3T3 and NHK NRU test method protocols. These IC_{50} values were used to evaluate the accuracy (also known as concordance - see **Section 6**) of the two *in vitro* cytotoxicity test methods for predicting *in vivo* GHS acute oral toxicity categories and their reliability (intra- and inter-laboratory reproducibility - see **Section 7**). The individual test data for the passing and failing tests are provided in **Appendix I** for the reference substances and the PC. The raw data for each test (in EXCEL® and PRISM® files) are available upon request from NICEATM on compact disk(s), as are the laboratory reports. Requests can be made by mail, fax, or e-mail to Dr. William S. Stokes, NICEATM, NIEHS, P. O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) niceatm@niehs.nih.gov.

Section 5.1 discusses the timeline for the validation study, the study participants, and their roles in the study. **Section 5.2** documents the use of coded reference substances and the GLP compliance by the participating laboratories. **Section 5.3** discusses the protocol revisions that were made during the study and the effect the revisions had on the results. **Section 5.4** presents the IC_{50} data collected during each phase to assess the reliability and accuracy (relevance) of the NRU methods. **Section 5.5** presents the statistical analyses performed. **Section 5.6** summarizes the results of IC_{50} comparisons of the 3T3 and NHK methods. **Section 5.7** offers information about the availability of all the data (e.g., raw OD data from all tests, laboratory reports), and **Section 5.8** presents the solubility test results for the reference substances from all laboratories.

5.1 Study Timeline and Participating Laboratories

5.1.1 Statements of Work (SOW) and Protocols

The SMT provided the laboratories with SOWs for each test method prior to initiation of testing (see **Appendix G**), and proposed dates for completion of the various aspects of the study (e.g., transfer of data, provision of reports). The SOWs defined the following:

- Project objectives
- Management and key personnel
- Required facilities, equipment, and supplies
- Quality assurance requirements
- Test phases and schedules
- Products (e.g., reports) required
- Report preparation

The SOW for BioReliance contained all of the above requirements, and also included requirements for:

- Reference substance acquisition, coding, preparation, and distribution
- Solubility testing

The SMT, in consultation with the laboratories, prepared Test Method Protocols for each phase of the study. Cytotoxicity testing in each phase of the validation study was initiated in each laboratory when the SMT received a signed protocol specific for that phase from the

Study Director. Solubility testing for the Phases I and II substances was performed prior to cytotoxicity testing for those substances; most of the solubility testing for the Phase III substances was performed toward the end of Phase II and during the early part of Phase III.

5.1.2 Study Timeline

The actual timeline of the study is shown in **Table 5-1**. The SMT modified the original timeline presented in the SOWs because of a number of factors, such as, protocol revisions, side studies, difficulties with acquisition of medium, etc.

Table 5-1 Validation Study Timetable

Event	BioReliance	ECBC	FAL	IIVS
Receipt of SOW from SMT	Jun 2002	Jun 2002	Jun 2002	Jun 2002
Procurement of Test Substances	Jul 2002 - Jan 2003	NA	NA	NA
Solubility Testing Completed	Jul 2002 - Jan 2003	Dec 2003	Dec 2003	Jan 2004
Distribution of Reference Substances Phase Ia Phase Ib Phase II Phase III	 Jul 2002 Sep 2002 Nov 2002 Feb - Mar 2003	NA	NA	NA
Initiation of Phase Ia	NA	Aug 2002	Aug 2002	Aug 2002
Completion of Phase Ia	NA	Nov 2002	Nov 2002	Oct 2002
Initiation of Phase Ib	NA	Dec 2002	Dec 2002	Dec 2002
Completion of Phase Ib	NA	May 2003	May 2003	May 2003
Initiation of Phase II	NA	Jun 2003	Jun 2003	Jun 2003
Completion of Phase II	NA	Nov 2003	Nov 2003	Nov 2003
Initiation of Phase III	NA	Dec 2003	Dec 2003	Dec 2003
Completion of Phase III	NA	Dec 2004	Dec 2004	Jan 2005

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SOW=Statement of Work; SMT=Study Management Team; NA=Not applicable.
Note: BioReliance distributed the reference substances and performed solubility testing. ECBC, FAL, and IIVS tested the reference substances for solubility and *in vitro* cytotoxicity.

5.1.3 Participating Laboratories

- BioReliance Corporation
  14920 Broschart Road
  Rockville, Maryland 20850-3349
  Study Director: Dr. Martin Wenk
- U.S. Army Edgewood Chemical Biological Center (ECBC)
  Molecular Engineering Team
  Aberdeen Proving Ground, MD 21010
  Study Director: Dr. Cheng Cao

- Institute for *In Vitro* Sciences (IIVS)
  21 Firstfield Road Suite 220
  Gaithersburg, MD 20878
  Study Director: Mr. Hans Raabe
- Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory (FAL)
  Queens Medical Centre, University of Nottingham
  Nottingham NG7 2UH
  United Kingdom
  Study Director: Dr. Richard Clothier

5.2 Coded Reference Substances and GLP Guidelines

5.2.1 Coded Reference Substances

BioReliance acquired 73 substances (72 reference substances and one PC substance) from reputable commercial sources (see **Appendix F1**). All but eight of the reference substances were >99% pure (see **Section 8.1.2.1**). BioReliance coded each substance with a unique, random identification number when repackaging them into smaller units for distribution to the laboratories. These units were given an additional code unique to the respective cytotoxicity laboratories, so that they could be provided in a blinded fashion (see **Section 3.4** for distribution procedures). The coded substance units were packaged and shipped such that their identities were concealed; however, all laboratories knew the identity of the positive control. The SMT revealed the codes for each phase after all laboratories had submitted their data and reports for that phase. The laboratories periodically required additional aliquots of reference substance, and BioReliance provided these aliquots from the original stock of reference substance in the same manner that the original aliquots were provided.

5.2.2 Lot-to-Lot Consistency of Reference Substances

Each substance was purchased as a single lot, and each laboratory received aliquots from this same lot throughout the validation study. The reference substance suppliers provided certificates of analysis for each lot, along with the MSDS documents containing substance, physical, and safety and handling information.

5.2.3 Adherence to GLP Guidelines

BioReliance, ECBC, and IIVS, followed GLP procedures for all testing, with the exception of tests designed to resolve technical challenges (e.g., formation of NR crystals; use of film plate sealers for volatile substances; slow growth of cells). The laboratories submitted all data to their respective quality assurance units (as per GLP requirements) and copies of the data were submitted to NICEATM. FAL followed most of the GLP guidelines, but did not employ independent quality assurance reviews of laboratory procedures or documentation. The Study Director for FAL performed all data reviews and provided copies to NICEATM. Hard copy printouts and electronic versions of all data are available at NICEATM.

5.3 3T3 and NHK NRU Test Method Protocols

The protocols for the 3T3 and NHK NRU test methods used during Phase III laboratory testing were the result of modifications and revisions to the *Guidance Document* (ICCVAM 2001b) protocols, the optimization of the protocols used in the laboratory evaluation Phases Ia and Ib, and the laboratory qualification phase (Phase II) (see **Section 2.6**). **Figure 1-2**

provides an outline of the study phases, and identifies where repeated observations were carried out to permit protocol evaluation and comparison. **Sections 2.2** and **2.3** address the similarities and differences between the 3T3 and NHK protocols. The remaining subsections in **Section 5.3** address the modifications to the protocols used in each phase, and how those modifications affected each data set.

5.3.1 Phase Ia: Laboratory Evaluation Phase

During Phase Ia, each laboratory established an historical database for the PC substance, SLS. No reference substances were tested in this phase. Ten concentration-response tests were performed using SLS and no more than two tests were performed/day. The resulting data were used to calculate the acceptable response limits for the SLS IC_{50} for use during Phase Ib testing.

Section 2.6.1 summarizes issues that occurred during Phase I and addresses protocol changes made after the initiation of Phase Ia. The specific changes to the protocols for both cell systems are summarized below, along with the impact these changes had on the test data. Changes made in the protocols during Phase Ia were incorporated into the Phase Ib protocols.

5.3.1.1 *Protocol Changes and the Effect on the Data*

- *NR Dye Crystals:* Reduced the NR dye concentration for both cell types. No subsequent tests failed because of NR crystal formation. The background OD values decreased and this was not interpreted as a negative effect on the data.
- *3T3 Cell Growth*: Modified cell culture conditions for 3T3 cells to improve cell growth characteristics. No apparent effect on the data was detected.
- *NHK Cell Growth (96-well plates):* Removed the cell culture refeeding step performed prior to reference substance addition. Although the OD values for the vehicle controls became higher, the SLS IC_{50} results were similar whether or not the cells were re-fed.
- *NHK Cell Growth (in culture flasks)*: FAL coated their culture flasks with fibronectin-collagen prior to seeding thawed cells. This may have affected the SLS data from FAL because it had the highest SLS IC_{50} values of the three laboratories (7.45 μg/mL vs. 4.03 μg/mL for ECBC and 3.68 μg/mL for IIVS). The fibronectin-collagen coating procedure was eliminated, and subsequent SLS data and IC_{50} results from FAL were comparable to the data from the other two laboratories.
- *OD Limits*: Eliminated the VC OD range as a test acceptance criterion. The SMT decided to accept tests that had VC ODs outside the originally preset range if all other test acceptance criteria were met. Test data were not adversely affected by relaxing this criterion.
- *Dilution Factor*: The SMT accepted data generated using dilution factors other than the recommended 1.47 for definitive tests if all other test acceptance criteria were met. The use of smaller dilution factors generally increased the number of data points between 10 - 90% viability, and the precision of the IC_{50} calculation was improved.

5.3.2 Phase Ib: Laboratory Evaluation Phase

Phase Ib was designed to determine whether the protocol revisions following Phase Ia were effective in improving intra- and inter-laboratory reproducibility, and to determine whether

the laboratories could obtain reproducible results when testing coded reference substances of various toxicities. Three coded reference substances representing the full range of toxicity were tested: arsenic trioxide (high toxicity: 5< LD_{50} ≤50 mg/kg), propranolol HCl (medium toxicity: 300< LD_{50} ≤2000 mg/kg), and ethylene glycol (low toxicity: LD_{50} >5000 mg/kg) (see **Section 3.3.5** for the selection of substances to be tested in Phases Ib and II). Because Phase Ib was part of the laboratory evaluation phase, the SMT decided that three substances would be sufficient, and that it was not necessary to represent all GHS acute oral toxicity categories. Each substance was tested in all laboratories at least once in a range finding experiment, and then in three, acceptable definitive tests performed on three different days. **Section 2.6.2** summarizes the technical challenges that arose during this phase and addresses protocol changes made after initiation of Phase Ib. The specific changes made in the 3T3 and NHK protocols, along with the effect the changes had on the test data, are summarized below.

5.3.2.1 *Protocol Changes and the Effect on the Data*

- *NR Dye Crystals*: Reduced the concentration of NR in the 3T3 method. The OD values and SLS IC_{50} results were similar in four exploratory experiments regardless of the NR concentration or NRU incubation time. The elimination of NR crystals reduced the background OD values without affecting the sensitivity of the procedure.
- *VC OD Range*: Used new VC OD ranges for guidance (e.g., as target values to assess cell growth), rather than as a test acceptance criterion, for the remainder of the study. This increased the number of tests that met the acceptance criteria. Relative toxicities did not change. The test data were not adversely affected by the removal of this criterion.

5.3.3 Phase II: Laboratory Qualification Phase

The results from Phase II were used to determine whether the protocol revisions from Phase Ib were effective in improving intra- and inter-laboratory reproducibility, and whether the laboratories could obtain reproducible results when testing a larger set of substances covering a wider range of physical/substance characteristics and toxicities. Nine coded reference substances were tested: aminopterin, cadmium chloride, chloramphenicol, colchicine, lithium carbonate, potassium chloride, 2-propanol, sodium fluoride, and sodium selenate. These substances (with the exception of sodium selenate) are included in the RC, and were selected because they fit the RC millimole regression line (i.e., they were within the acceptance intervals established by Halle [1998, 2003]). The RC is a database of acute oral LD_{50} values for rats and mice obtained from RTECS® and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for substances with known molecular weights (Halle 1998, 2003). Sodium selenate was selected because of its high toxicity, despite the fact that it was not in the RC, because there were no other substances in the highest GHS acute oral toxicity category, other than aminopterin, that were within the RC millimole regression acceptance intervals. Each laboratory tested each substance at least once in a range finding experiment, and then in three acceptable definitive tests performed on different days.

Section 2.6.2 summarizes the technical issues that arose during this phase and the protocol changes made prior to Phase II. The specific changes made in the 3T3 and NHK NRU protocols, along with the effect the changes had on the test data, are summarized below.

5.3.3.1 *Protocol Changes and the Effect on the Data*

- *Blank Wells*: Added reference substance to blank wells of the test plate to determine if reference substance affected (i.e., increased OD values) compared to medium-filled blank wells. There was no apparent effect on the test data as there were no noticeable differences in OD values between blanks with culture medium or culture medium and reference substance.
- *VC OD Range*: Eliminated the VC OD range as an acceptance criterion. There was no apparent effect on test data from not restricting the OD values to a pre-set range.
- *Harmonization of Laboratory Techniques*: Made revisions to the Phase II protocols as a result of the harmonization training by the testing laboratories (see **Section 2.6.2.6**). There was no apparent effect on the test data from IIVS and ECBC, but there was an improvement in the FAL data quality (e.g., fewer lost OD values due to cell seeding errors, more uniform OD values for six replicate wells per reference substance).
- *3T3 Cell Seeding Density*: Added a range of cell seeding densities to be used by the laboratories. This optimized the cell confluence at the end of chemical exposure and no apparent effects on the data were detected because of this modification.
- *NHK Cell Growth from Cryopreserved Stock Cells*: Eliminated the use of fibronectin-collagen coating of 80-cm^2 flasks for the initial propagation of NHK cells. By doing this, FAL achieved better cell growth, lower IC_{50} values for the PC, and better agreement of the mean SLS IC_{50} values with those of the other laboratories.
- *Volatile Substances*: Added the use of a CO_2 permeable plate sealer to control volatility (as identified by cross contamination of the control wells). The use of plate sealers for volatile substances was incorporated into the Phase III protocols.
- *R^2 Acceptance Criterion*: Relaxed the R^2 criterion for the fit of the dose-response data to the Hill function. Some tests that did not meet the original criterion were accepted by the SMT after determining that even though the curve fit was not optimum, it adequately conveyed the toxicity of the substance (i.e., an IC_{50} could be calculated with an adequate number of toxicity points between 0 and 100% viability).
- *Unusual Concentration-Response*: Revised the Hill function calculation to address substances that produced a concentration-response in which toxicity plateaued before reaching 0% viability. This modification allowed for a curve fit to the Hill function for such substances, and thus a better estimation of their IC_{50} values.
- *PC IC_{50} Range*: Expanded the SLS IC_{50} acceptable range, which resulted in additional tests in Phase II being acceptable. Expanding the PC range reduced the number of reference substance retests, and thereby qualified additional

definitive tests as acceptable because they would not fail simply because the PC was out of the pre-set range.

5.3.4 Phase III: Main Validation Phase

The purpose of Phase III was to generate high quality *in vitro* cytotoxicity data using the 3T3 and NHK NRU test methods with protocols that were optimized based on the experience and results in Phases I and II. Sixty coded reference substances were tested; 46 of these were RC substances that covered a broad range of toxicity. The reference substances in Phase III spanned all five GHS toxicity categories and unclassified substances. Each substance was tested in each laboratory at least once in a range finding experiment, and then in three acceptable definitive tests performed on different days.

Section 2.6.4 addresses protocol changes made before the initiation of Phase III. The specific changes made in the 3T3 and NHK protocols, along with the effect the changes had on the test data, are summarized below.

5.3.4.1 *Protocol Changes and the Effect on the Data*

- *Prequalification of NHK Culture Medium*: Included a protocol for prequalifying NHK culture medium and supplements. This prevented the participating laboratories from using medium and supplements that did not support adequate growth of the cells.
- *Stopping Rule for Testing*: Added this rule for reference substances that were insoluble (i.e., <200 μg/mL) and/or did not produce sufficient cytotoxicity for the calculation of an IC_{50}. This rule allowed testing to end for substances that produced no IC_{50} data after three definitive tests. Substances for which an IC_{50} was not produced by one or more laboratories are presented in **Table 5-2**. Carbon tetrachloride did not produce an IC_{50} in any of the laboratories in either the 3T3 or the NHK NRU test methods, and methanol did not produce an IC_{50} in the 3T3 NRU test method.
- *Acceptable Range for Dose-Response Data Points:* Modified the test acceptance criterion for the number of data points required on the toxicity curve. The criterion was changed from requiring a minimum of two points (at least one >0% and ≤50% viability, and at least one >50% and <100% viability) to one point >0% and <100% viability, if the smallest practical dilution factor (i.e., 1.21) was used, and all other test acceptance criteria were met. This reduced the number of failed experiments for substances with very steep concentration-response curves, without reducing the quality of the IC_{50} data. For the 3T3 NRU test method, diquat dibromide (1/9 definitive tests), epinephrine bitartrate (2/9 definitive tests), and 1,1,1-trichloroethane (2/8 definitive tests) had such steep dose-responses that some acceptable tests met these revised criteria. None of the NHK NRU tests needed the revised criteria.
- *R^2 Acceptance Criterion*: Rescinded the R^2 criterion for the fit of the Hill function. The SMT determined that the R^2 criterion was best used to characterize the shape of the concentration-response curve rather than to establish a criterion for test acceptability. This reduced the number of failed experiments without affecting the calculation of the IC_{50} values as long as an

adequate number of toxicity points between 0 and 100% viability were obtained.

- *PC Acceptance Criteria*: Modified the PC acceptance criterion for Hill function fit.
- *Hill Function Analysis*: Altered the PRISM® template for the Hill function analysis to perform calculations for IC_x values in two ways: (1) constraining Bottom parameter to zero, and (2) fitting the Bottom parameter. As a result of the changes and efforts by the laboratories to use dilution schemes that captured the entire concentration-response range, very few tests in Phase III had $R^2 < 0.9$.
- *Biphasic Dose-Response in Range Finder Test*: Provided guidance for proceeding with definitive testing when a biphasic dose-response was obtained in the range-finder test. The definitive test was to focus on the lowest concentrations that produced responses around 50% viability (See **Section 2.6.3.2**).

Table 5-2 Reference Substances Affected by Stopping Rule[1]

Reference Substance	Testing Stopped -- No IC_{50} Data					
	3T3 NRU Test Method			NHK NRU Test Method		
	ECBC	FAL	IIVS	ECBC	FAL	IIVS
Carbon tetrachloride	X	X	X	X	X	X
Disulfoton		X				
Gibberellic acid		X				
Methanol	X	X	X	X		
1,1,1-Trichloroethane	X				X	X
Valproic acid			X			
Xylene	X	X		X	X	

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

[1]Substances that did not provide sufficient cytotoxicity for the calculation of an IC_{50} in one or more laboratories (identified by X).

5.4 Data Used to Evaluate Test Method Accuracy and Reliability

This section first presents the acceptable PC data and IC_{50} results from each laboratory for each phase of the validation study, and then presents the reference substance IC_{50} results and Hill Slopes from each phase. The individual test data for both passing and failing tests are provided in **Appendix I** for the PC and reference substances. Accuracy (concordance for the prediction of GHS acute oral toxicity category) and reliability assessments are provided in **Sections 6** and **7**, respectively.

5.4.1 PC Data

A summary of the acceptable SLS data IC_{50} results used to calculate quality control acceptance limits for each test method in each laboratory are provided in **Table 5-3**. The SLS IC_{50} results were used to calculate acceptable limits for each laboratory to use in subsequent study phases. One of the test acceptance criteria for each reference substance test was that the associated SLS IC_{50} must be within the acceptance limits. The individual test data for both passing and failing PC tests are provided in **Appendix I3** for the 3T3 and in **Appendix I4** for the NHK methods.

Table 5-3 Positive Control (PC)[1] IC_{50} Results by Study Phase

Study Phase	ECBC				FAL				IIVS			
	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N	Mean IC_{50} (µg/mL)	Standard Deviation (µg/mL)	Acceptance Limits	N
3T3 NRU												
Ia[2]	38.3	4.71	28.8 – 47.7	15	42.3	8.56	25.2 – 59.5	25	40.9	3.19	34.5 – 47.3	12
Ib[3]	41.3	5.99	26.4 – 56.3	12	43.2	4.68	31.5 – 54.9	17	42.1	3.40	33.6 – 50.6	13
II[4]	41.2	4.20	30.8 – 51.6	29	45.9	7.50	27.2 – 64.7	36	40.6	3.50	31.8 – 49.3	21
III[5]	41.6	3.41	NA	65	41.1	6.23	NA	26	41.5	3.74	NA	22
NHK NRU												
Ia[2]	4.03	1.32	1.40 – 6.67	15	7.45	3.07	1.34 – 13.6	18	3.68	0.555	2.57 – 4.79	30
Ib[3]	3.65	0.98	1.22 – 6.10	11	5.35	2.32	0[6] – 11.1	15	3.57	0.59	2.10 – 5.04	17
II[4]	3.59	1.41	0.07 – 7.11	22	3.20	1.05	0.57 – 5.82	15	3.78	0.73	1.94 – 5.61	26
III[5]	3.03	0.75	NA	57	3.45	0.90	NA	35	3.12	0.53	NA	20

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; N=Number of acceptable tests; NA=Not applicable

[1]PC was sodium lauryl sulfate (SLS).

[2]Values generated from Phase Ia data were used as acceptance criteria for Phase Ib tests; Acceptance limits = Mean ±2 X standard deviation.

[3]Values generated from Phases Ia and Ib data were used as acceptance criteria for Phase II tests; Acceptance limits = Mean ±2.5 X standard deviation.

[4]Values generated from Phases Ia, Ib, and II data were used as acceptance criteria for Phase III tests; Acceptance limits = Mean ±2.5 X standard deviation.

[5]Values generated from Phase III test data.

[6]Calculation of lower limits yielded a negative value, so that lower limit was set at 0 and later revised to 0.1 µg/mL.

5.4.1.1 *Phase Ib PC Data Acceptance Limits*

The SLS IC_{50} acceptance limits for Phase Ib testing were calculated using the Phase Ia data. The data sets from each laboratory were examined for outliers using the method of Dixon and Massey (1981), but none were identified. The acceptance limits for the SLS IC_{50} values for each laboratory and test method were the mean ±2 SD.

5.4.1.2 *Phase II PC Data Acceptance Limits*

The IC_{50} values from the Phase Ia and Ib SLS tests were used to calculate laboratory-specific and test method-specific quality control acceptance limits for Phase II. Phase Ib tests that had SLS IC_{50} values outside of the acceptance limits were considered acceptable if they met all other test acceptance criteria. For any day during which there was more than one SLS test (for any one method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with multiple values from a single day. Outliers at the 99% level were removed and the remaining values were used to calculate the mean ±2.5 SD acceptance limits. The acceptance limits were expanded from 2 SD in Phase Ib to 2.5 SD for Phase II to allow for the fact that the SDs decrease as more data are collected.

5.4.1.3 *Phase III PC Data Acceptance Limits*

The IC_{50} values from the Phase I and II SLS tests were used to calculate laboratory-specific and method-specific quality control acceptance limits for Phase III data. The SLS IC_{50} values outside the acceptance limits were considered acceptable if the tests met all other acceptance criteria. For any day for which there was more than one SLS test (for any one method and laboratory), the IC_{50} values were averaged to better reflect day-to-day variation and avoid overweighting the overall mean with multiples values from a single day. ANOVA was used to compare the Phase Ia, Ib, and II data within each laboratory to determine whether the SLS IC_{50} for each method and laboratory was changing over the course of the study. For PC data that were not significantly different from phase to phase at $p < 0.05$, the IC_{50} values were used to calculate the mean ±2.5 SD as the acceptance limits for Phase III. The only significant differences in SLS values seen between study phases ($p < 0.0002$) were the FAL results for NHK. This difference was attributed to the changes in cell culture practices between Phases Ib and II (see **Section 5.3.3**). Thus, only the Phase II SLS IC_{50} values were used to calculate the acceptance limits for Phase III NHK data at FAL.

5.4.2 Reference Substance Data

Reference substance data and results from the individual 3T3 and NHK tests (both acceptable and unacceptable) from each laboratory are presented in **Appendices I1** and **I2**. **Tables 5-4** and **5-5** summarize the IC_{50} and Hill Slope data from the acceptable 3T3 and NHK tests, respectively, for each reference substance and laboratory. The Hill Slope data are provided for supplemental information on the concentration-response characteristics for each reference substance, but were not used for reliability or accuracy analyses. These tables are organized alphabetically by substance name and provide substance class (based on the NLM Medical Subject Heading [MeSH index]), arithmetic mean IC_{50} and SD for each laboratory, arithmetic mean Hill Slope and SD for each laboratory, and the number of tests used to produce the mean values. **Figure 5-1** graphically presents the 3T3 IC_{50} data from **Table 5-4**, and **Figure 5-2** presents the NHK IC_{50} data from **Table 5-5**. The reference substances in **Figures 5-1** and **5-2** are ordered by ascending IC_{50} (lowest value [most toxic] to highest value [least toxic]) using the 3T3 IC_{50} values from IIVS (the lead laboratory for the study). This allows a simple

comparison of each reference substance value from each laboratory. **Table 5-6** provides the numerical key to the reference substances in **Figures 5-1** and **5-2**.

Because of their low toxicity and/or low solubility, some substances were not sufficiently toxic for calculation of an IC_{50} value. For the 3T3 NRU test method, no IC_{50} values were obtained for carbon tetrachloride or methanol in any laboratory (see **Table 5-4**). ECBC was the only laboratory that obtained IC_{50} values for lithium carbonate, and IIVS was the only laboratory that obtained IC_{50} values for xylene. Only one acceptable test (and IC_{50} value) was obtained for disulfoton at FAL, for 1,1,1-trichloroethane at ECBC, and for valproic acid at IIVS. FAL did not achieve sufficient toxicity for the calculation of an IC_{50} for gibberellic acid in any 3T3 NRU tests performed. For the NHK NRU test method (see **Table 5-5**), there was insufficient toxicity in all tests in all laboratories for a calculation of an IC_{50} for carbon tetrachloride. Only one laboratory achieved sufficient toxicity for the calculation of an IC_{50} for 1,1,1-trichloroethane (ECBC) and xylene (IIVS). One laboratory, ECBC, failed to achieve sufficient toxicity for the calculation of an IC_{50} for methanol. All of these substances, with the exception of methanol, produced precipitate in the cell culture medium. The solvent used for methanol was DMSO, and because the amount of DMSO that could be used in the cell culture was limited to 0.5%, the amount of DMSO that could be used to dissolve methanol was also limited. The differences among laboratories regarding their ability to attain a high enough concentration to achieve an IC_{50} for some substances may be due to the differing perceptions of the laboratory personnel regarding whether or not the substance was sufficiently dissolved, or differences in the techniques used to dissolve the substances.

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Acetaminophen	Amide	III	40.8	9.12	3	-1.53	0.354	66.2	23.0	3	-1.23	0.503	43.4	11.4	3	-1.55	0.165
Acetonitrile	Nitrile	III	6433	129	3	-2.29	0.648	9690	5634	3	-1.55	0.196	9330	1217	3	-2.63	0.245
Acetylsalicylic acid	Carboxylic Acid; Phenol	III	646	61.5	3	-1.75	0.473	1234	298	3	-1.99	0.393	401	62.0	3	-1.31	0.167
Aminopterin	Heterocyclic	II	0.005	0.001	3	-2.00	0.395	0.012	0.005	3	-3.36	1.59	0.005	0.001	3	-1.46	0.198
5-Aminosalicylic acid	Carboxylic Acid; Phenol	III	1467	203	3	-1.82	0.267	2070	334	3	-2.33	0.809	1557	179	3	-1.64	0.326
Amitriptyline HCl	Polycyclic	III	6.03	1.38	3	-2.47	0.668	7.86	2.20	3	-2.98	0.446	7.81	1.38	3	-4.48	0.916
Arsenic III Trioxide	Arsenical	Ib	2.41	0.782	4	-1.94	0.204	1.04	0.070	4	-3.02	2.09	4.09	2.23	3	-1.62	0.285
Atropine sulfate	Heterocyclic	III	54.1	29.6	3	-1.32	0.480	133	41.1	3	-2.20	0.695	70.0	5.7	3	-1.27	0.165
Boric acid	Boron compound; Acid	III	1497	484	3	-1.14	0.039	3987	693	3	-1.86	0.654	1202	581	3	-1.71	0.677
Busulfan	Alcohol; Sulfur compound; Acyclic hydrocarbon	III	40.4	19.3	3	-0.515	0.003	321	180	3	-1.14	0.802	43.7	1.77	3	-0.627	0.164
Cadmium II chloride	Cadmium compound; Chlorine compound	II	0.480	0.066	3	-1.85	0.529	0.400	0.129	3	-3.05	0.743	0.817	0.427	3	-2.45	0.449
Caffeine	Heterocyclic	III	133	13.3	3	-1.11	0.097	157	81.7	3	-0.866	0.250	191	14.4	3	-1.27	0.077
Carbamazepine	Heterocyclic	III	83.0	12.0	3	-1.94	0.539	152	56.9	3	-3.50	1.27	91.8	11.0	3	-2.34	0.307
Carbon tetrachloride	Halogenated hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Chloral hydrate	Alcohol	III	151	15.6	3	-1.73	0.172	241	25.1	3	-2.16	0.597	170	19.9	3	-1.68	0.084
Chloramphenicol	Alcohol; Nitro compound; Cyclic hydrocarbon	II	55.3	12.4	4	-0.779	0.057	273	82.2	4	-1.16	0.249	156	27.9	3	-0.952	0.036
Citric acid	Carboxylic acid	III	473	138	3	-1.89	0.423	1148	143	4	-3.68	0.407	865	160	3	-2.51	0.530

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Colchicine	Polycyclic	II	0.021	0.002	4	-1.69	0.049	0.093	0.042	3	-1.61	1.80	0.028	0.0003	3	-1.69	0.255
Cupric sulfate pentahydrate	Sulfur compound; Metal	III	82.7	3.18	3	-4.85	0.700	123	54.0	4	-17.7	15.5	5.72	1.75	3	-5.71	1.14
Cycloheximide	Heterocyclic	III	0.125	0.057	3	-1.19	0.167	0.647	0.451	3	-1.53	0.128	0.109	0.025	3	-0.937	0.158
Dibutyl phthalate	Carboxylic acid	III	23.5	3.98	3	-3.37	1.27	191	94.5	4	-0.965	0.140	20.7	1.37	3	-2.62	0.283
Dichlorvos	Organophos-phorous	III	9.83	3.42	3	-1.32	0.297	32.8	2.07	3	-3.42	1.00	18.3	2.09	3	-2.13	0.439
Diethyl phthalate	Carboxylic acid	III	85.5	29.0	3	-1.11	0.340	147	37.8	3	-2.03	0.422	106	25.3	3	-2.35	0.824
Digoxin	Polycyclic; Carbohydrate	III	351	137	3	-2.11	2.05	892	319	3	-3.26	2.21	317	67.9	2	-3.04	1.52
Dimethyl-formamide	Amide; Carboxylic acid	III	5343	515	3	-1.96	0.087	5483	517	3	-1.80	0.143	4900	183	3	-1.87	0.102
Diquat dibromide monohydrate	Heterocyclic	III	3.87	0.887	3	-1.59	0.197	36.1	35.5	3	-11.5	10.1	5.39	1.36	3	-3.00	0.784
Disulfoton	Organophos-phorous; Sulfur compound	III	137	74.9	3	-2.06	1.88	11200	NA	1	-1.22	NA	60.4	52.5	3	-2.23	1.08
Endosulfan	Heterocyclic Sulfur compound	III	5.27	3.01	3	-0.669	0.243	15.2	11.9	4	-0.762	0.221	3.61	1.53	3	-0.871	0.636
Epinephrine bitartrate	Alcohol; Amine	III	51.5	6.16	3	-5.99	3.08	63.4	6.63	3	-45.1	32.0	63.4	1.91	3	-4.74	1.51
Ethanol	Alcohol	III	5360	1754	3	-1.33	0.104	8420	1205	3	-1.88	0.128	6413	345	3	-1.99	0.372
Ethylene glycol	Alcohol	Ib	18325	1658	4	-3.79	4.08	31650	7453	4	-1.70	0.166	25900	3081	3	-1.67	0.079
Fenpropathrin	Nitrile; Ester; Ether	III	22.6	2.41	3	-2.54	0.350	42.4	26.8	4	-1.44	0.645	16.7	2.03	3	-2.53	0.495
Gibberellic acid	Polycyclic	III	8027	908	3	-1.95	0.678	NA	NA	-	NA	NA	7657	745	3	-1.66	0.087
Glutethimide	Heterocyclic	III	167	7.00	3	-1.3	0.045	284	20.7	3	-1.47	0.131	125	9.25	4	-1.20	0.163
Glycerol	Alcohol	III	20000	2987	3	-2.02	0.273	38878	28238	4	-2.27	1.29	27833	10882	3	-1.87	0.306
Haloperidol	Ketone	III	5.32	0.649	3	-2.34	0.445	7.99	0.655	3	-4.99	0.378	5.47	0.654	3	-1.86	0.048

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Hexachlorophene	Cyclic hydrocarbon Phenol	III	5.02	2.41	3	-1.62	0.189	5.35	1.75	3	-1.17	0.322	3.06	0.289	3	-1.66	0.217
Lactic acid	Carboxylic acid	III	2943	315	3	-4.13	1.54	3487	561	3	-6.62	3.23	2790	259	3	-3.64	1.09
Lindane	Halogenated hydrocarbon	III	125	119	3	-0.737	0.231	266	94.8	4	-1.26	1.283	90.4	111	5	-1.46	0.262
Lithium I carbonate	Alkalies; Inorganic carbon; Lithium compound	II	564	67.6	3	-1.59	0.313	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Meprobamate	Carboxylic acid	III	353	49.7	3	-1.16	0.438	877	128	4	-1.32	0.270	386	9.02	3	-1.12	0.133
Mercury II chloride	Mercury compound; Chlorine compound	III	3.45	0.177	3	-4.18	0.988	5.99	1.87	3	-4.34	1.11	3.51	0.120	3	-4.16	1.31
Methanol	Alcohol	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Nicotine	Heterocyclic	III	272	65.3	3	-1.58	0.357	412	136	3	-12.0	6.99	450	54.7	3	-49.6	70.9
Paraquat	Heterocyclic	III	21.3	7.29	3	-1.32	0.341	24.9	16.5	3-	-4.10	3.13	23.7	15.2	3	-1.92	0.581
Parathion	Organophos-phorous; Sulfur compound	III	22.7	12.1	3	-1.89	1.33	141	98.7	4	-1.62	0.520	22.0	4.94	3	-1.55	0.562
Phenobarbital	Heterocyclic	III	634	134	3	-1.43	0.177	726	255	3	-1.84	0.851	476	111	4	-1.67	0.418
Phenol	Phenol	III	50.2	10.9	3	-1.46	0.318	104	24.8	3	-1.55	0.205	58.1	6.78	3	-1.41	0.259
Phenylthiourea	Sulfur compound; Urea	III	30.1	19.8	3	-0.781	0.218	239	65.8	3	-0.890	0.206	89.0	21.9	3	-1.40	0.127
Physostigmine	Carboxylic acid; Heterocyclic	III	28.2	14.9	3	-1.51	0.595	37.8	1.93	3	-7.22	1.04	20.4	6.71	4	-1.70	0.157
Potassium I chloride	Potassium compound; Chlorine compound	II	3352	468	4	-3.32	1.17	3842	1198	5	-4.31	2.27	3710	417	3	-2.87	0.147

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Potassium cyanide	Potassium compound; Nitrogen compound	III	15.3	3.76	3	-1.48	0.677	159	81.9	3	-1.03	0.152	18.9	0.950	3	-3.43	0.488
Procainamide HCl	Carboxylic acid; Amide	III	400	15.3	3	-12.4	1.91	431	4.73	3	-45.6	18.4	497	39.3	3	-19.9	13.1
2-Propanol	Alcohol	II	2610	240	2	-1.80	0.001	3970	139	3	-1.65	0.241	4110	161	3	-1.93	0.160
Propranolol HCl	Alcohol	Ib	13.6	4.37	4	-2.54	0.627	13.5	6.85	4	-3.31	2.53	17.6	3.78	3	-3.45	1.44
Propylparaben	Carboxylic acid; Phenol	III	20.9	3.33	3	-1.23	0.259	51.8	14.8	3	-1.45	0.442	17.1	2.10	3	-1.24	0.245
Sodium arsenite	Sodium compound; Arsenical	III	0.496	0.028	3	-1.43	0.087	1.44	0.819	3	-3.79	1.22	0.683	0.117	3	-1.90	0.535
Sodium chloride	Sodium compound; Chlorine compound	III	4790	233	3	-1.55	0.182	4625	611	4	-2.67	0.620	4877	457	3	-2.03	0.366
Sodium dichromate dihydrate	Sodium compound; Chromium compound	III	0.603	0.087	3	-1.64	0.136	0.657	0.244	3	-5.01	1.51	0.547	0.092	3	-1.93	0.194
Sodium I fluoride	Sodium compound; Fluorine compound	II	61.3	5.55	3	-5.06	1.50	96.1	17.7	3	-4.40	0.971	82.0	5.81	3	-2.73	0.850
Sodium hypochlorite	Sodium compound Oxygen compound; Chlorine compound	III	823	108	3	-2.57	1.12	805	367	3	-4.13	3.05	2005	872	4	-3.20	0.279
Sodium oxalate	Sodium compound; Carboxylic acid	III	42.0	17.3	3	-1.83	0.380	31.0	8.66	3	-3.11	0.367	49.5	26.3	4	-2.32	0.592
Sodium selenate	Sodium compound; Selenium compound	II	12.7	1.62	3	-1.59	0.217	54.2	10.4	3	-3.76	0.968	36.5	5.23	3	-1.65	0.112

Table 5-4 3T3 NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2] (IC_{50})	N	Hill Slope[3]	SD[4]
Strychnine	Heterocyclic	III	389	80.9	3	-2.51	0.728	124	20.3	3	-5.85	0.922	83.5	5.35	3	-6.49	2.12
Thallium I sulfate	Sulfur compound; Metal	III	2.81	0.671	3	-1.02	0.201	13.4	10.4	4	-0.714	0.302	6.27	1.75	3	-0.752	0.081
Trichloroacetic acid	Carboxylic acid	III	762	99.1	3	-1.66	0.118	1220	72.1	3	-2.22	0.089	801	114	3	-1.77	0.130
1,1,1-Trichloro-ethane	Halogenated hydrocarbon	III	41100	NA	1	-2.38	NA	21250	2357	3	-31.5	32.1	9827	180	3	-21.8	8.47
Triethylene-melamine	Heterocyclic	III	0.086	0.009	3	-0.567	0.018	1.45	0.265	3	-1.88	1.04	0.169	0.049	3	-0.615	0.138
Triphenyltin hydroxide	Organo-metallic compound	III	0.026	0.004	3	-1.66	0.257	0.026	0.021	3	-4.78	3.37	0.015	0.008	3	-1.46	0.149
Valproic acid	Carboxylic acid; Lipids	III	547	67.1	3	-2.24	0.742	1807	175	3	-4.07	0.766	574	NA	1	-1.24	NA
Verapamil HCl	Amine	III	32.2	5.82	3	-4.43	1.362	34.6	1.72	3	-29.1	18.6	38.9	4.20	3	-5.00	0.935
Xylene	Cyclic hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	724	87.1	3	-1.91	0.473

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SD=Standard deviation; N=Number of data points; NA=Not available (i.e., IC_{50} values or Hill Slope values could not be generated [see notes in **Appendix I** for more information])

[1]Arithmetic mean.

[2]Standard deviation of IC_{50}.

[3]Arithmetic Mean of Hill Slope values.

[4]Standard deviation of Hill Slope values.

[5]Chemical class assigned is based on the classification of the National Library of Medicine's Medical Subject Heading (MeSH), http://www.nlm.nih.gov/mesh/meshhome.html.

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Acetaminophen	Amide	III	558	80.7	3	-1.09	0.108	447	83.7	3	-1.09	0.646	571	79.0	3	-1.20	0.154
Acetonitrile	Nitrile	III	10868	7824	4	-2.61	0.424	10153	1960	4	-5.95	3.34	9290	413	3	-2.79	0.306
Acetylsalicylic acid	Carboxylic Acid; Phenol	III	631	19.9	3	-1.94	0.367	694	98.3	3	-1.85	0.324	514	79.1	3	-1.97	0.083
Aminopterin	Heterocyclic	II	889	182	3	-2.03	0.375	545	42.2	3	-1.27	0.225	611	70.7	2	-1.72	0.547
5-Aminosalicylic acid	Carboxylic Acid; Phenol	III	29.9	6.52	3	-3.45	0.806	78.2	42.3	3	-7.96	6.90	48.8	7.90	3	-3.66	0.629
Amitriptyline HCl	Polycyclic	III	10.8	3.34	3	-1.79	0.236	7.57	5.43	3	-1.43	0.479	10.9	1.04	3	-2.27	0.278
Arsenic III Trioxide	Arsenical	Ib	7.77	2.54	4	-2.67	0.470	2.55	1.92	6	-1.78	1.14	20.9	6.4	3	-2.02	0.338
Atropine sulfate	Heterocyclic	III	85.4	10.5	3	-1.26	0.307	104	88.2	3	-2.90	3.48	83.2	21.0	3	-1.21	0.101
Boric acid	Boron compound; Acid	III	440	138	3	-1.19	0.233	517	378	3	-0.752	0.117	464	11	3	-1.33	0.194
Busulfan	Alcohol; Sulfur compound; Acyclic hydrocarbon	III	253	68.2	3	-0.783	0.323	268	193	3	-1.50	0.357	313	37.2	3	-1.66	0.459
Cadmium II chloride	Cadmium compound; Chlorine compound	II	2.20	0.823	5	-4.01	1.25	1.88	1.22	3	-3.36	3.14	1.86	0.151	3	-4.65	1.38
Caffeine	Heterocyclic	III	817	256	3	-1.44	0.504	591	186	3	-1.06	0.499	574	7.81	3	-1.28	0.117
Carbamazepine	Heterocyclic	III	66.1	8.4	3	-1.15	0.307	253	325	3	-2.57	2.53	63.9	5.27	3	-1.34	0.444
Carbon tetrachloride	Halogenated hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Chloral hydrate	Alcohol	III	140	34.2	3	-1.55	0.378	159	50.1	3	-1.33	0.105	112	1.73	3	-1.42	0.123
Chloramphenicol	Alcohol; Nitro compound; Cyclic hydrocarbon	II	318	142	3	-1.51	0.794	414	182	4	-1.16	0.091	367	79.7	3	-0.917	0.249
Citric acid	Carboxylic acid	III	526	82.4	3	-1.62	0.158	312	51.6	4	-1.25	0.249	433	22.3	3	-1.62	0.080

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Colchicine	Polycyclic	II	0.005	0.002	3	-2.15	1.39	0.008	0.001	3	-3.16	1.96	0.008	0.002	3	-13. 8	11.0
Cupric sulfate pentahydrate	Sulfur compound; Metal	III	190	19.6	3	-6.16	3.16	195	12.5	3	-3.85	0.328	207	7.09	3	-5.69	0.871
Cycloheximide	Heterocyclic	III	0.053	0.012	3	-1.24	0.152	0.120	0.094	3	-0.850	0.388	0.071	0.013	3	-1.54	0.178
Dibutyl phthalate	Carboxylic acid	III	28.3	7.64	3	-1.40	0.295	47.4	34.3	3	-1.02	0.352	22.0	1.32	3	-1.33	0.197
Dichlorvos	Organophos-phorous	III	8.56	2.28	3	-1.17	0.147	12.4	3.74	3	-2.29	2.33	12.2	0.416	3	-1.50	0.214
Diethyl phthalate	Carboxylic acid	III	174	14.4	3	-2.21	0.358	71.5	67.3	3	-1.67	0.637	189	33.1	3	-1.97	0.242
Digoxin	Polycyclic; Carbohydrate	III	0.0054	0.0007	3	-2.00	0.127	0.0001	0.00002	3	-1.38	0.684	0.004	0.0003	3	-4.59	1.73
Dimethyl-formamide	Amide; Carboxylic acid	III	9353	155	3	-3.67	0.273	7817	100	3	-2.85	0.590	6397	202	3	-3.00	0.161
Diquat dibromide monohydrate	Heterocyclic	III	3.59	0.825	3	-1.44	0.051	6.77	3.73	4	-1.38	0.488	3.84	0.313	3	-1.10	0.139
Disulfoton	Organophos-phorous; Sulfur compound	III	140	27.0	3	-1.65	1.15	808	213	3	-0.841	0.452	186	59.2	3	-0.836	0.209
Endosulfan	Heterocyclic Sulfur compound	III	3.44	0.573	3	-1.68	0.438	1.42	0.701	4	-1.19	0.369	2.19	0.437	3	-2.20	0.242
Epinephrine bitartrate	Alcohol; Amine	III	115	10.8	3	-7.37	2.10	81.7	28.4	3	-8.39	5.81	75.0	12.2	3	-4.90	2.81
Ethanol	Alcohol	III	8290	390	3	-2.13	0.035	12013	2286	3	-1.82	0.635	10250	867	3	-2.29	0.185
Ethylene glycol	Alcohol	Ib	38000	4681	3	-3.22	0.650	49800	4371	3	-3.02	0.188	40000	5341	4	-2.56	0.444
Fenpropathrin	Nitrile; Ester; Ether	III	3.73	1.01	3	-1.42	0.486	2.23	0.616	3	-4.37	4.45	1.82	0.310	3	-1.78	0.617
Gibberellic acid	Polycyclic	III	2850	402	3	-2.45	0.372	2940	276	3	-5.90	2.69	2807	121	3	-3.30	1.104
Glutethimide	Heterocyclic	III	187	64.3	3	-1.47	0.616	170	24.1	3	-1.29	0.145	176	27.5	3	-1.54	0.237
Glycerol	Alcohol	III	34267	15399	3	-3.32	1.97	18023	8334	3	-1.62	0.521	29033	4596	3	-2.69	0.511
Haloperidol	Ketone	III	3.69	1.01	3	-0.964	0.206	3.72	1.81	3	-0.732	0.097	3.29	1.15	3	-0.840	0.100

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Hexachlorophene	Cyclic hydrocarbon Phenol	III	0.027	0.004	3	-2.21	0.301	0.046	0.020	3	-2.91	0.662	0.021	0.002	3	-2.36	0.059
Lactic acid	Carboxylic acid	III	1290	52.9	3	-2.36	0.306	1320	60.8	3	-3.25	0.328	1313	138	3	-3.23	0.408
Lindane	Halogenated hydrocarbon	III	19.1	3.14	3	-3.02	0.969	23.2	7.09	3	-2.24	0.315	15.6	2.4	3	-2.61	0.265
Lithium I carbonate	Alkalies; Inorganic carbon; Lithium compound	II	411	119	3	-1.95	0.456	486	95.7	3	-1.78	1.31	535	31.6	3	-2.64	0.164
Meprobamate	Carboxylic acid	III	761	116	3	-1.90	0.695	163	189	3	-0.806	0.206	624	84.2	3	-2.04	0.170
Mercury II chloride	Mercury compound; Chlorine compound	III	6.87	1.04	3	-16.3	4.95	5.4	1.02	3	-17.8	13.1	5.35	0.09	3	-17.8	3.31
Methanol	Alcohol	III	NA	NA	-	NA	NA	1133	213	3	-1.79	0.874	2100	226	3	-1.86	0.297
Nicotine	Heterocyclic	III	94.3	24.7	3	-0.654	0.092	134	78.4	3	-0.668	0.077	112	27.7	3	-0.733	0.047
Paraquat	Heterocyclic	III	48.3	6.03	3	-1.04	0.158	96.6	37.2	3	-1.34	0.326	53.4	5.52	3	-1.47	0.034
Parathion	Organophos-phorous; Sulfur compound	III	34.0	10.0	3	-1.60	0.640	31.2	11.9	3	-1.18	0.200	29.0	8.34	3	-1.85	0.956
Phenobarbital	Heterocyclic	III	693	180	3	-1.10	0.214	360	95.5	3	-0.976	0.229	381	69.9	3	-1.68	0.353
Phenol	Phenol	III	59.1	21.4	3	-0.919	0.084	93.2	5.97	3	-1.15	0.209	80.8	5.12	3	-0.915	0.029
Phenylthiourea	Sulfur compound; Urea	III	363	58	3	-1.55	0.726	401	83.6	3	-3.49	1.91	272	71.7	3	-1.00	0.053
Physostigmine	Carboxylic acid; Heterocyclic	III	164	5.51	3	-3.05	0.552	212	238	3	-3.81	2.44	139	8.74	3	-2.97	0.135
Potassium I chloride	Potassium compound; Chlorine compound	II	2560	432	3	-2.23	0.383	2287	631	3	-1.09	0.163	1990	161	3	-2.05	0.165

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Potassium cyanide	Potassium compound; Nitrogen compound	III	29.3	6.9	3	-1.21	0.241	89.0	100	3	-1.10	0.319	16.9	2.21	3	-1.37	0.154
Procainamide HCl	Carboxylic acid; Amide	III	1480	200	3	-3.56	0.813	1787	221	3	-4.22	1.57	2027	229	3	-4.42	0.459
2-Propanol	Alcohol	II	5263	583	3	-2.01	0.173	4273	1139	3	-2.31	0.211	7087	480	3	-3.01	0.406
Propranolol HCl	Alcohol	Ib	38.3	4.54	3	-3.44	0.559	43.8	2.52	3	-2.72	1.461	28.6	3.28	4	-2.09	0.413
Propylparaben	Carboxylic acid; Phenol	III	18.1	2.42	3	-1.18	0.122	18.6	2.84	3	-1.58	0.399	13.8	1.21	3	-1.20	0.065
Sodium arsenite	Sodium compound; Arsenical	III	0.79	0.248	3	-1.69	0.222	0.336	0.187	3	-1.54	0.317	0.470	0.066	3	-1.96	0.197
Sodium chloride	Sodium compound; Chlorine compound	III	3583	263	3	-2.43	0.153	1118	1388	3	-1.96	0.371	3470	300	3	-2.47	0.208
Sodium dichromate dihydrate	Sodium compound; Chromium compound	III	0.784	0.113	3	-2.35	0.282	0.851	0.302	4	-3.52	1.49	0.576	0.100	3	-2.32	0.199
Sodium I fluoride	Sodium compound; Fluorine compound	II	48.7	6.92	3	-2.50	0.263	39.7	9.61	3	-2.60	1.04	53.7	6.82	4	-2.71	0.150
Sodium hypochlorite	Sodium compound Oxygen compound; Chlorine compound	III	1863	581	3	-5.19	1.14	1243	576	3	-2.78	1.27	1633	180	3	-3.86	0.211
Sodium oxalate	Sodium compound; Carboxylic acid	III	355	54.9	3	-4.00	1.99	350	147	4	-6.10	6.40	360	94.6	3	-3.13	0.555
Sodium selenate	Sodium compound; Selenium compound	II	7.47	0.861	3	-1.78	0.529	16.1	9.55	3	-3.07	0.456	10.0	1.33	3	-1.75	0.226

Table 5-5 NHK NRU Test Method IC_{50} and Hill Slope Data by Laboratory

Substance	Chemical Class[5]	Phase in which Tested	ECBC					FAL					IIVS				
			IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]	IC_{50}[1] µg/mL	SD[2]	N	Hill Slope[3]	SD[4]
Strychnine	Heterocyclic	III	100	76.6	4	-1.30	0.729	52.5	28.0	3	-1.60	0.260	55.1	3.43	3	-1.47	0.466
Thallium I sulfate	Sulfur compound; Metal	III	0.198	0.100	3	-2.08	1.01	0.153	0.031	3	-2.64	0.639	0.127	0.020	3	-2.90	0.338
Trichloroacetic acid	Carboxylic acid	III	348	63.5	3	-1.36	0.241	541	150	3	-1.34	0.411	394	50.8	3	-1.48	0.103
1,1,1-Trichloro-ethane	Halogenated hydrocarbon	III	8137	591	3	-14.0	6.08	NA	NA	-	NA	NA	NA	NA	-	NA	NA
Triethylene-melamine	Heterocyclic	III	1.69	0.950	3	-0.838	0.076	2.03	0.471	3	-1.37	0.471	2.13	0.480	3	-1.95	0.369
Triphenyltin hydroxide	Organo-metallic compound	III	0.021	0.007	3	-2.46	0.698	0.007	0.007	3	-3.55	1.68	0.011	0.003	3	-3.34	0.396
Valproic acid	Carboxylic acid; Lipids	III	468	116	3	-1.31	0.252	702	160	3	-1.83	0.455	430	71.5	3	-1.24	0.115
Verapamil HCl	Amine	III	60.5	13.6	3	-1.72	0.238	79.4	33.9	3	-1.88	0.915	66.2	5.57	3	-2.53	0.221
Xylene	Cyclic hydrocarbon	III	NA	NA	-	NA	NA	NA	NA	-	NA	NA	486	185	3	-2.88	1.99

Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; SD=Standard deviation; N=Number of data points; NA=Not available (i.e., IC_{50} values or Hill Slope values could not be generated [see notes in **Appendix I** for more information])

[1]Arithmetic mean.

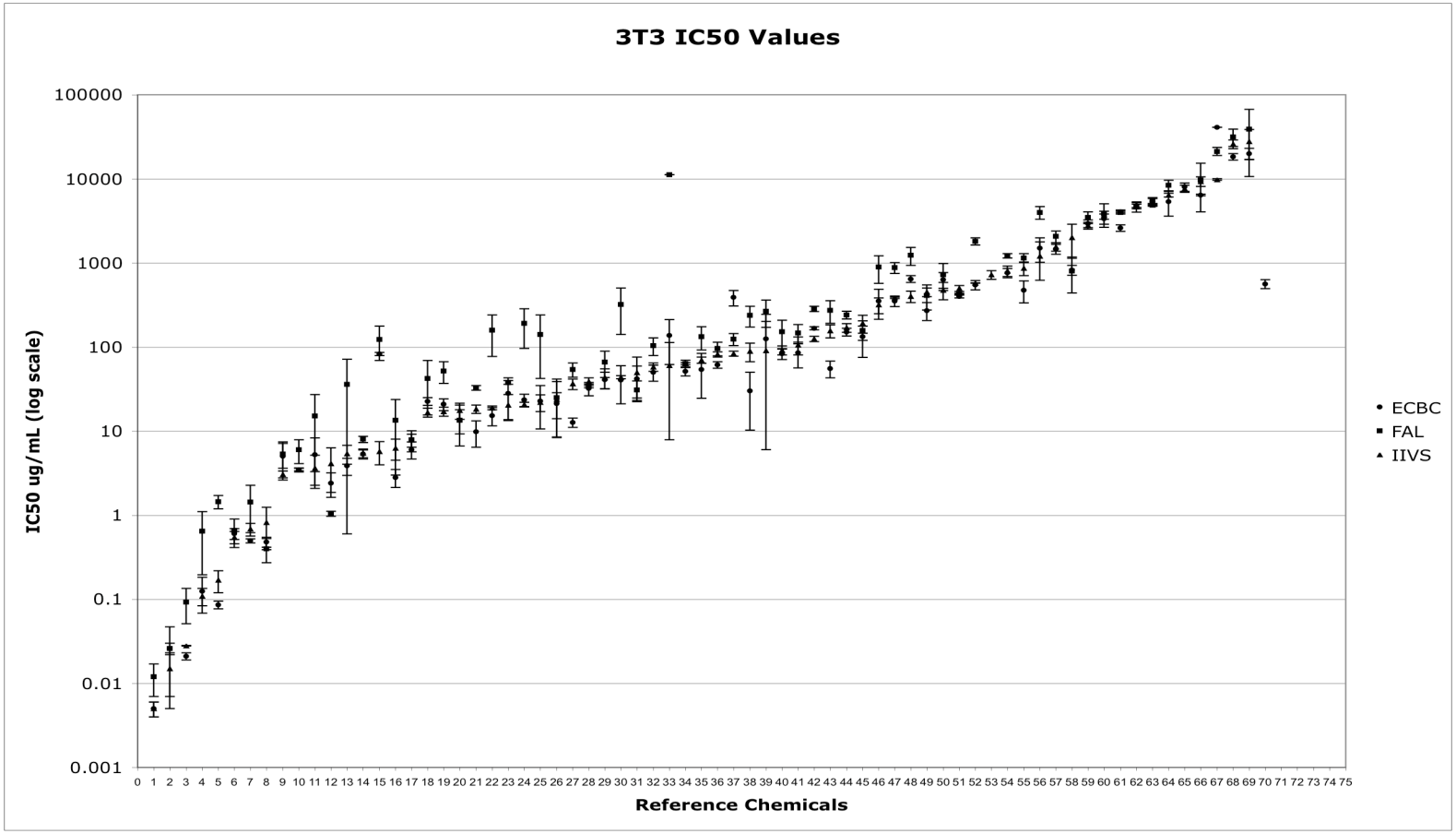
[2]Standard deviation of IC_{50}.

[3]Arithmetic Mean of Hill Slope values.

[4]Standard deviation of Hill Slope values.

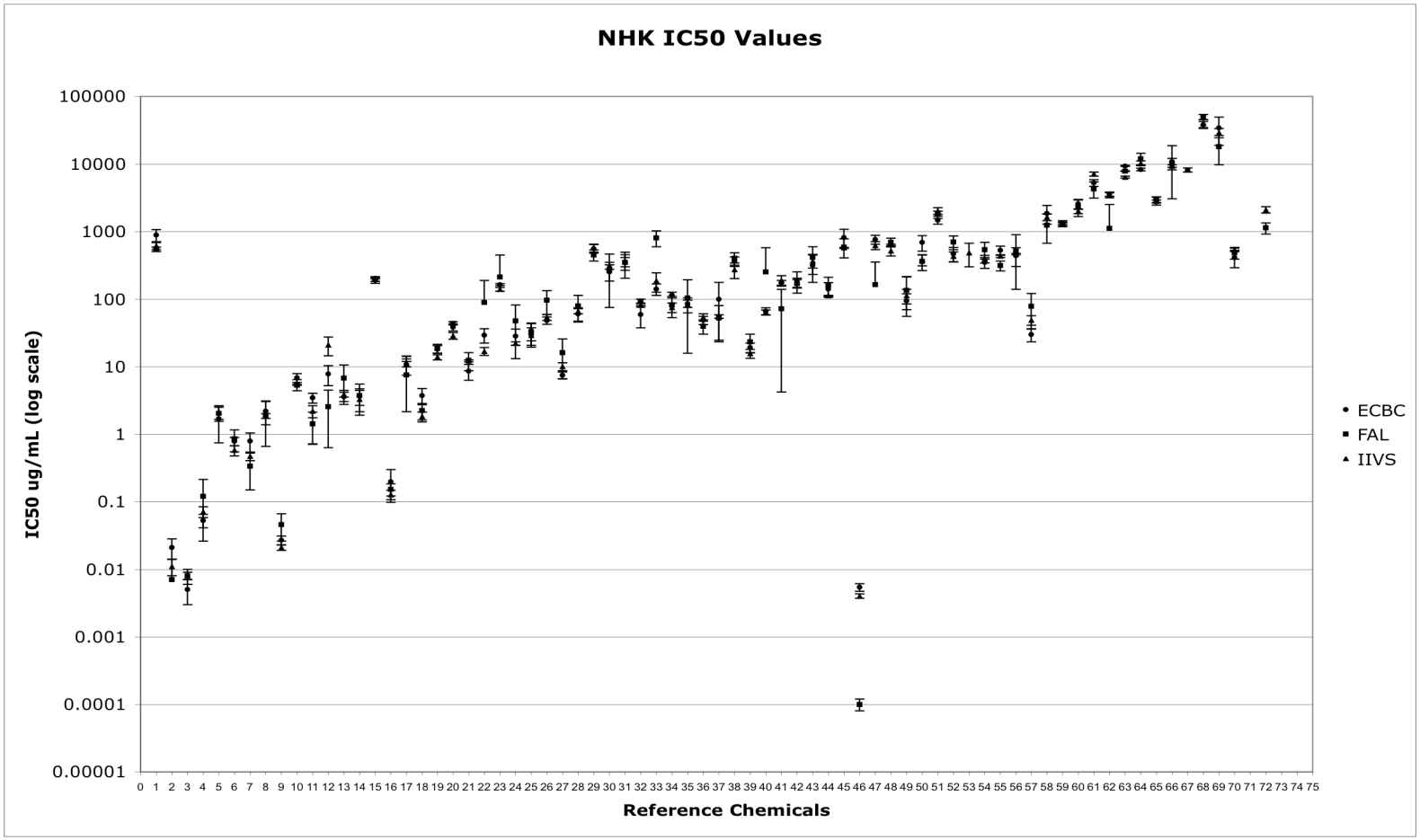
[5]Chemical class assigned is based on the classification of the National Library of Medicine's Medical Subject Heading (MeSH), http://www.nlm.nih.gov/mesh/meshhome.html.

Figure 5-1 Reference Substance IC_{50} Results for the 3T3 NRU Test Method by Laboratory



Abbreviations: ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.
Points show the mean arithmetic IC_{50} (µg/mL) for each reference substance from each laboratory. Error bars show the standard deviation. Data were sorted in ascending order of 3T3 IC_{50} values from IIVS (lead laboratory in the validation study). **Table 5-6** provides the numerical key for reference substance identification.

Figure 5-2 Reference Substance IC_{50} Results for the NHK NRU Test Method by Laboratory



Abbreviations: ECBC=Edgewood Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

Points show the mean arithmetic IC_{50} (µg/mL) for each reference substance from each laboratory. Error bars show the standard deviation. Data were sorted in ascending order of 3T3 IC_{50} values from IIVS (lead laboratory in the validation study). **Table 5-6** provides the numerical key for reference substance identification.

Table 5-6 Key to Validation Study Reference Substances[1]

No	Reference Substance	No	Reference Substance	No	Reference Substance	No	Reference Substance
1	Aminopterin	19	Propylparaben	37	Strychnine	55	Citric acid
2	Triphenyltin hydroxide	20	Propranolol HCl	38	Phenylthiourea	56	Boric acid
3	Colchicine	21	Dichlorvos	39	Lindane	57	5-Aminosalicylic acid
4	Cycloheximide	22	Potassium cyanide	40	Carbamazepine	58	Sodium hypochlorite
5	Triethylenemelamine	23	Physostigmine	41	Diethyl phthalate	59	Lactic acid
6	Sodium dichromate dihydrate	24	Dibutyl phthalate	42	Glutethimide	60	Potassium I chloride
7	Sodium arsenite	25	Parathion	43	Chloramphenicol	61	2-Propanol
8	Cadmium II chloride	26	Paraquat	44	Chloral hydrate	62	Sodium chloride
9	Hexachlorophene	27	Sodium selenate	45	Caffeine	63	Dimethylformamide
10	Mercury II chloride	28	Verapamil HCl	46	Digoxin	64	Ethanol
11	Endosulfan	29	Acetaminophen	47	Meprobamate	65	Gibberellic acid
12	Arsenic III trioxide	30	Busulfan	48	Acetylsalicylic acid	66	Acetonitrile
13	Diquat dibromide monohydrate	31	Sodium oxalate	49	Nicotine	67	1,1,1-Trichloroethane
14	Haloperidol	32	Phenol	50	Phenobarbital	68	Ethylene glycol
15	Cupric sulfate pentahydrate	33	Disulfoton	51	Procainamide HCl	69	Glycerol
16	Thallium I sulfate	34	Epinephrine bitartrate	52	Valproic acid	70	Lithium I carbonate
17	Amitriptyline HCl	35	Atropine sulfate	53	Xylene	71	Carbon tetrachloride
18	Fenpropathrin	36	Sodium I fluoride	54	Trichloroacetic acid	72	Methanol

Abbreviations: No=Number.
[1]As used in **Figures 5-1** and **5-2**.

5.5 Statistical Approaches to the Evaluation of 3T3 and NHK Data

The statistical approaches used for data evaluation are reviewed in the following sections for each phase of the validation study. **Section 2.2.3** discussed the endpoint measurements for the 3T3 and NHK test methods. The OD values of each of six replicate wells ([minimum of four] in the 96-well plate) per test concentration (eight concentrations/reference substance or PC) were used to determine relative cell viability in relation to the mean VC OD on the same plate. The cell viability values calculated for the replicate wells for each concentration were used to determine the concentration-response curve (percent viability vs. log concentration) for each test. The IC_{50} value was determined from fitting the curve to a Hill function.

5.5.1 Statistical Analyses for Phase Ia Data

The laboratories reported the IC_{50} results for SLS in μg/mL. The SMT used the results from the acceptable tests to calculate means and SDs for each method at each laboratory.

5.5.1.1 *Outlier Determination for Replicate Well Concentration Data*

A test for outliers at the 99% level (Dixon and Massey 1981) was used to determine the presence of outlier OD values among the six replicate wells for each reference substance concentration. The SMT applied the outlier test to the Phase Ia data when extreme values were noted. Outliers were excluded from the data set, and the IC_{50} was recalculated. The raw data files include all data provided by the laboratories, including the excluded outlier OD values. Because the protocol required a minimum of four acceptable test wells per reference substance concentration, no more than two wells of the six replicates could be excluded.

5.5.1.2 *Curve Fit Criteria*

After the completion of Phase Ia testing, a curve fit criterion was implemented for test acceptance following a visual review of the fit of the OD data to the Hill function curve. The SMT considered the fit of the concentration-response curve to the Hill function to be acceptable when $R^2 > 0.9$. A fit of $R^2 < 0.8$ was considered unacceptable and the data from that test were rejected. Curves with a fit of $0.8 < R^2 < 0.9$ were evaluated visually for goodness of fit and accepted if the SMT concluded that there were sufficient data points between 0 and 100% cytotoxicity, and a reasonable shape to the curve, to calculate a reasonably accurate IC_{50} value. Each test with a curve fit in this range was analyzed on a case-by-case basis, and no standard pass/fail criterion was developed. [Note: The use of a curve fit criterion was reevaluated in Phases Ib and II, and was eliminated as a test acceptance criterion for Phase III test results. An R^2 value ≥0.85 was maintained as a test acceptance criterion for the PC because its fit to the Hill function was well characterized.]

5.5.1.3 *Reproducibility Analyses for PC IC_{50} Values*

To evaluate reproducibility of the IC_{50} values for the PC for each test method, within and between the laboratories, the SMT considered the American Society of Testing and Materials (ASTM) Standard E691-99, *Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method* (ASTM 1999). This method uses two statistics, *h* and *k*, to judge the consistency of means and variances between laboratories. However, a minimum of six laboratories is required for this type of analysis and the SMT decided that it could not be appropriately applied to three laboratories. The variability of the PC IC_{50} results obtained from each test and laboratory was assessed using CV analysis and one-way ANOVA. Dividing the SD by the arithmetic mean IC_{50} value, and multiplying by 100 produced the CV. CV values were calculated for the acceptable tests within each laboratory to determine intralaboratory reproducibility. To compare the variation among laboratories, the CV was calculated using the arithmetic mean IC_{50} values from each of the three laboratories. Although no criterion for an acceptable CV was determined for this study, ECVAM recently used CV <30% as an acceptable range for both intra- and inter-laboratory reproducibility (Zuang et al. 2002; Fentem et al. 2001). Although CV <30% was intended to reflect an acceptable maximum for normal biological variability, the range was not supported by data.

For the ANOVA, IC_{50} values were first converted to mM units and then log-transformed to obtain normal distributions. One-way ANOVA was performed with SAS PROC GLM

software (SAS Institute 1999; see **Appendix D1** for example SAS code). A significance level of $p < 0.01$ was used to test results between the laboratories in order to be conservative with respect to identifying laboratory differences.

5.5.2 Statistical Analyses of Phase Ib Data

5.5.2.1 *Outlier Determination for Replicate Well Concentration Data*
For consistency of replicate well concentration data, the SMT applied the same outlier test used for the Phase Ia data (Dixon and Massey 1981) when extreme OD values were noted. If the extreme value was an outlier at the 99% level, it was excluded from the data set, and the IC_{50} was recalculated. All data are available in the data files provided by the laboratories, including the excluded outlier OD values.

5.5.2.2 *Reproducibility Analyses of the Reference Substance IC_{50} Values*
One-way ANOVA and CV analyses were used to assess method reproducibility within and among laboratories. For the ANOVA, the IC_{50} values were first converted to mM units and then log-transformed to obtain normal distributions. One-way ANOVA was performed with SAS PROC GLM (SAS Institute 1999; see **Appendix D1** for example SAS code). A significance level of $p < 0.01$ was used to test results between the laboratories in order to be conservative with respect to identifying laboratory differences. When the ANOVA detected significant differences among the laboratories, contrast analyses were performed to determine which laboratory was different from the others. These analyses compared the results of each laboratory with those of the other two laboratories. A significant difference in response among the laboratories was indicated by $p < 0.01$.

CV values were calculated for each reference substance by dividing the SD by the arithmetic mean IC_{50} value and multiplying by 100. CV values were calculated for the acceptable tests in each laboratory to determine intralaboratory reproducibility. To compare the variation among laboratories, the CV was calculated using the arithmetic mean IC_{50} values from each of the three laboratories.

As an additional approach to the assessment of interlaboratory reproducibility for each test substance, the maximum:minimum IC_{50} ratios (i.e., the maximum arithmetic mean laboratory IC_{50} value compared to the minimum arithmetic mean laboratory IC_{50} value) were calculated. This approach is similar to the calculation of maximum:minimum LD_{50} ratios for examining reproducibility of reference LD_{50} values (see **Section 4.4.1**).

5.5.3 Statistical Analyses of Phase II Data

5.5.3.1 *Outlier Determination for Replicate Well Concentration Data*
The Dixon and Massey (1981) outlier test was incorporated into the EXCEL® templates to assess the consistency of replicate well data for each reference substance concentration. Outliers at the 99% level were highlighted and the Study Director was offered the option of removing the value from subsequent calculations (e.g., mean OD of the six replicates; % viability; IC_{50}).

5.5.3.2 *Reproducibility Analyses of the Reference Substance IC_{50} Values*
The intra- and inter-laboratory reproducibility of the IC_{50} values were assessed using the acceptable tests to calculate the mean IC_{50}, SD, and CV for each substance, method, and

laboratory, as described in **Section 5.5.2.2**. One-way ANOVAs and calculations of maximum:minimum IC_{50} ratios were performed as described in **Section 5.5.2.2**.

5.5.3.3 *Comparison of 3T3 and NHK Test Results with the RC Millimole Regression*
To compare the 3T3 and NHK test results for the reference substances to those of the RC millimole regression, each IC_{50} value was transformed to mM units for the calculation of geometric mean IC_{50} values. The use of geometric means corresponded with the approach used to obtain single IC_{50} values from multiple IC_{50} values for the RC millimole regression (Halle 1998, 2003). The log geometric mean IC_{50} values (in mM) of the 11 RC substances tested during Phases Ib and II (see **Table 3-8**) were used with the log RC LD_{50} values, after transformation to log mmol/kg units (see **Appendices J1** and **J2**), to calculate least squares linear regressions for the data from each test method and laboratory. Each of these method/laboratory regressions was compared to the RC millimole regression using an F test with SAS PROC REG (SAS Institute 1999; see **Appendix D2** for example SAS code). An F test with a significance level of $p < 0.01$ was used to determine whether the joint comparison of slope and intercept indicated that the method/laboratory regressions were significantly different from the RC millimole regression.

As an alternate analysis, a least squares linear regression using IC_{50} and LD_{50} values from the RC was constructed for the 11 RC substances (*the RC-11 regression*) tested in Phases Ib and II. Each of these method/laboratory regressions was compared to the RC-11 regression using an F test with SAS PROC GLM (SAS Institute 1999; see **Appendix D2** for example SAS code) at a significance level of $p < 0.01$. This was used to determine whether the comparisons of slope and intercept indicated that the laboratory regressions were significantly different from the RC-11 regression.

5.5.4 Statistical Analyses of Phase III Data

5.5.4.1 *Outlier Determination for Replicate Well Concentration Data*
The laboratories used the Dixon and Massey (1981) outlier test at the 99% level that was incorporated into the EXCEL® templates to test for outlier values among replicate well data at the different reference substance concentrations. The Study Director had the option of excluding the outliers from the data set, which were highlighted by the template, and subsequent calculations. All data are available in the data files provided by the laboratories, including the outlier OD values.

5.5.4.2 *Reproducibility Analyses of the PC IC_{50} Data*
A number of analyses were performed to determine whether the SLS IC_{50} values were reproducible across study phases. The SLS IC_{50} values used to access variability were different from those shown in **Table 5-3**. To get an assessment of the true variation of SLS IC_{50} values, the reproducibility analyses included additional IC_{50} values from SLS tests that did not meet the IC_{50} acceptance limits (see **Table 5-3**) for each laboratory and study phase if they passed all other test acceptance criteria. If more than one SLS test was performed on a single day (for any test method and laboratory), the IC_{50} values were averaged to determine a single IC_{50} for the day. This prevented multiple data values from a single day from overly influencing the mean for each phase. CV analyses were performed as described in **Section 5.5.1** using the arithmetic mean SLS IC_{50} values for each method, laboratory, and study phase.

For the remaining analyses of reproducibility, the IC_{50} values were first log-transformed to obtain normal distributions. One-way ANOVAs were performed with SAS PROC GLM (SAS Institute 1999; see **Appendix D1** for example SAS code) for each method using study phase and laboratory as individual variables. A significance level of $p < 0.01$ was used to test for a statistical difference among the laboratory and/or phase results.

To determine whether there was a linear time trend for the SLS IC_{50} data, linear regression analyses using a least squares method were performed for each laboratory and method using SAS PROC REG (SAS Institute 1999). Time was expressed as an index for each test. The index number of each SLS test reflected its order of testing without respect to the time lapsing between tests. For example, the first SLS test was assigned a time index of 1 and the second SLS test was assigned a time index of 2 whether it occurred the day after the first test or one week after the first test. The slopes of the linear regressions were judged to be statistically significant at $p < 0.05$, which indicated that the IC_{50} had changed significantly over time.

5.5.4.3 *Reproducibility Analyses of the Reference Substance IC_{50} Values*

CV, one-way ANOVA analyses, and maximum:minimum IC_{50} ratios were performed to assess the intra- and/or inter-laboratory reproducibility of the Phase III reference substance data, as described in **Section 5.5.2.2**. An additional evaluation to determine whether normalizing the reference substance IC_{50} to the SLS IC_{50} would reduce interlaboratory variability was performed using five substances (for each test method) for which the ANOVAs indicated significant interlaboratory differences. The reference substance IC_{50} values were normalized to the SLS IC_{50} by calculating the reference substance IC_{50}:SLS IC_{50} ratio. CVs were calculated for each substance using the mean ratios from each laboratory. To determine whether this normalization reduced variability among the laboratories, the CVs for the substance IC_{50}:SLS IC_{50} ratios were compared to the CVs for the substance IC_{50}.
In addition, the geometric mean IC_{50} values were used to calculate least squares linear regression models after log transforming the data. Linear regressions were fit for each method and laboratory using the log-transformed reference LD_{50} values from **Table 4-2 (**in mmol/kg), with log IC_{50} in mM. To detect differences among the linear regressions in each laboratory, two models were fit for each method. The first was a full model that included effects for laboratory and interactions, and generated a regression line for each substance in each laboratory, by test method. The second model, which was considered to be a reduced model, assumed that one model fit all the laboratories. A goodness of fit F test was performed to compare the full and reduced models for each method. A significance level of $p < 0.01$ was used to test whether the regressions among laboratories were significantly different from one another. The following criteria were established for selection of data for use in the regression analyses for each test method:

- The substance was included in the RC
- All three laboratories reported IC_{50} values
- There was an associated rat oral reference LD_{50} value (see **Table 4-2**)

There were 47 reference substances that fit these criteria for the 3T3 and 51 test substances that fit the criteria for the NHK test methods.

5.5.4.4 *Comparison of 3T3 and NHK Results with the RC Millimole Regression*

To determine whether the IC_{50} values determined in the validation study were significantly different from the RC values, the laboratory-specific regression values for each method were combined using the geometric means of the laboratory-specific geometric mean IC_{50} values in mM and the reference LD_{50} in mmol/kg. Thus, there was one regression analysis with pooled laboratory data for the 3T3 NRU test method and another regression analysis (also with pooled data) for the NHK NRU test method. A third linear regression was calculated using the IC_{50} and LD_{50} values from the RC. The IC_{50} values and LD_{50} values were log-transformed for the regression calculations. The following criteria were established for the selection of substances to be used for the regression analyses:

- The substance was included in the RC
- All three laboratories reported IC_{50} values for both the 3T3 and NHK NRU test methods
- There was an associated rat oral reference LD_{50} value (see **Table 4-2**)

Forty-seven substances met these criteria. Two models were fit for each test method to detect differences between the NRU regression and the 47 RC substance regression. The first regression model was a full model that included effects for the RC and the NRU regression, and generated one regression line each for the RC and the NRU test method. The second (reduced) model assumed that a single model fit the combined RC and NRU IC_{50} data. The RC regression for the 47 reference substances was compared to the combined laboratory regression for each NRU test method using an F test to simultaneously compare slopes and intercepts. The NRU regressions were statistically different from the RC regressions if $p < 0.01$.

To assess the accuracy of the NRU methods and the associated IC_{50}-LD_{50} regressions, a predicted LD_{50} was calculated for each reference substance using its laboratory geometric mean IC_{50} in two analyses:

- The RC rat-only millimole regression calculated from the 282 RC substances with rat LD_{50} values, using units of mM for the IC_{50} and mmol/kg for the LD_{50} (see **Section 6.4.2**)
- The RC rat-only weight regression calculated from the 282 RC substances with rat LD_{50} values, using units of μg/mL for the IC_{50} and mg/kg for the LD_{50} (see **Section 6.4.3**)

The LD_{50} values predicted from the regression analyses were used to predict GHS acute oral toxicity categories (see **Section 6.4**). The accuracy of the predictions was determined by calculating the proportion of substances for which the predicted GHS toxicity category matched the GHS toxicity category. The LD_{50} predictions from these regression models were also used to determine starting doses for acute systemic toxicity test simulations for the purpose calculating animal use and savings that would be achieved using the NRU test methods. The simulation modeling methods, and results from the UDP and ATC methods, are described in **Section 10**.

5.5.5 Summary of the Data Used for Statistical Analyses

Table 5-7 summarizes the number of substances that were tested and the number of substances used for the various analyses performed to determine the accuracy and reliability of the *in vitro* NRU test methods.

Table 5-7 Datasets Used for Validation Study Analyses[1]

Use	3T3 NRU Test Method[1]	NHK NRU Test Method[1]	Characteristics of Dataset
Testing	72	72	Substances tested
Comparison of laboratory IC_{50}-LD_{50} regressions to one another	47	51	RC substances with IC_{50} values from all laboratories and reference rat oral LD_{50} values
Comparison of combined-laboratory IC_{50}-LD_{50} regressions to a regression calculated with RC data	47	47	RC substances with IC_{50} values for both test methods from all laboratories and rat oral reference LD_{50} values
Prediction of GHS accuracy using IC_{50} values in IC_{50}-LD_{50} regressions; prediction of starting doses for acute oral toxicity test (UDP and ATC) simulations	67	68	Substances with IC_{50} values from at least one laboratory
Reproducibility of acceptable rat oral LD_{50} values	NA	NA	62 substances with more than one acceptable rat oral LD_{50} value
Reproducibility of IC_{50} values	64	68	Substances with IC_{50} values from all laboratories
Comparison of reproducibility of IC_{50} values with reproducibility of LD_{50} values	53	57	Substances with IC_{50} values from all laboratories and more than one acceptable rat oral LD_{50} value

Abbreviations: RC=Registry of Cytotoxicity; 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; NA=Not applicable.
[1]Number of substances.

5.6 Summary of NRU Test Results

Table 5-8 shows the 3T3 and NHK IC_{50} values as geometric means of the geometric mean laboratory values, as a basis to compare the 3T3 and NHK NRU IC_{50} values for each reference substance. The substances in **Table 5-8** are organized by ascending 3T3 NRU IC_{50} values (as was done for **Figures 5-1** and **5-2**). For each method, the table provides the geometric mean IC_{50} (combined across laboratories) in μg/mL, the ratio of the geometric mean IC_{50} to the SLS IC_{50}, and the 3T3 IC_{50}:NHK IC_{50} ratios. Geometric means were used for this comparison because they were used for both the IC_{50} and LD_{50} regression analyses (see **Sections 5.5.3.3**, **5.5.4.3,** and **5.5.4.4**). The 3T3 and NHK NRU IC_{50} values were compared using the ratios of their geometric means. The IC_{50} values for each reference substance were also compared to the IC_{50} for SLS using the ratio of reference substance geometric mean IC_{50} to SLS geometric mean IC_{50}.

Table 5-8 Comparison of 3T3 and NHK NRU IC_{50} Geometric Means

Reference Substance	3T3 NRU		NHK NRU		
	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	IC_{50} Ratios 3T3:NHK
Carbon tetrachloride	NA	NA	NA	NA	NA
Methanol	NA	NA	1529[3]	383.2	NA
Aminopterin	0.006	0.0001	669	167.7	0.00001
Triphenyltin hydroxide	0.017	0.0004	0.01	0.003	1.7
Colchicine	0.034	0.001	0.007	0.002	4.9
Cycloheximide	0.187	0.004	0.073	0.02	2.6
Triethylenemelamine	0.272	0.007	1.85	0.5	0.1
Cadmium II chloride	0.518	0.01	1.84	0.5	0.3
Sodium dichromate dihydrate	0.587	0.01	0.721	0.2	0.8
Sodium arsenite	0.759	0.02	0.477	0.1	1.6
Arsenic trioxide	1.96	0.05	5.26	1.3	0.4
Mercury II chloride	4.12	0.1	5.8	1.5	0.7
Hexachlorophene	4.19	0.1	0.029	0.01	144.5
Thallium I sulfate	5.74	0.1	0.152	0.04	37.8
Haloperidol	6.13	0.1	3.36	0.8	1.8
Endosulfan	6.35	0.2	2.13	0.5	3.0
Amitriptyline HCl	7.05	0.2	8.96	2.2	0.8
Diquat dibromide monohydrate	8.04	0.2	4.48	1.1	1.8
Propranolol	13.9	0.3	35.3	8.8	0.4
Dichlorvos	17.7	0.4	10.7	2.7	1.7
Paraquat	20.1	0.5	61.6	15.4	0.3
Fenpropathrin	24.2	0.6	2.43	0.6	10.0
Physostigmine	25.8	0.6	88.5	22.2	0.3
Propylparaben	26.1	0.6	16.6	4.2	1.6
Sodium selenate	29	0.7	10.2	2.6	2.8
Potassium cyanide	34.6	0.8	29	7.3	1.2
Verapamil HCl	34.9	0.8	66.5	16.7	0.5
Parathion	37.4	0.9	30.3	7.6	1.2
Sodium oxalate	37.7	0.9	337	84.5	0.1
Sodium lauryl sulfate (SLS)*	***41.7***	***1.0***	***3.99***	***1.0***	***10.5***
Cupric sulfate pentahydrate	42.1	1.0	197	49.4	0.2
Acetaminophen	47.7	1.1	518	129.8	0.1
Dibutyl phthalate	49.7	1.2	28.7	7.2	1.7
Epinephrine bitartrate	59	1.4	87.4	21.9	0.7
Phenol	66.3	1.6	75	18.8	0.9
Atropine sulfate	76	1.8	81.8	20.5	0.9
Busulfan	77.7	1.9	260	65.2	0.3
Sodium I fluoride	78	1.9	49.8	12.5	1.6
Phenylthiourea	79	1.9	336	84.2	0.2
Carbamazepine	103	2.5	83.2	20.9	1.2

Table 5-8 Comparison of 3T3 and NHK NRU IC_{50} Geometric Means

Reference Substance	3T3 NRU		NHK NRU		
	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	Geometric Mean[1] IC_{50} (µg/mL)	Ratio Geometric Mean IC_{50} to SLS IC_{50}	IC_{50} Ratios 3T3:NHK
Diethyl phthalate	107	2.6	120	30.1	0.9
Lindane	108	2.6	18.7	4.7	5.8
Chloramphenicol	128	3.1	348	87.2	0.4
Disulfoton	133	3.2	270	67.7	0.5
Caffeine	153	3.7	638	159.9	0.2
Strychnine	158	3.8	62.5	15.7	2.5
Glutethimide	174	4.2	174	43.6	1.0
Chloral hydrate	183	4.4	133	33.3	1.4
Nicotine	361	8.7	107	26.8	3.4
Procainamide HCl	441	10.6	1741	436.3	0.3
Digoxin	466	11.2	0.001	0.0003	466000.0
Meprobamate	519	12.4	357	89.5	1.5
Lithium I carbonate	562[2]	13.5	468	117.3	1.2
Phenobarbital	573	13.7	448	112.3	1.3
Acetylsalicylic acid	676	16.2	605	151.6	1.1
Xylene	721[2]	17.3	466[2]	116.8	1.5
Citric acid	796	19.1	400	100.3	2.0
Trichloroacetic acid	902	21.6	413	103.5	2.2
Valproic acid	916	22.0	512	128.3	1.8
Sodium hypochlorite	1103	26.5	1502	376.4	0.7
5-Aminosalicylic acid	1667	40.0	46.7	11.7	35.7
Boric acid	1850	44.4	421	105.5	4.4
Lactic acid	3044	73.0	1304	326.8	2.3
Potassium I chloride	3551	85.2	2237	560.7	1.6
2-Propanol	3618	86.8	5364	1344.4	0.7
Sodium chloride	4730	113.4	1997	500.5	2.4
Dimethylformamide	5224	125.3	7760	1944.9	0.7
Ethanol	6523	156.4	10018	2510.8	0.7
Gibberellic acid	7810[3]	187.3	2856	715.8	2.7
Acetonitrile	7951	190.7	9528	2388.0	0.8
1,1,1-Trichloroethane	17248	413.6	8122[2]	2035.6	2.1
Ethylene glycol	24317	583.1	41852	10489.2	0.6
Glycerol	24655	591.2	24730	6198.0	1.0

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; SLS=Sodium lauryl sulfate; NA=Not available.
Reference substances are ordered by 3T3 NRU IC_{50} values.
[1]Geometric mean IC_{50} of the laboratory geometric mean values.
[2]Data available from only one laboratory.
[3]Data available from only two laboratories.
*Acceptable positive control (SLS) values from all study phases: N=293 for the 3T3 NRU and N=281 for the NHK NRU.

Table 5-8 shows that there are nine reference substances for which the 3T3 and NHK NRU IC_{50} values differ by at least one order of magnitude (i.e., 3T3 IC_{50}:NHK IC_{50} $\leq$0.1 or $\geq$10): aminopterin, triethylenemelamine, hexachlorophene, thallium sulfate, fenpropathrin, sodium oxalate, acetaminophen, digoxin, and 5-aminosalicylic acid. The IC_{50} values for SLS, also differed by slightly more than one order of magnitude in the two NRU test methods (41.7 μg/mL for 3T3 and 3.99 μg/mL for NHK). One test method was not more consistently sensitive (i.e., produced lower IC_{50} values) than the other for these nine reference substances. The 3T3 NRU test method was more sensitive than the NHK NRU test method for four of the nine substances: aminopterin, triethylenemelamine, sodium oxalate, and acetaminophen. The NHK NRU test method was more sensitive than the 3T3 NRU test method for five substances: hexachlorophene, thallium sulfate, fenpropathrin, digoxin, and 5-aminosalicylic acid. Despite the normalization procedure, the reference substance IC_{50}:SLS IC_{50} ratios for the two methods were still greater by at least one order of magnitude for six of the nine substances (aminopterin, triethylenemelamine, hexachlorophene, sodium oxalate, acetaminophen, and digoxin) and the order of magnitude difference increased for all six substances. A number of factors could potentially be responsible for these differences between the 3T3 and NHK NRU IC_{50} values:

- Cell culture conditions (i. e., the 3T3 treatment medium contains serum while the NHK treatment medium does not; differences in cell density in the treatment medium)
- Differences in sensitivity between the fibroblast cell line and primary keratinocytes
- Differences in sensitivity between human and mouse cells
- Differences in metabolic activity between the cell types

These factors may affect the results for some substances more than others. For example, a substance that binds to serum proteins would be less available to the 3T3 cells (which have serum in their growth medium) than to NHK cells (which are grown without serum). No additional testing was performed to investigate the differences between the 3T3 and NHK NRU IC_{50} values.

Two substances, digoxin and aminopterin, have IC_{50} values that differ by five orders of magnitude between the two NRU test methods. Digoxin was much more toxic to the NHK cells and aminopterin was more toxic to the 3T3 cells. Both substances are known substrates for organic anionic transporters (OAT) (ICCVAM 2006). Such transporters are important for *in vivo* toxicity responses in terms of the ability of challenge substances to be absorbed, reach target tissues, accumulate, or be excreted. The differential susceptibilities of the 3T3 and NHK cells may be explained by differential functioning of OAT between the cell types. Although species and tissue differences in OAT have been reported (Sekine et al. 2000; Miyazaki et al. 2004), the reason for these differential sensitivities is not known.

The 3T3 IC_{50}:NHK IC_{50} ratios shown in **Table 5-8** were used to determine the frequency distributions shown in **Table 5-9.** These distributions indicate that the 3T3 and NHK NRU IC_{50} values were within one order of magnitude of each other for 85% of the reference substances (obtained by adding 38.9% and 45.8% for the 0.1 < IC_{50} ratio $\leq$1 and 1 < IC_{50} ratio <10 ranges). Ninety-three percent of the reference substances have 3T3 and NHK NRU

IC_{50} values within two orders of magnitude of each other (obtained by adding 4.2% each for the $10 \leq IC_{50}$ ratio ≤ 100 and $0 < IC_{50}$ ratio ≤ 0.1 ranges to the 85% above).

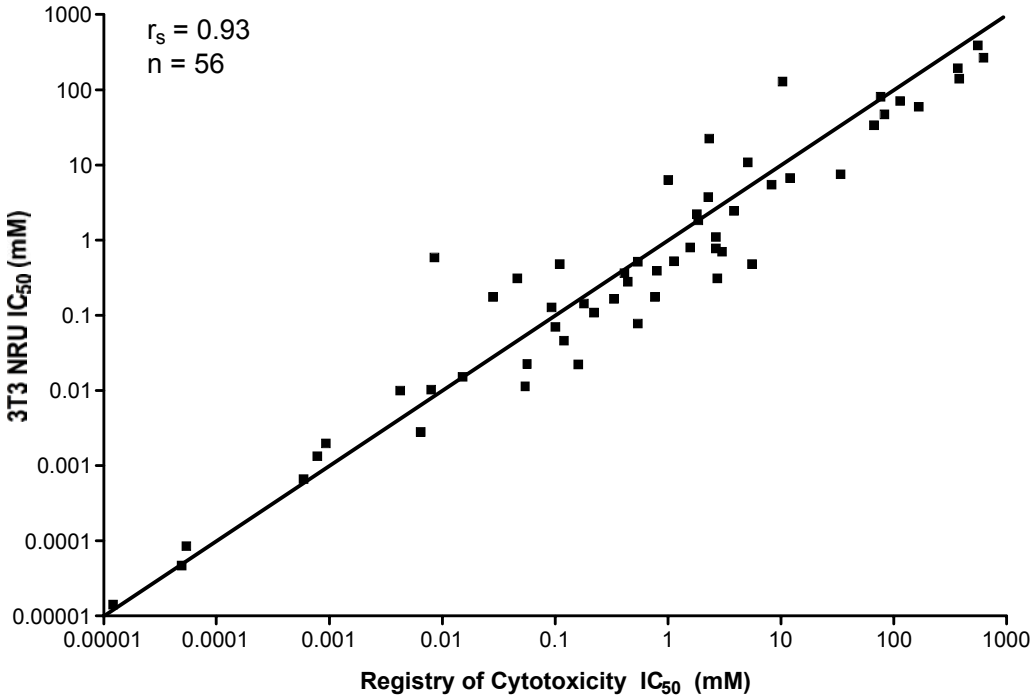
Table 5-9 Frequency of 3T3:NHK IC_{50} Ratios[1] for Reference Substances

3T3:NHK IC_{50} Ratio Range	Number of Substances	% of Substances
IC_{50} Ratio <0.00001	1	1.4
$0 < IC_{50}$ Ratio ≤ 0.1	3	4.2
$0.1 < IC_{50}$ Ratio ≤ 1	28	38.9
$1 < IC_{50}$ Ratio <10	33	45.8
$10 \leq IC_{50}$ Ratio <100	3	4.2
$100 \leq IC_{50}$ Ratio <1000	1	1.4
IC_{50} Ratio ≥ 1000	1	1.4
Not Available	2	2.8

Abbreviations: 3T3=Neutral red uptake using BALB/c 3T3 fibroblasts; NHK= Neutral red uptake using normal human epidermal keratinocytes.
Note: Compiled using reference substance data from **Table 5-7**.

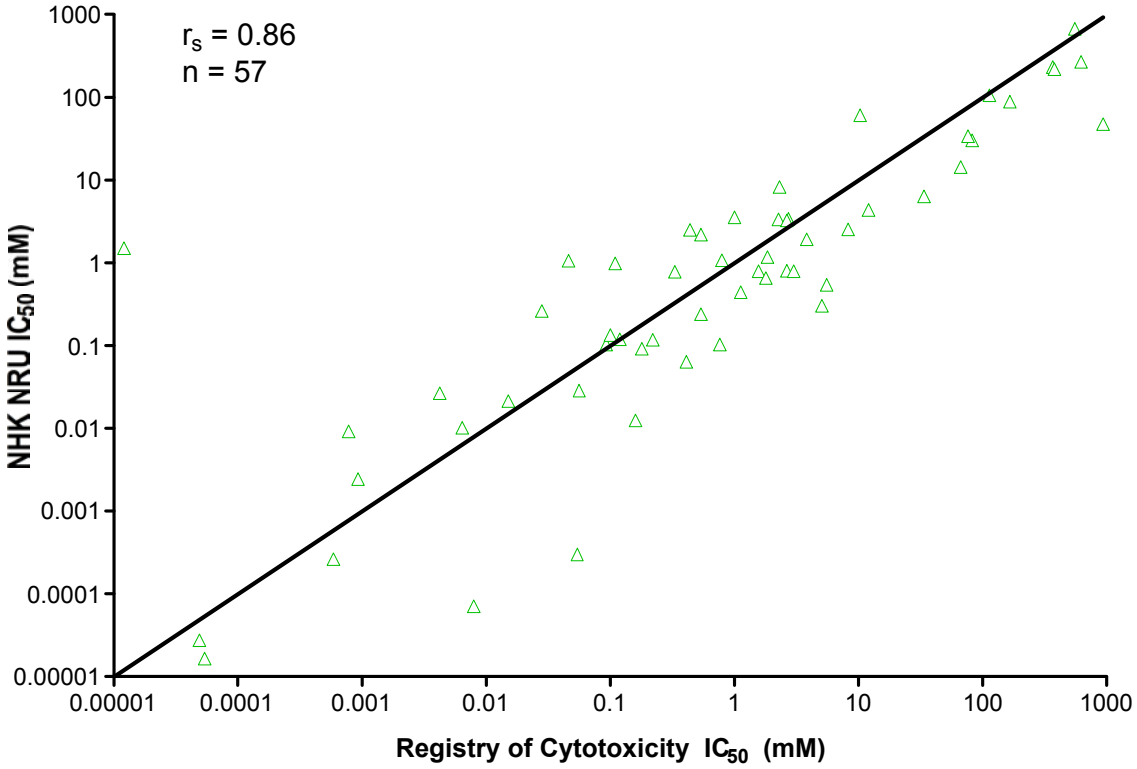
Correlations of the mean IC_{50} values for the reference substances common to the RC database with the IC_{50} values (i.e., geometric mean of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types) from the RC (Halle 1998, 2003) are shown in **Figure 5-3** (3T3 values) and **Figure 5-4** (NHK values). Although the validation study tested 58 RC substances in common with the RC, IC_{50} values were obtained for 56 substances using the 3T3 NRU test method and 57 substances using the NHK NRU test method. Spearman correlation analyses of the log-transformed IC_{50} data (in mM) indicated that the NRU IC_{50} values were significantly correlated with the RC IC_{50x} values ($p<0.001$, for both the 3T3 and NHK NRU test methods). The Spearman correlation coefficient, r_s, was 0.93 for the 3T3 values and 0.86 for the NHK values.

Figure 5-3 RC IC_{50} Values vs 3T3 NRU IC_{50} Values for 56 Substances in Common



Abbreviations: RC=Registry of Cytotoxicity; 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; r_s=Spearman correlation coefficient; n=Number of substances; mM=Millimolar.
The diagonal line indicates the predicted values for a 1:1 correspondence. No IC_{50} values were obtained for carbon tetrachloride or methanol because of insufficient toxicity. The Registry of Cytotoxicity IC_{50} values are geometric means of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types.

Figure 5-4 RC IC_{50} Values vs NHK NRU IC_{50} Values for 57 Substances in Common



Abbreviations: RC=Registry of Cytotoxicity; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; r_s=Spearman correlation coefficient; n=Number of substances; mM=Millimolar.
The diagonal line indicates the predicted values for a 1:1 correspondence. No IC_{50} values were obtained for methanol because of insufficient toxicity. The Registry of Cytotoxicity IC_{50} values are geometric means of IC_{50} values obtained from the literature for various basal cytotoxicity endpoints and cell types.

5.7 Availability of Data

All data were provided to the SMT as electronic files and paper copies. The laboratories also maintained copies of all raw data and the electronic files. The individual test data and IC_{50} results for both passing and failing tests are provided in **Appendix I** for the reference substances and the PC.

5.8 Solubility Test Results

A solubility protocol (see **Section 2-8** and **Appendix B3**) designed to identify the solvent that would provide the highest concentration of a reference substance for *in vitro* testing was evaluated. Each laboratory performed solubility tests on all reference substances. However, to avoid the use of different solvents by the laboratories when testing the same substance, which might increase the variability of the IC_{50} results among the laboratories, the SMT assigned the solvents to be used (see **Table 5-10**). The objectives of the solubility testing were to evaluate the utility and appropriateness of the solubility protocol, and to evaluate the concordance among laboratories in selecting the solvents for each of the 72 reference substances.

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Phase I																	
Arsenic III trioxide	0.25	0.05	<2	<2	Medium	***0.025***[6]	***0.025***[6]	***<0.2***	***<0.2***	***0.135***[6]	***0.135***[6]	***<0.2***	***<0.2***	***<0.02***[6]	***<0.02***[6]	***<0.2***	***<0.2***
Ethylene glycol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propranolol HCl	<2	10	200	20	DMSO	0.2	2	200	NT	20	20	200	NT	20	2	NT	NT
Phase II																	
Aminopterin	2	2	NT	NT	DMSO	2.0	<2	200	NT	<2	2	200	NT	0.2	0.2	200	NT
Cadmium II chloride	<2	<2	200	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	<20
Chloramphenicol	2	2	400	<200	DMSO	2.0	<2	200	NT	<2	<2	200	NT	0.2	0.2	20	20
Colchicine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lithium I carbonate	0.25	10	<2	NT	Medium	0.2	2.0	<20	<20	0.2	2	<200	<200	0.2	2	<2	<2
Potassium I chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
2-Propanol	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium I fluoride	20	20	<200	<200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium selenate	200	200	<200	<200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phase III																	
Acetaminophen	10	10	400	<200	DMSO	2	2	NT	NT	2	2	NT	NT	<2	<2	200	NT
Acetonitrile	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Acetylsalicylic acid	10	10	400	200	DMSO	2	2	NT	NT	<2	<2	200	NT	2	2	NT	NT
5-Aminosalicylic acid	2	2	<200	<200	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Amitriptyline HCl	200	200	NT	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	0.2	0.2	200	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Atropine sulfate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Boric aid	40	40	200	<200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Busulfan	<2	<2	40	<200	DMSO	<2	<2	200	NT	<2	<2	50[6]	<200	<0.2	<0.2	20	<200
Caffeine	10	10	20	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Carbamazepine	<2	<2	40	<200	DMSO	0.2	0.2	20	20	<2	<2	200	NT	<0.2	<0.2	2	<20
Carbon tetrachloride	2	10	NT	NT	DMSO	20	20	NT	NT	<0.2	<0.2	2	NT	20	20	NT	NT
Chloral hydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Citric acid	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Cupric sulfate pentahydrate	1	0.5	<2	2	Medium	2	0.2	<200	<200	2	2	NT	NT	0.2	0.2	<200	NT
Cycloheximide	20	20	400	<200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Dibutyl phthalate	<2	<2	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Dichlorvos	10	10	NT	NT	DMSO	2	2	NT	NT	<2	<2	200	NT	2	2	NT	NT
Diethyl phthalate	<2	<2	400	400	DMSO	<2	<2	200	NT	0.2	<0.2	200	NT	<2	<2	200	NT
Digoxin	0.05	0.05	200	< 200	DMSO	<2	<2	200	NT	<0.2	<0.2	200	NT	<2	<2	200	NT
Dimethylformamide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Diquat dibromide monohydrate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Disulfoton	<2	<2	500	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Endosulfan	<0.05	<0.05	40	NT	DMSO	<0.2	<0.2	20	<200	<0.2	<0.2	2	<200	<0.2	<0.2	20	<200
Epinephrine bitartrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Ethanol	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Fenpropathrin	<20	<20	500	NT	DMSO	<2	<2	200	NT	<0.2	<0.2	200	NT	<2	<2	200	NT
Gibberellic acid	10	10	NT	NT	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Glutethimide	<2	<2	500	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Glycerol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Haloperidol	<20	<20	40	NT	DMSO	<0.2	<0.2	20	<20	<0.2	<0.2	20	<20	<2	<2	20	<20
Hexachlorophene	0.05	<0.05	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Lactic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lindane	<0.05	<0.05	400	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	<200
Meprobamate	1	1	200	NT	DMSO	2	2	200	NT	2	2	200	NT	<0.2	<0.2	200	NT
Mercury II chloride	0.125	0.125	400	<200	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	200	NT
Methanol	40	40	400	400	DMSO	20	20	NT	NT	20	20	NT	NT	<2	<2	200	NT
Nicotine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Paraquat	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Parathion	0.05	<0.05	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Phenobarbital	2	2	200	<200	DMSO	2	2	NT	NT	<2	<2	200	NT	<2	<2	200	NT
Phenol	40	40	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phenylthiourea	2	2	400	<200	DMSO	2	<2	200	NT	20	20	NT	NT	<2	<2	200	NT
Physostigmine	2	2	400	200	DMSO	2	2	NT	NT	<2	<2	200	NT	<2	<2	200	NT
Potassium cyanide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Procainamide HCl	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT

Table 5-10 Solubility Test Results (mg/mL)

Reference Substance	BioReliance[1]				SMT[2] Selection	ECBC[3]				FAL[3]				IIVS[3]			
	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH		3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH	3T3[4] Medium	NHK[5] Medium	DMSO	ETOH
Propylparaben	0.25	0.25	400	400	DMSO	<2	<2	200	NT	<2	<2	200	NT	<2	<2	200	NT
Sodium arsenite	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium dichromate dihydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium hypochlorite	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium oxalate	<0.05	20	0.125	<0.05	Medium	<0.2	20	0.2	<2	20	20	NT	NT	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Strychnine	< 2	<2	2	2	Medium	0.2	<0.2	2	2	0.2	0.2	<200	<200	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Thallium I sulfate	1	0.5	<2	<2	Medium	0.2	0.2	<200	<200	***<0.2***	***<0.2***	***<0.2***	***<0.2***	0.2	0.2	<20	<200
Trichloroacetic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
1,1,1-Trichloroethane	10	10	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Triethylenemelamine	<2	<2	2	<20	DMSO	0.2	0.2	<200	<200	<0.2	<0.2	2	<2	***<0.2***	***<0.2***	***<0.2***	***<0.2***
Triphenyltin hydroxide	<0.05	<0.05	10	<20	DMSO	<0.2	<0.2	2	<20	<0.2	<0.2	2	<200	<2	<2	2	<20
Valproic acid	10	2	NT	NT	DMSO	2	2	NT	NT	<2	<2	200	NT	2	<2	200	NT
Verapamil HCl	<0.05	0.25	200	NT	DMSO	<2	<2	200	NT	<2	<2	200	NT	<0.2	<0.2	20	NT
Xylene	1	1	500	NT	DMSO	<2	<2	200	NT	2	<2	200	NT	<2	<2	200	NT

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; SMT=Study Management Team; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; DMSO=Dimethyl sulfoxide; ETOH=ethanol; NT=Not tested.
Note: Table sorted by study phase and alphabetical by substance.
[1]The solubility protocol used was different from that used by the testing laboratories.
[2]Solvents selected by the SMT for cytotoxicity testing. The BioReliance results were used to determine solvents for Phases I and II. Results from all laboratories were used to determine solvents for Phase III. 3T3 and NHK media were treated as a single solvent. If a substance insoluble in one medium, and not the other, and soluble in DMSO, then DMSO was selected for use with both cell types.
[3]Used protocol in **Figure 2-7**.
[4]Dulbecco's Modification of Eagle's Medium.
[5]Keratinocyte Growth Medium (KGM® from CAMBREX Clonetics®).
[6]The results were obtained using a deviation from the standard protocol.
Laboratories agreed on solvent. Laboratories did not agree on solvent. **bold** Protocol did not provide enough guideline information to select a single solvent.

5.8.1 Solubility Data

BioReliance evaluated the solubility of the reference substances, first in media, then in DMSO, and then in ETOH, at 400 and 200 mg/mL. Based on their experience, a solubility protocol was developed for the testing laboratories. This revised protocol required testing at lower concentrations, and use of the various solvents at concentrations that would be equivalent when applied to the cell cultures (see **Table 2-5**). The solubility flow chart (**Figure 2-7**) illustrates the tests for solubility in 3T3 and NHK medium, DMSO, and ETOH. **Table 5-10** provides the solubility test results.

5.8.2 Solubility and Volatility Effects in the Cytotoxicity Tests

The laboratories reported solubility results for the stock solutions of reference substance for each 3T3 and NHK test. Prior to the addition of the NR dye medium, the laboratories visually observed the test cultures and documented noticeable precipitate. **Table 5-11** illustrates the existence of solubility issues (in both the 3T3 and NHK NRU test methods) as evidenced by the observation of precipitates with some reference substances. **Sections 3.2.6** and **5.4.2** provide additional information on ability of the laboratories to achieve sufficient toxicity for the calculation of an IC_{50} in the presence of limited solubility. **Table 5-11** also notes the presence of volatility, as indicated by the use of film plate sealers during incubation.

Table 5-11 Reference Substances with Precipitate (PPT) and Volatility Issues[1]

Reference Substances	3T3 NRU Test Method				NHK NRU Test Method			
	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility
Acetonitrile				X				X
Aminopterin		X			X			
5-Aminosalicylic acid	X							
Arsenic III trioxide	X				X			
Cadmium II chloride		X					X	
Carbamazepine			X					
Carbon tetrachloride			X		X			
Citric acid						X		
Cupric sulfate pentahydrate						X		
Dibutyl phthalate		X					X	
Dichlorvos				X				X
Diethyl phthalate	X						X	
Digoxin			X					
Dimethylformamide						X		
Disulfoton			X				X	
Endosulfan	X			X				X
Ethanol				X				X
Fenpropathrin			X				X	
Gibberellic acid	X				X			
Glutethimide					X			
Lindane			X	X			X	
Lithium I carbonate	X				X			
Nicotine				X				X
Parathion	X						X	
Phenol				X				X
Potassium I chloride		X						
Potassium cyanide		X		X				X

Table 5-11 Reference Substances with Precipitate (PPT) and Volatility Issues[1]

Reference Substances	3T3 NRU Test Method				NHK NRU Test Method			
	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility	PPT in 2X Stock Dilutions	PPT in 1X Plate Dilutions	PPT in Stock and Plate Dilutions	Volatility
2-Propanol				X				X
Sodium arsenite		X						X
Sodium chloride						X		
Sodium I fluoride		X				X		
Sodium hypochlorite				X				
Sodium oxalate			X			X		
Strychnine	X				X			
Trichloroacetic acid						X		
1,1,1-Trichloroethane	X						X	
Valproic acid	X							
Verapamil HCl					X			
Xylene	X				X			

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; PPT=Precipitate.
Note: Table sorted alphabetical by reference substance.
[1]Results are based on at least one laboratory having precipitate or volatility issues with a substance. Volatility was denoted by the use of plate sealers during testing. 2X stock dilutions are prepared for each of 8 test substance concentrations. 1X plate dilutions are the result of diluting the 2X stock solutions with medium in the 96-well plates.

5.9 Summary

- The BioReliance, ECBC, and IIVS laboratories performed the 3T3 and NHK NRU tests in compliance with GLP guidelines.
- The quality and consistency of the reference substances was maintained during the study by the central purchase and distribution of individual lots of reference substances to the testing laboratories.
- Modifications and revisions made to the protocols during Phases I and II contributed to the optimization of the final protocols used in Phase III of the study. As a general rule, the protocol changes enhanced the performance of the methods and allowed more tests to meet the acceptance criteria.
- FAL improved the quality of its NHK data prior to Phase II testing by modifying the methods used to propagate the cells. Positive control IC_{50} data in Phases II and III from FAL more closely resemble the data from the other laboratories.
- Summary test data and IC_{50} results are presented in tabular and graphic formats. Comparisons of 3T3 NRU IC_{50} values to NHK NRU IC_{50} values show that the values for 85% of the reference substances are within one order of magnitude of each other. Digoxin and aminopterin yielded differences of up to five orders of magnitude when the IC_{50} values of the 3T3 and NHK NRU test methods were compared.
- Although each laboratory followed the same solubility protocol, they sometimes obtained different results. This may have been due to the subjective judgment of whether or not solubility was achieved. Additionally, the laboratories may have used solubility procedures that were beyond the level of detail in the solubility protocol.