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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<sub>50</sub> 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<sub>50</sub> values to human blood/serum LC.

**Table 3-1** shows the GHS scheme for classifying substances into six toxicity categories (five with measured LD<sub>50</sub> ranges and an unclassified category with LD<sub>50</sub> values greater than 5000 mg/kg) based on acute rodent oral LD<sub>50</sub> 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 <sub>50</sub> (mg/kg)
1	LD <sub>50</sub> ≤ 5
2	5 < LD <sub>50</sub> ≤ 50
3	50 < LD <sub>50</sub> ≤ 300
4	300 < LD <sub>50</sub> ≤ 2000
5	2000 < LD <sub>50</sub> ≤ 5000
Unclassified	LD <sub>50</sub> > 5000

Abbreviations: UN=United Nations; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

LD<sub>50</sub>=Dose that produces lethality in 50% of the test animals.

For the purposes of the initial toxicity classification, the rodent oral LD<sub>50</sub> values for the individual substances were obtained from readily available toxicological databases. These rodent oral LD<sub>50</sub> values were re-evaluated in **Section 4** for the purpose of identifying the most appropriate reference LD<sub>50</sub> 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<sub>50</sub> data were preferred because:

- The current acute oral toxicity test guidelines recommend using rats (OECD 2001a, c, d; EPA 2002a)
- The majority of LD<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> values that came largely from the 1983/84 RTECS<sup>®</sup> (Halle 1998, 2003). The RC is a database of acute oral LD<sub>50</sub> values for rats and mice obtained from RTECS<sup>®</sup> and IC<sub>50</sub> values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for chemicals with known molecular weights.
- The current RTECS<sup>®</sup> (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<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> 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

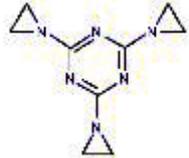
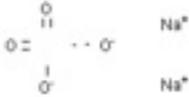
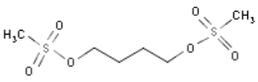
The physical/chemical and toxicological information in **Appendix F** may be useful for characterizing the performance of the *in vitro* NRU test methods for various chemical types (e.g., chemical class, toxic effect class). **Appendix F1** lists the reference substances in alphabetical order with information 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

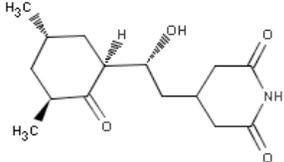
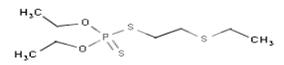
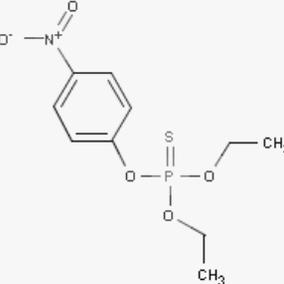
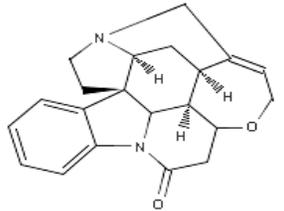
The primary sources of substances were well represented in the final list of reference substances. **Table 3-3** shows the distribution of reference substances by GHS category from each of the source lists. Forty-two (58%) of the 72 substances were MEIC chemicals (17 of the 42 MEIC chemicals [40%] were 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.

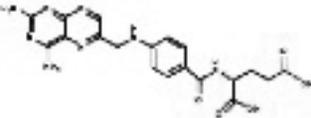
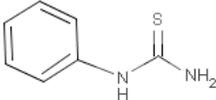
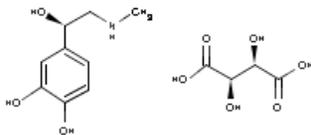
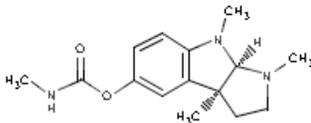
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
<i>LD<sub>50</sub> ≤ 5 mg/kg</i>							
Mercury II chloride	1	MEIC, EDIT, RC (outlier), TESS, NTP	Preservative; Manufacturing; Insecticide	271.50	0.22	Inorganic compound; Mercury compound; Chlorine compound	Cl—Hg
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	

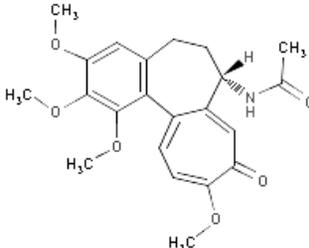
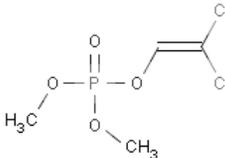
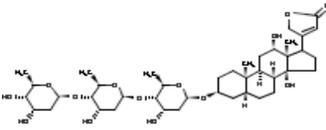
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Cycloheximide	2	RC (outlier), NTP	Antibiotic Fungicide	281.40	0.55	Organic compound; Heterocyclic compound	
Disulfoton	2	RC (outlier), EPA, NTP	Pesticide (insecticide)	274.42	4.02	Organic compound; Organophosphorous compound; Sulfur compound	
Parathion	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	

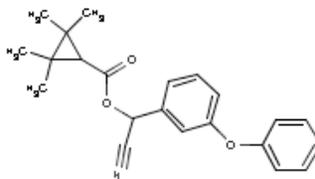
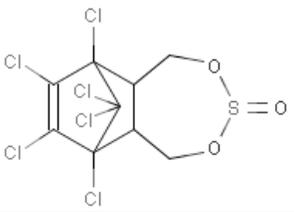
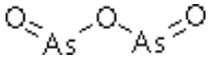
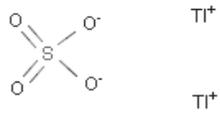
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Aminopterin	3**	RC	Pharmaceutical (antineoplastic); Pesticide (rodenticide)	476.45	NA	Organic compound; Heterocyclic compound	
Phenylthiourea	3	RC (outlier), NTP	Pesticide (rodenticide)	152.20	0.71	Organic compound; Sulfur compound; Urea	
Epinephrine 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	

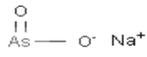
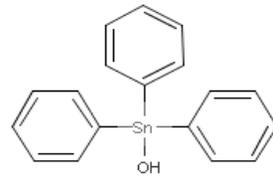
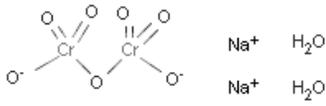
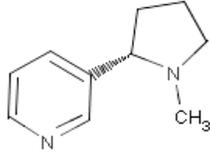
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<i>5 &lt; LD<sub>50</sub> ≤ 50 mg/kg</i>							
Colchicine	6**	MEIC, RC, TESS, NTP	Pharmaceutical (gout suppressant)	399.45	1.03	Organic compound; Polycyclic compound	
Potassium cyanide	10	MEIC, EDIT, RC (outlier), TESS	Electroplating	65.12	NA	Inorganic compound; Potassium compound; Nitrogen compound	$K \text{---} \text{C} \equiv \text{N}$
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	

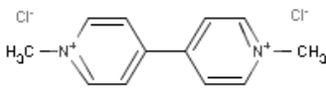
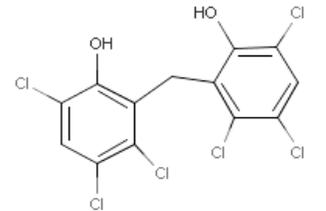
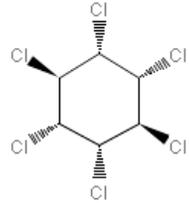
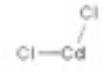
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GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Fenpropathrin	18*	EPA	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	

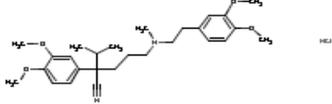
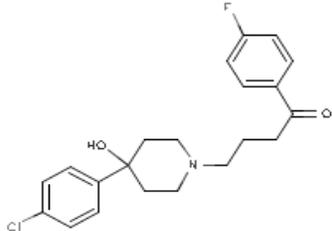
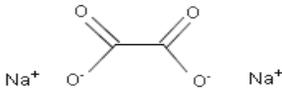
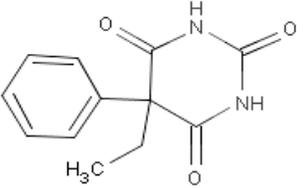
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GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Sodium arsenite	41*	TESS, 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	

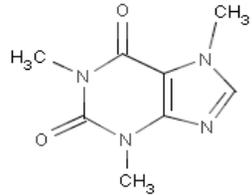
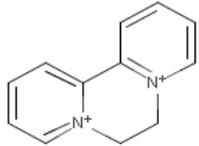
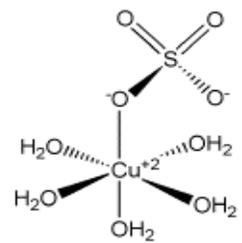
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<i>50 &lt; LD<sub>50</sub> ≤ 300 mg/kg</i>							
Paraquat	58	MEIC, EDIT, RC (outlier), TESS, EPA	Pesticide (herbicide)	257.20	-4.22 @ pH 7.4	Organic compound; Heterocyclic compound	
Hexachlorophene	61	MEIC, RC, TESS, NTP	Disinfectant	406.91	6.91	Organic 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	

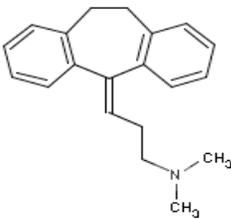
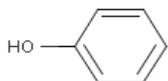
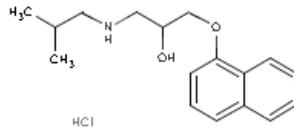
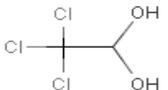
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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	

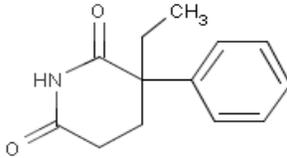
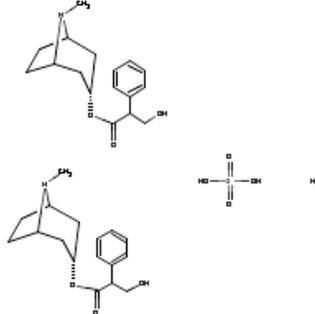
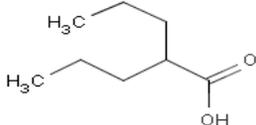
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Sodium I fluoride	180	MEIC, RC, TESS, EPA, NTP	Electroplating; Water fluoridation	41.99	NA	Inorganic compound; Sodium compound; Fluorine compound	Na <sup>+</sup> F <sup>-</sup>
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	 H <sub>2</sub> O Br <sup>-</sup>
Cupric sulfate * 5 H <sub>2</sub> O	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 <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
<i>300 &lt; LD<sub>50</sub> ≤ 2000 mg/kg</i>							
Amitriptyline HCl	319	MEIC, EDIT, RC, TESS	Pharmaceutical (antidepressant)	313.90	5.04	Organic compound; Polycyclic compound	 HCl
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	 HCl
Chloral hydrate	479	MEIC, RC, TESS, NTP	Pharmaceutical (sedative)	165.40	0.99	Organic compound; Alcohol	

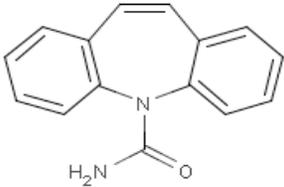
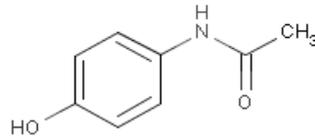
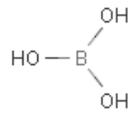
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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	

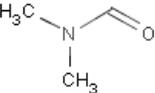
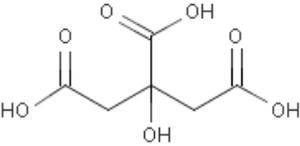
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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 <sup>7</sup>	MEIC, RC, TESS, NTP (Cl salt)	Pharmaceutical (mood stabilizer)	73.89	NA	Inorganic compound; Lithium compound; Alkylies; Carbon compound	
Procainamide	1950*	MEIC, TESS	Pharmaceutical (antiarrhythmic)	271.79	NA	Organic compound; Carboxylic acid; Amide	

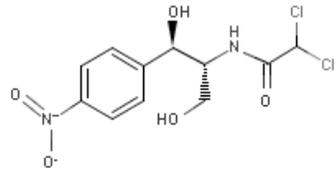
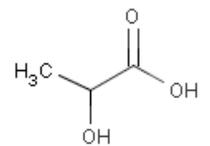
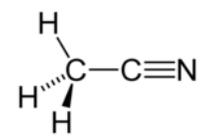
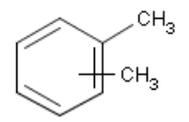
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Carbamazepine	1957*	MEIC, TESS	Pharmaceutical (antiepileptic)	236.30	2.45	Organic compound; Heterocyclic compound	
<i>2000 &lt; LD<sub>50</sub> ≤ 5000 mg/kg</i>							
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	K <sup>+</sup> Cl <sup>-</sup>
Boric acid	2660*	TESS, EPA, NTP	Pesticide (insecticide)	61.83	NA	Inorganic compound; Boron compound; Acids	

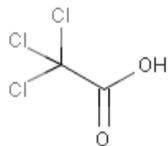
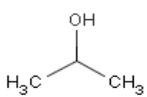
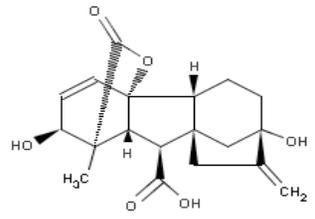
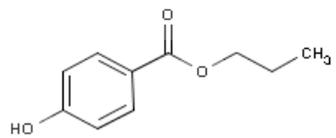
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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	Na <sup>+</sup> Cl <sup>-</sup>
Citric Acid	3000*	EPA, NTP, HPV	Food additive	192.10	-1.72	Organic compound; Carboxylic acid	

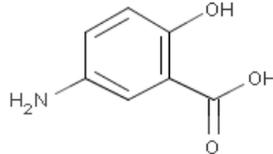
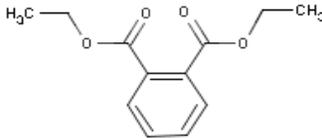
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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	

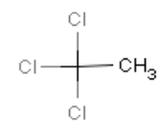
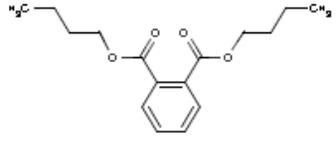
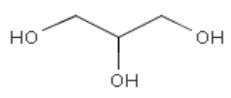
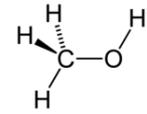
**Table 3-2 Reference Substances Used in the 3T3 and NHK NRU Test Methods Validation Study - Sorted by Toxicity**

GHS Category <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Trichloroacetic acid	4999	RC, NTP	Fixative	163.40	1.33	Organic compound; Carboxylic acid	
<i>LD<sub>50</sub> &gt;5000 mg/kg</i>							
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 <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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 <sup>8</sup>	TESS, NTP	Disinfectant	74.44	NA	Inorganic compound; Sodium compound; 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 <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log Kow <sup>5</sup>	Chemical Class <sup>6</sup>	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 <sup>1</sup> /Substance	Rodent Oral LD <sub>50</sub> <sup>2</sup> (mg/kg)	Source <sup>3</sup>	Product/Use <sup>4</sup>	Molecular Weight (g/mol)	log K <sub>ow</sub> <sup>5</sup>	Chemical Class <sup>6</sup>	Molecular Structure
Ethanol	14008	MEIC, RC (outlier), TESS, EPA, NTP, HPV	Solvent	46.07	-0.31	Organic compound; Alcohol	<chem>CCO</chem>

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); LD<sub>50</sub>=Dose that produces lethality in 50% of the test animals; K<sub>ow</sub>=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<sup>®</sup>=Registry of Toxic Effects of Chemical Substances.

<sup>1</sup>From RTECS<sup>®</sup> (MDL Information Systems 2002).

\*\*Mouse.

<sup>1</sup>GHS category designation for the substance (e.g., LD<sub>50</sub> <5 mg/kg)

<sup>2</sup>LD<sub>50</sub> data are from the Registry of Cytotoxicity (Halle 1998, 2003) and are for rats, unless otherwise noted. The LD<sub>50</sub> values are rounded to the nearest whole number.

<sup>3</sup>Sources used to identify candidate chemicals.

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

<sup>5</sup>From HSDB (NLM 2001, 2002) or Material Safety Data Sheets.

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

<sup>7</sup>Mouse data for lithium sulfate (Halle 1998, 2003).

<sup>8</sup>From HSDB (NLM 2002).

**Table 3-3 Distribution of Candidate Substances and Reference Substances by Source<sup>1</sup> 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 <sub>50</sub> ≤ 5	12/13	2/2	1/1	3/3	5/9	0/0
5 < LD <sub>50</sub> ≤ 50	12/15	6/6	5/5	9/10	8/11	2/5
50 < LD <sub>50</sub> ≤ 300	12/26	11/17	4/5	11/19	9/18	1/3
300 < LD <sub>50</sub> ≤ 2000	12/38	12/29	3/5	12/27	5/23	1/5
2000 < LD <sub>50</sub> ≤ 5000	12/12	6/6	2/2	6/6	12/12	6/6
LD <sub>50</sub> > 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<sub>50</sub>=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.

<sup>1</sup>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<sup>1</sup> by Toxicity Category**

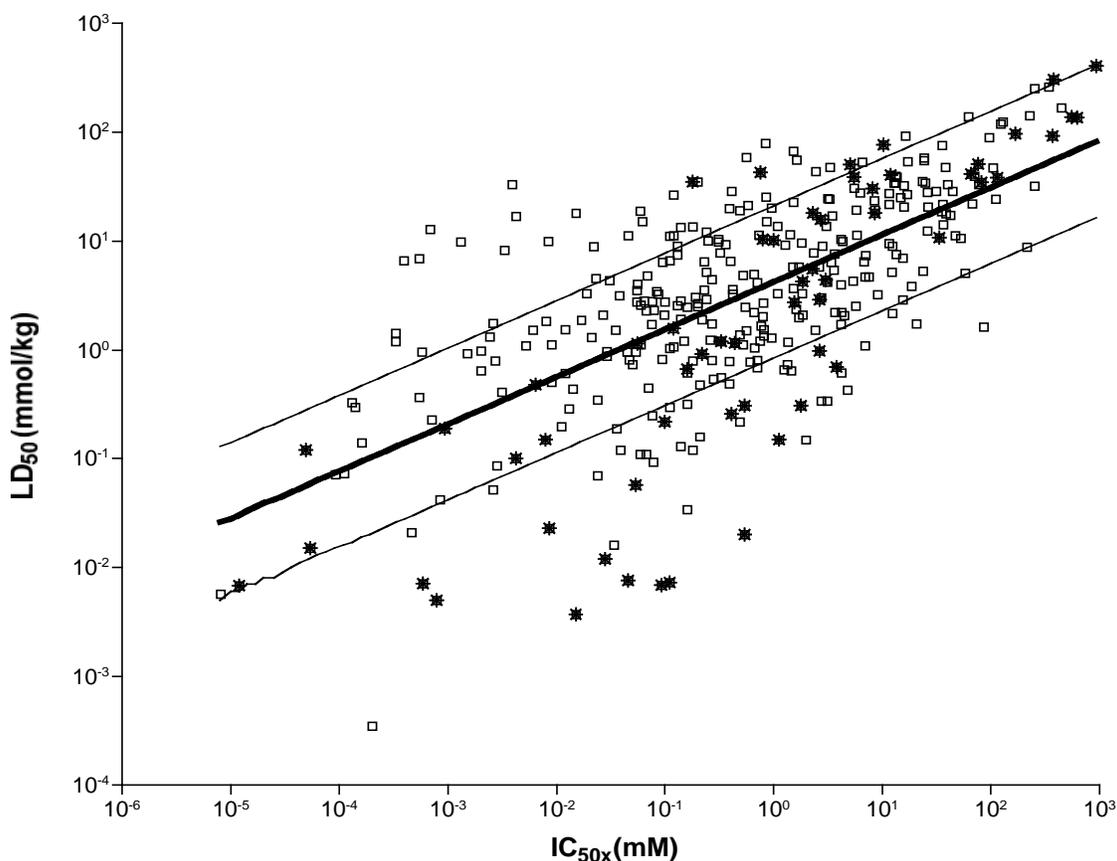
GHS Category (mg/kg)	RC Outliers/ Total Chemicals	Candidate and Selected Substances		
		Candidate Substances	RC Reference / RC Candidates	RC Reference Outliers/ RC Reference Chemicals
LD <sub>50</sub> ≤ 5	10/11 (91%)	13	9/10	8/9 (89%)
5 < LD <sub>50</sub> ≤ 50	15/26 (58%)	15	8/10	4/8 (50%)
50 < LD <sub>50</sub> ≤ 300	24/70 (34%)	26	11/18	5/11 (45%)
300 < LD <sub>50</sub> ≤ 2000	14/139 (10%)	38	9/29	0/9 (0%)
2000 < LD <sub>50</sub> ≤ 5000	12/57 (21%)	12	10/10	0/10 (0%)
LD <sub>50</sub> > 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<sub>50</sub>=Dose that produces lethality in 50% of the test animals.

<sup>1</sup>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<sup>1</sup> was underpredicted for 17 (77%) of these outlier substances and overpredicted (i.e., predicted LD<sub>50</sub> was lower than measured *in vivo* LD<sub>50</sub>) 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<sub>50</sub>=Dose that produces lethality in 50% of the test animals; IC<sub>50</sub>=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,  $\log(\text{LD}_{50}) = 0.435 \times \log(\text{IC}_{50x}) + 0.625$ , is shown by the bold line. The lighter lines show the  $\pm \log 5$  (i.e.,  $\pm 0.699$ ) prediction interval (Halle 1998, 2003). The open boxes represent the 289 chemicals not included in the validation study.

<sup>1</sup> Toxicity is inversely proportional to LD<sub>50</sub>. High LD<sub>50</sub> values reflect low toxicity and low LD<sub>50</sub> values reflect high toxicity

### 3.2.2 Chemical Classes Represented by the Selected Reference Substances

Medical subject heading (MeSH<sup>®</sup>) 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  $LD_{50} \leq 2000$  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<sup>®</sup> (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  $300 < LD_{50} \leq 2000$  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 <sup>1</sup>	GHS Acute Oral Toxicity Category (mg/kg)						Total
	LD <sub>50</sub> ≤5	5 < LD <sub>50</sub> ≤50	50 < LD <sub>50</sub> ≤300	300 < LD <sub>50</sub> ≤2000	2000 < LD <sub>50</sub> ≤5000	LD <sub>50</sub> >5000	
<b>Organic</b>							
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
<b>Total Organics</b>	<b>17</b>	<b>11</b>	<b>11</b>	<b>16</b>	<b>11</b>	<b>14</b>	<b>79</b>

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

Chemical Class <sup>1</sup>	GHS Acute Oral Toxicity Category (mg/kg)						Total
	LD <sub>50</sub> ≤5	5 < LD <sub>50</sub> ≤50	50 < LD <sub>50</sub> ≤300	300 < LD <sub>50</sub> ≤2000	2000 < LD <sub>50</sub> ≤5000	LD <sub>50</sub> >5000	
<b>Inorganic</b>							
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
<b>Total Inorganic</b>	<b>4</b>	<b>9</b>	<b>7</b>	<b>2</b>	<b>6</b>	<b>3</b>	<b>31</b>

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

<sup>1</sup>Based on the Medical Subject Heading [MeSH<sup>®</sup>] 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<sup>1</sup> Class for the 72 Reference Substances by Toxicity Category**

Product/Use Class <sup>1</sup>	GHS Acute Oral Toxicity Category (mg/kg)						Total
	LD <sub>50</sub> ≤5	5 < LD <sub>50</sub> ≤50	50 < LD <sub>50</sub> ≤300	300 < LD <sub>50</sub> ≤2000	2000 < LD <sub>50</sub> ≤5000	LD <sub>50</sub> >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).

<sup>1</sup>Product/use information from Hazardous Substances Data Bank (NLM 2002) or Registry of Toxic Effects of Chemical Substances ([RTECS<sup>®</sup>], 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<sub>50</sub> (mmol/kg) must be within the prediction interval ( $\pm 0.699$ ) 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<sub>50</sub> 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 <sub>50</sub> <sup>1</sup> (mg/kg)	Observed – Predicted log LD <sub>50</sub> <sup>2</sup>
<b>LD<sub>50</sub> ≤ 5 mg/kg</b>					
Aminopterin	54-62-6	3	NA	3	-0.652
Sodium selenate	13410-01-0	NA	NA	1.6 <sup>3</sup>	NA
<b>5 &lt; LD<sub>50</sub> ≤ 50 mg/kg</b>					
Colchicine	64-86-8	6	60	6 <sup>4</sup>	-0.593
Arsenic III trioxide	1327-53-3	153	26	20	-0.591
<b>50 &lt; LD<sub>50</sub> ≤ 300 mg/kg</b>					
Cadmium II chloride	10108-64-2	81	NA	88	0.011
Sodium I fluoride	7681-49-4	106	14	180	-0.109
<b>300 &lt; LD<sub>50</sub> ≤ 2000 mg/kg</b>					
DL-Propranolol HCl	350-60-90	54	23	470 <sup>4</sup>	-0.023
Lithium I carbonate	544-13-2	327 <sup>4</sup>	20	1187 <sup>4,5</sup>	-0.256 <sup>4</sup>
<b>2000 &lt; LD<sub>50</sub> ≤ 5000 mg/kg</b>					
Potassium I chloride	7447-40-7	346	50	2602	0.085
Chloramphenicol	56-75-7	91	45	3393	0.441
<b>LD<sub>50</sub> &gt; 5000 mg/kg</b>					
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.

<sup>1</sup>From the RC (Halle 1998, 2003) unless otherwise indicated. Data are for rats unless otherwise indicated.

<sup>2</sup>Available only for substances included in the RC. This figure characterizes the log LD<sub>50</sub> deviation from the RC regression. Outliers are > ±0.699 from the regression line.

<sup>3</sup>RTECS® (MDL Information Systems 2002).

<sup>4</sup>Mouse data.

<sup>5</sup>For lithium sulfate.

Only nine of the 72 reference substances met all three criteria. In the most toxic category (i.e., LD<sub>50</sub> ≤ 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<sub>50</sub> ≤ 300 mg/kg category, cadmium chloride was selected over the MEIC chemicals cupric sulfate 5H<sub>2</sub>O, diquat dibromide, sodium oxalate, and hexachlorophene because it fit the RC millimole regression better than the four MEIC chemicals (the observed LD<sub>50</sub> minus log predicted LD<sub>50</sub> 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<sub>50</sub> 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<sub>50</sub> data from ECBC, FAL, or IIVS)
- Xylene (no 3T3 or NHK NRU IC<sub>50</sub> data from ECBC or FAL)
- Methanol (no 3T3 NRU IC<sub>50</sub> data from ECBC, FAL, or IIVS; no NHK NRU IC<sub>50</sub> data from ECBC)
- Lithium carbonate (no 3T3 NRU IC<sub>50</sub> data from FAL or IIVS)
- 1,1,1-Trichloroethane (no 3T3 NRU IC<sub>50</sub> data from FAL or IIVS; no NHK NRU IC<sub>50</sub> data from ECBC)
- Valproic acid (no 3T3 NRU IC<sub>50</sub> data from ECBC or FAL; no NHK NRU IC<sub>50</sub> 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<sub>50</sub> 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, antipyrine, 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<sub>50</sub> 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).