Final Results of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/European Centre for the Validation of Alternative Methods (ECVAM) Validation Study of *In Vitro* Cytotoxicity Test Methods for Estimating Rat Acute Oral Toxicity

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Introduction

Poisoning is a more serious public health problem than is generally recognized. The Institute of Medicine estimates that more than 4 million poisoning episodes occur annually in the United States (Institute of Medicine [IOM] 2004). In 2001, poisoning (30,800 deaths) placed second behind automobile accidents (42,433 deaths) as the leading cause of injury-related death (IOM 2004). To reduce the risk for accidental poisonings, regulatory agencies in the United States (e.g., the Environmental Protection Agency [EPA], the Consumer Products Safety Commission [CPSC]) require acute oral toxicity testing of marketed products to determine the potential for harmful effects from ingestion. Increasing societal concerns about animal use for such testing have led to the development and evaluation of alternative *in vitro* test methods that might refine, reduce, or replace acute oral toxicity test methods[1].

In October 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity reviewed the validation status of *in vitro* methods directed toward reducing and refining the use of laboratory animals for acute oral systemic toxicity (i.e., lethality) testing (ICCVAM 2001a). Workshop participants reviewed data demonstrating that animal use could be reduced for sequential *in vivo* testing procedures such as the Up-and-Down Procedure (UDP, EPA 2002; OECD 2001a) and the Acute Toxic Class method (ATC, OECD 2001b), if the starting dose was close to the oral LD_{50}. Spielmann et al. (1999) suggested that *in vitro* basal cytotoxicity assays could be used to predict starting doses for *in vivo* acute systemic toxicity assays. Thus, workshop recommendations for reducing and refining the use of animals for acute systemic toxicity assays included the development of guidance for using *in vitro* basal cytotoxicity assays to estimate the starting doses for acute oral lethality assays (ICCVAM 2001b), and a validation study of these assays to determine their usefulness and limitations for estimating acute oral lethality.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM) subsequently designed and initiated an international, multi-laboratory validation study using the approach described in the *Guidance Document* (ICCVAM 2001b). One goal of the study was to characterize the potential reduction and refinement in animal use that would occur when *in vitro* neutral red uptake (NRU) basal cytotoxicity test methods are used to estimate starting doses for acute toxicity testing using the UDP and ATC methods.

[1] **Reduction alternative:** A new or modified test method that reduces the number of animals required. **Refinement alternative:** A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being. **Replacement alternative:** A new or modified test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one.

Methods

NRU assays using BALB/c mouse 3T3 fibroblasts (3T3) and normal human epidermal keratinocytes (NHK) were used to determine IC_{50} values (i.e., the concentration at which cell viability is reduced by 50% compared with the controls) for 72 reference chemicals that were selected based on their human exposure potential and the existence of human and/or rodent acute oral toxicity data. The IC_{50} values were used in IC_{50}-LD_{50} regression formulas that established the relationship between IC_{50} values and LD_{50} values to calculate the predicted LD_{50} values, which were then used to determine starting doses for computer simulated UDP and ATC tests.

The first regression for determining starting doses was calculated from the *in vitro* IC_{50} values and *in vivo* oral LD_{50} values for the 282 chemicals in the Registry of Cytotoxicity (RC) that were associated with rat oral LD_{50} values. The RC is a database that contains LD_{50} values for mice and rats from the Registry of Toxic Effects for Chemical Substances (RTECS®) and geometric mean IC_{50} values from published *in vitro* cytotoxicity assays using various cell lines and cytotoxicity endpoints for 347 chemicals (Halle 1998). Millimole units were used for both the IC_{50} and LD_{50} since the mole is the most appropriate unit for chemical activity.

Regression 1. RC Rat-Only Millimole Regression

$\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621

The molar units were changed to μg/mL for IC_{50} and mg/kg for LD_{50} so the approach could be applied to mixtures and products with no known molecular weight.

Regression 2. RC Rat-Only Weight Regression

$\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024

In Vivo Acute Systemic Toxicity Test Methods

The UDP is a sequential test in which one animal is dosed at a time (EPA 2002; OECD 2001a). If the first animal dies, the dose administered to the next animal is decreased. If the first animal survives, the dose administered to the next animal is increased. The recommended starting dose is one dose progression step below the analyst's best estimate of the LD_{50}. The default starting dose of 175 mg/kg is used if there is no information on which to base a starting dose. The entire default dosing scheme is 1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (EPA 2002; OECD 2001a). Dosing single animals proceeds until one of the "stopping rules" is met (see Step 6 in simulation modeling procedure for the UDP). Then, the LD_{50}, with confidence limits, is calculated.

The ATC is based on the stepwise administration of test substances, at one of four fixed doses (i.e., 5, 50, 300, or 2000 mg/kg), to three animals at a time (OECD 2001b). The starting dose is selected so that at least some of the animals die at that dose. If there is no information on which to base a starting dose, the default starting dose of 300 mg/kg is used. The next step, which may be to (1) stop testing, (2) test at the same dose, (3) test at the next higher dose, or (4) test at the next lower dose, is determined by the outcome of the three animals tested at the starting dose. For example, if the starting dose is 300 mg/kg and two to three animals die or are in a moribund state, the next step is to administer 50 mg/kg to three more animals. Testing proceeds until the chemical can be classified into an acute oral toxicity category (OECD 2001b).

Simulation Modeling Procedure

The simulation process for testing animals using acute oral systemic toxicity methods was performed using SAS® version 8 (SAS Institute, Cary, NC) software for the UDP and MATLAB® (The MathWorks, Inc. Natick, MA) software for the ATC. The simulation procedures follow the relevant test guidelines (EPA 2002; OECD 2001a; OECD 2001b) and use the assumption that the dose-mortality response follows a log-normal distribution. The lowest dose at which an animal dies in response to the administration of a toxic substance varies from animal to animal. For an entire population of animals, mortality is assumed to have a log-normal distribution with the mean equal to the log of the "true" LD_{50}. Sigma (σ), which reflects the variability of the simulated population, is the inverse of the slope of the dose-mortality curve. For any given dose, the probability that an animal will die is computed by the following log-normal cumulative distribution:

$$\text{Equation 1: Probability (death)} = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{\log\ dose} e^{\frac{-(x-\log\ true LD_{50})^2}{2\sigma^2}} dx$$

Due to a lack of information for the real dose-mortality curves, the simulations assumed several different values of the slope (i.e., the inverse of σ): 0.5, 0.8, 2, 4, and 8.3. Results presented are for dose-mortality slope = 2 only. The results for the remaining slopes are available at http://iccvam.niehs.nih.gov/ in *Background Review Document: In Vitro Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity* (ICCVAM 2006).

To model the variability of the NRU IC_{50} values within and between laboratories, the values were log-transformed to normalize the distribution of values for each test chemical. The mean and variance of the log-transformed values were used to generate a log-normal distribution from which to randomly select an IC_{50} value.

The simulation procedures used the following steps for each test chemical:

1. The reference LD_{50} value (determined from literature search/evaluation) served as the "true" LD_{50} value and the choices of assumed slope were entered as the "true" slope for the dose-mortality curve.
2. An IC_{50} value was selected from a distribution identified by the mean and variance of the IC_{50} values computed from the data. This value reflects that different laboratories produce different IC_{50} values in different situations.
3. The IC_{50} value from Step 2 was used in the regression model being evaluated in order to compute a predicted LD_{50} value. This value was in turn used for determining the starting dose for the simulated acute oral systemic toxicity assay.
4. The dosing simulation was run two times: once with the default starting dose (i.e., 175 mg/kg for the UDP and 300 mg/kg for the ATC) and once at the next default dose below the LD_{50} estimated by the NRU test method and regression.

For the UDP

5. For each simulated trial (10,000 for each chemical and starting dose), the animals are dosed sequentially. For each animal there is a corresponding dose that is administered to the animal. For the first animal in each trial, it is the starting dose for that trial. For each subsequent animal, the dose is dependent on the previous dose and the previous animal's response. The subsequent dose is lowered by a factor of 3.2 if the first animal dies, or is increased by a factor of 3.2 if the first animal lives. For test animal, the probability of response is computed with the cumulative log-normal distribution at that dose (see Equation 1). This probability is used to sample one observation from a binomial distribution with this probability of success.
6. Dosing simulation is stopped when one of the following stopping rules is satisfied:
   - Three consecutive animals survive at the 5000 mg/kg upper limit dose
   - Five reversals of outcome occur in any six consecutive animals tested
   - Four or more animals have followed the first reversal of outcome and the specified likelihood-ratios exceed the critical value
   - If none of the above conditions is met, dosing stops after 15 animals have been used.

For the ATC

5. For every simulated dose group of three animals (2000 for each chemical and starting dose), one observation was sampled from a binomial distribution with the probability of death calculated by the probability equation (see Equation 1) for a population of three. The sampled value, referred to as N1, indicates the number of animals, 0, 1, 2, or 3, in the dosing group that die.
6. If N1 ≤ 1, step 5 is repeated with the same dose. Now the sampled value from the binomial distribution is referred to as N2.
7. If N2 ≤ 1 and the dose is the highest dose tested, or the dose has already been decreased, the toxicity category is assigned and testing is terminated. If the dose is not the highest dose tested, and if the dose has not been decreased, the dose is increased to the next fixed dose and step 5 is repeated.
8. If N1 > 1 or N2 ≥ 2, and the dose is the lowest dose tested, or the dose has already been increased, the toxicity category is assigned and testing is terminated. If the dose is not the lowest dose tested, and if the dose has not already been increased, the dose is decreased to the next fixed dose and step 5 is repeated.

Results

Table 1 Mean Animal Savings for the UDP and the ATC Using Starting Doses Determined with NRU Test Methods

Assay/Regression	N[1]	With Default Starting Dose[2,3]	With NRU-Based Starting Dose[4]	Animals Saved[5]	With Default Starting Dose[4]	With NRU-Based Starting Dose[4]	Animals Saved[5]
3T3 NRU Test Method		**UDP**			**ATC**		
RC Rat-Only Millimole Regression[2]	67	9.35 ± 0.16	8.80 ± 0.17	0.54* (5.8%)	10.89 ± 0.12	10.27 ± 0.24	0.62* (5.7%)
RC Rat-Only Weight Regression[2]	67	9.36 ± 0.16	8.70 ± 0.16	0.66* (7.0%)	10.89 ± 0.12	9.85 ± 0.24	1.04* (9.6%)
NHK NRU Test Method		**UDP**			**ATC**		
RC Rat-Only Millimole Regression[2]	68	9.36 ± 0.16	8.86 ± 0.18	0.50* (5.3%)	10.91 ± 0.11	10.11 ± 0.24	0.80* (7.3%)
RC Rat-Only Weight Regression[2]	68	9.36 ± 0.16	8.80 ± 0.17	0.56* (6.0%)	10.91 ± 0.11	9.95 ± 0.24	0.96* (8.8%)

[1]Number of chemicals that (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

[2]Numbers are mean numbers of animals and standard errors for 10,000 (UDP) or 2000 (ATC) simulations for each chemical. Results for dose-mortality slope of 2 are presented.

[3]Default starting dose = 175 mg/kg for the UDP and 300 mg/kg for the ATC.

[4]Starting dose = one default dose lower than the NRU-based LD_{50} calculated using the NRU IC_{50} values in the specified regression. The IC_{50} value for each chemical was randomly selected from a distribution of values obtained for each test method.

[5]Difference between mean animal use with the default starting dose and mean animal use with the NRU-based starting dose. All differences denoted by * were statistically significant by one-sided Wilcoxon signed rank tests with p ≤ 0.05. Percentage difference is shown in parentheses.

[6]$\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621.

[7]$\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024.

Abbreviations: 3T3 = BALB/c mouse 3T3 fibroblasts; ATC = Acute Toxic Class method; NHK = Normal human epidermal keratinocytes; NRU = Neutral red uptake; RC = Registry of Cytotoxicity; UDP = Up-and-Down Procedure.

Table 2 Mean Animal Savings for the UDP by GHS Toxicity Category[1] Using Starting Doses Determined by NRU Test Methods and IC_{50} – LD_{50} Regressions[2]

Toxicity Category[1]	N[3]	RC Rat-Only Millimole Regression			RC Rat-Only Weight Regression		
		With Default Starting Dose[4,5]	With NRU-Based Starting Dose[4,6]	Animals Saved[7]	With Default Starting Dose[4,5]	With NRU-Based Starting Dose[4,6]	Animals Saved[7]
3T3 NRU Test Method							
LD_{50} ≤ 5 mg/kg	6	11.32 ± 0.20	10.19 ± 0.70	1.14 (10.0%)	11.29 ± 0.20	10.38 ± 0.62	0.90 (8.0%)
5 < LD_{50} ≤ 50 mg/kg	11	9.68 ± 0.23	9.74 ± 0.45	-0.07 (-0.7%)	9.71 ± 0.22	9.58 ± 0.42	0.13 (1.3%)
50 < LD_{50} ≤ 300 mg/kg	12	7.76 ± 0.10	8.18 ± 0.21	-0.42 (-5.5%)	7.74 ± 0.10	7.99 ± 0.18	-0.25 (-3.3%)
300 < LD_{50} ≤ 2000 mg/kg	16	8.53 ± 0.21	8.14 ± 0.21	0.38 (4.5%)	8.52 ± 0.21	8.16 ± 0.19	0.35 (4.1%)
2000 < LD_{50} ≤ 5000 mg/kg	10	10.73 ± 0.10	9.46 ± 0.15	1.28* (11.9%)	10.78 ± 0.11	9.14 ± 0.24	1.64* (15.2%)
LD_{50} > 5000 mg/kg	12	9.87 ± 0.34	8.29 ± 0.49	1.58* (16.0%)	9.87 ± 0.34	8.23 ± 0.48	1.65* (16.7)%
NHK NRU Test Method							
LD_{50} ≤ 5 mg/kg	6	11.21 ± 0.24	10.47 ± 0.71	0.75 (6.7%)	11.21 ± 0.24	10.49 ± 0.71	0.72 (6.4%)
5 < LD_{50} ≤ 50 mg/kg	11	9.65 ± 0.16	9.99 ± 0.45	-0.34* (-3.5%)	9.70 ± 0.18	9.78 ± 0.41	-0.07 (-0.8%)
50 < LD_{50} ≤ 300 mg/kg	12	7.78 ± 0.11	8.12 ± 0.21	-0.34 (-4.4%)	7.75 ± 0.11	7.99 ± 0.21	-0.24 (-3.1%)
300 < LD_{50} ≤ 2000 mg/kg	16	8.55 ± 0.22	8.03 ± 0.23	0.52* (6.1%)	8.54 ± 0.21	8.20 ± 0.22	0.34 (3.9%)
2000 < LD_{50} ≤ 5000 mg/kg	10	10.75 ± 0.08	9.54 ± 0.20	1.21* (11.3%)	10.77 ± 0.08	9.40 ± 0.25	1.38* (12.8%)
LD_{50} > 5000 mg/kg	13	9.87 ± 0.32	8.41 ± 0.44	1.47* (14.8%)	9.88 ± 0.32	8.34 ± 0.44	1.54* (15.6)%

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

[2]RC rat-only millimole regression: $\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621. RC rat-only weight regression: $\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024.

[3]Number of chemicals in each category that (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

[4]Numbers are mean number of animals used and standard errors for 10,000 simulations for each chemical. Results for dose-mortality slope = 2 are presented.

[5]Default starting dose = 175 mg/kg.

[6]Starting dose was one default dose lower than the LD_{50} predicted by the NRU IC_{50} and the regression evaluated. The IC_{50} value for each chemical was randomly selected from a distribution of values obtained for each test method.

[7]Difference between mean animal use with default starting dose and mean animal use with NRU-determined starting dose. Statistically significant differences by one-sided Wilcoxon signed rank tests at p < 0.05 are noted by *.

Abbreviations: 3T3 = BALB/c mouse 3T3 fibroblasts; NHK = Normal human epidermal keratinocytes; NRU = Neutral red uptake; RC = Registry of Cytotoxicity; UDP = Up-and-Down Procedure.

Table 3 Mean Animal Savings for the ATC by GHS Toxicity Category[1] Using Starting Doses Determined by NRU Test Methods and the IC_{50} – LD_{50} Regressions[2]

Toxicity Category[1]	N[3]	RC Rat-Only Millimole Regression			RC Rat-Only Weight Regression		
		With Default Starting Dose[4,5]	With NRU-Based Starting Dose[4,6]	Animals Saved[7]	With Default Starting Dose[4,5]	With NRU-Based Starting Dose[4,6]	Animals Saved[7]
3T3 NRU Test Method							
LD_{50} ≤ 5 mg/kg	6	9.77 ± 0.17	7.09 ± 1.09	2.68 (27.4%)	9.77 ± 0.17	7.56 ± 1.03	2.21 (22.6%)
5 < LD_{50} ≤ 50 mg/kg	11	11.56 ± 0.21	10.39 ± 0.52	1.17* (10.2%)	11.56 ± 0.21	10.06 ± 0.38	1.51* (13.0%)
50 < LD_{50} ≤ 300 mg/kg	12	10.81 ± 0.20	10.39 ± 0.17	0.42 (3.9%)	10.81 ± 0.20	10.35 ± 0.18	0.47* (4.3%)
300 < LD_{50} ≤ 2000 mg/kg	16	9.75 ± 0.07	10.67 ± 0.48	-0.92* (-9.5%)	9.75 ± 0.07	10.67 ± 0.50	-0.93 (-9.5%)
2000 < LD_{50} ≤ 5000 mg/kg	10	11.22 ± 0.08	11.14 ± 0.08	0.08 (0.7%)	11.22 ± 0.08	9.80 ± 0.51	1.43* (12.7%)
LD_{50} > 5000 mg/kg	12	11.85 ± 0.04	9.82 ± 0.78	2.03* (17.1%)	11.85 ± 0.04	8.83 ± 0.83	3.02* (25.5%)
NHK NRU Test Method							
LD_{50} ≤ 5 mg/kg	6	9.74 ± 0.16	6.78 ± 1.31	2.96 (30.4%)	9.74 ± 0.16	6.87 ± 1.28	2.87 (29.4%)
5 < LD_{50} ≤ 50 mg/kg	11	11.56 ± 0.21	10.38 ± 0.35	1.18* (10.2%)	11.56 ± 0.21	10.31 ± 0.19	1.25* (10.8%)
50 < LD_{50} ≤ 300 mg/kg	12	10.83 ± 0.21	10.39 ± 0.29	0.44 (4.0%)	10.83 ± 0.21	10.41 ± 0.28	0.42 (3.8%)
300 < LD_{50} ≤ 2000 mg/kg	16	9.77 ± 0.06	10.37 ± 0.49	-0.60 (-6.1%)	9.77 ± 0.62	10.46 ± 0.50	-0.69 (-7.1%)
2000 < LD_{50} ≤ 5000 mg/kg	10	11.22 ± 0.08	11.25 ± 0.12	-0.03 (-0.3%)	11.22 ± 0.09	10.69 ± 0.37	0.53 (4.7%)
LD_{50} > 5000 mg/kg	13	11.86 ± 0.03	9.43 ± 0.73	2.43* (20.5%)	11.86 ± 0.03	8.91 ± 0.78	2.94* (24.8%)

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

[2]RC rat-only millimole regression: $\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621. RC rat-only weight regression: $\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024.

[3]Number of chemicals in each category that (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

[4]Numbers are mean number of animals used and standard errors for 2000 simulations for each chemical.

[5]Default starting dose = 300 mg/kg.

[6]Starting dose was the next fixed dose lower than the LD_{50} predicted by the NRU IC50 and the regression evaluated. The IC_{50} value for each chemical was randomly selected from a distribution of values obtained for each test method.

[7]Difference between mean animal use with default starting dose and mean animal use with NRU-determined starting dose. Statistically significant differences by one-sided Wilcoxon signed rank tests at p < 0.05 are noted by *.

Abbreviations: 3T3 = BALB/c mouse 3T3 fibroblasts; ATC = Acute Toxic Class method; NHK = Normal human epidermal keratinocytes; NRU = Neutral red uptake; RC = Registry of Cytotoxicity.

Results

Table 4 Animal Deaths for the UDP and ATC Using Starting Doses Predicted by the 3T3 and NHK NRU Test Methods

Regression	UDP						ATC					
	Default Starting Dose[1]			NRU-Based Starting Dose[2]			Default Starting Dose[1]			NRU-Based Starting Dose[2]		
	Used[3]	Dead[3]	% Dead[4]	Used[3]	Dead[3]	% Dead[4]	Used[3]	Dead[3]	% Dead[4]	Used[3]	Dead[3]	% Dead[4]
3T3 NRU Test Method												
RC Rat-Only Millimole Regression[5]	9.35	4.11	44.0%	8.80	4.09	46.5%	10.89	3.77	34.6%	10.27	3.31	32.2%
RC Rat-Only Weight Regression[5]	9.36	4.11	43.9%	8.70	4.05	46.6%	10.89	3.77	34.6%	9.85	3.27	33.2%
NHK NRU Test Method												
RC Rat-Only Millimole Regression[5]	9.36	4.08	43.6%	8.86	4.07	45.9%	10.91	3.72	34.1%	10.11	3.19	31.6%
RC Rat-Only Weight Regression[5]	9.36	4.08	43.6%	8.80	4.02	45.7%	10.91	3.72	34.1%	9.95	3.21	32.3%

[1]Default starting dose = 175 mg/kg for the UDP and 300 mg/kg for the ATC.

[2]Starting dose was one default dose lower than the NRU-predicted LD_{50}.

[3]Numbers are mean numbers of animals for 10,000 (UDP) or 2000 (ATC) simulations for each chemical. Results for 67 chemicals in the 3T3 NRU and 68 chemicals in the NHK NRU test method. These chemicals (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

[4]Proportion of simulated animals that died compared to number of simulated animals used.

[5]$\log LD_{50}$ (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621.

[6]$\log LD_{50}$ (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024.

Abbreviations: 3T3 = BALB/c mouse 3T3 fibroblasts; ATC = Acute Toxic Class method; NHK = Normal human epidermal keratinocytes; NRU = Neutral red uptake; RC = Registry of Cytotoxicity; UDP = Up-and-Down Procedure.

Conclusions

- For the two regressions evaluated (i.e., the RC rat-only millimole and RC rat-only weight regressions), animal savings were similar when they were used with the NRU test methods to determine starting doses for the UDP (see Tables 1 and 2).
- For the ATC, animal savings were often greater (up to 1.3 animals for some comparisons) using the RC rat-only weight regression to determine starting doses compared to using the RC rat-only millimole regression (see Tables 1 and 3).
- Mean animal savings were similar for the 3T3 and NHK NRU test methods (see Table 1).
  - Mean savings for the UDP were 0.54 (5.8%) animals for the 3T3 NRU and 0.50 (5.3%) animals for the NHK NRU with the RC rat-only millimole regression.
  - Mean animal savings for the UDP were 0.66 (7.0%) animals for the 3T3 NRU and 0.56 (6.0%) animals for the NHK NRU with the RC rat-only weight regression.
  - Mean animal savings for the ATC were 0.62 (5.7%) animals for the 3T3 NRU and 0.80 (7.3%) animals for the NHK NRU with the RC rat-only millimole regression.
  - Mean animal savings for the ATC were 1.04 (9.6%) animals for the 3T3 NRU and 0.96 (8.8%) animals for the NHK NRU with the RC rat-only weight regression.
- For the UDP, there were no animal savings for chemicals in the GHS toxicity category that included the default starting dose of 175 mg/kg (i.e., 50 < LD_{50} ≤ 300 mg/kg), as would be expected if the NRU predictions were accurate, or for chemicals with 5 < LD_{50} ≤ 50 mg/kg. Animal savings were largest for the least toxic chemicals (i.e., 2000 < LD_{50} ≤ 5000 mg/kg and LD_{50} > 5000 mg/kg) (see Table 2). Mean animal savings for these categories ranged from 1.21 (11.3%) to 1.65 (16.7%).
- For the ATC, there were no animal savings for chemicals in the GHS toxicity category for 300 < LD_{50} ≤ 2000 mg/kg, as would be expected if the NRU predictions were accurate since this category is near the default starting dose of 300 mg/kg. Using the RC rat-only millimole regression, there were also no animal savings for chemicals with 2000 < LD_{50} ≤ 5000 mg/kg. Statistically significant animal savings were largest for chemicals with LD_{50} > 5000 mg/kg. Mean animal savings for chemicals in this category ranged from 2.03 (17.1%) to 3.02 (25.5%) animals (see Table 3). Mean animal savings were also statistically significant for chemicals with 5 < LD_{50} ≤ 50 mg/kg, ranging from 1.17 (10.2%) to 1.51 (13.0%) (see Table 3).
- When using the NRU test methods to determine starting doses for the simulated UDP, fewer animals were used, but approximately the same number of animals died relative to simulations using the default starting dose. For the ATC, use of the NRU test methods resulted in fewer animals used and fewer animal deaths relative to simulations using the default starting dose (see Table 4).
- The actual animal savings for chemicals tested in the future will depend on the distribution of the chemicals into the different GHS toxicity categories. Considering that approximately 85% of the chemicals in the European New Chemicals database have LD_{50} > 2000 mg/kg (S. Casati, personal communication, 2005), animal savings using this approach will likely be closer to 14% (the average of UDP and ATC animal savings for those categories). However, the extent to which these industrial chemicals represent the entire range of substances in commerce is not known.

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NIEHS National Institute of Environmental Health Sciences

U.S. Department of Health and Human Services National Institutes of Health

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/