Reducing Animal Use in Acute Systemic Toxicity Testing by Using *In Vitro* Cytotoxicity Assays for Estimating Starting Doses

JA Strickland[1,2], MW Paris[1,2], S Casati[3], RR Tice[1], WS Stokes[1], R Lee[4], E Harvey[4], J Haseman[5], H Raabe[6], C Cao[7], R Clothier[8], G Mun[6], A Sizemore[6], G Moyer[6], J Madren-Whalley[7], C Krishna[7], M Owen[8], N Bourne[8], M Wenk[9], and M Vallant[10]

[1]National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), NIEHS/NIH/DHHS, Research Triangle Park, North Carolina, USA; [2]Integrated Laboratory Systems, Inc., Research Triangle Park, North Carolina, USA; [3]ECVAM, JRC, Ispra, Italy; [4]Constella Group, Durham, NC; [5]Consultant, Raleigh, NC; [6]IIVS, Gaithersburg, MD; [7]US Army ECBC, Aberdeen Proving Ground, MD; [8]Univ. of Nottingham, Nottingham, UK, [9]BioReliance Corp, Rockville, MD; [10]NIEHS, RTP, NC

Introduction

Accidental 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 2004). In 2001, 30,800 deaths placed poisoning as the second leading cause of injury-related death behind automobile accidents (42,433 deaths) (Institute of Medicine 2004). The hazard potential for poisoning in humans is assessed by acute oral toxicity testing in rodents, which is a regulatory requirement for many substances and products. However, ethical and societal demands call for decreasing the numbers of animals used for such studies.

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 Acute Toxic Class (ATC; OECD 2001b) methods, if the starting dose was close to the oral LD_{50}. Spielmann et al. (1999) showed that *in vitro* basal cytotoxicity assays could be used to predict starting doses for *in vivo* acute systemic toxicity assays. Thus, one of the workshop recommendations for reducing and refining the use of animals for acute systemic toxicity assays was the publication of guidance for using *in vitro* basal cytotoxicity assays to estimate the starting doses for acute oral lethality assays (ICCVAM 2001b). This guidance provides details and examples on how to execute such an approach (ICCVAM 2001b).

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 a multi-laboratory validation study using the approach described in the *Guidance Document* (ICCVAM 2001b). One goal of the study was to characterize the 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.

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

ICCVAM
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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. (See poster 1970 for information on the reproducibility of these assays). The IC_{50} values were used in IC_{50}-LD_{50} regression formulas to calculate the predicted LD_{50}, which was then used to determine starting doses for the UDP and the ATC test methods.

The first regression used for determining starting doses was the Registry of Cytotoxicity (RC) regression, which was developed using rat and mouse oral LD_{50} values from the Registry of Toxic Effects for Chemical Substances® (RTECS) and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for 347 chemicals (Halle 1998) (see poster 1969 for more information on the regressions). A modified RC regression was calculated as follows:

- Only chemicals with rat oral LD_{50} data were used since (a) most oral systemic toxicity assays are performed with rats and (b) rats and mice may have different sensitivity to individual chemicals
- 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/products with no known molecular weight.
- Chemicals with known mechanisms of toxicity that were not expected to be active in the 3T3 or NHK cell cultures were excluded. Such chemicals included neurotoxins and cardiotoxins, and those that interfere with energy utilization, or alkylate macromolecules.

RC Regression:	$\log LD_{50}$ (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625
Modified RC Regression:	$\log LD_{50}$ (mg/kg) = 0.357 log IC_{50} (µg/mL) + 2.194

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 2002b). 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 the following fixed doses - 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 implement the distributional assumptions underlying the dose-mortality response. 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:

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

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 only for dose-mortality slope = 2 are presented.

The simulation procedure 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 randomly selected from a distribution identified by the mean and variance of the IC_{50} values computed from the data to reflect 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 to compute a predicted LD_{50} value to use 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 (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. The dosing simulations were repeated 2000 times for each chemical for each starting dose.

For the UDP

5. For each simulated trial (each chemical and starting dose), the animals are dosed sequentially. For each animal(i) there is a corresponding dose(i) 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 lower by 3.2 if the first animal dies, or is increased by 3.2 if the first animal lives. For test animal(i), 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 dose group of three animals, 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 $\leq$ 1, step 4 is repeated with the same dose. Now the sampled value from the binomial distribution is referred to as N2.
7. If N2 $\leq$ 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, or if the dose has not been decreased, the dose is increased to the next fixed dose and step 4 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, or if the dose has not already been increased, the dose is decreased to the next fixed dose and step 4 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-Determined Starting Dose[2,3]	Animals Saved[4]	With Default Starting Dose[3]	With NRU-Determined Starting Dose[3,5]	Animals Saved[4]
3T3 NRU Test Method			**UDP**			**ATC**	
RC Regression[6]	46	9.77 ± 0.010	8.79 ± 0.010	0.97 (10.0%)	10.90 ± 0.005	9.76 ± 0.009	1.13 (10.4%)
Modified RC Regression[7]	46	9.80 ± 0.010	8.64 ± 0.010	1.16 (11.8%)	10.90 ± 0.005	9.00 ± 0.008	1.90 (17.4%)
NHK NRU Test Method			**UDP**			**ATC**	
RC Regression[6]	47	9.75 ± 0.010	8.93 ± 0.010	0.82 (8.4%)	10.93 ± 0.005	9.72 ± 0.008	1.21 (11.1%)
Modified RC Regression[7]	47	9.78 ± 0.010	8.73 ± 0.010	1.05 (10.7%)	10.93 ± 0.005	9.25 ± 0.008	1.68 (15.4%)

[1]Number of chemicals.
[2]Numbers are mean numbers of animals and standard errors for 2000 simulations for each chemical. Results for dose-mortality slope of 2 are presented. The small differences in the number of animals used for the default starting dose reflect different simulation runs.
[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-predicted LD_{50} calculated using the NRU IC_{50} values in the specified regression.
[5]Difference between mean animal use with default starting dose and mean animal use with NRU-predicted starting dose.
[6]Percentage difference is shown in parentheses.
[6]$\log LD_{50}$ (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625.
[7]$\log LD_{50}$ (mg/kg) = 0.357 log IC_{50} (µg/mL) + 2.194.
Abbreviations: UDP – Up-and-Down Procedure; ATC – Acute Toxic Class method; NRU – neutral red uptake; RC- Registry of Cytotoxicity

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]	RC Regression N[3]	RC Regression With Default Starting Dose[4]	RC Regression With NRU-Determined Starting Dose[4]	RC Regression Animals Saved[5]	Modified RC Regression N[3]	Modified RC Regression With Default Starting Dose[4]	Modified RC Regression With NRU-Determined Starting Dose[4,6]	Modified RC Regression Animals Saved[5]
3T3 NRU Test Method								
$LD_{50} \leq 5$ mg/kg	4	11.78 ± 0.020	10.8 ± 0.025	0.95 (8.1%)	4	11.68 ± 0.020	11.28 ± 0.023	0.42 (3.6%)
$5 < LD_{50} \leq 50$ mg/kg	7	9.06 ± 0.023	9.15 ± 0.026	-0.09 (-1.0%)	7	9.05 ± 0.023	9.03 ± 0.025	0.02 (0.3%)
$50 < LD_{50} \leq 300$ mg/kg	5	7.70 ± 0.018	7.61 ± 0.018	0.09 (1.2%)	5	7.82 ± 0.018	7.84 ± 0.018	-0.02 (-0.2%)
$300 < LD_{50} \leq 2000$ mg/kg	9	8.76 ± 0.020	7.91 ± 0.018	0.84 (9.6%)	9	8.81 ± 0.021	7.81 ± 0.017	1.00 (11.4%)
$2000 < LD_{50} \leq 5000$ mg/kg	9	10.75 ± 0.020	9.23 ± 0.021	1.52 (14.1%)	9	10.84 ± 0.020	8.62 ± 0.021	2.22 (20.5%)
$LD_{50} > 5000$ mg/kg	12	9.59 ± 0.024	8.05 ± 0.026	1.54 (16.1%)	12	9.59 ± 0.024	7.71 ± 0.027	1.88 (19.6%)
NHK NRU Test Method								
$LD_{50} \leq 5$ mg/kg	4	11.54 ± 0.020	11.79 ± 0.022	-0.25 (-2.2%)	4	11.55 ± 0.020	11.90 ± 0.020	-0.35 (-3.0%)
$5 < LD_{50} \leq 50$ mg/kg	7	9.34 ± 0.024	8.48 ± 0.023	0.86 (9.2%)	7	9.28 ± 0.024	8.30 ± 0.022	0.98 (10.6%)
$50 < LD_{50} \leq 300$ mg/kg	5	7.82 ± 0.018	7.88 ± 0.019	-0.06 (-0.7%)	5	7.87 ± 0.018	8.03 ± 0.019	-0.16 (-2.0%)
$300 < LD_{50} \leq 2000$ mg/kg	9	8.74 ± 0.020	7.93 ± 0.018	0.81 (9.3%)	9	8.76 ± 0.020	7.86 ± 0.017	0.90 (10.3%)
$2000 < LD_{50} \leq 5000$ mg/kg	9	10.73 ± 0.020	9.29 ± 0.021	1.45 (13.5%)	9	10.82 ± 0.020	8.84 ± 0.021	1.98 (18.3%)
$LD_{50} > 5000$ mg/kg	13	9.52 ± 0.022	8.17 ± 0.025	1.35 (14.2%)	13	9.52 ± 0.022	7.75 ± 0.026	1.77 (18.6%)

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
[2]RC regression: $\log LD_{50}$ (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625. Modified RC regression: $\log LD_{50}$ (mg/kg) = 0.357 log IC_{50} (µg/mL) + 2.194.
[3]Number of chemicals in each category.
[4]Numbers are mean number of animals used and standard errors for 2000 simulations for each chemical. Results for dose-mortality slope of 2 are presented.
[5]Default starting dose = 175 mg/kg for the UDP.
[6]Starting dose was one default dose lower than the LD_{50} predicted by the NRU IC_{50} and the regression evaluated.
[7]Difference between mean animal use with default starting dose and mean animal use with NRU-determined starting dose.
Abbreviations: UDP – Up-and-Down Procedure; ATC – Acute Toxic Class method; NRU – neutral red uptake; RC- Registry of Cytotoxicity

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]	RC Regression N[3]	RC Regression With Default Starting Dose[4]	RC Regression With NRU-Determined Starting Dose[4,5]	RC Regression Animals Saved[6]	Modified RC Regression N[3]	Modified RC Regression With Default Starting Dose[4]	Modified RC Regression With NRU-Determined Starting Dose[4,5]	Modified RC Regression Animals Saved[6]
3T3 NRU Test Method								
$LD_{50} \leq 5$ mg/kg	4	9.35 ± 0.008	6.60 ± 0.022	2.75 (29.5%)	4	9.35 ± 0.008	7.23 ± 0.020	2.12 (22.6%)
$5 < LD_{50} \leq 50$ mg/kg	7	12.22 ± 0.015	11.12 ± 0.020	1.10 (9.0%)	7	12.22 ± 0.015	10.52 ± 0.019	1.70 (13.9%)
$50 < LD_{50} \leq 300$ mg/kg	5	10.70 ± 0.020	10.01 ± 0.016	0.69 (6.5%)	5	10.70 ± 0.020	9.92 ± 0.015	0.79 (7.3%)
$300 < LD_{50} \leq 2000$ mg/kg	9	9.79 ± 0.011	10.04 ± 0.015	-0.24 (-2.5%)	9	9.79 ± 0.011	9.77 ± 0.012	0.02 (0.2%)
$2000 < LD_{50} \leq 5000$ mg/kg	9	11.18 ± 0.009	11.02 ± 0.011	0.16 (1.4%)	9	11.18 ± 0.009	9.50 ± 0.017	1.67 (15.0%)
$LD_{50} > 5000$ mg/kg	12	11.90 ± 0.004	9.58 ± 0.021	2.32 (19.5%)	12	11.90 ± 0.004	7.82 ± 0.019	4.08 (34.3%)
NHK NRU Test Method								
$LD_{50} \leq 5$ mg/kg	4	9.37 ± 0.008	7.62 ± 0.025	1.76 (18.7%)	4	9.37 ± 0.008	8.11 ± 0.017	1.27 (13.5%)
$5 < LD_{50} \leq 50$ mg/kg	7	12.20 ± 0.016	9.77 ± 0.019	2.43 (19.9%)	7	12.20 ± 0.016	9.87 ± 0.019	2.33 (19.1%)
$50 < LD_{50} \leq 300$ mg/kg	5	10.75 ± 0.020	10.32 ± 0.019	0.43 (4.0%)	5	10.75 ± 0.020	10.19 ± 0.018	0.55 (5.2%)
$300 < LD_{50} \leq 2000$ mg/kg	9	9.79 ± 0.011	9.81 ± 0.012	-0.02 (-0.2%)	9	9.79 ± 0.011	9.79 ± 0.012	-0.005 (-0.05%)
$2000 < LD_{50} \leq 5000$ mg/kg	9	11.19 ± 0.009	10.81 ± 0.012	0.38 (3.4%)	9	11.19 ± 0.009	9.96 ± 0.016	1.23 (11.0%)
$LD_{50} > 5000$ mg/kg	13	11.92 ± 0.003	9.58 ± 0.021	2.34 (19.7%)	13	11.92 ± 0.003	8.13 ± 0.019	3.79 (31.8%)

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
[2]RC regression: $\log LD_{50}$ (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625. Modified RC regression: $\log LD_{50}$ (mmol/kg) = 0.357 log IC_{50} (µg/mL) + 2.194.
[3]Number of chemicals in each category.
[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 IC_{50} and the regression evaluated.
[7]Difference between mean animal use with default starting dose and mean animal use with NRU-determined starting dose.
Abbreviations: UDP – Up-and-Down Procedure; ATC – Acute Toxic Class method; NRU – neutral red uptake; RC- Registry of Cytotoxicity

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

Regression	UDP Default Starting Dose[1] Used[2]	UDP Default Starting Dose[1] Dead[2]	UDP Default Starting Dose[1] % Dead[3]	UDP NRU-Determined Starting Dose[4] Used[2]	UDP NRU-Determined Starting Dose[4] Dead[2]	UDP NRU-Determined Starting Dose[4] % Dead[3]	ATC Default Starting Dose[1] Used[2]	ATC Default Starting Dose[1] Dead[2]	ATC Default Starting Dose[1] % Dead[3]	ATC NRU-Determined Starting Dose[4] Used[2]	ATC NRU-Determined Starting Dose[4] Dead[2]	ATC NRU-Determined Starting Dose[4] % Dead[3]
3T3 NRU Test Method												
RC Regression[5]	9.77	4.16	42.6%	8.79	3.95	44.9%	10.90	3.55	32.6%	9.76	2.87	29.4%
Modified RC Regression[5]	9.80	4.18	42.7%	8.64	4.03	46.6%	10.90	3.55	32.6%	9.00	2.92	32.4%
NHK NRU Test Method												
RC Regression[5]	9.75	4.10	42.0%	8.93	3.96	44.3%	10.93	3.47	31.8%	9.72	2.82	29.0%
Modified RC Regression[5]	9.78	4.12	42.1%	8.73	3.99	45.8%	10.93	3.47	31.8%	9.25	2.91	31.5%

[1]Default starting dose = 175 mg/kg for the UDP and 300 mg/kg for the ATC.
[2]Starting dose was one fixed dose lower than the NRU-predicted LD_{50}.
[3]Numbers are mean numbers of animals for 2000 simulations for each chemical. Results for 46 chemicals in the 3T3 NRU and 47 chemicals in the NHK NRU test method.
[4]Proportion of simulated animals that died compared to number of simulated animals used.
[5]$\log LD_{50}$ (mmol/kg) = 0.435 log IC_{50} (mM) + 0.625.
[6]$\log LD_{50}$ (mg/kg) = 0.357 log IC_{50} (mM) + 2.194.
Abbreviations: UDP – Up-and-Down Procedure; ATC – Acute Toxic Class method; NRU – neutral red uptake; RC- Registry of Cytotoxicity

Conclusions

- For both the UDP and ATC methods, more animals were saved when using the NRU test methods with the modified RC regression to determine starting doses compared to using the NRU test methods with the RC regression (see Tables 1, 2, and 3).
- Mean animal savings were similar for the 3T3 and NHK NRU test methods. Mean savings for the UDP were 0.97 (10.0%) animals for the 3T3 NRU and 0.82 (8.4%) animals for the NHK NRU with the RC regression. Mean animal savings for the UDP were 1.16 (11.8%) animals for the 3T3 NRU and 1.05 (10.7%) animals for the NHK NRU with the modified RC regression. Animal savings for the ATC were 1.13 (10.4%) animals for the 3T3 NRU and 1.21 (11.1%) animals for the NHK NRU with the RC regression. Animal savings for the ATC were 1.90 (17.4%) animals for the 3T3 NRU and 1.68 (15.4%) animals for the NHK NRU with the modified RC regression (see Table 1).
- 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., $50 < LD_{50} \leq 300$ mg/kg). Animal savings were largest for the least toxic chemicals (i.e., $LD_{50} > 300$ mg/kg) (see Table 2). Mean animal savings for these categories ranged from 0.81 (9.3%) to 2.22 (20.5%).
- For the ATC, there were no animal savings for chemicals in the GHS toxicity category for $300 < LD_{50} \leq 2000$ mg/kg, which was near the default starting dose of 300 mg/kg. Animal savings were largest for chemicals with $LD_{50} > 5000$ mg/kg. Mean animal savings for chemicals in this category ranged from 2.32 (19.5%) to 4.08 (34.3%) animals (see Table 3). Mean animal savings were also considerable for chemicals with $LD_{50} < 50$ mg/kg. Mean animal savings for chemicals in the $LD_{50} \leq 5$ mg/kg and $5 < LD_{50} \leq 50$ mg/kg categories were 1.10 (9.0%) to 2.75 (29.5%) animals for the 3T3 NRU and 1.27 (13.5%) to 2.43 (19.9%) animals for the NHK NRU (see Table 3).
- When using the NRU test methods to determine starting doses for the simulated UDP and ATC, fewer animals were used and fewer animals died compared to using the default starting dose. The animal savings for the UDP was primarily in live animals, however, the animal savings for the ATC was primarily in dead animals (see Table 4).
- Considering that 95% of the chemicals in the European New Chemicals database have $LD_{50} > 300$ mg/kg, and 85% of the chemicals have $LD_{50} > 2000$ mg/kg (S. Casati, personal communication, 2005), animal savings using this approach will likely be greater than the average of 1 + animals found in this study. However, the extent to which these industrial chemicals represent the entire range of substances in commerce is not known.

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