In Vitro Cytotoxicity Test Methods for Estimating Rat Acute Oral Toxicity: Prediction of GHS Acute Oral Hazard Categories

M Paris[1], J Strickland[1], S Casati[2], R Tice[3], W Stokes[3]

[1]ILS, Inc., Contractor Supporting NICEATM, RTP, NC, USA; [2]ECVAM, JRC, Ispra, Italy; [3]NICEATM/NIEHS/NIH/DHHS, RTP, NC.

Abstract

An international validation study assessed the use of IC_{50} values from two *in vitro* neutral red uptake (NRU) basal cytotoxicity test methods (3T3 murine fibroblasts or normal human keratinocytes) for predicting acute oral hazard categories. Accuracy of these predictions (based on the United Nations Globally Harmonized System [GHS] hazard classifications) was evaluated using these IC_{50} values in two IC_{50}-LD_{50} regressions developed from the 282 rat oral LD_{50} values and corresponding IC_{50} values from the Registry of Cytotoxicity (database of 347 chemicals with rodent data). Regressions were based on molar units and weight units of chemicals. Predicted LD_{50} values, calculated using NRU IC_{50} values in the regressions, were used to classify 67-68 category based on the *in vivo* rat oral LD_{50} value for the *in vitro* predictions of GHS category. Accuracy of both methods and regressions for predicting GHS categories ranged from 29% (20/68) - 31% (21/67). Toxicity was overpredicted for 33% (22/67) - 40% (27/68) and underpredicted for 31% (21/68) - 36% (24/67) of the chemicals. Accuracy was highest for chemicals in the 300< LD_{50} ≤2000 mg/kg range (75% [12/16] - 81% [13/16]). Toxicity for this range was overpredicted for 19% (3/16) - 25% (4/16) and underpredicted for 0% (0/16) - 6% (1/16) of these chemicals. Accuracy was lowest for chemicals with LD_{50} ≤5 mg/kg (0% [0/6]). This study indicates that *in vitro* basal cytotoxic tests are not sufficiently accurate when used alone for predicting GHS classification. However, all *in vitro* predictions for the nontoxic GHS category, LD_{50} >5000 mg/kg, matched the *in vivo* category, suggesting that acute oral toxicity testing for such chemicals could proceed directly to the limit test and thus reduce the number of animals tested. Supported by: NIEHS contracts N01-ES-55504, N01-ES-75408; EPA IAG DW-75-93893601-0; European Commission 19416-2002-04 F2ED ISP GB.

Introduction

Acute oral toxicity testing in laboratory animals is used to characterize the potential hazard associated with human exposure to a substance. In October 2000, the panel experts at an ICCVAM 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 toxicity testing (ICCVAM 2001a). Participants recommended that two *in vitro* cytotoxicity assays using human cells and animal cells should undergo validation to determine the extent that they could predict acute *in vivo* lethality (ICCVAM 2001b).

An international validation study organized by 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) was undertaken to evaluate the extent that cytotoxicity data from rodent and human cells could estimate rodent *in vivo* LD_{50} values (i.e., the dose that produces lethality in 50% of the animals tested) (Spielmann et al. 1999; ICCVAM 2006). This study assessed the use of *in vitro* LD_{50} values (i.e., the concentration at which cell viability is reduced by 50% compared with the controls) from two *in vitro* neutral red uptake (NRU) basal cytotoxicity test methods (murine fibroblasts [3T3] and normal human epidermal keratinocytes [NHK]) for predicting acute oral hazard categories.

Accuracy of these predictions (based on the United Nations Globally Harmonized System [GHS] hazard classifications [UN 2005]) was evaluated using these IC_{50} values in two IC_{50}-LD_{50} regressions developed from the 282 rat oral LD_{50} values and corresponding IC_{50} values from the Registry of Cytotoxicity ([RC] database of 347 chemicals with rodent data [Halle 2003]). Regressions were based on either molar units or weight units of chemicals.

Predicted LD_{50} values, which were calculated using NRU IC_{50} values in the regressions, were used to classify chemicals into GHS hazard categories (**Table 1**). In this evaluation, accuracy is defined as the proportion of correct *in vitro* predictions of GHS hazard categories; the correct outcome is the GHS hazard category that would be assigned using the *in vivo* rat oral LD_{50} value.

Methods

In the NICEATM/ECVAM validation study, 72 reference chemicals were tested in NRU assays using either 3T3 or NHK (ICCVAM 2006) to determine IC_{50} values.

These chemicals 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 to calculate predicted LD_{50} values which were used to assign predicted GHS hazard categories for acute oral toxicity (UN 2005) (**Table 1**).

The first regression for calculating predicted LD_{50} values was derived using the *in vitro* IC_{50} values and *in vivo* oral LD_{50} values for the 282 chemicals in the RC that were associated with rat oral LD_{50} values (**Figure 1**). The RC is a database that contains LD_{50} values for mice and rats obtained from the Registry of Toxic Effects for Chemical Substances (RTECS®), which publishes the most toxic values available, and the geometric mean IC_{50} values from published reports for *in vitro* cytotoxicity assays that used various cell lines and cytotoxicity endpoints for 347 chemicals (Halle 2003).

Millimole units were used for both the IC_{50} values and LD_{50} values since the mole is the most appropriate unit for chemical activity.

For the second regression, the molar units were converted to μg/mL for IC_{50} and mg/kg for LD_{50}, so the approach would be applicable to mixtures and products with no known molecular weight (**Figure 2**).

The predicted LD_{50} values for both NRU test methods and IC_{50}-LD_{50} regressions were used to assign predicted GHS categories.

Table 1

GHS Hazard Classification Scheme for Acute Oral Toxicity

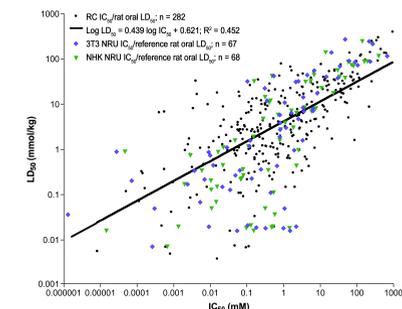| Category	LD_{50} (mg/kg)
1	LD_{50} ≤ 5
2	5 < LD_{50} ≤ 50
3	50 < LD_{50} ≤ 300
4	300 < LD_{50} ≤ 2000
5	2000 < LD_{50} ≤ 5000
Unclassified	LD_{50} > 5000

Abbreviations: UN=United Nations; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
LD_{50}=Dose that produces lethality in 50% of the animals tested.
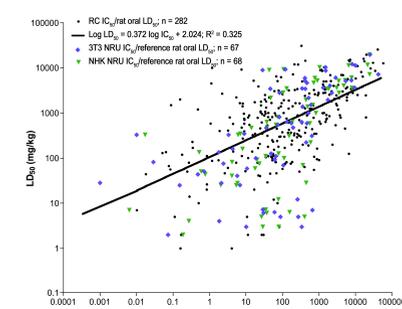
Figure 1

RC Rat-only Millimole Regression

- RC IC_{50}/rat oral LD_{50}; n = 282
- Log LD_{50} = 0.439 log IC_{50} + 0.621; R^2 = 0.452
- 3T3 NRU IC_{50}/reference rat oral LD_{50}; n = 67
- NHK NRU IC_{50}/reference rat oral LD_{50}; n = 68

Abbreviations: IC_{50}=Concentration at which cell viability is reduced by 50% compared with the controls; LD_{50}=Dose that produces lethality in 50% of the animals tested; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; RC=Registry of Cytotoxicity; 3T3=BALB/c murine fibroblasts; UN=United Nations.

The RC rat-only millimole regression was calculated using IC_{50} and rat oral LD_{50} values from 282 chemicals in the RC. Also shown are IC_{50} and rat oral LD_{50} values for chemicals tested with NRU.

Figure 2

RC Rat-only Weight Regression

- RC IC_{50}/rat oral LD_{50}; n = 282
- Log LD_{50} = 0.372 log IC_{50} + 2.024; R^2 = 0.325
- 3T3 NRU IC_{50}/reference rat oral LD_{50}; n = 67
- NHK NRU IC_{50}/reference rat oral LD_{50}; n = 68

Abbreviations: IC_{50}=Concentration at which cell viability is reduced by 50% compared with the controls; LD_{50}=Dose that produces lethality in 50% of the animals tested; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; RC=Registry of Cytotoxicity; 3T3=BALB/c murine fibroblasts.

The RC rat-only weight regression was calculated using IC_{50} and rat oral LD_{50} values from 282 chemicals in the RC. Also shown are IC_{50} and rat oral LD_{50} values for chemicals tested with NRU.

Table 2

Prediction of GHS Acute Oral Hazard Category by the 3T3 and NHK NRU Test Methods and the RC Rat-Only Millimole Regression[1]

Reference Rat Oral LD_{50}[2] (mg/kg)	3T3 NRU - Predicted Hazard Category (mg/kg)						Total[3]	Accuracy	Hazard Over-predicted	Hazard Under-predicted
	LD_{50} < 5	5 < LD_{50} ≤ 50	50 < LD_{50} ≤ 300	300 < LD_{50} ≤ 2000	2000 < LD_{50} ≤ 5000	LD_{50} > 5000				
LD_{50} <5	0	2	0	4	0	0	6	0%	0%	100%
5 < LD_{50} ≤50	0	1	6	3	1	0	11	9%	0%	91%
50 < LD_{50} ≤300	0	0	5	7	0	0	12	42%	0%	58%
300 < LD_{50} ≤2000	0	1	2	13	0	0	16	81%	19%	0%
2000 < LD_{50} ≤5000	0	0	0	10	0	0	10	0%	100%	0%
LD_{50} >5000	0	0	0	8	2	2	12	17%	83%	0%
Total	0	4	13	45	3	2	67	31%	34%	34%
Predictivity	0%	25%	38%	29%	0%	100%				
Category Overpredicted	0%	25%	15%	40%	67%	0%				
Category Underpredicted	0%	50%	46%	31%	33%	0%				

Reference Rat Oral LD_{50}[2] (mg/kg)	NHK NRU - Predicted Hazard Category (mg/kg)						Total[3]	Accuracy	Hazard Over-predicted	Hazard Under-predicted
	LD_{50} < 5	5 < LD_{50} ≤ 50	50 < LD_{50} ≤ 300	300 < LD_{50} ≤ 2000	2000 < LD_{50} ≤ 5000	LD_{50} > 5000				
LD_{50} <5	0	1	2	3	0	0	6	0%	0%	100%
5 < LD_{50} ≤50	0	2	5	3	1	0	11	18%	0%	82%
50 < LD_{50} ≤300	0	1	6	5	0	0	12	50%	8%	42%
300 < LD_{50} ≤2000	0	1	2	12	1	0	16	75%	19%	6%
2000 < LD_{50} ≤5000	0	0	0	10	0	0	10	0%	100%	0%
LD_{50} >5000	0	0	0	7	6	0	13	0%	100%	0%
Total	0	5	15	40	8	0	68	29%	40%	31%
Predictivity	0%	40%	40%	30%	0%	0%				
Category Overpredicted	0%	40%	13%	43%	75%	0%				
Category Underpredicted	0%	20%	47%	28%	25%	0%				

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); IC_{50}=Concentration at which cell viability is reduced by 50% compared with the controls; LD_{50}=Dose that produces lethality in 50% of the animals tested; NRU=Neutral red uptake; RC=Registry of Cytotoxicity; 3T3=BALB/c murine fibroblasts; UN=United Nations.
[1]The RC rat-only millimole regression is: log LD_{50} (mmol/kg) = log IC_{50} (mM) x 0.439 + 0.621. Numbers in table represent numbers of substances. Shaded cells are those containing the correct predictions.
[2]Reference rat oral LD_{50} values in mg/kg from geometric means of acceptable values identified during literature searches.
[3]Number of chemicals that (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

Table 3

Prediction of GHS Acute Oral Hazard Category by the 3T3 and NHK NRU Test Methods and the RC Rat-Only Weight Regression[1]

Reference Rat Oral LD_{50}[2] (mg/kg)	3T3 NRU - Predicted Hazard Category (mg/kg)						Total[3]	Accuracy	Hazard Over-predicted	Hazard Under-predicted
	LD_{50} < 5	5 < LD_{50} ≤ 50	50 < LD_{50} ≤ 300	300 < LD_{50} ≤ 2000	2000 < LD_{50} ≤ 5000	LD_{50} > 5000				
LD_{50} <5	0	0	2	4	0	0	6	0%	0%	100%
5 < LD_{50} ≤50	0	1	5	5	0	0	11	9%	0%	91%
50 < LD_{50} ≤300	0	0	4	8	0	0	12	33%	0%	67%
300 < LD_{50} ≤2000	0	1	3	12	0	0	16	75%	25%	0%
2000 < LD_{50} ≤5000	0	0	0	6	4	0	10	40%	60%	0%
LD_{50} >5000	0	0	0	5	7	0	12	0%	100%	0%
Total	0	2	14	40	11	0	67	31%	33%	36%
Predictivity	0%	50%	29%	30%	36%	0%				
Category Overpredicted	0%	50%	21%	28%	64%	0%				
Category Underpredicted	0%	0%	50%	43%	0%	0%				

Reference Rat Oral LD_{50}[2] (mg/kg)	NHK NRU - Predicted Hazard Category (mg/kg)						Total[3]	Accuracy	Hazard Over-predicted	Hazard Under-predicted
	LD_{50} < 5	5 < LD_{50} ≤ 50	50 < LD_{50} ≤ 300	300 < LD_{50} ≤ 2000	2000 < LD_{50} ≤ 5000	LD_{50} > 5000				
LD_{50} <5	0	1	2	3	0	0	6	0%	0%	100%
5 < LD_{50} ≤50	0	1	5	5	0	0	11	9%	0%	91%
50 < LD_{50} ≤300	0	1	5	6	0	0	12	42%	8%	50%
300 < LD_{50} ≤2000	0	1	2	13	0	0	16	81%	19%	0%
2000 < LD_{50} ≤5000	0	0	0	9	1	0	10	10%	90%	0%
LD_{50} >5000	0	0	0	6	6	1	13	8%	92%	0%
Total	0	4	14	42	7	1	68	31%	37%	32%
Predictivity	0%	25%	36%	31%	14%	100%				
Category Overpredicted	0%	50%	14%	36%	86%	0%				
Category Underpredicted	0%	25%	50%	33%	0%	0%				

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); IC_{50}=Concentration at which cell viability is reduced by 50% compared with the controls; LD_{50}=Dose that produces lethality in 50% of the animals tested; NRU=Neutral red uptake; RC=Registry of Cytotoxicity; 3T3=BALB/c murine fibroblasts; UN=United Nations.
[1]The RC rat-only weight regression is: log LD_{50} (mg/kg) = log IC_{50} (µg/mL) x 0.372 + 2.024. Numbers in table represent numbers of substances. Shaded cells are those containing the correct predictions.
[2]Reference rat oral LD_{50} values in mg/kg from geometric means of acceptable values identified during literature searches.
[3]Number of chemicals that (a) yielded IC_{50} values and (b) were associated with rat oral LD_{50} values.

Table 4

Prediction of GHS Acute Oral Hazard Category by the RC IC_{50} Values and the RC Regression[1]

In Vivo Rodent Oral LD_{50}[2] (mg/kg)	IC_{50}-Predicted Hazard Category (mg/kg)[3]						Total[3]	Accuracy	Hazard Over-predicted	Hazard Under-predicted
	LD_{50} < 5	5 < LD_{50} ≤ 50	50 < LD_{50} ≤ 300	300 < LD_{50} ≤ 2000	2000 < LD_{50} ≤ 5000	LD_{50} > 5000				
LD_{50} <5	0	5	3	4	0	0	12	0%	0%	100%
5 < LD_{50} ≤50	0	4	13	9	0	0	26	15%	0%	85%
50 < LD_{50} ≤300	0	9	20	38	2	0	69	29%	13%	58%
300 < LD_{50} ≤2000	0	4	24	97	14	1	140	69%	20%	11%
2000 < LD_{50} ≤5000	0	1	5	36	14	0	56	25%	75%	0%
LD_{50} >5000	0	0	1	19	19	5	44	11%	89%	0%
Total	0	23	66	203	49	6	347	40%	34%	26%
Predictivity	0%	17%	30%	48%	29%	83%				
Category Overpredicted	0%	61%	45%	27%	39%	0%				
Category Underpredicted	0%	22%	24%	25%	33%	17%				

Abbreviations: GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); IC_{50}=Concentration at which cell viability is reduced by 50% compared with the controls; LD_{50}=Dose that produces lethality in 50% of the animals tested; NRU=Neutral red uptake; RC=Registry of Cytotoxicity; 3T3=BALB/c murine fibroblasts; UN=United Nations.
[1]The RC regression is: log LD_{50} (mmol/kg) = log IC_{50} (mM) x 0.435 + 0.625. Numbers in table represent numbers of substances. Shaded cells are those containing the correct predictions.
[2]Rat (282 values) and mouse (65 values) oral LD_{50} values, mostly from the 1983/84 RTECS® (Halle 2003).
[3]IC_{50} values from the RC are geometric mean IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints (Halle 2003).

Conclusions

- The overall accuracy for GHS acute oral toxicity hazard category predictions for the 67 (3T3 NRU) or 68 (NHK NRU) chemicals when using the NRU IC_{50} values ranged from 29% to 31% for both RC rat-only millimole and RC rat-only weight regressions (**Tables 2** and **3**).

  - These low accuracy values may be due to the inability of *in vitro* cell cultures to mimic the kinetics and dynamics (e.g., metabolism; receptor-specific mediated effects) of the substances in an *in vivo* system.

  - This overall accuracy is deemed inadequate for this method to be used for regulatory hazard classification (**Tables 2** and **3**). Additional *in vitro* tests in a test battery will be necessary to improve the accuracy.

- However, with the 3T3 millimolar approach, the proportion of *in vivo* GHS category matches for all substances with *in vitro* predictions in the nontoxic category (LD_{50} > 5000 mg/kg) was 100% (two substances; see **Table 2**). With the NHK weight approach, the proportion was 100% (one substance; see **Table 3**). This suggests that acute oral toxicity testing for such chemicals could proceed directly to the limit test and thus reduce the number of animals tested. However, a larger database is necessary to adequately validate this approach.

- The accuracy for the RC IC_{50} values predicting the LD_{50} values in the RC regression was 40% (**Table 4**). The RC IC_{50} values are the average of values obtained from several different cell lines and this issue precludes using the data as a benchmark for comparison.

References

Halle W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD_{50}) zur Einsparung von Tierversuchen. Life Sciences/ Lebenswissenschaften, Volume 1, Jülich: Forschungszentrum Jülich.

Halle W. 2003. The Registry of Cytotoxicity: Toxicity testing in cell cultures to predict acute toxicity (LD_{50}) and to reduce testing in animals. Altern Lab Anim 31:89-198. (*English translation of Halle 1998*)

ICCVAM. 2001a. Report Of The International Workshop On *In Vitro* Methods For Assessing Acute Systemic Toxicity. NIH Publication No. 01-4499. Research Triangle Park, NC: National Institute for Environmental Health Sciences. http://iccvam.niehs.nih.gov/

ICCVAM. 2001b. Guidance Document On Using *In Vitro* Data To Estimate *In Vivo* Starting Doses For Acute Toxicity. NIH Publication No. 01-4500. Research Triangle Park, NC: National Institute for Environmental Health Sciences. http://iccvam.niehs.nih.gov/

ICCVAM. 2006. ICCVAM Test Method Evaluation Report (TMER): *In Vitro* Cytotoxicity Test Methods for Estimating Starting Doses For Acute Oral Systemic Toxicity Testing. NIH Publication No. 07-4518. Research Triangle Park, NC: National Institute for Environmental Health Sciences. http://iccvam.niehs.nih.gov/

Spielmann H, Genschow E, Liebsch M, Halle W. 1999. Determination of the starting dose for acute oral toxicity (LD_{50}) testing in the up and down procedure (UDP) from cytotoxicity data. Altern Lab Anim 27:957-966.

UN. 2005. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), First Revised Edition. [ST/SG/AC.10/30/Rev.1]. New York and Geneva:United Nations. http://www.unece.org/trans/danger/publi/ghs/ghs_rev01/01files_e.html

Acknowledgements