The Use of Mouse Fibroblast (3T3) and Normal Human Epidermal Keratinocyes (NHK) Cytotoxicity Assays for Estimating Acute Oral Toxicity of Formulations and Mixtures.

JM Charles[1], JA Strickland[1], MW Paris[1], RR Tice[2], WS Stokes[2].

[1]Integrated Laboratory Systems, Inc., Research Triangle Park, North Carolina, USA; [2]National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), NIEHS/NIH/DHHS, Research Triangle Park, North Carolina, USA.

Abstract

The Registry of Cytotoxicity (RC), which consists of *in vivo* acute oral toxicity data from rats and mice and *in vitro* cytotoxicity data from multiple mammalian cell lines for 347 chemicals, was published in 1998 (Halle 1998). A regression model constructed from these data was proposed by ZEBET, the German National Center for the Documentation and Evaluation of Alternative Methods to Animal Experiments, as a method to reduce animal use by identifying the most appropriate starting doses for acute oral systemic toxicity tests (Halle 1998; Spielmann et al. 1999). The regression line, however, presents LD_{50} data in mmoles/kg body weight making it unsuitable for formulations and mixtures. NICEATM/ECVAM has performed a validation study to evaluate 3T3 and NHK cells in basal cytotoxicity assays with a neutral red uptake (NRU) cell viability endpoint to predict starting doses for the acute oral Up-and-Down Procedure (UDP). Linear regression models were determined using the log rat LD_{50} (mg/kg) versus the log IC_{50} (µg/mL) for the 55 RC chemicals evaluated in this study. This regression line was compared to a similarly derived regression utilizing the RC dataset for these chemicals and was found to be in agreement. A review of the NICEATM/ECVAM data and the RC data confirms that those chemicals with known mechanisms of toxicity (i.e., cholinesterase inhibitors, etc.) or those requiring metabolic activation are often discordant with the model. By removing these chemicals from the analysis, a newly derived linear regression utilizing the RC dataset of rat LD_{50} values (285/347 chemicals) in mg/kg body weight may be suitable for estimating the starting doses for rat LD_{50} determinations for formulations and mixtures using the UDP. A similar model can be derived for the mouse utilizing the RC dataset (242/347 chemicals). Supported by NIEHS contract N01-ES-35504.

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 vitro basal cytotoxicity test methods are not currently suitable as replacements for acute oral systemic toxicity assays; however, such test methods have been evaluated as a means to reduce[1] and refine[2] the use of the animals in toxicity testing. The Registry of Cytotoxicity (RC), a database of 347 substances that currently consists of *in vivo* acute toxicity data from rats and mice and *in vitro* cytotoxicity data from multiple cell lines, was published in 1998 (Halle 1998). A regression formula, hereafter referred to as the RC regression, constructed from these data was proposed by ZEBET, the German National Center for the Documentation and Evaluation of Alternative Methods to Animal Experiments, as a method to reduce animal use by identifying the most appropriate starting doses for acute oral systemic toxicity tests (Halle 1998; Spielmann et al. 1999).

This is one of three abstracts describing the results of a validation study conducted in three laboratories to determine the usefulness and limitations of two *in vitro* basal cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity assays, and to determine the extent that the use of cytotoxicity data might reduce the total number of animals used for *in vivo* tests. Abstract 1970 reports the reproducibility of these *in vivo* tests and abstract 1968 characterizes the reduction of animal use. The validation study was organized by the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Center for the Validation of Alternative Methods (ECVAM) and is hereafter referred to as the NICEATM/ECVAM validation study.

This poster describes improvements to the linear regression models developed to enhance the prediction of the most appropriate starting dose for acute oral systemic toxicity tests.

[1]Reduction alternative: A new or modified test method that reduces the number of animals required.

[2]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.

Comparison of 3T3/NHK Regressions to the RC Regression

The NICEATM/ECVAM validation study tested 58 RC chemicals. Since the regressions generated by the three laboratories were not statistically different from one another, the data were combined (using a geometric mean IC_{50} of the three individual laboratory geometric mean IC_{50} values) into a single regression for each test method (3T3 and NHK). A graphical comparison of the RC regression for the 58 chemicals to the 3T3 and NHK combined-laboratory regressions is shown in **Figure 1**. A statistical comparison of slope and intercept (simultaneously) using an F test showed that neither the 3T3 regression (p = 0.929) nor the NHK regression (p = 0.144) was different from the 58 RC chemical regression.

Improving the Prediction of *In Vivo* Rat LD_{50} Values From *In Vitro* NRU IC_{50} Data

Since the RC and the 3T3 and NHK NRU IC_{50} – rodent acute oral LD_{50} regressions were not significantly different, the next step was an attempt to improve upon the RC millimole regression for the prediction of LD_{50} values from IC_{50} values. The RC data were used to develop three new regressions. For reference, the original RC regression (in millimoles) log (LD_{50}[mmol/kg]) = 0.435 x log (IC_{50x} [mM]) + 0.625 (Halle 1998) is shown in **Figure 2a**.

The RC Rat-Only Regression

The first regression used the RC data only for the 282 chemicals with rat LD_{50} data (i.e., the regression excluded the chemicals with mouse LD_{50} data) using the original units of mM for IC_{50} and mmol/kg for LD_{50} **Figure 2b**. Rat data only were used because:

- rats and mice may not have the same sensitivity to individual chemicals
- the majority of LD_{50} data used in the RC regression were from studies using rats (282 values versus 65 mouse values) (Halle 1998)
- the vast majority of acute oral systemic toxicity testing is performed with rats

Table 1 shows that the regression using rat LD_{50} data only was almost identical to the original RC regression which used both rat and mouse LD_{50} data.

The RC Rat-Only Regression

The second regression used the same RC data for the 282 chemicals with rat LD_{50} data, but was calculated with weight units rather than millimolar units (see **Table 1** and **Figure 3a**). Weight units (i.e., mg/kg for the LD_{50} and µg/mL for the IC_{50}) were selected for the units of measurement because

- millimole units are not applicable to mixtures and unknown substances
- they are more practical (i.e., in all regulatory systems, hazard classification is based on LD_{50} values expressed in mg/kg)

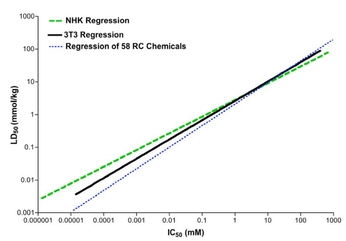
The Modified RC Regression

The third regression refined the weight-unit regression by excluding chemicals for which the mechanisms of toxic action were not expected to be active in the 3T3 and NHK cell cultures. Mechanism of action data for the 282 RC chemicals with rat LD_{50} values were obtained from summary sources (i.e., toxicology textbook and publicly available chemical databases). Examination of the RC rat database revealed the following items:

- 26% (73/282) of the chemicals were outliers (i.e., log observed – log predicted LD_{50} > 0.699 as defined for the RC millimole regression)
- for 55% (40/73) of the chemicals, *in vivo* toxicity was underpredicted; for 45% (33/73) of the chemicals, *in vivo* toxicity was overpredicted
- all underpredicted chemicals were very toxic, with $LD_{50} \leq 200$ mg/kg
- the discordant status of 65% (26/40) of the underpredicted chemicals could be explained by four general mechanisms
  - neurotoxic (i.e., cholinesterase inhibitor, affects CNS nicotinic receptor, or otherwise neurotoxic by a mechanism other than membrane destabilization such as that produced by a solvent)
  - interferes with energy utilization (i.e., interferes with ATP synthesis, inhibits ADP phosphorylation, or uncouples oxidative phosphorylation, or is a metabolic poison)
  - cardiotoxic via specific mechanisms (i.e., positive inotropic action, or calcium channel blocker)
  - alkylates cellular proteins and other macromolecules (i.e., covalently binds to enzymes and other proteins to disrupt normal function)

Such chemicals would not be expected to exert their toxic mechanisms in 3T3 and NHK cells and thus, they would be expected to poorly fit the RC millimole regression, as evidenced by their discordant status. A new regression was calculated after the exclusion of 50 chemicals in the RC database known to act by these four mechanisms (see **Table 1** and **Figure 3b**); this included the 26 underpredicted chemicals and 24 other chemicals that were not identified as outliers (see **Table 2**). The third regression was significantly different (p < 0.05) from the RC rat-only weight regression when slopes and intercepts were simultaneously compared (F test; p = 0.0063).

Figure 1. Regression for the 58 RC Chemicals with the 3T3 and NHK NRU Regressions



Regression for the 58 RC chemicals using RC data is log LD_{50} = 0.659 log IC_{50} + 0.323 (adjusted R^2 = 0.683). The combined-laboratory 3T3 NRU regression is log LD_{50} = 0.589 log IC_{50} + 0.425 (adjusted R^2 = 0.524). The combined-laboratory NHK NRU regression is log LD_{50} = 0.510 log IC_{50} + 0.452 (adjusted R^2 = 0.455). These regressions are not significantly different at p<0.05.

Figure 2. RC Regression (a) and RC Rat Regression (b) Using Millimole Units

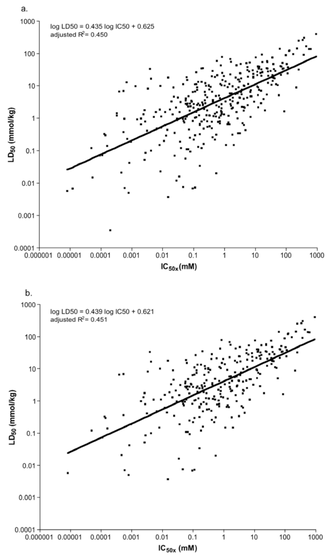


Table 1. Linear Regression Analyses to Improve the Prediction of Rodent LD_{50} from *In Vitro* NRU IC_{50} Using the RC Regression[a]

Data Used	Slope	Intercept	Adjusted R^2
347 RC chemicals with rat and mouse LD_{50} data – millimole units[b]	0.435	0.625	0.450[d]
282 RC chemicals – with rat LD_{50} data – millimole units[b]	0.439	0.621	0.451
282 RC chemicals with rat LD_{50} data – weight units[b]	0.372	2.024	0.322
232 RC chemicals with rat LD_{50} data (excluded 50 chemicals with specific mechanisms of action) – weight units[b]	0.357	2.194	0.353

[a]Slopes of all regressions were significantly different (p < 0.05) from zero at p < 0.0001.
[b]Simultaneous comparison of slopes and intercepts using an F test. Significance denoted by p < 0.05.
[c]IC_{50} in mM; LD_{50} in mmol/kg.
[d]Calculated from RC data (i.e., not reported by Halle [1998]).
[e]IC_{50} in µg/mL; LD_{50} in mg/kg.
[f]See the text for the applicable mechanisms and **Table 2** for the identified chemicals.

Figure 3. RC Rat-Only Regression (a) and RC Rat-Only Regression Excluding 50 Chemicals with Specific Mechanisms of Toxicity (b) Using Weight Units

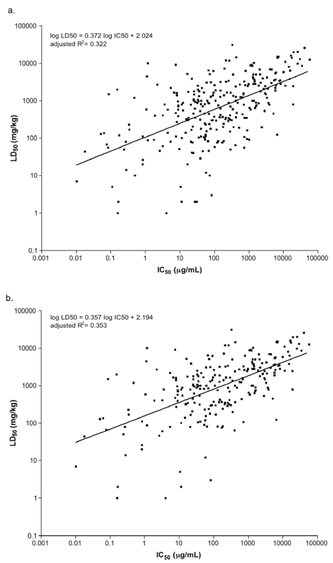


Table 2. Excluded Chemicals with Specific Mechanisms of Action

RC No	Chemical	CASRN	Outlier Status[f]	IC_{50} µg/mL[e]	LD_{50} mg/kg[e]	Predicted LD_{50} mg/kg[e]	Toxicity Mechanism[g]
51	Disulfoton	298-04-4	Neg	30.19	2	443	Cholinesterase inhibitor
49	Parathion	56-38-2	Neg	27.09	2	437	Cholinesterase inhibitor
137	Triethyltin chloride	994-31-0	Neg	0.11	5	36	Neurotoxic
252	Potassium cyanide	151-50-8	Neg	72.93	10	288	General enzyme inhibition. Interferes with ATP synthesis. High affinity for Fe+++; inhibits cell respiration by inhibition of cytochrome oxidase.
148	Nitrogen mustard * HCl	55-86-7	Neg	0.50	10	61	Alkylating agent
192	1,3-Bis(2-chloroethyl)- 1-nitrosourea	154-93-8	Neg	16.70	20	298	Alkylating agent
68	2,4-Dinitrophenol	51-28-5	Neg	38.67	29	394	Interferes with ATP synthesis
43	Aldrin	309-00-2	Neg	24.45	40	475	Neurotoxic
185	Heptachlor	76-44-8	Neg	22.02	41	460	Neurotoxic
241	Sodium azide	26628-22-8	Neg	46.16	45	236	Interferes with ATP synthesis (like potassium cyanide)
207	Dieldrin	60-57-1	Neg	68.56	46	762	Neurotoxic
103	Nicotine	54-11-5	Neg	290.45	50	881	CNS nicotinic receptor; cholinergic block causing polarization of CNS and PNS synapses
173	Pentachlorophenol	87-86-5	Neg	9.59	51	264	Uncouples oxidative phosphorylation
262	Amphetamine sulfate	60-13-9	Neg	726.02	55	2087	Neurotoxic
8	Digitoxin	71-63-6		0.08	56	61	Cardiotoxic; neurotoxic; causes hyperkalemia
235	Paraquat	4685-14-7	Neg	100.58	58	601	Multisystem failure due to depletion of superoxide dismutase, with formation of free radicals & lipid peroxidation. Lung fibrosis due to myofibroblast proliferation; interferes with ATP synthesis.
223	Lindane	58-89-9	Neg	119.24	76	832	CNS depression through inhibition of GABA receptor linked chloride channel at the picrotoxin binding site, leading to blockade of chloride influx into neurons?
190	Chlorambucil	305-03-3	Neg	23.12	76	418	Alkylating agent
220	m-Dinitrobenzene	99-65-0	Neg	65.57	82	471	Interferes with ATP synthesis
160	N-Methyl-N'-nitro-N-nitroso-guanidine	70-25-7		1.77	90	91	Alkylating agent; teratogen; affects liver
283	Mitomycin	78415-72-2	Neg	1007.61	91	1758	Cardiac-positive ionotropic action
116	Cyclophosphamide * H2O	6055-19-2 50-18-0	Neg	870.89	95	1931	Alkylating agent
217	Amrinone	60719-84-8		52.42	101	454	Cardiac drug; calcium channel blocker
196	VerapamilHCl	152-11-4	Neg	49.11	108	761	Inhibition of transmembrane Ca++ flux in excitatory tissues. Cardiac-Ca++ channel blocker. Also alpha-adrenergic blockade.
167	p,p'-DDD	72-54-8		7.68	112	266	Carcinogenic; neurotoxic
61	p,p'-DDT	50-29-3	Neg	56.72	113	674	Inhibits ADP phosphorylation
138	Tributyltin chloride	1461-22-9		0.18	120	52	Neurotoxic
69	Secobarbital sodium	309-43-3		54.66	125	557	Affects respiratory functions; CNS depression; Enhancement of inhibition occurs primarily at synapses where neurotransmission is mediated by GABA acting at GABAa receptors
27	Chlorpromazine	50-53-3		4.46	140	210	Alpha-adrenergic antagonistic activity; CNS depression
96	Cygon	60-51-5	Neg	284.29	151	1062	Inhibits cholinesterase
118	Phenobarbital	50-06-6	Neg	884.91	163	1753	Neurotoxic; CNS depression through Inhibition of GABA synapses? Inhibits hepatic NADH cytochrome oxidoreductase;
64	Bendiocarb	22781-23-3		40.19	179	447	Cholinesterase inhibitor
112	Caffeine	58-08-2	Neg	512.74	192	1249	Inhibition of phosphodiesterase leading to AMP accumulation. Translocation of intracellular Ca++? Adenosine receptor antagonism?; neurotoxic
318	Trifluoroacetic acid	76-05-1	Neg	2337.62	200	1789	Metabolic poison
87	Pentobarbital sodium	57-33-0		176.29	201	902	Affects respiratory functions
73	Carbaryl	63-25-2		52.32	250	472	Cholinesterase inhibitor
38	Imipramine * HCl	113-52-0		17.11	304	375	Antidepressant; affects parasympathetic nervous system, CNS and cardiovascular system
282	(-)-Phenylephrine	59-42-7		744.17	350	1350	CNS stimulant; alpha-receptor stimulant
278	Phenylephrine * HCl	61-76-7		847.35	350	1597	CNS stimulant; alpha-receptor stimulant
75	Trichlorfon	52-68-6		69.51	451	614	carcinogenic; inhibits cholinesterase- (its entire anticholinesterase action is the result of its nonenzymatic conversion to form dichlorvos.)
53	Quinidine sulfate	50-54-4		50.70	456	708	myocardial depressant; anticholinergic properties
264	Chloral hydrate	302-17-0		438.31	480	1066	potentiation of $GABA_A$ receptor activity, inhibition of Na+/K+ ATPase activity, & modulation of 5-hydroxytryptamine, receptor-mediated depolarization of the vagas nerve; CNS Depressant
26	Kelthane	115-32-2		4.45	574	228	Inhibits the ATPase associated with oxidative phosphorylation & cation transport in plasma membranes.
101	Glutethimide	77-21-4		338.97	600	1112	CNS depression; anticholinergic activity
70	Atropine sulfate	55-48-1		148.92	623	1477	Antimuscarinic, anticholinergic action. Competitive antagonism of anticholinesterase at cardiac & CNS muscarinic
63	Diazepam	439-14-5		45.56	709	541	Causes potentiation of the neural inhibition that is mediated by GABA; CNS depressant
114	Natulan * HCl	366-70-1		706.37	784	1685	Alkylating agent; inhibits DNA, RNA, and protein synthesis; antineoplastic agent
285	Caffeine sodium benzoate	8000-95-1		1918.33	859	3035	CNS stimulant; Hypothetical: Inhibition of phosphodiesterase leading to AMP accumulation. Translocation of intracellular Ca++? Adenosine receptor antagonism?
67	Malathion	121-75-5		66.08	885	692	Cholinesterase inhibitor
251	Scopolamine * HBr	6533-68-2 114-49-8		415.05	1268	1676	CNS depressant; antagonization of acetylcholine at the muscarinic receptor; belladona alkaloid

Chemicals for which the primary mechanism of toxicity is expected to require specialized cells; includes alkylating agents, cholinesterase inhibitors, neurotoxins, energy inhibitors, and cardiotoxic compounds.

[f]Chemicals that fall outside of the ± log 5 acceptance interval; outlier determination: if log of observed LD50 value (in mmol/kg) minus log of predicted LD50 value > 0.699 (or log 5), then positive (Pos) outlier; if < -0.699 (or -log 5) then negative (Neg) outlier

[g]IC50 value from the RC; converted from mmol/L to µg/mL

[h]LD50 value from the RC; collected from ECB4 RTECS

[i]calculated from the RC regression: log (LD50) = 0.435 x log (IC50x) + 0.625; converted from mmol/kg to mg/kg.

[j]Information obtained from databases (Hazardous Substance Database [HSDB]; Haz-Map; Pesticide Action Network [PAN] Pesticides Database; IPCS INTOX Database) and Casarett & Doull's Toxicology (2001)

[k]Identification made by RC author (Halle 2003).

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

Table 3. Comparison of Regressions and *In Vitro* NRU Test Methods for Performance in Predicting GHS[a] Toxicity Categories

Regression	N[b]	Accuracy
RC –millimole units	46 47	3T3 – 26% NHK – 28%
Modified RC (rat only excluding chemicals with specific mechanisms of action) –weight units	46 47	3T3 – 46% NHK – 38%

[a]Globally Harmonized System of Classification and Labelling of Chemicals with LD_{50} in mg/kg (UN 2005).
[b]Number of chemicals evaluated

Conclusions

- Substances with specific toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic, cardiotoxic, interfere with energy utilization, or alkylate proteins and other macromolecules) will likely be underpredicted by these *in vitro* basal cytotoxicity test methods. Therefore, the results from basal cytotoxicity testing with such substances may not be appropriate.
- The regression formula to use for determining starting doses should be the revised Registry of Cytotoxicity (RC) regression line [with IC_{50} values in g/mL and LD_{50} values in mg/kg] developed with the RC chemicals using rat LD_{50} data only and excluding chemicals with mechanisms of action that are not expected to be active in *in vitro* basal cytotoxicity test methods.
- This revised regression can be applied to rat oral LD_{50} testing of mixtures and unknowns where the molecular weight is not applicable or not known, respectively.
- The revised RC regression improves the regression's ability to predict Globally Harmonized System (GHS) Classification Toxicity Categories utilizing the 3T3 NRU assay from 26% to 46% and the NHK NRU Assay from 28% to 38% (see **Table 3**)

References

Canadian Centre for Occupational Health and Safety. 2005. IPCS INTOX Database. Available: http://www.intox.org/databank/index.htm. [accessed 29 November 2005].

Halle W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD_{50}) zur Einsparung von Tierversuchen. Life Sciences/ Lebenswissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich. English translation: 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 Laboratory Anim 31:89-198.

Klaassen CD, ed. 2001. Casarett and Doull's Toxicology, The Basic Science of Poisons. 6th Edition. New York:McGraw-Hill.

National Library of Medicine (NLM). 2005. Haz-Map, Occupational Exposure to Hazardous Agents, Specialized Information Services, National Institutes of Health, Department of Health & Human Services. Available: http://hazmap.nlm.nih.gov/. [accessed 29 November 2005].

National Library of Medicine. 2000, 2001, 2002, 2005. Hazardous Substances Data Bank. Accessed online via TOXNET. National Institutes of Health, Department of Health & Human Services. Available: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB. [accessed 2 June 2005].

Pesticide Action Network North America. 2005. Pesticide Action Network [PAN] Pesticides Database. Available: http://www.pesticideinfo.org/Index.html. [accessed 29 November 2005]

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 Laboratory Anim **27**:957-966.

Acknowledgements

This poster was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences. ILS staff supported by NIEHS contract N01-ES 35504. The views expressed above do not necessarily represent the official positions of any federal agency.

U.S. Department of Health and Human Services
National Institutes of Health
National Institute of Environmental Health Sciences