ESTIMATING SYSTEMIC TOXICITY IN VITRO USING AN ADENOSINE TRIPHOSPHATE CYTOTOXICITY (ATP) ASSAY IN NORMAL HUMAN EPIDERMAL KERATINOCYTES

Raabe, Hans A.[1]; Hilberer, Allison[1]; Kelly, Jeffrey[2]; Moyer, Gregory O.[1]; Powers, Mark[2]; Curren, Rodger D.[1],
[1]Institute for In Vitro Sciences, Inc. (IIVS), Gaithersburg, MD, USA. [2]Lonza, Walkersville, MD, USA.

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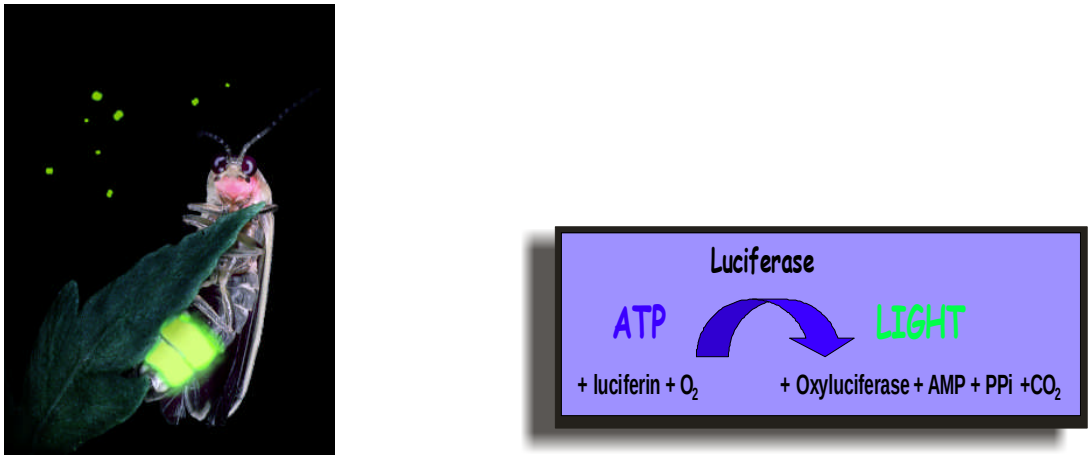
ABSTRACT

Ekwall et al. (ATLA 17:83-100, 1989) have proposed that ˜80% of chemical-induced systemic toxicity is the result of disruption of basic cellular processes common to most cell types in the body, and that systemic toxicity for many chemicals could be estimated in in vitro cultures. Strickland et al (The Toxicologist, Abs#761, 2003) reported on a joint European/USA validation to evaluate two cytotoxicity assays in NHEK and BALB/c 3T3 cells to predict acute systemic toxicity using a neutral red uptake (NRU) viability endpoint. We report on a two-lab validation study to evaluate the ATP viability endpoint using the optimized protocol from the aforementioned validation. Cytotoxicity was measured as a dose-dependent reduction in ATP from which an IC_{50} was determined. Initially, 20 chemicals from the Multicenter Evaluation of In Vitro Cytotoxicity (MEIC) program were tested to determine the relationship between the ATP IC50 values in vitro and the human LC_{50} values [log LC_{50} µM = 0.794(log IC_{50} µM)+0.176; r_2 = 0.887]. Subsequently, 50 chemicals from Strickland et al were tested to determine the relationship between the ATP IC_{50} values and the rodent oral LD_{50} values [log LD_{50} mmol/kg = 0.495 (log IC_{50} mM)+0.413; r_2 = 0.399] and is similar to the prediction model published in the Registry of Cytotoxicity (Halle, 1998). A high correlation (r_2 = 0.930) was demonstrated between ATP IC_{50} values and NRU_{50} values obtained in the cytotoxicity validation study. The results demonstrate that the NHEK assay with the ATP endpoint may be used to predict systemic toxicity, or rodent oral LD_{50} doses, and that the ATP endpoint is an acceptable alternative to the NRU endpoint.

INTRODUCTION

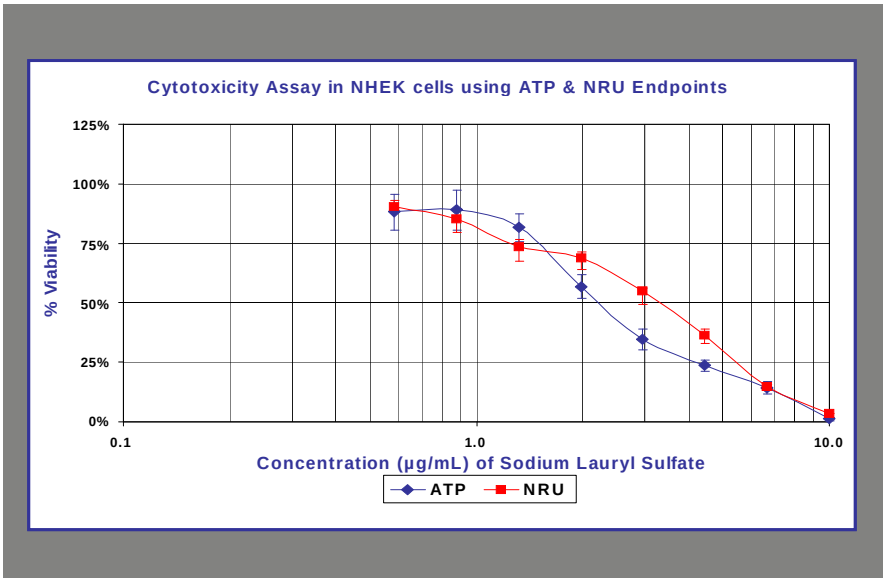
The adenosine triphosphate (ATP)-based cytotoxicity assay procedure is a cell survival/viability assay which utilizes the bioluminescent measurement of ATP present in metabolically active cells to assess cell viability (Crouch, et al., 1993). The ViaLight® Plus ATP assay is used to assess the viability of NHEK cultures treated with test chemicals according to the current protocol (Phase III) In Vitro Cytotoxicity Validation Study (ICCVAM, 2003). During the 48-hour treatment phase, untreated NHEKs typically divide and proliferate. Exposure to a toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate or viability as reflected by cell number. Since within each cell type there is a reasonably uniform quantity of ATP, the ATP assay provides a direct measure of the number of viable cells present. Cytotoxicity is expressed as a concentration-dependent reduction in the bioluminescent measurement of ATP after chemical exposure. The ATP assay takes less than 25 minutes to conduct whereas the NRU endpoint takes over 3.5 hours. The ATP assay also uses fewer, less complex steps making it ideal for robotic and high throughput screening systems.

Figure 1. The bioluminescent assay uses an enzyme (luciferase) which catalyzes the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the amount of ATP present.



Sodium lauryl sulfate (SLS) was used as the positive control. The current historic response range of 1.68 to 3.65 µg/mL is extremely similar to the range determined using the NRU endpoint (Fig. 2).

Figure 2. Positive control (SLS) dose response in NHEKs using ATP and NRU endpoints



In an initial study, 20 chemicals for which human lethal serum data were available [MEIC program (Ekwall et al., 1998)] were selected to represent a wide acute toxicity range. The results of this phase were used to develop the prediction equation to estimate human lethal serum LC_{50} values, and were compared to those reported previously using an NRU endpoint. In a subsequent study, 50 chemicals for which published rodent oral LD_{50} values were available were tested. The results were also compared to those reported previously using an NRU endpoint (Strickland et al. The Toxicologist, Abs#761, 2003). At least 3 trials for each chemical were tested in GLP compliance at IIVS and at Cambrex (Lonza).

MATERIALS AND METHODS

NHEK cells were cultured and treated with test chemicals according to the current protocol (Phase III) In Vitro Cytotoxicity Validation Study (ICCVAM, 2003). The ATP assay was used to replace the neutral red uptake endpoint according to the procedures described below.

Cells

Normal Human Epidermal Keratinocytes (NHEK), pooled neonatal (Lonza)

Chemicals

Test chemicals were of the highest purity available from Sigma Aldrich

Reagents

- Growth / Treatment Media: Keratinocyte Growth Medium without Ca^{++} (KGM, Lonza, CC-3104) supplemented with Calcium Chloride to 0.1 mM
- Dulbecco's Phosphate Buffered Saline (D-PBS) (Quality Biological, 114-059-101)
- Calcium and Magnesium-free Hanks' Balanced Salt Solution (CMF-HBSS) (Quality Biological, 114-052-101)
- HEPES Buffered Saline Solution (HEPES-BSS) (Lonza, CC-5022)
- Trypsin Neutralizing Solution (TNS) (Lonza, CC-5002)
- Trypsin/EDTA 0.025% (Lonza, CC-5012)
- Dimethylsulfoxide (DMSO) 99.9% HPLC Grade (Sigma Aldrich)
- ViaLight® Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza, LT27-102) includes lyophilized AMR PLUS Reagent, Assay Buffer, and Cell Lysis Reagent
- White-walled Clear-bottom 96-well Tissue Culture Plates (Lonza, LT27-102)

NHEK Culture

Freshly thawed NHEK cells were cultured in T25 flasks at 37 ± 1ºC in a humidified atmosphere containing 5 ± 1% CO_2 in air (standard culture conditions) until 50 to 80% confluent. Cultures were refed every 2 to 3 days with fresh warmed KGM.

NHEKs were subcultured into the inner 60 wells of the 96-well plates, and incubated until the cultures were approximately 20+% confluent.

Test Chemical Preparation

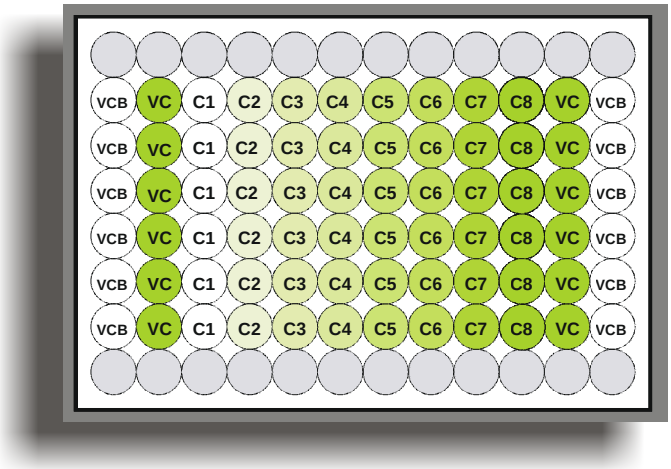
Fresh dosing solutions were prepared on the day of use

- The positive control (SLS) and the aqueous-soluble test chemicals were diluted directly in KGM, to prepare a series of 2X dosing solutions
- The remaining test chemicals were first dissolved and diluted in DMSO, and then transferred to KGM, to prepare the 2X dosing solutions series
- Dose range finding assays used eight dilutions with 1 log dilution intervals
- Definitive assays used eight dilutions with typically 1/3 to 1/4 log dilution intervals to address the full range of survival responses.

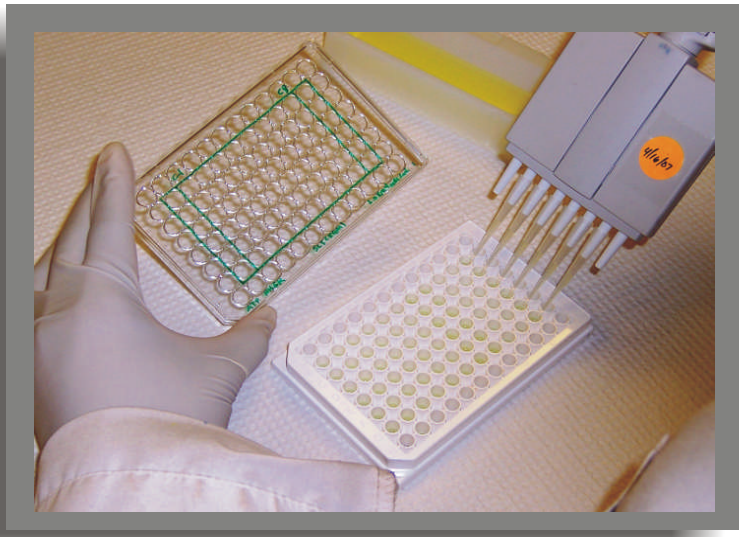
Test Chemical Treatment

The eight serial 2X dosing solutions were dispensed into the corresponding wells of 8-well reservoirs. Each test chemical 2X dosing solution was treated in six wells and incubated for 48 ± 0.5 hours at standard culture conditions.

Figure 3. 96-Well White-walled Clear Bottom Plate Configuration



VC = Vehicle Control
C1 – C8 = Test Chemicals at 8 concentrations
VCB = Vehicle Control Blanks (contain no cells)



Treatment Termination / ATP Determination

- After 48 ± 0.5 hours of exposure, the culture plates were removed from the incubator and allowed to cool to room temperature (RT) for at least 5 minutes.
- The dosing solutions were removed by inverting the plates.
- The wells were rinsed with 250 µL D-PBS. The rinsing solution was removed by inverting the plates and gently blotting on sterile absorbent paper.

- 50 µL of Cell Lysis Reagent were added to each well, and the plates were incubated for at least 10 minutes.
- 100 µL of AMR PLUS reagent were added to each well, and the plates were incubated for at least 2 minutes.
- The light emission (565 nm) of each well was measured using a Berthold Detection Systems Orion II luminometer. Data were recorded as relative light units (RLUs).

Presentation of Data

- Mean RLU_{565} values for the vehicle control-treated blank wells (VCB) were calculated
- Corrected RLU_{565} values for each culture well were calculated by subtracting the mean VCB RLU_{565} value from the individual RLU_{565} values
- The mean corrected RLU_{565} value for the vehicle control (VC) was calculated.
- Individual % of Control values were calculated for each culture well, by the following equation:

% of Control = (Individual corrected RLU_{565} / mean corrected VC RLU_{565}) X 100

- The mean % of Control values for each dose group were calculated
- Dose response curves were plotted presenting % of Control vs. test chemical concentration
- IC_{50} values were interpolated from the dose response curves

Criteria for Determination of a Valid Test

The bioassays were accepted when the positive control, SLS, induced an IC_{50} that fell within 2.5 standard deviations of the current historical mean established at IIVS (1.68 to 3.65 µg/mL).

RESULTS

ASSAY RELEVANCE

Mean IC_{50} values (in µg/mL) were calculated from at least 3 independent trials for each test chemical in both the initial human serum LC50 and the subsequent rodent oral LD_{50} studies. Molar equivalent IC_{50} values were calculated and are presented in Tables 1 and 2. Regression analyses were performed relating the in vitro log IC_{50} values to log molar human lethal serum LC_{50} values or rodent oral LD_{50} values.

Human Lethal Serum LC_{50} Prediction Study

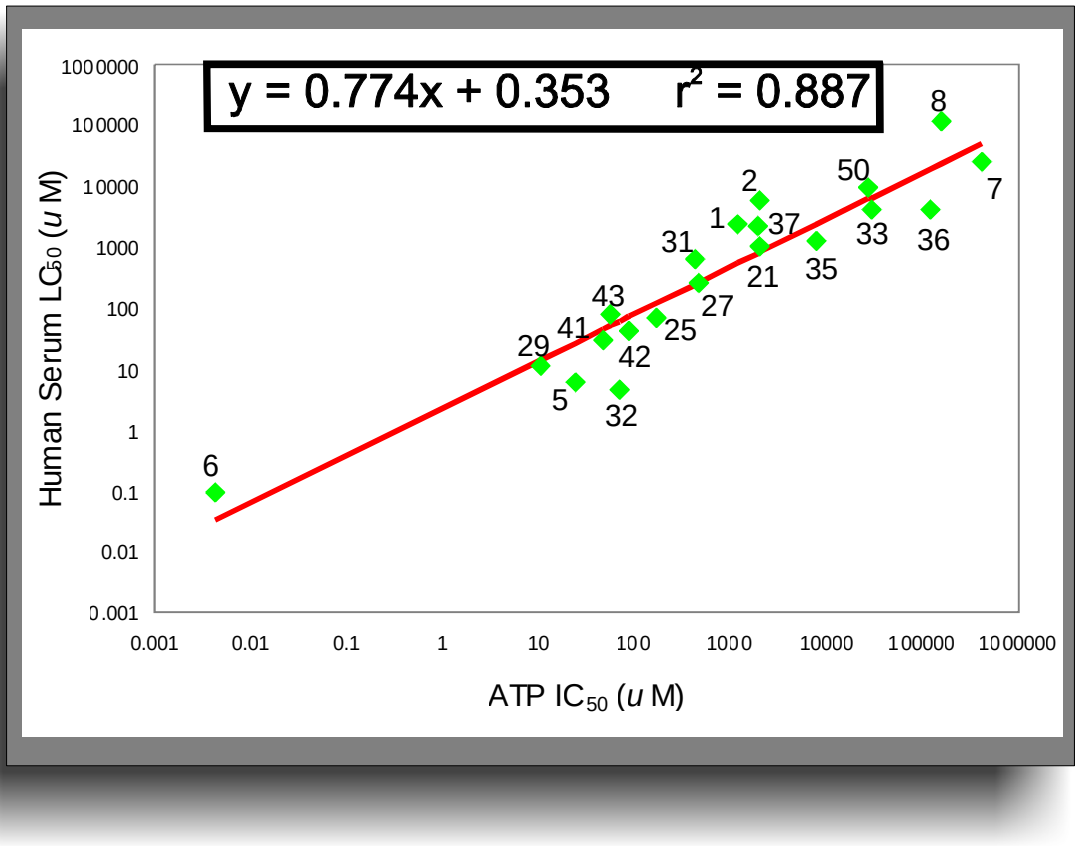
Table 1

MEIC #	Chemical	F.W.	IIVS NHEK IC_{50} (µM)	Cambrex NHEK IC_{50} (µM)	Human Serum LC_{50} (µM)
6	Digoxin	780.9	0.00424	0.00509	0.09
29	Thioridazine HCl	407	10.5		11
5	Amitriptyline	313.9	23.9	27.65	6
41	Chloroquine phosphate	515.9	46.5		29
43	Quinidine Sulfate	782.9	55.2		80
32	Lindane	290.83	68.5		4.5
42	Orphenadrine HCL	305.85	88.2		42
25	Paraquot	257.16	167		68
31	Warfarin	308.3	420	219.2	649
27	Cupric Sulfate	249.69	461	617	248
1	Acetaminophen	151.2	1189	1223	2368
37	Barium Nitrate	261.35	1920		2226
2	Acetylsalicylic Acid	180.2	1983	1650	5944
21	Theophyline	180.2	1990	1443	1000
35	Isoniazid	137.1	7910		1219
50	Potassium Chloride	74.55	26729		9615
33	Chloroform	119.4	28867	26159	4118
36	Dichloromethane	84.93	119027		4047
8	Ethanol	46.07	159768	147511	118069
7	Ethylene Glycol	62.07	410289	379974	24960

Molar equivalent in vitro IC_{50} values are presented in Table 1. The log of the in vitro IC_{50} values and the in vivo human lethal serum values from 20 chemicals from the MEIC program (Ekwall, 1998) were calculated. A regression analysis of

the in vitro log IC_{50} (µM) values obtained at IIVS and the reported log LC_{50} values was performed and is presented graphically in Fig. 4. The resulting relationship was log LC_{50} µM = 0.774(log IC_{50} µM) + 0.353 [r_2 = 0.887].

Figure 4. **ATP IC_{50} MEIC (20 Materials)**



Rodent Oral LD50 Prediction Study

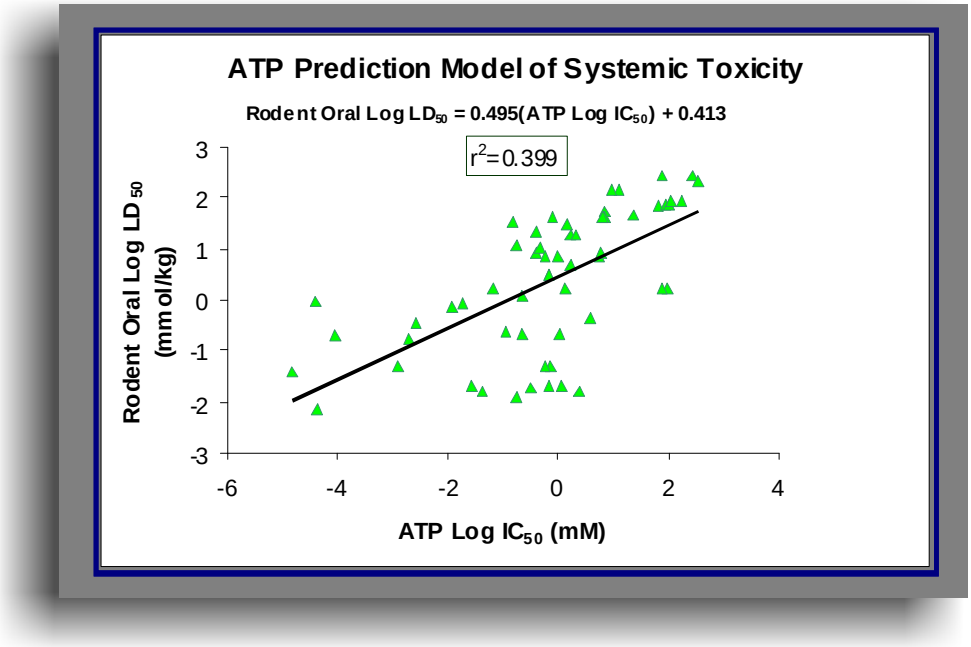
Molar equivalent in vitro IC_{50} values are presented in Table 2. The log of the in vitro IC_{50} values and the published rodent oral log LD_{50} (mmol/kg) values from 45 chemicals from the ICCVAM/ECVAM Phase III In Vitro Cytotoxicity Validation Study were calculated. A regression analysis of the ATP IC_{50} values in vitro and published rodent oral LD_{50} values was performed and is presented in Fig. 5. The resulting relationship was log LD_{50} (mmol/kg) = 0.495(log IC_{50} mM) + 0.413 [r_2 = 0.399].

Table 2.

MEIC Number	Chemical	Mean ATP IC_{50} (μM) IIVS	Mean ATP IC_{50} (μM) Cambrex	ATP Log IC_{50} (mM) IIVS	ATP Log IC_{50} (mM) Cambrex	NRU_{50} (μM)	Log NRU_{50} (mM)	Rodent Oral LD_{50} (mmol/kg)	Rodent Log Oral LD_{50} (mmol/kg)
43	Colchicine		0.015		-4.82	0.020	-4.70	0.038	-1.43
51	Triphenyltin Hydroxide	0.041		-4.39		0.028	-4.55	0.896	-0.047
31	Cycloheximide		0.043		-4.37	0.251	-3.60	0.007	-2.15
57	Hexachlorophene	0.0886	0.0743	-4.05	-4.13	0.052	-4.28	0.202	-0.696
53	Sodium Dichromate Dihydrate	1.93		-2.71		1.91	-2.72	0.169	-0.771
47	Thallium Sulfate	1.25		-2.90		0.251	-3.60	0.050	-1.31
49	Sodium (Meta) Arsenite	2.68		-2.57		3.63	-2.44	0.336	-0.474
59	Cadmium Chloride	11.5		-1.94		10.2	-1.99	0.738	-0.132
56	Haloperidol	19.0	43.5	-1.72	-1.36	8.32	-2.08	0.272	-0.566
27	Sodium Selenate Decahydrate	43.5	80.1	-1.36	-1.10	52.5	-1.28	0.016	-1.80
44	Propanolol HCl		66.5		-1.18	95.5	-1.02	1.58	0.197
58	Verapamil HCl		111		-0.955	135	-0.870	0.226	-0.646
16	Dibutyl Phthalate	148		-0.830		79.4	-1.10	32.0	1.50
39	Epinephrine Bitartrate		170		-0.770	224	-0.650	0.012	-1.92
32	Carbamazepine	179		-0.747		269	-0.570	11.9	1.07
40	Atropine Sulfate Monohydrate	226	248	-0.646	-0.606	117	-0.930	1.18	0.071
41	Physostigmine	326	232	-0.487	-0.635	501	-0.300	0.018	-1.74
18	5-Aminosalicylic Acid		388		-0.411	316	-0.500	22.4	1.35
46	Phenol	415		-0.382		851	-0.070	8.10	0.908
28	Chloramphenicol	469		-0.329		1122	0.050	10.8	1.03
38	Valproic Acid	602	973	-0.220	-0.012	2951	0.470	6.91	0.839
33	Parathion	655	27.7	-0.184	-1.558	97.7	-1.01	0.021	-1.68
50	Sodium Fluoride	663	660	-0.178	-0.180	1259	0.100	3.02	0.480
10	Diethyl Phthalate		771		-0.113	851	-0.070	41.9	1.62
29	Busulfan	737	581	-0.133	-0.236	1259	0.100	0.049	-1.31
5	Fenpropathrin	1076	228	0.032	-0.642	5.13	-2.29	0.217	-0.664
37	Phenylthiourea	1110		0.045		1738	0.240	0.020	-1.71
48	Caffeine	1298	1413	0.113	0.150	2951	0.470	1.60	0.203
15	Citric Acid		1433		0.156	2239	0.350	30.9	1.49
21	Trichloroacetic Acid	1667	2103	0.222	0.323	2399	0.380	19.1	1.28
54	Oxalic Acid Disodium		1692		0.228	2630	0.420	4.72	0.674
35	Aminopterin	2505		0.399		1380	0.140	0.016	-1.80
55	Nicotine	3748		0.574		676	-0.170	0.430	-0.367
34	Procainamide HCl		5654		0.752	7413	0.870	7.17	0.856
36	Lithium Carbonate	5955		0.775		7244	0.860	7.98	0.902
11	Boric Acid	6809		0.833		7586	0.880	55.4	1.74
13	Lactic Acid	7057	6642	0.849	0.822	14454	1.16	40.4	1.61
60	Sodium Hypochlorite	12926	9041	1.11	0.956	21878	1.34	139	2.14
19	Xylene	23547	*	1.37		4365	0.640	43.9	1.64
30	Sodium Chloride		63655		1.80	58884	1.77	69.3	1.84
22	Methanol	78027	276945	1.89	2.44	64565	1.81	272	2.43
17	Dimethylformamide	87415	104150	1.94	2.02	87096	1.94	72.6	1.86
25	2-Propanol	95591	75208	1.98	1.88	117490	2.07	1.58	0.197
23	Acetonitrile	109216	172229	2.04	2.24	223872	2.35	87.6	1.94
14	Glycerol		349924		2.54	316228	2.50	215	2.33

* - No data available

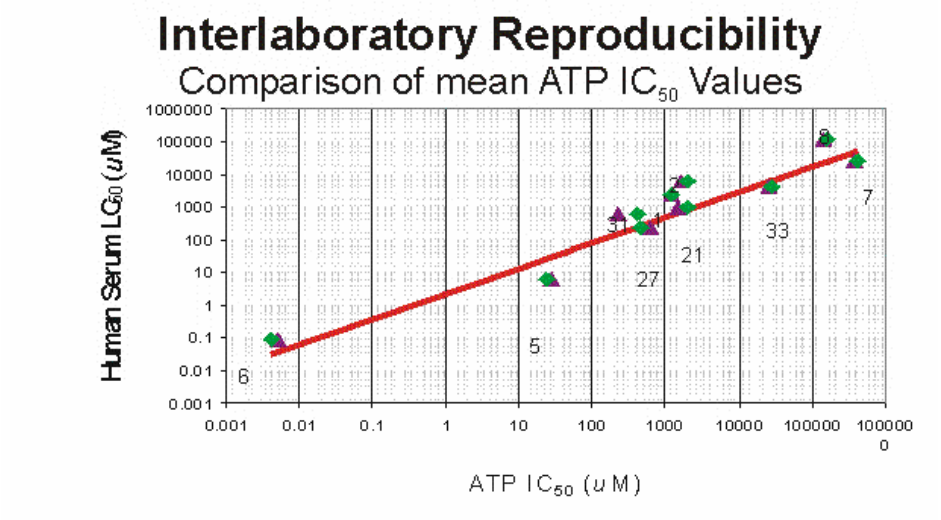
Figure 5.



REPRODUCIBILITY

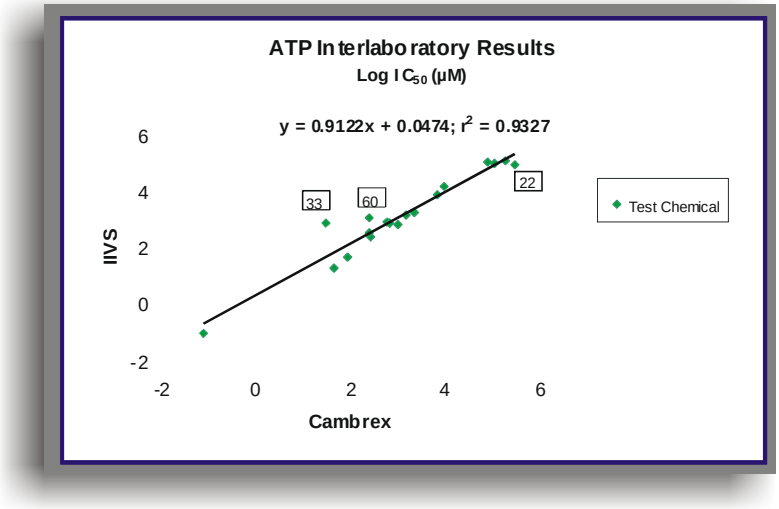
In the initial study to predict human serum LC_{50} values, 10 of the 20 chemicals were tested in common in both laboratories. In the subsequent study to predict rodent oral LD_{50} values, 20 of the 50 chemicals were tested in common in both laboratories. These 30 chemicals, as well as the positive control SLS were used to evaluate interlaboratory reproducibility. Intralaboratory reproducibility was evaluated solely from the results of 30 of the 35 chemicals assayed at IIVS in the second study.

Figure 6. Interlaboratory Reproducability - Initial Human Serum LC50 Prediction Study.
A comparison of the mean log IC_{50} values from the 10 MEIC chemicals tested at both laboratories are presented in Fig. 6. The greatest difference for mean IC_{50} values between the labs was for Warfarin (MEIC #31) (0.28 log difference).



A regression analysis comparing the mean log IC_{50} values obtained from both laboratories of the 20 chemicals tested in common in the subsequent rodent oral LD_{50} prediction study is presented in Fig. 7.

Figure 7. Interlaboratory Reproducibility Rodent Oral LD_{50} Prediction Study

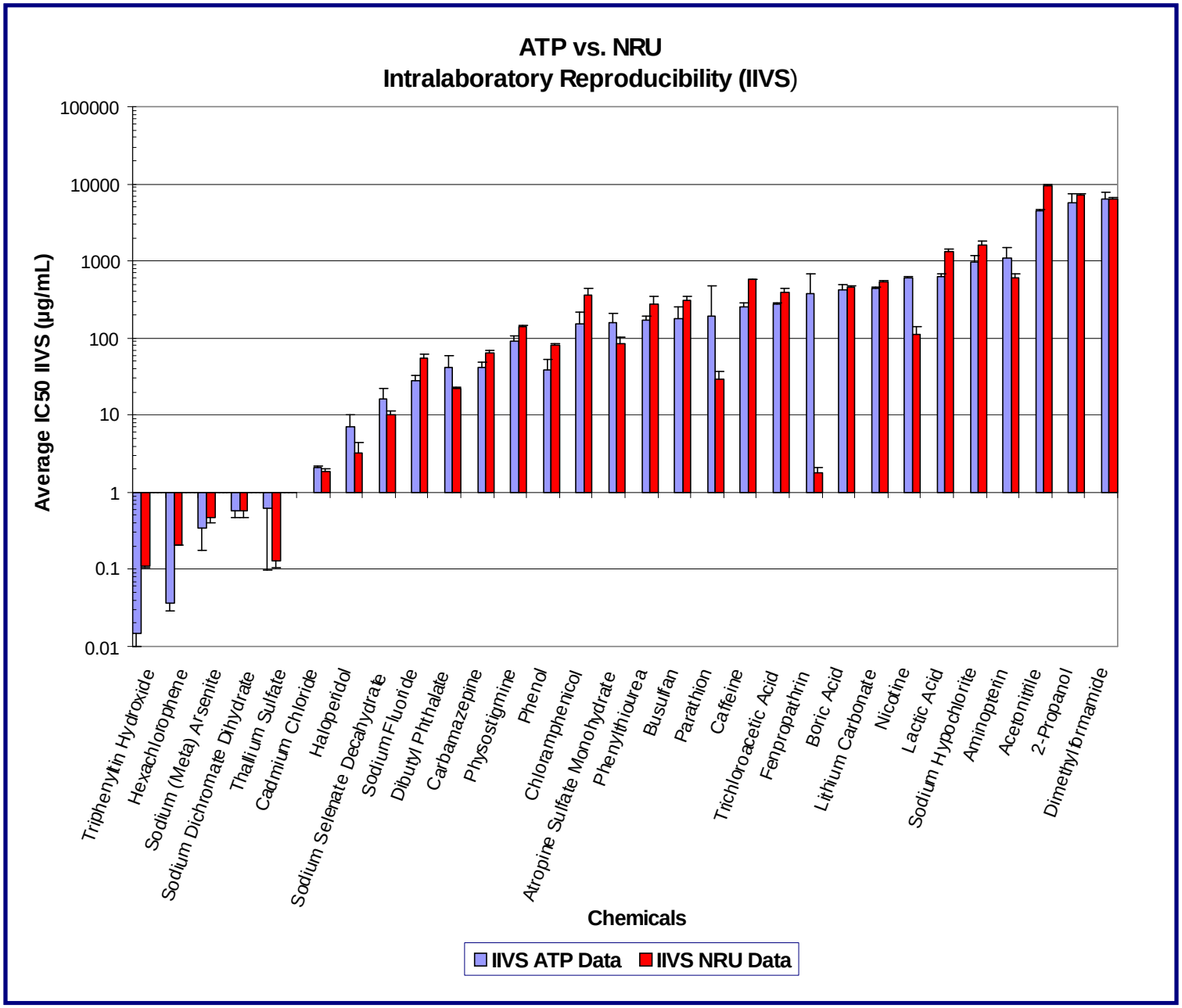


The positive control, SLS, was assayed in every trial in both laboratories in both the initial human serum LC_{50} and the subsequent rodent oral LD_{50} studies. The mean IC_{50} ± 2 standard deviation values for acceptable definitive trials conducted in each lab are presented in Table 3.

Table 3. Interlaboratory Reproducibility of the Positive Control

Laboratory	Study	SLS Mean IC50 (µg/mL)	2 Standard Deviations
Cambrex (Lonza)	initial (human LC50 prediction)	2.42	0.56
	subsequent (rodent oral LD50)	2.71	0.8
IIVS	initial (human LC50 prediction)	3.04	0.8
	subsequent (rodent oral LD50)	2.37	0.3

Figure 8 Intralaboratory Reproducibility Rodent Oral LD50 Prediction Study



Thirty-five (35) chemicals were tested in three trials at IIVS using the ATP endpoint. To present the reproducibility of the results graphically, the mean IC_{50} values and 1 standard deviation bars of 30 of the 35 chemicals are presented in Fig. 8 (the remaining 5 chemicals were non-toxic, and hence did not result in an IC_{50} value). The same 30 chemicals were also tested in three trials using the NRU endpoint (Paris, et al, 2003), and are presented for comparison.

COMPARISON BETWEEN ATP AND NRU ENDPOINTS

Figure 9. The in vitro IC_{50} prediction of rodent oral LD_{50} using in vitro values obtained from ATP and NRU endpoints is extremely similar [ATP (green) and NRU (red) regression formulas are noted].

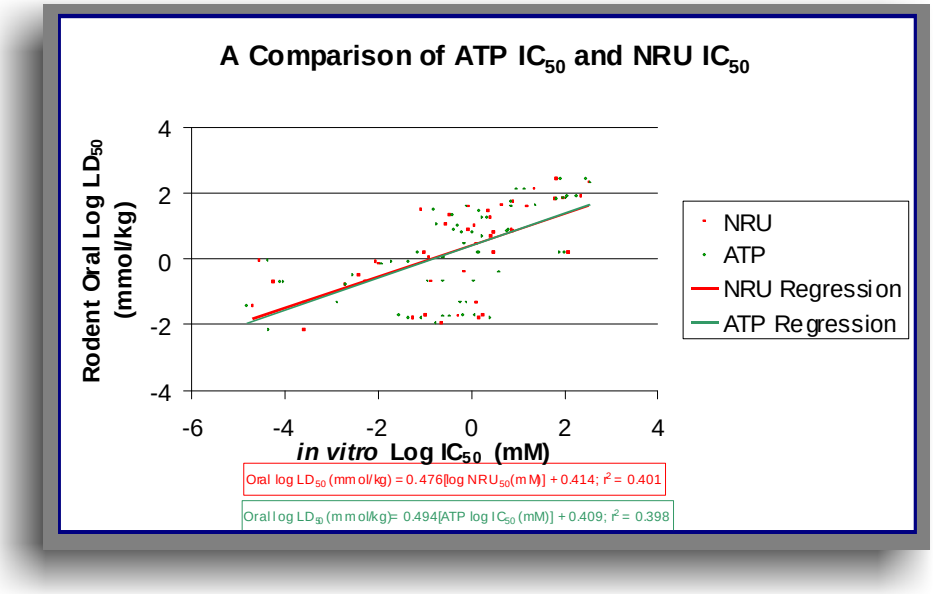
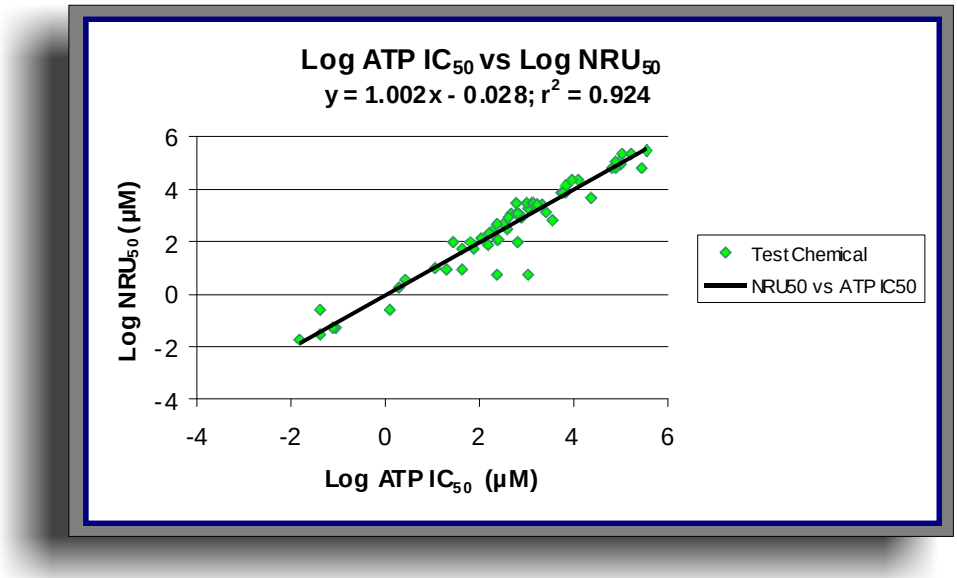


Figure 10. Further comparison of the in vitro log IC_{50} values showed a strong correlation between the ATP and NRU endpoints (r_2 = 0.924). Using the ATP endpoint, the test chemical, Fenpropathrin (60), resulted in a notably higher IC_{50} value.



CONCLUSIONS

- The ATP endpoint is faster and requires fewer steps than the NRU endpoint, hence making this endpoint very appropriate for high throughput screening
- Demonstrated relationship between in vitro IC_{50} values and human lethal serum LC50 values (r2 = 0.887) and published rodent oral LD50 (r2 = 0.399)
- Very high interlaboratory reproducibility of mean IC_{50} values (r2 = 0.933, rodent oral LD50 prediction study)
- Considering the dynamic range of the assay (potential responses over 8-logs), the differences in positive control reproducibility between labs considered biologically insignificant
- Very high correlation (r2 = 0.979, initial study; r2 = 0.924, subsequent study) between ATP IC50 and NRU50 values indicates that the ATP endpoint identified essentially the same IC_{50} as the neutral red uptake endpoint
- Results show that the NHEK assay with the ATP endpoint shows promise in the early evaluation of potential systemic toxicity
- Since the bioassay is designed to detect cytotoxic effects in the target cells, certain classes of materials shown to be neurotoxic (Organophosphates, Pesticides, and Insecticides) have been shown to be underpredicted using both the NRU and ATP viability endpoints.

REFERENCES

Crouch S, Kozlowski R, Slater K et al. (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods 160:8188

Curren RD, Wallace KA, Janus J, and Harbell JW. (1992) The use of normal human keratinocytes and the neutral red uptake bioassay to assess the first 30 MEIC compounds. 10th Anniversary Symposium of the CAAT:C16.

Ekwall B, Bondesson I, Castell JV, Gómez-Lechón MJ, Hellberg S, Högberg J, Jover R, Ponsoda X, Romert L, Stenberg K, and Walum E. (1989). Cytotoxicity evaluation of the first ten MEIC chemicals: Acute lethal toxicity in man predicted by cytotoxicity in five cellular assays and by oral LD50 tests in rodents. ATLA 17(2):83-100.

Ekwall B, Clemedson C, Crafoord B, Ekwall B, Hallander S, Walum E, and Bondesson I. (1998). MEIC Evaluation of acute systemic toxicity: Part V. Rodent and human toxicity data for the 50 reference chemicals. ATLA 26:571-619.

ICCVAM (2003) “Test Method Protocol for the NHK Neutral Red Uptake Cytotoxicity Test A Test for Basal Cytotoxicity for an In Vitro Validation Study Phase III”. In Vitro Cytotoxicity Test Method Protocols for Estimating Acute Oral Systemic Toxicity.
http://iccvam.niehs.nih.gov/methods/invitro.htm

Paris M, Strickland J, et al, (2006). Reproducibility Analyses for In Vitro Neutral Red Uptake Methods from a Validation Study to Evaluate In Vitro Cytotoxicity Assays for Estimating Rodent Acute Systemic Toxicity, ABS #1970, Society of Toxicology Annual Meeting, San Diego. March 5-9.

Strickland JA, Paris MW, Tice RR, Stokes WS, Balls M, Worth A, Casati S. (2002). Selection of Reference Chemicals for the Validation of In Vitro Cytotoxicity Assays for Predicting In Vivo Acute Systemic Toxicity. Fourth World Congress on Alternatives and Animal Use in the Life Sciences, New Orleans, August 11-15.

Strickland JA, Stokes S, Casati S, Paris MW, Worth AP, Raabe H, Cao C, Clothier R, Harbell J, Curren R, Haseman J, and Tice RR. (2003). Design Of A Validation Study To Evaluate In Vitro Cytotoxicity Assays For Predicting Rodent And Human Acute Systemic Toxicity. ABS #761, Society of Toxicology Annual Meeting, March 10-14.

Wallace KA, Harbell JW, Janus J, and Curren RD. (1993) The use of normal human keratinocytes and the neutral red uptake bioassay to assess MEIC compounds number 31-50. In Vitro Cell. and Devel. Biol. 29(3):89.

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Rd., Suite 100
Gaithersburg, MD 20878
Ph: 301.947.6523
Fx: 301.947.6538
For information, please contact: aulrey@iivs.org or visit us at www.iivs.org

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