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Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity

Based on Recommendations from an International Workshop
Organized by the Interagency Coordinating Committee
on the Validation of Alternative Methods (ICCVAM)
and the
National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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

THE INTERAGENCY COORDINATING COMMITTEE ON THE VALIDATION OF ALTERNATIVE METHODS AND THE NTP INTERAGENCY CENTER FOR THE EVALUATION OF ALTERNATIVE TOXICOLOGICAL METHODS

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1997 by the Director of the National Institute of Environmental Health Sciences (NIEHS) to implement NIEHS directives in Public Law 103-43. P.L. 103-43 directed NIEHS to develop and validate new test methods, and to establish criteria and processes for the validation and regulatory acceptance of toxicological testing methods. P. L. 106-545, the ICCVAM Authorization Act of 2000, established ICCVAM as a permanent committee. The Committee is composed of representatives from 15 Federal regulatory and research agencies and programs that generate, use, or provide information from toxicity test methods for risk assessment purposes. The Committee coordinates cross-agency issues relating to development, validation, acceptance, and national/international harmonization of toxicological test methods.

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Additional Information

Additional information can be found at the ICCVAM/Center Website: http://iccvam.niehs.nih.gov and in the publication: *Validation and Regulatory Acceptance of Toxicological Test Methods, a Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods* (NIH Publication No. 97-3981, or you may contact the Center at telephone 919-541-3398, or by e-mail at iccvam@niehs.nih.gov. Specific questions about ICCVAM and the Center can be directed to the ICCVAM Co-chairs:

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- National Library of Medicine
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National Institute of Environmental Health Sciences
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List of Acronyms/Abbreviations

ATC	Acute Toxic Class
ATCC	American Type Culture Collection
BALB/c	Inbred strain of mouse
BgVV	Federal Institute for Health Protection of Consumers and Veterinary Medicine (Germany)
BMFT	Ministry of Research and Technology (Germany)
BSS	Balanced Saline Solution
b.w.	Body weight
CAS	Chemical Abstract Service
CHO	Chinese hamster ovary cell line (epithelial)
CFR	Code of Federal Regulations
CMF-HBSS	Calcium/Magnesium-Free Hanks’ Balanced Salt (Saline) Solution
COLIPA	The European Cosmetic, Toiletry and Perfumery Industry
CS	Calf serum (bovine)
CTFA	The Cosmetic, Toiletry, and Fragrance Association (USA)
CTLU	Cytotoxicology Laboratory, Uppsala
DMEM	Dulbecco’s Modification of Eagle’s Medium without L-Glutamine
DMSO	Dimethyl Sulfoxide
ECACC	European Collection of Cell Cultures
EC/HO	European Commission/British Home Office
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
ETOH	Ethanol
EZ4U	Non-radioactive cell proliferation and cytotoxicity assay (Biomedica Gruppe)
FBS	Fetal Bovine Serum
FDP	Fixed-Dose Procedure
F_G	Empirical linear-shaped prediction interval
FL	Fluorescein Leakage
FRAME	Fund for the Replacement of Animals in Medical Experiments
GLP	Good Laboratory Practice Regulations
h	Hour(s)
HBSS	Hanks’ Balanced Salt (Saline) Solution
HEL-30	Murine keratinocyte cell line
Hepa-1	Mouse hepatoma cell line (epithelial)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HET-CAM	Hen’s Egg Test-Chorioallantoic Membrane
HTD	Highest tolerated dose
IC50	Inhibitory concentration estimated to affect endpoint in question by 50%
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Congress for Harmonization
IIVS	Institute for In Vitro Sciences
INVITTOX	ECVAM database
ISO 5725	A program for analysis and reporting of proficiency tests and method evaluation studies

ISO	International Standards Organization
KB	Kenacid Blue
KGM	Keratinocyte Growth Medium
L929	Mouse fibrosarcoma cell line (fibroblast)
LD50	Dose producing lethality in 50% of the animals
LDH	Lactate Dehydrogenase
MDCK	Madin Darby canine kidney cells
MEIC	Multicentre Evaluation of In Vitro Cytotoxicity
MEMO	MEIC Monographs, CTLU
MIT-24	Metabolic Inhibition Test
MSDS	Material Safety Data Sheet
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBCS	New-born Calf Serum (bovine)
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences/NIH
NIH	National Institutes of Health/DHHS
NIOSH	National Institute for Occupational Safety and Health
NHEK	Normal Human Epidermal Keratinocytes
NHK	Normal Human Keratinocytes
NR	Neutral Red dye
NR50	Mean concentration of test substance reducing viability of cells to 50% of the viability of controls
NRU50	The concentration of the test article which inhibits the uptake of neutral red by 50%
NRR	Neutral Red Release
NRU	Neutral Red Uptake
NTP	National Toxicology Program
OD_{540}	Optical density at 540nm
OECD	Organization for Economic Cooperation and Development
PBS	Phosphate Buffered Saline
PC	Positive Control
PHOTO-32	Software for concentration response analysis from 96-well plates
PT	Phototoxicity Test
RC	Registry of Cytotoxicity/ZEBET
RTECS	Registry of Toxic Effects of Chemical Substances
SIS	ECVAM Scientific Information System
SLS	Sodium Lauryl Sulfate
SOP	Standard Operating Procedures
TG 420	Test Guideline 420 (Acute Oral Toxicity - Fixed Dose Method) [OECD]
TG 423	Test Guideline 423 (Acute Oral toxicity - Acute Toxic Class Method) [OECD]
TG 425	Test Guideline 425 (Acute Oral Toxicity: Up-and-Down Procedure) [OECD]

TNS	Trypsin Neutralizing Solution
UDP	Up-and-Down Procedure
V79	Chinese hamster lung fibroblast cell line
VC	Vehicle Control
XTT	Sodium 3,3-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate
ZEBET	German Centre for the Documentation and Validation of Alternative Methods (at BgVV)
3Rs	Refinement, Reduction, and Replacement (of Animal Use)
3T3	BALB/c mouse fibroblast cells
3T3 A31	BALB/c mouse fibroblast cells – clone A31

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Drs. Fentem, Curren, and Liebsch are acknowledged for their significant contributions to the guidance document. They along with the following scientists were invited to serve on the Breakout Group 1 panel for the International Workshop on *In Vitro* Methods for Assessing Acute Toxicology, October 17-20, 2000.

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Preface

Acute systemic toxicity testing is conducted to determine the relative health hazard of chemicals and various products. Substances found to cause lethality in animals at or below prescribed doses are labeled to identify their hazard potential. While acute toxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between *in vitro* and *in vivo* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting *in vivo* acute toxicity.

An International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was convened on October 17-20, 2000, to review the validation status of available *in vitro* methods for predicting acute toxicity, and to develop recommendations for future research and development efforts that might further enhance the use of *in vitro* assessments of acute systemic toxicity. The Workshop was organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The U.S. Environmental Protection Agency (U.S. EPA), the NTP, and the National Institute of Environmental Health Sciences (NIEHS sponsored the workshop. Breakout Groups, comprised of invited scientific experts and ICCVAM agency scientists, developed conclusions and recommendations for four topics:

- *In Vitro* Screening Methods for Assessing Acute Toxicity;
- *In Vitro* Methods for Toxicokinetic Determinations;
- *In Vitro* Methods for Predicting Organ Specific Toxicity; and
- Chemical Data Sets for Validation of *In Vitro* Acute Toxicity Test Methods.

The Breakout Group that addressed the first topic, "*In Vitro* Screening Methods," was charged with evaluating the current validation status of basal cytotoxicity methods, and recommending whether and how these methods might be used to reduce and refine animal use for acute toxicity testing. The Group concluded that *in vitro* cytotoxicity data could be useful in estimating starting doses for *in vivo* acute toxicity testing, which will reduce the number of animals required for such determinations. Their conclusions were based on several studies but primarily those by Drs. Horst Spielmann and Willi Halle, and their colleagues at the German Centre for the Documentation and Evaluation of Alternatives to Testing in Animals. Halle compiled a Registry of Cytotoxicity containing *in vivo* acute toxicity data and *in vitro* cytotoxicity data for 347 chemicals. These data were used to construct a regression model to estimate LD_{50} values from cytotoxicity data. They subsequently proposed that using these estimates as starting doses for *in vivo* acute toxicity studies such as the Up-and-Down Procedure or the Acute Toxic Class method could reduce the number of animals used by as much as 30 percent. In addition, the Group recommended that this guidance document be prepared to provide practical guidance on how to generate and use basal cytotoxicity data to predict starting doses for *in vivo* acute toxicity assays. Drs. Manfred Liebsch, Rodger Curren, and Julia Fentem volunteered to draft this document and after the Workshop they worked with NICEATM to develop it. This guidance document has been reviewed by ICCVAM, the ICCVAM Workshop Organizing Committee, and those participating in the Breakout Group on *In Vitro* Screening Methods.

The workshop results have been published as the *Report on the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity* (NIH Publication No. 01-4499). The Organizing Committee and ICCVAM developed test recommendations to forward with these publications to Federal agencies for their consideration in accordance with Public Law 106-545. The ICCVAM recommendations are provided in the Workshop Report. Both publications are available at the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov), or a copy may be requested from NICEATM at P.O. Box 12233, MD EC-17, Research Triangle Park, NC 27709 (mail), 919-541-3398 (phone), 919-541-0947 (fax), or NICEATM@niehs.nih.gov (email).

On behalf of the ICCVAM, we gratefully acknowledge the efforts of the Breakout Group on *In Vitro* Screening Methods for their comprehensive evaluation of existing data and methods that served as the impetus for this guidance document. We extend our sincere appreciation to the contributing authors, Drs. Manfred Liebsch, Rodger Curren, and Julia Fentem, for their considerable efforts and contributions to this document. The efforts of the NICEATM staff in coordinating the preparation and publication of the document are acknowledged and appreciated, particularly those of Dr. Judy Strickland and Mr. Michael Paris, who worked diligently with the authors to produce the final version.

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1.0 INTRODUCTION

1.1 Purpose and Scope of this Guidance Document

This guidance document describes how to use *in vitro* cytotoxicity tests to estimate starting doses for acute oral lethality assays. Development of this document was recommended by participants in the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, held October 17-20, 2000, in Arlington, VA, U.S.A. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) convened the workshop to evaluate the validation status of available *in vitro* methods for assessing acute toxicity. A workshop breakout group reviewed the use of *in vitro* screening methods to estimate acute *in vivo* toxicity (i.e., LD_{50} values) and recommended the development of this guidance document which was written by three of its members.

This introduction summarizes background information about the correlation between *in vitro* cytotoxicity and acute lethality, explains the purpose of using *in vitro* cytotoxicity assays to predict starting doses for *in vivo* acute lethality assays, and describes a general approach for evaluating *in vitro* test performance. Chapter 2 describes the basic elements of *in vitro* assays for basal cytotoxicity and describes what investigators should consider before applying the results of these assays to their own situations. Chapter 3 describes the use of the Registry of Cytotoxicity (RC) prediction model to evaluate a candidate cytotoxicity assay. The RC prediction model is a regression analysis of LD_{50} values (the median lethal dose, i.e., the dose that produces lethality in 50% of the animals tested) and *in vitro* cytotoxicity IC_{50} values (i.e., concentration at which cell viability is inhibited by 50%) for 347 chemicals. Chapter 4 describes two candidate tests recommended for use with this method: neutral red uptake (NRU) assays using the mouse fibroblast cell line BALB/c 3T3 and normal human keratinocytes (NHK). Appendix A contains the RC data in spreadsheet format. Appendix B contains a list of test protocols for basal cytotoxicity from the Scientific Information System (SIS) of the European Centre for the Validation of Alternative Methods (ECVAM). Appendices C-G provide detailed stand-alone protocols for BALB/c 3T3 and NHK NRU assays, additional guidance for implementing the protocols, and a standard template for data collection.

1.2 The Correlation between Basal Cytotoxicity and Acute Lethality

Acute oral toxicity testing is typically the first step in identifying and characterizing the hazards associated with a particular chemical. Information derived from acute toxicity tests in laboratory animals (mainly rodents) is used for several purposes, including: (a) hazard classification and labeling of chemicals in accordance with national and international regulations (e.g., 49 Code of Federal Regulations [CFR] 173; 16 CFR 1500; 29 CFR 1910; 40 CFR 156; Organisation for Economic Co-operation and Development [OECD], 1998a); (b) risk assessments pertaining to the acceptability of acute exposures in the workplace, at home, and upon accidental release; (c) clinical diagnosis, treatment and prognosis of acute human poisoning cases; and (d) design (e.g., dose-setting, identification of potential target organs) of longer-term (e.g., 28-day) toxicity studies. Historically, lethality estimated by the LD_{50} test has been a primary toxicological endpoint in acute toxicity tests, although more detailed toxicological information is sometimes collected. More recently, the conventional test procedure has been modified in various ways to refine and reduce animal use (OECD, 1992, 1996, 1998b). Aiding the acceptance of these alternative methods has been the recognition that the LD_{50} is not a biological constant, but is influenced by many factors (Klaassen and Eaton, 1991). For most purposes, the LD_{50} only needs to be characterized "within an order of magnitude range," according to Klaassen and Eaton (1991).

The use of cell cultures *in vitro* as alternatives to predict acute lethality *in vivo* has been under study for almost 50 years (Pomerat and Leake, 1954; Eagle and Foley, 1956; Smith et al., 1963).

Numerous demonstrations of strong correlations between cytotoxicity *in vitro* and animal lethality *in vivo* exist. (For reviews see Phillips et al., 1990, and Garle et al., 1994). Recently, several major international *in vitro* initiatives have been directed toward reducing the use of laboratory animals for acute toxicity testing (Curren et al., 1998; Ekwall et al., 2000; Ohno et al., 1998a, 1998b, 1998c; Seibert et al., 1996; Spielmann et al., 1999). The status of these initiatives was reviewed at the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity, jointly sponsored by the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program (NTP), and the U.S. Environmental Protection Agency (U.S. EPA). Conclusions and recommendations from the workshop are published in the *Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity* (NIEHS, 2001).

The RC has made a major contribution to the knowledge of the correlation between *in vitro* cytotoxicity and *in vivo* lethality (Halle and Spielmann, 1992; Halle, 1998). The most recent RC compilation (Halle, 1998) contains *in vitro* cytotoxicity information (1,912 single IC_{50} values averaged for each of 347 chemicals [i.e., one IC_{50x} value/chemical from multiple reports in the literature]) paired with 347 *in vivo* acute oral LD_{50} values (mmol/kg) for rats (282 values) or mice (65 values) from the National Institute for Occupational Safety and Health (NIOSH) Registry of Toxic Effects of Chemical Substances (RTECS). (See Appendix A for the RC data.) Criteria for data to be included in the RC database are fully described by Halle (1998) and briefly described by Spielmann et al. (1999). The combination of rat and mouse data was justified, since it yielded a regression that was not significantly different from those obtained with either rat data or mouse data alone. The RC data clearly demonstrate a strong relationship between *in vitro* cytotoxicity and acute lethality in rodents (Figure 1).

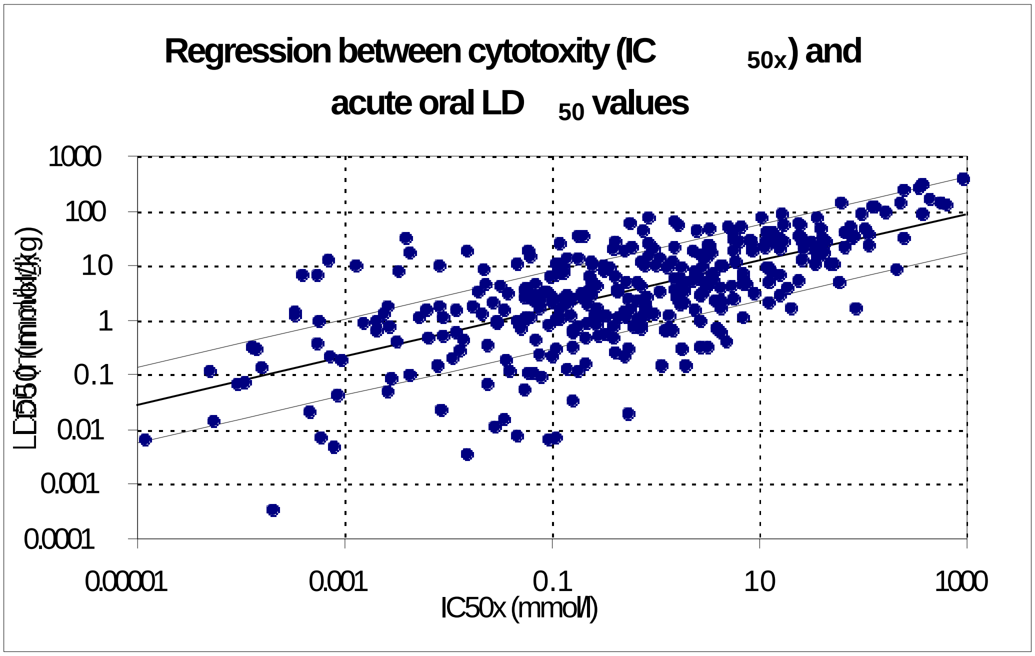

Figure 1. Registry of Cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral LD_{50} values for 347 chemicals.
The heavy line shows the fit of the data to a linear regression model, log (LD_{50}) = 0.435 x log (IC_{50x}) + 0.625; r=0.67. The other lines show the empirical $F_G = \pm$ log 5 acceptance interval for the prediction model (Spielmann et al., 1999), which is based on the anticipated precision of LD_{50} values from rodent studies Halle (1998).

1.3 *In Vitro* Determination of Starting Dose for *In Vivo* Tests

Spielmann et al. (1999) have proposed – as an initial step – that the relationship found with the RC regression be used with *in vitro* data to predict starting doses for subsequent *in vivo* acute lethality assays. They suggest that before initiating any *in vivo* lethality assay for a chemical, an *in vitro* cytotoxicity assay should be conducted to estimate the LD_{50} for that chemical. The LD_{50} predicted from the RC regression equation should then be used to choose the most appropriate starting dose for the *in vivo* assay. The LD_{50} estimate from the RC regression is based on molar amounts of the chemical, specifically a value in mmol/kg. This value must first be converted to a weight measurement expression, such as mg/kg, before using conventional LD_{50} dosing calculations. Using this estimate should make the conduct of *in vivo* assays much more efficient and result in savings both in the number of animals and in the amount of time required to obtain the final results. The workshop report (NIEHS, 2001) includes a discussion of the potential number of animals saved, based on several currently available *in vivo* protocols, e.g., protocols that use new sequential dosing methods such as the Acute Toxic Class method (ATC, OECD TG 423; OECD, 1996) and the Up-and-Down Procedure (UDP, OECD TG 425; OECD, 1998b). In these tests, using the fewest animals possible depends upon the correct choice of starting dose since, on average, the number of consecutive dosing steps is minimal if the starting dose is close to the true toxicity class (ATC) or to the true LD_{50} (UDP).

1.4 Determination of *In Vitro* Test Performance Characteristics

Before the results obtained with any *in vitro* cytotoxicity test are used with the RC regression to generate an expected LD_{50} value, the performance characteristics of the new method should be determined and compared with those of the RC information as discussed in Section 3.1. Section 3.2 suggests a set of reference chemicals that should be tested with the candidate *in vitro* cytotoxicity method. The resultant regression line should then be compared with that of the current RC regression line. If the line falls within the ± log 5 boundaries indicated in Figure 1, then the regression parameters of the RC may be used to predict the LD_{50} starting dose. Section 3.3 describes experimental trials, using two different cell types, performed after the workshop with the set of recommended reference chemicals. These experimental trials are included as examples of how to determine test performance for any *in vitro* test for basal cytotoxicity and to confirm the applicability of the test for use with the RC regression.

2.0 ELEMENTS OF A STANDARD TEST FOR BASAL CYTOTOXICITY

It is likely that many different *in vitro* cytotoxicity methods could be used to help select the *in vivo* starting dose for an acute lethality assay. Two decades of experience indicate that *in vitro* basal cytotoxicity data determined in various primary cells, as well as in various permanent non-differentiated finite or transformed cell lines, generally show comparable cytotoxic concentrations of the same xenobiotic, regardless of the type of toxic endpoints investigated. The RC data, which consist of information from many different *in vitro* methods that vary in both cell type and cytotoxicity endpoint (i.e., specific protocol), indicated that exceptions to this "rule" were observed only for those chemicals (some insecticides, neurotropic chemicals, and chemicals requiring metabolic activation) that require specific cell types to express their toxicity (Halle, 1998). Thus, a recommendation cannot be made for the "most relevant" or "most typical" *in vitro* test for basal cytotoxicity.

Currently the ECVAM SIS lists 20 different test protocols for basal cytotoxicity. (Appendix B and http://www.ivtip.org/protocols.html#basalcyto.) Several *in vitro* tests listed in the SIS as "specific" for a certain purpose, such as prediction of eye and skin irritancy, in fact provide only basal cytotoxicity information.

Nonetheless, since the responsiveness of all cell culture test systems to xenobiotics can be influenced significantly by test design and culture conditions, there is a consensus among *in vitro* toxicologists to give preference to protocols that are highly responsive. For example, while increasing exposure times (e.g., from 1 hour [h] up to 48 h) will usually increase the responsiveness of the test, an increase in serum in the culture medium (e.g., from 5% up to 20%) will generally decrease the responsiveness of a cytotoxicity test.

2.1 Selection of Cell Lines / Cells

Analyses performed before or during the workshop (NIEHS, 2001) did not reveal significant differences between the basal cytotoxicity results obtained using permanent mammalian cell lines, primary human cells, or using the IC_{50X} approach of Halle and Spielmann (Halle, 1998; Spielmann et al., 1999; Halle and Spielmann, 1992). Thus, primary cells, as well as many currently available mammalian cell lines could be used, provided they are of sufficient quality to assure reproducibility over time. However, rodent (i.e., rat or mouse) or human cells are expected to be most useful for this approach. Established rodent cell lines are recommended because: 1) it is assumed that rodent cells would give the best prediction of rodent acute lethality, and 2) the use of a standard cell type for this *in vitro* cytotoxicity technique will hasten the generation of a database that can be used to analyze the usefulness of this approach. There are also arguments for utilizing human cell lines to assess basal cytotoxicity. For example, an analysis of the RC rodent acute lethality data relative to cytotoxicity data generated using human cell lines in the MEIC program showed that both were highly correlative (R^2=0.90) (NIEHS, 2001). A long-term advantage of using human cells is that the human cell cytotoxicity data derived from this approach can be added to human toxicity databases to facilitate the development of methods that may later predict acute human lethality.

Of the rodent cell lines used for basal cytotoxicity, the mouse fibroblast cell line BALB/c 3T3 A31 is probably the most frequently used. Thus, a stable background of historical data exists, including data from controlled and blinded validation studies (Gettings et al., 1991, 1992, 1994a, 1994b; Spielmann et al., 1991, 1993, 1996; Balls et al., 1995; Brantom et al., 1997). Other rodent cell lines that have been used in basal cytotoxicity assays are described by Clemedson et al. (1996).

Of the human cells used for basal cytotoxicity, NHK or fibroblasts are probably the cells most frequently used with good results in validation studies (Willshaw et al., 1994; Sina et al., 1995; Gettings et al., 1996; Harbell et al., 1997).

Fish cell lines or invertebrate cell lines are not recommended for determining basal cytotoxicity

(Ekwall et al., 1998). Although, according to the concept of basal cytotoxicity, they are expected to show failure of the same basic cell functions as mammalian cells would show at comparable chemical concentrations, it is not easy to create test designs that are highly responsive to xenobiotics. For example, due to doubling times of up to several days, the responsive growth inhibition protocol cannot be used easily.

Highly differentiated cells may not give the best prediction of acute lethality for the large variety of chemicals likely to be tested for acute toxicity (Ekwall et al., 1998). For example, to eliminate the possibility of metabolic activation or inactivation of chemicals, neither hepatocyte nor hepatoma cytotoxicity data were included in the RC. This does not preclude the use of hepatocytes in future studies, however, either to estimate cytotoxicity or to investigate the effect of metabolism or cell-specific toxicity (Seibert et al., 1996). Hepatocytes are essential to investigations of metabolism-mediated toxicity that will be required to meet the longer-term goal of replacing *in vivo* acute lethality testing with *in vitro* methods (Seibert et al., 1996).

Whether rodent or human cells are used, they should be capable of active division (population doubling time of approximately 30 h or less) so that chemicals that exert their toxicity primarily during cell division will be adequately detected in these relatively short-term assays. As described in Section 2.3, chemical exposure should last at least one full cell cycle.

Finally, selection of a cell line always should be made in the context of the intended cytotoxicity endpoint to be measured. For example, if NRU is the intended measurement endpoint, the cells used must possess a significant amount of lysosomes to incorporate neutral red dye. Embryonic stem cells, for example, do not contain the requisite organelles, and NRU cannot be used to determine cytotoxicity in these cells.

Both the mouse and human cells mentioned above are easily obtainable from commercial sources. Cytotoxicity data from both the BALB/3T3 A31 cell line and NHK cells are presented in Section 3.3 of this document as examples of how to qualify new cytotoxicity protocols for use with the RC method for predicting starting doses for acute lethality assays *in vivo*.

2.2 Recommended Measurement Endpoints for Basal Cytotoxicity

Many measurement endpoints for cytotoxicity are well established and have been used to assess basal cytotoxicity. For inclusion of IC_{50} values in the RC, the following endpoints were accepted as sufficiently characteristic of basal cytotoxicity (Spielmann et al., 1999; Halle, 1998):

1) Inhibition of cell proliferation:
- Cell number
- Cell protein
- DNA content, DNA synthesis
- Colony formation

2) Cell viability - metabolic markers:
- Metabolic inhibition test (MIT-24)
- Mitochondrial reduction of tetrazolium salts into insoluble dye (MTT test), or, more recently, into soluble dye (MTS test or XTT test [e.g., “EZ4U”]).

3) Decreased cell viability - membrane markers:
- NRU into cell lysosomes
- Trypan Blue exclusion
- Cell attachment, cell detachment

4) Differentiation markers
- Functional differentiation within cell islets
- Morphological differentiation within cell islets
- Intracellular morphology

Markers of the release of intracellular components, such as the enzyme lactate dehydrogenase (i.e., LDH release test), or of dye introduced into the cells previous to chemical exposure (e.g., fluorescein leakage [FL] test or Neutral Red Release [NRR] test) were not considered to be characteristic for basal cytotoxicity because they specifically detect damage of the outer cell membrane and generally are associated with short-term chemical exposure. A chemical that specifically damages only cell membranes, however, will be detected correctly in one of the tests for basal cytotoxicity listed above.

2.3 Recommendations for Cytotoxicity Test Protocols

Since the RC was constructed with data from many different *in vitro* protocols, a number of different *in vitro* cytotoxicity protocols might produce correlations with *in vivo* acute lethality similar to the correlation produced by the RC. It is strongly suggested, however, that any proposed *in vitro* protocol incorporate the following conditions:

(a) Use a cell line (or primary cells) that divides rapidly with doubling times of less than 30 h under standard culture conditions, preferably with normal serum types, e.g., calf serum (CS), newborn calf serum (NBCS), or serum-free medium.

(b) Use only cells in the exponential phase of growth. Never use cells immediately after thawing them from frozen stock. Allow cells to grow 1-2 passages before they are used in the cytotoxicity test.

(c) The chemical exposure period should be at least the duration of one cell cycle, i.e., 24 – 72 h (Riddell et al., 1986).

(d) Initial seeding should be done at a density that allows rapid growth throughout the exposure period.

(e) Use appropriate positive and vehicle control materials for which cytotoxicity, or lack of cytotoxicity, has been well characterized by the performing laboratory.

(f) Use solvents only at levels previously shown not to cause cytotoxicity to the cell system over the entire period of the assay.

(g) Use a measurement endpoint that is well established and that has good interlaboratory reproducibility. Give preference to endpoints that determine either cell proliferation or cell viability (e.g., NRU, MTT, XTT). Simple endpoints such as total protein content are not recommended, as they may under-predict the toxicity of certain test chemicals by staining dead cells.

(h) The protocol should be compatible with 96-well plates and apparatus such as spectrophotometers that allow a quick and precise measurement of the endpoint.

(i) Complete a detailed concentration-response experiment using a progression factor that yields graded effects between no effect and total cytotoxicity. Any desired toxicity measure can be derived from a well-designed concentration-response experiment. Experiments that seek to detect only a marker concentration, such as the highest tolerated dose or the lowest cytotoxic dose, are characterized by a lack of information and a low level of accuracy.

3.0 PROCEDURE FOR QUALIFYING A CYTOTOXICITY TEST FOR USE WITH THE REGISTRY OF CYTOTOXICITY (RC) PREDICTION MODEL

Workshop participants agreed that tests of basal cytotoxicity were sufficiently predictive for the rodent LD_{50} such that cytotoxicity tests could be used to predict a starting dose for an *in vivo* lethality assay (NIEHS, 2001). This section discusses how to proceed. Theoretically, any *in vitro* test capable of determining basal cytotoxicity could be used to determine the best estimate of a starting dose for acute testing in the UDP (OECD, 1998b), the ATC method (OECD, 1996), or the Fixed Dose Procedure (FDP; TG 420, OECD, 1992). In addition, if the LD_{50} predicted from cytotoxicity is high (i.e., ≥ 2,000 mg/kg b.w.), a range-finding study for the ATC or FDP may not be necessary, as testing could be initiated using the limit test of 2,000 or 5,000 mg/kg.

Before using a candidate *in vitro* cytotoxicity test to predict starting doses, the correlation between the *in vitro* test and the *in vivo* test must be established quantitatively. This can be achieved either by (1) *in vitro* testing of a large number of chemicals with known LD_{50} values and deriving a regression formula based on the correlation between *in vivo* and *in vitro* data, or by (2) testing a smaller number of chemicals and applying Halle's RC prediction model (i.e., regression formula), which is derived from the correlation of *in vivo* and *in vitro* data for 347 chemicals (Halle, 1998; Spielmann et al., 1999). In the latter case, *in vitro* data for a small number of reference chemicals from the RC are compared with *in vitro* data from the RC to determine the adequacy of the test method.

Section 3.1 explains this procedure. Section 3.2 provides a set of 11 recommended reference chemicals from the RC. Section 3.3 presents experimental data from testing these 11 reference chemicals in the NRU cytotoxicity assay with both NHK cells and BALB/c 3T3 cells.

3.1 Procedure to Determine Whether a Candidate Cytotoxicity Test Can Use the RC Prediction Model

To determine whether predicted LD_{50} values from a basal cytotoxicity method can be used as starting doses for routine testing of acute oral toxicity with the ATC or the UDP methods, Spielmann et al. (1999) suggested a procedure which is shown in Figure 2. Ten to twenty reference chemicals are selected from the RC (Halle, 1998) and tested in a standardized cytotoxicity test (Figure 2, Step 1). A promising candidate would be the BALB/c 3T3 NRU test (see Appendix C for the Standard Operating Procedure [SOP]), which has been highly reproducible in several validation studies (Gettings et al., 1991, 1992, 1994a, 1994b; Spielmann et al., 1991, 1993, 1996; Balls et al., 1995; Brantom et al., 1997). An alternative test, less frequently used, which has also yielded good results in validation studies, is the NHK NRU assay (Willshaw et al., 1994; Sina et al., 1995; Gettings et al., 1996; Harbell et al., 1997).

To allow comparison of the regression obtained with the candidate test (Figure 2, Step 2), selected reference chemicals should cover the entire range of cytotoxicity and be as close as possible to the RC regression line. (Section 3.2 presents a table with 11 reference chemicals from the RC and their corresponding LD_{50} values.) The regression equation from the candidate test is calculated by linear regression (least square method) using the candidate IC_{50} values and the corresponding LD_{50} values from the RC (given in Table 1 in Section 3.2). The resulting regression is then compared with the RC regression (Figure 2, Step 3).

If the regression line obtained with the candidate cytotoxicity test parallels the RC regression and is within the ± log 5 interval, then the test is considered suitable to generate IC_{50} data to use with the RC regression for estimating starting doses (Figure 2, Step 4). The rationale for using the RC regression rather than the regression from the candidate cytotoxicity test is that the RC regression is based on data from 347 chemicals, while the candidate regression is based on data from only 10-20 chemicals. To predict an LD_{50} starting dose, the IC_{50} (in mmol/l) for the trial chemical is entered into the regression equation to calculate an LD_{50} in mmol/kg b.w. Multiplying by the molecular weight of the trial chemical transforms the mmol/kg b.w. value into mg/kg b.w.

If the regression from the candidate test shows a significantly higher or lower slope than the RC regression, then it may be possible to adjust the candidate cytotoxicity test to a higher or lower slope. (<u>Note</u>: *This option was added post hoc publication of Spielmann et al., 1999.)* However, a more efficient approach is likely to be to use one of the recommended cell lines (Section 2.1) and protocols (e.g., Appendices C and D). These are expected to produce results similar to the RC data. Two examples are given in Section 3.3.

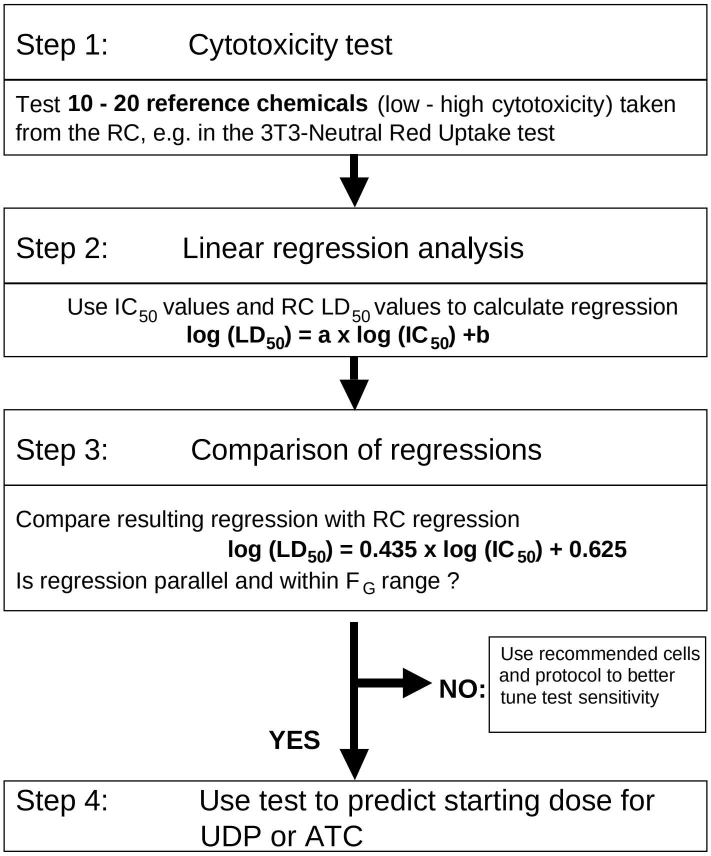

Figure 2. Procedure for evaluating a cytotoxicity test for tiered *in vitro*/*in vivo* testing for acute oral toxicity testing (slightly modified from Spielmann et al., 1999).

3.2 Recommended Reference Chemicals from the RC for Test Qualification

To compare a regression obtained from a candidate cytotoxicity test with the RC, 11 reference chemicals (Table 1) from Halle's RC (Halle, 1998) were selected using the following criteria:

- Cytotoxicity range must cover 5 - 6 logs from low to high toxicity.
- Chemical data points (IC_{50x}/LD_{50}) must be very close to the RC regression line.

- Chemicals must be available internationally, preferably from one supplier.
- Available purity of chemicals must be ≥ 95%.
- Handling of chemicals must be acceptable with regard to sufficient solubility, low volatility, and safe use (e.g., avoid the use of known carcinogens).

Table 1. Recommended reference chemicals for evaluating a cytotoxicity test for use with the RC prediction model

Chemical Name	**IC_{50x}** (mmol/ liter)	**LD_{50}** (mmol/ kg b.w.)	**LD_{50}** (mg/ kg b.w.)	**Molecular Weight** (g)	**CAS Number**	**Sigma-Aldrich Puchase #**	**Purity**	**Possible hazards; risk phrases from MSDS**
Sodium dichromate (VI) dihydrate	0.00093	0.19	49.8	298.0	7789-12-0	S9791	99.5%	Very toxic, corrosive, possible carcinogen.
Cadmium II chloride	0.0064	0.48	88.0	183.3	10108-64-2	C2544	>99.0%	May cause cancer. Harmful if swallowed. Prolonged exposure through inhalation or skin contact may cause serious health damage.
p-Phenylene-diamine	0.05	0.74	80.0	108.16	106-50-3	P6001	N/A	Toxic, irritant, possible mutagen.
DL-Propranolol HCl	0.12	1.59	470.4	295.84	3506-09-0	P0884	N/A	Toxic.
Trichlorfon	0.27	1.75	450.5	257.44	52-68-6	T5015	N/A	Toxic by inhalation. May cause sensitization by skin contact.
Ibuprofen	0.52	4.89	1008.9	206.31	15687-27-1	I4883	N/A	Harmful if swallowed. Possible risk of harm to unborn child.
Nalidixic acid	1.5	5.81	1349.4	232.26	389-08-2	N8878	N/A	Possible risk of harm to unborn child. May cause sensitization by inhalation, skin contact.
Salicylic acid	3.38	6.45	890.9	138.13	69-72-7	S6271	>99.0%	May cause harm to unborn child. Harmful if swallowed. Irritating to eyes, respiratory system, skin.
Antipyrene	11.6	9.56	1799.7	188.25	60-80-0	A5882	N/A	Irritant.
Dimethyl formamide	114	38.3	2800.1	73.11	68-12-2	D8654	>99.8%	Irritant, teratogen.
Glycerol	624	137	12,691.1	92.11	56-81-5	G8773	>99%	Irritating to eyes, skin.

3.3 Results Obtained with the Recommended Reference Chemicals in Two Standard Tests for Basal Cytotoxicity with Human and Rodent Cells

The approach of using the RC regression (i.e., the RC prediction model) to estimate an LD_{50} using data from a qualified cytotoxicity test was based on experience with comparable data obtained with various basal cytotoxicity tests (provided they followed the principles described previously). To convince even skeptical readers that cytotoxicity data for a small number of well selected reference chemicals would provide a candidate regression sufficiently comparable to the RC regression, the Institute for In Vitro Sciences (IIVS, Gaithersburg, MD) subsequently tested the 11 reference chemicals recommended in Section 3.2 using two candidate NRU test protocols (see SOPs in Appendices C and D). The cells used in this exercise were NHK obtained commercially from Clonetics Corp (Walkersville, MD, USA) and mouse BALB/c 3T3 clone A31 cells. Each of the 11 reference chemicals was tested in three independent test trials with each of the two cell types.

The outcomes of the experiments are shown in Figure 3 for the NHK and in Figure 4 for the BALB/c 3T3. Both figures depict the RC regression ± log 5 interval (black lines) and the 11 reference chemicals (triangles). Other chemicals from the RC were omitted for clarity. The new IC_{50} values (means of the three trials) obtained with the NHK NRU test (Figure 3), or 3T3 NRU test (Figure 4) are shown (squares), as well as the new linear regression lines determined from these data (gray dashed line). The new regression lines obtained with NHK and 3T3 cells are within the ± log 5 interval of the RC, and, though slightly steeper, are almost parallel to the RC regression function. Thus, intercepts and regression coefficients of the experimentally obtained new regressions do not differ significantly from the literature-based RC regression equation:

RC regression:
$\log (LD_{50}) = 0.435 \times \log (IC_{50x}) + 0.625$

New NHK NRU regression:
$\log (LD_{50}) = 0.498 \times \log (IC_{50}) + 0.551$
$[R^2 = 0.9356]$

New 3T3 NRU regression:
$\log (LD_{50}) = 0.506 \times \log (IC_{50}) + 0.475$
$[R^2 = 0.9848]$

As expected, due to selection of reference chemicals with data points close to the RC regression, the determination coefficients (R^2) of the new NHK and 3T3 regressions are very high.

In conclusion, by testing only 11 well selected reference chemicals from the RC, both the NHK NRU and 3T3 NRU tests yielded regression equations very close to the regression equation of the RC. Thus, both candidate cytotoxicity tests met the acceptance criteria of a test for basal cytotoxicity defined by Spielmann et al. (1999).

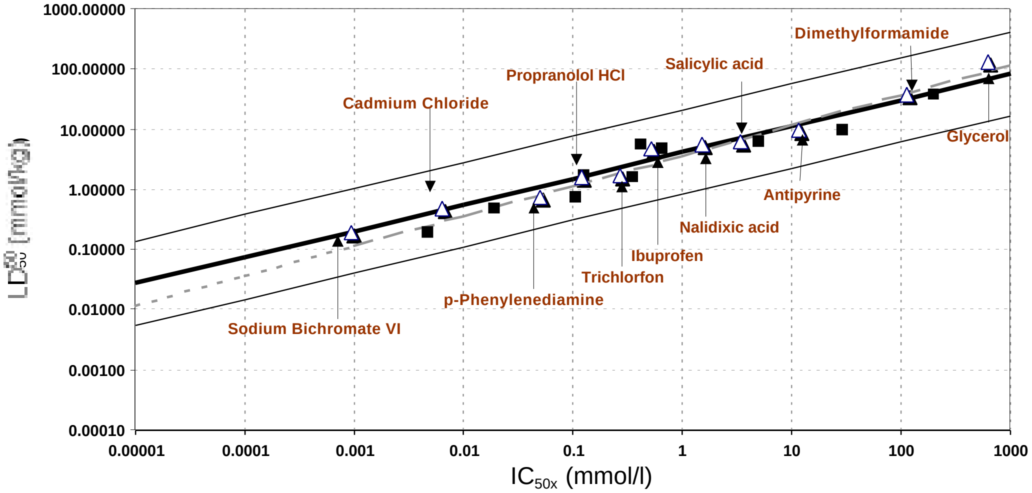

Figure 3. Regression obtained by testing the recommended reference chemicals from the RC with human keratinocytes in the NHK NRU cytotoxicity test

Figure shows the RC prediction regression (black bold line) ± log 5 interval (black thin lines) and the 11 reference chemicals (triangles). The new IC_{50} /LD_{50} points obtained with the NHK NRU test are shown (black squares) with the new linear regression line determined from these data (gray dashed line).

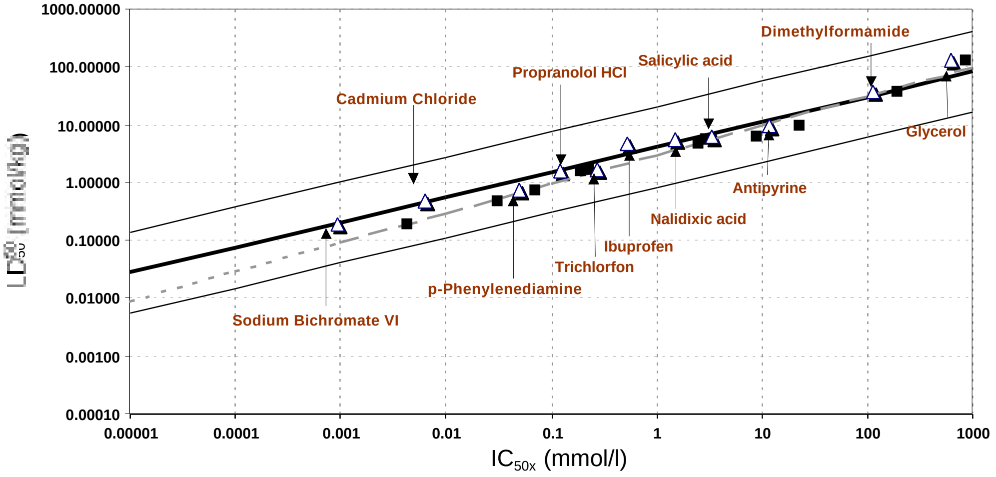

Figure 4. Regression obtained by testing the recommended reference chemicals from the RC with mouse fibroblasts in the BALB/c 3T3 NRU cytotoxicity test.

Figure shows the RC prediction regression (black bold line) ± log 5 interval (black thin lines) and the 11 reference chemicals (triangles). The new IC_{50} /LD_{50} points obtained with the BALB/c 3T3 NRU test are shown (black squares) with the new linear regression line determined from these data (gray dashed line).

4.0 RECOMMENDED BASAL CYTOTOXICITY TESTS: BALB/C 3T3 AND NORMAL HUMAN KERATINOCYTE (NHK) NEUTRAL RED UPTAKE (NRU) TESTS

4.1 Validation Status of the 3T3 NRU Test

The BALB/c 3T3 NRU test is probably the cytotoxicity test that has been used most frequently in formal validation programs, all of which were aimed at evaluation of cytotoxicity in predicting eye irritancy. Large-scale studies to be mentioned here are Phases I, II, and III of the Cosmetic, Toiletry, and Fragrance Association (CTFA) validation program (Gettings et al., 1991, 1992, 1994a, 1994b); the German eye irritation validation study (Spielmann et al., 1991, 1993, 1996); the European Commission/British Home Office (EC/HO) eye irritation validation study (Balls et al., 1995); and the European Cosmetic Toiletry and Perfumery Association (COLIPA) eye irritation study (Brantom et al., 1997). The 3T3 NRU Phototoxicity Test is a modification of the BALB/c 3T3 NRU test and involves a shorter chemical exposure and the additional application of light. The 3T3 NRU Phototoxicity Test has been fully validated (Spielmann et al., 1998a,b) and has gained regulatory acceptance.

For the purpose of evaluating the NRU test, and specifically the BALB/c 3T3 NRU test, as a standard test for basal cytotoxicity, all results available from these studies regarding the reliability (reproducibility within and between laboratories and over time) should be used to avoid wasting resources in repeating the establishment of reliability. Section 4.2 contains an example of establishing reliability of the BALB/c 3T3 NRU test from one of these studies.

4.2 Reliability of the 3T3 NRU Test

To establish interlaboratory reproducibility in the first phase of the German eye irritation validation study (Spielmann et al., 1991), 32 chemicals were tested in 12 laboratories using two tests: the hen's egg test-chorioallantoic membrane (HET-CAM) and the BALB/c 3T3 NRU test. (NRU tests using 3T3 cells were done in accord with the SOP presented in Appendix C.) Five independent repeat tests were conducted per laboratory. Of these 32 chemicals, three compounds [n-hexane, aluminum hydroxide, and di-(2-ethyl-hexyl)phthalate] showed unacceptably high inter-laboratory variability. For the other 29 chemicals, interlaboratory variability was acceptable (Table 2). Interlaboratory reproducibility was assessed with a standard procedure recommended by ISO 5725 (a program for analysis and reporting of proficiency tests and method evaluation studies). ISO 5725 describes reproducibility as an estimate of the limit below which the absolute value of the difference between two results determined in two different laboratories can be expected to fall, with a probability of 95%. The value tabulated in the far right column in Table 2 represents the span of about four standard deviations.

Table 2. Interlaboratory reproducibility of the 3T3 NRU cytotoxicity test determined according to ISO 5725 in 12 laboratories for 29 chemicals[a]

Substance	CAS No.	NR_{50} [b] (mg/ml)	Interlaboratory reproducibility[c] (mg/ml)
Dimethylsulphoxide	67-68-5	44.06	18.36
Propylene glycol	57-55-6	36.27	25.40
Acetone	67-64-1	18.41	14.74
Ethanol	64-17-5	18.01	14.69
Acetonitrile	75-056-8	13.72	15.38
Sodium chloride	7647-14-5	7.74	3.66
Thiourea	62-56-6	6.41	5.49
2-Butoxyethanol	111-76-2	5.43	8.73
Nicotinamide	98-92-0	5.36	5.78
Glutamic acid	56-86-0	4.84	2.01
Lactic acid	598-82-3	4.16	1.56
Pyridine	110-86-1	3.71	4.78
Benzoic acid	65-85-0	3.09	1.67
Isobenzoic furano dione	85-44-9	2.47	0.63
Cyclohexanol	108-93-0	1.89	2.07
Toluene	108-88-3	1.72	3.96
Salicylic acid	69-72-7	1.63	2.04
Tin(II) chloride	7772-99-9	1.55	2.35
Nitrobenzene	98-95-3	1.39	1.33
Tetrachlorethene	127-18-4	1.08	2.35
Aniline	62-53-3	1.07	1.25
EDTA-Na salt	13235-36-4	0.95	0.50
Ascorbic acid	50-81-7	0.49	0.81
Phenol	108-95-2	0.35	0.74
Acrylamide	79-06-1	0.29	0.19
Copper (II) sulfate	7758-98-7	0.10	0.05
Sodium lauryl sulfate	151-21-3	0.093	0.09
2-Propane-1-ol	107-18-6	0.05	0.06
Benzalkonium chloride	8001-54-5	0.01	0.01

[a] From Spielmann et al., 1991.

[b] NR_{50}= mean concentration of test substance reducing the viability of cells to 50% of the viability of controls.

[c] ISO 5725 describes reproducibility as an estimate of the limit below which the absolute value of the difference between two results determined in two different laboratories can be expected to fall, with a probability of 95%.

The second phase of the German eye irritation validation study was a blind trial for database development and involved the testing of 150 chemicals (Spielmann et al., 1993, 1996). Each chemical was assigned at random to two of the 12 total laboratories, since reproducibility of the BALB/c 3T3 NRU test was not an issue at this stage of the study. The final publication (Spielmann et al., 1997) on this phase focused on predictivity and test strategies for identification of severe eye irritants. The data from this publication have been re-analyzed for the present guidance document in the following way: since each chemical was tested in two different laboratories, the IC_{50} values obtained in two laboratories were plotted against each other, as shown in Figure 5 for 147 of the 150 chemicals. (Three chemicals had to be excluded because they were not tested according to the SOP.) Note that "Lab 1" represents the total of all participating laboratories, as does "Lab 2". Thus, Figure 5 does not show the comparability of results between two given laboratories. Rather, it shows the comparability of data obtained under routine conditions between randomly selected laboratories performing the BALB/c 3T3 NRU test according to the same SOP.

Results of the correlation analysis shown in Figure 5 are quite promising, since the linear correlation line (black) deviates only slightly from the ideal line (gray line at 45° angle). The linear correlation coefficient of $r = 0.88$ ($R^2 = 0.775$) shows a sufficient comparability of the data. Outliers, where data of the two laboratories differed by more than 1 log, occurred for less than 10% of the chemicals. A predominant reason for these interlaboratory deviations, discussed in Spielmann et al. (1997), was that one laboratory had used an adequate solvent for a test chemical, while the other laboratory had tested the chemical in media at concentrations above the aqueous solubility of the chemical. Thus, concentrations reported by the second laboratory were nominal rather than actual. As a consequence of this experience, later validation studies (Spielmann et al., 1998a,b) emphasized guidance for the use of solvents.

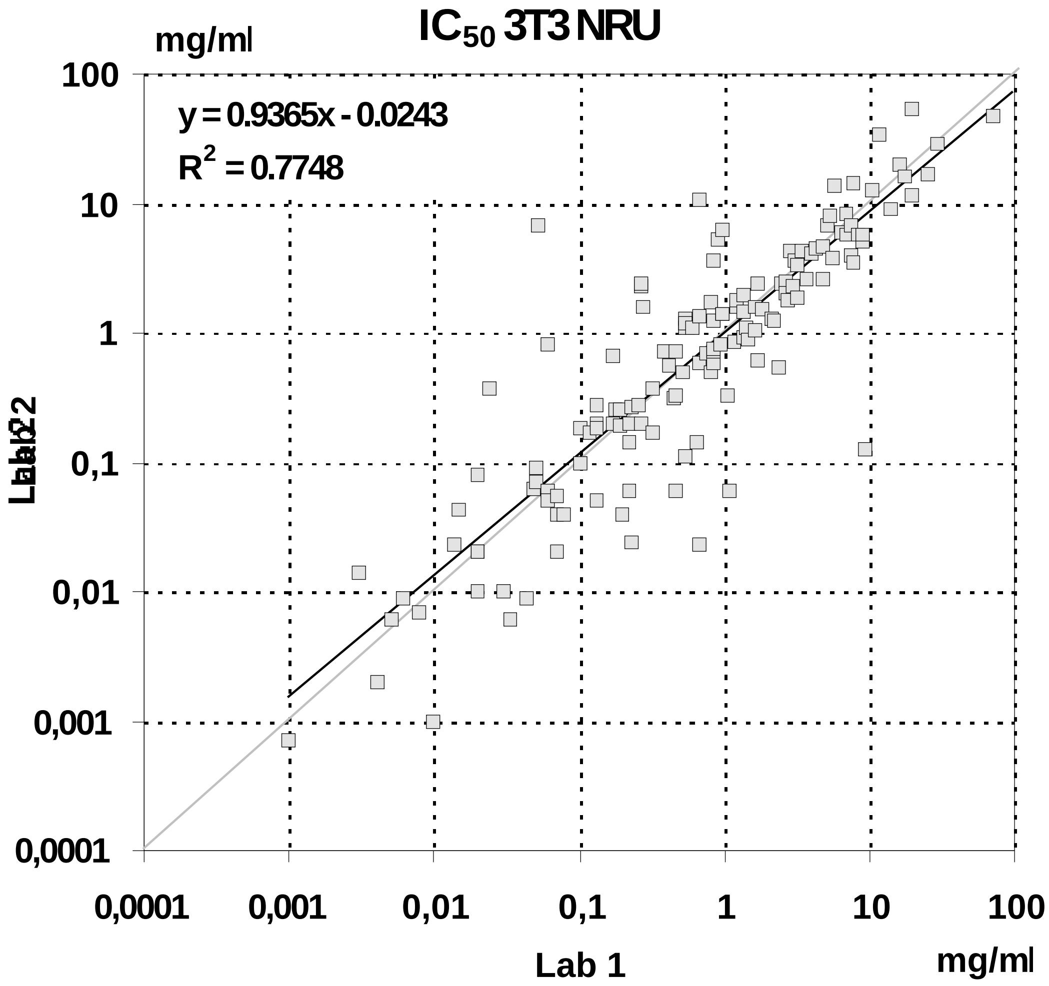

Figure 5. Interlaboratory comparability of the 3T3 NRU cytotoxicity test for 147 test chemicals in 2 different laboratories per chemical.
(Note: see text for explanation of the term "two laboratories per chemical".)

4.3 Validation Status of the NHK NRU Test

Although the NHK NRU test has been used less frequently in validation studies than has the BALB/c 3T3 NRU, the NHK NRU has been evaluated in several studies for its ability to predict eye irritation potential as reflected by Draize scores. It was used in Phases I, II, and III of the CTFA evaluation program (hydroalcoholic formulations, oil-and-water emulsions and surfactants and surfactant-containing formulations) (Gettings et al., 1991, 1994,1996); Phase III of the Soap and Detergent Manufacturers study using primarily neat surfactants and surfactant-containing formulations

(Bagley et al., 1994); as well as an independent study of surfactants and surfactant-containing formulations (Triglia et al., 1989). Many of these studies were subsequently reviewed by the Interagency Regulatory Alternatives Group, as part of a workshop review to evaluate the results of voluntary data submissions of *in vitro* methods to predict Draize scores (Harbell et al., 1997).

Gettings et al. (1996) evaluated the results of 34 different *in vitro* assays in testing 25 surfactant-based formulations for the prediction of Draize scores. The *in vitro* tests were ranked by discordance and separation index (i.e., the ability of the test to rank the toxicity of the 25 chemicals with the same relative rank as the Draize test). The NHK NRU test was not among the *in vitro* tests with the lowest discordance and highest separation index. Triglia et al. (1989), testing 12 surfactant-based formulations, suggested that sensitivity and specificity of the NHK NRU were sufficient for the test to be used as a screening tool as part of a battery of *in vitro* tests. Likewise, Harbell et al. (1997), in evaluating six data sets containing 9-45 surfactant or surfactant-containing materials, concluded that the NHK NRU had sufficient performance in predicting Draize scores that the assay could be used as a screen or adjunct over the range of toxicities found in personal care and household products.

4.4 Reliability of the NHK NRU Test

The reliability of the NHK NRU assay has been less well documented than that of the 3T3 NRU assay; however several reports have described the intralaboratory and interlaboratory variability of the test. Triglia et al. (1989) reported that 10 cytotoxicity trials in a single laboratory using the surfactant sodium lauryl sulfate (SLS) at five different concentrations produced coefficients of variation (CVs) <18% for all but the lowest concentration. (The average NRU_{50} [i.e., concentration reducing NRU to 50 % of control value] from one laboratory in these trials was 4.4 µg/ml; twelve years later the same laboratory has an average NRU_{50} for SLS of 4.4 +/- 0.97 µg/ml). Triglia et al. (1989) also reported interlaboratory variability for 12 compounds replicated in four laboratories. The interlaboratory CVs for the NRU_{50} means ranged from 19% - 60%. More recently, as part of the Interagency Regulatory Alternatives Group evaluation, Harbell et al. (1997) analyzed data from two laboratories that tested 22 materials in a blind fashion. NRU_{50} values for these materials showed an excellent interlaboratory correlation of 0.99. Dickson et al. (1993) also reported on variability for the NHK NRU assay and found that the NRU_{50} values for SLS tested in four different keratinocyte isolates were nearly identical at 66.7, 67.5, 70.9 and 73.4 µg/ml. Dickson et al. (1993) used a 24 h exposure rather than the 48 h exposure used for the other tests described in Sections 4.3 and 4.4.

5.0 CONCLUSION

This document provides guidance for using *in vitro* basal cytotoxicity assays to reduce the number of animals required for the conduct of *in vivo* lethality assays. The recommended approach takes advantage of the relationship between *in vitro* IC_{50}s and *in vivo* LD_{50}s derived from the RC for 347 chemicals (Halle and Spielmann, 1992; Halle, 1998). Detailed protocols for two recommended NRU assays, one using a rodent cell line, BALB/c 3T3 cells, and one using primary human cells, NHK, are included. Guidance is also provided for qualifying these tests, or any other *in vitro* cytotoxicity assay, for use with the RC regression to predict the starting dose for lethality assays.

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APPENDIX A

Registry of Cytotoxicity
List of 347 Chemicals
(Sorted by IC50 and LD50 Values)

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by IC50 (mM)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
2		Actinomycin D	0.0000081	0.0057	7.2
3		Aminopterin	0.000012	0.0068	3.0
132		Triphenyltin hydroxide	0.000049	0.12	44.0
6		Colchicine	0.000054	0.015	6.0
133		Cytochalasin D	0.000092	0.071	36.0
8		Digitoxin	0.00011	0.073	55.8
134		Rotenone	0.00013	0.33	130.2
9		Amethopterin	0.00014	0.3	136.4
10		Emetine	0.00016	0.14	67.3
135		2,3,7,8-Tetrachlorodibenzo-p-dioxin	0.0002	0.00035	0.1
11		Doxorubicin * HCl	0.00033	1.2	696.0
12		Puromycin	0.00033	1.43	674.4
136		Diethyldithiocarbamate sodium* 3H20	0.00039	6.66	1500.7
137		Triethyltin chloride	0.00046	0.021	5.1
138		Tributyltin chloride	0.00054	0.37	120.4
139		Retinol	0.00054	6.98	1999.8
140		6-Thioguanine	0.00057	0.96	160.5
13		Cycloheximide	0.00059	0.0071	2.0
141		Cytosine arabinoside	0.00068	12.9	3137.9
142		Methylmercury chloride	0.00071	0.23	57.7
143		Triethylene melamine	0.00078	0.005	1.0
14		Mitomycin C	0.00084	0.042	14.0
144		Sodium bichromate VI	0.00093	0.19	49.8
15		8-Azaguanine	0.0013	9.86	1500.1
145		Potassium chromate VI	0.0015	0.93	180.6
146		Potassium bichromate VI	0.002	0.65	191.2
16		Azaserine	0.002	0.98	169.7
147		Mitoxantrone	0.0024	1.32	586.8
148		Nitrogen mustard * HCl	0.0026	0.052	10.0
17		5-Fluorouracil	0.0026	1.77	230.3
149		Chromium VI trioxide	0.0027	0.8	80.0
150		Cis-platinum	0.0028	0.086	25.8
151		Hexachlorocyclopentadiene	0.0031	0.41	111.8
152		8-Hydroxyquinoline	0.0033	8.27	1200.6
18		Captan	0.0039	33.3	10009.6
153	26	Arsenic III trioxide	0.0042	0.1	19.8
154		Maneb	0.0042	16.9	4500.6
155		Benzalkonium chloride	0.0052	1.1	401.5
156		Stearyltrimethylammonium chloride	0.006	1.54	536.1
20		Cadmium II chloride	0.0064	0.48	88.0
157	38	Hexachlorophene	0.0079	0.15	61.0
21		6-Mercaptopurine	0.008	1.84	280.0
158		Dichlorophene	0.0083	10	2691.3
22	6	Digoxin	0.0085	0.023	18.0
23		Daraprim	0.0089	0.51	126.9
159		Hexadecyltrimethylammonium bromide	0.0089	1.12	408.3
25		Thio-TEPA	0.011	0.2	37.8
160		N-Methyl-N'-nitro-N-nitrosoguanidine	0.012	0.61	89.7
26		Kelthane	0.012	1.55	574.2
161		Silver I nitrate	0.013	0.29	49.3
27		Chlorpromazine	0.014	0.44	140.3
29	28	Mercury II chloride	0.015	0.0037	1.0
162		Chlorhexidine	0.015	18.2	9200.5
31	41	Chloroquine diphosphate	0.017	1.88	969.9
164		Oxatomide	0.019	3.31	1412.1
163		Cetyltrimethylammonium chloride	0.021	1.31	474.4
165		Isoproterenol * HCl	0.022	8.96	2219.8
166		Triisooctylamine	0.023	4.58	1620.2
33		p-Chloromercuribenzoic acid	0.024	0.07	25.0
167		p,p'-DDD	0.024	0.35	112.0
168		Dicoumarol	0.027	2.11	709.6
169		Epinephrine bitartrate	0.028	0.012	4.0
170	29	Thioridazine * HCl	0.029	0.88	358.2
35		Flufenamic acid	0.029	0.97	272.8
171		Fumagillin	0.031	4.36	1999.5
37		Aflatoxin B1	0.034	0.016	5.0
172		Nabam	0.035	1.54	394.8
173	39	Pentachlorophenol	0.036	0.19	50.6
174		Ambazone	0.038	3.16	749.9
175		Norepinephrine	0.039	0.12	20.3
176		Papaverine	0.045	0.96	325.8
177		Busulphan	0.046	0.0076	1.9
178		Salicylanilide	0.046	11.3	2409.7

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by IC50 (mM)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
179		Acrolein	0.047	0.82	46.0
180		p-Phenylenediamine	0.05	0.74	80.0
181	30	Thallium I sulfate	0.054	0.057	28.8
38		Imipramine * HCl	0.054	0.96	304.2
182		Triton X-100	0.055	2.78	1798.7
39		2,4-Dichlorophenol	0.055	3.56	580.3
183	5	Amitriptyline	0.056	1.15	319.1
184		Butylated hydroxytoluene	0.056	4.04	890.4
185		Heptachlor	0.059	0.11	41.1
186		Zineb	0.059	18.9	5211.3
40		Chlordan	0.06	1.12	458.9
41		Chloroquine sulfate	0.06	2.6	1086.8
42		p-Aminophenol	0.062	15.2	1658.9
187		4-Hexylresorcinol	0.064	2.83	549.9
43		Aldrin	0.067	0.11	40.1
44		Hydroxyzine * HCl	0.067	2.31	950.4
188		t-Butyl hydroquinone	0.069	4.81	799.6
189		Antimycin	0.07	0.45	112.6
45		Quinine * HCl	0.075	1.72	620.8
190		Chlorambucil	0.076	0.25	76.1
191		Dimenhydrinate	0.076	2.81	1320.8
192		1,3-Bis(2-chloroethyl)- 1-nitrosourea	0.078	0.093	19.9
193		5-Azacytidine	0.079	2.34	571.5
47		Naftipramide	0.084	3.45	1029.7
48		Mefenamic acid	0.087	3.27	789.1
49		Parathion	0.093	0.0069	2.0
194		p-Toluylendiamine	0.094	0.83	101.4
50		Trypan blue	0.095	6.43	6204.2
195		p,p'-DDA	0.099	2.1	590.4
196	40	VerapamilHCl	0.1	0.22	108.0
197		p,p'-DDE	0.1	2.77	880.9
51		Disulfoton	0.11	0.0073	2.0
198		Ioxynil	0.11	0.3	111.3
199		Cupric chloride	0.11	1.04	139.8
52		all-trans-Retinoic acid	0.11	6.66	2001.2
200		Dimethylaminoethyl methacrylate (polymer)	0.11	11.1	1745.4
53	43	Quinidine sulfate	0.12	1.08	456.3
54	23	Propranolol * HCl	0.12	1.59	470.4
201		13-cis-Retinoic acid	0.12	11.3	3395.4
202		Formaldehyde	0.12	26.6	798.8
55		Zinc II chloride	0.13	2.57	350.2
56		Manganese II chloride *4 H2O	0.13	7.5	1484.4
57		L-Dopa	0.13	9.03	1780.8
203		Thallium I acetate	0.14	0.13	34.2
204		Azathioprine	0.14	1.93	535.2
58		Dihydralazine sulfate	0.14	2.84	818.8
59		Tetracycline * HCl	0.14	13.4	6444.6
205		Versalide	0.15	1.22	315.3
60		Indomethacin	0.16	0.034	12.2
61		p,p'-DDT	0.16	0.32	113.4
62		Cobalt II chloride	0.16	0.62	80.5
206		Diquat dibromide	0.16	0.67	230.5
63	4	Diazepam	0.16	2.49	709.1
207		Dieldrin	0.18	0.12	45.7
64		Bendiocarb	0.18	0.8	178.6
208		Undecylenic acid	0.18	13.6	2506.6
209		Propylparaben	0.18	35.1	6325.7
65		Oxyphenbutazone	0.19	3.08	999.2
210		p-Nitrophenol	0.2	2.52	350.6
67	15	Malathion	0.2	2.68	885.4
211		Catechol	0.2	35.3	3887.2
68		2,4-Dinitrophenol	0.21	0.16	29.5
69		Secobarbital sodium	0.21	0.48	124.9
70	49	Atropine sulfate	0.22	0.92	622.7
212		p-Cresol	0.22	1.91	206.6
213		Ammonium persulfate	0.23	3.59	819.3
214		Thymol	0.23	6.52	979.6
71		Diphenhydramine * HCl	0.24	2.93	855.1
215		Chlorotetracycline	0.24	5.22	2500.0
72		Butylated hydoxyanisole	0.24	12.2	2199.3
216		Refortan	0.25	10.1	3162.3
73		Carbaryl	0.26	1.24	249.5
74		Nickel II chloride	0.27	0.81	105.0

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by IC50 (mM)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
75		Trichlorfon	0.27	1.75	450.5
76		Sodium dodecyl sulfate	0.27	4.45	1288.0
217		Amrinone	0.28	0.54	101.1
218		o-Phenylenediamine	0.31	9.89	1069.7
78		6-Methylcoumarin	0.31	10.5	1681.9
79		Phenylbutazone	0.32	1.22	376.3
80		2-Thiouracil	0.32	7.8	999.6
219		Hydralazine	0.33	0.56	89.7
81	27	Cupric sulfate * 5 H2O	0.33	1.2	299.6
238		Imidazolidinyl urea	0.36	9.34	2598.9
220		m-Dinitrobenzene	0.39	0.49	82.4
82	44	Diphenylhydantoin	0.39	0.79	199.3
221		2-Nitro-p-phenylenediamine	0.39	20.1	3078.5
222		Glibenclamide	0.4	6.58	3250.8
223	32	Lindane	0.41	0.26	75.6
224		n-Butyl benzoate	0.41	28.8	5133.6
225		Ammonium sulfide	0.42	3.29	168.2
226		Dodecylbenzene sodiumsulfonate	0.42	3.62	1261.6
227	46	Sodium oxalate	0.44	1.16	155.4
228		2,4,5-Trichlorophenoxyacetic acid	0.44	1.17	298.9
229	22	Dextropropoxyphene * HCl	0.49	0.22	82.7
230	42	Orphenadrine * HCl	0.49	1.39	425.2
231		Tween 80	0.49	19.1	25021.0
232		o-Cresol	0.52	1.12	121.1
233		Ibuprofen	0.52	4.89	1008.9
234		Phenylthiourea	0.54	0.02	3.0
235	25	Paraquat	0.54	0.31	57.7
83		Thiopental	0.55	2.48	601.1
84		Amobarbital	0.56	1.52	344.0
236		Hydrogen peroxide 90%	0.56	58.8	2000.4
85		Metamizol	0.58	21.5	7189.2
237		Beryllium II sulfate	0.61	0.78	82.0
239		m-Cresol	0.66	2.24	242.3
240		Pentoxifylline	0.66	4.98	1386.2
86	31	Warfarin	0.67	1.05	323.8
241		Sodium azide	0.71	0.69	44.9
87		Pentobarbital sodium	0.71	0.81	201.1
242		1,2,4-Trichlorobenzene	0.71	4.17	756.6
243		p-Anisidine	0.73	11.4	1404.1
244		Doxylamine succinate	0.75	1.21	470.1
88		Dibutyl phthalate	0.76	43.1	11998.2
89	16	2,4-Dichlorophenoxyacetic acid	0.77	1.67	369.1
90		Iproniazid	0.79	2.04	365.7
91	45	Chloramphenicol	0.79	10.5	3393.1
245		Resorcinol	0.8	2.73	300.6
246	37	Barium II nitrate	0.81	1.36	355.4
247		(+)-Thalidomide	0.81	1.55	400.3
92		Di(2-ethylhexyl)phthalate	0.84	79.4	31015.2
93		Sulfisoxazole	0.85	25.4	6790.2
248		m-Aminophenol	0.86	15.2	1658.9
94		Menthol	0.95	20.3	3172.9
249		3-Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)	0.96	1.3	346.2
250		Valproate sodium	1	10.2	1695.4
251		Scopolamine * HBr	1.08	3.3	1268.2
95		Salicylamide	1.08	13.8	1892.7
252	19	Potassium cyanide	1.12	0.15	9.8
96		Cygon	1.24	0.66	151.3
97		Phenacetin	1.27	9.21	1650.8
253		Isoxepac	1.33	0.74	198.5
254		Buflomedil	1.35	1.19	365.8
98		Methylparaben	1.42	11.5	1749.8
255		Sodium monochloroacetate	1.45	0.65	75.7
99		Nalidixic acid	1.5	5.81	1349.4
256		Tin II chloride	1.51	3.69	699.6
257		Isononylaldehyde	1.52	22.8	3243.8
100		L-Ascorbic acid	1.52	67.6	11907.1
101		Glutethimide	1.56	2.76	599.7
102		Acrylamide	1.61	2.39	169.9
258		Diethyl sebacate	1.63	56	14470.4
259		Methyl salicylate	1.7	5.83	887.1
260		Coumarin	1.71	2	292.3
103	18	Nicotine	1.79	0.31	50.3

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by IC50 (mM)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
104		Tolbutamide	1.81	9.62	2601.1
105	21	Theophylline	1.83	3.33	600.0
261	3	Ferrous sulfate	1.85	2.1	319.0
106	14	Sodium I fluoride	1.85	4.29	180.1
262	47	Amphetamine sulfate	1.97	0.15	55.3
107	2	Acetylsalicylic acid	2.27	5.55	999.9
108		Gibberellic acid	2.3	18.2	6304.7
109		Frusemide	2.33	7.86	2599.8
110		Acrylonitrile	2.42	1.54	81.7
263		Acetaldehyde	2.45	43.8	1929.8
111		Clofibric acid	2.61	5.82	1249.3
112	48	Caffeine	2.64	0.99	192.3
264		Chloral hydrate	2.65	2.9	479.7
113	1	Acetaminophen	2.71	15.9	2403.8
265		Streptomycin sulfate	2.73	0.34	495.6
114		Natulan * HCl	2.74	3.04	783.7
266		Potassium hexacyanoferrate III	2.82	9.02	2970.0
267		p-Hydroxybenzoic acid	2.92	15.9	2196.3
115	12	Phenol	3.01	4.4	414.1
268		1-Octanol	3.06	13.7	1784.6
116		Cyclophosphamide * H2O	3.12	0.34	94.9
269		Potassium I fluoride	3.13	4.22	245.2
117		Di(2-ethylhexyl)adipate	3.15	24.6	9117.7
270		Propionaldehyde	3.25	24.3	1411.6
271		Styrene	3.3	48	4999.7
272		Salicylic acid	3.38	6.45	890.9
273		Bromobenzene	3.46	17.2	2700.7
274		L-Cysteine	3.56	5.45	660.4
275		Nitrilotriacetic acid	3.61	7.69	1470.0
276		Ambuphylline	3.67	2.23	600.7
118	24	Phenobarbital	3.81	0.7	162.6
277		Potassium cyanate	4.14	10.4	843.6
278		Phenylephrine * HCl	4.16	1.72	350.3
279		Thioacetamide	4.17	4.01	301.3
280		Theophylline sodium acetate	4.19	2.22	582.2
281		1,2-Dibromomethane	4.2	0.62	107.8
119		Sodium salicylate	4.33	9.99	1599.5
282		(-)-Phenylephrine	4.45	2.09	349.5
283		Milrinone	4.77	0.43	90.8
120		5-Aminosalicylic acid	5.07	50.6	7749.4
121		Aminophenazone	5.39	4.32	999.3
284		Ammonium chloride	5.52	30.8	1647.8
122		Diethyl phthalate	5.52	38.7	8601.5
285		Caffeine sodium benzoate	5.67	2.54	859.4
286		Benzylpenicillin sodium	5.73	19.4	6914.2
287		Benzylalcohol	5.81	11.4	1232.9
288		1-Heptanol	6.25	28	3254.4
289		Tetrachloroethene	6.54	53.4	8854.8
290		Sodium sulfite	6.78	6.51	820.5
291		Aniline	6.9	4.72	439.6
292		Allylalcohol	6.94	1.1	63.9
293		Diisopropylamine dichloroacetate	7	7.39	1700.9
123	35	Isoniazid	7.49	4.74	650.1
294		Trichloroacetic acid	8.19	30.6	4999.4
295		2,5-Hexanedione	8.45	23.7	2705.6
124		Acetazolamide	8.49	19.3	4289.6
125	34	Carbon tetrachloride	8.51	18.2	2799.3
296		Homatropine methylbromide	9	3.24	1199.9
297	11	1,1,1-Trichloroethane	10.3	77.2	10298.5
298		Dichloroacetic acid	11.5	21.9	2823.8
299		Imidazole	11.5	27.6	1879.3
300		Antipyrine	11.6	9.56	1799.7
301	17	Xylene	12	40.5	4300.3
302		Nitrobenzene	12.2	5.2	640.2
303		Theophylline sodium	12.4	2.19	445.0
304		Calcium II chloride	12.4	9.01	999.9
305		n-Butanal	12.8	34.5	2488.1
306		Anisole	13.2	34.2	3698.7
307		2-Ethylbutanal	13.2	39.7	3977.1
308	33	Chloroform	13.4	7.61	908.4
309		Isobutanal	13.5	39	2812.7
126		Triethyl citrate	14.7	25.3	6990.9
310		Tributylamine	15.4	2.91	539.5

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by IC50 (mM)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
311		1-Hexanol	15.4	7.04	719.5
312		Benzoic acid	15.7	20.7	2528.1
313		Xanthinol nicotinate	15.8	32.5	14121.6
314		Saccharin	16.4	92.8	17000.0
315		Isobenzoic furano dione	17	27.1	4014.1
316		Toluene	17.1	54.3	5003.7
317		Barbital sodium	18.6	3.88	800.1
318		Trifluoroacetic acid	20.5	1.75	199.6
127		Dimethyl phthalate	23.4	35.5	6894.1
319		Methylpentinol	23.8	5.35	525.2
320		N,N-Dimethylacetamide	24.2	58.4	5089.0
321		Acetic acid	24.3	55.1	3309.3
322		1-Pentanol	24.9	34.4	3033.0
323		Urethan	25.9	28.1	2504.0
324		2-Butoxyethanol	26	12.5	1477.5
325		Cyclohexanol	26.3	20.6	2063.7
326		Halothane	31.1	28.8	5684.8
327	20	Lithium I sulfate	33.7	10.8	1187.4
328	36	Dichloromethane	34.9	18.8	1596.7
329		Sodium cyclamate	35.4	75.8	15254.0
330		Sulfuric acid	36	21.8	2138.1
331		Strontium II chloride	36.4	14.2	2251.0
332		1,4-Dioxane	38.1	47.7	4203.3
333		Lithium I chloride	38.6	17.9	758.8
334		Isobutanol	40.1	33.2	2461.4
335		Potassium hexacyanoferrate II	42.3	17.4	6409.6
336		Nicotinamide	44.4	28.7	3505.4
337		Pyridine	46.9	11.3	893.9
338		1-Butanol	52.5	10.7	793.3
339		1-Nitropropane	57.9	5.11	455.4
340		Diethylene glycol	62.1	139	14753.5
341		Lactic acid	66	41.4	3729.7
342		Piperazine	67.2	22.1	1904.1
343		Magnesium II chloride * 6 H2O	70.4	39.8	8092.5
344	13	Sodium chloride	75.9	51.3	2998.0
345		Sodium I bromide	77.4	33.4	3504.3
346	50	Potassium I chloride	82	34.9	2601.8
347		Thiourea	86	1.64	124.9
348		1-Propanol	96.5	89.8	5397.9
349		Ethyl methyl ketone	104	47.1	3396.9
350		Tetrahydrofurfuryl alcohol	111	24.5	2502.7
351		Dimethylformamide	114	38.3	2800.1
352		1,2,6-Hexanetriol	123	119	15969.8
353		Ethyl acetate	128	125	11015.0
128	10	2-Propanol	167	97.2	5842.7
354		1,3,5-Trioxane	213	8.88	800.0
355		D-Glucose	226	143	25765.7
356		2-Methoxyethanol	251	32.3	2458.4
129		Dimethyl sulfoxide	252	252	19691.3
357		Propylene glycol	342	263	20016.9
358		Acetonitrile	368	92.5	3798.1
130	9	Ethanol	379	304	14008.3
359		Acetone	444	168	9759.1
360	7	Ethylene glycol	555	138	8567.0
131		Glycerol	624	137	12619.1
361	8	Methanol	930	406	13012.3

Reference

Halle, W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD50) zur Einsparung von Tierversuchen. Life Sciences/Lebenswissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich.

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by LD50 (mg/kg)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
135		2,3,7,8-Tetrachlorodibenzo-p-dioxin	0.0002	0.00035	0.1
29	28	Mercury II chloride	0.015	0.0037	1.0
143		Triethylene melamine	0.00078	0.005	1.0
177		Busulphan	0.046	0.0076	1.9
13		Cycloheximide	0.00059	0.0071	2.0
51		Disulfoton	0.11	0.0073	2.0
49		Parathion	0.093	0.0069	2.0
3		Aminopterin	0.000012	0.0068	3.0
234		Phenylthiourea	0.54	0.02	3.0
169		Epinephrine bitartrate	0.028	0.012	4.0
37		Aflatoxin B1	0.034	0.016	5.0
137		Triethyltin chloride	0.00046	0.021	5.1
6		Colchicine	0.000054	0.015	6.0
2		Actinomycin D	0.0000081	0.0057	7.2
252	19	Potassium cyanide	1.12	0.15	9.8
148		Nitrogen mustard * HCl	0.0026	0.052	10.0
60		Indomethacin	0.16	0.034	12.2
14		Mitomycin C	0.00084	0.042	14.0
22	6	Digoxin	0.0085	0.023	18.0
153	26	Arsenic III trioxide	0.0042	0.1	19.8
192		1,3-Bis(2-chloroethyl)- 1-nitrosourea	0.078	0.093	19.9
175		Norepinephrine	0.039	0.12	20.3
33		p-Chloromercuribenzoic acid	0.024	0.07	25.0
150		Cis-platinum	0.0028	0.086	25.8
181	30	Thallium I sulfate	0.054	0.057	28.8
68		2,4-Dinitrophenol	0.21	0.16	29.5
203		Thallium I acetate	0.14	0.13	34.2
133		Cytochalasin D	0.000092	0.071	36.0
25		Thio-TEPA	0.011	0.2	37.8
43		Aldrin	0.067	0.11	40.1
185		Heptachlor	0.059	0.11	41.1
132		Triphenyltin hydroxide	0.000049	0.12	44.0
241		Sodium azide	0.71	0.69	44.9
207		Dieldrin	0.18	0.12	45.7
179		Acrolein	0.047	0.82	46.0
161		Silver I nitrate	0.013	0.29	49.3
144		Sodium bichromate VI	0.00093	0.19	49.8
103	18	Nicotine	1.79	0.31	50.3
173	39	Pentachlorophenol	0.036	0.19	50.6
262	47	Amphetamine sulfate	1.97	0.15	55.3
8		Digitoxin	0.00011	0.073	55.8
235	25	Paraquat	0.54	0.31	57.7
142		Methylmercury chloride	0.00071	0.23	57.7
157	38	Hexachlorophene	0.0079	0.15	61.0
292		Allylalcohol	6.94	1.1	63.9
10		Emetine	0.00016	0.14	67.3
223	32	Lindane	0.41	0.26	75.6
255		Sodium monochloroacetate	1.45	0.65	75.7
190		Chlorambucil	0.076	0.25	76.1
149		Chromium VI trioxide	0.0027	0.8	80.0
180		p-Phenylenediamine	0.05	0.74	80.0
62		Cobalt II chloride	0.16	0.62	80.5
110		Acrylonitrile	2.42	1.54	81.7
237		Beryllium II sulfate	0.61	0.78	82.0
220		m-Dinitrobenzene	0.39	0.49	82.4
229	22	Dextropropoxyphene * HCl	0.49	0.22	82.7
20		Cadmium II chloride	0.0064	0.48	88.0
219		Hydralazine	0.33	0.56	89.7
160		N-Methyl-N'-nitro-N-nitrosoguanidine	0.012	0.61	89.7
283		Milrinone	4.77	0.43	90.8
116		Cyclophosphamide * H2O	3.12	0.34	94.9
217		Amrinone	0.28	0.54	101.1
194		p-Toluylendiamine	0.094	0.83	101.4
74		Nickel II chloride	0.27	0.81	105.0
281		1,2-Dibromomethane	4.2	0.62	107.8
196	40	VerapamilHCl	0.1	0.22	108.0
198		Ioxynil	0.11	0.3	111.3
151		Hexachlorocyclopentadiene	0.0031	0.41	111.8
167		p,p'-DDD	0.024	0.35	112.0
189		Antimycin	0.07	0.45	112.6
61		p,p'-DDT	0.16	0.32	113.4
138		Tributyltin chloride	0.00054	0.37	120.4
232		o-Cresol	0.52	1.12	121.1

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by LD50 (mg/kg)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
347		Thiourea	86	1.64	124.9
69		Secobarbital sodium	0.21	0.48	124.9
23		Daraprim	0.0089	0.51	126.9
134		Rotenone	0.00013	0.33	130.2
9		Amethopterin	0.00014	0.3	136.4
199		Cupric chloride	0.11	1.04	139.8
27		Chlorpromazine	0.014	0.44	140.3
96		Cygon	1.24	0.66	151.3
227	46	Sodium oxalate	0.44	1.16	155.4
140		6-Thioguanine	0.00057	0.96	160.5
118	24	Phenobarbital	3.81	0.7	162.6
225		Ammonium sulfide	0.42	3.29	168.2
16		Azaserine	0.002	0.98	169.7
102		Acrylamide	1.61	2.39	169.9
64		Bendiocarb	0.18	0.8	178.6
106	14	Sodium I fluoride	1.85	4.29	180.1
145		Potassium chromate VI	0.0015	0.93	180.6
146		Potassium bichromate VI	0.002	0.65	191.2
112	48	Caffeine	2.64	0.99	192.3
253		Isoxepac	1.33	0.74	198.5
82	44	Diphenylhydantoin	0.39	0.79	199.3
318		Trifluoroacetic acid	20.5	1.75	199.6
87		Pentobarbital sodium	0.71	0.81	201.1
212		p-Cresol	0.22	1.91	206.6
17		5-Fluorouracil	0.0026	1.77	230.3
206		Diquat dibromide	0.16	0.67	230.5
239		m-Cresol	0.66	2.24	242.3
269		Potassium I fluoride	3.13	4.22	245.2
73		Carbaryl	0.26	1.24	249.5
35		Flufenamic acid	0.029	0.97	272.8
21		6-Mercaptopurine	0.008	1.84	280.0
260		Coumarin	1.71	2	292.3
228		2,4,5-Trichlorophenoxyacetic acid	0.44	1.17	298.9
81	27	Cupric sulfate * 5 H2O	0.33	1.2	299.6
245		Resorcinol	0.8	2.73	300.6
279		Thioacetamide	4.17	4.01	301.3
38		Imipramine * HCl	0.054	0.96	304.2
205		Versalide	0.15	1.22	315.3
261	3	Ferrous sulfate	1.85	2.1	319.0
183	5	Amitriptyline	0.056	1.15	319.1
86	31	Warfarin	0.67	1.05	323.8
176		Papaverine	0.045	0.96	325.8
84		Amobarbital	0.56	1.52	344.0
249		3-Cyano-2-morpholino-5-(pyrid-4-yl)-pyridine (Chemical 122)	0.96	1.3	346.2
282		(-)-Phenylephrine	4.45	2.09	349.5
55		Zinc II chloride	0.13	2.57	350.2
278		Phenylephrine * HCl	4.16	1.72	350.3
210		p-Nitrophenol	0.2	2.52	350.6
246	37	Barium II nitrate	0.81	1.36	355.4
170	29	Thioridazine * HCl	0.029	0.88	358.2
90		Iproniazid	0.79	2.04	365.7
254		Buflomedil	1.35	1.19	365.8
89	16	2,4-Dichlorophenoxyacetic acid	0.77	1.67	369.1
79		Phenylbutazone	0.32	1.22	376.3
172		Nabam	0.035	1.54	394.8
247		(+)-Thalidomide	0.81	1.55	400.3
155		Benzalkonium chloride	0.0052	1.1	401.5
159		Hexadecyltrimethylammonium bromide	0.0089	1.12	408.3
115	12	Phenol	3.01	4.4	414.1
230	42	Orphenadrine * HCl	0.49	1.39	425.2
291		Aniline	6.9	4.72	439.6
303		Theophylline sodium	12.4	2.19	445.0
75		Trichlorfon	0.27	1.75	450.5
339		1-Nitropropane	57.9	5.11	455.4
53	43	Quinidine sulfate	0.12	1.08	456.3
40		Chlordan	0.06	1.12	458.9
244		Doxylamine succinate	0.75	1.21	470.1
54	23	Propranolol * HCl	0.12	1.59	470.4
163		Cetyltrimethylammonium chloride	0.021	1.31	474.4
264		Chloral hydrate	2.65	2.9	479.7
265		Streptomycin sulfate	2.73	0.34	495.6
319		Methylpentinol	23.8	5.35	525.2

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by LD50 (mg/kg)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
204		Azathioprine	0.14	1.93	535.2
156		Stearyltrimethylammonium chloride	0.006	1.54	536.1
310		Tributylamine	15.4	2.91	539.5
187		4-Hexylresorcinol	0.064	2.83	549.9
193		5-Azacytidine	0.079	2.34	571.5
26		Kelthane	0.012	1.55	574.2
39		2,4-Dichlorophenol	0.055	3.56	580.3
280		Theophylline sodium acetate	4.19	2.22	582.2
147		Mitoxantrone	0.0024	1.32	586.8
195		p,p'-DDA	0.099	2.1	590.4
101		Glutethimide	1.56	2.76	599.7
105	21	Theophylline	1.83	3.33	600.0
276		Ambuphylline	3.67	2.23	600.7
83		Thiopental	0.55	2.48	601.1
45		Quinine * HCl	0.075	1.72	620.8
70	49	Atropine sulfate	0.22	0.92	622.7
302		Nitrobenzene	12.2	5.2	640.2
123	35	Isoniazid	7.49	4.74	650.1
274		L-Cysteine	3.56	5.45	660.4
12		Puromycin	0.00033	1.43	674.4
11		Doxorubicin * HCl	0.00033	1.2	696.0
256		Tin II chloride	1.51	3.69	699.6
63	4	Diazepam	0.16	2.49	709.1
168		Dicoumarol	0.027	2.11	709.6
311		1-Hexanol	15.4	7.04	719.5
174		Ambazone	0.038	3.16	749.9
242		1,2,4-Trichlorobenzene	0.71	4.17	756.6
333		Lithium I chloride	38.6	17.9	758.8
114		Natulan * HCl	2.74	3.04	783.7
48		Mefenamic acid	0.087	3.27	789.1
338		1-Butanol	52.5	10.7	793.3
202		Formaldehyde	0.12	26.6	798.8
188		t-Butyl hydroquinone	0.069	4.81	799.6
354		1,3,5-Trioxane	213	8.88	800.0
317		Barbital sodium	18.6	3.88	800.1
58		Dihydralazine sulfate	0.14	2.84	818.8
213		Ammonium persulfate	0.23	3.59	819.3
290		Sodium sulfite	6.78	6.51	820.5
277		Potassium cyanate	4.14	10.4	843.6
71		Diphenhydramine * HCl	0.24	2.93	855.1
285		Caffeine sodium benzoate	5.67	2.54	859.4
197		p,p'-DDE	0.1	2.77	880.9
67	15	Malathion	0.2	2.68	885.4
259		Methyl salicylate	1.7	5.83	887.1
184		Butylated hydroxytoluene	0.056	4.04	890.4
272		Salicylic acid	3.38	6.45	890.9
337		Pyridine	46.9	11.3	893.9
308	33	Chloroform	13.4	7.61	908.4
44		Hydroxyzine * HCl	0.067	2.31	950.4
31	41	Chloroquine diphosphate	0.017	1.88	969.9
214		Thymol	0.23	6.52	979.6
65		Oxyphenbutazone	0.19	3.08	999.2
121		Aminophenazone	5.39	4.32	999.3
80		2-Thiouracil	0.32	7.8	999.6
304		Calcium II chloride	12.4	9.01	999.9
107	2	Acetylsalicylic acid	2.27	5.55	999.9
233		Ibuprofen	0.52	4.89	1008.9
47		Naftipramide	0.084	3.45	1029.7
218		o-Phenylenediamine	0.31	9.89	1069.7
41		Chloroquine sulfate	0.06	2.6	1086.8
327	20	Lithium I sulfate	33.7	10.8	1187.4
296		Homatropine methylbromide	9	3.24	1199.9
152		8-Hydroxyquinoline	0.0033	8.27	1200.6
287		Benzylalcohol	5.81	11.4	1232.9
111		Clofibric acid	2.61	5.82	1249.3
226		Dodecylbenzene sodiumsulfonate	0.42	3.62	1261.6
251		Scopolamine * HBr	1.08	3.3	1268.2
76		Sodium dodecyl sulfate	0.27	4.45	1288.0
191		Dimenhydrinate	0.076	2.81	1320.8
99		Nalidixic acid	1.5	5.81	1349.4
240		Pentoxifylline	0.66	4.98	1386.2
243		p-Anisidine	0.73	11.4	1404.1
270		Propionaldehyde	3.25	24.3	1411.6

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by LD50 (mg/kg)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
164		Oxatomide	0.019	3.31	1412.1
275		Nitrilotriacetic acid	3.61	7.69	1470.0
324		2-Butoxyethanol	26	12.5	1477.5
56		Manganese II chloride *4 H2O	0.13	7.5	1484.4
15		8-Azaguanine	0.0013	9.86	1500.1
136		Diethyldithiocarbamate sodium* 3H20	0.00039	6.66	1500.7
328	36	Dichloromethane	34.9	18.8	1596.7
119		Sodium salicylate	4.33	9.99	1599.5
166		Triisooctylamine	0.023	4.58	1620.2
284		Ammonium chloride	5.52	30.8	1647.8
97		Phenacetin	1.27	9.21	1650.8
42		p-Aminophenol	0.062	15.2	1658.9
248		m-Aminophenol	0.86	15.2	1658.9
78		6-Methylcoumarin	0.31	10.5	1681.9
250		Valproate sodium	1	10.2	1695.4
293		Diisopropylamine dichloroacetate	7	7.39	1700.9
200		Dimethylaminoethyl methacrylate (polymer)	0.11	11.1	1745.4
98		Methylparaben	1.42	11.5	1749.8
57		L-Dopa	0.13	9.03	1780.8
268		1-Octanol	3.06	13.7	1784.6
182		Triton X-100	0.055	2.78	1798.7
300		Antipyrine	11.6	9.56	1799.7
299		Imidazole	11.5	27.6	1879.3
95		Salicylamide	1.08	13.8	1892.7
342		Piperazine	67.2	22.1	1904.1
263		Acetaldehyde	2.45	43.8	1929.8
171		Fumagillin	0.031	4.36	1999.5
139		Retinol	0.00054	6.98	1999.8
236		Hydrogen peroxide 90%	0.56	58.8	2000.4
52		all-trans-Retinoic acid	0.11	6.66	2001.2
325		Cyclohexanol	26.3	20.6	2063.7
330		Sulfuric acid	36	21.8	2138.1
267		p-Hydroxybenzoic acid	2.92	15.9	2196.3
72		Butylated hydoxyanisole	0.24	12.2	2199.3
165		Isoproterenol * HCl	0.022	8.96	2219.8
331		Strontium II chloride	36.4	14.2	2251.0
113	1	Acetaminophen	2.71	15.9	2403.8
178		Salicylanilide	0.046	11.3	2409.7
356		2-Methoxyethanol	251	32.3	2458.4
334		Isobutanol	40.1	33.2	2461.4
305		n-Butanal	12.8	34.5	2488.1
215		Chlorotetracycline	0.24	5.22	2500.0
350		Tetrahydrofurfuryl alcohol	111	24.5	2502.7
323		Urethan	25.9	28.1	2504.0
208		Undecylenic acid	0.18	13.6	2506.6
312		Benzoic acid	15.7	20.7	2528.1
238		Imidazolidinyl urea	0.36	9.34	2598.9
109		Frusemide	2.33	7.86	2599.8
104		Tolbutamide	1.81	9.62	2601.1
346	50	Potassium I chloride	82	34.9	2601.8
158		Dichlorophene	0.0083	10	2691.3
273		Bromobenzene	3.46	17.2	2700.7
295		2,5-Hexanedione	8.45	23.7	2705.6
125	34	Carbon tetrachloride	8.51	18.2	2799.3
351		Dimethylformamide	114	38.3	2800.1
309		Isobutanal	13.5	39	2812.7
298		Dichloroacetic acid	11.5	21.9	2823.8
266		Potassium hexacyanoferrate III	2.82	9.02	2970.0
344	13	Sodium chloride	75.9	51.3	2998.0
322		1-Pentanol	24.9	34.4	3033.0
221		2-Nitro-p-phenylenediamine	0.39	20.1	3078.5
141		Cytosine arabinoside	0.00068	12.9	3137.9
216		Refortan	0.25	10.1	3162.3
94		Menthol	0.95	20.3	3172.9
257		Isononylaldehyde	1.52	22.8	3243.8
222		Glibenclamide	0.4	6.58	3250.8
288		1-Heptanol	6.25	28	3254.4
321		Acetic acid	24.3	55.1	3309.3
91	45	Chloramphenicol	0.79	10.5	3393.1
201		13-cis-Retinoic acid	0.12	11.3	3395.4
349		Ethyl methyl ketone	104	47.1	3396.9
345		Sodium I bromide	77.4	33.4	3504.3
336		Nicotinamide	44.4	28.7	3505.4

Appendix A

Registry of Cytotoxicity: List of 347 Chemicals Sorted by LD50 (mg/kg)

RC No	MEIC No	Chemical	IC 50x (mM)	Oral Rat or Mouse LD50 (mmol/kg)	Oral Rat or Mouse LD50 (mg/kg)
306		Anisole	13.2	34.2	3698.7
341		Lactic acid	66	41.4	3729.7
358		Acetonitrile	368	92.5	3798.1
211		Catechol	0.2	35.3	3887.2
307		2-Ethylbutanal	13.2	39.7	3977.1
315		Isobenzoic furano dione	17	27.1	4014.1
332		1,4-Dioxane	38.1	47.7	4203.3
124		Acetazolamide	8.49	19.3	4289.6
301	17	Xylene	12	40.5	4300.3
154		Maneb	0.0042	16.9	4500.6
294		Trichloroacetic acid	8.19	30.6	4999.4
271		Styrene	3.3	48	4999.7
316		Toluene	17.1	54.3	5003.7
320		N,N-Dimethylacetamide	24.2	58.4	5089.0
224		n-Butyl benzoate	0.41	28.8	5133.6
186		Zineb	0.059	18.9	5211.3
348		1-Propanol	96.5	89.8	5397.9
326		Halothane	31.1	28.8	5684.8
128	10	2-Propanol	167	97.2	5842.7
50		Trypan blue	0.095	6.43	6204.2
108		Gibberellic acid	2.3	18.2	6304.7
209		Propylparaben	0.18	35.1	6325.7
335		Potassium hexacyanoferrate II	42.3	17.4	6409.6
59		Tetracycline * HCl	0.14	13.4	6444.6
93		Sulfisoxazole	0.85	25.4	6790.2
127		Dimethyl phthalate	23.4	35.5	6894.1
286		Benzylpenicillin sodium	5.73	19.4	6914.2
126		Triethyl citrate	14.7	25.3	6990.9
85		Metamizol	0.58	21.5	7189.2
120		5-Aminosalicylic acid	5.07	50.6	7749.4
343		Magnesium II chloride * 6 H2O	70.4	39.8	8092.5
360	7	Ethylene glycol	555	138	8567.0
122		Diethyl phthalate	5.52	38.7	8601.5
289		Tetrachloroethene	6.54	53.4	8854.8
117		Di(2-ethylhexyl)adipate	3.15	24.6	9117.7
162		Chlorhexidine	0.015	18.2	9200.5
359		Acetone	444	168	9759.1
18		Captan	0.0039	33.3	10009.6
297	11	1,1,1-Trichloroethane	10.3	77.2	10298.5
353		Ethyl acetate	128	125	11015.0
100		L-Ascorbic acid	1.52	67.6	11907.1
88		Dibutyl phthalate	0.76	43.1	11998.2
131		Glycerol	624	137	12619.1
361	8	Methanol	930	406	13012.3
130	9	Ethanol	379	304	14008.3
313		Xanthinol nicotinate	15.8	32.5	14121.6
258		Diethyl sebacate	1.63	56	14470.4
340		Diethylene glycol	62.1	139	14753.5
329		Sodium cyclamate	35.4	75.8	15254.0
352		1,2,6-Hexanetriol	123	119	15969.8
314		Saccharin	16.4	92.8	17000.0
129		Dimethyl sulfoxide	252	252	19691.3
357		Propylene glycol	342	263	20016.9
231		Tween 80	0.49	19.1	25021.0
355		D-Glucose	226	143	25765.7
92		Di(2-ethylhexyl)phthalate	0.84	79.4	31015.2

Reference

Halle, W. 1998. Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD50) zur Einsparung von Tierversuchen. Life Sciences/Lebenswissenschaften, Volume 1, 94 pp., Jülich: Forschungszentrum Jülich.

APPENDIX B

List of Test Protocols for Basal Cytotoxicity

European Centre for the Validation of Alternative Methods (ECVAM) Scientific Information System (SIS)

Appendix B

List of Test Protocols for Basal Cytotoxicity

European Centre for the Validation of Alternative Methods (ECVAM)

Scientific Information System (SIS) http://www.ivtip.org/protocols.html#basalcyto

THE FRAME MODIFIED NEUTRAL RED UPTAKE CYTOTOXICITY TEST
The cytotoxic effect of chemicals upon cells in culture is measured by cell viability (neutral red uptake) method. Topics: Basal Cytotoxicity. Contact: Dr. Richard H. Clothier, Queen's Medical Centre, UK Last update: September 1990. Protocol no: 3.

HUMAN LYMPHOCYTE CYTOTOXICITY ASSAY
This method measures the leakage of DNA and lactate dehydrogenase (LDH, EC. 1.1.1 27) from lymphocytes into the surrounding medium as an indicator of cytotoxicity. This method also includes an assay of intracellular (mitochondrial) diaphorase as a measure of cellular activity (MTT assay). Topics: Basal Cytotoxicity. Contact: Prof. Jorgen Clausen, Roskilde University, DK. Last update: May 1991. Protocol no: 6.

THE USE OF MEMBRANE PERMEABILITY AS A MEASURE OF CYTOTOXICITY IN PERFUSED CELL CULTURES
Membrane permeability of perfused cell cultures, as determined by the afflux of [3H]-2-deoxy-D-glucose-6-phosphate, is used as an indicator of the cytotoxic effect of chemicals. Topics: Basal Cytotoxicity. Contact: Dr. E. Walum, Bioscience Centre, SEK. Last update: June 1989. Protocol no: 9.

HEL-30 CYTOTOXICITY TEST
The ability of cultured cells to synthesize protein is used to assess the effect of a test compound on cellular anabolic competence. Topics: Basal Cytotoxicity Contact: Dr. Marina Marinovich, Universita di Milano, I. Last update: April 1990. Protocol no: 14.

THE FRAME CYTOTOXICITY TEST (KENACID BLUE)
The cytotoxic effect of chemicals upon cells in culture is measured by the change in total cell protein arising from the inhibition of cell proliferation (Kenacid Blue R dye binding method). Topics: Basal Cytotoxicity. Contact: Dr. Richard H. Clothier, Queen's Medical Centre, UK. Last update: July 1992. Protocol no: 15.

CYTOTOXICITY AND GENOTOXICITY IN PRIMARY CULTURES OF HUMAN HEPATOCYTES
This test determines the cytotoxic and genotoxic effect of test compounds on primary cultures of human hepatocytes, by measuring cell viability, DNA damage, and unscheduled DNA synthesis. Topics: Basal Cytotoxicity, Mutagenicity. Contact: Prof. Giovanni Brambilla, University of Genoa, I. Last update: May 1992. Protocol no: 16.

MTT ASSAY
This method outlines a simple assay to determine the viability/number of cells in culture, through the formation of a colored product (in a mitochondria-dependent reaction) to which the cell membrane is

impermeable. Topics: Basal Cytotoxicity. Contact: Dr. Rosanna Supine, Istituto Nadonale Tumori, I. Last update: April 1990. Protocol no:17.

CYTOSKELETAL ALTERATIONS AS A PARAMETER FOR ASSESSMENT OF TOXICITY
Changes in the balance of cytoskeletal proteins after exposure to test compounds can be detected by indirect immunofluorescence microscopy and quantitative biochemical methods. Topics: Basal Cytotoxicity. Contact: ECVAM SIS. Last update: July 1991. Protocol no: 24.

YEAST GROWTH RATE CYTOTOXICITY TEST
The cytotoxic effect of chemicals upon yeast (Saccharomyces cerevisiae) cells in culture is determined by inhibition of cell proliferation, as measured by cell density. Topics: Basal Cytotoxicity. Contact: Dr. Ingolf Cascorbi, Institute of Clinical Pharmacology, D. Last update: January 1994. Protocol no: 33.

YEAST PLASMA MEMBRANE H+-ATPASE TOXICITY TEST
The effect of chemicals on the activity of the plasma membrane-bound H+-ATPase, isolated from yeast (Saccharomyces cerevisiae) cells, is used as a measure of their toxicity. Topics: Basal Cytotoxicity. Contact: Dr. Ingolf Cascorbi, Humboldt-University, D. Last update: January 1994. Protocol no: 34.

CHINESE HAMSTER OVARY CELL NA+/K+-ATPASE TEST
The effect of chemicals on the activity of the plasma membrane-bound Na+/K+ -ATPase isolated from Chinese Hamster Ovary (CHO) cells is used as a measure of their toxicity. Topics: Basal Cytotoxicity Contact: Dr. Ingolf Cascorbi, Humboldt-University, D. Last update: January 1994. Protocol no: 35.

CHINESE HAMSTER OVARY (CHO) CELL PROLIFERATION TEST
The inhibition of CHO cell proliferation provides an overall assessment of the toxicity of the test substance. Topics: Basal Cytotoxicity Contact: Dr. Ingolf Cascorbi, Humboldt-University, D. Last update: January 1994. Protocol no: 36.

LS-L929 CYTOTOXICITY TEST
This simple cell culture-based cytotoxicity test (in which cell viability is determined by uptake of the dyes ethidium bromide and fluorescein acetate) has been developed as a general test for acute toxicity. Topics: Basal Cytotoxicity, Eye Irritation. Contact: Dr. R.B. Kemp, University College of Wales, UK. Last update: July 1992. Protocol no: 38.

V79 CYTOTOXICITY/ TEST FOR MEMBRANE DAMAGE
The cytotoxic effect of test chemicals in V79 cell culture can be determined by assessing damage to the plasma membrane as determined by a nucleic acid leakage assay. Topics: Basal Cytotoxicity. Contact: Prof. Vera Bianchi, University of Padova, I. Last update: June 1990. Protocol no: 39.

BALB/C 3T3 CYTOTOXICITY TEST
The cytotoxic effect of chemicals upon Balb/c 3T3 cells in culture is measured by cell viability (Neutral Red Uptake) and total cell protein (Kenacid Blue R dye binding method). Topics. Basal Cytotoxicity, Eye Irritation. Contact: Dr. med. Horst Spielmann, ZEBET BgVV, D. Last update: January 1992. Protocol no: 46, German EGA Validation Study Protocol.

QUANTITATIVE VIDEO MICROSCOPY OF INTRACELLULAR MOTION AND MITOCHONDRIA-SPECIFIC FLUORESCENCE
AVEC-DIC microscopy in combination with mitochondria-specific fluorescence allows a quantitative analysis of cell organelle dynamics and fine structure in cell cultures exposed to test compounds. Topics: Basal Cytotoxicity. Contact: Dr. Toni Lindl, Inst. f. Angewandte Zellkultur, D. Last update: April 1992. Protocol no: 52.

UV ABSORPTION AS AN APPROXIMATION FOR CELL NUMBER
The absorption of UV at 260nm in a fixed volume of solubilized cells is proportional to the cell number, and therefore can be used as a simple means of obtaining a cell count. Cell counts obtained in this way can be combined with measurements of the inhibition of DNA synthesis ([3H]-thymidine incorporation) by test compounds, to produce an index of cytotoxicity. Topics: Basal Cytotoxicity. Contact: Dr. Ming J.W. Chang, Chang Gung Medical College, Rep. of China. Last update: September 1992. Protocol no: 58.

***IN VITRO* PREDICTION OF THE MAXIMUM TOLERATED DOSE**
The results of cytotoxicity tests in primary cultures of rat hepatocytes and in MDCK and McCoy cells can be used to predict the *in vivo* 4-wk maximum tolerated dose in rats and dogs. A correlation between *in vitro* cytotoxicity, as measured in this system, and LD50 values in rats and mice has also been established. Topics: Basal Cytotoxicity, Acute Systemic Toxicity. Contact: Dr. R. Shrivastava, RL-CERM, F. Last update: February, 1992. Protocol no: 66.

TWO-COMPARTMENT HUMAN TISSUE CYTOTOXICITY TEST
The activating system (human liver microsomes) is separated by a semi-permeable membrane from the target cells (human mononuclear leukocytes or red cells) in order to identify cytotoxic metabolites that are capable of diffusing away from the site of production. Topics: Basal Cytotoxicity, Hepatotoxicity I Metabolism - Mediated Toxicity. Contact: Dr. M.D. Tingle, University of Liverpool, UK. Last update: January 1994. Protocol no: 73.

TETRAHYMENA ASSAY FOR MEMBRANE-STABILIZING ACTIVITY
The effect of a test compound on lipid structure and protein ion channels in biological membranes can be determined by using video image analysis to assess its effect on the swimming speed of the ciliated protozoan, Tetrahymena pyriformis. Topics: Basal Cytotoxicity, Ecotoxicity, Aqueous contamination. Contact: Dr. S.L. Cassidy, Dow Corning Corporation, USA. Last update: February 1994. Protocol no: 76.

CYP1A1-INDUCING POTENCY AND CYTOTOXICITY TEST IN THE HEPA-1 MOUSE HEPATOMA CELL LINE
This bioassay utilizes cultured Hepa-lclc7 (Hepa-l) mouse hepatoma cells to assess the CYP1A1-inducing potency or cytotoxicity of pure test chemicals or environmental samples. In the Hepa-l induction test, the CYP1A1-inducing potency of the test sample is detected as increased aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin-O-deethylase (EROD) activities. In the Hepa-l cytotoxicity test, the effect of the sample on cell viability is measured. Topics: Basal Cytotoxicity, Ecotoxicity. Contact: Dr. Sirpa Kärenlampi, Dr. Riitta Torronen, Dr. Paivi Kopponen, University of Kuopio, FIN. Last update: October 1995. Protocol no: 112, MEIC Project Protocol.

APPENDIX C

Standard Operating Procedure (SOP) for
the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test
(A Test for Basal Cytotoxicity)

Appendix C
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1.0 STANDARD OPERATING PROCEDURE (SOP) FOR THE BALB/C 3T3 NEUTRAL RED UPTAKE CYTOTOXICITY TEST - A TEST FOR BASAL CYTOTOXICITY

1.1 Background and Introduction

The present *in vitro* SOP based on Borenfreund and Puerner (1985) was originally elaborated in 1990 by ZEBET (German National Centre for the Documentation and Evaluation of Alternatives to Animal Experimentation) in co-operation with participants of the German BMFT (Ministry of Research and Technology) sponsored "BGA (Federal Health Institute) eye irritation validation study" (Spielmann et al., 1991). The SOP was used in the second phase of the study, data base development, to assess the cytotoxicity of 150 test chemicals under blind conditions (Spielmann et al., 1996). The test had successfully undergone an interlaboratory assessment phase in which 35 chemicals were tested in 12 laboratories with five independent repeat tests per laboratory (Spielmann et al., 1991). The SOP was submitted in 1992 to INVITTOX, where it is still available as Protocol No. 46 (FRAME, 1992) and published in a methods handbook (Liebsch and Spielmann, 1995).

For the present purpose of being a recommended standard test for basal cytotoxicity, the protocol was refined by adding some paragraphs and appendices, none of which change the original method. The additions are based on experience made with a modification of the test, the 3T3 Neutral Red Uptake Phototoxicity Test (3T3NRU-PT), which has meanwhile gained regulatory acceptance. The additions cover test acceptance criteria and recommendations on the concentration series to be tested. The RC regression for prediction of acute oral systemic rodent toxicity (Halle, 1998; Spielmann et al., 1999) is included as the prediction model in Section 1.8 for the specific used described in this document. Two deletions were made with regard to the original SOP. The second endpoint, a cell protein staining with Kenacid Blue (KB), was deleted because it did not contribute to the test. For about 90% of the chemicals tested, the KB_{50} values were close or even identical to the NR_{50} values, but in several cases where necrotic cells were fixed to the plastic material of the plates and then stained with KB, the KB_{50} values led to an under-prediction of cytotoxicity. The second deletion is the microscopic determination of the "highest tolerated dose" (HTD), since this measure turned out to be too subjective and yielded insufficient interlaboratory comparability in the validation study.

1.2 Rationale

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy BALB/c 3T3 cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the vital dye, NR, after one day (= one cell cycle) of chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

1.3 Basic Procedure

BALB/c 3T3 cells are seeded into 96-well plates and maintained in culture for 24 hours (h) (~ 1 doubling period) to form a semi-confluent monolayer (see Section 1.6.1 for more information on cell maintenance and culture procedures). They are then exposed to the test compound over a range of eight concentrations. After 24 h exposure, NRU is determined for each treatment concentration and compared to that determined in control cultures. For each treatment (i.e., concentration of the test chemical) the percent inhibition of growth is calculated. The IC_{50} (a.k.a., the concentration producing 50% reduction of NR uptake) is calculated from the concentration-response and expressed as µg/ml or mmol/l.

1.4 Test Limitations

- Volatile chemicals tend to evaporate under the conditions of the test; thus the IC_{50} may be variable, especially when the toxicity of the compound is fairly low. This can be overcome if plates are sealed with CO_2 permeable plastic film, which is impermeable to volatile chemicals.
- Other chemicals that are difficult to test include those that are unstable or explosive in water.
- Due to low metabolic capacity of the BALB/c 3T3 cells, the test is likely to underestimate the toxicity of chemicals that require metabolic activation to a toxic intermediary or product.
- The *in vivo* toxicity of substances that specifically attack dividing cells may be overestimated.
- The toxicity of substances that bind to serum proteins may be underestimated. This is overcome to a certain extent by lowering the serum content from 10% to 5% during chemical exposure. Theoretically, serum-free media can be developed for any cell line, but does not yet exist for the BALB/c 3T3 cells.
- It is possible that low cell viability readings may result in those cases where a chemical has a relatively selective effect upon the lysosomes/endosomes of the cell. An example of this would be chloroquine sulfate, which alters the pH of lysosomes/endosomes, an effect that inhibits NRU.
- Red chemicals absorbing in the range of NR might interfere with the test, provided they are present in sufficient amounts within the cells after washing, and are soluble in the NR solvent.

1.5 Material

1.5.1 *Cell Lines*

BALB/c 3T3 cells, clone 31 (e.g., ECACC # 86110401, European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK; CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

1.5.2 *Technical Equipment*

- Incubator: 37ºC, humidified, 7.5 % CO_2/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath: 37ºC
- Inverse phase contrast microscope
- Laboratory burner
- Centrifuge (optionally: equipped with microtiter plate rotor)
- Laboratory balance
- 96-Well plate photometer equipped with 540 nm filter
- Shaker for microtiter plates
- Cell counter or hemacytometer
- Pipetting aid
- Pipettes, 8-channel-pipettes, dilution block
- Cryotubes
- Tissue culture flasks (80 cm^2, 25 cm^2)
- 96-Well tissue culture microtiter plates (e.g., Nunc # 167 008)

1.5.3 *Chemicals, Media, and Sera*

- Dulbecco’s Modification of Eagle’s Medium (DMEM) without L-Glutamine
  (e.g., ICN-Flow Cat. No. 12-332-54)

- L-Glutamine 200 mM
  (e.g., ICN-Flow # 16-801-49)

- New Born Calf Serum (NBCS)
  (e.g., Biochrom # SO 125)

Note: Due to lot variability of NBCS, first check a lot for growth stimulating properties with 3T3 cells (20-25 hrs doubling time) and then reserve sufficient amount of NBCS.

- Trypsin/EDTA solution
  (e.g., ICN-Flo, # 16891-49)

- Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)

- PBS with Ca^{2+} and Mg^{2+}(for rinsing)

- Penicillin/streptomycin solution
  (e.g. ICN-Flow # 16-700-49)

- Neutral Red (NR)

- Dimethyl sulfoxide (DMSO), analytical grade

- Ethanol (ETOH), analytical grade

- Glacial acetic acid, analytical grade

- Distilled H_2O or any purified water suitable for cell culture

1.5.4 *Preparations*

Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

1.5.4.1 ***Media***

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

(A) for Freezing

20 %	NBCS
7 - 10 %	DMSO

(B) for Routine Culture

10 %	NBCS
4 mM	Glutamine
100 IU	Penicillin
100 µg/ml	Streptomycin

(C) for Treatment with Test Chemicals

5 %	NBCS
4 mM	Glutamine
100 IU	Penicillin
100 µg/ml	Streptomycin

Note: The serum concentration of treatment medium is reduced to 5%, since serum proteins may mask the toxicity of the test substance. Serum cannot be totally excluded because cell growth is markedly reduced in its absence.

Complete media should be kept at 4° C and stored for no longer than two weeks.

1.5.4.2 ***Neutral Red (NR) Stock Solution***

0.4 g	NR Dye
100 ml	H_2O

Make up prior to use and store dark at room temperature for up to two months.

1.5.4.3 ***Neutral Red (NR) Medium***

1 ml	NR Stock Solution
79 ml	DMEM

Note: The NR medium should be incubated overnight at 37ºC and centrifuged at 600 x g for 10 min (to remove NR crystals) before adding to the cells. Alternative procedures (e.g., Millipore filtering) can be used as long as they guarantee that NR medium is free of crystals.

1.5.4.4 ***Ethanol/Acetic Acid Solution (NR Desorb)***

1 %	Glacial acetic acid solution
50 %	Ethanol
49 %	H_2O

Prepare immediately prior to use. Do not store for longer than 1h.

1.5.4.5 *Preparation of Test Chemicals*

1. Depending on the solubility, dissolve test chemical either in sterile treatment medium, or ETOH, or DMSO, as appropriate - at 100-fold the desired final concentration in the case of solvents. Other solvents may be used provided they have been tested to be non-cytotoxic at the final concentration used in the test. The final solvent concentration should be kept at a constant level of 1-2 % (v/v) in the vehicle controls and in all of the eight test concentrations. This means, the test chemical is dissolved in the solvent first, and then 1-2 part(s) of this stock solution is added to 98-99 parts of sterile pre-warmed (37°C) medium. Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce the highest test concentration, if necessary.
2. Measure the pH of the highest concentration of the test chemical. If strong acids or bases have changed the pH of the medium, they should be neutralized with 0.1N NaOH or 0.1N HCl. In this case, prepare highest concentration of the chemical in ~ 80% of final volume, measure pH, neutralize, and add medium to desired final volume.
3. Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble chemicals should range from the soluble to the precipitating dose.

Note: Test chemical must be freshly prepared immediately prior to use. Preparation under red light may be necessary, if rapid photodegradation is likely to occur.

1.6 Methods

1.6.1 *Cell Maintenance and Culture Procedures*

BALB/c 3T3 cells are routinely grown as a monolayer in 80 cm^2 tissue culture grade flasks, at 37°C in a humidified atmosphere of 7.5 % CO_2. The cells should be examined on a daily basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted. Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found.

1.6.1.1 *Routine Culture of BALB/C 3T3 Cells*

- When cells approach confluence they should be removed from the flask by trypsinization:
  - Decant medium, rinse cultures with ~5 ml PBS (without Ca^{2+}, Mg^{2+}) per 25 cm^2 flask.
  - Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.
  - Discard the washing solution.
  - Add 1-2 ml trypsin-EDTA solution to the monolayer for a few seconds.
  - Remove excess trypsin-EDTA solution and incubate the cells at 37°C.
  - After 2-3 minutes (min), lightly tap the flask to detach the cells into a single cell suspension.

1.6.1.2 *Cell Counting*

After detaching the cells, add 0.1-0.2 ml of routine culture medium/cm flask, i.e., 2.5 ml for a 25 cm^2 flask. Disperse the monolayer by gentle trituration. It is important to obtain a single cell suspension for exact counting. Count a sample of the cell suspension obtained using a hemocytometer or cell counter.

1.6.1.3 Subculture

After determination of cell number, the culture can be sub-cultured into another flask or seeded into a 96-well microtiter plate. BALB/c 3T3 cells are routinely passaged at a cell density of ~ 1 x 10^6 cells in 80 cm^2 flasks every 3-4 days (average doubling time is 20-24 h).

Note: It is important that cells have overcome the lag growth phase when they are used for the test.

1.6.1.4 *Freezing*

Stocks of BALB/c 3T3 cells can be stored in sterile, freezing tubes in liquid nitrogen. DMSO is used as a cryoprotective agent.

- Centrifuge trypsinized cells at 200 x g.
- Suspend the cells in routine culture medium, containing 20 % NBCS, at a concentration of 1-5x10^6 cells/ml.
- Aliquot 120-180 µl of cooled DMSO into freezing tubes and fill to 1.8 ml with the cell suspension.
- Place the tubes into a freezer at -80°C for 24 h. This gives a freezing rate of 1°C/min.
- Place the frozen tubes into liquid nitrogen for storage.

1.6.1.5 *Thawing*

Thaw cells by putting ampules into a water bath at 37°C. Leave for as brief a time as possible.

- Resuspend the cells and transfer into routine culture medium.
- Incubate at 37°C in a humidified 7.5 % CO_2 atmosphere.
- When the cells have attached to the bottom of the flask (this may take up to 4 h), decant the supernatant and replace with fresh medium. Culture as described above.
- Passage two to three times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells should be thawed out approximately every two months. This period resembles a sequence of about 18 passages.

1.6.2 *Quality Check of Assay (I): Positive Control (PC)*

Of the many chemicals backed by sufficient history or intra- and interlaboratory repeat tests (e.g., those shown in Section 3.2 of the report) Sodium Lauryl Sulfate (SLS, CAS # 151-21-3) is one of the most frequently tested, and is therefore recommended as a PC. If a laboratory has not built a historical database on SLS, it is recommended that SLS be tested in a full-scale concentration-response test (at 8 concentrations), according to Section 1.6.5.2, concurrently with each experiment. Once historical data prove reproducibility, the PC might be applied in just one concentration (IC_{50}) on the same plate together with the test chemical. For the latter procedure, the 95% confidence interval (CI) of the IC_{50} of SLS has to be established and defined as an acceptance criterion for test sensitivity in the SOP.

The historical mean IC_{50} of SLS (Spielmann et. al., 1991) is **0.093 mg/ml**.

The 95% CI is **0.070 - 0.116 mg/ml.**

A test meets acceptance criteria, if the IC_{50} for SLS is within the 95% CI

1.6.3 *Quality Check of Assay (II): Vehicle Control (VC)*

The absolute value of optical density (OD_{540} of NRU) obtained in the untreated vehicle control indicates whether the 1×10^4 cells seeded per well have grown exponentially with normal doubling time during the two days of the assay.

A test meets acceptance criteria if the mean OD_{540} of VCs is ≥ 0.3

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11) of the 96-well plate (see Appendix E):

A test meets acceptance criteria if the left and the right mean of the VCs do not differ by more than 15% from the mean of all VCs.

Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent. Microscopic evaluation obviates the need for two rows of VCs.

1.6.4 *Quality Check of Concentration-Response*

The IC_{50} derived from the concentration-response should be backed by at least two, or if possible, three responses between 10 and 90% inhibition of NRU. If this is not the case, and the concentration progression factor can be easily reduced, reject the experiment and repeat it with a smaller progression factor.

1.6.5 *Concentrations of Test Chemical*

1.6.5.1 *Range Finder Experiment*

Test eight concentrations of the test chemical by diluting the stock solution with a constant factor (e.g., $\sqrt[2]{10} = 3.16$, see Appendix F), covering a large range, e.g.,:

1 ⇒3.16 ⇒10 ⇒31.6 ⇒100 ⇒316 ⇒1000 ⇒3160 µg/ml

1.6.5.2 *Main Experiment*

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., $\sqrt[6]{10} = 1.47$). Try to cover the relevant concentration range (between 10% and 90% effect) with at least three points of a graded effect, avoiding too many non-cytotoxic and/or 100%-cytotoxic concentrations.

Experiments revealing less than three cytotoxic concentrations in the relevant range, shall be repeated, where possible, with a smaller dilution factor. (Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor achievable.)

1.6.6 *Test Procedure*

See Table C.1 for a flow chart of the test procedure. Appendix G contains a recommended template for documenting the relevant data generated by the BALB/c 3T3 NRU assay.

1st day after growing up the cells from frozen stock:

1. Prepare a cell suspension of $1x10^5$ cells/ml in culture medium. Using a multi-channel pipette, dispense 100 µl culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 µl of a cell suspension of $1x10^5$ cells/ml (= $1x10^4$ cells/well). Prepare one plate per chemical to be tested and one plate for the PC.

[Note: Individual plates for the PC are for establishing an historical database. Once an IC_{50} mean has been determined, one need only include that PC concentration in the test material plate.]

2. Incubate cells for 24 h (7.5% CO_2, 37ºC) so that cells form a half-confluent monolayer. This incubation period assures cell recovery and adherence and progression to exponential growth phase.

3. Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental errors.

2nd day

1. After 24 h incubation, aspirate culture medium from the cells.

2. Per well, add 100 µl of treatment medium containing either the appropriate concentration of test chemical, or the PC, or nothing but vehicle (VC).

3. Incubate cells for 24 h (7.5% CO_2, 37ºC).

3rd day

A) Microscopic Evaluation

After 24 h treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record changes in morphology of the cells due to cytotoxic effects of the test chemical, but do not use these records for the calculation of HTD or any other quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay.

B) Measurement of NRU

This method is based upon that of Ellen Borenfreund (Borenfreund and Puerner, 1985). The uptake of NR into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability.

1. Wash the cells with 150 µl pre-warmed PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37°C in a humidified atmosphere of 7.5 % $C0_2$ for 3 h.

2. After incubation, remove the NR medium, and wash cells with 150 µl PBS.

3. Decant and blot PBS totally. (Optionally: centrifuge the reversed plate.)

4. Add exactly 150 µl NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

5. Shake microtiter plate rapidly on a microtiter plate shaker for 10 min until NR has been extracted from the cells and formed a homogeneous solution.

6. Measure the absorption of the resulting colored solution at 540 nm in a microtiter plate reader, using the blanks as a reference. Save raw data in a file format (e.g., ASCII, TXT, XLS) appropriate for further analysis of the concentration-response and calculation of IC_{50}.

Table C.1. 3T3 NRU Cytotoxicity Test: Flow Chart

TIME (h)	PROCEDURE
00:00	Seed 96-well plates: 1x10^4 cells / 100 µl DMEM culture medium / well
	Incubate (37°C / 7.5% CO_2 / 22-24 h)
	↓
23:00	Remove culture medium
	↓
24:00	Treat with 8 concentrations of test chemical in treatment medium (100 µl) (untreated vehicle control = treatment medium)
	Incubate (37°C / 7.5% CO_2 / 24 h)
	↓
48:00	Microscopic evaluation of morphological alterations Remove treatment medium wash once with 150 µl PBS Add 100 µl NR medium
	Incubate (37°C / 7.5% CO_2 / 3 h)
	↓
51:00	Discard NR Medium Wash once with 150 µl PBS Add 150 µl NR desorbing fixative (ETOH/Acetic acid solution)
51:40	↓
51:50	Shake plate for 10 min
	Detect NR Absorption at 540 nm (i.e., cell viability)

1.7 Data Analysis

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six replicate values per test concentration. This value is compared with the mean NRU of all VC values (provided VCs have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each compound tested should span the range of no effect up to total inhibition of cell viability.

Where possible, the concentration of a test chemical reflecting a 50% inhibition of cell viability (i.e., the IC_{50}) is determined from the concentration-response. This can be done either by applying:

- A manual graphical fitting method. The use of probability paper with "x = log" and "y = probit" scales is recommended because in most cases the concentration-response function will become almost linear in the relevant range. Semi-log paper could also be used for this technique.

or

- any appropriate non-linear regression procedure (preferably a Hill function* or a logistic regression) to the concentration-response data. Before using the IC_{50} for further calculations, the quality of the fit should be appropriately checked. (* = Hill functions are monotonous and sigmoidal in shape and represent an acceptable model for many dose response curves.)

More sophisticated programs specially developed for concentration-response analysis from 96-well plates can also be used. An example is PHOTO-32, which uses a nonmonotonous curve fitting algorithm (Holzhütter and Quedenau, 1995) and addresses the influence of variability on the IC_{50} by bootstrapping procedures performed on concentration replicates (Holzhütter, 1997).

Before using the IC_{50} information in any subsequent estimation of rodent LD_{50}, be sure that the IC_{50} data are expressed as mmol/l since the prediction model described in this guidance document is based on the relationship between the LD_{50} (in mmol/kg) and the IC_{50} (in mmol/l).

1.8 Prediction Model

In general, basal cytotoxicity is highly valuable information per se, which can be used in combination with other information, e.g., bioavailability, for many purposes in the process of safety or risk evaluation. For the purpose of this document, basal cytotoxicity is to be used to predict starting doses for *in vivo* acute oral LD_{50} values in rodents. After testing the reference chemicals recommended in Section 3.2 of this guidance document and qualifying the test as described in Section 3.1 (see Section 3.3 of the report for examples with two different cell types), best estimates of starting doses for *in vivo* acute oral toxicity tests are predicted according to the following prediction model:

$$\log (LD_{50}\ [\text{mmol/kg}]) = 0.435 \times \log (IC_{50}\ [\text{mmol/l}]) + 0.625$$

1.9 References

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APPENDIX D

Standard Operating Procedure (SOP) for the Normal Human Epidermal Keratinocyte Neutral Red Uptake Cytotoxicity Test

(A Test for Basal Cytotoxicity)

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1.0 STANDARD OPERATING PROCEDURE (SOP) FOR THE NORMAL HUMAN EPIDERMAL KERATINOCYTE NEUTRAL RED UPTAKE CYTOTOXICITY TEST - A TEST FOR BASAL CYTOTOXICITY

1.1 Background and Introduction

This SOP, based on a NRU assay by Borenfreund and Puerner (1984) using epidermal keratinocytes (Heimann and Rice, 1983), was obtained from the Institute of *In Vitro* Sciences (IIVS). Formulations for the media and solutions correspond to Cloneticsâ products by BioWhittaker, Inc. For the present purpose of being a recommended standard test for basal cytotoxicity, the protocol from IIVS was embellished by adding details on equipment, media and reagent components, and experimental procedure to make it easier for novice users to follow. For the specific purpose of this guidance document, the RC regression for prediction of acute oral systemic rodent toxicity (Halle, 1998; Spielmann et al., 1999) is included as the prediction model in Section 1.8.

1.2 Rationale

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy normal human keratinocytes (NHK) cells, when appropriately maintained in culture in a sub-confluent state, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in cell death and/or a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the vital dye, NR, after two days of chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

1.3 Basic Procedure

NHK cells are seeded into 96-well plates and maintained in culture until cells form a 30-50% confluent monolayer. They are then exposed to the test compound over a range of six to eight concentrations. After 48 hours (h) exposure, NRU is determined for each treatment concentration and compared to that determined in control cultures. For each treatment (i.e., concentration of the test chemical) the percent inhibition of growth is calculated. The IC_{50} (a.k.a., NRU_{50}, the concentration producing 50% reduction of NR uptake) is calculated from the concentration-response and expressed as µg/ml or mmol/l.

1.4 Test Limitations

- Volatile chemicals tend to evaporate under the conditions of the test; thus the IC_{50} may be variable, especially when the toxicity of the compound is fairly low. This can be overcome if plates are sealed with CO_2 permeable plastic film, which is impermeable to volatile chemicals.
- Materials that are not readily soluble in serum-free aqueous media may be difficult to test, and their *in vivo* toxicity potentially underestimated.
- Other chemicals that are difficult to test include those that are unstable or explosive in water.
- The *in vivo* toxicity of substances that specifically attack dividing cells may be overestimated.
- It is possible that low cell viability readings may result in those cases where a chemical has a relatively selective effect upon the

lysosomes/endosomes of the cell. An example of this would be chloroquine sulphate, which alters the pH of lysosomes/endosomes, an effect that inhibits NRU.

- Red chemicals absorbing in the range of NR might interfere with the test, provided they are present in sufficient amounts within the cells after washing, and are soluble in the NR solvent.

1.5 Material

1.5.1 *Cell Lines*

NHK cells (e.g., **Clonetics #CC-2507** for cryopreserved cells or **Clonetics #CC-2607** for proliferating cells**,** BioWhittaker, Inc., USA)

1.5.2 *Technical Equipment*

- Incubator: 37º ± 1ºC, humidified, 5 ± 1 % CO_2/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath: 37º ± 1ºC
- Inverse phase contrast microscope
- Centrifuge
- Laboratory balance
- 96-Well plate photometer equipped with 540 or 550 nm filter
- Shaker for microtiter plates
- Cell counter or hemocytometer
- Pipetting aid
- Pipettes, 8-channel-pipettes, dilution block
- Cryotubes
- Tissue culture flasks (80 cm^2, 25 cm^2)
- 96-Well tissue culture microtiter plates (e.g., Nunc # 167 008)

Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of NHK.

1.5.3 *Chemicals, Media, and Sera*

- Keratinocyte Growth Medium (KGM) complete with epidermal growth factor, insulin, hydrocortisone, antimicrobial agents and supplemented with bovine pituitary extract (e.g., Clonetics # CC-3001)
- HEPES Buffered Saline Solution (HEPES-BSS) (e.g., Clonetics # CC-5022)
- 0.025% Trypsin/EDTA solution (e.g., Clonetics # CC-5012)
- Trypsin Neutralizing Solution (TNS) (e.g., Clonetics # CC-5002)
- Phosphate Buffered Saline (PBS)
- 10% fetal bovine serum (FBS)
- Neutral Red (NR)
- Dimethyl sulfoxide (DMSO), analytical grade
- Ethanol (ETOH), analytical grade
- Glacial acetic acid, analytical grade
- Hanks' Balanced Saline Solution without Ca^{2+} or Mg^{2+} (CMF-HBSS) (e.g., Invitrogen # 14170)
- Formaldehyde
- Calcium chloride
- Distilled H_2O or any purified water suitable for cell culture

1.5.4 *Preparations*

Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

1.5.4.1 *Culture and Treatment Medium*

KGM supplemented with**:**

0.1 ng/ml	Human recombinant epidermal growth factor
5 g/ml	Insulin
0.5 g/ml	Hydrocortisone
50 g/ml	Gentamicin
50 ng/ml	Amphotericin B
0.15 mM	Calcium
2 ml	7.5 mg/ml Bovine pituitary extract

Complete media should be kept at 4°C and stored for no longer than two weeks.

1.5.4.2 *Neutral Red (NR) Stock Solution*

0.4 g	NR Dye
100 ml	H_2O

Make up prior to use and store dark at room temperature for up to two months.

1.5.4.3 *Neutral Red (NR) Medium*

1 ml	NR Stock Solution
79 ml	KGM

Note: The NR medium should be incubated overnight at 37ºC and centrifuged at 600 x g for 10 min (to remove NR crystals) before adding to the cells. Alternative procedures (e.g., Millipore filtering) can be used as long as they guarantee that NR medium is free of crystals.

1.5.4.4 *Wash/Fix Solution*

0.5%	Formaldehyde
1.0%	Calcium chloride
98.5%	H_2O

1.5.4.5 *Ethanol/Acetic Acid Solution (NR Desorb)*

1 %	Glacial acetic acid solution
50 %	Ethanol
49 %	H_2O

Prepare immediately prior to use. Do not store for longer than 1 h.

1.5.4.6 *Preparation of Test Chemicals*

1. The test article should be dissolved in KGM, deionized distilled water, ETOH, DMSO, acetone, or other appropriate solvent. Other solvents may be used provided they have been tested to be non-cytotoxic at the final concentration used in the test. If the solvent is something other than KGM, a 100X concentrate of each desired final concentration of test article should be prepared. This 100X concentrated dosing solution is then diluted 1:100 directly into sterile pre-warmed (37°C) KGM. This should ensure that the final solvent concentration in culture wells should not exceed 1% (v/v) in the vehicle controls and in all of the eight test concentrations. Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce highest test concentration, if necessary. The stability of the test article under the actual experimental conditions should be determined for each experiment.
2. Measure the pH of the highest concentration of the test chemical. If strong acids or bases have changed the pH of the medium, they should be neutralized with 0.1N NaOH or 0.1N HCI. In this case, prepare highest concentration of the chemical in ~ 80% of final volume, measure pH, neutralize, and add KGM to desired final volume.
3. Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble chemicals should range from the soluble to the precipitating dose.

Note: Test chemical must be freshly prepared immediately prior to use. Preparation under red light may be necessary, if rapid photodegradation is likely to occur.

1.6 Methods

A good discussion of the techniques used in the multiple-well plate assays, such as those described in this section, is given by Harbell (2001).

1.6.1 *Cell Maintenance and Culture Procedures*

1.6.1.1 *Receipt of Keratinocytes*

Upon receipt of proliferating keratinocytes, the cultures will be observed microscopically for signs of distress (e.g., floating cells, excessive debris, or lack of mitotic figures). Cultures exhibiting these properties will be discarded. Then perform the following:

- Decontaminate the outside of the culture flasks with 70% ETOH.
- Incubate the cultures at 37º ± 1ºC for a minimum of 60 minutes (min) to allow the temperature of the medium to equilibrate.
- Aseptically remove the medium and replace with fresh KGM warmed to approximately 37º C.
- Unless otherwise specified, the cultures are then incubated at 37º ± 1ºC and 5 ± 1% CO_2 in air.

Upon receipt of cryopreserved keratinocytes, the cells should be stored in liquid nitrogen.

1.6.1.2 *Thawing Cryopreserved Keratinocytes*

- Thaw cells by putting ampules into a water bath at 37°C for as brief a time as possible. Do not thaw cells at room temperature or by hand. Seed the thawed cells into culture flasks as quickly as possible and with minimal handling.
- Slowly (taking approximately 1-2 min) add 9 ml of KGM to the cells suspended in the cryoprotective solution and transfer 3500 cells/cm^2 into flasks containing routine pre-warmed culture medium.
- Incubate the cultures at 37º ± 1ºC until the cells attach to the flask, at which time the KGM should be removed and replaced with fresh KGM.
- Unless otherwise specified, the cells should be incubated at 37º ± 1ºC and 5 ± 1% CO_2 in air and fed every 2-3 days until they are 50-80% confluent.

1.6.1.3 *Subculturing the Keratinocytes*

- When the keratinocyte culture in a 25 cm^2 flask is 50 to 80% confluent, remove the medium and rinse the culture twice with 5 ml HEPES-BSS. Discard the washing solution.
- Add 2 ml trypsin/EDTA solution to each flask and remove after 15 to 30 seconds. Incubate the flask at room temperature for 3 to 7 min. When more than 50% of the cells become dislodged, rap the flask sharply against the palm of the hand.
- When most of the cells have become detached from the surface, rinse the flask with 5 ml of room temperature TNS.
- Then rinse the flask with 5 ml CMF-HBSS and transfer the cell suspension to a centrifuge tube.
- Pellet the cells by centrifugation for 5 min at approximately 220 x g. Remove the supernatant by aspiration.
- Resuspend the keratinocyte pellet by gentle trituration (to have single cells) in KGM. Count a sample of the cell suspension obtained using a hemacytometer (Trypan Blue exclusion) or cell counter.
- Prepare a suspension of 0.8 to 1.0×10^4 cells/ml in KGM. Transfer the cells into flasks containing pre-warmed growth medium at 3500 cells/cm^2. The keratinocyte cultures may be sustained through approximately three passages.

For subculturing into 96-well plates, obtain the cell suspension as described above. Add 250 µl cell suspension to the appropriate wells on each 96-well plate. (Note: evaporation of the medium can be a problem; therefore, the edge wells should receive 250 µl PBS. Incubate the cultures in a humidified incubator at 37° ± 1°C and 5 ± 1% CO_2 in air.

1.6.1.4 *Freezing Keratinocytes*

- Harvest the cells as above and resuspend the single cells in cold freezing solution (e.g., 80% growth medium, 10% fetal bovine serum [FBS], 10% DMSO) at $5x10^5$ to $2x10^6$ cells per ml. Aliquot to freezing vials.
- Insulate the vials and place into a –70°C freezer overnight (12-24 h).
- Place vials into liquid nitrogen for storage.

1.6.2 *Quality Check of Assay (I): Positive Control (PC)*

Of the many chemicals backed by sufficient history or intra- and interlaboratory repeat tests (e.g., those shown in Section 3.2 of the report) **Sodium Lauryl Sulfate (SLS, CAS # 151-21-3)** is one of the most frequently tested, and is therefore recommended as a PC. If a laboratory has not built a historical database on SLS, it is recommended that SLS be tested in a full-scale concentration-response test (at six to eight concentrations), according to Section 1.6.5.2, concurrently with each test article experiment. Once historical data prove reproducibility, the PC might be applied in just one concentration (IC_{50}) on the same plate together with the test chemical (also noted in Section 1.6.6). For the latter procedure, two standard deviations of the IC_{50} for SLS is the acceptance criterion for test sensitivity.

A test meets acceptance criteria, if the IC_{50} for SLS is within 2 standard deviations of the historical mean.

1.6.3 *Quality Check of Assay (II): Vehicle Control (VC)*

The <u>absolute value</u> of optical density (OD_{540} of NRU) obtained in the untreated vehicle control indicates whether the 0.8 to $1x10^4$ cells/ml seeded in each well have grown exponentially with normal doubling time during the three to five days of the assay.

A test meets acceptance criteria if the mean OD_{540} of VCs is ≥ 0.3

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row **2**) and the right side (row **11**) of the 96-well plate (see Appendix E):

A test meets acceptance criteria if the left and the right mean of the VCs do not differ by more than 15% from the mean of all VCs.

Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent. Microscopic evaluation obviates the need for two rows of VCs.

1.6.4 *Quality Check of Concentration-Response*

If possible, the test article concentrations for the definitive assay will be chosen such that at least six treatments will be available for the determination of the IC_{50}. Ideally, two concentrations should result in expected survivals lower than 50%, one concentration should result in an expected survival of approximately 50% and two concentrations should result in expected survivals greater than 50%.

1.6.5 *Concentrations of Test Chemical*

1.6.5.1 *Range Finder Experiment*

In this preliminary NR bioassay, six to eight decreasing concentrations of the test material are selected based upon the available information for the test material. The test article should be dissolved in KGM, water, DMSO, acetone, ETOH, or other appropriate solvent. The maximum solvent concentration (other than water or KGM) should be 1%. One way to determine concentrations of the chemical to be tested is to dilute the stock solution several times by a constant factor (e.g., $\sqrt[2]{10} = 3.16$, see Appendix F), covering a large range, e.g.:

1 ⇒3.16⇒10 ⇒31.6 ⇒100 ⇒316⇒ 1000 ⇒ 3160 µg/ml

1.6.5.2 *Main Experiment*

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., $\sqrt[6]{10} = 1.47$) to avoid too many non-cytotoxic and/or 100%-cytotoxic concentrations. Experiments revealing less than three cytotoxic concentrations in the relevant range shall be repeated, **where possible**, with a smaller dilution factor. (Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor achievable.)

1.6.6 *Test Procedure*

See Table D.1 for a flowchart of the test procedure. Appendix G contains a template recommended for documenting the relevant data.

1st day *after growing up the cells from frozen stock:*

1. Prepare a suspension of 0.8-1x10^4 cells/ml in KGM. Using a multi-channel pipette, dispense 250 µl cell suspension to the appropriate wells on each 96-well tissue culture microtiter plate. [Note: evaporation of the medium can be a problem; therefore, the edge wells should receive 250 µl PBS for blanks.] Prepare one plate per chemical to be tested and one plate for the PC.

[Note: Individual plates for the PC are for establishing an historical database. Once an IC_{50} mean has been determined, only that PC concentration need be included in the test material plate.]

2. Incubate cells (37 ± 1°C and 5 ± 1% CO_2) until a 30-50% confluent monolayer is produced (~24-72 h). This incubation period assures cell recovery and adherence and progression to the exponential growth phase.

3. Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify systematic cell seeding errors.

2nd day:

1. After 24-72 h incubation, remove culture medium from the cells by inverting the uncovered 96-well plates over a liquid discard container and then gently blotting the plates several times on sterile paper towels.

2. Immediately add 125 µl fresh KGM to each well. Add 125 µl of the test article dilutions, positive control dilutions and solvent control dilution to the appropriate wells. Wells designated as blanks receive 125 µl KGM.

3. Incubate cells for 48 h (37 ± 1°C and 5 ± 1% CO_2).

3rd day:

A) Microscopic Evaluation

After 48 h treatment, examine each plate under a phase contrast microscope to identify test chemical precipitate, systematic cell seeding errors and growth characteristics of control and treated cells. Record changes in morphology of the cells due to cytotoxic effects of the test chemical, but do not use these records for the calculation of any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay.

B) Measurement of NRU

This method is based upon that of Ellen Borenfreund (Borenfreund and Puerner, 1985). The uptake of the NR into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability.

1. Remove the treatment medium and add 250 µl NR medium to each well, except for blanks, which receive 250 µl KGM. Incubate at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 for 3 h.

2. After incubation, decant the NR medium, and add 250 µl Wash/Fix solution to each well.

3. After 2 min, decant and add 100 µl NR Desorb solution to all wells, including blanks.

4. Shake microtiter plate rapidly on a microtiter plate shaker for a minimum of 20 min at room temperature.

5. Measure the absorption of the resulting colored solution at **540-550 nm** in a microtiter plate reader, using the blanks as a reference. Save raw data in a file format (e.g., ASCII, TXT, XLS) appropriate for further analysis of the concentration-response and calculation of IC_{50}.

Table D.1. NHK NRU Cytotoxicity Test: Flow Chart

ASSAY PHASE	PROCEDURE
CELL GROWTH (24:00-72:00 h) [30–50% monolayer confluency]	• Seed 96-well plates: 2.0 to 2.5 x 10^3 cells/250 µl KGM culture medium/well • Incubate (37° ± 1°C, 5 ± 1% CO_2, 24-72 h)
TEST MATERIAL TREATMENT (48:00 h)	• Remove culture medium/add fresh KGM culture medium (125 µl/well) • Treat cells with 6-8 concentrations of test material in treatment medium (125 µl/well) [test material is 2X concentration before adding to wells] for 48 h treatment
PRELIMINARY NEUTRAL RED BIOASSAY (3:00 hours)	• Microscopic evaluation of morphological alterations • Remove treatment medium and add 250 µl/well NR medium • Incubate (37° ± 1°C, 5 ± 1% CO_2, 3 h)
NEUTRAL RED BIOASSAY (0:20 hours)	• Discard NR medium • Add 250 µl/well Wash/Fix solution for 2 min • Remove Wash/Fix solution • Add 100 µl/well NR Desorb (ETOH/acetic acid solution) • Shake plate for 20 min • Detect NR absorption at $OD_{540-550}$

1.7 Data Analysis

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six to eight replicate values per test concentration. This value is compared with the mean NRU of all VC values (provided VCs have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the six to eight concentrations of each compound tested should span the range of no effect up to total inhibition of cell viability.

Where possible, the concentration of a test chemical reflecting a 50% inhibition of cell viability (IC_{50}) is determined from the concentration-response. This can be done either by applying the following:

- a manual graphical fitting method. The use of probability paper with "x = log" and "y = probit" scales is recommended because in most cases the concentration-response function will become almost linear in the relevant range. Alternatively, semi-log paper could also be used for this technique.

or

- any appropriate non-linear regression procedure (preferably a Hill function* or a logistic regression) to the concentration-response data. Before using the IC_{50} for further calculations, the quality of the fit should be appropriately checked. (* = Hill functions are monotonous and sigmoidal in shape and represent an acceptable model for many dose response curves.)

More sophisticated programs specially developed for concentration-response analysis from 96-well plates can also be used.

Before using the IC_{50} information in any subsequent estimation of rodent LD_{50}, be sure that the IC_{50} data are expressed as mmol/l since the prediction model described in this guidance document is based on the relationship between the LD_{50} (in mmol/kg) and the IC_{50} (in mmol/l).

1.8 Prediction Model

In general, basal cytotoxicity is highly valuable information *per se*, which can be used in combination with other information, e.g., bioavailability, for many purposes in the process of safety or risk evaluation. For the purpose of this document, basal cytotoxicity is to be used to predict starting doses for *in vivo* acute oral LD_{50} values in rodents. After testing the reference chemicals recommended in Section 3.2 of this guidance document and qualifying the test as described in Section 3.1 (see Section 3.3 of the report for examples with two different cell types), best estimates of starting doses for *in vivo* acute oral toxicity tests are predicted according to the following prediction model:

$$\log (LD_{50}\ [\text{mmol/kg}]) = 0.435 \times \log (IC_{50}\ [\text{mmol/l}]) + 0.625$$

1.9 References

Borenfreund, E. and J. Puerner. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/ NR-90). J. Tissue Culture Meth. 9(1): 7-9.

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Spielmann, H., E. Genschow, M. Liebsch, and W. Halle. 1999. Determination of the starting dose for acute oral toxicity (LD50) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA 27: 957-966.

APPENDIX E

96-Well Plate Configuration

Appendix E

96-Well Plate Configuration

Note: The plate configuration shown below is a recommendation, based on experience in two validation studies. Other plate map designs are possible and are discussed by Harbell (2001). Plate configurations must be fixed in the SOP. To avoid errors, plate configurations should be kept constant if reader files have to be transferred to secondary software for computational concentration-response analysis.

Note: Since evaporation (during opening the door of the incubator) may take place in the peripheral wells, it is recommended to use these wells for blanks only. Since modern incubators are able to compensate the drop in humidity much quicker than older ones, columns 1 and 12 may be used for other purposes (e.g., two typical concentrations of the PC), while cells A2-A11 and H2-H11 can be used for the blanks.

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
C	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
D	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
E	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
F	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
G	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

VC = untreated VEHICLE CONTROL
(mean viability set to 100%)

C_1 - C_8 = TEST CHEMICAL at eight concentrations
(C1 = lowest, C8 = highest)

b = BLANKS
(containing no cells, but treated with NR medium
and with NR Desorb solution)

APPENDIX F

Decimal Geometric Concentration Series

Appendix F
Decimal Geometric Concentration Series

Note: Whereas geometric concentration series (as opposed to arithmetic concentration series) are regarded as a requirement in for any cytotoxicity assay that is based on concentration response analysis, the decimal geometric concentration series described below is just a recommendation.

In general **dose-response relationships** of many pharmacological or toxicological endpoints investigated have a **nonlinear**, often sigmoidal shape, which can be linearized to some extent by logarithmic transformation of the x-axis. This usually has to be done when IC$_{50}$ values are calculated either by regression analysis or by graphical estimation for the current NRU assay.
If the concentration series is done with arithmetic progression steps, transformation of the x-axis will result in an unequal distribution of measurements. Therefore, the use of a geometric concentration series (= constant dilution / progression factor) is recommended. The simplest geometric series are **dual geometric series**, e.g., a factor of 2. These series have the disadvantage of numerical values that permanently change between logs of the series (e.g., *log0*-2, 4, 8; *log1*- 16, 32, 64; *log2*- 128, 256, 512; *log3*- 1024, 2048,).

The **decimal geometric series**, first described by Hackenberg and Bartling (1959) for use in toxicological and pharmacological studies has the advantage that independent experiments with wide or narrow dose factors can be easily compared because they share identical concentrations. Furthermore, under certain circumstances, experiments can even be merged together:

EXAMPLE:

10						**31.6**						**100**
10				**21.5**				**46.4**				**100**
10		**14.7**		**21.5**		**31.6**		**46.4**		**68.1**		**100**
10	**12.1**	**14.7**	**17.8**	**21.5**	**26.1**	**31.6**	**38.3**	**46.4**	**56.2**	**68.1**	**82.5**	**100**

The dosing factor of **3.16** (= $^{2}\sqrt{10}$) divides a log into 2 equidistant steps, a factor of **2.15** (= $^{3}\sqrt{10}$) divides a decade into 3 steps. The factor of **1.47** (= $6\sqrt{10}$) divides a log into 6 equidistant steps, and the factor of **1.21** (= $^{12}\sqrt{10}$) divides the log into 12 steps.

For an easier biometrical evaluation of several related concentration response experiments use decimal geometric concentration series rather than dual geometric series. The technical production of decimal geometric concentration series is simple. An example is given for factor 1.47:

Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

Reference:

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol. Pharmakol. 235: 437-463.

APPENDIX G

Standard Test Reporting Template

Appendix G

Standard Test Reporting Template

This template is recommended to compile the data necessary to check the performance of a NRU test. Additional data, (e.g., temperature, CO_2, and humidity of incubators, or temperature of refrigerators, calibration of scales and pipettes, etc.), are not included since GLP laboratories usually record these in master records for the whole laboratory.

TEST SUBSTANCE		
Name	CAS-No. (if known)	
Laboratory Code	Molecular Weight (gram)	
Storage Conditions (tick _)	_ deep frozen	_ room temperature
	_ refrigerated	_ dark
Expiration date (if known)		

PREPARATION OF TEST SUBSTANCE								
Name of Solvent (if used)								
Percent Solvent (v/v) present in all wells								
Aids used to dissolve (tick _)	_ magnetic stirrer				_ ultra-sonication			
	_ vortex				_ heating to ....°C			
pH (measured at highest test concentration)								
Was neutralization necessary? (tick _)	_ NO		_ YES, with HCl			_ YES, with NaOH		
Concentration series (specify in µg/ml)								
Concentration series (specify in µmol/ml)								

CELL LINE		
Name:	Supplier:	
Total Passage No. (if known):	No. of Passages after Thawing:	

CELL CULTURE CONDITIONS		
Name of Medium:	Supplier:	Lot No.:
Name of Serum:	Supplier:	Lot No.:
Serum Concentration	During growth: ....%	During Exposure: ....%

TEST ACCEPTANCE CRITERIA			
VC: mean absolute OD540 (specify and _)	Mean OD = ...	_ ACCEPT	_ REJECT
VC: diff. betw. columns 2 and 11 (specify and _)	Difference = ...%	_ ACCEPT	_ REJECT
PC: IC_{50} of concurrent SLS test (specify and _)	IC_{50} = ...μg /ml	_ ACCEPT	_ REJECT
PC: specify where PC data are recorded:			

TEST RESULTS			
Chem. Conc. (μmol/ml)	OD540 MEAN ± SD	**Viability (%) MEAN ± SD**	Template reports trial No. .... of the test substance **NRU RESULT:** IC_{50} = ... μmol/ml [equals mmol/l]
VC = ZERO		100	
C1 =			
C2 =			
C3 =			**PREDICTED LD_{50}:** log LD_{50} = ...mmol/kg b.w. LD_{50} = ...mmol/kg b.w. LD_{50} = ...mg/kg b.w. **PREDICTED STARTING DOSE: UDP** one step (factor 3.2) below LD_{50} =............mg/kg Signature:............................. Date:.............................
C4 =			
C5 =			
C6 =			
C7 =			
C8 =			