

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 LD₅₀ 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 LD₅₀ 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 LD₅₀ 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 LD₅₀ 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 LD₅₀ values.) The regression equation from the candidate test is calculated by linear regression (least square method) using the candidate IC₅₀ values and the corresponding LD₅₀ 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 $\pm \log 5$ interval, then the test is considered suitable to generate IC₅₀ 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 LD₅₀ starting dose, the IC₅₀ (in mmol/l) for the trial chemical is entered into the regression equation to

calculate an LD₅₀ 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. (*Note: 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.

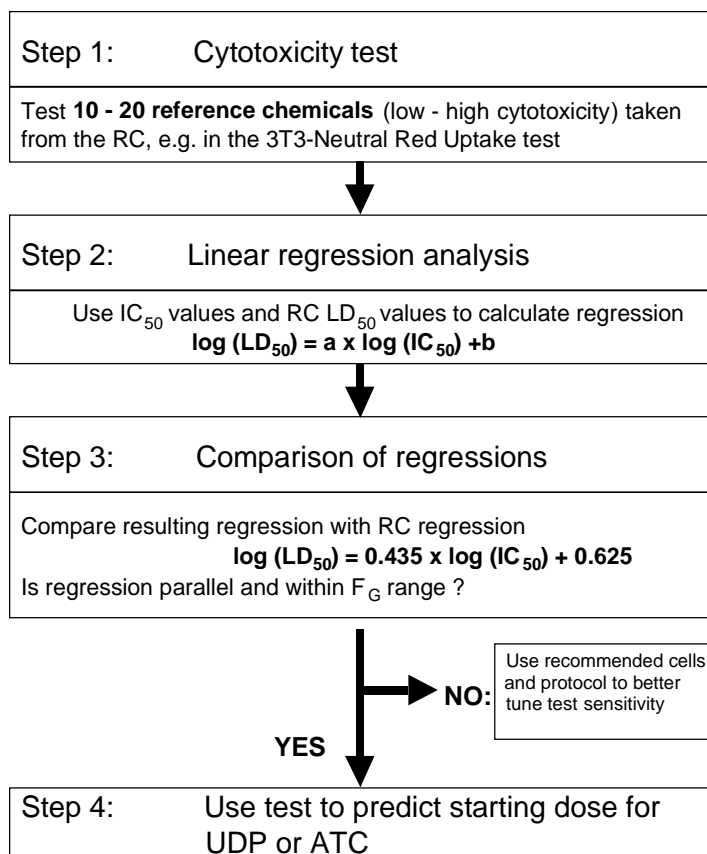


Figure 2. Procedure for evaluating a cytotoxicity test for tiered *in vitro/in vivo* testing for acute oral toxicity testing (slightly modified from Spielmann et al., 1999).

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 (IC₅₀/LD₅₀) 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)	LD₅₀ (mmol/ kg b.w.)	LD₅₀ (mg/ kg b.w.)	Molecular Weight (g)	CAS Number	Sigma-Aldrich Purchase #	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.
Antipyrine	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 LD₅₀ 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 \pm log 5 interval (black lines) and the 11 reference chemicals (triangles). Other chemicals from the RC were omitted for clarity. The new IC₅₀ 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 \pm 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:

$$\log(\text{LD}_{50}) = 0.435 \times \log(\text{IC}_{50x}) + 0.625$$

New NHK NRU regression:

$$\log(\text{LD}_{50}) = 0.498 \times \log(\text{IC}_{50}) + 0.551$$
$$[\text{R}^2 = 0.9356]$$

New 3T3 NRU regression:

$$\log(\text{LD}_{50}) = 0.506 \times \log(\text{IC}_{50}) + 0.475$$
$$[\text{R}^2 = 0.9848]$$

As expected, due to selection of reference chemicals with data points close to the RC regression, the determination coefficients (R²) 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).

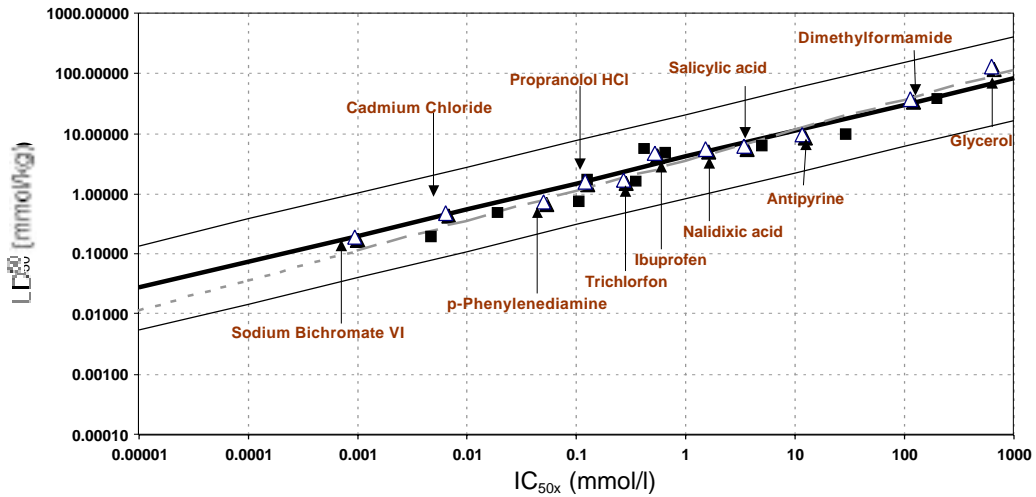


Figure 3. Regression obtained by testing the recommended reference chemicals from the RC with human keratinocytes in the NHK NRU cytotoxicity test

Figure shows the RC prediction regression (black bold line) \pm 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).

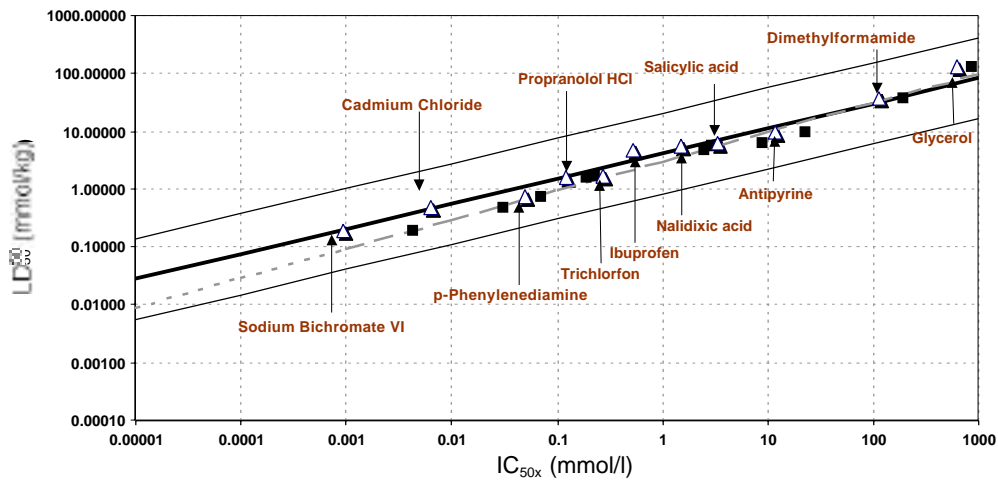


Figure 4. Regression obtained by testing the recommended reference chemicals from the RC with mouse fibroblasts in the BALB/c 3T3 NRU cytotoxicity test.

Figure shows the RC prediction regression (black bold line) \pm 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).

