Using *In Vitro* Cytotoxicity Data to Determine When Rat Acute Oral Toxicity Testing Should Start with the Limit Test

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Introduction

Poisoning is a more serious public health problem than is generally recognized. The Institute of Medicine estimates that more than 4 million poisoning episodes occur annually in the United States (Institute of Medicine [IOM] 2004). To reduce the risk for accidental poisonings, regulatory agencies require acute oral toxicity testing of marketed products to determine the potential for harmful effects from ingestion. Societal concerns about animal use for such testing have led to the development and evaluation of alternative *in vitro* test methods that might refine, reduce, or replace the use of animals in acute oral toxicity test methods[1].

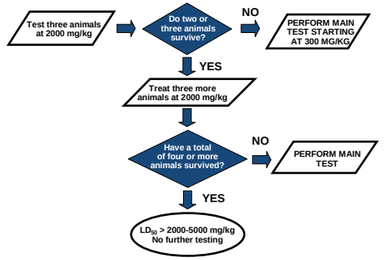
Acute toxicity test guidelines such as the Up-and-Down Procedure (UDP; OECD 2001a; EPA 2002), the Acute Toxic Class method (ATC; OECD 2001b), and the Fixed Dose Procedure (FDP; OECD 2001c) require 5-15 animals per test. However, these test guidelines provide for testing a reduced number of animals for substances that are expected to be nontoxic (i.e., with LD_{50} >2000 mg/kg or LD_{50} >5000 mg/kg, depending on the regulatory authority). This is accomplished in a limit test, which is a separate procedure from the main test and requires testing three to six animals to confirm suspected lack of toxicity.

The Registry of Cytotoxicity (RC) from the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment (BfR) compiled acute oral rat and mouse LD_{50} values from the NIOSH RTECS® database. The RC paired these values with published IC_{50} values from *in vitro* cytotoxicity assays using a variety of cell lines and cytotoxicity endpoints to create a significant linear regression for the IC_{50} prediction of LD_{50} values (Halle 2003). This poster provides analyses to determine whether IC_{50} values from *in vitro* cytotoxicity assays can provide useful information to determine when acute oral testing should begin at a limit dose of 2000 or 5000 mg/kg.

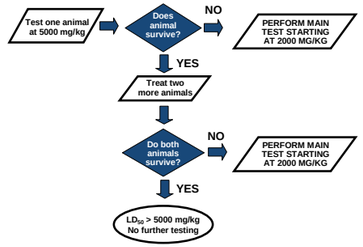
[1] **Reduction alternative:** A new or modified test method that reduces the number of animals required. **Refinement alternative:** A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being. **Replacement alternative:** A new or modified test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one.

Conduct of Limit Tests for the Acute Toxic Class Method (ATC)

ATC Limit Test at 2000 mg/kg



ATC Limit Test at 5000 mg/kg

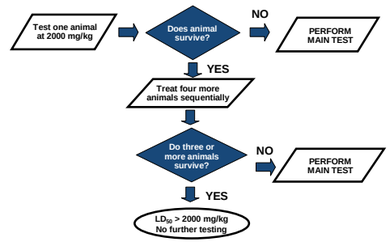


Conduct of Limit Tests for the Fixed Dose Procedure (FDP)

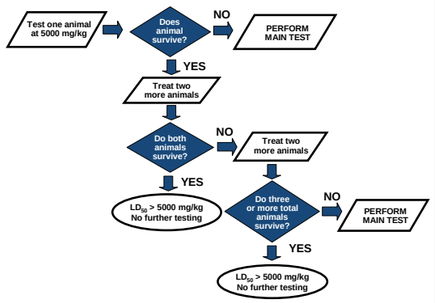
FDP Limit Test at 2000 or 5000 mg/kg

Conduct of Limit Tests for the Up-and-Down Procedure (UDP)

UDP Limit Test at 2000 mg/kg



UDP Limit Test at 5000 mg/kg



Methods

The rat oral LD_{50} and IC_{50} values for 418 chemicals from the ZEBET expanded RC were used to determine the most appropriate IC_{50} value to indicate when animal testing should start at the limit test of 2000 mg/kg or 5000 mg/kg. The IC_{50} values were geometric mean values (i.e., IC_{50x}) obtained from two to 32 *in vitro* cytotoxicity tests using various mammalian cell types and cytotoxicity endpoints. **Table 1** lists the criteria for the inclusion of IC_{50} data into the RC and the types of cytotoxicity endpoints and cells included.

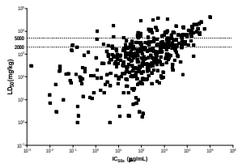
Table 1. Characteristics of the *In Vitro* Cytotoxicity Data Included in the Registry of Cytotoxicity

Acceptance Criteria for *In Vitro* IC_{50} Values	Cytotoxicity Endpoints Accepted	Example Cell Types
• At least two different IC_{50} values available, either from different cell types, different cell lines, or different cytotoxicity endpoints • From mammalian cells only • Cytotoxicity data from hepatocytes were not acceptable • Chemical exposure time was at least 16 hours	• Cell proliferation (cell number, cell protein, DNA content, DNA synthesis, colony formation) • Cell viability, metabolic indicators such as metabolic inhibition test, metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) • Cell viability, membrane indicators such as neutral red uptake, Trypan blue exclusion, cell attachment, cell detachment • Differentiation indicators	• Rat neonatal fibroblasts • Mouse 3T3 fibroblasts • Chinese hamster lung • Human KB • Chinese hamster V79 • Human lymphocytes • Syrian hamster BHK • Human skin fibroblasts • Rat myocytes • Mouse P-815 (mastocytoma) • S-180 mouse adenocarcinoma • Human HEPM cells (embryonic mesenchyme) • Human G (conjuctival) • Human HeLa • Bovine Madin-Darby kidney • Mouse neuro-2a • Human McCoy (epithelial synovial) • Human HEp-2 • Rat H9c2 (heart) • Rat skeletal muscle • Calf BKEz-7

The range of IC_{50x} values in the RC was 0.0015 µg/mL to 100,046 µg/mL and the range of LD_{50} values was 1 to 42,000 mg/kg (**Figure 1**). Counts of chemicals above and below various IC_{50} cutoff values were used to calculate performance characteristics (i.e., proportion of substances with hazard correctly predicted and proportions with hazard overpredicted and hazard underpredicted) for predicting LD_{50} >2000 mg/kg or LD_{50} >5000 mg/kg.

Figure 1

IC_{50} and LD_{50} values for 418 Registry of Cytotoxicity Chemicals with Rat Oral LD_{50} Data



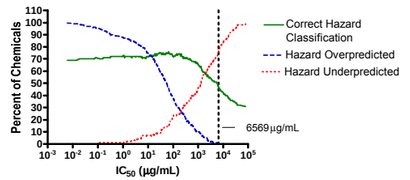
IC_{50} values are the geometric means of multiple IC_{50} values from *in vitro* cytotoxicity tests from various cell types and endpoints. The limit test doses for the acute oral toxicity test methods are shown by the dotted lines.

Results

- **Figures 2** and **3** show the proportion of substances for which hazard (i.e., toxicity) was correctly predicted and the proportions of substances for which hazard was overpredicted and underpredicted for the limit doses of 2000 mg/kg and 5000 mg/kg, respectively.
- The correct hazard classification rate for the limit dose of 2000 mg/kg was 69% at very low IC_{50} values, peaked at 76% as the IC_{50} increased, and then decreased to 31% at the highest IC_{50}.
- The correct hazard classification rate for the limit dose of 5000 mg/kg was 85% at very low IC_{50} values, peaked at 87% as the IC_{50} increased, and then decreased to 16% at the highest IC_{50}.

Figure 2

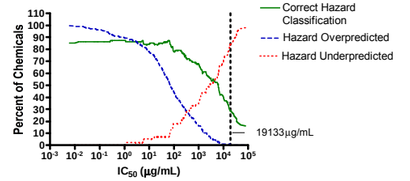
Rates for Correct Hazard Classification and Hazard Over- and Under-prediction of LD_{50} >2000 mg/kg at Various IC_{50} Values



Analysis based on the IC_{50} (geometric mean) and rat oral LD_{50} values for 418 substances in the Registry of Cytotoxicity: 289 substances with LD_{50} ≤2000 mg/kg and 129 substances with LD_{50} >2000 mg/kg. Hazard overprediction indicates that the IC_{50} predicted that toxicity was higher than observed (i.e., predicted LD_{50} ≤2000 while observed LD_{50} >2000 mg/kg). Hazard underprediction indicates that the IC_{50} predicted that toxicity was lower than observed (i.e., predicted LD_{50} >2000 while observed LD_{50} ≤2000 mg/kg). IC_{50} = 6569 µg/mL is highlighted to show where hazard overprediction rate = 0%.

Figure 3

Rates for Correct Hazard Classification and Hazard Over- and Under-prediction of LD_{50} >5000 mg/kg at Various IC_{50} Values



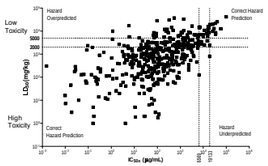
Analysis based on the IC_{50} (geometric mean) and rat oral LD_{50} values for 418 substances in the Registry of Cytotoxicity: 357 substances with LD_{50} ≤5000 mg/kg and 61 substances with LD_{50} >5000 mg/kg. Hazard overprediction indicates that the IC_{50} predicted that toxicity was higher than observed (i.e., predicted LD_{50} ≤5000 while observed LD_{50} >5000 mg/kg). Hazard underprediction indicates that the IC_{50} predicted that toxicity was lower than observed (i.e., predicted LD_{50} >5000 while observed LD_{50} ≤5000 mg/kg). IC_{50} = 19133 µg/mL is highlighted to show where hazard overprediction rate = 0%.

Results (cont'd)

- If chemicals with LD_{50} values greater than the limit test doses (i.e., the "non-toxic chemicals") are incorrectly predicted to have lower LD_{50} values (i.e., hazard is overpredicted), additional animals will be tested.
  - For the UDP, if testing starts at the default dose of 175 mg/kg, two (at 175 and 550 mg/kg for 2000 mg/kg limit test) to three (175, 550, and 1750 mg/kg for 5000 mg/kg limit test) more animals will be used for testing the chemicals for which hazard is overpredicted.
  - For the ATC, three (300 mg/kg for 2000 mg/kg limit test) to six (300 and 2000 mg/kg for the 5000 mg/kg limit test) more animals will be used for testing the chemicals for which hazard is overpredicted.
  - For the FDP, one (300 mg/kg for 2000 mg/kg limit test) to two (300 and 2000 mg/kg for the 5000 mg/kg limit test) more animals will be used for testing the chemicals for which hazard is overpredicted.
- If hazard is underpredicted for the chemicals with LD_{50} values less than the limit test doses (i.e., the "toxic" chemicals), then more animals will likely experience treatment-related death or be humanely killed because testing will start at the limit dose rather than at the default dose. More of the test animals will die as the dose is decreased in steps (i.e., 5000, 1750, 550, 175 mg/kg, etc., for the UDP, or 5000, 2000, 300 mg/kg, etc., for the ATC and FDP).
- For this evaluation, the optimum IC_{50} values were considered to be those that produced the lowest hazard overprediction rate (0%) because no animals are expected to die as a result of receiving a high dose of test chemical.
  - For the limit test at 2000 mg/kg, the hazard overprediction rate of 0% (0/129) occurred at IC_{50} = 6569 µg/mL. At this IC_{50}, the correct hazard classification rate was 47% (198/418) and the hazard underprediction rate was 76% (220/289).
  - For the limit test at 5000 mg/kg, the hazard overprediction rate of 0% (0/61) occurred at IC_{50} = 19133 µg/mL. At this IC_{50}, the correct classification rate was 29% (120/418) and the hazard underprediction rate was 84% (298/357).
  - **Figure 4** shows these IC_{50} cutoffs in relation to the database of 418 chemicals and the limit test cutoffs or 2000 and 5000 mg/kg.

Figure 4

Hazard Classifications for 418 Chemicals With Optimum IC_{50} for Determining to Test with the Limit Doses of 2000 mg/kg and 5000 mg/kg



The horizontal dashed lines distinguish the "toxic" (below the line) and "nontoxic" chemicals (above the lines). The IC_{50} values illustrated by the vertical dashed lines are the optimum IC_{50} values determined in the analysis to determine the rates for correct hazard prediction and under- and over-prediction of hazard. Chemicals with IC_{50} values below the given values are predicted to be toxic (i.e., LD_{50} ≤ 2000 mg/kg or ≤ 5000 mg/kg) and chemicals with IC_{50} values above the given values are predicted to be nontoxic (i.e., LD_{50} > 2000 mg/kg or > 5000 mg/kg). The IC_{50} values that resulted in 0% overprediction of hazard for the 2000 mg/kg and 5000 mg/kg limit doses are shown by the vertical dotted lines.

Conclusions

- IC_{50} values that resulted in 0% overprediction of hazard were selected as the optimum IC_{50} values from *in vitro* cytotoxicity tests to predict that chemicals should be tested at the limit dose in acute oral systemic toxicity tests because animal deaths are not expected at this level.
  - IC_{50} = 6569 µg/mL was selected to predict the limit test of 2000 mg/kg
  - IC_{50} = 19133 µg/mL was selected to predict the limit test of 5000 mg/kg
- Both under- and over-prediction of hazard would increase the number of animals tested for a particular chemical.
- Prediction of starting doses for acute oral systemic toxicity tests using *in vitro* basal cytotoxicity test methods are not applicable to chemicals with specific toxic mechanisms (i.e., cardiotoxicity or neurotoxicity) that are not expected to be active in cell cultures (ICCVAM 2006).

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