Refinement and Reduction of Acute Oral Toxicity Testing: Critical Review of the Use of Cytotoxicity Data

Arnhild Schrage, Katja Hempel, Markus Schulz, Susanne N. Kolle, Ben van Ravenzwaay, and Robert Landsiedel

BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany

First Author:

Arnhild Schrage

BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany

Tel.: +49 621 60-56754, Fax: +49 621 60-58134, Email: arnhild.schrage@basf.com

Corresponding Author:

Robert Landsiedel

BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany

Tel.: +49 621 60-56203, Fax: +49 621 60-58134, Email: robert.landsiedel@basf.com

Acknowledgements:

We thank the members of the laboratories for *in vitro* and short-term toxicology, especially Viola Cetto, Uwe Oberholz, Dominik Reinhard, Nicole Straessner and Andreas Verlohner for performing the routine testing and their help compiling the data. We also thank Martina Dammann for her help with the statistic evaluation.

Abstract:

Acute oral toxicity testing is still required for classification and labelling of chemicals, agrochemicals and formulations thereof. There were increasing efforts over the last two decades to reduce the number of animals needed according to the 3R concept.

To evaluate the utility of the *in vitro* cytotoxicity test in our routine testing for acute oral toxicity, we have implemented the neutral red uptake (NRU) method after 48 hours exposure in Balb/c 3T3 fibroblasts recommended by ICCVAM (ICCVAM Report 07-4519, 2006) in our laboratory. Initially, we tested 16 substances with already existing *in vivo* and *in vitro* data, to prove our technical proficiency for the *in vitro* test. Then, testing was performed with 187 test substances including a broad variety of chemicals, agrochemicals and formulations thereof. The starting dose for acute oral systemic toxicity assays in rats (LD_{50}) was estimated with the prediction model presented in this ICCVAM Validation Study and subsequently compared to the results obtained by *in vivo* testing according to or similar to the OECD guideline 423.

Comparing all 203 predicted LD_{50} values, which were deduced from the *in vitro* IC_{50} values, with the *in vivo* results from oral toxicity studies in rats, resulted in a low overall concordance of 35%. The cytotoxicity assay achieved a good concordance of 74% only for the low toxic substances (EU-GHS Cat. 4). This was mainly based on the fact that 71% of the substances were classified as low toxic *in vitro*.

We further analyzed the utility of the *in vitro* test for predicting the starting dose for the *in vivo* study and animal savings. For virtually non-toxic substances (LD_{50} > 2000 mg/kg bw), only a predicted starting dose of 2000 mg/kg would minimize the number of animals in the actual *in vivo* study. For substances with an $LD_{50} \leq 2000$ mg/kg bw (Cat. 1-4), selecting a starting dose one category higher or lower than the actual *in vivo* LD_{50} would minimize the number of animals. In this regard the prediction by the cytotoxicity test was useful for 59% of the substances. But using a standard starting dose of 300 mg/kg bw by default (without

previous cytotoxicity testing) would have been almost as useful (50%). In contrast, the prediction by an experienced toxicologist was correct for 95% of the substances. However, this was only performed for 40% of the substances, mainly of no to low toxicity. Calculating the animal numbers needed in several scenarios supported these results.

The additional analysis considering some physico-chemical data (solubility, molecular weight, log P_{OW}), substance class and mode of action revealed no specific applicability domains.

In summary, the use of the 3T3 NRU cytotoxicity data alone did not sufficiently contribute to the refinement and reduction of acute oral toxicity testing for the substances portfolio tested routinely in our laboratory.

(462 words)

Keywords:

In vitro, cytotoxicity, Balb/c 3T3 fibroblasts, neural red uptake, *in vivo*, acute oral systemic toxicity

Introduction:

Acute oral toxicity historically has been the initial test for the evaluation of the toxic characteristics of a substance. But today an acute toxicity study is no longer needed for pharmaceuticals (1) and all acute animal experiments for testing cosmetic products or their contents have been banned within the EU since March 2009 under the Directive 2003/15/EC (2).

Up to now, testing for acute oral toxicity is still required for the toxicological assessment of chemicals and agrochemicals worldwide. In Europe, the regulatory data requirement for acute toxicity testing of industrial chemicals is given by the regulation 1907/2006 ("REACH regulation") with specification of the data requirements in the Annexes VII - XI (3) and for agrochemicals by the regulation 1107/2009 with specified data requirements in annexes II and III (4). Additionally, regulations for biocides and medicinal products may apply. Comparable regulations are in force nearly all over the world, enabled by the respective competent authorities in the United States, Japan, China, Brazil, and other countries.

One of the most important goals as identified by Seidle *et al.* to be achieved with the study results is to allow classification and labeling (5). Usually therefore an exact LD_{50} in the test is not needed. Thus, testing is performed at the borders of hazard classification levels. With the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (6) coming into force the dose levels tested are usually 2000 mg/kg bw, 300 mg/kg bw, 50 mg/kg bw and 5 mg/kg bw, as described in the OECD guideline 423: Acute Toxic Class Method (7).

In the light of the 3R concept (Replacement, Reduction and Refinement), initially described by Russell & Burch (8), over the last two decades refinements of the acute oral toxicity guideline have been implemented (i.e. OECD guideline 423 instead of 401 (9)) leading to significant lowered animal numbers. Furthermore, attempts have been made to identify possible alternative methods for the prediction of acute oral toxicity, such as correlating rodent and/or human acute lethal toxicity with *in vitro* cytotoxicity (10-16). Methods for

predicting other important components of acute toxicity such as type, onset, duration and reversibility of the toxic as well as toxicokinetics and metabolism are still at the research level (17). Besides, there is ongoing research on quantitative structure-activity relationship (QSAR) modeling (18;19) or the Integrated Project AcuteTox and the approach using information from 28-days repeated dose toxicity studies, if available by ECVAM (20).

For evaluating the usefulness and limitations of two specific *in vitro* cytotoxicity test methods, an international, multi-laboratory validation study was organized by ICCVAM/NICEATM and ECVAM: three laboratories tested 72 reference substances for neutral red uptake (NRU) in BALB/c 3T3 mouse fibroblasts (3T3) and normal human epidermal keratinocytes (NHK). The resulting data were used to estimate starting doses for rodent acute oral toxicity testing, based on linear regressions developed from the Registry of Cytotoxicity database (10). It was concluded that the 3T3 and NHK NRU test methods were not sufficiently accurate to predict acute oral toxicity for regulatory hazard classification, but these *in vitro* methods might be used in a weight-of-evidence approach to determine the starting dose for the current acute oral toxicity protocols (i.e. the ATC method). Furthermore, the starting doses for substances with certain toxic mechanisms that were not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic or cardiotoxic) would likely be underestimated by these basal cytotoxicity test methods (16).

However, computer simulation of the ATC method testing showed that, for the substances tested in the validation study, NRU test methods resulted in average savings of 0.5 – 1.1 animals (5 – 10%) per test. Although the 3T3 NRU test method was less reproducible than the NHK NRU test method, it produced slightly higher animal savings and accuracy for prediction of GHS acute oral toxicity category using the IC_{50} and the revised RC regressions evaluated for the prediction of LD_{50} (16).

To contribute to animal welfare and based on these ICCVAM recommendation, we implemented the Balb/c 3T3 NRU test method in the routine assessment of oral acute toxicity in rats and collected *in vitro* and *in vivo* data for 187 substances. Retrospectively, we analyzed the utility of the *in vitro* method for assessing oral acute toxicity. We estimated its usefulness for the *in vivo* classification and for predicting the starting dose for the subsequent *in vivo* test, thereby also calculating the animal numbers to estimate possible animal savings. Additionally, we tried to identify specific applicability domains, hoping to improve the predictivity of the cytotoxicity test.

Material and methods:

Data set:

For 203 substances (16 substances from the Halle Register 2003 and 187 in-house test substances, including a broad variety of chemicals, agrochemicals and formulations thereof), we collected data about the cytotoxicity and the acute oral toxicity in rats, applicable for comparing the *in vitro* and *in vivo* results (Table 1A+B). Of all substances, 70% were agrochemical active ingredients (AI) or formulations: Sixty-one fungicide AI and 19 formulations, 9 herbicide AI and 9 formulations, 39 insecticide AI and 5 formulations. The remainder contained 40 chemicals, including the 16 substances from the Halle Register 2003, 8 mixtures, as well as some polymers and dyes, additives and coatings.

The substances from the Halle Register 2003 (Table 1A) were tested only *in vitro*, as the *in vivo* data already existed. For the others, both tests were performed using identical batches, except the substances tested pre-GLP or marked with an asterisk.

Acute oral toxicity

In total 203 acute oral toxicity studies in rats were compared to *in vitro* testing. Data for 16 test substances were obtained from literature; 187 test substances were tested in routinely performed acute oral toxicity testing *in vivo* according to the provisions of the German Animal Welfare Act and the European Council Directive 86/609/EEC in our AAALAC certified laboratory or a partner institute located in Germany. 87 studies were performed for registration purpose according to the OECD test guideline for Acute Oral Toxicity under Good Laboratory Practice (GLP) conditions; the other 100 were screening studies.

Testing according to OECD 423 ("Acute Toxic Class Method", ATC) (7;21-23) determines the acute oral toxicity of test substances after single administration in rats. Briefly, one or several fixed doses of a test substance (5, 50, 300, 2000 mg/kg bw) were administered by gavage to young adult Wistar rats (Crl:WI (Han), Charles River Wiga GmbH, Sulzfeld,

Germany) using a stepwise procedure with the use of 3 animals per step. Starting dose levels were determined by expert judgement based on information of the substance class or comparable formulations. Subsequent dose levels were based on decision tree in Annex 2 of OECD 423. For 75 of these GLP guideline test substances the starting doses were proposed by the same expert. These tests were therefore used for comparison of animal numbers used by expert or cytotoxicity and potential animal savings. Since "the guideline is not intended to allow the calculation of a precise LD_{50}, but does allow for determination of a range of exposures where lethality is expected", the estimated LD_{50} ranges were used for ranking of the compounds based on the classification and labeling categories of the Global Harmonized System (GHS). Eight of the 87 substances were tested according to the former valid guideline 401, not containing fixed doses.

Ninety-four test substances were tested for their acute oral toxicity in screening studies (non-GLP) following in principle the testing procedure of OECD 423. We used the same rat strain of the same age and also the recommended animal numbers (3 per dose level). Administration was via gavage and the observation period lasted for 14 days. Starting dose levels were also identified by expert judgement based on information of the substance class or comparable formulations, but since these studies were performed for development, the starting or following dose levels were in some cases also based on estimated risk assessment (exposure) or limited by substance availability. In these cases (substances no. 47 or 51 with LD50 > 50 mg/kg bw and 42 of the substances no. 63-124 with LD_{50} > 300 mg/kg bw) testing was performed up to a cut-off criteria value and not continued to either higher or lower level, if the test substance was not considered suitable for further development. Six other substances (used for validation of cytotoxicity test in house) were tested before 1989 (pre-GLP) and therefore also regarded as non-GLP screening substances.

Classification:

The classifications used for comparison of *in vitro* and *in vivo* results were categories 1-4 and "not required" for the highest class (category 5). Based on animal welfare considerations, testing for category 5 (testing at 5000 mg/kg bw) according to EU-GHS is not performed (GHS 2009, section 3.1.2.1 (g)(6). Moreover, there were no test substances falling into category 1 in our data set and hence, category 1 (< 5 mg/kg bw) and 2 (5-50 mg/kg bw) were grouped together.

Balb/c 3T3 NRU cytotoxicity test

The NRU test was performed according to the ICCVAM Report 2006 (16) in our GLP certified laboratory. Except as otherwise noted, materials were obtained from Biochrom, Berlin, Germany or Sigma-Aldrich, Steinheim, Germany. Balb/c 3T3 fibroblasts (clone A31, ECACC, Salisbury, Wiltshire SP4 OJG, UK) were cultivated for 24 h in cDMEM (DMEM, complemented with 10% Newborn Calf Serum, 4 mM L-glutamine, 100 IU penicillin, 100 µg/mL streptomycin) in a 96 well plate at 37°C and 5% CO_2. Usually, the maximal concentration for screening substances was 100 µg/ml and for GLP guideline substances 2154 µg/mL, corresponding to a predicted LD_{50} of 586 or 1836 mg/kg bw, respectively, *in vivo*. Eight selected concentrations, 6 replicates per concentration, were tested to obtain a reliable concentration-effect-curve. The substances were diluted in DMEM, DMSO, THF or Ethanol (the latter both from Riedel-de Haen, Seelze, Germany) with a maximal solvent concentration of 0.5%. After 48 h incubation and rinsing off the test substance with PBS, the cells were incubated with 50 µg/ml neutral red for 3 h at 37°C and 5% CO_2. Another rinse with PBS was followed by a 10 min lysis with neutral red desorption solution (deionised water with 50% Ethanol and 1% glacial acetic acid, Merck, Darmstadt, Germany). Then the OD_{550nm} was determined with a Wallac 1420 multilabel counter (Perkin Elmer, Waltham, Massachusetts, US) and the relative cell viability was calculated as percentage of the negative control (vehicle control = 100%). Finally, the concentration which led to 50% reduction of cell

growth compared to the control, the IC_{50} (IC = Inhibiting Concentration in µg/mL or mmol/L), was estimated.

Calculations/statistics

The predicted LD_{50} for lethal oral toxicity in rats was initially described for substances with known molecular weight (10):

- log LD_{50} (mmol/kg) = 0.439 * log IC_{50} (mM) + 0.621.

In order to develop a prediction model, which is applicable to mixtures or other substances without a known molecular weight, the data forming the basis for this millimole regression were converted to a weight basis (16):

- log LD_{50} (mg/kg) = 0.372 * log IC_{50} (µg/mL) + 2.024.

For the estimation of animal savings the theoretical animal numbers used for the calculation of animal numbers in GLP guideline and screening studies are shown in Table 2. To address different dose response curves, the animal numbers for GLP guideline studies were not only estimated using first the minimum amount of animals needed to reach the final *in vivo* result, but also by using a probit model based on a logarithmic dose scale with slopes of 2 and 8, in line with the ICCVAM publication (16). Slope 2 in general led to slight increases in animal numbers, but did not change the overall conclusion and slope 8 led to nearly the same results as for using the minimum animal numbers by stepwise procedure according to the OECD guideline 423 (Table 2A). Thus, for further evaluation (screening studies) only the latter one was used.

For the substances, which were tested in GLP guideline studies and for which expert proposal of starting dose was available (n = 75), we calculated the absolute number of animals when using the default starting dose of 2000, 300, and 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test or starting dose given by an experienced toxicologist and estimated the savings of animals. Expert proposal was given by a toxicologist with more than

15 years of experience in acute toxicicity testing and background in BASF test items by taking into account all available test substance information i.e. comparable formulations, structure similarities etc.

Nearly all calculations and graphical presentations were done with Microsoft Office Excel 2003. For correlations Pearson´s correlation coefficient and R^2 were determined. Furthermore, the scatterplots for comparing IC_{50}, predicted LD_{50} and LD_{50} obtained *in vivo* in Figure 1B and 2 were done with a 30-Days Demo Version of GraphPadPrism 5 (September 2010: http://www.graphpad.com/demos/). The log P_{OW} was calculated with the SPARC on line calculator v4.5 (September 2010: http://sparc.chem.uga.edu/sparc/) for pH 7, as this is close to the pH of the culture medium *in vitro*: pH 7.4 (data not shown).

Results:

At the time of analysis a data set of 203 substances, which were tested in the NRU cytotoxicity test and the acute oral toxicity test in rats, was available in our laboratory. Table 1A and B show *in vivo* and *in vitro* results for all substances; also some substance information such as physical form, molecular weight and log P_{OW}, are listed. Calculating the predicted LD_{50} was generally done using Halle´s mass based formula, as the molecular weight was not applicable for some test substances, e.g. the formulations. Nevertheless, a molar analysis was performed for the substances with a known molecular weight (see below).

Reproducibility and reliability of the cytotoxicity test:

To demonstrate our technical proficiency with the 3T3 NRU test method, we tested 16 substances with published IC_{50} and LD_{50} values (oral, rat/mouse, [mg/kg bw]) (10): 1x EU-GHS category 2, 3x category 3, 7x category 4 and 5 unclassified substances (Table 1A). Three of these can also be found in ICCVAM´s list of recommended Reference Standards and two substances are structural similar to two other substances of this list (16). Our IC_{50} results were in very good correlation with the published IC_{50} values with Pearson´s correlation coefficient of 0.9829 (Figure 1A). Comparison with the *in vivo* results showed a good accuracy of 69% (11/16) with only two under- and 3 overpredicted substances; 13% or 19%, respectively (Figure 1B).

Overall concordance of the *in vitro* and the *in vivo* test

Of all tested substances 39% (79/203) were classified as virtually non-toxic *in vivo* (LD_{50} > 2000 mg/kg bw, no category according to EU-GHS) and 39% (80/203) as low toxic (> 300 – 2000 mg/kg bw, Cat. 4). Only about one fifth of all substances were identified as moderate to very toxic: 12% (24/203) with a LD_{50} > 50 – 300 mg/kg (Cat. 3) and 10% (20/203) with $LD_{50} \leq 50$ mg/kg bw (Cat. 1-2). In contrast, the *in vitro* test identified 71% (145/203) of all

tested substances as low toxic (Cat. 4). Of the remainder 4% (8/203) were virtually non-toxic (no category), 24% (48/203) were toxic (Cat. 3) and 1% (2/203) were very toxic (Cat. 1-2). Therefore, the cytotoxicity assay showed a good prediction only for the low toxic substances (Cat. 4) with a concordance of 74% (59/80), but not for the other classes, resulting in a low overall concordance of 35% (71/203) (Table 3A and Figure 2A). In contrast, the overall concordance for only the 16 substances from the Halle Register, 2003 was rather good with 69% (11/16) (Table 3A).

Subgrouping the in-house substances by *in vivo* GLP guideline or screening studies showed that the guideline studies were mainly performed for non/weak toxic substances, whereas the toxic substances were detected mainly in screening studies, which are performed in an early phase of product development (Figure 2B+C).

Utility of predicting the *in vivo* starting dose

Selecting a starting dose which matches the *in vivo* classification of substances reduces the use of animals in the actual *in vivo* study independent of the study conditions (GLP guideline or screening study). As mentioned above, this was the case for only 35% (71/203) of the substances. For substances with an $LD_{50} \leq 2000$ mg/kg bw (Cat. 1-4), selecting a starting dose one category higher or lower than the actual *in vivo* LD_{50} may still save some animals. In this regard the prediction by the cytotoxicity test was useful for 58% (118/203) of the substances (Table 1A+B). But using a standard starting dose of 300 mg/kg bw by default (without previous cytotoxicity testing) would have been almost as useful (50%, 102/203). The prediction by an experienced toxicologist was correct for 95% (71/75) of the respective substances. This was, however, only performed for 36% of the substances, mainly of not too low toxicity.

Estimation of animal savings

For GLP animal studies, the minimum number of animals used by these tests was calculated to be 516, if the category had always been predicted correctly. This number was compared to the the animal studies where an expert proposal of a starting dose was available (n = 75). Additionally, we calculated the number of animals we would have used, if we had selected a default starting dose of 2000, 300, or 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test (Table 4 and Figure 3).

It can be clearly seen for the GLP animal studies that using a starting dose of 2000 mg/kg bw by default resulted in the same number of animals per test (6.9) as the calculation of minimum numbers (100% predictivity) and thus, results in the lowest animal numbers by using this approach in our laboratory. This is due to the fact that the prevalence of this category for the test substance data set in our laboratory for GLP studies is 70% and thus a correlation is high. The actual starting doses for the animal studies were selected by expert judgements and about the same number of animals per test (7.3) was actually used. If the starting doses were selected based on the cytotoxicity test, this would have led to 9.1 animals per test (a total of 165 additional animals used in the animal studies for the 75 substances) compared to default starting dose of 2000 mg/kg bw.Whereas selecting a default starting dose of 300 mg/kg bw or 50 mg/kg bw would have increased the number of animals used in the animal studies studies.

As shown in Table 2B+C, we also estimated the expected numbers of animals for some assumed LD_{50} with a slope of 2 or 8, which is in line with the calculations of the ICCVAM publication (16). Slope 2 in general led to slight increases in animal numbers, but did not change the overall conclusion and slope 8 led to nearly the same results as for using the minimum animal numbers by stepwise procedure according to the OECD guideline 423 (Table 2A). Thus, for further evaluation (screening studies) only the minimum animal numbers were used.

For screening studies *in vivo* testing in many cases was not continued up to the highest category, but stopped at a cut-off level. Since 42 of 100 fell into the category > 300 mg/kg bw

two scenarios have been made for calculation of animal numbers. For scenario A it was assumed that all > 300 mg/kg bw would have been in the range of > 2000 mg/kg bw and for scenario B it was assumed that all > 300 mg/kg bw would have been in the category > 300 - 2000 mg/kg bw (Table 5 and Figure 4). Using this estimation model, the animal numbers for scenario A were lower when starting with 2000 mg/kg bw and for scenario B lower when starting with 300 mg/kg bw. Prediction of starting dose by the cytotoxicity assay would have led to higher animal numbers in both scenarios (plus 34% or 10%, respectively).

Specific characteristics / applicability domains

As the cytotoxicity test was not very predictive in general, we analyzed specific characteristics of the test substances, in order to find applicability domains: limitation of the test substance concentration, MW based predicted LD_{50} thereby excluding formulations, log P_{OW}, substance class or mode of action (MoA).

One parameter, which might influence the cytotoxicity *in vitro*, is the solubility of the test substances. The highest concentration used *in vitro* was limited for 40 substances (Table 1B and 6): Testing with no response or no dose-response relationship, which is necessary for the IC_{50} calculation [µg/mL], was performed up to 100 µg/mL for 25 substances, up to 1000 µg/mL or 2154 µg/mL for 7 substances each and up to 4642 µg/mL for one substance. Assuming the IC_{50} value would be the highest *in vitro* test concentration plus 1 µg/ml, the predicted LD_{50} [mg/kg bw] was correct for 25% (10/40). This is a decreased concordance compared to the overall concordance. However, the predicted LD_{50} values were helpful for determining the starting dose for 80% (32/40) of these substances. Therefore, limiting the highest *in vitro* concentration, which was mainly due to restricted amount of test substance in the screening studies, had no negative effect on the prediction of the starting dose for the GLP *in vivo* studies.

As we also tested formulations during the routine testing, we generally calculated weight based IC_{50} values [µg/mL]. However, the predicted LD_{50} for rodents was initially calculated with the molecular weight (MW) based IC_{50} values [µmol/mL] (10).

In order to investigate, if the MW based procedure might be more predictive, we first calculated the MW based IC_{50} and predicted LD_{50} values [µmol/mL or mmol/kg bw, respectively] for all substance with known MW (n = 142); being mainly below 500 g/mol. Then we calculated the respective weight based LD_{50} values and compared them with the predicted LD_{50}, calculated with weight based IC_{50} values (Figure 5). The MW based calculation was slightly less sensitive. Nevertheless, the correlation between both predicted LD_{50} values [mg/kg bw], based on weight and MW IC_{50} values, respectively, was very high with Pearson´s correlation coefficient of 0.9263 for the logarithmic LD_{50} values.

Additionally, we analyzed size and lipophilicity for one third of the substances (69/203), reflected by MW and log P_{OW}, respectively; which influence the substance uptake *in vitro* and *in vivo*. The MW was < 500 g/mol for all selected substances, hence favouring the GIT absorption (REACH Guidance document 7c. section 12.3. p. 157). According to the log P_{OW} values, which were calculated for pH 7, eight substances were hydrophilic (log Pow: < -1), 40 were favourable for absorption by passive diffusion (log P_{OW} > -1 – +4), and 21 were lipophilic (log P_{OW} > +4) (data not shown). For the hydrophilic substances the accuracy for the LD_{50} prediction by the NRU cytotoxicity test was comparable to the overall accuracy (38%) and only slightly better for the other two groups (about 50% each).

Another possibility to determine specific applicability domains might be the sub-grouping according to the substance class or mode of action. Focusing on the agrochemical active ingredients (AI) and formulations, we could not find certain applicability domains among the chemical classes (data not shown). Another subgrouping due to the MoA was possible for

some MoA with a reasonable number of substances, but again no applicability domains could be found (data not shown).

In summary, the additional analysis of our data set considering specific properties [see above: water-solubility, molecular weight, lipophilicity, substance class (agrochemical formulations and active ingredients) or mode of action] revealed no specific applicability domains.

Discussion:

Since the number of test substances that can be evaluated in a validation process is limited, an evaluation of alternative tests after the validation and its utility for routine testing is indispensable. Thus, we used a data set of 203 substances for which *in vivo* and *in vitro* data from routine testing in our laboratory was available and evaluated the utility of routinely performed cytotoxicity testing for acute oral toxicity assessment and its benefit for animal welfare.

The data set

The distribution of test substances was typical for the substances tested at our laboratory within approximately one year plus the substances used for validation. The overall prevalence of non-toxic substances with LD_{50} values > 2000 mg/kg bw in our laboratory was 39% (79 out of 203) which is much lower than numbers calculated by Bulgheroni in 2009 with 87% of 4219 substances in the EU´s New Chemicals Database being non-toxic (24). This might be due to the fact that screening studies were included into the data set which were found to be more toxic than the chemicals that were tested for registration purpose. The further development of a new product candidate may be discontinued if it exhibited high acute toxicity in screening studies. Moreover, testing in screening studies was sometimes not performed up to the highest limit of classification (EU; 2000 mg/kg bw) due to limited substance availability. In these cases, the highest dose tested was usually 300 mg/kg bw (Cat. 4). When all substances from screening studies with a LD_{50} > 300 (n = 42) that could theoretically have also a higher LD_{50} are included into the overall prevalence of non-toxic substances, the overall prevalence increases to 121 substances (79 + 42), which corresponds to an overall prevalence of 60%. This is still lower than given in the publication of Bulgheroni, 2009 (see above).

Correlation of *in vivo* and *in vitro* data; improvements due to applicability domains

Cytotoxicity models are limited by their incomplete modeling of the various cell and organ types. structures and functions as they occur *in vivo* (25). Therefore, the low concordance of the NRU cytotoxicity assay compared to the acute oral toxicity in rats, which was already described in the ICCVAM validation study in 2006 and was confirmed by our data set, was not surprising. In order to improve the predictivity of this *in vitro* test, we analyzed specific subgroups of our data set regarding physico-chemical properties (limited substance concentration *in vitro*, molecular weight and log P_{OW}), the substance class or mode of action, but could not identify specific applicability domains.

Whereas our data set covered the broad range of toxicity *in vivo*, the cytotoxicity test mainly predicted substances of moderate toxicity (predicted EU-GHS cat. 4). Therefore, the *in vitro* test was often underpredictive, maybe by not detecting functional toxicity or the lack of toxifying metabolism. But in other cases it was overpredictive, maybe due to the lack of detoxification capacity or toxicokinetic. Therefore, *in vitro* test batteries including organ-specific and biokinetic tests, such as the MEA assay (26;27) or hepatocytes for metabolization (28;29) seem to be necessary for improving the *in vitro* test, in order to be able to reduce or replace acute oral toxicity testing. However, conventional cytotoxicity assays are still the basis for further functional testing *in vitro*, as they determine the maximal useful test substance concentrations.

Animal savings

For our data set the prediction of acute toxicity resulted in an overall low concordance of values. Calculation of animal numbers for our data set in routine acute toxicity testing representative for testing of substances in our laboratory resulted in increased animal numbers when using cytotoxicity data for prediction of starting doses. This is in line with the overall low concordance but differs from the published data of the validation set of ICCVAM report,

where using the cytotoxicity assay for estimating the starting dose for the *in vivo* testing would reduce animal number by about 10%. For our data set using high default starting doses (2000 or 300 mg/kg bw) and starting doses based on expert judgement lead to the highest animal savings, while using starting doses predicted from cytotoxicity would have led to higher animal numbers and is therefore not in line with animal welfare considerations.

Conclusion:

In summary, the overall concordance of cytotoxicity data from 3T3 NRU test with *in vivo* acute oral toxicity data was unconvincing. The use of cytotoxicity data to predict starting doses in our laboratory would not have contributed to the refinement and reduction in acute oral toxicity testing. In contrast, if the data would have been used in our laboratory to predict starting doses more animals would have been used, than by predicting the starting dose by expert judgement.

Attempts to improve the predictivity of the *in vitro* cytotoxicity by additional analysis considering some physico-chemical data (solubility, molecular weight, log P_{OW}), substance class and mode of action revealed no specific applicability domains so far.

References:

(1) ICH Expert Working Group. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2). 11-6-2009. 9-9-2010.

(2) EU. Directive 2003/15/EG of the European Parliament and of the Council of 27.02.2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal of the European Union 2003; 46(L 66):26-35.

(3) EU. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union 2006; 49(L 396):1-849.

(4) EU. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union 2009; 52(L 309):1-50.

(5) Seidle T, Robinson S, Holmes T et al. Cross-Sector Review of Drivers and Available 3Rs Approaches for Acute Systemic Toxicity Testing. Toxicological Sciences 2010; 116(2):382-396.

(6) UN. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), third revised edition. Third revised edition ed. 2009.

(7) OECD. OECD Guidelines for the Testing of Chemicals, Test No. 423: Acute Oral Toxicity - Acute Toxic Class Method. 2001.

(8) Russell WMS, Burch RL. The Principles of Humane Experimental Technique. Special Edition: 1992; 1. Edition: 1959 ed. Universities Federation for Animal Welfare (UFAW), 1959.

(9) OECD. OECD Guidelines for the Testing of Chemicals, Test No. 401: Acute Oral Toxicity, deleted on 17th December 2002. 1984.

(10) Halle W. The registry of cytotoxicity: Toxicity testing cultures to predict acute toxicity (LD50) and testing in animals
3. Atla-Alternatives to Laboratory Animals 2003; 31(2):89-198.

(11) Wallace KA, Harbell JW, Accomando N, Triana A, Valone S, Curren RD. Evaluation of the Human Epidermal Keratinocyte Neutral Red Release and Neutral Red Uptake Assay Using the 1St-10 Meic Test Materials
1. Toxicology in Vitro 1992; 6(4):367-371.

(12) Roguet R, Cotovio J, Gaetani Q, Dossou KG, Rougier A. Cytotoxicity of 28 Meic Chemicals to Rat Hepatocytes Using 2 Viability End-Points - Correlation with Acute Toxicity Data in Rat and Man
81. Atla-Alternatives to Laboratory Animals 1993; 21(2):216-224.

(13) Jirova D, Kejlova K, Brabec M, Bendova H, Kolarova H. The benefits of the 3T3 NRU test in the safety assessment of cosmetics: long-term experience from pre-marketing testing in the Czech Republic
51. Toxicology in Vitro 2003; 17(5-6):791-796.

(14) King AV, Jones PA. In-house assessment of a modified in vitro cytotoxicity assay for higher throughput estimation of acute toxicity
50. Toxicology in Vitro 2003; 17(5-6):717-722.

(15) Sjostrom M, Kolman A, Clemedson C, Clothier R. Estimation of human blood LC50 values for use in modeling of in vitro-in vivo data of the ACuteTox project
15. Toxicology in Vitro 2008; 22(5):1405-1411.

(16) ICCVAM. ICCVAM Test Method Evaluation Report (TMER): In Vitro Cytotoxicity Test Methods for Estimating Starting Doses For Acute Oral Systemic Toxicity Testing, NIH Publication No. 07-4519. 2006.

(17) Lilienblum W, Dekant W, Foth H et al. Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH). Archives of Toxicology 2008; 82(4):211-236.

(18) Sazonovas A, Japertas P, Didziapetris R. Estimation of reliability of predictions and model applicability domain evaluation in the analysis of acute toxicity (LD50). Sar and Qsar in Environmental Research 2010; 21(1-2):127-148.

(19) Zhu H, Martin TM, Ye L, Sedykh A, Young DM, Tropsha A. Quantitative Structure-Activity Relationship Modeling of Rat Acute Toxicity by Oral Exposure. Chemical Research in Toxicology 2009; 22(12):1913-1921.

(20) Kinsner-Ovaskainen A, Bulgheroni A, Hartung T, Prieto P. ECVAM's ongoing activities in the area of acute oral toxicity. Toxicology in Vitro 2009; 23(8):1535-1540.

(21) EU. Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), Part B: Methods for the determination of toxicity and other health effects: Acute Oral Toxicity - Acute Toxic Class Method. Official Journal of the European Union 2008; 51(L 142):145-173.

(22) U.S.EPA. Health Effects Test Guidelines OPPTS 870.1100: Acute Oral Toxicity. EPA 712–C–02–190, 1-35. 31-12-2002. Washington D.C., USA. 10-9-2010.

(23) MAFF Japan. Notification No. 12-Nousan-8147: "Data Requirements for Supporting Registration of Pesticides". Agricultural Production Bureau, MAFF, Japan, 2000.

(24) Bulgheroni A, Kinsner-Ovaskainen A, Hoffmann S, Hartung T, Prieto P. Estimation of acute oral toxicity using the No Observed Adverse Effect Level (NOAEL) from the 28 day repeated dose toxicity studies in rats. Regulatory Toxicology and Pharmacology 2009; 53(1):16-19.

(25) O'Brien PJ, Irwin W, Diaz D et al. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening
2. Archives of Toxicology 2006; 80(9):580-604.

(26) Gopal KV, Miller BR, Gross GW. Acute and sub-chronic functional neurotoxicity of methylphenidate on neural networks in vitro. Journal of Neural Transmission 2007; 114(11):1365-1375.

(27) Vato A, Bonzano L, Chiappalone M et al. Spike manager: a new tool for spontaneous and evoked neuronal networks activity characterization. Neurocomputing 2004; 58:1153-1161.

(28) Farkas D, Tannenbaum SR. In vitro methods to study chemically-induced hepatotoxicity: A literature review. Current Drug Metabolism 2005; 6(2):111-125.

(29) Gomez-Lechon MJ, Tolosa L, Castell JV, Donato MT. Mechanism-based selection of compounds for the development of innovative in vitro approaches to hepatotoxicity studies in the LIINTOP project. Toxicology in Vitro 2010; 24(7):1879-1889.

(30) EU. *Regulation (EC) No 1272/2008* of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing *Directives 67/548/EEC* and *1999/45/EC*, and amending *Regulation (EC) No. 1907/2006* (Text
with EEA relevance). Annex I, Section 3.3, 91-95. Official Journal of the European Union 2008; 51(L 353):1-1355.

Tables:

Table 1: LD_{50} (acute, oral, rat), IC_{50} and predicted LD_{50}, as well as some general information: A) of the substances from the Halle Register 2003 and B) of the BASF in-house substances. Data are sorted by LD_{50} value [mg/kg bw].

A) Substances with published *in vivo* and *in vitro* data, tested *in vitro* (n = 16). *In vivo* and *in vitro* data from the Halle Register, 2003 as well as our own *in vitro* results are shown. MW: molecular weight; R/M: rat/mouse; n. req.: not requested. * Recommended reference standards (ICCVAM 2006); ** Similar structure to recommended reference standards.

Substance information					Halle Register, 2003				BASF SE	
No.	**Name**	**CAS no.**	**Physical form**	**MW [g/mol]**	**LD_{50} (oral, R/M) [mg/kg bw]**	**EU-GHS category (oral. R/M)**	**IC_{50} [µg/ml]**	**Pred. LD_{50} [mg/kg bw]**	**IC_{50} [µg/ml]**	**Pred. LD_{50} [mg/kg bw]**
1	Nicotine	54-11-5	liquid	162.2	50	2	290	882	700	1209
2	N-Methyl-N´-nitro-N-nitrosoguanidine	70-25-7	solid	147.1	90	3	2	91	4	179
3	Acrylamide	79-06-1	liquid	71.1	170	3	114	369	81	542
4	p-Cresol	106-44-5	solid	108.2	207	3	24	236	10	244
5	Phenol *	108-95-2	liquid	94.1	414	4	283	641	172	717
6	Aniline	62-53-3	liquid	93.1	440	4	672	928	693	1204
7	1,2,4-Trichlorobenzene	120-82-1	liquid	181.4	757	4	129	659	49	450
8	Salicylic acid	69-72-7	solid	138.1	891	4	467	989	590	1134
9	Acetylsalicylic acid *	50-78-2	solid	180.2	1000	4	420	1098	483	1053
10	Ibuprofene	15687-27-1	solid	206.3	1009	4	109	660	267	845
11	Sodium dodecyl sulfate	151-21-3	solid	289.4	1288	4	67	644	54	465
12	Toluene	108-88-3	liquid	92.2	5004	n.req.	1576	1336	134	654
13	Diethyl phthalate **	84-66-2	liquid	222.3	8602	n.req.	1227	1971	345	929
14	Captan	133-06-2	solid	300.6	10010	n.req.	1	114	1	102
15	Ethanol *	64-17-5	liquid	46.1	14008	n.req.	17464	2572	14974	3778
16	1,2-Propandiol **	57-55-6	liquid	76.1	20017	n.req.	26030	4062	16024	3874

B) BASF in-house substances, tested *in vitro* and *in vivo* (n = 187). Data are sorted by EU-GHS category and predicted LD_{50} value [mg/kg bw]. Abbreviations: formul.: formulation; n.a.: not applicable; MW: molecular weight (rounded to 50 units); n.kn.: not known; n.req.: not requested; preGLP: The *in vivo* study was performed before 1989, regarded as a non-GLP screening study. * The *in vivo* study was performed according to OECD guideline 401, prior in force.

Substance information				*in vitro* data (Balb/c 3T3 NRU)				*in vivo* data (acute oral rat)			recommended starting dose	
No.	**Group**	**Physical form**	**MW [g/mol]**	**Max. test conc. [µg/ml]**	**Precipitate [µg/ml] at:**	**IC_{50} [µg/ml]**	**pred. LD_{50} [mg/kg]**	**GLP, OECD 423**	**LD_{50} [mg/kg]**	**EU-GHS category [mg/kg]**	**by *in vitro* test**	**by expert judgement**
17	chemical	solid	300	298	n/a	3	153	preGLP	50	2	50	
18	fungicide	solid	400	100	46.4	15	291	-	< 50	2	300	
19	fungicide	solid	450	100	100	16	293	-	< 50	2	300	
20	insecticide	solid	250	100	100	16	298	-	about 50	2	300	
21	fungicide	solid	400	100	100	19	314	-	< 50	2	300	
22	insecticide	solid	250	100	100	20	320	-	< 50	2	300	
23	fungicide	solid	450	100	100	20	322	-	< 50	2	300	
24	fungicide	solid	400	100	100	25	350	-	< 50	2	300	
25	fungicide	solid	400	100	100	33	387	+	< 50	2	300	
26	fungicide	solid	450	100	100	38	409	-	< 50	2	300	
27	fungicide	solid	450	100	100	41	420	-	< 50	2	300	
28	fungicide	liquid	450	100	100	56	471	-	< 50	2	300	
29	fungicide	solid	450	100	100	62	490	-	< 50	2	300	
30	fungicide	solid	400	100	100	62	492	-	< 50	2	300	
31	insecticide	solid	200	100	100	68	509	-	> 5 - 50	2	300	
32	fungicide	solid	400	100	100	86	554	-	< 50	2	300	
33	fungicide	solid	400	100	100	> 100	> 586	-	< 50	2	2000	
34	fungicide	solid	400	100	100	> 100	> 586	-	about 50	2	2000	
35	insecticide	solid	300	100	100	> 100	> 586	-	< 50	2	2000	
36	insecticide	solid	300	100	100	3	151	-	> 50 - 300	3	50	

37	chemical	solid	300	10	100	3	154	+	> 50 - 300	3	50	
38	chemical	liquid	350	1000	100	11	255	+	> 50 - 300	3	300	300
39	insecticide	solid	450	437	100	11	257	+*	290	3	50	
40	fungicide	solid	n.kn.	100	100	18	309	-	> 50 - 300	3	300	
41	fungicide	solid	450	100	100	18	310	-	about 300	3	300	
42	herbicide	solid	250	257	100	23	337	preGLP	57	3	300	
43	fungicide	solid	n.kn.	100	100	32	382	-	> 50 - 300	3	300	
44	fungicide	solid	400	100	100	32	384	-	> 50 - 300	3	300	
45	fungicide	solid	450	100	100	33	387	-	> 50 - 300	3	300	
46	herbicide	solid	300	100	100	37	404	-	> 50 - 300	3	300	
47	fungicide	solid	450	100	100	42	423	-	> 50	3	300	
48	fungicide	solid	450	100	100	71	516	-	< 300	3	300	
49	fungicide	solid	450	100	100	> 100	> 586	-	< 300	3	2000	
50	fungicide	solid	n.kn.	100	100	> 100	> 586	-	> 50 - 300	3	2000	
51	fungicide	solid	500	100	100	> 100	> 586	-	> 50	3	2000	
52	fungicide	solid	450	100	100	> 100	> 586	-	< 300	3	2000	
53	insecticide	liquid	450	100	100	> 100	> 586	-	> 60 - 300	3	2000	
54	fungicide	solid	450	100	100	106	598	-	> 50 - 300	3	300	
55	insecticide	liquid	400	100	100	151	684	-	about 300	3	300	
56	chemical	liquid	100	81000	100	5620	2624	preGLP	76	3	2000	
57	fungicide	solid	300	5	100	0.04	31	-	> 300 - 2000	4	5	
58	fungicidal formul.	liquid	n.a.	10	100	0.1	42	+	> 500 - 2000	4	50	300
59	fungicidal formul.	liquid	n.a.	5	100	0.3	66	+	> 300 - 500	4	50	300, 500
60	fungicidal formul.	liquid	n.a.	10	100	0.3	67	+	about 500	4	50	2000
61	fungicidal formul.	liquid	n.a.	10	100	0.49	81	+	> 500 - 2000	4	50	500
62	fungicidal formul.	liquid	n.a.	10	100	1	109	+	> 500 - 2000	4	50	2000
63	fungicide	solid	400	100	100	1.3	115	-	> 300	4	50	
64	insecticide	solid	250	100	100	1	115	-	> 300	4	50	
65	insecticide	solid	300	100	100	1.6	127	-	> 300	4	50	
66	insecticide	liquid	250	100	100	2	139	-	> 300	4	50	
67	insecticide	solid	450	100	100	2.1	140	-	> 300	4	50	
68	fungicide	solid	400	100	100	3	156	-	> 300	4	50	
69	insecticide	solid	300	100	100	3	165	-	> 300	4	50	
70	fungicidal formul.	solid	n.a.	5	100	4.8	189	+	> 300 - 2000	4	50	2000
71	insecticide	solid	600	100	100	6.9	217	-	> 300	4	50	

72	insecticide	solid	350	100	100	7	223	-	> 300	4	300	
73	insecticide	solid	300	100	100	13	273	-	> 300	4	300	
74	insecticide	solid	550	100	100	15	286	-	> 300	4	300	
75	fungicide	solid	500	100	100	15	290	-	> 300	4	300	
76	biocide	liquid	n.kn.	100	100	15	291	+	> 300 - 2000	4	300	2000
77	fungicide	solid	500	100	100	18	307	-	> 300	4	300	
78	insecticide	solid	300	100	100	19	319	-	> 300	4	300	
79	insecticide	solid	350	100	100	20	321	-	> 300	4	300	
80	insecticidal formul.	liquid	n.a.	2154	100	21	327	+	> 300 - 2000	4	300	2000
81	fungicide	solid	450	100	100	26	354	-	> 300	4	300	
82	fungicide	solid	500	100	100	27	360	-	> 300	4	300	
83	fungicide	solid	500	100	100	28	367	-	> 300	4	300	
84	chemical	solid	n.kn.	2154	100	29	370	+	> 300 - 2000	4	300	2000
85	fungicide	solid	500	100	100	30	376	-	> 300	4	300	
86	fungicide	solid	450	100	100	31	379	-	> 300	4	300	
87	insecticide	solid	250	100	100	31	381	-	> 300	4	300	
88	insecticide	solid	350	100	100	32	382	-	> 300	4	300	
89	insecticide	solid	350	100	100	37	407	-	> 300	4	300	
90	insecticide	solid	450	100	100	38	407	-	> 300	4	300	
91	chemical	liquid	n.kn.	2154	100	39	414	+	> 300 - 2000	4	300	2000
92	fungicide	solid	450	100	100	41	422	-	> 300	4	300	
93	fungicide	solid	450	100	100	42	426	-	> 300	4	300	
94	fungicide	liquid	300	298	100	44	431	preGLP	~1000	4	300	
95	chemical	solid	400	2154	100	49	450	+	> 300 - 2000	4	300	300
96	herbicidal formul.	liquid	n.a.	2154	100	57	475	+	> 300 - 2000	4	300	2000
97	fungicide	solid	450	100	100	62	491	-	> 300	4	300	
98	fungicide	solid	450	100	100	63	495	-	> 300	4	300	
99	fungicide	solid	450	100	100	64	498	-	> 300	4	300	
100	fungicide	liquid	450	100	100	67	504	-	> 300	4	300	
101	fungicide	solid	450	100	100	68	509	-	> 300	4	300	
102	insecticide	solid	250	100	100	71	516	-	> 300 - 2000	4	300	
103	insecticide	solid	200	100	100	78	534	-	> 300 - 2000	4	300	
104	fungicide	liquid	450	100	100	80	538	-	> 300	4	300	
105	fungicide	solid	500	100	100	80	540	-	> 300	4	300	
106	insecticidal formul.	liquid	n.a.	1000	100	84	550	+	> 300 - 2000	4	300	500

107	fungicide	solid	450	100	100	> 100	> 586	-	> 300	4	300	
108	fungicide	solid	500	100	100	> 100	> 586	-	> 300	4	300	
109	fungicide	solid	500	100	100	> 100	> 586	-	> 300	4	300	
110	fungicide	solid	450	100	100	> 100	> 586	-	> 300	4	2000	
111	insecticide	solid	300	100	100	> 100	> 586	-	> 300 - 2000	4	2000	
112	insecticide	solid	250	100	100	> 100	> 586	-	> 300	4	2000	
113	insecticide	liquid	250	100		> 100	> 586	-	> 300 - 2000	4	2000	
114	insecticide	solid	300	100		> 100	> 586	-	> 300 - 2000	4	2000	
115	fungicide	solid	450	100	100	112	612	-	> 300	4	300	
116	herbicide	solid	300	298	100	115	617	preGLP	~1000	4	300	
117	fungicidal formul.	liquid	n.a.	1000	463.2	124	635	+	> 500 - 2000	4	300	2000
118	insecticide	solid	300	100	-	126	640	-	> 300	4	300	
119	insecticide	solid	250	100	-	131	647	-	> 300	4	300	
120	chemical	solid	250	2154	-	166	707	+	> 300 - 2000	4	300	2000
121	chemical	solid	250	2154	1000	168	710	+	> 300 - 2000	4	300	2000
122	chemical	liquid	100	10000	-	315	898	+	> 300 - 2000	4	300	300
123	herbicide	solid	250	2403	n/a	352	936	preGLP	~1200	4	300	
124	fungicide	solid	500	100	10	431	1009	-	> 300	4	300	
125	chemical	solid	200	1000	-	> 1000	> 1380	+	> 500 - 2000	4	2000	300
126	herbicidal formul.	liquid	n.a.	2154	-	1062	1412	+	> 300 < 2000	4	2000	2000
127	insecticidal formul.	liquid	n.a.	2154	215.4	1805	1720	+	> 300 < 2000	4	2000	300
128	chemical	liquid	200	10000	-	1976	1778	+	> 300 - 2000	4	2000	2000
129	herbicide	liquid	150	149660	n/a	5909	2673	+*	~1000	4	2000	
130	fungicidal formul.	liquid	n.a.	10	215.4	0.4	72	+	> 2000	n.req.	50	2000
131	fungicide	solidified	400	388	n/a	0.4	74	+*	> 5000	n.req.	50	
132	fungicidal formul.	liquid	n.a.	10	215.4	1	84	+	> 2000	n.req.	50	2000
133	fungicide	solid	350	326	n/a	1	90	+*	> 5000	n.req.	50	
134	fungicidal formul.	liquid	n.a.	10	100	1.1	111	+	> 2000	n.req.	50	2000
135	fungicidal formul.	liquid	n.a.	10	215.4	2	135	+	> 2000	n.req.	50	2000
136	insecticide	solid	350	100	21.5	2.2	143	-	> 2000	n.req.	50	
137	chemical	solid	n.kn.	10	21.5	2.3	145	+	> 2000	n.req.	50	2000
138	fungicidal formul.	liquid	n.a.			N/A	161	+	> 2000	n.req.	50	500
139	fungicide	solid	300	313	n/a	4	173	+*	> 5000	n.req.	50	
140	chemical	liquid	n.kn.	10	-	4	178	+	> 2000	n.req.	50	2000
141	fungicide	solid	400	100	46.4	4.6	186	-	> 2000	n.req.	50	

142	fungicidal formul.	solid	n.a.	5	4.6	4.8	190	+	> 2000	n.req.	50	2000
143	fungicide	solid	350	10	46.4	7	215	+	> 2000	n.req.	50	
144	fungicidal formul.	solid	n.a.	46	21.5	7	218	+	> 2000	n.req.	300	2000
145	fungicidal formul.	liquid	n.a.	10	215.4	7	224	+	> 2000	n.req.	50	2000
146	mixture	liquid	n.a.	1000	46.4	9	235	+	> 2000	n.req.	300	2000
147	fungicide	solid	400	100	100	8.7	236	-	> 2000	n.req.	50	
148	additive	solid	350	1000	100	16	297	+	> 2000	n.req.	300	
149	fungicidal formul.	solid	n.a.	2154	10	21	330	+	> 2000	n.req.	300	2000
150	additive	liquid	n.kn.	100	-	29	368	+	> 2000	n.req.	300	2000
151	chemical	liquid	200	100	46.4	34	393	+	> 2000	n.req.	300	300
152	mixture	liquid	n.a.	1000	464.2	37	405	+	> 2000	n.req.	300	2000
153	fungicide	solid	350	330	n/a	59	481	+*	> 5000	n.req.	300	
154	fungicide	solid	400	100	-	68	508	-	> 2000	n.req.	300	
155	dye, mixture	liquid	n.a.	2154	-	71	516	+	> 2000	n.req.	300	2000
156	additive	solid	350	1000	4.6	80	541	+	> 2000	n.req.	300	300
157	chemical	solid	600	2154	46.4	87	556	+	> 2000	n.req.	300	2000
158	chemical	solid	650	100	4.6	> 100	> 586	+	> 2000	n.req.	2000	2000
159	herbicide	solid	350	100	46.4	> 100	> 586	-	> 2000	n.req.	2000	
160	insecticide	solid	300	100	-	> 100	> 586	-	> 2000	n.req.	2000	
161	insecticide	solid	350	100	-	> 100	> 586	-	> 2000	n.req.	2000	
162	insecticide	solid	400	100	100	> 100	> 586	-	> 2000	n.req.	2000	
163	insecticide	solid	450	100	-	> 100	> 586	-	> 2000	n.req.	2000	
164	insecticide	solid	250	100	-	> 100	> 586	-	> 2000	n.req.	2000	
165	insecticide	solid	300	100		> 100	> 586	-	> 2000	n.req.	2000	
166	insecticide	solid	300	100	-	> 100	> 586	-	> 2000	n.req.	2000	
167	architetural coating	liquid	n.a.	2154	-	131	648	+	> 2000	n.req.	300	2000
168	fungicide	solid	350	100	-	147	677	-	> 2000	n.req.	300	
169	fungicidal formul.	liquid	n.a.	2154	100	186	738	+	> 2000	n.req.	300	2000
170	insecticidal formul.	liquid	n.a.	2154	100	209	771	+	> 2000	n.req.	300	2000
171	architetural coating	solid	n.a.	1000	100	225	792	+	> 5000	n.req.	300	5000
172	fungicidal formul.	liquid	n.a.	2154	-	287	868	+	> 2000	n.req.	300	2000
173	mix - adhesive	solid	n.a.	2154	463.2	307	889	+	> 2000	n.req.	300	2000
174	herbicide	solid	200	4432	n/a	499	1066	+*	> 2000	n.req.	300	
175	chemical	solid	550	1000	-	557	1110	+	> 2000	n.req.	300	2000
176	herbicidal formul.	liquid	n.a.	1000	4.6	665	1186	+	> 2000	n.req.	300	2000

177	herbicide	solid	200	2216	n/a	666	1187	+*	> 5000	n.req.	300	
178	chemical	solid	110	1000	-	757	1244	+	> 2000	n.req.	300	2000
179	architetural coating	solid	n.a.	2154	464.2	903	1329	+	> 2000	n.req.	300	2000
180	additive	solid	1150	1000	1000	> 1000	> 1380	+	> 2000	n.req.	2000	2000
181	additive	liquid	n.a.	1000	464.2	> 1000	> 1380	+	> 2000	n.req.	2000	2000
182	herbicide	solid	300	1000	-	> 1000	> 1380	+	> 2000	n.req.	2000	2000
183	herbicidal formul.	solid	n.a.	1000	215.4	> 1000	> 1380	+	> 2000	n.req.	2000	2000
184	insecticidal formul.	solid	n.a.	1000	10	> 1000	> 1380	+	> 2000	n.req.	2000	300
185	mixture	liquid	n.a.	1000	-	> 1000	> 1380	+	> 2000	n.req.	2000	2000
186	fungicidal formul.	liquid	n.a.	2154	100	1073	1417	+	> 2000	n.req.	2000	2000
187	architetural coating	liquid	n.a.	2150	100	1784	1712	+	> 2000	n.req.	2000	2000
188	chemical	solid	n.kn.	2154	-	1806	1720	+	> 2000	n.req.	2000	2000
189	herbicidal formul.	liquid	n.a.	2154	-	1900	1753	+	> 2000	n.req.	2000	2000
190	chemical	liquid	n.a.	2154	-	2117	1825	+	> 2000	n.req.	2000	2000
191	chemical	liquid	n.a.	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	2000
192	polymer	liquid	n.a.	2154	46.4	> 2154	> 1836	+	> 2000	n.req.	2000	2000
193	dye, mixture	solid	n.a.	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	2000
194	herbicidal formul.	liquid	n.a.	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	2000
195	herbicidal formul.	liquid	n.a.	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	2000
196	chemical	solid	150	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	2000
197	mix - yeast extract	liquid	n.a.	2154	-	> 2154	> 1836	+	> 2000	n.req.	2000	5000
198	polymer	liquid	n.a.	2154	2154.4	> 2154	> 1836	+	> 2000	n.req.	2000	2000
199	herbicidal formul.	solid	n.a.	10000	4642	2473	1933	+	> 2000	n.req.	2000	2000
200	architetural coating	liquid	n.a.	2154	-	2715	2002	+	> 2000	n.req.	2000	2000
201	herbicidal formul.	liquid	n.a.	10000	-	3662	2237	+	> 2000	n.req.	2000	2000
202	chemical	liquid	50	4642	-	> 4642	> 2444	+	> 2000	n.req.	2000	2000
203	polymer	liquid	14000	10000	-	5397	2584	+	> 2000	n.req.	2000	2000

Table 2: Calculation of needed animal number per starting dose and LD_{50}. The animal number for GLP guideline and screening studies were estimated using first the minimum amount of animals needed to reach the final *in vivo* result (A) and by using a probit model based on a logarithmic dose scale with slopes of 2 and 8 (B or C, respectively), according to the ICCVAM publication (16).

A)		**Starting dose [mg/kg bw]**		
		2000	300	50
LD_{50} [mg/kg bw]	> 2000	6	12	18
	> 300 - 2000	9	9	15
	> 50 - 300	12	9	9
	≤ 50	15	12	9

B) Slope 2		**Starting dose [mg/kg bw]**		
		2000	300	50
LD_{50} [mg/kg bw]	2500	7.9	11.2	17.2
	2000	9.0	10.5	16.5
	1000	9.1	9.1	15.1
	300	12.0	9.8	11.3
	200	12.3	9.3	9.4
	100	12.2	9.2	9.1
	50	15.0	12.0	9.8
	5	13.5	10.5	7.5

C) Slope 8		**Starting dose [mg/kg bw]**		
		2000	300	50
LD_{50} [mg/kg bw]	2500	6.0	12.0	18.0
	2000	9.0	10.5	16.5
	1000	9.0	9.0	15.0
	300	12.0	9.8	11.3
	200	12.0	9.0	9.0
	100	12.0	9.0	9.0
	50	15.0	12.0	9.8
	5	13.5	10.5	7.5

Table 3: Comparing the *in vivo* hazard category and the predicted LD_{50} A) of all tested substances, B) of substances from the Halle Register, 2003 and C) of the BASF in-house substances. The *in vivo* acute, oral hazard category (30) was compared to predicted LD_{50} derived from the *in vitro* IC_{50} for all test substances. The absolute numbers of correct, under- or overestimated predictions are shown. The bold framed cells show the number of correct predictions. OP: overpredicted; UP: underpredicted.

A) all tested substances (n = 203)

LD_{50} rat, oral [mg/kg bw]	**≤ 50**	**> 50 - 300**	**> 300 - 2000**	**> 2000**	**total**	**total [%]**	**predictivity**	**catgory OP**	**category UP**
EU-GHS cat.	**Cat. 1-2**	**Cat. 3**	**Cat. 4**	**n. req.**					
> 2000	0	1	1	6	8	4%	75%	25%	-
Pred. LD_{50} > 300 - 2000	16	17	59	53	145	71%	41%	23%	37%
[mg/kg bw] > 50 - 300	4	6	18	20	48	24%	13%	8%	79%
≤ 50	0	0	2	0	2	1%	not calc.	-	not calc.
total	20	24	80	79					
total [%]	10%	12%	39%	39%					
accuracy	0%	25%	74%	8%					
toxicity OP	-	0%	25%	92%					
toxicity UP	100%	75%	1%	-					

B) Substances from the Halle Register, 2003 (n = 16)

LD_{50} rat, oral [mg/kg bw]	**≤ 50**	**> 50 - 300**	**> 300 - 2000**	**> 2000**	**total**	**total [%]**	**predictivity**	**catgory OP**	**category UP**
EU-GHS cat.	**Cat. 1-2**	**Cat. 3**	**Cat. 4**	**n. req.**					
> 2000	0	0	0	2	2	13%	100%	0%	-
Pred. LD_{50} > 300 - 2000	1	1	7	2	11	69%	64%	18%	18%
[mg/kg bw] > 50 - 300	0	2	0	1	3	19%	67%	0%	33%
≤ 50	0	0	0	0	0	0%	not calc.	-	not calc.
total	1	3	7	5					
total[%]	6%	19%	44%	31%					

accuracy	0%	67%	100%	40%
toxicity OP	-	0%	0%	60%
toxicity UP	100%	33%	0%	-

C) BASF in-house substances (n = 187)

LD_{50} rat, oral [mg/kg bw]		**≤ 50**	**> 50 - 300**	**> 300 - 2000**	**> 2000**	**total**	**total [%]**	**predictivity**	**catgory OP**	**category UP**
EU-GHS cat.		**Cat. 1-2**	**Cat. 3**	**Cat. 4**	**n. req.**					
	> 2000	0	1	1	4	6	3%	67%	33%	-
Pred. LD_{50}	**> 300 - 2000**	15	16	52	51	134	72%	39%	23%	38%
[mg/kg bw]	**> 50 - 300**	4	4	18	19	45	24%	9%	9%	82%
	≤ 50	0	0	2	0	2	1%	not calc.	-	not calc.
total		19	21	73	74					
total [%]		10%	11%	39%	40%					
accuracy		0%	19%	71%	5%					
toxicity OP		-	0%	27%	95%					
toxicity UP		100%	81%	1%	-					

Table 4: Estimation of animal numbers for GLP guideline studies. For the 75 substances, which were tested in GLP guideline studies and for which expert proposal of starting dose was available, we calculated the absolute number of animals when using the default starting dose of 2000, 300, and 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test or given by an experienced toxicologist and estimated the savings of animals. Finally, we calculated the minimum number, assuming always using the correct starting dose.

Starting dose			Animal number	
			sum	per test
If always	2000 mg/kg bw	then	519	6.9
If always	300 mg/kg bw	then	834	11.1
If always	50 mg/kg bw	then	1278	17.0
Expert judgement		then	546	7.3
Cytotoxicity test		then	684	9.1
If always correct		Minimum of animals	516	6.9

Table 5: Estimation of animal numbers for screening studies. As described in Table 4, we calculated the absolute number of animals when using the default starting dose of 2000, 300, and 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test and estimated the savings of animals. Two scenarios were calculated for the 100 screening studies: **A)** It was assumed that all > 300 mg/kg bw would have been in the range of > 2000 mg/kg bw or **B)** it was assumed that all > 300 mg/kg bw would have been in the categorie > 300 - 2000 mg/kg bw.

A)

Starting dose			Animal number	
			sum	per test
If always	2000 mg/kg bw	then	891	8.9
If always	300 mg/kg bw	then	1116	11.2
If always	50 mg/kg bw	then	1455	14.6
Cytotoxicity test		then	1191	11.9

B)

Starting dose			Animal number	
			sum	per test
If always	2000 mg/kg bw	then	1017	10.2
If always	300 mg/kg bw	then	990	9.9
If always	50 mg/kg bw	then	1329	13.3
Cytotoxicity test		then	1089	10.9

Table 6: Limitation of predicting the correct LD_{50} [mg/kg bw]due to limited solubility or highest concentration *in vitro*? The limited highest concentration *in vitro* restricted the calculation of the IC_{50} [µg/ml] and therefore the predicted LD_{50} [mg/kg bw] for 20% (40/203) of the substances. Among these substances the predicted LD_{50} was correctly compared to the *in vivo* determined hazard category for 25% (10/40). Nevertheless, the predicted starting dose would have been helpful for 80% (32/40).

	IC_{50} [µg/ml]	**pred. LD_{50} [mg/kg bw]**	**correct pred. LD_{50}***	**helpful starting dose***
A	> 100	> 586	32% (8/25)	68% (17/25)
B	> 1000	> 1380	14% (1/7)	100% (7/7)
C	> 2154	> 1836	0% (0/7)	100% (7/7)
D	> 4642	> 2444	100% (1/1)	100% (1/1)

* despite suboptimal highest concentrations *in vitro*

Figures:

Figure 1: Comparing the *in vitro* data with published *in vitro* results and *in vivo* hazard categories. The reliablity of the cytotoxicity test was proofed with 16 test substances from the Halle Register 2003. **A)** Correlation of published and own IC_{50} values with Pearson´s correlation coefficient of 0.9829. **B)** Comparison of the predicted LD_{50} with the *in vivo* results. The substances were grouped by the *in vivo* acute, oral hazard categories (30) and then plotted against the respective predicted LD_{50} derived from the *in vitro* IC_{50}. The grey markings show the correct predictions.

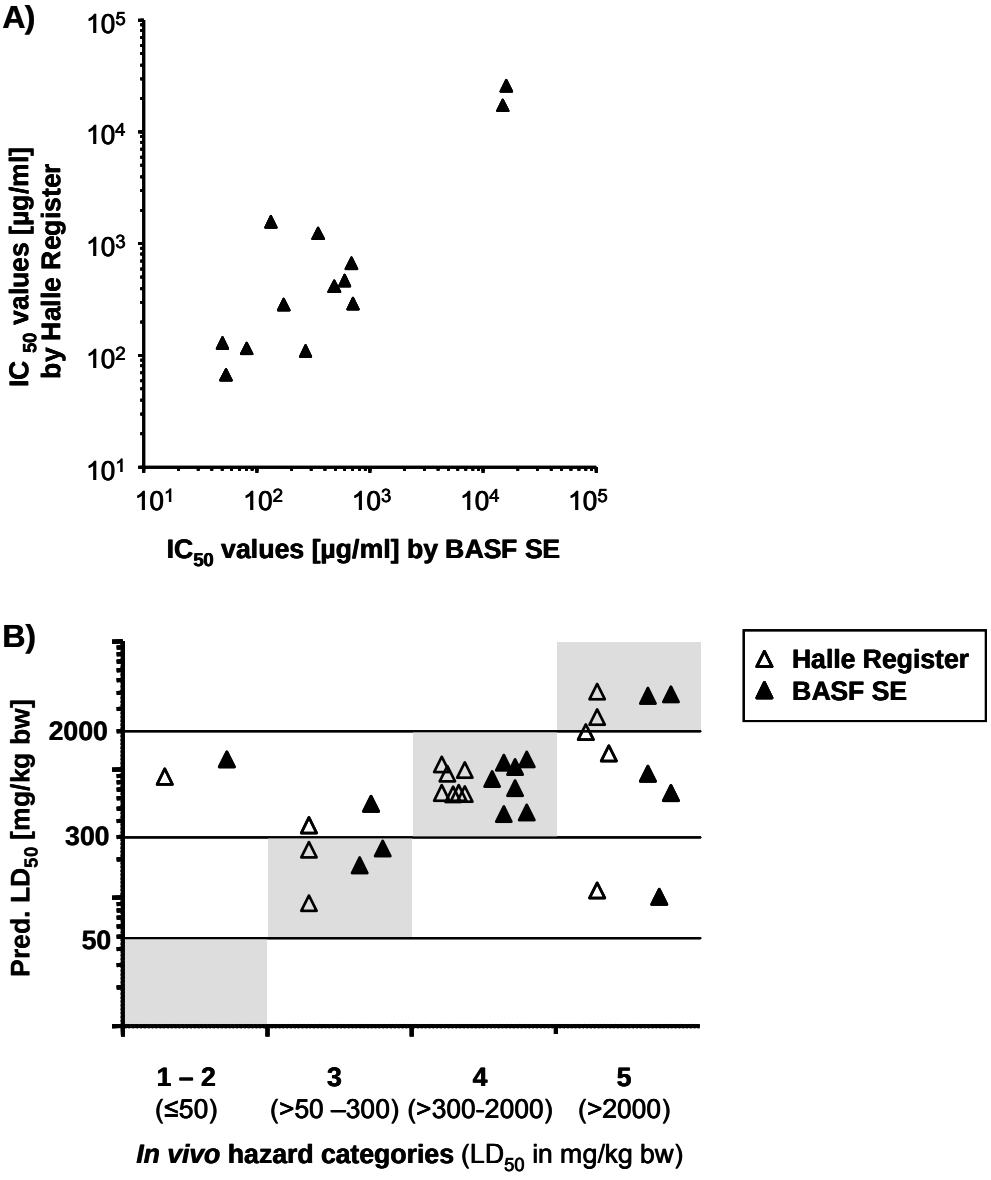


Figure 2: Comparing the predicted LD_{50} values with the *in vivo* hazard categories: A) all substances (n = 203). B) the GLP guideline studies (n = 87) and C) the screening studies (n = 100). The substances were grouped by the *in vivo* acute, oral hazard categories (30) and then plotted against the respective predicted LD_{50} derived from the *in vitro* IC_{50}. The grey markings show the correct predictions.

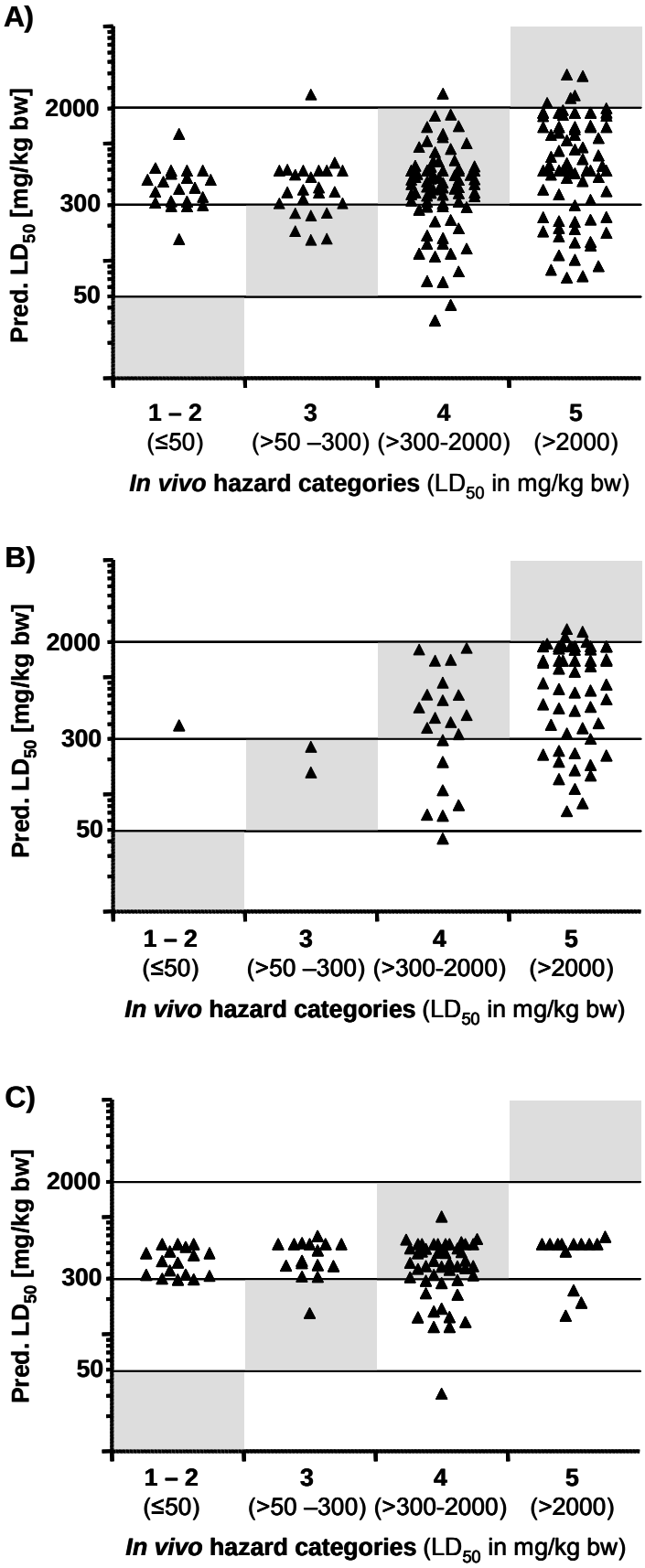


Figure 3: Estimated animal numbers for GLP guideline studies. For the 75 substances, which were tested in GLP guideline studies and for which expert proposal of starting dose was available, the absolute number of animals were added together when using the default starting dose of 2000, 300, and 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test or given by an experienced toxicologist (more than 15 years of experience in acute toxicity testing and background in BASF test items) and estimated the savings of animals.

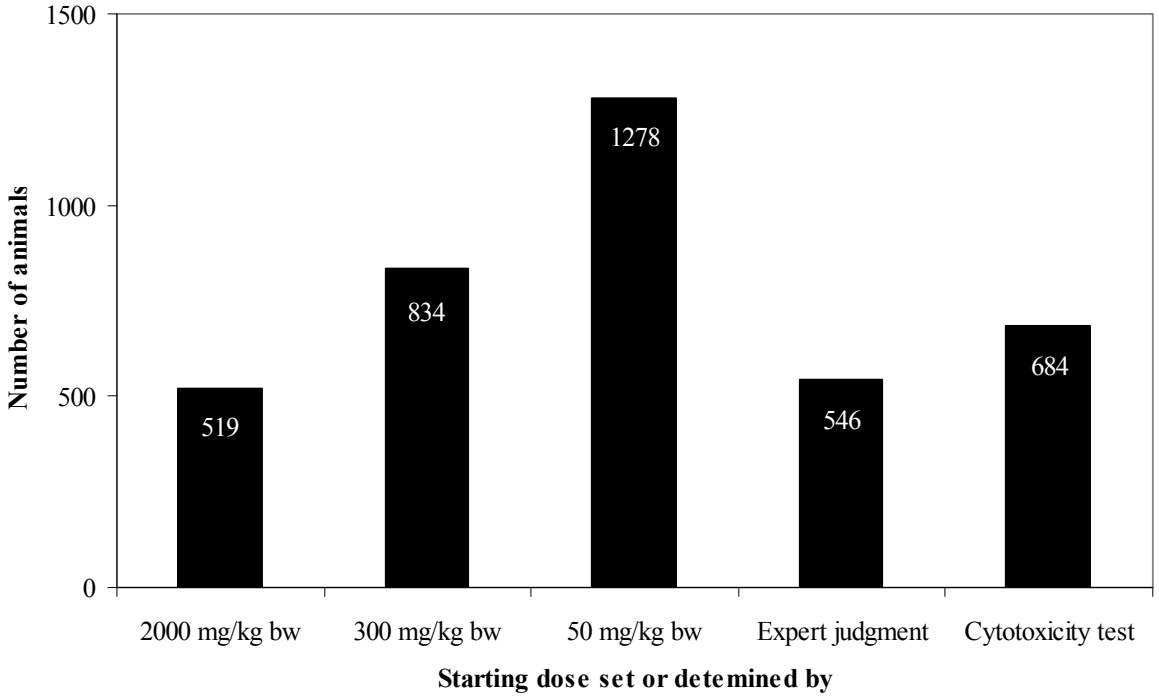
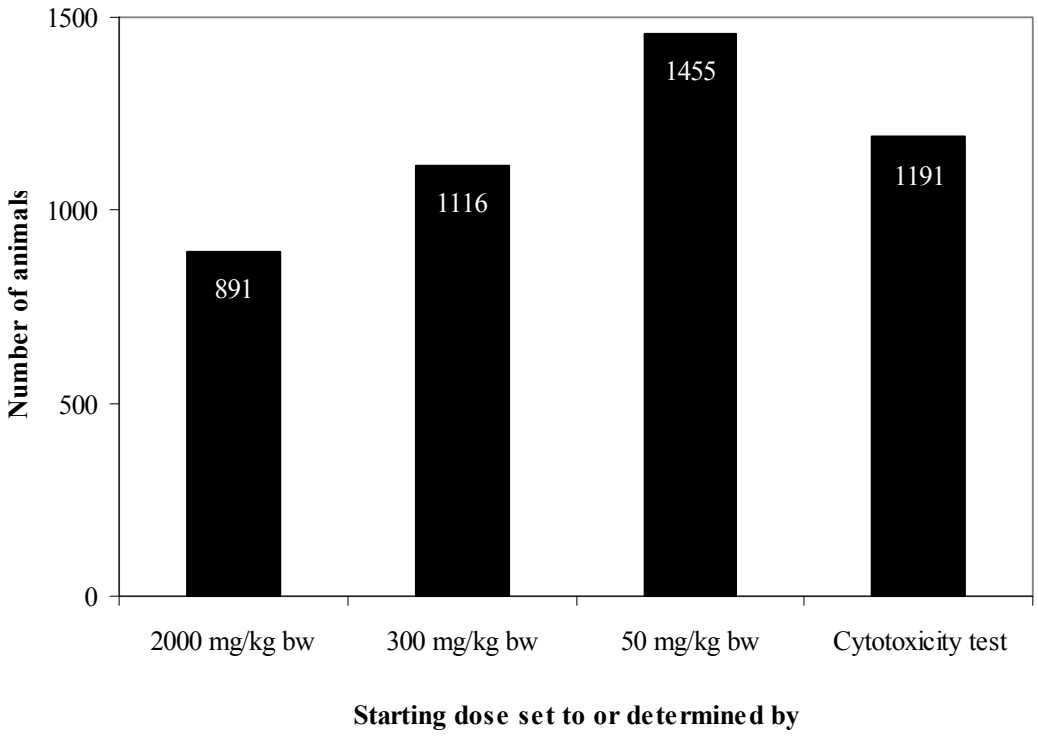


Figure 4: Estimated animal numbers for screening studies. The absolute number of animals was added together when using the default starting dose of 2000, 300, and 50 mg/kg bw or the predicted starting dose, derived from the *in vitro* test. Two scenarios were calculated for the 100 screening studies: **A).** It was assumed that all > 300 mg/kg bw would have been in the range of > 2000 mg/kg bw or **B)** that all > 300 mg/kg bw would have been in the category > 300 - 2000 mg/kg bw.

A)



B)

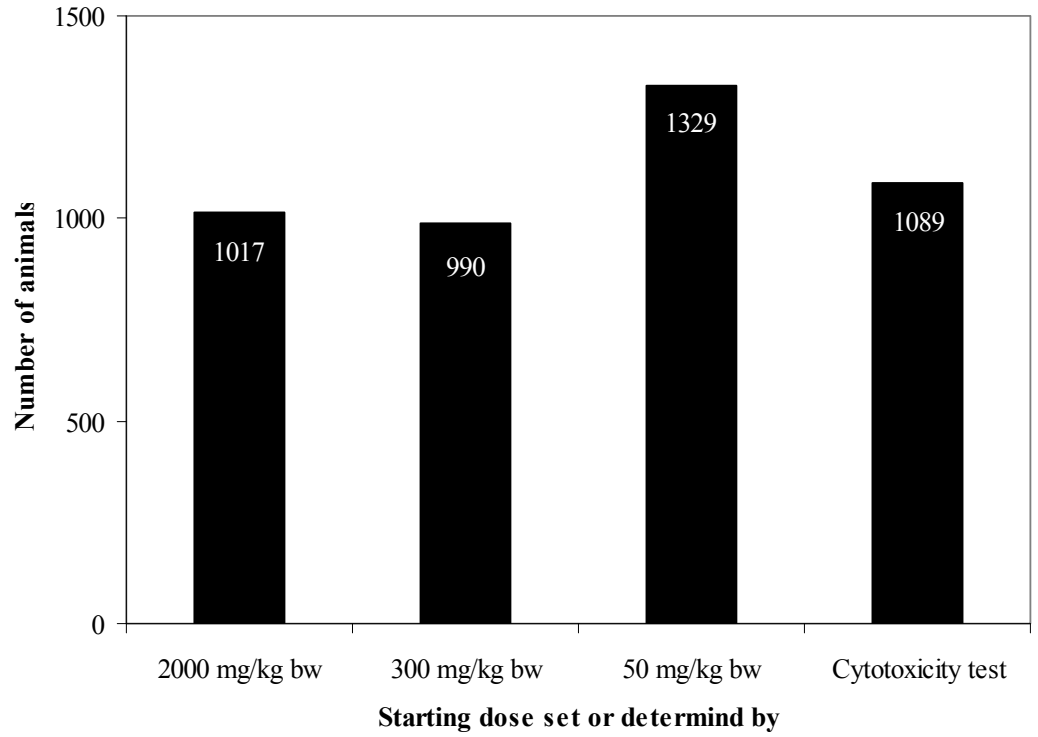


Figure 5: Comparing the predicted LD_{50} [mg/kg bw]. derived from weight or MW based IC_{50}. respectively [µg/ml or µmol/ml]. As the MW was known for 142 substances. the predicted LD_{50} was additionally calculated by the MW based formula [ICCVAM 2006] and plotted against the respective weight based LD_{50}. The Pearson´s correlation coefficient for the correlation of the logarithmic LD_{50} values was 0.9289.