A Novel Two-Step Hierarchical Quantitative Structure–Activity Relationship Modeling Work Flow for Predicting Acute Toxicity of Chemicals in Rodents

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BACKGROUND: Accurate prediction of in vivo toxicity from in vitro testing is a challenging problem. Large public–private consortia have been formed with the goal of improving chemical safety assessment by the means of high-throughput screening.

OBJECTIVE: A wealth of available biological data requires new computational approaches to link chemical structure, in vitro data, and potential adverse health effects.

METHODS AND RESULTS: A database containing experimental cytotoxicity values for in vitro half-maximal inhibitory concentration (IC₅₀) and in vivo rodent median lethal dose (LD₅₀) for more than 300 chemicals was compiled by Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (ZEBET; National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments). The application of conventional quantitative structure–activity relationship (QSAR) modeling approaches to predict mouse or rat acute LD₅₀ values from chemical descriptors of ZEBET compounds yielded no statistically significant models. The analysis of these data showed no significant correlation between IC₅₀ and LD₅₀. However, a linear IC₅₀ versus LD₅₀ correlation could be established for a fraction of compounds. To capitalize on this observation, we developed a novel two-step modeling approach as follows. First, all chemicals are partitioned into two groups based on the relationship between IC₅₀ and LD₅₀ values: One group comprises compounds with linear IC₅₀ versus LD₅₀ relationships, and another group comprises the remaining compounds. Second, we built conventional binary classification QSAR models to predict the group affiliation based on chemical descriptors only. Third, we developed k-nearest neighbor continuous QSAR models for each subclass to predict LD₅₀ values from chemical descriptors. All models were extensively validated using special protocols.

CONCLUSIONS: The novelty of this modeling approach is that it uses the relationships between in vivo and in vitro data only to inform the initial construction of the hierarchical two-step QSAR models. Models resulting from this approach employ chemical descriptors only for external prediction of acute rodent toxicity.

KEY WORDS: acute toxicity, computational toxicology, IC₅₀, LD₅₀, LOAEL, NOAEL, QSAR.

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Development of accurate and predictive in vitro toxicity testing methods that could be used as alternatives for lengthy and costly in vivo experiments has long been an elusive goal for both industry and regulatory agencies (National Research Council 2007). New, bold research programs were recently established at the National Toxicology Program (Xia et al. 2008) and the U.S. Environmental Protection Agency (U.S. EPA) (Dix et al. 2007) and coordinated at the interagency level by the U.S. government (Collins et al. 2008) to address this important challenge in a systematic way. The overall goal of these initiatives is to explore a diverse array of in vitro toxicity assays, such as cell-based and cell-free high-throughput screening (HTS) techniques, as well as toxicogenomic technologies, to evaluate the toxic potential of chemicals and prioritize candidates for animal testing. However, the utility of in vitro data as indicators of in vivo effects will be fully realized only if rigorous correlation between the toxicity of chemicals in vitro and in vivo can be established (National Research Council 2007; Rabinowitz et al. 2008).

Many previous studies have indicated that the correlation between the in vitro toxicity results and animal toxicity test data (e.g., acute, subacute, subchronic, and chronic rodent toxicity test results) is generally poor. Most notably, in 2001, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) hosted a workshop to assess the relationship between cytotoxicity and rodent acute toxicity for > 300 diverse compounds; the data were compiled by the Zentralstelle zur Erfassung und Bewertung von Ersatz-und Ergänzungsmethoden zum Tierversuch (ZEBET; the National Center for Documentation and Evaluation of Alternative Methods to Animal Experiments) [ICCVAM and National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) 2001]. It was concluded that there is no clear correlation between cytotoxicity [half-maximal inhibitory concentration (IC₅₀)] and acute toxicity [median lethal dose (LD₅₀)] data in rodents. Similarly, poor correlation was found between in vitro cytotoxicity and in vivo rodent carcinogenicity, even when a diverse set of in vitro end points from HTS was used (Xia et al. 2008; Zhu et al. 2008).

Cheminformatics approaches such as quantitative structure–activity relationship (QSAR) modeling have been widely used in toxicology (Dearden 2003; Johnson et al. 2004). Several software packages, such as Toxicity Prediction by Komputer Assisted Technology (TOPKAT) (Venkatapathy et al. 2004) and Multiple Model-Automated Computer-Aided Evaluation (Multicase) (Matthews et al. 2006), have been developed and actively used by both industry and regulatory agencies. However, existing modeling tools generally do not achieve good external accuracy of prediction for compounds not used in model development, and few QSAR models have been successful in predicting in vitro toxicity end points for diverse sets of environmental compounds (Benigni et al. 2007; Stouch et al. 2003).
Materials and Methods

Data sets. The ZEBET database consists of data for 361 chemicals compiled from literature studies and published in a consolidated ICCVAM report (ICCVAM and NICEATM 2001). Every compound in this data set has at least one cytotoxicity result (IC50) and at least one type of rodent acute toxicity value (rat or mouse LD50). We defined ZEBET criteria to select cytotoxicity data for this data set as follows: a) either at least two different IC50 values were available, or from different cell types or from different cytotoxicity endpoints; b) cytotoxicity data were obtained with mammalian cells; c) cytotoxicity data obtained with hepatocytes were not acceptable; and d) chemical exposure time in the cytotoxicity tests was at least 16 hr. Furthermore, only the results obtained from the following cytotoxicity tests were accepted: a) cell proliferation measured by cell number, protein, DNA content, DNA synthesis, or colony formation; b) cell viability and metabolic indicators, including metabolic inhibition test (MIT-24), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, and sodium 3,3,3-(1-[phenylaminocarbonyl]-3,4-tetrazolium)-bis(4-enthoxy-6-nitro)benzene sulfonic acid hydrate (XTTC); c) cell viability and membrane indicators, including neutral red uptake, trypan blue exclusion, cell attachment, and cell detachment; and d) differentiation indicators.

For the purpose of this work, we curated the data set to select the subset of organic compounds and excluded inorganic and organometallic compounds, as well as compound mixtures, because conventional chemical descriptors used in QSAR studies could not be computed in these cases. There were 254 and 235 compounds that had rat or mouse LD50 (millimole/kilogram-body weight/day) values, respectively. Only LD50 values published in the Registry of Toxic Effects of Chemical Substances (RTECS) (Norager et al. 1978; Ruden and Hansson 2003) were used. The distributions of log(1/LD50) values of ZEBET compounds, with the exception of a single outlier, were from −2.61 to 2.30 for the rat and from −2.50 to 2.19 for the mouse. We considered one compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (CAS 1746-01-0), an activity outlier because its log(1/LD50) value was −4.21 for the rat, which deviated significantly from the activity range of the data set. After excluding this single outlier, the data sets used for modeling consisted of 253 compounds for the rat and 235 compounds for the mouse. An additional set of 115 compounds with complete data (both LD50 and IC50) for the rat was recently released by ICCVAM, which we used for validation (referred to as the ICCVAM data set). [For raw data, see Supplemental Material, Table 1 (doi:10.1289/ehp.0800471.S1)].

The data on rat chronic lowest observed adverse effect levels (LOAELs) and rat chronic no observed adverse effect levels (NOAELs) were compiled from an internal low-dose toxicity data set established in our laboratory [see Supplemental Material, Table 2 (doi:10.1289/ehp.0800471.S1)]. These data include a combination of multiple toxicity phenotypes, such as liver toxicity and kidney toxicity. Compared with the ZEBET data set, 42 unique compounds have both rat LOAEL and in vitro IC50 values, and 41 compounds have both NOAEL and in vitro IC50 values. Because of limited availability of LOAEL and NOAEL data, we used these two data sets only to illustrate the data partitioning algorithm and did not build any QSAR models for them.

QSAR modeling approaches. We used the k-nearest neighbor (6NN) QSAR modeling approach that has been developed in our group (Zheng and Tropsha 2000). In brief, the method is based on the 6NN principle and the variable selection procedure. It employs the leave-one-out cross-validation procedure (LOO-CV) and a simulated-annealing algorithm for the variable selection. The procedure starts with the random selection of a predefined number of descriptors from all descriptors. If 6NN > 1, the estimated activities ŷ of compounds excluded by the LOO procedure are calculated using the following formula:

\[ ŷ_{ij} = \sum_{j=1}^{k} \frac{y_j w_{ij}}{\sum_{j=1}^{k} w_{ij}} = \frac{\sum_{j=1}^{k} y_j w_{ij}}{\sum_{j=1}^{k} w_{ij}}, \]  

where ŷ is the activity of the jth compound. We define weights \( w_{ij} \) as

\[ w_{ij} = 1 - \frac{d_{ij}}{\sum_{j=1}^{k} d_{ij}}, \]

where \( d_{ij} \) is the Euclidean distance between compound i and its jth nearest neighbor. Further details of the algorithms and work flow are provided elsewhere (Medina-Franco et al. 2005; Ng et al. 2004; Shen et al. 2002; Zheng and Tropsha 2000).

We developed rat and mouse LD50 QSAR models for ZEBET compounds using DRAGON chemical descriptors (DRAGON
for Windows, version 5.4; Teleste s.r.l., Milan, Italy). Before model construction, 23 compounds with rat LD$_{50}$ results and 24 compounds with mouse LD$_{50}$ results were selected at random to serve as external validation sets. The remaining 230 rat and 211 mouse compounds were used as modeling sets, and each was divided multiple times into training/test sets using the sphere exclusion approach (Golbraikh et al. 2003). We characterized the statistical significance of the models with the standard LOO-CV $R^2$ ($q^2$) for the training sets and the conventional $R^2$ for the test sets when modeling real values (i.e., continuous QSAR). For classification modeling, we used correct classification rates expressed as a fractional value between 0 and 1. The model acceptability cutoff values of the LOO-CV accuracy of the training sets and the prediction accuracy for test sets were both set at 0.65 for classification models. For continuous models, the acceptability thresholds for LOO-CV regression $q^2$ for the training sets and $R^2$ values for the test set were both set at 0.5. Models that did not meet both training and test set cutoff criteria were discarded.

Moving M-regression for data partitioning. We used a novel approach related to a class of M-regression methods (Andersen 2007), which we termed “moving M-regression,” to select compounds for which there is a strong correlation between I$_{C_{50}}$ and LD$_{50}$ values (class 1). The approach is a variant of the least squares regression that takes into account only those data points contained in a band around the regression line $y = ax + b$. For each $y$, only the points within the interval $[y - d_1, y + d_1]$ are candidates for class 1, whereas points outside of this band are excluded from class 1. If the line $y = ax + b$ is moved, some new points will enter the band, whereas other points will leave it, which may result in a higher regression $R^2$; this also explains why we use the term “moving M-regression.” For each point, we define

$$\eta(x, y) = \begin{cases} 1, & \text{if } y \in [ax + b - d_1, ax + b + d_1] \\ 0, & \text{otherwise.} \end{cases}$$

Thus, the moving M-regression line can be found by minimizing the following expression:

$$F(a, b) = \sum_{i=1}^{n} \eta(x_i, y_i)(y_i - ax_i - b)^2.$$  

Function $F$ is not differentiable at all points $(x, y)$ such that $y_1 = ax_1 + b - d_1$ and $y_2 = ax_1 + b + d_1$. For practical purposes, we approximate $\eta(x, y)$ by sums of two sigmoid functions:

$$\eta(x, y) \sim \frac{1}{2} + \frac{1}{1 + \exp \left[-P_1(y, -ax_i - b + d_1)\right]} + \frac{1}{1 + \exp \left[P_2(y, -ax_i - b - d_2)\right]}.$$  

where $P_1$ and $P_2$ are large (-100) positive parameters. Indeed, as $P_1$ and $P_2$ approach infinity, the expression on the right side of Equation 5 approaches the right side of Equation 3. Small approximation errors in the vicinity of points $\{ax_i + b - d_1, y_i\}$ and $\{ax_i + b + d_1, y_i\}$ approach zero as both $P_1$ and $P_2$ approach infinity. It is as if the data points are gradually included within, or excluded from, the band when the regression line is moving. Finally, replacing $\eta(x_i, y_i)$ by Equation 3, we obtain

$$F(a, b) = \sum_{i=1}^{n} \frac{1}{2} \left[ 1 + \exp \left[-P_1(y_i - ax_i - b + d_1)\right] + 1 + \exp \left[P_2(y_i - ax_i - b - d_2)\right] \right] (y_i - ax_i - b)^2.$$  

To optimize $F(a, b)$, the following system of equations is to be solved:

$$\begin{align*}
\frac{\partial F}{\partial a} &= 0 \\
\frac{\partial F}{\partial b} &= 0.
\end{align*}$$

Equations 7 are nonlinear, so depending on the data set and parameters $P_1, P_2, d_1,$ and $d_2$, they can have multiple solutions $(a, b)$.

In these studies, $x$ and $y$ were the in vitro log(1/I$_{C_{50}}$) and in vivo log(1/LD$_{50}$) values, respectively, for a data set of compounds under study. Instead of using Equation 6, we determined the compounds that belong to class 1 by maximizing the number of data points within the band. With this correction, our target function takes the form

$$F^*(a, b) = \sum_{i=1}^{n} \left[ 1 + \exp \left[-P_1(y_i - ax_i - b + d_1)\right] + 1 + \exp \left[P_2(y_i - ax_i - b - d_2)\right] \right] (y_i - ax_i - b)^2.$$  

To obtain the baseline toxicity regression (see Results), we opted to minimize the number of outliers below the regression line. For this purpose, we added additional terms for the lower border of the band weighted by an arbitrary parameter $\alpha$. Thus, we minimized the following target function:

$$F^{**}(a, b) = \sum_{i=1}^{n} \left[ 1 + \frac{1}{1 + \exp \left[-P_1(y_i - ax_i - b + d_1)\right]} + 1 + \exp \left[P_2(y_i - ax_i - b - d_2)\right] \right] (y_i - ax_i - b)^2.$$  

The initial point $(a, b)$ for minimization of $F^{**}$ was selected manually. $P_1$ and $P_2$ were equal to 100, $d_1$ and $d_2$ were equal to 0.4, and $\alpha$ was equal to 1. To optimize Equation 9, the system of Equations 7 in which $F$ is replaced by $F^{**}$ should be solved. Figure 1 summarizes the data analytical work flow that we employed in this study for rodent acute toxicity modeling.

**Model validation.** We validated training set models by evaluating their external predictive power on the test sets as described above.

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**Figure 1.** The work flow of the two-step $k$NN QSAR LD$_{50}$ modeling.
Furthermore, a 5-fold external CV analysis was performed for the original ZEBET data set: the data set was randomly split into five equal-size subsets of compounds and five independent sets of calculations were conducted each time using 80% of the whole data set as a modeling set and the remaining 20% compounds as a test set. In addition, robustness of QSAR models was verified using a Y-randomization (randomization of response) approach as follows. We randomly divided the modeling set compounds into class 1 and class 2 subsets and developed 4NN QSAR LD₅₀ models for each subset using the same protocol and the same cutoff criteria (r² and R² > 0.5) as for compounds in classes 1 and 2 that we generated by means of the moving regression. The purpose of this was to see if statistically significant QSAR models could be obtained for any random division of the original data into two classes. Independently, we applied the test to compounds in unique classes 1 and class 2 by randomizing their LD₅₀ values and redeveloping training set models. Both Y-randomization tests were repeated 10 times.

**Results**

**Failure of conventional QSAR modeling of rodent acute toxicity.** The modeling set including 230 compounds with known rat LD₅₀ data was partitioned into 32 training and test sets and the conventional 4NN QSAR modeling approach was applied to all training sets as detailed in “Materials and Methods.” We characterized each training set model by its r² value; for the five best training set models, these values ranged between 0.5 and 0.57. These five models were used for predicting LD₅₀ values for the respective external validation set (23 compounds). However, for each of these models the R² value for this external set was < 0.5. When we used other types of in-house or commercial QSAR methods (e.g., support vector machine or partial least squares) and other types of descriptors (e.g., MolConnZ descriptors or molecular operating environment descriptors), we obtained no statistically significant predictive QSAR models (data not shown). Likewise, modeling of the mouse data set (211 modeling compounds and 24 external validation compounds) was unsuccessful (data not shown). This negative result corroborates the well-known inability of conventional QSAR modeling approaches to arrive at statistically significant and externally predictive models of *in vivo* toxicity.

**Data partitioning using the moving M-regression approach.** It is well known that *in vitro* cytotoxicity correlates poorly with *in vivo* toxicity end points when any relatively large set of compounds is considered. The ZEBET data set is no exception; cytotoxicity (IC₅₀) correlates with acute toxicity (LD₅₀) for only a fraction of the compounds in either the rat (Figure 2A) or mouse (Figure 2B) data sets. Most of the compounds are more toxic *in vivo* than *in vitro*. Similar patterns could be found between cytotoxicity and other *in vivo* toxicity end points, for example, rat chronic LOAEL and rat chronic NOAEL (Figures 2C, D).

To devise a mathematical means for identifying compounds with strong *in vitro/in vivo* correlation, we extended concepts that have been previously employed in calculating the “baseline regression” that correlated the aquatic toxicity of (some) chemicals with the logarithm of the n-octanol/water partition coefficient (log P) (Klopman et al. 1999, 2000; Mayer and Reichenberg 2006). Here, we have developed a novel approach, termed “moving M-regression,” to identify a subset of compounds with strong IC₅₀ versus LD₅₀ correlation. Using this method, we have partitioned compounds in the modeling set into two classes: class 1, compounds with acute toxicity that linearly correlate with cytotoxicity; and class 2, compounds with acute toxicity that do not correlate well with cytotoxicity, with these points positioned above the regression line.

This analysis for the rat ZEBET data set resulted in 122 compounds assigned to class 1, that is, within the linear regression correlation band between LD₅₀ and IC₅₀ values. The points corresponding to 93 out of 108 remaining compounds are located above the regression line band and are classified as class 2, whereas 15 compounds fall below the regression line (Figure 2A). Although these compounds are likely to be activity outliers, in the absence of an objective rationale for their outlier status, we merged them into class 1 to obtain the highest coverage of the resulting models and to provide a more realistic measure of external predictivity. Figure 2A and Equation 10 show the correlation between the LD₅₀ and IC₅₀ values of the resulting 137 class 1 compounds:

\[ \log(1/LD_{50}) = -1.1 + 0.4 \times \log(1/IC_{50}) \]  

with R² = 0.74, SE = 0.36, and n = 137. We also applied this approach to analyze the relationship between the *in vitro* IC₅₀ and other *in vivo* toxicity data, including mouse LD₅₀, rat chronic LOAEL, and rat chronic NOAEL.

![Figure 2](image_url)
Two-step QSAR for acute rodent toxicity prediction

(Table 1). The same trend was found for all data sets, that is, in all cases the data were partitioned into two classes: a) points on the baseline and b) points off the baseline (Table 1, Figure 2). We found the ratio of class 1 to class 2 compounds to be similar for each of the four in vitro/in vivo toxicity data sets. This result further supports the generality of the “moving M-regression” approach.

Hierarchical QSAR modeling of the partitioned rodent toxicity data. Using class assignments from the data partitioning described above, we employed a two-step QSAR approach (Figure 1) for a) classification modeling (i.e., establishing that compounds assigned to classes 1 and 2 based on their biological activity data could be subdivided into the same classes based on their chemical structure), and b) predictive continuous modeling for all compounds in each class (i.e., estimation of the LD50 based on chemical structure, not IC50 data). For ZEBET rat data, we generated three modeling sets: set 1, 230 compounds (137 class 1 vs. 93 class 2) for classification modeling set 2, 137 class 1 compounds; and set 3, 93 class 2 compounds for developing two continuous rat LD50 models. The analysis of these three data sets resulted in 252 classification models, as well as 1,207 continuous LD50 models for class 1 compounds and 40 continuous LD50 models for class 2 compounds that satisfied the statistical significance threshold criteria. Table 2 lists the statistical figures of merit for the best kNN models obtained for these three modeling sets.

To demonstrate that these QSAR models have significant external prediction accuracy, we have employed several concurrent approaches for model validation. First, following our general model validation work flow (Tropsha and Golbraikh 2007), we used 23 compounds excluded randomly from the entire data set as an external validation set. The following two-step prediction protocol for external compounds was used: a) kNN classification models were used to assign compounds to class 1 or class 2; and b) depending on the outcome, the respective class-specific continuous QSAR models was employed to predict the LD50 values for each compound. The results demonstrate that the overall accuracy of prediction for this external set is reasonably good. In the first step, the classification model had 65% prediction accuracy (the fraction of correctly identified class 1 and class 2 compounds). In the second step, we obtained $R^2 = 0.70$, mean absolute error(MAE) = 0.39, and prediction coverage (i.e., the fraction of the external set compounds within the applicability domains of the models) of 74% for the external test set when combining the predictions for class 1 and class 2 compounds.

Second, we performed a 5-fold external CV analysis to test the robustness of the modeling outcome using 253 rat ZEBET compounds. The dataset was randomly split into five equal-size subsets of compounds and the modeling procedure was repeated five times, using each subset as a test set and the remaining four subsets as training set, as detailed in “Materials and Methods.” The statistical results of this exercise were as follows: slope (reg.) = 0.45 ± 0.01, $R^2_{reg}$ = 0.71 ± 0.04, $R^2_{ext}$ = 0.55 ± 0.05, MAE = 0.44 ± 0.04, coverage = 73 ± 39.

Third, we performed Y-randomization tests to establish whether our models are statistically robust. Random partitioning of the compounds into two classes (10 times) produced only three (for class 1) and 28 (for class 2) models that satisfied the criteria of $q^2/R^2 > 0.5$, compared with 1,207 and 40, respectively, models for “moving M-regression” assisted partitioning. Randomizing LD50 data generated no model with $q^2/R^2 > 0.5$ for class 1 and class 2 compounds.

Fourth, we performed additional Y-randomization analyses by randomly moving or rotating the correlation line (including negative correlation) and redefining compounds into classes 1 and 2. The randomly assigned class 1 and class 2 sets were used to develop QSAR LD50 models individually and the procedure was repeated 10 times. We found that at most, a very small number (< 7) of acceptable ($Q^2 > 0.5$, $R^2 > 0.5$) models could be developed.

Similar modeling results were obtained using the ZEBET mouse LD50 data. After partitioning 211 modeling set compounds into 119 class 1 compounds and 92 class 2 compounds, we developed 843 classification models for class 1 versus 2, 236 continuous LD50 models for class 1 compounds, and 356 models for class 2 compounds. A two-step prediction protocol for evaluation of the 24 external compounds resulted in similarly good external prediction accuracy: $R^2 = 0.69$, MAE = 0.42, and prediction coverage of 54%.

As a true external validation challenge, we have used our model to make predictions for the 115 compounds with rat LD50 data in the new ICCVAM data set. We compiled this data set after we finished the development of the above-described QSAR LD50 models, so it could be viewed as a true “blind” validation test. The statistical parameters of the prediction results for these compounds were $R^2 = 0.57$, MAE = 0.48, and prediction coverage of 70%. Although somewhat less accurate than the results of the previous external prediction, this validation reinforces the statistical significance and utility of the model.

Y-randomization tests were also performed for the mouse LD50 data set. Similar to the rat data, after 10 random assignments of compounds into the two classes, we developed, at most, 4 (for class 1) and 38 (for class 2) models ($q^2/R^2 > 0.5$), compared with 843 and 236 models, respectively, when we used a classification model. Randomization of LD50 values produced no significant models.

Stability of the in vitro and in vivo moving M-regression parameters. Because the regression correlation between in vitro (IC50) and in vivo (LD50) data is required to classify the modeling set compounds and, subsequently, to create the kNN classification models, this linear correlation is an essential factor to determine the robustness of our final models. Hence, the slope of the correlation and associated correlation coefficient ($R^2$) should remain stable when new compounds are added into the modeling set. To validate this

| Model | N-training | Pred-training | N-test | Pred-test | NNN |
|-------|------------|---------------|--------|-----------|-----|
| The best kNN classification model for 137 class 1 versus 93 class 2 compounds |
| 1 | 173 | 0.84 | 55 | 0.73 | 1 |
| 2 | 147 | 0.86 | 74 | 0.70 | 1 |
| 3 | 193 | 0.83 | 37 | 0.73 | 1 |
| 4 | 165 | 0.86 | 59 | 0.70 | 1 |
| 5 | 173 | 0.81 | 55 | 0.75 | 1 |
| The best kNN continuous model for 137 class 1 compounds |
| 1 | 103 | 0.66 | 34 | 0.81 | 3 |
| 2 | 103 | 0.73 | 34 | 0.71 | 2 |
| 3 | 111 | 0.71 | 26 | 0.74 | 3 |
| 4 | 115 | 0.65 | 23 | 0.79 | 5 |
| 5 | 177 | 0.73 | 60 | 0.71 | 2 |
| The best kNN continuous model for 93 class 2 compounds |
| 1 | 80 | 0.61 | 13 | 0.84 | 2 |
| 2 | 77 | 0.67 | 16 | 0.77 | 1 |
| 3 | 80 | 0.69 | 13 | 0.74 | 1 |
| 4 | 80 | 0.65 | 13 | 0.76 | 2 |
| 5 | 79 | 0.63 | 14 | 0.78 | 2 |

Abbreviations: NNN; number of the nearest neighbors used for prediction; N-test, number of compounds in the test set; N-training, number of compounds in the training set; Pred-test, the overall predictivity of the test set (correct classification rate for classification models, $R^2$ for continuous models); Pred-training, the overall predictivity of the training set (correct classification rate for classification models, $q^2$ for continuous models).
supposition, we compiled all available ZEBET and ICCVAM compounds with rat LD<sub>50</sub> data to create a new modeling set, including the original modeling set (230 compounds), the external validation set (23 compounds), and additional data (115 compounds). We also included the compounds previously not used for modeling (inorganic, organometallic, and mixtures) because we used no chemical descriptors in this validation. Using the moving M-regression approach for all 425 compounds with IC<sub>50</sub> and LD<sub>50</sub> values, the resulting in vitro/in vivo correlation parameters are similar to those obtained from our original modeling set in Equation 10:

\[
\text{Log}(1/LD_{50}) = -1.1 + 0.36 \times \log(1/IC_{50}).
\]  

with \(R^2 = 0.71, \text{SE} = 0.37, \text{and } n = 258\). The proportions of class 1 and class 2 compounds and outliers among these 425 compounds were also comparable to those of the original modeling set of 230 compounds (Table 1). We conclude that adding new compounds into the modeling set, which should be important to improve the final model by enriching its chemical and biological diversity, does not affect the in vitro/in vivo regression statistics.

**Comparison between the two-step hierarchical LD<sub>50</sub> QSAR model and TOPKAT.** We compared the performance of our modeling approach with that of TOPKAT software, version 6.1 (Accelrys 2009; Ensilver 1988). Two types of comparison were considered. First, we have analyzed 27 of the 115 ICCVAM compounds that have been used neither for modeling (inorganic, organometallic, and mixtures) because we used no chemical descriptors in this validation. Using the moving M-regression approach for all 425 compounds with IC<sub>50</sub> and LD<sub>50</sub> values, the resulting in vitro/in vivo correlation parameters are similar to those obtained from our original modeling set in Equation 10:

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\]  

with \(R^2 = 0.71, \text{SE} = 0.37, \text{and } n = 258\). The proportions of class 1 and class 2 compounds and outliers among these 425 compounds were also comparable to those of the original modeling set of 230 compounds (Table 1). We conclude that adding new compounds into the modeling set, which should be important to improve the final model by enriching its chemical and biological diversity, does not affect the in vitro/in vivo regression statistics.

**Comparison between the two-step hierarchical LD<sub>50</sub> QSAR model and TOPKAT.** We compared the performance of our modeling approach with that of TOPKAT software, version 6.1 (Accelrys 2009; Ensilver 1988). Two types of comparison were considered. First, we have analyzed 27 of the 115 ICCVAM compounds that have been used neither for modeling (inorganic, organometallic, and mixtures) because we used no chemical descriptors in this validation. Using the moving M-regression approach for all 425 compounds with IC<sub>50</sub> and LD<sub>50</sub> values, the resulting in vitro/in vivo correlation parameters are similar to those obtained from our original modeling set in Equation 10:

\[
\text{Log}(1/LD_{50}) = -1.1 + 0.36 \times \log(1/IC_{50}).
\]  

with \(R^2 = 0.71, \text{SE} = 0.37, \text{and } n = 258\). The proportions of class 1 and class 2 compounds and outliers among these 425 compounds were also comparable to those of the original modeling set of 230 compounds (Table 1). We conclude that adding new compounds into the modeling set, which should be important to improve the final model by enriching its chemical and biological diversity, does not affect the in vitro/in vivo regression statistics.
in the activity space because external compound activity is predicted by averaging the activities of nearest-neighbor compounds in the training set as described in “Materials and Methods.” The MAE for the prediction of RTECS compounds that have experimental activity above 2 or below –2 is 1.14 log units. On the other hand, the MAE for the prediction for RTECS compounds that have experimental activity between –2 and 2 is considerably lower, 0.52 log units. The likely explanation for the better performance of our models in the latter range is that more than 90% of our modeling set compounds have rat LD50 activity in the same range, between –2 and 2. Increasing the diversity and activity range of compounds in the modeling set should significantly improve the prediction accuracy of our models.

Discussion

The conventional wisdom in mechanistic and regulatory toxicology is that predictions of the in vivo toxicity end points from in vitro measures, even within the same species, are difficult. However, an approximate linear correlation between in vitro IC50 and rodent LD50, two of the widely acceptable benchmark parameters used for regulatory purposes, can be established for a significant fraction of the compounds. Indeed, we confirmed this notion by quantitative analysis of the IC50/LD50 relationships and devised an objective, computational means to partition compounds into two groups: those having good linear fit within a defined band, or those falling outside the band and exhibiting, for the most part, higher in vivo than in vitro toxicity. Our hypothesis to explain this observation is that, whereas cytotoxicity assays can reflect some of the toxicity mechanisms resulting in adverse health effects at the whole-animal level, the in vitro tests cannot fully reproduce the complex mechanisms of the in vivo toxicity. For example, it is well known that many compounds are not toxicants themselves but have metabolites that are toxic. We argue that the two-step prediction model based on chemical descriptors only that we developed in our studies also assists in identification of the compound subset that may act directly (i.e., without being biotransformed) and through mechanisms likely to be predictive of the potential in vivo effects. A similar argument was presented previously in ecotoxicity research where log P was found to be a mechanistically relevant predictor (Verhaar et al. 2000).

To further substantiate this argument, we considered the top 10 chemical fragment descriptors that were used most frequently in statistically significant QSAR models, that is, descriptors with the highest discriminatory power [see Supplemental Material, Table 3 (doi:10.1289/ehp.0800471.S1)]. It is noteworthy that the aromatic primary amine, “hydrazine,” and “sulfonamide” moieties, found within compounds that are known to be toxic both in vitro and in vivo (Alaejos et al. 2008; Carr et al. 1993; Toth 1988), were found predominantly in compounds of class 1. On the other hand, “pyrrolidine” and “aromatic tertiary amine” moieties, which require biotransformation (Domagala 1994), were predictors for class 2. We have also demonstrated that this objective division of the data set into two major groups affords robust hierarchical QSAR models, an assertion further supported by successive challenges to the models with external data sets, CV, and randomization of data.

The approach advocated in this study for biologically informed partitioning of structure–activity relationship data differs from conventional cheminformatics clustering approaches. Traditional methods partition compounds into multiple subgroups based on their chemical structure properties only (i.e., chemical descriptors). The underlying reasoning for chemically based clustering is that similar structures are expected to have similar biological properties and mechanisms of activity. However, it is a well-known limitation of structure–activity relationships that the absence or presence of a functional group or other minor change of the chemical structure may result in a large change of biological activity (Maggiora 2006). In our studies, the conventional chemical structure–based clustering method did not yield any statistically meaningful models, either global or local. The distribution of pairwise chemical similarities for all compounds within the modeling sets (class 1 vs. class 2) of rat LD50 values using DRAGON descriptors is very similar (data not shown). This observation reconfirms that chemical clustering would not have partitioned compounds in a way similar to the biological data-based partitioning.

Conclusions

Although the cytotoxicity data generally show weak correlation with rodent acute toxicity, we have demonstrated that these data can be used to inform and improve QSAR modeling of in vivo acute toxicity. We have developed a novel two-step kNN QSAR modeling approach that affords a successful prediction of acute toxicity (LD50) values from chemical structure for both rats and mice. Furthermore, we predicted LD50 values for external compounds with accuracy, exceeding that of previously published QSAR models developed with the commercial (TOPKAT) software. It should be stressed that although in vitro cytotoxicity data have been used to establish the rules for partitioning most compounds into two classes, the ultimate models, both classification and continuous, employ chemical descriptors only. This vital feature of our approach makes it possible to achieve accurate predictions of rodent acute toxicity directly from chemical structure alone, even bypassing the need for in vivo studies of new compounds. We believe that this biological-data–based partitioning approach using in vitro toxicity data for the modeling set only, coupled with subsequent chemical–structure–based classification and continuous QSAR modeling techniques, holds promise for modeling other complex in vivo toxicity end points. This approach charts a future course for combining in vitro screening methods and QSAR modeling to prioritize chemicals for in vitro animal toxicity testing.

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