



## Predicting genotoxicity of aromatic and heteroaromatic amines using electrotopological state indices<sup>☆</sup>

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### Abstract

A quantitative structure–activity relationship (QSAR) model relating electrotopological state (E-state) indices and mutagenic potency was previously described by Cash [Mutat. Res. 491 (2001) 31–37] using a data set of 95 aromatic amines published by Debnath et al. [Environ. Mol. Mutagen. 19 (1992) 37–52]. Mutagenic potency was expressed as the number of *Salmonella typhimurium* TA98 revertants per nmol (LogR). Earlier work on the development of QSARs for the prediction of genotoxicity indicated that numerous methods could be effectively employed to model the same aromatic amines data set, namely, Debnath et al.; Maran et al. [Quant. Struct.-Act. Relat. 18 (1999) 3–10]; Basak et al. [J. Chem. Inf. Comput. Sci. 41 (2001) 671–678]; Gramatica et al. [SAR QSAR Environ. Res. 14 (2003) 237–250]. However, results obtained from external validations of those models revealed that the effective predictivity of the QSARs was well below the potential indicated by internal validation statistics (Debnath et al., Gramatica et al.). The purpose of the current research is to externally validate the model published by Cash using a data set of 29 aromatic amines reported by Glende et al. [Mutat. Res. 498 (2001) 19–37; Mutat. Res. 515 (2002) 15–38] and to further explore the potential utility of using E-state sums for the prediction of mutagenic potency of aromatic amines.

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## 1. Introduction

A principal objective in developing quantitative structure–activity relationship (QSAR) models to predict mutagenicity is to obtain knowledge regarding the potential potency of substances that have not been tested, or for which reliable experimental data are not available. In addition, QSARs may be used to screen new R&D chemicals for toxicological safety with the intent to save time and money by pre-empting the further development of hazardous chemicals. However, the benefits of using a QSAR model for hazard screening may only be realized once the reliability, uncertainty, and predictivity of the model have been assessed. The predictivity of a QSAR is often evaluated using only internal performance measures such as the coefficient of determination ( $r^2$ ) or internal validation results ( $Q_{\text{LOO}}^2$ ). There is often no attempt to validate the model using external validation methods, which may provide a more accurate assessment of the predictivity of a model.

In this present research, a QSAR model was developed to predict the mutagenic potency of chemicals using data for 95 aromatic and heteroaromatic amines published by Debnath et al. [1] and the methods described by Cash [2]. The Debnath data set has been previously modeled using a number of different QSAR building techniques that utilized molecular, constitutional, topological, and quantum mechanical descriptors [1,3–5]. While statistics from internal validation of these models indicate good correlations with the training set (correlation coefficients ranged from 0.89 to 0.91), the results from external validation exercises indicate that the QSAR equations lack predictivity for chemicals not used in the development of the models [1,5–7]. Additionally, the complex molecular descriptors typically chosen for the previous models resulted in a heavy demand of computer resources, making the models unsuitable for situations that require rapid screening of a large number of chemicals.

An example regulatory program which requires the rapid assessment of chemicals to identify potential health hazards is the U.S. Environmental Protection Agency's (U.S. EPA's) New Chemicals Program, which was created under the Toxic Substances Control Act (TSCA) of 1976. Under TSCA, the assessment of new industrial chemicals and the retrospective assessment of an inventory of existing chemicals are

within the purview of U.S. EPA's Office of Pollution Prevention and Toxics (OPPT), formerly the Office of Toxic Substances. OPPT is charged with assessing, and if necessary, regulating all phases of the life cycle of industrial chemicals. The office has reviewed about 42,000 premanufacture notification (PMN) chemicals and currently receives approximately 2000 submissions per year [8,9]. Because TSCA does not require testing of these chemicals prior to submission, fast and accurate SAR and QSAR methods are employed by OPPT for the subsequent assessments of PMN chemicals. If a QSAR is used in this type of regulatory decision-making process, it is imperative that the model be reliable as well as fast and easily employed. In an attempt to design a model for mutagenicity which could be used to rapidly screen large numbers of chemicals, while maintaining the use of simplified molecular descriptors which describe aspects of shape and electronic configurations similar to the traditional quantum mechanical descriptors, a method to predict mutagenic potency was investigated [2] using Electrotopological State (E-state) Indices to model the data set.

These descriptors encompass the same general type of molecular and topological information, although in a much more approximate form, as the more complex descriptors used in previous models, but require significantly fewer computer resources. There is certainly a trade-off between level of approximation and demand on resources. The success of this trade-off depends on the question to be addressed and cannot be predicted a priori. The E-state indices are a family of atom level molecular descriptors that quantify the electron accessibility of each atom type as described by Kier and Hall [10]. Both a representation of the electron density and the accessibility of those electrons to participate in intermolecular interactions are expressed in the E-state index. E-state indices may be thought of as refinements of valence connectivity indices, incorporating information about atom types, electronic states, and connectivities. The indices also take into account the structural configuration of the nearest neighbors surrounding the atom and, thus, contain some shape information, although in a secondary fashion. Calculation of these sums from Simplified Molecular Input Lines Entry System (SMILES) notation [11] is exceptionally straightforward on even a modest desktop computer. The purpose of the current research was to validate the E-state QSAR model published by Cash [2] by deriving

internal validation statistics and assessing the model's predictive accuracy using an external validation set.

The E-state indices derived by Cash [2] adequately described the training set of aromatic amines as revealed by the statistical parameters of the model ( $r=0.88$ ;  $r_{\text{adj}}^2=0.76$ ). Internal validation indicated that the QSAR model was expected to be a reliable predictor of LogR ( $Q_{\text{LOO}}^2=0.70$ ). However, external validation of the model using data published by Glende et al. [6,7] revealed that the E-state equation was a poor predictor of LogR, consistent with the results of previous methods developed for the prediction of mutagenicity of aromatic and heteroaromatic amines [1,3,4]. Upon inspection of the validation results, observations were made regarding important differences in the structural components of the training and validation sets. To address the issue, a new QSAR was designed using randomized sets of the combined Debnath et al. [1] and Glende et al. [6,7] data in an attempt to produce a more robust model and a more representative validation set. Randomization of the aromatic amines data set resulted in better structural distribution for the training and validation sets, but external validation results still showed poor predictive accuracy for the model. It was proposed that smaller sub-classes of chemicals within the training set may have been presenting more complex variations than could be appropriately captured during model development using such a broad class as the aromatic and heteroaromatic amines. To investigate this theory, sub-classes of the data were formed from the original training set and class-specific QSARs were designed and externally validated. Again, validation results from the class specific QSARs showed poor predictive accuracy for the models and a further investigation of the training set was conducted to identify possible trends in the data that were not being appropriately captured by the E-state descriptors. The methods employed for the derivation of the E-state equations and possible reasons for the lack of predictive accuracy of the QSAR models are discussed herein.

Although this research did not result in a reliable method for the rapid prediction of mutagenicity, it highlights the need for external validation of a model to evaluate the effective prediction power of a QSAR. Evaluating a model's predictive capabilities using only internal performance statistics derived from the training set alone may give neither a true indication of the predictive accuracy of the model nor the potential need

to re-evaluate the chemical groupings because of unexpected trends in the data set. Additionally, a mechanistic analysis of the process in question was performed, which led to some interesting results. The potential implications this may have on the model are discussed below.

## 2. Materials and experimental methods

### 2.1. Data

Mutagenicity data for the training and validation sets were originally published by Debnath et al. [1] and Glende et al. [6,7]. A total of 95 chemicals were used from the Debnath data set to create a training set for the method and an additional 29 chemicals (Table 1) were chosen from the Glende et al. publications to derive the external validation set. Mutagenic potency was reported as log reversions per nmol of compound (LogR) in *Salmonella typhimurium* TA98 with the addition of an exogenous metabolic activation system (S9). The LogR values reported by Debnath et al. [1] were derived from studies conducted by a number of different laboratories, while Glende et al. [6,7] reported LogR values obtained from studies conducted at a single laboratory by the publishing authors. No effort was made to ensure that the laboratory conditions used to derive the LogR values were comparable, and no attempt to confirm mutagenic potency was undertaken for this analysis. However, in the three cases where data between the two sets overlapped, the difference between these values was observed to be less than one log unit. E-state sums were generated for 34 different atom types for all chemicals in the data sets using SMILES notation as the structural identifier and in the manner pioneered by Kier and Hall [10,12].

### 2.2. Data analysis

The raw data used to derive the original E-state equation was supplied by the author and the QSAR equation was recreated using the same multiple linear regression (MLR) techniques and E-state indices as outlined in Cash [2]. All regressions were performed using Statgraphics Plus for Windows Version 4.0. Variable selection was completed using forward selection stepwise MLR (F-to-enter = 3). Additionally, a genetic algorithm method was also employed for

Table 1  
Results for Eq. (1): experimental and predicted LogR values for the validation set chemicals

Chemical name	Experimental LogR	Predicted LogR (Eq. (1))	Log units difference	Atom types present
<b>Biphenyls</b>				
4'- <i>n</i> -Butyl-4-aminobiphenyl	-0.74	-0.72	0.02	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3,5-Diisopropyl-4-aminobiphenyl	-1.95	-2.14	0.19	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
4'-Ethyl-4-aminobiphenyl	-0.14	-0.76	0.62	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3,5-Dimethyl-4-aminobiphenyl	-1.34	-0.63	0.71	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3,5'-Dimethyl-4-aminobiphenyl	0.35	-0.51	0.86	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3- <i>n</i> -Butyl-4-aminobiphenyl	0.17	-0.73	0.90	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
4'- <i>i</i> -Propyl-4-aminobiphenyl	-0.72	-1.62	0.90	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3-Ethyl-4-aminobiphenyl	0.17	-0.79	0.96	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3,5-Diethyl-4-aminobiphenyl	-1.71	-0.42	1.29	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
4'- <i>t</i> -Butyl-4-aminobiphenyl	-1.17	-2.51	1.34	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3'-Trifluoromethyl-4-aminobiphenyl	-0.38	0.98	1.36	C <sub>subst-aromatic</sub> , F
4'-Methyl-4-aminobiphenyl	0.64	-0.83	1.47	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3'-Methyl-4-aminobiphenyl	0.74	-0.82	1.56	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3- <i>i</i> -Propyl-4-aminobiphenyl	-0.01	-1.63	1.62	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
4'-Trifluoromethyl-4-aminobiphenyl	-0.55	1.25	1.80	C <sub>subst-aromatic</sub> , F
3- <i>t</i> -Butyl-4-aminobiphenyl	-0.39	-2.52	2.13	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
3,5'-Ditrifluoromethyl-4-aminobiphenyl	-0.78	2.03	2.81	C <sub>subst-aromatic</sub> , F
<b>Fluorenes</b>				
1- <i>t</i> -Butyl-2-aminofluorene	0.07	0.16	0.09	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1-Ethyl-2-aminofluorene	1.85	1.94	0.09	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
7- <i>t</i> -Butyl-2-aminofluorene	-0.20	0.13	0.33	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
7-Methyl-2-aminofluorene	1.47	1.82	0.35	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1- <i>n</i> -Butyl-2-aminofluorene	2.86	2.06	0.80	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1- <i>i</i> -Propyl-2-aminofluorene	2.23	1.08	1.15	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
7-Trifluoromethyl-2-aminofluorene	0.76	3.05	2.29	C <sub>subst-aromatic</sub> , F
7-Adamantyl-2-aminofluorene	-0.64	3.17	3.81	C <sub>subst-aromatic</sub> , F
<b>Naphthalenes</b>				
1- <i>n</i> -Butyl-2-aminonaphthalene	-0.29	-0.60	0.31	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1- <i>t</i> -Butyl-2-aminonaphthalene	-2.00	-2.42	0.42	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1- <i>i</i> -Propyl-2-aminonaphthalene	-0.62	-1.53	0.91	CH <sub>3</sub> , C <sub>subst-aromatic</sub>
1-Ethyl-2-aminonaphthalene	0.36	-0.68	1.04	CH <sub>3</sub> , C <sub>subst-aromatic</sub>

variable selection, which identified the same key descriptors as the MLR method. Internal validation of the models was done using a leave-one-out cross validated  $Q^2$  ( $Q_{LOO}^2$ ), which was calculated using standard statistical techniques of Eriksson et al. [13]. The E-state QSAR equation was then subject to external validation using a data set of structures not included in the original training set for the model.

The external validation set was used to evaluate the predictive accuracy of the E-state QSAR model and the results of the external validation were evaluated using standard statistical methods. The predictive accuracy of the model was qualitatively evaluated by assessing the LogR values. Also, predictive accuracy was quantitatively evaluated by assessing the coefficient of determination ( $r^2$ ) derived by regressing

the experimental LogR values against LogR values predicted by the QSAR equations. The results of the external validation were then compared to the results from the internal validation ( $Q_{LOO}^2$ ).

### 3. Results and discussion

A QSAR equation was originally developed by Cash [2] using the mutagenicity data for 95 aromatic and heteroaromatic amines presented by Debnath et al. [1]. Although E-state sums for 34 atom types were considered for the analysis, only 17 remained after removing those atom types that contained E-state sums of zero for all 95 chemicals in the data set. The 17 remaining atom types were methyl, methylene, methine, quaternary

sp<sup>3</sup>-hybridized carbons, substituted and unsubstituted aromatic carbons, primary amine, secondary amine, aromatic nitrogen, nitro-group nitrogen, hydroxyl oxygen, ether oxygen, nitro-group oxygens, sulfide sulfur, and the three lightest halogens. Stepwise MLR analysis was performed on E-state sums for all 17 atom types in Eq. (1). For this regression, 8 of the possible 17 atom types met the criteria for inclusion in the model: methyl and substituted aromatic carbons, nitro and ether oxygens, secondary amine and aromatic amine nitrogens, fluorine, and chlorine. Eq. (1) here is slightly different from that given in [2] because a few transcription errors in experimental LogR values have been corrected

$$\begin{aligned} \text{LogR} = & -3.85 - 0.38C_{\text{methyl}} + 0.84C_{\text{subst-aromatic}} \\ & + 0.075O_{\text{nitro}} + 0.17O_{\text{ether}} - 0.38N_{\text{sec-amine}} \\ & + 0.10F + 0.093N_{\text{aromatic-amine}} + 0.10Cl \end{aligned} \quad (1)$$

$n=95$ ,  $r^2=0.78$ ,  $r_{\text{adj}}^2=0.76$ ,  $s=0.95$ ,  $F=37.9$ .

The goodness-of-fit parameters for the equation indicated that the training set was described relatively well by these eight E-state descriptors. Results from the internal validation of the equation gave a  $Q_{\text{LOO}}^2$  value of 0.70, indicating that Eq. (1) was expected to be a good predictor of LogR.

Eq. (1) was subjected to an external validation using LogR values for an additional 29 aromatic amines

obtained from a subset of data published by Glende et al. [6,7]. These differ from the original set of 95 in having an alkyl or trifluoromethyl substituent either ortho to the amine group or else at some location in the aromatic system distant from the amino group. The results from this analysis indicated that the predicted LogR values were within an order of magnitude of the experimental value for only 55% (16/29) of the chemicals in the validation set (Table 1). When the LogR values predicted by Eq. (1) were regressed against the experimental values (Fig. 1), the resulting correlation coefficient ( $r$ ) was 0.52, which indicates that a positive correlation exists between these variables. However, the  $r^2$  of the regression was 0.27, indicating that the model had poor predictive accuracy for determining the LogR of the chemicals in the validation set.

After reviewing the results from the external validation of Eq. (1), we noted that approximately 60% of the chemicals in the validation set contained a biphenyl group. This high degree of representation by one type of chemical class was considered a potential source of structural bias in the external validation set as compared to the training set for this model. Additionally, many of the chemicals in the validation set had more varied substitutions patterns on the aromatic backbone than the molecules in the training set. The validation set contained chemicals with branched functional groups, such as *t*-butyl and isopropyl, as well as strongly electron-withdrawing

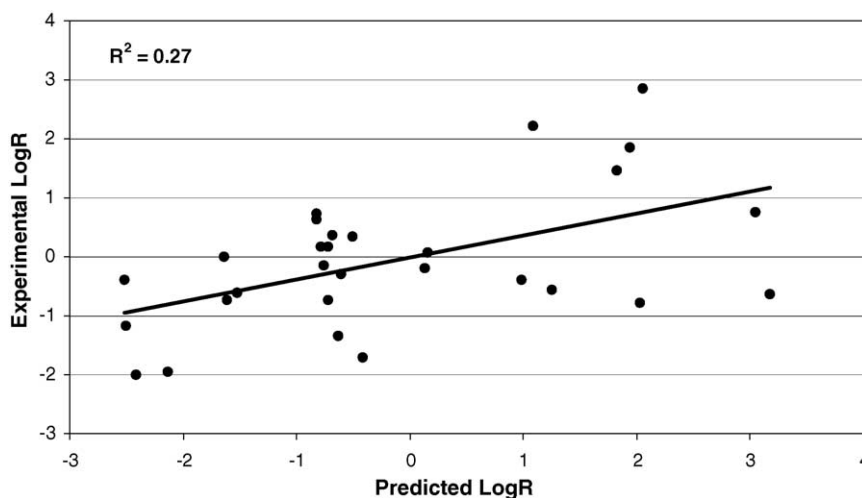


Fig. 1. External validation of Eq. (1): predicted LogR values vs. experimental LogR values.

groups such as trifluoromethyl and bistrifluoromethyl substitutions that were not represented by the training set. We also noticed in the validation results that highly fluorinated structures such as 3',5'-distrifluoromethyl-4-aminobiphenyl were being substantially over-predicted (experimental LogR = -0.78, predicted LogR = 2.03). Additionally, compounds with branched side-chains, such as 4'-*i*-propyl-4-aminobiphenyl (experimental LogR = -0.72, predicted LogR = -1.62) and 4'-*t*-butyl-4-aminobiphenyl (experimental LogR = -1.17, predicted LogR = -2.51), were being substantially under-predicted. Closer examination of the results from the external validation revealed that only three of the eight E-state descriptors used in Eq. (1) were represented in the validation set, indicating that the validation set may not have been adequately representative of the training set.

Based on these observations, we concluded that many compounds in the validation set may have been outside the valid prediction space of the training set. To test this idea, we combined and randomized the training and validation sets to create new training and validation sets with better structural distribution and more importantly, to obtain a more representative validation set for the method. This operation produced new training and validation sets, and a new E-state QSAR equation was derived using the randomized training set. The model was evaluated both internally and externally using the same methods employed for the evaluation of Eq. (1).

Stepwise multiple linear regression performed on the new training set resulted in Eq. (2). The following six atom types met the criteria for inclusion in the model ( $F$ -to-enter = 3): methyl and substituted aromatic carbons, nitro oxygens, secondary and aromatic amine nitrogens, and fluorine. Thus, chlorine and ether oxygen, which were included in Eq. (1), were not determined to be statistically significant descriptors for Eq. (2)

matic carbons, nitro oxygens, secondary and aromatic amine nitrogens, and fluorine. Thus, chlorine and ether oxygen, which were included in Eq. (1), were not determined to be statistically significant descriptors for Eq. (2)

$$\begin{aligned} \text{LogR} = & -3.19 - 0.28C_{\text{methyl}} + 0.73C_{\text{subst-aromatic}} \\ & + 0.055O_{\text{nitro}} - 0.42N_{\text{sec-amine}} \\ & + 0.12N_{\text{aromatic-amine}} + 0.052F \end{aligned} \quad (2)$$

$$n = 95, r^2 = 0.77, r_{\text{adj}}^2 = 0.75, s = 0.89, F = 48.$$

The goodness-of-fit parameters for Eq. (2) are comparable to Eq. (1) and indicate that LogR was described relatively well by these six E-state indices. Combining and randomizing the data sets resulted in better structural distribution as results indicate that all six of the atom types used to derive Eq. (2) were represented in the structures of the validation set (Table 2). A resultant  $r$  value of 0.66 indicates that there was a positive correlation between predicted and experimental LogR values, but an  $r^2$  value of 0.44 indicates that Eq. (2) did not adequately predict LogR (Fig. 2). The leave-one-out  $Q^2$  value was 0.70. The predicted LogR values were within one order of magnitude of the published experimental values for 59% (17/29) of the chemicals in the validation set for Eq. (2). These results are comparable with those obtained for Eq. (1) and indicate that, although combining and randomizing the data sets did result in a more structurally comparable training and validation set, there was not substantial improvement in the model's predictive abilities when subject to external validation.

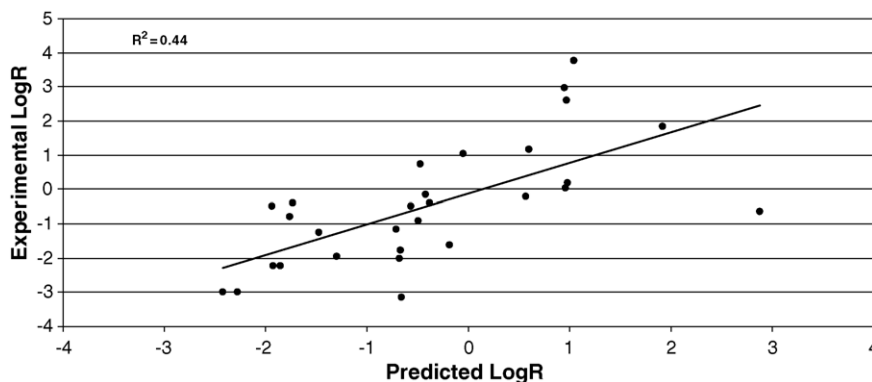


Fig. 2. External validation of Eq. (2): predicted LogR values vs. experimental LogR values.

Table 2  
Experimental and predicted LogR values for the 29 aromatic amines used to validate Eq. (2)

Chemical name	Experimental LogR	Predicted LogR (Eq. (2))	Log units difference	Atom types present
<b>Biphenyls</b>				
3'-Trifluoromethyl-4-aminobiphenyl	-0.38	-0.38	0.00	C <sub>aromatic</sub> , F
4'-Ethyl-4-aminobiphenyl	-0.14	-0.42	0.28	C <sub>methyl</sub> , C <sub>aromatic</sub>
2,4'-Diaminobiphenyl	-0.92	-0.49	0.43	C <sub>aromatic</sub>
3,5-Diisopropyl-4-aminobiphenyl	-1.95	-1.30	0.65	C <sub>methyl</sub> , C <sub>aromatic</sub>
4-Amino-4'-nitrobiphenyl	1.04	-0.05	1.09	C <sub>aromatic</sub> , O <sub>nitro</sub>
3'-Methyl-4-aminobiphenyl	0.74	-0.47	1.21	C <sub>methyl</sub> , C <sub>aromatic</sub>
3- <i>t</i> -Butyl-4-aminobiphenyl	-0.39	-1.73	1.34	C <sub>methyl</sub> , C <sub>aromatic</sub>
<b>Diphenylenes</b>				
4,4'-Methylenedianiline	-1.60	-0.19	1.41	C <sub>aromatic</sub>
<b>Anilines</b>				
4-Cyclohexylaniline	-1.24	-1.47	0.23	C <sub>aromatic</sub>
2,4-Dimethylaniline	-2.22	-1.93	0.29	C <sub>methyl</sub> , C <sub>aromatic</sub>
4-Chloro-2-nitroaniline	-2.22	-1.85	0.37	C <sub>aromatic</sub> , O <sub>nitro</sub>
2,4-Diaminoisopropylbenzene	-3.00	-2.42	0.58	C <sub>methyl</sub> , C <sub>aromatic</sub>
2-Chloroaniline	-3.00	-2.27	0.73	C <sub>aromatic</sub>
3-Amino- $\alpha,\alpha,\alpha$ -trifluorotoluene	-0.80	-1.76	0.96	C <sub>aromatic</sub> , F
4,4'-Methylenebis( <i>o</i> -isopropylaniline)	-1.77	-0.67	1.10	C <sub>methyl</sub> , C <sub>aromatic</sub>
4-Chloro-1,2-phenylenediamine	-0.49	-1.94	1.45	C <sub>aromatic</sub>
<b>Chemicals with two fused aromatic rings</b>				
8-Aminoquinoline	-1.14	-0.71	0.43	C <sub>aromatic</sub> , N <sub>aromatic</sub>
5-Aminoquinoline	-2.00	-0.68	1.32	C <sub>aromatic</sub> , N <sub>aromatic</sub>
3-Aminoquinoline	-3.14	-0.66	2.48	C <sub>aromatic</sub> , N <sub>aromatic</sub>
<b>Chemicals with three fused aromatic rings</b>				
1-Ethyl-2-aminofluorene	1.85	1.92	0.07	C <sub>methyl</sub> , C <sub>aromatic</sub>
3-Aminocarbazole	-0.48	-0.56	0.08	C <sub>aromatic</sub> , N <sub>sec-amine</sub>
2-Amino-7-acetamidofluorene	1.18	0.60	0.58	C <sub>methyl</sub> , C <sub>aromatic</sub> , O <sub>nitro</sub> , N <sub>sec-amine</sub>
7- <i>t</i> -Butyl-2-aminofluorene	-0.20	0.57	0.77	C <sub>methyl</sub> , C <sub>aromatic</sub>
1,6-Diaminophenazine	0.20	0.98	0.78	C <sub>aromatic</sub> , N <sub>aromatic</sub>
1,9-Diaminophenazine	0.04	0.96	0.92	C <sub>aromatic</sub> , N <sub>aromatic</sub>
2-Aminoanthracene	2.62	0.97	1.65	C <sub>aromatic</sub>
9-Aminophenanthrene	2.98	0.95	2.03	C <sub>aromatic</sub>
3-Aminophenanthrene	3.77	1.05	2.72	C <sub>aromatic</sub>
7-Adamantyl-2-aminofluorene	-0.64	2.88	3.52	C <sub>aromatic</sub>

It was surprising to us that neither Eq. (1) nor Eq. (2) showed acceptable predictive accuracy when subject to external validation because the internal validation statistic ( $Q_{LOO}^2$ ) indicated that both equations would be expected to predict LogR well. We initially theorized that E-state sums would be suitable descriptors for modeling the mutagenicity of this data set because they performed well on the original  $n=95$  data set [2], and because the descriptors were thought to encompass many of the electronic characteristics that influence the proposed mechanism of action for aromatic and heteroaromatic amines. The mutagenicity of aromatic

amines is thought to be related to the formation of a reactive intermediate that forms via oxidation of the amine to a functionalized hydroxylamine, which is then converted to a cation intermediate, as shown in Fig. 3. Mutagenicity of aromatic amines is believed to be related to the stability of this reactive intermediate.

Based on results from the validation of Eqs. (1) and (2), we considered the possibility that the original grouping of aromatic and heteroaromatic amines may have been too broad of a chemical class for modeling this endpoint. There may have been

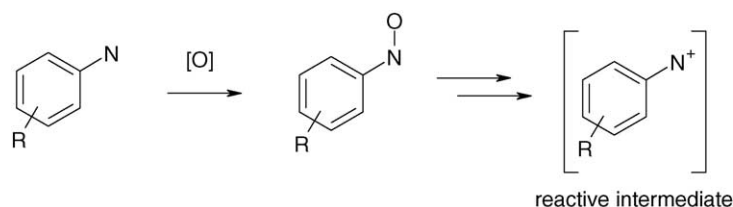


Fig. 3. Mutagenicity of aromatic amines and the formation of a reactive intermediate that forms via oxidation of the amine to a functionalized hydroxylamine and converted to a cation intermediate.

sub-trends in the data sets specific to smaller chemical classes whose contributions could not be appropriately captured during model development when using such a broad chemical class as the aromatic and heteroaromatic amines. To investigate this hypothesis, we subdivided the data sets into smaller chemical classes based on structural backbone (e.g., biphenyls,

naphthalenes, fluorenes), and generated new QSARs for each of the chemical sub-classes.

Results from dividing the Debnath et al. [1] and Glende et al. [6,7] data sets into smaller structure-specific classes are presented in Table 3. The results indicate that further subdividing the data set generally did not improve the predictiveness of the models

Table 3  
Comparison of experimental LogR values and E-state sums for five diaminophenazine isomers

Structure	Chemical name	Experimental LogR	Eq. (1)	Eq. (2)	Atom type E-state sums	
					C <sub>subst-aromatic</sub>	N <sub>aromatic</sub>
	2,7-Diamino phenazine	3.97	0.90	1.26	4.7	9.0
	2,8-Diamino phenazine	1.12	0.89	1.25	4.7	9.0
	1,7-Diamino phenazine	0.75	0.73	1.12	4.4	9.0
	1,6-Diamino phenazine	0.20	0.57	0.98	4.3	8.9
	1,9-Diamino phenazine	0.04	0.54	0.96	4.2	8.9



when the results were compared to those for the validation of Eqs. (1) and (2). In the external validation of the structure-specific QSARs, we noted that the mutagenicity of one particular chemical, 7-adamantyl-2-aminofluorene, was consistently and dramatically over-predicted by the models. Removal of this chemical from the validation sets resulted in substantial improvements in the  $r^2$  values, though true predictiveness of these models may not have been accurately characterized because of the small number of chemicals used for the validations. Larger data sets for these sub-classes of aromatic amines need to be assessed in order to properly determine if these class-specific models are appropriate.

With respect to the specific problem with 7-adamantyl-2-aminofluorene, we considered the possibility that the steric bulk of the adamantyl group interferes with passive transport across biological membranes, leading to a much lower experimental LogR than predicted. Using a computer program developed to predict this effect by modeling the effective cross-sectional diameters of molecules [14], we found, however, that 7-adamantyl-2-aminofluorene has about the same effective cross-sectional diameter as the other substituted aminofluorenes. The reason for this is, the adamantyl group extends almost parallel to the longest axis of the fluorene nucleus, so it has the effect of making the molecule longer and thicker, but it does not substantially increase the minimum size of the middle dimension. This middle dimension is the effective diameter of the smallest pore in a membrane through which a molecule might be passively transported.

An interesting observation noted during development of these sub-class models was that there was no significant relationship between LogR and E-state sums for the sub-class of biphenyls. A notable structural difference between the biphenyls and the other aromatic amines included in the data set is that the biphenyls have the ability to rotate about the axis of the central carbon-carbon bond, potentially causing steric effects and consequently affecting stabilization of the reactive intermediate. The other polycyclic molecules included in the data set have rigid planar backbones. E-state sums only account for the influence of the nearest neighbor substitutions within a structure and, therefore, do not account for the influences of three-dimensional rotation. If this ability to rotate and form an out-of-plane structure is involved in the docking of

the molecule during induction of mutagenicity, then this would be a characteristic influencing mutagenicity that traditional E-state sums cannot capture.

Re-grouping the data set by similar chemical backbone also revealed structural isomers in the data sets that were noted as having vastly different LogR values within a narrow chemical class (Table 4). An example is the diaminophenazines. These positional isomers contain identical chemical moieties, but the arrangement of the atoms on the phenazine backbone is slightly different, as shown in the structures presented in Table 3. The experimental LogR values for the five diaminophenazine isomers span almost four orders of magnitude, ranging from 0.04 to 4.0. However, they possess identical atom types leading to similar E-state sums because the positional variations of the functional groups on the aromatic backbone do not drastically affect the calculation of the E-state sums. Therefore, it might be expected that models based solely on E-state sums would predict that these five structural isomers would have similar LogR values, and this is indeed the case. Similar trends also were noted for other classes of positional isomers.

Looking at these results, we concluded that E-state sums may not be able to characterize the observed differences in mutagenicity of these isomers because mutagenic potency appears to be influenced by factors other than electron accessibility and functional substitutions of the parent structure. Inspection of the phenazines sub-class, and factors involved in the mechanism proposed for aromatic amines, gives evidence that extended conjugation and steric factors within the molecule may play an important role in the resulting mutagenicity of the parent compound [15,16]. Stabilization of the reactive intermediates may be altered by extended conjugation in the molecule via the aromatic system, as well as the steric factors associated with certain positional configurations of the amine substitutions and their spatial relation to other substituents on the ring system. These combined influences may lead to an increase or decrease in reactivity of the bioactivated intermediate, thereby affecting the residence time of the metabolite in the body. This may in turn lead to potential differences in mutagenic potency, even within a very narrow sub-class such as the diaminophenazines. Stability of the reactive intermediate may affect mutagenic potency because, all other factors being equal, increased stability allows the chemical to have a

Table 4  
Results of grouping the randomized data set by chemical backbone

Training set			Validation set			
Description of subset <sup>a</sup>	<i>N</i>	Equation	Training set, $R_{adj}^2$	<i>N</i>	Percentage of predictions within one log unit	Validation <sup>b</sup>
All chemicals with fused rings	43	$-2.34 - 0.32C_{methyl} + 0.64C_{subst-aromatic} - 0.53N_{amine}$	0.66	14	50 (7/14)	0.31, 0.71
Chemicals without fused ring systems	52	$-4.38 + 0.13C_{aromatic} + 0.36C_{subst-aromatic} + 0.34C_{Quat} + 0.070O_{nitro} + 0.12O_{ether} + 0.088F + 0.19Cl$	0.56	16	50 (8/16)	0.26
All chemicals with $\geq 3$ aromatic rings (e.g., anthracenes, pyrenes)	33	$-1.189 - 0.25C_{methyl} + 0.47C_{subst-aromatic} + 0.082O_{nitro} - 0.59N_{amine}$	0.49	10	50 (5/10)	0.11, 0.62

<sup>a</sup> Subsets containing exactly 2 ( $n=9$ ) or 4 ( $n=9$ ) fused aromatic rings are not presented because the training subsets were considered too small to provide a reliable equation. The subset containing exactly three aromatic rings ( $n=25$ ) was not included because the resulting model included only one E-state descriptor. Subsets containing only biphenyls ( $n=28$ ) or only anilines ( $n=18$ ) did not have a statistically significant correlation between LogR and E-state sums and are, therefore, not included in the table.

<sup>b</sup> An  $r^2$  value for the entire validation set is the first value presented. An  $r^2$  value for the validation set with one chemical (7-adamantyl-2-aminofluorene) removed are presented second. This chemical was removed from the analysis because it possesses large, bulky, and non-flexible substituents that may have affected mutagenicity in a manner not likely to be described by E-state sums. The above E-state models severely over-predicted LogR for this chemical and, consequently, drastically affected the  $r^2$  value of the validation set.

longer residence time in the body, thereby increasing the probability for reactions with DNA. The structures presented for the sub-class of phenazines in Table 3 support these proposed influences.

The most highly mutagenic chemical in the table is 2,7-diaminophenazine. In examining this chemical more closely, it is observed that there is a high degree of symmetry in the molecule and the orientation of the amine groups on the aromatic backbone of the molecule effectively stabilize the resulting carbocation formed upon activation, as shown in Fig. 4. For the least mutagenic compound, 1,9-diaminophenazine, no resulting

stabilized resonance form of the active intermediate may be drawn. We note that the mutagenic potency of 2,7-diaminophenazine is greatly underpredicted by all models that have been developed for this data set [1–5], not just the E-state models.

Finally, it should be noted that the phenazines containing the amino groups in the 2, 7, and 8 positions on the rings of the molecule are more mutagenic based on the experimental values. This could reflect a steric relationship necessary for intercalation in the DNA, which is not accounted for using only E-states in the QSAR analysis.

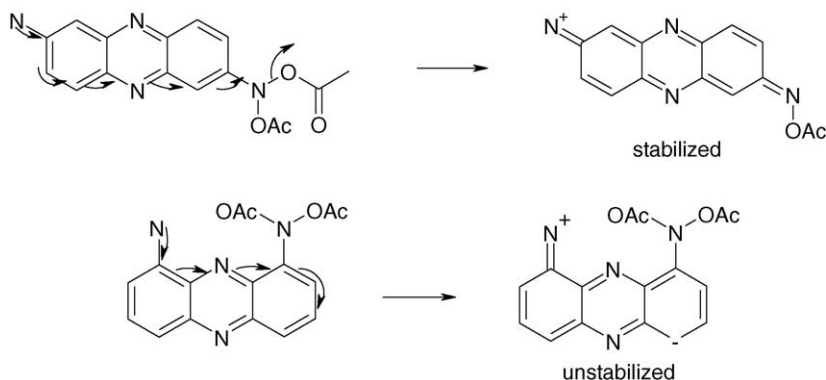


Fig. 4. High degree of symmetry in the molecule and the orientation of the amine groups on the aromatic backbone of the molecule effectively stabilize the resulting carbocation formed upon activation.

Table 5

Chemicals used in the training and validation sets and their experimental and predicted LogR values<sup>a</sup>

Chemical	Chemical class	Experimental LogR	Predicted LogR (Eq. (1))	Predicted LogR (Eq. (2))
Eq. (1): training set/ Eq. (2): training set				
4-Ethoxyaniline	Aniline	-2.30	-2.35	-2.54
2-Amino-5-nitrophenol	Aniline	-2.52	-2.58	-2.29
4-Chloroaniline	Aniline	-2.52	-2.58	-2.12
3-Methoxy-4-methylaniline	Aniline	-1.96	-2.12	-2.25
2-Methoxy-5-methylaniline	Aniline	-2.05	-2.22	-2.32
4-Bromoaniline	Aniline	-2.70	-2.51	-1.83
2,4-Difluoroaniline	Aniline	-2.70	-2.51	-2.91
2,4-Diamino- <i>n</i> -butylbenzene	Aniline	-2.70	-2.51	-1.77
4-Methoxy-2-methylaniline	Aniline	-3.00	-3.19	-2.22
2-Amino-4-chlorophenol	Aniline	-3.00	-3.19	-2.52
2,5-Dimethylaniline	Aniline	-2.40	-2.64	-1.95
4-Amino-2'-nitrobiphenyl	Aniline	-0.92	-0.42	-0.43
2,6-Dichloro-1,4-phenylenediamine	Aniline	-0.69	-1.28	-1.96
2,4-Dinitroaniline	Aniline	-2.00	-1.13	-1.57
2-Bromo-4,6-dinitroaniline	Aniline	-0.54	-1.44	-1.51
2,4,5-Trimethylaniline	Aniline	-1.32	-2.29	-1.55
4-Fluoroaniline	Aniline	-3.32	-2.34	-2.32
2-Amino-4-methylphenol	Aniline	-2.10	-3.19	-2.52
4-Phenoxyaniline	Aniline	0.38	-0.93	-1.46
4,4'-Ethylenebis(aniline)	Aniline	-2.15	-0.26	-0.09
9-Aminoanthracene	Anthracene	0.87	0.83	0.86
1-Aminoanthracene	Anthracene	1.18	0.86	0.95
3,3'-Dichlorobenzidine	Biphenyl	0.81	0.84	-0.20
3,3'-Dimethylbenzidine	Biphenyl	0.01	-0.14	0.21
Benzidine	Biphenyl	-0.39	-0.64	-0.41
2-Aminobiphenyl	Biphenyl	-1.49	-1.24	-0.93
3-Amino-3'-nitrobiphenyl	Biphenyl	-0.55	-0.23	-0.27
3,3'-Dimethoxybenzidine	Biphenyl	0.15	0.48	-0.78
3,4'-Diaminobiphenyl	Biphenyl	0.20	0.57	-0.42
2-Amino-4'-nitrobiphenyl	Biphenyl	-0.62	-0.16	-0.20
2-Amino-3'-nitrobiphenyl	Biphenyl	-0.89	-0.35	-0.37
3-Amino-2'-nitrobiphenyl	Biphenyl	-1.30	-0.71	-0.49
3,3'-Diaminobiphenyl	Biphenyl	-1.30	-0.71	-0.47
2,2'-Diaminobiphenyl	Biphenyl	-1.52	-0.90	-0.64
3-Amino-4'-nitrobiphenyl	Biphenyl	0.69	-0.05	-0.11
4-Aminobiphenyl	Biphenyl	-0.14	-1.12	-0.83
4-Amino-3'-nitrobiphenyl	Biphenyl	1.02	-0.15	-0.20
1-Aminocarbazole	Carbazole	-1.04	-0.57	-0.65
4-Aminocarbazole	Carbazole	-1.42	-0.58	-0.66
2-Aminocarbazole	Carbazole	0.60	-0.46	-0.55
6-Aminochrysene	Chrysene	1.83	3.08	2.79
4-Aminophenyl-ether	Diphenyl ether	-1.14	-1.18	-1.46
4-Aminophenyl disulfide	Diphenyl sulfide	-1.03	-1.07	-0.79
4,4'-Diaminophenyl sulfide	Diphenyl sulfide	0.31	-0.54	-0.33
4,4'-Methylenebis( <i>o</i> -fluoroaniline)	Diphenylene	0.23	-0.50	-1.25
4,4'-Methylenebis( <i>o</i> -ethylaniline)	Diphenylene	-0.99	0.29	0.59
8-Aminofluoranthene	Fluoranthene	3.80	3.46	3.13
1-Aminofluoranthene	Fluoranthene	3.35	3.32	3.00
3-Aminofluoranthene	Fluoranthene	3.31	3.38	3.05
2-Aminofluoranthene	Fluoranthene	3.23	3.41	3.08
7-Aminofluoranthene	Fluoranthene	2.88	3.33	3.02

Table 5 (Continued)

Chemical	Chemical class	Experimental LogR	Predicted LogR (Eq. (1))	Predicted LogR (Eq. (2))
4-Aminofluorene	Fluorene	1.13	1.36	1.32
2-Bromo-7-aminofluorene	Fluorene	2.62	2.36	2.18
2-Aminofluorene	Fluorene	1.93	1.48	1.40
3-Aminofluorene	Fluorene	0.89	1.46	1.40
2-Hydroxy-7-aminofluorene	Fluorene	0.41	1.20	1.18
2-Amino-7-nitrofluorene	Fluorene	3.00	2.15	1.79
1-Aminofluorene	Fluorene	0.43	1.41	1.36
2,7-Diaminofluorene	Fluorene	0.48	1.92	1.79
2-Amino-1-nitronaphthalene	Naphthalene	-1.17	-0.89	-0.84
2-Aminonaphthalene	Naphthalene	-0.67	-1.10	-0.81
1-Aminonaphthalene	Naphthalene	-0.60	-1.16	-0.87
1-Amino-4-nitronaphthalene	Naphthalene	-1.77	-0.59	-0.58
1-Aminophenanthrene	Phenanthrene	2.38	0.99	0.99
2-Aminophenanthrene	Phenanthrene	2.46	1.08	1.07
2-Aminophenazine	Phenazine	0.55	0.57	0.98
1,7-Diaminophenazine	Phenazine	0.75	0.73	1.12
2,8-Diaminophenazine	Phenazine	1.12	0.89	1.25
1-Aminophenazine	Phenazine	-0.01	0.35	0.80
2,7-Diaminophenazine	Phenazine	3.97	0.90	1.26
4-Aminopyrene	Pyrene	3.16	3.24	2.94
2-Aminopyrene	Pyrene	3.50	3.36	3.04
1-Aminopyrene	Pyrene	1.43	3.29	2.98
6-Aminoquinoline	Quinoline	-2.67	-1.05	-0.61
Eq. (1): training set/ Eq. (2): validation set				
3-Amino-alpha,alpha,alpha-trifluorotoluene	Aniline	-0.80	-0.70	-1.76
2,4-Diaminoisopropylbenzene	Aniline	-3.00	-3.19	-2.42
2-Chloroaniline	Aniline	-3.00	-3.19	-2.27
2,4-Dimethylaniline	Aniline	-2.22	-2.61	-1.93
4-Chloro-2-nitroaniline	Aniline	-2.22	-2.61	-1.85
4-Cyclohexylaniline	Aniline	-1.24	-1.86	-1.47
4-Chloro-1,2-phenylenediamine	Aniline	-0.49	-1.83	-1.94
2-Aminoanthracene	Anthracene	2.62	2.36	0.97
2,4'-Diaminobiphenyl	Biphenyl	-0.92	-0.42	-0.49
4-Amino-4'-nitrobiphenyl	Biphenyl	1.04	0.02	-0.05
3-Aminocarbazole	Carbazole	-0.48	-0.47	-0.56
4,4'-Methylenedianiline	Diphenylene	-1.60	-0.38	-0.19
4,4'-Methylenebis(o-isopropylaniline)	Diphenylene	-1.77	-0.59	-0.67
2-Amino-7-acetamidofluorene	Fluorene	1.18	0.86	0.60
9-Aminophenanthrene	Phenanthrene	2.98	0.94	0.95
3-Aminophenanthrene	Phenanthrene	3.77	1.05	1.05
1,6-Diaminophenazine	Phenazine	0.20	0.57	0.98
1,9-Diaminophenazine	Phenazine	0.04	0.54	0.96
8-Aminoquinoline	Quinoline	-1.14	-1.18	-0.71
5-Aminoquinoline	Quinoline	-2.00	-1.13	-0.68
3-Aminoquinoline	Quinoline	-3.14	-1.11	-0.66
Eq. (1): validation set/ Eq. (2): validation set				
3,5-Diisopropyl-4-aminobiphenyl	Biphenyl	-1.95	-2.14	-1.30
4'-Ethyl-4-aminobiphenyl	Biphenyl	-0.14	-0.76	-0.42
3'-Trifluoromethyl-4-aminobiphenyl	Biphenyl	-0.38	0.98	-0.38
3'-Methyl-4-aminobiphenyl	Biphenyl	0.74	-0.82	-0.47
3- <i>t</i> -Butyl-4-aminobiphenyl	Biphenyl	-0.39	-2.52	-1.73
1-Ethyl-2-aminofluorene	Fluorene	1.85	1.94	1.92

Table 5 (Continued)

Chemical	Chemical class	Experimental LogR	Predicted LogR (Eq. (1))	Predicted LogR (Eq. (2))
7- <i>t</i> -Butyl-2-aminofluorene	Fluorene	−0.20	0.13	0.57
7-Adamantyl-2-aminofluorene	Fluorene	−0.64	3.17	2.88
Eq. (1): validation set/ Eq. (2): training set				
4′- <i>n</i> -Butyl-4-aminobiphenyl	Biphenyl	−0.74	−0.72	−0.38
3,5-Dimethyl-4-aminobiphenyl	Biphenyl	−1.34	−0.63	−0.21
3′,5′-Dimethyl-4-aminobiphenyl	Biphenyl	0.35	−0.51	−0.11
3- <i>n</i> -Butyl-4-aminobiphenyl	Biphenyl	0.17	−0.73	−0.39
4′- <i>i</i> -Propyl-4-aminobiphenyl	Biphenyl	−0.72	−1.62	−1.05
3-Ethyl-4-aminobiphenyl	Biphenyl	0.17	−0.79	−0.45
3,5-Diethyl-4-aminobiphenyl	Biphenyl	−1.71	−0.42	−0.03
4′- <i>t</i> -Butyl-4-aminobiphenyl	Biphenyl	−1.17	−2.51	−1.71
4′-Methyl-4-aminobiphenyl	Biphenyl	0.64	−0.83	−0.48
3- <i>i</i> -Propyl-4-aminobiphenyl	Biphenyl	−0.01	−1.63	−1.07
4′-Trifluoromethyl-4-aminobiphenyl	Biphenyl	−0.55	1.25	−0.13
3′,5′-Ditrifluoromethyl-4-aminobiphenyl	Biphenyl	−0.78	2.03	−0.88
1- <i>t</i> -Butyl-2-aminofluorene	Fluorene	0.07	0.16	0.59
7-Methyl-2-aminofluorene	Fluorene	1.47	1.82	1.81
1- <i>n</i> -Butyl-2-aminofluorene	Fluorene	2.86	2.06	2.02
1- <i>i</i> -Propyl-2-aminofluorene	Fluorene	2.23	1.08	1.28
7-Trifluoromethyl-2-aminofluorene	Fluorene	0.76	3.05	1.40
1- <i>n</i> -Butyl-2-aminonaphthalene	Naphthalene	−0.29	−0.60	−0.28
1- <i>t</i> -Butyl-2-aminonaphthalene	Naphthalene	−2.00	−2.42	−1.64
1- <i>i</i> -Propyl-2-aminonaphthalene	Naphthalene	−0.62	−1.53	−0.98
1-Ethyl-2-aminonaphthalene	Naphthalene	0.36	−0.68	−0.35

<sup>a</sup> Experimental LogR values originally reported by Debnath et al. [1] and Glende et al. [6,7].

#### 4. Conclusions

The results of this analysis indicate that E-state indices performed as well as other complex molecular descriptors at modeling mutagenicity (Table 5). However, none of the E-state models presented in this article or previously published research showed acceptable predictive accuracy when subject to external validation. Results for all the models indicated that the training set data could be described relatively well using a number of descriptors. However, results from external validations of these mutagenicity models indicated possible over-fitting of the model data resulting in good internal validation statistics, but poor predictive accuracy. On the other hand, a data:predictor ratio of >10:1, as in all the models discussed here, should be sufficient to avoid this problem.

After investigating the results of this research, we noted that stabilization of the resulting carbocation formed upon activation to the bioactive intermediate, in addition to steric factors hindering the reactivity of the molecule, may play a significant role in affecting

the mutagenic potential of the compounds. The statistical assessment performed on the data set allowed identification of these important spatial parameters not investigated in the initial assessment. The mechanistic considerations presented in this paper are not considered novel for this type of toxicity. However, even with the identification of these potential mitigating factors, as well as others previously identified such as water solubility and  $K_{ow}$ , it is interesting to note that many QSAR authors attempting to model this data set have traditionally relied only on descriptors deemed as “statistically significant” to the trends in the training set, without providing a further inspection of the potential mechanisms for the data set. For the effective application of a toxicity QSAR, it is important that the descriptors have a physicochemical interpretation that is consistent with a known mechanism of biological action for the endpoint of interest. Without this additional step of reconciliation, it is often found that models fall short when subject to external validation.

These results highlight the need to perform an external validation of a model to assess its true predictive

ability. Training set statistics and internal validation techniques alone may be very misleading when trying to evaluate the true predictive accuracy of a model. With this in mind, it is important to understand the differences between a model's fit and a model's predictive ability. If interest is focused only on an understanding of the mechanistic interpretation of the data, a model with a good fit to the underlying data may be very useful. However, this kind of model may not be appropriate or representative for other, new compounds which were not included in the original training set. What was particularly surprising in the present study was that the model developed in [2] did a poor job of predicting compounds described in [7], which differed only in having an alkyl substituent far away from the amine function. It is not intuitively obvious that such substitution would greatly affect experimental LogR, but it did. Models such as those developed here may be used to derive trends in the data set or to give insight into the appropriate grouping of chemicals for compounds that would not typically be combined if one looked only at structural similarities. These assessments may be used to give the researcher a better indication of the types of descriptors that could be used more effectively to model an endpoint of interest.

Additional work is needed in order to determine if E-state indices are appropriate descriptors for modeling mutagenicity of aromatic amines. The E-state sums may not adequately describe key positional features that may influence the stability of the reactive intermediates, whereby affecting mutagenicity of the amines. E-state sums only account for the electronic characteristics and influences of the nearest neighbor substitutions for each atom in the parent compound and do not characterize such factors as steric effects or resonance stability affecting the reactivity of the metabolites. With this in mind, it is thought that E-state sums may be more appropriate predictors for the mutagenicity for chemicals that do not require metabolic activation.

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