An in Silico Method for Predicting Ames Activities of Primary Aromatic Amines by Calculating the Stabilities of Nitrenium Ions

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In this paper, we describe an in silico first principal approach to predict the mutagenic potential of primary aromatic amines. This approach is based on the so-called "nitrenium hypothesis", which was developed by Ford et al. in the early 1990s. This hypothesis asserts that the mutagenic effect for this class of molecules is mediated through the transient formation of a nitrenium ion and that the stability of this cation is correlated with the mutagenic potential. Here we use quantum mechanical calculations at different levels of theory (semiempirical AM1, ab initio HF/3-21G, HF/6-311G(d,p), and DFT/B3LYP/6-311G(d,p)) to compute the stability of nitrenium ions. When applied to a test set of 257 primary aromatic amines, we show that this method can correctly differentiate between Ames active and inactive compounds, and furthermore that it is able to rationalize and predict SAR trends within structurally related chemical series. For this test set, the AM1 nitrenium stability calculations are found to provide a good balance between speed and accuracy, resulting in an overall accuracy of 85%, and sensitivity and specificity of 91% and 72%, respectively. The nitrenium-based predictions are also compared to the commercial software packages DEREK, MULTICASE, and the MOE-Toxicophore descriptor. One advantage of the approach presented here is that the calculation of relative stabilities results in a continuous spectrum of activities and not a simple yes/no answer. This allows us to observe and rationalize subtle trends due to the different electrostatic properties of the organic molecules. Our results strongly indicate that nitrenium ion stability calculations should be used as a complementary approach to assist the medicinal chemist in prioritizing and selecting nonmutagenic primary aromatic amines during preclinical drug discovery programs.

INTRODUCTION

In the process of drug discovery it is important that compounds not only have high potency but that they also possess the right ADMET (absorption−distribution−metabolism−excretion−toxicology) properties for the target.[1] Optimized ADMET properties become more important during the lifetime of a drug discovery program; therefore, it is highly desirable to discover potential ADMET liabilities early in the drug discovery process. Identifying ADMET liabilities at later stages can be extremely expensive, both in terms of lost time and money.

One of the important toxicological end points typically addressed early in drug discovery is mutagenicity. The most frequently used test for assessing in vivo mutagenicity is the bacterial reverse mutation test, more commonly referred to as the Ames assay.[2,3] The Ames test is an in vitro assay for assessing mutations using different *Salmonella typhimurium* strains that are sensitive to particular types of mutations and different classes of chemical mutagens. Compounds are tested with and without an enzymatic activation system (often Arochlor-induced rat liver S9-fraction) to allow detection of pro-mutagens. The Ames assay is considered one of the more reliable early predictors of carcinogenic potential. The correlation between bacterial mutagenicity and rodent carcinogenicity established by Ames has been reported to be around 90%.[2] Snyder and Green report a concordance value of 63% between Ames genotoxicity and rodent carcinogenicity for a set of 198 compounds from the PDR database.[4] For a small set of 13 to 39 compounds, the interlaboratory reproducibility of Ames data with and without S9-fraction shows concordance values from 79% to 92% and 65% to 90%, respectively.[5] Kazius et al.[6] report an interlaboratory Ames test error of 15%. Performing Ames tests for a large set of compounds is relatively resource expensive. Though high-throughput formats for this assay exist,[7] it is highly desirable to be able to accurately predict the potential for mutagenicity by computational means to support early drug discovery.

In recent years numerous papers have been published that address this issue. Kazius et al. used a test set of 4337 compounds and derived a set of 29 toxicophores to predict mutagenicity.[6] The overall accuracy of this model was 82% for the training set and 85% for an external test set of 535 compounds. The statistics for 441 compounds with the specific aromatic amine toxicophore shows an accuracy of

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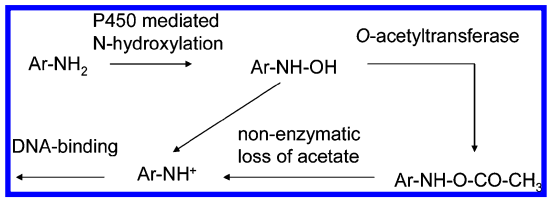
Scheme 1. Mechanism for Aromatic Amine Genotoxicity Adapted from Ford and Griffin[21]



86%. Votano et al. used artificial neural net, *k*-nearest neighbor (*k*-NN) and decision forest approaches to predict bacterial mutagenicity.[8] These authors used a training set of 3363 diverse compounds and validated their models on an external test set of 400 compounds. These models yielded an average accuracy of 88% for the training set and 82% for the test set. Votano et al. compared their results to those generated with DEREK[9] and MULTICASE[10,11] and found that their models were significantly more accurate than either of these commercially available programs. Overall DEREK and MULTICASE suffered from a high proportion of false positives. In a similar study on 334 aromatic and secondary amines, Mattioni et al. reported a similar performance.[12] Recently Langham and Jain published the results of *k*-NN, RuleFit, and support vector machine (SVM) models trained on the Organon data set.[13] They obtained accuracies between 74% and 79% for the training set and 71% to 77% for the external test set. Serafimova et al. reported a hybrid model, TIMES, which couples a metabolic activation simulator together with an expert system based on structure-toxicity rules. Their model based on a training set of 1314 structures from the National Toxicology Program and 1626 chemicals proprietary to BASF showed an overall sensitivity of 82% and a specificity of 89%.[5] Contrera et al. used E-state indices and MDL QSAR software to generate models for predicting bacterial mutagenicity. These authors used a large training set of 3338 compounds and a test set of over 1400 compounds. With their methodology these authors were able to achieve a specificity of 76% and a sensitivity of 81%.[14] White et al. showed that a combination of TOPKAT, DEREK, and CASETOX can lead to an improvement in accuracy, sensitivity, and specificity for mutagenicity predictions.[15] Following the nomenclature of Riffenburgh,[16] the term sensitivity is defined as the ratio of true positives/all experimental positives. Similarly specificity is the ratio of true negatives/all experimental negatives and accuracy is defined as (true positives + true negatives)/sample size. The accuracies reported in the literature and the interlaboratory variability give a good frame of reference for what can be reasonably achieved with an in silico method.

Mutagenicity of Aromatic Amines. A very well researched class of mutagenic compounds of particular importance in the pharmaceutical industry is that of aromatic amines. These moieties occur frequently as substructures in drug candidates. The quantitative structure activity relationship (QSAR) of mutagenic and carcinogenic aromatic amines has been reviewed by Benigni et al.[17] The most widely accepted mutagenic mechanism for primary aromatic amines is summarized in Scheme 1.

To exert their mutagenic effect, aromatic amines are activated through *N*-oxidation by cytochrome P450s to form *N*-hydroxylamines. These *N*-hydroxylamines then undergo N−O bond cleavage to form the nitrenium ion either directly or following conjugation of the hydroxylamine with acetate or sulfate. The nitrenium ion intermediate then forms an adduct with DNA, which results in miscoding during DNA replication. According to this mechanism the stability of the nitrenium ion is fundamental in determining the extent of that mutagenic effect. We note that, in addition to aromatic amines Wild and co-workers have used evidence from photolysis experiments to suggest that the genotoxicity observed for arylazides and nitroaryl compounds is also the result of the stability of the common nitrenium intermediate formed by these compounds.[18] In addition to DNA adduct formation intercalation into the DNA without the formation of a covalent bond is another mechanism known to lead to genotoxicity. Structure-based computational approaches to assess the intercalation potential have been developed.[19,20]

In 1992, Ford et al. built a predictive model, for a small set of aromatic amine molecules encountered in cooked foods, that correlates the Ames activity to the stability of the nitrenium ion.[21,22] These authors used the semiempirical AM1 method to calculate the stability of the nitrenium ion relative to that of the baseline molecule, aniline. It was shown that only the formation energy of the final nitrenium ion, that is, the overall enthalpy change associated with reaction (1) below, varied significantly with the nature of the aryl group, whereas the energies associated with schematic reactions (2) and (3) were much less sensitive to changes of the aryl group.

$$\mathrm{ArNH_2} + \mathrm{PhN^+H} \rightarrow \mathrm{ArN^+H} + \mathrm{PhNH_2} \quad (1)$$

$$\mathrm{ArNH_2} + \mathrm{PhNHOH} \rightarrow \mathrm{ArNHOH} + \mathrm{PhNH_2} \quad (2)$$

$$\mathrm{ArNHOH} + \mathrm{PhN^+H} \rightarrow \mathrm{ArN^+H} + \mathrm{PhNHOH} \quad (3)$$

$$\Delta\Delta E = \Delta E_{\mathrm{ArNH^+}} + \Delta E_{\mathrm{PhNH_2}} - \Delta E_{\mathrm{ArNH_2}} - \Delta E_{\mathrm{PhNH^+}} \quad (4)$$

From reaction (1), one can then calculate relative energies $\Delta\Delta E$ according to equation (4). A negative value for $\Delta\Delta E$ indicates that the nitrenium ion for the aromatic amine of interest is more stable than that for the reference aniline, and a positive value for $\Delta\Delta E$ indicates a less stable nitrenium ion for the aromatic amine of interest relative to the reference aniline. According to the nitrenium hypothesis negative values of $\Delta\Delta E$ should correlate with an Ames positive compound, whereas positive values of $\Delta\Delta E$ should correlate with an Ames negative compound.

Ford and Griffin used the semiempirical AM1 calculated heat of formation ΔH_f for ΔE in equation 4. Depending on the *Salmonella* strain in which mutagenicity was assessed experimentally, these authors were able to generate QSAR models with correlation coefficients that ranged between 0.593 and 0.992.[21]

In a number of studies a group at Lawrence Livermore National Laboratory found better correlations between the mutagenicity and the LUMO energy of the parent amine than with the nitrenium ion stability.[23–25] The mechanistic reason for the observed correlation of the mutagenic potential with the LUMO energy is not entirely clear. However, in two recent DFT-studies Borosky reaffirmed the importance of the nitrenium stability in determining the mutagenic potency.[26,27] Although no satisfactory overall correlation

between the mutagenic potency and the nitrenium ion stability was found good correlations were observed for structurally related compounds.

Though many primary aromatic amines show Ames activity, this moiety is nevertheless present in 89 launched drugs and an additional 131 molecules that have entered phase I, II, or III clinical studies, based on a substructure search of the MDDR database.[28] Also a search of the ACD library[29] revealed that approximately 58,000 structures with primary aromatic amine functionality are commercially available for purchase. When synthesizing new drugs, it is important to use those building blocks that are less likely to have Ames liabilities. A fast and reliable in silico prediction of Ames activity for this class of molecules would therefore be of considerable benefit to medicinal chemists when prioritizing and selecting compounds for synthesis.

The goal of the research we describe in this paper was to apply the original nitrenium hypothesis to a set of primary aromatic amines to understand how this hypothesis can best be used to select primary amine molecules that are less likely to be mutagenic. For this purpose, a test set was constructed from the 4337 compounds used in the recent study by Kazius et al.[6] Various approaches to estimate the nitrenium ion stability have been investigated, as well as their concomitant effect on the sensitivity, specificity, and accuracy of the mutagenic potential predictions. Additionally, we expected that a computational approach based on the underlying physical mechanism of genotoxicity might be better able to predict subtle changes in SAR than methods based solely on structural alerts or descriptors. Therefore, we compared the nitrenium approach with other standard in silico procedures: DEREK, MULTICASE, and Kazius et al.'s toxicophore within MOE (referenced herein as MOE-t).

Technical Details. The set of primary aromatic amines used in this study was extracted from the data set of 4337 molecules compiled by Kazius et al.[6] (Organon Ames Data set). Only those aromatic amines were retained that had (i) no formal charge, (ii) a molecular weight below 500, (iii) no more than one stereocenter, (iv) less than 10 rotatable bonds, and (v) only one aromatic amine functionality. We

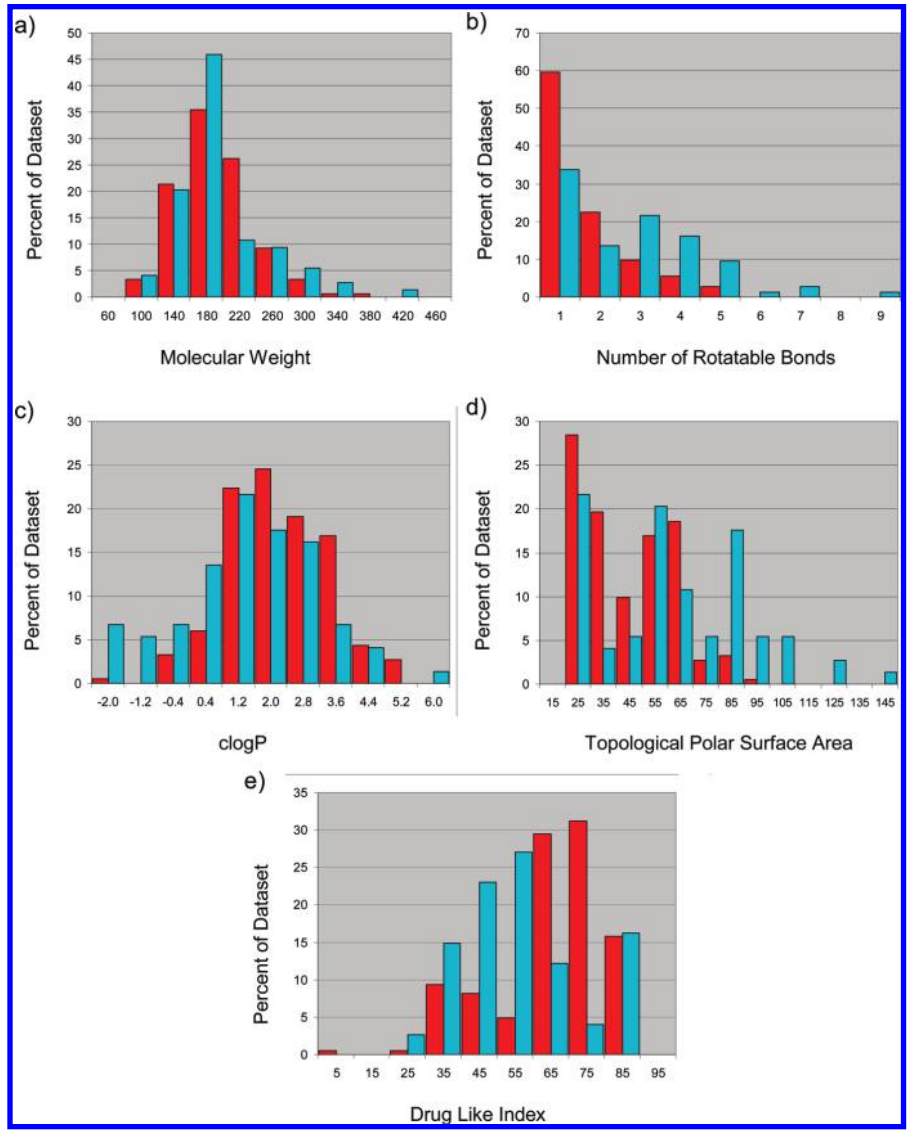


Figure 1. Distribution of physicochemical properties (a) molecular weight, (b) number of rotatable bonds, (c) clogP, (d) topological polar surface area, and (e) drug-like index for mutants (red bars) and nonmutants (light blue bars). The *y*-axis is the percent of the mutant or nonmutant subset. The clogP was calculated using Sybyl, version 7.3, the number of rotatable bonds was calculated using QikProp,[40] the polar surface area is calculated using an in-house application of the method described by Ertl[41] and the drug-like-index is based on the publication by Xu and Stevenson.[42]

also removed compounds with aromatic nitro groups as these could exhibit Ames toxicity because of their nitro-moiety. This filtering was performed with a Accelrys Pipeline Pilot[30] script. For each entry, only the largest fragment was retained to remove counterions and solvents. Hydrogen atoms were added, and the molecules standardized so that ionizable groups, for example, basic amines and carboxylic acids are in their neutral form. At this stage, the subset of molecules that passed the outlined selection criteria included 269 molecules of which 192 are mutagens and 77 are nonmutagens according to the original assignment by Kazius et al.[6] During a visual inspection of the subset, ten additional compounds were removed that could lead to Ames toxicity because of groups other than primary aromatic amines or because they had undesirable chemical features. These compounds included five azo-compounds, one purine *N*-oxide, one azide, one hydroxyl-amine, one nitrosourea, and one phosphorus-containing compound. Lastly, the baseline compound aniline was removed. We also removed compound CAS number 67199-66-0 because there was ambiguity about its molecular structure. The final data set thus consists of 257 molecules of which 183 are mutagens and 74 are nonmutagens. The complete data set is available from the Supporting Information.

In addition to the neutral form of the aromatic amine, the syn and anti geometries of the nitrenium ion are also required in Ford and Griffin's method. Three dimensional structures for all three species were generated using Schrödinger's premin script[31] with the MMFFs94 forcefield.[32] Full geometry optimizations for the neutral molecules as well as the syn and anti forms of their nitrenium ions were then carried out using Gaussian.[33] The fact that the calculations are done in the gas phase and not in solvent is not expected to result in significant error, as the final energies are calculated relative to aniline. We treated all compounds with potentially ionizable groups in their respective neutral form to minimize the impact of large concentrated charge on the calculation. Four different levels of theory were employed to obtain the geometries and energies for the different molecules: (i) semiempirical AM1, (ii) HF/3-21G, (iii) HF/6-311G(d,p), and (iv) DFT/B3LYP/6-311G(d,p). Following Ford and Griffin stability energies for the nitrenium ions ($\Delta\Delta E$) were calculated relative to that of the reference molecule aniline using equation (4). When assessing the Ames activity of each molecule, the most stable form of the two nitrenium ion conformations (syn and anti) was selected for the final $\Delta\Delta E$ determination. To develop a simple classification scheme compounds were then assigned Ames positive when $\Delta\Delta E <$ 0.0 kcal/mol and Ames negative when $\Delta\Delta E > 0.0$ kcal/mol; this choice was based on an inspection of the distributions of $\Delta\Delta E$ values that were obtained for the entire data set (see Results and Discussion).

Ford and Griffin used the AM1 approach in their original publication, which is calibrated to give heats of formation. For the ab initio calculations, we used the total energies and did not attempt to convert these values into heats of formation. We believe that this is justified as we are only concerned with relative energies and are not comparing absolute values between different calculations. This approach has also been adopted by others.[27]

The $\Delta\Delta E$ values presented in this paper were also corroborated using a second implementation of the nitrenium ion stability technique. Briefly, this employs three-dimensional structures generated by Corina,[34] customized SVL scripting, semiempirical AM1 calculations with MOPAC,[35] and ab initio calculations with Q-Chem.[36]

To compare the methods presented in this paper with other approaches the Ames activities for the 257 compounds were also predicted using Lhasa Ltd.'s Derek for Windows (DEREK)[37] and MultiCASE Inc.'s MC4PC (MULTICASE).[38] In addition the performance of a MOE-toxicophore (MOE-t) descriptor[39] model was assessed. The DEREK analysis was conducted using version 10.0.2, processing against all alerts and constraining the species to *Salmonella*. MULTICASE analysis was conducted using MULTICASE PC, version 2.0.0.95. Compounds were processed against the overall *Salmonella* model and submodels for *Salmonella* strains TA98, TA100, TA1535, TA1537, and TA102 plus *Escherichia coli*. Compounds were processed with and without activation in all MULTICASE models. The MULTICASE models with catalog numbers A2H, A2K, A2L, A2M, A2O, A2 V, A2W, A2X, A2Z, A6E, A9A, and A9G were employed. For MULTICASE assessments, the ICSAS expert method call was used as the final result for comparison purposes.

RESULTS AND DISCUSSION

Physical Property Profiles of the Data Set. Analysis of the initial 257 compound data set by physicochemical properties shows that although the ratio between mutants and nonmutants is about 2.5:1 the overall distribution of physical chemical properties is similar. A selection of physicochemical properties derived from the data set is shown in Figure 1. This analysis shows that the data set consists of relatively small compounds with physicochemical property ranges that may be considered drug-like and certainly of relevance to

Table 1. Truth Tables for Ames Predictions with Commercial Software[a]

	Ames positive predicted DEREK	Ames negative predicted DEREK
Ames positive experimental	152 (183)	31
Ames negative experimental	10	64 (74)

	Ames positive predicted MULTICASE	Ames negative predicted MULTICASE
Ames positive experimental	103 (156)	53
Ames negative experimental	7	62 (69)

	Ames positive predicted MOE-t	Ames negative predicted MOE-t
Ames positive experimental	180 (183)	3
Ames negative experimental	36	38 (74)

[a] The numbers in parentheses are the experimental assignments from the Organon data set by Kazius et al.[6] The number of compounds for MULTICASE is reduced as 27 Ames positive and 5 Ames negative compounds were predicted "indeterminate" in MULTICASE.

Table 2. Truth Tables for Ames Predictions Using the Nitrenium Hypothesis at Different Levels of Theory[a]

	Ames positive predicted AM1[b]	Ames negative predicted AM1[b]	Ames positive predicted AM1[c]	Ames negative predicted AM1[c]
Ames positive experimental	167 (183)	16	155 (164)	9
Ames negative experimental	21	53 (74)	14	43 (57)

	Ames positive predicted HF/3-21G[b]	Ames negative predicted HF/3-21G[b]	Ames positive predicted HF/3-21G[c]	Ames negative predicted HF/3-21G[c]
Ames positive experimental	161 (183)	22	150 (167)	17
Ames negative experimental	17	57 (74)	12	48 (60)

	Ames positive predicted HF/6-311G (d,p)[b]	Ames negative predicted HF/6-311G (d,p)[b]	Ames positive predicted HF/6-311G (d,p)[c]	Ames negative predicted HF/6-311G (d,p)[c]
Ames positive experimental	161 (183)	22	151 (165)	14
Ames negative experimental	19	55 (74)	12	48 (60)

	Ames positive predicted DFT/B3LYP/ 6-311G(d,p)[b]	Ames negative predicted DFT/B3LYP/ 6-311G(d,p)[b]	Ames positive predicted DFT/B3LYP/ 6-311G(d,p)[c]	Ames negative predicted DFT/B3LYP/ 6-311G(d,p)[c]
Ames positive experimental	171 (183)	12	160 (167)	7
Ames negative experimental	28	46 (74)	18	26 (44)

[a] The numbers in parentheses are the experimental assignments from the data set by Kazius et al.[6] The first set. [b] Results from calculations using a cutoff of 0.0 kcal/mol between Ames positive and Ames negative compounds. [c] The second set results from calculations using a cutoff of 0.0 kcal/mol between Ames positive and Ames negative compounds, but excluding any compounds within ±5.0 kcal/mol.

Table 3. Summary Statistics for the Different Approaches to Predict Ames Activity[a]

	DEREK	MULTI CASE	MOE-t	nitrenium AM1	nitrenium HF/ 3-21G	nitrenium HF/ 6-311G (d,p)	nitrenium DFT/B3LYP/ 6-311G (d,p)
accuracy	0.840	0.733	0.848	0.856 [0.896]	0.848 [0.872]	0.840 [0.884]	0.844 [0.882]
false positive rate	0.135	0.101	0.486	0.284 [0.246]	0.230 [0.200]	0.257 [0.200]	0.378 [0.409]
false negative rate	0.169	0.340	0.017	0.087 [0.055]	0.120 [0.102]	0.120 [0.085]	0.066 [0.042]
sensitivity	0.831	0.660	0.984	0.913 [0.945]	0.880 [0.898]	0.880 [0.915]	0.934 [0.958]
specificity	0.865	0.899	0.514	0.716 [0.754]	0.770 [0.800]	0.743 [0.800]	0.622 [0.591]

[a] Numbers in square brackets are calculated using the second set of numbers from Table 2 with the ±5.0 kcal/mol safety window.

the types of molecules that are routinely encountered in preclinical drug discovery programs.

Comparison with Other Commercial Software Packages. To assess the predictive power of our methods we summarized the results in the following 2×2 matrices also called truth tables following the nomenclature of Riffenburgh,[16] and compared them to those obtained using DEREK, MULTICASE, and the MOE-t, which is based on the Organon descriptor (Table 1).[6]

DEREK performed reasonably well for this test set by correctly predicting 152 of the 183 Ames positive compounds and 64 of 74 Ames negative compounds, thereby resulting in an overall accuracy of 84%, a sensitivity of 83%, and a specificity of 87% (Table 3). MULTICASE could not unequivocally predict 32 of the 257 compounds. Within the remaining 225 compounds, MULTICASE clearly suffered in the prediction of Ames positive compounds correctly identifying only 103 of 156. This is reflected in a low sensitivity of only 66%. The MOE-t descriptor approach performed extremely well in identifying the Ames positive structures (Table 1). It only misses three out of the 183 Ames positive molecules. For the Ames negative compounds, on the other hand, this approach misclassifies 36 of 74 compounds, nearly 50% of the molecules. As a result, the MOE-t approach had impressive accuracy (85%) and sensitivity (98%) statistics, but its specificity was poor (51%).

Within the MOE-t results, one could make the argument that false positives are less severe than false negatives, as nearly 100% of the Ames positive compounds are identified and it is unlikely that an Ames positive compound is carried forward. Though this argument may hold for the later stage of drug development, this may not be the case in the earlier stages of drug discovery and preclinical development. Here, the large number of false positives could actually be detrimental since such a classification could lead to the deprioritization of molecules, and without sufficient resources to actually test the compounds in vitro, could inadvertently lead to the wrong decision to abandon an otherwise promising molecule. Thus it would be desirable to have an approach that gives a more balanced result than the MOE-t descriptor.

The results for the nitrenium hypothesis at different levels of theory are summarized in Table 2, and the overall statistics for all models investigated are compared in Table 3. Even at the AM1 level of theory we see a good performance for the nitrenium hypothesis, which is comparable to that of DEREK. The performance is slightly superior for the Ames positive compounds and slightly worse for the Ames negative compounds. In addition to using a hard cutoff for $\Delta\Delta E$ of

Table 4. Primary Aromatic Amines Incorrectly Predicted to Be Ames Positive by the Nitrenium Stability Calculations[a]

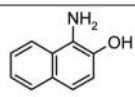
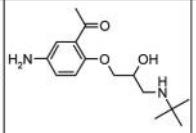
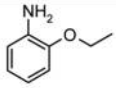
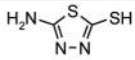
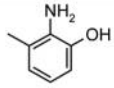
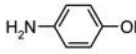
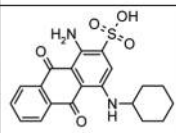
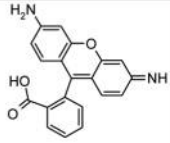
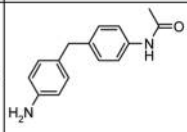
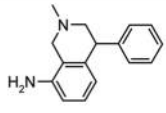
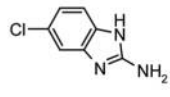
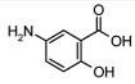
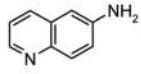
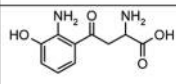
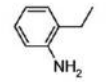
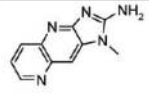
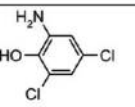
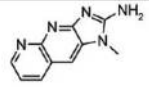
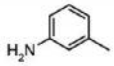
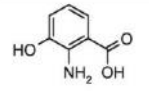
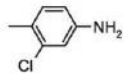
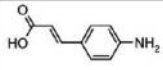
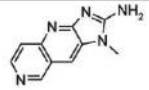
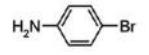
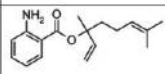
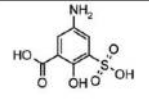
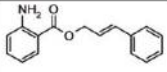
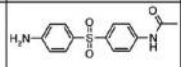
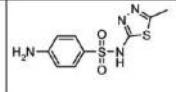
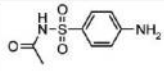
ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LY/ 6-311G(d,p) [kcal/mol]
184		1198-27-2	nonmutagen	mutagen	nonmutagen	nonmutagen	-25.1	-34.6	-30.9	-32.7
185		56980-94-0	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-26.5	-26.2	-27.5
186		94-70-2	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-21.7	-19.9	-20.7
187		2349-67-9	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.5	26.4	11.8	0.3
188		2835-97-4	nonmutagen	mutagen	nonmutagen	nonmutagen	-14.1	-20.9	-18.8	-19.5
189		123-30-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-13.0	-18.6	-24.0	-21.1
190		4368-56-3	nonmutagen	mutagen	nonmutagen	mutagen	-12.8	-19.8	-22.6	-24.9
191		13558-31-1	nonmutagen	mutagen	mutagen	mutagen	-12.4	-17.6	-17.5	-27.1
192		24367-94-0	nonmutagen	mutagen	mutagen	nonmutagen	-9.7	-14.7	-13.3	-20.3
193		32795-47-4	nonmutagen	mutagen	nonmutagen	indeterminate	-9.2	-15.6	-13.0	-17.3
194		5418-93-9	nonmutagen	mutagen	mutagen	indeterminate	-9.0	-1.8	-4.2	-11.2
195		89-57-6	nonmutagen	nonmutagen	nonmutagen	nonmutagen	-5.9	-13.5	-14.6	-14.3
196		580-15-4	nonmutagen	mutagen	mutagen	mutagen	-5.4	-7.6	-9.1	-7.5
197		484-78-6	nonmutagen	mutagen	nonmutagen	nonmutagen	-5.4	-2.4	-2.7	-28.9

Table 4. Continued

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LY/ 6-311G(d,p) [kcal/mol]
198		578-54-1	nonmutagen	mutagen	nonmutagen	nonmutagen	-4.6	-10.5	-8.6	-9.3
199		157730-37-5	nonmutagen	mutagen	mutagen	indeterminate	-3.1	2.3	-1.1	-7.3
200		527-62-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-2.0	7.2	3.5	0.0
201		166664-83-1	nonmutagen	mutagen	mutagen	indeterminate	-1.9	3.3	0.3	-6.7
202		638-03-9	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.7	-2.8	-1.6	-3.3
203		548-93-6	nonmutagen	nonmutagen	nonmutagen	nonmutagen	-0.7	-1.1	-3.6	-3.5
204		7745-89-3	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	0.4	-2.2
206		2393-18-2	nonmutagen	mutagen	nonmutagen	nonmutagen	1.1	-1.4	-1.8	-5.4
208		157730-36-4	nonmutagen	mutagen	mutagen	mutagen	2.1	8.6	7.4	-0.9
212		106-40-1	nonmutagen	mutagen	nonmutagen	nonmutagen	4.2	3.3	2.2	-2.9
213		7149-26-0	nonmutagen	nonmutagen	nonmutagen	nonmutagen	4.4	2.1	1.8	-2.6
214		6201-87-2	nonmutagen	nonmutagen	nonmutagen	nonmutagen	4.8	2.8	-0.9	-1.3
220		87-29-6	nonmutagen	nonmutagen	nonmutagen	nonmutagen	7.3	4.9	5.1	-1.0
228		565-20-8	nonmutagen	nonmutagen	nonmutagen	nonmutagen	9.2	15.5	11.4	-0.4
251		144-82-1	nonmutagen	nonmutagen	nonmutagen	nonmutagen	21.0	29.7	8.4	-17.8
252		144-80-9	nonmutagen	nonmutagen	nonmutagen	nonmutagen	21.2	30.2	11.9	-3.7

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

Table 5. Primary Aromatic Amines Incorrectly Predicted to Be Ames Negative by Nitrenium Stability Calculations[a]

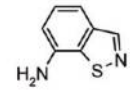
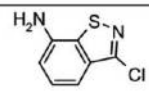
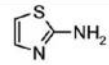
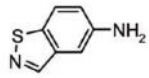
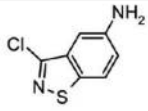
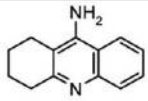
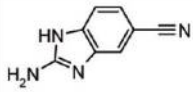
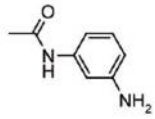
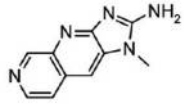
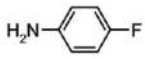
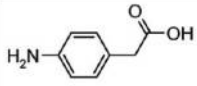
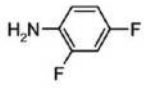
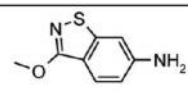
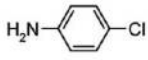
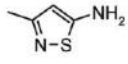
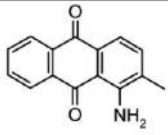
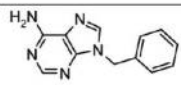
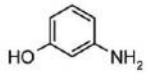
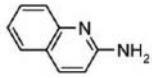
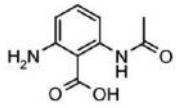
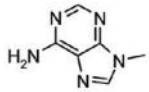
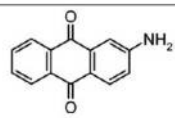
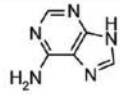
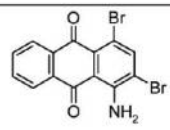
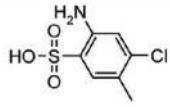
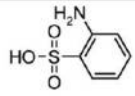
ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
117		89795-79-9	mutagen	mutagen	Mutagen	mutagen	-10.7	2.4	-2.2	-6.3
127		148193-31-1	mutagen	mutagen	Mutagen	nonmutagen	-9.1	9.0	2.7	-2.5
132		96-50-4	mutagen	mutagen	nonmutagen	nonmutagen	-8.3	-6.7	8.5	0.5
133		53473-85-1	mutagen	mutagen	Mutagen	mutagen	-8.2	1.0	-4.3	-7.1
149		148193-30-0	mutagen	mutagen	Mutagen	mutagen	-6.1	8.7	1.2	-2.7
156		1684-40-8	mutagen	mutagen	Mutagen	nonmutagen	-4.9	-5.3	0.7	-9.2
158		63655-40-3	mutagen	mutagen	Mutagen	indeterminate	-4.0	3.1	5.0	-1.5
165		102-28-3	mutagen	mutagen	Mutagen	nonmutagen	-1.0	-1.5	0.7	-3.8
166		157730-35-3	mutagen	mutagen	Mutagen	mutagen	-0.6	5.4	3.5	-3.7
167		371-40-4	mutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	-2.8	-3.2
168		1197-55-3	mutagen	mutagen	nonmutagen	nonmutagen	0.5	-6.5	-4.9	-7.1
169		367-25-9	mutagen	mutagen	nonmutagen	indeterminate	0.6	6.6	0.1	-1.0
170		148193-33-3	mutagen	mutagen	Mutagen	mutagen	0.6	9.0	8.3	-4.1
171		20265-96-7	mutagen	mutagen	nonmutagen	nonmutagen	0.6	8.1	1.9	-1.7
172		52547-00-9	mutagen	mutagen	Mutagen	nonmutagen	0.7	20.4	17.6	4.6

Table 5. Continued

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
173		82-28-0	mutagen	mutagen	nonmutagen	mutagen	1.5	0.8	0.8	1.7
174		4261-14-7	mutagen	mutagen	nonmutagen	nonmutagen	4.5	14.8	16.7	13.2
175		591-27-5	mutagen	mutagen	nonmutagen	nonmutagen	5.1	6.8	6.4	0.4
176		580-22-3	mutagen	mutagen	Mutagen	indeterminate	5.4	9.2	10.6	4.3
177		5623-11-0	mutagen	nonmutagen	Mutagen	nonmutagen	6.5	5.0	6.5	-8.1
178		700-00-5	mutagen	mutagen	nonmutagen	nonmutagen	8.0	19.5	19.3	16.4
179		117-79-3	mutagen	mutagen	Mutagen	mutagen	9.5	12.9	12.9	8.7
180		73-24-5	mutagen	mutagen	nonmutagen	nonmutagen	12.4	26.1	25.6	22.1
181		81-49-2	mutagen	mutagen	nonmutagen	mutagen	13.3	15.1	14.6	7.0
182		88-51-7	mutagen	nonmutagen	nonmutagen	mutagen	19.2	24.8	19.3	14.3
183		88-21-1	mutagen	nonmutagen	nonmutagen	nonmutagen	21.6	25.0	21.3	18.2

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

0.0 kcal/mol between the Ames positive and Ames negative compounds, we calculated the off-diagonal elements in the truth tables using an uncertainty interval of $\Delta\Delta E = \pm 5.0$ kcal/mol. With this measure we see a significant improvement in the false positive and false negative rates.

Going from the semiempirical to the ab initio Hartree−Fock (HF) level does not significantly improve the predictive power of the nitrenium hypothesis (see Tables 2 and 3). Even using a larger basis set like 6-311G(d,p) only slightly improves the specificity. To assess the effect of going beyond

Table 6. Truth Tables for Ames Predictions Using Different Consensus-Scoring Schemes[a]

	Ames positive predicted AM1 + MOE-t (consensus)	Ames negative predicted AM1 + MOE-t (consensus)	indeterminate
Ames positive experimental	167 (183)	3	13
Ames negative experimental	18	35 (74)	21

	Ames positive predicted AM1 + MOE-t + DEREK (majority)	Ames negative predicted AM1 + MOE-t + DEREK (majority)	indeterminate
Ames positive experimental	171 (183)	12	0
Ames negative experimental	21	53 (74)	0

	Ames positive predicted AM1 + MOE-t + DEREK (consensus)	Ames negative predicted AM1 + MOE-t + DEREK (consensus)	indeterminate
Ames positive experimental	147 (183)	2	34
Ames negative experimental	6	36 (74)	32

[a] The numbers in parentheses are the experimental assignments from the data set by Kazius et al.[6]

the HF-level, the $\Delta\Delta E$ values were calculated using density functional theory (DFT) with the 6-311G(d,p) basis set and the B3LYP functional. We found an improvement in the prediction of Ames positive compounds with a sensitivity of 93%, but the prediction for Ames negative compounds deteriorated (specificity 62%). There is overlap between the false positive and false negative compounds predicted by the nitrenium calculations at different levels of theory. Overall 16 compounds are incorrectly predicted to be Ames positive and 11 compounds are incorrectly predicted to be Ames negative at all four levels of theory. The DFT calculations could have some problems in correctly predicting the Ames activities for sulfone compounds, but seem to do better in predicting the activities for aminobenzothiazoles (e.g., compound 117, mutagenic) compared with the semiempirical and HF calculations. As the computational effort for the DFT calculations increases significantly we feel that the AM1 approach offers a good balance between speed and accuracy (see Tables 4 and 5).

Consensus-Scoring. As the MOE-t approach exhibits a very small false negative rate (Tables 1 and 3), the possibility of a consensus scoring method was tested. In particular, the performance of three consensus-scoring schemes is summarized in Table 6. Taking the consensus-score between the predictions from the nitrenium-hypothesis at the AM1 level of theory and the MOE-t approach leads to a significant reduction in the false negative compounds, 3 compared to 18 when using the nitrenium-hypothesis alone. But, this is achieved at the price of not being able to predict 34 compounds unequivocally, as MOE-t and the nitrenium hypothesis give opposing results. Adding DEREK as a third method and predicting compounds as Ames positive when two out of the three approaches predict a compound to be positive and as negative when two out of three predict it to be negative, results in a scoring scheme that can now unequivocally predict all compounds. For this scoring scheme the improvement in the number of false negatives, 12, is small compared to using the nitrenium hypothesis alone, where 16 false negatives are found. No change is observed for the experimentally Ames negative compounds. Using a consensus-score where all three methods (MOE-t, DEREK, nitrenium hypothesis AM1) have to agree, the number of false positives and false negatives can be drastically reduced compared to using the nitrenium hypothesis alone. With this approach only two false negatives and six false positives are found. The price for this is that 66 compounds, or 26% of the complete data set, cannot unequivocally be classified. MULTICASE was not considered in the consensus scoring schemes because of its low overall accuracy and sensitivity (Table 3).

Global Trends in $\Delta\Delta E$. The distribution of $\Delta\Delta E$ energies obtained for the Ames positive and negative compounds in

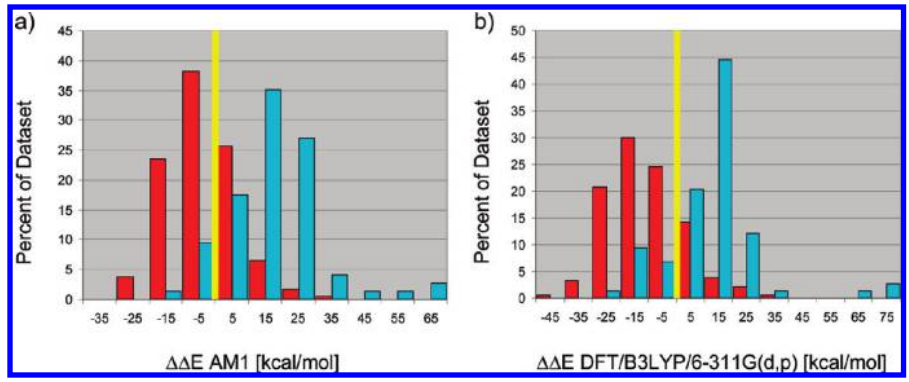


Figure 2. Distribution of calculated $\Delta\Delta E$ values for Ames positive (red bars) and Ames negative compounds (light blue bars). The yellow line marks the border between predicted Ames positive compounds (negative $\Delta\Delta E$ values) and predicted Ames negative compounds (positive $\Delta\Delta E$ values). (a) $\Delta\Delta E$ AM1 [kcal/mol] vs percent of mutant and nonmutant data set and (b) $\Delta\Delta E$ DFT/B3LYP/6-311G(d,p) [kcal/mol] vs percent of mutant and nonmutant data set.

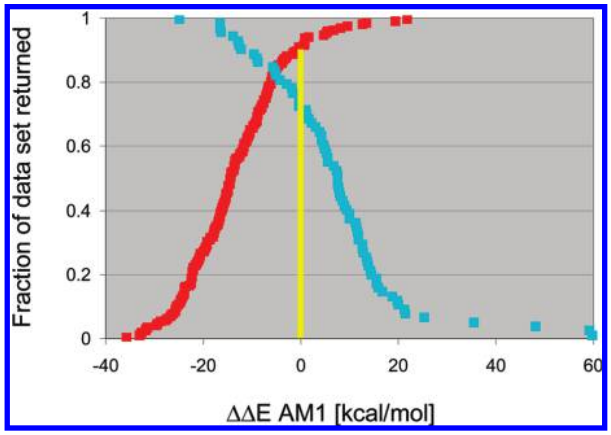


Figure 3. Fraction of returned Ames positive (red) and Ames negative (light blue) compounds at a given AM1 $\Delta\Delta E$ value. At $\Delta\Delta E = 0.0$ kcal/mol 91% of all Ames positive compounds have a lower $\Delta\Delta E$ and 73% of all Ames negative compounds have a higher $\Delta\Delta E$.

the data set are shown in Figure 2. To derive guidelines on how to use the nitrenium hypothesis and interpret the $\Delta\Delta E$ values, we have calculated confidence intervals for the AM1 calculations. At the semiempirical AM1 level of theory, the Ames positive compounds have a mean $\Delta\Delta E = -13.73$ kcal/mol with a standard deviation of 10.05 kcal/mol and the Ames negative compounds have a mean $\Delta\Delta E = 7.00$ kcal/mol with a standard deviation of 14.92 kcal/mol. Assuming a *t*-distribution of the calculated values for Ames positive and Ames negative compounds confidence intervals can be calculated. Given an AM1 calculated $\Delta\Delta E < -30.3$ kcal/mol (1.653 * −10.05 − 13.73), one can be 95% confident that the compound would be mutagenic if tested; with an AM1 calculated $\Delta\Delta E > 31.9$ kcal/mol (1.666 * 14.92 + 7.00) one can be 95% confident that the compound would be nonmutagenic if tested. Examination of the test data set, indicates that there is only a single Ames negative compound, compound 185, that is incorrectly predicted to be Ames positive out of 68 with an AM1 calculated $\Delta\Delta E < -17.0$ kcal/mol. Similarly for compounds with an AM1 calculated $\Delta\Delta E > 13.5$ kcal/mol we find only two incorrect predictions. No misclassifications were found for $\Delta\Delta E < -25.1$ kcal/mol and $\Delta\Delta E > 21.7$ kcal/mol.

Another way of analyzing the performance of the nitrenium hypothesis and developing guidance on interpretation of specific $\Delta\Delta E$ values is to examine the percentage of Ames positive and Ames negative compounds identified at a given $\Delta\Delta E$ value. Figure 3 shows these curves for the semiempirical AM1 calculations. At $\Delta\Delta E = 0.0$ kcal/mol 91% of all Ames positive compounds have a lower $\Delta\Delta E$ and 73% of all Ames negative compounds have a higher $\Delta\Delta E$. One can also see that shifting the cutoff between Ames positive and Ames negative values downward to −5.5 kcal/mol leads to an even more balanced approach. In this case, 84% of all Ames positive compounds have a lower $\Delta\Delta E$ and 84% of all Ames negative compounds have a higher $\Delta\Delta E$.

We propose to use the nitrenium hypothesis for selecting and prioritizing Ames negative compounds. Using a large, diverse data set, the results of the validation study suggest that the AM1 level of theory is appropriate for calculation of nitrenium ion stabilities, as no clear advantage was seen for the more computationally expensive ab initio HF or DFT calculations. It is recommended to use a cutoff of $\Delta\Delta E = 0.0$ kcal/mol between Ames positive and Ames negative predictions. In certain cases, for example, some 5-ring heteroaromatic primary aromatic amines where ring-openings are observed during optimization at the AM1-level of theory, it may be justified to go to a higher level of theory for calculating $\Delta\Delta E$ values. For a set of

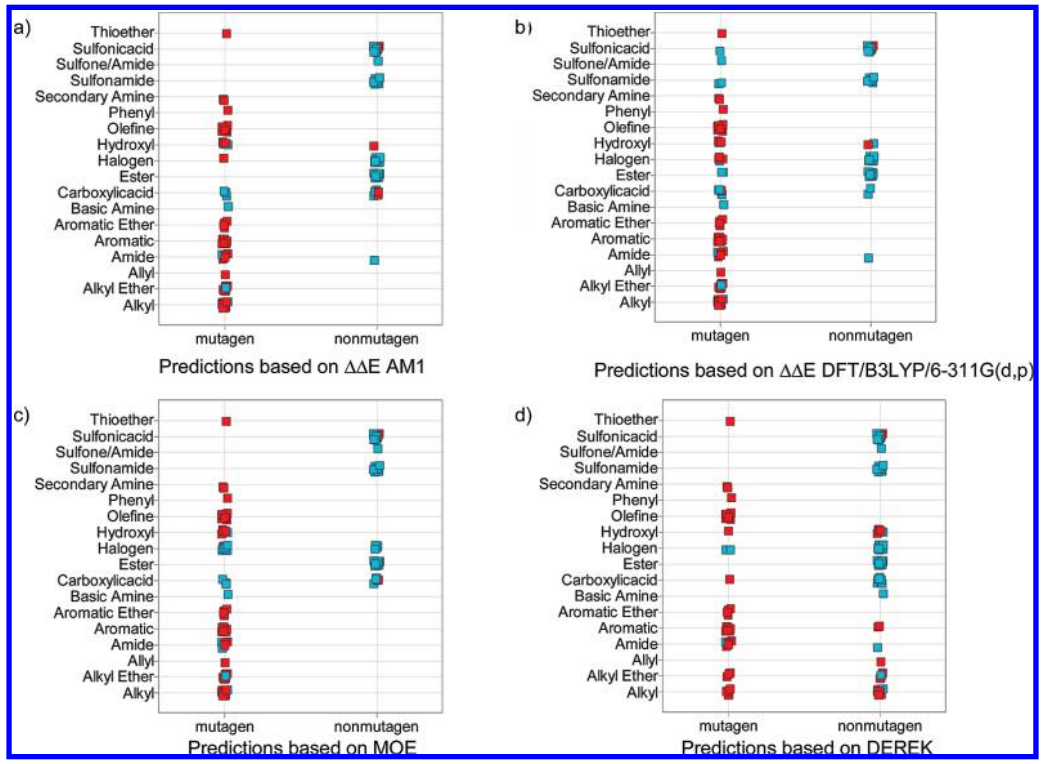


Figure 4. Ames predictions for substituted primary phenyl amines categorized by functional group of substitution (a) the nitrenium hypothesis semiempirical AM1, (b) ab initio DFT/B3LYP/6-311G(d,p), (c) MOE-t, (d) DEREK. Ames positive compounds red, Ames negative compounds light blue.

Table 7. Truth Tables of Ames Predictions for Primary Phenyl Amines with Commercial Software and the AM1-Based Nitrenium Hypothesis[a]

	Ames positive predicted DEREK	Ames negative predicted DEREK
Ames positive experimental	38 (60)	22
Ames negative experimental	3	52 (55)

	Ames positive predicted MULTICASE	Ames negative predicted MULTICASE
Ames positive experimental	17 (44)	27
Ames negative experimental	1	53 (54)

	Ames positive predicted MOE-t	Ames negative predicted MOE-t
Ames positive experimental	57 (60)	3
Ames negative experimental	20	35 (55)

	Ames positive predicted AM1	Ames negative predicted AM1
Ames positive experimental	53 (60)	7
Ames negative experimental	12	43 (55)

	Ames positive predicted DFT/B3LYP/ 6-311G(d,p)	Ames negative predicted DFT/B3LYP/ 6-311G(d,p)
Ames positive experimental	57 (60)	3
Ames negative experimental	19	36 (55)

[a] The numbers in parentheses are the experimental assignments from the Organon[6] data set. The numbers for MULTICASE are reduced as it categorized one Ames negative compound and 16 Ames positive compounds indeterminate.

closely related compounds a better agreement with experimental results may be achieved when changing the reference compound aniline, used here, to a different aromatic amine. This basically means moving the cutoff between Ames positive and Ames negative compounds to a value of $\Delta\Delta E$ either larger or smaller than 0.0 kcal/mol.

Trends in $\Delta\Delta E$ for Specific Aryl Amine Chemotypes. *Substituted Anilines.* The largest class of compounds is substituted anilines (phenyl amines), with 115 members, 60 Ames positive and 55 Ames negative compounds. For this subclass the overall performance for the different approaches is summarized in Figure 4 and Table 7. From the truth tables it can be seen that DEREK and MULTICASE suffer from relatively low sensitivity, but have relatively high specificity. Conversely, MOE-t does extremely well in identifying the Ames positive compounds, but has low specificity. This behavior was already seen in the analysis of the complete test set, but is even more pronounced for this subset of substituted anilines. The nitrenium hypothesis at the AM1 level of theory shows a similar performance to the MOE-t descriptor for the Ames positive compounds but is superior for the Ames negative compounds. This is reflected in the overall accuracy values for the different approaches where the nitrenium hypothesis at the AM1 level gives 84% compared to 80% for MOE-t, 78% for DEREK and only 71% for MULTICASE. Overall the nitrenium approach (semiempircal AM1) gives the most balanced result with a sensitivity of 88% and a specificity of 78% compared to 63% and 95% for DEREK, 39% and 98% for MULTICASE, and 95% and 64% for MOE-t (Table 8).

Analyzing this subset in more detail we find that of the 60 Ames positive compounds seven are misclassified by the AM1 calculations (compounds 168, 169, 171, 175, 177, 182, and 183), three only by a $\Delta\Delta E$ of less than 1 kcal/mol. Of the 55 Ames negative compounds twelve are misclassified by the AM1 calculations as Ames positive (compounds 185, 186, 188, 189, 192, 195, 197, 198, 200, 202, 203, and 204), three only by a $\Delta\Delta E$ of less than 1 kcal/mol (Table 9). DEREK misclassified only three Ames negative compounds, and MOE-t twenty. For the Ames positive compounds DEREK misclassified 22 and MOE-t only three (Table 10). Ten of the wrongly predicted Ames negative compounds by MOE-t are correctly predicted by the AM1 calculations. On the other hand two compounds correctly predicted to be nonmutagenic by MOE-t are misclassified by the AM1 calculations. There is an overlap in 10 of the misclassified Ames negative compounds between AM1 and MOE-t. Four compounds correctly classified by MOE-t as Ames positive, are Ames negative based on the AM1 calculations. All three of the misclassified Ames positive compounds by MOE-t are also misclassified by the AM1 calculation. Two of the misclassified Ames negative compounds by DEREK are correctly predicted by the AM1 calculations. Sixteen of the misclassified Ames positive compounds by DEREK are corrected by the AM1 calculations.

Fused Ring Systems. The nitrenium hypothesis does well for larger molecules with four or five fused rings (Figures 5 and 6). All of these 15 compounds in the data set are correctly predicted to be Ames positive by the nitrenium stability calculations, as well as DEREK and MOE-t. Out of the 80 compounds with three fused ring systems, three

Table 8. Summary Statistics for the Different Approaches to Predict Ames Activity for the Subset of Primary Phenyl Amines

	DEREK	MULTI CASE	MOE-t	nitrenium AM1	nitrenium DFT/B3LYP/6-311G (d,p)
accuracy	0.783	0.714	0.800	0.835	0.809
false positive rate	0.055	0.019	0.364	0.218	0.345
false negative rate	0.367	0.614	0.050	0.117	0.050
sensitivity	0.633	0.386	0.950	0.883	0.950
specificity	0.945	0.981	0.636	0.782	0.655

Table 9. Primary Phenyl Amines Incorrectly Predicted by AM1 Nitrenium Stability Calculations[a]

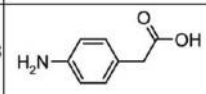
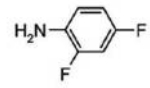
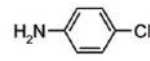
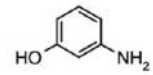
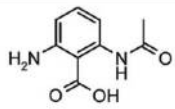
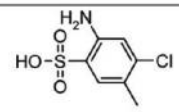
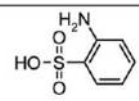
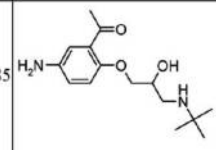
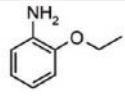
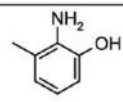
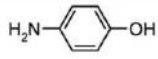
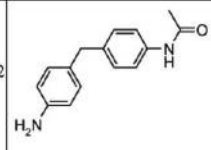
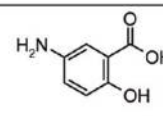
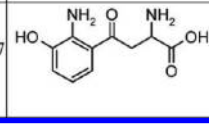
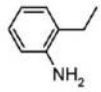
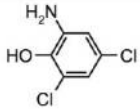
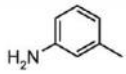
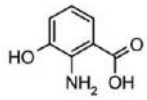
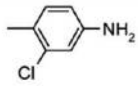
ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
168		1197-55-3	mutagen	mutagen	nonmutagen	nonmutagen	0.5	-6.5	-4.9	-7.1
169		367-25-9	mutagen	mutagen	nonmutagen	indeterminate	0.6	6.6	0.1	-1.0
171		20265-96-7	mutagen	mutagen	nonmutagen	nonmutagen	0.6	8.1	1.9	-1.7
175		591-27-5	mutagen	mutagen	nonmutagen	nonmutagen	5.1	6.8	6.4	0.4
177		5623-11-0	mutagen	nonmutagen	mutagen	nonmutagen	6.5	5.0	6.5	-8.1
182		88-51-7	mutagen	nonmutagen	nonmutagen	mutagen	19.2	24.8	19.3	14.3
183		88-21-1	mutagen	nonmutagen	nonmutagen	nonmutagen	21.6	25.0	21.3	18.2
185		56980-94-0	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-26.5	-26.2	-27.5
186		94-70-2	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-21.7	-19.9	-20.7
188		2835-97-4	nonmutagen	mutagen	nonmutagen	nonmutagen	-14.1	-20.9	-18.8	-19.5
189		123-30-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-13.0	-18.6	-24.0	-21.1
192		24367-94-0	nonmutagen	mutagen	mutagen	nonmutagen	-9.7	-14.7	-13.3	-20.3
195		89-57-6	nonmutagen	nonmutagen	nonmutagen	nonmutagen	-5.9	-13.5	-14.6	-14.3
197		484-78-6	nonmutagen	mutagen	nonmutagen	nonmutagen	-5.4	-2.4	-2.7	-28.9

Table 9. Continued

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
198		578-54-1	nonmutagen	mutagen	nonmutagen	nonmutagen	-4.6	-10.5	-8.6	-9.3
200		527-62-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-2.0	7.2	3.5	0.0
202		638-03-9	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.7	-2.8	-1.6	-3.3
203		548-93-6	nonmutagen	nonmutagen	nonmutagen	nonmutagen	-0.7	-1.1	-3.6	-3.5
204		7745-89-3	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	0.4	-2.2

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

anthraquinones (compounds 173, 179, and 181) are incorrectly predicted to be Ames negative by the nitrenium stability calculations at the AM1 level of theory. Four compounds (compounds 190, 191, 199, and 201), one of them an anthraquinone (compound 190), are incorrectly predicted as Ames positive at the AM1 level of theory. Very similar behavior is observed at the higher level of calculations. Again the results from the nitrenium calculations mirror those from DEREK, which incorrectly predicts compounds 75, 138, 181, and 173 as Ames negative and compounds 191, 208, 199, and 201 as Ames positive. The fact that so many anthraquinones are incorrectly predicted, four out of six, could point to the fact that issues other than the stability of the nitrenium ion lead to the observed Ames activity.[43,44] Good predictions are also observed for acridines, carbazoles, fluoranthrenes, fluorenes, phenanthrenes, anthracenes, and other large aromatic systems. Only one (compound 191) out of 52 compounds is incorrectly predicted. The only problematic class is that of the above-mentioned anthraquinones.

Sulfur-Containing Molecules. The most severe outliers for Ames positive compounds with the AM1 calculations are two benzenesulfonic acids, compounds 182 and 183, but there does not seem to be a general problem with this class of compounds as others are predicted correctly. The most severe outlier for Ames negative compounds with the AM1 calculations is the 1-amino-naphthalen-2-ol, compound 184. In some cases, problems were observed in the geometry optimization for the nitrenium ions. The 2-aminothiazole compound 132 and the thiadiazole compound 187 show ring-opening for one nitrenium ion conformation at the AM1 level of theory (Figure 7). In the case of compound 187, this is likely the reason for incorrectly predicting this compound as Ames positive. At the DFT/B3LYP/6-311G(d,p) and HF/6-311G(d,p) levels of theory several sulfur containing compounds exhibited problems in the geometry optimization of the nitrenium ions. It is known in the literature that DFT/B3LYP/6-31G* calculations result in extremely large C−S bond lengths.[45] Ring-opening and bond breaking were observed in the geometry optimization of the nitrenium ions for compounds 132, 187, 251, and 252. The DFT/B3LYP/6-311G(d,p) geometry for one of the nitrenium ions of 177 shows a ring closure. Problems with the geometry optimizations are the likely reason for at least some of the observed incorrect predictions.

Charged Molecules. In our study, we considered all primary aromatic amines to be neutral. Figure 8 presents the performance of the nitrenium hypothesis where the data is organized based on the potential ionization state of the aromatic amine. Though quite a few incorrect predictions are observed with the nitrenium hypothesis for potentially charged molecules, these incorrect predictions are not uniform for a whole class of compounds. In addition the predictions for charged molecules with MOE-t and DEREK are broadly similar. Therefore, it seems to be justified to assume the primary aromatic amine in its neutral state.

Tautomers. Another interesting question regarding this quantum chemical approach is how well it can deal with different tautomers. For a small sample of four compounds the nitrenium stabilities were calculated for two different tautomers (Table 11). For three of these compounds the differences are relatively small, ∼2 kcal/mol. Only for compound 180 a larger difference of 6.5 kcal/mol is

Table 10. Primary Aromatic Phenyl Amines Incorrectly Predicted by MOE-t or DEREK[a]

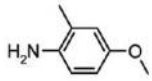
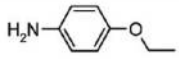
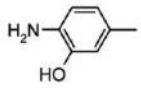
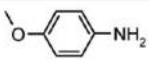
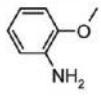
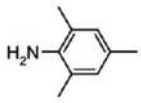
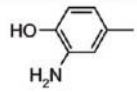
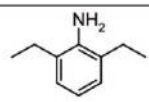
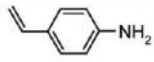
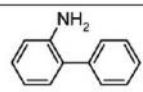
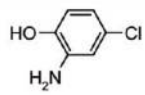
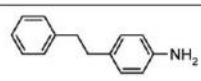
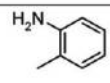
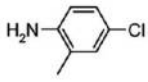
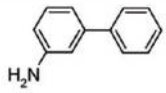
ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
54		102-50-1	mutagen	mutagen	nonmutagen	indeterminate	-19.4	-30.8	-30.3	-30.4
64		156-43-4	mutagen	mutagen	nonmutagen	nonmutagen	-17.8	-26.7	-26.7	-26.9
78		2835-98-5	mutagen	mutagen	nonmutagen	mutagen	-16.0	-22.4	-21.5	-21.9
80		20265-97-8	mutagen	mutagen	nonmutagen	nonmutagen	-15.8	-24.7	-24.8	-24.8
91		134-29-2	mutagen	mutagen	nonmutagen	nonmutagen	-14.7	-19.8	-18.1	-18.6
96		88-05-1	mutagen	mutagen	nonmutagen	indeterminate	-14.4	-24.1	-22.8	-22.2
108		95-84-1	mutagen	mutagen	nonmutagen	indeterminate	-12.0	-16.4	-15.5	-17.1
125		579-66-8	mutagen	mutagen	nonmutagen	nonmutagen	-9.2	-17.7	-15.9	-16.3
129		1520-21-4	mutagen	mutagen	nonmutagen	nonmutagen	-8.9	-12.8	-11.9	-13.5
131		2185-92-4	mutagen	mutagen	nonmutagen	nonmutagen	-8.6	-10.3	-9.8	-16.3
146		95-85-2	mutagen	mutagen	nonmutagen	nonmutagen	-6.4	-2.5	-5.0	-6.8
148		13024-49-2	mutagen	mutagen	nonmutagen	nonmutagen	-6.2	-12.0	-10.8	-14.5
157		636-21-5	mutagen	mutagen	nonmutagen	nonmutagen	-4.6	-8.7	-7.0	-7.3
160		95-69-2	mutagen	mutagen	nonmutagen	indeterminate	-3.7	-0.6	-4.8	-8.3
163		2243-47-2	mutagen	mutagen	nonmutagen	nonmutagen	-1.9	-2.7	-1.7	-4.4

Table 10. Continued

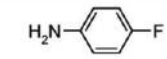
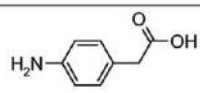
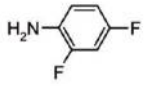
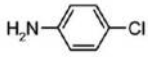
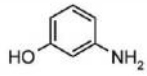
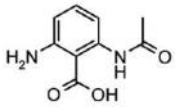
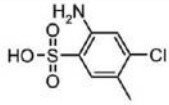
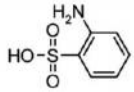
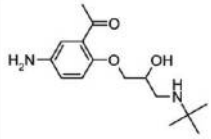
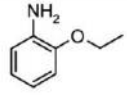
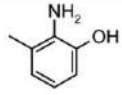
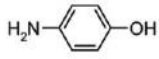
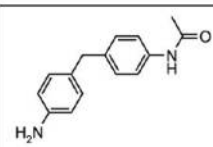
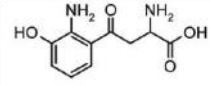
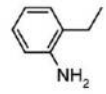
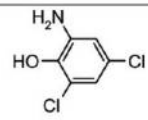
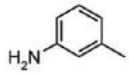
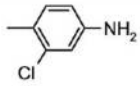
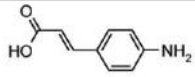
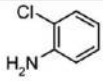
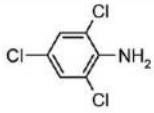
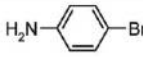
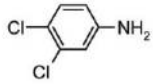
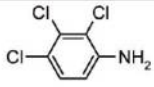
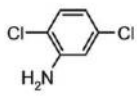
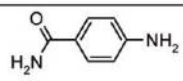
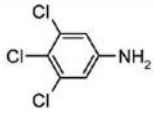
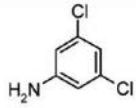
ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
167		371-40-4	mutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	-2.8	-3.2
168		1197-55-3	mutagen	mutagen	nonmutagen	nonmutagen	0.5	-6.5	-4.9	-7.1
169		367-25-9	mutagen	mutagen	nonmutagen	indeterminate	0.6	6.6	0.1	-1.0
171		20265-96-7	mutagen	mutagen	nonmutagen	nonmutagen	0.6	8.1	1.9	-1.7
175		591-27-5	mutagen	mutagen	nonmutagen	nonmutagen	5.1	6.8	6.4	0.4
177		5623-11-0	mutagen	nonmutagen	mutagen	nonmutagen	6.5	5.0	6.5	-8.1
182		88-51-7	mutagen	nonmutagen	nonmutagen	mutagen	19.2	24.8	19.3	14.3
183		88-21-1	mutagen	nonmutagen	nonmutagen	nonmutagen	21.6	25.0	21.3	18.2
185		56980-94-0	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-26.5	-26.2	-27.5
186		94-70-2	nonmutagen	mutagen	nonmutagen	nonmutagen	-16.7	-21.7	-19.9	-20.7
188		2835-97-4	nonmutagen	mutagen	nonmutagen	nonmutagen	-14.1	-20.9	-18.8	-19.5
189		123-30-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-13.0	-18.6	-24.0	-21.1
192		24367-94-0	nonmutagen	mutagen	mutagen	nonmutagen	-9.7	-14.7	-13.3	-20.3
197		484-78-6	nonmutagen	mutagen	nonmutagen	nonmutagen	-5.4	-2.4	-2.7	-28.9

Table 10. Continued

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
198		578-54-1	nonmutagen	mutagen	nonmutagen	nonmutagen	-4.6	-10.5	-8.6	-9.3
200		527-62-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-2.0	7.2	3.5	0.0
202		638-03-9	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.7	-2.8	-1.6	-3.3
204		7745-89-3	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	0.4	-2.2
206		2393-18-2	nonmutagen	mutagen	nonmutagen	nonmutagen	1.1	-1.4	-1.8	-5.4
207		95-51-2	nonmutagen	mutagen	nonmutagen	nonmutagen	1.4	9.6	5.6	2.8
210		634-93-5	nonmutagen	mutagen	nonmutagen	nonmutagen	3.6	25.3	12.2	4.2
212		106-40-1	nonmutagen	mutagen	nonmutagen	nonmutagen	4.2	3.3	2.2	-2.9
215		95-76-1	nonmutagen	mutagen	nonmutagen	nonmutagen	5.1	17.2	9.9	4.1
217		634-67-3	nonmutagen	mutagen	mutagen	nonmutagen	5.2	22.4	12.4	4.6
218		95-82-9	nonmutagen	mutagen	nonmutagen	nonmutagen	6.5	20.6	14.7	9.7
225		2835-68-9	nonmutagen	mutagen	nonmutagen	nonmutagen	8.1	9.1	8.8	5.0
229		634-91-3	nonmutagen	mutagen	mutagen	nonmutagen	9.7	25.7	17.5	9.5
232		626-43-7	nonmutagen	mutagen	nonmutagen	nonmutagen	11.2	22.7	19.2	15.0

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

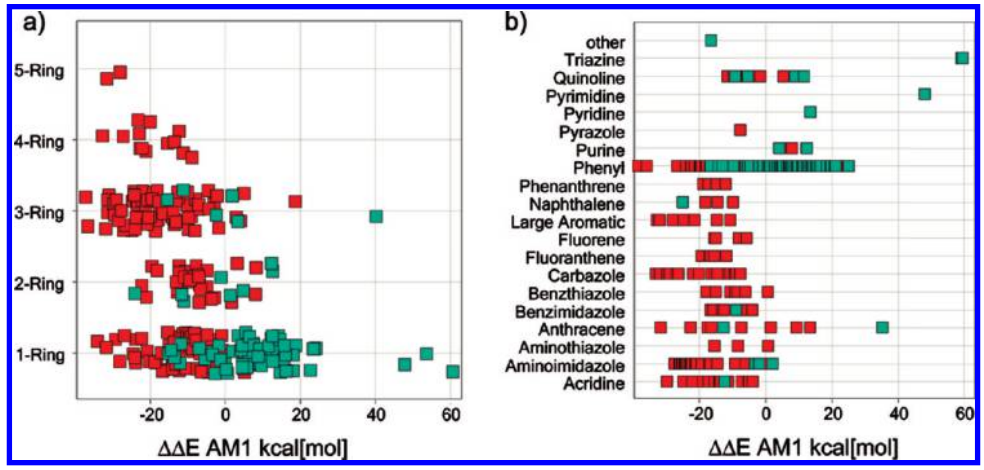


Figure 5. (a) $\Delta\Delta E$ AM1 [kcal/mol] vs number of fused aromatic rings and (b) $\Delta\Delta E$ AM1 [kcal/mol] vs compound class. Ames positive compounds are red; Ames negative compounds are cyan.

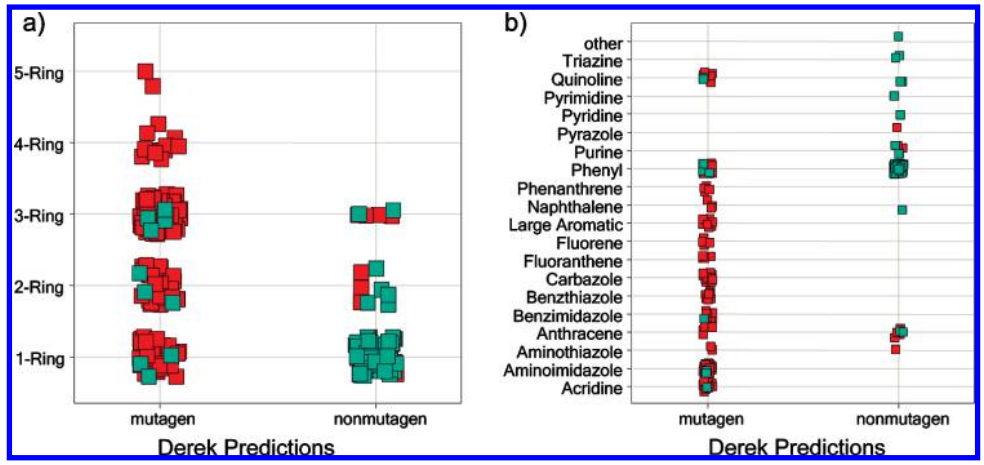


Figure 6. (a) DEREK predictions vs number of fused aromatic rings and (b) DEREK predictions vs compound class. Ames positive compounds are red; Ames negative compounds are cyan.

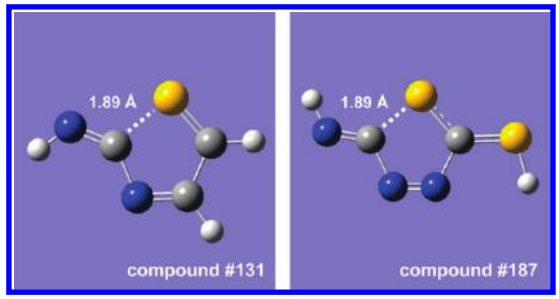


Figure 7. AM1 optimized geometries for nitrenium ions of compounds 132 and 187 resulting in bond breaking.

observed. Even in this case the predicted Ames activity is independent of the tautomeric form used in the calculation. It is plausible that the predictions for two tautomers however could straddle the classification point. It should be noted that DEREK accounts for tautomerism in query compounds.

$\Delta\Delta E$ as a Parameter for the Evaluation of Mutagenicity SAR. The data used in our study is derived from the Organon data set[6] and as such cannot be expected to provide complete SAR coverage for a particular series of molecules. Nevertheless some conclusions can be drawn from the series of para-substituted anilines. The AM1 nitrenium hypothesis nicely captures the trend going from Ames positive to Ames negative for the *p*-fluoro-, *p*-chloro-, and *p*-bromoaniline, though the exact $\Delta\Delta E$ value for the *p*-chloroaniline predicts this compound to be Ames negative. This approach does clearly better than DEREK and MULTICASE, which predict these compounds to be Ames negative throughout and MOE-t which predicts all to be Ames positive. Of the para-substituted anilines the *p*-*N*,*N*-dimethylaniline (compound 2) is predicted to be the most mutagenic in this series, followed by *N*-(*p*-aminophenyl)-acetamide (compound 44), *p*-methoxy-phenylamine (compound 80), and *p*-phenylaniline (compound 98). The mutagenic potential of these compounds can be explained by their ability to stabilize the nitrenium ion through mesomeric effects. The *p*-aminobenzamide (compound 225), *p*-aminobenzoic acid (compound 239), *p*-aminobenzenesulfonamide (compound 247), and *p*-aminobenzenesulfonic acid (compound 253) cannot perform a similar stabilization of the nitrenium ion and are thus predicted to be not mutagenic. The only drastic outlier in this series is *p*-aminophenol (compound 189) (Table 12).

Two other examples where this methodology shows its strength are the series of methyl and chloro substituted anilines (Table 13). The methyl group has a stabilizing effect on the nitrenium ion as can be seen from the decreasing $\Delta\Delta E$ going from aniline (compound 0, nonmutagenic) to 2-methylaniline (compound 157, mutagenic), 2,5-dimethylaniline (compound 147, mutagenic), 3,4-dimethylaniline (compound 142, mutagenic), and 2,4,6-trimethylaniline (compound 96, mutagenic). All compounds are correctly predicted to be Ames positive,

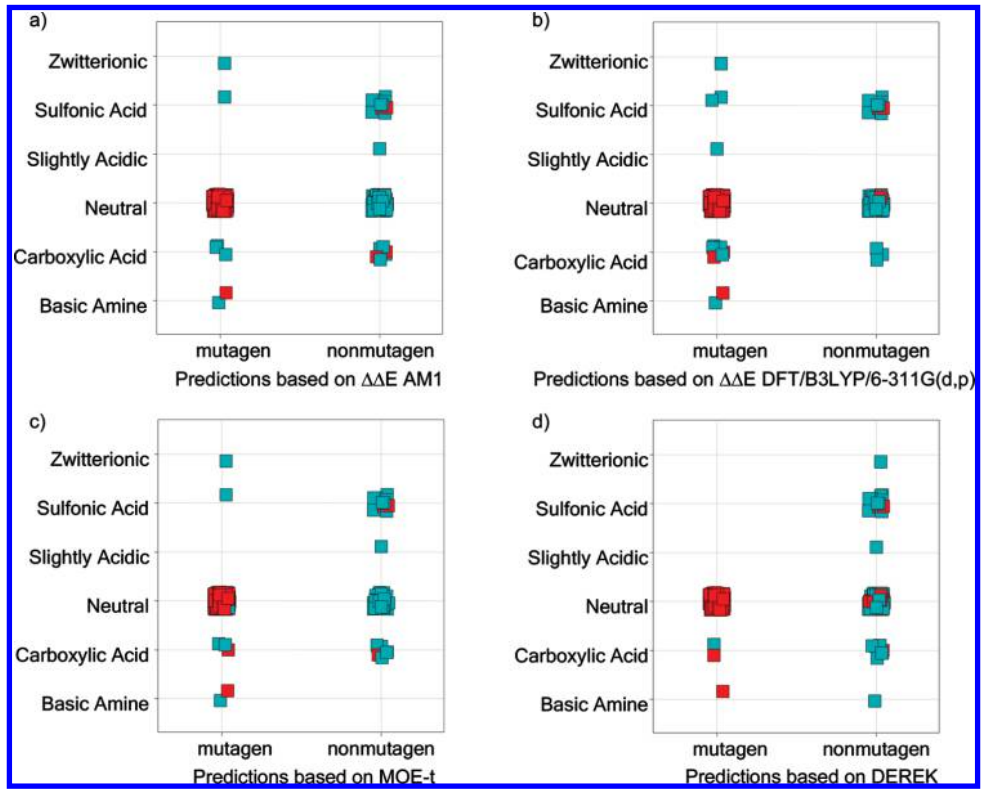
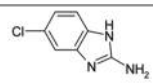
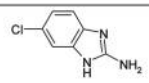
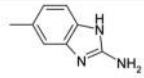
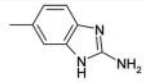
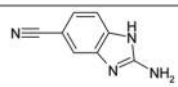
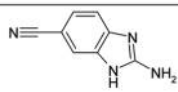
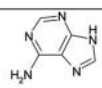
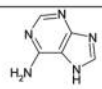


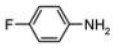
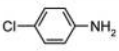
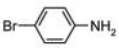
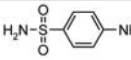
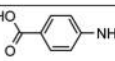
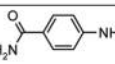
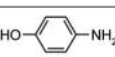
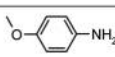
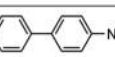
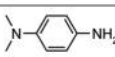
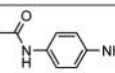
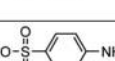
Figure 8. Ames predictions categorized by charged state for (a) the nitrenium hypothesis semiempirical AM1, (b) ab initio DFT/B3LYP/6-311G(d,p), (c) MOE-t, and (d) DEREK. Ames positive compounds red, Ames negative compounds light blue.

Table 11. AM1 Calculated $\Delta\Delta E$ Values and Ames Classification for Different Tautomers[a]

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTICASE	ΔΔE AM1 [kcal/mol]
194		5418-93-9	Nonmutagen	mutagen	mutagen	indeterminate	-9.0
194a		5418-93-9_a	Nonmutagen	mutagen	mutagen	indeterminate	-10.8
104		6285-68-3	Mutagen	mutagen	mutagen	mutagen	-13.1
104a		6285-68-3_a	Mutagen	mutagen	mutagen	mutagen	-15.2
158		63655-40-3	Mutagen	mutagen	mutagen	indeterminate	-4.00
158a		63655-40-3_a	Mutagen	mutagen	mutagen	indeterminate	-3.35
180		73-24-5	Mutagen	mutagen	nonmutagen	nonmutagen	12.36
180a		73-24-5_a	Mutagen	mutagen	nonmutagen	nonmutagen	5.79

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

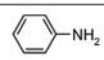
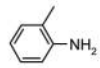
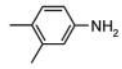
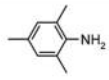
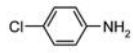
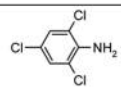
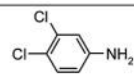
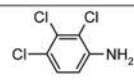
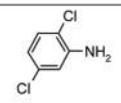
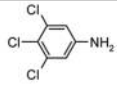
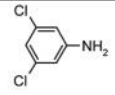
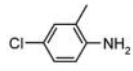
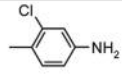
Table 12. Impact of para Substitutions on Nitrenium Stabilities of Primary Aromatic Phenyl Amines[a]

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
0		Aniline	nonmutagen	mutagen	nonmutagen	nonmutagen	0.0	0.0	0.0	0.0
167		371-40-4	mutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	-2.8	-3.2
171		20265-96-7	mutagen	mutagen	nonmutagen	nonmutagen	0.6	8.1	1.9	-1.7
212		106-40-1	nonmutagen	mutagen	nonmutagen	nonmutagen	4.2	3.3	2.2	-2.9
247		63-74-1	nonmutagen	nonmutagen	nonmutagen	nonmutagen	16.5	23.8	17.3	9.8
239		150-13-0	nonmutagen	nonmutagen	nonmutagen	nonmutagen	13.2	13.8	13.9	9.5
225		2835-68-9	nonmutagen	mutagen	nonmutagen	nonmutagen	8.1	9.1	8.8	5.0
189		123-30-8	nonmutagen	mutagen	nonmutagen	nonmutagen	-13.0	-18.6	-24.0	-21.1
80		20265-97-8	mutagen	mutagen	nonmutagen	nonmutagen	-15.8	-24.7	-24.8	-24.8
98		92-67-1	mutagen	mutagen	mutagen	mutagen	-13.9	-17.2	-18.9	-21.8
2		99-98-9	mutagen	mutagen	mutagen	indeterminate	-33.3	-47.4	-44.3	-44.8
44		122-80-5	mutagen	mutagen	mutagen	nonmutagen	-21.4	-26.1	-24.4	-24.5
253		121-57-3	nonmutagen	nonmutagen	nonmutagen	nonmutagen	25.1	30.4	25.0	18.2

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

whereas DEREK predicts only the two dimethylanilines to be Ames positive. While methylation of the aniline ring has a stabilizing effect on the nitrenium ion, chlorination has a largely destabilizing effect. The 4-chloroaniline (compound 171, mutagenic) and the 2-chloroanline (compound 207, nonmutagenic) have a $\Delta\Delta E$ of 0.6 kcal/mol, and 1.4 kcal/mol, respectively. Substitution with a chloro group in the meta-position of the aniline ring has an even larger destabilizing effect on the nitrenium ion, as is evident from the calculated $\Delta\Delta E = 11.2$ kcal/mol for the 3,5-dichloroaniline (compound 232, nonmutagenic). The $\Delta\Delta E$ values for the 2,5-dichloroaniline (compound 218, nonmutageinc), 3,4-dichloroaniline (compound 215, nonmutagenic), 3,4,5-trichloroaniline (compound 229, nonmutagenic), 2,4,6-trichloroaniline (compound 210, nonmutagenic), and 2,3,4-trichloroanline (compound 217, nonmutagenic) fall between 3.6 and 9.7 kcal/mol. All of these chloro-substituted aniline compounds, with the exception of 4-chloroaniline, are correctly predicted by the nitrenium hypothesis. Interestingly MOE-t only correctly predicts the 4-chloroaniline, completely missing the trend for the chloro-substituted anilines. DEREK also predicts three (compounds 171, 217, 229) out of these eight chloro-substituted aniline compounds incorrectly. Two interesting compounds are the 2-methyl-4-chloroaniline (compound 160, mutagenic) which has $\Delta\Delta E = -3.7$ kcal/mol and the 3-chloro-4-methylaniline (compound 204, nonmutagenic), which has $\Delta\Delta E = -0.6$ kcal/mol. The $\Delta\Delta E$ value for compound 160 is close to the sum of the $\Delta\Delta E$ values of the 2-methylaniline (compound 157, mutagenic, $\Delta\Delta E = -4.6$ kcal/mol) and 4-chloroaniline (compound 171, mutagenic, $\Delta\Delta E = 0.6$). This indicates an additive effect of the chloro and methyl substituent on the stability of the nitrenium ion. Similarly, assuming an additive substitution effect on the stability for the nitrenium ion one would expect compound 204 to be less mutagenic. Though the calculated $\Delta\Delta E = -0.6$ kcal/mol, which classifies this compound still as mutagenic contrary to the experimental data, the nitrenium hypothesis gives the correct trend. This is something DEREK,

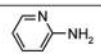
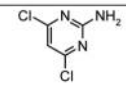
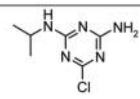
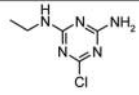
Table 13. Mutagenicity SAR of Ames Predictions for Chloro and Methyl Substituted Anilines[a]

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
0		62-53-3	nonmutagen	mutagen	nonmutagen	nonmutagen	0.0	0.0	0.0	0.0
157		636-21-5	mutagen	mutagen	nonmutagen	nonmutagen	-4.6	-8.7	-7.0	-7.3
147		95-78-3	mutagen	mutagen	mutagen	indeterminate	-6.2	-11.5	-10.1	-11.4
142		95-64-7	mutagen	mutagen	mutagen	nonmutagen	-7.0	-11.9	-11.2	-12.7
96		88-05-1	mutagen	mutagen	nonmutagen	indeterminate	-14.4	-24.1	-22.8	-22.2
171		20265-96-7	mutagen	mutagen	nonmutagen	nonmutagen	0.6	8.1	1.9	-1.7
207		95-51-2	nonmutagen	mutagen	nonmutagen	nonmutagen	1.4	9.6	5.6	2.8
210		634-93-5	nonmutagen	mutagen	nonmutagen	nonmutagen	3.6	25.3	12.2	4.2
215		95-76-1	nonmutagen	mutagen	nonmutagen	nonmutagen	5.1	17.2	9.9	4.1
217		634-67-3	nonmutagen	mutagen	mutagen	nonmutagen	5.2	22.4	12.4	4.6
218		95-82-9	nonmutagen	mutagen	nonmutagen	nonmutagen	6.5	20.6	14.7	9.7
229		634-91-3	nonmutagen	mutagen	mutagen	nonmutagen	9.7	25.7	17.5	9.5
232		626-43-7	nonmutagen	mutagen	nonmutagen	nonmutagen	11.2	22.7	19.2	15.0
160		95-69-2	mutagen	mutagen	nonmutagen	indeterminate	-3.7	-0.6	-4.8	-8.3
204		7745-89-3	nonmutagen	mutagen	nonmutagen	nonmutagen	-0.6	1.6	0.4	-2.2

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

MULTICASE, and MOE-t are not able to do; they classify compounds either as mutagenic or nonmutagenic. It is the continuous nature of the $\Delta\Delta E$ output of the present approach that allows one to interpret the data to see how drastically the electronics of an aniline ring needs to be changed to alter the mutagenic potential of the system. MOE-t, DEREK, and MULTICASE give only "yes" or "no" predictions, thus no further learning can be gained

Table 14. Ames Predictions for Pyridine. Pyrimidine, and Triazine Compounds[a]

ID	Structure	CAS Number	Ames Categorization Organon	MOE-t	DEREK	MULTI CASE	ΔΔE AM1 [kcal/mol]	ΔΔE HF 3-21G [kcal/mol]	ΔΔE HF 6-311G(d,p) [kcal/mol]	ΔΔE DFT/B3LYP/ 6-311G(d,p) [kcal/mol]
240		504-29-0	nonmutagen	mutagen	nonmutagen	nonmutagen	13.5	18.2	19.5	17.6
255		56-05-3	nonmutagen	mutagen	nonmutagen	nonmutagen	48.0	72.5	72.2	57.8
256		6190-65-4	nonmutagen	mutagen	nonmutagen	nonmutagen	59.1	84.2	89.1	61.5
257		1007-28-9	nonmutagen	mutagen	nonmutagen	nonmutagen	59.5	84.8	90.0	64.2

[a] Experimental values are from Kazius et al.[6] Cells are highlighted green when the prediction is in agreement with the experiment and magenta when it is not.

by interpretation of their output. DEREK does provide some context around most structural alerts, but this requires the user to manually examine the alert description and is therefore not suitable for high throughput analysis.

The series of *N*-heteroaromatic amines (pyridine, compound 240, pyrimidine, compound 255, and triazines, compounds 256 and 257) offers interesting insight into the advantages of this quantum chemical method over a simple toxicophore approach. From Table 14 it is apparent that MOE-t predicts all these compounds as Ames positive. This is the result of using the aromatic amine as a specific toxicophore and not excluding the above compounds with a specific SMARTS pattern.[6] Our quantum chemical approach on the other hand correctly predicts these compounds not to be mutagenic. In fact compounds 255, 256, and 257 give the highest positive $\Delta\Delta E$ values in this data set. DEREK and MULTICASE correctly predicted these compounds to be nonmutagenic.

SUMMARY AND CONCLUSION

We have shown that the nitrenium hypothesis offers a powerful tool for the assessment of the mutagenic potential of primary aromatic amines. For the test set of 257 primary aromatic amines, the approach of calculating the nitrenium ion stabilities at the AM1 level of theory achieved an accuracy of 86%, which is comparable to MOE-t, 85%, and DEREK, 84%, and clearly better than MULTICASE at 73%. The sensitivity and false negative rate for the AM1 calculations (91%, 9%) are slightly worse than MOE-t (98%, 2%) but better than DEREK (83%, 17%) and significantly better than MULTICASE (66%, 34%). Though MOE-t has an excellent sensitivity at 98% (as might be expected since its underlying toxicophores were derived from an extended version of the set of molecules considered here), it also has the worst false positive rate and specificity of all four methods, with values of 49% and 51%, respectively.

In summary, the overall performance of the nitrenium hypothesis considered here using the data set of 257 primary aromatic amines is broadly comparable to the MOE-t model. Compared to DEREK and MULTICASE the nitrenium approach showed clear advantages in the prediction of Ames positive compounds because it returned lower false negative rates, and compared to MOE-t it gives a significantly higher specificity and lower false positive rates. However, we note that these other approaches are not specifically tailored to the aromatic amines class of molecules that we have considered exclusively in this work. The AM1 approach has proven itself to be successful and applicable to monocyclic and polycyclic aromatic ring systems.

An attractive feature of the nitrenium hypothesis is that it not only classifies molecules as mutagens/nonmutagens but also quantifies relative stabilities and gives a continuous set of values, which allows the investigation of trends within a series of structurally related compounds. As an example we showed that the approach, even with the simplest level of electronic structure theory considered here, AM1, correctly mirrored the trend of Ames activity for para-substituted anilines and dichloroanilines. We also noted the predictions for other substitutions, for which our approach identified correct trends, though not always giving a classification in complete agreement with the experiment. It has been shown by others that predictive regression models can be generated, correlating the relative nitrenium stabilities with the number of revertants measured in the Ames assay.

On the basis of the results presented here and elsewhere, we expect that $\Delta\Delta E$ based analyses may be broadly applicable to assess the mutagenic potential of aromatic amine molecules. In contrast to knowledge-based approaches, the nitrenium stability parameter $\Delta\Delta E$ is unsupervised and is not dependent on a parametrization scheme that is derived from a training set, rather it has a physical basis in the underlying mechanism of toxicity for arylamines. The nitrenium hypothesis might therefore be expected to be more broadly applicable to arylamine chemotypes that are presently under-represented in training sets for the knowledge-based methods.

Electronic structure packages are now an integral part of computational drug discovery. The availability of these com-

puter programs and scripting procedures means that the nitrenium ion based approach of predicting Ames activities is amenable to automation and can be incorporated into desktop applications for use by medicinal chemists. For example, it has been recently coded into an Accelrys' Pipeline Pilot web-based application within Boehringer Ingelheim. We are currently applying and obtaining value from this technology in the selection and prioritization of aromatic amine molecules in preclinical drug discovery programs.

For practical purposes, we suggest using AM1 calculations to calculate the stability of the nitrenium ions. For cases where AM1 geometry optimizations fail, switching to a higher level of theory may be necessary. The more negative/positive the calculated $\Delta\Delta E$ value the more reliable is the prediction for the compound to be experimentally Ames positive/Ames negative. For $\Delta\Delta E$ values close to zero the user might want to impose an indeterminate range. One of the strengths of our approach is that it allows to identify subtle SAR trends within a series of related compounds. This gives the user the opportunity to investigate how structural changes impact the mutagenic potential of a compound even in cases where the predictions based on $\Delta\Delta E$ values are not in total agreement with the experiment. We believe that the nitrenium hypothesis is a valuable tool to assess the mutagenic potential of primary aromatic amines and propose its use in early drug discovery to prioritize compounds and establish structure activity relationships to avoid mutagenicity.

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Supporting Information Available: The complete data set. This material is available free of charge via the Internet at http://pubs.acs.org.

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