Hemoglobin Binding of Aromatic Amines: Molecular Dosimetry and Quantitative Structure–Activity Relationships for N-Oxidation

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Aromatic amines are important intermediates in industrial manufacturing. N-Oxidation to N-hydroxyarylamines is a key step in determining the genotoxic properties of aromatic amines. N-Hydroxyarylamines can form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner. The determination of hemoglobin adducts is a useful tool for biomonitoring exposed populations. We have established the hemoglobin binding index (HBI) [(nmole compound/mole hemoglobin)/(nmole compound/kg body weight)] of several aromatic amines in female Wistar rats. Including the values from other researchers obtained in the same rat strain, the logarithm of hemoglobin binding (logHBI) was plotted against the following parameters: the sum of the Hammett constants (C = aP + cM), pKa, logP (octanol/water), the half-wave oxidation potential (E0), and the electronic descriptors of the amines and their corresponding nitrenium ions obtained by semi-empirical calculations (MNDQAM1, AM1, and PM3), such as atomic charge densities, energies of the highest occupied molecular orbit and lowest occupied molecular orbit and their coefficients, the bond order of C–N, the dipole moments, and the reaction enthalpy [MNDQAM1, AM1HF or PM3HF = Hf(nitrenium) – Hf(amino)]. The correlation coefficients were determined from the plots of all parameters against log HBI for all amines by means of linear regression analysis. The amines were classified in three groups: group 1, all para-substituted amines (maximum, n = 9); group 2, all amines with halogens (maximum, n = 11); and group 3, all amines with alkyl groups (maximum, n = 13). For the amines of group 1, logHBI correlates with cP, AM1HF, E0, the pKa, and the logP with r = 0.84, 0.73, 0.72, –0.69 and 0.50, respectively. For the amines of group 2, logHBI correlates with pKa, cP, MNDQAM1, AM1HF, E0, and logP with r = 0.81, –0.80, –0.55, –0.46, and –0.20, respectively. For the amines of group 3, logHBI correlates with E0, PM3HF, cP, pKa, and logP with r = 0.92, 0.89, 0.75, 0.19 and 0.12, respectively. This investigation shows for a large variety of aromatic amines the bioavailability of N-hydroxyarylamine (the genotoxic metabolite) and the utility of electronic descriptors for prediction of N-oxidation.

Introduction

Aromatic amines are important intermediates in industrial manufacturing. N-Oxidation to N-hydroxyarylamines is a key step determining the genotoxic properties of aromatic amines. N-Hydroxyarylamines can form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner (1,2). N-Hydroxyarylamines are oxidized in erythrocytes to nitrosarenes, which react with the β-93 cysteine of hemoglobin. The resulting N-hydroxy sulfonimide then rearranges to the more stable sulfonic acid amide (1,3). Recently it has been found that 4-aminobiphenyl is mainly oxidized by cytochrome P-450 IAD and that cytochrome P-450 IAD expression correlates with hemoglobin binding (3). Furthermore, humans with the fast-oxidative phenotype have higher hemoglobin adduct levels. Therefore, hemoglobin adducts should be an appropriate dosimeter for N-hydroxyarylamines in biological systems. The main goal of our work is to determine the bioavailability of the potential ultimate carcinogen—the hydroxyarylamine—for a large array of substituted aromatic amines by measuring the amount of hydrolyzable hemoglobin adducts in rats.

Materials and Methods

Amines of the highest available purity were obtained from Riedel-de Haen (Selze, Germany), Aldrich (Steinheim, Germany), Fluka (Ulm, Germany), and Merck (Darmstadt, Germany). The purity of the amines was checked by GC–MS [Hewlett Packard instrument 5988, DB 1701 column (10 m × 0.32 mm × 1 μm)]. For each compound, two female Wistar rats were given 0.5 mmole/kg of amine by gavage. The rats were killed 24 hr later. The hemoglobin was isolated, hydrolyzed in 0.1 M NaOH, and extracted in hexane as described previously (4,9). The hexane fraction was analyzed by GC–MS with electron impact ionization in the single ion mode. Structure identification was based on the retention time and on the mass spectrum or the ratio of the main mass fragments. Amines with small hemoglobin binding (HBI ≤1) were derivatized with pentafluoropropionic acid anhydride and analyzed by GC–MS. To establish whether

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the aromatic amines recovered from the alkaline hydrolysis were
covalently bound, all samples were also extracted with hexane at
neutral pH and analyzed by GC–MS.

Results and Discussion

The most hemoglobin binding (Table 1) was obtained with a
halogen or a trifluoromethyl group in the para position. A chloro
atom in the ortho position reduces the formation of hemoglobin
adds drastically (1000-fold, for 2-CA [chloroaniline] compared
to 4-CA). This is not a result of the para position being free
for hydroxylation because 2,4-DCA (dichloroaniline) has an HBI
equal to that of 2-CA. An additional ortho chloro atom as in
2,6-DCA or PCA (pentachloroaniline) abolishes hemoglobin
binding. A chlorine in the meta position decreases hemoglobin
binding by a factor of 45 (3-CA compared with 4-CA). An addi-
tional chlorine in the meta position lowers the HBI further
(20-fold).

For the methyl- or ethyl-substituted amines, we found the
following relationships: all alkyl-substituted amines have a lower
HBI than aniline. Only the HBI of 3,5-DMA (dimethylaniline)
is comparable with that of aniline. The HBI of 3-EA (ethylaniline)
is higher than that of 2-EA or 4-EA. This might be explained by the
fact that the oxidation of alkyl groups in the ortho or para posi-
tion to an amino group is facilitated compared to that of alkyl
groups in the meta position. This trend is also seen in the dimethyl-substituted amines, where all amines show lower
hemoglobin binding than the monosubstituted compounds except
for 3,5-DMA. Two methyl groups in the ortho position, as in
2,6-DMA or 2,4,6-TMA (trimethylaniline), almost abolish
hemoglobin binding.

In quantitative structure–activity studies, several descriptors
are used. For our investigation we considered a) lipophilicity, b)
electronic parameters that include ionization constants, the Ham-
matt values, and the descriptors from molecular orbital calcu-
lations, and c) steric parameters. For our correlation analyses we
included HBI values of other researchers that were determined
in the same rat strain. For all amines, the coefficient of correla-
tion between each parameter and the logarithm of HBI (logHBI)
was determined by means of linear regression. The best corre-
lation coefficient was found for PM3HF, with r = 0.47. Therefore,
the amines were classified in three groups: group 1, all para-
substituted amines (maximum, n = 9); group 2, all amines with
halogens (maximum, n = 11); and group 3, all amines with alkyl
groups (maximum, n = 13).

Table 1. Hemoglobin binding of aromatic amines.

| Compound                  | HBI   |
|--------------------------|-------|
| 4-Methylmercaptoaniline   | 3.8 ± 0.5 |
| 4-Methylaniline (2)       | 4.3 ± 1.0 |
| 4-Ethylaniline            | 5.8 ± 1.4 |
| Aniline (2)               | 22.0 ± 3.0 |
| 4-Fluoroaniline           | 33.0 ± 9.0 |
| 4-Iodoaniline             | 2960 ± 200 |
| 4-Bromoaniline            | 3410 ± 14.0 |
| 4-Chloroaniline (2)       | 5690 ± 460 |
| 4-Trifluoromethylaniline  | 1480 ± 600 |

| Compound                  | HBI   |
|--------------------------|-------|
| 2-Chloroaniline           | 0.5 ± 0.1 |
| 3-Chloroaniline (4)       | 12.5 ± 2.0 |
| 4-Chloroaniline (2)       | 5690 ± 460 |
| 2,4-Dichloroaniline       | 0.6 ± 0.2 |
| 2,6-Dichloroaniline       | 0.8 ± 0.2 |
| 3,4-Dichloroaniline (2)   | 90 ± 2.0 |
| 3,5-Dichloroaniline (4)   | 0.6 ± 0.1 |
| 2,3,4,5,6-Pentachloroaniline (4) | — |
| 3-Chloro-4-fluoroaniline  | 30.7 ± 2.4 |
| 2,4-Fluoroaniline         | 32.0 ± 6.0 |
| 4-Fluoroaniline           | 330 ± 9.0 |
| 4-Iodoaniline             | 2960 ± 200 |
| 4-Bromoaniline            | 3410 ± 14.0 |

| Compound                  | HBI   |
|--------------------------|-------|
| 2-Methylaniline (2)       | 4.0 ± 1.0 |
| 2-Ethylaniline            | 5.1 ± 1.1 |
| 2-Ethylamine              | 5.1 ± 1.1 |
| 3-Methylaniline (2)       | 4.9 ± 0.2 |
| 3-Ethylaniline            | 12.7 ± 1.5 |
| 4-Methylaniline (2)       | 4.3 ± 1.0 |
| 4-Ethylamine              | 5.8 ± 1.6 |
| 4-Ethylamine              | 5.8 ± 1.6 |
| 2,4-Dimethylaniline       | 2.3 ± 1.0 |
| 2,5-Dimethylaniline       | 7.3 ± 1.0 |
| 2,6-Dimethylaniline       | 11.1 ± 0.3 |
| 3,4-Dimethylaniline       | 0.7 ± 0.3 |
| 3,5-Dimethylaniline       | 14.0 ± 1.9 |
| 2,4,5-Trimethylaniline (2) | 0.7 ± 0.2 |
| 2,4,6-Trimethylaniline    | 0.2 ± 0.04 |

HBI, hemoglobin binding index [mmole (compound)/mole HBI]/[mmole (compound)/kg(body weight)].

Correlation of logP with logHBI. We included all octanol-
water partition coefficients (logP) available in the literature for
aromatic amines. The best correlation of logP with logHBI was
obtained for the amines of group 1, with r = 0.50 (n = 8), fol-
lowed by group 2, with r = −0.20 (n = 10), and group 3, with
r = 0.12 (n = 11). Analysis of the data with the Hansch equation
also revealed poorly fitting curves. The partition coefficient
alone is not sufficient to predict hemoglobin binding.

Correlation of the pKa with logHBI. The pKa and logHBI
values correlate for group 2, with r = 0.87 (n = 11) (logHBI =
−2.452 + 1.127 pKa) for group 1, with r = −0.69 (n = 9), and
for group 3, with 0.19 (n = 13). Thus, hemoglobin binding is
indirectly proportional and directly proportional to the pKa values
of the amines for para-substituted compounds and for halogen-
substituted compounds, respectively.

Correlation of the Hammett Constants with logHBI. For
multiply substituted compounds, the parameters were added.
Group 1 correlates best with the logHBI, with r = 0.84 (n = 9),
followed by group 2, with r = −0.80 (n = 8), and group 3, with
r = 0.75 (n = 6). The Hammett constants are a useful tool for
predicting hemoglobin binding of groups 1 and 2. The analysis
is restricted by the lack of classical Hammett constants for ortho-
substituted compounds.

Correlation of the Half-Wave Oxidation Potential with
logHBI. Group 3 correlates with r = 0.92 (n = 8), followed by
group 1 with r = 0.72 (n = 8), and group 2, with r = −0.46 (n
= 11). This was the best correlation found for the alkyl-
substituted amines.

Correlation of the Electronic Properties Calculated with
MNDO, AM1, and PM3 with logHBI. We used semi-empirical
molecular orbital calculations to obtain electronic parameters
for all amines. The amines and nitroamine [the nitroamine ion is
the best intermediate to describe the product distribution of
cytochrome P-450 oxidations of amines (5)] were calculated
without changing any of the default parameters with the three
methods MNDO, AM1, and PM3 (10). We listed the heat of for-
mation, the eigenvalues of all lowest occupied molecular orbits
(LOMO) and highest occupied molecular orbits (HOMO), the
coefficients of the molecular orbitals in the LOMO of the
nitrenium ions, and the molecular orbitals of the HOMO of the amines, the charge densities, the charge densities of the π-bonding system (pz), the bond length densities C–N, and all dipole moments. All values were plotted against logHBI. The heat of formation and the charges on the nitrogen atom gave the best results [the full data set and the comparison of the different calculation methods have been published elsewhere (9)]. The values of the calculated heat of formation (ΔHf) have to be expressed on a common basis in order to be compared. The process of nitrenium formation was described by the equation XRNH₂→XRNH⁻⁺+H⁺+2e⁻. MNDOHF, AMIHF, and PM3HF are the formal reaction enthalpies of this process [ΔHf(nitrenium)-ΔHf(amine)]. The loss of a proton and two electrons, which is the same for all calculated amines, and the entropy term are not included in these reaction enthalpies. Excellent correlations were found between MNDOHF or AMIHF and the Hammett constants or E₁/₂ (Fig. 1). The calculated parameters can replace the Hammett parameters, which are not available for all amines. The values obtained with MNDO correlate well with AMIHF (r = 0.93) but poorly with PM3HF, especially with the values of group 2. The electron charge densities obtained with PM3 calculations for the nitrenium ions are different from the results obtained with MNDO and AM1. We used all the calculated values to find the best fit with logHBI. LogHBI correlates with MNDOHF or AMIHF of group 1 and group 3, with r = 0.71 [E0.73] (n = 9) and 0.75 [0.84] (n = 13), respectively. By eliminating one outlier in group 1 (4-TFMA), the correlation increases to 0.96, (logHBI = -40.269 + 0.189 MNDOHF). PM3HF correlates, with r = 0.88, with the logHBI of group 3. MNDO or AM1 calculations are a good predictive tool for amines of group 1. PM3 calculations predict well for group 3 (Fig. 2).

Are these structure–activity relationships found in rats transposable to other species? A larger set of amines has been studied regarding their capability to form methemoglobin (MetHb). Neumann (2) demonstrated that for several amines the extent of MetHb formation in rats and mice is similar to the extent of hemoglobin binding (r = 0.93). The structure–activity relationship for the MetHb response in cats (6) is similar to the HBI values in the present study. Compared to humans and mice, rats have two additional cysteine groups, which might react with nitrosoarenes (7), in the α-chain. Neumann (2) found similar structure–activity relationships in mice and rats, but 2–10 times lower hemoglobin binding in mice. Bryant et al. (8) demonstrated a comparable dose response for hemoglobin binding of 4-aminobiphenyl in rats and humans. Assuming that the human dose response to the amines (most of them from group 3) which has been found (8) is comparable to that of the rat, we estimated the daily exposure to alkylated amines to be between 0.014 and 23 μg per day per 70 kg nonsmoker. If these levels of exposure are realistic, the rat model could also be suitable for predicting effects in humans of amines other than 4-aminobiphenyl.

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