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Investigating the Mechanisms of Aromatic Amine-Induced Protein Free Radical Formation by Quantitative Structure−Activity Relationships: Implications for Drug-Induced Agranulocytosis

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Aromatic amine drugs have been associated with agranulocytosis (neutrophil depletion) for which the mechanism is unknown. We have previously shown that the metabolism of two aromatic amine drugs by human myeloperoxidase (MPO) results in phenyl radical metabolite formation and also in protein free radical formation on MPO. Because the concentration of drug required to produce a maximum signal for MPO protein free radical (MPO•) detection was different for each drug, this prompted us to consider that other aromatic amines may also show varying degrees of ability to induce MPO• formation. Immunoassay experiments using the immuno-spin-trapping technique were performed, which evaluated the potency of different aromatic amines containing the aniline substructure to generate the MPO•. Each reaction contained equal amounts of H_2O_2, 5,5-dimethyl-1-pyrroline-*N*-oxide, MPO, and variable concentrations of aniline derivatives. Several physicochemical parameters for aniline derivatives were used to derive quantitative structure−activity relationship equations, which showed that the Hammett constant (σ) best correlated with the MPO• formation for all aniline derivatives. More statistically robust equations were derived if the anilines were separated into mono- and disubstituted groups. However, some aniline derivatives did not induce MPO• formation. Using electron spin resonance spectroscopy, we evaluated the ability of all aniline derivatives tested to produce phenyl radical metabolites, as previously shown by spin trapping for the aromatic amine drugs. Interestingly, we found that only those aniline derivatives that produced a phenyl radical also formed MPO•. We propose that the phenyl radical is the reactive free radical metabolite responsible for generating the MPO•.

Introduction

Aromatic amine xenobiotics are associated with numerous acute toxicities, including methemoglobinemia and hemolytic anemia, whereas chronic exposure to certain aromatic amines (such as 4-aminobiphenyl) is associated with cancer. Occupational exposure has been a focal point for the determination of biomarkers to evaluate human exposure. Drug molecules containing the aromatic amine or aniline substructure are also associated with toxic side-effects, including agranulocytosis, the depletion of neutrophils (<500/ μL), which increases the risk of sepsis. The mechanism of drug-induced agranulocytosis is not clear; however, it is believed that reactions resulting from the formation of reactive drug metabolites are involved (*3, 4*). The reaction center for aromatic amines is the aniline moiety. In the instance of procainamide-induced lupus, *N*-acetylprocainamide, which also has pharmacological activity, was shown to significantly delay the onset of antinuclear antibody formation (*5*). The *N*-acetyl group is electron withdrawing and, therefore, deactivating with respect to oxidation, suggesting that oxidation of the aromatic amine of procainamide to a reactive metabolite may play an etiological role. Furthermore, procainamide-induced lupus developed more rapidly in slow acetylator phenotype individuals (*6*). No similar findings exist for drug-induced agranulocytosis, which is due in part to the low frequency of this side-effect as well as the lack of suitable models of the condition.

The possibility of the involvement of myeloperoxidase (MPO)-generated free radical metabolites in drug-induced agranulocytosis has been proposed for some time (*7*). Peroxidase enzymes are capable of oxidizing numerous substrates to free radical metabolites (*8*). Since MPO is located in neutrophils, it has been suggested as a catalyst for reactive metabolite generation that could lead to neutrophil death (*9*). It was recently found that in a case of dapsone-induced agranulocytosis, the patient presented an absence of neutrophils, eosinophils, and CD34+ progenitor cells, but basophil levels were unchanged (*10*); this finding was attributed to reactive metabolite formation by peroxidases (MPO and eosinophil peroxidases) since basophils are typically low in peroxidase activity.

We previously found that myeloperoxidase catalyzes the oxidation of two aromatic amine drugs, which leads to the formation of protein free radicals on MPO in the HL-60 cell line as well as in purified enzyme preparations (*1, 2*). In this study, we build upon our previous findings to investigate the factors responsible for these observations. We focus on the physicochemical properties of aniline derivatives (representative structures shown in Figure 1a) that lead them to generate protein free radicals, and developed quantitative structure activity relationships (QSARs, Hansch analysis) that relate their potency in generating protein free radicals to their physicochemical properties. In addition, we examine the relationship

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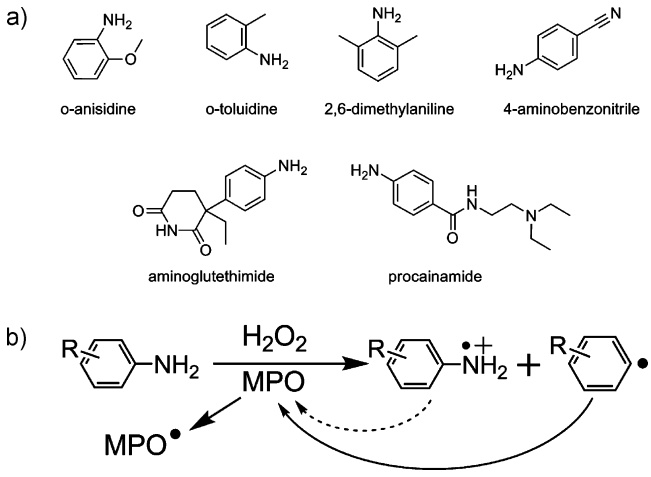

Figure 1. a. Representative structures of compounds tested in the present study. b. Phenyl radical metabolite involvement in MPO free radical formation (MPO•). The dashed line from the cation radical indicates possible involvement, but based on the results of this study, we propose that the phenyl radical metabolite is more significantly involved in MPO• formation.

between the detection of phenyl radicals formed from aromatic amines and their ability to form protein free radicals (shown in Figure 1b).

Experimental Procedures

Reagents. Human MPO was purchased from Athens Research & Technology (Athens, GA) and was dissolved in 0.1 M phosphate buffer, pH 7.4, and dialyzed twice for 12 h each time. MPO concentration was determined by its extinction coefficient (178 mM^{-1} cm^{-1} at 429 nm) (*11*). Horseradish peroxidase type VI (HRP, Sigma Chemical Co., St. Louis, MO) was desalted in a PD-10 desalting column (GE Healthcare, Waukesha, WI) prior to use and its concentration was determined by 102 mM^{-1} cm^{-1} at 402 nm. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Alexis Biochemicals (San Diego, CA), purified twice by vacuum distillation at room temperature, and stored under argon atmosphere at −80 °C until use. Hydrogen peroxide (30% v/v, H_2O_2, Fisher Scientific Co., Fair Lawn, NJ) was assayed by its extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm (*12*). All aniline compounds, 2-methyl-2-nitrosopropane (MNP), and diethylenetriaminepentaacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest grade available. MNP was dissolved in buffer (2 mg/mL), protected from light, sealed, and shaken overnight at 32 °C. Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA) was mixed with purified water overnight, and filtered before use. Phosphate buffer used in experiments was mixed with Chelex-100 resin overnight to remove metal contaminants, and the resin was removed by vacuum filtration. Diethylenetriaminepentaacetic acid (100 mM) was included in the buffer to prevent metal-catalyzed reactions.

Enzyme-Linked Immunosorbent Assay (ELISA) and MPO Protein Free Radical Detection. Ninety-six-well ELISA plates (Greiner Labortechnik, Germany) were used to carry out reactions in order to detect the DMPO-MPO adducts. These reactions were carried out in buffer and contained 100 mM DMPO, 100 μM H_2O_2, 50 nM MPO, and varying concentrations of anilines. The reactants were mixed in 1.5 mL centrifuge tubes, then 100 μL of this reaction was transferred to each of three wells containing 200 μL water. This reaction was carried out for 2 h in an Eppendorf Thermomixer R (Germany) at 37 °C with mixing at 500 rpm. At the end of the incubation period, the plate was washed and blocked overnight with 4% cold fish gelatin, and anti-DMPO detection of MPO−DMPO was carried out as described previously (*1*). The concentration of aniline derivative necessary to induce a 2-fold increase above background for detection of the myeloperoxidase protein free radical (MPO•) was designated the EC_2 value.

Electron Spin Resonance Spectroscopy Detection of Aniline Free Radical Metabolites. Free radical metabolites were detected by electron spin resonance (ESR) spectroscopy using the spin-trapping technique (*13*). The spin trap we used was MNP, which we previously found provided significant detail for revealing the structure of the free radical that was formed (*1, 2*). ESR spectra were recorded using an Elexsys E500 spectrometer (Bruker Biospin Ltd., Billerica, MA) using an ER 4122 SHQ cavity. The settings for most spectra were recorded at 9.78 GHz, 100 kHz modulation field, 20 mW power, 0.4 G modulation amplitude, 1024 points, and 6.32×10^5 receiver gain. In certain cases where the spectra were not well resolved, we used the following modifications to these settings: 1 mW power, 0.07 G modulation amplitude, conversion time and time constant of 2621.44 ms, 2048 data points, and 5368 s sweep time. In order to generate free radical metabolites, we added 10 mM MNP, 5 mM aniline, 1.7 μM HRP, and 1 mM H_2O_2 in 200 μL buffer (described above), briefly vortexed the reaction, and added it to a quartz flat cell for recording spectra. We chose these concentrations based on preliminary experiments.

Physicochemical Parameters and QSAR Derivation. The Hammett constant (σ) values were obtained from (*14*); lipophilicity (experimental values of the log octanol/water partition coefficient, or log P) were retrieved from the ALOGPS 2.1 program (*15*); and ionization potential of the parent aniline (IP-P), electron affinity of the parent aniline (EA-P), and ionization potential and electron affinity of the corresponding phenyl radical metabolites (IP-R, EA-R, respectively) were calculated using MOPAC with PM3 parameters after molecular mechanics geometry optimization (MM3/PM3) in Sybyl 7.1. Free radical enthalpy of formation values has been previously calculated with PM3 parameters (*16*). QSAR equations were derived using SigmaPlot 11.0. Best subset regression was used to identify the physicochemical parameter that best correlated with the EC_2 values, and the inclusion of additional parameters was evaluated to see whether or not they would improve the statistical robustness of the equation.

Results

Aniline-induced MPO Free Radical Formation. The aniline derivatives used in this study were assessed for their ability to induce anti-DMPO detection (formation of MPO•) that was 2-fold greater than control levels (defined as the EC_2 value). As shown in Table 1, we were able to derive EC_2 values for most aniline compounds used in this study. The EC_2 values ranged from 2.4 μM to 733 μM. The most potent inducers of protein free radical formation were 2,4-dimethylaniline and 4-ethylaniline, and the least potent was 2,6-dichloroaniline. We were not able to derive EC_2 values for *p*-anisidine, 3,4-dimethoxyaniline, 2,4-dimethoxyaniline, and 3-nitro- or 2-nitroaniline because, for these compounds, there was no increase in anti-DMPO detection significantly greater than the control found in the concentration range between 0.1 μM − 500 μM of aniline derivative. The physicochemical parameters were obtained as described in the legend and Experimental Procedures. A selection of representative structures from the current test set in Table 1 is shown in Figure 1a.

Table 1. EC_2 Values for Aniline-induced MPO Free Radical Formation and Physicochemical Parameters for Anilines

CASRN	NAME	Class	EC_2 (μM)	log EC_2	ó[1]	log P[2]	EA-R[3]	IP-R[3]	IP-P[3]	EA-P[3]
104−94−9	*p*-anisidine	mono	-*	-	−0.28	0.95	5.01	10.04	8.21	−0.55
125−84−8	**aminoglutethimide**	**mono**	**1.5**	**0.176**	**−0.15**[4]	**1.1**	**4.90**	**9.93**	**8.3**	**−0.67**
589−16−2	4-ethylaniline	mono	2.4	0.38	−0.15	1.96	4.92	9.94	8.33	−0.65
106−49−0	*p*-toluidine	mono	3.1	0.49	−0.14	1.39	4.92	9.95	8.35	−0.61
108−44−1	*m*-toluidine	mono	8.9	0.95	−0.06	1.4	4.9	9.89	8.48	−0.59
62−53−3	aniline	mono	20.5	1.31	0	0.9	4.96	9.97	8.53	−0.64
90−04−0	*o*-anisidine	mono	3.6	0.56	0	1.18	5.04	10.06	8.26	−0.57
371−40−4	4-fluoroaniline	mono	16.3	1.21	0.06	1.15	5.27	10.34	8.55	−0.29
95−53−4	*o*-toluidine	mono	3.6	0.56	0.1	1.32	4.91	9.93	8.44	−0.6
536−90−3	*m*-anisidine	mono	6.8	0.83	0.11	0.93	5.03	10.01	8.5	−0.62
106−47−8	4-chloroaniline	mono	19.3	1.29	0.24	1.83	5.24	10.26	8.6	−0.28
51−06−9	**procainamide**	**mono**	**3.2**	**0.505**	**0.36**[5]	**0.88**	**5.35**	**10.40**	**8.43**	**0.34**
108−42−9	3-chloroaniline	mono	248	2.39	0.37	1.88	5.26	10.22	8.73	−0.26
95−51−2	2-chloroaniline	mono	61.2	1.79	0.67	1.9	5.32	10.31	8.62	−0.29
873−74−5	4-aminobenzonitrile	mono	279	2.45	0.7	0.9	5.49	10.53	8.87	0.19
99−09−2	3-nitroaniline	mono	--	--	0.74	1.37	5.82	10.88	9.39	0.75
100−01−6	4-nitroaniline	mono	345	2.54	0.78	1.39	5.89	10.93	9.27	0.78
88−74−4	2-nitroaniline	mono	--	--	1.72	1.85	5.94	10.96	9.25	0.79
2735−04−8	2,4-dimethoxyaniline	di	--	--	−0.28	1.05[6]	5.10	10.07	8.35	−0.65
95−64−7	3,4-dimethylaniline	di	2.7	0.43	−0.2	1.84	4.88	9.88	8.32	−0.61
6315−89−5	3,4-dimethoxyaniline	di	--	--	−0.17	1.08[6]	5.07	10.04	8.33	−0.63
95−68−1	2,4-dimethylaniline	di	2.4	0.38	−0.04	1.68	4.89	9.92	8.28	−0.61
87−62−7	2,6-dimethylaniline	di	7.3	0.86	0.2	1.84	4.89	9.92	8.35	−0.6
95−69−2	4-chloro-2-methylaniline	di	3.64	0.56	0.34	2.1	5.2	10.23	8.51	−0.29
133−15−3	4-aminosalicylic acid	di	29.8	1.47	0.57	0.89	6	11.1	9.47	0.52
95−76−1	3,4-dichloroaniline	di	455	2.66	0.61	2.69	5.49	10.46	8.74	0
554−00−7	2,4-dichloroaniline	di	67.6	1.83	0.91	2.78	5.58	10.58	8.68	−0.01
608−31−1	2,6-dichloroaniline	di	733	2.87	1.34	2.76	5.67	10.64	8.73	0.01

[1] From Ref *14*. [2] From Ref *15* (http://www.vcclab.org). [3] Values were determined by MOPAC calculations as described in Experimental Procedures. EA-R, electron affinity of the radical metabolite; IP-R, ionization potential of the radical metabolite; IP-P, ionization potential of the parent aniline; EA-P, electron affinity of the parent aniline. Values are in eV. [4] Value for $-C(CH_3)_3$. [5] Value for $-CONHCH_3$. [6] Calculated logP (AlogP).

Table 2. Pearson Correlation (correlation matrix) for the Physicochemical Parameters used to Derive QSAR Equations in This Study

	log P	EA-R	IP-R	IP-P	EA-P
σ	0.47[a]	**0.83**	**0.795**	**0.648**	**0.754**
	0.033	**3.1×10^{-7}**	**1.7×10^{-6}**	**0.00150**	**7.8×10^{-5}**
log P		0.16	0.107	−0.109	0.0736
		0.48	0.643	0.638	0.751
EA-R			**0.996**	**0.918**	**0.962**
			3.7×10^{-21}	**4.8×10^{-9}**	**4.0×10^{-12}**
IP-R				**0.925**	**0.965**
				2.1×10^{-9}	**1.5×10^{-12}**
IP-P					**0.93**
					6.4×10^{-10}

[a] Each pair of values corresponds to the correlation (r) and significance (p) between the physicochemical parameters that are compared. Values in bold typeface show highly significant correlation.

Derivation of QSAR Equations. Before derivation of QSARs it is important to determine the relationship between the physicochemical parameters to be used in QSAR derivation (intercorrelation) to select the fewest number of parameters that could potentially account for the most variance in the data. In Table 2 we show the pairwise Pearson correlations between the different physicochemical parameters computed for the 21 compounds in Table 1 having measured EC_2 values. These values indicate that the calculated quantum chemical parameters are highly collinear (>92%), whereas log P is not significantly correlated with any other parameters (<16%). The σ parameter, on the other hand, shows variable correlation with quantum mechanical parameters, ranging from 65% (IP-P) to 83% (EA-R). Hence, only 3 parameters were considered in subsequent QSAR development: σ, IP-P (which had the least colinearity with σ), and log P. QSAR derivation is intended to reveal whether one or more parameters correlate significantly with activity; hence, only compounds with experimental EC_2 values are included in model development. In addition, to provide a small test set for final QSAR evaluation, we excluded from all model training sets the two monosubstituted aniline drugs, aminoglutethimide and procainamide.

Table 3 shows the best 1- and 2-parameter QSAR equations derived from the aniline activities used in this study. Equations 1, 2, and 3 were derived from the combined set of 21 mono and disubstituted anilines for which EC_2 values were experimentally determined. Interestingly, the best overall 1-parameter fit, by a significant margin, was obtained using σ, and not log P or IP-P (Figure 2). Equations 4−6 and 7−9 were derived for the subsets of 13 mono- and 8 disubstituted anilines, respectively. In both cases, a 1-parameter QSAR based on σ provided reasonable statistics with improved r^2 when the mono- and disubstituted anilines were modeled as separate subsets (eqs 4 and 7; Figures 3 and 4) compared to eq 1. Interestingly, in the case of the monosubstituted anilines, a 1-parameter QSAR based on IP-P (eq 7) gave slightly better overall statistics, whereas this parameter alone gave poor statistics in modeling either the full data set or the subset of disubstituted anilines. The inclusion of 4-aminosalicylic acid in the data set may have led to less robust equations since the latter was the only aniline containing a strong acid group which could have different values for its physicochemical parameters based on its ionization.

In addition, we performed a best subset regression that revealed whether more than one parameter would contribute to improving the statistics of the QSAR expression. In the case of the full set of 21 anilines, addition of IP-P with σ (eq 2, Table 3) provided slight improvement in overall statistics. However, replacing σ with EA (eq 3, Table 3) produced the best two-parameter QSAR equation; EA was not a significant parameter when the data set was separated into mono- and disubsituted anilines. In the case of the 13 monosubstituted anilines, however, inclusion of IP-P with σ provided significant overall improvement (eq 6), consistent with the finding that IP-P alone gave the best 1-parameter correlation for this subset. For the 8

Table 3. QSAR Equations for Aniline-induced MPO Free Radical Formation

Eqn	Compound Sets		n	r^2	F-ratio	SE
	All anilines					
	log EC2 = 0.794(±0.126) + 1.74 σ(±0.249)	(1)	21	0.719	48.6	0.458
	log EC2 = -4.21(±3.56) + 1.452 σ(±0.319) + 0.593 IP-P(±0.426)	(2)	21	0.747	26.6	0.447
	log EC2 = -21.351(±2.94) + 0.877 EA-R(±0.154) + 2.117 IP-P(±0.295)	(3)	21	0.772	30.5	0.423
	Monosubstituted anilines					
	log EC2 = 0.851(±0.126) + 2.12 σ(±0.338)	(4)	13	0.781	39.3	0.379
	log EC2 = -21.2(±3.46) + 2.63 IP-P(±0.403)	(5)	13	0.795	42.5	0.368
	log EC2 = -11.7(±5.81) + 1.08 σ(±0.563) + 1.48 IP-P(±0.639)	(6)	13	0.852	28.8	0.327
	Disubstituted anilines					
	log EC2 = 0.578(±0.256) + 1.73 σ(±0.386)	(7)	8	0.769	20.0	0.515
	log EC2 = -0.404(±5.41) + 1.68 σ(±0.489) + 0.639 IP-P(±0.639)	(8)	8	0.770	8.36	0.563
	log EC2 = -17.5(±4.35) + 1.89 IP-P(±0.478) + 1.25 LogP(±0.281)	(9)	8	0.844	13.6	0.464

disubstituted anilines, although the most significant 1-parameter correlation was also based on σ, the best 2-parameter equation was based on IP-P and log P. Given the small number of cases (8) in this subset, however, use of a 2-parameter correlation is not recommended due to high probability of chance correlation. However, these results do seem to support the observation of differences in physicochemical determinants of activity between the mono- and disubstituted anilines.

For each of the QSAR equations reported in Table 3, we considered cases of statistical outliers as well as compounds whose activities had large leverage on the resulting statistics (not necessarily the same). In the case of outliers (all having Studentized Residuals >2.4), no consistent or generalizable pattern could be identified to justify their exclusion, which could artificially inflate the statistics in relation to prospective predictivity of the equation. In addition, we examined cases where individual compound activities exerted large leverage on the statistics. The importance of this distinction is that excluding outliers almost always improves a statistical correlation (r^2), whereas if a compound is not an outlier but exerts large leverage on the statistics, its exclusion generally degrades the overall statistics but provides a more realistic estimate of the prospective predictivity of the equation. For example, 2,6-dichloroaniline was identified as exerting large leverage on both eqs 1 and 2 in Table 3 (e.g., its exclusion from eq 1 decreased the r^2 value from 0.720 to 0.678; results not shown). However, this same compound did not exert large leverage on eqs 7−9. Given the relatively small size of the present data set and the relatively

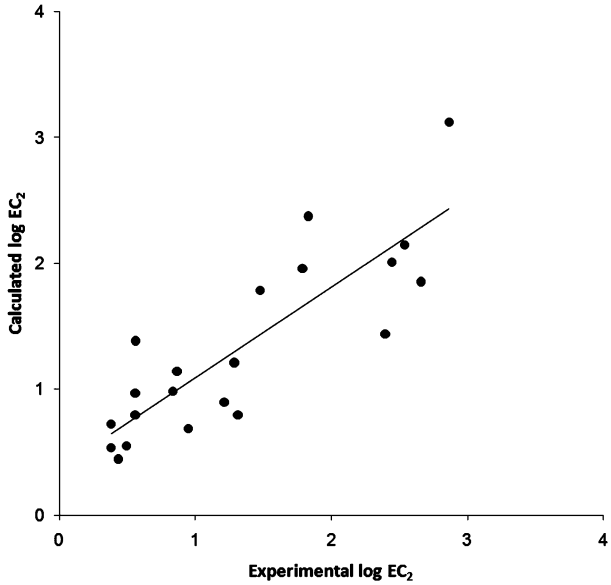

Figure 2. Experimental vs Calculated log EC_2 values for anilines. Each data point was derived by applying eq 1 (Table 3), which contained both mono- and disubstituted anilines. Circles represent data points that were used in the regression.

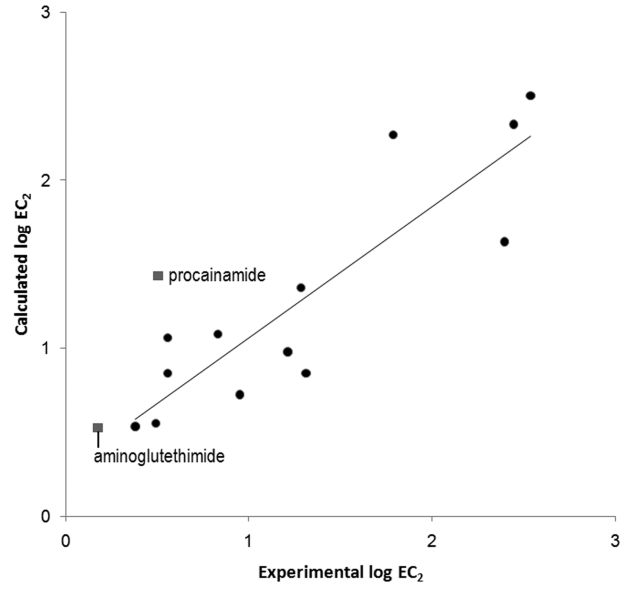

Figure 3. Experimental vs Calculated log EC_2 values for monosubstituted anilines. Each data point was derived by applying eq 4, Table 3. Circles represent anilines that were used in derivation of the QSAR equation. The EC2 values predicted by this equation for aminoglutethimide and procainamide are plotted as squares.

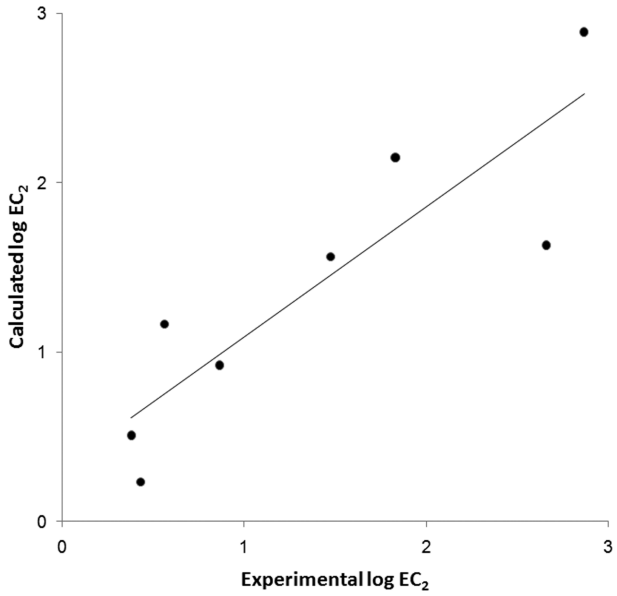

Figure 4. Experimental vs Calculated log EC_2 values for disubstituted anilines. Circles represent the data points derived using eq 7 from Table 3.

modest effect of excluding compounds with high leverage, no compounds were excluded from the present analysis and equations in Table 3.

As a limited prediction challenge, we applied the QSAR equations in Table 3 for the full set of anilines (eqs 1−3) and for the monosubstituted anilines (eqs 4−6) to predict the log EC_2 values for the previously studied monosubstituted aniline drugs, aminoglutethimide and procainamide (*1, 2*). As shown in Table 4, absolute values of the log EC_2 predictions in each case differ significantly from the experimental values. It should be noted, however, that the experimental log EC_2 value for aminoglutethimide (0.176) was a factor of 2 lower than the lowest log EC_2 value in the entire QSAR training set (0.38). Hence, this compound represented a true extrapolation of the QSAR equations outside of the training set range of activities. Given this consideration, the QSAR predictions, which all fall in the lowest third of the activity range, are reasonable. In addition, the relative order of predicted activities is correct for each QSAR equation, i.e., log EC_2 (procainamide) > logEC_2 (aminoglutethimide), and the ratio of experimental values (2.8) is reproduced quite accurately (2.3−3.0) in all but eq 5 (1.5). This last result further argues against use of IP-P alone in modeling the disubstituted anilines.

Detection of HRP-catalyzed Phenyl Radical Metabolite Formation from Anilines. HRP/H_2O_2-catalyzed aniline free radical metabolites were spin-trapped using MNP, and the coupling constants obtained from their spectra were compared with the ESR coupling constants for tertiary butyl aryl nitroxide radicals (obtained from reference tables in the Landolt-Bornstein book series) (*17*−*19*). As shown in Table 5, we were able to detect free radical metabolites of most anilines; however, the complete structure (i.e., what atom of the free radical reacted with MNP) could not be determined for many of the anilines (indicated by "unresolved H-splittings"). In addition, some anilines did not produce any detectable adducts. The compounds that produced well-defined ESR spectra were aniline, *p*-toluidine, 4-chloroaniline, 4-fluoroaniline, 4-ethylaniline, 2-chloroaniline, and 4-aminobenzonitrile. These compounds all had substitutions on the benzene ring *para* to the amine, except for 2-chloroaniline. The ESR spectra from 4-, 3-, and 2-chloroaniline including their simulated spectra are shown in Figure 5.

Discussion

The mechanism of drug-induced agranulocytosis is unknown, but is believed to have many components, with reactive metabolites being a major one. We previously determined that aminoglutethimide and procainamide, both of which contain aniline substructures, produce protein free radicals upon oxidation by myeloperoxidase. In addition, it was found that both drugs generate a phenyl radical metabolite (*1, 2*). The relationship between the phenyl radical metabolite and protein radical formation is unknown. Moreover, the capacity for aniline compounds to induce MPO$^\bullet$ formation was not reported previously. We thus undertook this study to identify the physicochemical parameters that may be involved in protein free radical formation and to determine whether phenyl radical metabolites could be implicated.

We have derived QSAR equations that demonstrate a relationship between two electronic parameters (σ, IP-P) of aniline compounds and the log of their EC_2 values for protein free radical formation. This suggests that the ease of oxidation of aniline derivatives was a significant determinant of activity. Aniline lipophilicity, as approximated by log P, was not found to be a significant determinant of activity, suggesting that hydrophobic binding differences are less important in accounting for protein free radical formation among the studied compounds. It has been shown previously that σ was inversely related to the rate constants for HRP Compound I reduction (*20*). We derived an equation from these published data, but instead of using the ratio of substituted aniline to aniline, we used the log of the rate constant (kX) and correlated this with σ values:

$$\log kX = -7.147\sigma + 6.739,\ r^2 = 0.94,\ s = 0.563,\ F = 92.4$$

This equation indicates that anilines that reduce HRP Compound I the fastest (i.e., the best HRP substrates) have more negative σ values. In the present study, the derived QSAR equations showed a similar relationship, with lower EC_2 values (more potent MPO$^\bullet$ inducers) corresponding to anilines with smaller or more negative σ values. This implies that better substrates, with higher rates of reactivity, are more effective MPO free radical generators.

In our experiments, however, we found that there were two groups of substrates that did not induce MPO free radical formation. One group, *p*-anisidine and 3,4-, and 2,4-dimethoxyaniline, would appear on the basis of relatively low σ values to be highly efficient donor substrates for peroxidases; *p*-anisidine has a $\sigma = -0.28$, 3,4-dimethoxyaniline $\sigma = -0.17$, and 2,4-dimethoxyaniline $\sigma = -0.28$. Interestingly, these compounds fall at the extreme of the σ range for the tested compounds, implying that there is an optimal range of σ values beyond which effectiveness drops off. Interestingly, *p*-toluidine ($\sigma = -0.14$) and aminoglutethimide ($\sigma = -0.15$) both induced MPO free radical formation with great efficacy. On the other hand, relatively inefficient peroxidase substrates also appeared to be capable of generating MPO$^\bullet$, e.g., dichloroanilines and nitroanilines. However, they did so with much less efficacy as indicated by their relatively high EC_2 values. Further evidence that an optimal range of σ values, or electronic factors, is necessary for MPO$^\bullet$ generation is provided by the inability to measure EC_2 values for 2- and 3-nitroaniline ($\sigma = 1.72$, 0.74, respectively), which both fall at the other extreme of large σ values (see Table 1). 4-Nitroaniline and 3,4-dichloroaniline were the third and second least effective in generating MPO$^\bullet$, respectively, and neither produced a detectable phenyl radical. Presumably, the rate of production of the precursor (nitrogen-

Table 4. Predicted log EC_2 Values for Aminoglutethimide and Procainamide

Method[1]	n	logEC_2-A aminoglutethimide	logEC_2-P procainamide	logEC_2-P/logEC_2-A
Experimental		0.176	0.505	2.8
QSAR eq *1* (σ)	21	0.526	1.43	2.7
QSAR eq *2* (σ, **IP-P**)	21	0.488	1.32	2.7
QSAR eq *3* (EA-R, IP-P)	21	0.517	1.18	2.3
QSAR eq *4* (σ)	8	0.525	1.62	3.0
QSAR eq *5* (**IP-P**)	8	0.629	0.971	1.5
QSAR eq *6* (σ, **IP-P**)	8	0.418	1.17	2.8

[1] QSAR Equations are listed in Table 3 based on the properties listed here, where n = total number of compounds used to derive the equation.

Table 5. Spin Trapping of Aniline-derived Free Radicals Formed by HRP/H_2O_2 Using MNP

	Coupling constants (aryl nitroxides) from references (G)*	Coupling constants using HRP/H_2O_2 (G)
aniline	a^N = 11.86, a^H_o = 2.16, a^H_m = 0.92, a^H_p = 2.21 (*22*)	a^N = 15, $a^H_{o,p}$ = 1.6, a^H_m = 0.92
p-toluidine	a^N = 13.6, a^H_o = a^H (CH_3) = 1.86, a^H_m = 0.93, (*23*)	a^N = 15, a^H_m = 0.99, $a^H_{o,\ p\text{-}CH3}$ = 1.7
m-toluidine	a^N = 13.45, $a^H_{o,p}$ = 1.8, a^H_m = $a^H_{(CH3)}$ = 0.6 (*24*)	a^N = 15.5, unresolved H-splittings
o-toluidine	a^N = 13.5, a^H_o = 0.8, a^H_m = 0.6, 0.7, a^H_p = 0.39, $a^H_{(CH3)}$ = 0.24, $a^H_{(tbu)}$ = 0.23# (*25*−*29*)	a^N = 15.6, unresolved H-splittings
p-anisidine	a^N = 13.9, a^H_o = 1.86, a^H_m = 0.93 (*23, 28, 30*)	Not detected
m-anisidine	a^N = 12.8, a^H_o = 1.8, a^H_p = 1.8, a^H_m = 0.8 (*24*)	a^N = 15.6, unresolved H-splittings
o-anisidine	a^N = 14.5, a^H_o = 0.99, $a^H_{(3)}$ = 0.61, $a^H_{(4)}$ = 0.41, $a^H_{(5)}$ = 0.87, $a^H_{(OCH3)}$ = 0.063, $a^H_{(tbu)}$ = 0.26# (*28*)	a^N = 15.6, unresolved H-splittings
3,4-dimethylaniline		a^N = 15.6, unresolved H-splittings
2,4-dimethylaniline	a^N = 13.5, $a^H_{(2,\ CH3)}$ = 0.24, a^H_3 = 0.685, $a^H_{(4,CH3)}$ = 0.41, a^H_5 = 0.685, a^H_6 = 0.8# (*26, 27*)	a^N = 15.6, unresolved H-splittings
2,6-dimethylaniline	a^N = 13.4, $a^H_{(2,\ CH3)}$ = $_{(6,CH3)}$ = 0.17, $a^H_{(3,5)}$ = 0.68, a^H_4 = 0.15, $a^H_{(tbu)}$ = 0.34# (*25*−*28, 31*)	a^N = 15.6, unresolved H-splittings
3,4-dimethoxyaniline	a^N = 14.5 (*32*)	Not detected
2,4-dimethoxyaniline		Not detected
4-chloroaniline	a^N = 13.0, a^H_o = 2.01, a^H_m = 0.98 (*23*)	a^N = 14.6, a^H_o = 1.8, a^H_m = 0.94
3-chloroaniline	a^N = 12.45, a^H_o = 1.9, a^H_p = 1.9, a^H_m = 0.8 (*24*)	a^N = 15.6, unresolved H-splittings
2-chloroaniline	a^N = 13.9, a^H_o = 0.74, a^H_m = 0.47,0.82, a^H_p = 0.29, $a^H_{(tbu)}$ = 0.25# (*28, 33*)	a^N = 15.3, a^H_o = 0.83, a^H_m = 0.50,0.87, a^H_p = 0.37, a^H_{tbu} = 0.23
4-fluoroaniline	a^N = 13.7, a^H_o = 1.72, a^H_m = 0.86, a^F = 3.5 (*23*)	a^N = 15.1, a^H_o = 1.68, a^H_m = 0.89, a^F = 3.1
2,4-dichloroaniline	a^N = 13.8# (*28, 29*)	a^N = 15.2, unresolved H-splittings
3,4-dichloroaniline		Not detected
2,6-dichloroaniline	a^N = 13.1, a^H_m = 0.69, a^H_p = 0.1, $a^H_{(tbu)}$ = 0.35 (*28*)	a^N = 14.8, unresolved H-splittings
4-nitroaniline		Not detected
3-nitroaniline		Not detected
2-nitroaniline		Not detected
4-ethylaniline	a^N = 13, a^H_o = 1.9, a^H_m = 0.9# (*25, 34*−*36*)	a^N = 15.0, a^H_o = 1.87, a^H_m = 1.1
4-aminosalicylic acid		a^N = 15.2, unresolved H-splittings
4-chloro-2-methylaniline		a^N = 15.4, unresolved H-splittings
4-aminobenzonitrile	a^N = 10.06, a^H_o = 2.46, a^H_m = 0.94, $a^N_{(CN)}$ = 0.41 (*22*)	a^N = 13.3, a^H_o = 2.1, a^H_m = 0.97, $a^N_{(CN)}$ = 0.27

* The a^N is typically lower in organic solvents. # Value for 2-chloro-4-bromoaniline.

centered radical) and formation of the phenyl radical were low and below the limits of detection. 2,6-Dichloroaniline, which was the least effective substrate (EC_2 = 733 μM), had a detectable (albeit weak) phenyl radical spectrum which was likely due to stabilization of the radical metabolite by the *o*-chloro groups.

Our initial studies on drug-induced MPO• formation involved the peroxidase metabolism of aminoglutethimide and procainamide, which are both aniline-based drugs. Having derived QSAR equations to describe the physicochemical relationship between aniline compounds and MPO formation, we wished to determine whether these equations would reasonably predict the EC_2 values of the drugs. The drugs were not used to derive the QSAR equations and therefore represented a small test set. Interestingly, equations using σ did not accurately predict the absolute values of EC_2, but did correctly and accurately predict their relative values. One reason for this disparity could be that the σ values for the drugs are not known (we used σ values from structurally similar para substituents). Another reason may be that the group in the *para* position for procainamide is electron withdrawing ($-CONH_2CH_2R$, σ = 0.36), and would be predicted to be a weak protein radical inducer and a relatively poor peroxidase substrate.

Identification of the presumed free radical metabolite that is responsible for generating the MPO• is technically challenging because it is difficult to differentiate the effects of the nitrogen-centered cation radical from those of the carbon-centered phenyl radical. In order to generate the phenyl radical metabolites, the nitrogen-centered radical has to be sufficiently thermodynamically unstable in order to break down into the phenyl radical. Formation of the phenyl radical requires cleavage of the C−N bond, which requires activation energy. This appears to be a two-step process; the first step, where an initial radical metabolite is formed (nitrogen-centered radical), is necessary but not sufficient for MPO• formation; the second step is the spontaneous formation of the phenyl radical (cleavage of the C−N bond) which appears to react with MPO, forming MPO•. Since we previously determined that aminoglutethimide and procainamide formed phenyl radicals, we wished to investigate whether the anilines used in this study also formed phenyl radicals. In addition, we wished to determine the relationship between the formation of phenyl radical metabolites and MPO•. With two exceptions (4-nitroaniline and 3,4-dichloroaniline) out of 28 aniline derivatives, it appeared that there was a qualitative relationship between phenyl radical metabolite formation and MPO•. More importantly, the aniline compounds for which we

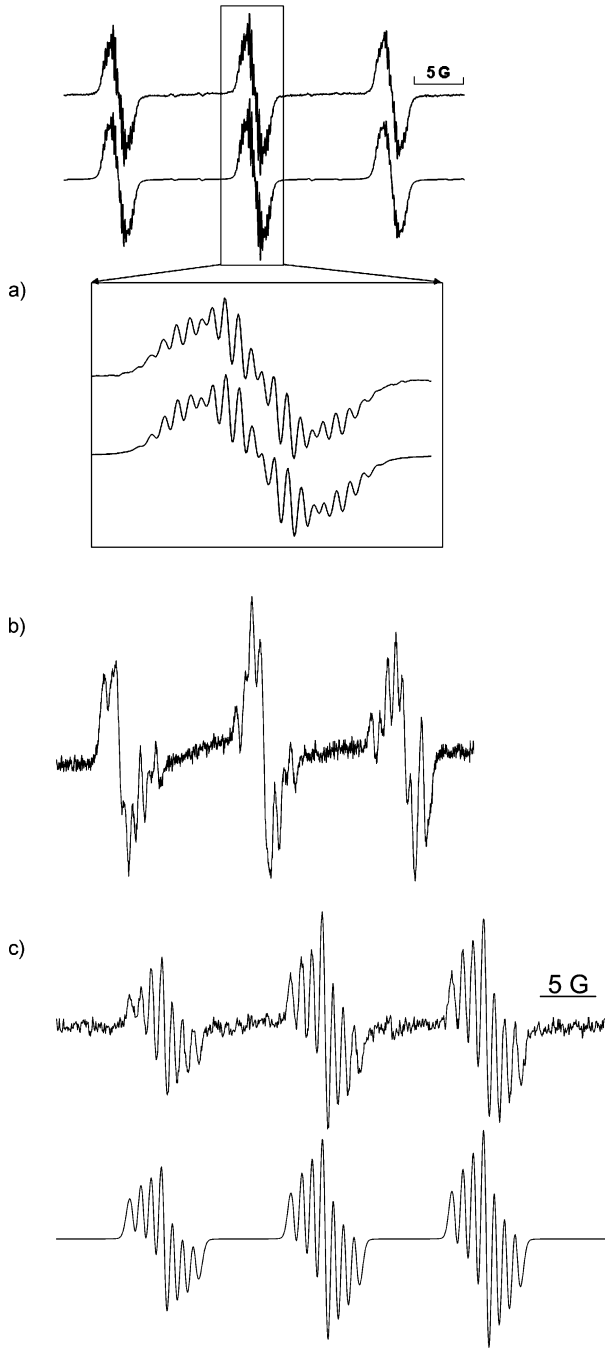

Figure 5. a. ESR spectrum of 2-chloroaniline when oxidized by HRP/H_2O_2. 2-chloroaniline, MNP, and H_2O_2 were mixed in Chelex-100-treated phosphate buffer, and the reaction was initiated by adding HRP. The reaction was transferred to a flat cell and placed in the ESR cavity for recording. The spectrum for 2-chloroaniline was not well resolved, which required specific parameters to be used (see Experimental Procedures). The simulation of this spectrum (correlation: $r = 0.99$) is shown below the experimental spectrum (top), and the center peak is zoomed to show the overlay. The hyperfine splitting constants are shown in Table 5. Figure 5b ESR spectrum of 3-chloroaniline oxidized by HRP/H_2O_2. The reaction was carried out as described in Figure 5a. In this case, the signal:noise was not as high as it was for 2-chloroaniline, which required 10 mW power to be used in order to obtain as resolved a spectrum. The other parameters were as described in the Experimental Procedures. This spectrum could not be simulated, presumably because of mixed product formation. Figure 5c. ESR spectrum obtained upon 4-chloroaniline oxidation by HRP/H_2O_2. The reaction was carried out as described in Figure 5a; however, spectrum resolution was attained using the following instrument settings: 20 mW power, modulation amplitude of 0.4 G, and conversion and time constant at 655.36 ms. The simulation of the experimental spectrum (correlation: $r = 0.97$) is shown below. The hyperfine splitting constants are shown in Table 5.

were unable to detect phenyl radicals did not form MPO$^{\bullet}$. This suggests that the phenyl radical is the metabolite responsible for MPO$^{\bullet}$ formation.

The mechanisms of drug-induced agranulocytosis are still unresolved and are considered to be multifactoral, i.e., a particular alignment of multiple parameters must be present for the adverse drug reaction to occur. In the absence of suitable models, investigations of such parameters have been hindered. It has been proposed for some time that leukocyte (MPO)-generated reactive metabolites may be involved in the etiology of drug-induced agranulocytosis (*21*); a role for MPO-generated free radical metabolites has also been proposed (*7*). We have focused on the physicochemical side of these reactions by evaluating the tendency of aromatic amines that generate phenyl radical metabolites to induce MPO$^{\bullet}$. This study established that the electronic withdrawing nature of the substitution on the aniline ring can determine the outcome of protein free radical formation on MPO. In the future, this research needs to establish the significance of MPO$^{\bullet}$ in the etiology of agranulocytosis and identify potential *in vivo* pathways of inducing and altering this reaction.

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