Comparison of Halo- and Nitro-benzene Toxicity in Rat and Human Hepatocytes

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Abstract

The toxicity of halobenzenes are mediated by metabolites formed by oxidative metabolism. Quantitative structure-activity relationships (QSAR) were applied to evaluate the toxicity of halobenzene congeners in rat and human hepatocytes. Toxicity was determined by incubation of individual compounds with hepatocyte suspensions followed by trypan blue exclusion or MTT reduction to assess viability. QSAR derivation indicated that halobenzene induced toxicity in fresh male SD rat hepatocytes strongly correlated with log P (hydrophobicity), EHOMO (energy of the highest molecular orbital) and dipole moment, μ : log LD_{50} = 8.503 -0.514 **log P** + 0.400 **EHOMO** - 0.0868 **μ**. In male human hepatocytes, a similar correlation was obtained: log LD_{50} = 7.071 - 0.462 **log P** + 0.261 **EHOMO** - 0.0523 **μ**. This suggests that halobenzenes with the highest toxicity in normal fresh rat hepatocytes and cryopreserved human hepatocytes depended on hydrophobicity, ease of oxidation and on asymmetric charge distribution according to arrangement of the halogen substituents. However, in phenobarbital induced cytochrome P450 fresh rat hepatocytes toxicity strongly correlated to log P and dipole moment, and not EHOMO: log LD_{50} = 4.604 – 0.365 **log P** – 0.383 **μ**, suggesting that oxidation by P450 is no longer rate limiting in induced hepatocytes. Nitrobenzene toxicity is mediated by reactive metabolites formed by reductive metabolism. Dinitrobenzene (DNB) toxicity studies with human and rat hepatocytes using MTT reduction to assess viability, showed that the order of toxicity towards rat hepatocytes was 1,4-DNB>>1,2-DNB>1,3-DNB. However, in human hepatocytes, no toxicity was observed at these doses. Nitrofurantoin, a nitroaromatic antimicrobial drug, was also toxic to rat but not human hepatocytes. Previously published studies have shown that both rats and hamsters have profound species differences in 1,3-DNB toxicity. These studies show that hamsters were much more resistant than rats to toxicity induced *in vivo* by 1,3-DNB. This was attributed to lower nitroreductase activity, higher aromatic hydroxylation and GSH reductase activities, higher acetylation capacity and more robust mitochondria in the hamster. Similar protective mechanisms may be responsible for the resistance of human hepatocytes to nitrobenzenes.

Introduction

Halobenzenes and nitrobenzenes are model compounds which require metabolic activation by the liver to form chemically reactive metabolites that mediate serious liver damage. Many studies on bioactivation have revealed that chemically inert substances can be converted to reactive metabolites by a variety of different mechanisms. Halobenzenes require oxidative metabolism while nitrobenzenes require reductive metabolism to form their toxic metabolites. Major interspecies variations are frequently observed in both the rates and the routes of drug metabolism between humans and animals. The resultant toxic effects of chemical compounds on the body exhibit certain specificity which depends on the compound's chemical structure and reactivity. Isolated hepatocytes are the most widely used model employed to screen compounds, and to characterize the nature and mechanisms by which compounds can cause hepatotoxicity. Due to ethical and economic considerations, as well as the pressures to reduce animal experimentation, an increasing interest is devoted to *in vitro* hepatic model systems, particularly human-derived ones, in toxicity testing. *In silico* methods such as Quantitative Structure Activity Relationships (QSAR) are also being used for screening new chemical compounds. QSAR provides an alternative test method capable of rapid screening for potential hazards and strategy to improve models of chemical reactivity. QSARs are derived mathematical formulas which correlate chemical structure and biological activity, describing the structural dependence of biological activities. In this study, cytotoxicity of halobenzene congeners towards phenobarbital P450 induced and uninduced fresh rat hepatocytes, and cryopreserved human hepatocytes, was determined. In addition, the cytotoxicity of dinitrobenzene isomers towards cryopreserved rat and human hepatocytes was determined. The physico-chemical parameters that underlie the cytotoxic effects of these compounds were also examined using QSAR.

Materials and Methods

Hepatocyte Cultures: Cryopreserved Male Sprague Dawley Rat Hepatocytes and Male Human Hepatocytes were obtained from BioreclamationIVT. Fresh Male Sprague Dawley Rat Hepatocytes were prepared by 2-step collagenase perfusion of the liver as described by Moldeus et al[1]. P450 induced fresh hepatocytes were prepared by injecting Rats I.P with 80 mg/kg phenobarbital for 3 days.

Hepatocyte Toxicity: Compounds were incubated with hepatocyte suspensions under 37°C and 5% CO_2. The concentration at which a compound caused 50% (LD_{50}) cell death after 2 hours incubation was determined by Trypan blue exclusion method or MTT reduction.

Trypan Blue Exclusion: Equal volumes of cell suspension and 0.4% Trypan blue solution were mixed and blue stained cells were counted as non viable under a light microscope.

MTT Assay: MTT (10X) was added to each well containing compounds or vehicle controls for a final concentration of 0.5 mg/ml. Plates were incubated in a 37°C, 5% CO_2, humidified tissue culture incubator for three hours, and 0.2 ml of acidified isopropanol was added to each well to dissolve the MTT formazan. Absorbance of MTT formazan was measured at 572 nm and 690 nm. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value.

QSAR: QSAR equations were derived by multiple linear regression analysis using Sigma Stat (V2.03, 1992-1997 SPSS Inc.) Dipole moment (μ), energy of the lowest unoccupied molecular orbital (ELUMO), and energy of the highest occupied molecular orbital (EHOMO) were calculated by PM5 method, using the CAChe Worksystem Pro (V6.1.1, 2000-2003 Fujitsu, Ltd.). Log P values were obtained from Hansch and Leo, 1995[2].

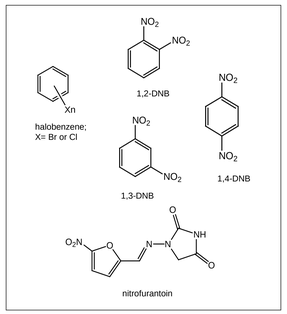

Figure 1. **a)** Representative structure of halobenzenes, X_n=Bromo or Chloro substituent, positioned on the benzene ring as indicated in Table1. **b)** Structures of dinitrobenzene isomers and nitroaromatic antimicrobial drug, nitrofurantoin.

Results

Table 1. Experimental values of toxicities and calculated physicochemical parameters for halobenzene compounds

	Halobenzene	log LD_{50} 2h HUMAN	log LD_{50} 2h RAT	log LD_{50} 2h RAT P450 induced	Log P	EHOMO	dipole moment μ
1	1,2,4-tribromobenzene (TBB)	2.68	2.30	2.30	4.98	-11.021	1.529
2	1,2,4-trichlorobenzene (TCB)	2.76	2.40	2.40	4.02	-10.561	1.608
3	1,3-dibromobenzene (1,3-DBB)	2.69	2.55	2.48	3.75	-9.946	1.355
4	1,2-dibromobenzene (1,2-DBB)	2.68	2.54	3.54	3.64	-9.862	2.143
5	1,2-dichlorobenzene (1,2-DCB)	2.97	2.70	2.70	3.43	-9.295	1.971
6	Bromobenzene (BB)	3.06	2.78	2.78	2.99	-9.806	1.449
7	1,3-dichlorobenzene (1,3-DCB)	2.93	2.78	2.78	3.53	-9.421	1.233
8	Chlorobenzene (CB)	no data	3.30	3.30	2.89	-9.389	1.306
9	1,4-dichlorobenzene (1,4-DCB)	3.06	3.30	3.30	3.44	-9.235	0.001
10	1,4-dibromobenzene (1,4-DBB)	2.75	3.34	3.34	3.79	-9.869	0.001

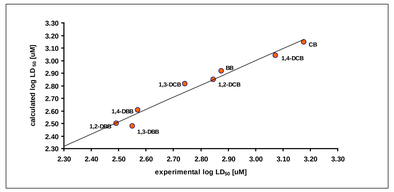

Figure 2. Calculated versus experimental LD_{50} values for fresh rat hepatocytes treated with halobenzenes. The line was derived by plotting the experimental values to calculated values determined by the dervived QSAR equation:
log LD_{50} = 8.503 - 0.514 log P + 0.400 EHOMO - 0.0868 μ; n=8.0, r^2=0.966, s=0.061 F=37.800, P = 0.002. Outliers: TBB; TCB

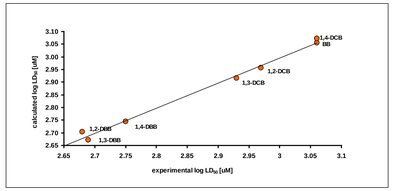

Figure 3. Calculated versus experimental LD_{50} values for cryopreserved human hepatocytes treated with halobenzenes. The line was derived by plotting experimental values to calculated values determined by the derived QSAR equation:
log LD_{50} = 7.071 - 0.462 log P + 0.261 EHOMO - 0.0523 μ; n=7.0, r^2=0.992, s=0.021 F=124.438, P=0.001. Outliers: TBB; TCB

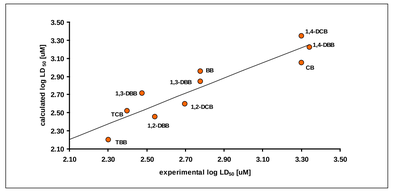

Figure 4. Calculated versus experimental LD_{50} values for P450 induced fresh rat hepatocytes treated with halobenzenes. The line was derived by plotting experimental values to calculated values determined by the derived QSAR equation:
log LD_{50} = 4.604 - 0.365 log P - 0.383 μ; n=10.0, r^2=0.846, s=0.175, F=19.164, P=0.001.

Table 2. Experimental values of toxicities and calculated physicochemical parameters for nitrobenzene compounds

	Nitrobenzene	LD_{50} 2h [uM] HUMAN	LD_{50} 2h [μM] RAT	Log P	ELUMO	dipole moment μ
1	1,2-dinitrobenzene (1,2DNB)	>1500	501	1.69	-2.418	7.73
2	1,3-dinitrobenzene (1,3-DNB)	>2500	1225	1.49	-2.282	5.02
3	1,4-dinitrobenzene (1,4-DNB)	>1000	204	1.46	-2.638	0.00
4	nitrofurantoin	>2000	1375	-0.47	-2.701	2.30

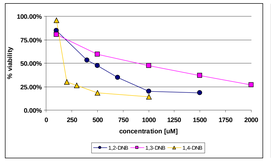

Figure 5. Toxicity of dinitrobenzene isomers at 2 hours in cryopreserved rat hepatocytes.

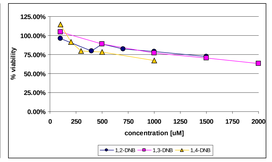

Figure 6. Toxicity of dinitrobenzene isomers at 2 hours in cryopreserved human hepatocytes.

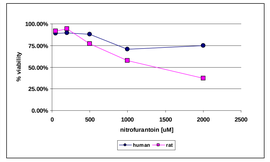

Figure 7. Toxicity of nitrofurantoin at 2 hours in cryopreserved human and rat hepatocytes.

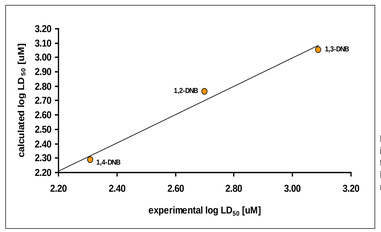

Figure 8. Calculated versus experimental LD_{50} values for rat hepatocytes treated dinitrobenzene isomers. The line was derived by plotting experimental values to calculated values determined by the derived QSAR equation:
log LD_{50} = 7.962 + 2.151 ELUMO ;
n=3.0, r^2=0.982, F=54

Conclusions

• QSAR analysis indicated that the cytotoxicity of halobenzene congeners towards fresh rat hepatocytes and cryopreserved human hepatocytes correlated to higher values of log P, and dipole moment and EHOMO. This suggests that compounds with unsymmetrical charge distribution, increased hydrophobicity and ease towards oxidation by P450 are more readily activated to their reactive intermediates. However, when hepatocytes from phenobarbital induced rats were used, cytotoxicity correlated best only with log P and dipole moment. EHOMO was now no longer significant in the underlying toxicity of phenobarbital induced hepatocytes. This suggests that ease of oxidation is only rate limiting when P450 levels are low, as in uninduced hepatocytes.

• Dinitrobenzene isomers were cytotoxic towards rat hepatocytes but not human hepatocytes. Similar results were observed with nitrofurantoin, a nitroaromatic antimicrobial drug (Figure 1). QSAR demonstrated that the cytotoxicity of dinitrobenzenes towards rat hepatocytes correlated to low values of ELUMO. Compounds with low ELUMO energies more readily accept electrons, suggesting that nitroreductase catalyzed formation of an electrophilic reductive metabolite such as nitrosonitrobenzene, is important in cytotoxicity. The resistance of human hepatocytes to nitrobenzenes may be attributed to lower nitroreductase activity[3], higher aromatic hydroxylation[4], GSH reductase activities[4], and higher acetylation capacity[4], as previously seen in the hamster[4]. Therefore, the species differences seen here may be due to a lower capacity of humans to reduce nitrobenzenes, which is required for cytotoxicity.

References

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