HEPATIC METABOLISM OF AROMATIC AMINE HAIR DYE COMPONENTS AS COMPARED WITH THAT OF 2-AMINOFLUORENE

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Introduction

Most epidemiology studies evaluating permanent hair dye use and bladder cancer show no significant association. However, one recent study did report an association which was modified by phenotypic or genotypic differences in CYP1A2, NAT1 and NAT2 enzymes[1]. Furthermore, because of the established role of these enzymes in arylamine activation and detoxification, these researchers concluded that the evidence suggested a causal association between arylamines used in permanent hair dyes and bladder cancer risk. *N*-Oxidation leading to the formation of an *N*-hydroxyarylamine is regarded as a critical step in the conversion of arylamines to proximate carcinogens. We have investigated the metabolism of two aromatic amines which are commonly used in hair dyes, *p*-phenylenediamine (PPD) and *p*-toluenediamine (PTD) in cryopreserved human hepatocytes, human liver microsomes, and heterologously expressed human CYPs. 2-Aminofluorene (2-AF), whose hepatic metabolism is well characterized was included as a positive control compound.

1. Gago-Dominguez, M., *et al* (2003). "Permanent hair dyes and bladder cancer: risk modification by cytochrome P4501A2 and N-acetyltransferases 1 and 2." Carcinogenesis **24**: 483-489.

Methods

- **Cryopreserved human hepatocytes** were obtained from *In Vitro* Technologies. Suspended hepatocytes (1 x 10^6 cells per ml) were incubated with substrate for 1, 2 or 4 hours. Aliquots (500 µl) were removed and the reaction stopped by the addition of 200µl acetonitrile prior to analysis.
- **Pooled human liver microsomes** were obtained from Gentest via BD Biosciences. Test compounds were incubated with pooled human liver microsomes (1 mg protein/ml) in the presence or absence of an NADPH regenerating system. The reaction was initiated by the addition of microsomes, incubated at 37°C and terminated by addition of acetonitrile as described above.
- **Recombinant human CYPs** (CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) were obtained from Cypex, Dundee, UK. Incubations were carried out as described. Bacterially expressed CYPs were used at a concentration of 2.25 – 12.65 pmol/ml.
- **Analysis** was carried out using a Gynkotek HPLC system with either a Gynkotek UVD170S/340S UV/visible detector or a Berthold Radioflow LB509 detector. The limits of detection were approximately 5% of parent compound metabolised for UV detection and 1% for radiochemical detection. A Waters QuattroMicro MS was used for Multiple Reaction Monitoring (MRM) of the positive parent ion and specific product ions. The limit of detection for MS was <1%.
- **Protein Binding:** ^{14}C-labelled aromatic amines were incubated with an NADPH-regenerating system and scintillation counting was used to quantify the amount of ^{14}C-PPD which remained bound to microsomal protein following repeated washes with acetonitrile.

Results

- When PPD was incubated with isolated human hepatocyes, metabolites corresponding to *N*-acetyl and *N,N'*-diacetyl PPD (Figure 1) were detected. When PTD was incubated with female human hepatocytes, monoacetylation, but not diacetylation, was detected. Neither compound gave rise to monohydroxylated or dihydroxylated metabolites, sulphates or glucuronides.
- A number of metabolites were observed when 2-AF was incubated with human liver microsomes in the presence of an NADPH regenerating system (Figure 2 (a)) or with recombinant CYPs from a bacterial expression system (Figure 2 (b,c) and Table 1). In contrast, no mono-oxygenated products were detected when ^{14}C-PPD was incubated with human liver microsomes, (Figure 3 (a)) or recombinant CYP isozymes. An example is shown in Figure 3 (b,c). As with PPD, incubation of PTD with pooled human liver microsomes revealed no evidence for the production of oxidative metabolites. Furthermore, when PTD was incubated with recombinant CYP isozymes no metabolites were detected.
- In protein binding experiments, neither PPD nor PTD bound to microsomal proteins in an NADPH-dependent fashion (Table 2).

Discussion

The results presented here indicate that PPD and PTD are metabolised to acetylated, but not hydroxylated, metabolites, over up to four hours' incubation with human hepatocytes. Furthermore, no evidence for the formation of mono-oxygenated metabolites of PPD or PTD was found using human microsomes or recombinant CYP isozymes. The results of our experiments provide no evidence that either PPD or PTD is *N*-hydroxylated by hepatic CYPs to a metabolite that could lead to a proximate carcinogen. These results are inconsistent with the hypothesis that these compounds play a causal role in the development of bladder cancer in users of permanent hair dyes via a mode of action involving hepatic metabolism to *N*-hydroxylarylamines.

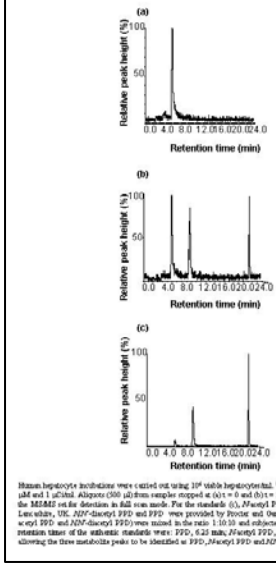
Table 1: Metabolism of 2-AF to hydroxylated metabolites by recombinant CYPs

	Specific activity (nmol metabolite formed/pmol P450/hour (%))				
	7-OH	**5-OH**	**3-OH**	**1/*N*-OH**	**Total**
CYP1A1	0.16 (14.0%)	BLQ (0%)	0.37 (32.5%)	0.61 (53.5%)	1.14 (100%)
CYP1A2	0.22 (20.4%)	0.04 (3.7%)	0.64 (59.3%)	0.18 (16.7%)	1.08 (100%)
CYP1B1	0.04 (9.8%)	BLQ (0%)	0.14 (34.1%)	0.23 (56.1%)	0.41 (100%)
CYP2C9	0.12 (100%)	BLQ (0%)	BLQ (0%)	BLQ (0%)	0.12 (100%)
CYP2C19	0.20 (33.9%)	BLQ (0%)	0.33 (55.9%)	0.06 (0.1%)	0.59 (100%)
CYP2D6	0.26 (89.7%)	BLQ (0%)	0.03 (10.3%)	BLQ (0%)	0.29 (100%)
CYP3A4	0.01 (50%)	BLQ (0%)	BLQ (0%)	0.01 (50%)	0.02 (100%)

Table 2: Covalent binding of ^{14}C labelled aromatic amines to microsomal proteins

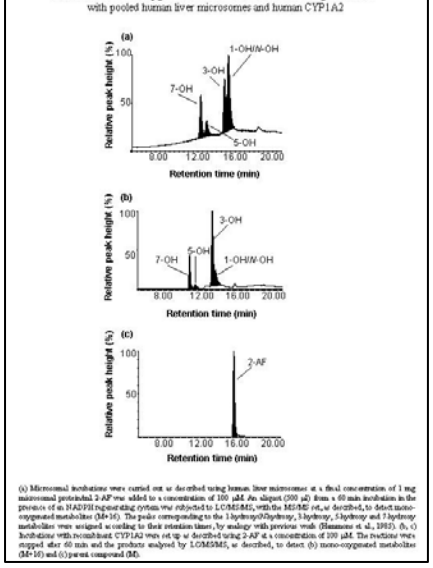
		Amount bound (pmol/mg protein/hour)	
(a) ^{14}C-PPD	**+/- NADPH regenerating system**	**Exp. 1**	**Exp. 2**
40 µM	+	271	273
	-	343	413
130 µM	+	845	779
	-	1077	1165
(b) ^{14}C-PTD		**Exp 1**	
10 µM	+	422	
	-	489	
100 µM	+	937	
	-	1154	

Figure 1
Identification of PPD metabolites produced by pooled male human hepatocytes



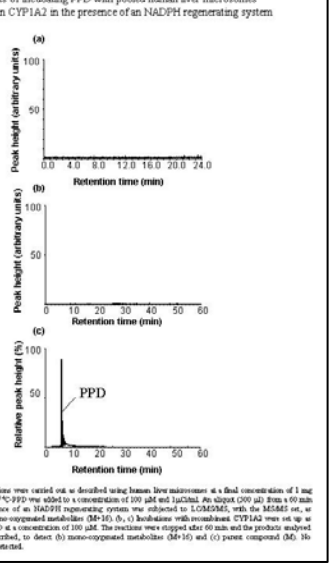
Human hepatocyte incubations were carried out using 10^6 viable hepatocytes/ml. ^{14}C-PPD was added to a concentration of 10 µM and 1 µCi/ml. Aliquots (500 µl from sampler stopped at (a) t = 0 and (b) t = 240 min were subjected to LC/MS/MS, with the MS/MS set for detection in full scan mode. For the standards (c), *N*-acetyl PPD was obtained from Lancaster Synthesis, Lancashire, UK. *N,N'*-diacetyl PPD and PPD were provided by Procter and Gamble, Cincinnati, Ohio. Standards (PPD, *N*-acetyl PPD and *N,N'*-diacetyl PPD) were mixed in the ratio 1:10:10 and subjected to LC/MS/MS analysis as described. The retention times of the authentic standards were: PPD, 6.25 min; *N*-acetyl PPD, 10.36 min; *N,N'*-diacetyl PPD, 22.91 min, allowing the three metabolite peaks to be identified as PPD, *N*-Acetyl PPD and *N,N'*-diacetyl PPD, respectively.

Figure 2
Detection of mono-oxygenated metabolites of 2-AF following incubation with pooled human liver microsomes and human CYP1A2



(a) Microsomal incubations were carried out as described using human liver microsomes at a final concentration of 1 mg microsomal protein/ml. 2-AF was added to a concentration of 100 µM. An aliquot (500 µl) from a 60 min incubation in the presence of an NADPH regenerating system was subjected to LC/MS/MS, with the MS/MS set, as described, to detect mono-oxygenated metabolites (M+16). The peaks corresponding to the 1-hydroxy/*N*-hydroxy, 3-hydroxy, 5-hydroxy and 7-hydroxy metabolites were assigned according to their retention times, by analogy with previous work (Hammond et al., 1993). (b, c) Incubations with recombinant CYP1A2 were set up as described using 2-AF at a concentration of 100 µM. The incubations were stopped after 60 min and the products analysed by LC/MS/MS, as described, to detect (b) mono-oxygenated metabolites (M+16) and (c) parent compound (M).

Figure 3
Results of incubating PPD with pooled human liver microsomes or human CYP1A2 in the presence of an NADPH regenerating system



(a) Microsomal incubations were carried out as described using human liver microsomes at a final concentration of 1 mg microsomal protein/ml. ^{14}C-PPD was added to a concentration of 100 µM and 1µCi/ml. An aliquot (500 µl) from a 60 min incubation in the presence of an NADPH regenerating system was subjected to LC/MS/MS, with the MS/MS set, as described, to detect mono-oxygenated metabolites (M+16). (b, c) Incubations with recombinant CYP1A2 were set up as described using ^{14}C-PPD at a concentration of 100 µM. The incubations were stopped after 60 min and the products analysed by LC/MS/MS, as described, to detect (b) mono-oxygenated metabolites (M+16) and (c) parent compound (M). No metabolite peaks were detected.