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Short communication

Pharmacogenetic determinants of human liver microsomal aminopyrine metabolism and the role of cytochrome P450 2D6

Salem O.A. Abdalla

Department of Pharmacology, Faculty of Pharmacy, Manara University, Latakia, Syria

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Abstract: Aminopyrine (AM) has been used as a model substrate for investigation of drug metabolism. The major metabolic route is N-demethylation that was confirmed in liver microsomes. The aim of the present study was to identify the human cytochrome P-450 enzyme (CYP) mediating the N-demethylation of 4-di-methylaminoantipyrine (4-DMAA) to 4-methylaminoantipyrine (4-MAA). The contribution of human CYP to the metabolism of (4-DMAA) to (4-MAA) in human was investigated using virus expressed human CYP, human liver microsomes with chemical inhibition studies. The substrate of 4-dimethylaminantipyrine was employed at different concentrations (11.5, 23, 46, 115 and 230 µmol per l) with varying concentrations of selective inhibitors of CYP (CYP1A2), (CYP3A4), (CYP2C8),(CYP2A6), (CYP2D6), (CYP2C19) and (CYP1A1). 4-DMAA and 4-MAA were analysed by HPLC and enzyme kinetic parameters (K_m and V_{max}) were calculated from the concentration data. The transformation of 4-dimethylaminoantipyrine to 4-methyaminoantipyrine by microsomes prepared from baculovirus-expressed human CYP was pronounced with CYP2D6. The metabolism of 4-dimethylaminoantipyrine was inhibited by 60.0% and 55.17% by a concentration of 100 µmo per l of the known CYP2D6 inhibitors quinidine and moclobemide. The corresponding K_i values were 0.050 and 0.11 mM, respectively. The corresponding IC_{50} values were 0.06 and 0.13 mM, respectively. The enzyme CYP2D6 apparently has an important role in N-demethyl-ation of 4-dimethylaminoantipyrine.

Introduction

Aminopyrine or amidopyrine 4-(dimethylamino)-1,2 dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one played a key role in the *in-vivo* study of human hepatic drug metabolism, because it was widely used as a probe of liver functionality [1, 2] and hepatocellular mass in several diseases such as cirrhosis, chronic hepatitis, hepato-carcinoma or liver ischemia [3 - 6]. Aminopyrine also called amidopyrine, pyrazolone, 4-dimethylaminoanti pyrine and antipyrine, is a pyrazolone class analgesic agent in otic solution (solution of ear) in combination with other analgesic such as benzocaine and phenylephrine [7, 8]. Amino-pyrine is a five-membered lactam ring compound containing two nitrogens and ketone in the same

molecule. The lactam structure is an active nucleus regarding pharmacological activity [9]. Cytochrome P450 (CYP) consists of a super-family of haemoproteins which act as the terminal oxidase of the mixed function oxidase system. Currently, not less than 500 CYP genes and 25 pseudogenes are exist across all the species. These genes are classified into families and subfamilies (designated by a letter) according to the amino acid identity of the encoded proteins [10]. Substrates for CYP2C9 include fluoxetine, losartan, phenytoin and numerous non-steriod anti-inflammatory drugs (NSAIDs) [11]. Thus, the aim of this study was to identify the human cytochrome P-450 enzyme mediating the N-demethylation of 4-di-methylaminoantipyrine (4-DMAA) to 4-methyl-aminoantipyrine (4-MAA).

Material and methods

All chemicals and reagents were of analytical grade unless stated otherwise. HPLC-grade acetonitrile and methanol were obtained from J. T. Baker (Mallinckrodt Baker, Holland), the other chemicals and reagents were obtained from following sources: Methyleaminoantipyrine, ketoconazole, alpha-naphthoflavone, omeprazole and sulphaphenazole were purchased from Sigma chemical (Steinheim, Germany) while coumarin and quinidine were obtained from (Fluka Steinheim, Germany). NADPH was purchased from Roche (Mannheim, Germany) (4-dimethylaminoantipyrine, 4-DMAA) was ordered from Sigma chemical (Steinheim, Germany). Potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany).

Microsomes and human P-450 isoforms: Baculovirus-derived microsomes expressing human P-450 CYP3A4/OR (Cat. No. P207, Lot 67), CYP3A5/OR (Cat. No. P235, Lot 21), CYP3A7/OR (Cat. No. P237, Lot 9), CYP1A1/OR (Cat. No. P211, Lot 22), CYP1A2/OR (Cat. No. P203, Lot 28), CYP2C9/OR (Cat. No. P242, Lot 3), CYP2C8/OR (Cat. No. P252, Lot 10), CYP2C19/OR (Cat. No. P219, Lot 19), CYP2D6/OR (Cat. No. P217, Lot 43), CYP2E1/OR (Cat. No. P206, Lot 19), CYP2A6/OR (Cat. No. P254, Lot 7) were all obtained from Gentest (Frankfurt, Main, Germany).

Preparation of microsomes: Human hepatic microsomes were prepared by fractionation as described previously [12]. Eight gram of liver per experiment was allowed to thaw at room temperature in homogenization buffer (Tris 20 mM, Na-EDTA 5 mM, sucrose 254 mM pH 7.4 in ice bath). The suspension was centrifuged at 9 000 g for 30 min and the resulting supernatant was further centrifuged at 105 000 g for 60 min in an ultracentrifuge. The microsomal pellets were suspended in 250 mM sodium/potassium phosphate buffer (pH 7.4) containing five mM EDTA and 30.0% glycerol (v/v). They were stored in aliquots at - 80 ^{o}C until used. Microsomal protein concentration was determined by the method of [12] with bovine serum albumin as a reference standard. The rat liver microsomes were prepared following the same procedures described for human liver microsomes and protein concentration was determined using the BCA method (Pierce Chemical Rocford, IL) [13].

*Incubation condition:*The HPLC system consisted of a L-600A pump (Merck, Hitachi Tokyo, Japan) and 655A - 40 auto sampler (Merck, Hitachi Tokyo, Japan). The system was equipped with LiChrospher 100 RP-8e select column (5 µm particle size, 100 Å pore size, 4 x 125 mm internal dimensions; Merck, Darmstadt, Germany) preceded by a pre-column (100 Diol, 5 µM). The mobile phase consisted of 80.0% (v/v) of 50 mM sodium phosphate buffer (pH 6.0), acetonitrile 19% (v/v) and 01.0% (v/v) methanol. The flow rate was 1.0 ml per min. The absorbance was measured at 254 nm, linked to computer data system with an ultraviolet (UV) detector (655 A Merck Hitachi Tokyo, Japan). The injection volume in these analyses was 40 µl, and the retention times of 4-methylaminoantipyrine (4-MAA), 4-dimethyl-aminoantipyrine (4-DMAA) and internal standard sulphaphenazole were 6.00, 7.70 and 9.6 minutes, respectively.

Results and discussion

Aminopyrine N-demethylation activity has been studied in humans mainly by use of the aminopyrine breath test, although in vitro studies remain to be done [14, 15]. Therefore, It is performed the complete *in-vitro* study on the metabolism of aminpyrine by human liver microsomes and specific human cytochrome P450 enzymes. Some formation of the 4-DMAA metabolite 4-MAA was observed in the incubations with CYP2D6, CYP2C19, CYP1A2, CYP1A1 and CYP1B1 and the highest formation of 4-MAA was observed with CYP2D6 as shown in **Figure 1**. The average of immuno-quantified levels of the various specific P-450s in human liver microsomal samples were 6.7, 25, 1.4, 1.2, 42, 33.63, 16.85, 31.29, 26.82, 96 and 2.0 pmol per mg proteins in human liver for the CYP2D6, CYP2C19, CYP1A1, CYP1B1, CYP1A2, CYP3A5, CYP2C8, CYP2E1, CYP2A6, CYP2C9, CYP3A4 and CYP3A7, respectively, [16, 17] and the extrapolated clearances via the specific human CYP enzymes were 6.67, 0.37, 1.14, 0.60, 0.34, 0.39, 0.19, 0.0, 0.0, 0.0, 0.0 and 0.0 l per min, respectively, as shown in **Figure 1**.

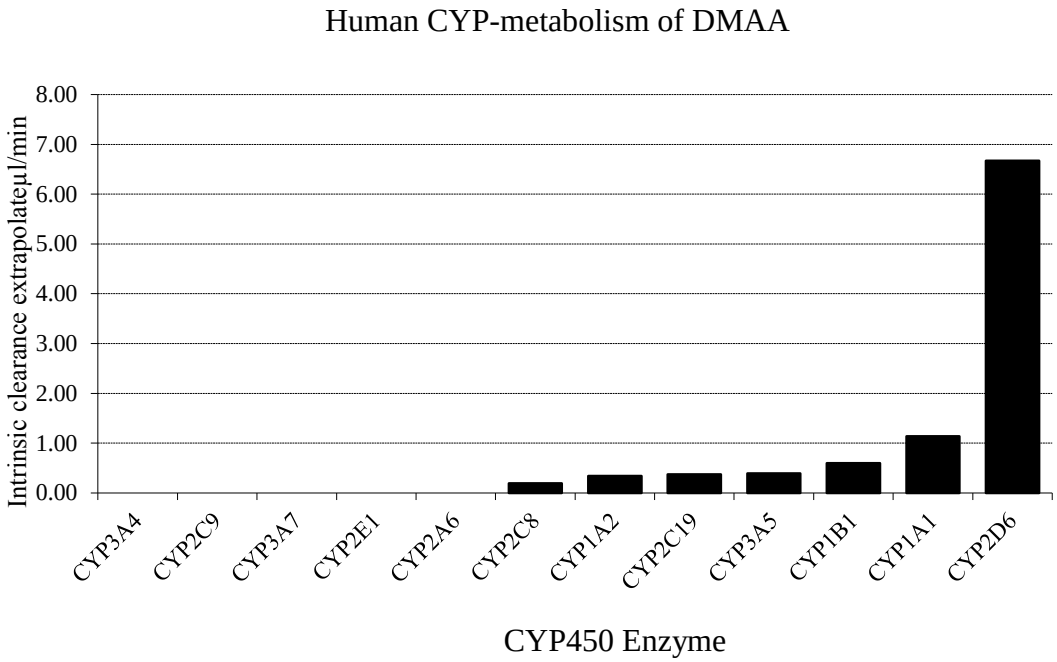

Figure 1: Calculated intrinsic clearance of 4-dimethylaminoantipyrine by specific human cytochrome P450 enzymes

The metabolism of 4-dimethylaminoantipyrine (4-DMAA) was studied in human liver microsomes [18, 19]. The formation of the 4-methylamin-oantipyrine (4-MAA) was measured by comparing the retention times with the synthetic standards. To determine the P450 reaction phenotyping of 4-dimethylamino-antipyrine, microsomes expressing individual recombinant human P450 isozymes (CYP1A1, CYP1B1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2-D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were incubated with different concentrations from 4-DMAA from 25 to 800 µmol per l in the presence of an NADPH-regenerating system at 37 °C for 20 min. Formation of 4-MAA was observed in the incubations with CYP2D6, CYP2C19, CYP1A2, CYP1A1 and CYP1B1 whereas the high formation of 4-MAA was observed only with CYP2D6 as shown in **Figure 2**.

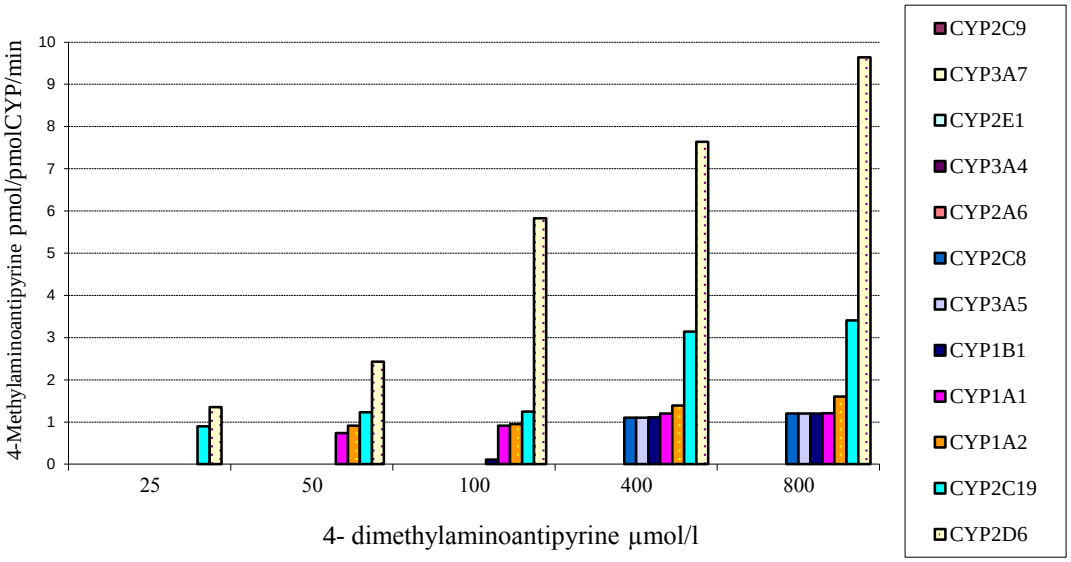

Figure 2: Cytochrome P450 isozymes involved in *in-vitro* demethylation of 4-dimethylaminoantipyrine

The formation rate of 4-methyaminoantipyrine (4-MAA) with rCYP2D6 was faster than with the other P450 isozymes. Also, the highest catalytic efficiency (intrinsic clearance, V_{max}/K_m) was observed with rCYP2D6 (0.011 µl/min/pmol) as illustrated in **Figure 1**. These *in-vitro* investigations indicate that cytochrome P4502D6 appeared to be the primary enzyme metabolizing 4-dimethylaminoantipyrine. And concerning CYP2D6 a similar and consistent effect was observed in all incubations in the present studies, namely, with chemical inhibitors and with the isolated enzymes as well. Since CYP2D6 is responsible for the metabolism of many commonly used drugs, this may mean that the so called poor metabolisers of substrates of CYP2D6 which is 07.0% in Caucasian populations (see introduction) are at a high risk for side effects of antipyrine. The present results obtained on the impact of CYP2D6 for the biotransformation and elimination of analgesic-antipyretic drugs complements with the results of many other previous studies showing that many antiarrhythmics, ß-receptor blockers, neuroleptics, anti-depressants, tamoxifen and codeine are metabolized by CYP2D6 [20, 21]. Thus, the current results are in line with the previous data that CYP2C19 is the most efficient enzyme in demethylation of aminopyrine and that CYP2C8 and CYP2D6 may also be involved [21].

Table 1: Inhibitory effects of CYP-specific inhibitors on 4-methylaminoantipyrine formation from 4-dimethylaminoantipyrine

Inhibitors	**HLM**		
	Inhibition (%)	K_i (mM)	IC_{50} (mM)
Quinidine	60.01	0.05	0.06
Moclobemide	55.17	0.11	0.13
Furafylline	28.25	0.22	0.26
Alpha-naphthoflavone	13.49	0.46	0.55
Ketoconazole	08.35	0.55	0.66
Coumarin	06.19	1.10	1.33

The extract from human liver microsomes obtained 20 min after incubation with 4-MAA with 50 µmol/l with 50 µM from chemical inhibitors was monitored by HPLC analysis

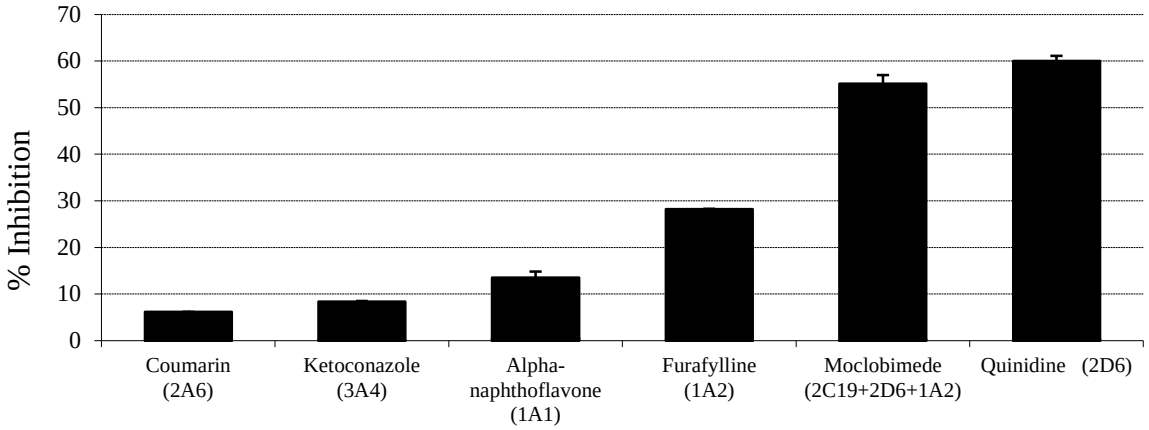

Figure 3: Inhibition of demethylation of 4-dimethylaminoantipyrine by selective chemical inhibitors of CYP450 isoenzymes

In **Figure 3**, the metabolism of 4-dimethyl-aminoantipyrine was inhibited by 60.0% and 55.17% by a concentration of 100 µmo per l of the known CYP2D6 inhibitors quinidine and moclobemide. The corresponding K_i values were 0.050 and 0.11 mM, respectively. The corresponding IC_{50} values were 0.06 and 0.13 mM, respectively. The IC_{50} values seen with furafylline, alpha-naphthoflavone, ketoconazole and coumarin were 0.26, 0.55, 0.66 and 1.33 mM and the k_i values were 0.22, 0.46, 0.55 and 1.10 mM, respectively, **Table 1**. This chemical inhibition data suggested that CYP2D6 enzyme was primarily responsible for the N-demethylation in the metabolism of 4-dimethylaminoantipyrine. The formation of 4-methylaminoantipyrine from dimethylaminoantipyrine was observed in the incubations with recombinant CYP2D6, CYP2C19, CYP1A1, CYP1B1, CYP1A2, CYP3A5 and CYP2C8 but the highest formation observed with CYP2D6 with an intrinsic clearance of 0.11 µl per pmol CYP per min. Intrinsic clearances via CYP2C19, CYP1A1 and CYP1B1 have significantly been lower with values of 0.02 µl per pmol CYP per min for all these three enzymes. In accord with the earlier data [21]. It is concluded that CYP2D6 may be clinically important enzyme responsible for the N-demethylation of 4-dimethyl-aminoantipyrine to 4-methylaminoanti-pyrine in hepatic biotrans-formation of amino-pyrine.

Conflict of interest: The author declares absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Ethical issues: Including plagiarism, informed consent, data fabrication or falsification and double publication or submission have completely been observed by authors.
Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.
Author declaration: The author confirm that all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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