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Study on the relationship between genetic polymorphisms of cytochrome CYP2C19 and metabolic bioactivation of dipyrone

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ABSTRACT

Dipyrone is a non-steroidal anti-inflammatory drug. It is a widely used and well tolerated analgesic drug which is however compromised by agranulocytosis as adverse effect.. The complex metabolism of dipyrone has been subject of many invivo studies. However, the specific cytochrome P450 enzymes involved catalysing the formation of 4-aminoantipyrine (4-AA) from 4-methylaminoantipyrine (4-MAA) is still not unequivocally identified. The aim of the present study was to identify the cytochrome P450 enzyme (CYP) mediating this reaction. The relevant CYP was identified using virus expressed isolated rat liver microsomes with chemical inhibition studies. The substrate of 4-methylaminantipyrine was employed at six different concentrations (25, 50, 100, 400, 800 and 1200 µmol/l) with 100 µmol/l of selective inhibitors of CYP1A2 (furafylline, fluvoxamine), CYP3A4 (ketoconazole), CYP2A6 (coumarin), CYP2D6 (Quinidine), CYP2C19 (omeprazole), CYP2C9 (sulphaphenazole) and CYP1A1 (alpha - naphthoflavone). 4-MAA and 4-AA were then analyzed by HPLC. The formation rates corresponding to the six substrate concentrations were subjected to a regression analysis in order to estimate KM and Vmax according to the Michaelis-Menten equation by nonlinear regression analysis with the program Sigma Plot. The results clearly demonstrated that the N-demethylation of 4-MAA by microsomes prepared from baculovirus-expressing CYP was pronounced with CYP2C19. Intrinsic clearance of the most active enzymes were 0.092, 0.027, and 0.026 for the CYP enzymes 2C19, 2D6 and 1A2 respectively. Metabolism by rat liver microsomes was strongly inhibited by omeprazole (IC50 of 0.05). The outcome of present study concluded that the enzyme CYP2C19 apparently has an important role in N-demethylation of 4- methylaminoantipyrine.

Key words: Dipyrone, 4-methylaminoantipyrine (4-MAA), 4- aminoantipyrine (4-AA), CYP2C19, Bioavailability of dipyrone.

INTRODUCTION

Dipyrone is a pyrazoline derivative available in oral and parenteral forms acting as cyclooxygenases inhibitor. It has been used as a non steroidal anti-inflammatory agent as well as a potent analgesic and antipyretic drug in many countries for more than 60 years [1]. Dipyrone, chemically [(2,3-dihydro-1,5 dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-yl) methylamino] methane sulphonic acid [2]. Dipyrone was first synthesized by the German company Hoechst

AG in 1920, and its mass production started in 1922. Oral doses of 0.5 to 1 g are effective in treating fever. Repeated doses (up to 4 times daily) can be administered, the maximum recommended dose is 3 to 4 g daily. Dipyrone is given orally as capsules or at tablets. Sometimes, users of dipyrone prefer to swallow the contents of an ampoule for parenteral administration, because they believe that the analgesic effect is quicker than with the usual solid oral forms [3]. In fact, dipyrone has many challenges. One of those challenges is the difficulties to ignore the fatal agranulocytosis. This serious side effect caused withdrawn dipyrone from the US market in 1979 [1]. It is non-enzymatically hydrolysis in the gastric juice to the active moiety 4-methylaminoantipyrine (4-MAA) [4,5]. After that the 4-MAA undergoes demethylation in the liver to 4-aminoantipyrine (4-AA) . Finally the 4-AA (Figure1) undergoes further phase-II biotransformation to acetyl-aminoantipyrine (4-AAA) by the polymorphic N-acetyltransferase [6,7,8]. CYP2C19 is a clinically important enzyme which metabolizes a wide variety of drugs, such as the anticonvulsant phenytoin [9], anti-ulcer drugs such as omeprazole [10], certain antidepressants [11], the anti malarial proguanil [12] and the anxiolytic drugs diazepam [13] . It is also partially responsible for the metabolism of a number of other drugs, such as the β-blocker propranolol. Those metabolized to a significant extent by CYP2D6 and/or CYP2C19, therefore potentially susceptible to genetic polymorphism [14]. In general, individuals can be characterized as extensive metabolizers or poor metabolizers of drugs metabolized by CYP2C19 in population studies. Poor metabolizers represent 2-5 % of Caucasians, 13- 23 % of Asian populations and as many as 38- 79 % of individuals of some of the islands of Polynesia and Micronesia [15]. African population have been studied less extensively, but the poor metabolizer trait has been reported to be approximately 40% in African-Americans from mid- Tennessee [16], Africans from Zimbabwe and Nigerians. Poor metabolizers can experience undesirable side- effects, such as prolonged sedation and unconsciousness after administration of diazepam. This can be a particular problem in Asian patients where the poor metabolizer phenotype is frequent. In contrast, omeprazole has been reported to produce a greater cure rate for gastric ulcers and accompanying Helicobacter pylori infections in CYP2C19 poor metabolizers than in extensive metabolizers because blood levels are higher in these individuals [17].

Interestingly, The biotransformation pathway of dipyrone is well established [18,19] but there is a little information in the literature about the specific CYP enzymes. The aim of the current study was to identify the specific cytochrome P450 enzymes involved catalysing the formation of 4-aminoantipyrine (4-AA) from 4-methylaminoantipyrine (4-MAA). We hypothesised that the knowledge of the relevant specific CYP enzymes may help in predicting drug-drug interactions and also might assist in elucidating the relevant bioactivation reactions which result in agranulocytosis in some individuals.

EXPERIMENTAL SECTION

Chemicals:

4-aminoantipyrine, and Methylaminoantipyrine were obtained from Sigma chemical, Steinheim, Germany. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest analytical grade available.

Microsomal Preparations:

Human liver microsomes (HLM) and rat liver microsomes (RLM) are used widely to characterize the role of cytochrome P450s (P450) and other enzymes in drug metabolism.

The differential centrifugation procedure used to prepare HLM is as follows: Typically, liver samples are homogenized and centrifuged at a lower force to form a crude pellet of cell debris, nuclei, peroxisomes, lysosomes, and mitochondria (premicrosomal pellet). The resulting supernatant is then centrifuged at a higher force to precipitate the microsomes. The microsomal pellet is resuspended in a final suspension buffer and is then ready for use in incubation studies. The samples of rat liver from male Wistar rats weighing between 180 to 220 g, aged 3 months were kindly provided by the Department of Toxicology at the University of Gottingen in Germany. Rat liver microsomes (RLM) pre-diluted in the same phosphate buffer (Sigma, UK) were added in a volume 25 µl following a previously described method [20].

Preparation of Dipyrone Solution

The protocol of samples were prepared by dissolving specified weight of dipyrone in 10 ml of deionized water to produce dipyrone solution (500mg/L). Dipyrone solution was then rediluted in 50 mM potassium phosphate buffer (pH 7.4). From the stock solution concentrations of 25 to 1200 µM. were prepared.

Incubations with heterologous expressed isolated CYP450s
Firstly, the recombinant CYP450 enzymes, pre-diluted in the 50 mM potassium phosphate buffer (pH 7.4). 25 µl of the recombinant cytochrome P450 enzymes (0.4 - 0.6 pmol/ µl) or RLM (5 - 10 mg protein /ml) were added to 50 µl of serial dilution of dipyrone solution (ranged from 25 to 1200 µM) prior to the start of the reaction with NADPH as a previously described method [20]. The reaction was initiated by the addition of 25 µl of freshly prepared 10 mM NADPH (in the same incubation buffer) to the reaction mixture in a final volume of 100 µl. The reaction allowed to proceed for 20 min at 37°C in opened Eppendorf tubes in a shaking water bath and then stopped by adding 100 µl of ice-cold acetonitrile (Sigma, UK). The resulting mixtures were centrifuged at 13000 rpm for 5 min. 100 µl of the produced metabolites were identified and quantified using HPLC analysis. Enzyme kinetic data analysis was finally used to determine enzyme kinetic parameters. The formation of 4-aminoantipyrine was linear with time between 6 and 10 min.

Determination of inhibition characteristics.
To further investigate the role of genetic polymorphisms of cytochrome CYP2C19 on metabolic bioactivation of dipyrone, biotransformation studies were also performed in the presence of selective chemical inhibitors. In briefly, the Dipyrone was incubated for 20 min together with 50 µmol/l of the selective chemical inhibitors ketoconazole (CYP3A4), alpha-naphthoflavone (CYP1A1), coumarin (CYP2A6), omeprazole (CYP2C19), quinidine (CYP2D6), tranylcypromine (CYP2C19), fluvoxamine (CYP1A2 and CYP2C19), furafylline (CYP1A2), moclobemide (CYP2C19) and (CYP2D6) and sulphaphenazole (CYP2C9).

Calculation of enzyme kinetic constants V_{max} and K_M
From the HPLC-quantified 4-AA the formation rate (pmol / mg / min) was calculated. The formation rates corresponding to the six substrate concentrations were then subjected to a regression analysis in order to estimate K_M and V_{max} according to the Michaelis- Menten equation by nonlinear regression analysis with the program Sigma Plot.

HPLC analysis and chromatographic conditions
HPLC for analysis of dipyrone metabolites was performed similar as described earlier [8, 21]. The incubation mixtures were centrifuged at 14000 rpm for 5 min. Supernatants were transferred into new tubes and 100 µl used for HPLC analysis. 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 a LiChrospher 100 (Å pore size) RP-8e select column with 5 µm particle size (Merck, Darmstadt, Germany) with internal dimensions of 4 mm x 125 mm preceded by a pre-column (100 Å, diol coated, 5 µM particle size) The mobile phase consisted of 75 % (v/v) of 50 mM sodium phosphate buffer (pH 6.0) and 25% (v/v) methanol. The flow rate was 1.0 ml/min. The absorbance was measured at 254 nm with an ultraviolet (UV) detector (655 A Merck Hitachi Tokyo, Japan) linked to computer data system. The injection volume in these analyses was 40 µl, and the retention times of 4-methylaminoantipyrine (4-MAA), 4-aminoantipyrine (4-AA), and internal standard 4-dimethylaminoantipyrine (4-DMAA) were 10.30, 7.70 and 16.75 minutes, respectively.

Statistical analysis
The results were statistically analyzed and the differences between groups was examined using one-way analysis of variance (ANOVA) and post-tests carried out using Fisher's pair wise comparisons via the statistical package Minitab TM 13 windows. Statistically significant differences were set at $p < 0.05$.

RESULTS AND DISCUSSION

Biotransformation was studied in the subcellular fraction termed microsomes, which is a fraction of membrane vesicles corresponding to the endoplasmic reticulum in the intact cell. Microsomes were isolated from rat liver tissues. The impact of genetic polymorphism in one of the enzymes apparently involved in biotransformation of the studied drug. CYP2C19 was analysed in the used rat liver samples and recombinant specific human CYP enzymes by allelic discrimination. The microsomes were incubated with the substrates dipyrone. The produced metabolites were identified and quantified using HPLC analysis.

Investigations of the metabolism of dipyrone by RLM
The 4-aminoantipyrine (4-AA) (peak 1, Fig. 2) and 4-methylaminoantipyrine (4-MAA) (peak 2, figure 2) have been measured with HPLC as illustrated in a chromatogram obtained from injection of 20 µl of standard solution with 25

µmol/l of 4-AA and 4-MAA, each. As shown in the figure 2, the separation was completed within 10 min. The retention times were 5.15 min for 4-AA and 6.78 min for 4-MAA.And the kinetic plot of demethylation of 4-methylaminoantipyrine as shown in the figure 3 by rat liver microsomes

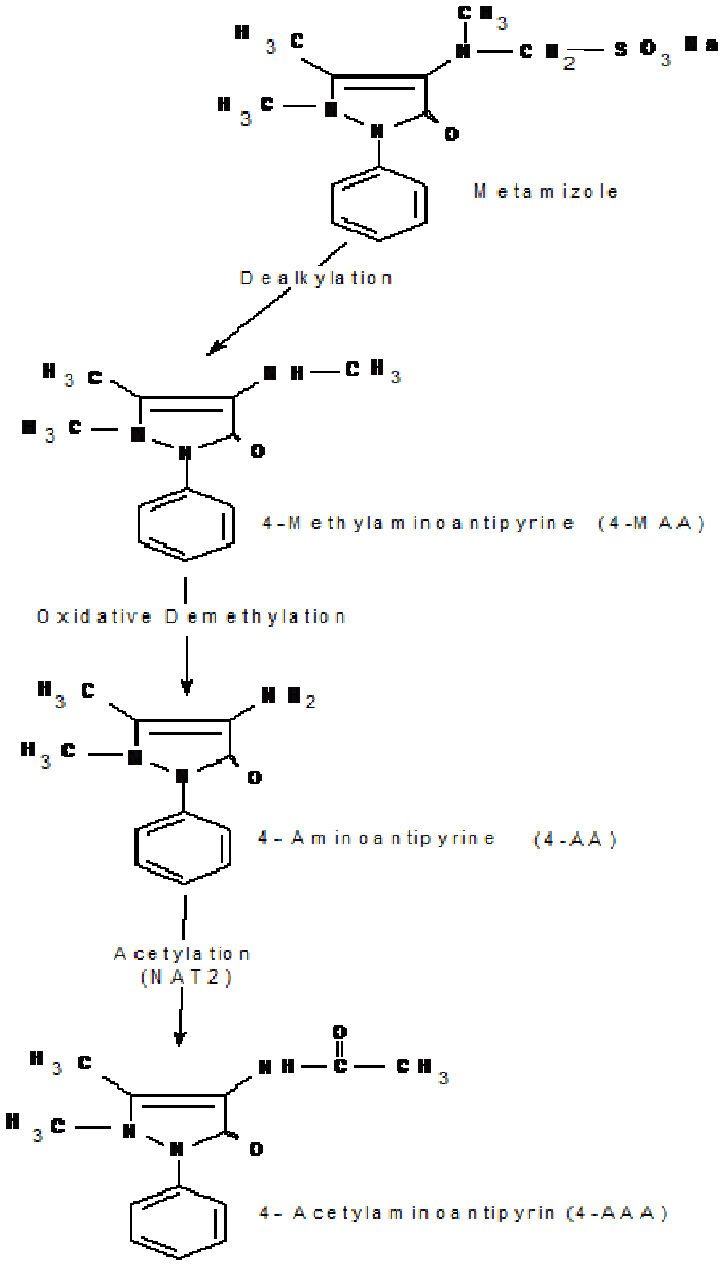

Figure 1: Structure and biotransformation of Dipyrone and its main metabolites in human, drawn according to (Geisslinger et al., 1996). Metamizole=Dipyrone

Investigations of the metabolism of dipyrone by recombinant CYP450s

To confirm the specific CYP enzyme involved in biotransformation of 4-methyl-aminoantipyrine the enzymes kinetics of metabolism of 4-metylaminoantipyrine were finally studied by recombinant specific human CYP enzymes, which appeared to be the enzyme CYP2C19 according to the inhibition study and according to one experiment with rat liver microsomes from a CYP2C19 deficient subject, microsomes expressing individual recombinant P450 isozymes (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6,

CYP2E1, CYP3A4, CYP3A5 and CYP3A7) were incubated with different concentrations of 4-MAA from 25 to 800 µmol/l in the presence of an NADPH-regenerating system at 37°C for 20 min. Some formation of 4-AA was observed in the incubations with (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7) whereas the high formation of 4-AA was observed only with CYP2C19 and with CYP1A2. the data are given in Figure 4.

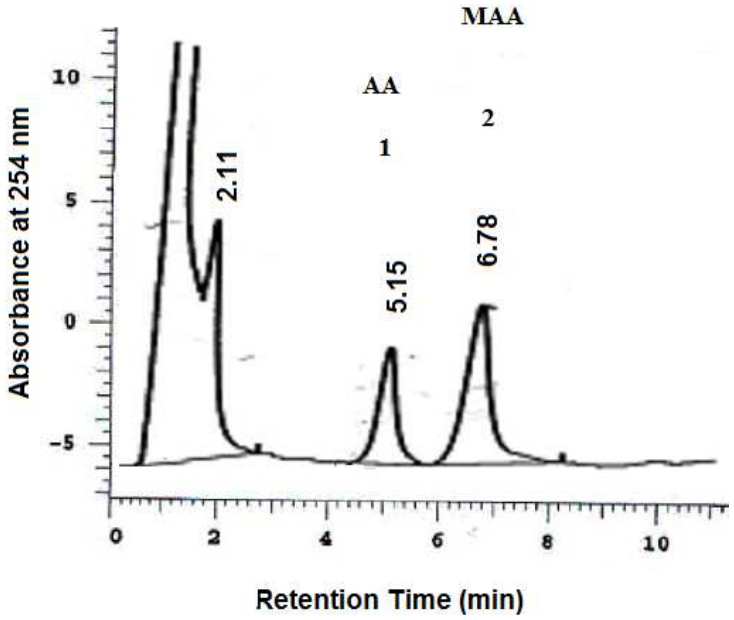

Figure 2: Shows the 4-methylaminoantipyrine and its metabolite formed by rat liver microsomes. A reaction mixture (200 µl) with 1.25 mg/ml (final concentration) of microsomal protein from male Wistar rats, 1.0 mg/ml of NADPH and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC. Peaks: 4-AA at 5.15 min and 4-MAA at 6.78 min

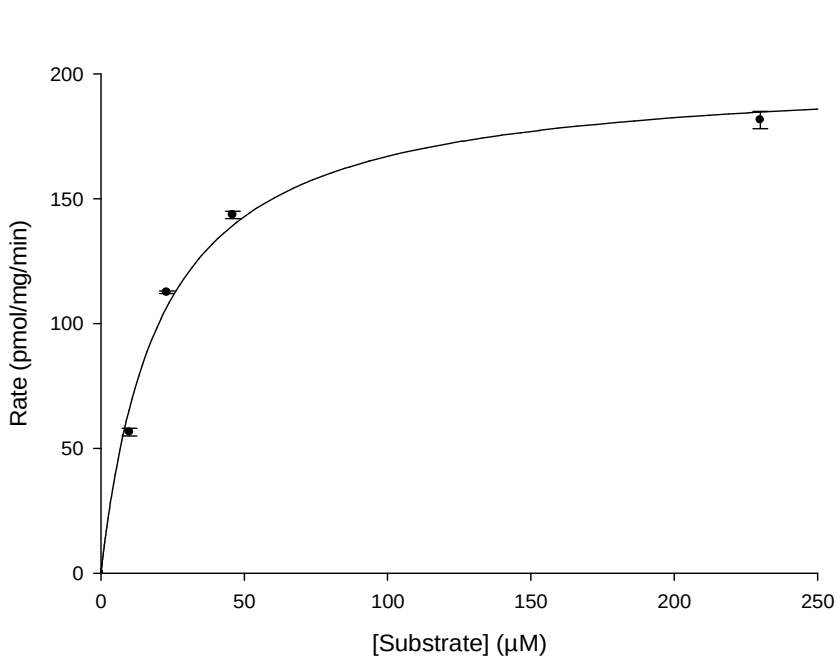

Figure 3: Kinetic plot of demethylation of 4-methylaminoantipyrine by RLM. The reaction mixture (200 µl) included 1.25 mg/ml (final concentration) of microsomal protein of male Wistar rats and 1.0 mg/ml of NADPH. 4-methylaminoantipyrine was incubated for 20 min at 37 °C in 25 mM potassium phosphate buffer (pH 7.4). 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC

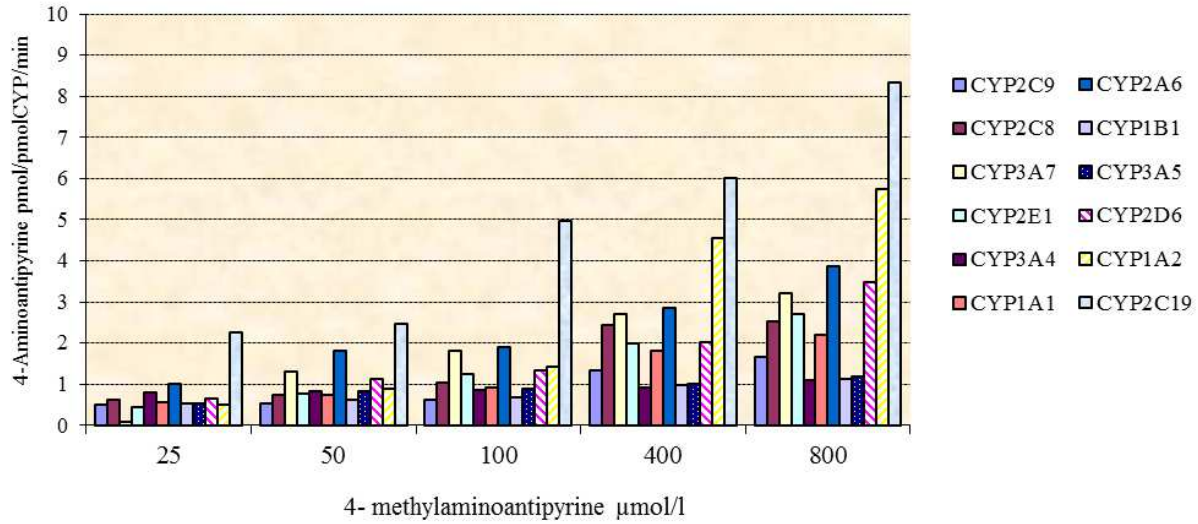

Figure 4: Cytochrome P450 isozymes obtained by *invitro* demethylation of methylaminoantipyrine. MAA was incubated with microsomes expressing human recombinant P450 isozymes (0.6 pmol/µl) for 20 min and the concentration of 4-methylaminoantipyrine was 25, 50, 100, 400, 800 µmol/l. The formation of 4-aminantipyrine AA was monitored by HPLC analysis with UV detection. Results are as average of duplicate incubation

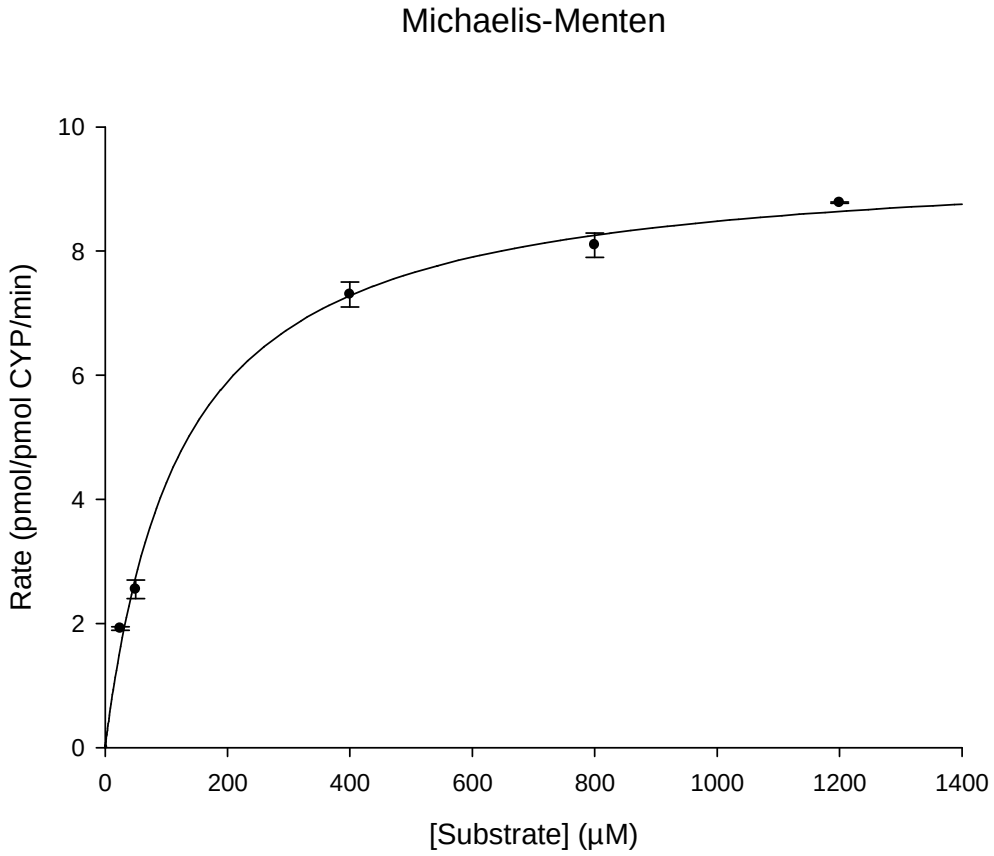

Figure 5: Kinetic plot of demethylation of 4-methylaminoantipyrine by recombinant Human CYP2C19 enzyme. MAA was incubated with human recombinant CYP2C19 enzymes (0.6 pmol/µl) for 20 min and the concentration of 4-methyaminoantipyrine was 25, 50, 100, 400, 800, 1200 µmol/l

The formation rates of 4-aminoantipyrine (AA) with rCYP2C19 were faster than that observed with the other P450 isozymes. Moreover, the highest catalytic efficiency (intrinsic clearance, V_{max}/K_m) was observed with CYP2C19, (0.077 µl/min/pmol) when compared with other P450 isozymes (Figure 5).

The average immunoquantified levels of the various specific P450s in rat liver microsomal samples were 25, 42, 1.2, 1.43, 6.7, 16.85, 17.88, 31.29, 2, 26.82, 33.63, and 96 pmol/mg proteins in rat liver for the (CYP1A1, CYP1B1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP3A7,CYP2E1, CYP3A4, CYP3A5 and CYP2C19 respectively). On the other hand, no metabolites were seen when 4-methylaminoantipyrine and microsomes were incubated without NADPH and with NADPH but without any incubation time (0 min). In rat liver microsomes, the metabolism of 4-methylaminoantipyrine was strongly competitively inhibited by a concentration of 50 µM omeprazole, the inhibition was (65.9 % inhibition) and to a lesser degree by ketoconazole (36.6 % inhibition) and but no inhibition was detected with alpha-naphthoflavone, coumarin, quinidine and sulphaphenazole as shown in Table.2. Our *invitro* investigations emphasised that, cytochrome P450 2C19 was a primary enzyme metabolizing of dipyrone (as shown in Table1). The data reported here pointed to the fact that CYP2C19 is clinically crucial enzyme responsible of the metabolism of a number of therapeutic agents . Our results were in agreement with that reported by Kappers et al [22] and by Shiraj et al [23].

Table 1: Enzyme kinetic parameters for Dipyrone demethylation by cytochrome P450. The dipyrone concentration range varied from 25 to 800 µM. The concentration of baculovirus-expressed enzymes was 0.6 pmol/µl. All data represent the mean of minimally two experiments

Enzyme	V_{max} (pmol/pmolCYP/min)	K_m (µmol/l)	Cl_{int} (µl/pmol CYP/min)	Cl extrapolated (l/min)
CYP2C19	9.5	123	0.077	4.634
CYP2D6	3.7	138	0.027	0.269
CYP1A2	8.1	317	0.026	1.199
CYP1A1	2.6	150	0.017	0.465
CYP2C8	4	245	0.016	0.413
CYP2A6	3.8	260	0.015	0.588
CYP2E1	2.3	169	0.014	0.510
CYP3A7	2.2	177	0.012	1.790
CYP2C9	2.2	216	0.010	0.031
CYP1B1	1.4	193	0.007	0.614
CYP3A5	1.5	263	0.006	0.012
CYP3A4	1.6	315	0.005	0.009

Table 2: Estimated % inhibition of the formation of 4-aminoantipyrine by selective chemical inhibitors added at a concentration of 50 µM inhibitor drugs. A reaction mixture (200 µl), 5 mg/ml of microsomal protein of male Wistar rats, and 50 µmol of 4-methylaminoantipyrine was incubated for 20 min at 37°C in 25 mM potassium phosphate buffer, (pH 7.4) 4-methylaminoantipyrine metabolites were extracted and analyzed by HPLC

RLM			
Inhibitors	**% Inhibition**	**K_i (mM)**	**IC_{50} (mM)**
Omeprazole	65.90	0.04	0.05
Ketoconazole	36.60	0.14	0.77
Sulphaphenazole	(no inhibition)	-	-
Coumarin	(no inhibition)	-	-
Quinidine	(no inhibition)	-	-
Alpha-naphthoflavone	(no inhibition)	-	-

CONCLUSION

CYP2C19 has been revealed to metabolize many clinically used drugs. Polymorphisms in this enzyme seriously affect the toxicity of drugs such as anxiolytic drugs diazepam and antiulcer drug omeprazole with increased efficacy at low doses of the drug. The outcome of present study point to conclusion that the enzyme CYP2C19 apparently has an important role in the metabolic biotransformation of a potent antipyretic drug dipyrone. These polymorphisms continue to be studied with respect to their effects on toxicity and efficacy of clinically used drugs.

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