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# Influence du cytochrome P450 2D6 sur le métabolisme de la TFMPP (1-(3-trifluoro-méthylphenyl)pipérazine) : étude à l'aide de modèles *in vitro* et *in vivo***

# *Influence of cytochrome P450 2D6 on the metabolism of TFMPP (1-(3-trifluoro-methyl-phenyl)piperazine) : studies using in vitro and in vivo techniques***

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# RÉSUMÉ

*La connaissance des isoenzymes du cytochrome P450 (CYP), qui sont responsables du métabolisme des xénobiotiques, est indispensable pour prédire la toxicocinétique et l'évaluation des risques toxicologiques. La 1-(3-trifluoro-méthyl-phenyl)pipérazine (TFMPP) est une nouvelle drogue synthétique. Des études antérieures ont montré qu'elle est principalement metabolisée en hydroxy-TFMPP (OH-TFMPP). L'objet de cette étude a été de vérifier, à partir de modèles* in vivo *et* in vitro, *si cette réaction est catalysée par le cytochrome CYP2D6. dans ce travail, nous avons comparé le rapport TFMPP / OH-TFMPP dans l'urine de rats femelles Dark Agouti (fDA, modèle du phénotype CYP2D6 métaboliseur lent), de rats Wistar (WI, modèle du phénotype CYP2D6 métaboliseur rapide) et de rats males Dark Agouti (mDA, modèle intermédiaire). Les résultats montrent clairement l'influence de cette enzyme. L'identification des CYP450 hépatiques humains a été réalisée in vitro à l'aide de microsomes hépatiques humaines et d'enzymes humaines recombinantes. Ces études ont montré que CYP1A2, CYP2D6 et CYP3A4 catalysent cette réaction, CYP2D6 étant l'enzyme la plus importante, responsable à 81 % de la clairance hépatique. Des études d'inhibition ont confirmé ces résultats. La formation de métabolites était par ailleurs, significativement plus basse dans les microsomes des métaboliseurs lents. Les concentrations plasmatiques des différentes souches des rats ont été comparées pour vérifier si les pharmacocinétiques étaient différentes. Les rats WI avaient les concentrations les plus faibles. Elles étaient déja plus élevées chez les rats mDA et de loin plus importantes chez les rats fDA.*


***MOTS-CLÉS :***
*1-(3-trifluoro-méthyl-phényl)pipérazine, TFMPP, métabolisme, cytochrome P450, rats Wistar, rats Dark Agouti.*


# SUMMARY

*Knowledge of the cytochrome P450 (CYP) isoenzymes responsible for the metabolism of xenobiotics is indispensable for prediction of toxicokinetics and for risk assessment. 1-(3-trifluoro-methyl-phenyl)piperazine (TFMPP) is a new designer drug. Previous studies had shown that TFMPP was mainly metabolized by aromatic hydroxylation to hydroxy TFMPP (HO-TFMPP). In this study,* in vivo *and* in vitro *methods were used in order to investigate whether this reaction is catalyzed by CYP2D6. Therefore, the peak area ratios TFMPP/HO-TFMPP in urine of female Dark Agouti rats (fDA), a model of the human CYP2D6 poor metabolizer phenotype, male Dark Agouti rats (mDA), an intermediate model and Wistar rats (WI), a model of the human CYP2D6 extensive metabolizer phenotype, were compared. The results suggested the influence of CYP2D6. In vitro methods, different human hepatic microsomal preparations and recombinant human enzymes, were used in order to identify the human hepatic CYPs. These studies showed that CYP1A2, CYP2D6 and CYP3A4 catalyzed the monitored reaction, with CYP2D6 being the most important enzyme responsible for 81 % of the net intrinsic clearance, which was confirmed by inhibition studies. Furthermore, metabolite formation was significantly lower in microsomes of a poor metabolizer. In order to study whether there are differences in the pharmacokinetic, blood plasma levels of the different rat strains were compared. WI showed the lowest blood levels, fDA the highest and mDA had blood levels on between.*


***KEY-WORDS :***
*1-(3-trifluoro-methyl-phenyl)piperazine, TFMPP, metabolism, cytochrome P450, Wistar rats, Dark Agouti rats.*

# Introduction

1-(3-trifluoromethylphenyl)piperazine (TFMPP) is a new designer drug of the class of piperazine-derived compounds, which was temporarily placed into Schedule I of the Controlled Substance Act in the United States of America (1). Its effects have been described to be similar to those of classical designer drugs (2). The involvement of particular cytochrome P450 (CYP) in the biotransformation of a new drug is needed for risk assessment. Previous in vivo studies in male Wistar rats (WI) showed that TFMPP was mainly metabolized by aromatic hydroxylation to hydroxy TFMPP (HO-TFMPP) (Figure 1) followed by partial glucuronidation or sulfatation (3). The first aim of the work presented here was to study, whether TFMPP hydroxylation may also be catalyzed by polymorphically expressed CYP2D6. For this purpose a rat model was used which has been proposed as a preliminary screening for CYP2D6 substrates (4, 5).

Female Dark Agouti rats (fDA) were chosen as a model of the human CYP2D6 poor metabolizer phenotype (PM), WI as the corresponding model of the human CYP2D6 extensive metabolizer phenotype (EM) (5, 6) and male Dark Agouti rats (mDA) as an intermediate model between the PM and EM models (7). The second aim was to identify the human hepatic CYPs catalyzing TFMPP hydroxylation, to determine the kinetic constants for this reaction, to draw conclusions concerning the intrinsic clearances, and to compare the metabolite formation in pooled human liver microsomes (pHLM) and in single donor human liver microsomes with PM genotype (PM HLM). Finally, plasma levels in the above mentioned rats were compared in order to get hints for possible differences in pharmacokinetics in human PM and EM subjects relevant for risk assessment.

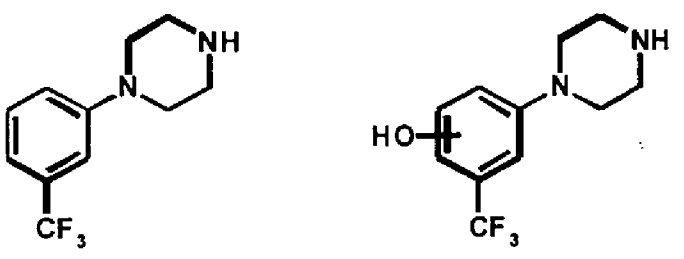


***Figure 1 :*** *Structure of TFMPP and its major metabolite HO-TFMPP.*

# Materials and Methods

## Materials

TFMPP-HCl and mCPP-HCl were obtained from Lancaster Synthesis, quinine and quinidine were obtained from Promochem, $NADP^+$ was obtained from Biomol, isocitrate and isocitrate dehydrogenase from Sigma, all other chemicals and reagents from Merck. The following microsomes were from Gentest and delivered by NatuTec: baculovirus-infected insect cell microsomes containing 1 nmol/ml human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 (Supersomes), wild-type baculovirus-infected insect cell microsomes (control Supersomes), pHLM (20 mg microsomal protein/ml, 400 pmol total CYP/mg protein) and PM HLM (20 mg microsomal protein/ml). After delivery, the microsomes were thawed at 37° C, aliquoted, snap-frozen in liquid nitrogen and stored at -80° C until use.

## Animals, treatments and collection of urine and blood samples

The rats were housed in metabolism cages for 24 hours, having water ad libitum The investigations have been performed as follows: urine samples (n = 8) were collected separately from the faeces over a 24 h period, blood samples (n = 4-6) were taken from the tail vein 1, 3, 5, 7, and 9 hours after administration of a single dose of TFMPP (20 mg/kg body mass, BM) in aqueous solution by gastric intubation. Furthermore, WI were pretreated with quinine (80 mg/kg BM) (8) before administration of TFMPP (20 mg/kg BM) and blood samples were taken as described above. All samples were directly analyzed as described below.

## Sample preparation of rat urine and rat plasma samples

The rat urine and blood plasma samples were prepared according to a validated procedure with modifications (9). The samples were extracted using mixed-mode HCX SPE cartridges and thereafter heptafluorobutyrylated. Details are described in ref. (10).

## Gas-chromatography-mass spectrometry (GC-MS) analysis of rat urine and plasma samples

The samples were analyzed using a Hewlett Packard (Agilent) HP 6890 Series GC system equipped with a HP-5MS capillary column (30 m x 0.25 mm I.D.), combined with an HP 5972 Series mass selective detector, an HP 6890 Series injector and an HP Chem Station G1701AA version A.03.00. Detailed information on the GC-MS conditions is given in ref.(10). In rat urine samples, the peak area ratios (PAR) between TFMPP and its metabolite HO-TFMPP were determined. In rat plasma samples the PAR between TFMPP and and mCPP (IS) were determined.

## Microsomal incubations

The incubations were conducted at 37° C. Typical incubation mixtures (final volume: 50 µl) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM $Mg^{2+}$, 5 mM isocitrate, 1.2 mM $NADP^+$, 2 U/ml isocitrate dehydrogenase, 200 U/ml superoxide dismutase and substrate which was added after dilution of a 250 mM methanolic stock solution in buffer. Reactions were started by addition of the ice-cold microsomes and terminated with 5 µl of 60 % (w/w) perchloric acid, 1 µl of 0.5 mM methanolic solution of mCPP was added as internal standard the incubation mixtures were centrifuged and the supernatants were transferred to autosampler vials.

## Initial screening studies

In order to investigate the involvement of particular CYPs in TFMPP metabolism, 200 µM TFMPP and 50 pmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 were incubated for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM or 90 mM Tris-buffer, according to the Gentest manuals.

## Enzyme kinetic studies

Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations (n = 2 each) with the following TFMPP concentration ranges, incubation times and protein concentrations: 1 - 1500 µM TFMPP with 50 pmol CYP1A2/ml for 20 min ; 0.5 - 600 µM TFMPP with 50 pmol CYP2D6/ml for 20 min ; 10 - 1300 µM TFMPP with 80 pmol CYP3A4/ml for 20 min and 1 - 1500 µM TFMPP with 0.5 mg pHLM protein/ml for 20 min. Less than 20 % of substrate were metabolized in all incubations. Apparent $K_m$ and $V_{max}$ values for single isoforms were estimated by nonlinear regression according to the Michaelis-Menten equation. A two site binding model was applied to the data of the HLM experiments (11, 12). The kinetic data were estimated using GraphPad Prism 3.02 software (San Diego, CA).

Unfortunately, no reference substances of the metabolites were available. Therefore, only relative estimations of $V_{max}$ values, expressed as dimensionless peak areas (PA) per minute and mg pHLM protein or pmol CYP for cDNA-expressed CYPs, could be obtained.

## Calculation of relative activity factors and of intrinsic clearance

Taking into account differences in functional levels of redox partners between the two enzyme sources, the relative activity factor (RAF) approach was used (13, 14). Reaction activities of probe substrates in both, insect cell microsomes and pHLM were taken from the supplier's data sheets. The RAFs and the percentage of intrinsic clearance by a particular isoform were calculated according to refs. (11, 13-16).

## Chemical inhibition studies

The effect of 3 µM quinidine on HO-TFMPP formation was assessed in incubations containing 0.5 mg pHLM protein/ml and 10 µM TFMPP. Controls contained no quinidine, but the same amount of methanol to control for any solvent effects (n = 6, each). Significance of inhibition was tested by a one-tailed unpaired t-test.

## Comparative studies between pHLM and PM HLM

Incubations were carried out at 10 µM TFMPP for 20 min using either 0.5 mg pHLM or PM HLM protein/ml. Significance of differences in metabolite formation was tested by a one-tailed unpaired t-test.

## Liquid chromatography-mass spectrometry (LC-MS) conditions and quantification in microsomal incubations

TFMPP, HO-TFMPP and mCPP were separated and quantified using an Agilent Technologies AT 1100 series atmospheric pressure chemical ionization (APCI) LC-MSD, SL version and a LC-MSD ChemStation using the A.08.03 software. Gradient elution was performed. The mobile phase consisted of ammonium formate (5 mM, adjusted to pH 3.0 with formic acid; eluent A) and acetonitrile (eluent B). Detailed information on the gradient, the flow rate, the APCI and MS conditions is given in ref. (10).

# Results

## Analysis of rat urine samples

In rat urine samples, the PAR of TFMPP vs. HO-TFMPP was determined (given values are the mean of the results of eight analyses ± standard error of the mean). The lowest ratio was determined in urine of WI (PAR TFMPP vs. HO-TFMPP: 0.08 ± 0.04) and the highest in urine of fDA (PAR TFMPP vs. HO-TFMPP: 3.48 ± 0.76). The ratio determined in urine of mDA lay between these two groups (PAR TFMPP vs. HO-TFMPP: 0.54 ± 0.18). The means of metabolic ratios of the three rat groups were significantly different among each other (comparison in pairs, $p < 0.0001$).

## LC-MS procedures

The applied LC-MS conditions provided baseline separation of HO-TFMPP, mCPP and TFMPP. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and IS) and zero samples (control microsomes without substrate, but with IS; data not shown).

## Initial screening studies

Among the nine CYPs tested only CYP1A2, CYP2D6 and CYP3A4 catalyzed the hydroxylation of TFMPP. HO-TFMPP was not detectable in incubations with the remaining cDNA-expressed CYPs or with insect cell control microsomes.

## Kinetic Studies

All incubations were carried out at initial rate conditions, a prerequisite for Michaelis-Menten kinetics (17). All of the kinetics with single cDNA-expressed CYPs showed a typical hyperbolic profile and the kinetic parameters are shown in Table I.

$V_{max}$ values could only be expressed as arbitrary units, because the metabolites could not be quantified without reference substances. The $V_{max}$ values are expressed as dimensionless PA per min and pmol CYP for cDNA-expressed CYPs or as PA per min and mg microsomal protein for HLM.

Calculation of the percentage of the different CYP isoforms to the predicted total TFMPP hydroxylation clearance by all individual CYPs in liver microsomes showed that CYP2D6 was the major isoform which catalyzed the monitored reaction. CYP2D6 accounted for 81 % of predicted total TFMPP hydroxylation clearance. CYP1A2 and CYP3A4 were responsible for only about 10 % each. More details are given in (10).

***Table I** : Kinetic data of TFMPP hydroxylation by CYP1A2, CYP2D6, CYP3A4 and pHLM.*

| | CYP1A2 | CYP2D6 | CYP3A4 | pHLM[a] |
|---|---|---|---|---|
| Apparent $K_m$ [µM] | 186.2 ± 32.3 | 7.8 ± 1.8 | 487.7 ± 98.9 | 11.2 ± 11.6 |
| Vmax [$min^{-1}$* pmol $CYP^{-1}$] or [$min^{-1}$* mg $HLM^{-1}$] | 2.3 ± 0.1 | 8.0 ± 0.4 | 1.5 ± 0.1 | 60.7 ±20.9 |

[a] Kinetic data estimated according to a two site binding.

## Chemical inhibition studies

In order to underline the importance of CYP2D6 in TFMPP hydroxylation, the CYP2D6 specific inhibitor quinidine (3 µM) was added to incubation mixtures and the metabolite formation rate compared with incubations without inhibitor. In presence of inhibitor and 10 µM TFMPP the metabolite formation was significantly inhibited by about 78 % ($p < 0.0001$).

## Comparative studies between pHLM and PM HLM

In order to further underline the importance of CYP2D6 in TFMPP hydroxylation and to demonstrate differences in PM and EM subjects, the metabolite formation rate of pHLM was compared to that of PM HLM. The metabolite formation rate of PM HLM was about 63 % lower ($p < 0.0001$) than that of pHLM.

## Analysis of rat plasma samples

In rat plasma samples, the PARs of TFMPP vs. mCPP were determined. As shown in Table. II, the blood plasma levels in fDA were significantly higher than those of WI ($p \leq 0.0013$) and of mDA ($p \leq 0.0164$). The blood plasma levels in mDA always lay in between fDA and WI and were always significantly higher than those of WI ($p \leq 0.004$). Pretreatment of WI with quinine led to significantly higher blood plasma levels at all time points ($p \leq 0.01$) compared to untreated WI.

***Table II** : Peak area ratios (PAR) of TFMPP/mCPP (IS) determined in blood plasma, after solid phase extraction and heptafluorobutyrylation, of fDA, mDA, and WI as well as of WI after pretreatment with 80 mg/kg BM of quinine taken 1, 3, 5, 7, and 9 hours after administration. Each value represents the mean of four samples ± standard error of the mean.*

| | 1 h | 3 h | 5 h | 7 h | 9 h |
|---|---|---|---|---|---|
| fDA | 0.66 ± 0.04 | 1.16 ± 0.27 | 1.437 ± 0.15 | 1.56 ± 0.19 | 0.85 ± 0.15 |
| mDa | 0.26 ± 0.07 | 0.53 ± 0.02 | 0.41 ± 0.01 | 0.17 ± 0.01 | 0.07 ± >0.01 |
| WI | 0.15 ± 0.01 | 0.22 ± 0.02 | 0.19 ± 0.01 | 0.08 ± >0.01 | 0.04 ± >0.01 |
| WI (pretreated) | 0.31 ± 0.04 | 0.53 ± 0.10 | 0.79 ± 0.19 | 0.73 ± 0.09 | 0.52 ± 0.11 |

# Discussion

Metabolism studies of new designer drugs are commonly conducted using rat models, due to ethical reservation about human studies (3, 18-21). In the current study, it was examined whether TFMPP hydroxylation may be catalyzed by CYP2D6 by comparing PAR of TFMPP vs. HO-TFMPP in urine from fDA, mDA and WI. These animals were proposed as a model allowing a preliminary screening for CYP2D6 substrates. The results showed that WI (EM model) excreted TFMPP mainly as the corresponding hydroxy metabolite, mDA (intermediate model) excreted TFMPP significantly less metabolized, with HO-TFMPP still being the main analyte and fDA (PM model) mainly excreted the unmetabolized parent compound TFMPP. These results suggested that TFMPP hydroxylation should be catalyzed by CYP2D6 in humans.

For confirmation, the human hepatic CYPs involved in TFMPP hydroxylation were identified using microsomal preparations from different sources. This method has already been described for other new designer drugs (11;16). According to the supplier's advice, the incubation conditions chosen were adequate to make a statement on a general involvement of a particular CYP isoform. Among the nine CYP isoforms tested only CYP1A2, CYP2D6 and CYP3A4 supported the hydroxylation of TFMPP.

The kinetic profiles of the reactions by these particular CYPs and pHLM were further investigated. As no reference substance of the monitored metabolite was available for its exact quantification, only PAs could be determined instead of absolute metabolite concentrations. However, this neither affected the conclusions drawn from the kinetic estimations, nor those from the inhibition studies. Linearity of the mass spectrometer response over the concentration range could be shown for structurally closely related compounds (data not shown), so that linearity of the mass spectrometer response of HO-TFMPP could be assumed.

As expected, classical hyperbolic Michaelis-Menten plots were found using cDNA-expressed CYPs. The apparent Km and Vmax values of the investigated CYPs were calculated by nonlinear regression fit according to the Michaelis-Menten equation. As more than one enzyme was involved in TFMPP hydroxylation, a two site binding model was applied to the data of the HLM experiments.

The RAF approach (11, 13, 14, 16, 22, 23) was used to correct recombinant CYP formation rates for native human liver enzyme activity and revealed that CYP2D6 accounted for 81 % of predicted total TFMPP hydroxylation clearance by all individual CYPs in pHLMs. CYP1A2 and CYP3A4 were responsible for only about 10 % each.

In order to confirm the role of CYP2D6 in TFMPP hydroxylation, inhibition studies with the CYP2D6 specific chemical inhibitor quinidine (6) were performed at a substrate concentration corresponding to the calculated Km,1 value in pHLM. The concentration of the inhibitor (3 µM) was based on average literature data (11, 16, 17, 24, 25). The results showed that the overall turnover was significantly inhibited by about

77 %, which was consistent with the observation, that CYP2D6 accounted for about 81 % of the net intrinsic clearance of TFMPP. Comparative studies using pHLM and PM HLM revealed significant differences between CYP2D6 PM and EM, with PM HLM showing 63 % lower metabolite formation rate.

In order to find out whether TFMPP plasma levels vary in PM and EM possibly resulting in different toxicological risks plasma levels in the described rat models were examined. The deficiency of fDA of hydroxylating TFMPP should led to increased blood plasma levels of the parent compound. In fact, fDA showed the highest and WI showed the lowest TFMPP blood plasma levels at all sample times and the plasma levels in mDA lay between these two groups. Pretreatment of WI with the the CYP2D specific inhibitor quinine (6, 26) resulted in significantly higher TFMPP plasma levels, a further confirmation that the differences could be attributed to the differences in CYP2D activity.

In summary, the studies showed that TFMPP hydroxylation is mainly catalyzed by CYP2D6. The animal studies indicated that CYP2D6 PM might exhibit a lower clearance than EM. Furthermore, simultaneous intake of potent CYP2D6 inhibitory drugs might also lead to a decreased clearance of TFMPP and, consequently, lead to elevated plasma concentrations. Whether this genetic polymorphism and/or drug interactions are of relevance for TFMPP pharmacokinetics and/or clinical outcome of intoxications, cannot be assessed at the moment due to lack of sufficient authentic human data.

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