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PHARMACOKINETICS AND DISPOSITION

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Inhibition of CYP2C9 by selective serotonin reuptake inhibitors: in vitro studies with tolbutamide and (S)-warfarin using human liver microsomes

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Abstract *Objective*: To investigate the in vitro potential of selective serotonin reuptake inhibitors (SSRIs) to inhibit two CYP2C9-catalysed reactions, tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation.
Methods: The formation of 4-hydroxytolbutamide from tolbutamide and that of 7-hydroxywarfarin from (S)-warfarin as a function of different concentrations of SSRIs and some of their metabolites was studied in microsomes from three human livers.
Results: Both tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation followed one enzyme Michaelis-Menten kinetics. Kinetic analysis of 4-hydroxytolbutamide formation yielded a mean apparent Michaelis-Menten constant (K_m) of 133 μM and a mean apparent maximal velocity (V_{max}) of 248 pmol · min^{-1} · mg^{-1}; formation of 7-hydroxywarfarin yielded a mean K_m of 3.7 μM and a mean V_{max} of 10.5 pmol · min^{-1} · mg^{-1}. Amongst the SSRIs and some of their metabolites tested, only fluvoxamine markedly inhibited both reactions. The average computed inhibition constant (K_i) values and ranges of fluvoxamine when tolbutamide and (S)-warfarin were used as substrate, were 13.3 (6.4–17.3) μM and 13.0 (8.4–18.7) μM, respectively. The average K_i value of fluoxetine for (S)-warfarin 7-hydroxylation was 87.0 (57.0–125) μM.
Conclusion: Amongst the SSRIs tested, fluvoxamine was shown to be the most potent inhibitor of both tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation. Fluoxetine, norfluoxetine, paroxetine, sertraline, desmethylsertraline, citalopram, desmethylcitalopram had little or no effect on CYP2C9 activity in vitro. This is consistent with in vivo data indicating that amongst the SSRIs, fluvoxamine has the greatest potential for inhibiting CYP2C9-mediated drug metabolism.

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Introduction

Selective serotonin reuptake inhibitors (SSRIs) interfere, both in vitro and in vivo, with cytochrome P450-mediated drug metabolism. Most studies on this subject concern interactions with SSRIs for pathways mediated by CYP1A2, CYP2C19, CYP2D6 and CYP3A4 [1]. Few data are available on the effect of SSRIs on CYP2C9-mediated drug biotransformation. Only one in vitro study investigated systematically the effect of SSRIs on CYP2C9 activity, using phenytoin as substrate [2]. p-Hydroxylation of phenytoin is mainly catalysed by CYP2C9 and to a lesser extent by CYP2C19, and therefore phenytoin cannot be considered as a model substrate for CYP2C9 [3]. The aim of this study was to evaluate the inhibitory potential of SSRIs and some of their metabolites on the in vitro metabolism of tolbutamide and (S)-warfarin using human liver microsomes. Tolbutamide was chosen as substrate because tolbutamide 4-methylhydroxylation is generally regarded as a marker of CYP2C9 activity [4]. (S)-warfarin, the pharmacologically active enantiomer of warfarin, was also studied as substrate because it is mainly metabolized by CYP2C9 to form 7-hydroxywarfarin [5]. We also examined in vitro, whether the potentiation of the anticoagulant effect of warfarin by some SSRIs, reported in vivo, can be explained by inhibition of (S)-warfarin's main metabolic pathway [6–8].

Materials and methods

Chemicals and reagents

β-NADP, D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, tolbutamide, 7-ethoxycoumarin, chlorpropamide and sulphaphenazole were purchased from Sigma Chemicals (St. Louis, Mo., USA). 4-Hydroxytolbutamide, (S)-warfarin and 7-hydroxywarfarin were obtained from Ultrafine Chemicals (Manchester,

UK). Sertraline hydrochloride and desmethylsertraline maleate (Pfizer Pharmaceuticals, New York, N.Y., USA), fluoxetine hydrochloride, norfluoxetine hydrochloride (Eli Lilly, Indianapolis, Ind., USA), fluvoxamine maleate (Solvay Duphar, Weesp, The Netherlands), citalopram hydrobromide, desmethylcitalopram hydrochloride (Lundbeck, Copenhagen, Denmark) and paroxetine hydrochloride (Smithkline Beecham Pharmaceuticals, Welwyn, UK) were obtained from the manufacturers.

Microsomal preparations

Liver samples from three healthy organ donors (aged 7, 32 and 62 years) were used, with the approval of the local medical ethics committee. The samples were sliced into portions and placed in vials, frozen in liquid nitrogen and stored at −80 °C until preparation of microsomes by differential ultracentrifugation [9]. The microsomal pellets were suspended in a 0.05 M potassium phosphate buffer (pH 7.4). The protein content of the microsomal preparations was determined by the method of Bradford [10]. Microsomal preparations were aliquoted, frozen and stored at −80 °C until use.

Incubation conditions for 4-methylhydroxylation of tolbutamide

Tolbutamide was incubated with microsomal protein (1 $mg \cdot ml^{-1}$) and glucose-6-phosphate dehydrogenase (2 $U \cdot ml^{-1}$) in 100 mM potassium phosphate buffer (pH 7.4). The final incubation volume was 500 µl. The 4-hydroxytolbutamide formation rate from tolbutamide was determined in a concentration range of 25 to 1000 µM, in three human livers (HL 7, HL 9 and HL 10). After pre-incubation (5 min at 37 °C) the reaction was initiated by the addition of 50 µl of a NADPH-generating system [NADP (1 mM), glucose-6-phosphate (10 mM) and $MgCl_2$ (5 mM), final concentrations]. After incubation at 37 °C for 30 min in a shaking water bath, the reaction was stopped by the addition of 50 µl $HClO_4$, 60%; 2.17 nmol chlorpropamide was added as internal standard. The samples were vortexed, followed by centrifugation at $16000 \times g$ for 4 min. The pH of the supernatant was adjusted with 5 N KOH to a value between 1.5 and 2. Samples were again vortexed and centrifugated at $16000 \times g$ for 4 min prior to injection of 25 µl of the supernatant onto the HPLC column. Because sulphaphenazole interfered in the HPLC assay of 4-hydroxytolbutamide, an extraction step was required (see below) after the addition of $HClO_4$.

In preliminary experiments it was ascertained that the formation of 4-hydroxytolbutamide from tolbutamide (1 mM) was linear up to 2 mg protein ml^{-1} incubation mixture during 30 min incubation time. The reaction was also linear throughout 40 min incubation for a microsomal protein content of 1 $mg \cdot ml^{-1}$.

Incubation conditions for 7-hydroxylation of (S)-warfarin

The incubation method for (S)-warfarin 7-hydroxylation was the same as that for tolbutamide 4-methylhydroxylation. (S)-warfarin was incubated in a final volume of 250 µl. The 7-hydroxywarfarin formation rate from (S)-warfarin was determined in a concentration range of 0.75–50 µM, in the same three human livers. After pre-incubation the reaction was initiated by the addition of 25 µl NADPH-generating system and terminated by the addition of 25 µl $HClO_4$, 60%. 7-Ethoxycoumarin (0.033 nmol) was added as internal standard. After centrifugation and pH adjustment as described for tolbutamide, 50 µl of the supernatant was injected onto the HPLC column.

In preliminary experiments it was ascertained that the formation of 7-hydroxywarfarin from (S)-warfarin (50 µM) was linear up to 2 mg protein ml^{-1} incubation mixture during 30 min incubation time. The reaction was also linear throughout 40 min incubation for a microsomal protein content of 1 $mg \cdot ml^{-1}$.

For the preparation of the (S)-warfarin stock solution [1 mM (S)-warfarin in 5 mM KOH], 0.0145 mmol (S)-warfarin was dissolved in 70 µl 1 M KOH and further diluted with water to a final volume of 14.5 ml. Each incubation mixture of (S)-warfarin contained a final concentration of 0.5 mM KOH; the final pH of the incubation mixtures was 7.4.

Inhibition experiments

The following compounds were tested in the same three livers for their inhibitory effect at substrate concentrations of 200 µM tolbutamide and 4 µM (S)-warfarin: sulphaphenazole (0.1–100 µM), citalopram (0.1–100 µM), desmethylcitalopram (0.1–100 µM), fluoxetine (0.1–100 µM), norfluoxetine (0.1–100 µM), fluvoxamine (0.1–100 µM), sertraline (0.1–100 µM), desmethylsertraline (0.1–30 µM) and paroxetine (0.1–100 µM). The experiments were repeated for those substances displaying marked inhibition, at different tolbutamide (25–1000 µM) and (S)-warfarin (0.75–50 µM) concentrations in order to determine the apparent inhibitory constant (K_i).

HPLC assay of 4-hydroxytolbutamide

4-Hydroxytolbutamide was quantified with an HPLC method according to Miners et al. with slight modifications [11]. The HPLC system consisted of a Gilson 307 pump and a Gilson 234 automatic injector (Gilson, Middelton, Wisc., USA) with a 100–µl loop (Rheodyne, Cotati, Calif., USA), a Waters 2487 tunable absorbance detector (Waters, Milford, Mass., USA) and an HP 3395 integrator (Hewlett Packard, Palo Alto, Calif., USA). A Symmetry C18 column (150 mm × 3.0 mm ID, 5 µm; Waters, Milford, Mass., USA) was used to separate 4-hydroxytolbutamide and the internal standard chlorpropamide at ambient temperature. The mobile phase consisted of a 10-mM sodium acetate buffer with 0.05 N triethylamine (adjusted to pH 4.3 with H_3PO_4), acetonitrile and methanol (71:25:4 v/v) mixture and was delivered at a flow rate of 0.6 $ml \cdot min^{-1}$. Detection was performed by UV absorbance at 230 nm. A calibration curve was constructed by spiking microsomes not containing the NADPH-generating system, with 4-hydroxytolbutamide from 0.49 to 13.90 µM. Height ratios were determined utilizing the internal standard method. The intra-day coefficients of variation ($n = 6$) at low (0.60 µM), medium (6.98 µM) and high (12.57 µM) 4-hydroxytolbutamide concentrations were all below 3%; the inter-day coefficients of variation ($n = 3$) at the low, medium and high concentrations were all below 7%.

The samples incubated with tolbutamide and sulphaphenazole were extracted after incubation and termination of the reaction by $HClO_4$. After centrifugation, tolbutamide, 4-hydroxytolbutamide and the internal standard were extracted from 400 µl incubation mixture in 2 ml ether by 3-min vortexing followed by centrifugation. The aqueous phase was discarded and the ether phase was evaporated to dryness; the residue was reconstituted in 300 µl mobile phase and 25 µl was injected onto the column. A calibration curve was also extracted.

HPLC assay of 7-hydroxywarfarin

7-Hydroxywarfarin was quantified with the HPLC method according to Lang and Böker with slight modifications [12]. The HPLC system consisted of a Gilson 307 pump and a Gilson 234 automatic injector (Gilson, Middelton, Wisc., USA) with a 100-µl loop (Rheodyne, Cotati, Calif., USA), a Waters 470 fluorescence detector (Waters, Milford, Mass., USA) and an HP 3395 integrator (Hewlett Packard, Palo Alto, Calif., USA). A Symmetry C18 column (150 mm × 3.0 mm ID, 5 µm; Waters, Milford, mass., USA) was used to separate 7-hydroxywarfarin and the internal standard 7-ethoxycoumarin at ambient temperature. The eluent consisted of a 0.05 N triethylamine (pH adjusted to 2.45 with H_3PO_4), acetonitrile and methanol (59:31:10 v/v) mixture; the flow rate was 0.8 $ml \cdot min^{-1}$. Detection was performed by fluorescence detection

at an excitation wavelength of 320 nm and an emission wavelength of 415 nm. A calibration curve was constructed by spiking microsomes not containing the NADPH-generating system, with 0.012 to 0.617 μM 7-hydroxywarfarin. Height ratios were determined using the internal standard method. The intra-day coefficients of variation ($n = 6$) at low (0.024 μM), medium (0.247 μM) and high (0.493 μM) 7-hydroxywarfarin concentrations were all below 2%; the inter-day coefficients of variation ($n = 3$) at the low, medium and high concentrations were all below 5%.

Data analysis

Formation rates were expressed as pmoles per minute per milligram of protein. The data points consisting of the 4-hydroxytolbutamide and 7-hydroxywarfarin formation velocities (v) at varying tolbutamide and (S)-warfarin concentrations, respectively, in the absence of inhibitor, were fitted by non-linear regression analysis to a one enzyme Michaelis-Menten equation:

$$v = \frac{V_{max} \times S}{K_m + S} \quad \text{(Eq. 1)}$$

V_{max} and K_m are the apparent maximal velocity and the apparent Michaelis-Menten constants, respectively. The drug concentration producing 50% decrease in metabolite formation rate (IC_{50}) was determined for the various inhibitors by visual analysis of plots of the percentage control activity vs inhibitor concentration.

For K_i determinations, data for inhibitors were also fitted to Eq. 1, yielding K'_m and V'_{max}, apparent kinetic parameters in the presence of inhibitor. K_i values were determined by least-squares linear regression analysis of secondary plots ("K'_m/V'_{max}" ratio as a function of inhibitor concentration) [13].

The data sets were fitted using an iterative program (Statistica, version 5.0, Statsoft, Tulsa, Okla., USA).

Results

Kinetic analysis of 4-hydroxytolbutamide and 7-hydroxywarfarin formation

As shown in Table 1, kinetic analysis of 4-hydroxytolbutamide formation from tolbutamide yielded a mean K_m of 133 μM and a mean V_{max} of 248 pmol · min^{-1} · mg^{-1}; formation of 7-hydroxywarfarin from (S)-warfarin yielded a mean K_m of 3.7 μM and a mean V_{max} of 10.5 pmol · min^{-1} · mg^{-1}. Eadie-Hofstee plots for both 4-hydroxytolbutamide and 7-hydroxywarfarin formation displayed a monophasic pattern (data not shown).

Inhibition experiments

In a first series of experiments, the formation rates of 4-hydroxytolbutamide and 7-hydroxywarfarin were measured in microsomal preparations of three different livers as a function of the concentration of sulphaphenazole and the various SSRIs, at a fixed tolbutamide (200 μM) and (S)-warfarin (4 μM) concentration respectively. A representative plot is shown in Fig. 1. Amongst the SSRIs tested, only fluvoxamine markedly inhibited both reactions with average IC_{50} values of 23 μM for tolbutamide 4-methylhydroxylation and 27 μM for (S)-warfarin 7-hydroxylation. The other substances tested, except desmethylsertraline, had IC_{50} values over 100 μM for both reactions studied. Desmethylsertraline was only tested up to 30 μM due to its limited water solubility. No apparent inhibition by desmethylsertraline was observed up to 30 μM. Sulphaphenazole, a potent prototypical CYP2C9 inhibitor, strongly inhibited both reactions with average IC_{50} values of 0.63 μM for tolbutamide hydroxylation and 0.28 μM for (S)-warfarin hydroxylation.

In a second series of experiments, fluvoxamine was retested at different tolbutamide and (S)-warfarin concentrations in order to determine the K_i value. A representative plot of the 4-hydroxytolbutamide and 7-hydroxywarfarin formation rate as a function of the respective substrate concentration in the absence and in the presence of different concentrations of fluvoxamine (0, 10, 20 μM and 40 μM), is shown in Fig. 2. The average computed K_i values of fluvoxamine were 13.3 μM for tolbutamide hydroxylation and 13.0 μM for (S)-warfarin hydroxylation (Table 1). For both substrates, inhibition by fluvoxamine was characterized by a concentration-dependent increase in K_m values, whereas

Table 1 Kinetic parameters of tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation; inhibition constants of fluvoxamine for inhibition of tolbutamide 4-hydroxylation and (S)-warfarin 7-hydroxylation and inhibition constant of fluoxetine for inhibition of (S)-warfarin 7-hydroxylation in three human liver microsomal preparations (HL 7, HL 9, HL 10). V_{max} apparent maximal velocity, K_m apparent Michaelis-Menten constant, K_i apparent inhibition constant

	HL 7	HL 9	HL 10	Mean
Tolbutamide 4-methylhydroxylation				
V_{max} (pmol · min^{-1} · mg^{-1})	160	369	217	248
K_m (μM)	205	84	110	133
K_i fluvoxamine (μM)	17.3	16.1	6.4	13.3
(S)-warfarin 7-hydroxylation				
V_{max} (pmol · min^{-1} · mg^{-1})	8.93	13.7	8.8	10.5
K_m (μM)	4.5	2.9	3.8	3.7
K_i fluvoxamine (μM)	18.7	8.4	11.9	13.0
K_i fluoxetine (μM)	125	80	56	87

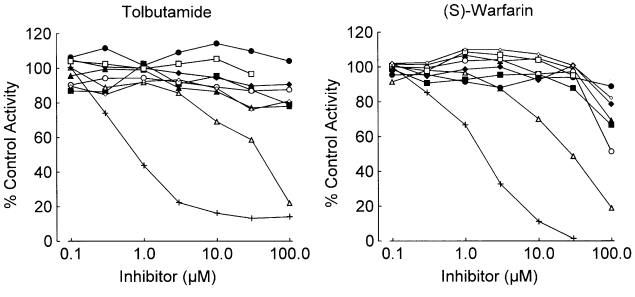

Fig. 1 Effect of citalopram (○), desmethylcitalopram (●), desmethylsertraline (□), fluoxetine (■), fluvoxamine (△), norfluoxetine (▲), paroxetine (◇), sertraline (◆) and sulfaphenazole (+) on 4-hydroxytolbutamide (*left*) and 7-hydroxywarfarin formation (*right*) at a tolbutamide concentration of 200 μM and an (S)-warfarin concentration of 4 μM. Activities are expressed as a percentage of control activity. A representative plot for liver HL 7 is shown

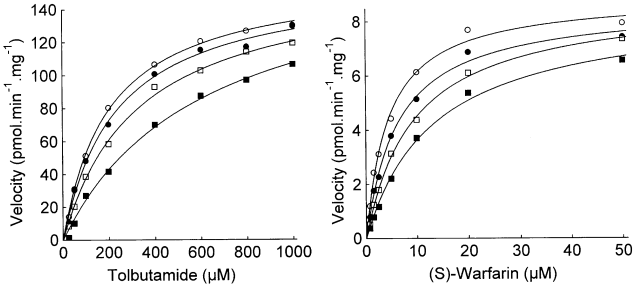

Fig. 2 Formation rates of 4-hydroxytolbutamide (*left*) and 7-hydroxywarfarin (*right*) in function of concentrations of tolbutamide or warfarin in a representative human liver microsomal preparation (HL 7). Incubations were performed without inhibitor (○) and in the presence of 10 μM (●), 20 μM (□) and 40 μM (■) fluvoxamine. All data were fitted to Eq. 1

V_{max} remained unaffected. Although no marked inhibition by fluoxetine was observed at a fixed (S)-warfarin concentration of 4 μM, the K_i value of fluoxetine for the 7-hydroxylation of (S)-warfarin was determined because some case reports suggested a possible interaction between warfarin and fluoxetine [7, 8]. The mean K_i value of fluoxetine (0, 50 μM and 100 μM) for the 7-hydroxylation of (S)-warfarin was 87 μM.

Discussion

As previously reported, tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation followed one enzyme Michaelis-Menten kinetics and the apparent kinetic parameters found in this study are similar to those reported by others [11, 14].

Amongst the SSRIs and some of their metabolites tested, only fluvoxamine was able to markedly inhibit both tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation. This is compatible with the results of Yamazaki and Shimada, who demonstrated inhibition of both reactions by 50 μM fluvoxamine [15]. The K_i values of fluvoxamine found for both reactions studied were comparable. For both reactions, inhibition by fluvoxamine was characterized by a concentration-dependent increase in K_m value with no effect on V_{max} value, compatible with a competitive inhibition model. The K_i values of fluvoxamine were also comparable to those reported when phenytoin was used as substrate [2]. Fluvoxamine has, however, besides a moderate inhibitory effect on CYP2C9 activity, the ability to inhibit CYP2C19 activity, which could explain the comparable K_i values of fluvoxamine for phenytoin p-hydroxylation, tolbutamide 4-methylhydroxylation and (S)-warfarin 7-hydroxylation [15–17]. Schmider et al. reported rather low K_i values of fluoxetine (19 μM) and norfluoxetine (17 μM) when phenytoin was used as a substrate [2]. A substantial number of case reports support the hypothesis that fluoxetine inhibits phenytoin kinetics in vivo [1]. Our in vitro results showed no marked inhibition of either tolbutamide 4-methylhydroxylation or (S)-warfarin 7-hydroxylation by fluoxetine and norfluoxetine, as assessed by IC_{50} determinations. Furthermore, the average K_i value of fluoxetine for the 7-hydroxylation of (S)-warfarin was quite high. An explanation for this apparent discrepancy between the effect of fluoxetine on phenytoin p-hydroxylation and its much smaller inhibitory effect when (S)-warfarin or tolbutamide are used as substrates, could be inhibition of the CYP2C19 component of phenytoin p-hydroxylation [3]. Fluoxetine and its metabolite norfluoxetine, have been shown to have a moderate inhibitory effect on CYP2C19 activity in vitro [18]; fluoxetine also moderately inhibited CYP2C19 activity in vivo [17].

No formal in vivo studies are available about the effect of fluvoxamine on warfarin metabolism but indirect clinical data supports our in vitro findings: fluvoxamine co-administration produced a substantial increase in racemic warfarin plasma concentrations (+65%) and an increase in prothrombin time [6].

The in vivo situation is, however, more complex than might be expected from the in vitro results. Warfarin is used clinically as the racemate, and the anticoagulant effect resides in the (S)-enantiomer. Whereas (S)-warfarin metabolism is mainly dependent on CYP2C9, the major contributor to (R)-warfarin metabolism is CYP1A2; CYP3A4 and CYP2C19 mediate minor pathways [5]. (R)-warfarin has been reported to be an inhibitor of (S)-warfarin 7-hydroylation [19]. Fluvoxamine is known to be a potent inhibitor of CYP1A2 activity in vitro and in vivo [20, 21]; there is also evidence that fluvoxamine can inhibit CYP3A4 and CYP2C19 to some extent [17, 22]. Some authors have suggested that fluvoxamine could produce an increase of (R)-warfarin plasma concentrations due to CYP1A2 (and CYP3A4 and CYP2C19) inhibition; this could in turn lead to inhibition of CYP2C9, leading to an increase in (S)-warfarin plasma concentrations and a potentiation of the anticoagulant effect [1]. However, clear evidence in favour of this hypothesis is lacking [5].

The fact that fluoxetine, paroxetine, sertraline and citalopram produced no or only minor changes in the pharmacokinetics of warfarin [23–26], is compatible with our in vitro results: these SSRIs had no or little effect on CYP2C9 activity. However, some case reports describe prolonged bleeding when warfarin and fluoxetine were co-administered; no interaction mechanism was suggested by the authors [7, 8]. On the other hand fluoxetine and sertraline were also reported to produce no clinically significant decrease in the clearance of tolbutamide [27, 28].

In summary, clinicians should carefully monitor therapy when SSRIs are added to a regimen involving drugs mainly metabolized by CYP2C9, e.g. tolbutamide and (S)-warfarin. This is of particular importance when fluvoxamine and warfarin are co-administered because of the narrow therapeutic index of warfarin.

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