Tizanidine is mainly metabolized by cytochrome P450 1A2 *in vitro*

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Aims
To identify the cytochrome P450 (CYP) enzyme(s) that catalyze the metabolism of tizanidine *in vitro*.

Methods
The effect of CYP isoform inhibitors on the elimination of tizanidine was studied using pooled human liver microsomes. The metabolism of the drug by a range of human recombinant CYP isoforms was then investigated.

Results
Incubation of tizanidine (80 nM) with human liver microsomes resulted in time- and NADPH-dependent substrate consumption with a half-life of 50 min, initial reaction velocity of 1.1 pmol min^{-1} mg^{-1} protein and intrinsic clearance of 17 ml min^{-1} kg^{-1}. The predicted *in vivo* hepatic clearance (CL$_h$) of tizanidine using the well-stirred and parallel-tube model was close (68% and 82%, respectively) to its estimated *in vivo* CL$_h$. Fluvoxamine and furafylline strongly inhibited tizanidine metabolism. Inhibitors specific to isoforms other than CYP1A2 had no substantial effect. Recombinant CYP1A2 metabolized tizanidine to a substantial degree (35% in 45 min), but other recombinant CYPs had little metabolic capacity for the drug.

Conclusions
CYP1A2 is primarily responsible for the metabolism of tizanidine. CYP1A2 inhibitors may inhibit its metabolism also *in vivo*.

Introduction

Tizanidine is a centrally acting skeletal muscle relaxant, used for the symptomatic treatment of painful muscle spasms and spasticity. Furthermore, tizanidine has antinociceptive properties and is used for the treatment of chronic headache [1, 2].

The oral bioavailability of tizanidine is about 21% mainly due to extensive first-pass metabolism and its mean elimination half-life is approximately 3 h [1]. Less than 3% of the dose is excreted as parent tizanidine in urine, in which several metabolites have been detected [3].

Tizanidine has been in clinical use for about 20 years.

However, the CYP enzymes involved in its metabolism are not known. This study aimed to determine which CYP enzymes catalyze the metabolism of tizanidine at therapeutic concentrations.

Methods

Chemicals and microsomes

Tizanidine hydrochloride (Novartis, Cork, Ireland), ketoconazole (Janssen, Olen, Belgium), troleandomycin, quinidine, trimethoprim, sulfaphenazole, furafylline, diethyldithiocarbamate (DDC), omeprazole and β-NADPH (Sigma Aldrich, St Louis, MO, USA), fluvox-

amine (Solvay Duphar, Weesp, Holland) and ciprofloxacin (Bayer, Leverkusen, Germany) were used in this study. Pooled human liver microsomes, human recombinant CYP isoforms (Supersomes™) and control supersomes were obtained from Gentest Corp. (Woburn, MA, USA). The human liver tissue had been obtained in accordance with all pertinent regulations. Other chemicals were from Merck (Darmstadt, Germany).

Incubation conditions

The depletion of tizanidine after its incubation with human liver microsomes or recombinant CYP isoforms was used as the measure of its metabolism. All incubations were conducted at 37 °C in duplicate (all the data were within 7% of their mean) in 0.1 M sodium phosphate buffer (pH 7.4), containing 5.0 mM MgCl$_2$. Tizanidine 80 nM (dissolved in methanol and diluted with water), solvent with or without inhibitor, buffer and β-NADPH (1.0 mM) were premixed, and incubations were commenced by addition of microsomes. The incubation mixture contained 1 mg ml^{-1} microsomal protein, 30 pmol CYP ml^{-1} (corresponding to the CYP1A2 activity in human liver microsomes), or the same protein content as with recombinant CYP1A2 (control supersomes). When substrate depletion as a function of incubation time was measured, the incubation volume was 2.5 ml and aliquots (0.4 ml) were removed at 0, 15, 30, 45, 60 and 75 min, added to 100 µl NaOH (0.4 M), and cooled on ice. In other experiments, the incubation volume was 1 ml and incubation time 45 min. No nonspecific microsomal binding of tizanidine was detected using equilibrium dialysis at 37 °C for 18 h (data not shown).

Furafylline, fluvoxamine and ciprofloxacin were used as inhibitors of CYP1A2, trimethoprim as an inhibitor of CYP2C8, sulfaphenazole as an inhibitor of CYP2C9, omeprazole as an inhibitor of CYP2C19, quinidine as an inhibitor of CYP2D6, DDC as an inhibitor of CYP2E1, and ketoconazole and troleandomycin as inhibitors of CYP3A4 [4–9]. Inhibitor concentrations are given in Figure 1. Stock solutions were prepared in water and methanol. Ciprofloxacin was dissolved in water and sodium hydroxide. Furafylline, troleandomycin and DDC were pre-incubated with human liver microsomes for 15 min, after which tizanidine was added. Control incubations contained an equal concentration of methanol (= 0.5%) or sodium hydroxide (0.01% w/v).

Measurement of tizanidine

After addition of the internal standard (clonidine 15 ng) the samples were extracted twice with 2.0 ml of ethyl
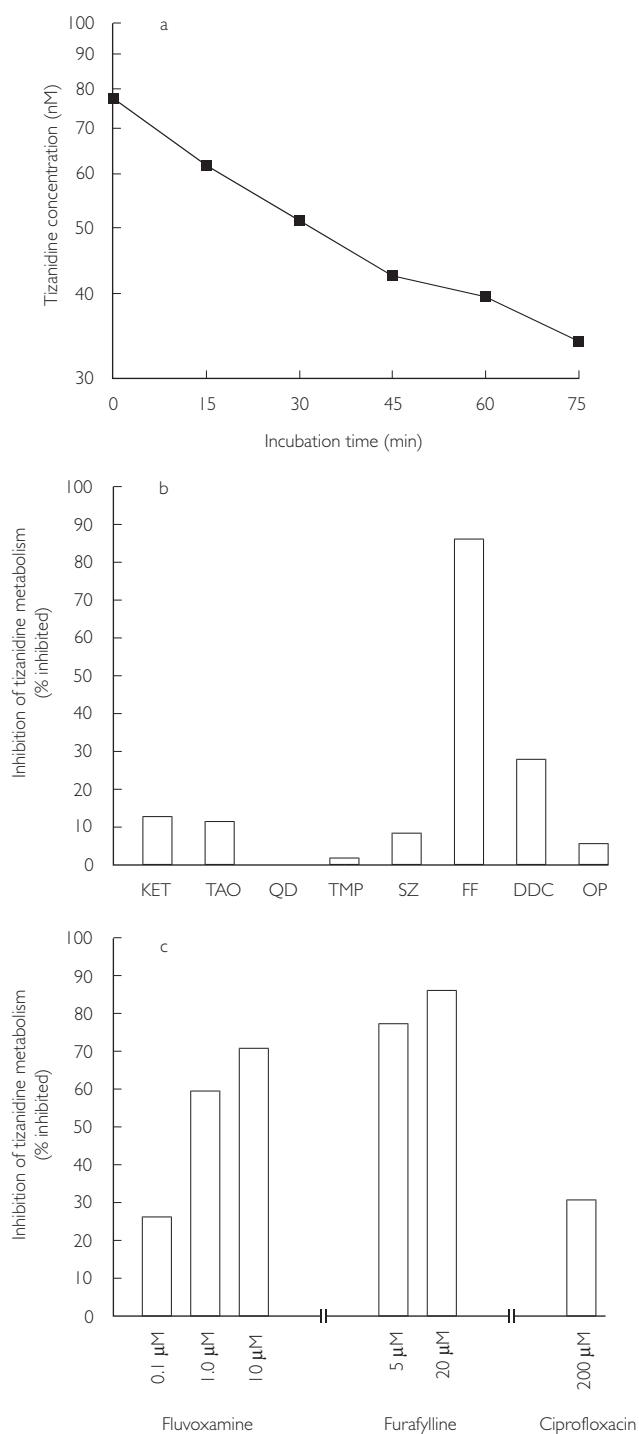
Figure 1

Metabolism of tizanidine (80 µM) by pooled human liver microsomes (a), and % of inhibition of tizanidine metabolism in pooled human liver microsomes by inhibitors of a range of CYP isoforms (b) and (c). The data points are the mean values from duplicate incubations. Key: KET, ketoconazole (1 µM); TAO, troleandomycin (100 µM); QD, quinidine (10 µM); TMP, trimethoprim (100 µM); SZ, sulfaphenazole (10 µM); FF, furafylline (20 µM); DDC, diethyldithiocarbamate (100 µM); OP, omeprazole (10 µM)

acetate for 10 min [10]. After centrifugation, the supernatant was evaporated to dryness under nitrogen. The residue was reconstituted with 100 µl of the HPLC mobile phase. Tizanidine concentration was determined by HPLC with UV detection (320 nm for tizanidine and 228 nm for clonidine). A HP 1100 System (Waldbrunn, Germany) and a Waters Xterra RP18 reversed-phase column, 3.9×100 mm (Milford, MA, USA) were used. The mobile phase was acetonitrile : ammonium acetate (10 mM, pH 9.5) 12 : 88 (v/v), the flow rate was 0.75 ml min^{-1} and HPLC was performed at 35 °C. The limit of quantification (peak to noise-ratio 3 : 1) was 10 nM, and the coefficient of variation was 2.4% at 20 nM and 1.3% at 80 nM ($n = 4$).

Data analysis

Pseudo-first order rate constants (k) were determined from the depletion of tizanidine as a function of incubation time using nonlinear regression analysis. The elimination half-life ($t_{1/2}$) was calculated by the equation $t_{1/2} = \ln 2/k$. Assuming that the concentration of tizanidine (80 nM) is much smaller than the K_m for its metabolism, the intrinsic clearance CL$_{\text{int, in vitro}}$ is equal to k/P_{MS}, where P_{MS} is the microsomal protein concentration [11]. These CL$_{\text{int, in vitro}}$ values were expressed per kilogram body weight by using the standard scaling factors of 48.8 mg microsomal protein g^{-1} liver and 25.7 g liver kg^{-1} body weight [12, 13].

Hepatic clearance (CL$_h$) values were calculated using the well stirred model [14]:

$$CL_h = Qf_u CL_{\text{int}}/(Q + f_u CL_{\text{int, in vitro}})$$

and the parallel-tube model:

$$CL_h = Q(1 - e^{-f_u \cdot CL_{\text{int, in vitro}}/Q})$$

where Q is the hepatic blood flow (20 ml min^{-1} kg^{-1}) [15] and f_u is the unbound fraction (0.7) [3] of tizanidine in blood. These values were compared with the *in vivo* clearance of tizanidine. A directly measured *in vivo* clearance was not available in the literature, and therefore it was estimated using the equation CL = $V/t_{1/2} \times \ln 2$, where V is the volume of distribution after intravenous administration (2.4 l kg^{-1}) and $t_{1/2}$ is the elimination half-life (2.5 h) for tizanidine [16].

Results

Incubation of tizanidine with human liver microsomes resulted in time- and NADPH-dependent substrate consumption. The decline in tizanidine concentration was log-linear until 45 min with a $t_{1/2}$ of 50 min and initial reaction velocity of 1.1 pmol min^{-1} mg^{-1} protein (Figure 1a). The CL$_{\text{int, in vitro}}$ for tizanidine was estimated to
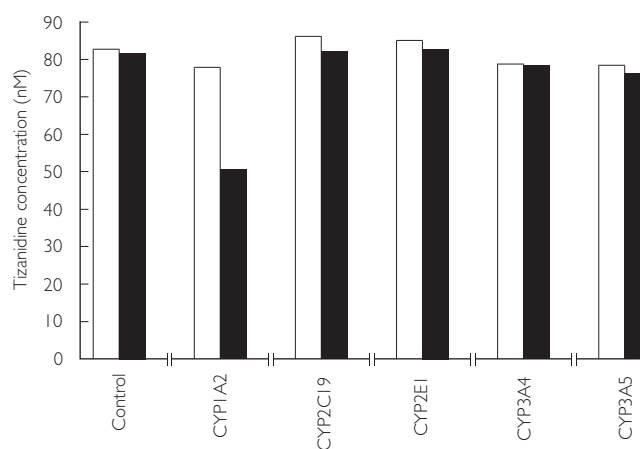
Figure 2

Metabolism of tizanidine (80 nM) by different human recombinant CYP isoforms. The data are the mean values from duplicate incubations. Incubation time: 0 min (□), 45 min (■)

be 17 ml min^{-1} kg^{-1}. The predicted CL$_h$ of tizanidine was 7.5 ml min^{-1} kg^{-1} using the well-stirred model and 9.1 ml min^{-1} kg^{-1} using the parallel-tube model. Human liver microsomes did not metabolize tizanidine in the absence of β-NADPH.

Furafylline, fluvoxamine, ciprofloxacin and DDC caused substantial inhibition of the metabolism of tizanidine (Figure 1b and 1c). Ketoconazole, troleandomycin, sulfaphenazole, and omeprazole had a minor effect and quinidine and trimethoprim had no effect on tizanidine metabolism (Figure 1b).

The concentration of tizanidine decreased by 35% following incubation with recombinant CYP1A2 for 45 min (Figure 2). Five % of drug or less was lost when incubated with CYP2C19, CYP2E1, CYP3A4 and CYP3A5. Control supersomes did not metabolize tizanidine to any appreciable extent.

Discussion

Our aim was to identify the contribution of different CYP enzymes to the total elimination of tizanidine *in vitro*. To this end, the disappearance of parent tizanidine was measured after its incubation in the presence of pooled human liver microsomes. Although the use of a single substrate concentration and pooled human liver microsomes has some limitations, our results strongly suggest that at clinically relevant concentrations tizanidine is metabolized mainly by CYP1A2 *in vitro* and that other CYPs do not contribute appreciably to its biotransformation.

Incubation of tizanidine with human liver microsomes and β-NADPH caused a log-linear deple-

tion in tizanidine concentration. The predicted CL_h of tizanidine using the well-stirred and parallel-tube model were 68% and 82%, respectively, of its estimated *in vivo* clearance (11.1 ml min^{-1} kg^{-1}) [16]. This suggests that the elimination of tizanidine mainly occurs via CYP-dependent metabolism.

The CYP1A2 inhibitors furafylline (selective) and fluvoxamine strongly inhibited the metabolism of tizanidine, whereas inhibitors of other CYP isoforms had no substantial effects. In addition, ciprofloxacin, a moderately potent CYP1A2 inhibitor [6], had a marked effect. Fluvoxamine at 1 µM inhibited tizanidine metabolism by 60%. However, at the concentrations used (up to 10 µM), fluvoxamine can also inhibit CYP2C9, CYP2C19, CYP3A4 and CYP2D6 [17]. DDC, an inhibitor of CYP2E1, also inhibits CYP1A2 by approximately 30% at a concentration of 100 µM [5], which may explain the finding that DDC inhibited tizanidine metabolism by 28% in the present study. These findings suggest that CYP1A2 is primarily responsible for the metabolism of tizanidine.

The activity of recombinant CYP1A2 in the experiments was chosen to correspond to that of CYP1A2 in human liver microsomes. The decline in tizanidine concentration following incubation with recombinant CYP1A2 (35% in 45 min) was almost as rapid as that with human liver microsomes. The other recombinant CYP isoforms metabolized tizanidine to a minor extent.

The use of relevant substrate concentrations is important in *in vitro* studies since the contributions of the various CYP isoforms may vary according to the concentration used [15, 18]. For example, lignocaine is metabolized *in vitro* principally by CYP3A4 when its concentration exceeds 100–1000-fold the therapeutic range [19, 20], but mainly by CYP1A2 when its concentration is within this range [18]. In humans, inhibition of CYP1A2 raises the plasma concentration of lignocaine markedly, whereas inhibition of CYP3A4 has only a marginal effect [21, 22]. The concentration of tizanidine (80 nM) used in this *in vitro* study corresponds to the peak plasma concentrations (20 ng ml^{-1}) in humans [1]. However, the relationship between the plasma concentration of a drug and its concentration at the enzyme active site in the hepatocyte is complex. Consequently, the use of a single, low substrate concentration can be misleading, for example, if enzyme kinetics *in vitro* are atypical (e.g. sigmoidal). However, our aim was not to study in detail the enzyme kinetics of tizanidine over a wide range of substrate concentrations or to identify all enzymes that can contribute to the individual metabolic pathways. We aimed to define the role of CYP enzymes in tizanidine metabolism at clinically relevant concentrations, in order to examine the need for further *in vivo* interaction studies.

Our findings suggest that fluvoxamine may impair tizanidine clearance *in vivo*, since plasma concentrations of the former [23] are comparable with concentrations that strongly inhibited tizanidine metabolism *in vitro* (present work).

In conclusion, our *in vitro* results indicate that CYP1A2 is primarily responsible for the metabolism of tizanidine. Tizanidine may be susceptible to *in vivo* interactions with inhibitors or inducers of CYP1A2.

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