Large interindividual variability in the *in vitro* formation of tamoxifen metabolites related to the development of genotoxicity

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Aims

To characterize the interindividual variability and the individual CYP involved in the formation of α -hydroxy-, N-desmethyl- and N-didesmethyl-tamoxifen from tamoxifen.

Methods

Microsomes from 50 human livers were used to characterize the interindividual variability in the α -hydroxylation, N-desmethylation and N-didesmethylation of tamoxifen. Selective inhibitors and recombinant enzymes were used to identify the forms of CYP catalysing these reactions.

Results

The rates of formation of α -hydroxy-, N-desmethyl- and N-didesmethyl-tamoxifen were highly variable, and correlated with each other ($P < 0.0001$). The respective ranges were 0.7–11.4, 25.7–411, and below the limit of quantification – 4.4 pmol mg⁻¹ protein min⁻¹. Formation of all metabolites was observed with expressed recombinant CYP3A4, inhibited by troleandomycin (65, 77 and 35%, respectively, $P < 0.05$) and associated with CYP3A4 expression ($r_s = 0.612$, $r_s = 0.585$ and $r_s = 0.430$, $P < 0.01$, respectively).

Conclusions

Formation of α -hydroxy-, N-desmethyl- and N-didesmethyl-tamoxifen *in vitro* is highly variable and mediated predominantly by CYP3A4.

Introduction

Tamoxifen is an antiestrogen that is used widely for the treatment of and prevention of breast cancer [1, 2]. Genotoxicity following tamoxifen treatment has been highlighted in a study that reported an increased incidence of endometrial cancer following tamoxifen treatment [3]. A proposed mechanism of genotoxicity is the formation of α -(N²-deoxyguanosinyl)-tamoxifen DNA

adducts via the O-sulphonated conjugate of α -hydroxy-tamoxifen (α -OH-tam) [4]. Conversely, N-didesmethyl-tamoxifen (N-didesmethyl-tam) has been suggested to protect against genotoxicity as it inhibits the covalent binding of tamoxifen *in vitro* to human liver microsomes [5]. In spite of these proposed roles in the development of genotoxicity, studies investigating the formation of α -OH-tam and N-didesmethyl-tam by individual

cytochrome P450 (CYP) isoform(s) have been limited. Therefore, the aims of this study were to determine the *in vitro* interindividual variability and contributions of CYP(s) to the formation of α -OH-tam and N-didesmethyl-tam. In addition, as the production of N-didesmethyl-tam is a two-step process from tamoxifen via N-desmethyl-tam, the formation of the latter metabolite was also investigated.

Methods

Materials

Z-N-didesmethyl-tam, Z-N-desmethyl-tam, and Z-tamoxifen were gifts from Klinge Pharma GmbH (Munich, Germany). Z- α -OH-tam was obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada). S-(+)-mephenytoin was a kind gift from W. Trager (Seattle, WA, USA). Other materials were obtained from the following sources: β -nicotinamide adenine dinucleotide phosphate (β -NADPH, tetrasodium salt, reduced form), coumarin, furafylline, quinidine (free-base) and sulphaphenazole from Sigma (Deisenhofen, Germany); troleandomycin from ICN Biomedicals Inc. (Aurora, OH, USA). Supersomes containing expressed recombinant wild-type CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 isoforms, and a monoclonal antibody raised against human CYP2B6 (MAB-2B6) were purchased from BD GENTEST™ (A BD Biosciences Company, Woburn, MA, USA). All other chemicals and solvents were of analytical grade.

Human liver microsomes

Human liver microsomes were used according to institutional guidelines and with the patients' written consent as described previously. The mean $\pm$ SD (range) age of the patients was 57.5 ± 16.8 (4–77) years, 32 were male and 18 were female [6]. Microsomes were characterized with respect to CYP2B6, CYP2C9, CYP2D6 and CYP3A4 expression and CYP2B6, CYP2C9 and CYP2D6 genotypes [6].

Formation of α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam by expressed CYP isoforms and human liver microsomes

The *in vitro* formation rates of α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam by recombinant CYPs and human liver microsomes were investigated using a method published previously [6]. Briefly, tamoxifen (10 μ M, 1% dimethyl sulfoxide) was incubated with 12.5 pmol expressed CYP or 10 μ g microsomal protein, 2 mM NADPH, 33 mM magnesium chloride and

100 mM potassium phosphate buffer (pH 7.5, microsomal incubation buffer) in a total volume of 100 μ l at 37 °C for 30 min, and the reaction was stopped with 40 μ l of acetonitrile. 3-OH-tam (internal standard, 10 μ l of a 5 μ M solution) was added, and samples were centrifuged at 1500 g for 5 min. The resultant supernatant was removed and 10, 30 or 50 μ l injected onto the LC-MS system. If quantifiable amounts of α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam were observed with initial incubations in the presence of expressed CYP, additional incubations with tamoxifen (0.0625–10 μ M) were performed to obtain an estimate of intrinsic clearance. The formation of N-didesmethyl-tam from N-desmethyl-tam (0.1875–10 μ M, 1% dimethyl sulfoxide) by expressed recombinant CYP3A4 was also investigated. Due to the inhibition of CYP2E1 activity by dimethyl sulfoxide, formation of metabolites by this isoform was investigated using a tamoxifen saturated (approximately 5 μ M) 100 mM potassium dihydrogen phosphate buffer, pH 7.5. The formation of all metabolites was linear with microsomal protein concentrations between 0.01 and 0.1 mg ml⁻¹ and up to an incubation time of 1 h.

Inhibition studies

Furafylline (25 μ M, CYP1A2), coumarin (100 μ M, CYP2A6), sulphaphenazole (5 μ M, CYP2C9), quinidine (1 μ M, CYP2D6), troleandomycin (10 μ M, CYP3A4) [7, 8], S-mephenytoin (100 μ M, CYP2C19) and MAB-2B6 (10 μ l 100 μ g⁻¹ microsomal protein, BD GENTEST™) were used to study the inhibition of α -hydroxy-tam, N-desmethyl-tam and N-didesmethyl-tam formation in microsomes ($n = 6$ –10) using a method published previously [6].

Analysis of metabolites

As described previously, α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam were quantified by LC-MS analysis using positive ionization with selected ion monitoring mode (SIM). N-desmethyl-tam was monitored at m/z 358, N-didesmethyl-tam at m/z 344, and α -OH-tam and 3-OH-tam (internal standard) at m/z 388 (HP1100 LC system) Agilent Technologies, Waldbronn, Germany) [6].

Data analysis

Quantification of metabolite formation was performed with HP Chemstation (98) software (Agilent Technologies) [6]. Formation rates of the metabolites were expressed as pmol.mg protein⁻¹ min⁻¹ or pmol pmol P450⁻¹ min⁻¹. Owing to the limited solubility of tamoxifen, metabolite formation by expressed CYPs did not

always reach saturation. Estimates of intrinsic clearance ($\mu\text{l min}^{-1} \text{ pmol P450}^{-1}$) were obtained from either the slope of the linear regression, or nonlinear regression of the single Michaelis-Menten model (as linear Eadie-Hofstee plots were obtained) (Prism®, Version 3.0, GraphPad Software Inc., San Diego, CA, USA). The extent of inhibition was expressed as a percentage of the controls, with significance determined by a two-tailed Wilcoxon signed rank test. Spearman rank correlations were used to investigate associations between the formation rates of each metabolite, and between the latter and CYP expression. All data are mean $\pm$ SD with a P -value ≤ 0.05 considered significant.

Results

LC-MS separation and validation

LC-MS retention times for α -OH-tam, 3-OH-tam, N-didesmethyl-tam and N-desmethyl-tam were 2.8, 10.1, 12.1 and 12.4 min, respectively. All intra- and interassay bias and coefficients of variation values were $< \pm 12\%$.

Formation of α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam by expressed recombinant CYPs and human liver microsomes

The intrinsic clearances of tamoxifen to its metabolites are shown in Table 1. α -OH-tam was formed by expressed CYP2B6 and CYP3A4, and N-desmethyl-tam was formed by expressed CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Figure 1). The formation of N-didesmethyl-tam from tamoxifen was mediated by expressed CYP1A2, CYP2C19, CYP2D6 and CYP3A4. The intrinsic clearance of tamoxifen to N-desmethyl-tam by expressed CYP3A4

was approximately sixfold higher than by other CYPs. Consequently, the conversion of N-desmethyl-tam to N-didesmethyl-tam by CYP3A4 was also investigated. The estimated Michaelis-Menten kinetic parameters for N-didesmethyl-tam formation from N-desmethyl-tam by expressed CYP3A4 were $K_m = 10.5 \mu\text{M}$, $V_{\max} = 0.061 \text{ pmol pmol P450}^{-1} \text{ min}^{-1}$, giving an intrinsic clearance of $7.6 \times 10^{-3} \mu\text{l min}^{-1} \text{ pmol P450}^{-1}$ (Figure 1).

Considerable variability was observed in α -OH-tam, N-desmethyl-tam and N-didesmethyl-tam formation in the 50 livers studied, with respective mean $\pm$ SD (range) of 3.7 ± 2.4 (0.7–11.4), 122 ± 75.1 (25.7–411), and 1.2 ± 0.9 (below limit of quantification (< 0.26)–4.4) $\text{pmol mg protein}^{-1} \text{ min}^{-1}$. CYP2B6, CYP2C9 and CYP2D6 genotypes had no influence on the formation rates of either metabolite (data not shown). There was a significant association between the rates of formation of the metabolites: α -OH-tam and N-didesmethyl-tam, $r_s = 0.839$, $P < 0.0001$; α -OH-tam and N-desmethyl-tam, $r_s = 0.972$, $P < 0.0001$; N-didesmethyl-tam and N-desmethyl-tam, $r_s = 0.904$, $P < 0.0001$. Furthermore, the ratio of the formation rates of α -OH-tam and N-didesmethyl-tam, thought to be important in predicting the development of genotoxicity, ranged widely from 1.64 to 10.7, with a mean of 4.59.

Inhibition studies

α -OH-tam formation from tamoxifen ($n = 10$) was inhibited by troleandomycin with a mean percentage [$\pm$ SD, 95% confidence interval (CI)] of formation compared to control of 35 (± 18 , 22–49, $P = 0.001$). N-didesmethyl-tam formation from tamoxifen was inhibited by troleandomycin, *S*-mephenytoin, coumarin, furafylline, sulphaphenazole, and quinidine with mean percentage ($\pm$ SD, 95% CI) of formation compared to

Table 1

Intrinsic clearance ($\text{ml min}^{-1} \text{ pmol P450}^{-1}$) for the metabolism of tamoxifen to α -OH-tam, N-didesmethyl-tam and N-desmethyl-tam from tamoxifen by expressed CYP isoforms

	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A4
α -OH-tam		0.21×10^{-3} (0.993)				1.80×10^{-3} (0.999)
N-didesmethyl-tam	4.6×10^{-3} (0.998)			5.6×10^{-3} (0.992)	2.2×10^{-3} (0.958)	1.8×10^{-3} (0.983)*
N-desmethyl-tam	12.2×10^{-3} (1.00)	0.43×10^{-3} (1.00)	4.38×10^{-3} (0.990)	25.9×10^{-3} (0.999)	5.66×10^{-3} (0.982)	71.1×10^{-3} (0.987)*

*Intrinsic clearance was calculated using nonlinear regression analysis of a single-enzyme Michaelis-Menten model.

Figure 1

Rate of formation (pmol pmol P450⁻¹ min⁻¹) of N-desmethyl-tam from tamoxifen (a) and N-desmethyl-tam from N-desmethyl-tam (b) by expressed CYP3A4. The Michaelis-Menten single enzyme model was fitted to the model

control of 66 (± 19, 46-86), 90 (± 6, 83-97), 81 (± 16, 64-97), 78 (± 23, 53-102), 84 (± 5, 79-88), and 82 (± 19, 62-102), respectively (all $P < 0.05$, $n = 6$ livers). N-desmethyl-tam formation from tamoxifen was inhibited by troleandomycin, *S*-mephenytoin, coumarin and furafylline with mean percentage (± SD, 95% CI) of formation compared to control of 42 (± 12, 33-51, $P = 0.001$), 91 (± 4, 88-94, $P < 0.001$), 91 (± 8, 86-97, $P < 0.01$), and 90 (± 13, 81-99, $P < 0.05$), respectively.

Relationship between CYP expression and metabolite formation

CYP isoform expression in microsomes from 50 livers is shown in Table 2, and has been reported previously [6]. CYP2B6 and CYP2C9 expression was associated with α -OH-tam formation ($r_s = 0.350$, $P < 0.05$ and $r_s = 0.327$, $P < 0.05$, respectively, Figure 2A,B).

Table 2

Extent of expression of CYP isoforms in human liver microsomes ($n = 50$)

	CYP expression (pmol/mg protein)	
	Mean ± SD	Range
CYP2B6	18.2 ± 18.1	1.30–95.9
CYP2C9	116 ± 48.1	32.6–224
CYP2D6	7.99 ± 6.59	0–30.8
CYP3A4	64.1 ± 68.7	6.6–295

Data previously reported [6].

Furthermore, there were significant associations between the expression of CYP3A4 and α -OH-tam formation ($r_s = 0.612$, $P < 0.0001$, Figure 2C), CYP3A4 and N-didesmethyl-tam formation ($r_s = 0.430$, $P < 0.01$, $n = 36$, Figure 2D) and CYP3A4 and N-desmethyl-tam formation ($r_s = 0.585$, $P < 0.0001$, Figure 2E). The formation of N-desmethyl-tam was also significantly associated with CYP2B6 and CYP2C9 expression ($r_s = 0.302$, $P < 0.05$ and $r_s = 0.289$, $P < 0.05$, respectively). No association between formation of the metabolites and CYP2B6, CYP2C9 and CYP2D6 genotypes was observed (data not shown).

Discussion

Tamoxifen genotoxicity is suggested to occur by bioactivation to α -OH-tam [4]. We observed a 16-fold variation in α -OH-tam formation amongst the 50 livers investigated. Our findings also indicated that CYP3A4 is the predominant isoform mediating this reaction. Although recombinant CYP2B6 catalysed the formation of α -OH-tam, the role of CYP2B6 is not predicted to be clinically relevant as this pathway was not inhibited by the CYP2B6 monoclonal antibody or influenced by CYP2B6 genotype. Although these findings confirm those reported previously in a smaller number of liver samples [9], this is the first study to report the substantial interindividual variability in the α -hydroxylation of tamoxifen in a large population ($n = 50$).

It has been postulated that N-didesmethyl-tam may provide protection against genotoxicity caused by α -OH-tam. Formation of this metabolite from tamoxifen is a two-step process and, in the present work, a large degree of interindividual variability in the rate of production of this metabolite was observed. Although the latter could not be estimated accurately, it will be directly influenced by the substantial degree (16-fold)

Figure 2

Spearman rank correlation between CYP2B6 (a), CYP2C9 (b) and CYP3A4 (c) expression (pmol mg protein⁻¹) and the rate of formation of α -OH-tam (V, pmol mg protein⁻¹ min⁻¹) ($n = 50$ livers), between CYP3A4 expression and the rate of formation of N-didesmethyl-tam (d, $n = 36$ livers), and between CYP3A4 expression and the rate of formation of N-desmethyl-tam (e) from tamoxifen ($n = 50$ livers) in human liver microsomes

of interindividual variability in the formation of N-desmethyl-tam, 16-fold in the present work, and 7–12-fold in previous studies [10, 11].

A number of previous studies have reported that the formation of N-desmethyl-tam is mediated predominantly by CYP3A4 [10–15]. In addition, the results of

the present study indicate that CYP1A2 and CYP2C19 may also play a minor role in its formation, findings that are in broad agreement with previous reports of N-demethyl-tam formation by various expressed CYP isoforms [13–16]. However, the most recent of these studies utilizing expressed CYPs observed that the rate

of formation of N-desmethyl-tam was higher with CYP2D6 than CYP3A4 [15, 16]. It is unlikely that this discrepancy can be explained by differences in expression systems, since both Boocock and colleagues [15] and the present study used baculovirus insect cell preparations. Furthermore, differences in organic solvents used for dissolving tamoxifen (<1% methanol or 1 : 1 acetone : ethanol compared with 1% dimethyl sulfoxide) are unlikely to inhibit the activity of either CYP2D6 or CYP3A4 to such a degree that the rank in their ability to form N-desmethyl-tam would change [17–19]. Although we observed formation of this metabolite by expressed CYP2D6, there was no association between N-desmethylation and CYP2D6 expression in liver microsomes, or significant inhibition of metabolism in the presence of quinidine. Consequently, the role of CYP2D6 in comparison with CYP3A4 was concluded to be minor.

Similarly, our findings suggest a predominant role for CYP3A4 and minor roles for CYP1A2, CYP2C19, CYP2C9 and CYP2D6 in the formation of N-didesmethyl-tam. However, it is difficult to separate the involvement of individual CYPs in each of the two steps leading to the formation of this metabolite, and further studies are required to clarify the individual contribution of the minor isoforms involved. Nonetheless, this is the first study to postulate CYP characterization of the formation of N-didesmethyl-tam.

The significant association between the formation rates of α -OH-tam and both N-didesmethyl-tam and N-desmethyl-tam, and N-didesmethyl-tam and N-desmethyl-tam is anticipated, as CYP3A4 predominantly mediates the formation of all three metabolites. This may be of clinical importance as N-didesmethyl-tam inhibits CYP3A4 activity *in vitro* [20], and therefore it may impair the conversion of tamoxifen to N-desmethyl-tam and α -OH-tam. Subsequently, the development of genotoxicity in different tissues in the body may be dependent on the ratio of the amount of α -OH : N-didesmethyl formed, which varied approximately sixfold in our liver samples.

In conclusion, we report high interindividual variability in the formation from tamoxifen of α -OH-tam, and N-didesmethyl-tam, and also N-desmethyl-tam. In addition, it was concluded that these reactions are predominantly mediated by CYP3A4. These data are of potential clinical significance, since the occurrence of genotoxicity following tamoxifen treatment may be related to the formation of these metabolites.

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