Genetic polymorphism of cytochrome P450 2D6 determines oestrogen receptor activity of the major infertility drug clomiphene via its active metabolites

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Clomiphene citrate is the most used drug for the treatment of female infertility, a common condition in western societies and developing countries. Despite dose escalation, up to 30% of women do not respond. Since clomiphene shares structural similarities with tamoxifen, which is predominantly bioactivated by the polymorphic cytochrome P450 (CYP) 2D6, we systematically explored clomiphene metabolism and action in vitro and in vivo by pharmacogenetic, -kinetic and -dynamic investigations. Human liver microsomes were incubated with clomiphene citrate and nine metabolites were identified by mass spectrometry and tested at the oestrogen receptor for their antagonistic capacity. (E)-4-hydroxyclomiphene and (E)-4-hydroxy-N-desethylclomiphene showed strongest inhibition of the oestrogen receptor activity with 50% inhibitory concentrations of 2.5 and 1.4 nM, respectively. CYP2D6 has been identified as the major enzyme involved in their formation using recombinant CYP450 isozymes as confirmed by inhibition experiments with CYP monoclonal antibodies. We correlated the CYP2D6 genotype of 30 human liver donors with the microsomal formation rate of active metabolites and observed a strong gene-dose effect. A healthy female volunteer study confirmed our in vitro data that the CYP2D6 polymorphism substantially determines the formation of the active clomiphene metabolites. Comparison of the C_max of (E)-4-hydroxyclomiphene and (E)-4-hydroxy-N-desethylclomiphene showed 8 and 12 times lower concentrations in subjects with non-functional CYP2D6 alleles. Our results highlight (E)-4-hydroxyclomiphene and (E)-4-hydroxy-N-desethylclomiphene as the active clomiphene metabolites, the formation of which strongly depends on the polymorphic CYP2D6 enzyme. Our data provide first evidence of a biological rationale for the variability in the response to clomiphene treatment.

INTRODUCTION

Infertility affects 15–20% of couples worldwide and within the past decades, there has been a steady rise in the treatment with clomiphene citrate (2-[4-[2-chloro-1,2-di(phenyl)ethenyl]phenoxy]-N,N-diethylisothalamine dihydrogen citrate). Clomiphene (CLOM) either alone or in combination with gonadotropin or metformin is used as first line therapy to induce ovulation in infertile women with polycystic ovarian syndrome but also in women with unexplained infertility (1–3). CLOM antagonizes the binding of 17-β-oestradiol to the oestrogen receptor at the hypothalamic arcuate nucleus leading to an increase in gonadotropin-releasing hormone thereby releasing follicle stimulating hormone and luteinizing hormone for the subsequent induction of ovulation (4). The standard CLOM therapy regimen consists of repeated cycles of increasing CLOM dosages from 50 to 150 mg/day until ovulation; however, 8–30% of treated women do not respond and fail to ovulate (5,6). Although there are known factors leading to failure of clomiphene treatment, such as...
hyperandrogenaemia and obesity (7,8), the variability of the response to clomiphene treatment is not yet fully understood.

Clomiphene shares the triphenylethylene structure with other selective oestrogen receptor modulators, such as tamoxifen, and therefore it is plausible that the cytochrome P450 (CYP) 2D6 enzyme, a key player of tamoxifen activation, is also involved in the clomiphene metabolism. Clomiphene is prescribed as a mixture of (E)- and (Z)-isomer; however, detailed knowledge on the isomer specific metabolism and biological activity of clomiphene is sparse. With respect to tamoxifen, it has been shown that the formation of the active metabolites strongly depends on CYP2D6 polymorphisms, thereby resulting in significantly different plasma concentrations of active metabolites (9,10). Moreover, despite some conflicting results (11–13), there is evidence that breast cancer patients with no or low CYP2D6 function are at risk for poor outcome (14–16). The CYP2D6 enzyme activity manifests in the population as ultrarapid (UM), extensive (EM), intermediate (IM) as well as poor metabolizer (PM) phenotypes with frequencies of 5–10%, 65–80%, 10–15% and 5–10%, respectively, in the Caucasian population (17,18). Underlying genetic polymorphisms are held responsible for the wide inter-individual variability of hepatic enzyme expression and function (19,20), resulting in highly variable pharmacokinetic parameters of CYP2D6 substrates, such as tamoxifen.

Given the structural and metabolic similarities between clomiphene and tamoxifen, it is essential to revisit and update the molecular basis of clomiphene pharmacokinetics and pharmacodynamics as well as the underlying genetic background. Here we show in vitro and in vivo data in support of the role of CYP2D6 genetics for the stereoselective bioactivation of clomiphene and provide a mechanistic explanation for the inter-individual variability of the efficacy to stimulate ovulation.

RESULTS

Identification of 4-hydroxy-clomiphene metabolites and their activity at the oestrogen receptor

To investigate whether clomiphene is subject to CYP-mediated N-de-alkylation and 4-hydroxylation, we performed incubation experiments with pooled human liver microsomes. Synthetic compounds including deuterated analogues were used as references within a high-resolution high performance liquid chromatography (HPLC) system coupled with tandem mass spectrometry. In addition to the parent compounds (E)- and (Z)-clomiphene, we identified nine metabolites including (E)-4-hydroxy-N-desethylclomiphene [(E)-4-OH-DE-CLOM], (E)-4-hydroxy-clomiphene [(E)-4-OH-CLOM] and (Z)-4-hydroxy-clomiphene [(Z)-4-OH-CLOM] (Fig. 1).

Using an oestrogen response element (ERE) reporter assay, (E)-4-OH-CLOM and (E)-4-OH-DE-CLOM showed highest inhibitory potency at the oestrogen receptor. IC50 values for the inhibition of the effect of oestriadiol were 2.5 and 1.4 nM, respectively. (Z)-4-OH-CLOM was less efficient with an IC50 of 31 nM (Fig. 2). Neither the parent drug isomers (E)-CLOM and (Z)-CLOM nor (E/Z)-N-desethylclomiphene (E/Z)-DE-CLOM inhibited the oestrogen effect up to a concentration of 100 nM. These data provide evidence that the 4-hydroxylated (E)-metabolites are at least 100 times more potent at the ER than their parent compounds.

Clomiphene metabolism and the role of cytochrome P450 enzymes in vitro

CYP2D6 has been shown to be the major enzyme in the metabolism of (E)-clomiphene catalysing the formation of (E)-4-OH-clomiphene, and to a minor extend (E)-DE-clomiphene, (Z)-4-OH-clomiphene, (E)-N,N-desethylclomiphene [(E)-DDE-clomiphene] and (E)-4-OH-DE-clomiphene (Fig. 3A). Although the de-ethylation of (E)-clomiphene by CYP3A4 was substantial, this effect was only observed in the presence of high amounts of cytochrome b5. Other relevant CYPs for the formation of (E)-DE-clomiphene were CYP2C19, CYP2C8 and CYP3A5, and for (E)-4-OH-DE-clomiphene CYP2B6 (Fig. 3A). (Z)-clomiphene was almost exclusively transformed into (Z)-DE-clomiphene mainly by CYP3A4 and CYP2D6 with negligible contribution by CYP2B6, CYP2C19, CYP2C8 and CYP1A2. Of note, no other (Z)-clomiphene metabolites were observed at significant levels (Fig. 3B). With respect to the de-ethylation of (E)-4-OH-clomiphene, the CYP3A5 showed highest activity followed by CYP3A4 plus cytochrome b5. CYP2D6, CYP2C19 and CYP2C8 (Fig. 3C). (E/Z)-DE-clomiphene was 4-hydroxylated mainly by CYP2D6 and further de-alkylation to DDE-clomiphene was catalysed by CYP2D6 with minor contributions by CYP2C19 and CYP3A4 (Fig. 3D).

The activity of specific isozymes was further tested with human liver microsomes using inhibitory monoclonal antibodies directed against respective CYP isoforms. The anti-CYP2D6 antibody inhibited the formation of (E)-4-OH-clomiphene and (E)-4-OH-DE-clomiphene by >80%. The antibody against CYP3A4 inhibited the de-ethylation of (E)-clomiphene by ~40%. Antibodies directed against other CYP isoforms did not significantly decrease formation rates.

The conversion of (E)-clomiphene to (E)-DE-clomiphene, (E)-4-OH-clomiphene and (Z)-4-OH-clomiphene and the conversion of (Z)-clomiphene to (Z)-DE-clomiphene in pooled human liver microsomes followed Michaelis–Menten kinetics (Supplementary Material, Fig. S1). The formation of (E)-4-OH-clomiphene showed the highest intrinsic clearance of 20.7 μl/min/mg followed by (E)-DE-clomiphene and (Z)-4-OH-clomiphene (7.7 and 7.5 μl/min/mg, respectively; Table 1).

CYP2D6 polymorphisms affect clomiphene metabolism in vitro

In a set of 30 human liver microsomes, the formation rates of (E)-4-OH-clomiphene from (E)-clomiphene and (E)-4-OH-declomiphene from DE-clomiphene were significantly correlated with the amount of microsomal CYP2D6 protein (linear regression: r2 = 0.74, P < 0.0001 and r2 = 0.37, P = 0.0003, respectively; Fig. 4A and B), whereas there was no correlation with expression levels of CYP3A4, 2B6, 2C8 and 2C19. In contrast, de-ethylation of both (E)-clomiphene and (Z)-clomiphene was associated with the protein levels of CYP3A4, 2B6 and 2C8 but not with CYP2D6 protein content (data not shown).

CYP2D6 genotypes of the human liver samples were classified on the basis of PM alleles (*3, *4, *5, *6, *7, *8), IM alleles (*9, *10, *41) and EM alleles (absence of any PM or IM allele), as PM/PM, PM/IM, IM/IM, PM/EM, IM/EM or EM/EM. Carriers of duplications of fully functional genes were referred to as UM. Stratification by the CYP2D6 genotype showed a strong gene-dose effect on the formation rate of (E)-4-OH-clomiphene (Spearman test: r = 0.83, P = 1.1 × 10−8).
PM/PM showed the lowest formation rates (2.1 ± 0.6 pmol/min/mg protein) when compared with EM/EM (31.4 ± 12.1 pmol/min/mg protein) or IM/IM (8.4 ± 0.25 pmol/min/mg protein), respectively. Carriers of gene duplications showed even higher formation rates (48 ± 18 pmol/min/mg protein) (Fig. 4C). Similar relationships were observed between the CYP2D6 genotype and metabolic activity for the formation of (E)-4-OH-DE-CLOM (Spearman test: \( r = 0.80, P = 8.9 \times 10^{-8} \); Fig. 4D).

The observed dramatic decrease in the intrinsic clearance of (E)-CLOM via 4-hydroxylation in pooled liver microsomes from PM donors when compared with a pool representative for the distribution of genotypes in a Caucasian population indicated a strong impact of the genetic variability of CYP2D6 on the in vitro metabolism of CLOM (Table 1 and Supplementary Material, Fig. S1).

CYP2D6 polymorphisms affect clomiphene metabolism in vivo

The in vivo influence of CYP2D6 genetic variability on CLOM exposure and metabolism was investigated in a clinical study in four EM and two PM female volunteers, who received a

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**Figure 1.** Human metabolism of (E)- and (Z)-clomiphene. Respective metabolic steps are labelled with CYPs contributing to this transformation as determined by the present study. CYPs predominantly involved are indicated in bold.

**Figure 2.** The anti-oestrogenic effect of 4-hydroxylated clomiphene metabolites at the oestrogen receptor. MCF-7 cells were transfected with an ERE reporter comprising five repeats of consensus EREs 5' to the thymidine kinase promoter and the luciferase gene. Cells were incubated with 10^{-10} M β-oestradiol and different concentrations of clomiphene metabolites as indicated. Luciferase activity was normalized to the control treated with oestradiol alone. Data are presented as means of triplicates ± SD.
Figure 3. Formation of clomiphene metabolites by recombinant CYPs. (A) (E)-clomiphene, (B) (Z)-clomiphene, (C) N-desethylclomiphene and (D) (E)-4-hydroxyclomiphene were incubated with a final concentration of 6 μM in the presence of microsomes containing 20 nM of recombinant CYPs as indicated. Other CYPs tested (i.e. 1E2, 2A6 and 2C9) did not show substantial formation of any metabolites. Data are presented as means of duplicates and range.

Table 1. Parameters of Michaelis–Menten kinetics for the formation of clomiphene metabolites from human liver microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>V\text{max} (± SE) (pmol/min/mg)</th>
<th>K\text{M} (± SE) (μM)</th>
<th>C\text{int} (μl/min/mg)</th>
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<tbody>
<tr>
<td>Human liver microsomes (pooled from 150 donors)</td>
<td>(E)-CLOM</td>
<td>(E)-4-OH-CLOM</td>
<td>102.4 (± 2)</td>
<td>4.9 (± 0.4)</td>
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<td></td>
<td>(E)-DE-CLOM</td>
<td>253.6 (± 4.4)</td>
<td>33.0 (± 1.2)</td>
<td>7.68</td>
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<td>(Z)-4-OH-CLOM</td>
<td>16.9 (± 0.5)</td>
<td>2.3 (± 0.3)</td>
<td>7.46</td>
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<td>(E)-4-OH-DE-CLOM</td>
<td>2.0 (± 0.1)</td>
<td>1.5 (± 0.5)</td>
<td>1.39</td>
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<tr>
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<td>(E)-DDE-CLOM</td>
<td>4.5 (± 0.5)</td>
<td>20.9 (± 5)</td>
<td>0.22</td>
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<tr>
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<td>(Z)-CLOM</td>
<td>(Z)-DE-CLOM</td>
<td>223.9 (± 14.9)</td>
<td>94.8 (± 10)</td>
</tr>
<tr>
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<td>(E)-4-OH-CLOM</td>
<td>5.9 (± 0.5)</td>
<td>27.8 (± 5.1)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>(Z)-4-OH-CLOM</td>
<td>0.88 (± 0.06)</td>
<td>14.6 (± 2.6)</td>
<td>0.06</td>
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<tr>
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<td>(Z)-DDE-CLOM</td>
<td>21.4 (± 14.2)</td>
<td>1165 (± 814)</td>
<td>0.06</td>
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<td></td>
<td>(E)-4-OH-CLOM</td>
<td>0.11 (± 0.01)</td>
<td>5.8 (± 1.5)</td>
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<td>(E)-4-OH-DE-CLOM</td>
<td>0.06 (± 0.01)</td>
<td>6.5 (± 1.5)</td>
<td>n.d.</td>
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<td></td>
<td>(E)-DDE-CLOM</td>
<td>20.6 (± 1)</td>
<td>62.2 (± 4.9)</td>
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<tr>
<td></td>
<td>(Z)-CLOM</td>
<td>(Z)-DE-CLOM</td>
<td>186.7 (± 11.5)</td>
<td>76.1 (± 7.5)</td>
</tr>
<tr>
<td></td>
<td>(E)-4-OH-CLOM</td>
<td>0.12 (± 0.02)</td>
<td>33.6 (± 11.7)</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(Z)-DDE-CLOM</td>
<td>4.56 (± 1.19)</td>
<td>288.0 (± 90.7)</td>
<td>0.02</td>
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<td></td>
<td>(Z)-4-OH-CLOM</td>
<td>0.31 (± 0.06)</td>
<td>144.4 (± 36.9)</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(E)-4-OH-CLOM</td>
<td>0.05 (± 0.07)</td>
<td>267.9 (± 489.2)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Human liver microsomes (pooled from eight carriers of two non-functional CYP2D6 alleles)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>V\text{max} (± SE) (pmol/min/mg)</th>
<th>K\text{M} (± SE) (μM)</th>
<th>C\text{int} (μl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-CLOM</td>
<td>(E)-4-OH-CLOM</td>
<td>3.4 (± 0.29)</td>
<td>25.3 (± 4.8)</td>
<td>0.13</td>
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<td></td>
<td>(E)-DE-CLOM</td>
<td>290.7 (± 14.9)</td>
<td>36.8 (± 3.7)</td>
<td>7.91</td>
</tr>
<tr>
<td></td>
<td>(Z)-4-OH-CLOM</td>
<td>0.3 (± 0.02)</td>
<td>17.2 (± 3.2)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(E)-4-OH-DE-CLOM</td>
<td>0.06 (± 0.01)</td>
<td>6.5 (± 1.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(E)-DDE-CLOM</td>
<td>3.3 (± 0.15)</td>
<td>62.2 (± 4.9)</td>
<td>0.05</td>
</tr>
<tr>
<td>(Z)-CLOM</td>
<td>(Z)-DE-CLOM</td>
<td>186.7 (± 11.5)</td>
<td>76.1 (± 7.5)</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>(E)-4-OH-CLOM</td>
<td>0.12 (± 0.02)</td>
<td>33.6 (± 11.7)</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(Z)-DDE-CLOM</td>
<td>4.56 (± 1.19)</td>
<td>288.0 (± 90.7)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(Z)-4-OH-CLOM</td>
<td>0.31 (± 0.06)</td>
<td>144.4 (± 36.9)</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(E)-4-OH-CLOM</td>
<td>0.05 (± 0.07)</td>
<td>267.9 (± 489.2)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not determined due to high variability and/or low activity.

*Intrinsic clearance was calculated as follows: C\text{int} = V\text{max}/K\text{M}.
single dose of 100 mg of CLOM citrate. Plasma levels of (E)- and (Z)-CLOM as well as their respective metabolites were monitored for a period of 7 days uncovering substantial differences between CYP2D6 phenotypes (Fig. 5). In PMs, the mean maximal plasma level (Cmax) of (E)-CLOM was approximately six times higher when compared with EMs, while Cmax of the 4-hydroxylated metabolite (E)-4-OH-CLOM was almost eight times lower (Table 2). The difference with respect to de-ethylated metabolites was even more pronounced: carriers of two non-functional CYP2D6 alleles showed a dramatic lack in the clearance of (E)-DE-CLOM resulting in a 50-fold higher area under the curve (AUC0–168) when compared with EMs (1.1 versus 13.1 nm). In contrast, exposure of (Z)-CLOM was only slightly affected by the CYP2D6 PM phenotype (30% higher Cmax and AUC0–168).

DISCUSSION

Although clomiphene (CLOM) has been in use as first line infertility drug for almost four decades, its metabolism and activity profile is poorly understood. Here we show that CLOM is a prodrug and requires bioactivation by 4-hydroxylation. In particular, CLOM action is mediated through oestrogen receptor binding of (E)-4-hydroxylated metabolite as demonstrated in vitro using the ERE reporter assay. These findings corroborate earlier studies of cell growth inhibition by CLOM metabolites in MCF-7 breast cancer cells (21).
reminiscent of those of the anticancer drug tamoxifen. The similarity in drug action is evident from the structural relationship between 4-OH-CLOM and 4-hydroxytamoxifen. X-ray crystallography of the oestrogen receptor-4-hydroxytamoxifen complex confirmed the binding of the 4-hydroxy moiety to the oestrogen receptor (22) and the relevance of the 4-hydroxy metabolites is evident from in vitro and in vivo observation (14,23–25). In breast cancer patients, 4-hydroxytamoxifen together with 4-hydroxy-N-desmethyltamoxifen (endoxifen) accounts for drug action (reviewed in26).

Figure 5. Pharmacokinetics of clomiphene and its metabolites in humans. Two female healthy volunteers homozygous for CYP2D6 PM alleles (in red) and four homozygous or heterozygous for CYP2D6 EM alleles (in blue) received 100 mg of clomiphene citrate po. Plasma levels of (A) (E)- and (Z)-clomiphene; (B) (E)-4-hydroxyclomiphene; (C) (E)- and (Z)-N-desethylclomiphene; (D) (E)-4-hydroxy-N-desethylclomiphene were determined up to 7 days. Data are presented as mean and range.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CYP2D6 EM (n = 4)</th>
<th>CYP2D6 PM (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c_{max} (nm), mean (range)</td>
<td>t_{1/2} (h), mean (range)</td>
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<tr>
<td>(Z)-CLOM</td>
<td>43.8 (34.7–60.6)</td>
<td>108 (102–113)</td>
</tr>
<tr>
<td>(E)-CLOM</td>
<td>19.6 (11.4–23.3)</td>
<td>106 (50–162)</td>
</tr>
<tr>
<td>(Z)-DE-CLOM</td>
<td>2.3 (1.9–3)</td>
<td>3 (2.3–3.6)</td>
</tr>
<tr>
<td>(E)-DE-CLOM</td>
<td>6 (3.6–8.6)</td>
<td>0.4 (0.4–0.4)</td>
</tr>
<tr>
<td>(Z)-DDE-CLOM</td>
<td>3.2 (30.2–39.5)</td>
<td>0.4 (0.4–0.4)</td>
</tr>
<tr>
<td>(E)-DDE-CLOM</td>
<td>4.7 (2.3–6.3)</td>
<td>3.4 (2.6–4.3)</td>
</tr>
<tr>
<td>(Z)-4-OH-CLOM</td>
<td>1.3 (1–2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(E)-4-OH-CLOM</td>
<td>22.8 (9.6–37.3)</td>
<td>82.6 (75.1–90)</td>
</tr>
<tr>
<td>4-OH-DE-CLOM</td>
<td>21.1 (17.5–25)</td>
<td>1.1 (1–1.1)</td>
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<tr>
<td>(Z)-CLOM-NO</td>
<td>1.8 (0.8–2.6)</td>
<td>347 (310–383)</td>
</tr>
<tr>
<td>(E)-CLOM-NO</td>
<td>1.9 (1.1–2.2)</td>
<td>19 (9–28)</td>
</tr>
</tbody>
</table>

Table 2. Pharmacokinetic parameters of clomiphene and its metabolites in female healthy volunteers following a single dose of clomiphene citrate

(E)-CLOM which makes up 40% of the (E/Z)-isomer mixture in the drug formulation has the same conformation as the clinically used (Z)-isomer of tamoxifen citrate. This suggests a similar metabolism for (Z)-tamoxifen and (E)-CLOM as indicated by animal studies (23,27–29) and human urine metabolite profiles (30). We showed that CYP2D6 is the key enzyme in the conversion of CLOM to its active 4-hydroxy metabolites and that CYP2D6 genotypes affect its metabolism in vitro and in vivo. A strong gene-dose effect was evident from the formation rate of both
(E)-4-OH-CLOM and (E)-4-OH-DE-CLOM in human liver microsomes. This is in line with the observed gene-dose effect for the formation of endoxifen in tamoxifen-treated breast cancer patients (10). With respect to de-ethylation, our in vitro results indicate that besides genetically determined variability in CYP2C19, CYP2D6 and CYP3A5 protein levels, the expression of cytochrome b5 may also influence formation rates. The strong impact of cytochrome b5 levels on CYP3A4 activity has been described previously for several other hydroxylation and de-alkylation reactions involved in the metabolism of vincristine, alfentanil, midazolam and amitriptyline (31–33).

While (Z)-CLOM represents 60% of the formulation, Ghobadi et al. (34) reported 20 times higher AUC0–72h when compared with (E)-CLOM following a single oral dose of 50 mg. An explanation could be provided by the strong stereoselectivity of CLOM metabolism: 4-hydroxylation occurs exclusively in the (E)-isomers of CLOM and its de-ethylated metabolites.

Here, we present for the first time CLOM pharmacokinetic data from healthy volunteers genotyped for CYP2D6. EM subjects showed extensive metabolism of (E)-CLOM as evident from their 10 times higher AUC of (Z)-CLOM compared with AUC of (E)-CLOM. In contrast, PM subjects lacking functional CYP2D6 showed equal AUCs for both CLOM isomers indicating a defective metabolism. The same relationship, albeit stronger, has been observed for (E)- and (Z)-isomers indicating a defective metabolism. The same relationship, albeit stronger, has been observed for (E)- and (Z)-isomers indicating a defective metabolism. The same relationship, albeit stronger, has been observed for (E)- and (Z)-isomers indicating a defective metabolism. The same relationship, albeit stronger, has been observed for (E)- and (Z)-isomers indicating a defective metabolism.

As previously shown for tamoxifen, patients homozygous for non-functional alleles (i.e. PM/PM) are not likely to reach sufficiently high plasma levels of 4-hydroxytamoxifen and endoxifen to completely block oestrogen receptor activity. Likewise, patients who are carriers of one non-functional allele in combination with one reduced function allele (i.e. PM/IM) showed low steady-state plasma levels of active tamoxifen metabolites (10). These two groups account for 13% of all patients. Another 48% had a compromised CYP2D6 activity (i.e. IM/IM, PM/EM, IM/EM). Given this genotype distribution, which is representative for a population of European descent, our data may help explain why, in some instances, CLOM response rates improved during dose escalation (6).

Very recently, this concept has been corroborated by a CYP2D6 genotype-adjusted tamoxifen dose escalation in breast cancer patients (35–37). Increasing the tamoxifen dosage in patients with low plasma endoxifen levels and/or a CYP2D6 IM or PM phenotype resulted in elevated concentrations of the active tamoxifen metabolites, particularly in IM.

In analogy, the large group of patients with compromised CYP2D6 activity may benefit from a genotype-guided optimized CLOM dose escalation strategy by faster response or an alternative therapy.

In conclusion, our findings have important translational applications particularly in the light of the time constraints infertile women are facing when they seek treatment to enhance ovulation. Clinical studies are now needed to validate this concept.

MATERIALS AND METHODS

Chemicals

Clomiphene citrate (E/Z 58:42) was obtained from Sigma. Pure (E)-Clomiphene (Enclopmiphene) and pure (Z)-Clomiphene (Zuclomiphene) were kind gifts from Dr. MS Lennard (University of Sheffield, Sheffield, UK). All other clomiphene metabolites including deuterated analogues as internal standards were synthesized either by transformation of commercially available (E/Z)-clomiphene citrate or by de novo synthesis involving the McMurry reaction (38). Purity of all compounds exceeded 95% as determined by nuclear magnetic resonance spectroscopy, elemental analysis and HPLC.

ERE reporter assay

Clomiphene and clomiphene metabolites were tested for their capacity to inhibit oestrogen action at the oestrogen receptor. To determine concentrations for the 50% blocking of the stimulating effect of 10−10 M 17β-oestradiol (IC50), (E)- and (Z)-clomiphene and respective metabolites were analysed by ERE reporter assays at concentrations of 24 × 10−10 to 2.5 × 10−7 M. Briefly, MCF-7 cells were oestrogen deprived for 3 days in phenol red free Dulbecco’s modified Eagle’s medium (Invitrogen, Darmstadt, Germany) supplemented with 10% charcoal treated fetal calf serum, 0.5 mM sodium pyruvate and 0.1% gentamycin, and seeded at 105 cells in 24-well microplates. Cells were transfected with 150 ng of an ERE reporter plasmid, i.e. a 5× tandem repeat of a consensus ERE 5′ to the minimal tyrosine kinase promoter inserted into the pGL3 basic luciferase reporter vector using Effectene Transfection Reagent (Qiagen, Hilden, Germany) (10). For normalization, cells were co-transfected with a beta-galactosidase plasmid (Promega) as described by Burk et al. (39).

Cytochromes P450 and human liver microsomes

Recombinant cytochromes P450 (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) coexpressed with nicotinamide adenine dinucleotide phosphate (NADPH)-P450 oxidoreductase in insect cells (supersomes) were purchased from BD Gentest (Woburn, MA, USA). Human liver microsomes were prepared from surgically removed liver tissue as described previously (40).

The study was approved by the ethics committees of the Medical Faculties of the Charité, Humboldt-University Berlin and written informed consent was obtained from each patient. Donators of liver samples were genotyped for CYP2B6, CYP2C8,
CYP2C9, CYP2C19, CYP2D6 and CYP3A5 and protein content of these enzymes was quantified in microsomal preparations by western blots (13). A pool of human liver microsomes was prepared by mixing random amounts of microsomes prepared from 150 different donators. Liver microsomes from eight donators with two non-functional CYP2D6 alleles were combined to obtain a PM pool.

**In vitro incubations with recombinant CYPs and human liver microsomes**

Incubation mixtures contained either recombinantly expressed CYP isoenzymes (20 pmol of CYP/ml) or human liver microsomes (0.25 mg of protein/ml), 0.1 mM potassium phosphate buffer, pH 7.4, 1% dimethyl sulfoxide (from substrate solution), NADPH-regenerating system (5 mM MgCl₂, 5 mM glucose 6-phosphate, 0.5 mM NADP⁺ and 4.0 μM glucose 6-phosphate dehydrogenase) and different concentrations of (E)-clomiphene, (Z)-clomiphene, (E/Z)-desethylclomiphene or 4-hydroxyclophelene as indicated in the figures in a total incubation volume of 100 μl. The reaction mixture was pre-incubated for 2 min at 37°C before starting by the addition of the NADPH-generating system. After an incubation period of 30 min, each reaction was terminated by adding 100 μl of ice-cold 1% acetic acid in acetonitrile containing a mixture of deuterated chloromphene metabolites as internal standards. Samples were centrifuged to separate precipitated protein and the supernatant was diluted with 0.1% formic acid prior to analysis by LC-MS/MS. Liver donors were genotyped for CYP2D6 and the phenotype was deduced from the CYP2D6 genotype assigning *3, *4, *5, *6, *7 and *8 as PM alleles, *9, *10 and *41 as IM alleles, carriers of no variant as EM alleles and more than two EM alleles as ultra-rapid metabolizer (13).

**Volunteer study**

All individuals were genotyped for the most relevant CYP2D6 variants in Caucasians (i.e. CYP2D6*3, *4, *5, *6, *7, *8, *9, *10, *41, gene amplification) by the use of 5' nuclease assays as previously described (41,42). The PM phenotype is defined by carriers of two loss-of-function CYP2D6 alleles, including the CYP2D6 deletion (study subjects *4/*4 and *4/*5), whereas CYP2D6-EMs are subjects carrying no or only one variant allele (study subjects: two individuals with each *1/*1 and *1/*4 genotype) (18,20). The volunteers aged 23–43 years, weight 48–74 kg and were 160–173 cm tall, respectively. The study protocol, patient information sheet and the consent form were approved by the Ethics Committee of the University of Tübingen, Germany and the German Federal Institute for Drugs and Medical Devices (BfArM). The participants gave their informed consent prior to the study. As defined by our study protocol, intake of any other drug including contraceptives or any foodstuffs or beverages which are known to interfere with CYPs or tobacco was not allowed.

Volunteers received a single dose of 100 mg of clomiphene citrate (two 50 mg tablets Clomifen-ratiopharm®, Ratiopharm GmbH, Ulm, Germany) with a glass of water at 8:00 AM after an overnight fast. Blood samples (9 ml) were taken immediately before and 1, 2, 4, 6, 8, 10, 12, 24, 72 and 168 h after the dose, and the exact time of sampling was recorded for subsequent kinetic analysis. Plasma was separated, frozen immediately and stored at −20°C until analysis.

**Quantification of clomiphene and its metabolites**

**Calibration and method validation.** Quantification of clomiphene and its metabolites in plasma samples was validated according to the Food and Drug Administration (FDA) guidance as described previously (38). In brief, eight calibration samples were prepared with final concentrations of analytes ranging from 0.25 to 50 nm for hydroxylated metabolites, from 0.5 to 100 nm for de-ethylated metabolites and from 1 to 200 nm for the parent drug isomers. Likewise, quality controls were prepared from different stock and working solutions and analysed together human samples. Lower limits of quantification (LLOQ) for (E)- and (Z)-clomiphene were 3 nm and for the metabolites LLOQ ranged from 0.15 to 0.3 nm. Inter-assay variability (coefficient of variation <10%) and accuracy (89.4–109.7%) were in agreement with the FDA guidance. For in vitro assays, nine calibration samples were prepared in incubation buffer with concentrations ranging from 0.075 to 1500 nm for 4-OH-DE-CLOM, 4-OH-CLOM and DDE-CLOM and from 0.35 to 7000 nm for DE-CLOM.

**Sample preparation and LC-MS/MS analysis.** Sample preparation from the volunteers’ plasma was carried out by protein precipitation with 1% acetic acid in acetonitrile containing the internal standards and subsequent dilution. Chromatographic separation of clomiphene isomers and their metabolites was performed using a rapid resolution column (ZORBAX Eclipse plus C18, 1.8 μm particle size, 100 mm x 2 mm I.D.; Agilent Technologies, Waldbronn, Germany) at a column temperature of 45°C, and a 1200 rapid resolution LC system (Agilent) with a binary pump using a gradient of acetonitrile in 0.1% formic acid in water. A 6460 triple quadrupole mass spectrometer (Agilent) equipped with a Jet Stream electrospray source (Agilent) was used for multiple reaction monitoring (38).

**Data analyses**

Linear regression was used to analyse the correlation between the amounts of metabolites formed and the protein content of specific CYPs in human liver microsomes (Prism 5.04 for windows, GraphPad Software Inc., La Jolla, CA, USA). Km and Vmax of Michaelis–Menten kinetics and AUC₀→∞ and Cₘₐₓ of in vivo kinetics were obtained by curve fitting using Prism. To test the influence of various CYP2D6 genotypes on the formation rates of (E)-4-OH-CLOM and (E)-4-OH-DE-CLOM in human liver microsomal incubations, Spearman tests were performed using R-2.11.1.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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