DMD Fast Forward. Published on October 18, 2016 as DOI: 10.1124/dmd.116.073437
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Title:

Application of Mice Humanised for Cytochrome P450 CYP2D6 to the Study of Tamoxifen Metabolism and Drug-Drug Interaction with Antidepressants

Authors:

A. Kenneth MacLeod, Lesley A. McLaughlin, Colin J. Henderson and C. Roland Wolf.

Affiliation:

Medical Research Institute, University of Dundee, Dundee DD1 9SY, United Kingdom.

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Running Title:

Tamoxifen and Antidepressants in Humanised Mice

Corresponding author:

C. Roland Wolf, Medical Research Institute, University of Dundee, Dundee DD1 9SY, United Kingdom. Tel +441382383134. Email: c.r.wolf@dundee.ac.uk

Number of text pages: 12

Number of tables: 2

Number of figures: 4 (+2 supplementary)

Number of references: 33

Number of words in the Abstract: 250

Number of words in the Introduction: 705

Number of words in the Discussion: 1483

Abbreviations

AD: anti-depressant

AUC: area under the curve

CAR: constitutive androstane receptor

CE: collision energy

Cmax: maximum plasma concentration

CV: cone voltage

CYP: cytochrome P450

DDI: drug-drug interaction

ER: Oestrogen receptor

HLM: human liver microsomes

hCYP2D6: CYP2D6-humanised mouse

KCl: potassium chloride

LC-MS/MS: liquid chromatography tandem mass spectrometry

MLM: mouse liver microsomes

NDT: N-desmethyltamoxifen

PBS: phosphate-buffered saline

PK: pharmacokinetic

PXR: pregnane X receptor

[S]: substrate concentration

SSRI: selective serotonin reuptake inhibitor

WT: wild-type

4-HT: 4-hydroxytamoxifen

Abstract

Tamoxifen is an oestrogen-receptor (ER) antagonist used in the treatment of breast cancer. It is a pro-drug, converted by several P450 enzymes to a primary metabolite, N-desmethyltamoxifen (NDT), which is then further modified by CYP2D6 to a pharmacologically potent secondary metabolite, 4-hydroxy-N-desmethyltamoxifen (endoxifen). Anti-depressants (ADs), which are often co-prescribed to patients receiving tamoxifen, are also metabolised by CYP2D6 and evidence suggests that a drug-drug interaction (DDI) between these agents adversely affects the outcome of tamoxifen therapy by inhibiting endoxifen formation. Here, we have evaluated this potentially important DDI *in vivo*, in mice humanised for CYP2D6 (hCYP2D6). The rate of conversion of NDT to endoxifen by hCYP2D6 mouse liver microsomes (MLM) *in vitro* was similar to that of the most active members of a panel of 13 individual human liver microsomes (HLM). Co-incubation with the CYP2D6 inhibitor, quinidine, ablated endoxifen generation by hCYP2D6 MLM. The NDT-hydroxylation activity of wild-type MLM was 7.4 times higher than that of hCYP2D6, while MLM from *Cyp2d* knockout animals were inactive. Hydroxylation of NDT correlated with that of bufuralol, a CYP2D6 probe substrate, in the HLM panel. *In vitro,* ADs of the selective serotonin reuptake inhibitor (SSRI) class were, by an order of magnitude, more potent inhibitors of NDT hydroxylation by hCYP2D6 MLM than were compounds of the tricyclic class. At a clinically-relevant dose, paroxetine pre-treatment inhibited the generation of endoxifen from NDT in hCYP2D6 mice *in vivo*. These data demonstrate the potential of ADs to affect endoxifen generation and, thereby, the outcome of tamoxifen therapy.

Introduction

Although tamoxifen has been approved for clinical use for over 40 years, only recently has it been identified as a potential pro-drug. Two hydroxylated metabolites in particular, endoxifen and 4-hydroxytamoxifen (4-HT), have been shown to be up to 100 times more potent ER-antagonists than the parent compound (Johnson et al., 2004) and are therefore likely to contribute to target inhibition and, thereby, the outcome of therapy (Figure 1). As endoxifen is several times more abundant in systemic blood samples than 4-HT, it is generally considered the more important of these metabolites (Stearns et al., 2003; Madlensky et al., 2011). Crucially, the rate-limiting step in the conversion of tamoxifen to endoxifen is catalysed by the highly polymorphic enzyme, CYP2D6 (Desta et al., 2004). Phenotypic status with regard to this enzyme profoundly influences the circulating level of endoxifen at steady-state (Stearns et al., 2003; Murdter et al., 2011). The clinical significance of these observations has been scrutinised intensely, with large numbers of retrospective studies finding both for and against an impact on therapeutic outcome. Perhaps most notably, a meta-analysis by the International Tamoxifen Pharmacogenomics Consortium found, when strict inclusion criteria were applied, a clear association of CYP2D6 poor metaboliser status with lower rates of invasive disease-free survival on tamoxifen therapy (Province et al., 2014).

In addition to the pharmacogenetic variability in CYP2D6 activity, this enzyme is also the focal-point for a number of clinically-significant DDIs. Between 10 and 25% of women with breast cancer experience depression (Fann et al., 2008), while as many as 70-80% experience hot flashes (hot flushes, (Day et al., 1999)). In both instances, ADs may be prescribed, many of which are both substrates for and inhibitors of CYP2D6. Available clinical data indicate a 45-58% decrease in plasma levels of endoxifen in individuals taking CYP2D6 inhibitors (Stearns et al., 2003; Jin et al., 2005; Borges et al., 2006). Two of the most commonly-used ADs, the SSRIs paroxetine and fluoxetine, are classed as strong inhibitors of CYP2D6 (FDA: Drug Development and Drug Interactions: Table of Substrates) and these have an even greater bearing on the amount of circulating endoxifen, particularly in individuals carrying allelic variants of CYP2D6 which confer extensive metaboliser

phenotype, with decreases of 64-71% (Stearns et al., 2003; Borges et al., 2006). In a retrospective population-based cohort study of 2430 individuals who received at least one antidepressant during tamoxifen therapy, paroxetine was found to be the only co-medication associated with an increased risk of death from breast cancer (Kelly et al., 2010), although this remains a contentious finding as other studies have yielded conflicting results (Lehmann et al., 2004; Chubak et al., 2008; Ahern et al., 2009; Dezentje et al., 2010; Lash et al., 2010).

Here, our aim was to utilise a CYP2D6-humanised mouse model to study tamoxifen metabolism, with a particular focus on whether an *in vivo* interaction of tamoxifen and its metabolites with ADs could be demonstrated.

Materials and Methods

Chemicals and reagents

High/low P450 activity HLM preparations from individual donors were purchased from BD Gentest (San Jose, CA, USA). Pooled human liver microsomes (150 donors) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Endoxifen and tamoxifen-d5 were obtained from Toronto Research Chemicals (Toronto, Canada). NADPH was purchased from Melford laboratories (Ipswich, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, UK).

Animal lines and husbandry

The generation and characterisation of Cyp2d-knockout and CYP2D6-humanised mice has been described previously (Scheer et al., 2012). Briefly, all nine functional murine *Cyp2d* genes were deleted to produce the Cyp2dKO line, and the hCYP2D6 line was generated by a targeted insertion of an expression cassette containing 9kb of the CYP2D6 promoter, along with all exons, introns and 5' and 3' untranslated regions, into the murine *Cyp2d* locus. These animals were obtained from Taconic (Cologne, Germany) and have been maintained by regular out-crossing to C57/BL6N, and back-crossed on the same genetic background for at least 6 generations. C57BL/6N mice were used as wild-type controls. Mice were housed on sawdust in solid-bottom, polypropylene cages and provided with an RM1 pelleted diet (Special Diet Services Ltd., Essex, UK) and drinking water ad libitum before and throughout the studies. The temperature was maintained within the range of 19–23°C, and the relative humidity was within the range of 40–70%. A 12-hour light/dark cycle was maintained. All animal procedures were carried out on 8- to 12-week old female mice under the auspices of the Animal (scientific Procedures) Act (1986), as amended by EU Directive 2010/63/EU, and after local ethical review.

Subcellular fractionation

Livers were excised and snap-frozen in liquid nitrogen for storage at -80°C until processing. These were thawed by the addition of 3 volumes of KCl buffer (1.15% w/v potassium chloride, 10mM

potassium phosphate, pH 7.4) and homogenised by rotor-stator. Debris was pelleted by centrifugation (11 000 x *g* at 4°C for 15 minutes) and the supernatant withdrawn for ultracentrifugation (100 000 x *g* at 4°C for 60 minutes). After ultracentrifugation, the pellet (microsomal fraction) was resuspended in KCl buffer containing 0.25 M sucrose. Protein content was quantified by Bradford assay (Bio-Rad, Hemel Hempstead UK).

***In vitro* studies**

All *in vitro* analyses were carried out in 100 mM potassium phosphate buffer, pH 7.4, containing 3.3 mM $MgCl_2$, with agitation at 400 rpm at 37°C on a thermo-shaker. All samples were handled in amber tubes under conditions of subdued light for the duration of the procedure. Incubations were initiated by the addition of NADPH to a final concentration of 1mM and terminated by transferring an aliquot of the reaction mixture, typically 50 µL, to two volumes of ice-cold acetonitrile containing internal standard (tamoxifen-d5 at 0.2 µg/mL), followed by vortexing (5s) and incubation on ice. Assays to determine the apparent kinetic parameters of endoxifen formation in hCYP2D6 MLM were performed in triplicate under conditions of linearity for time (6 min) and hCYP2D6 protein (0.0625 mg/mL). All subsequent assays were carried out under the same conditions, irrespective of the microsome source, with 5 µM NDT as substrate. For AD inhibition assays, fluoxetine, amitriptyline, clomipramine and imipramine were co-incubated with substrate. Paroxetine (at 2x concentration) was pre-incubated with MLM (0.125 mg/mL) and NADPH (1mM) for 20 minutes before addition of an equal volume of buffer containing NDT (10 µM) and fresh NADPH (2mM), therefore concentrations of all components in the final reaction mixture were the same for all ADs. Solvent (methanol) concentrations were 0.2% or lower in all incubations.

***In vivo* studies**

All animal work was carried out on 8- to 12-week-old female mice. Paroxetine maleate salt was dissolved to 2.16 mg/mL in PBS to give a solution of 1.6 mg/kg paroxetine which was administered at a dose of 8 mg/kg *p.o.* (5 µL per g body weight). NDT was suspended in corn oil at 2 mg/mL for *p.o.* administration at 10 mg/kg (5 µL per g body weight). For sample collection for pharmacokinetic (PK)

analysis, 10 µL of whole blood was withdrawn from the tail vein at the indicated time points. Samples were immediately added to a tube containing heparin solution (10 µL, 15 IU/mL) and stored at -20°C until processing.

Sample processing for LC-MS/MS

In vivo PK samples were thawed by the addition of 70 µL acetonitrile containing IS (tamoxifen-d5 at 0.2 µg/mL). After incubation on ice for ten minutes, *in vivo* and *in vitro* samples were vortexed (5s) and centrifuged for 10 minutes at 16 000 x *g*. The supernatant was added to 96-well plates for LC-MS/MS. As with microsomal incubations, all samples were handled in amber tubes under conditions of subdued light throughout the procedure.

LC-MS/MS

Analysis of *in vitro* incubation and *in vivo* blood PK samples was carried out on a Waters Acquity UPLC and Micromass Quattro Premier mass spectrometer (both Micromass, Machester, UK). LC separation was performed on a Kinetex 1.7µ C19 100A; 50 x 2.1 mm column (Phenomenex, Macclesfield, UK) at a temperature of 45°C with an injection volume of 5 µL and flow rate of 0.5 mL/min. Mobile phases were water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). Gradient elution was carried out from 70%/30% A/B to 30%/70% A/B over 2 minutes. Multiple reaction monitoring data in ESI-positive mode were acquired for NDT (358.20 > 57.98, cone voltage (CV) = 40 V, collision energy (CE) = 25 kV), endoxifen (374.22 > 58.04, CV = 42 V, CE = 23 kV) and tamoxifen-d5 (377.22 > 71.87, CV = 45 V, CE = 26 kV). Acquired data were analysed in Quanlynx relative to analyte standard curves spanning the range of concentrations under study. Analyte recovery was high (~90%) and the LC-MS/MS assay was highly reproducible between runs as reflected in the continuity of signal from the calibration standards.

Data analysis

In vitro kinetic data exhibited a substrate-inhibition profile and therefore was fitted with the following equation: $V=V_{max}*[S]/(K_{S1}+[S]*(1+[S]/K_{S2}))$, using GraFit version 7 (Erithacus Software, Horley, UK). Spearman's rank correlations and inhibition parameters of ADs were calculated using Graphpad Prism version 6 (La Jolla, CA, USA). PK parameters of *in vivo* data were calculated with a simple non-compartmental model using using PK Functions in Microsoft Excel and *P* values were calculated using an unpaired, one-tailed *t*-test.

Results

NDT is converted to endoxifen by CYP2D6 in hCYP2D6 MLM in vitro.

Under conditions of linearity for time and protein, formation of endoxifen from NDT in hCYP2D6 liver microsomes exhibited a kinetic profile suggestive of substrate inhibition (Figure 2A). Apparent kinetic parameters were obtained: K_{S1}: 5.1 ± 0.4 µM (dissociation constant for productive enzyme: substrate complex), K_{S2}: 3.9 ± 0.3 µM (dissociation constant for substrate bound to inhibitory site), V_{max} 1128 ± 57 pmol/min/mg. Under the same incubation conditions, and with 5 µM NDT as substrate, co-incubation with 1 µM quinidine reduced endoxifen formation by >95% (Figure 2B). Liver microsomes from Cyp2dKO mice did not produce detectable levels of endoxifen, while liver microsomes from wild-type mice produced 7.4-fold more endoxifen than hCYP2D6 liver microsomes. In incubations with a high/low P450 activity HLM panel, hydroxylation of NDT correlated most strongly with that of bufuralol, the probe drug for CYP2D6, although a statistically significant correlation was also observed with the probe for CYP2B6 (Table 1 and Supplemental Figure 1).

ADs inhibit the conversion of NDT to endoxifen by hCYP2D6 liver microsomes.

To determine whether ADs could inhibit the formation of endoxifen from NDT in hCYP2D6 liver microsomes, two SSRIs – paroxetine and fluoxetine – and three tricyclic anti-depressants – amitriptyline, clomipramine and imipramine – were individually titrated into the optimised reaction mixture. As paroxetine is a mechanism-based inhibitor of CYP2D6 (Bertelsen et al., 2003), a 20-minute pre-incubation step was carried out for this compound as described in Materials and Methods. All compounds inhibited the reaction with Ki values as follows; paroxetine: 57 nM (95% CI: 36.9 – 88.2) nM, fluoxetine: 59 nM (39 – 89), amitriptyline: 720 nM (515 – 1008), clomipramine: 489 nM (351 – 680), imipramine: 838 nM (576 – 1218) (Figure 3).

Paroxetine inhibits the CYP2D6-mediated conversion of NDT to endoxifen in hCYP2D6 mice in vivo.

In patients receiving the most common clinical dose of 20 mg tamoxifen per day, the average blood plasma $C_{max,ss}$ of NDT is approximately 200 ng/mL (Lien et al., 2013; Jager et al., 2014). Preliminary studies in our lab (data not shown) indicated that single doses of NDT required to achieve similar concentrations were not well-tolerated by all animals. Hence a dose of 10 mg/kg NDT was utilised here to give a reasonable level of exposure, at approximately half of steady-state human levels, while avoiding toxic effects. For paroxetine, although $C_{max,ss}$ is not dose-proportional (Sindrup et al., 1992), in patients receiving the relatively high dose of 40 mg/day it is approximately 150 ng/mL (GSK, 2008). We found that a single dose of 8 mg/kg paroxetine was appropriate to achieve maximum plasma concentrations in hCYP2D6 mice which were similar to this reported human value. Therefore, in order to establish whether the *in vitro* interaction of NDT with paroxetine occurred in hCYP2D6 mice *in vivo*, six animals were administered paroxetine and six were administered PBS vehicle alone, one hour prior to all 12 receiving NDT. Plasma levels of both of these compounds, and of endoxifen, were monitored over the following 48 hour period. There were no observed differences in the PK profile or apparent parameters of NDT between groups (Figure 4A). However, pre-treatment with paroxetine resulted in decreased exposure to endoxifen, with a highly significant reduction in AUC_{all} and moderate but non-significant decrease in C_{max} (Figure 4B, Table 2). Monitoring of paroxetine levels in the pre-treated group confirmed that the intended level of exposure had been reached (Figure 4C).

Discussion

Tamoxifen has been in clinical use for the treatment of cancer since the 1970s but the relatively recent discovery of endoxifen (Stearns et al., 2003), coupled with an increased understanding of the phenotypic variability of CYP2D6 (Zanger et al., 2004), has suggested opportunities for further optimisation of therapy. There is a general consensus that the interaction of tamoxifen with CYP2D6 and strong inhibitors thereof may have some bearing on the outcome of therapy, but prospective trials are yet required in order to determine the true extent of these effects, whether genotype-guided therapy should be adopted, and whether co-administration of certain anti-depressants with tamoxifen should be avoided (Kelly et al., 2010; Province et al., 2014).

Reported steady-state concentrations of tamoxifen metabolites in patient serum are highly variable but, with mean values for NDT, endoxifen and 4-HT of approximately 200, 10-50 and 2-5 ng/mL, respectively, metabolism through NDT is defined as the major route (Figure 1) (Stearns et al., 2003; Madlensky et al., 2011; Lien et al., 2013; Jager et al., 2014). However, in preliminary experiments with a single 15 mg/kg oral dose of tamoxifen in hCYP2D6 mice, we observed a 2.7-fold higher value for blood concentrations of 4-HT than for NDT (C_{max} for 4-HT and NDT were 235 and 86 ng/mL, respectively, data not shown), indicating that the major and minor pathways were reversed in this species. Hence we deemed it necessary to bypass this conflict in primary metabolism by using NDT as the substrate in our experiments. Recently, this same problem has been encountered in a mouse model containing humanisations for pregnane X receptor (PXR), constitutive androstane receptor (CAR), CYP3A4/3A7 and CYP2D6 (hPXR/hCAR/h3A4/3A7/2D6) (Chang et al., 2016). After administration of a single dose of 20 mg/kg tamoxifen to this complex model, 4-HT levels were found to be 5.4-fold higher than those of NDT (C_{max} for 4-HT and NDT were 248 and 46 ng/mL, respectively). As far as we are aware, the mouse enzymes which mediate this preferential hydroxylation of tamoxifen are yet to be identified.

There are over 100 identified allelic variants of *CYP2D6* (P450_Nomenclature_Database), with varying levels of activity in the metabolism of compounds of both endogenous and exogenous origin

(Yu et al., 2004; Zanger et al., 2004). Dependent on an individual's CYP2D6 status, they may be classed as either a poor, intermediate, extensive or ultrarapid metaboliser (Cascorbi, 2003; De Gregori et al., 2010). This phenotypic status can be determined through the analysis of the urinary ratio of debrisoquine to its CYP2D6-generated metabolite, 4-hydroxydebrisoquine (Dalen et al., 1999). In a previous study, we found that WT and Cyp2dKO mice were representative of human poor metabolisers in this regard, while hCYP2D6 mice were representative of extensive metabolisers (Scheer et al., 2012). In the current study we show that, *in vitro*, liver microsomes from Cyp2dKO mice are incapable of generating endoxifen from NDT, while WT MLM do so at a rate which greatly exceeds that of HLM. This comparatively high activity of murine P450s has been seen for other drugs and may, at least in part, be due to the multiplicity of these enzymes (Hrycay and Bandiera, 2009; Hasegawa et al., 2011; Scheer et al., 2012). Humanisation for CYP2D6 both removes this high activity murine component and incorporates the functional human component, rendering the amount of endoxifen produced more in line with that of the of the members of the HLM panel which possess the highest level of activity toward the CYP2D6 probe (bufuralol). As with the urinary ratio of debrisoquine to 4-hydroxydebrisoquine, therefore, it appears that Cyp2dKO and hCYP2D6 mice are at either end of the CYP2D6 phenotypic spectrum in relation to NDT metabolism *in vitro*. Our data for hCYP2D6 MLM yielded a K_{S1} value of 5.1 ± 0.4 µM, which is consistent with the K_m of 4.5 and 5.9 µM reported by Desta *et. al.* in their analyses of two individual HLM preparations (Desta et al., 2004). These authors also noted that, in a third HLM preparation, endoxifen was formed at a very slow rate. Our observations are also consistent with those of Chang *et. al.*, who reported a K_m value of 5.9 µM for 4-HT hydroxylation in their recent work with liver microsomes from the hPXR/hCAR/h3A4/3A7/2D6 model (Chang et al., 2016). Furthermore, we found that NDT-hydroxylation correlated most strongly with bufuralol hydroxylation in a panel of HLM, confirming the wider importance of CYP2D6 in this context. We also observed, however, a correlation with S-mephenytoin N-demethylation, the probe activity for CYP2B6. Although this correlation barely achieved statistical significance, this raises the possibility that there may be a role for this enzyme in the generation of endoxifen from NDT, which deserves further investigation.

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Here, the SSRIs paroxetine and fluoxetine inhibited the CYP2D6-dependent formation of endoxifen in hCYP2D6 MLM incubations with Ki values of 57 and 59 nM, respectively. These values are somewhat lower than the 360 nM (paroxetine) and 240 nM (fluoxetine) previously reported by Bertelsen *et. al.* (Bertelsen et al., 2003) in the inhibition of dextromethorphan O-demethylation by HLM but, in the case of paroxetine, other reported values are closer to our observed value (Crewe et al., 1992; Fogelman et al., 1999). Tricyclic ADs were an order of magnitude less effective in inhibiting NDT hydroxylation. Paroxetine is a strong inhibitor of CYP2D6 (FDA: Drug Development and Drug Interactions: Table of Substrates) and is the most likely AD to exert a negative influence on the outcome of tamoxifen therapy (Borges et al., 2006; Kelly et al., 2010). Here, we observed a substantial decrease in exposure (AUC_{all}) to endoxifen in hCYP2D6 mice treated with NDT and paroxetine, relative to control, demonstrating that this DDI occurs *in vivo*. Crucially, the maximum plasma concentrations of NDT and paroxetine were similar to those evident in patients at steady-state (GSK, 2008; Lien et al., 2013; Jager et al., 2014). We did not observe the converse effect with NDT, *i.e.* an increased exposure in the presence of paroxetine. This suggests that the routes of elimination for NDT and endoxifen may be different, and is in agreement with data from the hPXR/hCAR/h3A4/3A7/2D6 model (Chang et al., 2016). Indeed, 4-OH metabolites of tamoxifen, including endoxifen, are known to be glucuronidated and sulphated (Poon et al., 1993; Kisanga et al., 2005). It would therefore be informative to determine, in future experiments, whether the profiles of these and other circulating and excreted conjugates are altered with paroxetine co-administration. Furthermore, it would be interesting to ascertain whether paroxetine has a more profound effect on endoxifen levels in hCYP2D6 mice at steady state. As discussed above, hCYP2D6 mice align with the CYP2D6 extensive metaboliser phenotype, yet we see only a decrease in C_{max} of only 21%, and in AUC_{all} of only 28%. While this has to be viewed in consideration of the rapid elimination of paroxetine in our study, at steady-state levels of both NDT and the paroxetine these values may be more in line with the 64-71% decrease seen in patients (Stearns et al., 2003; Borges et al., 2006). Although plasma levels of paroxetine in hCYP2D6 mice were an order of magnitude higher than the observed *in vitro* Ki for inhibition of NDT hydroxylation (670 nM versus 57 nM), significant quantities of endoxifen were still produced. One possible explanation for this is the high level of

binding of paroxetine with murine plasma proteins which, at > 96%, is similar to the reported human value, as inhibition of CYP2D6 may be dependent on the free drug concentration (van Harten, 1993; Qin et al., 2016). Consistent with the *in vitro* analyses which indicated that hCYP2D6 liver microsomes were at least 2.6-fold more efficient than any member of the HLM panel in generating endoxifen, it should be noted that endoxifen levels were higher (between 2.5- and 13-fold) than in human subjects (Jin et al., 2005; Lien et al., 2013; Jager et al., 2014).

In the *in vivo* study presented here we attempted to incorporate a pharmacodynamic endpoint by Western blotting for ER targets (Cdc2, Mad2 and p21) in the endometrium, where tamoxifen is known to exert pro-oestrogenic effects, but no changes were observed (data not shown). This may be because of the short-term nature of the study: chronic administration of tamoxifen/NDT may be required to generate the pre-cancerous changes observed in human patients. We also investigated the utility of the C57BL/6-derived E0771 cell line for potential syngeneic tumour studies. As observed by Gu *et. al.*, this cell line expresses ERα at a level far lower than in the oestrogen-dependent MCF7 human cell line (Gu et al., 2009). Indeed, we found that E0771 cells exhibited no dependency whatsoever on oestradiol for their growth *in vitro* (Supplemental Figure 2). Future work incorporating pharmacodynamic endpoints, such as the anti-tumour activity of NDT in xenografted immunodeficient hCYP2D6 and Cyp2dKO mice, would allow the further evaluation of tamoxifen interactions with *CYP2D6* phenotype and ADs.

In summary, we have shown that humanisation for CYP2D6 is both necessary and sufficient to render the mouse human-like in its disposition to NDT. In modelling human-specific aspects of tamoxifen metabolism *in vivo* we have demonstrated that ADs, particularly those of the SSRI class, have the capacity to alter systemic exposure to pharmacologically potent metabolites, which may influence therapeutic outcome. Our work exemplifies the utility of humanised mouse models for the non-clinical study of drug metabolism.

Acknowledgements

We thank Dr. Yury Kapelyukh for help with kinetic analyses and Mrs. Julia Carr for assistance with animal work. We thank Taconic for their collaboration in the generation of the Cyp2dKO and hCYP2D6*2 mice used in this study.

Authorship Contributions

Participated in research design: MacLeod, McLaughlin, Henderson and Wolf.

Conducted experiments: MacLeod and McLaughlin.

Performed data analysis: MacLeod.

Wrote or contributed to the writing of the manuscript: MacLeod, Henderson and Wolf.

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Footnotes

Financial support:

This work was supported by Cancer Research UK [C4639/A10822].

Figure Legends

Figure 1. Phase I metabolism of tamoxifen. Cytochromes P450 metabolise tamoxifen through the "major" (N-demethylation followed by 4-hydroxylation) or "minor" (4-hydroxylation followed by N-demethylation) pathway, so denoted due to the relative abundance of each metabolite in plasma samples (Stearns et al., 2003; Madlensky et al., 2011). Conversion of NDT to endoxifen is catalysed exclusively by CYP2D6 (Desta et al., 2004).

Figure 2. CYP2D6 converts NDT to endoxifen in hCYP2D6 mice. (A) Kinetic analysis of endoxifen formation in hCYP2D6 MLM (pooled from four animals). Data represent combined mean ± SD of ten technical replicates carried out on four separate occasions. **(B)** Endoxifen formation in MLM and HLM. MLM were pooled from three (Cyp2dKO, WT) or four (hCYP2D6) individual animals. HLM were individual (13 donors) or pooled (150 donors). Data represent mean ± SD of duplicate incubations and are representative of an experiment carried out on two separate occasions.

Figure 3. Conversion of NDT to endoxifen in hCYP2D6 MLM is inhibited by ADs. Paroxetine was pre-incubated with MLM before addition of NDT, as described in Materials and Methods. All other compounds were co-incubated with NDT. Data represent mean of duplicate incubations and are representative of an experiment carried out on two separate occasions.

Figure 4. Paroxetine inhibits conversion of NDT to endoxifen in hCYP2D6 *in vivo*. Animals were dosed with paroxetine (8 mg/kg, n = 6) or vehicle (n = 6) then, one hour subsequently, all were dosed with NDT (10 mg/kg). Data shown are mean ± SEM.

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Table 1. Spearman's rank correlation of conversion of NDT to endoxifen with P450 probe substrate metabolism in a panel of 13 HLM. Spearman's rank correlation coefficient (SRCC) of NDT hydroxylation with metabolism of P450 probe substrates is shown. Probe substrate values were provided by the vendor.

Enzyme	Probe substrate activity	SRCC with NDT hydroxylation	p value
1A2	phenacetin O-deethylation	-0.289	0.360
2A6	coumarin 7-hydroxylation	0.161	0.600
2B6	s-mephenytoin N-demethylation	0.572	0.045
2C8	paclitaxel 6-alphahydroxylation	0.380	0.186
2C9	diclofenac 4'-hydroxylation	0.526	0.076
2C19	s-mephenytoin 4'-hydroxylation	-0.220	0.444
2D6	bufuralol 1'-hydroxylation	0.698	0.010
2E1	chlorzoxazone 6-hydroxylation	0.311	0.281
3A4	testosterone 6-betahydroxylation	-0.005	0.993
4A11	lauric acid 12-hydroxylation	-0.290	0.316

Table 2. **PK parameters of endoxifen in hCYP2D6 mice.** Parameters (mean ± SD) are shown for vehicle (n = 6) and paroxetine (n = 6) pre-treated groups.

	$t_{1/2}$ (hr)	C_{max} (ng/ml)	AUC_{all} (hr*ng/mL)
Vehicle	14.3 ± 4.7	132 ± 46	3508 ± 797
Paroxetine	13.8 ± 3.1	104 ± 12	2525 ± 321**
p-value	0.416	0.094	0.009

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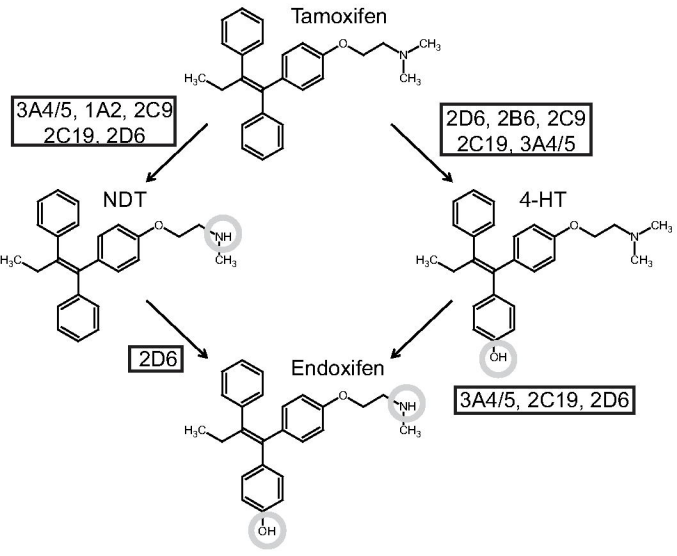

Figure 1

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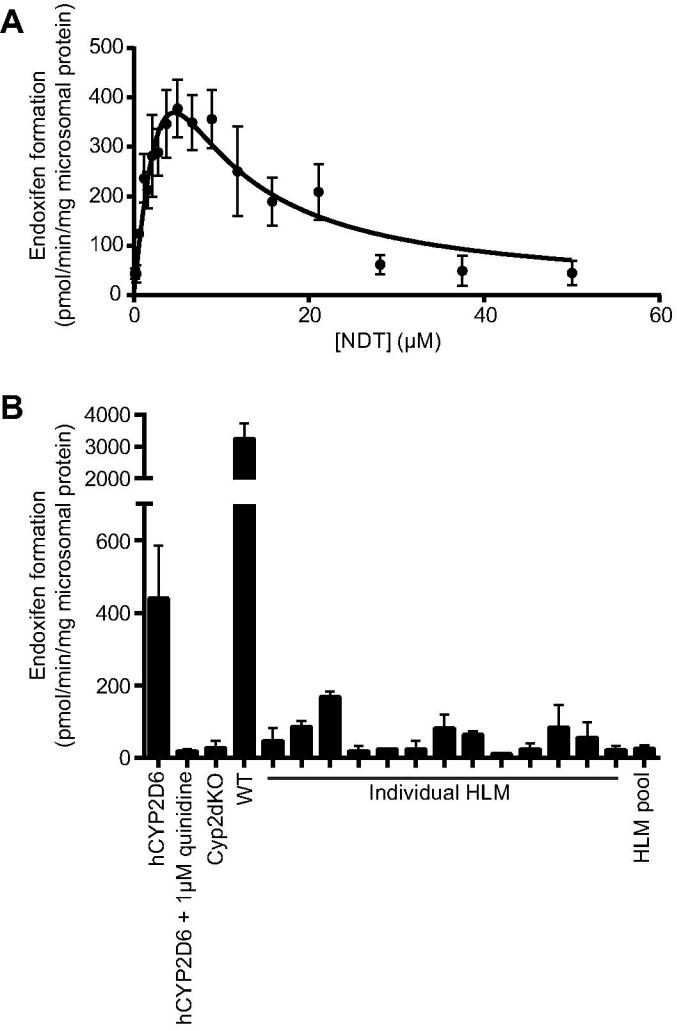

Figure 2

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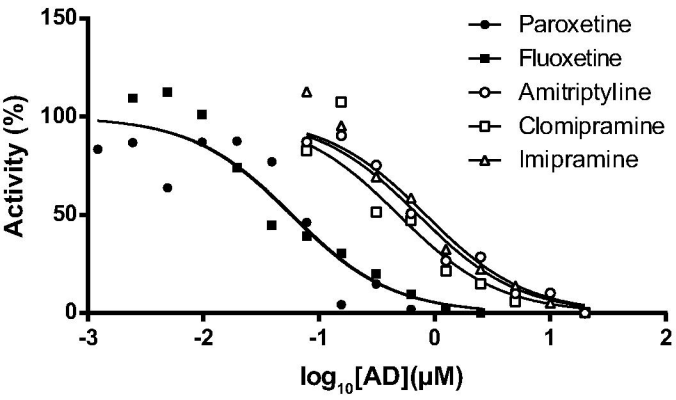

Figure 3

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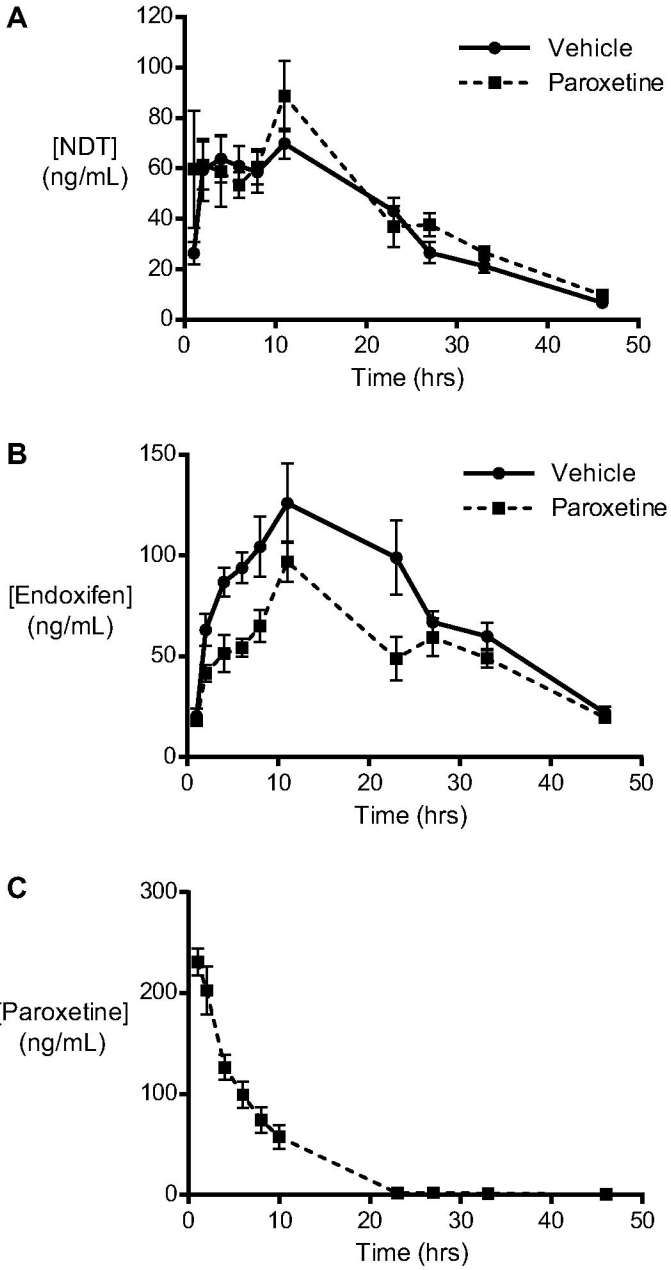


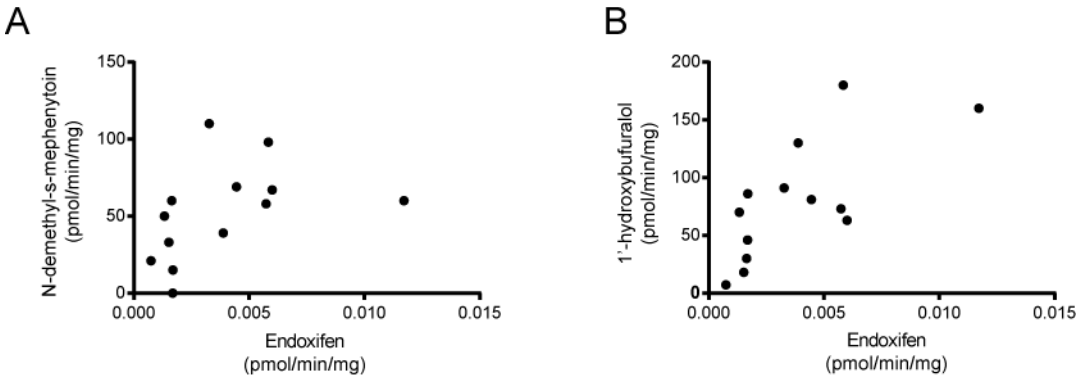
Figure 4

Supplemental Figures

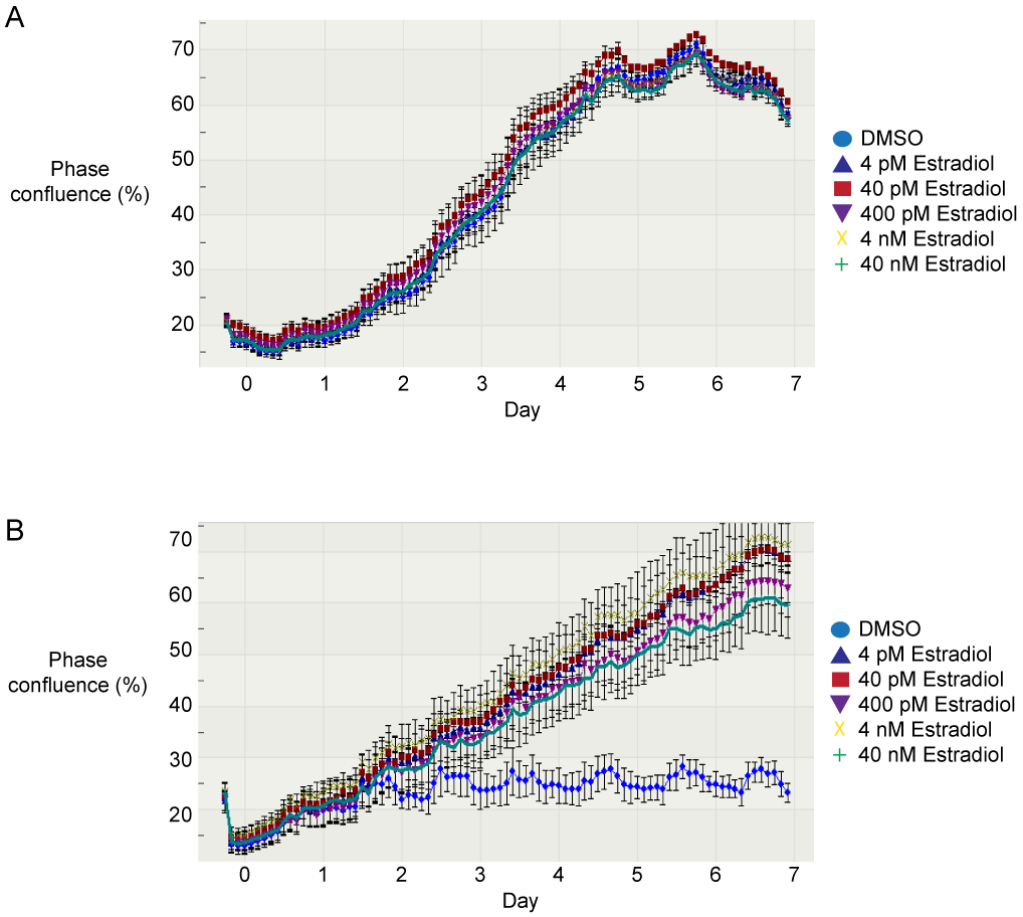
Application of Mice Humanised for Cytochrome P450 CYP2D6 to the Study of Tamoxifen Metabolism and Drug-Drug Interaction with Antidepressants

A. Kenneth MacLeod, Lesley A. McLaughlin, Colin J. Henderson and C. Roland Wolf.

Drug Metabolism and Disposition



Supplemental Figure 1. Spearman's rank correlation of NDT hydroxylation with CYP2B6 and and CYP2D6 probe activities. As detailed in Table 1, conversion of NDT to endoxifen correlated with **(A)** S-mephenytoin N-demethylation (CYP2B6 activity, SRCC = 0.572, p = 0.045) and **(B)** bufuralol 1'-hydroxylation (CYP2D6 activity, SRCC = 0.698, p = 0.010).



Supplemental Figure 2. E0771 cell proliferation is independent of oestradiol *in vitro*. (A) E0771 and **(B)** MCF7 (positive control) cells were cultured in the presence of a range of oestradiol concentrations, or with the vehicle (DMSO) only, for seven days. The experimental protocol was adapted from those of Johnson (Johnson et al., 2004) and Aakvaag (Aakvaag et al., 1990). Briefly, cells were routinely cultured in DMEM containing 10% foetal bovine serum (FBS, both Thermo Fisher Scientific). On the day before seeding, cell monolayers (70% confluency) were washed once every hour for five hours with experimental medium (phenol red-free DMEM containing 10% charcoal-stripped FBS, Thermo Fisher Scientific) then incubated in this medium overnight. The following morning, cells were trypsinised (phenol red-free trypsin-EDTA, Thermo Fisher Scientific) and seeded into 96-well plates at a density of 1.6 x 10^4 cells per well. Five hours later, medium was replaced with experimental medium containing a range of concentrations of oestradiol (Sigma) and growth monitored by measurement of phase confluency on the InCucyte ZOOM platform (Essen BioScience, Ann Arbor, MI, USA) every two hours.

Supplemental References

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