Tamoxifen Pharmacokinetics Beyond the Genotyping Era

Tamoxifen farmacokinetiek na het tijdperk van genotypering

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Chapter 1

Introduction

INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy in women in almost all countries worldwide and is the primary cause of cancer death among women. Approximately 1.7 million new cases of breast cancer and about 522,000 deaths from breast cancer occurred around the world in 2012.[1] Changes in the understanding of the biology of the disease have contributed to improvement in therapy. Breast cancer is not a single disease, but is composed of distinct subtypes associated with diverse clinical outcomes. This finding has contributed to the ending of one-size-fits-all therapies for breast cancer. The personalization (or individualization) of breast cancer treatment is based on a patient's unique biologic profile that should guide therapy. The ultimate goal is increased efficacy, ideally with less drug-related toxicity.[2, 3] The expression of estrogen and/or progesterone receptors was one of the first identified tumor characteristics that may predict response to therapy.[3] About two-thirds of breast cancers express estrogen receptors (ERs) and are dependent on estrogen for growth. Selective estrogen receptor modulators and aromatase inhibitors are effective therapies for hormone receptor-positive breast cancers as they deprive breast cancer cells of estrogens or estrogenic growth stimuli. Tamoxifen (approved by the FDA in 1977) is a selective estrogen receptor modulator which acts by blocking the action of estrogen in breast tissue by competing with estrogen for binding to the ER.[4] For almost forty years, tamoxifen has been widely used as endocrine therapy for estrogen receptor-positive breast cancer, both in the adjuvant setting and for metastatic disease. Five years of tamoxifen is currently the standard of care for premenopausal women with early breast cancer. In postmenopausal women tamoxifen is used in sequence with aromatase inhibitors or for women with contraindications to aromatase inhibitors.[5, 6]

Tamoxifen for 5 years has been shown to be highly effective in the adjuvant treatment of ER-positive breast cancer, reducing breast cancer recurrence by nearly forty percent and cancer-specific mortality by approximately a third during the first 15 years.[5] Further reductions in breast cancer recurrence and mortality have been observed by continuing tamoxifen therapy for 10 years.[7] In metastatic disease, tamoxifen has been associated with prolonged remission and survival.[8]

Although tamoxifen has proven to be an effective drug, not all women with estrogen receptor-positive tumors derive benefit from tamoxifen therapy. Women who all receive tamoxifen at the same dose, as in current daily practice, can have different clinical outcomes. In addition, occurrence of side effects may also differ between individuals, with

some individuals experiencing treatment-limiting side effects, while others do not notice any side effects.[5, 9]

Identifying predictive biomarkers of response to tamoxifen, other than expression of ERs, and the use of these markers to individualize tamoxifen therapy has become a field of intensive research.[4] Response or resistance to tamoxifen may be influenced by variation in expression of ERs, estrogen independent growth and amplification of HER2, among others.[9] In addition to these tumor-associated factors, much attention has been given to genetic polymorphisms in drug-metabolizing enzymes involved in the metabolic activation of tamoxifen into endoxifen, especially cytochrome P450 (CYP)2D6, as important host factors associated with resistance to tamoxifen.[4, 9]

Tamoxifen is a pro-drug and undergoes extensive metabolism to form its active metabolite 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). Several CYP enzymes are involved in the metabolic pathways from tamoxifen to endoxifen, including CYP2D6, CYP3A4, CYP3A5, CYP2C9, CYP2C19, CYP2B6, with key roles for CYP2D6 and CYP3A.[10, 11] Endoxifen is 30-100 times more active as anti-estrogen than its mother compound tamoxifen and believed to be responsible for the efficacy of tamoxifen.[12] Sufficient exposure to endoxifen seems therefore be important for therapeutic benefit. Recent studies suggest that endoxifen systemic concentrations should exceed a minimal threshold level to achieve optimal therapeutic effect from tamoxifen.[13, 14] Knowledge of factors influencing endoxifen exposure and taking into account of these factors contribute to the individualization of tamoxifen therapy.

Although polymorphisms in genes of drug-metabolizing enzymes do influence tamoxifen pharmacokinetics, contributing to variability in response to tamoxifen, this genetic variation accounts for only part of the large inter-patient variability in endoxifen concentrations.[11] Variability in tamoxifen pharmacokinetics is more likely the result of the relationship between patient-related factors, genetic factors, and environmental factors.[9] Thus, environmental factors including co-medication, lifestyle and adherence are also major contributors to the variability in endoxifen exposure.

In this thesis, environmental factors that may influence tamoxifen pharmacokinetics and contribute to inter-patient and intra-patient variability in endoxifen exposure, and ways to individualize and optimize tamoxifen therapy are studied.

The first chapter (**Chapter 2**) outlines strategies that could be used for the individualization of tamoxifen therapy. *CYP2D6* genotype has been suggested as a biomarker for tamoxifen efficacy, however, studies have reported inconsistent results. Also, it was found that *CYP2D6* genotype could only explain a minor part of the variability in endoxifen concentrations.

A phenotyping strategy could be used to individualize tamoxifen therapy, but therapeutic drug monitoring (TDM) is likely to be the best and most practical approach, as it takes into account all genetic and non-genetic factors influencing endoxifen exposure by simply measuring systemic concentrations. For this purpose, the relationship between endoxifen systemic exposure and clinical efficacy is highly essential.

For a good interpretation of the influence of genetic and environmental factors on tamoxifen pharmacokinetics and to investigate the association between tamoxifen metabolite concentrations and clinical outcome it is important to have a sensitive and selective analytical method for accurate measurement of systemic levels of tamoxifen, endoxifen and other metabolites. Since it has been discovered that 4'-hydroxymetabolites do not exhibit inhibitory effect on the ER, chromatographical separation of 4'-hydroxymetabolites and 4-hydroxymetabolites and quantification of the active metabolites is important for interpretation of the results of clinical studies. The same holds true for (E)-isomers and (Z)-isomers of 4-hydroxylated metabolites, as (E)-isomers have less than 1% of the ER affinity as compared with (Z)-isomers. In **Chapter 3** the development and validation of a highly sensitive and selective ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay for the quantification of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and (Z)-endoxifen in plasma is described. We also tested the light sensitivity of tamoxifen and its metabolites. The analytical assay has been used for the measurements in subsequent pharmacokinetic studies.

Because response to tamoxifen is probably related to endoxifen exposure, an approach to increase endoxifen concentrations in women receiving tamoxifen was examined. We hypothesized that endoxifen exposure could be increased by induction of CYP enzymes involved in the metabolism of tamoxifen (especially CYP3A). We therefore used the well-known inducer rifampicin. In **Chapter 4** the effects of CYP induction by rifampicin on tamoxifen pharmacokinetics were evaluated in a randomized cross-over study.

Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) are widely used for the treatment of depression, anxiety disorders and alleviation of hot flashes. Both are frequently co-prescribed in women receiving tamoxifen. However, these antidepressants inhibit CYP2D6 to varying degrees. Potent CYP2D6-inhibiting antidepressants, such as paroxetine and fluoxetine, have been shown to significantly reduce endoxifen concentrations and may interfere with tamoxifen efficacy. This has resulted in recommendations to avoid these potent CYP2D6-inhibiting SSRIs in tamoxifen-treated individuals. In **Chapter 5** dispensing data for tamoxifen and six frequently used SSRIs and venlafaxine were monitored to assess changes in co-prescription

of these antidepressants in tamoxifen-treated women over time (2005 – 2010). For this study, data from a community pharmacy database (PHARMO-Institute for Drug Outcome Research) were used.

Antidepressants that are weak inhibitors of CYP2D6, such as escitalopram, are likely to be safer alternatives in women receiving tamoxifen. In women who use paroxetine or fluoxetine concomitantly with tamoxifen, the potent CYP2D6-inhibiting antidepressant could be switched to the weak CYP2D6 inhibitor escitalopram. In **Chapter 6** we assessed the effects of switching these antidepressants on tamoxifen pharmacokinetics and evaluated whether switching resulted in relevant rises in endoxifen exposure in these women.

The pharmacokinetics of tamoxifen may not only vary between individuals, but also within individuals who take the same dose of the drug. Circadian variations in biochemical and physiological functions may influence the pharmacokinetics of drugs such as tamoxifen. In **Chapter 7** we evaluated circadian variation in tamoxifen pharmacokinetics by assessing pharmacokinetic differences among three different dosing times: in the morning, afternoon and evening. In this way, variation in endoxifen systemic exposure within women could be identified. Circadian variation in the pharmacokinetics of tamoxifen was also evaluated in mice, examining 6 different administration times. Variation in plasma pharmacokinetics as well as organ exposure was assessed. Additionally, mRNA expression of key CYP enzymes involved in the metabolism of tamoxifen was quantified to detect potential daily rhythmicity.

In **Chapter 8** an update is given of the available evidence of genetic and non-genetic factors influencing tamoxifen pharmacokinetics and which have been associated with breast cancer outcomes. The metabolism of tamoxifen is quite complex and only a small part of the pharmacokinetic variability can be explained by impaired CYP2D6 activity due to genetic polymorphisms. The activity of other CYP enzymes involved in phase I metabolism of tamoxifen as well as UDP-glucuronosyltransferases and sulfotransferases can also be affected by genetic polymorphisms and co-medication, which may influence tamoxifen pharmacokinetics and clinical efficacy. In addition, environmental factors such as age, body mass index, adherence, seasonal variation and circadian variation are other factors contributing to pharmacokinetic differences of tamoxifen. Because all these factors may affect the levels of the pharmacologically active metabolite endoxifen, which is thought to be effective against breast cancer recurrence above a minimal threshold level, the direct measurement of endoxifen concentrations seems the most promising approach for treatment individualization. Barriers to apply TDM for tamoxifen are discussed in the review.

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Chapter 2

Individualization of tamoxifen treatment for breast carcinoma

Binkhorst L, van Gelder T, Mathijssen RH.

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ABSTRACT

Traditionally, all patients treated with tamoxifen receive a standard dose. A number of studies claimed a clinically relevant impact of cytochrome P450 2D6 (*CYP2D6*) genotype on outcome, and therefore genotyping before tamoxifen therapy was advocated. Recent data showed that adequate exposure to the active metabolite endoxifen is important and that genotype only partially explains inter-individual differences in endoxifen concentrations. Phenotyping approaches, as well as therapeutic drug monitoring (TDM) strategies, are now being tested to individualize tamoxifen treatment.

TAMOXIFEN TREATMENT AND METABOLISM

Tamoxifen is an oral endocrine therapy for early and advanced estrogen receptor-positive breast cancer that has been commonly used for more than 30 years. In breast tissue, tamoxifen and its metabolites exert estrogen antagonist effects, reducing the risk of disease recurrence and breast cancer mortality. Besides predominant anti-estrogen effects in breast tissue, tamoxifen has favorable effects on bone density and the cardiovascular system by functioning as a partial estrogen agonist in these tissues. Currently, patients receive a fixed daily dose of tamoxifen regardless of the characteristics of an individual patient. Given the wide inter-patient variability in tamoxifen pharmacokinetics, it is not surprising that the response to tamoxifen in terms of clinical efficacy and adverse events greatly varies among patients.

Tamoxifen is extensively metabolized in humans, into at least twenty-two metabolites, involving various oxidative and conjugation routes catalyzed by many phase I and phase II metabolizing enzymes (Figure 1).[1] Cytochrome P450 (CYP) CYP2D6 and CYP3A play a dominant role in phase I metabolism of tamoxifen. Tamoxifen is metabolized into 4-hydroxytamoxifen and endoxifen, both of which have a 2-log higher affinity for the estrogen receptor as compared with the mother compound itself. Endoxifen is also formed through an alternative metabolic route, with *N*-desmethyltamoxifen as an important intermediate metabolite. Because of the relatively high plasma concentrations of endoxifen, this metabolite is believed to be of extreme importance for the efficacy of tamoxifen treatment. To further complicate the metabolism of tamoxifen, it was recently found that only (Z)-endoxifen is active, in contrast to its inactive (Z)-4'-isomer.[1]

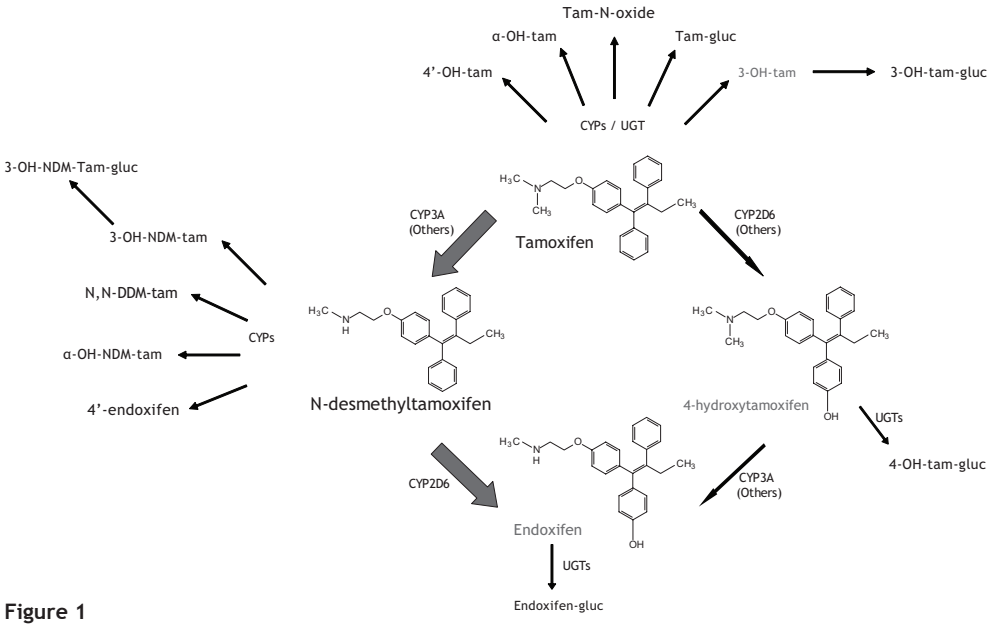

Figure 1

Tamoxifen is metabolized by CYP2D6 into 4-hydroxytamoxifen and then by CYP3A4 into endoxifen. As an alternative pathway, tamoxifen is metabolized by CYP3A4 into *N*-desmethyltamoxifen and then by CYP2D6 into endoxifen. Phase I and phase II enzymes will further metabolize these metabolites into active (in gray) and inactive metabolites (in black). The size of the names of these metabolites reflect the abundance in human plasma.

GENOTYPING AND PHENOTYPING STRATEGIES TO INDIVIDUALIZE TAMOXIFEN TREATMENT

The CYP2D6 enzyme, encoded by a highly polymorphic gene, is of undeniable importance for the formation of endoxifen (Figure 1). Polymorphisms in *CYP2D6*, in particular *CYP2D6*4*, which results in absent enzyme activity, have been associated with lower endoxifen plasma concentrations and diminished tamoxifen efficacy. *CYP2D6* genotype was therefore suggested as a useful marker to predict treatment outcome of tamoxifen in individual patients. Hence, for a long time there was interest in studying *CYP2D6* genotyping in order to individualize tamoxifen therapy. Although several studies found poorer disease-free survival in tamoxifen-treated patients with reduced or non-functioning *CYP2D6* alleles, the use of genotyping to predict response to tamoxifen treatment was not supported by others. Two recently published large prospective studies - the Breast International Group (BIG) 1-98 trial and the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial - are generally

believed to be decisively unsupportive for *CYP2D6* genotyping in the adjuvant setting.[2] No associations were found between impaired CYP2D6 phenotype (predicted by genotype) and breast cancer recurrence. Endoxifen concentrations were not measured in these studies. Although genetic variation in CYP2D6 activity has been related to endoxifen concentrations, discrepancy between *CYP2D6* genotype-predicted phenotype and endoxifen concentrations, as observed,[3] may contribute to the conflicting results on the role of *CYP2D6* genotyping in individualizing tamoxifen therapy.

Only ~39% of the inter-individual variability in endoxifen plasma concentrations could be clarified by *CYP2D6* genotype, leaving unexplained a very large proportion of variability in endoxifen concentrations.[1] In addition to *CYP2D6* genotype, other factors are of influence on endoxifen systemic concentrations. Use of concomitant medication, especially drugs associated with CYP2D6 inhibition (e.g., antidepressants), was not always taken into account in studies relating *CYP2D6* genotype and clinical outcome. Also, factors that influence CYP3A enzyme activity may contribute in important ways to altered endoxifen concentrations. Although CYP3A activity is less sensitive for genetic variation, environmental factors and co-medication may strongly affect the oxidative capacity of this iso-enzyme. Because *CYP2D6* genotyping does not account for the use of CYP2D6-inhibiting co-medication and the influence of CYP3A enzyme activity on tamoxifen pharmacokinetics, an alternative approach for individualization of tamoxifen therapy has been proposed. This approach, called phenotyping, makes use of a 'probe' drug to predict tamoxifen pharmacokinetics.

Dextromethorphan, a known probe for combined CYP2D6 and CYP3A activity (Figure 2), was chosen in the phenotyping approach. Plasma exposure of this compound showed a good correlation with endoxifen exposure and trough concentrations.[3] By using this phenotyping strategy, endoxifen exposure could be predicted more accurately, as compared with the more widely investigated *CYP2D6* genotyping method. Although phenotyping is still quite complex to perform and facilitation is required, time will tell whether this test is of additional value in tailored tamoxifen treatment.

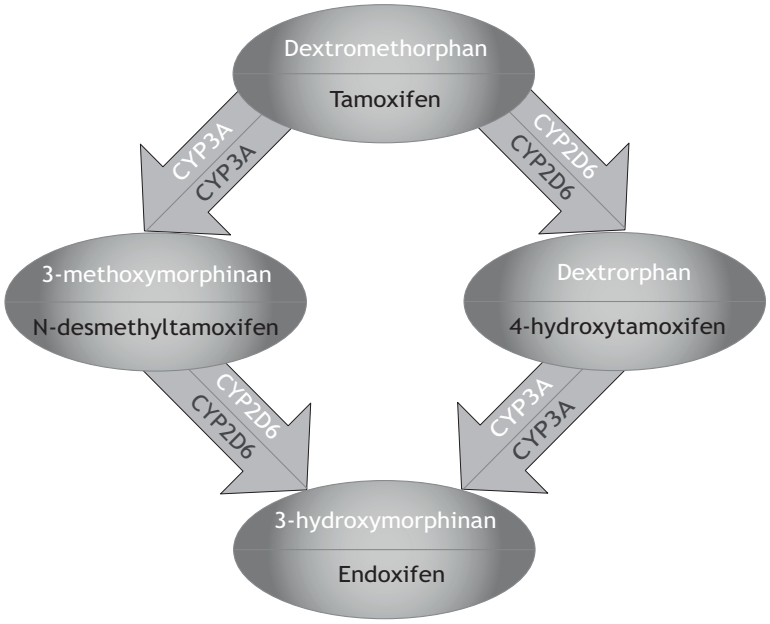

Figure 2

Metabolism of dextromethorphan 'projected' on the metabolism of tamoxifen. Similar to tamoxifen, dextromethorphan is metabolized by CYP3A4 and CYP2D6 into intermediate metabolites (3-methoxymorphinan and dextrorphan, respectively) and then by CYP2D6 and CYP3A4 into 3-hydroxymorphinan.

TARGET CONCENTRATION AND TDM

A crucial issue in individualizing tamoxifen therapy is the association between plasma exposure of endoxifen and efficacy of tamoxifen treatment. Associations between tamoxifen-metabolite plasma concentrations and long-term treatment outcomes have been investigated in 1,370 participants of The Women's Healthy Eating and Living (WHEL) study.[4] No (linear) associations with treatment outcome were observed for tamoxifen, 4-hydroxytamoxifen, or *N*-desmethyltamoxifen. However, a threshold effect for endoxifen was suggested, after distribution of endoxifen concentrations into quintiles. A higher risk of disease recurrence was found in women with endoxifen concentrations belonging to the lowest quintile.[4] Clearly, more studies are required to confirm these associations between active (Z)-endoxifen plasma concentrations and treatment outcome.

Because a threshold concentration for endoxifen to achieve therapeutic benefit from tamoxifen has been suggested, strategies to predict whether this minimal effective level

will be attained are important. *CYP2D6* genotyping appeared to be less useful, as 24% of the patients with poor metabolizer genotype still achieved higher than threshold endoxifen concentrations in the WHEL study.[4] Although the probe dextromethorphan predicted endoxifen systemic concentrations quite accurately,[3] factors not related to CYP2D6 and CYP3A activity may also potentially affect tamoxifen pharmacokinetics. For example, reduced CYP2C9 enzyme activity has also been shown to affect endoxifen plasma concentrations, emphasizing the role of other enzymes in endoxifen formation.[1] TDM is therefore suggested as another strategy to optimize tamoxifen therapy. Because genotyping and phenotyping strategies could not explain all variability in endoxifen exposure, monitoring endoxifen plasma concentrations, instead of predicting them, seems to be the most appropriate approach to individualize tamoxifen therapy. Importantly, in the TDM strategy a clear relationship between plasma concentrations of the drug of interest and clinical effect should exist, and measured plasma concentrations ought to be useful for decision making in clinical practice. Currently, it is not clear what the exact therapeutic threshold for endoxifen is, making this strategy less efficient. Moreover, plasma concentrations may not reflect concentrations in target tissue because metabolizing enzymes expressed in breast (cancer) tissue may seriously affect intracellular concentrations and possibly alter the estrogen receptor-inhibiting capacity of endoxifen. TDM also has practical limitations because steady state plasma concentrations of tamoxifen-metabolites are not reached until ~4 months after start of therapy or dose adjustment.[1,5] In line with the phenotyping strategy, TDM is laborious, requiring multiple sample collections and quantification of plasma concentrations. In addition, samples must be taken at precisely determined time points, and patient's compliance is of great importance for a reliable interpretation of measured endoxifen plasma concentrations, indicating that this strategy is also susceptible to inaccuracy.

DOSE INCREASE OF TAMOXIFEN

In theory, a higher tamoxifen dose does not necessarily lead to higher levels of endoxifen, particularly in patients with impaired CYP2D6 enzyme activity, due to either inactive alleles or co-medication. Recently, it was shown that endoxifen concentrations can be increased in patients with reduced CYP2D6 activity by escalating the standard tamoxifen dose of 20 mg to 40 mg.[5] Higher endoxifen levels were found in patients classified as intermediate metabolizers for *CYP2D6*, and even in patients with *CYP2D6* poor metabolizer status. Dose

increase was also shown to be safe, as adjustments in these patients did not result in more side effects (e.g., hot flashes). However, the change in endoxifen concentrations varied widely among patients, with a strong increase in a minority of intermediate metabolizer patients, and no change at all in some others.[5] Although compliance could have played a role in these observations, results of this study suggest that escalating the dose does not lead to dose-proportional increases in endoxifen concentrations in individual patients.[5]

FUTURE PERSPECTIVES

First, it is important that associations between endoxifen plasma concentrations and tamoxifen treatment outcome be confirmed in prospective studies and that endoxifen target concentrations be established. Next, strategies for individualization of tamoxifen therapy can be examined and should be compared with current practice, again prospectively. These studies should identify the best strategy to improve response to tamoxifen treatment.

Serious toxicity is less common in tamoxifen-treated patients, but not totally absent. For instance, the appearance of endometrial cancer due to tamoxifen treatment raises the question whether a maximum systemic concentration of tamoxifen should also be determined. And should the tamoxifen dose be decreased in patients with very high endoxifen concentrations? In addition, with the discovery of new metabolites, it is also possible that other active metabolites will be found in the near future. In that case, we should not only focus on endoxifen exposure but also take other metabolites into account. Possibly, the sum of (active) metabolites should be determined and related to clinical efficacy. Clearly, more research is needed to answer all these remaining questions and define the best strategy, or strategies, for individualization of tamoxifen treatment.

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Chapter 3

Quantification of tamoxifen and three of its phase-I metabolites in human plasma by liquid chromatography / triple-quadrupole mass spectrometry

Binkhorst L, Mathijssen RH, Ghobadi Moghaddam-Helmantel IM, de Bruijn P, van Gelder T, Wiemer EA, Loos WJ.

J Pharm Biomed Anal 2011;56:1016-23.

ABSTRACT

In view of future pharmacokinetic studies, a highly sensitive ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method has been developed for the simultaneous quantification of tamoxifen and three of its main phase 1 metabolites in human lithium heparinized plasma. The analytical method has been thoroughly validated in agreement with FDA recommendations. Plasma samples of 200 µL were purified by liquid-liquid extraction with 1 mL *n*-hexane/isopropanol, after deproteination through addition of 50 µL acetone and 50 µL deuterated internal standards in acetonitrile. Tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen were chromatographically separated on an Acquity UPLC® BEH C18 1.7 µm 2.1 mm x 100 mm column eluted at a flow-rate of 0.300 mL/min on a gradient of 0.2 mM ammonium formate and acetonitrile, both acidified with 0.1% formic acid. The overall run time of the method was 10 minutes, with elution times of 2.9, 3.0, 4.1 and 4.2 minutes for endoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen and tamoxifen, respectively. Tamoxifen and its metabolites were quantified by triple-quadrupole mass spectrometry in the positive ion electrospray ionization mode. The multiple reaction monitoring transitions were set at 372>72 (*m*/*z*) for tamoxifen, 358>58 (*m*/*z*) for *N*-desmethyltamoxifen, 388>72 (*m*/*z*) for 4-hydroxytamoxifen and 374>58 (*m*/*z*) for endoxifen. The analytical method was highly sensitive with the lower limit of quantification validated at 5.00 nM for tamoxifen and *N*-desmethyltamoxifen and 0.500 nM for 4-hydroxytamoxifen and endoxifen, which is equivalent to 1.86, 1.78, 0.194 and 0.187 ng/mL for tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, respectively. The method was also precise and accurate, with within-run and between-run precisions within 12.0% and accuracy ranging from 89.5 to 105.3%. The method has been applied to samples from a clinical study and cross-validated with a validated LC-MS/MS method in serum.

INTRODUCTION

The selective estrogen receptor modulator tamoxifen remains an important drug in the treatment of estrogen receptor (ER)-positive breast cancer. In the United States tamoxifen is also approved for the prevention of breast cancer in women at high-risk.[1-4] Tamoxifen reduces the risk of recurrence and the risk of mortality, however, not all women benefit from tamoxifen therapy, and treatment-related adverse reactions also vary greatly between patients. Inter-individual variability in metabolism of tamoxifen, which is influenced by both genetic and environmental factors, contributes to the differences in efficacy and toxicity of tamoxifen.[1, 2, 5-7]

Tamoxifen is a pro-drug and undergoes biotransformation into several metabolites, including *N*-desmethyltamoxifen, which is the most abundant metabolite, and its active metabolites 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). The cytochrome P450 enzymes CYP3A4 and CYP2D6 play a dominant role in the biotransformation of tamoxifen, with other CYP enzymes (CYP2B6, CYP2C9 and CYP2C19) playing a minor role.[7-9] The anti-estrogenic potency of 4-hydroxytamoxifen and endoxifen, regarding ER-binding and suppression of estrogen-dependent proliferation of breast cancer cells, is 30-100-fold higher compared with tamoxifen. As plasma concentrations of endoxifen are 5-10 times higher than of 4-hydroxytamoxifen, endoxifen is thought to be of most importance for the pharmacological activity of tamoxifen treatment.[7, 10, 11]

Several studies have shown that genetic variation in CYP2D6 enzymes and the concomitant use of CYP2D6 inhibitors influence endoxifen plasma concentrations.[7, 12-14] In addition, the activity of other CYP enzymes (CYP3A4/5, CYP2C9 and CYP2C19), which may also be affected by genetic polymorphisms and concomitant medication, may be responsible for the large inter-patient variability in endoxifen plasma concentrations. Therefore, monitoring endoxifen plasma concentrations rather than *CYP2D6* genotype testing is suggested to be a better approach to personalize tamoxifen therapy.

To assess the effects of genetic polymorphisms in cytochrome P450 enzymes and influences of co-medication on the plasma concentrations of tamoxifen and its metabolites and for monitoring of endoxifen plasma concentrations, quantification of these compounds with a sensitive and validated analytical method is important. For this purpose, the development of bioanalytical methodologies for the quantification of tamoxifen and its metabolites in human serum, plasma, urine and tissue have been reported in various publications, reviewed by Teunissen *et al.*.[15] However, not all analytical assays included tamoxifen and its three main metabolites (*N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen). In addition,

not all assays have been thoroughly validated, which is important for its use in clinical pharmacokinetic studies and clinical practice.[15]

Although a few LC-MS/MS assays have been adequately validated and included at least the three main phase I metabolites,[16-18] the sensitivity of the methods may not be enough for the determination of low metabolite concentrations. One of these validated LC-MS/MS methods[16] was used for the quantification of tamoxifen and its metabolites in a recent study, in which dextromethorphan was used as a phenotyping probe to predict endoxifen exposure in patients using tamoxifen.[19] In several patients, serum levels of the tamoxifen metabolites 4-hydroxytamoxifen and endoxifen were below the lower limits of quantification of 1.13 and 2.69 ng/mL, respectively, and could not be reliable determined.

In view of future pharmacokinetic studies, we developed a highly sensitive and selective ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay for tamoxifen and its main phase I metabolites. The method requires only 200 µL plasma and involves a liquid-liquid extraction procedure for the purification of the plasma samples. The method is fully validated according to the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA, with lower limits of quantitation of 1.86, 1.78, 0.194 and 0.187 ng/mL for tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, respectively.

EXPERIMENTAL

Chemicals

Pure Z *(cis)*-isomers of tamoxifen, *N*-desmethyltamoxifen and 4-hydroxytamoxifen, the stable labeled deuterated internal standards tamoxifen-d5, *N*-desmethyltamoxifen-d5, 4-hydroxytamoxifen-d5 and a racemic mixture of the Z- and E-isomers (1:1) of 4-hydroxy-*N*-desmethyltamoxifen-d5 were obtained from Toronto Research Chemicals (North York, ON, Canada). The pure Z *(cis)*-isomer of 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) was kindly provided by Jina Pharmaceuticals Inc. (Libertyville, IL). All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were from Biosolve BV (Valkenswaard, The Netherlands). Dimethylsulphoxide (DMSO), ammonium formate, glycine and *n*-hexane were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), sodium hydroxide and 2-propanol from Merck (Darmstadt, Germany) and formic acid from J.T. Baker (Deventer, The Netherlands). Blank human lithium heparinized plasma was obtained from Biological Specialty Corporation (Colmar, PA).

Preparation of stock solutions, calibration standards and quality control samples

Stock solutions containing 1.00 mM free base of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in DMSO were prepared individually. Following preparation, stock solutions were stored at T < -70°C. Individual stock solutions of tamoxifen and its metabolites were used for the preparation of a working stock solution, containing 200 µM tamoxifen, 200 µM *N*-desmethyltamoxifen, 20 µM 4-hydroxytamoxifen and 20 µM endoxifen in DMSO. The working stock solution was divided into 150 µL aliquots, which were used for the construction of calibration curve standards during the validation. Separate stock solutions (i.e., independent weightings) of tamoxifen and its metabolites were used for the preparation of the pools of quality control (QC) samples. The variation between the stock solutions of tamoxifen and its metabolites used for the construction of the calibration standards and QC samples was in all cases < 5%.

Deuterated internal standards were dissolved in DMSO separately, to obtain internal standard stock solutions at a concentration of 1 mg/mL free base, which subsequently were aliquotted and stored at T < -70°C. Aliquots of 10 µL of the individual stock solutions were concurrently 10,000-fold diluted in acetonitrile, resulting in an internal standard working solution containing 100 ng/mL tamoxifen-d5, *N*-desmethyltamoxifen-d5, 4-hydroxy tamoxifen-d5 and 4-hydroxy-*N*-desmethyltamoxifen-d5, which was stored at T < 8°C for a maximum of 3 months.

Calibration curve standards were freshly prepared (in duplicate) for each run, by addition of 10 µL aliquots of appropriate dilutions of the working stock solution in acetonitrile/DMSO (1:1, v/v) to 190 µL aliquots of human lithium heparinized plasma (excepted of calibration standard 7, which was prepared by addition of 45 µL diluted working stock solution to 955 µL plasma) at the following concentrations: 5.00, 10.0, 50.0, 100, 250, 500, 900, and 1000 nM for tamoxifen and *N*-desmethyltamoxifen and 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, 90.0, and 100 nM for 4-hydroxytamoxifen and endoxifen.

A total of five pools of quality control (QC) samples were prepared by spiking appropriate dilutions of stock solutions of tamoxifen and its metabolites to human lithium heparinized plasma at concentrations of 5.00 nM (LLQ), 15.0 nM (QC-Low), 400 nM (QC-Middle), 800 nM (QC-High) and 16,000 nM (QC-Diluted) for tamoxifen and *N*-desmethyltamoxifen and at 0.500 nM (LLQ), 1.50 nM (QC-Low), 40.0 nM (QC-Middle), 80.0 nM (QC-High) and 1,600 nM (QC-Diluted) for 4-hydroxytamoxifen and endoxifen. QC-Diluted was processed after a 20-fold dilution in blank human lithium heparinized plasma. Pools of QC samples were aliquotted and stored at T < -70°C until analysis.

Plasma sample preparation

Aliquots of 50 µL of internal standard working solution and 50 µL of acetone were added to 200 µL of plasma samples in 1.5 mL microcentrifuge tubes and vigorously vortexed for 5 minutes. The samples were then centrifuged at 18,000 x *g* at ambient temperature for 10 minutes. Subsequently, the supernatant was transferred into 2 mL microcentrifuge tubes and 100 µL aliquots of glycine buffer (pH 11.5) and 1 mL aliquots of *n*-hexane/2-propanol (95:5, v/v) were added. Hereafter, the samples were again vortexed and centrifuged under the previously mentioned conditions. Aliquots of 800 µL of the organic phase were transferred into 4.5 mL glass tubes and evaporated to dryness under nitrogen at T = 60°C. The residues were reconstituted in 100 µL aliquots of acetonitrile/water/formic acid (40:60:0.1, v/v/v) and centrifuged for 30 seconds at 4,000 x *g*. The supernatants were transferred into 350 µL 96-well plates, which were placed into a chilled (T = 10°C) autosampler, from which aliquots of 5 µL were injected onto the UPLC column.

Equipment

The UPLC-MS/MS system was composed of a Waters Acquity UPLC Sample Manager coupled to a Waters TQ Detector (Waters, Etten-Leur, The Netherlands). The MassLynx V4.1 SCN627 software package was used for the acquisition and processing of data. Quantification was performed using QuanLynx as implemented in the MassLynx software.

Chromatographic conditions

An Acquity UPLC® BEH C18 column 1.7 µm, 100 mm x 2.1 mm, (Waters, Etten-Leur, The Netherlands), thermostatted at T = 50°C, was used for the separation of the analytes. Aqueous ammonium formate (0.2 mM) and acetonitrile, both acidified with 0.1% formic acid, were used as mobile phase A and mobile phase B, respectively. Using these mobile phases, a gradient at a flow-rate of 0.300 mL/min was achieved. A linear gradient separation was used, with 30-80% of mobile phase B from 0 to 6 minutes, then 80-30% of mobile phase B over 2 minutes, which was held for 2 minutes for re-equilibration of the system. An autosampler (at 10°C) injected volumes of 5 µL onto the UPLC column. The overall run time was 10 minutes. The needle of the autosampler was washed using a strong needle wash solvent (water/acetonitrile/2-propanol/methanol/formic acid, 25:25:25:25:0.1 v/v/v/v/v) and a weak needle wash solvent (30% acetonitrile in water). The column effluent was introduced to the mass spectrometer and monitored.

Mass spectrometry

Tandem mass spectrometry was performed in the positive ion electrospray ionization mode. Mass transitions of *m/z* were optimized for tamoxifen, its metabolites and the deuterated internal standards of tamoxifen and its metabolites by infusion of the respective analytes in acetonitrile/water/formic acid (40:60:0.1, v/v/v) via combined infusion. Optimal MS settings were adjusted manually. The desolvation gas was set at 800 L/h, the cone gas at 25 L/h (nitrogen) and the ionspray voltage was kept at 1.50 kV. The cone voltage was kept at 45 V for tamoxifen, endoxifen and their deuterated internal standards, 42 V for *N*-desmethyltamoxifen and *N*-desmethyltamoxifen-d5 and 47 V for 4-hydroxytamoxifen and its internal standard, with a source temperature of T = 150°C and desolvation temperature of T = 350°C. The dwell times were set at 50 ms and the inter-channel delay at 10 ms. Multiple reaction monitoring (MRM) mode was applied for the quantitation with the parameters as presented in Table 1. The collision cell pirani pressure was set at ~5e ^{3} mbar (argon).

Table 1. MS/MS settings

Analyte	Scan window (minutes)	Parent *(m/z)*	Daughter *(m/z)*	Collision (V)
Tamoxifen	3.50 – 5.00	372	72	25
Tamoxifen-d5	3.50 – 5.00	377	72	25
N-desmethyltamoxifen	3.50 – 5.00	358	58	21
N-desmethyltamoxifen-d5	3.50 – 5.00	363	58	21
4-OH-tamoxifen	2.50 – 3.50	388	72	25
4-OH-tamoxifen-d5	2.50 – 3.50	393	72	25
Endoxifen	2.50 – 3.50	374	58	23
Endoxifen-d5	2.50 – 3.50	379	58	23

Quantitation

Calibration curves were constructed by plotting the peak area ratios of the components to internal standards versus the known concentrations with a weight factor of 1/concentration^{2}.

Light sensitivity of tamoxifen and its metabolites

An experiment in which tamoxifen and its metabolites were exposed to several light sources was conducted to investigate the stability during sample handling and preparation. A solution of tamoxifen and its metabolites was prepared by addition of 50 µL working stock solution to 20 mL human lithium heparinized plasma. Subsequently four groups of samples were prepared using this solution. The first group was protected from light for 6 hours, the second group was exposed for 6 hours to UV-light (254 nm), the third group was exposed for 6 hours to daylight (~350-700 nm) in 1.5 mL microcentrifuge tubes and the fourth group was exposed for 6 hours to daylight (~350-700 nm) in 1.5 mL amber-colored microcentrifuge tubes. The four groups of plasma samples were analyzed by UPLC-MS/MS using the conditions described in the experimental sections.

Method validation

The UPLC-MS/MS method was validated in agreement with the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

Blank human lithium heparinized plasma samples of ten different lots were analyzed to determine the potential presence of endogenous contaminating compounds that may interfere with the assay. Potential clinical co-administered drugs were investigated for possible interference with the analytical method, including aprepitant, citalopram, dexamethasone, dextromethorphan, domperidon, ibuprofen, lorazepam, metoclopramide, oxazepam, pantoprazole, paracetamol, paroxetine, ranitidine, rifampicin and venlafaxine. All drugs have been dissolved and/or diluted in water to a concentration of 1 mg/mL and subsequently 200-fold diluted in human lithium heparinized plasma to provide final concentrations of 5 µg/mL. Aliquots of QC-Diluted (i.e., 16,000 nM for tamoxifen and *N*-desmethyltamoxifen and 1,600 nM for 4-hydroxytamoxifen and endoxifen) have subsequently been diluted in the plasma containing the above mentioned drugs at concentrations of QC-High (in triplicate), which have been processed and compared to equal dilutions of QC-Diluted in blank human lithium heparinized plasma.

For the determination of the LLQ, blank human lithium heparinized plasma samples of 10 different donors were spiked at a concentration of 5.00 nM for tamoxifen and *N*-desmethyltamoxifen and 0.500 nM for the other two metabolites and analyzed during one run. Accuracy (ACC), within-run precision (WRP) and the between-run precision (BRP) were determined by analyzing 5 replicates of pools of LLQ and QC samples independently over a

three-run period, with the calibration curve standards processed in duplicate. The ACC, WRP and BRP at the level of the LLQ and QC samples were calculated by one-way analysis of variance, using the run as the variable as earlier described.[20, 21]

The evaluation of the matrix effect for tamoxifen and its metabolites was tested by comparing the MS/MS response of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen at a concentration of 25.0 nM and 80.0 nM spiked in triplicate in acetonitrile/ water/formic acid (40:60:0.1, v/v/v) to the MS/MS responses of the analytes spiked in triplicate into extracts of blank human lithium heparinized plasma, as described recently.[21, 22]

Extraction recovery (RE) was determined by comparing the MS/MS response of tamoxifen and its metabolites at 25.0 nM and 80.0 nM spiked in triplicate into six different lots of blank lithium heparinized plasma before extraction, to the MS/MS responses of the analytes spiked in triplicate into extracts of blank human lithium heparinized plasma after extraction, corrected for the evaporated volume of organic phase.[21, 22]

The stability of tamoxifen and its metabolites in human lithium heparinized plasma was tested in triplicate at the concentrations of QC-Low, QC-High and QC-Diluted during overnight (i.e., ~18 hours) incubation at ambient temperature, following three freeze-thaw cycles, in which the samples were thawed for at least 15 minutes followed by refreezing for at least 18 hours. The storage stability of processed samples in the autosampler was tested using samples at the same concentrations. QC samples were processed in triplicate and repeatedly injected at different time points (within a period of 24 hours).

Application of the method to clinical samples

The method has been cross-validated with a validated LC-MS/MS method for the analysis of tamoxifen and its metabolites in serum. A total of 76 samples of patients using tamoxifen (see www.trialregister.nl; NTR study number 1751), from which serum samples have been analyzed using the method as published by Teunissen *et al.*[16] and from which also plasma aliquots were available, were quantitated by the method as described here.

The described analytical method has also been applied to pharmacokinetic samples, derived from the previously mentioned clinical study, with serum levels of 4-hydroxytamoxifen and endoxifen below the lower limits of quantification of the analytical method (1.13 and 2.69 ng/mL, respectively).

RESULTS AND DISCUSSION

LC-MS/MS conditions and method development

The tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen product ion spectra (Figure 1) yield abundant product ions suitable for use in multiple reactions monitoring. The selected product ions and collision energies of tamoxifen, its metabolites and their respective deuterated internal standards are presented in Table 1.

Because of its direct influence on the sensitivity and selectivity of the method, sample pre-treatment is of great importance in the development of an analytical method. As protein precipitation results in less clean extracts, remaining endogenous compounds may cause ion-suppression and thereby negatively affect the sensitivity of the assay. Solid phase and liquid-liquid extraction lead to more purified extracts and are, therefore, appropriate sample pre-treatment procedures. Solid phase extraction has, if not automated, disadvantages including poor reproducibility and is, compared to liquid-liquid extraction, relatively laborious.[15, 23] In this method, a liquid-liquid extraction procedure was applied with acetonitrile, acetone and *n*-hexane/isopropanol, which resulted in clean extracts.

By applying a linear gradient, tamoxifen and its three metabolites were adequately base-line separated and separated from early eluting hydrophilic, potentially interfering matrix components, while maintaining a relative short injection to injection time of 10 minutes with elution times of 2.9 minutes for endoxifen, 3.0 minutes for 4-hydroxytamoxifen, 4.1 minutes for *N*-desmethyltamoxifen and 4.2 minutes for tamoxifen (Figure 2). Two additional peaks were detected in the chromatograms of 4-hydroxytamoxifen and endoxifen, with elution times of approximately 3.3 and 3.2 minutes, respectively, which are 4'-hydroxytamoxifen and 4'-hydroxy-*N*-desmethyltamoxifen.[17, 18]

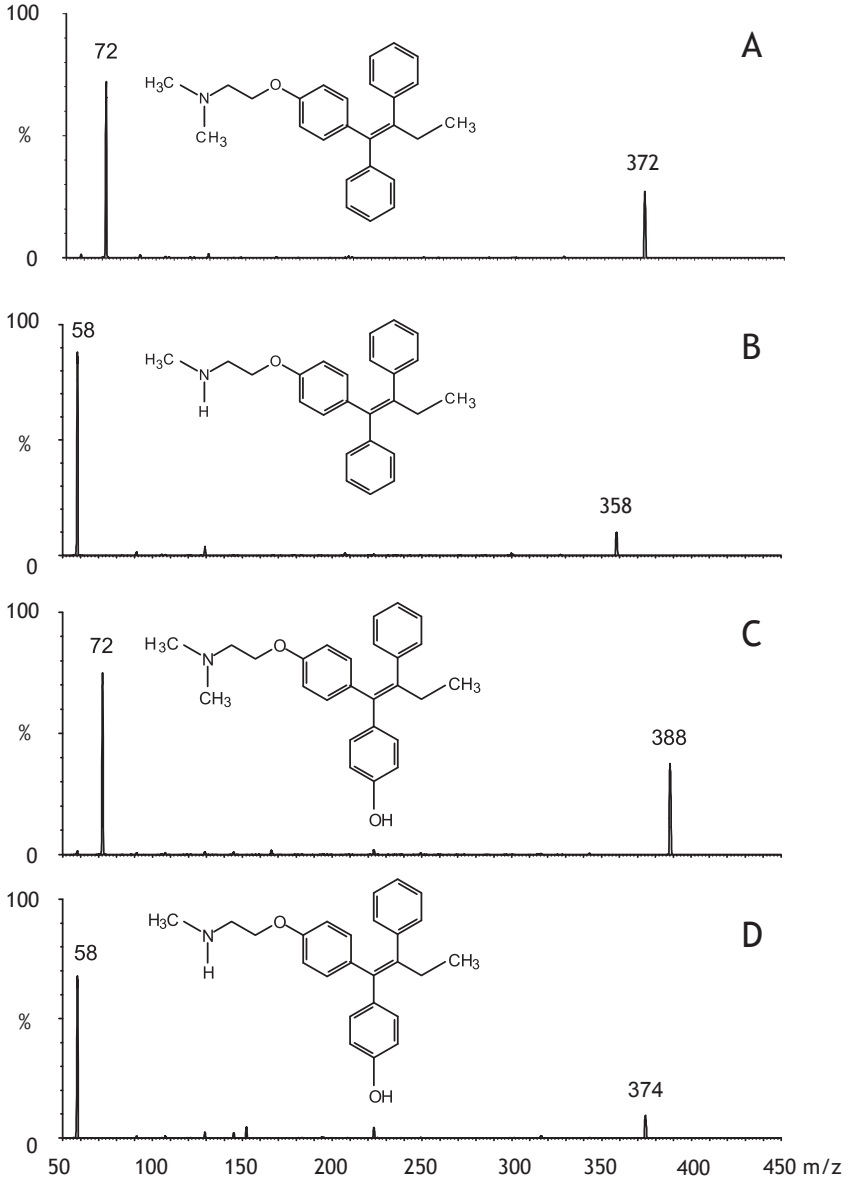

Figure 1

Mass spectrum and chemical structures of tamoxifen (A), *N*-desmethyltamoxifen (B), 4-hydroxytamoxifen (C) and endoxifen (D).

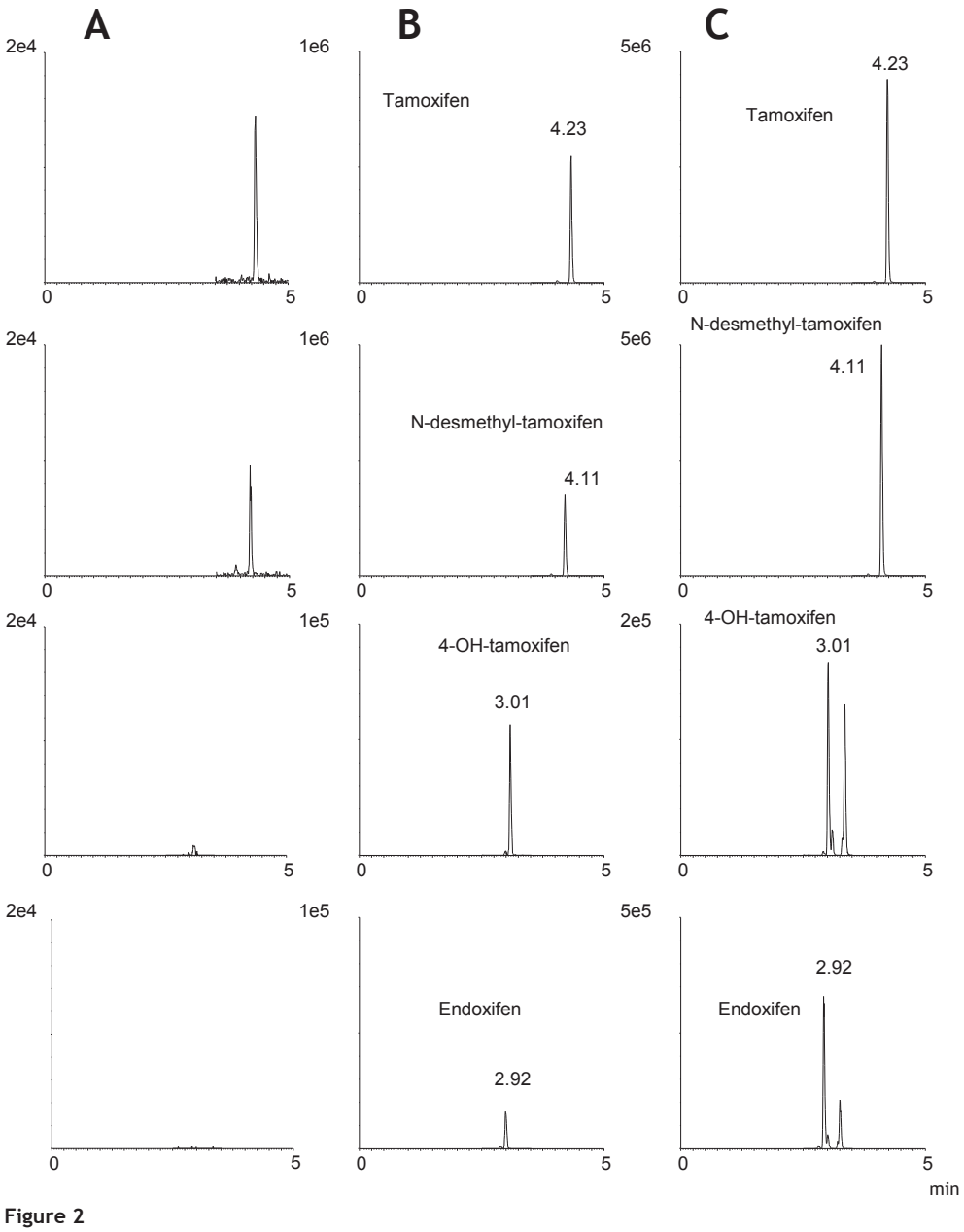

Figure 2

Representative chromatograms of a double blank processed plasma sample (A), a plasma sample spiked at the concentration of the LLQ (B) and a plasma sample collected 4 hours after 20 mg tamoxifen administration on steady state containing 143 nM tamoxifen, 229 nM *N*-desmethyltamoxifen, 4.29 nM 4-hydroxytamoxifen and 20.1 nM endoxifen (C).

Light sensitivity of tamoxifen and its metabolites

It has been reported that tamoxifen is light sensitive and should be protected from light during sample handling and preparation.[16] Data on light sensitivity of tamoxifen and its metabolites are, however, lacking. To investigate the sensitivity of tamoxifen and its metabolites to light, the extent of degradation of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen under different light source conditions was determined. One group of samples was for 6 hours protected from light, the second group was exposed for 6 hours to UV-light (254 nm), the third group was exposed for 6 hours to daylight (~350-700 nm) in 1.5 mL microcentrifuge tubes and the last group was exposed for 6 hours to daylight (~350-700 nm) in 1.5 mL amber-colored microcentrifuge tubes. Samples were analyzed and compared to samples which were immediately stored at T < -70°C after preparation (reference samples). Tamoxifen and its metabolites were very light sensitive under UV-light (254 nm). No degradation of tamoxifen or its metabolites was observed when the samples were exposed to daylight in 1.5 mL (transparent) microcentrifuge tubes (Figure 3). Sample handling and preparation could therefore be conducted under normal laboratory conditions.

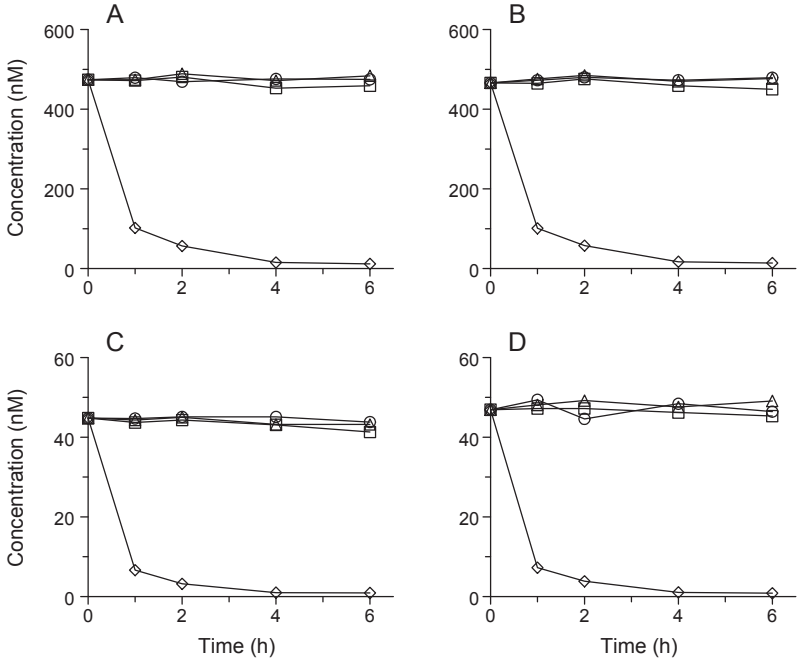

Figure 3

Exposure of tamoxifen (A), *N*-desmethyltamoxifen (B), 4-hydroxytamoxifen (C) and endoxifen (D) to UV-light (lozenges), daylight in transparent microcentrifuge tubes (squares), daylight in amber-colored microcentrifuge tubes (triangles) and protected from light (circles).

Assay performance

The method results were linear ($r^2 \geq 0.995$) in the concentration range of 5.00 to 1,000 nM for tamoxifen and *N*-desmethyltamoxifen and of 0.500 to 100 nM for 4-hydroxytamoxifen and endoxifen in human lithium heparinized plasma and none of the blank plasma samples showed potential interference for tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen, endoxifen or any of the deuterated internal standards.

None of the tested, potentially co-administered drugs interferes with the quantitation of tamoxifen or its metabolites.

The LLQ was validated at 5.00 nM for tamoxifen and *N*-desmethyltamoxifen and at 0.500 nM for 4-hydroxytamoxifen and endoxifen, which is equivalent to 1.86, 1.78, 0.194 and 0.187 ng/mL for tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, respectively. The LLQ has been validated in separate runs. In one validation run, analytes were spiked to 10 different lots of human lithium heparinized plasma. In three other runs, a pool of LLQ samples was processed as QC samples. For tamoxifen, measured concentrations in 9 of 10 independently spiked plasma samples fell within the acceptable range of accuracy of 80-120%, with an average measured concentration of 4.78 ± 0.554 nM. The measured concentrations of *N*-desmethyltamoxifen for all 10 independent heparinized plasma samples fell within the acceptable range of accuracy, with an average observed concentration of 4.98 ± 0.454 nM. The average concentration for 4-hydroxytamoxifen in the 10 independent samples (8 in acceptable range) was 0.554 ± 0.053. For endoxifen, measured concentrations in 9 of 10 independent samples fell within the acceptable range of accuracy, with an average concentration of 0.496 ± 0.053 nM.

The within-run and between-run precisions and the accuracies at five tested concentrations, including at the level of the LLQ, are summarized in Table 2 and all fell within the accepted ranges as specified by the FDA.

The extraction recovery (RE) and matrix effect (ME) were determined in six different lots of lithium heparinized plasma, spiked with tamoxifen and its metabolites at a concentration of 25.0 nM and 80 nM. The mean measured extraction efficiencies and matrix effect are shown in Table 3. As shown, no matrix effect was observed for tamoxifen or its metabolites. The recoveries ranged from 64% for tamoxifen to 87% for 4-hydroxytamoxifen.

Tamoxifen and its metabolites were stable in lithium heparinized plasma during overnight incubation at ambient temperature, following three freeze-thaw cycles and as processed samples in the chilled (T = 10°C) autosampler for at least 24 hours.

Table 2. Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples[a]

Sample	Spiked (nM)	GM (nM)	ACC (%)	WRP (%)	BRP (%)	n[c]
Tamoxifen						
LLQ	5.00	4.55	91.0	6.10	2.01	15 of 15
Low	15.0	13.7	91.3	3.76	2.78	14 of 15
Middle	400	371	92.8	2.97	0.75	15 of 15
High	800	724	90.5	2.70	#[b]	15 of 15
Diluted	16,000	15,588	97.4	3.83	7.72	14 of 15
***N*-desmethyltamoxifen**						
LLQ	5.00	4.51	90.2	3.90	5.63	15 of 15
Low	15.0	14.0	93.3	4.81	#[b]	14 of 15
Middle	400	376	94.0	3.90	1.94	15 of 15
High	800	734	91.8	2.48	1.73	15 of 15
Diluted	16,000	15,466	96.7	3.53	4.47	15 of 15
4-OH-tamoxifen						
LLQ	0.500	0.520	104.8	6.08	5.44	15 of 15
Low	1.50	1.52	101.3	3.60	2.63	15 of 15
Middle	40.0	41.1	102.8	3.52	#[b]	15 of 15
High	80.0	80.5	100.6	3.03	#[b]	15 of 15
Diluted	1,600	1,684	105.3	2.97	5.17	15 of 15
Endoxifen						
LLQ	0.500	0.457	91.4	12.0	8.19	12 of 15
Low	1.50	1.35	90.0	4.06	1.05	13 of 15
Middle	40.0	36.7	91.8	3.54	2.67	14 of 15
High	80.0	71.6	89.5	2.84	2.29	13 of 15
Diluted	1,600	1,517	94.8	5.00	5.83	15 of 15

Abbreviations: GM, grand mean; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy;

[a] n=5 in 4 separate runs (3 runs at the LLQ).

[b] No additional variation observed by performing the assay in different runs.

[c] Number of individual samples falling within acceptable range of accuracy of 85-115% (80-120% at LLQ).

Table 3. Extraction recovery (RE) and matrix effect (ME) in lithium heparinized plasma from six different lots spiked with all analytes at a concentration of 25.0 nM and 80.0 nM.

	25.0 nM		80.0 nM	
Analyte	ME (%)	RE (%)	ME (%)	RE (%)
Tamoxifen	126 ± 7.4	64 ± 6.8	103 ± 2.2	79 ± 13.6
N-desmethyltamoxifen	111 ± 10.0	83 ± 4.5	91 ± 2.4	95 ± 16.0
4-OH-tamoxifen	108 ± 0.7	87 ± 5.3	107 ± 1.3	90 ± 8.4
Endoxifen	96 ± 5.0	76 ± 4.2	97 ± 5.6	81 ± 8.3

Data presented as mean ± s.d. (n=6)

Clinical application

As shown in Figure 4, concentrations of tamoxifen and its metabolites quantitated in serum using the method of Teunissen *et al.*[16] and in lithium heparinized plasma by our method are comparable, with random errors across all concentrations for all compounds. Differences in quantitated concentrations of tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen between plasma and serum analysis were determined. Respectively 96%, 91%, 96% and 93% of the samples fell within a range of 30% difference and respectively 92%, 79%, 86% and 80% of the samples even fell within a range of 20% difference.

The described analytical method was also applied to samples from a previous clinical study, with serum concentrations below the lower limits of quantification for 4-hydroxytamoxifen and endoxifen (1.13 and 2.69 ng/mL, respectively).[16] We observed concentrations as low as 1.18 nM (0.46 ng/mL) for 4-hydroxytamoxifen and 2.39 nM (0.891 ng/mL) for endoxifen, stressing the need for highly sensitive analytical methods.

CONCLUSION

A highly sensitive, selective, accurate and precise method has been developed and validated for the simultaneous analysis of tamoxifen and its three main phase I metabolites, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, in human lithium heparinized plasma. As tamoxifen and its three main metabolites were stable in daylight in transparent microcentrifuge tubes, sample handling and preparation can be conducted under normal laboratory conditions.

The validation method meets the current requirements of bioanalytical method validation and the method is one of the most sensitive methods, especially for endoxifen, published so far with lower limits of quantitation of 1.86, 1.78, 0.194 and 0.187 ng/mL for tamoxifen,

N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, respectively. The analytical method has been successfully cross-validated with a validated LC-MS/MS method for the analysis of tamoxifen and its metabolites in serum.

By using the described analytical method, we were able to quantify low concentrations of 4-hydroxytamoxifen and endoxifen. As observed in some patients in a previous clinical study, quantification of low endoxifen concentrations is important in view of future pharmacokinetic studies and for monitoring of endoxifen plasma concentrations.

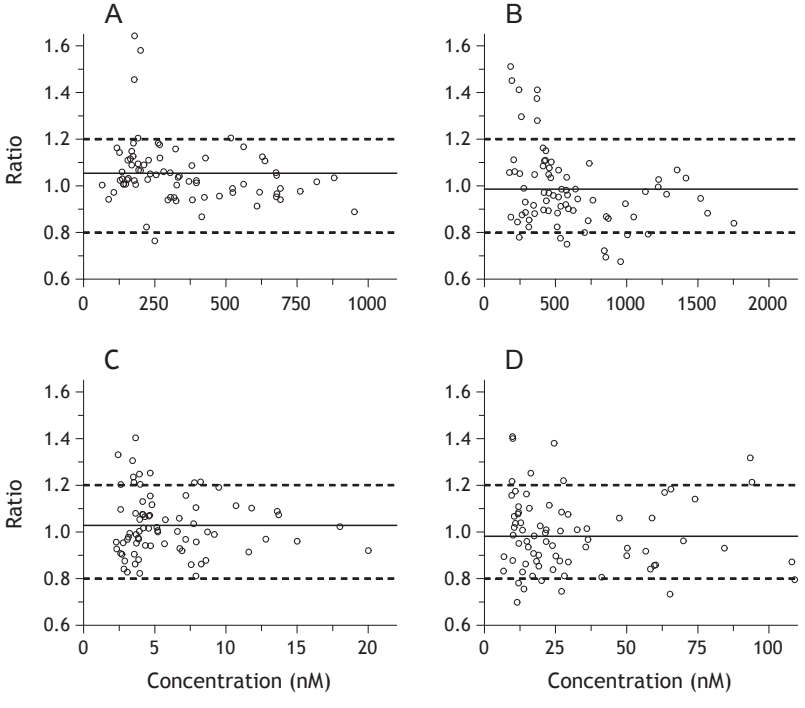

Figure 4

Cross validation results of the analysis of 76 samples analyzed in serum by the method published recently[16] and the current method in plasma for tamoxifen (A), *N*-desmethyltamoxifen (B), 4-hydroxytamoxifen (C) and endoxifen (D). On the X-axis, the serum concentrations are plotted and on the Y-axis the ratios C_{plasma}/C_{serum}. The solid line represents the average ratio (i.e., 1.0 is equal), while the dotted lines represents the 20% difference between the plasma and serum analysis.

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Chapter 4

Effects of CYP induction by rifampicin on tamoxifen exposure

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ABSTRACT

Tamoxifen undergoes biotransformation into several metabolites, including endoxifen. Differences in metabolism contribute to the inter-individual variability in endoxifen concentrations, potentially affecting treatment efficacy. We evaluated the effects of cytochrome P450 (CYP) induction by rifampicin on the exposure of tamoxifen and its metabolites. Co-administration of rifampicin resulted in markedly reduced (up to 86%, $P \leq 0.040$) exposure to tamoxifen and its metabolites. Given the extensive metabolism of tamoxifen, several factors may have contributed to this effect. Similar drug-drug interactions may exist between tamoxifen and other strong CYP inducers.

INTRODUCTION

The selective estrogen receptor modulator tamoxifen, a drug frequently used in breast cancer treatment, reduces the risk of recurrence of disease as well as mortality.[1] However, there is a large variability in response to tamoxifen in terms of efficacy and toxicity.[1, 2] Inter-individual variability in bioactivation of tamoxifen into its active metabolites, which is influenced by both genetic and environmental factors, may contribute to these differences.[3-5] Tamoxifen undergoes biotransformation into several metabolites (Figure 1), with the formation of *N*-desmethyltamoxifen being the predominant metabolization route, and the formation of 4-hydroxytamoxifen a minor route.[6, 7] Further metabolism of both metabolites results in the formation of 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), which is considered to be the most important metabolite contributing to the pharmacologic activity of tamoxifen.[8-10] Tamoxifen and *N*-desmethyltamoxifen can also be converted to 4'-hydroxytamoxifen and 4'-hydroxy-*N*-desmethyltamoxifen isomers, respectively; these have been associated with ~10% of the activity of 4-hydroxytamoxifen and endoxifen.[11, 12] Phase I metabolism of tamoxifen into its main metabolites is catalyzed predominantly by cytochrome P450 (CYP) iso-enzymes CYP3A and CYP2D6, although others, such as CYP2C9, CYP2C19 and CYP2B6, are also involved.[6]

CYP2D6 genotype and concomitant use of CYP2D6 inhibitors have been related to alterations in endoxifen plasma concentrations[3, 13] and efficacy of tamoxifen treatment,[5, 14-16] although contradictory results on a possible association between *CYP2D6* genotype and clinical outcome have been reported.[17, 18] Endoxifen concentrations have been shown to vary greatly among patients, even after correcting for *CYP2D6* genotype and the use of CYP2D6 inhibitors,[11, 13, 19, 20] indicating an important role for other enzymes in the formation of endoxifen. Recent studies have shown that the activity of CYP2C9 and CYP2C19 may also be associated with altered endoxifen levels and treatment outcome.[11, 15, 21] In addition, CYP3A activity may be important in the biotransformation of tamoxifen into endoxifen.

Rifampicin, a rifamycin antibiotic, induces multiple drug-metabolizing enzymes and transporters, including several CYP enzymes, UDP-glucuronosyltransferases, and ABCB1, but it has the most potent effects on CYP3A4. These effects, among others, are mediated via action on the pregnane X receptor.[22] Because of its strong CYP-inducing effects, rifampicin is frequently used in drug interaction studies. In view of the fact that CYP enzymes involved in the metabolism of tamoxifen are induced by rifampicin, we hypothesized that rifampicin could alter the pharmacokinetics of tamoxifen, favoring conversion of the drug into endoxifen. The CYP-inducing effects of rifampicin could thereby potentially correct the

endoxifen concentrations in women with low endoxifen exposure. We therefore evaluated the effects of CYP induction by rifampicin on the plasma exposure of tamoxifen and its metabolites.

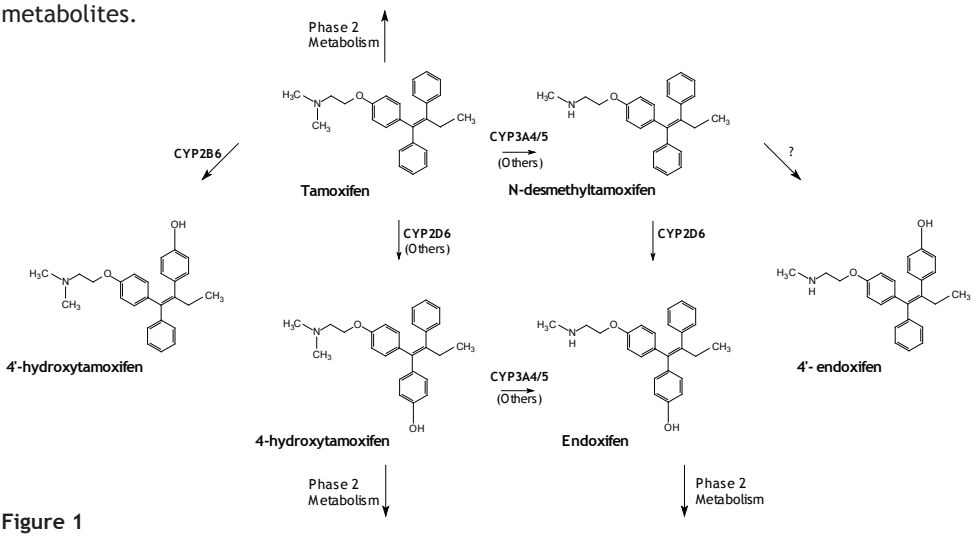

Figure 1

Metabolism of tamoxifen into its metabolites *N*-desmethyltamoxifen, 4-hydroxytamoxifen, endoxifen, 4'-hydroxytamoxifen and 4'-endoxifen. Phase 2 metabolism indicates further metabolism via glucuronidation and/or sulfation.

RESULTS

For safety reasons a pre-planned interim analysis was performed after enrolling four evaluable patients. The ages of these patients ranged from 28 to 51 years, and body weight varied from 60 to 88 kg. Three patients were classified as extensive metabolizers and one patient as an intermediate metabolizer, according to their *CYP2D6* genotypes.

The pharmacokinetic parameters of tamoxifen and its main metabolites during treatment with tamoxifen, alone and after CYP induction by rifampicin, are listed in Table 1. The decreases in the area under the concentration-time curve ($AUCss_{0\text{-}24h}$) of tamoxifen and its three metabolites following rifampicin administration are shown in Figure 2. As compared with the values after administration of tamoxifen alone, co-administration of rifampicin resulted in markedly reduced plasma exposures of tamoxifen (reductions of 81%-86%, $P < 0.001$), *N*-desmethyltamoxifen (reductions of 62%-74%, $P = 0.002$), and 4-hydroxytamoxifen (reductions of 59%-83%, $P = 0.008$). However, in contrast to our expectations, after induction by rifampicin, the AUCs of endoxifen also decreased by 28%-85% ($P = 0.040$). When rifampicin was co-administered with tamoxifen, trough levels of all four compounds – tamoxifen,

Table 1. Effects of rifampicin on tamoxifen pharmacokinetics

Parameter	Tamoxifen	Tamoxifen with rifampicin	Ratio (with/without rifampicin)	P-value[d]
Tamoxifen				
C_{max} (nM)	212 (178-269)	45.5 (34.7-83.0)	0.21 (0.15-0.31)	0.002
C_{trough} (nM)	99.4 (74.7-163)	16.3 (10.2-26.5)	0.16 (0.14-0.21)	<0.001
AUC_{0-24h} (nM * h)	3,099 (2,518-4,274)	505 (391-803)	0.16 (0.14-0.19)	<0.001
NDM				
C_{max} (nM)	297 (235-386)	94.1 (67.5-159)	0.32 (0.25-0.41)	0.002
C_{trough} (nM)	201 (145-317)	57.7 (37.7-102)	0.29 (0.22-0.41)	0.003
AUC_{0-24h} (nM * h)	4,974 (3,671-6,814)	1,562 (1,274-2,549)	0.31 (0.26-0.38)	0.002
4-OH				
C_{max} (nM)	3.29 (2.71-4.26)	1.07 (0.77-1.61)	0.33 (0.18-0.59)	0.019
C_{trough} (nM)	2.18 (1.94-2.41)	0.55 (0.38-0.77)	0.25 (0.17-0.37)	0.007
AUC_{0-24h} (nM * h)	57.6 (51.0-66.6)	15.5 (11.2-21.1)	0.27 (0.17-0.41)	0.008
Endoxifen				
C_{max} (nM)	15.3 (5.60-30.4)	4.60 (4.40-4.73)	0.30 (0.16-0.78)	0.039
C_{trough} (nM)	10.4 (4.41-17.3)	3.26 (2.52-4.27)	0.31 (0.15-0.72)	0.048
AUC_{0-24h} (nM * h)	259 (103-469)	79.1 (72.2-94.4)	0.31 (0.15-0.72)	0.040
Ratios				
Met/Tam[a]	1.72 (1.58-2.01)	3.28 (3.19-3.47)	1.90 (1.73-2.03)	<0.001
NDM/Tam	1.60 (1.46-1.81)	3.09 (2.96-3.26)	1.93 (1.80-2.03)	<0.001
Endox/NDM	0.052 (0.015-0.094)	0.051 (0.029-0.067)	0.97 (0.60-1.92)	0.931
4-OH/Tam	0.019 (0.012-0.024)	0.031 (0.026-0.038)	1.65 (1.17-2.20)	0.031
3-HM/DM[b]	0.22 (0.04-0.86)	0.54 (0.17-1.42)	2.49 (1.65-4.10)	0.017
4ß-OH/chol[c]	7.74 (6.44-8.91)	20.1 (15.9-24.6)	2.60 (2.48-2.98)	<0.001

Abbreviations: C_{max}, maximum concentration; C_{trough}, concentration before dosing (average of two measurements at T=0h and T=24h); AUC, area under the concentration-time curve; Met, metabolites; Tam, tamoxifen; NDM, *N*-desmethyltamoxifen; Endox, endoxifen; 4-OH, 4-hydroxytamoxifen; 3-HM, 3-hydroxymorphinan; DM, dextromethorphan; 4ß-OH, 4ß-hydroxycholesterol; chol, cholesterol.

Parameters are presented as geometric mean (range).

Parameters of two patients using 40 mg tamoxifen were dose-corrected to 20 mg.

[a] AUC_{0-24h} ratio of NDM, 4-OH, and endoxifen to tamoxifen.

[b] AUC_{0-6h} ratio of 3-hydroxymorphinan to dextromethorphan.

[c] 4ß-hydroxycholesterol to cholesterol ratio (average of three measurements at T=0.5h, T=6h, and T=12h).

[d] Two-sided paired *t*-test (after natural log-transformation).

N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen – decreased with similar extents, and their maximum concentrations (C_{max}) also decreased significantly.

Almost two-fold increases were observed in the metabolites-to-tamoxifen AUC ratios (metabolic ratios) after rifampicin administration. Dextromethorphan was administered to the patients as a probe drug for CYP2D6 and CYP3A activity.[19] Concomitant rifampicin administration resulted in an ~2.5-fold increase in 3-hydroxymorphinan-to-dextromethorphan AUC ratio relative to administration of tamoxifen alone. A nearly three-fold increase in the 4β-hydroxycholesterol-to-cholesterol ratio (endogenous CYP3A4/5 marker) was observed in all study participants. This ratio altered from 6.4-8.9 during treatment with tamoxifen alone to 15.9-24.6 following rifampicin administration, reflecting CYP3A4/5 induction by rifampicin.[23]

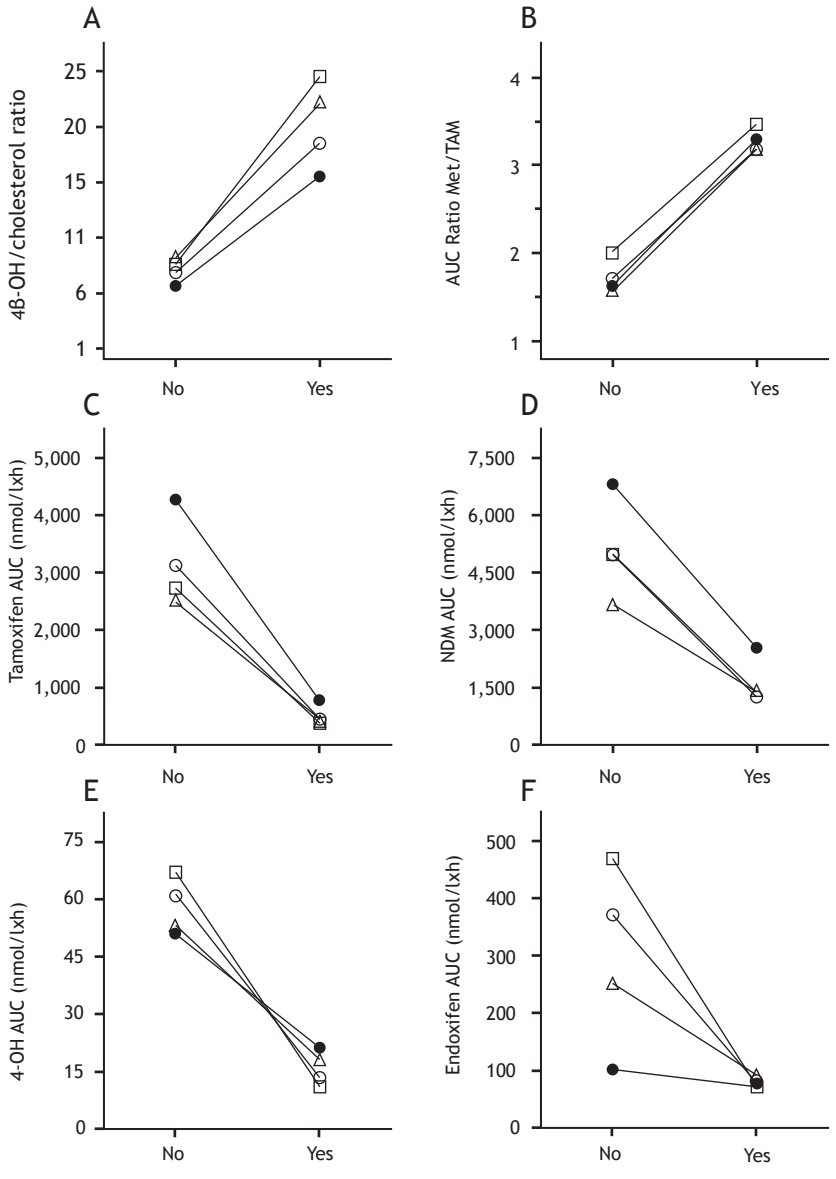

Figure 2

The effects of rifampicin on the ratios of 4β-hydroxycholesterol-to-cholesterol (endogenous CYP3A4/5 marker) (A), the ratios of the AUCs of the three metabolites to the AUC of tamoxifen (metabolic ratio) (B), and plasma exposures ($AUCss_{0\text{-}24h}$) of tamoxifen (C), *N*-desmethyltamoxifen (D), 4-hydroxytamoxifen (E) and endoxifen (F). One patient was classified as a CYP2D6 intermediate metabolizer (IM; closed circles); the other patients were classified as CYP2D6 extensive metabolizers (EM; open circles). AUC, area under the concentration-time curve.

DISCUSSION

Induction by rifampicin resulted in strong reductions in plasma concentrations of tamoxifen and its main metabolites. Endoxifen exposure in plasma fell by 28-85%, showing the clinical relevance of this drug-drug interaction. Given the potentially harmful effects of rifampicin administration on tamoxifen efficacy, the trial was permanently closed to patient accrual after the interim analysis.

The reductions in plasma exposure of tamoxifen and *N*-desmethyltamoxifen are in line with the findings of Kivisto *et al.*,[24] but they did not study other metabolites in their analysis. Potentially, tamoxifen and *N*-desmethyltamoxifen are metabolized further into endoxifen. However, in our patients, endoxifen plasma concentrations also showed a strong decrease. In addition, in contrast to the findings by Kivisto *et al.*, the C_{max} of *N*-desmethyltamoxifen decreased.

Rifampicin is a well-known inducer of CYP enzymes. The cholesterol metabolite 4ß-hydroxycholesterol has been shown to be a suitable endogenous biomarker for CYP3A4/5 activity.[23] In this study, we determined 4ß-hydroxycholesterol-to-cholesterol ratios to detect CYP3A4/5 induction. A strong increase in the 4ß-hydroxycholesterol-to-cholesterol ratio was observed in all study participants, confirming CYP3A4/5 induction. Evidence of the induction of CYP-mediated metabolism of tamoxifen was seen in the increases in the metabolites-to-tamoxifen AUC ratios (metabolic ratios) in all the patients after rifampicin co-administration (ratios of (AUC of *N*-desmethyltamoxifen + AUC of 4-hydroxytamoxifen + AUC of endoxifen) / (AUC of tamoxifen)). The observed increase in the 3-hydroxymorphinan-to-dextromethorphan AUC ratio, a metabolic route catalyzed by CYP2D6 and CYP3A4, suggests CYP induction as well. However, it is difficult to identify which specific iso-enzymes were affected by rifampicin. The increase in the *N*-desmethyltamoxifen-to-tamoxifen AUC ratio, alongside the observed increase in the 4ß-hydroxycholesterol-to-cholesterol ratio, indicates induction of CYP3A4. The increase in 4-hydroxytamoxifen-to-tamoxifen AUC ratio suggests induction of other CYP enzymes, potentially CYP2D6 (because this iso-enzyme has a major role in the metabolic conversion of tamoxifen into 4-hydroxytamoxifen). However, the endoxifen-to-*N*-desmethyltamoxifen AUC ratio, which indicates metabolic conversion by CYP2D6, was unchanged in most patients. Nevertheless, given that endoxifen can be metabolized further, the metabolic ratios can potentially be affected.

Reductions in drug and metabolite AUCs were seen in all the participants but were less pronounced with respect to 4-hydroxytamoxifen and endoxifen in the one patient who was classified as an intermediate CYP2D6 metabolizer. The fact that the 4ß-hydroxycholesterol-

to-cholesterol ratio was also lower in this same patient, suggests that the activity of both CYP2D6 and CYP3A4/5 may have influenced the effects of rifampicin.

Endoxifen exposure was significantly reduced by co-administration of rifampicin, and there are several potential explanations for this. Glucuronidation, catalyzed by UDP-glucuronosyltransferases, is the main elimination route for tamoxifen and its metabolites.[7, 25] Given that rifampicin has also been shown to induce several UDP-glucuronosyltransferases,[22] reductions in metabolite concentrations may be explained by induced hepatic and intestinal metabolism of tamoxifen and its metabolites into glucuronide conjugates, thereby promoting their excretion into the bile and feces.[7, 25] Additive rifampicin-mediated induction of efflux transporters (i.e., ABCB1) may also have contributed to the excretion of tamoxifen glucuronides and the unconjugated compounds into the bile. These hypotheses are supported by the observed second peaks in plasma concentrations of tamoxifen and its metabolites, which are more pronounced following rifampicin administration (Figure 3). Decreased primary intestinal absorption due to increased intestinal secretion by ABCB1-mediated efflux might also play a role in decreasing the tamoxifen exposure. However, although it has been shown that the active tamoxifen metabolites are transported by ABCB1, *in vivo* experiments in mice have indicated that the bioavailability of orally administered tamoxifen and the concentrations of its metabolites were unchanged in the absence of ABCB1.[26] The contribution of induced efflux transport by ABCB1 to plasma exposure of tamoxifen metabolites is therefore unclear.

In addition to the metabolites mentioned, several other inactive phase I metabolites have recently been identified in human plasma.[11, 27] These are formed via various metabolic pathways. Co-administration of rifampicin may also induce the metabolism of tamoxifen into other metabolites. We were able to estimate the concentrations of 4'-hydroxytamoxifen and 4'-hydroxy-*N*-desmethyltamoxifen in plasma (Figure 3) because they were chromatographically separated from 4-hydroxytamoxifen and endoxifen,[28] and found that the ratios of the AUCs of these metabolites to that of tamoxifen were also increased when rifampicin was co-administered with tamoxifen.

Overall, given the extensive metabolism of tamoxifen, the interaction between rifampicin and tamoxifen is complex and multifactorial. The results of this study strongly indicate that metabolic pathways other than the one related to CYP2D6 are crucial in the formation of endoxifen. Given that rifampicin co-administration resulted in strongly reduced concentrations of endoxifen, the concurrent use of rifampicin and tamoxifen should be avoided. Potentially, similar drug-drug interactions may exist between tamoxifen and other potent inducers, such as St John's wort and carbamazepine.

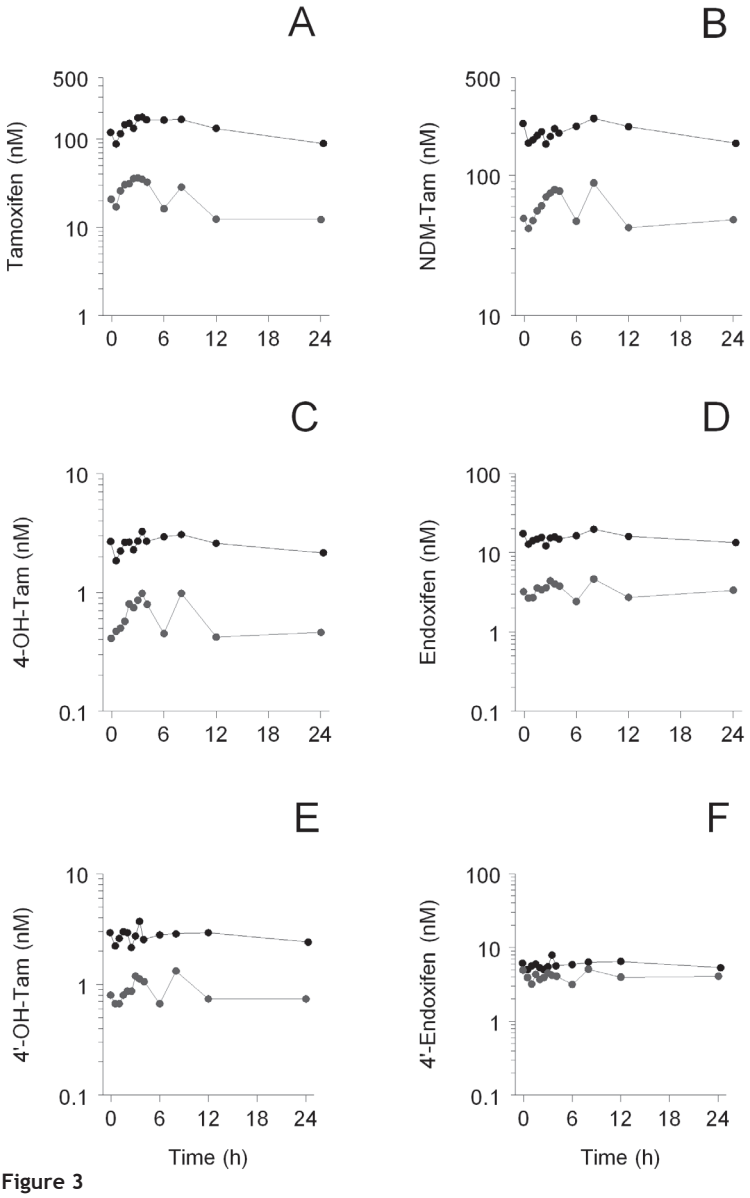

Figure 3

Plasma concentration-time curves of tamoxifen (A) and its metabolites *N*-desmethyltamoxifen (B), 4-hydroxytamoxifen (C), endoxifen (D), 4'-hydroxytamoxifen (E) and 4'-endoxifen (F) during treatment with tamoxifen alone (black line) and after co-administration of rifampicin (gray line) in a representative patient. Following rifampicin administration, secondary peaks were more pronounced for tamoxifen and the metabolites. Since 4'-hydroxytamoxifen and 4'-endoxifen were not formally validated in the applied analytical method, these metabolites are presented as equivalents to 4-hydroxytamoxifen and endoxifen concentrations, respectively.

METHODS

Subjects

The participants in this study were patients with breast cancer who were treated with 20 or 40 mg of tamoxifen once daily for at least four weeks. The inclusion criteria were normal blood cell counts and normal hepatic/renal function, no potentially interacting co-medication and/or dietary supplements, and no contra-indications for rifampicin use. Pregnant/lactating patients were excluded, and the consumption of grapefruit juice (CYP3A4 inhibitor) and alcohol was not allowed during the study period. Before the commencement of the study, medical histories and information about co-medication were obtained from all the participants. They underwent routine blood analyses during the study, and blood samples were collected for *CYP2D6* genotype testing.

Study design

This was a randomized cross-over pharmacokinetic study to investigate the effects of CYP induction by rifampicin on the plasma pharmacokinetics of tamoxifen and its metabolites. The study (Dutch Trial Registry; NTR2709) was approved by the ethics review boards of the Erasmus University Medical Center and was performed in compliance with the Declaration of Helsinki. Informed consent forms were signed by all participants before study entry. The subjects underwent two periods of 24-h blood sampling, once while on tamoxifen alone and once after 15 days of oral rifampicin at a dose of 600 mg per day taken in combination with tamoxifen. The participants were randomized for sampling sequence; tamoxifen alone followed by tamoxifen with co-administration of rifampicin versus tamoxifen with co-administration of rifampicin followed by tamoxifen alone, separated by a 4-week wash-out period. In the combination treatment, tamoxifen was administered first, followed after an interval of 12 hours by rifampicin. Two hours after tamoxifen intake, an oral dose of 30 mg dextromethorphan hydrobromide was administered as a marker to determine CYP2D6 and CYP3A4 activity,[19] and 4ß-hydroxycholesterol-to-cholesterol concentration ratios were measured to monitor CYP3A4/5 induction by rifampicin.[23]

During both periods, blood samples for the quantification of tamoxifen and its metabolites and dextromethorphan and its metabolites were collected just before and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12 and 24 hours after the daily administration of tamoxifen. The samples were processed to plasma (by centrifugation for 10 minutes at 2,500*g*) and stored at -70°C until analysis. Ratios of 4ß-hydroxycholesterol-to-cholesterol were measured in three

plasma samples from each patient (taken at 0.5, 6 and 12 hours after tamoxifen intake), during both sampling periods.

For safety reasons, patients underwent routine blood testing on day 7 of rifampicin co-administration and during both sampling periods. Adverse effects were recorded by the participants.

Genotyping

Genotyping was performed for *CYP2D6*3, *4, *5, *6, *10*, and **41* polymorphisms, associated with either no or reduced enzyme activity, using TaqMan allelic discrimination assays (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands) on an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA).

Pharmacokinetic analysis

Plasma concentrations of tamoxifen and its main metabolites (*N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen) as well as of dextromethorphan and its metabolites (dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan) were quantitated using validated UPLC-MS/MS methods.[28, 29] By using the analytical method for tamoxifen and its metabolites, we were able to separate 4-hydroxytamoxifen and endoxifen isomers. Low endoxifen concentrations could be quantitated because the analytical method was highly sensitive, with the lower limits of quantification being 1.86, 1.78, 0.194 and 0.187 ng/mL for tamoxifen, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, respectively. The $AUCss_{0\text{-}24h}$ was calculated by noncompartmental analysis using the Phoenix WinNonlin 6.1 program (Pharsight Corporation, Mountain View, CA), whereas C_{max} and trough concentrations were visually determined. The pharmacokinetic parameters of patients using 40 mg tamoxifen were dose-corrected to 20 mg. Two-sided paired *t*-tests were performed to compare natural log-transformed pharmacokinetic parameters, using PASW Statistics 17.0 (SPSS Inc., Chicago, IL). A *P*-value of ≤ 0.05 was considered statistically significant.

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Chapter 5

Unjustified prescribing of CYP2D6 inhibiting SSRIs in women treated with tamoxifen

Binkhorst L, Mathijssen RH, van Herk-Sukel MP, Bannink M,Jager A, Wiemer EA, van Gelder T.

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ABSTRACT

Tamoxifen is a largely inactive pro-drug, requiring metabolism into its most important metabolite endoxifen. Since the cytochrome P450 (CYP) 2D6 enzyme is primarily involved in this metabolism, genetic polymorphisms of this enzyme, but also drug-induced CYP2D6 inhibition can result in impaired endoxifen formation and as a consequence may affect the efficacy of tamoxifen treatment. Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) have been effectively used for the treatment of depression and hot flashes, both of which occur frequently in tamoxifen-treated women. Due to the drug-drug interaction considerably reduced endoxifen concentrations by inhibition of CYP2D6 will be the result. Evidence of a significant influence of strong CYP2D6-inhibiting drugs on the pharmacokinetics of tamoxifen has resulted in recommendations to avoid potent CYP2D6-inhibiting antidepressants (e.g., paroxetine, fluoxetine) in patients treated with tamoxifen for breast cancer. Nevertheless, dispensing data for tamoxifen and seven regularly used SSRIs/SNRIs in the period between 2005 and 2010, obtained from a large community pharmacy database in the Netherlands (3,000,000 people), show that the potent CYP2D6-inhibiting drug paroxetine remains one of the most frequently used antidepressants in tamoxifen-treated patients. Moreover, trends in the use of SSRIs/SNRIs in the population of all women were similar with trends in women using tamoxifen. Apparently, the recommendations to avoid paroxetine in tamoxifen-treated women have not been implemented into clinical practice. Several reasons may underlie continued use of this drug-drug combination. Contrary to *CYP2D6* polymorphisms, drug-induced CYP2D6 inhibition can easily be avoided, because alternative drugs are available. In clinical practice, one should strive to avoid potent CYP2D6 inhibitors as much as possible in tamoxifen-treated patients to reduce the risk of compromising the efficacy of the endocrine therapy. Co-medication should be reviewed by both physicians and pharmacists and potent CYP2D6 inhibitors ought to be switched to weaker alternatives.

TAMOXIFEN AND ANTIDEPRESSANTS

Tamoxifen is an important and effective endocrine therapy in patients with estrogen receptor- positive breast cancer. However, the success story of this old drug is limited by the fact that a considerable percentage of patients experience a relapse of disease or disease progression.[1,2]

The Achilles' heel of this anti-estrogen therapy is that tamoxifen is a largely inactive pro-drug, requiring metabolism into the active metabolites 4-hydroxytamoxifen and in particular endoxifen to reach its effect. This metabolism is catalyzed by the cytochrome P450 (CYP) system, with a crucial role for CYP2D6.[3] Partly due to the highly polymorphic nature of the *CYP2D6* gene, with more than eighty different alleles known, mainly associated with reduced or absent enzyme activity, the extent of metabolic conversion of tamoxifen into endoxifen varies greatly between patients. This most probably affects the efficacy of tamoxifen treatment.[4] As observed recently, endoxifen concentrations possibly need to exceed a minimum threshold concentration to achieve therapeutic effect.[5,6]

Also drug-induced CYP2D6 inhibition can seriously disrupt the formation of the active tamoxifen metabolite and as a consequence may interfere with the efficacy against breast cancer.[7] Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) - both antidepressant drugs - are frequently co-prescribed in patients on tamoxifen therapy for the treatment of a range of mental disorders. Breast cancer patients commonly suffer from hot flashes as a consequence of breast cancer treatment, including tamoxifen, for which SSRIs and SNRIs may also be used. In addition to clonidine and the anticonvulsant gabapentin, several SSRIs and SNRIs, including paroxetine, fluoxetine, citalopram, and venlafaxine, effectively reduce the incidence and severity of hot flashes.[8-10] Unfortunately, all these antidepressant drugs inhibit CYP2D6 enzyme function, thereby reducing endoxifen plasma concentrations, although the degree of inhibition varies among the different compounds.[11-13] Paroxetine and fluoxetine have been associated with the greatest ability to inhibit CYP2D6 activity and significant, up to 66% reduced endoxifen plasma concentrations were observed in tamoxifen-treated patients receiving these drugs concomitantly.[4,13] As observed by Goetz *et al.*, co-administration of these CYP2D6 inhibitors seems to reduce the efficacy of tamoxifen treatment.[14] The effects of CYP2D6 inhibitor use on the efficacy of tamoxifen treatment have been examined in subsequent studies. For instance, it was found that women receiving paroxetine concurrently with tamoxifen appeared to have a higher risk of breast cancer mortality, with increases in mortality risk related to the duration of concomitant use.[15] Also, an increased risk of recurrence with the

concomitant use of moderate/strong CYP2D6-inhibiting drugs has been reported,[16] although this was not found by others.[17,18] As a safe alternative, antidepressants with limited CYP2D6-inhibiting properties, such as venlafaxine and (es)citalopram, may be used because these drugs lead to less or no interference with tamoxifen metabolism.[4,13]

TRENDS IN THE USE OF ANTIDEPRESSANTS

Based on the evidence of a clinically relevant influence of strong CYP2D6 inhibitors on tamoxifen metabolism and the strong biological rationale, caution is warranted for concomitant use of CYP2D6 inhibitors in tamoxifen-treated patients. Combined use of tamoxifen and potent CYP2D6-inhibiting SSRIs should be avoided in patients receiving tamoxifen for breast cancer, which has been recommended in clinical guidelines and incorporated in a changed drug label for tamoxifen.[4,7,10,13,14,19-21] Given these recommendations, one would expect to see minimization of the use of these antidepressants among tamoxifen-treated women over the last years. Surprisingly, this is not the case. We have closely monitored dispensing data for tamoxifen and seven commonly used antidepressants, associated with CYP2D6 inhibition, in the period from 2005 to 2010. Dispensing data were derived from a community pharmacy database (PHARMO-Institute for Drug Outcome Research, Utrecht, the Netherlands). This database contains complete drug-dispensing histories from community pharmacies of more than three million people of 48 carefully selected geographic regions (urban and rural) in the Netherlands and forms a representative sample for the Western European society. Patients with all types of health insurance and regardless of prescriber are registered in these community pharmacies. All patients can be followed from the first drug dispensing in a PHARMO community pharmacy until the end of follow-up (loss to follow-up in PHARMO community pharmacy or death) or end of study period (31 December 2010). Data that were available included dispensed drug, coded according to the Anatomical Therapeutic Chemical (ATC) Classification, dose regimen, dispensed quantity, date of dispensing and estimated duration of use.

In the period between 2005 and 2010, dispensing data of ~1.5 million women were available in the community pharmacy database of PHARMO. Tamoxifen use in this population ranged from 3,885 users in 2005 to 3,509 women receiving tamoxifen in 2010. The prevalence of use of the seven antidepressant drugs in the population of women receiving tamoxifen as well as in the population of all women was assessed. The number of women receiving tamoxifen and an antidepressant drug during the same period was determined per calendar

year, defining concomitant users. Duration of (concomitant) use of both drugs was determined using dispensing dates and dispensed quantities. To distinguish between occasional versus regular use of antidepressants during tamoxifen treatment, regular or long-term use was defined as concomitant use of an antidepressant and tamoxifen for three months (≥ 90 days) or longer. In the population of women using tamoxifen, approximately 14% appeared to receive one of the seven antidepressants concurrently (ranging from 11.8% in 2005 to 14.9% in 2009). The largest proportion of these women, around 80.9% (ranging from 78.9% to 82.5%), received regular antidepressant treatment (concomitant use for at least 90 days). The use of the antidepressants in the population of all women appeared to be about 4.6% (ranging from 4.3% to 4.9%). Trends in the use of antidepressants over time in all women were compared with trends in women receiving tamoxifen.

Figure 1 shows that within the population of women receiving tamoxifen, the number of women receiving the strong CYP2D6 inhibitor paroxetine decreased over time, while there was an increase in the use of venlafaxine and (es)citalopram. However, similar trends in the use of these antidepressants were observed in the population of all women (Figure 2), with a drop in paroxetine use. The proportion of decrease in paroxetine use was comparable in both populations, with a reduction around 30% (33.7% versus 28.7%) in the period from 2005 to 2010. This suggests that the observed changes may have been related to marketing activities or to changes in guidelines for the general population, and not specifically related to new insights in the treatment of hot flashes or depression in tamoxifen-treated patients. In 2010, in this population-based study of ~1.5 million Dutch citizens, no less than 156 out of 3,509 women treated with tamoxifen received paroxetine or fluoxetine concomitantly, while this number should be close to zero.

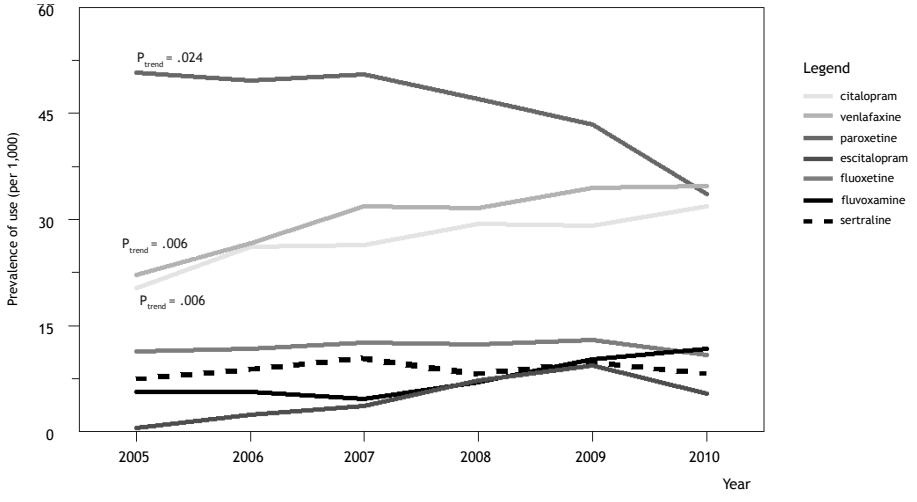

Figure 1

Prevalence of use of seven commonly used antidepressants, associated with CYP2D6 inhibition, per 1,000 women receiving tamoxifen in the period between 2005 and 2010. The following antidepressants are shown, in order to their CYP2D6-inhibiting properties, paroxetine; fluoxetine; sertraline; fluvoxamine; citalopram; escitalopram; and venlafaxine. P-values for trends are shown for paroxetine, citalopram and venlafaxine.

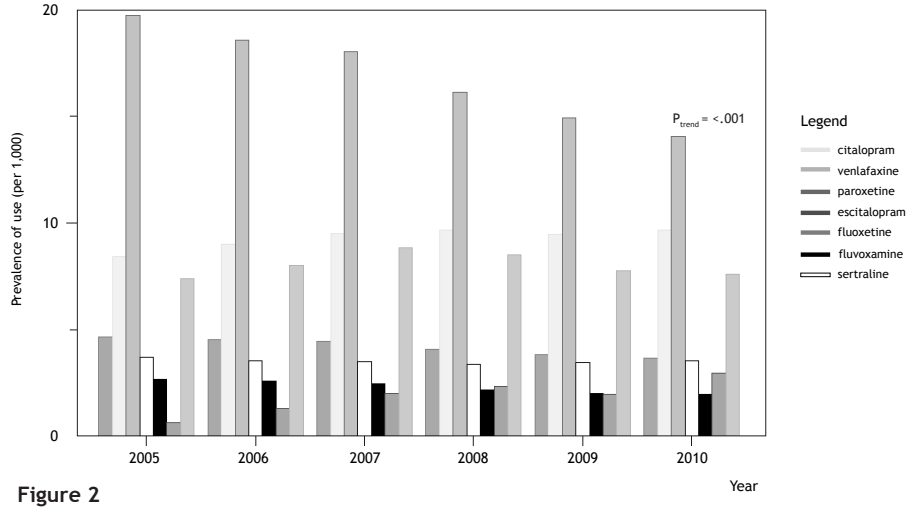

Figure 2

Prevalence of use of seven commonly used antidepressants, associated with CYP2D6 inhibition, in the population of all women in the period between 2005 and 2010 (PHARMO-dataset). Prevalence of use are shown for paroxetine; fluoxetine; sertraline; fluvoxamine; citalopram; escitalopram; and venlafaxine. P-value for trend is shown for paroxetine.

CO-PRESCRIPTION OF POTENT CYP2D6-INHIBITING ANTIDEPRESSANTS AMONG TAMOXIFEN USERS

Despite the recommendations to avoid potent CYP2D6-inhibiting drugs and changes in the drug label for tamoxifen including information on CYP2D6 inhibitor use, complete avoidance of strong CYP2D6-inhibiting antidepressants is far from realized, as we show in the PHARMO database-analysis. Although we did observe a trend towards a decrease in concomitant use of paroxetine and tamoxifen, this highly potent CYP2D6 inhibitor continues to be one of the most frequently used antidepressants in women treated with tamoxifen. Several plausible reasons may contribute to this observation.

First of all, this drug-drug interaction can be easily ignored, since it has no direct adverse consequences, and the antidepressant helps to reduce the severity of hot flashes. In contrast to drug-drug interactions which cause increased exposure of the drug, resulting in an increased risk of developing side effects, drug-drug interactions leading to impaired efficacy of the drug due to reduced bioactivation, as with the pro-drug tamoxifen, appear to be overlooked.[22] The clinical effect of reduced efficacy, in this case a higher risk of breast cancer recurrence, takes some time to occur.

Second, the two drugs are most often prescribed by different physicians. Tamoxifen would be initiated by the oncologist, while paroxetine is most likely started by the general practitioner. Patients may also receive their medication from different pharmacies, for example, a patient may receive one drug from an outpatient pharmacy and the other drug from a community pharmacy.[23] As a result the drug-drug interaction remains unnoticed. In the Netherlands, pharmacy information systems of outpatient pharmacies and community pharmacies are not always coupled to each other. Therefore, both pharmacists and physicians should be extremely alert on identifying co-medication in patients who receive or who will receive tamoxifen treatment. In clinical practice, the use of co-medication, especially antidepressants, should always be inquired with the patient by both healthcare providers.

Third, the combination of drugs is believed to be inevitable by the patient or physician, especially in patients already on paroxetine treatment at the time of starting tamoxifen. Patients are comfortable with the treatment, and discontinuation of the drug may not be preferred. Stopping or switching antidepressant drugs may be rather difficult, as observed in clinical practice. When continuation of therapy with an antidepressant drug is desired, an alternative antidepressant which adequately controls a patient's symptoms is important. Because a discontinuation syndrome is well-known for antidepressants, particularly for

paroxetine, antidepressants have to be cross-tapered. For these reasons, it is important to stop or switch antidepressant drugs under careful supervision of an experienced psychiatrist.

DISCUSSION AND RECOMMENDATIONS

Currently available data regarding the effects of potent CYP2D6-inhibiting drugs on breast cancer recurrence and mortality are conflicting.[14-18] Nevertheless, no information on endoxifen levels was available in these studies. Potential confounding by drug indication may have influenced the results of these studies as patients suffering from depression or tamoxifen-related hot flashes may be less compliant with endocrine therapy. Genotyping for *CYP2D6* was also not performed in most of the studies. Similarly, inconsistent results have been found in studies evaluating the influence of genetic variation in *CYP2D6* on the efficacy of tamoxifen therapy.[14, 24-27] Two large prospective trials, ATAC and BIG 1-98, did not find an association between *CYP2D6* genotype and breast cancer outcome,[26,27] however, the validity of genotype data in these studies has been questioned.[28] Nevertheless, in tamoxifen-treated patients, the importance of endoxifen exposure is becoming more and more recognized. Endoxifen concentrations possibly have to be above a minimum level for achieving its protective effect.[5] More evidence that support concentration-dependent effects of endoxifen in women treated with tamoxifen has recently been published. In this study, molecular mechanisms of endoxifen, 4-hydroxytamoxifen, and a pure anti-estrogen were investigated. It was shown that mechanisms of action differed between endoxifen and 4-hydroxytamoxifen. Gene expression profiles of MCF7 cells differed between the substances as well as between different endoxifen concentrations.[6] Therefore, it seems likely that potent CYP2D6-inhibiting drugs affect the efficacy of tamoxifen treatment by reducing endoxifen concentrations and should not be used along with tamoxifen to increase the likelihood of receiving optimal benefit from tamoxifen therapy.

In contrast to a diminished CYP2D6 enzyme activity due to genetic polymorphisms, impaired CYP2D6 metabolism by inhibiting co-medication can easily be avoided, especially in case of antidepressants, because there is a broad range of alternatives available when there is a strong indication for these compounds. Despite risks and difficulties associated with switching or stopping antidepressants this should be considered in most women. When non-CYP2D6-inhibiting alternatives are not available or unsuitable for the patient, weak or moderate CYP2D6-inhibiting antidepressants may be prescribed as a ‘second best’ alternative. Venlafaxine and citalopram as well as the s-enantiomer of citalopram,

escitalopram, are considered to be safe(r) alternatives for the treatment of depression or hot flashes in tamoxifen-treated patients, because their CYP2D6-inhibiting potential is either mild or absent.[10-12] Only slightly decreased endoxifen concentrations were found in patients receiving weak CYP2D6 inhibitors, including citalopram, compared with patients receiving no CYP2D6-inhibiting drugs. Venlafaxine appeared to have no effect on endoxifen concentrations when used concomitantly with tamoxifen; both in patients carrying two functional *CYP2D6* alleles and in patients with variant alleles.[13] However, an intra-patient comparison has not been performed yet. In a Danish population, no increased breast cancer recurrence rate was observed in tamoxifen-treated patients receiving citalopram or escitalopram concomitantly compared to women taking tamoxifen without these antidepressants.[29] Yet, none of the studies found higher recurrence rates or increased risk of death in patients receiving tamoxifen and venlafaxine at the same time.[15, 17-18]

Moderate inhibitors of CYP2D6, such as sertraline, can decrease endoxifen plasma concentrations, however, not to a similar extent as paroxetine.[13] Nevertheless, current evidence on the clinical effects of the use of weak/moderate CYP2D6 inhibitors in tamoxifen-treated patients is scarce, complicating clinical decision-making whether to use these drugs concurrently with tamoxifen. Recommendations on avoidance of weak/moderate CYP2D6 inhibitors in patients with reduced CYP2D6 activity, according to genotype,[10,19] should be interpreted with caution since low endoxifen concentrations also have been found in patients with two functional alleles,[30] making these patients less suitable for receiving CYP2D6-inhibiting drugs as well. The direct impact of various CYP2D6-inhibiting drugs on the pharmacokinetics of tamoxifen in relation with clinical outcome has to be prospectively evaluated, with inclusion of a patient's *CYP2D6* genotype. Such a trial is currently ongoing at our cancer center, where patients using the combination of tamoxifen and a strong CYP2D6-inhibiting antidepressant are switched to a drug with little or no CYP2D6-inhibiting properties (Dutch trial registry number NTR3125). Following switching, changes in the pharmacokinetics of active tamoxifen metabolites are examined within individual patients. Awaiting these study results, one should use weak/moderate CYP2D6 inhibitors with caution in patients receiving tamoxifen, especially moderate CYP2D6 inhibitors.

On the other hand, occurrence of side effects (e.g., hot flashes) may result in poorer adherence to tamoxifen, which has been associated with worse treatment outcome.[17,31] In clinical practice, persistence to adjuvant endocrine therapy was demonstrated to be suboptimal, ranging from only 27% to 69%,[32] with higher discontinuation rates in patients suffering from treatment-related side effects.[33] Effective therapies for the treatment of hot flashes may therefore be essential to solve problems regarding adherence. In addition,

depressive disorder may negatively affect adherence. The use of antidepressants which have been shown to be effective in the treatment of depressive disorder or alleviation of hot flashes, but possess weak or moderate CYP2D6-inhibiting properties, may therefore be advocated (i.e., venlafaxine, citalopram).

Besides antidepressants, clonidine and gabapentin show benefit in controlling hot flashes and may safely be used in combination with tamoxifen, regarding their pharmacokinetic interaction potential. Nevertheless, disadvantages of these drugs include the occurrence of side effects (e.g., sleep disturbances) in case of clonidine and frequent administration when gabapentin is used, because only a high dose (900 mg/day) appeared to be effective.[8, 34,35] In general, when antidepressants or other drugs are used for the treatment of hot flashes, the benefit of the drug should outweigh possible negative effects.

In postmenopausal women, necessitating the use of a potent CYP2D6-inhibiting drug, tamoxifen therapy could be replaced with an aromatase inhibitor, however, this should be carefully considered by the medical oncologist, taking into account the tolerability of this endocrine treatment by the patient.

The problem with tamoxifen is, however, more complicated. Even if all tamoxifen-treated patients would stop using strong CYP2D6 inhibitors, this does not imply that the problem is solved. In addition to adherence and genetic polymorphisms in *CYP2D6*, which may have a significant effect on the pharmacokinetics of tamoxifen,[13] other drug-metabolizing enzymes and drugs which are able to modulate these enzymes may also affect tamoxifen metabolism.[36] This should be taken into account too. In addition, impaired metabolism may partly explain variability in response to tamoxifen, however, other mechanisms (e.g., alterations in estrogen receptor expression and function) also underlie tamoxifen resistance.[37]

There are some limitations in this study. First, only drug-dispensing data derived from a single community pharmacy database of PHARMO was available, with no validation by other community databases. No demographic or pathological characteristics of the patients were available, lacking diagnoses for breast cancer. In addition, we did not have information on indication of antidepressant treatment (e.g., depression, hot flashes), co-morbidity and concomitant use of drugs (other than antidepressants and tamoxifen), factors that might have influenced the selection for a particular (potent CYP2D6-inhibiting) antidepressant drug, which is another important limitation of the study.

CONCLUSION

In conclusion, despite the strong biological rationale and recommendations to avoid potent CYP2D6-inhibiting co-medication in tamoxifen-treated patients, paroxetine is still frequently prescribed concurrently with tamoxifen, which is undesirable in most cases regarding the efficacy of tamoxifen treatment. Further steps should be taken to avoid the concomitant use of these drugs as much as possible to increase the chance of effective endocrine therapy. Pharmacists and physicians should be alert in reviewing co-medication in patients receiving tamoxifen. It is advised that strong CYP2D6 inhibitors are switched to little or no CYP2D6-inhibiting alternatives, whenever possible, and this should be supervised by an experienced psychiatrist. Studies prospectively examining the precise impact of various CYP2D6-inhibiting antidepressants on the pharmacokinetics of tamoxifen in individual patients and in relation with clinical outcome are strongly required.

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Chapter 6

Augmentation of endoxifen exposure in tamoxifen-treated women following SSRI-switch

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ABSTRACT

Background

Potent CYP2D6-inhibiting antidepressants can seriously disrupt tamoxifen metabolism, probably influencing the efficacy of tamoxifen therapy. In a prospective pharmacokinetic study we studied the effects of switching potent CYP2D6-inhibiting antidepressants to the weak CYP2D6 inhibitor escitalopram on tamoxifen pharmacokinetics.

Methods

Under close supervision of a psychiatrist, women who received tamoxifen and paroxetine or fluoxetine concomitantly for at least 4 weeks, were switched to a weak CYP2D6-inhibiting antidepressant. Blood sampling for pharmacokinetics was performed before and after switching. Tamoxifen and its metabolites were quantitated by a validated assay. The Wilcoxon signed-rank test was used for statistical analysis.

Results

Ten women were switched to escitalopram without psychiatric problems and no antidepressant-related adverse events were reported. During concomitant use of escitalopram, endoxifen exposure was considerably higher than during paroxetine or fluoxetine co-treatment (median, 387 nM*h (range 159-637 nM*h) vs. 99.2 nM*h (range 70.0-210 nM*h); P = 0.012). Plasma exposure to 4-hydroxytamoxifen was 34% (P = 0.017) higher following switching. Ratios of endoxifen-to-*N*-desmethyltamoxifen and 4-hydroxytamoxifen-to-tamoxifen increased by 3.3 and ~1.5-fold, reflecting higher CYP2D6 activity during co-treatment with the weak CYP2D6 inhibitor.

Conclusions

Switching to the weak CYP2D6 inhibitor escitalopram was safe and feasible and resulted in clinically relevant rises of endoxifen concentrations. We therefore strongly advise to switch paroxetine and fluoxetine to escitalopram in patients using tamoxifen.

INTRODUCTION

Tamoxifen, a selective estrogen receptor modulator, is the standard endocrine treatment for premenopausal women with hormone-sensitive breast cancer. In sequence with, or as an alternative to aromatase inhibitors, tamoxifen can be given to postmenopausal women.[1]

Tamoxifen reduces the 15-year risk of recurrence and breast cancer death in patients with early disease and prolongs survival in the metastatic setting. However, recurrence of disease and disease progression is observed in a substantial proportion of patients. Resistance to tamoxifen may be attributable to variability in exposure to the active metabolite.[2, 3]

Tamoxifen is a pro-drug and undergoes metabolic activation to 4-hydroxytamoxifen and endoxifen, catalyzed by cytochrome P450 enzymes (e.g., CYP2D6, CYP3A, CYP2C9).[4, 5] Endoxifen is considered to be the principal active metabolite of tamoxifen and systemic concentrations of this metabolite probably need to exceed a threshold level for clinical efficacy in women with breast cancer.[6-8]

The CYP2D6 enzyme has a key role in the metabolism of tamoxifen into endoxifen. It has been shown that patients carrying variant alleles of *CYP2D6* produce little endoxifen[5, 9] and, although not consistently shown, may have poorer clinical outcome.[10-13] CYP2D6-inhibiting medications may also interfere with tamoxifen therapy by reducing endoxifen concentrations. Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) are known to inhibit CYP2D6 to varying degrees. Because depressive disorder is common in breast cancer patients, but also for other indications, these antidepressant drugs are often co-prescribed in tamoxifen-treated individuals.[9, 14, 15] Paroxetine and fluoxetine are potent CYP2D6 inhibitors, which have been shown to markedly reduce endoxifen formation[7, 9] and to negatively affect clinical outcome in women receiving tamoxifen.[16, 17]

Venlafaxine and escitalopram have been proposed as safer options in patients using tamoxifen regarding their effects on endoxifen formation.[9, 14, 15] However, an intra-patient comparison is lacking so far. Therefore, we investigated the effects of switching potent CYP2D6-inhibiting antidepressants to a weak CYP2D6-inhibiting alternative on the plasma pharmacokinetics of tamoxifen and its metabolites in breast cancer patients in a pharmacokinetic study.

METHODS

Subjects

Women who were treated with 20 or 40 mg tamoxifen in combination with a potent CYP2D6-inhibiting antidepressant (paroxetine or fluoxetine) for at least 4 weeks were included in the study. Other inclusion criteria were: age >18 years; WHO performance score < 1; and adequate hematological, renal, and hepatic functions. The principal exclusion criteria were contra-indications for venlafaxine or escitalopram use, Congenital Long QT Syndrome, or suicidal ideation. The concomitant use of medications and/or supplements that could interact with tamoxifen or the antidepressant drugs was not allowed. Standard laboratory tests and an electrocardiogram were performed before start of the study and blood samples were obtained for *CYP2D6* genotype determination. Informed consent forms were signed by all study participants before study entry and the Erasmus MC review board approved the study protocol (Dutch Trial Registry; NTR3125).

Study design

Under careful supervision of a psychiatrist (MB), patients were switched from the potent CYP2D6-inhibiting antidepressant (paroxetine or fluoxetine) to treatment with a weak CYP2D6-inhibiting antidepressant (escitalopram or venlafaxine). The antidepressant therapy was individually adjusted and switching strategies were supervised by the psychiatrist. Adverse effects and the use of concomitant medication were recorded by the patients during the study.

Once during the concomitant use of tamoxifen and the potent CYP2D6-inhibiting antidepressant and once during co-treatment with the weak CYP2D6 inhibitor, blood was collected for pharmacokinetic analyses of tamoxifen and its metabolites. Both periods were separated by an adequate wash-out period. Since switching between the antidepressants required dose-tapering, the second day of blood sampling was dependent on the last day of paroxetine/fluoxetine intake.

Laboratory tests were performed on both days of blood sampling, and an additional electrocardiogram was obtained during the second sampling day, because patients were using the new antidepressant at that time.

Measurement of tamoxifen and its main metabolites in plasma

Blood samples (4 mL; lithium-heparin) for the measurement of tamoxifen and its main metabolites were collected just before and at 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 hours after administration of tamoxifen. Plasma was isolated by centrifugation of the samples for 10 minutes at 2,500 *g*, and stored at -70° C until analysis. The measurement of tamoxifen and its main metabolites in plasma was performed as described elsewhere.[18]

Individual pharmacokinetic parameters of tamoxifen and its metabolites were estimated by noncompartmental analysis using Phoenix WinNonlin 6.1 (Pharsight Corporation, Mountain View, CA). Estimated parameters of patients who used 40 mg tamoxifen were corrected to 20 mg. The metabolic ratios were computed as $AUC_{0\text{-}24\ metabolite}$ / $AUC_{0\text{-}24\ tamoxifen}$.

CYP2D6 genotyping

Genomic DNA was isolated from whole blood and genotype analyses for *CYP2D6 *3, *4, *5,*6, *10, *17,* and **41* were performed as previously described.[19]

Statistics

To detect a 25% difference in the $AUC_{0\text{-}24}$ of endoxifen between co-administration of a potent CYP2D6 inhibitor and a weak CYP2D6 inhibitor, with a two-sided 5% significance level and a power of 80%, thirteen study participants were required. This was based on a within-patient variation of 20% in pharmacokinetics of endoxifen.

Pharmacokinetic data are presented as median and ranges. The differences in pharmacokinetic parameters, before and after switching, were evaluated using Wilcoxon signed-rank tests for related samples. *P*-values ≤ 0.05 were regarded as statistically significant. Statistical tests were performed using IBM SPSS statistics, version 21 (SPSS Inc., Chicago, IL).

RESULTS

Pharmacokinetic data were available for ten patients (Table 1). Due to problems with blood sampling for pharmacokinetic analysis, only trough samples (C_{trough}) were available for two of these patients. Most women received adjuvant tamoxifen at a dose of 20 mg. Two women received a dose of 40 mg; one woman for metastatic disease and one because of extreme overweight.

The women received antidepressants for the treatment of depressive disorder (n=6) or

anxiety disorder (n=4), which was diagnosed before initiation of tamoxifen therapy. Eight women used paroxetine at a dose ranging from 15 to 60 mg per day; two women received fluoxetine at a dose of 20 and 30 mg. Nine women were switched to escitalopram; seven patients received a dose of 10 mg per day and two patients received a higher dose of 15 and 20 mg because of the nature of their conditions. One woman received, by mistake, citalopram at a dose of 10 mg. The age of the study participants ranged from 41 to 62 years (median 51 years) and the body mass index varied from 23.0 to 45.2 kg/m^2 (median 30.0 kg/m^2).

The pharmacokinetic parameters of tamoxifen and its three main metabolites during co-administration of paroxetine or fluoxetine and during escitalopram co-administration are listed in Table 2. Plasma concentration versus time profiles of tamoxifen and endoxifen and individual changes in plasma exposure following switching are shown in Figure 1. Following switching from the potent CYP2D6-inhibiting antidepressant to escitalopram, endoxifen plasma exposure increased markedly from 99.2 nM*h (range, 70.0-210 nM*h) to 387 nM*h (range, 159-637 nM*h; P = 0.012). The trough concentration (C_{trough}) and maximum concentration (C_{max}) of endoxifen were also ~3-fold higher during escitalopram co-administration. The area under the curve (AUC_{0-24}), C_{trough} and C_{max} of 4-hydroxytamoxifen increased by 34% (P = 0.017), 40% (P = 0.017), and 42% (P = 0.036) after switching. However, the pharmacokinetic parameters of tamoxifen and *N*-desmethyltamoxifen were not significant different between co-administration of paroxetine/fluoxetine and escitalopram.

Switching from the potent CYP2D6 inhibitor to the weak CYP2D6 inhibitor resulted in a more than 3-fold higher AUC ratio (metabolic ratio) of endoxifen-to-*N*-desmethyltamoxifen and ~1.5-fold higher 4-hydroxytamoxifen-to-tamoxifen ratio.

Adverse effects that were reported by the study participants included hot flashes, insomnia, nausea, and joint pain. Adverse effects were mild and appeared not to be associated with antidepressant use. However, following switching to the weak CYP2D6-inhibiting antidepressant, some individuals reported an increase in incidence and severity of hot flashes.

Table 1. Patient characteristics

Patient	Age (years)	BMI (kg/m²)	Tamoxifen dose (mg)	Setting	Mental disorder [d]	First antidepressant + dose [a]	Second antidepressant + dose	*CYP2D6* genotype
1	43	24.1	20	Adjuvant	Depressive disorder	Paroxetine 15 mg	Escitalopram 15 mg	*1/*1 (EM)
2	54	23.0	20	Adjuvant	Depressive disorder	Paroxetine 60 mg	Escitalopram 20 mg	*1/*1 (EM)
3	49	32.9	20	Adjuvant	Depressive disorder	Paroxetine 20 mg	Citalopram 10 mg [b]	*1/*1 (EM)
4	43	29.4	20	Adjuvant	Anxiety disorder	Paroxetine 20 mg	Escitalopram 10 mg	*1/*4 (IM)
5	55	33.6	20	Adjuvant	Anxiety disorder	Fluoxetine 20 mg	Escitalopram 10 mg	*1/*1 (EM)
6[c]	48	45.2	40[e]	Adjuvant	Depressive disorder	Paroxetine 20 mg	Escitalopram 10 mg	*1/*1 (EM)
7	59	26.4	20	Adjuvant	Anxiety disorder	Paroxetine 20 mg	Escitalopram 10 mg	NA
8	41	29.3	40	Metastatic	Depressive disorder	Fluoxetine 30 mg	Escitalopram 10 mg	*1/*1 (EM)
9	53	30.7	20	Adjuvant	Anxiety disorder	Paroxetine 40 mg	Escitalopram 10 mg	*4/*41 (IM)
10[c]	62	32.0	20	Adjuvant	Depressive disorder	Paroxetine 20 mg	Escitalopram 10 mg	*4/*41 (IM)

[a] Paroxetine and fluoxetine are equally potent inhibitors of CYP2D6 ([20]).

[b] One woman received citalopram instead of escitalopram, however, the weak CYP2D6-inhibiting properties of the compounds are similar ([21]).

[c] Due to problems with blood sampling, only trough samples (C_{trough}) available.

[d] Diagnosed before initiation of tamoxifen therapy.

[e] Due to high body mass index.

Table 2. Effects of potent and weak CYP2D6-inhibiting SSRIs on tamoxifen pharmacokinetics

Parameter	Tamoxifen + potent CYP2D6-inhibiting SSRI	Tamoxifen + weak CYP2D6-inhibiting SSRI	Ratio weak/potent CYP2D6-inhibiting SSRI	P-value[a]
Tamoxifen				
C_{max} (nM)	369 (189-667)	366 (177-516)	0.97 (0.67-1.29)	0.889
C_{trough} (nM)[b]	278 (128-557)	290 (123-375)	0.95 (0.67-1.38)	0.575
AUC_{0-24h} (nM * h)	6422 (3574-12182)	6958 (3226-9567)	0.98 (0.79-1.18)	0.674
ND-Tam				
C_{max} (nM)	528 (395-977)	631 (365-955)	1.09 (0.77-1.75)	0.484
C_{trough} (nM) [b]	446 (306-807)	560 (312-704)	0.97 (0.76-1.37)	0.953
AUC_{0-24h} (nM * h)	10149 (7744-20107)	11500 (7441-16113)	1.09 (0.75-1.27)	0.674
4-OH-Tam				
C_{max} (nM)	3.46 (1.36-4.95)	4.09 (2.42-8.25)	1.42 (0.81-2.05)	0.036
C_{trough} (nM)[b]	2.47 (1.29-4.65)	3.25 (1.99-6.06)	1.40 (0.82-2.06)	0.017
AUC_{0-24h} (nM * h)	63.8 (27.4-98.2)	85.8 (51.1-148)	1.34 (0.88-1.87)	0.017
Endoxifen				
C_{max} (nM)	5.46 (3.86-11.1)	23.1 (9.05-33.2)	2.96 (1.50-7.44)	0.012
C_{trough} (nM)[b]	5.20 (3.48-10.6)	16.3 (7.05-30.8)	2.80 (1.02-6.33)	0.005
AUC_{0-24h} (nM * h)	99.2 (70.0-210)	387 (159-637)	2.98 (1.67-6.82)	0.012
Ratios				
End/ND-Tam	0.0113 (0.0065-0.014)	0.0311 (0.018-0.057)	3.33 (1.56-5.37)	0.012
4-OH-Tam/Tam	0.0109 (0.0053.0.014)	0.0149 (0.0084-0.020)	1.51 (1.08-1.67)	0.012
End/Tam	0.0213 (0.0057-0.029)	0.0559 (0.034-0.10)	2.85 (1.96-6.42)	0.012

Abbreviations: ND-Tam, *N*-desmethyltamoxifen; 4-OH-Tam, 4-hydroxytamoxifen; End, endoxifen; Tam, tamoxifen; AUC, area under the curve; C_{trough}, concentration before dosing; C_{max}, maximum concentration. Potent CYP2D6-inhibiting SSRI, paroxetine or fluoxetine; weak CYP2D6-inhibiting SSRI, escitalopram (in one woman citalopram).

Data are presented as median and range.

Parameters of one patient were dose-corrected to 20 mg.

[a] Wilcoxon signed-rank test.

[b] C_{trough} - data of ten patients, parameters of two patients were dose-corrected to 20 mg.

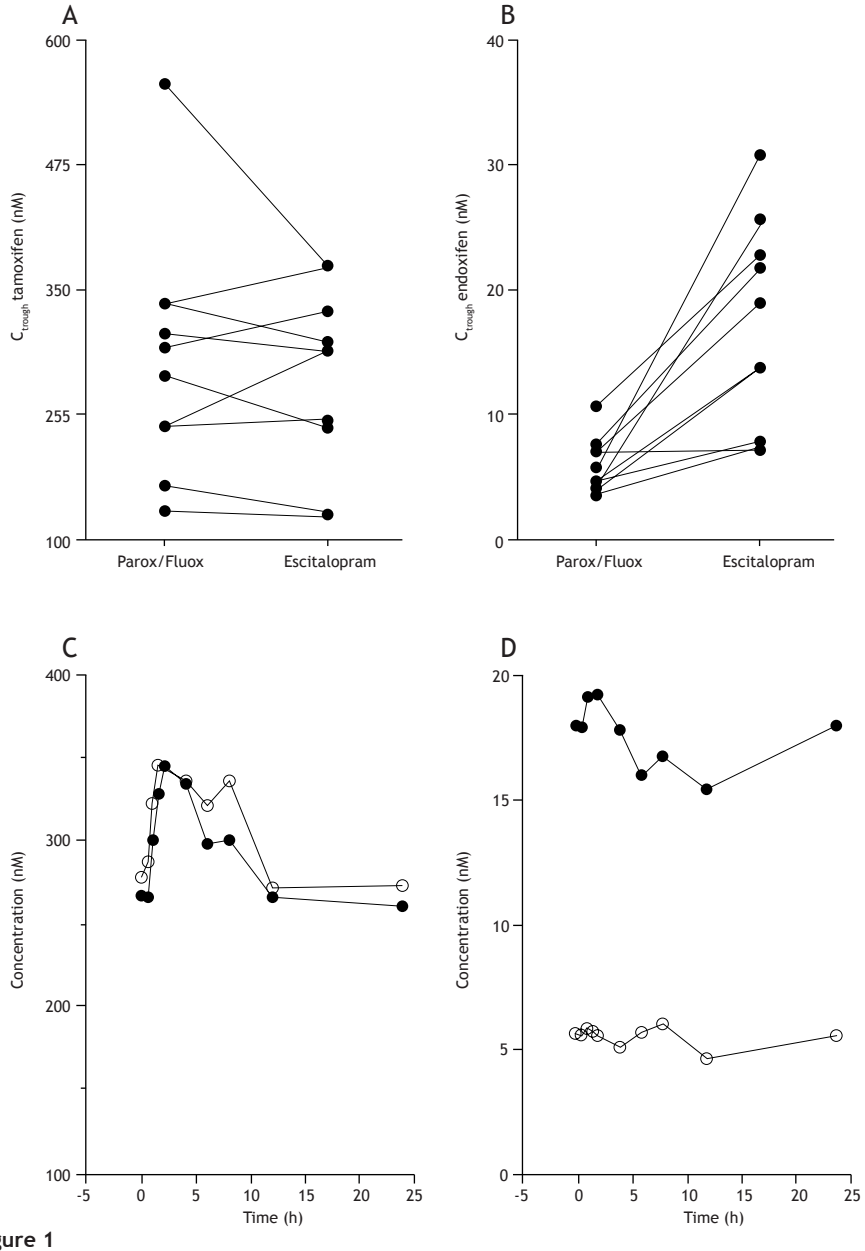

Figure 1

A and B: Individual changes in trough concentration (C_{trough}) for tamoxifen (A) and endoxifen (B) following switching from a potent CYP2D6-inhibiting antidepressant (paroxetine or fluoxetine) to a weak CYP2D6-inhibiting antidepressant (escitalopram).

C and D: Mean plasma concentration-time profiles for tamoxifen (C) and endoxifen (D) during concomitant use of paroxetine or fluoxetine (open circles) and during concomitant use of escitalopram (closed circles).

DISCUSSION

In this study, we evaluated for the first time whether switching from paroxetine or fluoxetine to escitalopram could increase endoxifen concentrations in women treated with tamoxifen. We observed that exposure to the active tamoxifen metabolites, particularly endoxifen, was considerably higher during co-administration of escitalopram with tamoxifen than during concomitant use of paroxetine or fluoxetine. Due to the lesser degree of CYP2D6 inhibition, or no inhibition at all, during the concomitant use of escitalopram, concentrations of 4-hydroxytamoxifen and endoxifen increased. This is further supported by the higher endoxifen-to-*N*-desmethyltamoxifen ratio, and to a lesser extent the 4-hydroxytamoxifen-to-tamoxifen ratio, during escitalopram co-administration, reflecting higher CYP2D6 activity. Although the increase in endoxifen exposure varied between individuals, probably depending on *CYP2D6* genotype, even in women with intermediate metabolizer genotype endoxifen exposure increased following SSRI switching.

The extremely low endoxifen concentrations during paroxetine co-administration were in line with the findings by Stearns *et al.*,[7] although the endoxifen concentrations were slightly higher than those observed in our study. Also, we found higher 4-hydroxytamoxifen concentrations after switching. These observations are remarkable because patients in the present study received the weak CYP2D6-inhibiting antidepressant, while women in the previous study did not receive any CYP2D6-inhibiting medication concomitantly during the control phase.[7] This might be explained by the use of higher doses of paroxetine (> 15 mg per day) in the current study, resulting in more potent CYP2D6 inhibition during paroxetine co-administration.[22]

Effective treatment of depression or anxiety disorders with antidepressants is vital; for the disorder itself, but it may also contribute to better adherence to tamoxifen.[23] The use of potent CYP2D6-inhibiting antidepressants along with tamoxifen is discouraged. Antidepressants with weak CYP2D6-inhibiting properties, such as escitalopram, have been recommended in tamoxifen-treated patients.[14] We demonstrated that during co-administration of escitalopram, women had endoxifen exposures that were similar to those observed in a genotype-matched cohort of tamoxifen-treated women without CYP2D6-inhibiting co-treatment.[19, 24]

Although we found that escitalopram had little or no effect on endoxifen formation, the effect on breast cancer outcome is not completely clear. However, evidence suggests that endoxifen systemic exposure is a predictor of tamoxifen efficacy. Madlensky *et al.*[8] reported a higher risk of breast cancer recurrence in patients having endoxifen concentrations below

a minimal threshold level. In our study, none of the women reached endoxifen concentrations above the proposed threshold concentration during co-treatment with the potent CYP2D6-inhibiting antidepressant. During escitalopram co-administration, five women with CYP2D6 extensive metabolizer genotype had endoxifen concentrations above the threshold. Three women who did not reach endoxifen concentrations above the threshold level after switching had impaired CYP2D6 metabolism according to genotype.

Although the study was not designed to detect differences in side effects, it is interesting to mention that hot flashes were reported particularly during escitalopram co-administration, which is most likely due to higher endoxifen levels.[25] In none of the ten women escitalopram treatment had to be discontinued.

Individuals were switched to escitalopram (10-20 mg/day); none of the patients received venlafaxine. Women were successfully switched, using cross-tapering, under careful supervision of an experienced psychiatrist. No antidepressant-related adverse events or psychiatric relapse were noticed.

A limitation of the study might be the small sample size, however, results were unequivocal. Lack of adherence to tamoxifen or the antidepressant therapy might have influenced the results of the study. In addition, steady state levels of tamoxifen metabolites were not reached in all patients, because not all women used tamoxifen for 4 months. However, this may have contributed to only small differences in concentrations of tamoxifen metabolites.

In conclusion, escitalopram seems to be a safe alternative in tamoxifen-treated patients requiring antidepressants and we strongly recommend to switch paroxetine and fluoxetine to escitalopram in these patients.

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Chapter 7

Circadian variation in tamoxifen pharmacokinetics

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Submitted for publication.

ABSTRACT

Background

Tamoxifen has been found to be successful in the treatment of hormone receptor-positive breast cancer, however, it is characterized by a large variability in response, partly due to differences in pharmacokinetics. Here, we examined circadian variation in tamoxifen pharmacokinetics in mice and breast cancer patients.

Methods

Pharmacokinetic analysis was performed in mice, dosed at 6 different times during a 24-h period. Tissue samples were used for mRNA expression analysis of drug-metabolizing enzymes. In patients, a prospective cross-over study was performed. During three 24-h periods, after tamoxifen dosing at 8 AM, 1 PM and 8 PM, for at least 4 weeks, blood samples were collected for pharmacokinetic measurements. Differences in pharmacokinetic parameters of tamoxifen and its metabolites between administration times were assessed.

Results

The mRNA expression levels of drug-metabolizing enzymes showed circadian variation in mouse liver and intestine. Tamoxifen exposure appeared to be highest after administration at midnight in mice. In humans, pharmacokinetic parameters of tamoxifen and its metabolites were higher following morning administration compared with evening administration: tamoxifen C_{max} and $AUC_{0\text{-}8h}$ were 20% higher ($P < 0.001$), and tamoxifen t_{max} was shorter (2.1h vs. 8.1h; $P = 0.001$), indicating variation in absorption. Systemic exposure ($AUC_{0\text{-}24h}$) to active metabolites was 12-15% higher ($P < 0.003$) following morning administration.

Conclusions

These results suggest that dosing time is of influence on tamoxifen pharmacokinetics. Whether or not the clinical outcome will be modulated by the time of drug intake needs to be confirmed in future clinical studies.

INTRODUCTION

Tamoxifen belongs to the selective estrogen receptor modulators, and has been used extensively in the treatment of hormone receptor-positive breast cancer since its introduction almost forty years ago. The drug and its metabolites antagonize the effects of estrogen in breast tissue, reducing rates of breast cancer recurrence and mortality in the adjuvant setting and prolonging survival in patients with metastatic disease.[1-3] However, its use is characterized by a large inter-individual variability in response, and non-response is observed in 30-50% of the patients. One of the mechanisms that may underlie the variable clinical response to tamoxifen is variability in pharmacokinetics.[1-4]

Tamoxifen is metabolized into various metabolites, catalyzed by phase I (cytochrome P450; CYP) and phase II (UDP-glucuronosyltransferases, sulfotransferases) metabolizing enzymes.[5] In humans, the parent compound is largely metabolized into *N*-desmethyltamoxifen, and to a lesser extent into active 4-hydroxytamoxifen. Both metabolites can be metabolized into 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), which is probably the most important active metabolite because it has a higher estrogen receptor affinity than the mother compound and reaches higher systemic levels than 4-hydroxytamoxifen.[5-7] Several CYP enzymes are involved in the metabolism of tamoxifen, including CYP2D6, CYP3A4, CYP2C9 and CYP2C19, of which CYP2D6 is the main enzyme for endoxifen formation.[5,8] Tamoxifen and its metabolites are subsequently metabolized into other phase I metabolites and conjugates and eliminated through the bile, faeces, and urine. Some compounds undergo enterohepatic recirculation.[5,9-10]

Endoxifen is considered to be responsible for the clinical effects of tamoxifen therapy. As recently suggested, a minimum threshold concentration for endoxifen should be attained to benefit from tamoxifen therapy.[11] Reduced endoxifen formation has been observed in tamoxifen-treated individuals carrying *CYP2D6* variant alleles and with the concomitant use of CYP2D6-inhibiting medications.[5,12-13] Both factors have also been associated with clinical outcome in tamoxifen-treated breast cancer patients, although the results of these studies have been inconsistent.[14-17] However, endoxifen formation not only depends on CYP2D6, as other enzymes, including CYP3A4 and CYP2C9, appear to be important as well. *CYP3A4*22* genotype and concomitant medication that alters CYP3A4 enzyme activity have both been shown to affect tamoxifen pharmacokinetics.[18-19] In addition to these factors, which contribute to inter-individual pharmacokinetic variability, tamoxifen pharmacokinetics may also differ within patients, known as intra-individual variability. In this way circadian rhythms may influence tamoxifen pharmacokinetics.

Many biochemical and physiological processes in organisms, including animals and humans, follow day-night rhythms. These daily rhythms are generated by an internal timing system known as the circadian clock. Circadian variations in gastrointestinal functions, hepatic and intestinal enzyme activity and organ blood flow may all affect absorption, distribution, metabolism and elimination of drugs, including tamoxifen. Accordingly, depending on the time of drug administration, endoxifen concentrations may vary, which is possibly important for the efficacy of tamoxifen therapy and occurrence of adverse effects.[20-25]

Although it has already been demonstrated for a variety of drugs that pharmacokinetic variation partly depends on the time of drug administration in a 24-h period,[20,25] circadian variation in tamoxifen pharmacokinetics remains to be examined. Here, we investigated circadian variation in the pharmacokinetics of tamoxifen in mice and breast cancer patients. Circadian changes in plasma and organ exposure to tamoxifen and its metabolites was studied in FVB mice, which were orally administered tamoxifen at six different times over a 24-h period. Additionally, we examined circadian rhythms in mRNA levels of essential CYP enzymes in tissues of FVB mice. To evaluate circadian variation in pharmacokinetics of tamoxifen in breast cancer patients, we examined three different administration times: morning (8 AM), afternoon (1PM) and evening (8 PM).

RESULTS

Study in mice

To evaluate potential circadian rhythms in CYP-mediated metabolism and the contribution to circadian variation in pharmacokinetics of tamoxifen, mRNA expression levels of CYP enzymes involved in the metabolism of tamoxifen were assessed in tissues of mice at six time points over a 24-h period. The daily mRNA expression patterns of components of the circadian clock (*Period 2 (Per2), Dbp, Bmal1, Cryptogene 1 (Cry1)* and *Rev-erb-a*) and drug-metabolizing enzymes (*Cyp2d10, Cyp2d22 and Cyp3a11*; orthologues of human CYP enzymes involved in tamoxifen metabolism) in mouse liver and small intestine (control group) are displayed in Supplemental Figure 1. As expected, cosinor analysis, used for the evaluation of 24-h rhythmicity, showed circadian oscillation in mRNA expression of components of the circadian clock. According to cosinor analysis, the mRNA expression of *Cyp2d10* and *Cyp2d22* in mouse liver appeared to oscillate rhythmically with a period of 24-hour ($P \leq 0.0413$). Highest expression levels of *Cyp2d10* and *CYP2d22* were observed at 4 AM (which was the

active phase of the mice in the experiment) and lowest at 4 PM (resting phase), with peak-trough ratios of 1.5 and 2.9. Expression of *Cyp3a11* mRNA showed 24-h variation in the proximal part of the small intestine in mice ($P = 0.0172$), and transcript levels peaked at midnight and were lowest at noon (peak-trough ratio 2.8).

We then examined circadian variation in plasma and organ exposure to tamoxifen and its metabolites in mice. The exposure to tamoxifen and its metabolites 4-hydroxytamoxifen (major metabolite in mice), *N*-desmethyltamoxifen and endoxifen in plasma, liver, and three consecutive parts of the small intestine for the six dosing-time groups are presented in Supplemental Table 1. Mean plasma concentration-time curves for tamoxifen following 6 administration times are displayed in Figure 1.

In mice, no significant differences in exposure to tamoxifen and its metabolites were observed in plasma or tissues after oral tamoxifen administration at six different time points in a 24-h period. Cosinor analysis did not show significant circadian rhythms in area under the curves (AUCs) of the compounds as a function of dosing time ($P \geq 0.1354$). A 12-h rhythm was also not found. However, although not statistically significant, a trend towards higher tamoxifen exposure in plasma and liver tissue, expressed as $AUC_{0\text{-LAST}}$, was seen following administration at midnight, which is the period in which mice are most active (Figure 2).

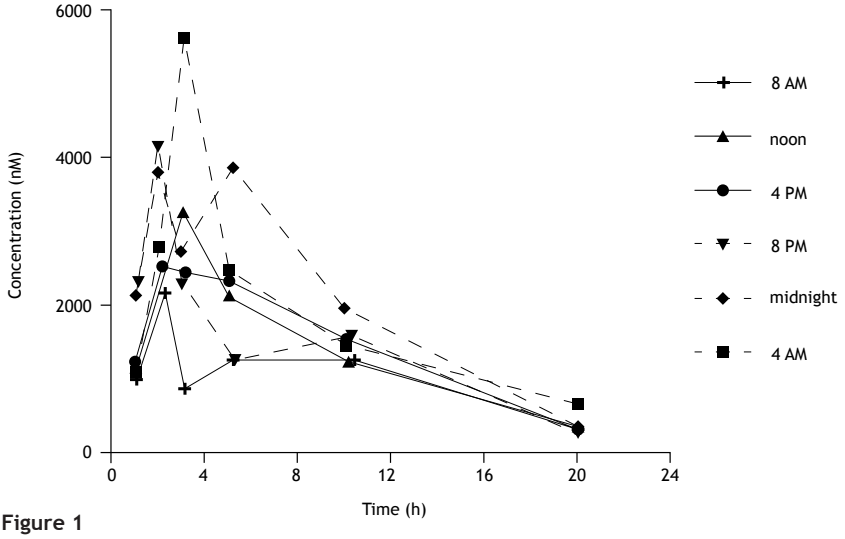

Figure 1

Plasma concentration-time curves of tamoxifen following drug administration at six different times in a 24-h period in mice. Tamoxifen was administered to mice at a dose of 4 mg by gavage at six different time points. Blood samples were collected at 1, 2, 3, 5, 10 and 20 hours after tamoxifen administration (3 mice/time point).

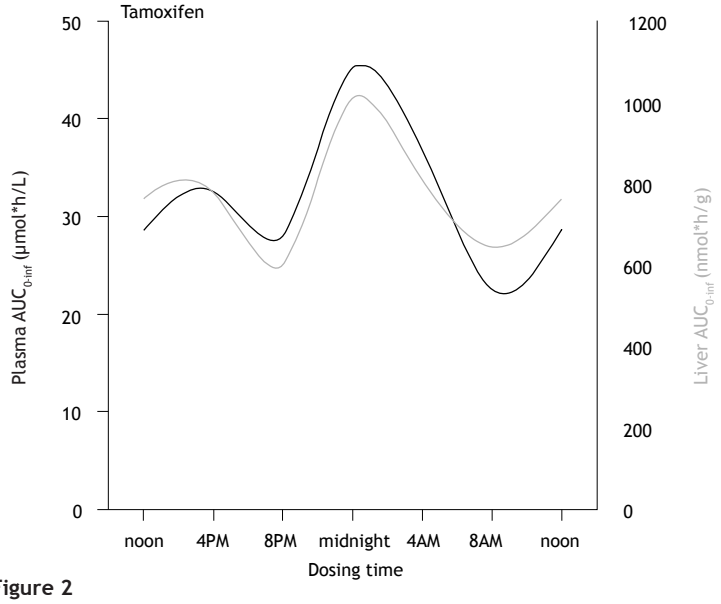

Figure 2

Exposure to tamoxifen ($AUC_{0\text{-}inf}$) in plasma and liver tissue of mice following tamoxifen administration at six different times. Tamoxifen was administered to 18 mice at six different time points. Blood was collected at six time points (3 mice/time point) after tamoxifen administration and area under the curves ($AUCs_{0\text{-}inf}$) were calculated. Data is presented as $AUC_{0\text{-}inf}$ (y-axis), derived from noncompartmental analysis on plasma concentration-time profiles of tamoxifen following dosing at six different times in a 24-h period (x-axis).

Clinical study

Between August 2012 and April 2014, a total of 27 female patients with (a history of) hormone receptor-positive breast cancer completed two study periods; administration of tamoxifen in the morning (8 AM) and in the evening (8 PM). Of these patients, 12 women completed the third period of tamoxifen administration in the afternoon (1 PM). Before study entry, based on personal preference, tamoxifen was used by 17 women in the morning (63%), and by 10 women in the evening (37%). Almost all study participants were postmenopausal, one woman was premenopausal, and most of the women received tamoxifen at a single dose of 20 mg; only one woman received a dose of 40 mg for metastatic disease. The mean age of the study participants was 53 ± 9 years and the mean body mass index was 27.5 ± 4.7 kg/m^2.

Adverse events observed in this study included hot flashes (n=25), mood swings (n=5), joint pain (n=5), and weight gain (n=3), which are all known side effects of tamoxifen. No serious

adverse events occurred during the study period. Hematological and biochemical parameters were not statistically or clinically relevant different between the days of pharmacokinetic blood collection and none of the patients showed signs of disturbed liver or kidney function.

Concerning differences between morning and evening administration (n=27), the mean plasma concentration-time profiles and pharmacokinetic parameters for tamoxifen and its metabolites are presented in Figure 3 and Table 1, respectively. Significant differences in pharmacokinetic parameters of tamoxifen and its three major metabolites were observed between morning and evening administration. For tamoxifen, the $AUC_{0\text{-}8h}$ and C_{max} were significantly higher and t_{max} was shorter after administration in the morning than after evening dosing. A 20% difference in $AUC_{0\text{-}8h}$ ($P < 0.001$) and C_{max} ($P < 0.001$) was observed for tamoxifen. The $AUC_{0\text{-}24h}$, C_{trough}, $t_{1/2}$, and CL/F did not significantly differ between morning and evening administration. For the metabolites *N*-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen, AUCs and C_{max} values were also higher and t_{max} shorter following morning administration. The $AUC_{0\text{-}8h}$ and C_{max} were 28% ($P < 0.001$) and 25% ($P = 0.001$) higher for 4-hydroxytamoxifen after morning dosing. Similar results were observed for the most important active metabolite endoxifen and the $AUC_{0\text{-}8h}$ and C_{max} were both 23% higher ($P < 0.001$) following administration in the morning. However, differences in the $AUCs_{0\text{-}24h}$ were small for these compounds, ranging from only 12% ($P = 0.003$) for 4-hydroxytamoxifen to 15% ($P < 0.001$) for endoxifen.

The metabolic ratios, endoxifen-to-*N*-desmethyltamoxifen, *N*-desmethyltamoxifen-to-tamoxifen, 4-hydroxytamoxifen-to-tamoxifen and total metabolites-to-tamoxifen differed only 6-9% between morning and evening administration (Table 1).

In the subgroup of women who completed three periods of different administration times (n=12), no significant differences in pharmacokinetic parameters or AUC ratios for tamoxifen and its metabolites were observed between dosing in the afternoon versus morning or dosing in the afternoon versus evening (Figure 4 and Table 2).

Results of genotyping for cytochrome P450 polymorphisms are summarized in Table 3. Endoxifen systemic exposure was relatively low in women who were *CYP2D6* poor metabolizer (two non-functional *CYP2D6* alleles), although two women had low endoxifen exposure despite their intermediate (one non-functional allele or two decreased functional alleles) and extensive (two active alleles) CYP2D6 metabolizer status. Differences in endoxifen concentrations between morning and evening administration seemed to be greater in women having extensive CYP2D6 metabolism compared with women having decreased CYP2D6 metabolism. The effects of administration time on plasma concentrations of endoxifen or other metabolites did not vary according to *CYP2C19* or *CYP3A4* genotypes.

However, the sample size was too small to detect significant differences between individuals with different genotypes.

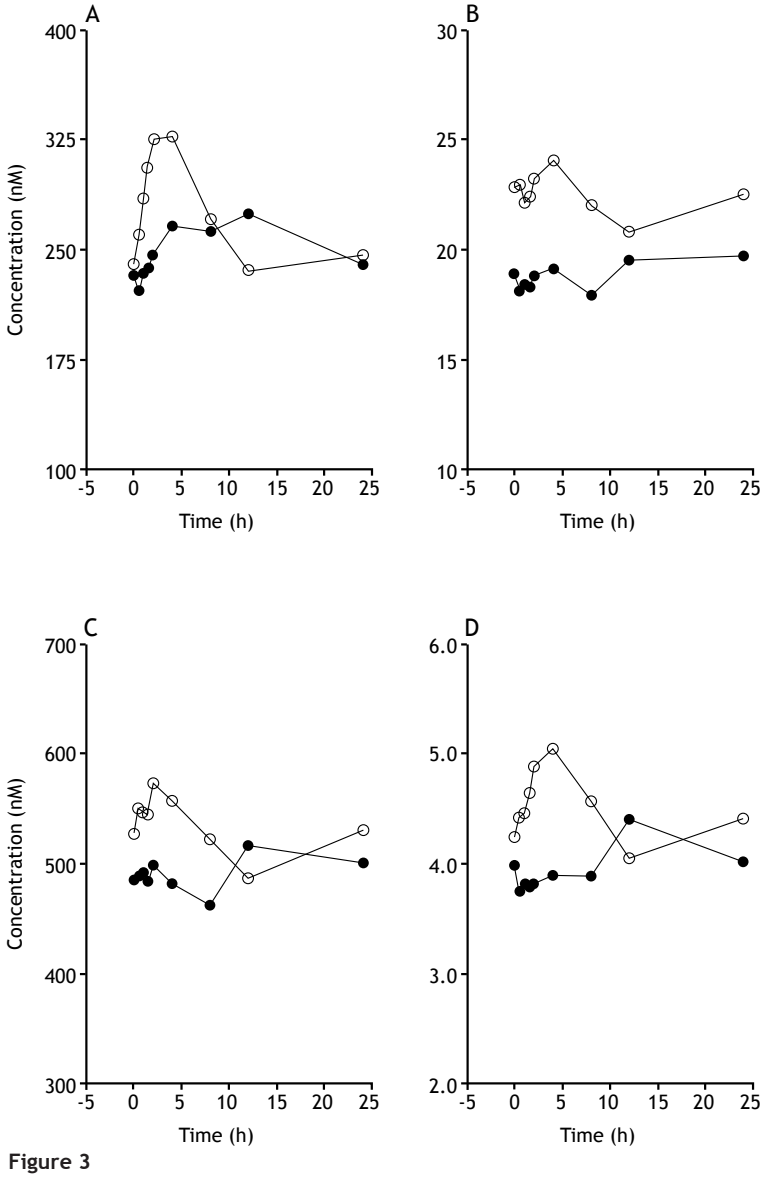

Figure 3

Mean plasma concentration-time profiles for tamoxifen (A), endoxifen (B), *N*-desmethyltamoxifen (C) and 4-hydroxytamoxifen (D) following tamoxifen administration in the morning at 8 AM (open circles) and in the evening at 8 PM (closed circles) in 27 women with (a history) of breast cancer.

Table 1. Pharmacokinetic parameters for tamoxifen and its metabolites following administration in the morning (8 AM) and evening (8 PM) in 27 women

	Morning administration (8 AM)	Evening administration (8 PM)	Ratio Morning vs Evening	Difference (95% confidence interval)	P-value[c]
Tamoxifen					
AUC_{0-8h} (nM*h)	2355 ± 709	1977 ± 587	1.20 ± 0.16	378 (273, 484)	<0.001
AUC_{0-24h} (nM* h)	6266 ± 1853	6138 ± 1764	1.03 ± 0.11	128 (-133, 389)	0.324
C_{24h} (nM)	246 ± 88.5	240 ± 75.1	1.04 ± 0.21	6.60 (-12.8, 26.0)	0.491
C_{max} (nM)	356 ± 107	298 ± 84.2	1.20 ± 0.20	58.0 (38.1, 77.8)	<0.001
t_{max} (h)	2.1 (1.0 – 24.0)	8.1 (1.5 – 24.0)			0.001
$t_{1/2}$ (h)[a]	48.0 ± 32.3	76.3 ± 51.0	0.86 ± 0.64	-28.4 (-58.1, 1.36)	0.060
CL/F (l/h)	9.40 ± 3.17	9.55 ± 3.04	0.99 ± 0.11	-0.16 (-0.59, 0.28)	0.471
ND-tamoxifen					
AUC_{0-8h} (nM*h)	4081 ± 1576	3496 ± 1263	1.16 ± 0.13	584 (400, 768)	<0.001
AUC_{0-24h} (nM* h)	11891 ± 4280	11092 ± 4017	1.08 ± 0.13	799 (316, 1283)	0.002
C_{24h} (nM)	519 ± 210	476 ± 176	1.09 ± 0.17	43.5 (8.99, 78.0)	0.015
C_{max} (nM)	615 ± 235	536 ± 207	1.16 ± 0.19	78.9 (44.9, 113)	<0.001
t_{max} (h)	2.1 (0.5 – 24.2)	4.1 (0.5 – 24.2)			0.019
4OH-tamoxifen					
AUC_{0-8h} (nM*h)	35.6 ± 10.6	28.6 ± 9.20	1.28 ± 0.30	7.01 (4.85, 9.19)	<0.001
AUC_{0-24h} (nM* h)	101 ± 30.0	92.8 ± 30.6	1.12 ± 0.24	8.50 (3.18, 13.83)	0.003
C_{24h} (nM)	4.24 ± 1.24	3.86 ± 1.35	1.14 ± 0.24	0.37 (0.10, 0.65)	0.010
C_{max} (nM)	5.26 ± 1.63	4.43 ± 1.48	1.25 ± 0.49	0.823 (0.356, 1.29)	0.001
t_{max} (h)	4.0 (0.5 – 24.0)	12.0 (1.0 – 24.1)			0.001

Endoxifen					
$AUC_{0\text{-}8h}$ (nM*h)	179 ± 84.3	144 ± 61.9	1.23 ± 0.14	34.9 (23.4, 46.4)	<0.001
$AUC_{0\text{-}24h}$ (nM* h)	524 ± 245	453 ± 203	1.15 ± 0.13	70.6 (45.4, 95.7)	<0.001
C_{24h} (nM)	22.5 ± 10.2	19.7 ± 9.05	1.16 ± 0.20	2.74 (1.41, 4.08)	<0.001
C_{max} (nM)	27.2 ± 12.5	22.0 ± 9.27	1.23 ± 0.21	5.20 (3.20, 7.19)	<0.001
t_{max} (h)	2.1 (0.5 – 24.2)	4.1 (0.5 – 24.2)			0.026
Ratios					
Endoxifen/ND-tam	0.052 ± 0.031	0.048 ± 0.028	1.07 ± 0.15	0.0040 (0.00061, 0.0075)	0.023
ND-tam/Tamoxifen	1.90 ± 0.35	1.80 ± 0.34	1.06 ± 0.081	0.094 (0.042, 0.15)	0.001
4OH-tam/Tamoxifen	0.017 ± 0.0057	0.016 ± 0.0049	1.09 ± 0.19	0.0015 (0.00043, 0.0025)	0.007
Metab/Tamoxifen[b]	2.01 ± 0.33	1.90 ± 0.32	1.06 ± 0.078	0.11 (0.053, 0.16)	<0.001

Abbreviations: ND-tam, *N*-desmethyltamoxifen; 4OH-tam, 4-hydroxytamoxifen; Metab, metabolites; AUC, area under the curve; C_{24h}, concentration before dosing (t=24h); C_{max}, maximum concentration; t_{max}, time to reach C_{max}; t½, elimination half-life; CL/F, apparent oral clearance.

Data are presented as mean and standard deviation.

t_{max}: expressed as median and range.

Parameters of one patient using 40 mg tamoxifen were dose-corrected to 20 mg.

[a] Based on results of 19 patients.

[b] $AUC_{0\text{-}24h}$ ratio of ND-tamoxifen, 4-OH-tamoxifen and endoxifen to tamoxifen.

[c] Paired Student's *t*-test (t_{max}: Wilcoxon signed-rank test)

Table 2. Pharmacokinetic parameters for tamoxifen and its metabolites following administration in the morning (8 AM), evening (8 PM) and afternoon (1 PM) in 12 women

	Morning administration (8 AM)	Evening administration (8 PM)	Afternoon administration[a] (1 PM)	P-value[c] vs .mor vs. eve
Tamoxifen				
AUC_{0-8h} (nM*h)	2464 ± 636	2174 ± 552	2301 ± 483	0.29
				0.51
AUC_{0-24h} (nM*h)	6488 ± 1482	6566 ± 1620	6314 ± 1090	1.0
				1.0
C_{24h} (nM)	248 ± 57.6	257 ± 74.5	259 ± 76.7	1.0
				1.0
C_{max} (nM)	371 ± 100	321 ± 77.0	347 ± 78.9	0.34
				0.35
t_{max} (h)	3.0 (1.1 - 4.1)	8.0 (2.0 - 24.0)	4.0 (2.0 - 24.0)	
ND-tamoxifen				
AUC_{0-8h} (nM*h)	4389 ± 1547	3792 ± 1308	4092 ± 1240	0.22
				0.06
AUC_{0-24h} (nM*h)	12531 ± 3990	11861 ± 4061	12371 ± 3591	1.0
				1.0
C_{24h} (nM)	531 ± 174	508 ± 178	510 ± 184	0.65
				1.0
C_{max} (nM)	653 ± 233	585 ± 220	627 ± 208	1.0
				0.45
t_{max} (h)	3.0 (1.0 - 24.2)	4.0 (1.0 - 24.0)	4.0 (0.5 - 24.0)	
4OH-tamoxifen				
AUC_{0-8h} (nM*h)	38.3 ± 9.93	32.6 ± 8.49	33.7 ± 10.7	0.17
				1.0
AUC_{0-24h} (nM*h)	107 ± 27.3	104 ± 27.3	98.7 ± 28.9	0.89
				1.0
C_{24h} (nM)	4.34 ± 1.16	4.29 ± 1.14	4.12 ± 0.90	1.0
				1.0
C_{max} (nM)	5.54 ± 1.54	5.00 ± 1.25	5.11 ± 1.59	0.63
				1.0
t_{max} (h)	3.0 (1.5 - 12.1)	12.0 (2.0 - 24.1)	8.0 (2.0 - 24.0)	

Endoxifen				
AUC_{0-8h} (nM*h)	189 ± 92.3	148 ± 67.1	166 ± 85.7	0.15
				0.17
AUC_{0-24h} (nM*h)	533 ± 258	471 ± 221	479 ± 249	0.63
				1.0
C_{24h} (nM)	22.6 ± 11.3	20.5 ± 9.75	20.6 ± 8.65	0.33
				1.0
C_{max} (nM)	28.2 ± 14.0	22.8 ± 10.1	25.2 ± 12.6	0.36
				0.34
t_{max} (h)	3.0 (0.5 - 24.2)	12.0 (1.0 - 24.1)	4.0 (1.1 - 24.1)	
Ratios				
Endoxifen/ND-tam	0.050 ± 0.033	0.048 ± 0.031	0.045 ± 0.031	0.16
				1.0
ND-tam/Tamoxifen	1.91 ± 0.35	1.79 ± 0.34	1.94 ± 0.34	0.36
				0.27
4OH-tam/Tamoxifen	0.017 ± 0.0063	0.017 ±0.0055	0.016 ± 0.0058	0.18
				1.0
Metab/Tamoxifen[b]	2.02 ± 0.32	1.88 ± 0.32	2.04 ± 0.31	0.17
				0.25

Abbreviations: ND-tam, *N*-desmethyltamoxifen; 4OH-tam, 4-hydroxytamoxifen; Metab, metabolites; AUC, area under the curve; C_{24h}, concentration before dosing (t=24h); C_{max}, maximum concentration; t_{max}, time to reach C_{max}.

Data are presented as mean and standard deviation.

t_{max}: expressed as median and range.

Parameters of one patient using 40 mg tamoxifen were dose-corrected to 20 mg.

[a] AUC_{0-24h} based on results of 10 patients.

[b] AUC_{0-24h} ratio of ND-tamoxifen, 4-OH-tamoxifen and endoxifen to tamoxifen.

[c] Repeated measures ANOVA with post hoc Bonferroni's test.

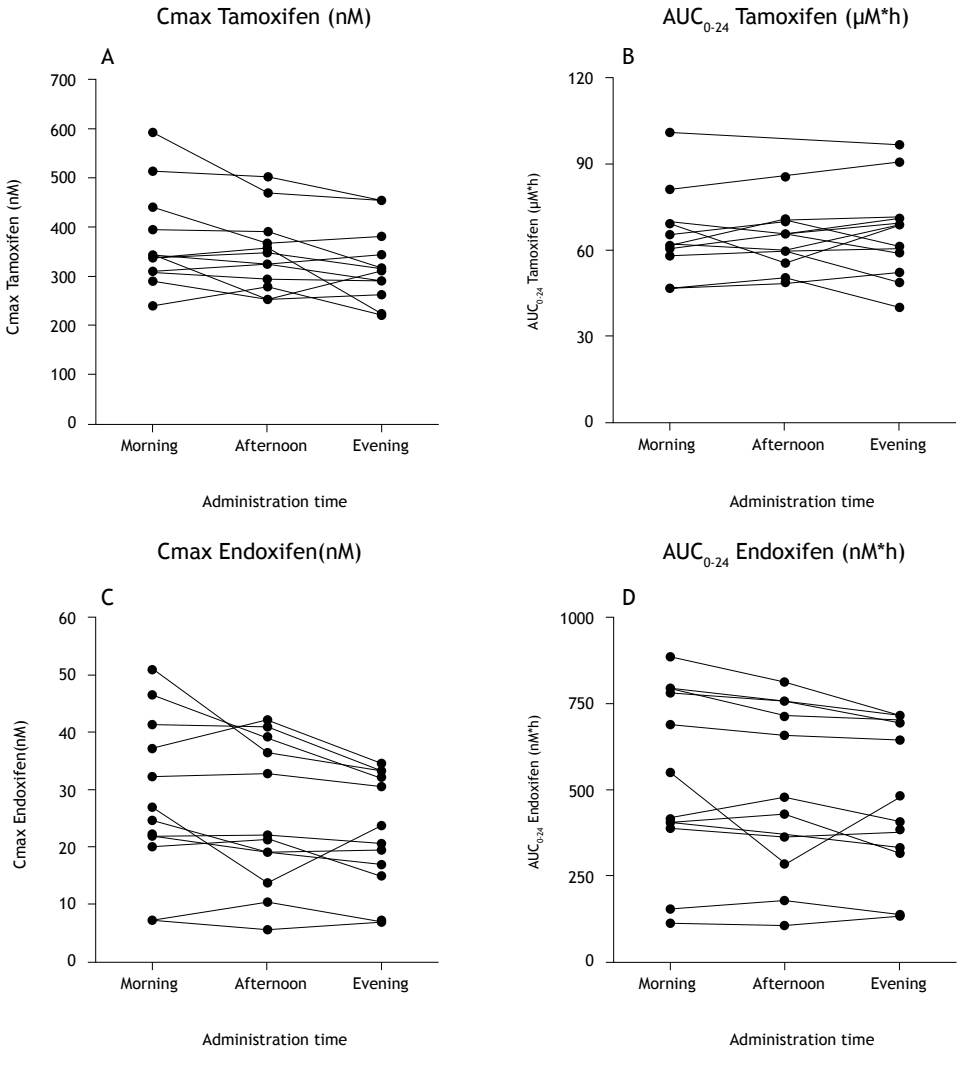

Figure 4

Individual changes in plasma exposure ($AUC_{0\text{-}24h}$) and maximum concentrations (C_{max}) for tamoxifen (A and B) and endoxifen (C and D) after tamoxifen administration at three different times; morning (8 AM), evening (8 PM) and afternoon (1 PM) in twelve women. For two patients, $AUC_{0\text{-}24h}$ data were lacking.

Table 3. Results of genotyping for cytochrome P450 polymorphisms

Gene	Alleles	Number (%)[b]
CYP2D6[a]	EM	15 (58%)
	IM	8 (31%)
	PM	3 (11%)
CYP2C19	*1/*1	13 (50%)
	*1/*2	6 (23%)
	*1/*17	2 (8%)
	*2/*2	1 (4%)
	*2/*17	4 (15%)
CYP3A4	*1/*1	10 (39%)
	*1/*22	12 (46%)
	*22/*22	4 (15%)

[a] EM, two active alleles; IM, one non-functional allele or two decreased function alleles; PM, two non-functional alleles.

[b] Results of one individual were missing.

DISCUSSION

In the preclinical study, circadian variation in mRNA expression levels of CYP enzymes, *Cyp2d10*, *Cyp2d22* and *Cyp3a11*, in mouse liver and small intestine was observed, which is in accordance with the findings by Zhang *et al.*[26] On the basis of mRNA expression levels, higher CYP enzyme activity could be expected during the active phase and lower activity during the resting phase.

We observed that exposure to tamoxifen appeared to be higher in mouse plasma and liver tissue after tamoxifen administration in the active phase, with the highest levels following administration at midnight, although differences were not statistically significant. However, a large variation in concentrations of tamoxifen and its metabolites between the individual mice was observed, which may be explained by several factors. First, the animals were given a fixed dose of 4 mg tamoxifen and small differences in bodyweight of the mice may have contributed to the variability in pharmacokinetics. In addition, tamoxifen was dissolved in peanut oil and given by oral gavage, which also may have influenced the absorption in mice.

In the exploratory clinical study, significant differences in pharmacokinetics of tamoxifen and its metabolites between morning and evening administration were observed. The tamoxifen C_{max} and AUC_{0-8h} were both higher after morning administration and t_{max} was reached earlier, suggesting an increased absorption rate following morning dosing compared with evening dosing. These results complement previous findings of more rapid absorption of lipophilic drugs after administration in the morning.[20] Increased rates of absorption, resulting in higher C_{max} levels, have been observed for diclofenac, verapamil, and nifedipine, among others.[20] The underlying mechanisms of a higher absorption rate in the early morning involve faster gastric emptying, higher gastrointestinal motility, and higher gastrointestinal blood flow in the morning than in the evening.[20,27-29] In addition, since higher gastric acidity lowers lipophilic drug absorption, the absorption of these drugs is generally lower in the evening due to increased gastric acid secretion at that moment of the day.[30]

Higher plasma exposure following morning administration was also observed for all three tamoxifen metabolites. For both 4-hydroxytamoxifen and endoxifen, morning administration resulted in higher C_{max} (25% and 23% higher) and AUC_{0-8h} (28% and 23% higher) and shorter t_{max} as compared with evening administration, which is similar to the parent compound.

The $AUCs_{0-24h}$ of the active metabolites were also significantly higher following morning dosing than following evening dosing, however, actual differences were only minor (differences in AUC_{0-24h}: 12-15%). Despite the higher metabolite concentrations, the difference in metabolic ratios of the three metabolites-to-tamoxifen between morning and evening dosing was small and clinically irrelevant (~6%), implying that the increase in metabolite concentrations is not associated with major changes in cytochrome P450-mediated metabolism. The higher tamoxifen concentrations due to increased absorption in the early morning have probably contributed to higher metabolite levels.

In the subgroup of twelve women who completed three cycles of different dosing times, no significant or clinically relevant differences in pharmacokinetic parameters of tamoxifen and its metabolites were observed after drug administration in the afternoon compared with administration in the morning or evening. Although not significantly different, it seemed that, as shown in Figure 4 and Table 2, the plasma exposure to tamoxifen and its three metabolites following administration in the afternoon was slightly higher than that observed after evening dosing but a little lower than after morning administration. This may be explained by the fact that the sleep/wakefulness cycle is the most important rhythm in humans and influences physiological functions.[21] Therefore, the greatest variation in processes, such as gastric emptying, gastrointestinal motility and gastric acid secretion, could be expected between morning and evening.[20,21]

In the preclinical study, plasma and liver exposure to tamoxifen appeared to be highest following administration at midnight. The results are in line with the observation of higher plasma levels of tamoxifen after dosing at 8 AM, the start of the active phase in humans. However, in humans, dosing in the afternoon did not result in the highest exposure to tamoxifen and its metabolites. Differences in physiological processes between mice and humans may be a plausible explanation for this discrepancy. In addition, we observed circadian variation in mRNA expression levels of CYP enzymes in mouse liver and intestine. Circadian variation of hepatic CYP3A4 activity in humans has been assumed from the observation of a 2.8-fold mean diurnal variation in the 6β-hydroxycortisol-to-cortisol ratio as a marker of CYP3A4 activity.[31] Although differences in tamoxifen pharmacokinetics in humans are most probably due to variation in absorption, the influence of circadian variation in metabolism cannot be totally excluded. Metabolic ratios did not show relevant differences between morning and evening dosing, however, this might be explained by circadian variation in elimination of tamoxifen metabolites through glucuronidation by UDP-glucuronosyltransferases.[32] However, we did not measure glucuronides of tamoxifen and its metabolites. A trend towards greater differences in endoxifen exposure between morning and evening administration in women with extensive CYP2D6 metabolism, according to genotype, was observed, which might suggest a possible influence of circadian variation in (CYP2D6) metabolism.

Besides variation in pharmacokinetics, we also assessed changes in adverse effects during the different dosing times. Hot flashes occurred in almost all women in this study, and is the most common side effect of tamoxifen.[33] Unfortunately, we were not able to detect significant differences in occurrence or severity of adverse effects due to the small number of study participants. However, a number of women reported changes in incidence and severity of hot flashes during the study, which may indicate a possible relation between the time of tamoxifen administration and occurrence of hot flashes. Possibly, the occurrence of hot flashes, and other adverse effects, are associated with peak plasma concentrations of tamoxifen or one of its metabolites. A shift in the time of maximum plasma concentration, due to a different administration time, may be a plausible explanation for the changes in the occurrence of hot flashes. This is in line with the findings of Lorizio *et al.*,[33] who observed a relation between endoxifen concentrations and the occurrence of adverse effects. The higher endoxifen levels, especially the peak plasma concentrations, following morning administration may theoretically lead to more or more severe adverse effects, although this could not be established in this study.

The results of this study may have clinical implications. Endoxifen is considered the active compound of tamoxifen and concentrations probably need to exceed a minimum critical level to be effective against breast cancer.[11,34] In the current study, morning dosing resulted in significantly higher endoxifen exposure compared with evening dosing, although the actual differences were limited (mean difference $AUC_{0\text{-}24h}$:~15%). However, a high inter-patient variation was observed and endoxifen plasma exposure was markedly increased, up to 45%, in few patients after morning dosing, which suggests that in some individuals the time of administration may be clinically relevant.

A definitive recommendation for the time of tamoxifen administration is difficult to make. Although morning administration resulted in higher endoxifen exposure, which may contribute to treatment efficacy,[11] the plasma concentrations of tamoxifen and its metabolites were less stable over a 24-hour period with higher maximum concentrations compared with evening administration (Figure 3). Evening dosing may therefore be more beneficial regarding the occurrence of adverse effects.[33] Whether or not the clinical outcome and/or side effects will be modulated by the time of tamoxifen administration needs to be confirmed in future clinical studies.

Therapeutic drug monitoring has been proposed for individualization of tamoxifen therapy as this drug is characterized by a large inter-individual variability in pharmacokinetics, partly due to variation in CYP2D6 metabolism as a result of genetic polymorphisms.[35] In the last years, many studies have been undertaken to identify genetic and environmental factors which may contribute to the inter-individual variability in pharmacokinetics.[5,7,11-13] Currently, the majority of studies focus on the relationship between *CYP2D6* genotype, endoxifen concentrations and clinical outcome or toxicity.[11,36] However, most studies do not take into account the time of tamoxifen administration and the time of the day of blood collection (intra-individual circadian variation), which may contribute to increased variability. Trough samples are not always collected,[5,11-13] as the long elimination half-lifes of tamoxifen and *N*-desmethyltamoxifen suggest no large differences in concentrations. However, the results of this study suggest that the time of tamoxifen administration as well as sampling time may be important and should be taken into consideration in studies relating endoxifen concentrations and clinical outcome and possibly in case of applying therapeutic drug monitoring for therapy individualization.

Potential limitations of our study include the lack of standardization of waking and sleeping hours of the study participants and standardization of the meals. Although the meals were not strictly controlled, it is unlikely that this may have influenced the results of the study given that food has not been shown to affect the pharmacokinetics of tamoxifen.[37]

In addition, meals were served on fixed times (at 7.30 AM, noon, and 5 PM), with the time of dinner in the evening three hours before tamoxifen administration, making the influence of food on the pharmacokinetics even more unlikely.

In conclusion, the present study shows a significantly higher absorption rate of tamoxifen following morning administration than after evening administration. Few patients had markedly, up to 45%, increased endoxifen exposure following morning dosing, which may be clinically relevant. However, systemic exposure to tamoxifen and its metabolites was more stable after evening dosing, which may be beneficial with regard to adverse effects. In addition, administration time should be taken into consideration in studies relating endoxifen concentrations and clinical outcome and possibly in therapeutic drug monitoring for therapy individualization.

METHODS

Animals and synchronization

One-hundred twenty-six female FVB mice with an age of 8-12 weeks were used in the experiments. Animals were housed under standardized conditions with a room temperature of 22˚C, relative humidity of 55%, and food and water *ad libitum*. For logistic reasons, the mice were kept in two rooms, under either a normal or inverted 12h light/12h dark regime (light 8 AM – 8 PM and dark 8 PM – 8 AM and *vice versa*). The animal experiments were approved by DEC consult, an independent Animal Ethical Committee (Dutch equivalent of the IACUC) and performed in accordance with local guidelines.

Study in mice

Tamoxifen was dissolved in peanut oil at a concentration of 4 mg/mL, which was freshly prepared before each administration time. At six different time points, at 8 AM, noon, 4 PM, 8 PM, midnight and 4 AM (taking into account normal and inverted light/dark regimes), a fixed dose of 4 mg tamoxifen (~200 mg/kg bodyweight) was administered orally by gavage into the stomach to a group of 18 mice. Mice were fasted for three hours prior to tamoxifen administration to minimize variation in absorption. Under anesthesia with isoflurane, blood samples were collected from the orbital sinus at 1, 2, 3, 5, 10 and 20 hours after tamoxifen administration. Three mice were sacrificed per time-point. After cervical dislocation, liver and small intestine (proximal, middle and distal part) were quickly removed. Tissue samples

were separately collected in 1.8 mL polypropylene tubes and snap frozen in liquid nitrogen and stored at -80˚C. Before analysis, tissue samples were 4-fold diluted with human plasma and homogenized using TissueLyzer (Qiagen, Germany) for 4 minutes at 30 Hz. Blood was collected in lithium-heparinized collection tubes and plasma was separated by centrifugation at 2,500*g* for 10 minutes at 4˚C. Plasma samples were immediately stored at -80˚C until the day of analysis.

In a control group of 18 mice, administered solvent orally by gavage, plasma, liver and small intestine samples were collected every 4 hour in a 24-h period (3 mice per time-point). Rhythmic mRNA expression of CYP enzymes was examined in mouse liver and three consecutive parts of the small intestine. Circadian fluctuations in mRNA expression of *Cyp3a11*, *Cyp2d10* and *Cyp2d22* were investigated, as these are the murine orthologues of the human CYP enzymes which are involved in the metabolism of tamoxifen. Expression of components of the circadian clock (*Period 2 (Per2), Dbp, Bmal1, Cryptogene 1 (Cry1)* and *Rev-erb-a*) was also determined, indicating the proper light entrainment of the mice.

Patients

Women using tamoxifen for breast cancer once daily for at least 4 months (to guarantee steady state) were included in the study. Additional eligibility criteria included: age >18 years; WHO performance score ≤ 1; normal blood cell counts and adequate renal and hepatic functions. Women were excluded if they were pregnant or lactating, used tamoxifen more than once a day, or suffered from any illness that would prohibit the understanding and giving of informed consent. The use of (herbal) supplements was not allowed during the whole study period. During clinical days, patients received standard hospital meals, served at 7.30 AM, noon and 5 PM.

Clinical study

This was a pharmacokinetic cross-over study, analyzing up to three different dosing times of tamoxifen. At the start of the study, patients were using tamoxifen once daily at an oral dose of 20 or 40 mg either in the morning or evening. No dose modifications were allowed before or during the study period. Due to the long half-life of tamoxifen, and hence the prolonged period for reaching steady state levels of tamoxifen metabolites, randomization was impractical, and patients were allocated to one of the two sequences (starting in the morning or evening), depending on the time of tamoxifen administration before the study.

After at least 4 weeks of dosing at either 8 AM or 8 PM, pharmacokinetic profiles of tamoxifen and its metabolites were assessed during a 24-h period. Patients were then switched to the other time of administration for at least 4 weeks followed by a second pharmacokinetic blood sampling period. The third time of pharmacokinetic blood sampling occurred after 4 weeks of tamoxifen dosing in the afternoon (1 PM). On each study day, blood samples for hematological and biochemical analysis were also obtained. In addition, information on adherence, dosing time, concomitant medication and adverse effects was collected on the days of pharmacokinetic blood sampling.

Through an indwelling catheter, blood samples were collected in lithium-heparinized collection tubes just before and at 0.5, 1, 1.5, 2, 4, 8, 12, and 24 hours after tamoxifen administration. Plasma was separated and stored as mentioned before. A blood sample for genotype testing was collected from all patients.

Pharmacokinetic analyses of tamoxifen and its metabolites

Concentrations of tamoxifen and its main metabolites *N*-desmethyltamoxifen, 4-hydroxytamoxifen, (Z)-endoxifen, 4'-hydroxytamoxifen and 4'-hydroxy-*N*-desmethyltamoxifen in plasma and tissue homogenates of the mice and in human plasma samples were measured using a validated UPLC-MS/MS method, as previously described.[38]

Pharmacokinetic parameters, including area under the plasma concentration-time curve (AUC), minimum plasma concentration (C_{trough}; t=24h), maximum concentration (C_{max}), the time to reach maximum plasma concentration (t_{max}) and apparent elimination half-life ($t_{1/2}$) of tamoxifen and its metabolites were derived from noncompartmental analysis on plasma concentration-time profiles using the program Phoenix WinNonlin 6.1 (Pharsight Corporation, Mountain View, CA). The apparent oral clearance was calculated from the equation CL/F = dose/AUC. Metabolic ratios were calculated as $AUC_{0\text{-}24\ metabolite}$ /$AUC_{0\text{-}24\ tamoxifen}$.

Genotyping for genes encoding cytochrome P450 enzymes

DNA was isolated from whole blood samples and genotype analyses were performed for *CYP2D6 *3, *4, *6, *10, *17, *41, CYP2C19*2, *3, *17* and *CYP3A4 *22* using TaqMan allelic discrimination assays and *CYP2D6* gene deletion (*5) and duplication using *CYP2D6* TaqMan Gene Copy Number Assay.

RNA isolation and cDNA preparation

Total RNA was extracted from mouse liver cells and three parts of the small intestine using RNA-Bee reagent (Bio-Connect) according to the manufacturer's protocol. The quality of RNA and concentrations were analyzed using the Nanodrop ND1000 (NanoDrop Technologies). A total of 1 microgram of RNA was used for cDNA preparation using iScript (Biorad) according to the manufacturer's protocol.

Semi-quantitative real-time PCR

Gene expression was analyzed by semi-quantitative real-time PCR using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's protocol on a Biorad C1000 Touch Thermal Cycler using a standard two-step amplification program with annealing/extension at 60˚C. Reactions for samples with housekeeping genes (*ß2 microglobulin (B2M), hypoxanthine guanine phosphoribosyltransferase (Hprt), glyceraldehyde 3-phosphate dehydrogenase* (Gapdh)) and for the genes of interest were always performed within the same plate. Data of qPCR represents the average of 3 housekeeping genes. The primers for the detection of housekeeping genes, clock genes and genes of drug-metabolizing enzymes are listed in Table 4. Relative expression of the genes of interest was calculated using the comparative C(t) method and was normalized to the relative expression at time point 8 AM (relative expression = 1).

Table 4. Primers for the detection of housekeeping genes, clock genes and genes of drug-metabolizing enzymes

Gene	Forward primer	Reverse primer
B2M	5'-CCGGCCTGTATCCAGAAA-3'	5'-ATTTCAATGTGAGGCGGGTGGAAC-3'
Hprt	5'-CGAAGTGTTGGATACAGGCC-3'	5'-GGCAACATCAACAGGACCTCC-3'
Gapdh	5'-CAGAACATCATCCCTGCATCC-3'	5'-GTCATCATACTTGGCAGGTTTCTC-3'
Bmal1	5'-GCACTGCCACTGACTACCAAGA-3'	5'- TCCTGGACATTGCATTGCAT-3'
Dbp	5'-ACCGTGGAGGTGCTAATGAC-3'	5'-CCTCTTGGCTGCTTCATTGTT-3'
Per2	5'-GGCTTCACCATGCCTGTTGT-3'	5'-GGAGTTATTTCGGAGGCAAGTGT-3'
Cry1	5'-CAGACTCTCGTCAGCAAGATG-3'	5'-CAAACGTGTAAGTGCCTCAGT-3'
Rev-erb-a	5'-ACCTTACTGCTCAGTGCCTGGAAT-3'	5'-TGGACCTTGACACAAACTGGAGGT-3'
Cyp3a11	5'- ACCTGGGTGCTCCTAGCAAT-3'	5'- ACCATCAAACAACCCCCATGT-3'
Cyp2d10	5'- CTCATCCCCAAGGGGTCAAT-3'	5'- ACCGGAAAAGGAAAGACACC-3'
Cyp2d22	5'- CCACGCTCTCAAGGTATAGTC-3'	5'- TTCAACAAGCCCAGTAGCCT-3'

Statistical analysis

A sample size of 18 women was required to detect a difference of 20% in endoxifen $AUC_{0\text{-}24h}$ between two different administration times, with 80% power and alpha (α) 0.05. This was based on the assumption of an intra-patient variability in endoxifen pharmacokinetics of 20%. However, an amendment was made to the study protocol to insert a third administration time (afternoon) in the study. Since a number of patients was not able (or willing) to accomplish the third pharmacokinetic period, new individuals were included in the study. As a relevant difference in pharmacokinetics between dosing in the afternoon versus dosing in the morning/evening should be seen in 12 individuals, we decided to include a smaller number of women first.

Pharmacokinetic data are presented as mean and standard deviation, unless stated otherwise. One-way analysis of variance (ANOVA) was used to compare the groups of mice. Post hoc Bonferroni's test was used to find differences in mean between the groups. Harmonic regression analysis of circadian oscillation was performed using CircWave Batch v5.0 for cosinor analysis with a 24-hour wave, with forward linear harmonic regression using an *F*-test. User defined alpha was 0.05.

Differences in pharmacokinetic parameters between morning and evening administration in humans were analyzed by paired Student's *t*-tests. For differences in t_{max}, Wilcoxon signed-rank tests were used. Repeated measures ANOVA with post hoc Bonferroni's test was used for the analysis of differences between morning, evening and afternoon administration. Statistical analyses were performed using IBM SPSS statistics, version 21 (SPSS Inc., Chicago, IL). In all analyses, a $P \leq 0.05$ was considered statistically significant.

Study approval

The study was performed at the Erasmus MC Cancer Institute, Rotterdam, the Netherlands. The local ethics committee approved the study protocol (Dutch Trial Registry; NTR3473) and the study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before study entry.

ACKNOWLEDGEMENT

We thank Anna Pagani, Anne-Joy de Graan, Annemieke Nieuweboer, Cindy Bolder, Dennis de Meulder, Dominique Kuiper, Ellen de Morree, Els Moltzer, Inge Ghobadi Moghaddam-Helmantel, Patricia van Kuijk, Stefanie Vester, Ton Boersma and Xander den Dekker for their specific contribution to this study.

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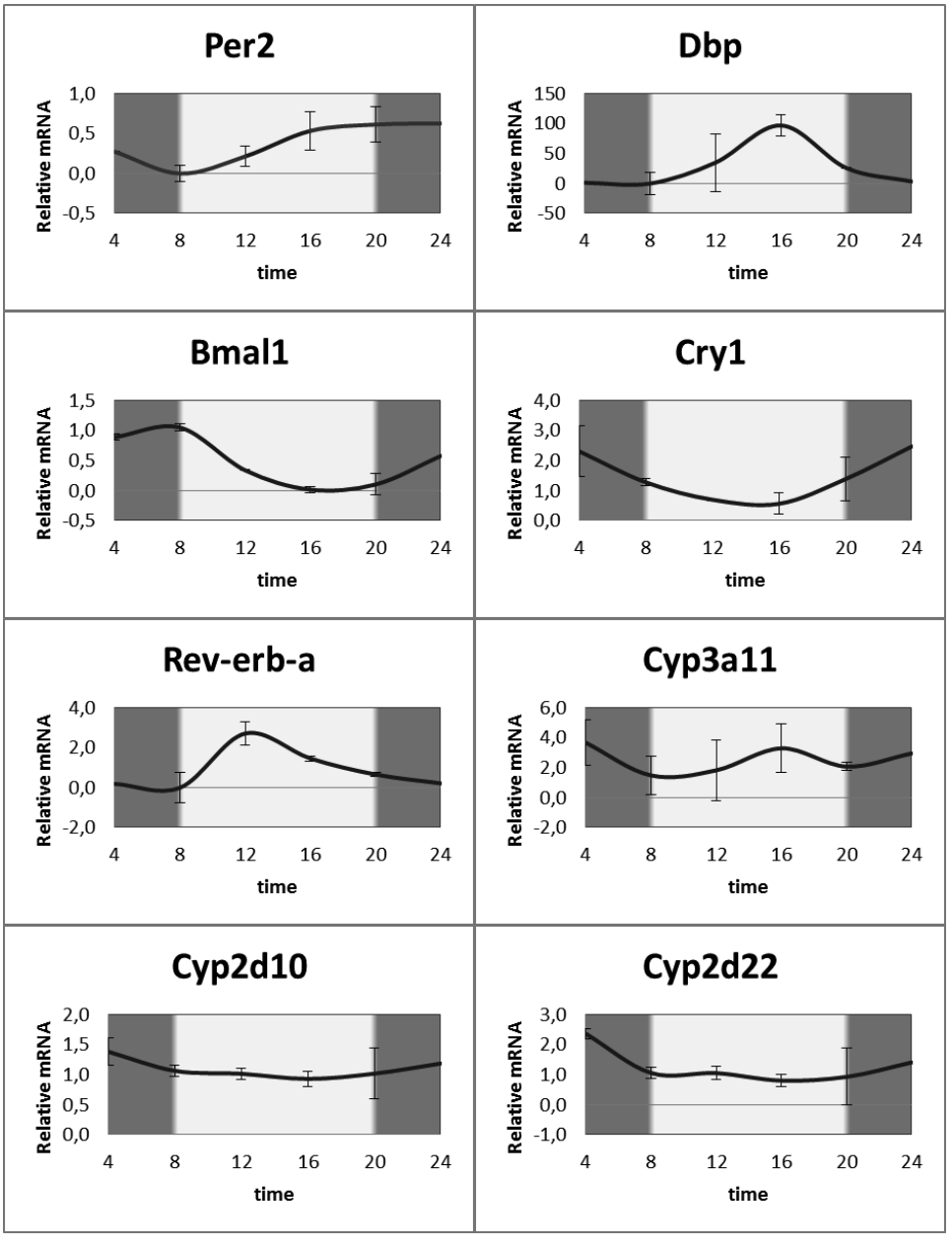
Supplemental Figure 1

Daily mRNA expression of components of the circadian clock and drug-metabolizing enzymes. Diurnal mRNA expression profiles of components of the circadian clock *(Period 2 (Per2), Dbp, Bmal1, Cryptogene 1 (Cry1)* and *Rev-erb-a*) and drug-metabolizing enzymes (*Cyp3a11, Cyp2d10* and *Cyp2d22*) in mouse liver and three consecutive parts of the small intestine. The dark phase is represented by the dark grey area and the light phase by the white area. Data is represented as relative expression to 8 AM (relative expression = 1).

Mouse liver


Per2
Relative mRNA
1,0
0,5
0,0
-0,5
4 8 12 16 20 24
time
Dbp
Relative mRNA
150
100
50
0
-50
4 8 12 16 20 24
time
Bmal1
Relative mRNA
1,5
1,0
0,5
0,0
-0,5
4 8 12 16 20 24
time
Cry1
Relative mRNA
4,0
3,0
2,0
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0,0
4 8 12 16 20 24
time
Rev-erb-a
Relative mRNA
4,0
2,0
0,0
-2,0
4 8 12 16 20 24
time
Cyp3a11
Relative mRNA
6,0
4,0
2,0
0,0
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4 8 12 16 20 24
time
Cyp2d10
Relative mRNA
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1,5
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4 8 12 16 20 24
time
Cyp2d22
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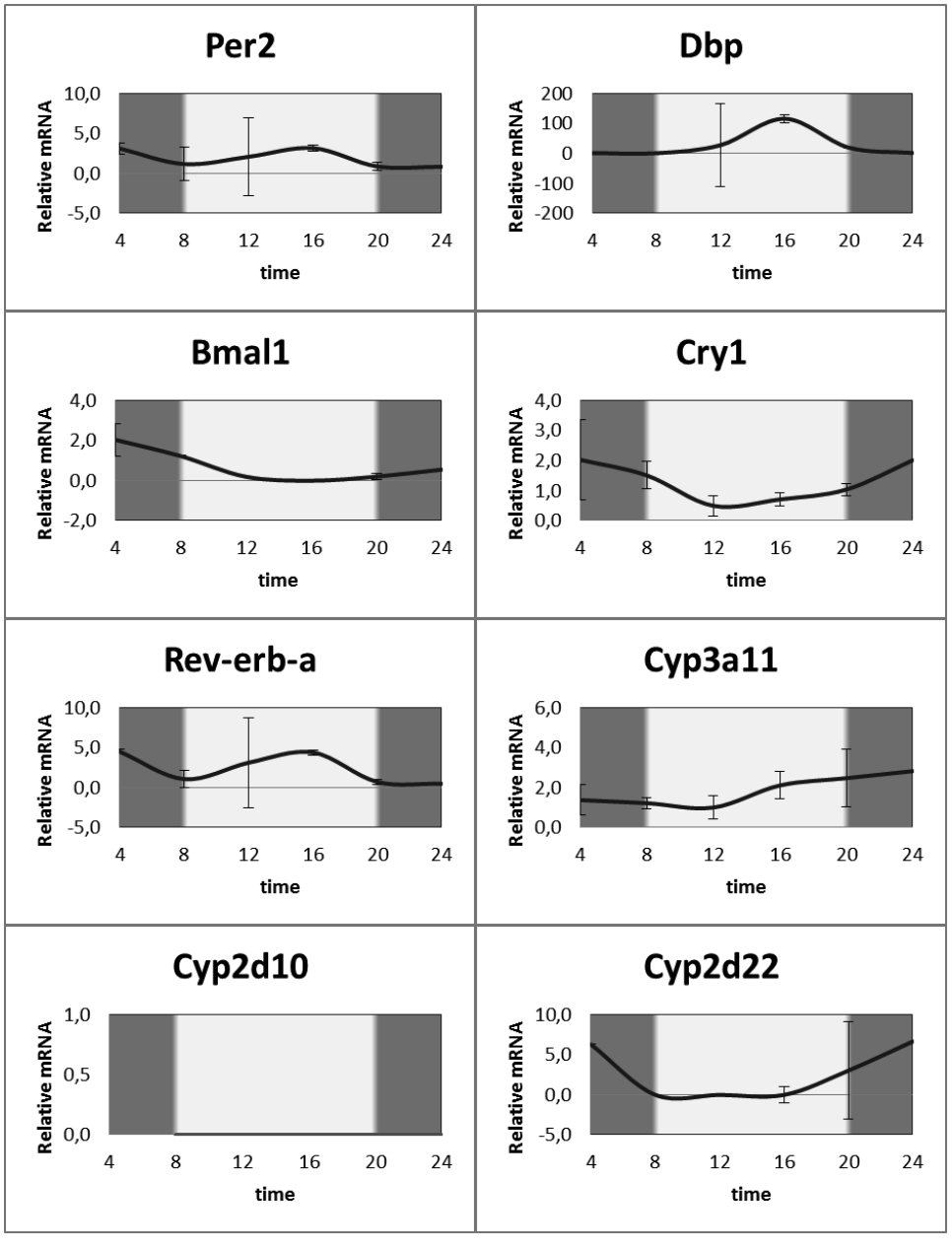
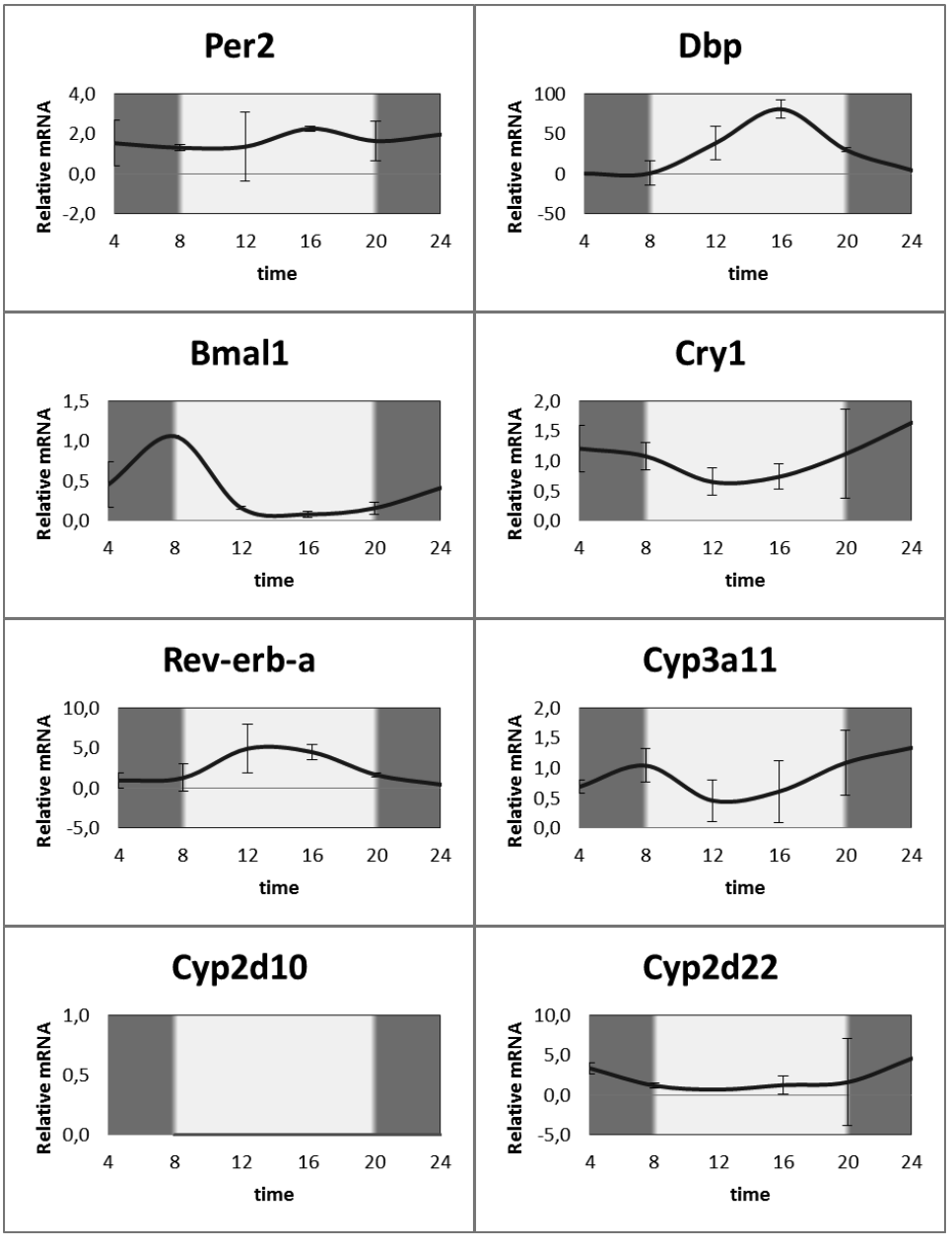
4 8 12 16 20 24
time

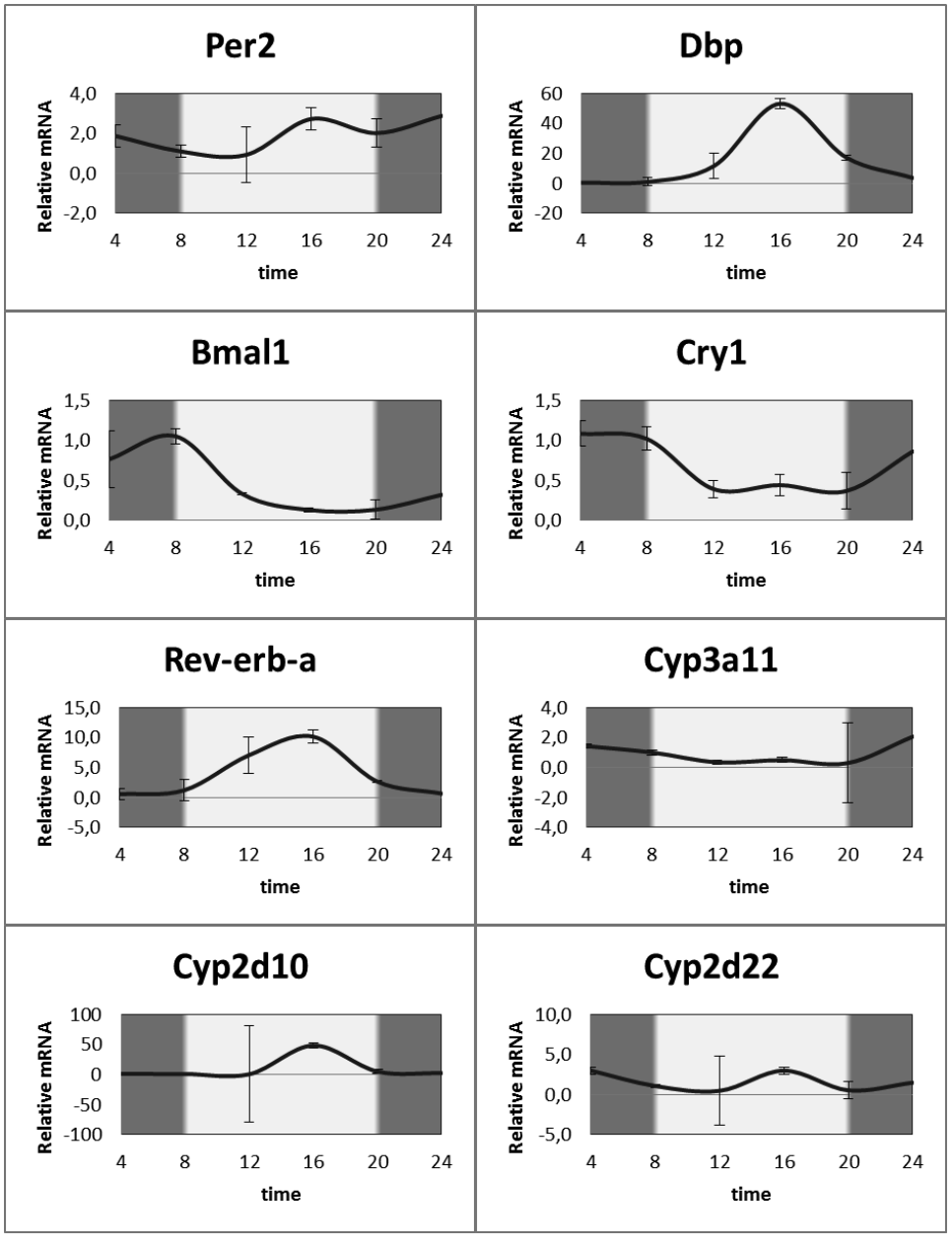
Mouse small intestine (proximal part)



Mouse small intestine (middle part)



Mouse small intestine (distal part)



Supplemental Table 1. Exposure to tamoxifen and its metabolites in mouse plasma, liver and three consecutive parts of the small intestine following six different administration times[a]

	8 AM	Noon	4 PM	8 PM	Midnight	4 AM
Plasma						
Tamoxifen						
$AUC_{0\text{-last}}$ (nM*h)	22500 ± 5629	28651 ± 10226	32533 ± 7637	27774 ± 1094	45282 ± 17350	36723 ± 2175
4-OH-Tam						
$AUC_{0\text{-last}}$ (nM*h)	18517 ± 4055	20628 ± 7609	22886 ± 5427	21020 ± 1071	28409 ± 7614	28714 ± 3799
ND-Tam						
$AUC_{0\text{-last}}$ (nM*h)	7161 ± 594	10752 ± 4991	13912 ± 1384	10935 ± 1928	14859 ± 4196	13655 ± 2261
Endoxifen						
$AUC_{0\text{-last}}$ (nM*h)	5094 ± 165	6305 ± 1935	8045 ± 870	7014 ± 1529	8034 ± 1365	8743 ± 1805
Liver						
Tamoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	644 ± 255	766 ± 125	781 ± 184	596 ± 35	1014 ± 405	808 ± 35
4-OH-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	308 ± 90	319 ± 60	344 ± 75	254 ± 17	390 ± 121	397 ± 50
ND-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	295 ± 43	425 ± 146	537 ± 69	387 ± 48	494 ± 86	485 ± 71
Endoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	98 ± 14	128 ± 31	156 ± 21	118 ± 9.9	147 ± 21	160 ± 38
Intestine (proximal)						
Tamoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	2144 ± 1294	2340 ± 443	1451 ± 566	1202 ± 338	2459 ± 336	3431 ± 1045
4-OH-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	1056 ± 537	1289 ± 176	899 ± 248	777 ± 123	1117 ± 72	1708 ± 174

ND-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	184 ± 32.6	286 ± 87.7	323 ± 68.9	218 ± 19.0	305 ± 44.4	347 ± 29.7
Endoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	157 ± 24.4	219 ± 36.6	258 ± 47.5	192 ± 12.2	213 ± 3.20	298 ± 37.8
Intestine (middle)						
Tamoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	2969 ± 1106	2958 ± 563	2455 ± 1262	2437 ± 236	3671 ± 1839	2557 ± 239
4-OH-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	1251 ± 359	1531 ± 242	1042 ± 221	950 ± 161	1418 ± 469	1373 ± 137
ND-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	227 ± 9.97	323 ± 105	373 ± 132	280 ± 31	418 ± 83.8	351 ± 43.3
Endoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	224 ± 32	365 ± 107	281 ± 89	287 ± 43.8	406 ± 63.3	331 ± 63
Intestine (distal)						
Tamoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	2671 ± 658	2368 ± 323	2798 ± 559	1985 ± 475	2196 ± 835	2338 ± 98
4-OH-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	640 ± 170	747 ± 76	744 ± 174	559 ± 119	721 ± 188	810 ± 37
ND-Tam						
$AUC_{0\text{-last}}$ (nmol/g *h)	191 ± 20	331 ± 115	449 ± 140	253 ± 42	362 ± 41	318 ± 72
Endoxifen						
$AUC_{0\text{-last}}$ (nmol/g *h)	172 ± 14	286 ± 58	341 ± 65	220 ± 45	259 ± 4.0	284 ± 67

Abbreviations: ND-Tam, *N*-desmethyltamoxifen; 4-OH-Tam, 4-hydroxytamoxifen.

Exposure is expressed as $AUC_{0\text{-last}}$. Each value represents the mean and standard deviation of triplo measurement.

[a] No significant differences were observed (one-way analysis of variance (ANOVA) with post hoc Bonferroni's test).

Chapter 8

Individualization of tamoxifen therapy: much more than just *CYP2D6* genotyping

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Cancer Treat Rev 2015;41:289-99.

ABSTRACT

Clinical response to tamoxifen varies widely among women treated with this drug for hormone receptor-positive breast cancer. The principal active metabolite – endoxifen – is generated through hepatic metabolism of tamoxifen, with key roles for cytochrome P450 (CYP) CYP2D6 and CYP3A. By influencing endoxifen formation, genetic variants of drug-metabolizing enzymes as well as co-medication may affect response to tamoxifen. After a decade of research, examining the effects of *CYP2D6* genetic variants on tamoxifen efficacy, there is still no agreement on the clinical utility of *CYP2D6* genotype as biomarker for the prediction of breast cancer outcome, as studies revealed conflicting results. However, tamoxifen metabolism is complex and involves several other drug-metabolizing enzymes. As evaluated in this review, genetic variants of other CYP enzymes, including *CYP3A4* and *CYP2C9*, as well as co-medication modulating the metabolic activity of CYP2D6 and CYP3A4 have been shown to affect endoxifen concentrations and may also contribute to the variability in response to tamoxifen. Phenotyping strategies can predict endoxifen exposure more accurately than *CYP2D6* genotype, but do not take into account all factors influencing endoxifen exposure. Therapeutic drug monitoring (TDM) is likely to be the optimal strategy for individualization of tamoxifen treatment. According to a growing amount of literature, endoxifen concentration seems to be a predictor of clinical outcome. The relationship between endoxifen systemic levels and breast cancer outcomes has to be replicated and confirmed and the value of TDM should be evaluated in prospective clinical trials. Caution is advised regarding the concomitant use of medications which could interact with tamoxifen, including inhibitors and inducers of CYP enzymes.

INTRODUCTION

Worldwide, breast cancer is the most common cancer among women, and 522,000 breast cancer related deaths in 2012 have been estimated by the World Health Organization.[1] About 60-70% of the breast cancers are hormone receptor-positive, and endocrine therapy with tamoxifen or aromatase inhibitors forms the backbone of treatment regimens in women with this type of breast cancer.[2, 3] The selective estrogen receptor modulator tamoxifen binds to the estrogen receptor (ER), preventing estrogen from binding, and inhibits the transcriptional activity of the ER in breast tissue by the recruitment of co-repressors instead of co-activators and consequently inhibits tumor growth.[4] Tamoxifen is the standard of care for premenopausal women with hormone receptor-positive breast cancer and is used in postmenopausal women in sequential treatment with an aromatase inhibitor or for those individuals who cannot tolerate aromatase inhibitors.[3, 5]

The drug has been successfully used for almost forty years in early and advanced disease. In the adjuvant setting it has been shown that five years of tamoxifen treatment reduces disease recurrence by almost forty percent and breast cancer mortality by thirty percent during the first 15 years.[2, 3] Extended tamoxifen therapy, for up to ten years, appeared to be superior to five years, with further reductions of 25% and 29% in recurrences and mortality.[6] Tumor shrinkage and prolonged survival have been observed in metastatic disease.[7] Nevertheless, disease recurs in approximately 30% of the patients and non-response is also observed in about 50% of the patients with metastatic disease.[3, 7]

Response or resistance to tamoxifen may be affected by several host, environmental and tumor factors. Variation in ER expression and ER function have been recognized as mechanisms of resistance. As host factors, genetic polymorphisms in drug-metabolizing enzymes involved in the metabolism of tamoxifen may affect clinical efficacy, because they contribute to the variability in systemic exposure to the principal active metabolite endoxifen.[8]

Since cytochrome P450 (CYP) 2D6 plays the most prominent role in the metabolism of tamoxifen into endoxifen, a lot of studies have focused on *CYP2D6* genotype as a predictive marker for clinical response and individualization of tamoxifen therapy.[9] Genetic variation in *CYP2D6* only explains partly the variability in endoxifen exposure. Polymorphisms in genes encoding for other drug-metabolizing enzymes, as well as concomitant medication, may also affect tamoxifen pharmacokinetics. Therefore, therapeutic drug monitoring (TDM) may be a better approach for individualization of tamoxifen therapy. In this review genetic and non-genetic factors which may influence tamoxifen pharmacokinetics and efficacy are extensively discussed. In addition, obstacles for applying TDM will be evaluated.

TAMOXIFEN METABOLISM

Tamoxifen has weak affinity to the ER and is regarded as a pro-drug. Tamoxifen is extensively metabolized into many metabolites by several members of the cytochrome P450 family and phase II conjugation enzymes (Figure 1).[10, 11] The major primary metabolite *N*-desmethyltamoxifen and the minor primary metabolite 4-hydroxytamoxifen are formed by *N*-demethylation and 4-hydroxylation of the parent drug, which is predominantly catalyzed by CYP3A and CYP2D6. Further CYP-mediated metabolism of these metabolites results in the formation of 4-hydroxy-*N*-desmetyltamoxifen (endoxifen).[10, 11] Endoxifen and 4-hydroxytamoxifen have equivalent anti-estrogenic potencies and are 30 to 100 times more active as anti-estrogens than tamoxifen and *N*-desmethyltamoxifen. Because of the 5 to 10-fold higher plasma concentrations of endoxifen compared to 4-hydroxytamoxifen, endoxifen is believed to be the principal active metabolite.[12, 13] In addition to CYP3A and CYP2D6, multiple other CYP enzymes, including CYP2C9, CYP2C19 and CYP2B6, are involved in the metabolism of tamoxifen.[10, 11]

The metabolism of tamoxifen is more complicated. Tamoxifen and *N*-desmethyltamoxifen can also be metabolized into 4'-hydroxymetabolites, which are very similar to 4-hydroxymetabolites, but lack inhibitory effect on the ER. In addition, not only (Z)-isomers of the 4-hydroxylated metabolites are formed, but also (E)-isomers, although to a lesser extent. The (E)-isomers have less than 1% of the ER affinity as compared with the (Z)-isomers and do not contribute to the pharmacological effect.[11]

Tamoxifen and its metabolites undergo further metabolism by UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) to form more soluble compounds that can be excreted via the bile and urine.[11, 14-16] Some of the compounds are subsequently reabsorbed via enterohepatic recirculation.[16] The glucuronidation of tamoxifen and its metabolites is catalyzed by UGT1A4, UGT1A8, UGT1A10, UGT2B7, and UGT2B15, and SULT1A1 plays a prominent role in the sulfation of tamoxifen metabolites.[14, 15]

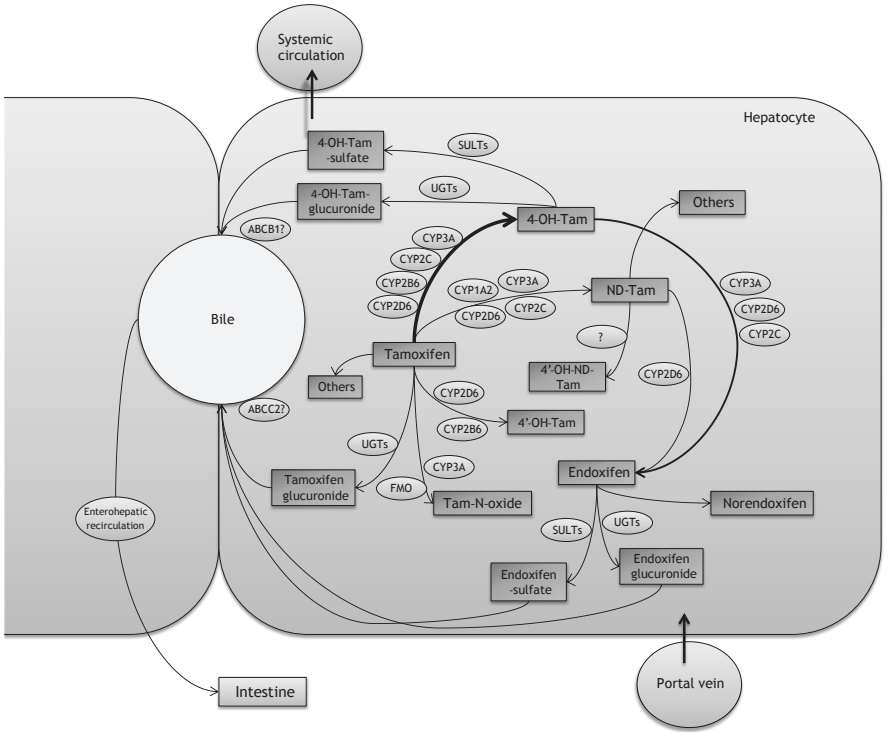

Figure 1 A

The hepatic metabolism of tamoxifen.

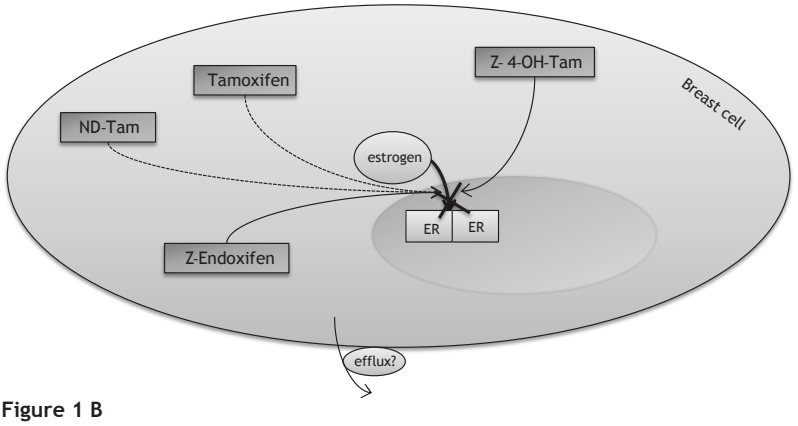

Figure 1 B

Tamoxifen and its metabolites competitively block the estrogen receptors and inhibit the transcriptional activity of the estrogen receptors and consequently inhibit tumor growth. Endoxifen and 4-hydroxytamoxifen are 30 to 100 times more active as anti-estrogens than tamoxifen and *N*-desmethyltamoxifen. Endoxifen is probably the principal active metabolite. Abbreviations; 4-OH-Tam, 4-hydroxytamoxifen; ND-Tam, *N*-desmethyltamoxifen.

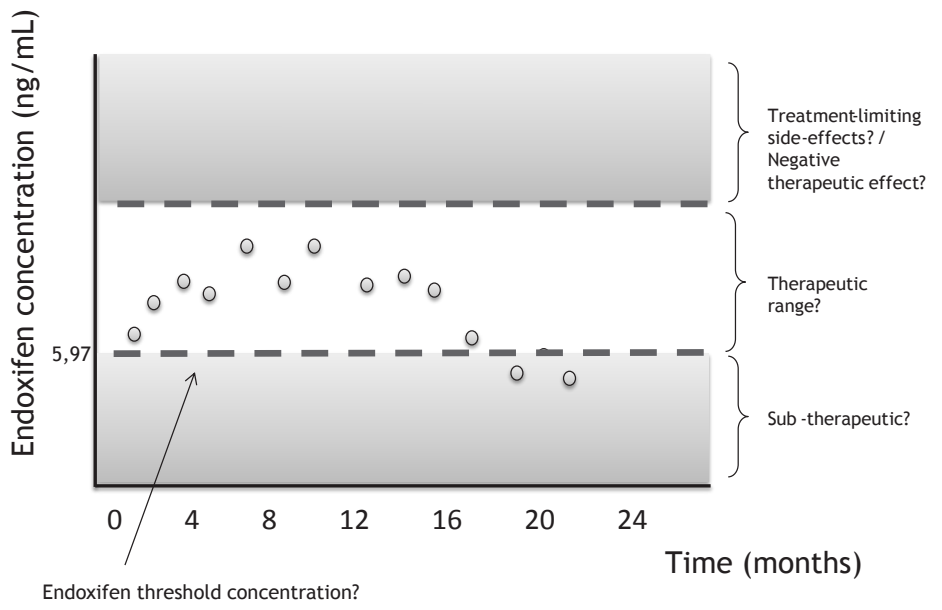

Figure 2

Endoxifen concentrations should probably be above a minimal threshold concentration for efficacy against breast cancer. Potentially, endoxifen concentrations should be within a therapeutic range, because of treatment-limiting side effects. By applying therapeutic drug monitoring, all factors influencing endoxifen exposure are taken into account. Regularly measurement of the endoxifen concentration may be important because of potential changes in exposure during long-term treatment or changes in co-medication or other environmental factors.

CYP2D6 GENOTYPE

CYP2D6 catalytic activity is highly important in tamoxifen-treated individuals, because this enzyme has a key role in the biotransformation of *N*-desmethyltamoxifen into endoxifen. The CYP2D6 enzyme is encoded by a highly polymorphic gene. Some of these allelic variants encode a reduced function CYP2D6 enzyme (e.g., *CYP2D6*9,*10,*17,*29,*36,*41*), whereas others result in no enzyme activity at all (e.g., *CYP2D6*3 *4,*5,*6,*40*). *CYP2D6*4* is the most common non-functional allele in Caucasians, while the reduced function allele *CYP2D6*10* is most common in the Asian population. Individuals can be classified into four phenotypic categories; poor metabolizers (PM, two non-functional *CYP2D6* alleles), intermediate metabolizers (IM, one functional allele or two reduced function alleles), extensive

metabolizers (EM, two functional alleles), and ultra-rapid metabolizers (UM, multiplication of functional alleles).[17, 18] In individuals with intermediate and poor CYP2D6 metabolism endoxifen concentrations have been shown to be up to 60% and 74% lower than in women with extensive CYP2D6 metabolism.[11, 19, 20] Gene-dose effects have been reported for endoxifen concentrations as well as for the metabolic ratios of *N*-desmethyltamoxifen-to-endoxifen.[11, 19] It has been hypothesized that women with impaired CYP2D6 metabolism and thus lower exposure to endoxifen have an unfavorable outcome compared to those with normal CYP2D6 metabolism. Numerous studies have been carried out to evaluate the association between impaired CYP2D6 function by genetic variants and breast cancer outcome in women treated with tamoxifen, however, have yielded conflicting results (Table 1).[20,26,28-58] Studies varied in many ways, such as clinical outcome, the number of studied *CYP2D6* variant alleles, the source of DNA for genotype analysis, co-treatment with chemotherapy, and duration of tamoxifen therapy, among others, and most of them did not account for CYP2D6-inhibiting co-medication and adherence[59] (Table 1). Therefore, it is difficult to compare the outcomes of these studies. Many studies have reported no association, including three large trials; the BIG 1-98 trial, the ATAC trial and the TEAM trial.[52-54] However, the first two trials have been criticized because DNA was derived from tumor tissue and potentially misclassification of *CYP2D6* genotypes due to loss of heterozygosity (LOH) might have influenced the results.[60] Nevertheless, similar results have been found in the TEAM trial, in which the possible influence of LOH was ruled out.[54] Despite these large trials, the search for a potential association between *CYP2D6* polymorphisms and tamoxifen efficacy is still ongoing. Some argue that *CYP2D6* genotyping may have a role in certain subgroups; only in premenopausal women,[53] only for women treated in the adjuvant setting,[35] or only in women meeting strict clinical and genotype criteria.[61] However, taken all data together, it is reasonable to believe that the efficacy of tamoxifen therapy cannot be predicted by *CYP2D6* genotype solely. Variation in activity of other drug-metabolizing enzymes, due to genetic polymorphisms or concomitant medications, involved in the metabolism of tamoxifen have to be taken into account too.

Table 1. The effects of genetic polymorphisms in *CYP2D6* on tamoxifen pharmacokinetics and clinical outcome

Gene	Allele(s)	Comparison(s)	Effects on tamoxifen pharmacokinetics[a]	Effects on clinical outcome[b]	Reference(s)
CYP2D6	combined	PM vs IM vs EM (vs UM)	PM vs EM: 30-74% lower endoxifen concentrations IM vs EM: up to 45% lower endoxifen concentrations		11, 19, 21-26
	*1,*10 (reduced function)	*10/*10 vs *1/*10 vs *1/*1	*1/*10 vs *1/*1: ~9% lower endoxifen concentrations *10/*10 vs *1/*1: 59-60% lower endoxifen concentrations		20, 27
	*1,*4 (non-functional)	PM vs EM		PM: worse outcome	28, 29
		PM vs IM/EM			
		PM vs EM		PM: worse BCS	30
	*1,*10 (reduced function)	*1/*1+*1/*10 vs*10/*10		*10/*10: worse outcome	31
		*1/*1+*1/*10 vs*10/*10		*10/*10: worse outcome MBC	20
	combined	PM vs EM		PM / IM, AS ≤0.5, activity <50%: worse outcome (Adjuvant + ABC)	26, 32-39
		IM vs EM			
		PM/IM vs EM			
		PM vs IM/EM			
		Activity <50% vs >50%			
		Activity score ≤0.5 vs ≥1			
		PM vs EM		PM / IM, activity <50%: worse BCS/OS (Adjuvant + ABC/MBC)	34, 35, 40

	PM/IM vs EM		
	Activity <50% vs >50%		
*1,*4 (non-functional)	PM vs EM	No association with outcome	41-44
	IM vs EM	PM/IM: better outcome	
	PM/IM vs EM		
	PM/IM vs EM	No association with OS	28, 41
*1,*10 (reduced function)	*1/*1+*1/*10 vs*10/*10	No association with outcome	45, 46
	*1/*1+*1/*10 vs*10/*10	No association with OS	46
combined	PM vs EM	No association with outcome	47-58
	IM vs EM		
	PM/IM vs EM		
	PM vs IM/EM		
	PM vs EM	No association with OS	32,33, 47-49
	IM vs EM		
	PM/IM vs EM		
	PM vs IM/EM		

PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; BCS, breast cancer survival; OS, overall survival; ABC, advanced breast cancer; MBC, metastatic breast cancer.

[a] Due to differences in analytical method, endoxifen concentrations vary widely among the studies.

[b] Clinical outcome: disease-free survival, relapse-free survival, progression-free survival, recurrence-free survival, time-to-progression, time-to-relapse.

CYP2D6-INHIBITING MEDICATION

Medications that are co-prescribed in tamoxifen-treated individuals may lead to pharmacokinetic drug-drug interactions by interfering with CYP-mediated metabolism of tamoxifen into endoxifen. Some medications have been associated with competitive or non-competitive inhibition of CYP2D6 enzyme activity. Selective serotonin reuptake inhibitors (SSRIs) and selective serotonin and norepinephrine reuptake inhibitors (SNRIs) are well-known for their CYP2D6-inhibiting properties, but are still frequently prescribed to women treated with tamoxifen for a wide range of mental disorders.[62-64] Approximately one quarter of women suffering from breast cancer experience depressive symptoms which requires antidepressant treatment.[65] In addition, SSRIs and SNRIs are important for treating tamoxifen-related hot flashes, since estrogen-based therapies are no option in breast cancer patients.[66]

CYP2D6 activity can be affected by these medications to varying degrees; some medications have been associated with potent inhibition, while others only slightly affect CYP2D6 activity.[63, 64] Stearns and colleagues demonstrated that endoxifen concentrations were more than 50% lower in women using tamoxifen after initiation of concomitant use of the potent CYP2D6 inhibitor paroxetine. The decrease in endoxifen concentrations was most evident in women with functional *CYP2D6* alleles.[12] Subsequent studies also revealed that endoxifen concentrations were up to 72% lower in tamoxifen-treated women who received potent CYP2D6-inhibiting antidepressants (paroxetine, fluoxetine) concomitantly than in women who did not receive any CYP2D6 inhibitor. The concurrent use of potent CYP2D6-inhibiting medications in women with *CYP2D6* extensive metabolizer genotype resulted in endoxifen concentrations as low as those observed in individuals without functional *CYP2D6* alleles.[19, 21, 23]

In women who were concomitantly treated with tamoxifen and a weak CYP2D6-inhibitor, including sertraline and citalopram, endoxifen concentrations were only slightly lower. The use of venlafaxine has not been associated with reductions in endoxifen levels. However, the effects of these weak CYP2D6-inhibiting antidepressants on endoxifen systemic concentrations have only been studied in prospective follow-up studies with a relatively small number of individuals using a specific SSRI or SNRI.[19, 21] Escitalopram has recently been studied in a small pharmacokinetic study in which women using tamoxifen along with a potent CYP2D6-inhibiting antidepressant were switched to the weak CYP2D6-inhibiting antidepressant. It appeared that endoxifen exposure during concomitant use of escitalopram was nearly 3-fold higher than during the period in which a potent CYP2D6 inhibitor was used.[67] These results suggest the safe use of escitalopram and possibly other weak CYP2D6

inhibitors along with tamoxifen.

The efficacy of tamoxifen may be negatively affected by the concomitant use of CYP2D6 inhibitors, especially potent inhibitors. Studies examining the effects of concomitant use of CYP2D6 inhibitors, mainly SSRIs, on breast cancer outcomes in tamoxifen-treated women have reported mixed and inconclusive results (Table 2).[40, 68-73] Nevertheless, these studies also varied widely regarding the source of information of CYP2D6 inhibitor use, the studied CYP2D6-inhibiting drugs (differences in potency of CYP2D6 inhibition), and defining overlapping periods of the use of tamoxifen and the CYP2D6 inhibitor. In addition, the studies lacked *CYP2D6* genotype data. These variations may have contributed to the discrepancy between study results.

Despite these conflicting results, potent CYP2D6-inhibiting antidepressants have consistently shown to reduce concentrations of endoxifen, and are therefore believed to decrease the likelihood of optimal tamoxifen therapy. Since there are numerous alternative drugs available, including antidepressants with weak CYP2D6 inhibitor potency or medications such as gabapentin and clonidine for alleviation of hot flashes, potent CYP2D6-inhibiting antidepressants should not be prescribed to tamoxifen-treated individuals.[62] As reported previously, SSRIs can be safely switched, however, to prevent problems concerning switching, this should be done under close monitoring by a psychiatrist. The concomitant use of moderate and weak CYP2D6 inhibitors is less clear. However, in general, the benefit of the use of the CYP2D6 inhibitor should outweigh potential negative effects on the efficacy of tamoxifen.

Although the use of potent CYP2D6-inhibiting antidepressants in tamoxifen users is discouraged, they appeared to be still co-administered in tamoxifen-treated women. An evaluation of changes in co-prescription of diverse CYP2D6-inhibiting antidepressants, classified according to their CYP2D6 inhibitor potency, among tamoxifen-treated women living in the U.S. revealed that co-prescription of potent CYP2D6 inhibitors dropped from 34% during the period 2004-2006 to 15% in 2010 (~44%). Compared to women using aromatase inhibitors, a more pronounced decrease in co-prescription of potent CYP2D6-inhibiting antidepressants was observed among women using tamoxifen.[74] Results of a Dutch study showed that the concurrent use of paroxetine among women treated with tamoxifen dropped by ~30% in the period between 2005 and 2010, however, this was similar to the decrease in paroxetine prescriptions observed in the reference population of all women included in the Dutch community pharmacy database.[62] Both studies showed that the concomitant use of weak CYP2D6-inhibiting antidepressants increased among tamoxifen users during the 6-year period.[62, 74] Similar trends were observed in a Belgian population,

with a decrease in the use of potent CYP2D6 inhibitors and an increase in weak CYP2D6 inhibitor use among women receiving tamoxifen concomitantly in the period 2006-2009.[75] These studies evaluated the co-prescription through 2010. It is expected that the downward trend in potent CYP2D6 inhibitor use persists, as observed in the Belgian study.[75] With the current alternatives available, one should strive to the complete avoidance of potent CYP2D6 inhibitor use among tamoxifen users.

Table 2. The effects of non-genetic factors on tamoxifen pharmacokinetics and clinical outcome

Factor	Comparison(s)	Effect on tamoxifen pharmacokinetics	Effects on clinical outcome[a]	Reference
CYP2D6-inhibiting medication	CYP2D6 inhibitor vs no CYP2D6 inhibitor	CYP2D6 inhibitor vs no CYP2D6 inhibitor: 32-72% lower endoxifen concentrations		12,19,21,23
	Potent CYP2D6 inhibitor vs weak CYP2D6 inhibitor	Potent CYP2D6 inhibitor vs weak CYP2D6 inhibitor: 68% lower endoxifen concentrations		67
	CYP2D6 inhibitor vs no CYP2D6 inhibitor		Use of CYP2D6 inhibitor: worse outcome	40, 68
	CYP2D6 inhibitor vs no CYP2D6 inhibitor		Use of (potent) CYP2D6 inhibitor: worse OS	40, 69
	CYP2D6 inhibitor vs no CYP2D6 inhibitor		No association with outcome	70-73
CYP-inducing medication	CYP-inducing medication vs no CYP-inducing medication	Rifampicin vs no rifampicin: 28-85% lower endoxifen exposure		78
	CYP-inducing medication vs no CYP-inducing medication	Phenytoin vs no phenytoin: 83% lower endoxifen concentrations		79
Age		Higher age: higher endoxifen concentrations and higher inter-patient variability in endoxifen concentration		85, 86
BMI		Higher BMI: lower endoxifen concentrations		25, 86
Season	Winter vs other seasons	Winter months: 20% lower endoxifen concentrations		23
Dosing time	Morning vs evening dosing	Morning dosing: 15% higher endoxifen exposure (AUC_{0-24}), 23% higher endoxifen C_{max}		87

[a] Clinical outcome: disease-free survival, relapse-free survival, progression-free survival, recurrence-free survival, time-to-progression, time-to-relapse.

CYTOCHROME P450 3A

In addition to CYP2D6, CYP3A4 and CYP3A5 are major enzymes involved in the primary and secondary steps of the metabolism of tamoxifen. Thus, polymorphisms in *CYP3A4* and *CYP3A5* as well as CYP3A interacting co-medication may also influence tamoxifen pharmacokinetics. An initial study by Jin and colleagues showed that individuals without functional *CYP3A5* alleles (*CYP3A5*3/*3* genotype) tend to have lower endoxifen concentrations than individuals carrying a functional *CYP3A5* allele (*CYP3A5*1/*1* or *CYP3A5*1/*3* genotype),[21] however, this was not confirmed by others.[11, 23, 24, 27, 76] Studies examining the effect of *CYP3A5*3* genotype on breast cancer outcomes have also reported conflicting results (Table 3).[28,32,43]

Although several *CYP3A4* polymorphisms (e.g., *CYP3A4*1B*) have been recognized, there is little evidence that these polymorphisms have clinical consequences. However, recently a new *CYP3A4* variant allele, *CYP3A4*22*, has been identified which is associated with lower CYP3A4 mRNA expression in the liver and reduced CYP3A4 enzyme activity.[77] Teft *et al*. confirmed the lower CYP3A4 enzyme activity *in vivo* using the endogenous biomarker 4ß-hydroxycholesterol-to-cholesterol. In the same study, concentrations of tamoxifen and its metabolites, including endoxifen, appeared to be significantly higher in patients carrying the *CYP3A4*22* allele.[23] This result is in agreement with previous findings of lower systemic exposure to tamoxifen and its main metabolites following induction of drug-metabolizing enzymes, especially CYP3A4, by rifampicin. The increased CYP3A4 activity, confirmed by higher 4ß-hydroxycholesterol-to-cholesterol ratios, resulted in 28-85% lower endoxifen exposure. This has led to the recommendation to avoid rifampicin, and potentially other potent CYP inducers, in patients treated with tamoxifen.[78]

The anti-epileptic drug phenytoin, which is also a potent inducer of drug-metabolizing enzymes including CYP3A4, appeared to reduce plasma concentrations of tamoxifen and endoxifen as well and should therefore also not be used concurrently with tamoxifen.[79] Although induction of phase II enzymes (e.g., UGTs) and efflux transporters (e.g., ABCB1) by rifampicin and phenytoin may have contributed to reduced levels of tamoxifen and its metabolites, this cannot explain the higher levels of these compounds in women carrying *CYP3A4*22* alleles. In addition, ABCB1 appeared to have no influence on levels of tamoxifen or its metabolites.[23, 80] Nevertheless, the metabolism of tamoxifen is more complex and, besides metabolic conversion into the three main metabolites, involves biotransformation into several other primary and secondary metabolites. Because CYP3A4 is involved in most of these metabolic pathways, induction or inhibition of this enzyme may stimulate or

negatively affect the formation of these metabolites. This may contribute to the availability of tamoxifen and its three major metabolites, including endoxifen.[11, 78]

Accordingly, the results of these studies show that CYP3A activity is also highly important in the metabolism of tamoxifen and should be taken into account. More importantly, one should be careful with the co-prescription of medications that can either inhibit or induce CYP3A activity, because these may influence the efficacy of tamoxifen therapy. Several drugs have been associated with CYP3A induction or inhibition, including some antiretrovirals, antifungals and cardiac drugs, however, whether these drugs indeed affect endoxifen exposure needs to be evaluated.

Table 3. The effects of genetic polymorphisms in drug-metabolizing enzymes on tamoxifen pharmacokinetics and clinical outcome

Gene	Allele(s)	Comparison(s)	Effects on tamoxifen pharmacokinetics	Effects on clinical outcome[a]	Reference(s)
CYP3A4	*1,*22 (reduced expression)	*1/*22 + *22/*22 vs *1/*1	*1/*22 + *22/*22: higher endoxifen concentrations		23
CYP3A5	*1,*3 (non-functional)	*1/*1 + *1/*3 vs *3/*3	*1/*1 + *1/*3: trend towards higher endoxifen concentrations		21
	*1,*3	*1/*3 vs *3/*3 *1/*1 vs *1/*3 , *3/*3 *1/*1 ,*1/*3 vs *3/*3	No differences in endoxifen concentrations		11, 23, 24, 27, 76
	*1,*3	*1/*1 +*1/*3 vs *3/*3		*3/*3: better outcome	43
	*1,*3	*1/*1 vs *1/*3 vs *3/*3		No association with outcome or OS	28, 32
CYP2C9	*1,*2, *3	*1/*1 vs carrier *2,*3	Carriers of *2,*3 alleles: lower endoxifen concentrations		11
	*1,*2, *3	*1/*1 vs carrier *2,*3	No differences in endoxifen concentrations		21, 23, 27
	*1,*2, *3	*1/*1 vs carrier *2,*3		No association with outcome or OS	32, 57

CYP2C19	*1,*2,*3,*17	*1/*1 vs *1/*2 vs *Vt/*17 vs *2/*2 vs *17/*17	No differences in endoxifen concentrations		11, 27, 76
		*1/*1 vs *1/*2, *2/*2,*1/*17, *1/*3, *3/*3			
		*1/*2 vs *2/*2			
	*1,*2,*3,*17	*17 carriers vs Wt (*1,*2,*3)		*17 carriers: better outcome	32
	*1,*2,*17	*1/*1 vs *2 carriers, *1/*1 vs *17 carriers, *1/*1 vs *2/*17, *2 carriers, *17 carriers		*2 carriers: better outcome MBC	81
	*1,*2,*3	*1/*1 vs *1/*2, *1/*3		No association with outcome	45, 54, 56, 57, 82
		*1/*1 vs *2/*2, Wt vs *17 carriers			
		*1/*1, *1/*2, *1,*3 vs *2/*2, *2/*3, *3/*3			
		Wt/Wt + Wt/*2 vs *2/*2			
UGT2B7	*1,*2	*1 vs *2	No differences in endoxifen concentrations		11
	*1,*2	*1/*1 vs *1/*2, *2/*2		No association with outcome	53, 83
UGT2B15	*1,*2	*1 vs *2	No differences in endoxifen concentrations		11

	*1,*2	*1/*1 vs *1/*2 + *2/*2		*1/*2 + *2/*2: better outcome	54
	*1,*2	*1/*1 vs *1/*2, *2/*2		No association with outcome	43, 83
		*1/*1 vs *1/*2 + *2/*2		*Combined with SULT1A1*2: 2 variant alleles: worse outcome and OS	41
UGT1A8	*1,*3	*1/*1 vs*1/*3 + *3/*3		No association with outcome	83
SULT1A1	*1,*2, copy number	*1/*1 vs *1/*2 vs *2/*2 Copy number 1-5	No differences in endoxifen concentrations		21, 22, 24
	*1,*2	*1/*1 + *1/*2 vs*2/*2,		SULT1A1*2/*2: worse OS	84
		*1/*1 vs *1/*2 +*2/*2		SULT1A1*1/*1: better outcome (combined with CYP2D6*4)	42
				*Combined with UGT2B15*2: 2 variant alleles: worse outcome and OS	41
	*1,*2, copy number	*1/*1 vs *1/*2 +*2/*2 Copy number 1-4+		No association with outcome	43, 82
SULT1A2	*1,*2, *3	*1/*1 vs *1/*2 vs *1/*3 vs *2/*2 vs *2/*3 vs *3/*3	SULT1A2*2,*3: higher endoxifen concentrations		24

[a] Clinical outcome: disease-free survival, relapse-free survival, progression-free survival, recurrence-free survival, time-to-progression, time-to-relapse.

CYTOCHROME P450 2C

Other important drug-metabolizing enzymes are members of the CYP2C family; CYP2C9 and CYP2C19.[11] Genetic variants of *CYP2C19* include *CYP2C19*2* and *CYP2C19*3*, which have been associated with decreased enzyme activity, and *CYP2C19*17*, a variant associated with increased activity due to enhanced gene transcription. Increased CYP2C19 enzyme activity (*CYP2C19*17* genotype) has been associated with increased formation of 4-hydroxytamoxifen from tamoxifen. However, concentrations of other metabolites or metabolic ratios did not differ among individuals with different *CYP2C19* genotypes.[76] Two other studies did not show an association between *CYP2C19* polymorphisms and concentrations of tamoxifen and its metabolites.[11, 27] Few studies have examined the association between *CYP2C19* genotype and breast cancer outcome, however, results were again conflicting (Table 3).[32,45,54,56,57,81,82]

Several studies did not find an association between *CYP2C9* polymorphisms and tamoxifen pharmacokinetics.[21, 23, 27] In contrast, Murdter and colleagues found that CYP2C9 appeared to be important in the formation of endoxifen via the metabolic conversion of tamoxifen into 4-hydroxytamoxifen, forming the precursor for endoxifen. Systemic concentrations of both active metabolites were lower and the tamoxifen-to-4-hydroxytamoxifen ratio was higher in individuals with *CYP2C9* variant alleles.[11] *CYP2C9* genotype had no impact on clinical outcome in studies investigating this association.[32, 57]

Although polymorphisms in *CYP2C9* and *CYP2C19* have been shown to modulate systemic levels of tamoxifen metabolites, no (relevant) drug-drug interactions have been described between tamoxifen and inhibitors of CYP2C9 or CYP2C19 (e.g., omeprazole, fluconazole). Theoretically these drugs might also affect tamoxifen pharmacokinetics, although probably not to a clinically relevant extent.

PHASE II ENZYMES

Major routes of elimination and inactivation of tamoxifen and its metabolites are via glucuronidation and sulfation, which is catalyzed by UGTs and SULTs.[11] Three UGTs, involved in the glucuronidation of 4-hydroxytamoxifen and endoxifen, are encoded by polymorphic genes. The variant allele *UGT2B7*2* has been associated with reduced activity, while an increased glucuronidation activity has been found for *UGT2B15*2*. The variant allele *UGT1A8*3* lacks activity.[83] However, no association has been observed between genetic variants of *UGT2B7* and *UGT2B15* and systemic levels of 4-hydroxytamoxifen or endoxifen in tamoxifen-treated individuals.[11] Few studies have examined the association between

polymorphic UGTs (e.g., *UGT2B7*2*, *UGT2B15*2*, *UGT1A8*3*) and clinical outcome of tamoxifen therapy, but most of them did not find a significant association.[43, 53, 83] The most common variant allele *SULT1A1*2* has been associated with decreased enzyme activity, but neither Jin and colleagues[21] nor Gjerde and colleagues[22] showed an association between *SULT1A1* genotype and concentrations of tamoxifen or its metabolites. Similar results were found in a subsequent study, however, in this study carriers of allelic variants of *SULT1A2* appeared to have higher levels of the active tamoxifen metabolites.[24] Poorer overall survival was found in tamoxifen-treated women carrying two variant *SULT1A1*2* alleles as compared with carriers of at least one high-activity *SULT1A1*1* allele.[84] Nevertheless, this was not confirmed in subsequent studies (Table 3).[24,41-43,82,84]

OTHER FACTORS INFLUENCING TAMOXIFEN PHARMACOKINETICS

Age

Tamoxifen metabolism could also be influenced by demographic factors and environmental factors other than co-medication. It has been shown that age is positively associated with systemic levels of tamoxifen, *N*-desmethyltamoxifen and endoxifen.[85, 86] Higher endoxifen concentrations, but also higher variability in endoxifen concentrations among individuals were observed in the oldest patients. This might be explained by decreased elimination of the compounds or due to more comorbidities and/or the concomitant use of medications in this age group.[85]

Body mass index (BMI)

BMI has been reported to be negatively associated with systemic levels of endoxifen.[25, 86] For instance, Wu and colleagues found a trend towards lower serum concentrations of tamoxifen and its metabolites in women with higher BMI values.[86] An increased volume of distribution and possibly enhanced phase II metabolism may underlie the lower systemic levels in individuals with a higher BMI. In daily practice, patients with a high BMI may therefore receive tamoxifen as adjuvant therapy at a dose of 40 mg.

Seasonal variation and circadian rhythm

Other environmental factors which have been found to affect tamoxifen pharmacokinetics are season and vitamin D levels. In a study performed by Teft and colleagues, endoxifen

concentrations appeared to be 20% lower during the winter compared with the mean endoxifen levels during the whole year. The lower endoxifen concentrations were also associated with lower levels of vitamin D. The exact mechanism underlying the differences in systemic exposure to endoxifen as a consequence of seasonal variation and vitamin D levels needs further evaluation.[23]

Tamoxifen pharmacokinetics may not only differ between individuals, but also within individuals. In women treated with tamoxifen, differences in exposure to tamoxifen and its metabolites have been found between morning and evening administration. While morning dosing resulted in higher endoxifen exposure than dosing in the evening, endoxifen plasma concentrations were more stable following dosing in the evening. The latter may be more beneficial regarding concentration-related side effects.[87]

EVIDENCE FOR ENDOXIFEN AS THE PRINCIPAL ACTIVE METABOLITE OF TAMOXIFEN

Endoxifen (or metabolite BX) has been identified by Lien and colleagues, approximately twenty-five years ago.[16] However, first 4-hydroxytamoxifen was considered to be the active metabolite of tamoxifen. Stearns and colleagues characterized endoxifen and found that it has identical anti-estrogen activities as 4-hydroxytamoxifen, and reaches higher plasma concentrations than 4-hydroxytamoxifen, suggesting an important role for this metabolite.[12] In addition, it has been demonstrated *in vitro* that endoxifen has different mechanisms of action in breast cancer cells than 4-hydroxytamoxifen. In contrast to 4-hydroxytamoxifen, endoxifen targets the ERα for degradation by the proteasome, resulting in reduced ERα protein levels.[88] Hawse and colleagues showed that gene expression profiles of breast cancer cells (MCF7 cells) were substantially different between endoxifen and 4-hydroxytamoxifen treated cells.[89] In addition, all of the effects of endoxifen appeared to be concentration dependent.

Madlensky and colleagues studied the relationship between *CYP2D6* polymorphisms, endoxifen concentrations and breast cancer outcomes in tamoxifen-treated women. The large cohort (n=1,370; participating in the Women's Healthy Eating and Living (WHEL) study) consisted of women with estrogen receptor-positive breast cancer who were treated with tamoxifen in the adjuvant setting. An association between endoxifen concentrations and breast cancer outcomes was found, such that women with endoxifen concentrations below a minimal threshold level appear to have an increased risk of disease recurrence. Neither

tamoxifen nor other main metabolites were associated with risk of recurrence, stressing the importance of endoxifen.[25] Recently, Saladores and colleagues investigated the association between endoxifen systemic concentrations and breast cancer outcome (measured as distant relapse-free survival) in over 300 premenopausal women with hormone receptor-positive breast cancer receiving adjuvant tamoxifen.[90] In line with Madlensky *et al.*, after classifying individuals into quartiles according to their endoxifen concentration, it was shown that women in the lowest quartile had a higher risk of worse clinical outcome (distant relapse) compared with women having endoxifen concentrations belonging to the upper quartile. Gong and colleagues did also support the hypothesis of a threshold concentration for endoxifen to achieve optimal tamoxifen efficacy. In this study, the relationship between endoxifen concentration and tumor growth inhibition was studied by performing PK-PD modeling on data obtained from a xenograft efficacy study in human breast cancer bearing mice.[91]

However, results of Love and colleagues suggest an optimal range for endoxifen concentrations, rather than a minimal threshold concentration, for efficacy against breast cancer. A small (n=48) nested case control (exploratory) study in tamoxifen-treated women revealed, unexpectedly, that not only low, but also high endoxifen levels (> 70 ng/mL) were associated with a higher recurrence risk.[92] This finding requires further investigation, but more importantly validation in future studies.

STRATEGIES TO INDIVIDUALIZE TAMOXIFEN THERAPY: PHENOTYPING AND ALGORITHMS

The contribution of factors other than *CYP2D6* polymorphisms to the inter-patient variability in endoxifen concentrations is evident as only 39% of this variability can be predicted by *CYP2D6* genotype.[11] Isolated *CYP2D6* genotyping therefore is an oversimplified approach for individualization of tamoxifen. Other strategies have been proposed for treatment individualization by predicting endoxifen exposure. For instance, an approach using dextromethorphan as a probe drug for both CYP2D6 and CYP3A activity has been suggested as an alternative strategy. Dextromethorphan appeared to be a good phenotyping probe to predict endoxifen exposure.[93] By developing a population pharmacokinetic model for tamoxifen, it was found that 54% of the variability in endoxifen exposure could be explained by dextromethorphan derived CYP2D6 and CYP3A4 phenotypes.[94] Additionally, the impact of CYP2D6 and CYP3A was separately investigated and it appeared that CYP3A activity was also

highly important in the metabolic conversion of tamoxifen into endoxifen. Similarly, CYP2D6 phenotype, as assessed by a ^{13}C-dextromethorphan breath test, has been shown to explain almost half of the inter-individual variability in endoxifen exposure.[95] However, both phenotyping approaches require dextromethorphan administration and blood or breath sampling. Teft and colleagues proposed the use of an algorithm to predict endoxifen levels, incorporating demographic variables, *CYP2D6* and *CYP3A4*22* genotype, the use of SSRIs and seasonal variability. It appeared that *CYP2D6* and *CYP3A4*22* genetic variation and non-genetic factors explained respectively 33% and 13% of the variation in endoxifen levels, which is similar to the phenotyping approaches.[23] Therefore, phenotyping and the use of the mentioned algorithm are better strategies than *CYP2D6* genotyping to predict tamoxifen efficacy, because endoxifen exposure is predicted more accurately.

THERAPEUTIC DRUG MONITORING FOR TAMOXIFEN

However, although better, phenotyping approaches cannot fully explain the variability in endoxifen exposure. From the data as presented in this review, it is clear that tamoxifen pharmacokinetics are influenced by many genetic and environmental factors, which individually explain only a (small) part of the variation in endoxifen exposure. Considering that endoxifen is the most important metabolite for clinical efficacy, monitoring of systemic concentrations of endoxifen is likely to be the most promising approach to personalize and optimize tamoxifen therapy. An important advantage of this approach is that by measuring the endoxifen concentrations all factors influencing the generation and clearance of endoxifen are taken into account, and can be corrected for. Regarding the high inter-patient variability in pharmacokinetics and the inability of monitoring efficacy and/or toxicity clinically, tamoxifen seems to be suitable for TDM.[96, 97] It has been shown that concentrations of tamoxifen and its metabolites in serum are correlated with those in breast tumor tissue, which suggests that endoxifen concentrations measured in serum give information about endoxifen levels at the target site of action.[98] The most important criterion to fulfill for TDM is the relationship between endoxifen systemic concentrations and tamoxifen efficacy. It may not only be important to attain a minimal threshold concentration of endoxifen, but concentrations should possibly also not be too high (Figure 2). Side effects of tamoxifen, including hot flashes, vaginal dryness, weight gain, and depression have been reported in ~73% of the patients,[99] with some of them being treatment-limiting or resulting in decreased adherence to tamoxifen. As endoxifen concentrations have been associated with the

occurrence of side effects,[99] although not consistently,[100, 101] the 'therapeutic window' for endoxifen may also be limited by toxicity. In addition, with long-term treatment (higher cumulative dose), tamoxifen may increase the risk of endometrial cancer.[102] However, more studies correlating (long-term) exposure to tamoxifen and its metabolites and adverse effects are needed.

Interpretation and clinical decision making based on measured endoxifen concentrations requires a validated and standardized analytical assay for the measurement of endoxifen, tamoxifen and other metabolites. Up till now, studies have reported highly variable endoxifen concentrations, which might be explained by the use of different analytical methods. As suggested, the inability of the analytical separation and individual quantification of endoxifen and the inactive metabolite 4'-hydroxy-*N*-desmethyltamoxifen may have resulted in higher endoxifen concentrations in some studies.[11] Also, (Z)-isomers and (E)-isomers of 4-hydroxytamoxifen and endoxifen should be individually measured. Accordingly, a highly sensitive and selective analytical method for the quantification of tamoxifen and its metabolites, especially (Z)-endoxifen, is needed for TDM.

In tamoxifen-treated women, individuals with low endoxifen concentrations may receive a higher tamoxifen dose. A few studies have demonstrated that a higher tamoxifen dose (30 or 40 mg daily) results in higher endoxifen concentrations, even in women with non-functional *CYP2D6* alleles.[97, 103, 104] In women with intermediate CYP2D6 metabolism, the higher tamoxifen dose resulted in endoxifen concentrations comparable to those observed in CYP2D6 extensive metabolizers. However, it appears that escalation of the tamoxifen dose does not lead to dose-proportional increases in endoxifen levels in individual women,[103, 104] which also supports the use of TDM.

Administration of endoxifen might be another option to achieve higher endoxifen systemic concentrations in women with initially low endoxifen exposure.[105] The use of endoxifen in breast cancer patients is now being tested in clinical trials. However, because tamoxifen and other metabolites may also contribute to the overall clinical effect of tamoxifen, the sole administration of endoxifen may not be the optimal strategy. Therefore, a dosing strategy of tamoxifen at a dose of 20 mg in combination with endoxifen (at a dose dependent on CYP2D6 phenotype) has been suggested to be a better approach.[106] Nevertheless, these approaches need further investigation.

ADHERENCE

We can introduce multiple strategies to individualize tamoxifen therapy, however, if patients do not take their medication, treatment will never be efficacious. In breast cancer patients, non-adherence to tamoxifen and discontinuation of therapy are major concerns and may contribute to poor treatment efficacy. Maximal adherence to tamoxifen therapy is far from realized, with reported adherence rates varying from 41 to 88%.[107, 108] Discontinuation of tamoxifen therapy is mostly seen during the first year, and only 50% of the breast cancer patients continue endocrine therapy for the recommended period of 5-years.[109, 110] Importantly, studies have reported that non-adherence and discontinuation are associated with an increased risk of recurrence and mortality.[70, 108] Many factors have been shown to be associated with non-adherence and discontinuation, including age of the patient (younger and older age), follow-up care with a general practitioner instead of oncologist, treatment-related side effects and higher CYP2D6 enzyme activity.[107, 110] TDM can be used to identify patients being non-adherent. However, more importantly, to improve adherence physicians should be attentive to side effects, and give proper information about the benefit of the therapy. Co-morbidity such as mental disorders should be adequately treated.

SUMMARY AND FUTURE PERSPECTIVES

Tamoxifen remains fundamental in the treatment of hormone receptor-positive breast cancer. Despite tamoxifen's proven efficacy, women who have hormone receptor-positive breast cancer and receive tamoxifen at the same dose, may have different clinical response. In addition, while some tamoxifen-treated women do not experience any side effects, other women experience severe side effects resulting in discontinuation of the therapy. Variability in exposure to endoxifen may be, at least partly, responsible for the inter-individual variability in response to tamoxifen.

Over the past decade, many studies have examined the association between *CYP2D6* genotypes and breast cancer outcomes. However, results of these studies have been inconsistent and even large trials did not provide enough evidence to end the discussion whether or not to use *CYP2D6* genotype as a predictor of tamoxifen efficacy. Meanwhile, the search for additional factors that may affect tamoxifen pharmacokinetics continued and, as discussed in this review, several other drug-metabolizing enzymes have been shown to influence tamoxifen therapy as well. Accordingly, genotype-predicted CYP2D6 activity alone seems to be inadequate to guide dose and drug selection. Phenotyping strategies can predict

endoxifen exposure more accurately than *CYP2D6* genotype, and may be useful in determining the initial tamoxifen dose prior to initiation of the therapy. This might be relevant as it takes about 4 months for tamoxifen metabolites to reach steady state levels.

The best way forward for individualization of tamoxifen therapy seems to be TDM. There is increasing evidence supporting the important role of endoxifen for tamoxifen efficacy. Further research is needed to establish the relationship between endoxifen systemic concentrations and efficacy and toxicity of tamoxifen and to define a 'therapeutic window' for endoxifen, if there is any. Preferably, the added value of TDM for tamoxifen should be studied in direct comparison with standard of care tamoxifen. In addition, TDM requires a validated and standardized analytical assay for the accurate measurement of tamoxifen and its metabolites. As it has been shown that dosing time influenced the pharmacokinetics of tamoxifen, the time of tamoxifen intake and the time of blood sampling are important and should be taken into account if TDM will be applied. Potential changes in exposure to endoxifen during long-term treatment, changes in a patient's co-medication or lifestyle, and problems concerning adherence indicate that systemic concentrations of endoxifen should be measured regularly during tamoxifen treatment. However, recently, it has been demonstrated that dried blood spot sampling can be used as a simple alternative to venous blood sampling to determine endoxifen concentrations, making it easier to perform TDM.[111]

Finally, medications that are used concomitantly with tamoxifen, such as CYP2D6-inhibiting antidepressants and inhibitors or inducers of CYP3A4, may affect tamoxifen pharmacokinetics. The concurrent use of potent CYP2D6-inhibiting antidepressants and potent inducers of CYP3A4 in women receiving tamoxifen is discouraged. One should be careful with the concomitant use of other (potential) interacting medications in tamoxifen users.

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Chapter 9

Summary

SUMMARY

Breast cancer is a major health concern. The disease is associated with high rates of morbidity and mortality worldwide. Treatment of breast cancer is continuously improving, as new therapies are developed, but also by individualization of treatment. The ultimate goal of individualized breast cancer treatment is improved effectiveness, ideally with less drug-related toxicity. Estrogen and/or progesterone receptor expression is an important tumor characteristic that is used to guide breast cancer therapy. Estrogen receptor (ER)-positive breast cancers require estrogen for tumor growth and can be treated with endocrine therapies, including tamoxifen. The selective estrogen receptor modulator tamoxifen has been proven efficacious in the treatment of ER-positive breast cancer; as adjuvant treatment and for metastatic disease. However, up to 50% of the patients do not benefit from tamoxifen therapy.

Not only tumor characteristics are important, including variation in ER expression, but also host factors and environmental factors may influence the response to tamoxifen. Genetic variation in drug-metabolizing enzymes responsible for tamoxifen metabolism is an important host factor. Polymorphisms in cytochrome P450 (CYP) genes have been associated with impaired formation of the principal active metabolite endoxifen from tamoxifen, which in turn may relate to tamoxifen efficacy. In this context, *CYP2D6* genotype has been studied extensively as predictor of treatment outcome. Environmental factors, such as co-medication that affect CYP enzyme activity, may also influence tamoxifen response by interfering with endoxifen formation.

Results from a large study, associating tamoxifen metabolite concentrations and breast cancer outcomes, suggest that tamoxifen efficacy is dependent on reaching a minimal threshold concentration of endoxifen. The studies in this thesis contribute to the knowledge of environmental factors that influence endoxifen exposure and may contribute to the individualization and optimization of tamoxifen therapy.

In **Chapter 2** we have discussed strategies to individualize tamoxifen therapy for patients with breast cancer. Obtaining *CYP2D6* genotype status prior to tamoxifen therapy has been proposed as a promising strategy for tamoxifen individualization. However, as it is unlikely that the response to tamoxifen is dependent on a single genetic variant, other strategies have been suggested. A phenotyping approach using dextromethorphan as a probe drug incorporates *CYP2D6* genotype as well as the use of CYP2D6-inhibiting co-medication and CYP3A phenotype, which makes it feasible to better predict endoxifen exposure.

Monitoring systemic concentrations of endoxifen seems to be the most feasible approach to individualize tamoxifen therapy, because this approach takes into account all factors influencing the formation and elimination of endoxifen. However, before this strategy can be applied for tamoxifen, several 'barriers' have to be overcome, of which the confirmation of an association between endoxifen concentrations and tamoxifen efficacy is the most important one.

For the measurement of tamoxifen and its main phase I metabolites, *N*-desmethyltamoxifen, 4-hydroxytamoxifen and (Z)-4-hydroxy-*N*-desmethyltamoxifen (endoxifen) in plasma, a highly sensitive and selective ultra-performance liquid chromatography tandem mass spectrometry assay was developed which is described in **Chapter 3**. The assay has been fully validated according to FDA recommendations. Especially for endoxifen, this assay was highly sensitive with a lower limit of quantification of 0.187 ng/mL. In addition, we were able to analytically separate 4-hydroxytamoxifen and endoxifen and the inactive 4'-hydroxylated metabolites. We investigated the light sensitivity of tamoxifen and its main metabolites, because this was not studied in detail before. No degradation was observed following six hour exposure to daylight in transparent tubes for all 4 compounds. This advocates normal sample handling during collection and preparation, without light protection. Also, a cross-validation was performed with serum samples obtained from a previous study.

Tamoxifen is a pro-drug and requires metabolic activation into endoxifen, which is catalyzed by several CYP enzymes. CYP2D6 has a major role in the conversion of tamoxifen into endoxifen, but other CYP enzymes such as CYP3A, CYP2C9 and CYP2C19 are also important. In **Chapter 4** we investigated whether a potent inducer of CYP enzymes could be used for the induction of tamoxifen metabolism, resulting in increased endoxifen systemic exposure. For this purpose, rifampicin was used as an inducer of several drug-metabolizing enzymes, but particularly CYP3A4. Dextromethorphan was used as a probe for CYP2D6/CYP3A4 activity and the 4ß-hydroxycholesterol-to-cholesterol ratio as a marker for CYP3A4/5. Both markers confirmed induction of CYP-mediated metabolism following rifampicin co-administration at a dose of 600 mg per day for 15 days. However, induction by rifampicin resulted in strongly reduced plasma exposure to tamoxifen and all three metabolites, including endoxifen. For endoxifen, 28-85% lower plasma exposure was observed after rifampicin administration in four individuals. The results of this study show the relevance of metabolic routes other than CYP2D6 in endoxifen formation. It is recommended that patients treated with tamoxifen should not receive rifampicin concomitantly.

Antidepressants are other, and probably the most important, examples of co-medication

that can interfere with the efficacy of tamoxifen by influencing the pharmacokinetics of the drug. Breast cancer patients frequently suffer from depression or have to deal with tamoxifen-related hot flashes. Selective serotonin reuptake inhibitors (SSRIs) and venlafaxine are commonly used antidepressants which can effectively be used for depression and hot flashes. The disadvantage of the use of these antidepressants in tamoxifen-treated patients is that they inhibit CYP2D6 enzyme activity, resulting in impaired endoxifen formation. Paroxetine and fluoxetine are potent CYP2D6 inhibitors that should not be used in combination with tamoxifen. In **Chapter 5** we retrospectively evaluated dispensing data for tamoxifen and seven antidepressants (six SSRIs and venlafaxine) associated with CYP2D6 inhibition. Dispensing data of these drugs were obtained from a community pharmacy database (PHARMO-Institute for Drug Outcome Research), which contains drug-dispensing histories of about 3 million individuals. We assessed changes in prescription of (potent) CYP2D6-inhibiting antidepressants in women with and without tamoxifen treatment during the period 2005-2010. It was found that approximately 14% of the women treated with tamoxifen received one of the seven antidepressants concomitantly. From 2005 to 2010, a trend towards a decrease in the concurrent use of tamoxifen and the potent CYP2D6 inhibitor paroxetine was observed. However, the antidepressant was still frequently used in combination with tamoxifen. For optimal benefit from tamoxifen therapy, one should strive to avoid potent CYP2D6 inhibitors, including paroxetine, as much as possible.

Because the use of potent CYP2D6-inhibiting SSRIs in combination with tamoxifen is discouraged, alternatives are needed. The use of antidepressants with weaker CYP2D6-inhibiting properties, including escitalopram, is recommended, because these drugs theoretically have less potential to interfere with the clinical efficacy of tamoxifen. In **Chapter 6** we investigated the effects of switching the potent CYP2D6 inhibitors paroxetine and fluoxetine to a weak CYP2D6-inhibiting antidepressant on the pharmacokinetics of tamoxifen in breast cancer patients. Switching to the weak CYP2D6 inhibitor escitalopram resulted in a ~3-fold higher endoxifen exposure. The plasma exposure to 4-hydroxytamoxifen was also higher during escitalopram co-administration. Switching was feasible and no antidepressant-related adverse effects or psychiatric relapse were noticed. The results of this study suggest that escitalopram can be safely used in combination with tamoxifen. In clinical practice, patients using paroxetine or fluoxetine concurrently with tamoxifen should be switched to escitalopram.

Besides co-medication, other environmental factors may influence tamoxifen pharmacokinetics. Pharmacokinetic processes are influenced by physiological functions, including gastrointestinal functions and enzyme activity, which in turn are subject to

circadian rhythms. Circadian rhythms may therefore contribute to variability in tamoxifen pharmacokinetics within an individual. The time of drug intake may be important, because it may influence endoxifen exposure. To assess circadian variation in tamoxifen pharmacokinetics, in **Chapter 7** we studied whether different dosing times resulted in differences in pharmacokinetics and endoxifen exposure in mice and breast cancer patients. Three different dosing times (morning, afternoon and evening) were evaluated in humans and six administration times were tested in mice. In mice, no significant differences in exposure to tamoxifen in plasma or tissues were observed. In humans, a significant higher absorption rate was observed following administration in the morning compared with evening dosing, resulting in higher plasma exposure to tamoxifen and its metabolites, although differences were relatively small in most individuals. A recommendation for the time of tamoxifen intake is difficult; morning dosing results in slightly higher endoxifen levels, but evening dosing may be more favorable regarding adverse effects. Given the results of this study, both dosing time and blood sampling time should be taken into account in clinical studies associating endoxifen concentrations and breast cancer outcomes.

In **Chapter 8** we have reviewed available literature on genetic and environmental factors influencing tamoxifen pharmacokinetics and clinical outcome. The influences of *CYP2D6* genotype and CYP2D6-inhibiting co-medication on tamoxifen metabolite concentrations and breast cancer outcomes have been extensively studied. Genetic polymorphisms in *CYP3A4, CYP3A5, CYP2C9, CYP2C19*, UDP-glucuronosyltransferases, and sulfotransferases have been studied as well. However, studies associating genetic factors or co-medication and clinical outcome have provided mixed results.

Evidence from preclinical and clinical studies supporting the theory that endoxifen is required for clinical efficacy and concentrations of this metabolite should be above a threshold concentration is growing. This advocates the application of therapeutic drug monitoring for individualization of tamoxifen therapy. For this, as mentioned earlier, confirmation of the endoxifen concentration – clinical efficacy relationship, and a validated and standardized analytical assay for the measurement of tamoxifen, endoxifen and other metabolites are highly important.

Appendix 1

Samenvatting

SAMENVATTING

Borstkanker is wereldwijd een groot gezondheidsprobleem. In 2012 werden er ongeveer 1,7 miljoen nieuwe gevallen van borstkanker ontdekt en zijn er meer dan een half miljoen personen overleden als gevolg van de ziekte. De behandeling van borstkanker wordt gelukkig steeds beter, waardoor de kans op langdurige overleving stijgt. Een verbeterde medicamenteuze antikanker behandeling levert hieraan een belangrijke bijdrage. Dit wordt niet alleen bewerkstelligd door de ontwikkeling van nieuwe therapieën, maar ook door het individualiseren van de antikanker behandeling. Door het individualiseren van de borstkankerbehandeling wordt er gestreefd naar optimale werkzaamheid met idealiter zo min mogelijk geneesmiddelgerelateerde bijwerkingen.

Expressie van oestrogeen- en/of progesteronreceptoren is een belangrijke tumorkarakteristiek die gebruikt wordt bij het bepalen van de borstkankerbehandeling. Oestrogeenreceptor-positieve borsttumoren zijn afhankelijk van oestrogeen voor hun groei en kunnen hierdoor behandeld worden met endocriene therapieën, waaronder tamoxifen. De selectieve oestrogeenreceptor modulator tamoxifen zorgt voor het blokkeren van de groeistimulerende effecten van oestrogeen in borstweefsel door competitief te binden aan oestrogeenreceptoren. Tamoxifen is effectief gebleken bij de behandeling van oestrogeenreceptor-positieve borstkanker; als adjuvante behandeling en bij gemetastaseerde ziekte. Als adjuvante behandeling zorgt tamoxifen ervoor dat het risico op terugkeer van de ziekte en op overlijden als gevolg van borstkanker wordt verminderd. Bij gemetastaseerde borstkanker zorgt tamoxifen voor het stoppen of vertragen van de groei van kankercellen en voor een langere overleving. Echter, ondanks de bewezen effectiviteit van tamoxifen, hebben 30-50% van de patiënten geen baat bij de tamoxifenbehandeling.

Niet alleen tumorkarakteristieken zijn belangrijk, waaronder variatie in oestrogeenreceptor expressie, maar ook patiëntgerelateerde factoren en omgevingsfactoren kunnen van invloed zijn op de effectiviteit van tamoxifen. Genetische variatie in activiteit van geneesmiddelmetaboliserende enzymen die verantwoordelijk zijn voor het metabolisme van tamoxifen is een belangrijke patiëntgerelateerde factor. Tamoxifen wordt gemetaboliseerd tot diverse actieve en inactieve metabolieten. Endoxifen wordt gezien als de belangrijkste actieve metaboliet en verantwoordelijk gehouden voor de effectiviteit van tamoxifen. Verschillende cytochroom P450 (CYP) enzymen zijn betrokken bij het metabolisme van tamoxifen in endoxifen, waaronder CYP2D6 en CYP3A. Polymorfismen in genen die coderen voor CYP- enzymen zijn geassocieerd met verminderde vorming van endoxifen, wat weer gerelateerd kan worden aan tamoxifeneffectiviteit. In deze context is

het CYP2D6-genotype vaak bestudeerd als voorspeller van de effectiviteit van tamoxifen. Ook geneesmiddelen die invloed hebben op de activiteit van CYP-enzymen en gelijktijdig gebruikt worden met tamoxifen kunnen de tamoxifeneffectiviteit beïnvloeden door te interfereren met de vorming van endoxifen.

Resultaten afkomstig van een grote studie, waarin de associatie tussen concentraties van tamoxifenmetabolieten en uitkomst is bestudeerd, suggereren dat de effectiviteit van tamoxifen afhankelijk is van het bereiken van een minimale therapeutische drempelwaarde van endoxifen. Het bestuderen van factoren die invloed hebben op de endoxifen blootstelling is hierdoor van belang. De studies in dit proefschrift leveren informatie over omgevingsfactoren die de endoxifen blootstelling kunnen beïnvloeden, wat kan bijdragen aan het individualiseren en optimaliseren van de tamoxifenbehandeling.

In hoofdstuk 2 worden strategieën besproken voor het individualiseren van de tamoxifenbehandeling voor borstkanker. Het bepalen van het CYP2D6-genotype voor start van de tamoxifenbehandeling is geopperd als een veelbelovende strategie voor het individualiseren van de tamoxifenbehandeling. Echter, aangezien het niet waarschijnlijk is dat de effectiviteit van tamoxifen afhankelijk is van één genetische variant, zijn andere strategieën voor het individualiseren gesuggereerd. Een voorbeeld hiervan is een fenotyperingsstrategie waarbij gebruik wordt gemaakt van dextromethorfan als een 'probe drug'. Op deze manier wordt er rekening gehouden met het CYP2D6-genotype evenals met gelijktijdig gebruik van CYP2D6-remmende medicatie en CYP3A fenotype, waardoor de endoxifen blootstelling beter voorspeld kan worden. Het monitoren van endoxifenconcentraties lijkt de meest veelbelovende strategie voor het individualiseren van de tamoxifenbehandeling, aangezien er op deze manier rekening wordt gehouden met alle factoren die van invloed zijn op de vorming en eliminatie van endoxifen. Echter, voordat deze strategie toegepast kan worden voor tamoxifen moet er aan een aantal randvoorwaarden worden voldaan. Een belangrijke voorwaarde is de bevestiging van de associatie tussen endoxifenconcentraties en tamoxifeneffectiviteit.

Een sensitieve en selectieve analysemethode ('ultra-performance liquid chromatography tandem mass spectrometry') voor het kwantificeren van tamoxifen en de voornaamste metabolieten, *N*-desmethyltamoxifen, 4-hydroxytamoxifen en (Z)-4-hydroxy-*N*-desmethyltamoxifen (endoxifen) in plasma is ontwikkeld en beschreven in hoofdstuk 3. De analysemethode is gevalideerd conform FDA richtlijnen. Zeer lage detectielimieten werden verkregen voor alle componenten, maar met name voor endoxifen (0,187 ng/ml). Ook bleek het mogelijk om 4-hydroxytamoxifen en endoxifen en de inactieve 4'-hydroxymetabolieten

analytisch te scheiden. De lichtgevoeligheid van tamoxifen en de metabolieten werd eveneens onderzocht, omdat dit nog niet eerder goed uitgezocht was. Voor alle vier de componenten werd er geen degradatie waargenomen na blootstelling aan daglicht gedurende 6 uur in transparante buisjes. Dit pleit voor een normale behandeling van de monsters tijdens afname en opwerken, zonder dat er lichtprotectie nodig is. Tevens werd er succesvol een cross-validatie uitgevoerd met serummonsters afkomstig vanuit een eerder uitgevoerde studie.

Tamoxifen is een prodrug en moet metabole activatie ondergaan om endoxifen te vormen wat gekatalyseerd wordt door verscheidene CYP-enzymen. CYP2D6 speelt een belangrijke rol bij de omzetting van tamoxifen naar endoxifen, maar ook andere CYP-enzymen zoals CYP3A, CYP2C9 en CYP2C19 zijn belangrijk. In hoofdstuk 4 is onderzocht of een potente CYP-inductor gebruikt kan worden voor inductie van het metabolisme van tamoxifen om uiteindelijk een hogere endoxifen blootstelling te kunnen verkrijgen. Voor dit doel werd rifampicine gebruikt, aangezien het een potente inductor is van verschillende geneesmiddelmetaboliserende enzymen, met name CYP3A4. Dextromethorfan werd in deze studie gebruikt als probe voor CYP2D6/CYP3A4 activiteit en de 4ß-hydroxycholesterol-cholesterol ratio werd gebruikt als marker voor CYP3A4/5 activiteit. Beide markers bevestigden de inductie van CYP-gemedieerd metabolisme na de inname van rifampicine in een dosering van 600 mg per dag voor een periode van 15 dagen. Echter, rifampicine-gemedieerde inductie resulteerde in een sterk verlaagde plasma blootstelling aan tamoxifen en alle metabolieten, waaronder endoxifen. Een 28-85% lagere endoxifen blootstelling werd gevonden na rifampicine inname in de 4 vrouwen die aan de studie deelnamen. De resultaten van deze studie laten zien dat er naast CYP2D6 ook andere metabole routes zijn die relevant zijn voor de vorming van endoxifen. Het wordt sterk aanbevolen om patiënten die behandeld worden met tamoxifen geen rifampicine gelijktijdig te laten gebruiken.

Antidepressiva zijn andere belangrijke geneesmiddelen die kunnen interfereren met de effectiviteit van tamoxifen door beïnvloeding van de farmacokinetiek. Regelmatig komt het voor dat patiënten met borstkanker te maken krijgen met een depressie of met tamoxifen-gerelateerde opvliegers. Selectieve serotonine-heropnameremmers (SSRI's) en venlafaxine zijn antidepressiva die regelmatig worden gebruikt en effectief zijn bij de behandeling van depressie en opvliegers. Het nadeel van het gebruik van deze antidepressiva bij patiënten die met tamoxifen worden behandeld is dat ze de activiteit van CYP2D6-enzymen kunnen remmen. Dit kan resulteren in verminderde vorming van endoxifen. Paroxetine en fluoxetine zijn sterke CYP2D6-remmers die niet in combinatie met tamoxifen gebruikt zouden moeten worden. Dat het gebruik van deze combinatie helaas nog wel regelmatig voorkomt, is

gebleken uit het onderzoek dat wordt beschreven in hoofdstuk 5. Retrospectief zijn aflevergegevens geëvalueerd van tamoxifen en zeven antidepressiva die geassocieerd zijn met CYP2D6-inhibitie (zes SSRI's en venlafaxine). Aflevergegevens van deze geneesmiddelen waren verkregen vanuit een openbare apotheek database (PHARMO-Institute for Drug Outcome Research). Deze database bevat de aflevergeschiedenis van geneesmiddelen van ongeveer 3 miljoen individuen. We hebben veranderingen in het voorschrijven van (potente) CYP2D6-remmende antidepressiva bestudeerd in vrouwen met en zonder tamoxifenbehandeling gedurende de periode 2005-2010. Er werd gevonden dat ongeveer 14% van de vrouwen die met tamoxifen behandeld werden ook een van de zeven antidepressiva gelijktijdig gebruikten. Een afname in het gelijktijdig gebruik van tamoxifen en de potente CYP2D6-remmer paroxetine werd waargenomen in de periode van 2005 tot 2010. Ondanks deze dalende trend bleek dat dit antidepressivum toch nog steeds regelmatig werd gebruikt in combinatie met tamoxifen. Om optimaal te profiteren van de tamoxifenbehandeling zou men moeten streven naar het zoveel mogelijk vermijden van potente CYP2D6-remmers, waaronder paroxetine.

Aangezien het gebruik van sterke CYP2D6-remmende SSRI's in combinatie met tamoxifen wordt ontraden, zijn er alternatieven nodig. Het gebruik van antidepressiva met minder CYP2D6-remmende eigenschappen wordt aanbevolen, waaronder escitalopram, aangezien deze antidepressiva theoretisch minder kans geven te interfereren met de effectiviteit van tamoxifen. In hoofdstuk 6 is het effect onderzocht van het switchen van de potente CYP2D6-remmers paroxetine en fluoxetine naar een zwak CYP2D6-remmend antidepressivum op de farmacokinetiek van tamoxifen in borstkanker patiënten. Het switchen naar de zwakke CYP2D6-remmer escitalopram resulteerde in een ~3-voudig hogere endoxifen blootstelling. De blootstelling aan 4-hydroxytamoxifen was ook hoger tijdens het gebruik van escitalopram. Het switchen bleek goed mogelijk te zijn en er werden geen antidepressiva-gerelateerde bijwerkingen of psychiatrische terugval gevonden. De resultaten van deze studie suggereren dat escitalopram veilig gebruikt kan worden in combinatie met tamoxifen. In de dagelijkse praktijk zouden patiënten die paroxetine of fluoxetine tegelijk gebruiken met tamoxifen geswitcht moeten worden naar escitalopram.

Naast co-medicatie kunnen andere omgevingsfactoren ook van invloed zijn op de farmacokintiek van tamoxifen. Farmacokinetische processen worden beïnvloed door fysiologische functies, zoals gastro-intestinale functies en enzymactiviteit, die onderhevig zijn aan circadiane ritmes. Hierdoor kunnen circadiane ritmes bijdragen aan variabiliteit in de farmacokinetiek van tamoxifen binnen een individu. Het tijdstip van inname is hierdoor van belang, aangezien de endoxifen blootstelling beïnvloed kan worden. Voor het bepalen

van circadiane variatie in de farmacokinetiek van tamoxifen hebben we in hoofdstuk 7 bestudeerd of verschillende innametijden resulteren in verschillen in farmacokinetiek en endoxifen blootstelling in muizen en borstkanker patiënten. Drie verschillende innametijden (ochtend, middag en avond) werden bestudeerd in mensen en zes toedieningstijden werden getest in muizen. In muizen werden geen significante verschillen in tamoxifen blootstelling in plasma en weefsels gevonden. Een significant hogere absorptiesnelheid werd gevonden na inname in de ochtend in vergelijking met inname in de avond in mensen. Dit resulteerde in een hogere plasma blootstelling aan tamoxifen en de metabolieten, hoewel de verschillen relatief klein waren bij de meeste individuen. Een aanbeveling voor het tijdstip van inname blijft lastig; inname in de ochtend resulteert in een licht verhoogde endoxifen blootstelling, maar inname in de avond kan mogelijk gunstiger zijn wat betreft bijwerkingen. De resultaten van de studie suggereren dat er rekening gehouden moet worden met zowel de innametijd als de tijd van bloedafname in klinische studies waarin de associatie tussen endoxifenconcentraties en klinische uitkomst wordt bestudeerd.

In hoofdstuk 8 hebben we de beschikbare literatuur over genetische- en omgevingsfactoren die invloed kunnen hebben op de farmacokinetiek van tamoxifen en de klinische uitkomst gereviewed. De invloed van het CYP2D6-genotype en CYP2D6-remmende co-medicatie op de concentraties van tamoxifenmetabolieten en uitkomst is uitgebreid bestudeerd. De invloed van polymorfismen in genen van CYP3A4, CYP3A5, CYP2C9, CYP2C19, UDP-glucuronosyltransferases en sulfotransferases is ook bestudeerd. Echter, de studies die een associatie tussen genetische factoren of co-medicatie en klinische uitkomst hebben onderzocht geven tegenstrijdige resultaten. Er komen steeds meer bewijzen uit preklinische en klinische studies die de theorie ondersteunen dat endoxifen van belang is voor de effectiviteit van tamoxifen en dat concentraties van deze metaboliet boven een bepaalde therapeutische grens moeten liggen. Dit pleit voor het toepassen van ´therapeutic drug monitoring´ (TDM). Zoals al eerder gesuggereerd is, is het voor het toepassen van TDM van belang dat de relatie tussen endoxifenconcentraties en klinisch effect bevestigd wordt en dat er een gevalideerde en gestandaardiseerde analytische methode beschikbaar is voor het kwantificeren van tamoxifen, (Z)-endoxifen en andere metabolieten.

Appendix 2

Dankwoord

DANKWOORD

Mijn proefschrift is afgerond en een mooie en leerzame periode van onderzoek wordt hiermee afgesloten. Dit proefschrift zou er niet zijn geweest zonder de hulp en bijdrage van velen. Al deze personen wil ik hiervoor graag bedanken, maar een aantal van hen wil ik in het bijzonder noemen.

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Lieve Suzanne, bedankt voor je steun en gezelligheid! We hebben tijdens de studie en de afgelopen jaren veel leuke dingen meegemaakt. Ik ben blij met onze vriendschap en hoop dat we nog veel mooie gebeurtenissen van en met elkaar meemaken!

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Appendix 3

Curriculum Vitae

CURRICULUM VITAE

Lisette Binkhorst werd op 21 maart 1985 geboren in Tholen. In 2003 heeft ze haar VWO diploma behaald aan het Mollerlyceum te Bergen op Zoom. Aansluitend is ze gestart met de studie diergeneeskunde aan de Universiteit van Antwerpen, België. In 2004 is ze gestopt met diergeneeskunde en begonnen met haar studie farmacie aan de Universiteit Utrecht. Tijdens haar studie heeft ze een stage gevolgd aan de James Cook University in Townsville, Australië. Ze heeft een literatuuronderzoek uitgevoerd naar de effecten van antidepressiva op glucose homeostase, gewicht en lipidenprofiel. In het kader van de masteropleiding heeft ze een wetenschappelijke stage gevolgd in de Apotheek Haagse Ziekenhuizen te Den Haag. Tijdens deze stage heeft ze retrospectief onderzoek gedaan naar de effecten van tramadol na overdosering. In augustus 2010 heeft ze haar masterdiploma behaald. In hetzelfde jaar is ze gestart met promotieonderzoek bij de ziekenhuisapotheek en het laboratorium Translationele Farmacologie van de afdeling Interne Oncologie in het Erasmus Medisch Centrum, onder begeleiding van prof. dr. T. van Gelder en prof. dr. A.H.J. Mathijssen. Dit onderzoek resulteerde in het huidig proefschrift. Haar onderzoek heeft ze op diverse (internationale) congressen gepresenteerd.

Appendix 4

Publications

PUBLICATIONS

Binkhorst L, Mathijssen RH, Ghobadi Moghaddam-Helmantel IM, de Bruijn P, van Gelder T, Wiemer EA, Loos WJ. Quantification of tamoxifen and three of its phase-I metabolites in human plasma by liquid chromatography/triple-quadrupole mass spectrometry. J Pharm Biomed Anal 2011;56:1016-23.

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Binkhorst L, van Gelder T, Loos WJ, de Jongh FE, Hamberg P, Ghobadi Moghaddam-Helmantel IM, de Jonge E, Jager A, Seynaeve C, van Schaik RH, Verweij J, Mathijssen RH. Effects of CYP induction by rifampicin on tamoxifen exposure. Clin Pharmacol Ther 2012;92:62-7.

Binkhorst L, van Gelder T, Mathijssen RH. Individualization of Tamoxifen Treatment for Breast Carcinoma. Clin Pharmacol Ther 2012;92:431-3.

Binkhorst L, Mathijssen RH, van Herk-Sukel MP, Bannink M, Jager A, Wiemer EA, van Gelder T. Unjustified prescribing of CYP2D6 inhibiting SSRIs in women treated with tamoxifen. Breast Cancer Res Treat 2013;139:923-9.

ter Heine R, Binkhorst L, de Graan AJ, et al. Population pharmacokinetic modelling to assess the impact of CYP2D6 and CYP3A metabolic phenotypes on the pharmacokinetics of tamoxifen and endoxifen. Br J Clin Pharmacol 2014;78:572-86.

Binkhorst L, Kloth JSL, de Wit AS, de Bruijn P, Ho Lam M, Chaves I, Burger H, van Alphen RJ, Hamberg P, van Schaik RHN, Jager A, Koch BCP, Wiemer EAC, van Gelder T, van der Horst GTJ, Mathijssen RHJ. Circadian variation in tamoxifen pharmacokinetics. Submitted for publication.

Binkhorst L, Bannink M, de Bruijn P, Ruit J, Droogendijk H, van Alphen RJ, den Boer TD, Ho Lam M, Jager A, van Gelder T, Mathijssen RHJ. Augmentation of endoxifen exposure in tamoxifen-treated women following SSRI-switch. Submitted for publication.

Binkhorst L, Mathijssen RHJ, Jager A, van Gelder T. Individualization of tamoxifen therapy: much more than just CYP2D6 genotyping. Cancer Treat Rev 2015;41:289-99

Kloth JSL, Binkhorst L, de Wit AS, de Bruijn P, Hamberg P, Ho Lam M, Burger H, Machado I, Wiemer EAC, van der Horst GTJ, Mathijssen RHJ. Sunitinib pharmacokinetics depends on administration time. Clin Pharmacokinet 2015.

Appendix 5

PhD portfolio

Name PhD student: Lisette Binkhorst	**PhD period:** 2010 - 2014
Erasmus MC Department: Hospital Pharmacy & Medical Oncology	**Promotor(s):** Prof.dr.T. van Gelder, Prof.dr. A.H.J. Mathijssen

1. PhD training

	Year	Workload (ECTS)
General courses		
- BROK course (Erasmus MC)	2011	1
- CPO mini course (Erasmus MC)	2011	0.3
- Masterclass English	2012	2
- Biomedical English Writing and Communication (Erasmus MC)	2014	3
Specific courses (e.g. Research school, Medical Training)		
- Basic Introduction Course on SPSS	2011	0.8
- Biostatistical Methods I: Basic Principles	2012	5.9
Seminars and workshops		
- NIH Principles of Clinical Pharmacology	2011	1
- Workshop Presence and Presentation	2011	0.4
- Workshop: “Systematic literature search in Pubmed”	2012	0.2
- Workshop on Photoshop and Illustrator CS5	2013	0.3
- PGx Workshop	2014	0.2
- Clinical Pharmacology meeting	2011-2014	1
- PhD- meeting Personalized Medicine	2010-2014	2
Presentations		
- San Antonio Breast Cancer Symposium, poster presentation	2011	1
- Pharma meeting, Rotterdam (oral presentation)	2012	0.4
- ESMO 2012, Vienna, poster presentation	2012	1
- NVKF&B Scientific Meeting (oral presentations)	2012, 2013	1
- ECCO, Amsterdam, poster presentation	2013	1
- ESMO, 2014, Madrid, poster presentation	2014	1
- Figon Dutch Medicine Days, poster presentation	2014	0.4

(International) conferences		
- San Antonio Breast Cancer Symposium , San Antonio	2011	0.4
- European Society for Medical Oncology (ESMO), Vienna	2012	0.4
- ECCO - European CanCer Organisation, Amsterdam	2013	0.4
- Figon Dutch Medicine Days	2011, 2014	0.2
- European Society for Medical Oncology (ESMO), Madrid	2014	0.4
Other		
- CMBD day pharmacogenetics	2011	0.2
- MOLMED day, Erasmus MC	2012	0.2
- Scientific Meeting Medical Oncology	2010-2014	0.4
- IKNL- network days	2010-2013	0.6
- NVKF&B scientific meeting	2012-2014	0.6
- PhD Day	2012, 2013	0.2
2. Teaching activities		
	Year	Workload (ECTS)
Lecturing		
- Education medical students	2010-2014	2
- PhD- meeting Personalized Medicine	2010-2014	1
- Clinical Pharmacology meeting	2011-2014	0.2
- Clinical Research Meeting (dept Medical Oncology)	2011-2012	0.2
- Clinical Lessons nurses	2011	0.2
- GAME, Utrecht University	2012	0.4