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General introduction

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INTRODUCTION

Breast cancer and tamoxifen

Breast cancer is the most common malignancy in women worldwide. In 2011 13,987 women were diagnosed with invasive breast cancer and 3,261 patients died of the disease in The Netherlands.[1] For decades the selective estrogen receptor modulator (SERM) tamoxifen has been used as part of the systemic treatment of metastatic and early hormone receptor positive breast cancer. In early breast cancer tamoxifen is the main endocrine therapy in premenopausal women; in postmenopausal women tamoxifen may be part of a sequential treatment before or after 2 to 3 years of treatment with an aromatase inhibitor. A recent study even demonstrated that continuing tamoxifen from five to ten years further reduces recurrence and mortality rates in early breast cancer.[2] Nonetheless, about half of estrogen receptor (ER) positive tumors in women with advanced breast cancer do not respond to tamoxifen therapy and one third of ER positive early breast cancer patients will have a disease recurrence after 5 years of tamoxifen treatment.[3-6] Furthermore, tamoxifen treatment can be complicated by bothersome side effects such as hot flashes and severe but less common adverse events like venous thrombo-embolisms and endometrial cancer. These observations stress the need for markers that are strongly predictive of both tamoxifen efficacy and side effects.

Tamoxifen metabolism

Tamoxifen is a prodrug that needs biotransformation to the metabolites 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) to become active. 4-Hydroxytamoxifen and N-desmethyltamoxifen are both primary metabolites. The latter is the most abundant in plasma (~90%). Hydroxylation of N-desmethyltamoxifen, mainly mediated by the Cytochrome P450 2D6 isoenzyme (CYP2D6), leads to formation of the secondary metabolite endoxifen,[7] depicted in Figure 1.1. Endoxifen and 4-hydroxytamoxifen exhibit a 30-100 fold higher anti-estrogen activity than tamoxifen. Both metabolites have the same potency with regard to ER-α and -β binding,[8] suppression of ER-dependent human breast cancer cell line proliferation[8-10] and global ER-responsive gene expression.[11] Additionally, endoxifen targets the estrogen receptor-α for degradation in breast cancer cells.[10] Endoxifen is considered the most important tamoxifen metabolite, because its plasma concentrations are 5-10-fold higher than 4-hydroxytamoxifen concentrations.[12, 13]

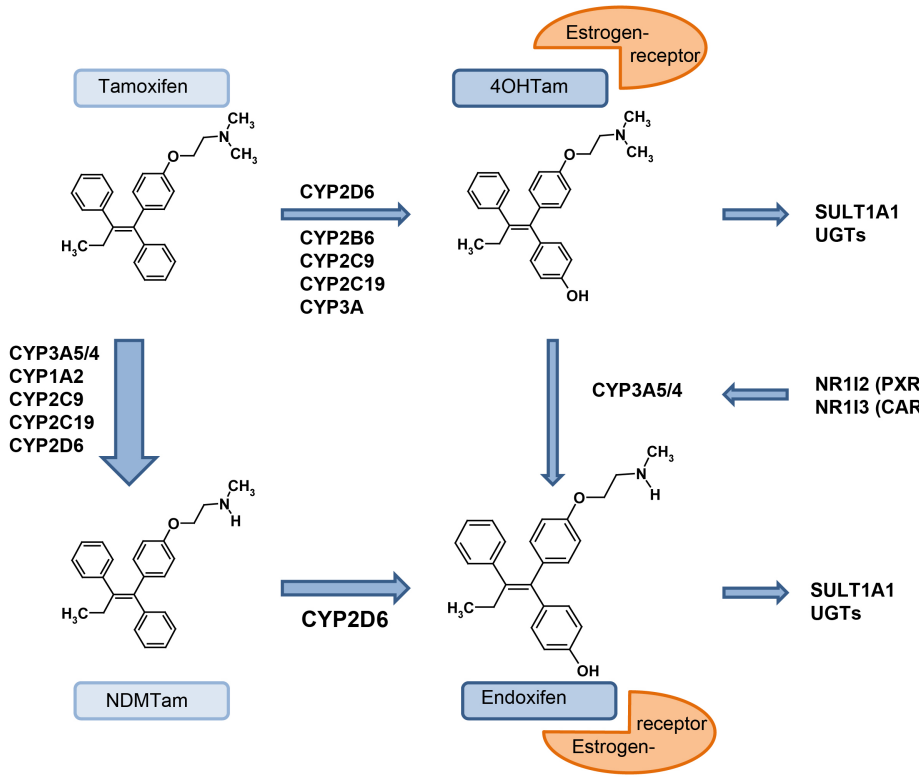

Figure 1.1 Tamoxifen metabolism. Abbreviations: 4OHTam, 4-hydroxytamoxifen; CYP, cytochrome P450 isoenzyme; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; NDMTam, N-desmethyltamoxifen; NR1, nuclear receptor subfamily 1; PXR, pregnane X receptor; CAR, constitutive androstane receptor.

CYP2D6 activity and endoxifen

The CYP2D6 gene is highly polymorphic. Specific alleles harbouring genetic variants (~80) encode enzymes with almost absent or impaired activity. Allelic frequencies are depending on ethnicity.[14] For example, the most common absent activity allele in Caucasians is CYP2D6*4 with an allelic frequency of 20%. The most frequent reduced activity allele in Asians is CYP2D6*10 with a 38% allelic frequency. The CYP2D6 genotype can be translated into a CYP2D6 phenotype (i.e. enzymatic activity). Patients can thus be classified into a poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM) or ultrarapid metabolizer (UM) phenotype. The heterozygous EM is sometimes distinguished as a separate CYP2D6 phenotype and encompasses those genotypes with one normal and one absent activity allele (e.g. *1/*4). Furthermore, certain pharmaceuticals

inhibit CYP2D6 activity (CYP2D6 inhibitors) and transform a genotype predicted EM to an IM or PM. Both CYP2D6 genotype and the concomitant use of CYP2D6 inhibitors are clearly associated with endoxifen blood concentrations in tamoxifen treated breast cancer patients. In PMs and IMs, treated with tamoxifen, significantly lower plasma endoxifen concentrations are found than in EMs.[12, 15] Still, the explained variance of plasma endoxifen concentration by CYP2D6 genotype or predicted phenotype is low (23%[15]).

CYP2D6 activity, tamoxifen efficacy and side effects

In 2005 Goetz et al. reported the first data showing that early breast cancer patients with decreased CYP2D6 activity based on CYP2D6 genotype had an increased risk for breast cancer recurrence compared to patients with a normal active CYP2D6.[16] Furthermore, patients with an EM predicted phenotype experienced more moderate and severe hot flashes, which is a common tamoxifen side effect.[16] This first positive association study launched a great amount of research with the aim to validate and elucidate the predictive value and thus clinical relevance of CYP2D6 activity predicted by genotype and concomitant CYP2D6 inhibitor use. At that moment many issues were unresolved. Would a broader CYP2D6 allelic coverage explain more of the variation in endoxifen concentration and would that better predict tamoxifen response? Do genetic variants of other metabolic enzymes contribute to the concentration of active tamoxifen metabolites and are these also clinically relevant? Is it unsafe to co-prescribe CYP2D6 inhibitors during tamoxifen therapy as this may decrease tamoxifen efficacy? Would a CYP2D6 phenotyping test be more accurate in predicting endoxifen levels as not only genetic but also epigenetic factors and drug interaction may influence CYP2D6 activity? Finally, the most important question is whether low endoxifen concentration as a result of decreased metabolism is associated with clinical outcome in tamoxifen treated breast cancer patients.

Endoxifen and tamoxifen efficacy

The hypothesis that CYP2D6 activity predicts tamoxifen efficacy is fully based on the assumption that endoxifen is the most active tamoxifen metabolite in tamoxifen treated breast cancer patients. This assumption was based on in vitro studies only. Patients with decreased or absent CYP2D6 activity exhibit lower endoxifen blood levels, but whether these levels are low enough to impair efficacy is uncertain. Only one recent study addressed this issue. In the Women's Healthy Eating and Living (WHEL) study the first retrospective correlation between endoxifen concentration and breast cancer survival was made.[17] Patients with endoxifen levels in the range of the lowest quintile had

a 35% higher rate of breast cancer events suggesting a threshold effect with a reported endoxifen threshold of 5.97 ng/ml (= 16.0 nM). In this study 76% of the CYP2D6 poor metabolizers had endoxifen levels within the range of the lowest quintile. This study however was not designed for this purpose and the borderline significant difference in outcome between the patients with the lowest endoxifen levels (lowest quintile) and the patients with higher endoxifen levels (HR=0.74; 95% CI, 0.55-1.00) should be interpreted with caution. Therefore, well designed prospective studies are needed to investigate the relation between endoxifen and clinical outcome.

AIM AND OUTLINE OF THIS THESIS

The general aim of this thesis was to study the variation in tamoxifen metabolism in relation to endoxifen serum concentration, tamoxifen efficacy and side effects with a focus on CYP2D6 activity and pharmacogenetics.

In **chapter 2** a systematic review is given of the studies published before 2009 addressing the relation between CYP2D6 genotype and tamoxifen efficacy. A critical appraisal of preclinical, but mostly clinical studies is given. Although the studies published after 2009 were not discussed, most of the criticism and remarks given in this review are also applicable to some of the more recent association studies.

Chapter 3 describes the method of pre-amplification of DNA in order to optimize genotyping results when DNA derived from formalin-fixed paraffin embedded (FFPE) tissue is used. DNA derived from FFPE tissue is often fragmented and crosslinked and therefore difficult to genotype. Pre-amplification enabled reliable genotyping analysis using Taqman probes with this source of DNA. This method was used in the studies described in chapter 5 and 6.

Chapter 4 describes the high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that was designed and validated in the Leiden University Medical Center to accurately detect and quantify tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen. The importance of accurate chromatographic separation of the many metabolites (measured and unmeasured) was stressed by the previous observation that endoxifen and 4-hydroxytamoxifen concentrations are overestimated by a factor 2-3 when chromatographic peaks are overlapping.[18, 19] Different analytical methods and procedures may have explained the differences in endoxifen concentrations between the separate previously reported studies. Therefore, it is very important that association studies on endoxifen serum and plasma concentrations use adequate and reproducible

detection methods enabling good interpretation and comparison of the data. This LC-MS/MS method was applied to the prospective CYPTAM and KINETAM studies, described in chapter 8 to 11.

CYP2D6 genotypes and the predicted phenotypes were related to disease free survival during tamoxifen use in early breast cancer patients in **chapter 5**. Patients who were enrolled in the Tamoxifen Exemestane Adjuvant Multinational (TEAM) Trial and randomized to treatment with tamoxifen with a planned switch to exemestane after 2.5 to 3 years were genotyped for five CYP2D6 alleles and 24 genetic variants of other metabolic enzymes and the estrogen receptor-1. Because CYP2D6 genotyping was done on DNA derived from tumor blocks, an additional microsatellite analysis was performed to exclude the influence of loss of heterozygosity (LOH) of the CYP2D6 gene on the called CYP2D6 genotype. A previous report was heavily criticized for using DNA derived from tumor blocks.[20] LOH in tumor tissue may have caused false CYP2D6 genotype assignment and critics stated that this may have caused the deviation of the Hardy Weinberg equilibrium (allele frequencies in a population should remain constant) reported in that study.[21] To our best knowledge, we report the first CYP2D6 genotype association study in which tumor blocks were used as source of DNA for CYP2D6 genotyping, but in which potential influence of LOH in tumor tissue was accounted for.

In **chapter 6** CYP2D6 genotypes and the predicted phenotypes were related to the occurrence of hot flashes during tamoxifen use in early breast cancer patients enrolled in the TEAM trial. Additionally, 24 genetic variants of other metabolic enzymes and the estrogen receptor-1 were investigated. The previously reported microsatellite analysis excluding influence of LOH made this the largest study thus far in which reliable CYP2D6 genotypes and predicted phenotypes were related to estrogen dependant side effects.

Concomitant use of a CYP2D6 inhibitor has been reported in up to 30% of patients with breast cancer.[22] In **chapter 7** the potential detrimental effect of concomitant CYP2D6 inhibitor use (e.g. paroxetine and fluoxetine) on tamoxifen efficacy in early breast cancer patients is addressed. For this study a large database was used in which pharmacy data, pathology report data and clinical data from the Dutch Medical Register (LMR) were linked. Overlapping tamoxifen and CYP2D6 inhibitor dispenses were related to disease recurrence. Additionally, this is the first study in which the adherence to tamoxifen was related to clinical outcome.

In **chapter 8** the pharmacokinetic sub-study of the prospective CYPTAM study is described. Tamoxifen treated early breast cancer patients who are CYP2D6 PM and IM were treated with a temporary pharmacokinetic guided tamoxifen dose escalation. The increased tamoxifen dose was calculated by dividing the individual's baseline endoxifen concentration by the median endoxifen

concentration in extensive metabolizers multiplied by 20 mg (120 mg maximum). The aim of this study was to investigate whether such dose escalation in PMs and IMs would increase endoxifen to a level similar to endoxifen levels observed in EMs without increasing toxicity.

Chapter 9 describes the KINETAM study which is a CYP2D6 genotype guided dose escalation study in early and metastatic breast cancer patients who were treated with tamoxifen. The aim of this study was to investigate whether a tamoxifen dose increase from 20 mg to a safe and registered 40 mg once daily dose in PMs and IMs would also sufficiently raise the endoxifen concentrations.

A new 13C-dextrometorphan breath test for phenotyping CYP2D6 is described in **chapter 10**. This simple breath test was developed to account for genetic variants, epigenetic and environmental factors and CYP2D6 inhibitor use that may all influence CYP2D6 activity. The 13C-Dextrometorphan breath test was related to CYP2D6 genotype and serum endoxifen concentrations in tamoxifen treated early breast cancer patients in a sub-study of the CYPTAM study.

In **chapter 11** the outline of the prospective multicenter CYPTAM study is discussed that included over 650 patients in the Netherlands and Belgium. The main aim of this documentation study is to relate CYP2D6 genotype, predicted phenotype and endoxifen serum concentrations to disease recurrence in tamoxifen treated early breast cancer patients. Preliminary results are presented showing the relation between CYP2D6 predicted phenotype based on a broad allelic coverage (33 alleles) using Roche's Amplichip and steady state endoxifen levels. This is one of the first prospective studies addressing the direct relation between endoxifen and clinical outcome. The final results of this study and similar ongoing studies are awaited and will be crucial to determine the need to further investigate the influence of alterations in the tamoxifen metabolism on efficacy. A positive association may lead to therapeutic drug monitoring, as well as the further development of endoxifen as a primary endocrine breast cancer therapeutic.

This thesis ends with concluding remarks and future prospects in **chapter 12** and a summary of the results is presented in **chapter 13**.

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