A Pharmacological Approach to Personalize the Use of Anti-Cancer Drugs

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A Pharmacological Approach to Personalize the Use of Anti-Cancer Drugs

Een farmacologische benadering voor het individualiseren van behandeling met antikanker middelen

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I have learned to be even more patient
Roger Federer

Voor mijn ouders

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INTRODUCTION

1

INTRODUCTION

Cancer is one of the leading causes of death in the western world and also the primary cause of death in the Netherlands. Over the past decades, the incidence of several cancer types has been increasing. In the Netherlands, each year there are approximately 80.000 newly diagnosed cases of cancer, of which breast cancer, colon cancer and skin cancer (excluding basal cell carcinoma) are most prevalent.[1] During the past years, the focus in cancer research has been on the newly targeted agents, but classic chemotherapy and anti-hormonal treatment still play an important part in the treatment of solid tumors. And although there has been much experience with these anti-cancer treatments, only limited progress has been made to individualize the treatment with these agents.

Most anti-cancer drugs are given in a flat-fixed or BSA adjusted dose, despite the wide inter-patient variability (IIV) in pharmacokinetics and toxicity. It is highly clinically relevant to be able to predict individual drug exposure because many drugs used to treat cancer have a small therapeutic window. This means that a low exposure could lead to an ineffective treatment, while a high exposure may lead to (extreme) toxicity. Knowledge of factors that influence the pharmacokinetics of these drugs could provide a way to individualize anti-cancer therapy. Examples of such factors are genetic variation in metabolizing enzymes or drug transporters, but also environmental factors like smoking could potentially influence drug exposure in an individual. Another way of predicting drug exposure of a specific anti-cancer drug is by phenotyping with a (endogenous or exogenous) probe drug that is metabolized in a similar way as the drug of interest.[2] In this thesis, different ways of individualizing widely used anti-cancer drugs are studied.

In **Chapter 2**, the influence of smoking on the pharmacokinetics and pharmacodynamics of the taxanes docetaxel and paclitaxel were studied. These classic anti-cancer agents act through stabilizing microtubule assembly in the cell, thereby stimulating apoptosis of a cancer cell.[3] These drugs are widely used in oncology practice and are registered for the treatment of cancers of the breast, ovary, cervix, lung, head and neck, esophagus and prostate. Docetaxel is metabolized by the liver enzymes CYP3A4 and CYP3A5, while paclitaxel is metabolized by CYP2C8 and CYP3A4.[4-6] For other anticancer agents such as irinotecan and erlotinib, smoking is known to influence the systematic exposure to these drugs.[7] As these taxanes are (partly) metabolized by the same enzymes as irinotecan and erlotinib, it is possible that the systemic exposure to docetaxel and paclitaxel is also altered by cigarette smoke.

Chapter 3 describes risk factors of developing neurotoxicity during paclitaxel therapy. Genetic polymorphisms and systemic exposure were investigated as potential predictors of systemic exposure and neurotoxicity of paclitaxel. Genetic variants include previously investigated single nucleotide polymorphisms in *CYP2C8* and *ABCB1*, but also a recently discovered reduced function polymorphism in intron 6 of *CYP3A4*, named *CYP3A4*22*.

In **Chapter 4**, a novel approach was used to identify patients with increased risk of paclitaxel-induced toxicity caused by decreased metabolism of paclitaxel. By using the drug metabolizing enzyme and transporter (DMET) chip, which enables simultaneous studying of 1.936 genetic variants in metabolic enzymes and transporters, we explored the possibility to identify genetic markers for decreased paclitaxel metabolism, which can lead to increased toxicity during treatment.

In **Chapter 5**, the contribution of the organic anion transporting polypeptide (OATP) transporters on the disposition of docetaxel were studied in cell lines, in knock-out mice, and finally in humans, providing translational research results in a single study.

Chapter 6 describes a potential manner to predict the capability of an individual patient to metabolize docetaxel or paclitaxel by using an endogenous marker. In the human body, cholesterol is converted in 4β-OH-cholesterol by CYP3A4/5. The level of 4β-OH-cholesterol in the systemic circulation and the ratio of 4β-OH-cholesterol: cholesterol has been proposed as a marker for CYP3A4/5 activity.[8] The potential of the endogenous 4β-OH-cholesterol marker to predict individual metabolic profiles of taxanes has not been investigated before and is assessed in this chapter.

In the last chapter of the thesis (**Chapter 7**) the anti-estrogenic drug tamoxifen is studied, which is used in both the adjuvant treatment and metastatic setting of breast cancer. Dextromethorphan, an exogenous probe drug, is used to predict the exposure to endoxifen, which is the most abundant active metabolite of tamoxifen.[9] Because tamoxifen is generally prescribed for as much as five years in the adjuvant setting it is important to ensure optimal therapeutic drug levels. There has been much debate in the literature on the way to provide individualized tamoxifen therapy using *CYP2D6* genotyping.[10,11] Determination of genetic variation or genotyping in individual patients aims to achieve this goal, but only predicts 23% of the variability in endoxifen levels.[12] Therefore, we proposed a different method to predict individual endoxifen concentrations by phenotyping with a probe drug. This method has the advantage to combine genetic and environmental factors. Dextromethorphan is the active ingredient in cough syrup and has a limited toxicity profile. Also, dextromethorphan is metabolized by the same cytochrome P450 enzymes as tamoxifen (CYP3A4 and CYP2D6). These characteristic makes this potentially a useful probe drug.

In conclusion, this thesis entitled *"A pharmacological approach to personalize the use of anti-cancer drugs"* explores ways to predicts the pharmacokinetics and pharmacodynamics of the anti-cancer drugs docetaxel, paclitaxel and tamoxifen to optimize therapeutic benefit and minimize toxicological ramifications of these therapies. The ultimate aim of these studies is to provide individual patients with "tailor made" anti-cancer therapy.

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INFLUENCE OF SMOKING ON THE PHARMACOKINETICS AND TOXICITY PROFILES OF TAXANE THERAPY

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ABSTRACT

Purpose

Cigarette smoke is known to interact with the metabolism of several anticancer drugs. It may also affect the incidence and severity of adverse events and efficacy of chemotherapy. The main objective of this study was to examine the effects of smoking on the pharmacokinetics and toxicities of patients treated with docetaxel or paclitaxel.

Experimental design

Smoking status, toxicity profiles, and pharmacokinetic parameters (calculated by nonlinear mixed-effect modeling population analysis) were determined in 566 patients (429 nonsmokers and 137 smokers) treated with docetaxel or paclitaxel.

Results

Smokers treated with docetaxel showed less grade IV neutropenia (35% vs. 52%; $P = 0.01$) than nonsmokers. Smokers treated with paclitaxel had less grade III-IV leukopenia than nonsmokers (12% vs. 25%; $P = 0.03$) and the white blood cell (WBC) nadir was lower in nonsmokers (median, 2.7 $\times 10^9$/L; range 0.05 x 10^9 to 11.6 x10^9/L) than in smokers (median, 3.3 x10^9/L; range 0.8 x10^9 to 10.2 x10^9/L; $P = 0.02$). Of interest, significantly lower WBC counts and absolute neutrophil counts at baseline were seen in nonsmoking patients treated with paclitaxel ($P = 0.0001$). Pharmacokinetic parameters were similar in smokers and nonsmokers for both taxanes.

Conclusion

Cigarette smoking does not alter the pharmacokinetic determinants of docetaxel and paclitaxel. Smokers treated with docetaxel and paclitaxel have less neutropenia and leukopenia, but further research is warranted to elucidate this potential protective effect.

TRANSLATIONAL RELEVANCE

The chemotherapeutic agents docetaxel and paclitaxel are known for their small therapeutic window and for their large interindividual variability in metabolism and toxicity profile. Several factors may influence the systemic exposure to these drugs, including genetic factors (i.e., polymorphisms in genes coding for drug transporters) and comedication (i.e., use of strong CYP3A inhibitors). Also, cigarette smoking was identified as a factor influencing the pharmacokinetics and pharmacodynamics of cytotoxic drugs metabolized by CYP3A, but was not studied thoroughly for taxanes before. Because of the small therapeutic window, an alteration in drug exposure may easily lead to unexpected toxicities or suboptimal therapeutic effects in individual patients with cancer; therefore, a knowledge of the effects of smoking on taxane therapy is important for the further individualized treatment of docetaxel and paclitaxel treatment.

INTRODUCTION

Smoking tobacco is the foremost preventable cause of cancer.[1] It has been directly or indirectly linked to more than 10 different tumor types and accounts for 30% of all cancer deaths.[2] Despite today's current insights on the extensive harmful effects of smoking its prevalence remains high. In 2008, 23.5% of men and 18.3% of women in the United States were smokers.[3] Worldwide there are over 1.3 billion tobacco smokers and this number is still increasing.[4]

Cigarette smoke contains many substances, including polycyclic aromatic hydrocarbons, which are known to induce cytochrome P450 (CYP) metabolic enzymes and some isoforms of the uridine diphosphate glucuronosyltransferase (UGT) family which are involved in glucuronic acid conjugation.[5] Therefore, smoking might potentially interfere with the pharmacokinetics of several drugs. Currently, it is known that smoking accelerates the metabolism of many different agents (i.e., clozapine, quinine, and propanolol). Especially extra-hepatic localized CYP1A2- and CYP1A1-mediated metabolism is influenced by cigarette smoke.[5,6] For example, smokers treated with clozapine have approximately 2.5 times lower serum drug concentrations than nonsmokers, indicating an enhanced clearance.[7]

In the recent literature, there are several suggestions that smoking can also modulate the pharmacokinetics of anticancer drugs. Erlotinib, a drug primarily metabolized by CYP3A4 but also by CYP1A2, has a 2.8-fold lower systemic exposure in smokers than in nonsmokers.[8] In addition, we have previously shown that smokers treated with irinotecan have an 18% higher clearance than nonsmokers. This prodrug is primarily metabolized by CYP3A4 and carboxylesterases, and its active metabolite SN-38 is glucuronidated by UGT1A1. The resulting altered balance in the complex metabolism leads to a 40% reduction of systemic exposure to SN-38. The higher relative extent of glucuronidation of SN-38 observed in smokers in this study can be explained by induction of UGT1A1.[9]

Both docetaxel and paclitaxel are antimicrotubular agents extensively metabolized by CYP3A, while CYP2C8 is also involved in the metabolism of paclitaxel.[10,11] These drugs are registered for and used in the treatment of a variety of cancers, such as ovarian, breast, prostate and non-small cell lung cancer.[12] There is a large interindividual variability in the pharmacokinetics of taxanes, their toxicity and therapeutic response.[13,14] This poses a serious issue for dosing within the narrow therapeutic window of both docetaxel and paclitaxel. Patients with a low docetaxel clearance are at a higher risk of severe adverse events such as febrile neutropenia, and other severe toxicities such as mucositis and skin toxicity.[14,15] A low paclitaxel clearance puts patients primarily at risk for hematological toxicities (mainly neutropenia) and peripheral neuropathy.[16] On the other hand, patients with high clearances are at risk of suboptimal systemic drug levels, potentially leading to a decreased therapeutic effect. Literature data suggest substantial influence by genetic, nutritional and environmental factors on the pharmacokinetics of paclitaxel.[17] At present, many factors contributing to this large inter-individual variability remain to be elucidated.

More extensive knowledge of factors influencing the metabolism of docetaxel and paclitaxel may give new prospects in developing individual dosing regimes. Against this background, we conducted a retrospective study to determine the potential effects of smoking behavior on the pharmacokinetics and hematological toxicities of both docetaxel and paclitaxel in a large cohort of patients with solid tumors.

PATIENTS AND METHODS

Patients

A total of 566 patients were included in this retrospective analysis. For docetaxel, patients were previously enrolled in 7 different prospective studies all involving pharmacokinetic analyses, of which one is still ongoing.[18-23] For paclitaxel, patients were enrolled in 3 different prospective studies involving pharmacokinetic analyses, of which one is still ongoing.[24,25] The cutoff date for the analysis was August 2011.

The common inclusion criteria for all mentioned studies were (i) a histological or cytological confirmed diagnosis of cancer treated with docetaxel or paclitaxel, (ii) aged 18 years or older, (iii) WHO performance score of 0 to 1, and (iv) adequate hematopoietic, hepatic and renal functions. Patients using drugs known to be potent CYP3A4 inducers or inhibitors were not included in these studies. The medical ethical committee of the Erasmus University Medical Center (Rotterdam, the Netherlands) approved all individual trials and patients provided written informed consent prior to participation in a trial.

Treatment

Docetaxel-treated patients generally received a 75 to 100 mg/m^2 dose, depending on tumor type or combination regimen. Paclitaxel-treated patients generally received either 90 mg/m^2 weekly or 175 mg/m^2 every 3 weeks. Patients receiving medication

known to influence the pharmacokinetics of docetaxel or paclitaxel were excluded from entry in the study.

Smoking status

Patients were categorized on smoking status according to smoking information from medical files recorded on the day of pharmacokinetic sampling. If smoking status was not entirely clear based on information in the medical records, patients were excluded from the analyses. Patients who had quit smoking within 4 weeks before pharmacokinetic sampling were also excluded, to guarantee a safe "washout period" for potential enzyme induction (or inhibition).

Pharmacodynamic and pharmacokinetic analysis for docetaxel and paclitaxel

Toxicity was graded according to National Cancer Institute–Common Terminology Criteria (NCI-CTC) criteria 4. Patients were evaluable for toxicity analysis when they received more than one treatment cycle. Occurrence of >grade III toxicity was scored for each patient during the whole period of treatment cycles. Baseline white blood cell (WBC) and absolute neutrophil counts (ANC) were recorded before start of taxane therapy. Nadir WBC and ANC values were determined as lowest WBC and ANC values during all treatment cycles.

Blood samples for pharmacokinetic analysis of docetaxel or paclitaxel were obtained in up to 3 treatment cycles per patient. Samples were collected in the presence of lithium heparin as anticoagulant. For docetaxel,[18-23] 121 and 169 patients, respectively, and for paclitaxel[24,25], 22 and 254 patients respectively were either extensively sampled after the end of infusion, or in a limited sampling strategy, with 4 to 5 samples in up to approximately 24 hours after the end of infusion.

Docetaxel has been quantitated in plasma by a validated high-performance liquid chromatography (HPLC) method with UV detection[26] or by validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods.[19,27] Paclitaxel has been quantitated by either a validated HPLC method with UV detection[28] or by a validated LC-MS/MS method based on the method described for docetaxel.[19]

On the basis of the measured plasma concentrations at different time points and previously developed population pharmacokinetic models for docetaxel[29] and paclitaxel[13] with population Cremophor concentrations,[30] individual pharmacokinetic parameters were estimated as empirical Bayes estimates using the nonlinear mixed-effect modeling software NONMEM version VI and 7 (Icon Development Solutions)

As Cremophor EL, the formulation vehicle of paclitaxel, causes a shift in the blood distribution of paclitaxel and a reduction in the availability of the free circulating fraction of paclitaxel, the total fraction of paclitaxel does not behave in a linear pharmacokinetic way in contrast to the "free" fraction. Therefore "unbound", instead of total clearance were used in the analysis.[25,31]

Statistical analysis

Data are presented as medians and ranges, unless stated otherwise. To test the difference in continuous variables between smokers and nonsmokers, the Kruskal-Wallis test was used. For the comparison between smoking status and nominal variables, χ^2 test was used to calculate a corresponding *P* value. To correct for the different tumor types, different dosing, and combination regimens, a logistic regression analysis was used for the pharmacodynamic analysis. A *P* value < 0.05 was considered to indicate a significant difference. All the statistical analyses were performed with Stata version 11.1.

RESULTS

Baseline parameters

In smokers treated with paclitaxel, significantly higher WBC (9.1 vs. 7.1 x 10^9/L; *P* = 0.0001) and ANC values before start of treatment (6.6 vs. 4.6 x 10^9/L; *P* = 0.0001) were observed (Table 1). Smokers treated with docetaxel had also higher WBC and ANC values than nonsmokers, but this difference was not significant (Table 2). In both docetaxel- and paclitaxel-treated patients, other basic demographic patient characteristics were quite similar between smokers and nonsmokers, except that in the paclitaxel-treated patients, smokers were younger than nonsmokers (Tables 1 and 2).

Smoking status and docetaxel-related toxicity

In the group of smokers, there was significantly less grade IV neutropenia (ANC < 0.5 x 10^9/L) in all treatment cycles than in the nonsmoking group (35% vs. 52%; *P* = 0.01, Table 3). When corrected in a multivariate analysis for tumor type, different dosing, and combination regimens, this effect remained apparent [OR, 0.48; 95% confidence interval (CI), 0.26-0.89; *P* = 0.02]. However, during docetaxel treatment, the incidence of neutropenic fever was similar in both groups (16% vs. 20%; *P* = 0.43; Table 3). Smokers treated with docetaxel also had significantly less grade III-IV leukopenia (WBC < 2.0 × 10^9/L) than nonsmokers (43% vs. 56%; *P* = 0.04; Table 3; Fig 1). When corrected in a multivariate analysis for tumor type, different dosing, and combination regimens, smokers still had fewer grade III-IV leukopenia, although this result was not significant (OR, 0.63; 95% CI, 0.34-1.15; *P* = 0.13).

Smoking status and paclitaxel-related toxicity

There was significantly less WBC grade III-IV toxicity in smokers (12% vs. 25%; *P* = 0.03; Table 3; Fig 1) than in nonsmokers treated with paclitaxel. In a multivariate analysis correcting tumor type, different dosing, and combination regimens, this effect remained significant (OR, 0.31; 95% CI 0.12-0.82; *P* = 0.02). The WBC nadir was also higher in smokers (3.3 x 10^9/L) than in nonsmokers (2.7 x 10^9/L; *P* = 0.02). In patients treated with paclitaxel there was a trend towards lower incidence of ANC grade III-IV

Table 1. Patient characteristics treated with paclitaxel

Parameter [a]	All patients		Nonsmokers		Smokers		*P* [c]
Number of patients [b]	276	(100)	214	(78)	62	(22)	
Gender [b]							
Male	140	(51)	112	(80)	28	(20)	
Female	136	(49)	102	(75)	34	(25)	
Age (years)	60	(18-82)	61	(18-82)	57	(26-73)	0.007
Tumor type [b]							
Esophageal/gastric	118	(43)	91	(77)	7	(23)	
Ovarian	39	(14)	33	(85)	6	(15)	
Breast	17	(6)	13	(76)	4	(24)	
Cervix	17	(6)	9	(53)	8	(47)	
Endometrium	17	(6)	16	(94)	1	(5.8)	
Lung	15	(5)	13	(87)	2	(13)	
Head/Neck	11	(4)	7	(64)	4	(36)	
(A)CUP	10	(4)	6	(60)	4	(40)	
Testis	6	(2)	4	(67)	2	(33)	
Bladder	6	(2)	3	(50)	3	(50)	
Sarcoma	4	(1)	4	(100)	0	(0)	
Melanoma	3	(1)	3	(100)	0	(0)	
Prostate	3	(1)	3	(100)	0	(0)	
Other	10	(5)	12	(92)	1	(8)	
Baseline Hematology							
Platelets ($\times 10^9$/L)	283	(65-735)	280	(77-735)	289	(65-643)	0.5
WBC ($\times 10^9$/L)	7.3	(2.3-24)	7.1	(2.3-24)	9.1	(4.8-19)	0.0001
ANC ($\times 10^9$/L)	4.8	(1.2-21)	4.6	(1.2-21)	6.6	(2.1-16)	0.0001
Hemoglobin, mmol/L	8.1	(3.4-11)	8.0	(3.4-11)	8.3	(4.1-10)	0.01

[a] All data are represented as median with range in parentheses, unless stated otherwise.
[b] Number with percentages in parentheses.
[c] Statistically tested with the two-sided Kruskal-Wallis test.
Abbreviations: (A)CUP, (adeno) carcinoma of unknown primary; WBC, white blood cell count; ANC, absolute neutrophil count

toxicity during all treatment cycles in smokers when compared to nonsmokers (27% *vs.* 40%; *P* = 0.06, Table 3; Fig. 1). The ANC nadir was higher in smokers than in nonsmokers (1.7 *vs.* 1.3 x 10^9/L; P = 0.04, Table 3). However, also during paclitaxel treatment, the incidence of neutropenic fever was similar in both groups (8% vs. 6%; *P* = 0.5; Table 3).

Table 2. Patient characteristics treated with docetaxel

Parameter [a]	All patients		Nonsmokers		Smokers		*P* [c]
Number of patients [b]	290	(100)	215	(74)	75	(26)	
Gender [b]							
Male	114	(39)	78	(68)	36	(32)	
Female	176	(61)	137	(78)	39	(22)	
Age (years)	55	(18-85)	56	(18-85)	54	(32-75)	0.4
Tumor type [b]							
Breast	131	(45)	102	(78)	29	(22)	
Prostate	34	(12)	26	(76)	8	(24)	
Head/Neck	31	(11)	21	(68)	10	(32)	
Melanoma	20	(7)	19	(95)	1	(5)	
Lung	18	(6)	10	(56)	8	(44)	
Sarcoma	16	(6)	11	(69)	5	(31)	
Esophageal	10	(3)	7	(70)	3	(30)	
(A)CUP	8	(3)	3	(38)	5	(63)	
Gastro-Intestinal	7	(2)	7	(100)	0	(0)	
Bladder	6	(2)	4	(67)	2	(33)	
Cervix	3	(1)	2	(67)	1	(33)	
Other	6	(2)	3	(50)	3	(50)	
Baseline Hematology							
Platelets ($\times 10^9$/L)	292	(66-930)	289	(66-930)	311	(118-620)	0.4
WBC ($\times 10^9$/L)	8.6	(2.9-29)	8.4	(2.9-29)	9.2	(3.4-27)	0.4
ANC ($\times 10^9$/L)	6.7	(1.7-27)	6.6	(1.7-27)	7.3	(2.1-24)	0.6
Hemoglobin, mmol/L	7.7	(4.4-11)	7.6	(4.4-11)	7.7	(5.8-9.9)	0.2

[a] All data are represented as median with range in parentheses, unless stated otherwise.
[b] Number with percentages in parentheses.
[c] Statistically tested with the two-sided Kruskal-Wallis test.
Abbreviations: (A)CUP, (adeno) carcinoma of unknown primary; WBC, white blood cell count; ANC, absolute neutrophil count

Table 3. Effect of smoking behavior on docetaxel and paclitaxel-induced toxicity

Parameter [a]	*N* [c]	All patients	Nonsmokers	Smokers	*P* [d]
Docetaxel					
White blood cell count					
Nadir (×10⁹/L)	213/75	1.8 (0.05-17)	1.73 (0.09-17)	2.5 (0.05-13)	0.5
Decrease WBC [f]	210/75	76 (0-100)	76 (0-98)	73 (0-100)	0.9
CTC grade 3–4 [b]	213/75	152 (53)	120 (56)	32 (43)	0.04[e]
Absolute neutrophil count					
Nadir (×10⁹/L)	213/75	0.6 (0.01-15)	0.5 (0.01-15)	0.8 (0.05-12)	0.6
Decrease ANC [f]	211/75	91 (0-100)	92 (0-100)	88 (0-100)	0.8
CTC grade 3–4 [b]	213/75	170 (59)	128 (60)	42 (56)	0.5[e]
CTC grade 4 [b]	213/75	137 (48)	111 (52)	26 (35)	0.01[e]
Neutropenic fever					
CTC grade 3–4 [b]	213/75	55 (19)	43 (20)	12 (16)	0.4[e]
Paclitaxel					
White blood cell count					
Nadir (×10⁹/L)	213/60	2.7 (0.05-12)	2.7 (0.05-12)	3.3 (0.08-10)	0.02
Decrease WBC [f]	213/60	61 (0-100)	61 (0-100)	63 (5.6-98)	0.3
CTC grade 3–4 [b]	213/60	61 (22)	54 (25)	7 (12)	0.03[e]
Absolute neutrophil count					
Nadir (×10⁹/L)	213/60	1.4 (0.05-9.3)	1.3 (0.05-9.3)	1.7 (0.05-7.5)	0.04
Decrease ANC [f]	212/60	69 (0-100)	69 (0-97)	72 (2.6-99)	0.9
CTC grade 3–4 [b]	213/60	101 (37)	85 (40)	16 (27)	0.06[e]
CTC grade 4 [b]	213/60	47 (17)	40 (19)	7 (12)	0.2[e]
Neutropenic fever					
CTC grade 3–4 [b]	210/59	18 (7)	13 (6)	5 (8)	0.5[e]

[a] Data are represented as median with range in parentheses, unless stated otherwise.
[b] Number with percentage in parentheses.
[c] Number of patients (nonsmokers / smokers).
[d] Statistically tested with the two-sided Kruskal-Wallis test, unless stated otherwise.
[e] Statistically tested with the χ^2-test (variable categories *vs.* smoking status).
[f] Percentage decrease nadir to baseline
Abbreviations: N, number; CTC, National Cancer Institute's Common Terminology Criteria for Adverse Events version 4.

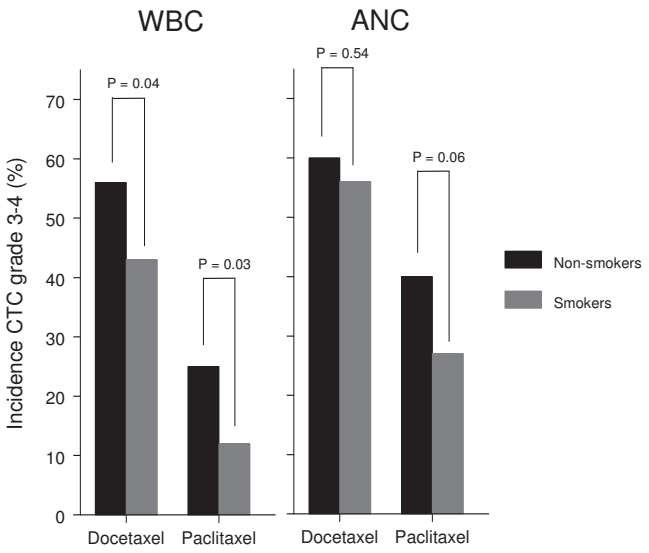

Figure 1. Incidence of NCI-CTC, version 4, grade III-IV WBC and ANC during docetaxel (n = 288) and paclitaxel (n = 273) treatment in nonsmokers and smokers. *P* values are calculated with univariate χ^2 tests.

Smoking in relation to taxane pharmacokinetics

There was no significant difference in docetaxel and paclitaxel clearance between smokers and nonsmokers. The clearance in patients treated with docetaxel was 39 L/h (range, 3.6-75 L/h) in smokers and 39 L/h (range 6.1-85 L/h) in nonsmokers. In the patients treated with paclitaxel, the unbound clearance was 463 L/h in smokers (range, 138-906 L/h) and 450 L/h (range, 157-1,037 L/h) in nonsmokers (Table 4).

Table 4. Effect of smoking behavior on docetaxel and paclitaxel pharmacokinetic parameters

Parameter [a]	N [b]	All patients	Nonsmokers	Smokers	P [c]
Docetaxel					
CL (L/h)	215/75	39 (3.6-85)	39 (6.1-85)	39 (3.6-75)	1.0
Paclitaxel (Unbound)					
CL Unbound (L/h)	214/62	457 (138-1037)	450 (157-1037)	463 (138-906)	0.8

[a] All data are represented as median with range in parentheses.
[b] Number of patients (nonsmokers / smokers).
[c] Statistically tested with the two-sided Kruskal-Wallis test
Abbreviations: N, number; CL, clearance, i.e., dose divided by AUC

DISCUSSION

This is the first study assessing the effects of smoking on the side effects and pharmacokinetics of taxane treatment in a large group of patients with cancer. Cigarette-smoking patients treated with docetaxel or paclitaxel appeared to have less drug-related neutropenia and leukopenia than nonsmokers, which is not explained by altered systemic exposure to these drugs, but could possibly be explained by a significant higher baseline WBC and ANC values in paclitaxel-treated patients and a trend towards higher baseline WBC in docetaxel-treated patients.

The difference in baseline WBC and ANC values between smokers and nonsmokers in paclitaxel-treated patients seen in our study is in line with literature data on healthy humans, where it has extensively been reported that smokers have higher baseline WBC and ANC values.[32-34] A possible explanation for this effect is that when alveolar macrophages are stimulated by cigarette smoke constituents, they produce proinflammatory markers such as TNF-α, interleukin (IL)-1, IL-6, IL-8 and several hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF).[33,35] The production of these factors results in increased proliferation and accelerated release of leucocytes from the bone marrow, resulting in a higher leukocyte count.[33] Apart from WBCs, other inflammatory markers such as C-reactive protein and fibrinogen are known to be elevated in healthy smokers.[36] As we did not study these parameters we cannot confirm these findings.

It has been reported that neutropenia and leukopenia experienced during the course of chemotherapy is independently associated with improved survival in different tumor types.[37-38] Therefore, the higher WBC and ANC found in smokers in our study could indicate a weaker biological effect of taxane treatment. Also, higher pretreatment neutrophil counts have been independently associated with shorter overall survival and progression free survival,[39-40] although the underlying mechanism is not clarified yet. These findings highlight the importance of smoking cessation in patients with cancer.

This study was designed to evaluate the effects of smoking on taxane pharmacokinetics. Therefore, patients who quit smoking more then 4 weeks before pharmacokinetic sampling were classified as nonsmokers. However, the higher baseline blood counts in smokers can persist for several years after quitting smoking,[32,41] which might potentially introduce misclassification in smoking status in the toxicity analysis. However, this misclassification is expected to be limited because many patients with cancer continue to smoke after diagnosis,[42] or already stopped smoking for other reasons that the discovery of a malignancy. In addition, this misclassification would only lead to an underestimation (instead of overestimation) of the effect of smoking on hematological counts before start of therapy. Also, nonsmokers who started smoking after pharmacokinetic sampling could potentially bias the results because the time course of de-induction of CYP enzymes is not precisely known. However, we expect this group to be very small or nonexisting at all.

A history of smoking appeared to reduce the incidence of gemcitabine-related severe neutropenia in more than 100 patients with cancer in a study by Kanai and colleagues.[43] Only 24% of their smoking patients (including ex-smokers) were found to have grade III-IV neutropenia after gemcitabine treatment, whereas in nonsmokers, this was 56%, suggesting a protective effect,[43] in line with our results. Van Erp and colleagues found no protective effect on hematological toxicities in smokers treated with imatinib.[44] Limiting factors of this study are that imatinib infrequently induces neutropenia and that it was a small study including only 15 smokers.

It is known that constituents of cigarette smoke are potent inducers of several drug-metabolizing enzymes. Cigarette smoke can therefore potentially modify the pharmacokinetics and clinical effects of certain drugs. The pharmacokinetics and pharmacodynamics of drugs mainly metabolized by CYP1A are known to be influenced by smoking.[5-7] Smoking has also been suggested to induce CYP3A in 2 *in vitro* studies[45,46] and in a small clinical study on quinine – a known CYP3A substrate.[47] Other studies, however, have not confirmed the influence of smoking on CYP3A.[48-50] Our group studied the effects of smoking on the adverse effects of irinotecan, which has a complex metabolism, including CYP3A involvement, but also other metabolic routes are involved.[9] In agreement with the findings reported here, in that study, a reduced incidence of hematological toxicity was observed in smokers. These patients had significantly less grade III-IV leukopenia and neutropenia than nonsmokers when treated with single agent irinotecan. In that study, the lower incidence of hematological toxicities in smokers was partly explained by significantly lower systemic exposure to irinotecan and its active metabolite SN-38, which might be related to altered CYP3A-mediated metabolism.[9] However, influences of other mechanisms, including ATP-binding cassette transporters responsible for the transport of irinotecan and its metabolites, and variation in uridine diphosphate glucuronosyltransferase 1A1 activity which is involved in the glucuronidation of SN-38, cannot be ruled out.

In conclusion, smoking does not alter the pharmacokinetic parameters of docetaxel and paclitaxel. It is therefore unlikely that smoking influences the CYP3A metabolism of drugs. In our study, smokers had less leukopenia and neutropenia than nonsmokers. Further research is warranted to clarify the underlying mechanism of this potential protective effect of smoking on hematological toxicities in taxane therapy.

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3

CYP3A4*22 GENOTYPE AND SYSTEMIC EXPOSURE AFFECT PACLITAXEL-INDUCED NEUROTOXICITY

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ABSTRACT

Purpose

Paclitaxel is used for the treatment of several solid tumors and displays a high inter-individual variation in exposure and toxicity. Neurotoxicity is one of the most prominent side-effects of paclitaxel. This study explores potential predictive pharmacokinetic and pharmacogenetic determinants for the onset and severity of neurotoxicity.

Experimental design

In an exploratory cohort of patients (n = 261) treated with paclitaxel, neurotoxicity incidence and severity, pharmacokinetic parameters and pharmacogenetic variants were determined. Paclitaxel plasma concentrations were measured by HPLC or LC-MS/MS, and individual pharmacokinetic parameters were estimated from previously developed population pharmacokinetic models by non-linear mixed effects modeling (NONMEM). Genetic variants of paclitaxel pharmacokinetics tested were *CYP3A4*22*, *CYP2C8*3*, *CYP2C8*4*, and *ABCB1 3435 C>T*. The association between *CYP3A4*22* and neurotoxicity observed in the exploratory cohort was validated in an independent patient cohort (n = 239).

Results

Exposure to paclitaxel ($_{log}$AUC) was correlated with severity of neurotoxicity ($P < 0.00001$). Female *CYP3A4*22* carriers were at increased risk of developing neurotoxicity ($P = 0.043$) in the exploratory cohort. *CYP3A4*22* carrier status itself was not associated with pharmacokinetic parameters (CL, AUC, C_{max}, or $T_{>0.05}$) of paclitaxel in males or females. Other genetic variants displayed no association with neurotoxicity. In the subsequent independent validation cohort, *CYP3A4*22* carriers were at risk of developing grade 3 neurotoxicity (odds ratio = 19.1; $P = 0.001$).

Conclusions

Paclitaxel exposure showed a relationship with the severity of paclitaxel-induced neurotoxicity. In this study, female *CYP3A4*22* carriers had increased risk of developing severe neurotoxicity during paclitaxel therapy. These observations may guide future individualization of paclitaxel treatment.

TRANSLATIONAL RELEVANCE

The chemotherapeutic agent paclitaxel is known for its small therapeutic window and large inter-individual variability in metabolism and toxicity profile. Peripheral neuropathy is a severe adverse event frequently seen during paclitaxel therapy. Pharmacogenetic and pharmacokinetic determinants have been suggested as predictive factors for this severe toxicity and could therefore potentially identify patients at risk. However, contradictory findings have been reported on the influence of genetic variants on the development of neurotoxicity. Also, the influence of pharmacokinetics on this potentially dose-limiting side-effect has not been studied in large cohorts of patients before. Furthermore, associations between the newly discovered *CYP3A4*22* polymophism and development of neurotoxicity during paclitaxel therapy has not been explored yet. More knowledge of factors that may predict neurotoxicity prior to taxane treatment could ultimately help choosing the appropriate therapy and dose for the individual patient.

INTRODUCTION

Paclitaxel is a highly active anti-microtubular agent used for the treatment of various solid tumors and has a large inter-patient variability in pharmacokinetics and toxicity.[1] Neurotoxicity is frequently observed during paclitaxel treatment and is often dose-limiting. The degree of neurotoxicity is highly variable between individual patients.[2,3] Axonal degeneration and demyelization are the primary underlying causes of this neurotoxicity.[4]

Genetic variants in enzymes involved in paclitaxel metabolism could contribute to inter-individual differences in toxicity and efficacy of paclitaxel treatment. Paclitaxel is metabolized by cytochrome 450 (CYPs) enzymes CYP2C8 and CYP3A4.[5,6] Recently, a new intron 6 single nucleotide polymorphism (SNP), encoding the *CYP3A4*22* variant allele, was discovered. This variant allele is associated with decreased CYP3A4 hepatic mRNA levels and consequently lower enzymatic activity.[7] *In vivo*, the *CYP3A4*22* variant allele was shown to be associated with altered therapeutic parameters in several CYP3A4 metabolized drugs (e.g., tacrolimus, simvastatin, and cyclosporine).[8-10]

The majority of patients treated with paclitaxel will develop peripheral neurotoxicity in the course of their treatment.[11] The incidence and severity of neurotoxicity has been associated with pharmacokinetic exposure parameters such as area under the curve (AUC), and time above total paclitaxel concentrations of 0.05 μmol/L ($T_{>0.05}$).[12] Mielke *et al* studied the association between paclitaxel pharmacokinetics and neurotoxicity in 24 patients and found that drug exposure (AUC x weeks of paclitaxel therapy) was higher in the group that developed neurotoxicity.[12] Furthermore, Green *et al* showed in 23 patients that paclitaxel pharmacokinetics and severity of neurotoxicity were correlated.[13] Studies in larger cohorts on the relationship between paclitaxel exposure and neurotoxicity have not been published so far.

The aim of the current study was to evaluate the influence of several SNPs in genes encoding drug metabolizing enzymes and transporters on the pharmacokinetics of

paclitaxel and development and severity of sensory neuropathy. In addition, we aimed to further clarify potential associations between paclitaxel pharmacokinetic parameters and the development and severity of peripheral neuropathy in a large cohort of patients.

PATIENTS AND METHODS

Patients

Exploratory and validation cohort

An exploratory cohort of cancer patients treated with paclitaxel for different tumor types within a prospective trial in which pharmacokinetics, pharmacodynamics and pharmacogenetics were studied (registered at www.trialregister.nl as NTR2311, ethics board study number MEC 03.264) were included in the exploratory cohort (n = 261). The influence of genetic variants on the pharmacokinetics and frequency and severity of paclitaxel-induced neurotoxicity were studied. The findings were subsequently validated in an independent cohort of paclitaxel-treated patients (n = 239) from whom whole blood for DNA analysis and neurotoxicity data were available (ethics board study number MEC 02.1002; this study involves a large data set of cancer patients who provided blood for DNA analysis for pharmacogenetic purposes). In this validation cohort the association between *CYP3A4*22* carrier status and development and severity of neuropathy were studied.

The inclusion criteria for the exploratory cohort were (i) histological or cytological confirmed diagnosis of cancer treated with paclitaxel, (ii) age 18 years or older, (iii) WHO performance score 0-1 and (iv) adequate hematopoietic, hepatic and renal functions. The use of CYP3A4 and CYP2C8 inducers or inhibitors was not allowed. In the validation cohort, patients were included if whole blood and neurotoxicity data were available. The trials were approved by the Ethics Board of the Erasmus University Medical Center and supported by the Dutch Cancer Society. All patients provided written informed consent prior to study participation.

Neurotoxicity

During the entire treatment course with paclitaxel, neurotoxicity was graded by the treating physician according to National Cancer Institute – Common Terminology Criteria for Adverse Events (NCI-CTCAE) criteria version 2-4. During each hospital visit the highest neurotoxicity score of the previous cycle was assessed. In both cohorts the highest neurotoxicity score during paclitaxel treatment was used in the analyses.

Pharmacokinetic analysis

Paclitaxel pharmacokinetics, using a validated limited sampling strategy, were assessed in up to three treatment cycles for each patient in the exploratory cohort. Pharmacokinetic sampling was allowed during any treatment cycle. Lithium heparin was used as anticoagulant for all samples. Paclitaxel was quantitated by a validated UV detection HPLC method [14] or by a validated LC-MS/MS method.[15]

Individual pharmacokinetic parameters were calculated based on measured plasma samples and a previously developed population pharmacokinetic model for paclitaxel.[16-18] Individual pharmacokinetic parameters were estimated as Empirical Bayes estimates within the non-linear mixed-effect modeling software NONMEM version 7 (Icon Development Solutions, Ellicott City, MD). AUCs were obtained by integrating the predicted concentration-time profile up to 96 h after start of the infusion. The time above 0.05 μmol/L ($T_{>0.05}$) was predicted for each patient.

Genotyping

Genomic DNA was isolated from 200 μL EDTA whole blood using MagnaPure LC (Roche Diagnostics GmbH, Mannheim, Germany). Genotyping was performed using TaqMan® (Applied Biosystems, Carlsbad, CA) assays for *CYP2C8*3* (rs10509681, C_25625782_20, 1196A>G), *CYP2C8*4* (rs1058932, C_361406_1, 792C>G), *ABCB1 3435 C>T* (rs1045642, C_7586657_20) and *CYP3A4*22* (rs35599367, C_59013445_10, intron 6 C>T), using 20 ng genomic DNA on the ABI PRISM 7500® fast real-time PCR Systems (Applied Biosystems) according to the manufactures instructions. Assays were validated by sequencing.

Expression of CYP3A4 in human dorsal root ganglia

Human dorsal root ganglia isolated from the lumbar position 4 (L4) were obtained from the National Disease Research Interchange (NDRI) and RNA was extracted using the RNEasy mini kit (Qiagen). Expression of *CYP3A4* was measured by qRT-PCR using SYBR green and the gene specific primers (Forward: 5'-CACAGATCCCCCTGAAATTAAGCTTA-3'; Reverse: 5'-AAAATTCAGGCTCCACTTACGGTG-3'). Gene expression was determined by C_t relative to the housekeeping gene, *GAPHD,* which was measured using a gene specific TaqMan probe (HS02758991_g1; Applied Biosystems).

Statistical analysis

Data are presented as medians with ranges, unless stated otherwise. To test whether patients with different grades of neurotoxicity had different PK parameters, the Kruskal-Wallis test was used. To study the relationship between genetic variants and severity of neurotoxicity, the Fisher exact test was used. To test the association between severity of neurotoxicity and *CYP3A4*22* carrier status, logistic regression was performed. The analysis was performed separately for males and females because of the reported gender difference in paclitaxel pharmacokinetic parameters.[19] To test if all studied genetic variants were in Hardy-Weinberg equilibrium, the chi-square test was used. A *P*-value below 0.05 was considered statistically significant. All statistical analyses were performed with SPSS (Armonk, NY) version 20.0.

RESULTS

Patients

Exploratory cohort

In the exploratory cohort, 261 patients (135 male, 126 female) were included. Median age was 61 years (range: 18-82 years) and 96% of patients were of Caucasians (Table 1). Esophageal cancer was the main diagnosis (46%) in this cohort. Patients were treated with a median dose of 180 mg paclitaxel during each cycle (range: 75-560 mg). The median cumulative dose in this cohort was 975 mg (range: 280-3,910 mg). In 7 patients genotyping could not be performed due to poor DNA quality.

Validation cohort

In the validation cohort, 239 patients (129 male, 110 female) were included. Median age was 63 years (range: 24-83 years) and 95% of patients were Caucasians. Most patients in this cohort were diagnosed with esophageal cancer (64%; Table 1). Patients were

Table 1. Patient characteristics[a]

Characteristic	Exploratory cohort	Validation cohort
Number of patients	261	239
Median age, years (range)	61 (18-82)	63 (24-83)
Gender, N (%)		
Male	135 (52)	129 (54)
Female	126 (48)	110 (46)
Ethnicity, N (%)		
Caucasian	250 (96)	226 (95)
Other	10 (4)	8 (4)
Unknown	1 (0)	5 (2)
Primary tumor site, N (%)		
Esophagus	121 (46)	152 (64)
Ovary	39 (15)	36 (15)
Cervix	18 (7)	6 (3)
Endometrial	15 (6)	6 (3)
Breast	13 (5)	26 (11)
Lung	12 (5)	2 (1)
Head/Neck	10 (4)	1 (0)
A(CUP)	9 (3)	4 (2)
Other	24 (9)	6 (3)

[a] Continuous data are given as median with range in parentheses, and categorical data are given as number of patients with percentage of the total population in parentheses.
Abbreviations: N, number; A(CUP), (adenoma)carcinoma of unknown origin.

treated with a median dose of 165 mg paclitaxel during each cycle (range: 70-480 mg). The median cumulative dose in this cohort was 1,140 mg (range: 200-2,975 mg). In 2 patients genotyping could not be performed due to poor DNA quality.

Paclitaxel dose

Patients in both cohorts received paclitaxel weekly or every 3 weeks in different combination regimens. Also patients receiving chemotherapy in combination with radiotherapy, as a preoperative regimen for resectable esophageal cancer, were included.[20] These patients received a weekly dose of 50 mg/m^2. The cumulative dose of paclitaxel did not differ between *CYP3A4*22* carriers and non-carrier in both cohorts together (P = 0.30). In the training set, the cumulative dose did not differ between *CYP3A4*22* carriers and non-carriers in males (P = 0.93) and females (P = 0.66). In the validation cohort, the cumulative dose was also not significantly different between *CYP3A4*22* carriers and non-carriers, both in males (P = 0.66) and females (P = 0.12).

Association pharmacokinetic parameters and development of toxicity

Exploratory cohort

Systemic exposure (AUC) of paclitaxel was significantly associated with severity of neurotoxicity in both females and males ($P \leq 0.001$; **Table 2**). Also, $T_{>0.05}$ and the maximum observed concentration after administration (C_{max}) were significantly associated with neurotoxicity ($P \leq 0.001$; Table 2). Paclitaxel exposure ($_{log}$AUC) and development and severity of neurotoxicity showed a relationship (R = 0.52; $P < 0.000001$).

Influence of genetic variants on neurotoxicity

All tested genetic variants were in Hardy-Weinberg equilibrium (**Suppl table 1**).

Exploratory cohort

In the exploratory cohort neurotoxicity was observed in 106 of 261 patients (41%). There were significantly more females than males who developed neurotoxicity (67% *vs.* 33%; $P < 0.0001$). In this cohort, severity of neurotoxicity was differently distributed between female *CYP3A4*22* carriers and non-carriers (P = 0.043), while male *CYP3A4*22* carriers and non-carriers had an even distribution of neurotoxicity (P = 0.90; **Table 3**). The other tested SNPs showed no association with severity of neurotoxicity (**Table 4**). *CYP3A4*22* carrier status in both males and females was not associated with pharmacokinetic parameters (unbound CL, AUC, $T_{>0.05}$ and C_{max}) of paclitaxel (data not shown). There was no influence of *CYP2C8*3* or *CYP2C8*4* carrier status on pharmacokinetics of paclitaxel (data not shown). Furthermore, the *ABCB1 3435C>T* SNP was also not associated with paclitaxel pharmacokinetics (data not shown). Cumulative dosages of patients with grade 3 neurotoxicity are summarized in **Table 5**.

Validation cohort

To confirm the relationship observed in the exploratory cohort between *CYP3A4*22* carrier status and severity of neurotoxicity, we studied this association in an independent

Table 2. Associations between pharmacokinetic parameters and neurotoxicity

	Neurotoxicity CTCAE				
PK parameters[a]	Grade 0	Grade 1	Grade 2	Grade 3	P-value[b]
Weekly schedule (N)	109	29	3	-	
AUC (ngxh/ml)	2.3 (1.3-11.7)	5.0 (1.6-11.3)	6.1 (5.6-6.2)	-	0.001
$T_{>0.05}$ (h)	7.6 (3.5-29.3)	12.9 (4.0-30.4)	15.2 (13.7-16.3)	-	0.007
C_{max} (ng/mL)	1,394 (174-7,860)	2,896 (850-4,656)	4,370 (3,500-4,431)	-	<0.0001
3-Weekly schedule (N)	46	57	15	2	
AUC (ngxh/ml)	11.6 (2.9-26.1)	13.9 (3.5-25.4)	14.1 (8.8-24.8)	16.5 (15.9-17.0)	0.003
$T_{>0.05}$ (h)	21.4 (8.3-31.8)	24.6 (12.7-32.8)	23.8 (18.2-32.1)	29.0 (25.3-32.7)	0.005
C_{max} (ng/mL)	3,211 (495-8,420)	4,346 (496-8,313)	3,991 (1,922-8,713)	5,033 (4,180-5,886)	0.001
Female (N)	55	57	12	2	
AUC (ngxh/ml)	4.5 (1.3-21.0)	13.3 (3.3-24.7)	13.5 (6.1-20.5)	16.5 (15.9-17.0)	<0.00001
CL (L/h)	466 (157-696)	394 (228-654)	402 (191-636)	193 (186-200)	0.003
$T_{>0.05}$ (h)	14.8 (3.7-29.3)	22.9 (9.2-32.2)	23.4 (15.2-28.9)	29.0 (25.3-32.7)	<0.00001
C_{max} (ng/mL)	1,602 (673-7,107)	3,989 (886-7,726)	4,401 (2,706-6,143)	5,033 (4,180-5,886)	<0.00001
Male (N)	100	29	6	-	
AUC (ngxh/ml)	2.5 (1.4-26.1)	6.2 (1.6-25.4)	13.7 (5.6-24.8)	-	0.0001
CL (L/h)	539 (142-1,037)	541 (273-886)	494 (278-550)	-	0.3
$T_{>0.05}$ (h)	8.3 (3.5-31.8)	15.4 (4.0-32.8)	22.9 (13.7-32.1)	-	0.001
C_{max} (ng/mL)	1,409 (174-8,420)	3,916 (496-8,313)	3,694 (1,922-8,713)		0.0001

[a] Data are represented as median with ranges

[b] P-values < 0.05 represents differentially distributed pharmacokinetic values between grades of neurotoxicity and are calculated with the Kruskal-Wallis test

Abbreviations: CTCAE, National Cancer Institute's Common Terminology Criteria for Adverse Events version 2-4; PK, pharmacokinetic; N, number; AUC, area under the curve; $T_{>0.05}$, time above 0.05 µmol/L; $C_{max,}$ maximum concentration.

Table 3. Association between CYP3A4*22 and neurotoxicity[a]

	No. of patients	Neurotoxicity CTCAE grade 0	Neurotoxicity CTCAE grade 1	Neurotoxicity CTCAE grade 2	Neurotoxicity CTCAE grade 3	P-value[b]
Exploratory Cohort	254					
Female	122					
C/C	105	50 (48)	46 (44)	8 (8)	1 (1)	
C/T+T/T	17	4 (24)	8 (47)	4 (24)	1 (6)	0.043
Male						
C/C	114	85 (75)	24 (21)	5 (4)	-	
C/T + T/T	18	13 (72)	4 (22)	1 (6)	-	0.90
Validation Cohort	237					
Female	110					
C/C	98	30 (31)	53 (54)	13 (13)	2 (2)	
C/T+T/T	12	6 (50)	4 (33)	-	2 (17)	0.036
Male	127					
C/C	113	80 (71)	28 (25)	5 (4)	-	
C/T + T/T	14	8 (57)	4 (29)	-	2 (14)	0.025

[a] All data are represented as absolute number with percentage in parentheses, unless stated otherwise
[b] P-values < 0.05 represents differentially distributed neurotoxicity scores between non-carriers and carriers of the variant allele and are calculated with the Fisher exact test.
Abbreviations: CTCAE, National Cancer Institute's Common Terminology Criteria for Adverse Events version 2-4

validation cohort. In this cohort, 113 of 239 patients (47%) developed neurotoxicity. Significantly more females than males developed neurotoxicity (65% *vs.* 29%; $P < 0.0001$). In this cohort, in both females and males, the grade of neurotoxicity was differently distributed in *CYP3A4*22* carriers than in *CYP3A4*22* non-carriers ($P = 0.036$ and $P = 0.025$, respectively; Table 3). The risk of developing grade 3 neurotoxicity was higher in *CYP3A4*22* carriers than in non-carriers (odds ratio = 19.1; $P = 0.001$; 95% confidence interval = 3.3-110), confirming the observation in females in the exploratory cohort and showing this time a comparable effect in males. Cumulative dosages of patients with grade 3 neurotoxicity are summarized in Table 5.

Additional exploratory analysis

Grade 3 neurotoxicity may be a result of the cumulative dose of paclitaxel, and is a reason to discontinue paclitaxel treatment. Therefore we also performed an exploratory Cox regression analysis in patients of both cohorts together because of the small number of neurotoxicity grade 3, taking cumulative dose into account. In this analysis, the occurrence of grade 3 neurotoxicity was included as the event, while the cumulative dose of paclitaxel was included as the time-to-event variable. The

Table 4. Associations between polymorphisms and neurotoxicity[a]

Gene and Variant	No. of patients	Neurotoxicity CTCAE				*P*-value[b]
		Grade 0	Grade 1	Grade 2	Grade 3	
CYP2C8*3	254					
Female	122					
*1/*1	92	41	40	9	2	
*1/*3 + *3/*3	30	13	14	3	-	1.0
Male	132					
*1/*1	105	79	21	5	-	
*1/*3 + *3/*3	27	19	7	1	-	0.84
CYP2C8*4	250					
Female	119					
*1/*1	108	47	48	11	2	0.65
*1/*4	11	7	4	-	-	
Male	131					
*1/*1	119	88	25	6	-	
*1/*4	12	10	2	-	-	1.0
ABCB1 3435 C>T	255					
Female	122					
C/C	30	16	13	1	-	
C/T	57	23	27	6	1	
T/T	35	15	14	5	1	0.69
Male	133					
C/C	36	26	8	2	-	
C/T	63	43	17	3	-	
T/T	34	30	3	1	-	0.24

[a] All data are represented as absolute number with percentage in parentheses, unless stated otherwise
[b] P-values < 0.05 represents differentially distributed neurotoxicity scores between non-carriers and carriers of the variant allele and are calculated with the Fisher exact test.
Abbreviations: CTCAE, National Cancer Institute's Common Terminology Criteria for Adverse Events version 2-4

prognostic impact of CYP3A4*22 was then evaluated, adjusted for cohort and gender. Again, neurotoxicity grade 3 was more often seen in *CYP3A4*22* carriers (hazard ratio = 22.1, 95% confidence interval = 4.7-105, P < 0.001).

Expression of CYP3A4 in human dorsal root ganglia

We found that CYP3A4 was expressed in human dorsal root ganglia in two separate patient samples as demonstrated by amplified products that were detected by qRT-PCR (**Figure 1**). CYP3A4 transcripts were expressed with a C_t value of 28.71 ± 0.074 in dorsal

Table 5. Patients with grade 3 neurotoxicity

Patient ID	Cohort	Gender	Age	Tumor type	CYP3A4*22	Cumulative Dose
1	Training	Female	54	Lung	CT	960
2	Training	Female	65	Ovarium	CC	2880
3	Validation	Male	64	Esophagus	CT	1060
4	Validation	Male	70	Esophagus	CT	1940
5	Validation	Female	25	Breast	CC	710
6	Validation	Female	46	Breast	CC	2635
7	Validation	Female	62	Breast	CT	1305
8	Validation	Female	71	Esophagus	CT	1760

root ganglia of patient 1 and 28.27 ± 0.009 in the dorsal root ganglia of patient 2, relative to the control gene, GAPDH, which was expressed with a C_t value of 21.96 ± 0.008 in dorsal root ganglia of patient 1 and 25.40 ± 0.090 in the dorsal root ganglia of patient 2.

DISCUSSION

In this study, we showed that systemic exposure to paclitaxel was highly correlated with the development of (severe) neurotoxicity. Importantly, systemic exposure to paclitaxel measured during one course is already predictive for both development and severity of neuropathy in males and females. This result is in line with the study of Mielke *et al* who observed that the time above the threshold of 0.05 μmol/L paclitaxel was associated with development of neuropathy[12] and the study of Green *et al*, reporting a relationship between paclitaxel exposure and neurotoxicity.[13]

In addition, we showed that females carrying the reduced function *CYP3A4*22* variant allele had an increased risk of developing severe neurotoxicity. This was demonstrated in our exploratory cohort, and subsequently confirmed in the independent validation cohort. Interestingly, in the exploratory cohort only female carriers of *CYP3A4*22* were found to have an increased risk of neurotoxicity, whereas in the validation cohort there was an increased risk of grade 3 neuropathy in both males and females carrying the *CYP3A4*22* allele. It should be noted that the low incidence of grade 3 neurotoxicity in our cohort makes the absolute risk of developing neurotoxicity during paclitaxel treatment difficult to interpret. The lack of statistical significance in the male *CYP3A4*22* carriers in the exploratory cohort could possibly be explained by the fact that there were no male patients with grade 3 neurotoxicity in this cohort. Because of the observed discrepancy between exploratory and validation cohorts, it is not yet possible to present a conclusion on the risk of neurotoxicity for male *CYP3A4*22* carriers.

Recently, it was shown that taxane-induced neuropathy is not a pharmacodynamic marker of treatment outcome.[21] Therefore, a predictive marker for neuropathy during

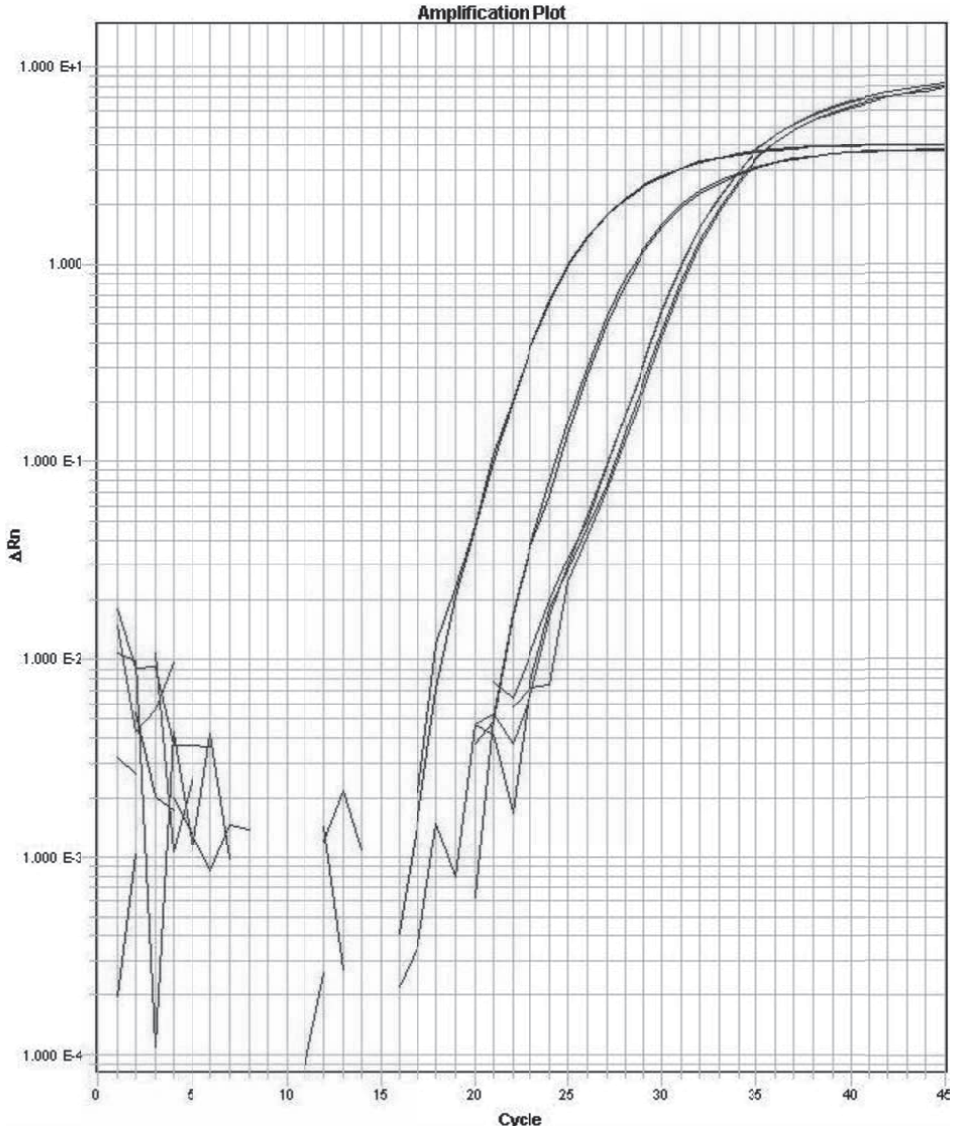

Figure 1. Amplification plot of CYP3A4 and GAPDH as determined by qRT-PCR from cDNA prepared from the L4 dorsal root ganglia isolated from 2 separate patients. Cycle is represented by the number of PCR cycles required for product amplification while ΔRn defines the fluorescent signal intensity for SYBR Green (CYP3A4) or Taqman probe (GAPDH).

paclitaxel therapy could be of particular clinical usefulness. *CYP3A4*22* carrier status has the potential to aid medical oncologists in selecting female patients sensitized to development of neurotoxicity during paclitaxel therapy. It would be clinically relevant to predict grade 3 (or higher) neurotoxicity because this toxicity often leads

to dose reductions or preliminary discontinuation of paclitaxel therapy. For a patient in whom severe neurotoxicity should absolutely be avoided (*e.g.* those with disabling peripheral neurological disorders, or those with pre-existing neuropathy from previous chemotherapy), pre-treatment knowledge of the *CYP3A4*22* carrier status might help choosing the appropriate (chemo-) therapy for an individual patient. If alternative drugs are available, these patients should preferably not be exposed to paclitaxel.

In this study, systemic pharmacokinetic parameters did not differ between *CYP3A4*22* carriers and non-carriers. This is in contrast with altered tacrolimus pharmacokinetics observed in *CYP3A4*22* carriers[9] and increased cholesterol reduction in simvastatin treated patients who are *CYP3A4*22* carriers.[8] It is also in contrast to the increased risk of delayed graft function and worse creatinine clearance in cyclosporine-treated kidney patients who carry the *CYP3A4*22* allele.[10] This discrepancy could possibly be due to the fact that CYP3A4 in the liver is only a minor elimination pathway of paclitaxel when compared to CYP2C8, which indeed has a 2.3-fold greater metabolite production than CYP3A4.[22] However, none of the CYP2C8 SNPs nor *ABCB1 C3435T* showed an association with paclitaxel pharmacokinetics or the development of paclitaxel-induced neuropathy in our study. These findings are in line with several other pharmacogenetic studies in paclitaxel treated patients.[1,23] Bergmann and colleagues also did not find an association between *CYP2C8*3*, and *ABCB1 C3435T* and sensory neuropathy and overall survival in ovarian cancer patients.[24] More recently, these authors reported that paclitaxel clearance was 11% lower in *CYP2C8*3* carriers than in non-carriers.[25] In our study, we did not observe pharmacokinetic differences between patients, also not in a subgroup analysis of ovarian cancer patients (data not shown). We also could not confirm the findings by Leskala *et al* and Green *et al*, who reported an association between *CYP2C8*3* and neurotoxicity in patients treated with paclitaxel.[13,26] Because of the discrepancy in results in these studies, the potential of genetic variants to predict individual paclitaxel pharmacokinetics is still under debate. We are currently performing a large study associating 1,936 relevant SNPs in drug metabolizing enzymes and transporters (DMET) with paclitaxel pharmacokinetics to elucidate this issue further.

A higher systemic exposure to paclitaxel could not explain the higher incidence of neurotoxicity seen in *CYP3A4*22* carriers. Therefore, a possible explanation might be that the effect of the *CYP3A4*22* SNP is not systemic but localized in the peripheral neurons. Gosh and colleagues suggested a potential cytoprotective role for *CYP3A4* in central nerves.[27,28] It was already known that CYP3A4 is expressed by endothelial cells in the blood brain barrier,[27] but these authors observed that CYP3A4 was expressed in approximately 75% of neurons of epileptic brain tissue.[28] In CYP3A4 transfected cells, incubated with toxic concentrations of carbamazepine, a remarkably reduced cell death was observed, suggesting a cytoprotective effect of CYP3A4.[28] In the current study, we found that CYP3A4 is also expressed in peripheral nerves, in particular dorsal root ganglia, and this could explain the possible cytoprotective mechanism against toxic CYP3A4 substrates, such as paclitaxel. This localization of CYP3A4 in peripheral neurons provides a potential mechanistic explanation for the observation that female carriers

of the *CYP3A4*22* variant allele, which is associated with reduced CYP3A4 function, have a higher risk to develop severe neuropathy in our study. The observation that CYP3A4*22 is also expressed in peripheral neurons is only an indication that CYP3A4 might protect against neurotoxicity during paclitaxel therapy. It is, however, too early to provide a mechanistic explanation for our observations. Further research into the underlying biological principles of this potential protective role of CYP3A4 is needed.

Unfortunately, multivariate analyses were not warranted in both cohorts because of the relatively low incidence of grade 3 neurotoxicity. Therefore, we cannot exclude the possibility that the effect of *CYP3A4*22* on neurotoxicity is influenced by confounders. Therefore, our preliminary findings have to be validated in future research to explore the clinical potential of *CYP3A4*22* as a marker for development of neurotoxicity.

Recently, several other SNPs identified in large genome wide association studies (GWAS) were associated with paclitaxel-induced neuropathy. Schneider *et al*, presented results of an interim analysis of their E5103 phase III trial, comparing chemotherapy plus concurrent bevacizumab, or chemotherapy with concurrent and sequential bevacizumab, as adjuvant treatment for early stage breast cancer.[29] They found that SNPs in RWDD3 (rs2296308) and TECTA (rs1829) were associated with the time of first reporting ≥grade 2 neuropathy. Not much is known about RWDD3 or TECTA, but involvement of TECTA in sensoric hear loss and cellular stress has been suggested,[30] making an association with the development of neuropathy biologically plausible. Bergmann *et al* aimed to validate these findings in an independent cohort but could not confirm any association between these SNPs and time to neurotoxicity.[31] In another GWAS, Baldwin *et al* found a SNP in FGD4 (rs10771973) to be associated with the onset of peripheral neuropathy and validated this finding in an independent European and African American cohort.[32] In this study, there was also evidence that two other markers in EPHA5 (rs7349683) and FZD3 (rs7001034) were associated with onset or severity of paclitaxel-induced neuropathy.

Neurotoxicity is not only a side effect attributable to taxanes, but is also frequently seen in treatments with several other drugs metabolized by CYP3A4. For example, bortezomib and thalidomide, used for the treatment of multiple myeloma, have incidences of grade ≥3 neurotoxicity of 8% and 5%, respectively.[33] Also, vincristine[34] and ixabepilone[35] have been reported to frequently cause severe peripheral neuropathy. Therefore, further clinical research should elucidate the possible effects of *CYP3A4*22* carrier status on the development of neurotoxicity during treatment with these agents.

In conclusion, we identified a relationship between *CYP3A4*22* carrier status in women and occurrence of neurotoxicity during paclitaxel therapy. In our study, female carriers of *CYP3A4*22* had an increased risk of neurotoxicity, although paclitaxel pharmacokinetics profiles were similar to those of non-carriers. This novel SNP could potentially be used as a predictive factor for paclitaxel-induced neurotoxicity in females, but further research is necessary to confirm our preliminary findings. Also, the predictive value of *CYP3A4*22* carrier status in other CYP3A4-metabolized drugs remains to be established.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Hardy Weinberg equilibrium of studied SNPs

Exploratory cohort	Chi-square	*P*-value
CYP3A4*22	0.37	0.54
CYP2C8*3	0.76	0.38
CYP2C8*4	0.58	0.45
ABCB1 3435 C>T	0.88	0.35
Validation cohort		
CYP3A4*22	0.80	0.37

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4

A PHARMACOGENETIC PREDICTIVE MODEL FOR PACLITAXEL CLEARANCE BASED ON THE DMET PLATFORM

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ABSTRACT

Purpose

Paclitaxel is used to in the treatment of solid tumors and displays high inter-individual variation in exposure. Low paclitaxel clearance (CL) could lead to increased toxicity during treatment. We present a genetic prediction model identifying patients with low paclitaxel CL, based on the Drug-Metabolizing Enzyme and Transporter (DMET)-platform, capable of detecting 1,936 SNPs in 225 metabolizing enzyme and drug transporter genes.

Experimental design

In 270 paclitaxel-treated patients, unbound plasma concentrations were determined and pharmacokinetic parameters were estimated from a previously developed population pharmacokinetic model (NONMEM). Patients were divided into a training and validation set. Genetic variants determined by the DMET platform were selected from the training set to be included in the prediction model when they were associated with low paclitaxel CL (1 SD below mean CL) and subsequently tested in the validation set.

Results

A genetic prediction model including 14 SNPs was developed on the training set. In the validation set, this model yielded a sensitivity of 95%, identifying most patients with low paclitaxel CL correctly. The positive predictive value of the model was only 22%. The model remained associated with low CL after multivariate analysis, correcting for age, gender and hemoglobin levels at baseline ($P = 0.02$).

Conclusions

In this first large-sized application of the DMET-platform for paclitaxel, we identified a 14 SNP model with high sensitivity to identify patients with low paclitaxel CL. However, due to the low positive predictive value we conclude that genetic variability encoded in the DMET-chip alone does not sufficiently explain paclitaxel CL.

TRANSLATIONAL RELEVANCE

During the last 20 years, paclitaxel has become a standard cytotoxic drug in the treatment of several malignancies, such as breast, ovarian and non-small cell lung cancer. However, due to its wide inter-patient variability in plasma exposure, efficacy and toxicity profiles of this compound are still quite unpredictable for the individual patient. Dose-limiting toxicities resulting from high systemic concentrations could lead to prematurely discontinuation of treatment or to treatment-related hospitalisations. It is therefore important to identify patients at risk for (extremely) high exposures to the drug, allowing these patients to be monitored more closely or to apply dose reductions. In the current study, we explored the potential association of 1,936 genetic polymorphisms in 225 drug metabolizing enzyme and drug transporter genes (DMET-platform) with paclitaxel unbound clearance.

INTRODUCTION

Paclitaxel is a highly active anti-cancer drug with a broad spectrum of activity. It is used in the treatment of several solid tumors, such as breast, ovarian, and non-small-cell lung cancer. Paclitaxel stabilizes cellular microtubules and thereby blocking chromosomal segregation and mitosis, eventually inducing apoptosis.[1]

Paclitaxel is metabolized in the liver by cytochrome P450 (CYP) CYP2C8 and 3A4. The anion organic transporting polypeptide OATP1B3 was identified as an important influx transporter,[2] while the efflux of paclitaxel was shown to be mediated by ABCB1 (P-glycoprotein) and ABCC2 transporters.[3,4] The pharmacokinetics of this agent are known for its large inter-individual variability, which could have important safety consequences and may also affect treatment outcome. The source of this large variability remains to be elucidated.

It has been suggested that genetic variation in the genes involved in the metabolism of paclitaxel could explain part of the mentioned variability. As a candidate gene approach, SNPs in *CYP2C8*3*, *CYP3A5* and *ABCB1* (*3435C>T*, *2677G>T* and *1236C>T*) have been tested, but these studies yielded contradictory results.[5-10]

Another approach to study genetic variants that could potentially influence paclitaxel pharmacokinetics and toxicity is by using a more broad approach, such as the Drug-Metabolizing Enzyme and Transporter (DMET) genetic platform (Affymetrix). This platform includes all potentially important SNPs for drug metabolism, and investigates 1,936 variants in 225 genes involved in drug metabolism and transport. The aim of this study was to develop a predictive signature for paclitaxel pharmacokinetics by use of the DMET genotyping platform.

PATIENTS AND METHODS

Patients

Patients treated with paclitaxel for different tumor types were included in a prospective trial studying pharmacokinetics, pharmacodynamics and pharmacogenetics (registered at www.trialregister.nl as NTR2311, study number MEC 03.264).

The inclusion criteria in this study were (*i*) a histological or cytological confirmed diagnosis of cancer treated with paclitaxel, (*ii*) aged 18 years or older, (*iii*) World Health Organization (WHO) performance score of 0 or 1 and (*iv*) adequate hematopoietic, hepatic and renal functions. CYP3A4 and CYP2C8 inducers and inhibitors were not allowed during the course of paclitaxel treatment. All patients provided written informed consent prior to study participation. The trial was approved by the medical ethical committee of the Erasmus University Medical Center.

Pharmacokinetic analysis

Paclitaxel pharmacokinetic limited sampling was performed one to three times during any cycle of paclitaxel therapy in each individual patient. For all samples lithium heparin was used as anticoagulant. A validated UV detection high-performance liquid chromatography (HPLC) method[11] or a validated LC-MS/MS method -- based on a method used for docetaxel[12] -- was used to quantitate paclitaxel in plasma. Measured plasma samples and a previously developed population pharmacokinetic model[13-15] were used to calculate paclitaxel pharmacokinetic parameters in each individual patient. It is known that the total fraction of paclitaxel does not have linear pharmacokinetics in contrast to the "free" systemic fraction. This is caused by the formulation vehicle of paclitaxel, Cremophor EL, which is responsible for disproportionate drug accumulation in the plasma fraction, and therefore causes a decrease in free circulating drug.[16,17] For this reason, we used "unbound" instead of total paclitaxel clearance in the analysis of this study. The individual pharmacokinetic parameters were estimated as Empirical Bayes estimates within the non-linear mixed-effect modeling software system NONMEM (version 7, Icon Development Solutions, Ellicott City, MD) and an area-under-the-curve (AUC) was obtained by integration of the predicted concentration-time profile up to 96h after the start of infusion.

SNP genotype analysis using the DMET platform

DNA of 293 patients was extracted from whole blood using MagnaPure LC (Roche Diagnostics GmbH) according to manufacturer's instructions. The Affymetrix DMET Plus Premier Pack (Affymetix, CA, USA) was used to genotype genomic DNA of all paclitaxel treated patients as described by Dumaual et al.[18] Genotypes of all single-nucleotide polymorphisms (SNPs) on the DMET assay were reported either as "call" or as "no call". Markers on the DMET assay with call rates less than 90% were excluded from analysis. After removal of duplicate measurements, patients with missing clearance data and removal of low-call rate assays, data for 270 patients were eligible for data analysis.

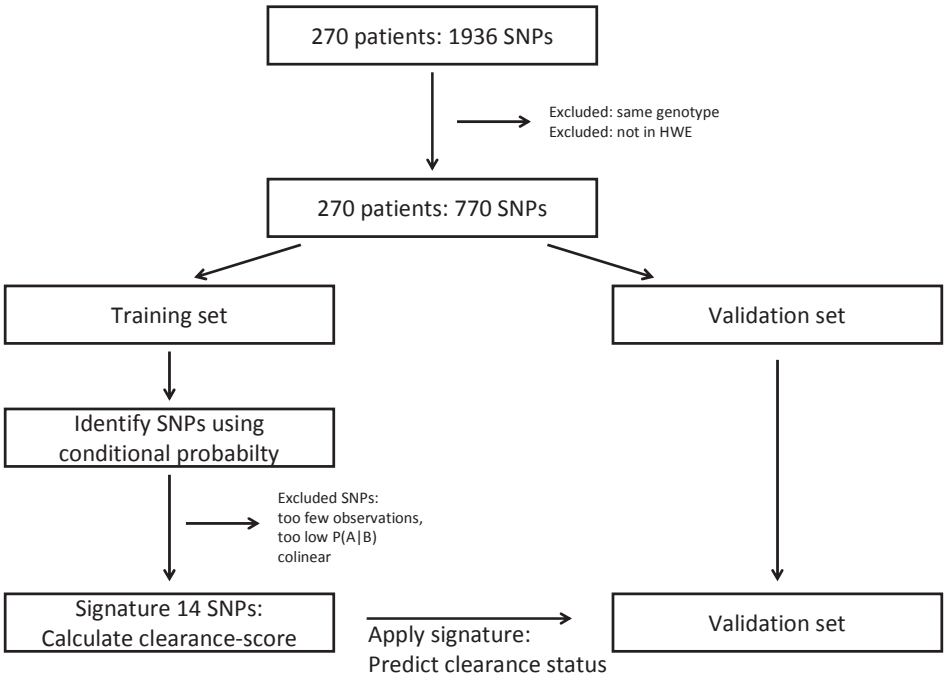

Figure 1. Flowchart of genetic marker selection.

Marker selection

As shown in the flowchart (**Figure 1**), SNPs were excluded from the analysis if the genotype was identical in all patients, leaving 1,048 SNPs reporting different genotypes for analysis. The remaining genotypes were tested for Hardy-Weinberg equilibrium and SNPs deviating from this equilibrium ($P > 0.05$) were excluded. This left 770 SNPs for the prediction analysis. The cohort of patients was split into a training and a validation set. To guarantee a sufficient number of minimal observations for analysis, each genotype (wild-type, heterozygote, or homozygote variant) of a particular SNP should occur in at least 5% of patients, which equals 7 patients in the training set. Identification of SNPs which were significantly associated with low clearance was performed using a Naïve Bayes formula in the training set. The conditional probability was estimated for each SNP:

$$P(A|B) = P(A \,\&\, B) / P(B).$$

Here, A is clearance, while B is the genotype. This expresses the chance of a patient having low clearance (coded as 1) while having a homozygote wild-type, a heterozygote, or a homozygote variant genotype. Each of the possible genotypes

has a probability of occurring, which is used as a weight in the prediction analysis. To select SNPs associated with paclitaxel clearance, a wild type or variant genotype should be present in at least half of the patients with low clearance in the training-set, and to have a P(A|B) > 0.2.

This resulted in the selection of 20 SNPs. Four SNPs (rs2359612, rs8050894, rs9934438, and rs9923231) were co-linear and belonged to the same gene (*VKORC1*). Of these SNPs, the SNP with the highest allelic frequency, rs9923231, was selected. Similarly, two other SNPs (rs7793861 and rs7797834) were co-linear in the CYP51A1 gene. Here, rs7797834 was selected because this SNP had the highest allelic frequency. Next, the remaining 16 SNPs were included in a multivariate logistic regression model. Each of these SNPs was added to the model one by one, and SNPs were included in the signature if they increased the likelihood ratio of the fitted model, leaving 14 SNPs for final analysis.

SNP signature

In developing the predictive model patients were divided in having either low clearance, thus assumed to have an increased risk of toxicity during paclitaxel therapy, or normal (or high) clearance. Low clearance was defined as a clearance one SD below the mean clearance of the entire cohort. Low clearance was coded as 1, the other clearances as 0. The 14 selected SNPs (Table 3) were used to build the SNP signature predicting low clearance in paclitaxel treated patients. For each of these SNPs, the conditional probabilities estimated in the training-samples were used. For each individual sample a total probability weighted score was calculated based on the sum of all 14 probabilities of having the selected SNPs. The scores of the samples in the training-cohort were associated with clearance in a ROC-curve. From this ROC-curve, we selected the threshold where all patients with truly low clearance were identified correctly (*i.e.* 100% sensitivity). Finally, the probability weighted scores of the samples from the validation-cohort were calculated and compared to the selected threshold. If the score was above the threshold the sample was predicted as "low clearance", otherwise as "rest". The predicted calls were then compared with the actual clearance data.

Statistics

Data are presented as median with ranges, unless stated otherwise. Differences between validation and training set were tested with the chi-square test for binary covariables and differences between validation and training set in continuous variables were tested with the Mann-Whitney test. Logistic regression was used to study the influence of covariables on the association between the SNP signature and a patient having low clearance. Variables tested in this model were age, gender and hemoglobine (Hb) levels before start of therapy because these variables have previously been shown to influence paclitaxel treatment. For example, male patients have higher paclitaxel metabolism than female patients.[19] Paclitaxel elimination is also negatively correlated with age.[19] Furthermore, Hb levels have been shown to be

a prognostic factor in cancer treatment.[20-22] *P*-values were all two-sided and *P*-values < 0.05 were considered statistically significant. Analysis were performed with STATA version 11 (StataCorp LP, College Station, TX) and SPSS version 20.0 (SPSS Inc, Armonk, NY).

RESULTS

Patients

In the analysis, 270 Caucasian patients treated with paclitaxel were included. This group was divided in a training (n = 140) and a validation set (n = 130), so that the numbers of patients in the low clearance category are equally distributed between the training and validation set. The training and validation set displayed the same patient characteristics (**Table 1**). The median age of the whole cohort was 61 years (range: 18-82 years) and esophageal cancer was the main diagnosis (49%). Patients were treated with a median dose of 170 mg (range: 50-560 mg). Patients received paclitaxel weekly or every 3 weeks in different combination regimens. Patients receiving a weekly dose of 50 mg/m^2 paclitaxel in combination with radiotherapy,

Table 1. Patient characteristics

Characteristic	All patients	Training set	Validation set
Number of patients	270	140	130
Median age, years (range)	61 (18-82)	61 (18-79)	61 (18-82)
Gender, N (%)			
Male	139(51.5)	74 (52.9)	65 (50)
Female	131 (48.5)	66 (47.1)	65 (40)
Median dose, mg (range)	170 (50-560)	170 (50-560)	168 (50-490)
Primary tumor site, N (%)			
Esophagus	131 (49)	68 (49)	63 (49)
Ovary	37 (14)	19 (14)	18 (14)
Cervix	20 (7)	10 (7)	10 (8)
Endometrial	13 (5)	8 (6)	5 (4)
Breast	18 (7)	8 (6)	10 (8)
Lung	9 (3)	5 (4)	4 (3)
Head/Neck	10 (4)	5 (4)	5 (4)
A(CUP)	8 (3)	4 (3)	4 (3)
Testis	6 (2)	2 (1)	4 (3)
Other	18 (7)	11 (8)	7 (5)

[a] Continuous data are given as median with range in parentheses, and categorical data are given as number of patients with percentage of the total population in parentheses.
Abbreviations: N, number; A (CUP), (adenoma) carcinoma of unknown origin.

Table 2. Paclitaxel clearance in both sets[a]

Paclitaxel clearance (L/h)[b]	All patients	N	Training set	N	Validation set	N
All patients	480 (138-1,037)	270	474 (138-1,037)	140	494 (239-618)	130
Low clearance-group	286 (138-339)	44	258 (138-328)	23	302 (239-339)	21
Not low clearance-group	512 (340-1,037)	226	505 (340-1,037)	117	518 (345-858)	109

[a] Data are represented as median with ranges
[b] Paclitaxel clearance in based on unbound concentrations
Abbreviations: N, number of patients

as a preoperative regimen for resectable esophageal cancer, were also included [23]. There was no statistical significant difference between the training and validation-set with respect to clearance (P = 0.66), gender (P = 0.64), tumor type (P = 0.98), smoking status (P = 0.56), hemoglobin levels (P = 0.31) or platelets counts (P = 0.72) at start of therapy, excluding potential selection bias between the training and validation set.

Genetic signature predicting low metabolism

The mean clearance of all patients was 488 ± 149 L/h. Therefore, the threshold for having low clearance was 339 L/h, which was 1 SD below the mean of the total cohort. In total 14 SNPs located on 11 different chromosomes were selected for the prediction model (Table 3). Included in the model were SNPs in the genes: *SLC22A11* (rs1783811), *GSTZ1* (rs7975), *SLC28A2* (rs1060896), VKORC1 (rs9923231), *PGAP3* (rs2952151), *CDA* (rs1048977), *EPHX1* (rs1051740), *CYP20A1* (rs1048013), *SLC6A6* (rs2341970), *CRIP3* (rs2242416), *GSTA4* (rs13197674), *AKAP9* (rs7785971), *CYP51A1* (rs7797834), and *CYP2D7P1* (rs28360521). The probabilities of having a homozygote wild-type, heterozygote, or homozygote variant for each SNP are listed in Table 3. The sum of these probabilities gives each patient a probability score and these scores were used to generate a ROC curve. From this curve a threshold was selected (2.12) at which point a 100% sensitivity was achieved to identify low-clearance patients in the training set. All patients with a probability score higher than 2.12 were scored as having low clearance and all patients with a probability score lower than 2.12 were scored as not having low clearance. Subsequently, we validated this predictive model in the validation cohort. With the cut off score of 2.12, 20 out of 21 patients with truly low clearance were predicted by the signature as having low clearance, yielding the model a sensitivity of 95% and a positive predictive value of 22% (Table 4). Patients with a positive SNP prediction model had an OR of 9.9 (95%CI 1.3-76.4; P = 0.028) of having low clearance. When tested in a multivariate logistic regression model to correct for the influence of age, gender and hemoglobin levels at start of therapy, the SNP prediction model was independently associated with low clearance (OR = 10.9; 95%CI 1.4-86.3; P = 0.024). None of the other tested variables significantly improved the model.

Table 3. Selected SNPs for the conditional probability gene signature and probability score

Genetic Variation	Gene (Chr)	SNP value = 0[a]	SNP value = 1[a]	SNP value = 2[a]	P(SNP=0| low clearance)[b]	P(SNP=1| low clearance)[c]	P(SNP=2| low clearance)[d]
rs1783811	SLC22A11 (Chr11)	AA	AG	GG	0.154	0.106	0.230
rs7975	GSTZ1 (Chr 14)	CC	CT	TT	0.205	0.16	0
rs1060896	SLC28A2 (Chr 15)	AA	AC	CC	0.213	0.133	0.105
rs9923231	VKORC1 (Chr16)	CC	CT	TT	0.235	0.119	0.136
rs2952151	PGAP3 (Chr17)	CC	CT	TT	0.203	0.136	0.111
rs1048977	CDA (Chr1)	CC	CT	TT	0.210	0.127	0.071
rs1051740	EPHX1 (Chr1)	CC	CT	TT	0.091	0.119	0.214
rs1048013	CYP20A1 (Chr2)	AA	AG	GG	0.226	0.106	0.2
rs2341970	SLC6A6 (Chr3)	CC	CT	TT	0.208	0.115	0.111
rs2242416	CRIP3 (Chr6)	AA	AG	GG	0.174	0.090	0.26
rs13197674	GSTA4 (Chr6)	CC	CT	TT	0.111	0.103	0.234
rs7785971	AKAP9 (Chr7)	AA	AT	TT	0.111	0.109	0.241
rs7797834	CYP51A1 (Chr7)	AA	AG	GG	0.241	0.095	0.167
rs28360521	CYP2D7P1 (Chr22)	CC	CT	TT	0.207	0.098	0.143

[a] SNP values as coded on the DMET platform
[b]Probability that a patient is homozygote wild type for each SNP, given that this patient has a clearance < 339.6 L/h
[c] Probability that a patient is heterozygote for each SNP, given that this patient has a clearance < 339.6 L/h
[d] Probability that a patient is homozygote for the variant allele for each SNP, given that this patient has a clearance < 339.6 L/h

Abbreviations: SLC22A1; solute carrier family 22 (organic anion/urate transporter) member 11, GSTZ1; glutathione transferase zeta 1, SLC28A2; solute carrier family 28, (sodium-coupled nucleoside transporter) member 2, VKORC1; Vitamin K epoxide reductase complex subunit 1, PGAP3; post-GPI attachement to proteins 3, CDA; cytidine deaminase, EPHX1; epoxide hydrolase 1 microsomal (xenobiotic), CYP20A1; cytochrome P450 family 20 subfamily a polypeptide 1, SLC6A6; solute carrier family 6 (neurotransmitter transporter taurine) member 6, CRIP3; cysteine-rich protein 3, GSTA4; gluthathione S-transferase alpha 4, AKAP9; A kinase anchor protein 9, CYP51A1; cytochrome P450 family 51 subfamily A polypeptide 1, CYP2D7P1; cytochrome P450 family 2 subfamily D polypeptide 7 pseudogene 1.

Table 4. Results of validation of SNP prediction model in validation set[a]

SNP prediction model	Truly low-clearance	Truly not low-clearance	N
Predicted low-clearance	20 (15)	73 (56)	93 (72)
Predicted not low-clearance	1 (1)	36 (28)	37 (28)
N	21 (16)	109 (84)	130 (100)

[a] cells represent absolute number of patients with percentage of total patients in parentheses
Abbreviations: N, number of patients

DISCUSSION

During the early years of the 21st century, emphasis has been on somatic tumor mutations that can predict disease course and treatment outcome and could therefore aid in selecting the appropriate therapy for an individual patient. However, germline genetic variation, as present in normal tissue can influence the pharmacokinetics and pharmacodynamics of an anti-cancer drug regardless of tumor type and therefore also affect treatment outcome and toxicity [24]. Knowledge of this germline variation could therefore significantly contribute to a truly individualized pharmacotherapy of anti-cancer drugs.

In the current study, we present the findings of a pharmacokinetic-pharmacogenetic study that relates multiple SNPs in metabolic enzymes and transporters to the unbound clearance of paclitaxel, in order to identify patients with low clearance who are potentially at risk for increased toxicity. This analysis resulted in the development of a genetic signature, predictive for low paclitaxel clearance, containing 14 SNPs, which yielded a high sensitivity, but a low positive predictive value, when tested in a validation cohort.

To the best of our knowledge, this is the first large-scale application of the DMET platform to explain the pharmacokinetic variability of a commonly used anti-cancer drug. The traditional candidate gene approach is most often used in pharmacogenetic cancer research.[25-27] However, this method is only able to identify a limited number of genetic variants which are plausible candidates within the current knowledge of the field. The DMET platform enables us to study genetic variants in all currently known drug metabolizing enzymes and transporters simultaneously, making this a tool with high potential for pharmacogenetic research.[24]

Our genetic prediction model yielded a sensitivity of 95% when tested in the validation cohort. However, the model had a positive predicting value of only 22%. This means that this model identifies almost all patients with truly low clearance, at the cost of a high percentage of false positives. Also, 36 out of 37 patients (97%) have truly no 'low-clearance' after testing negative for the genetic prediction model, meaning that they do not have an increased toxicity risk. To be clinically applicable, we reasoned that a prediction model should have besides a high sensitivity also a high positive predictive value. The latter is not reached in our study.

In previous studies several SNPs have been associated with paclitaxel clearance or toxicity, but contradictory findings have been reported. For example, *ABCB1 3435 C>T* was associated with paclitaxel clearance[28] and *ABCB1 2677 G>T/A* was associated with response to paclitaxel.[29] The combination of *ABCB1 3435 C>T* and *ABCB1 2677 G>T/A* has been linked to neutropenia and patients with at least one *ABCB1 3435 C>T* showed a trend towards more development of neurotoxicity during paclitaxel therapy.[6] Also, SNPs in *CYP2C8* have been related to paclitaxel therapy response. For example, *CYP2C8*3* carriers had higher rates of complete response than non-carriers.[30] On the same note, *CYP2C8*3* carriers were found to have lower paclitaxel clearance than non-carriers[8] and a higher risk of neurotoxicity.[9,10] However, none of these previous identified associations were found in other studies.[5,31]

Interestingly, in the currently developed model, none of the SNPs that were previously associated with paclitaxel clearance or toxicity were included. However, epoxide hydrolase 1 (EPHX1) and glutathione S-transferase alpha 4 (GSTA4) have previously been associated with docetaxel clearance in a small study which related selected genes in metabolism and signaling of reactive oxygen species (ROS).[32,33] In addition, it was found that EPHX1 was upregulated in gemcitabine resistant non-small cell lung cancer cells.[34] None of the other 12 SNPs that were selected for the prediction model have previously been associated with clearance or toxicity of taxanes. Because of the discrepancy between the previously associated candidate SNPs with paclitaxel clearance and the outcome of this DMET analysis, we presume it is unlikely that common inherited genetic variability in drug metabolizing enzymes and transporters contributes in a clinically relevant way to the variability in paclitaxel clearance.

To conclude, we developed a validated genetic prediction model in a large cohort of paclitaxel-treated patients to identify patients at risk of low clearance. Although this validated prediction model for paclitaxel clearance had a high sensitivity, its positive predictive value was too low to be of direct clinical use. Strikingly, the genes that are reported to influence paclitaxel pharmacokinetics were not identified in this analysis; we therefore conclude that genetic variability in DMET genes does not substantially contribute in explaining a large part of the interpatient variability in paclitaxel clearance. If these genes can explain the interpatient variability in the pharmacokinetics of other drugs should be explored in further studies.

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5

INFLUENCE OF POLYMORPHIC OATP1B-TYPE CARRIERS ON THE DISPOSITION OF DOCETAXEL

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ABSTRACT

Purpose

Docetaxel is extensively metabolized by CYP3A4 in the liver but mechanisms by which the drug is taken up into hepatocytes remain poorly understood. We hypothesized that (i) liver uptake of docetaxel is mediated by the polymorphic solute carriers OATP1B1 and OATP1B3, and (ii) that inherited genetic defects in this process may impair systemic drug elimination.

Experimental Design

Transport of docetaxel was studied *in vitro* using various cell lines stably transfected with OATP1B1*1A (wild-type), OATP1B1*5 [c.521T>C (V174A); rs4149056], OATP1B3, or the mouse transporter Oatp1b2. Docetaxel clearance was evaluated in wild-type and Oatp1b2-knockout mice as well as in two cohorts of patients with multiple variant transporter genotypes (n = 213).

Results

Docetaxel was found to be a substrate for OATP1B1, OATP1B3, and Oatp1b2 but was not transported by OATP1B1*5. Deficiency of Oatp1b2 in mice was associated with an 18-fold decrease in docetaxel clearance (P = 0.0099), which was unrelated to changes in intrinsic metabolic capacity in mouse liver microsomes. In patients, however, none of the studied common reduced function variants in OATP1B1 or OATP1B3 were associated with docetaxel clearance (P > 0.05).

Conclusions

The existence of at least two potentially redundant uptake transporters in the human liver with similar affinity for docetaxel supports the possibility that functional defects in both of these proteins may be required to confer substantially altered disposition phenotypes. In view of the established exposure-toxicity relationships for docetaxel, we suggest that caution is warranted if docetaxel has to be administered together with agents that potently inhibit both OATP1B1 and OATP1B3.

TRANSLATIONAL RELEVANCE

Docetaxel is widely used for the treatment of multiple solid tumors, including cancers of the breast, lung, head and neck, stomach, and prostate. The interindividual pharmacokinetic variability seen with docetaxel treatment remains high, and this phenomenon may have important ramification for the agent's clinical activity and toxicity. We speculated that differential expression of polymorphic transporters involved in the hepatic elimination of docetaxel plays a crucial role in explaining this pharmacologic variability. Here, we investigated the contribution of organic anion transporting polypeptides to the disposition of docetaxel using an array of *in vitro* and *in vivo* model systems. Our results indicate the existence of at least two potentially redundant uptake transporters in the human liver with similar affinity for docetaxel (OATP1B1 and OATP1B3) that regulate the initial, rate-limiting step in the elimination of docetaxel. In view of the established exposure-toxicity relationships for docetaxel, our results suggest that caution is warranted if docetaxel has to be administered together with agents that potently inhibit both these transporters.

INTRODUCTION

The antimicrotubular agent docetaxel is a widely used chemotherapeutic agent that has been approved for the treatment of multiple malignant diseases, including cancers of the breast, lung, head and neck, stomach, and prostate. The disposition properties of docetaxel are characterized by up to 10-fold differences in drug clearance between patients receiving the same therapeutic regimen.[1] The high degree of interindividual pharmacokinetic variability observed with docetaxel has important toxicological ramification. In particular, it was previously demonstrated that a mere 50% decrease in docetaxel clearance is associated with a more than 4-fold increase in the odds of developing severe neutropenia, the dose-limiting toxicity.[2,3]

Despite the established exposure-pharmacodynamic relationships for docetaxel, the mechanisms underlying the agent's unpredictable pharmacokinetics remain largely unexplained. It has been speculated that a critical determinant of docetaxel's pharmacokinetic variability is associated with differential expression of polymorphic drug-metabolizing enzymes and/or transporters at sites of elimination. However, several recent analyses indicated that the contribution of genetic variants in obvious candidate genes encoding enzymes or ATP-binding cassette transporters to explaining pharmacokinetic variability of docetaxel is rather limited.[4-11]

The mechanisms by which docetaxel is taken up into human liver cells are still largely unknown. Previous *in vitro* screens have provided evidence that cellular uptake of the related compound paclitaxel may be regulated, in part, by the polymorphic organic anion transporting polypeptides OATP1B1 (gene name, *SLCO1B1*) and/or OATP1B3 (gene name, *SLCO1B3)*.[12,13] These transporters are expressed at high levels in the liver, where their localization is restricted to the basolateral membrane of hepatocytes, and they have been implicated in the liver uptake of multiple structurally

diverse endogenous molecules and xenobiotics.[14] In the current study, we tested the hypothesis that inherited variation in OATP1B1 and OATP1B3 is associated with the disposition of docetaxel and that these transporters collectively contribute to interindividual differences in the clearance of docetaxel in patients with cancer.

MATERIAL AND METHODS

In vitro transport studies

Xenopus laevis oocytes injected with OATP1B1, OATP1B3, or rat Oatp1b2 cRNA along with water-injected controls were obtained from BD Biosciences. Chinese hamster ovary (CHO) cell lines stably expressing OATP1B1 or OATP1B3[15] and Flp-In T-Rex293 cells transfected with OATP1B1*1A (wild-type), OATP1B1*1B [c.388A>G (N130D); rs2306283], OATP1B1*5 [c.521T>C (V174A); rs4149056], or OATP1B1*15 (N130D, V174A) have been described previously.[16] OATP1B1 or OATP1B3 overexpressing human embryonal kidney (HEK293) cells were created by stably transfecting the respective cDNA fragments spliced from TrueClone plasmids (OriGene Technologies) cloned into a pIRES2-EGFP vector (BD Biosciences). Mouse Oatp1b2 overexpressing HEK293 cells were created similarly from a commercial cDNA cloned into a pDream2.1/MCS vector (GenScript). Overexpression of transporters in HEK293 cells was confirmed using TaqMan probes (Applied Biosystems).

Uptake experiments were performed as described previously,[3] with results normalized to uptake values in cells transfected with an empty vector. Preliminary experiments indicated that Phenol Red, a pH indicator in trypsin used to resuspend cultured cells, influenced OATP1B-mediated uptake of docetaxel in Flp-In T-Rex293 cells (Supplementary Fig. S1), and therefore these studies were conducted in Phenol Red-free conditions.

Animal Experiments

Adult male Oatp1b2-knockout mice[17] and age-matched wild-type mice (Taconic), both on a DBA1/lacJ background, were housed in a temperature-controlled environment with a 12-hour light cycle and given a standard diet and water *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Docetaxel, formulated in polysorbate 80 (Taxotere) and diluted in normal saline, was administered by tail vein injection at a dose of 10 mg/kg, and plasma, liver, and kidney from each animal were collected at 5, 15, 30, 60, 120, 240, and 480 minutes. Urine was collected from animals housed in metabolic cages for 48 hours after docetaxel administration. Samples were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS-MS)[18] and noncompartmental parameters calculated using PK Solutions 2.0 (Summit Research Services). Tissue concentrations of docetaxel were corrected for contaminating plasma.[19] Gene expression patterns in livers were assessed using the Mouse 430v2 GeneChip array (Affymetrix). Microsomal metabolism of docetaxel in liver samples from wild-type and

Oatp1b2-knockout mice was conducted as described [20] in the presence or absence of the Cyp3a inhibitor, ketoconazole.

Determination of Docetaxel Concentrations

Docetaxel was quantified using a validated method involving reversed-phase liquid chromatography coupled to tandem mass-spectrometric detection. Sample extracts were injected onto an Alltima HP C18 HL 3 µm column (50×2.1mm internal diameter, Alltech Applied Science, Breda, The Netherlands) by a Waters 2795 Separation Module (Milford, MA). The mobile phase was composed of acetonitrile and water containing formic acid (0.1% v/v), and was delivered using linear gradient settings at a flow rate of 0.2 mL/min. Detection was performed with a MicroMass Quatro Micro triple-quadrupole mass spectrometer (Waters) in the positive ion mode. The electrospray ionization was set at 3.8 kV and the cone voltage at 18V. The dwell times were set at 150 ms and the inter-channel delay at 50 ms. Multiple reaction monitoring mode was applied for the quantitation with the following parameters: m/z 808>527, collision energy at 9 eV for docetaxel and m/z 813>532, collision energy at 10 eV for the internal standard docetaxel-d5. The collision cell pressure was set at ~4×10-3 mbar (argon).

Patient Studies

Patients were enrolled onto a prospective pharmacokinetic study (Dutch trial registry: NTR2311). Inclusion criteria included a confirmed diagnosis of a solid tumor for which docetaxel (formulated in polysorbate 80; Taxotere) was a reasonable therapeutic option, age 18 years or older, World Health Organization (WHO) performance score of 0 or 1, and adequate hematopoietic, hepatic, and renal functions, as described previously.[21] Concurrent use of agents known to induce or inhibit CYP3A4 was not allowed. The study was approved by the Erasmus University Medical Center review board, and all patients provided written informed consent.

Blood collection for pharmacokinetic analyses was conducted using a limited-sampling strategy where 4 or 5 samples were obtained over a 24-h period after the end of infusion. Docetaxel concentrations in plasma were determined as described (Supplementary Methods). Pharmacokinetic parameters were estimated using a previously developed population model,[22] in NONMEM version 7 (Icon Development Solutions). There was no statistically significant influence of sex, administered dose, or tumor type on the clearance of docetaxel ($P > 0.05$), and thus pharmacokinetic data of all patients were pooled in subsequent correlation studies without further correction or consideration of subgroup analyses.

Genomic DNA was isolated from whole blood using MagnaPure LC (Roche Diagnostics GmbH). Allelic discrimination analysis was conducted for the determination of several variants in OATP1B1 and OATP1B3 that were selected from the literature on the basis of their relatively high predicted allelic frequency and/or the known or suspected influence on functional properties of the encoded proteins (Supplementary

Table S1).[16] The analyses were performed using TaqMan assays on an ABI PRISM 7500 system (Applied Biosystems) according to the manufacturer's instructions. Confirmatory genotyping analyses on select candidate variants, including the c.388G>A and c.521T>C variants in OATP1B1, and the c.334T>G and c.699G>A variants in OATP1B3, were done on previously collected samples from a similar, predominantly white cohort of 72 patients that had received docetaxel-based chemotherapy.[4]

Statistical Considerations

Data are presented as mean with SD, unless stated otherwise. Statistical calculations were done using analysis of variance or Student *t* test in SPSS version 17 (SPSS Inc), depending on the number of groups, and $P < 0.05$ was considered significant.

RESULTS

Docetaxel transport *in vitro*

Experiments assessing the interaction of docetaxel with human OATP1B1 and OATP1B3 indicated that drug uptake is dependent on cell context, with both proteins being able to take up docetaxel when expressed in HEK293 cells or CHO cells, but no noticeable transport occurring by OATP1B1 when expressed in *Xenopus laevis* oocytes (Figure 1A). Docetaxel was also found to be transported into cells expressing the mouse mOatp1b2 or rat rOatp1b2 transporters (Figure 1B).

The transport of docetaxel into CHO cells transfected with OATP1B1 or OATP1B3 was found to be time-dependent and saturable with a Michaelis-Menten constant (K_m) of 7.6 ± 3.0 and 2.2 ± 0.6 µmol/L, respectively, and a maximum velocity (V_{max}) of 30.7 ± 5.7 and 27.2 ± 2.4 pmol/mg/min, respectively (Figures 1C-F), and similar results were obtained for paclitaxel (Supplementary Figure S2). Compared with cells overexpressing the wild-type OATP1B1 (OATP1B1*1A), *in vitro* transport activity of cells transfected with constructs carrying the c.521C substitution (OATP1B1*5 and OATP1B1*15) was completely lost (Figure 2). Interestingly, the presence of the docetaxel excipient polysorbate 80 (Tween 80), at levels that can be achieved in patients,[23] abrogated the OATP1B1-genotype-dependent transport of docetaxel (Figure 2).

Docetaxel pharmacokinetics in Oatp1b2-knockout mice

We next evaluated the possible importance of these transporters for docetaxel in mice with a genetic deletion of Oatp1b2 [Oatp1b2(-/-) mice]. The area under the curve (AUC) for docetaxel in these animals was dramatically increased compared with that observed in wild-type mice (8,826 ± 845 vs. 336 ± 96.9 ng x h/mL; $P = 0.0066$) as a result of a more than 18-fold decrease in systemic clearance (1.08 ± 0.097 vs. 19.9 ± 7.08 L/h/kg; $P = 0.0099$). The respective concentration-time profiles of docetaxel in mice (Figure 3A) suggests that the slow clearance in the Oatp1b2(-/-) mice is due to a distribution defect rather than an event occurring in the terminal

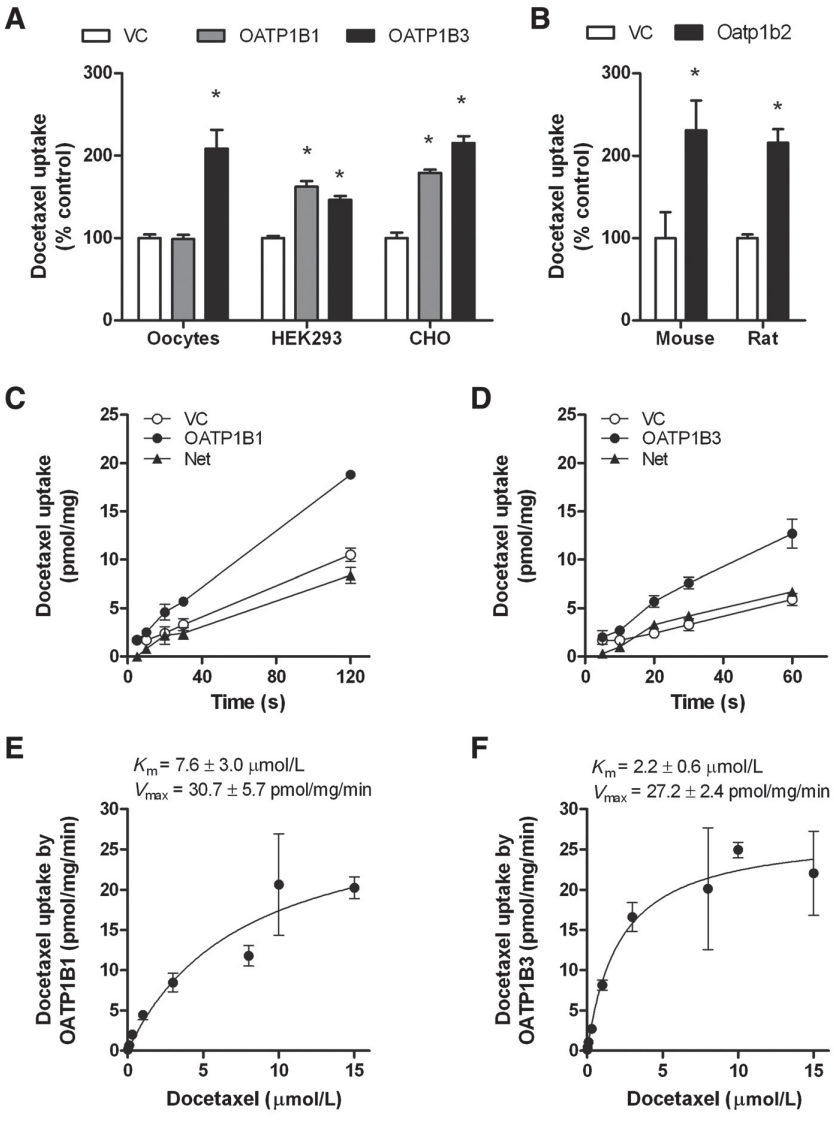

Figure 1. *In vitro* transport studies of docetaxel. (A) Transport of docetaxel by human OATP1B1 and OATP1B3 was evaluated with constructs transfected in *Xenopus laevis* oocytes (docetaxel concentration, 2 μmol/L; 30 minutes incubations), HEK293 cells (2 μmol/L; 30 minutes), or CHO cells (1 μmol/L; 2 minutes). (B) Transport of docetaxel by mouse Oatp1b2 transfected in HEK293 cells (0.1 μM; 60-min) or rat Oatp1b2 transfected in *Xenopus laevis* oocytes (2 μmol/L; 30 minutes). Data represent the mean of 2 to 32 observations and are expressed as the average percent of uptake values in cells transfected with an empty vector (VC). The star (*) denotes a significant difference from VC ($P < 0.05$). Time dependence of transport by OATP1B1 (C) and OATP1B3 (D) and concentration dependence of transport by OATP1B1 (E) and OATP1B3 (F) was evaluated in CHO cells, where data represent the mean of 2 to 4 independent experiments in cells stably expressing OATP1B1, OATP1B3, or in control cells (VC), and the net difference. K_m denotes the Michaelis–Menten constant and V_{max} the maximum velocity. Error bars represent the SE.

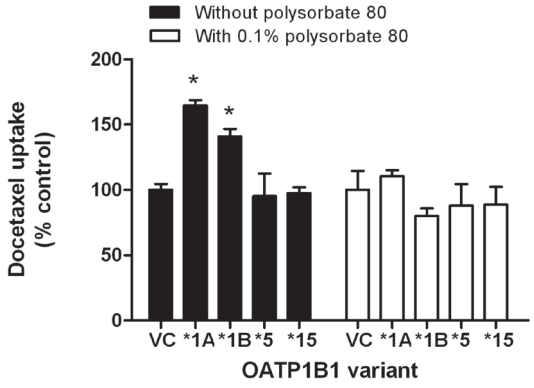

Figure 2. Influence of OATP1B1 variants on docetaxel transport *in vitro*. Transport of docetaxel (concentration, 0.1 µmol/L; 60 minute incubations) was evaluated in Flp-In T-Rex293 cells transfected with OATP1B1*1A (wild-type), OATP1B1*1B [c.388A>G (N130D); rs2306283], OATP1B1*5 [c.521T>C (V174A); rs4149056], or OATP1B1*15 (N130D, V174A). Data represent the mean of 6 observations and are expressed as the average percent of uptake values in cells transfected with an empty vector (VC) in the absence or presence of 0.1% of polysorbate 80. Error bars represent the SE. The star (*) denotes a significant difference from VC ($P < 0.05$).

elimination phase. Indeed, the terminal half-lives of docetaxel were not significantly different in Oatp1b2(-/-) mice and wild-type mice (2.41 ± 0.151 vs. 2.44 ± 0.533 h; $P = 0.87$).

As anticipated, the liver/plasma AUC ratio was significantly reduced in Oatp1b2(-/-) mice (1.32 ± 0.088 vs. 8.14 ± 2.39; $P = 0.0079$). The kidney/plasma AUC ratio was also reduced in Oatp1b2(-/-) mice (5.14 ± 0.363 vs. 43.0 ± 12.5; $P = 0.0063$), although there was limited shunting of docetaxel in the knockout mice to urine (urinary excretion, 1.68 ±0.758 %dose vs. 1.03 ± 0.676 %dose; $P = 0.15$). To rule out potentially altered, compensatory expression of enzymes and transporters in the liver of Oatp1b2(-/-) mice at baseline, microarrays were used to evaluate differential expression profiles of 839 probe sets for 463 genes, including 49 ATP-binding cassette transporters, 78 cytochrome P450 enzymes, and 336 solute carriers. Compared to levels in liver of wild-type mice, besides probe sets for the Oatp1b2 gene *Slco1b2*, only transcripts of the enzyme *Cyp2b10* were decreased in the Oatp1b2(-/-) mice (Figure 3B). Because taxanes are not known to be metabolized by Cyp2b10, this genetic alteration is unlikely to directly or indirectly influence docetaxel handling by the liver. Furthermore, there were no potentially compensatory changes in hepatic Cyp3a activity, the main metabolic route for docetaxel, as Oatp1b2-knockout had no influence on the hepatic microsomal metabolism of docetaxel *ex vivo* in the presence or absence of the Cyp3a inhibitor ketoconazole (Figure 3C).

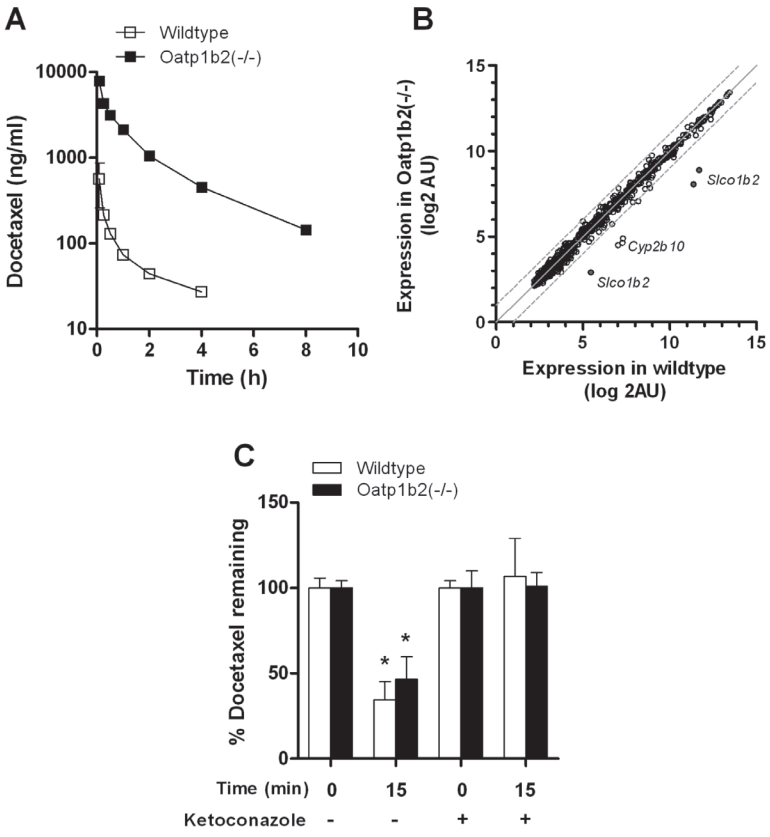

Figure 3. Influence of Oatp1b2-knockout on docetaxel pharmacokinetics. (A) Plasma concentration-time profile of docetaxel in wildtype and Oatp1b2(-/-) mice (i.v. dose, 10 mg/kg). Data represent the mean of at least 6 observations per time point, and error bars represent the SE. (B) Comparative expression of 839 probe sets for 463 genes, including 49 ATP-binding cassette transporters, 78 cytochrome P450 enzymes, and 336 solute carriers, at baseline in livers of wild-type mice and Oatp1b2(-/-) mice (n = 5 per group). Each symbol represents a single probe set, the solid line is the line of identity, and the dotted lines are the 95% confidence intervals. (C) Influence of Oatp1b2 knockout on the *ex vivo* liver microsomal metabolism of docetaxel (concentration, 2 µmol/L; 15 minutes incubations) in the absence or presence of ketoconazole (20 µmol/L). Under these conditions, formation of the main murine metabolite of docetaxel (M2) was not different between liver microsomes from wild-type or Oatp1b2(-/-) mice (P = 0.29). Data represent the mean of 8 independent observations per group and are expressed as the percent of drug added to the microsomes at time zero. Error bars represent the SE. The star (*) denotes a significant difference from time zero ($P < 0.05$).

Docetaxel pharmacokinetics in patients with different transporter genotypes

To provide preliminary evidence for a possible role of OATP1B1 and OATP1B3 in the clinical pharmacology of docetaxel, an exploratory pharmacogenetic association analysis was conducted in human subjects with cancer undergoing docetaxel-based chemotherapy. To this end, pharmacokinetic and pharmacogenetic data was obtained from 141 predominantly white patients (87 females and 54 males) with a median age of 55 years (Supplementary Table S2). The average clearance of docetaxel in the study population was 41.8 ± 12.3 L/h, with a 6.3-fold difference between the lowest and highest values.

The relative frequencies of the variant alleles in our patient population were comparable with previously reported estimates,[16] the distributions of all polymorphisms were in Hardy-Weinberg equilibrium, and demographic characteristics at baseline were similar for individuals carrying 0, 1, or 2 variant alleles at the loci of interest. Despite the observed functional impact of the OATP1B1 c.521C substitution *in vitro*, none of the individual polymorphisms in OATP1B1 were found to be associated with the clearance of docetaxel *in vivo* (Figure 4). Significant associations were also not observed between docetaxel clearance and the studied variants in OATP1B3 (Figure 5), and associations did not improve when individuals were clustered on the basis of observed diplotypes (Supplementary Figure S3). In consideration of the relatively few individuals carrying polymorphism predicted to be associated with altered docetaxel transport, we conducted additional genotyping on the c.388G>A and c.521T>C variants in OATP1B1 and 284 the c.334T>G and c.699G>A variants

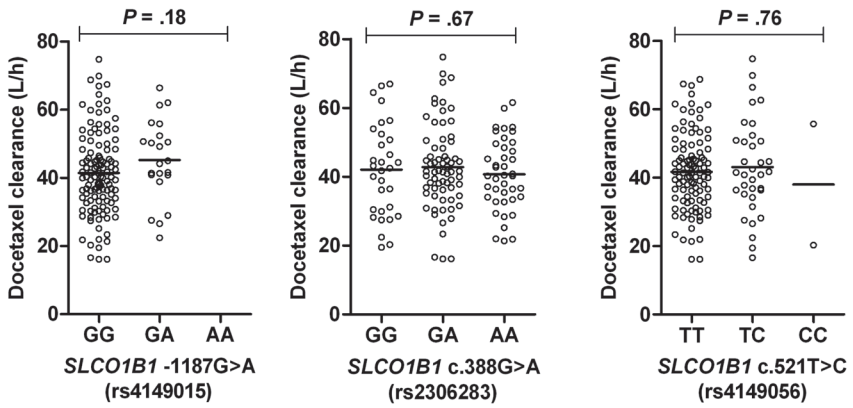

Figure 4. Docetaxel clearance as a function of observed OATP1B1 (*SLCO1B1*) genotypes. Data were obtained in 141 predominantly white patients with cancer receiving docetaxel-based chemotherapy. Each symbol represents an individual patient, and horizontal lines indicate median values. The *P*-value denotes a statistical comparison of the clearance of docetaxel in the different genotype group.

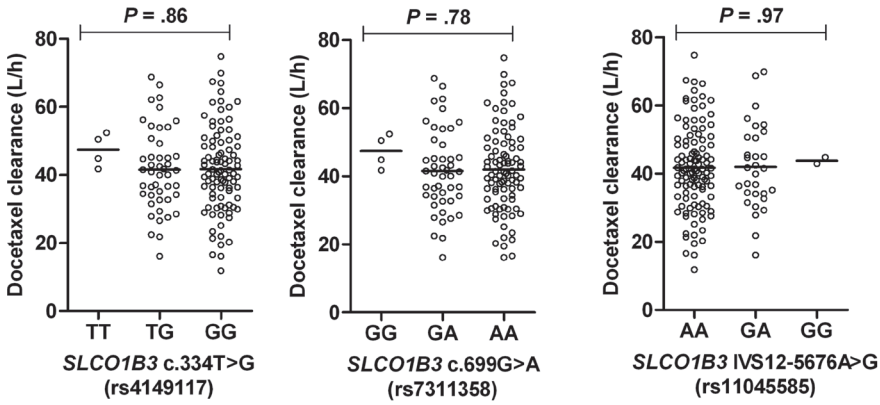

Figure 5. Docetaxel clearance as a function of observed OATP1B3 (*SLCO1B3*) genotypes. Data were obtained in 141 predominantly white cancer patients receiving docetaxel-based chemotherapy. Each symbol represents an individual patient, and horizontal lines indicate median values. The *P*-value denotes a statistical comparison of the clearance of docetaxel in the different genotype group.

in OATP1B3 in a 285 separate cohort of 72 patients.[4] However, enrichment of these candidate polymorphisms also did not result in statistically significantly improved genetic associations with docetaxel clearance (Supplementary Figure S4).

DISCUSSION

The current study provides support for a growing body of knowledge that solute carriers belonging to the family of organic anion transporting polypeptides can have a dramatic impact on the hepatic accumulation and systemic clearance of CYP3A4 substrates. Using an array of *in vitro* transport assays, including intracellular accumulation studies in multiple transfected model systems, docetaxel was identified as a high-affinity substrate for human OATP1B1 and OATP1B3. We found that the interaction of docetaxel with OATP1B1 and OATP1B3 was strongly dependent on cell context and culture medium composition, and this has obvious implications for future screening strategies aimed at identifying novel substrates for these transporters.

Our *in vitro* studies also suggest that docetaxel is a transported substrate of mouse Oatp1b2 and rat Oatp1b2. The rodent Oatp1b2 transporters share more than 60% amino acid sequence homology to human OATP1B1 and OATP1B3, and on the basis of their shared basolateral localization in hepatocytes and overlapping substrate specificity,[24] it is possible that in rodents Oatp1b2 fulfils the same function in the liver as OATP1B1 and OATP1B3 in humans. On the basis of this premise, we evaluated the pharmacokinetic properties of docetaxel in a mouse model with a genetic deletion of Oatp1b2. One possible limitation of this model is that fact that, unlike in humans, mouse

hepatocytes express multiple members of Oatp1a, a related subfamily of transporters that can potentially provide compensatory restoration of function when Oatp1b2 is lost.[25] Despite this limitation, compared with wild-type mice, the systemic exposure to docetaxel in the Oatp1b2(-/-) mice was remarkably increased by more than 26-fold. Gene expression profiling and Cyp3a activity measurements in liver samples excluded alterations in alternate transport mechanisms or metabolic pathways as a possible cause of the delayed clearance phenotype in Oatp1b2(-/-) mice. These findings suggest that Oatp1b2-mediated transport of docetaxel is likely a critically important rate-limiting process in the elimination of this drug in mice. This supposition is consistent with the notion that the change in clearance of docetaxel observed here in Oatp1b2(-/-) animals is at least as dramatic as compared with phenotypic changes associated with complete deficiency of metabolic Cyp3a activity in mice.[20] Nonetheless, considering the relatively low amino acid homology between OATP1B1 or OATP1B3 and Oatp1b2 (about 64%) and between CYP3A4 and Cyp3a11 (about 73%), additional investigation is required using humanized models for these proteins to provide direct evidence for involvement of OATP1B-type carriers in the hepatic uptake of docetaxel.

It is interesting to note that a previous study demonstrated that mice deficient for all Oatp1a and Oatp1b genes display only a rather modest 2-fold increase in concentrations of paclitaxel in plasma, presumably due to decreased uptake of the drug into the liver compared to wild-type mice.[26] The reasons underlying the apparent differences in outcome of the study with paclitaxel and our current results for docetaxel are not entirely clear. It is possible that the background strains onto which these respective knockout mice were developed (FVB vs. DBA1/lacJ, respectively) differentially impact any resulting phenotypes for structurally similar xenobiotics. Regardless of the exact mechanism, the observations made in the mice provide further evidence that hepatic OATP transporters can affect the pharmacokinetic properties of a remarkably broad range of substrates that include charged organic anions (e.g., methotrexate), charged organic cations (e.g., imatinib), polar zwitterions (e.g., fexofenadine), and uncharged hydrophobic agents (e.g., taxanes).

On the basis of *in vitro* uptake studies, multiple functionally different haplotypes, including OATP1B1*5 and OATP1B1*15, were found to have a detrimental impact on docetaxel transport. This finding is consistent with previously studies showing substantially diminished transport activity of several OATP1B1 substrates by these particular variants when transfected into mammalian cells.[27] *In vivo*, these variants have been associated with altered systemic exposure and toxicity in response to multiple substrate drugs.[28]

Interestingly, the relevance of these genetic variants in OATP1B1 could not be confirmed in our prospectively conducted pharmacogenetic-association study done in a group of predominantly white patients with cancer receiving treatment with docetaxel. It is possible that additional rare genetic variants or haplotypes in OATP1B1 of importance to the transport docetaxel in this population are yet to be discovered and that much larger numbers of patients are then needed to more precisely quantify genotype-phenotype associations.

We also considered the possibility that the interaction of docetaxel with OATP1B1 may be masked by the pharmaceutical vehicle polysorbate 80 (Tween 80), which is used to solubilize docetaxel in clinical preparations. Indeed, the presence of polysorbate 80, even in relatively low amounts, completely nullified the genotype-dependent transport of docetaxel by OATP1B1 observed in the absence of polysorbate 80. Although further investigation is required to confirm direct involvement of polysorbate 80-mediated inhibition of OATP1B1 as the primary mechanistic basis for the observed *in vivo* effects, it is of note that similarly altered hepatic uptake has been described for colchicine in the presence of Solutol HS15[29] and for paclitaxel in the presence of Cremophor.[12] If confirmed, these observations suggest that the impact of reduced function variants of OATP1B1 on the clearance of docetaxel may be much more pronounced for polysorbate 80-free formulations of the drug, such as nab-docetaxel (ABI-008).

In our study, several genetic variants in OATP1B3 were also not significantly associated with the pharmacokinetics of docetaxel. This is in line with previously published data that we collected in another predominantly white, independent cohort of patients.[4] It should be pointed out that this finding is at odds with several other investigations performed in patients of Asian descent. For example, homozygosity (GG) for rs11045585 was associated with reduced clearance of docetaxel, compared with patients carrying the AA or AG genotypes.[5] In another study, a particular OATP1B3 genotype combination comprising the reference allele at IVS4+76G>A (rs4149118) and variant alleles at 699G>A (rs7311358), IVS12+5676A>G (rs11045585), and *347_*348insA (rs3834935)indel was also linked with reduced clearance of docetaxel.[30] It is possible that differences in outcome with our study are associated with the fact that such variants may occur at different frequencies between Asians and Caucasians, and/or on different, ethnicity-dependent haplotype structures.

Regardless of any potential ethnic considerations, the existence of at least 2 potentially redundant uptake transporters in the human liver with similar affinity for docetaxel supports the possibility that functional defects in both of these proteins may be required to confer substantially altered disposition phenotypes such as those seen in the Oatp1b2(-/-) mice. While complete functional deficiency of either OATP1B1 or OATP1B3 has been recorded to occur,[31] deficiency of both transporters is very rare, with an estimated frequency in the human population of about 1 in a million.[32] It can thus be postulated that intrinsic physiologic and environmental variables influencing OATP1B1- or OATP1B3-mediated uptake of docetaxel into hepatocytes may have a more profound influence on the clearance of docetaxel in the general population than do defective genetic variants. This recognition is particularly relevant in the context of the recent guidelines offered by The International Transporter Consortium regarding preclinical criteria needed to trigger the conduct of clinical studies to evaluate drug-transporter interactions.[33] Indeed, it is conceivable that instances of idiosyncratic hypersensitivity to docetaxel are the result of currently unrecognized drug-drug interactions at the level of hepatocellular uptake mechanisms (see Supplementary Table S3 for examples).

Collectively, our findings show the importance of OATP1B-type solute carriers as the initial, rate-limiting step in the elimination of docetaxel. Our results suggest that genetic defects leading to impaired function of both OATP1B1 and OATP1B3 may be required to confer substantially reduced clearance of this drug in humans. In view of the established exposure-toxicity relationships for docetaxel, we suggest that caution is warranted if docetaxel has to be administered together with agents that potently inhibit both of these transporters.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declared no conflict of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

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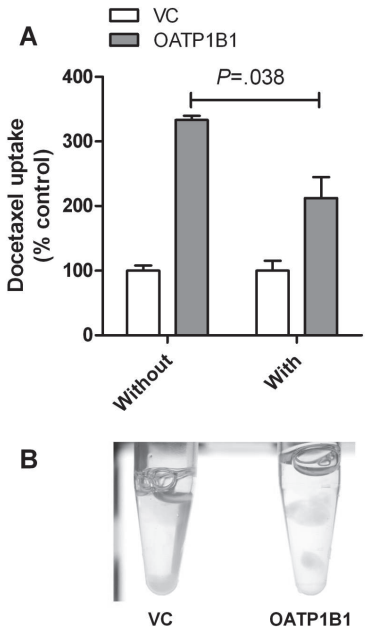
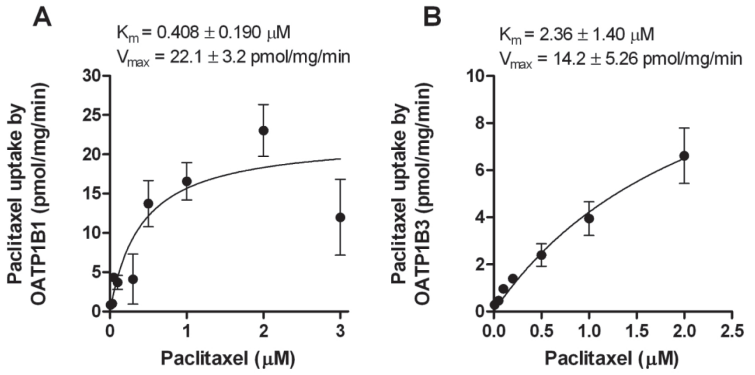
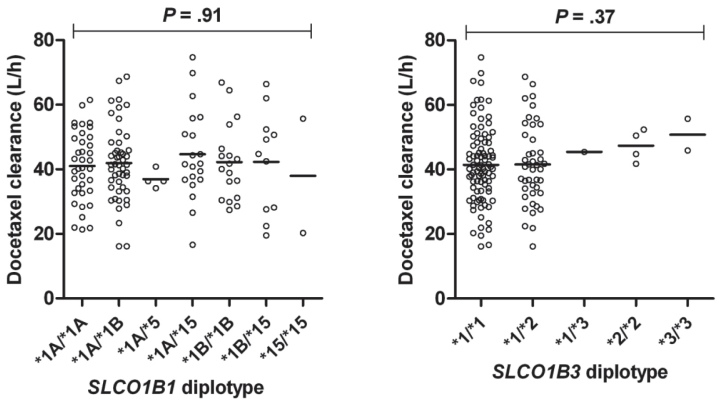
SUPPLEMENTARY MATERIALS



Supplementary figure 1. (A) Influence of Phenol Red on OATP1B1-mediated transport of docetaxel *in vitro*. Transport of docetaxel (concentration, 0.1 μM ; 60-min incubations) was evaluated in Flp-In T-Rex293 cells transfected with OATP1B1 in the absence or presence of Phenol Red at a concentration of 10 μg/mL (26.4μM). Data represent the mean of 3 observations, and are expressed as the average percent of uptake values in cells transfected with an empty vector (VC). Error bars represent the standard error. The *P*-value denotes a statistical comparison of differences in uptake of docetaxel by OATP1B1 in the absence or presence of Phenol Red. (B) Visualization of Flp-In T-Rex293 cells transfected with an empty vector (VC) or OATP1B1 cultured in DMEM containing Phenol Red (10 μg/mL) indicating accumulation of Phenol Red in cells expressing OATP1B1.



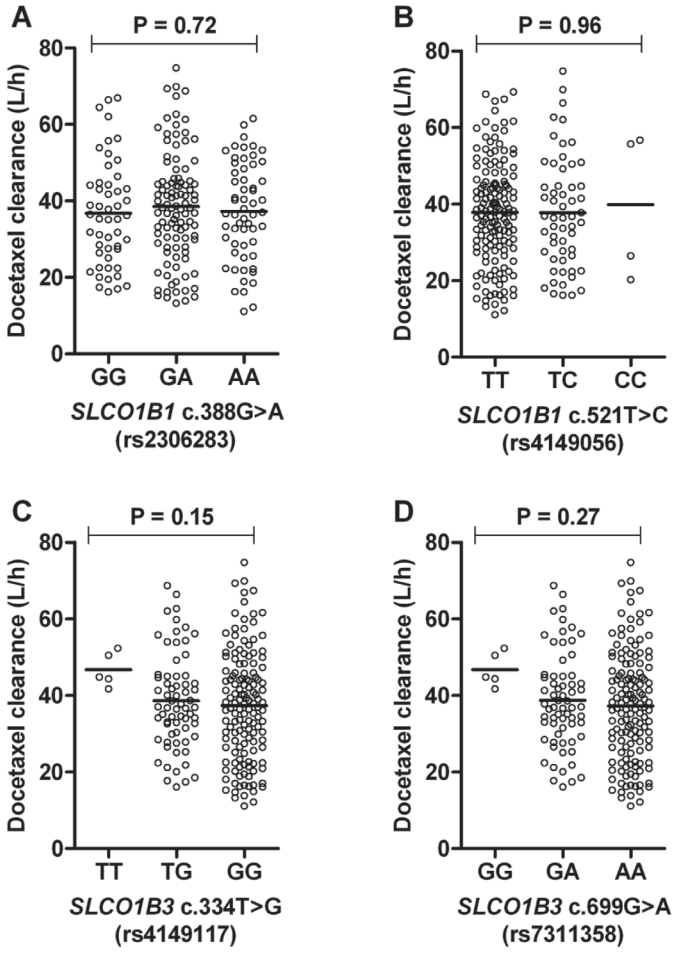
Supplementary figure 2. Characterization of paclitaxel concentration-dependent transport by OATP1B1 (A) and OATP1B3 (B) in CHO cells. Data represents the mean and standard deviation of 2 to 5 independent experiments in cells stably expressing OATP1B1, OATP1B3, or in control cells (VC), and the net difference. K_m denotes the Michaelis-Menten constant, and V_{max} the maximum velocity.



	Ramsey et al Freq	Current Freq	c.388	c.521
*1A	0.540	0.521	A	T
*1B	0.140	0.339	G	T
*5	0.005	0.014	A	C
*15	0.120	0.125	G	C

	Smith et al Freq	Current Freq	c.344	c.699
*1	0.804	0.799	G	A
*2	0.168	0.183	T	G
*3	0.022	0.018	G	G
*4	0.006	0.000	T	A

Supplementary figure 3. Docetaxel clearance as a function of observed OATP1B1 (*SLCO1B1*) or OATP1B3 (*SLCO1B3*) diplotypes. Data were obtained in 141 predominantly white patients with cancer receiving docetaxel-based chemotherapy. Each symbol represents an individual patient, and horizontal lines indicate median values. The *P* value denotes a statistical comparison of the clearance of docetaxel in the different diplotype groups. The composition and frequencies (Freq) of the observed haplotypes in OATP1B1 and OATP1B3 are shown below the figures and compared with reported frequency values in the literature. Ramsey et al. refers to: Ramsey LB, Bruun GH, Yang W, et al: Rare versus common variants in pharmacogenetics: SLCO1B1 variation and methotrexate disposition. Genome Res 22:1-8, 2012; Smith et al. refers to: Smith NF, Marsh S, Scott-Horton TJ, et al: Variants in the SLCO1B3 gene: interethnic distribution and association with paclitaxel pharmacokinetics. Clin Pharmacol Ther. 81:76-82, 2007.



Supplementary figure 4. Docetaxel clearance as a function of observed OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) genotypes. Data were pooled from the prospective cohort of 141 patients and a retrospective analysis on a cohort of 72 patients receiving docetaxel-based chemotherapy (Baker SD, Verweij J, Cusatis GA, *et al*. Pharmacogenetic pathway analysis of docetaxel elimination. Clin Pharmacol Ther 85:155-63, 2009). Each symbol represents an individual patient, and horizontal lines indicate median values. The P-value denotes a statistical comparison of the clearance of docetaxel in the different genotype group.

Supplementary table 1. Genotyped variants in OATP1B1 *(SLCO1B1)* and OATP1B3 *(SLCO1B3)*

Gene	Position	Location	Effect[a]	Activity[b]	NCBI ID	MAF[c]	MAF[d]	Assay[e]	References[f]
SLCO1B1	c.388A>G	Exon 4	p.Asn130Asp	Increased	Rs2306283	0.46 A	0.41 A	C__1901697_20	1-6
	c.521T>C	Exon 5	p.Val174Ala	Decreased	Rs4149056	0.14 C	0.12 C	C__30633906_10	1,6-14
	g.-11187 G>A	Promotor	-	Decreased	Rs4149015	0.08 A	0.07 A	C__32325356_10	6,14,15
SLCO1B3	c.334 T>G[g]	Exon 3	p.Ser112Ala	Unchanged/Decreased	Rs4149117	0.19 T	0.29 T	C__25639181_40	16-21g
	c.699 G>A[g]	Exon 6	p.Met233Ile	Unchanged	Rs7311358	0.19 G	0.29 G	C__25765587_40	16-20g
	IVS12-567A>G	Intron 12	-	Decreased	Rs11045585	0.13 G	0.17 G	C__31106434_10	22-23

[a] Number represents amino-acid codon
[b] Functional activity in vivo of the variant allele relative to the reference allele
[c] MAF in studied population
[d] MAF in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP)
[e] TaqMan® (Applied Biosystems, CA, USA) genotyping assays used
[f] References for activity in vivo of the variant allele relative to the reference allele
[g] c.699 G>A and c.334 T>G are in complete linkage disequilibrium
Abbreviations: MAF; minor allele frequency, NCBI ID; National Center for Biotechnology Information identification number

Supplementary table 2. Patient characteristics[a]

Characteristic	Value
Number of patients	141
Age, years	55 (18-85)
Gender	
Male	54 (38)
Female	87 (62)
BSA (m^2)	1.86 (1.37-2.60)
Etnicity	
Causcasian	131 (93)
Sub-Saharian	2 (1.4)
Northern African	1 (0.7)
Asian	3 (2.1)
Hindustan	3 (2.1)
Unknown	1 (0.7)
Dose mg/m^2	75 (30-100)
Dose mg	150 (50-230)
Primary tumor site	
Breast	74 (53)
Prostate	21 (15)
Melanoma	11 (7.8)
Head/Neck	10 (7.1)
Sarcoma	7 (5.0)
Lung	5 (3.5)
Other	13 (9.2)
total	141

[a] Continuous data are given as median with range in parentheses, and categorical data are given as number of patients with percentage of the total population in parentheses.

Supplementary table 3. Inhibitors of OATP1B1 and OATP1B3

	OATP1B1		OATP1B3	
Compound	IC_{50} (μM)[a]	Ref	IC_{50} (μM)[a]	Ref
Beclomethasone	6.7	[1]	1.4	[1]
Bromocryptine	0.7	[1]	1.8	[1]
Clarithromycin	8.26[b]-96	[2,3]	32	[2]
Cyclosporine	0.2[b]-2.2	[3-9]	0.06	[9]
Ergocryptine	0.8	[1]	2.2	[1]
Erythromycin	11.4[a]-217	[2,3]	34	[2]
Estropipate	0.06	[1]	19.3	[1]
Everolimus	4.1	[10]	3.7	[10]
Moricizine	8.1	[1]	2.7	[1]
Niflumic acid	3.7	[1]	22.0	[1]
Ramipril	4.0	[1]	3.3	[1]
Repaglinide	1.1-2.2	[1,11]	4.8	[1]
Resveratrol	11.2	[1]	23.7	[1]
Rifampicin	0.477[b]-17[b]	[1,3,4,6,12-14]	1.5-5[b]	[1,13]
Roxithromycin	153	[2]	37	[2]
Sirolimus	9.8	[10]	1.3	[10]
Telithromycin	121	[2]	11	[2]
Ursolic acid	12.5	[1]	2.3	[1]

[a] Inhibitor concentration producing 50% inhibition of transporter activity
[b] Ki provided instead of IC_{50}

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6

4β-HYDROXYCHOLESTEROL AS AN ENDOGENOUS CYP3A MARKER IN CANCER PATIENTS TREATED WITH TAXANES

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Submitted for publication

ABSTRACT

Background

Taxanes are anti-cancer agents used to treat several types of solid tumors. They are metabolized by cytochrome P450 (CYP) 3A, displaying a large pharmacokinetic (PK) variability. In this study, we evaluated the endogenous CYP3A4 marker 4β-hydroxycholesterol (4β-OHC) as a potential individual taxane PK predictor.

Methods

Serum 4β-OHC and cholesterol levels were determined in 291 paclitaxel and 151 docetaxel-treated patients, and were subsequently correlated with taxane clearance.

Results

In the patients treated with paclitaxel, no clinically relevant correlations between 4β-OHC or 4β-OHC:cholesterol ratio and paclitaxel clearance were found. In the patients treated with docetaxel, 4β-OHC concentration was weakly correlated with docetaxel clearance in males (R = 0.35 *P* = 0.01). Of the 10% patients with taxane outlier clearances, 4β-OHC did correlate with docetaxel clearance in males (R = 0.76, *P* = 0.03).

Conclusions

Neither 4β-OHC nor 4β-OHC:cholesterol ratio were clinically relevant correlated with taxane clearance.

INTRODUCTION

The anti-microtubular agents paclitaxel and docetaxel are widely used for the treatment of breast, non small-cell lung, ovarian, and prostate cancer.[1] These taxanes display a large interindividual variability in pharmacokinetics, toxicity profiles and effectivity.[2] This large variability makes dosing within a small therapeutic window of these agents difficult. Therefore, patients with high taxane clearance are at risk for a suboptimal therapeutic effect due to low systemic drug concentrations. On the other hand, patients with low taxane clearance are at a higher risk of severe adverse events. For example, patients with low paclitaxel clearance are at risk of peripheral neuropathy and hematological toxicities[3], while docetaxel patients with low clearance develop febrile neutropenia, mucositis, and skin toxicity more frequently.[4,5] Factors causing this large interindividual pharmacokinetic variation are still largely unknown.

The cytochrome P450 (CYP) 3A family is responsible for the metabolism of a large number of drugs.[6] Paclitaxel and docetaxel are both metabolized by CYP3A4. For docetaxel, CYP3A5 has a minor contribution to this phase I metabolic route, whereas for paclitaxel CYP2C8 plays a prominent role (**Suppl. Figure 1**).[7,8] Therefore, knowledge of CYP3A metabolic capacity of an individual patient could aid in the development of a personalized dosing strategy, especially for anti-cancer agents with a narrow therapeutic index. Predicting individual metabolic profiles by using a phenotypic marker could potentially allow for individual dose adjustments during successive courses.[9] CYP3A metabolic activity can be measured by determining the clearance of a marker specifically metabolized by CYP3A. The value and selection of a suitable CYP3A phenotyping method for clinical use has extensively been discussed.[10,11] Several CYP3A substrates such as midazolam, erythromycin, cortisol, alprazolam, alfentanil, dextromethorphan, nifedipine, lidocaine and dapsone have been suggested as probe drugs.[9,12,13] All these methods are laborious and require exogenous drug administration, which potentially limits their clinical use.

Recently, the endogenous compound 4β-hydroxycholesterol (4β-OHC) has been proposed as a marker for CYP3A activity because *in vivo* levels of 4β-OHC are thought to reflect CYP3A4/5 activity.[14-16] The conversion of cholesterol to 4β-OHC is exclusively by CYP3A4 (Suppl. Figure 1).[16] An advantage of this marker is the long plasma half-life of 4β-OHC (~17 days). Therefore, changes over time in plasma concentrations within individuals will be relatively low. At the same time, this long half life also limits its potential as a marker to predict a rapid CYP3A4 change.[17,18] It has already been shown that treatment with strong CYP3A4 inducers (e.g., carbamazepine, phenytoin, and phenobarbital) increased plasma concentrations of 4β-OHC approximately 10-fold.[16] Also, treatment with the weak CYP3A4 inducer ursodeoxycholic acid resulted in a modest increase in 4β-OHC,[16] indicating the ability of the endogenous marker to distinguish between weak and strong inducers. The ability of 4β-OHC and the 4β-OHC:cholesterol ratio to predict individual taxane pharmacokinetic profiles has not been investigated yet.

The aim of this study was to investigate correlations between the endogenous marker 4β-OHC and the clearance of the taxanes paclitaxel and docetaxel to assess the potential of 4β-OHC as a phenotyping method in taxane therapy. The association between taxane clearance and 4β-OHC parameters was also assessed in the 10% patients with outlier clearance values, because in these patients availability of a strategy that allows *a priori* dose adjustments would be especially important. Because of previously published differences in CYP3A4/5 activity between males and females the correlations were analyzed separately for gender.[19,20] Also, paclitaxel metabolism has been described to be lower in females than in males.[21]

MATERIALS & METHODS

Patients

Patients treated with paclitaxel or docetaxel whom were enrolled in a pharmacokinetic study (Dutch trial register, www.trialregister.nl, NTR2311) were included in this analysis. Inclusion criteria were as described previously.[22,23] In brief, patients included had (i) a histological or cytological confirmed diagnosis of cancer treated with paclitaxel or docetaxel, (ii) were aged ≥18 years, (iii) had WHO performance score of 0-1 and (iv) had adequate hematopoietic, hepatic and renal functions. During the study, CYP3A4 and CYP2C8 inducers or inhibitors were not allowed. The trial was approved by the medical ethical committee of the Erasmus University Medical Center and all patients participating in this trial provided written informed consent.

Treatment

Patients treated with docetaxel were mainly administrated weekly a 75 to 100 mg/m^2 dose intravenously, which depended on the tumor type and combination regimen used. Patients treated with paclitaxel were mainly administrated a intravenous dose of 50 mg/m^2, 90 mg/m^2 weekly or 175 mg/m^2 every 3 weeks. Patients did not receive other chemotherapy for 4-6 weeks before start of docetaxel or paclitaxel treatment.

Pharmacokinetic analysis

Pharmacokinetic sampling was performed in any treatment cycle according to a limited sampling strategy and docetaxel and paclitaxel quantification in plasma as described previously.[22,23] Samples were drawn pre-treatment, before the end of infusion, and in the elimination phase of the drug. Docetaxel was quantitated in plasma by a validated high-performance liquid chromatography (HPLC) method with UV detection[24] or by validated LC MS/MS method.[25,26] Paclitaxel was quantitated by a validated UV detection HPLC method[27] or by a validated LC-MS/MS method based on the method described for docetaxel.[25] Individual pharmacokinetic parameters were based on a previously developed population pharmacokinetic models for docetaxel[28] or paclitaxel[2] with population Cremophor concentrations.[29] The pharmacokinetic parameters were estimated as Empirical Bayes estimates with the non-linear mixed-effect modeling

software NONMEM version VI and 7 (Icon Development Solutions, Icon Development Solutions, Ellicott City, MD). Unbound clearance of paclitaxel was used in the analysis instead of total paclitaxel, because the formulation vehicle of paclitaxel, Cremophor EL, causes the total fraction of paclitaxel to have non-linear pharmacokinetics.[30]

Measurement of 4β-hydroxycholesterol

Measurement of 4β-OHC was performed during the same paclitaxel cycle as the pharmacokinetic sampling. Blood samples were collected and centrifuged immediately after collection and then stored at -70°C until the day of analysis. After the addition of 50 µL of internal standard solution (1000 ng/mL 4β-hydroxycholesterol-d7 in water) and 500 µL of 1 *M* ethanolic potassium hydroxyde to 50 µL plasma, oxyesterols and the internal standard were saponificated for 30 minutes at T = 37°C. After saponification, 300 µL of water was added to the solution and extracted twice with 1-mL n-hexane. The organic phase was evaporated at 45°C under reduced pressure. Hereafter, oxyesterols and the internal standard were derivated based on the mixed-anhydride method previously described by Yamashita et al[31] with minor modifications. Amounts of 10 mg 2-methyl-6-nitrobenzoic anhydride, 3 mg of 4-dimethylaminopyridine and 8 mg of picolinic acid were dissolved in 150 µL of pyridine solution and added to the evaporated samples following addition of 20 µL of triethylamine. Hereafter the samples were incubated for 45 minutes at 37°C. The oxysterols were extracted after the addition of 500 µL of water and 1-mL of n-hexane. The organic phase was evaporated and the residu was resuspended in 200 µL acetonitrile/methanol/water (3:6:1.8, v/v/v) and stored at 4°C until analysis. Oxysterols were separated by Ultra Performance Liquid Chromatography on a Acquity BEH Pheny™ 1.7 µm column (Waters, Etten-Leur, The Netherlands) with a mobile phase composed of water acidified with 0.1% formic acid and acetonitrile/methanol (1:2, v/v) acidified with 0.1% formic acid (21:79, v/v) eluted at a flow-rate of 0.300 mL/min. Baseline separation was achieved for the 4α-hydroxycholesterol and the 4β-OHC. Column effluents were analysed by mass spectrometry with atmospheric pressure electropray ionization. The source temperature and the desolvation temperature were set at 130°C and 350°C respectively. The desolvation gas flow was set at 800 L/hr and the cone capillary voltage was kept at 1.5 kV. Multiple reaction monitoring (MRM) mode was applied for the quantitation of 4β-hydroxycholesterol and the internal standard with the following parameters: m/z 635 > 146, collision energy at 25 eV and m/z 642 > 146, collision energy at 15 eV respectively. The cone voltage was 38V for all compounds and dwell times were set at 100 ms. Calibration curves were linear over a range of 2-650 ng/mL. The total relative standard deviation (CV) were less than 8%. To correct for cholesterol levels, total cholesterol was measured on a Roche Modular P800 analyser (Roche Diagnostic Corp., Indianapolis, IN).

Statistics

Pharmacokinetic data are presented as median values with ranges unless stated otherwise. To test the associations between the pharmacokinetic parameters of

paclitaxel or docetaxel and 4β-OHC, cholesterol and the 4β-OHC:cholesterol ratio, the Spearman correlation test was used. Patients with the 10% lowest and highest docetaxel and paclitaxel clearances were selected for separate analysis to test the correlation between the 4β-OHC marker and clearance in patients with pharmacokinetic outlier values. Because of previously seen difference in 4β-OHC levels between males and females, indicating a gender difference in CYP3A4/5 activity between males and females,[19,20] the data were also analyzed separately for males and females. All *P*-values are two-sided, and a *P*-value < 0.05 was considered statistically significant. Analysis was conducted with SPSS version 20.0 (SPSS Inc, Armonk, NY) and Stata release 12 (StataCorp LP, College Station, TX).

RESULTS

Patients

291 patients treated with paclitaxel and 151 patients treated with docetaxel were included in this study. In both cohorts patients were mostly of Caucasian origin (paclitaxel cohort 96%, docetaxel cohort 92%; **Table 1**). The median age in the paclitaxel cohort was 61 years (range, 18-82 years), 49% of these patients were female and esophageal cancer was the most frequent primary tumor type (50%; Table 1). In the docetaxel treated group, the median age was 56 years (range, 18-80 years), most patients were female (68%) and breast cancer was the most frequent primary cancer (64%); Table 1). Patients on paclitaxel received a median administered dose of 170 mg and patients on docetaxel a median dose of 160 mg (Table 1).

Paclitaxel cohort

Pharmacokinetic parameters and 4β-OHC of paclitaxel treated patients are summarized in **Table 2**. Paclitaxel clearance differed approximately 8-fold between individual patients. The median 4β-OHC levels were 19.4 ng/mL (range, 2.9-155 ng/mL) and the median 4β-OHC:cholesterol ratio was 4.1 (range, 1.0-26.0). The median 4β-OHC concentrations were higher in females than in males (21.0 ng/mL *versus* 17.5 ng/mL; $P = 0.02$). The median 4β-OHC:cholesterol ratio was similar in males and females (3.9 range, 1.0-19.0 and 4.3 range, 1.0-26.0, respectively).

There were no significant correlations between the clearance of paclitaxel and 4β-OHC levels in both males and females (**Table 3; Figure 1**), except for a weak correlation between cholesterol levels and paclitaxel clearance (R = -0.13; $P = 0.03$). There were also no correlations between the 4β-OHC:cholesterol ratio and paclitaxel clearance ($P > 0.4$; Table 3). And, when comparing patients with the 10% highest or lowest paclitaxel clearances (n = 58), there were no correlations between the clearance of paclitaxel and 4β-OHC parameters ($P > 0.05$). This outcome did not change when the data were analyzed separately for gender (males: $P \geq 0.5$ and females $P > 0.2$).

Table 1. Patient Characteristics[a]

Characteristic	Paclitaxel cohort	Docetaxel cohort
Number of patients	291	151
Gender[b]		
Male	149 (51)	49 (33)
Female	142 (49)	102 (68)
Ethnicity		
Caucasian	278 (96)	139 (92)
Other	11 (4)	6 (4)
Unknown	2 (1)	6 (4)
Age, y	61 (18-82)	56 (18-80)
BSA (m^2)	1.89 (1.4-2.8)	1.89 (1.4-2.6)
Dose (mg)	170 (70-560)	160 (50-230)
Tumor type[b]		
Esophageal	144 (50)	-
Ovary	45 (16)	-
Breast	14 (5)	96 (64)
Cervix	21 (7)	-
Endometrial	15 (5)	-
Lung	11 (4)	4 (3)
Head/Neck	11 (4)	5 (3)
(A)CUP	8 (3)	-
Testis	6 (2)	-
Melanoma	2 (1)	6 (4)
Prostate	1 (0)	28 (19)
Other	13 (5)	12 (8)

[a] All data are represented as median with range in parentheses, unless stated otherwise.
[b] Number with percentages in parentheses.
Abbreviations: BSA, body surface area; (A)CUP, (adeno)carcinoma of unknown primary

Docetaxel cohort

The docetaxel pharmacokinetic parameters and the 4β-OHC parameters in the docetaxel treated cohort are shown in Table 2. There was almost a 6-fold difference in docetaxel clearance between patients. The median 4β-OHC levels were 20.7 ng/mL (range, 6.3-193 ng/mL). The median 4β-OHC:cholesterol ratio was 4.0 (range, 1.0-27.0). The median 4β-OHC levels did not differ between males and females (20.1 ng/mL, range, 6.3-42.2 ng/mL and 21.2 ng/mL, range, 8.0-193 ng/mL, respectively). Also, the median 4β-OHC: cholesterol ratio was similar in males and females (4.4 range, 2.0-9.0 and 3.9 range, 1.0-27.0, respectively).

Table 2. Summary of docetaxel and paclitaxel pharmacokinetics and 4β-OH-cholesterol pharmacokinetic parameters

	Paclitaxel cohort			Docetaxel cohort		
Parameter	No	Median	Range	No	Median	Range
CL (L/h)[a] taxane	291	477	138-1,037	151	44.1	16.2-95.9
4β-OH cholesterol (ng/mL)	291	19.4	2.9-155	151	20.7	6.3-193
Cholesterol (mmol/L)	291	4.7	2.1-20.0	151	5.4	2.7-10.7
Ratio[b]	291	4.1	1.0-26.0	151	4.0	1.0-27.0
Male						
CL (L/h)[a] taxane	149	540	142-1,037	49	45.0	26.6-95.9
4β-OH cholesterol (ng/mL)	149	17.5	2.9-77.9	49	20.1	6.3-42.2
Cholesterol (mmol/L)	149	4.3	2.1-20.0	49	4.7	2.7-7.7
Ratio[b]	149	3.9	1.0-19.0	49	4.4	2.0-9.0
Female						
CL (L/h)[a] taxane	142	425	138-906	102	42.1	16.2-84.9
4β-OH cholesterol (ng/mL)	142	21.0	6.2-155	102	21.2	8.0-193
Cholesterol (mmol/L)	142	5.1	2.4-8.6	102	5.7	3.1-10.7
Ratio[b]	142	4.3	1.0-26.0	102	3.9	1.0-27.0

[a] For paclitaxel treated patients, unbound clearance is used.
[b] Ratio 4β-OH-cholesterol:cholesterol
Abbreviations: CL, clearance, that is, dose divided by area under the curve

There was no correlation between 4β-OHC parameters and docetaxel in the total cohort ($P \geq 0.4$; **Figure 2**). In males treated with docetaxel (n = 49), there was a significant but weak correlation between docetaxel clearance and 4β-OHC levels (R = 0.35; $P = 0.01$; Table 3). This correlation was not found in females. There were no correlations between the 4β-OHC:cholesterol ratio and docetaxel clearance ($P > 0.2$; Table 3). Of the patients with the 10% lowest and highest docetaxel clearances, only males showed a significant correlation between docetaxel clearance and 4β-OHC (R = 0.76; $P = 0.03$).

DISCUSSION

Recently, the metabolic conversion of cholesterol into 4β-OHC has been described as a useful tool to predict CYP3A4 activity after treatment with strong CYP3A inducers such as cabamazepine, phenytoin or phenobarbital.[16] Treatment with these inducers resulted in highly elevated plasma concentrations of 4β-OHC, as a result of an intensified conversion of cholesterol into 4β-OHC.[16] On the other hand, treatment with CYP3A4 inhibitors, such as ritonavir or itraconazole, lead to decreased plasma levels of 4β-OHC. Taken together, these results suggest a potential use of this marker to assess CYP3A activity after enzyme

Table 3. Correlations between 4β-OH-cholesterol parameters and paclitaxel and docetaxel clearance (L/h)

	R[b]	*P*-value
Paclitaxel (n = 291)		
4β-OH-cholesterol (ng/mL)	-0.06	0.29
Cholesterol (mmol/L)	-0.13	0.03
Ratio[a]	0	0.98
Male (n = 149)		
4β-OH-cholesterol (ng/mL)	0.03	0.75
Cholesterol (mmol/L)	-0.01	0.88
Ratio[a]	0	0.98
Female (n = 142)		
4β-OH-cholesterol (ng/mL)	0	1.0
Cholesterol (mmol/L)	-0.05	0.57
Ratio[a]	0.06	0.47
Docetaxel (n = 151)		
4β-OH-cholesterol (ng/mL)	0.07	0.40
Cholesterol (mmol/L)	-0.01	0.90
Ratio[a]	0.04	0.67
Male (n = 49)		
4β-OH-cholesterol (ng/mL)	0.35	0.01
Cholesterol (mmol/L)	0.11	0.45
Ratio[a]	0.18	0.22
Female (n = 102)		
4β-OH-cholesterol (ng/mL)	-0.04	0.70
Cholesterol (mmol/L)	-0.02	0.81
Ratio[a]	-0.04	0.69

[a] Ratio 4β-OH-cholesterol:cholesterol
[b] Spearman rank correlation coefficient was used to evaluate associations between 4β-OH-cholesterol and docetaxel and paclitaxel pharmacokinetics. All statistical tests were two sided.

induction or inhibition.[15] Our current study is the first to test the ability of the endogenous marker 4β-OHC and the 4β-OHC:cholesterol ratio to predict individual clearance profiles in cancer patients treated with the taxanes docetaxel and paclitaxel.

The 4β-OHC values measured in our study were comparable to 4β-OHC reported in another study in healthy Caucasians (mean 20.5 ng/mL).[32] Also, gender differences in 4β-OHC levels in the paclitaxel treated patients were similar to those previously reported, as were the higher 4β-OHC levels in female we found.[19,20] The variability observed of 4β-OHC measurements was large in both the docetaxel and the paclitaxel-treated patients.

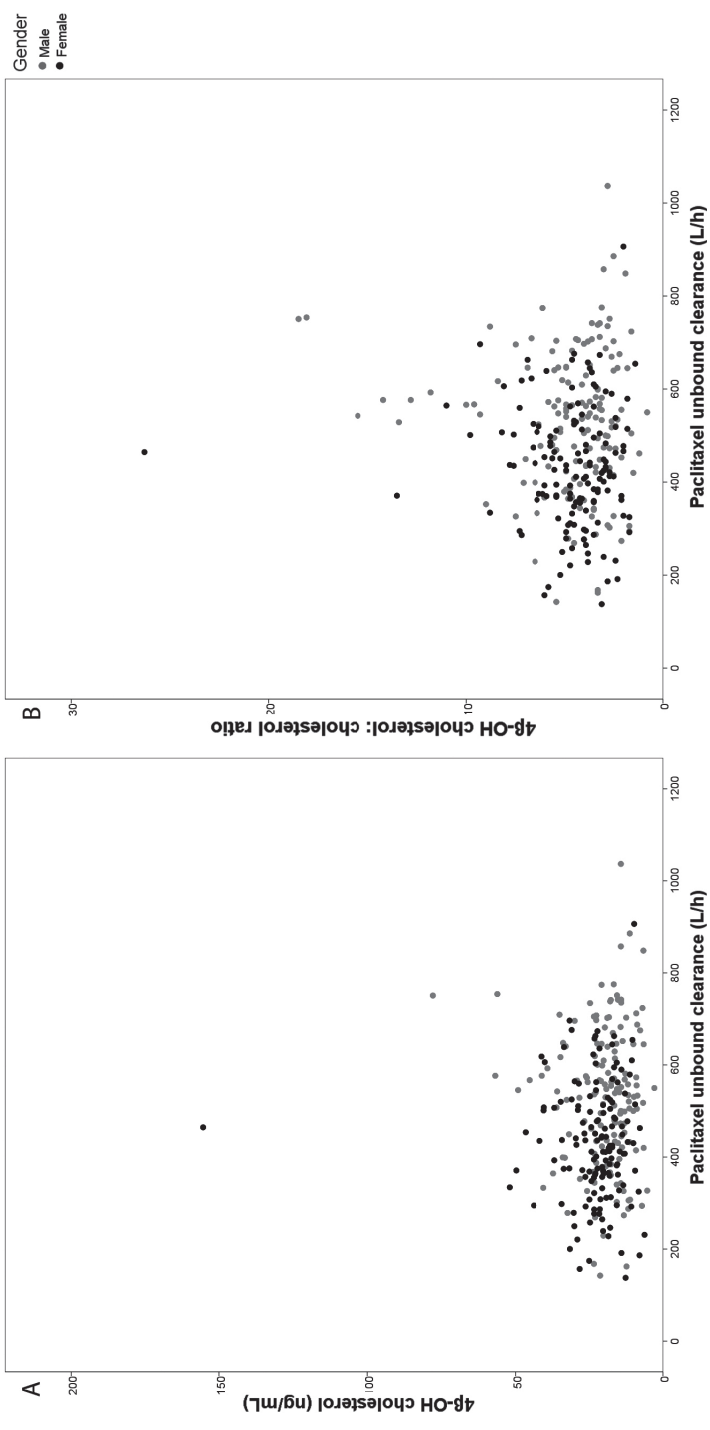

Figure 1. A. Scatter plot of 4β-OHC concentration (ng/mL) *versus* paclitaxel unbound clearance (L/h). B. Scatter plot of 4β-OHC: cholesterol ratio *versus* paclitaxel unbound clearance (L/h).

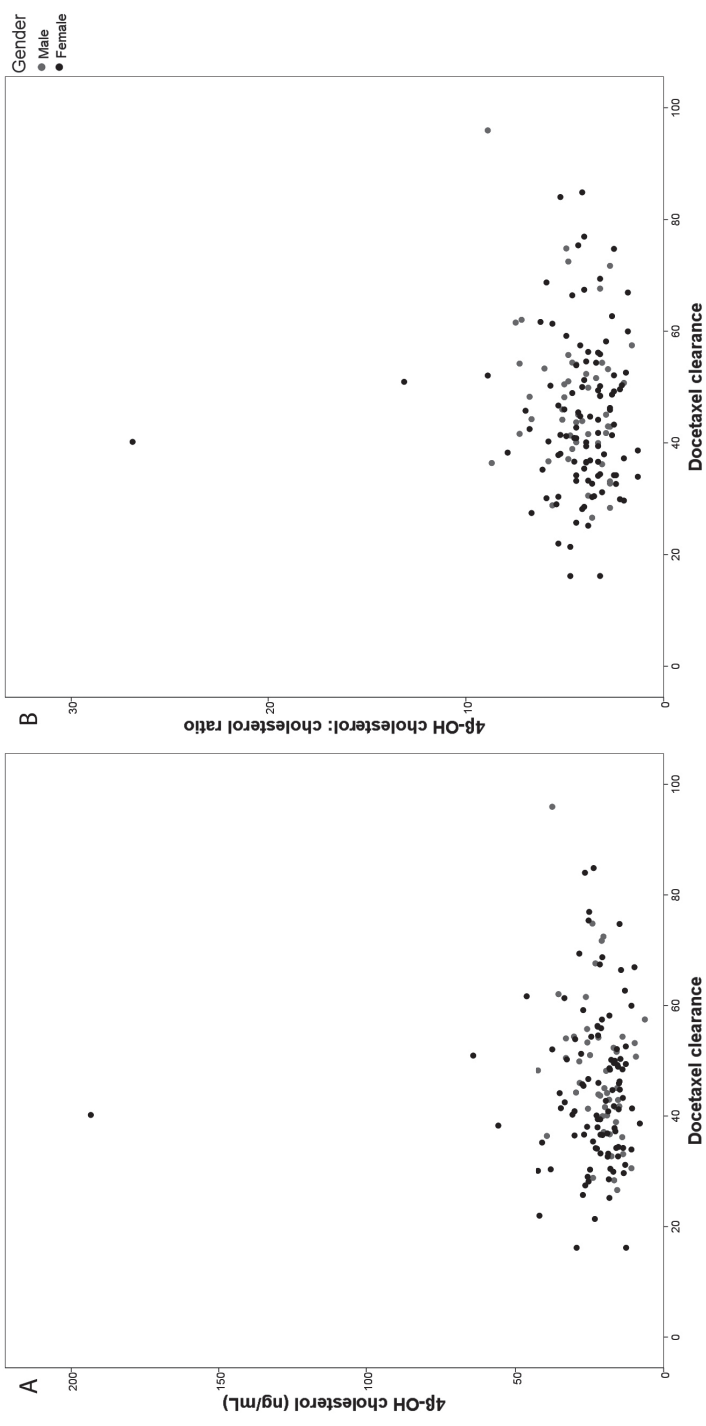

Figure 2. A. Scatter plot of 4β-OHC concentration (ng/mL) *versus* docetaxel clearance (L/h). B. Scatter plot of 4β-OHC: cholesterol ratio *versus* docetaxel clearance (L/h).

Table 4. Correlations between 4β-OH-cholesterol parameters and docetaxel and paclitaxel clearance (L/h) in patients with 10% outlier clearance values

	R[b]	*P*-value
Paclitaxel (n = 58)		
4β-OH-cholesterol	-0.15	0.27
Cholesterol	-0.25	0.06
Ratio[a]	-0.09	0.48
Male (n = 35)		
4β-OH-cholesterol	-0.06	0.71
Cholesterol	-0.08	0.63
Ratio[a]	-0.12	0.50
Female (n = 23)		
4β-OH-cholesterol	0.05	0.83
Cholesterol	-0.25	0.26
Ratio[a]	0.02	0.92
Docetaxel (n = 30)		
4β-OH-cholesterol	0.09	0.64
Cholesterol	0.05	0.79
Ratio[a]	0.03	0.86
Male (n = 8)		
4β-OH-cholesterol	0.76	0.03
Cholesterol	-0.22	0.60
Ratio[a]	0.52	0.19
Female (n = 22)		
4β-OH-cholesterol	-0.08	0.71
Cholesterol	0.10	0.66
Ratio[a]	-0.17	0.45

[a] Ratio 4β-OH-cholesterol: Cholesterol
[b] Spearman rank correlation coefficient was used to evaluate associations between 4β-OH-cholesterol and docetaxel and paclitaxel pharmacokinetics. All statistical tests were two sided.

This could potentially be due to the use of CYP3A4 inhibiting or inducing comedication before start of docetaxel or paclitaxel treatment. Because of the long half-life of the effect of CYP3A4 inhibition of induction could affect 4β-OHC concentrations during therapy.

Unfortunately, paclitaxel and docetaxel clearance could not be predicted accurately by the 4β-OHC marker, which thus precludes clinical use. Although a correlation was seen in a small subgroup of men with the lowest and highest docetaxel clearances, this correlation may result from chance, and has limited predictive value for individual patients. As the conversion of cholesterol to 4β-OHC is exclusively mediated by CYP3A, it is understandable that paclitaxel clearance - which is mediated by both CYP3A and CYP2C8 - is not predicted

by this endogenous marker. For docetaxel clearance this is more surprising, as docetaxel is also exclusively metabolized by CYP3A, although drug transporters may also affect the exposure to this drug.[23] Also, we did not normalize 4β-OHC concentrations to individual cholesterol levels as was suggested by Yang and colleagues[17] to control for CYP3A inhibition or induction. However, the use of CYP3A4 and CYP2C8 inducers and inhibitors was strictly prohibited during the study, making this potential explanation for a lack of correlation less plausible. Another explanation for the lack of correlation in the docetaxel-treated patients with 4β-OHC parameters could be that docetaxel metabolism is not solely dependent on CYP3A4, but may include other enzymes and transporters. For example, it was recently reported that administration of the CYP3A inhibitor imatinib did not affect docetaxel clearance, while it was shown to inhibit CYP3A4.[33]

Although a lot of research in the field of 'phenotyping' has already been done, a perfect phenotyping method for CYP3A4 still has not been found. Multiple exogenous CYP3A phenotyping methods have been proposed, including the midazolam clearance test, the erythromycin breath test, and the administration of cortisol. Clear correlations have been observed between midazolam clearance or erythromycin clearance and hepatic CYP3A concentrations and amount of CYP3A4 protein levels, respectively, making these drug potentially usable probe-drugs.[34,35] Midazolam metabolism has also been associated with the induction and inhibiton of CYP3A4 activity in patients receiving rifampin and erythromycin, respectively.[36] Also, midazolam metabolism has been shown to be highly correlated to the clearance of cyclosporine and irinotecan, while erythromycin and cortisol were correlated with docetaxel metabolism.[13,34,37-39] However, in another recent study, the utility of the erythromycin test to predict docetaxel pharmacokinetics could not be confirmed.[40] The use of these phenotyping methods is still not widely adopted. There are several explanations for this. First of all, the administration of an exogenous compound and additional blood sampling is a burden to the patient and is time consuming. Secondly, the administration of radioactively labeled material, as is the case for erythromycin, makes this method even less attractive. Finally, all these methods are expensive and complex, and may not be suitable for every individual patient. We may therefore wonder if an exogenous drug will ever be adopted as a clinically implementable probe-drug, or that we should focus more on endogenous options. Although this first study on correlations between an endogenous marker and two anti-cancer drugs may be disappointing, we should further explore other options including other endogenous markers and other drugs. Whether the 4β-OHC marker could be of potential clinical use for other CYP3A4 substrates remains to be elucidated.

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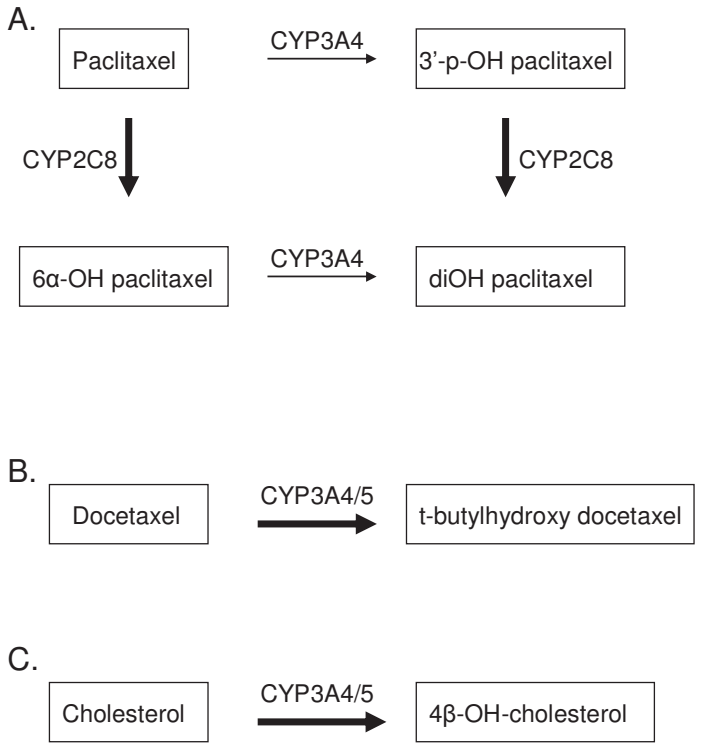
SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. A. Scheme explaining the metabolism of paclitaxel, primarily mediated by CYP2C8 and to a minor extent by CYP3A4. B. Scheme explaining the metabolism of docetaxel, primarily mediated by CYP3A4/5. C. Schematic conversion of cholesterol into 4β-OHC, primarily mediated by CYP3A4/5.

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7

DEXTROMETHORPHAN AS A PHENOTYPING TEST TO PREDICT ENDOXIFEN EXPOSURE IN PATIENTS ON TAMOXIFEN TREATMENT

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ABSTRACT

Purpose

Tamoxifen, a widely used agent for the prevention and treatment of breast cancer, is mainly metabolized by CYP2D6 and CYP3A to form its most abundant active metabolite, endoxifen. Interpatient variability in toxicity and efficacy of tamoxifen is substantial. Contradictory results on the value of CYP2D6 genotyping to reduce the variable efficacy have been reported. In this pharmacokinetic study, we investigated the value of dextromethorphan, a known probe drug for both CYP2D6 and CYP3A enzymatic activity, as a potential phenotyping probe for tamoxifen pharmacokinetics.

Methods

In this prospective study, 40 women using tamoxifen for invasive breast cancer received a single dose of dextromethorphan 2 hours after tamoxifen intake. Dextromethorphan, tamoxifen, and their respective metabolites were quantified. Exposure parameters of all compounds were estimated, log transformed, and subsequently correlated.

Results

A strong and highly significant correlation (r = -0.72; P < .001) was found between the exposures of dextromethorphan (0 to 6 hours) and endoxifen (0 to 24 hours). Also, the area under the plasma concentration–time curve of dextromethorphan (0 to 6 hours) and daily trough endoxifen concentration was strongly correlated (r = -0.70; P < .001). In a single patient using the potent CYP2D6 inhibitor paroxetine, the low endoxifen concentration was accurately predicted by dextromethorphan exposure.

Conclusion

Dextromethorphan exposure after a single administration adequately predicted endoxifen exposure in individual patients with breast cancer taking tamoxifen. This test could contribute to the personalization and optimization of tamoxifen treatment, but it needs additional validation and simplification before being applicable in future dosing strategies.

INTRODUCTION

The antiestrogenic agent tamoxifen is frequently used for the treatment of breast cancer, both in adjuvant and metastatic settings. Although this therapy has proven efficacy, 30% to 50% of patients treated with adjuvant tamoxifen therapy ultimately experience a relapse of breast cancer, and de novo or acquired resistance is also seen in metastatic disease.[1]

The clinical activity of tamoxifen as well as treatment-related toxicity differ greatly between individual patients. This is, at least partially, due to the interpatient variability in the pharmacokinetics of the agent,[1-3] which, in turn, is influenced by many factors, such as comedication,[3-5] lifestyle factors, and genetic variation in metabolizing enzymes.[1,2,6-8] This highlights the need for an accurate and predictive marker to personalize and optimize tamoxifen therapy.[9]

Tamoxifen is a prodrug that requires transformation into its 100-fold more potent metabolite endoxifen (N-desmethyl-4-hydroxy-tamoxifen) by CYP3A4/5 and CYP2D6 isozymes (Fig 1).[10] Both CYP2D6 and CYP3A play a crucial role in the activation of tamoxifen into this most abundant active metabolite.

The CYP2D6 gene is a highly polymorphic gene, the activity of which is mainly determined by the presence of variant alleles. The variant CYP2D6*4 allele, encoded by a single nucleotide polymorphism in the gene encoding for CYP2D6, is the most prevalent CYP2D6 dysfunctional allele among Caucasians, but many other polymorphisms also result in an altered enzymatic activity.[11] In case of one or two nonfunctional alleles, CYP2D6 enzyme function is reduced or absent, respectively. Patients with only one functional allele are referred to as intermediate metabolizers (IMs), whereas individuals with two nonfunctional alleles are poor metabolizers (PMs). Individuals with two functional alleles or with one functional and one decreased-function allele are called extensive metabolizers (EMs). Individuals with duplications of the functional CYP2D6 gene do express higher amounts of the enzyme and are classified as ultrarapid metabolizers (UMs).[12,13]

Variation in CYP2D6 enzyme activity has been related to the efficacy of tamoxifen therapy. Several studies indicate that patients with reduced functioning CYP2D6 alleles have a worse therapeutic outcome compared with patients with normal enzyme functionality.[1,12,14-18] However, other studies reported contradictory results,[19-28] which renders interpretation of the clinical relevance of genetic testing highly complex. Besides genotyping, several studies also showed the impact of CYP2D6 inhibiting comedication, resulting in decreased enzyme function and, consequently, in therapeutic outcome.[5,11,29,30]

Whether variations in CYP3A4 and/or CYP3A5 influence tamoxifen treatment outcome is still largely unclear.[31] CYP3A activity is highly susceptible to induction and inhibition by comedication,[32] lifestyle, and environmental factors[33] so theoretically may contribute to the interpatient variability in tamoxifen pharmacokinetics and treatment outcome.

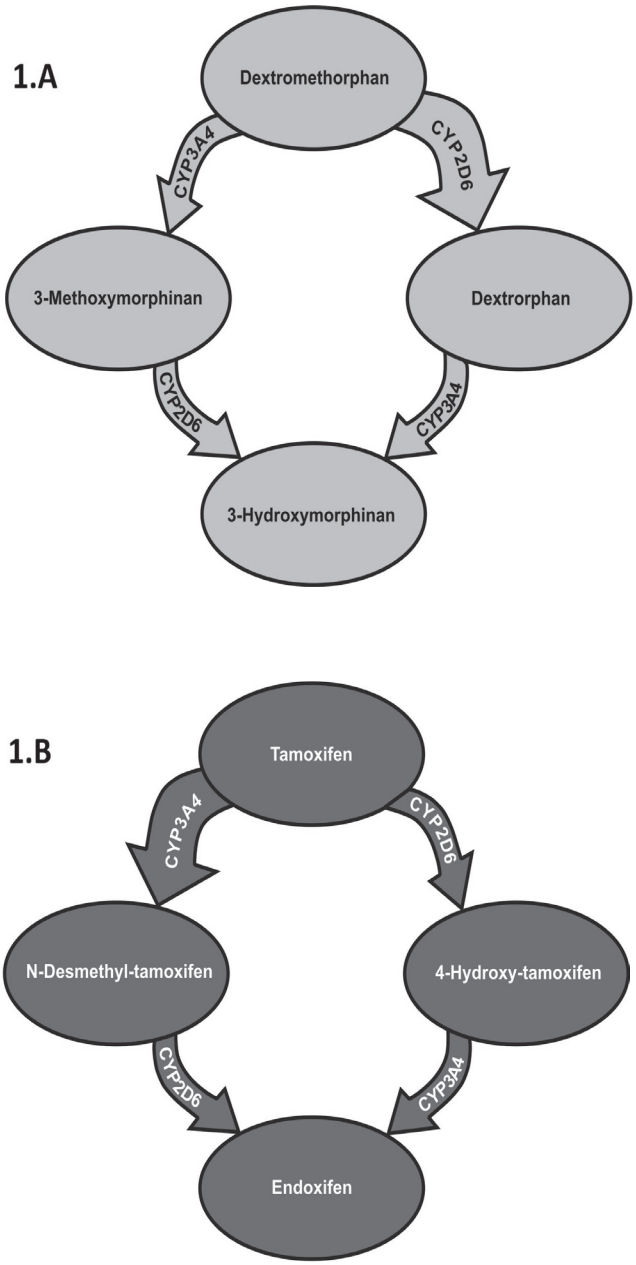

Figure 1. Scheme for both dextromethorphan and tamoxifen metabolism. Dextromethorphan is mainly converted by CYP2D6 (figure 1A) to dextrorphan, and tamoxifen is mainly converted by CYP3A4 (figure 1B) into N-desmethyl tamoxifen. Afterwards, dextrorphan is converted by CYP3A4 into 3-hydroxymorphinan and N-desmethyl tamoxifen is converted by CYP2D6 into endoxifen.

Overall, it may be an oversimplification to solely implement CYP2D6 genetic variability to predict tamoxifen pharmacokinetics and treatment outcome. A combined phenotyping approach for CYP2D6 and CYP3A activity, therefore, might be a better approach to more accurately predict the pharmacokinetics, toxicity profile, and therapeutic effects of tamoxifen.[33] Dextromethorphan is a known CYP2D6 and CYP3A phenotyping probe that can be used as a simple and safe drug for monitoring the combined CYP3A and CYP2D6 activity.[34] Here, we report the outcomes of a prospective clinical trial to study the relationship between the pharmacokinetics of dextromethorphan and the pharmacokinetics of tamoxifen in female patients with breast cancer.

METHODS

Treatment of patients

Eligible patients had known invasive breast cancer and used 20 or 40 mg of tamoxifen daily, either as adjuvant therapy or as treatment for metastatic disease, respectively. Patients had to use tamoxifen for at least 3 weeks to ensure steady-state concentrations. Other inclusion criteria were age older than 18 years; good WHO performance score (ie, 0 to 1); and adequate hematologic, renal, and hepatic functions. No concomitant medication known to be moderate or strong inducers or inhibitors of CYP3A, the drug transporters ABCB1 (P-glycoprotein), and/or ABCG2 (Breast Cancer Resistance Protein) was allowed (Appendix Table A1, online only) for at least 3 weeks before pharmacokinetic sampling. All patients provided written informed consent before study entrance and filled out a diary providing information on the amount of tamoxifen and time of intake to assess compliance to therapy. The study protocol was approved by the institutional review board and was registered in the Dutch trial registry (No. NTR1751).

Pharmacokinetic sampling and analysis

To investigate pharmacokinetic relationships between tamoxifen and its putative probe dextromethorphan, patients were given a single dose of dextromethorphan 30 mg orally, 2 hours after oral tamoxifen intake. Subsequently, blood samples for pharmacokinetic analyses of tamoxifen and its metabolites (4-hydroxy-tamoxifen, *N*-desmethyl-tamoxifen, and endoxifen) and dextromethorphan and its metabolites (dextrorphan, 3-methoxymorphinan, and 3-hydroxymorphinan) were collected for 24 hours via an indwelling intravenous catheter. For this study procedure, all patients were hospitalized at the Erasmus Medical Center, Rotterdam, the Netherlands. For the tamoxifen pharmacokinetic analysis, a 4-mL blood sample was collected in the absence of any anticoagulant in serum gel tubes at predose and at 30 minutes and 1, 1.5, 2, 4, 8, 12, and 24 hours after the administration of the daily dose of tamoxifen. Blood samples were placed at ambient temperature for 30 minutes to allow for coagulation. Then, the samples were centrifuged for 10 minutes at 2,800 to 3,000 x *g* at 4°C. Subsequently, serum was transferred into amber colored vials and was stored at temperature colder than -70°C until analysis by a validated assay occurred.[35]

For dextromethorphan pharmacokinetic analyses, 4-mL blood samples were collected in tubes containing lithium heparin as anticoagulant at predose and at 30 minutes and 1, 1.5, 2, 4, 6, 10, and 22 hours after the administration of a 30-mg dose. Blood samples were centrifuged within 15 minutes after collection for 10 minutes at 2,800 to 3,000 x g at 4°C. Plasma was stored at temperature colder than -70°C until additional analysis occurred. Dextromethorphan and its metabolites were measured by using a validated assay, as recently reported.[36]

Individual pharmacokinetic parameters for tamoxifen, dextromethorphan, and their respective metabolites were estimated by using noncompartmental analysis with the software program Phoenix WinNonlin, 6.1 (Scientific Consultant, Apex, NC; Pharsight, Mountain View, CA).

Genotyping procedures

All patients were genotyped for the CYP2D6*3, *4, *5 and *6 polymorphisms, associated with no enzyme activity, to detect more than 95% of CYP2D6 poor metabolizers. Also CYP2D6*10, *17, and *41, which are associated with reduced enzyme activity, were determined.9 In addition, for patients identified with substantial discrepancies between CYP2D6 genotype and phenotype (as measured by endoxifen area under the curve [AUC]), an additional Amplichip CYP450 Test (Roche Diagnostics, Pleasanton, CA) was performed to identify rare but relevant polymorphisms in the CYP2D6 gene. Next to the above-mentioned polymorphisms, variants *2 (normal function) and *7, *8, *9, *11, *14A, *14B, *15, *19, *20, *25, *26, *29, *30, *31, *35, *36, *40, 1xN, 2xN, 4xN, 10xN, 17xN, 35xN, and 41xN were determined to detect greater than 99% of all IMs and PMs.

Statistical analysis

Pharmacokinetic data are presented as median values with ranges unless stated otherwise. Pharmacokinetic parameters for the 40-mg dose group were corrected to a 20-mg dose. Before statistical data analysis, parameter values were log transformed. To test associations between AUC and steady-state concentration before dosing (ie, C_{trough} T = 0) of tamoxifen metabolites and dextromethorphan metabolites, Pearson's correlation coefficient (r) was used. A power analysis to detect a correlation in clearance of .75 with 90% power required at least 37 patients. $P \leq .05$ was considered statistically significant. All statistical tests were two sided. Statistical analysis was performed with SPSS version 15.0 (SPSS Inc, Chicago, IL).

RESULTS

Patients and treatment

A total of 40 women using tamoxifen on steady-state with a median age of 53 years (range, 22 to 71 years) were recruited between July 2009 and April 2010 (Table 1). Of these women, 28 received adjuvant tamoxifen at a dose of 20 mg daily, and

Table 1. Patient demographics and clinical characteristics

Characteristics	Value
No. of eligible patients	40
Age [y], median (range)	53 (22-71)
Height [m], median (range)	1.69 (1.56-1.79)
Weight [kg], median (range)	72.7 (48.5-114)
Body surface area [m^2], median (range)	1.86 (1.45-2.3)
Body mass index, median (range)	25.6 (19.7-40.9)
≤50 years	24.9 (19.7-40.9)
>50 Years	26.3 (20.1-39.0)
Dose tamoxifen, number (%)	
20 mg (adjuvant)	28 (70)
40 mg (metastatic)	12 (30)
Performance score (WHO), number (%)	
0	35 (87.5)
1	5 (12.5)

12 women received tamoxifen for metastatic disease dosed at 40mgonce daily. One of the women used paroxetine as an antidepressant agent on a daily basis. Because the exclusion criteria of the study did not mention CYP2D6 inhibitors, this patient was not excluded from the analysis. Two patients were not included in the correlation analysis (except for C_{trough} T = 0 values) because of incomplete blood sampling for pharmacokinetic analysis.

Tamoxifen and dextromethorphan pharmacokinetics

Summaries of the pharmacokinetics of tamoxifen and dextromethorphan are presented in Tables 2 and 3. The AUC_{tau} (area under the plasma concentration–time curve for a dosing interval) of tamoxifen had a median of 6,303 nmol_h/L (range 2,402 to 17,775 nmol_h/L), and the median endoxifen AUC_{tau} was 424 nmol_h/L (range, 146 to 1,220 nmol_h/L). At T = 0 hours, the median tamoxifen C_{trough} levels were 216 nmol/L (range, 81 to 389 nmol/L), and median endoxifen C_{trough} levels were 21.3 nmol/L (range, 4.9 to 46.0 nmol/L). As shown, the AUCtau of tamoxifen varied more than sevenfold, and the endoxifen AUCtau varied more than eight-fold. Furthermore, median Ctrough levels of endoxifen varied almost 10-fold, indicating a large variability in tamoxifen metabolism between patients (Table 2). The values of metabolites were excluded from analysis if they were less than the lower limit of quantitation of the assays. The dextromethorphan AUC from 0 to 6 hours had a median of 18.9 nmolxh/L (range, 4.4 to 604 nmolxh/L), and the median AUC from 0 to 24 hours was 61.6 nmolxh/L (range, 10.3 to 2,045 nmolxh/L; Table 3).

Table 2. Summary of tamoxifen and dextromethorphan pharmacokinetic parameters

Parameter[a]	Tamoxifen	No.[b]	4-Hydroxy-tamoxifen	No.[b]	N-desmethyl-tamoxifen	No.[b]	Endoxifen	No.[b]
AUC (0-24h) nmol*h/L	6,303 (2,402-17,775)	37	105 (61-370)	31	11,682 (5,084-36,401)	37	424 (146-1,220)	35
C_{trough} T = 0 nmol/L	216 (81-389)	40	4.26 (2.3-7.8)	35	448 (167-1,108)	40	21.3 (4.9-46.0)	38

[a] Parameters given as median with ranges, AUC and C_{trough} concentrations were dose corrected to 20 mg tamoxifen
[b] Represents number of patients evaluable
Abbreviations: AUC, area under the plasma concentration versus time curve; C_{trough} T = 0, steady state concentration before dosing

Table 3. Summary of dextromethorphan pharmacokinetic parameters

Parameter	Dextromethorphan	No.[a]	Dextrorphan	No.[a]	3-Methoxymorphinan	No.[a]	3-Hydroxymorphinan	No.[a]
AUC nmol*h/L								
0-6 hours	18.9 (4.4-604)	39	37.5 (8.5-94.3)	38	6.4 (3.2-24.0)	11	6.1 (3.0-13.6)	33
0-12 hours	40.8 (10.1-1,169)	34	50.6 (20.1-156)	37	13.9 (8.1-62.0)	7	11.8 (5.9-24.4)	17
0-24 hours	61.6 (10.3-2,045)	31	54.9 (27.7-131)	37	25.8 (14.6-34.9)	5	16.3 (5.9-41.4)	6

[a] Represents number of patients evaluable
Abbreviation: AUC, area under the curve

Genotyping analysis

CYP2D6 genotypes and resulting phenotype classification are summarized in Table 4. Of the 40 patients, one patient with three functional alleles was classified as UM, 20 patients were classified as EMs, 18 patients were IMs, and one patient with two non-functional alleles was scored as a PM. All alleles were in Hardy-Weinberg equilibrium, except for *17, probably because of the relatively small sample size for this allele.

In six patients, an additional genotype analysis with the AmpliChip was applied. However, no rare CYP2D6 genetic variants were identified in those patients, thereby almost completely excluding the possibility that the unexpectedly low endoxifen concentrations in these patients were caused by rare CYP2D6 allelic variants.

Table 4. CYP2D6 genotype results and predicted phenotype from genotype

CYP2D6 Genotype	Predicted phenotype[a]	Number of patients (%)	Patients with 25% lowest endoxifen AUC_{tau}
*1 XN/*2	UM	1 (2.5%)	1
*1/*1	EM	18 (45%)	1
*1/*41	EM	2 (5%)	
*1/*3	IM	2 (5%)	
*1/*4	IM	10 (25%)	2[b]
*1/*5	IM	1 (2.5%)	
*1/*6	IM	1 (2.5%)	1
*4/*41	IM	2 (5%)	2
*5/*41	IM	1 (2.5%)	1
*5/*17	IM	1 (2.5%)	1
*4/*4	PM	1 (2.5%)	1

[a] Phenotypic interpretation of CYP2D6 genotype
[b] Including one patient who was taking the strong CYP2D6 inhibitor paroxetine
Abbreviations: AUC_{tau}, area under the plasma concentration-time curve for a dosing interval; XN, extra number of allele as a result of gene duplication; UM, ultrarapid metabolizer; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer

Association between tamoxifen and dextromethorphan pharmacokinetics

Several significant correlations were observed between dextromethorphan pharmacokinetics and endoxifen exposure (Table 5). When there was a high correlation ($r > 0.90$; $P < .001$) between the dextromethorphan AUC at 0 to 6 hours and AUC_{tau}, only the more clinically applicable AUC at 0 to 6 hours is presented in Table 5. Also, only correlations between groups with greater than 20 patient occurrences are shown in Table 5. Highly significant correlations were found between the exposures

Table 5. Correlations between log-transformed dextromethorphan and tamoxifen pharmacokinetic parameters

Dextromethorphan parameter	Tamoxifen parameter	r[a]	p	No.[b]	95% CI
AUC 0-6h					
Dextromethorphan	N-desmethyl-tamoxifen AUC_{tau}	0.49	.002	37	0.20 to 0.70
Dextromethorphan	N-desmethyl-tamoxifen C_{trough} T=0	0.44	.005	39	0.14 to 0.66
Dextromethorphan	4-Hydroxy-tamoxifen AUC_{tau}	-0.36	.045	31	-0.64 to -0.01
Dextromethorphan	4-Hydroxy-tamoxifen C_{trough} T=0	-0.48	.0005	33	-0.70 to -0.17
Dextromethorphan	Endoxifen AUC_{tau}	-0.72	.0001	35	-0.85 to -0.51
Dextromethorphan	Endoxifen C_{trough} T=0	-0.70	.0001	37	-0.83 to -0.48
Dextrorphan	N-desmethyl-tamoxifen AUC_{tau}	0.21	.23	36	-0.13 to 0.50
Dextrorphan	N-desmethyl-tamoxifen C_{trough} T=0	0.26	.13	36	-0.08 to 0.54
Dextrorphan	4-Hydroxy-tamoxifen AUC_{tau}	-0.01	.94	30	-0.37 to 0.35
Dextrorphan	4-Hydroxy-tamoxifen C_{trough} T=0	0.06	.74	31	-0.30 to 0.41
Dextrorphan	Endoxifen AUC_{tau}	0.06	.76	34	-0.29 to 0.39
Dextrorphan	Endoxifen C_{trough} T=0	0.16	.35	36	-0.18 to 0.47
3-Hydroxymorphinan	N-desmethyl-tamoxifen AUC_{tau}	-0.035	.85	32	-0.32 to 0.32
3-Hydroxymorphinan	N-desmethyl-tamoxifen C_{trough} T=0	0.043	.81	33	-0.30 to 0.38
3-Hydroxymorphinan	4-Hydroxy-tamoxifen AUC_{tau}	-0.29	.15	27	-0.60 to 0.10
3-Hydroxymorphinan	4-Hydroxy-tamoxifen C_{trough} T=0	-0.18	.35	28	-0.52 to 0.20
3-Hydroxymorphinan	Endoxifen AUC_{tau}	-0.24	.20	30	-0.55 to 0.13
3-Hydroxymorphinan	Endoxifen C_{trough} T=0	-0.10	.58	31	-0.44 to 0.26

[a] A Pearson's correlation coefficient was used to evaluate associations between dextromethorphan and tamoxifen pharmacokinetics. All statistical tests were two-sided.
[b] Number of patients evaluable for correlation analysis
Abbreviations: AUC, area under the plasma concentration versus time curve; AUC_{tau}, area under the plasma concentration-time curve for a dosing interval; C_{trough} T=0, steady state trough concentration before dosing.

to dextromethorphan and the steadystate of endoxifen (AUCtau) and daily trough endoxifen (Ctrough T = 0) concentrations (r > -0.63; *P* < .001; Table 5; Fig 2). When subanalysis was performed on the 20-mg and 40-mg treated groups, the exposure relationships did not differ between the groups.

Interestingly, the single patient (Fig 2, box) using the strong CYP2D6 inhibitor paroxetine indeed had a relative low endoxifen AUC_{tau} (186 nmolxh/L) compared with the median of the group (424 nmolxh/L) and was classified as an IM with one functional allele and one dysfunctional allele. This low endoxifen exposure, in all likelihood the result of paroxetine-mediated inhibition of CYP2D6, was accurately predicted by the exposure to dextromethorphan.

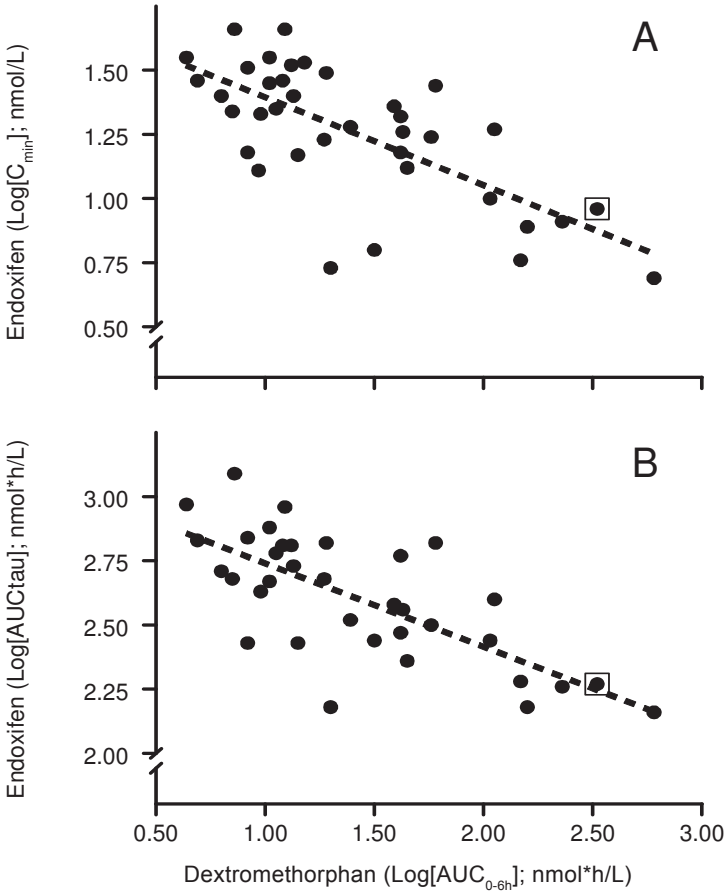

Figure 2. (A) Correlation between dextromethorphan AUC 0-6h and endoxifen C_{trough} T=0 in 38 patients. The dashed line represents a linear regression line (r = -0.70, p < .0001). (B) Correlation between dextromethorphan AUC 0-6h and endoxifen AUC_{tau} in 35 patients (r = -0.72, p < .0001). All statistical tests were two-sided. The box in both A and B represents the single patient that used the strong CYP2D6 inhibitor paroxetine.

Association between drug concentrations and CYP2D6 genotype

When performing a Spearman rank correlation test between CYP2D6 genotype (coded as PM = 1, IM = 2, EM = 3, UM = 4) and endoxifen AUC_{tau}, we found a correlation (r = 0.55; *P* = .001; 95% CI, 0.27 to 0.75).

We identified the 10 patients (25%) with the lowest endoxifen AUC_{tau} values of our study group, because, for these patients, the correlation between predicted phenotype and real exposure to endoxifen probably is most essential. Interestingly, in this group of patients, one was identified as an EM and even one as a UM, indicating a

large difference between genotypic prediction and observed phenotype (Table 4). The additional Amplichip analysis did not alter the genotypically estimated parameters.

The patient using the CYP2D6 inhibitor paroxetine (Fig 2, box) had a low endoxifen AUC at 0 to 24 hours (186 nmolxh/L), within the lowest quartile of endoxifen drug levels, and was classified as an IM with one functional allele. Genotyping alone would not have predicted the low observed concentrations of endoxifen in this patient.

Association between dextromethorphan pharmacokinetics and tamoxifen-related toxicity

Patient diaries and medical files were screened for tamoxifen related toxicity. However, the amount of tamoxifen-related toxicity was relatively mild. Toxicity showed no relationship with dextromethorphan or tamoxifen pharmacokinetics.

DISCUSSION

In this study, dextromethorphan plasma exposure was highly and significantly correlated with endoxifen serum exposure, providing an alternative method to predict tamoxifen pharmacokinetics. To our knowledge, these are the first data indicating that dextromethorphan is a good phenotyping probe to predict tamoxifen pharmacokinetics, providing a valuable tool for future studies to additionally personalize tamoxifen therapy for patients with breast cancer.

Until now, many efforts have been undertaken to investigate the relation between CYP2D6 genotype and efficacy of tamoxifen treatment, eventually aiming at additional refinement and optimization of tamoxifen therapy. Several reports described a relation between variation in CYP2D6 genotype and differences in adverse effects (eg, hot flashes[12]) and clinical outcome (disease recurrence, disease free survival, and/or overall survival[1,12,14-18]). However, other studies, could not confirm these findings.[19-24,26-28,37] In line with this, a meta-analysis that used pooled data from 10 studies (N = 3,205) failed to show an association between CYP2D6 genotype and disease-free or overall survival.[23] These discrepant findings could partly be explained by the retrospective nature of most studies. In addition, more rare variant alleles of CYP2D6 were not tested for in most studies. Also, it cannot be excluded that genetically identified Ems may in fact be CYP2D6 PMs because of the use of CYP2D6 inhibition resulting from comedication, which in most studies was insufficiently assessed. Recently, new data from the prospective ATAC (Arimidex, Tamoxifen, Alone or in Combination) and BIG 1-98 (Breast International Group 1-98) trials, presented at San Antonio Breast Cancer Symposium 2010, also showed no association between CYP2D6 genotype and survival.[26,27] Therefore, at this moment, too much controversy remains for routine CYP2D6 testing.

The formation of endoxifen is not only possible through CYP2D6 metabolism but also by CYP3A (Fig 1B). This might be an underestimated factor, which also could affect tamoxifen phenotype. In fact, CYP2D6 genotyping has been shown to predict only 23% of the variability in tamoxifen pharmacokinetics.[4]

The utility of phenotyping probes has been explored before in oncology for several cytotoxic drugs, including irinotecan and docetaxel.[33,38,39] Here, we studied dextromethorphan as a potential phenotyping probe drug for tamoxifen therapy. Dextromethorphan is, similar to tamoxifen, metabolized by both CYP2D6 and CYP3A, rendering it potentially an excellent probe drug[34,40-42] to explore the pharmacokinetics of tamoxifen in relation to efficacy, eventually, to individualize and optimize treatment. Ample experience with dextromethorphan as probe drug has been gathered in other disciplines of medicine.[43-46] So far, there are no data yet on the value of dextromethorphan in relation to tamoxifen therapy in patients with breast cancer.

Currently, dextromethorphan is also under investigation in another trial, exploring its usefulness as probe drug in relationship to tamoxifen therapy. At the MayoClinic, a trial has recently been started to identify CYP2D6 PMs by use of a 13C dextromethorphan breath test and to correlate breath test results to the pharmacokinetics of tamoxifen.

Also, alternative phenotypic outcome values have been suggested, such as a scoring system for CYP2D6 activity by Borges et al[5] incorporating CYP2D6 genotype and CYP2D6 inhibitor use. In contrast with the dextromethorphan data, however, this scoring system does not account for potential CYP3A influence and, therefore, might be less complete.

For several patients in our study, a firm discrepancy between genotype and observed phenotype (eg, endoxifen AUC_{tau}) was observed. These patients had a functional CYP2D6 enzyme according to the results of the genotyping tests; despite that, they had endoxifen concentrations that belonged to the 25% lowest values of the total group. In some instances, the concentration of endoxifen was even less than the lower limit of quantitation of the analytic method. The single patient classified as a UM had a low endoxifen exposure despite carrying three functional alleles. One of the two patients classified as EM also belonged to the 25% lowest endoxifen concentrations, whereas this was the case for seven of the 18 patients classified as IM phenotype. The use of the Amplichip did not alter genotypically predicted CYP2D6 phenotype. Thus, the dextromethorphan phenotyping test was demonstrated a more reliable test to predict endoxifen concentrations. This was particularly the case for a patient who was using the strong CYP2D6 inhibitor paroxetine. The low systemic exposure of endoxifen in this patient was accurately predicted by the phenotyping test and not by the assessed genotype. The latter observation underscores the importance of collecting complete information on comedication taken in this type of study to allow for additional correction for these variables. Our data indicate that CYP2D6 genotyping alone is insufficiently explanatory regarding the activity of tamoxifen for an individual patient with breast cancer.

The data of the current analysis also showed that a dextromethorphan AUC of 0 to 6 hours predicted the endoxifen concentration in individual patients as well as an AUC of 0 to 24 hours. This means that a pharmacokinetic sampling strategy over a time period of 6 hours in an outpatient setting may be implemented. The next step in the continued clinical development is to validate the test in an independent patient

cohort. As mentioned earlier, this phenotyping method is quite laborious, because multiple samples need to be taken to enable the estimation of dextromethorphan and tamoxifen pharmacokinetics. Therefore, a feasible limited sampling strategy needs to be developed for clinical use, with the detection of ideally one single sample being predictive for a dextromethorphan AUC. In this regard, the CYP3A phenotyping probe midazolam is a good example.[47] Population pharmacokinetic modeling and simulation are in progress to address this issue. Hereafter, a dose-adjusting model of tamoxifen that is based on the dextromethorphan phenotyping test will be developed. Eventually, the test will be repeated over time, correcting the dose for possible changes in phenotype, providing patients a tailor-made therapy.

In conclusion, this dextromethorphan phenotyping probe was a good predictor for endoxifen exposure during tamoxifen treatment, because it incorporated the impact of CYP2D6 as well as CYP3A activity. This test could aid in future studies on the association of tamoxifen and CYP2D6 genotype and phenotype and, ultimately, in the additional personalization and optimization of tamoxifen treatment for breast cancer.

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8

SUMMARY

SUMMARY

The individualization of anti-cancer therapy has been given much attention over the past decade. There has been a focus on differences between patients in tumor characteristics, but also the individualization of the given dose is extremely important. Until now, the clinically used dose of most anti-cancer drugs is traditionally determined in phase I studies. In such studies the anti-cancer drug is given to small heterogeneous groups of patients exposed to increasing dose levels of the drug. The dose level below the one in which unacceptable toxicity is observed is chosen as the recommended phase II dose and will finally become the registered dose level if the new drug is registered for clinical use. However, with current knowledge of individual factors that influence the pharmacology of anti-cancer drugs we may seriously question if this traditional dosing regimen is still feasible. This is particularly important because many anti-cancer agents have a very narrow therapeutic window, which means the range between drug concentrations at which severe toxicity is observed during treatment and the levels at which the drug has sub-therapeutic effects is small. As a result of these small therapeutic margins, the individual variability in toxicity during treatment and efficacy of treatment is large. Identifying factors responsible for this variability is of utmost clinical importance to be able to accurately predict the pharmacokinetics of the drug of interest and consequently the occurrence and severity of toxicity during anti-cancer therapy and the therapeutic effects of the treatment.

The work described in this thesis provides a pharmacological approach contributing to the knowledge of factors influencing the metabolism and toxicity profiles of three anti-cancer agents known for their large inter-individual variation in pharmacokinetics and pharmacodynamics: the taxanes docetaxel and paclitaxel, and the anti-hormonal agent tamoxifen. Both environmental factors (i.e. smoking) as well as genetic factors (polymorphisms in genes coding for metabolizing enzymes and uptake/efflux transporters) were investigated in translational studies, including cell line experiments, mice experiments, and studies in cancer patients. Finally, both an endogenous marker and exogenous marker were correlated with taxane and tamoxifen pharmacokinetics, respectively.

In Chapter 2 we investigated the influence of smoking on the pharmacokinetics and hematological toxicity in patients treated with paclitaxel and docetaxel. We found that the systemic exposure to both taxanes were similar in smokers and nonsmokers. However, smokers were found to have less haematological toxicity than nonsmokers. Paclitaxel treated patients had less grade 3 or 4 leukopenia than nonsmokers, and docetaxel treated patients had less grade 4 neutropenia. Interestingly, in paclitaxel treated patients, the white blood cell counts and absolute neutrophil counts at start of therapy were significantly higher in smokers than in nonsmokers, and a similar trend was seen in the docetaxel treated patients, suggesting induction of the bone marrow by cigarette smoke as the underlying cause of this protective effect.

In **Chapter 3**, genetic variants in a selection of metabolizing enzymes and transporting proteins were tested for their influence on paclitaxel-induced neurotoxicity. The novel *CYP3A4*22* single-nucleotide polymorphism (SNP) was associated with an increased risk of severe neurotoxicity, while the other tested variants did not. Also, the systemic exposure to paclitaxel, measured during one course of paclitaxel treatment, was highly associated with the severity of neurotoxicity. However, *CYP3A4*22* carrier status was not associated with an altered systemic exposure to paclitaxel, indicating a local effect of *CYP3A4*22* in the peripheral nerves. This will be explored further in future studies.

In **Chapter 4**, the drug metabolizing and transporting (DMET) array was used to screen 1,936 pharmacologically relevant SNPs in 225 genes to discover genetic variants that are associated with low paclitaxel clearance. A predictive genetic model of 14 SNPs was developed in a training set of paclitaxel treated patients and subsequently tested in a validation cohort. The developed model had a very high sensitivity (95%), identifying almost all patients with an increased risk of low paclitaxel metabolism. However, the trait-off of this model is the large number of screened patient to identify all patients at risk of low paclitaxel clearance. Therefore, we believe that the current model is not directly implementable in clinical practice. Further research with the DMET array will focus on paclitaxel-related toxicity and docetaxel pharmacokinetics and pharmacodynamics.

In **Chapter 5** we hypothesized that differential expression of polymorphic transporters involved in the hepatic elimination of docetaxel may play an important role in the pharmacologic variability seen in docetaxel treatment. Here, we investigated the contribution of organic anion transporting polypeptides (OATPs) to the disposition of docetaxel using *in vitro* and *in vivo* model systems. We found that docetaxel was not only transported by OATP1B3, in line with earlier published work, but also by OATP1B1. In OATP1B2 knock out mice, docetaxel systemic exposure was highly increased, suggesting a crucial role of OATP1B2 in docetaxel uptake. However, human genetic variants in OATP1B1 and OATP1B3 did not seem to influence the pharmacokinetics of docetaxel. Perhaps a combined decreased functioning of both OATP1B1 and OATP1B3 is required to create an effect on the systemic exposure of docetaxel. These results suggest that caution is warranted if docetaxel has to be administered together with agents that potently inhibit both these transporters.

In **Chapter 6,** we evaluated 4β-hydroxycholesterol as an endogenous marker for CYP3A function to predict individual taxane pharmacokinetics. This is the first study that studies the use of an endogenous marker to predict anti-cancer drug pharmacokinetics in a large set of cancer patients. Unfortunately, the 4β-hydroxycholesterol parameters did not show clinically relevant correlations with taxane metabolic profiles. If other endogenous markers are better predictors for taxane pharmacokinetics will be tested in future studies.

In **Chapter 7**, we prospectively evaluated the feasibility of a phenotyping probe drug to predict the metabolism of tamoxifen. Tamoxifen is currently given in

a fixed dose: 20 mg in the adjuvant treatment of breast cancer and 40 mg if the disease is metastasized. However, many factors contribute to the variability seen in tamoxifen pharmacokinetics, and therefore a flat-fixed dose may not be optimal. Dextromethorphan, which is the active ingredient in cough syrup, has a metabolism which is comparable to the metabolism of tamoxifen, as both drugs are converted by CYP3A4 and CYP2D6. Therefore, dextromethorphan was tested as a putative probe drug for tamoxifen metabolism. We observed that dextromethorphan concentrations were highly correlated to endoxifen exposure. This dextromethorphan test provides a proof of principle for using dextromethorphan as a phenotyping test for tamoxifen and may potentially contribute to the personalization and optimization of tamoxifen therapy.

FUTURE PERSPECTIVES

With the knowledge obtained from this thesis, future studies should focus on further exploring factors that may influence systemic exposure of anti-cancer agents with a small therapeutic window. Ultimately, bringing these factors together in a predictive model that can be tested and validated in large cohorts of cancer patients should lead to more evidence-based dosing regimens for these drugs.

A.1

APPENDIX 1
SAMENVATTING

SAMENVATTING

De afgelopen jaren is veel onderzoek gedaan naar het individualiseren van antikanker therapie. Dat wil zeggen dat iedere patiënt een therapie op maat hoort te krijgen. De nadruk in dit onderzoek lag veelal op het bestuderen van verschillen in eigenschappen van tumoren, maar daarnaast is ook het geven van de juiste dosering van het antikanker middel voor een individuele patiënt erg belangrijk. Gewoonlijk wordt de dosering van antikanker middelen bepaald tijdens zogenaamd "fase I" onderzoek. In dit soort onderzoek wordt het antikanker middel gegeven aan kleine groepen patiënten in oplopende dosering. Het doseringsniveau net onder het niveau waarbij ontoelaatbare bijwerkingen optreden wordt gekozen als dosering voor verder onderzoek en wordt meestal ook de dosering die na registratie van het medicijn gebruikt gaat worden. Dit is nauwelijks persoonsgericht te noemen.

Er komt gelukkig steeds meer informatie beschikbaar over individuele factoren die de behandeling met antikanker middelen kunnen beïnvloeden. Dit is vooral belangrijk voor middelen tegen kanker omdat deze medicijnen een zogenaamde "kleine therapeutisch breedte" hebben. Dat betekent dat doseren zeer nauwkeurig moet gebeuren omdat de dosering die nodig is om het medicijn werkzaam te laten zijn en de doserering waarbij bijwerkingen optreden dicht bij elkaar liggen. Hoewel de therapeutische breedte dus klein is bij antikanker middelen, is de variatie in werkzaamheid en bijwerkingen van deze therapie vaak opvallend groot. Dit onderstreept het klinische belang van het vinden van factoren die de hoeveelheid blootstelling aan het antikanker middel kunnen voorspellen. Als deze kennis over een individuele patiënt bij de start van een behandeling met een antikanker middel al aanwezig is, dan kan de behandeling hierop aangepast worden. Op deze manier is zogenaamde geïndividualiseerde antikanker therapie mogelijk, waarbij het aantal bijwerkingen kan worden teruggedrongen en de werkzaamheid kan worden geoptimaliseerd.

In dit proefschrift wordt een klinisch farmacologische benadering van het individualiseren van antikanker therapie beschreven. "Klinische farmacologie" houdt zich bezig met het effect van het lichaam op het medicijn, de farmacokinetiek, en het effect van het middel op het lichaam, de farmacodynamiek. De antikanker middelen die onderzocht worden in dit proefschrift zijn docetaxel en paclitaxel (taxanen), en het antihormonale middel tamoxifen. Dit zijn alle drie veel gebruikte middelen in de oncologische praktijk en de beschreven studies zijn hierdoor extra relevant voor het klinisch handelen. Zowel omgevingsfactoren (b.v. roken) als genetische factoren (genetische variatie in metaboliserende enzymen en medicijn transporters) worden in dit proefschrift onderzocht. Dit gebeurt in translationeel onderzoeksverband met experimenten in cellijnen, experimenten met proefdieren en klinisch onderzoek in kanker patiënten. Verder is ook onderzocht of de effectiviteit vaan de omzetting van taxanen voorspeld kan worden door de omzetting van een lichaamseigen stof. Deze lichaamseigenstof, cholesterol, heeft een omzetting die vergelijkbaar is met de twee bestudeerde antikanker middelen. Ook is geprobeerd de effectiviteit van de

omzetting van het middel tamoxifen te voorspellen met behulp van de omzetting van dextrometorfan, de actieve stof in hoestdrank. Deze stof wordt niet door het lichaam gevormd en moet dus toegediend worden. Maar ook deze stof kent een omzetting welke lijkt op de omzetting van het anti-kanker middel.

In Hoofdstuk 2 hebben we de invloed van roken op de farmacokinetiek van paclitaxel en docetaxel onderzocht. Ook is de invloed van roken op het beenmerg tijdens therapie met paclitaxel en docetaxel onderzocht. Het bleek dat de blootstelling aan beiden taxanen gelijk was in rokers en niet-rokers. Opvallend was echter dat rokers minder ernstige beenmerg onderdrukking kregen als gevolg van de chemotherapie dan niet-rokers. Patiënten behandeld met paclitaxel en docetaxel hadden minder vaak een ernstig tekort aan (een subtype) van witte bloedcellen dan niet-rokers. Mogelijk komt dit beschermende effect doordat sigarettenrook de aanmaak van witte bloedcellen in het beenmerg stimuleert. Op deze manier zouden er bij rokers meer witte bloedcellen in de bloedbaan komen. Verder onderzoek is echter vereist om dit mechanisme te verduidelijken.

In Hoofdstuk 3 wordt de invloed van genetische variatie in een selectie van metaboliserende enzymen en transport eiwitten op neurotoxiciteit tijdens paclitaxel behandeling onderzocht. Neurotoxiciteit is een (vaak pijnlijke) beschadiging van de zenuwen die kan optreden tijdens/na behandeling met paclitaxel. Uit dit onderzoek blijkt dat een recent ontdekte genetische variant, genaamd *CYP3A4*22*, bijdraagt aan een verhoogd risico op ernstige neurotoxiciteit tijdens paclitaxel therapie. De andere in dit onderzoek bestudeerde genetische varianten lieten dit verhoogde risico niet zien. Daarnaast bleek dat de blootstelling aan paclitaxel, gemeten in het lichaam tijdens één paclitaxel kuur, gerelateerd is aan de ernst van de neurotoxiciteit die optrad. Het verhoogde risico van *CYP3A4*22* dragers op neurotoxiciteit kon niet verklaard worden door verschil in blootstelling aan paclitaxel in *CYP3A4*22* dragers en niet-dragers. Dit suggereert een lokaal effect van het enzym CYP3A4 in de perifere neuronen en zal verder onderzocht worden in toekomstige studies.

In Hoofdstuk 4 is een zogenaamde DMET (Drug Metabolizing Enzymes and Transporters) chip met 1936 genetische variaties in 225 farmacologisch relevante genen gebruikt om een model te ontwikkelen dat kan voorspellen welke patiënten paclitaxel (te) langzaam afbreken en daardoor meer risico lopen op ernstige bijwerkingen van de therapie. In een grote groep patiënten behandeld met paclitaxel is een model bestaand uit 14 genetische variaties ontwikkeld. Dit model is vervolgens getest op een tweede groep patiënten. Het model identificeerde 95% van de patiënten met lage paclitaxel afbraak op een correcte wijze. Helaas moet wel een groot aantal patiënten getest worden om alle patiënten met lage paclitaxel klaring te kunnen aanwijzen. Daardoor is het huidige model niet direct bruikbaar in de klinische praktijk. Vervolgonderzoek met de DMET chip zal zich allereerst richten op het voorspellen van bijwerkingen tijdens paclitaxel therapie. Ook zal de DMET chip gebruikt worden om docetaxel farmacokinetiek en bijwerkingen te voorspellen. Uiteindelijk zal dit

onderzoek uit moeten wijzen of genetische variatie voldoende verschillen tussen patiënten kan verklaren om daaruit de farmacokinetiek en bijwerkingenprofiel te kunnen voorspellen.

Hoofdstuk 5 beschrijft een translationele studie waarin de invloed van de lever transporteiwitten OATP1B1 en OATP1B3 op de farmacologische variatie van docetaxel wordt onderzocht. OATP1B1 en OATP1B3 zijn zogenaamde transport eiwitten, die een medicijn een levercel in kunnen pompen die het vervolgens omzet of afgebreekt. In deze studie worden cellijn- en muizenmodellen gebruikt, maar wordt ook de invloed van genetische variatie in OATP1B1 en OATP1B3 in een grote groep met paclitaxel behandelde patiënten onderzocht. Er werd gevonden dat docetaxel niet alleen door OATP1B3 de levercel ingepompt wordt maar dat ook OATP1B1 hierin een belangrijke rol speelt. We zagen dat in muizen zonder deze OATP transport eiwitten, de blootstelling aan docetaxel ernstig was verhoogd, dit kan wijzen op een belangrijke rol van OATP in het metabolisme van docetaxel. In paclitaxel behandelde patiënten kon het effect van genetische variatie in de OATP transport eiwitten op docetaxel metabolisme niet worden bevestigd. Een verklaring hiervoor kan zijn dat een genetisch defect in beiden transporters noodzakelijk is om een belangrijk effect te bewerkstelligen.

In **Hoofdstuk 6** hebben we de lichaamseigen stof 4β-hydroxycholesterol getest als voorspeller voor het metabolisme van paclitaxel en docetaxel in individuele patiënten die behandeld werden met deze middelen. Deze stof zou een goede voorspeller kunnen zijn voor de effectiviteit van de afbraak van taxanen omdat de productie van 4β-hydroxycholesterol door middel van CYP3A plaatsvindt, net als de afbraak van paclitaxel en docetaxel. Dit is de eerste studie in patiënten met kanker die een lichaamseigen stof probeert te relateren aan de omzetting van antikanker middelen. Helaas kan 4β-hydroxycholesterol niet in de kliniek gebruikt worden als voorspeller omdat de relatie tussen de marker en taxanen metabolisme niet sterk genoeg is. Of andere lichaamseigen stoffen betere voorspellers zijn voor het metabolisme van taxanen dient nog onderzocht te worden in vervolg onderzoek.

Hoofdstuk 7 beschrijft tenslotte een prospectieve studie waarin de stof dextrometorfan als voorspeller getest wordt voor het metabolisme van tamoxifen. Tamoxifen wordt normaliter gebruikt voor de behandeling van borstkanker in een dosering van 20 mg of 40 mg. Het is echter bekend dat vele factoren bijdragen aan de variatie in tamoxifen farmacokinetiek en daarom is een vaste dosering voor iedere patiënt zeker niet de beste doseringswijze. Dextrometorfan is de actieve stof in hoestsiroop en heeft een vergelijkbaar metabolisme als tamoxifen want beide stoffen worden omgezet door de leverenzymen CYP3A4 en CYP2D6. Deze dextrometorfan test blijkt in deze studie het tamoxifen metabolisme accuraat te voorspellen en zou daarom mogelijk gebruikt kunnen worden om de behandeling met tamoxifen te individualiseren.

TOEKOMSTIG ONDERZOEK

Met de kennis die verkregen is uit dit proefschrift zal verder onderzoek zich moeten richten op het bestuderen van factoren die de blootstelling aan antikanker middelen met een smalle therapeutische breedte kunnen verklaren. Uiteindelijk is het doel om met deze factoren een voorspellend model voor de omzetting van antikanker middelen te ontwikkelen dat getest kan worden in grote groepen van patiënten met kanker. Uiteindelijk zou dit kunnen leiden tot meer therapie op maat voor een individuele patiënt.

A.2

APPENDIX 2
DANKWOORD

DANKWOORD

En dan is het eindelijk klaar! Wat een gek maar fantastisch gevoel om deze periode af te sluiten. Ik zei vroeger altijd: je leert jezelf pas echt kennen op een tennisbaan, maar inmiddels weet ik dat dit pas echt opgaat tijdens een promotietraject. Ik besef me dat ik dit niet bereikt zou hebben zonder een heleboel mensen die ik dan ook heel graag wil bedanken.

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Karel, bedankt voor alle lol op G4, maar ook voor je wijze raad. Voor mij was het erg prettig een ervaren PhD-er naast me te hebben zitten die het klappen van de zweep al kende.

Ellen, Annemieke, Johan, Lisette, Jessica, Wendy, Caroline, Marijn en Sander, dank jullie wel voor alle momenten van gezelligheid en goede adviezen en koffie. Een klankbord vinden bij je naaste collega's is een absolute must in een promotietraject als je het mij vraagt! Dames (en inmiddels 1 heer) van ons PhD eetclubje: hopelijk mag ik ook nog gezellig mee-eten als ik klaar ben!

Lieve Es, we kennen elkaar nu al behoorlijk lang en onze vriendschap is ontzettend belangrijk voor mij. Het eerste wat ik bedacht toen ik ging promoveren is dat jij mijn paranimf hoort te zijn. Alles is leuk met jou. Super fijn dat je er vandaag bij bent als

paranimf, en ik hoop dat we bij alle andere belangrijke gebeurtenissen in elkaars leven zullen zijn.

Lieve Jes, ik weet nog heel goed dat we in onze eerste week samen in de rij stonden om studieboeken te halen. Als ik toen eens had geweten dat je nu mijn paranimf zou zijn. Bedankt voor al je steun, gezelligheid en dat je altijd voor me klaar staat. Super fijn dat je mijn paranimf wilt zijn!

Lieve vriendinnen, Zoes, Nien, Davina, Hannah, Marlies, Mado, en Hes. Bedankt voor alle gezelligheid in onze (studie) jaren en daarna. Voor een (high) theetje, drankje, etentje en een luisterend oor stonden jullie altijd klaar. Wat fijn om met jullie mijn (promotie) ups and down te kunnen delen!

Groep 14! In mijn jaar met jullie is mijn liefde voor onderzoek geboren denk ik achteraf. Maar gelukkig hebben we dat jaar heel wat meer gedaan dan gezamenlijk door microscopen turen en beesten ontleden. Wat een goede herinneringen heb ik aan de BMW feesten en al de andere leuke dingen! Ik hoop dat ons kerstdiner definitief traditite wordt!

Verder wil ik iedereen van tennis bedanken die me van de broodnodige ontspanning hebben voorzien. Voor mij is tennis altijd een super goede uitlaatklep geweest.

A.2

Lieve Papa en Mama. Ik heb mijn proefschrift niet voor niets aan jullie opgedragen. Jullie onvoorwaardelijke steun en liefde zijn voor mij een van de belangrijkste dingen in mijn leven. Altijd zijn jullie bereid naar me te luisteren en mij te helpen bij wat dan ook. Het voelt super dat jullie zo trots op me zijn. Heerlijk om met jullie (aan een Frans zwembad) over onderzoek en de kliniek te kunnen kletsen.

Lieve Cuja, ook jouw liefde gedurende mijn hele leven is ontzettend belangrijk voor mij. Als ik over mezelf zou denken zoals jij soms over mij denkt, dan zou ik vast en zeker naast mijn schoenen gaan lopen. Heerlijk om af en toe zo opgehemeld te worden.

Lieve Roland en Mirte, fijne gesprekken met jullie zijn me veel waard en verbreden mijn horizon. Zonder jullie bij me zou mijn dag niet compleet zijn.

Mijn schoonfamilie. Lieve Karin, Coen en Eline, Lisan en Samuel, Jur en Coby, bedankt voor jullie lieve woorden, kaartjes en interesse over de jaren heen. Ik kan me geen fijnere schoonfamilie wensen! Ook jullie horen er voor mij echt bij op deze dag.

Lieve Bas. Helemaal aan het begin van mijn promotie leerden we elkaar kennen en je hebt alle kanten van mijn traject van heel dichtbij meegemaakt. Super fijn om samen de successen te vieren maar je ving me ook op als de dingen minder lekker liepen. Bedankt voor je liefde, eindeloze steun, en relativeringsvermogen. Het is heerlijk om met jou door het leven te gaan. Ik hou van jou, M.G!

A.3

APPENDIX 3
CURRICULUM VITAE

CURRICULUM VITAE

Anne-Joy Margreet de Graan werd geboren op 28 juni 1986 in Amersfoort. Op het Eemland College in Amersfoort haalde zij haar VWO diploma. Aansluitend werd ze toegelaten aan het St. Mary's College in California, VS, waar ze Engels en verschillende (bio) medische vakken heeft gevolgd. Tijdens dit jaar heeft ze in het tennis team van de universiteit gespeeld in de eerste divisie. In 2004 werd Anne-Joy uitgeloot voor toelating aan de studie geneeskunde. Hierop is ze biomedische wetenschappen aan de Universiteit Utrecht gaan studeren. In dat jaar heeft ze meegedaan met de decentrale selectie procedure voor toelating voor de studie geneeskunde aan het Erasmus MC alwaar ze aangenomen werd. In 2005 is ze begonnen met haar doctoraal geneeskunde. Naast haar studie had Anne-Joy verschillende bijbanen in verpleeghuizen, zorgcentra, en op de afdeling "Vaat en transplantatie chirurgie" in het Erasmus MC. Tijdens haar doctoraal studie is ze geselecteerd voor de onderzoeksmaster "Clinical Research". Tijdens deze master heeft zij vakken gevolgd aan de Johns Hopkins University, Baltimore, VS. In het kader van deze onderzoeksmaster is zij daarna een wetenschappelijke stage van een jaar op de afdeling Interne Oncologie in het Erasmus MC onder begeleiding van prof. dr. A.H.J. Mathijssen gaan doen. Het diploma van deze onderzoeksmaster heeft zij in 2010 behaald. Uit de wetenschappelijke stage bij de Interne Oncologie is een promotie traject voortgevloeid onder supervisie van prof. dr. J. Verweij, prof. dr. A.H.J. Mathijssen en dr. R.H.N. van Schaik over de farmacologie van antikanker middelen beschreven in dit proefschrift. In 2011 heeft zij een stage gevolgd in het st. Jude Children's Hospital in Memphis, VS, onder supervisie van dr. A. Sparreboom. In het kader van haar promotie traject heeft ze haar onderzoek op diversen grote internationale congressen gepresenteerd. In 2012 kreeg ze een travel award van de European Society of Medical Oncology (ESMO) voor haar onderzoek. Per maart 2013 is Anne-Joy begonnen aan haar coschappen om haar studie geneeskunde af te ronden.

A.4

APPENDIX 4
PUBLICATIES

PUBLICATIES

Lammers LA, Mathijssen RH, van Gelder T, Bijl MJ, de Graan AJ, Seynaeve C, van Fessem MA, Berns EM, Vulto AG, van Schaik RH. The impact of CYP2D6-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer. *Br J Cancer 103(6):765-71, 2010*

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de Graan AJ, Sparreboom A, de Bruijn P, de Jonge E, van der Holt B, Wiemer EA, Verweij J, Mathijssen RH, van Schaik RH. 4β-Hydroxycholesterol as an endogenous CYP3A marker in cancer patients treated with taxanes. *Submitted*.

A.5

APPENDIX 5
PHD PORTFOLIO

PHD PORTFOLIO

1. PhD training	Year	Workload (Hours/ECTS)
General courses		
• Biomedical English Writing and Communication, Erasmus Medical Center	2011	4 ECTS
• BROK ('Basiscursus Regelgeving Klinisch Onderzoek'), Erasmus Medical Center	2010	1 ECTS
Specific courses (e.g. Research school, Medical Training)		
• Basic and Translational Oncology (MolMed)	2009	1.8 ECTS
• SNP's and Human Disease (MolMed)	2009	2 ECTS
• Research management for PhD student (MolMed)	2010	1 ECTS
• Introduction to data analysis using a population approach with NONMEM (King's College London)	2010	2 ECTS
Seminars and workshops		
• Journal Club pharmacogenetics	2010-2012	2 ECTS
• NIH cursus "Principles of Clinical Pharmacology"	2010-2011	1 ECTS
• End-note workshop, Erasmus Medical Center	2011	0.1 ECTS
• DNA course, department of Clinical Chemistry, Erasmus Medical Center	2011	1 ECTS
• Workshop: "Graphic style and plot vs table" by Curtis Barrett, Erasmus Medical Center	2012	0.1 ECTS
• Workshop: "Patient gebonden onderzoek", Erasmus Medical Center	2012	0.2 ECTS
• PhD meeting Personalized Medicine ("OIO overleg")	2010-2013	2 ECTS
• COIG workshop: "Clinical Pharmacology"	2012	1 ECTS
Presentations		
• Research Meeting dept. of Medical Oncology, oral presentations	2010/2012	2 ECTS
• MOLMED day, Erasmus Medical Center, poster presentation	2011	0.5 ECTS
• Josefine Nefkens Institute Lab Meetings; oral presentation	2010	1 ECTS
• EORTC- NCI-AACR, molecular target and therapies, Berlin Germany; poster presentation	2010	1 ECTS
• AACR-NCI-EORTC, molecular target and therapies, San Francisco, USA; poster presentation	2011	1 ECTS
• American Society of Clinical Pharmacology and Therapeutics, annual meeting, National Harbour, Maryland, USA; poster presentation	2012	1 ECTS
• ESMO 2012, Vienna, Austria; poster presentation	2012	1 ECTS
• MOLMED day, Erasmus Medical Center, poster presentation	2012	1 ECTS
• Daniel den Hoed research day 2012; oral presentation	2012	1 ECTS
• Oncologiedagen 2012 Nederland en Vlaanderen; oral presentation	2012	1 ECTS

(Inter)national conferences		
• Figon Dutch Medicine Days	2009	0.2 ECTS
• EORTC- NCI-AACR, molecular target and therapies, Berlin Germany.	2010	0.4 ECTS
• AACR-NCI-EORTC, molecular target and therapies, San Francisco, USA	2011	0.4 ECTS
• American Society of Clinical Pharmacology and Therapeutics, annual meeting, National Harbour, Maryland, USA	2012	0.4 ECTS
• ESMO 2012, Vienna, Austria	2012	1 ECTS
Other		
• Medelingendag NVKFB Jaarbeurs Utrecht	2010	0.2 ECTS
• PhD day Erasmus Medical Center	2010-2012	0.4 ECTS
• Symposium Translational Oncology KWF	2010	0.2 ECTS
• Wetenschapsmiddag Interne Oncologie	2010-2012	0.3 ECTS
• IKR netwerkdagen Bergen op Zoom/Vlissingen	2010-2011	0.8 ECTS
• MOLMED day, Erasmus Medical Center	2012	0.2 ECTS
• Internship department of Pharmaceutical Sciences, St Jude Children Research Hospital, Memphis, TN, USA	2011	2 ECTS
• Internship department of Clinical Chemistry, Erasmus MC	2011	2 ECTS
• Daniel den Hoed Research Day	2012	0.2 ECTS
• Oncologiedagen Nederland en Vlaanderen	2012	0.2 ECTS

2. Teaching	Year	Workload (Hours/ECTS)
Lecturing		
• Clinical research meetings department Medical Oncology	2010/2013	0.4 ECTS
• Farmacogenetica-Journal Club	2010	0.2 ECTS
• PhD meeting Personalized Medicine ("OIO"overleg)	2010-2012	1 ECTS
• AIOS lecture Ikazia ziekenhuis	2011	0.4 ECTS
• Clinical lesson verpleegkundigen	2011	0.2 ECTS
• Pharmacology meeting Sophia Children's Hospital	2011	0.2 ECTS
• Meeting dept. Clincial Chemistry, Erasmus Medical Center	2012	0.2 ECTS
Supervising Master's theses		
• Supervising thesis Marina Hooghart (biology and medical research student)	2010	1 ECTS
• Supervising thesis A. Nieuweboer (medical student)	2011	1 ECTS

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