Pharmacogenetics and pharmacokinetics in high-dose alkylating chemotherapy

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Pharmacogenetics and pharmacokinetics in high-dose alkylating chemotherapy

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(met een samenvatting in het Nederlands)

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Contents

Preface

Preface

High-dose chemotherapy has widely been used in the treatment of several solid tumours. A frequently applied combination in the Netherlands consists of 4-day cycles with cyclophosphamide, thiotepa and carboplatin (CTC). A large study of high-dose chemotherapy in the adjuvant treatment of high-risk primary breast cancer showed that high-dose chemotherapy with cyclophosphamide, thiotepa and carboplatin caused a trend for better relapse-free survival. Especially in patients with HER2/neu-negative disease both a relapse-free and overall survival advantage were seen [1]. Furthermore, this study and several other studies of high-dose chemotherapy suggested that a subgroup of breast cancers may exist, possibly 20 to 30% of all cases, that are exquisitely sensitive to these alkylating agents [2].

In the past few years, new data has become available pertaining to the mechanism of action of alkylating agents. Bifunctional alkylating agents (such as cyclophosphamide, thiotepa and carboplatin) form inter- and intrastrand DNA cross-links that can be repaired by a DNA repair mechanism called homologous recombination. This is probably the only repair mechanism that can repair the DNA adducts formed by bifunctional alkylating agents. Homologous recombination deficiency has been shown to be present in about 30% of sporadic breast cancers [3]. It is speculated that the patients that especially benefited from high-dose chemotherapy with cyclophosphamide, thiotepa and carboplatin are patients whose tumours contain homologous recombination deficiency. Studies are underway to confirm of refute this speculation.

High-dose chemotherapy is demanding and expensive for both the patient and care-givers. The use of effective supportive care with anti-emetics, haematopoietic growth factors and peripheral blood progenitor cell transplantation has contributed greatly to the feasibility and safety of high-dose chemotherapy. But despite this, severe and sometimes life-threatening toxicity may occur, such as severe mucositis, haemorrhagic cystitis, veno-occlusive disease of the liver, ototoxicity, neuropathy, cardiotoxicity and pulmonary toxicity [4].

The incidence and severity of toxicity of the CTC regimen shows a wide interpatient variability: it may be severe in some patients and non-existent in others. If one could identify patients whose tumours are extremely sensitive to high-dose chemotherapy and if one could also reduce the toxicity observed, high-dose chemotherapy with alkylating agents might become important again for some breast cancer patients.

Previous studies have shown that the occurrence of toxicity is partly related to the pharmacokinetics of the CTC agents [5;6]. In order to reduce the variability in pharmacokinetics between patients, therapeutic drug monitoring has been applied for the CTC regimen. Using this strategy, the variability in exposure to the cytotoxic agents was clearly reduced and a reduction in the occurrence of severe liver toxicity was suggested [7]. However, therapeutic drug monitoring proved to be labour-intensive and difficult to apply in most hospital settings. Furthermore, during the first days of the course all patients will receive an unadjusted standard dose. Strategies for *a priori* identification of patients at risk for severe toxicity would therefore be preferable.

Cyclophosphamide and thiotepa are both metabolised by enzymes (e.g. cytochrome P450, glutathione S-transferase, aldehyde dehydrogenase) of which polymorphisms have been

described. Certain polymorphisms cause increased, diminished or even complete absence of function of these enzymes, which may have impact on the exposure to cyclophosphamide and thiotepa. Therefore, the genotype of these enzymes is expected to be a relevant determinant for pharmacokinetic variability.
The objectives of this thesis were to identify relations between the genotype of the metabolising enzymes involved in cyclophosphamide, thiotepa and carboplatin metabolism, pharmacokinetics and toxicity in order to identify patients at risk for severe toxicity or under-treatment and to develop a strategy for safe dosing of the agents included in the high-dose CTC regimen.
The first chapter gives an overview of relations between polymorphisms in drug metabolising enzymes and drug transporters and survival after cancer drug treatment. The second chapter describes a bioanalytical method for the simultaneous quantification of cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide. This method has been used for the pharmacogenetic and pharmacokinetic studies in the subsequent chapters. In chapter 3, pharmacogenetic studies of thiotepa, cyclophosphamide and carboplatin are described. The influence of polymorphisms in drug metabolising enzymes on the pharmacokinetics of thiotepa (Chapter 3.1) and cyclophosphamide (Chapter 3.2) is discussed. Furthermore, relations between polymorphisms in drug metabolising enzymes and toxicity of the compounds of the CTC regimen are identified (Chapter 3.3). Chapter 4 describes other factors that can influence the pharmacokinetics of the compounds of the CTC regimen, such as serum creatinine (Chapter 4.1), weight (Chapter 4.2), alteration of the administration regimen (Chapter 4.3), co-medication (Chapter 4.4) and renal insufficiency (Chapter 4.5).

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Chapter 1

Introduction

Chapter 1.1

An overview of the relations between polymorphisms in drug metabolising enzymes and drug transporters and survival after cancer drug treatment

Cancer Treatment Reviews (in press)

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Abstract

A wide interindividual variability in survival after cancer treatment is observed. This is attributable to many factors, including tumour and patient related factors. Genetic polymorphisms in drug metabolising enzymes and drug transporters may be one of these factors. Drug metabolising enzymes are responsible for the activation, inactivation and detoxification of many chemotherapeutic agents. Deficiencies in these enzymes may result in altered exposure (both extracellular and intracellular) to the chemotherapeutic agents, thereby influencing the efficacy of treatment. Drug transporters are important in the uptake and excretion of chemotherapeutic agents. Polymorphisms in drug transporter genes may influence the bioavailability and disposition of these agents.

Studies have shown that variability in survival can (partly) be explained by polymorphisms in genes encoding drug metabolising enzymes and drug transporters. This review will discuss the role of genetic polymorphisms in drug metabolising enzymes and drug transporters in relation to survival after cancer treatment.

The most important polymorphisms shown to influence survival after cancer treatment are polymorphisms in the genes encoding the phase II detoxification enzymes glutathione S-transferases (GSTs). It appears that GSTM1 null and GSTT1 null have a clear association with longer overall survival in patients with different malignancies who are treated with substrates for these GSTs (mostly alkylating agents and platinum compounds). Genetic polymorphisms in GSTP1 and GSTA1 are also associated with an increased overall survival in patients with different malignancies.

Most of the current data on the relation between treatment response and pharmacogenetics is derived from retrospective and exploratory studies. Prospective studies will be necessary.

Introduction

Differences in drug response among patients after cancer treatment are common. This variability in drug response is partly due to variability in pharmacokinetics. Factors responsible for this variability include ethnicity, age, gender, diet, smoking, alcohol consumption, renal and liver function, concomitant disease and co-medication. In many cases however, genetic factors are shown to have an even greater influence on drug disposition. It is estimated that genetic variability accounts for 20-95% of the variability in therapeutic response and toxic effects [1]. These differences in genetic factors, for instance observed in genes encoding drug metabolising enzymes and drug transporters, can influence the pharmacokinetic and pharmacodynamic profile of anticancer drugs, leading to differences in response and development of severe toxicities.

Genetic variation in the human genome is a common phenomenon and approximately 1 out of 1000 basepairs differs between any two individuals [2]. Most of these variations are single nucleotide polymorphisms (SNPs). These single nucleotide differences account for >90% of the genetic variation. Insertions and deletions, tandem repeats and microsatellites account for the remaining 10% [3]. The number of polymorphisms identified in genes encoding drug metabolising enzymes and drug transporters is rapidly increasing, probably leading to a better understanding of the observed variation in efficacy and toxicity of anticancer drugs in patients.

Drugs are metabolised by drug metabolising enzymes and drug transporters play a role in the disposition of drug in the body. These can be classified into three main categories. The first category consists of phase I enzymes. These include reductases, oxidases and hydrolases. The cytochrome P450 enzymes (CYPs) belong to this category. Most drugs are metabolised by CYPs either as a route to detoxification or as an activation pathway for an inactive prodrug. The second category is called phase II enzymes. These enzymes usually conjugate phase I products, but can also conjugate other reactive intermediates or the parent compound, with various endogenous moieties such as glucuronic acid, glutathione or sulphate. These enzymes also contribute to the intracellular metabolism of many substrates. The phase II enzymes include UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), and sulfotransferases (SULTs) [4]. The last category consists of drug transporters. These transporters are membrane-bound proteins that control drug uptake and excretion. Drug transporters greatly influence the bioavailability and disposition of drugs. Examples of genes that encode these transporters are MDR1 (ABCB1), which encodes P-glycoprotein, and ABCG2, which encodes Breast Cancer Resistance Protein (BCRP) [5].

Polymorphisms in genes encoding drug metabolising enzymes may decrease the intracellular enzyme concentration, lead to a dysfunctional protein, or may structurally alter the enzyme with consequent changes in enzyme function. Polymorphisms in drug transporter genes can influence the uptake and excretion capability of the protein. Together this may alter the pharmacokinetic and pharmacodynamic profile of a drug. Therefore, polymorphisms in genes encoding proteins involved in drug metabolism and disposition may be important for treatment response after cancer treatment. Especially the influence of polymorphisms on survival after cancer treatment is important since this is the ultimate outcome measure.

This review will focus on the influence of genetic polymorphisms in phase I and II enzymes and drug transporters on survival after cancer treatment. A review of the literature of studies reporting significant relations between survival and polymorphisms in drug metabolising enzymes and drug transporters is provided. Furthermore, the clinical relevance of these polymorphisms in predicting outcome is discussed.

Methods

A literature search was carried out using PubMed for publications concerning the influence of polymorphisms in drug metabolising enzymes and drug transporters on survival. Furthermore, reference lists of publications were screened on other relevant studies. Studies that reported significant relations were included in this review, limiting results to human research published in English.

Phase I enzymes

Cytochrome P450

CYP enzymes are important in the biosynthesis and degradation of endogenous compounds such as steroids, lipids and vitamins. They metabolise many drugs as well as chemicals present in the diet and environment. The CYP enzymes are responsible for the metabolism of over 90% of clinically prescribed drugs. CYPs reduce or alter the pharmacologic activity of many drugs and facilitate their elimination. Three families of encoded proteins, CYP1, CYP2, and CYP3 contribute mainly to the metabolism of drugs [6].

Individual CYP enzymes each have unique substrate specificity. However, considerable overlap may be present. Thus, drugs may be metabolised by a single CYP enzyme or a variety of CYP enzymes may contribute.

The liver is the major site of CYP mediated metabolism, but the CYP enzymes are also expressed in the enterocytes in the epithelium of the small intestine, kidney and lung. Genetic polymorphisms can result in inhibition or induction of the involved enzymes. This can markedly influence blood levels of a given drug, leading to under-treatment or toxicity. Differences among patients in drug metabolism in the liver and intestine are common and are often major contributors to differences in drug response and adverse effects [7].

Several human CYP enzymes have been correlated with survival after cancer treatment. The effects of polymorphisms in these enzymes and their association with survival are described below. Table 1 gives an overview of studies describing a relation between polymorphisms in CYP genes and survival.

CYP2D6

The CYP2D6 gene is a well studied member of the CYP superfamily, with over 60 allelic variants described [8]. They can be classified based upon their effects on enzyme activity, either to increase, decrease or totally eliminate CYP2D6 activity. CYP2D6 gene duplication exists in the population and this is correlated with ultrarapid metabolism [6]. Most of the variation in CYP2D6 activity is explained by a subgroup of CYP2D6 SNPs. The non-

functional alleles *3, *4, *5 and *6 are responsible for >98% of CYP2D6 poor metabolisers in Caucasians [8].
CYP2D6 is involved in the metabolism of numerous drugs. With respect to anti-cancer drugs, CYP2D6 is involved in the conversion of tamoxifen to the 50-100 fold more potent 4-hydroxytamoxifen and endoxifen. Thus, for treatment with tamoxifen, CYP2D6 activity might be of importance. Polymorphisms in the CYP2D6 gene may lead to less formation of 4-hydroxytamoxifen and endoxifen and therefore result in shorter survival times for cancer patients treated with tamoxifen.
Several studies have indeed found that breast cancer patients, who are carriers of non-functional alleles of CYP2D6, like CYP2D6 *4, *5, *10, and *41, have shorter relapse-free periods, worse event-free survival and lower overall survival [9-11] (Table 1). For example, Goetz et al. [12] demonstrated that breast cancer patients, who were treated with tamoxifen and who were homozygous for CYP2D6*4 experienced significantly shorter relapse-free time (P=0.023) and poorer disease-free survival (P=0.012) compared with women who had at least one wild-type CYP2D6 allele, although results were only significant in univariate analysis.
On the other hand, Wegman et al [13] found that possession of at least one CYP2D6*4 allele leads to an improved survival rate in breast cancer patients treated with tamoxifen. This is in contrast with the results of other studies and with the hypothesis that wild-type homozygous patients are supposed to generate the active metabolite 4-hydroxytamoxifen more readily and thereby have improved response of tamoxifen. However, the results were obtained from a small number of patients, and therefore the association may be a coincidence.

CYP3A4, CYP3A5
The CYP3A subfamily represents the majority of CYP protein in the human liver and is involved in the metabolism of many drugs. CYP3A4 is the major hepatic CYP3A enzyme and is also present in the intestinal epithelium. CYP3A4 activity shows wide inter-individual variation of up to 40-fold [14].
The first genetic CYP3A4 polymorphism described is the promoter variant allele CYP3A4*1B (A-392G) with a large interethnic variation. The allele frequency is 2-9% in Caucasians and 35-67% in African-Americans. The CYP3A4*1B allele is not identified in Asian subjects [6]. Although the CYP3A4*1B allele was initially shown to have a 1.5-fold increase in transcription in vitro, other reports indicate no change in enzyme activity [6;15].
Although a number of genetic variants have been identified in CYP3A4, it is generally accepted that most of the known SNPs in the coding and 5'-flanking regions of CYP3A4 are not the main determinants for the large interindividual variability of CYP3A4 expression or activity [16].
Another important member of the CYP3A subfamily is CYP3A5, which is expressed in only 10-40% of Caucasians. CYP3A5 is expressed in the liver as well as in other organs (prostate, kidney, adrenal, pituitary). A genetic polymorphism in intron 3 of the CYP3A5 gene (A6986G) results in loss of expression of the enzyme, which was named the CYP3A5*3 allele. It appeared that 80% of the Caucasian population and 30% of the African American population are homozygous for this inactive CYP3A5 allele and are thus deficient in CYP3A5 activity [6].

Because CYP3A4 and CYP3A5 have overlapping substrate specificities, the contribution of each CYP3A4 and CYP3A5 to total CYP3A activity will depend on both the drug under investigation and the individual [6].

CYP3A4 and CYP3A5 play a role in the activation of cyclophosphamide to 4-hydroxycyclophosphamide, the major metabolic pathway [17]. Petros et al [18] showed that polymorphisms in CYP3A4 and CYP3A5 were associated with a reduced systemic clearance of cyclophosphamide and a significant poorer overall clinical response (Table 1). Median survival of 85 breast cancer patients with the CYP3A4*1B polymorphism was 1.3 years (95% CI 0.6 to 2.1) compared to 2.7 years (95% CI 1.8 to 4.1) for patients with both copies of the common allele (P=0.043). Similar differences in median survival were observed for polymorphisms in CYP3A5. Patients with the wild-type allele CYP3A5*1 had a significantly shorter median survival than patients with both copies of the CYP3A5*3 allele (P=0.002). However, 29 polymorphisms in 17 drug metabolising genes were tested and no adjustments were made to account for the number of statistical tests conducted.

Tamoxifen is metabolised to N-desmethyltamoxifen by CYP3A4 and CYP3A5. N-desmethyltamoxifen is a weak anti-oestrogen and a precursor of the active metabolite endoxifen [19]. Wegman et al [19] showed that the genotype of CYP3A5 may contribute to tamoxifen response. They found an improved relapse-free survival in patients homozygous for CYP3A5*3. This is rather unexpected since this genotype represents an inactive form of the enzyme and should therefore not catalyse the formation of the primary metabolite N-desmethyltamoxifen. This outcome gives rise to questions about the hypothesis that genotypes contributing to the biosynthesis of the active metabolites improve the outcome of tamoxifen treatment. However, the metabolism of tamoxifen is complex and the mechanisms responsible for the resistance are therefore unlikely to be explained by a single polymorphism but rather by a combination of several mechanisms.

Table 1. Association studies of polymorphisms in CYP genes and survival.

Genes	SNP	Subjects	Treatment	Effect on end point	Risk measure (95% CI)	Ref
Breast cancer						
CYP2D6	CYP2D6*4	190	TAM	DFS↓	CYP2D6*4/*4 HR=2.44 (1.22-4.90)	[9;12]
CYP2D6	CYP2D6*10	21	TAM	TTP↓	CYP2D6*10/*10 HR=3.68 (1.23-11.04)	[10]
CYP2D6	CYP2D6*4, *5, *10, *41	206	TAM	EFS↓	CYP2D6 *4, *5, *10 or *41 HR=1.89 (1.10-3.25)	[11]
CYP2D6	CYP2D6*4	24	TAM	RFS↑	CYP2D6*4 RR=0.28 (0.11-0.74)	[13]
CYP3A4	CYP3A4*1B	85	CP and platinum	OS↓	CYP3A4*1B P=0.043	[18]
CYP3A5	CYP3A5*1	85	CP and platinum	OS↓	CYP3A5*1 HR=2.6 (P=0.0035)	[18]
CYP3A5	CYP3A5*3	108	TAM	RFS↑	CYP3A5*3/*3 HR=0.13 (0.02-0.86)	[19]

CP, cyclophosphamide; TAM, tamoxifen; DFS, disease-free survival; TTP, time to disease progression; EFS, event-free survival; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; RR, relative risk

Phase II enzymes

Glutathione S-transferase

The GSTs are phase II detoxification enzymes. GSTs catalyse the conjugation of glutathione to a wide range of substrates, including mutagens, carcinogens and chemotherapeutic agents like alkylating agents and anthracyclines [18;20-25].

The detoxification capability plays an important role in the protection of the cell from environmental, genotoxic and oxidative stress, but is also associated with drug resistance [22;23;26]. The detoxification of reactive oxygen species, which may act as intermediates in the cytotoxicity of many chemotherapeutic agents, may modulate the response to a specific drug, even when the chemotherapeutic agent itself is not a substrate.

The most important human GST genes are GSTM1, GSTT1, GSTP1 and GSTA1 [22;23;27;28].

Human GST genes contain different polymorphisms; deletions as well as single nucleotide polymorphisms. Loss of GSTM1 and GSTT1 enzyme function is ascribed to a homozygous deletion resulting in the GSTM1 null and GSTT1 null genotype, respectively. The GSTM1 gene is absent in approximately 50% of the Caucasian population. The GSTT1 gene is absent in approximately 15% of the Caucasian population [20].

Four different alleles of the GSTP1 gene, GSTP1*A, *B, *C and *D have been described, arising from nucleotide transitions that change codon 105 from Ile to Val and codon 114 from Ala to Val. The different GSTP1 proteins differ in their ability to metabolise anticancer agents. The Val105 and the Val114 allele are associated with reduced catalytic activity [27;29].

The gene GSTA1 has two important alleles: *A and *B. A promoter point mutation leads to decreased promoter activity in carriers of the GSTA1*B allele, thereby reducing its expression [21;22;27].

Differences in detoxification of treatment agents or GST-mediated protection against oxidative damage during treatment due to polymorphisms in GST genes can influence survival. Individuals with GSTM1/T1 null genotypes or GSTA1/P1 genotypes with reduced activity would experience a higher effective dose of chemotherapeutic agents and/or more reactive oxidant damage to tumour tissue. Therapy might then be more effective in these patients, in which case longer survival for this group would be observed. However, shorter survival for patients with reduced capacity for GST-mediated detoxification might be explained by more severe therapy-related toxicity.

In this section the association between polymorphisms in GST genes and survival after cancer treatment will be discussed for several malignancies. Table 2 gives an overview of studies describing a relation between polymorphisms in GST genes and survival.

Acute myeloid leukaemia

Standard therapy for patients with acute myeloid leukaemia (AML) consists of an anthracycline (e.g. daunorubicin) combined with agents like cytarabine and etoposide. Anthracyclines are substrates for GSTs [30]. Studies show that deletions in two of the most important GST subfamily genes, GSTM1 and GSTT1 influence the outcome of treatment with induction therapy in patients with AML. A study by Voso et al [31] showed that patients

with AML and GSTM1 null or GSTT1 null genotype had a significantly lower response to anthracycline-based induction therapy (P=0.04) and a shorter survival (P=0.04) compared to patients with an undeleted genotype (Table 2). These results were confirmed in a study with Chinese male patients with de novo AML [32]. Also Barragan et al [33] found that the GSTM1 null genotype was associated with a poorer survival in AML patients.
A significant relation between an increased risk for death due to toxicity and GSTT1 null genotype in patients with AML was reported by two different groups [20;34]. Naoe et al [34] showed that the GSTT1 null genotype was associated with a worse prognosis in adults, mainly due to increased early death after initial chemotherapy. Davies et al [20] found similar results in children. Children with AML and GSTT1 null genotype experienced more toxicity and reduced survival after standard induction therapy compared to children with at least one GSTT1 allele [20].
These results show that GSTM1 null or GSTT1 null are associated with a worse prognosis in patients with AML. Although one might expect an improved survival in patients with GSTM1 null or GSTT1 null due to a higher effective dose of chemotherapy, in AML reduced GST activity may lead to a worse prognosis, probably due to an increase in toxic deaths.

Breast cancer
Chemotherapy regimens for breast cancer frequently consist of GST substrates such as anthracyclines and cyclophosphamide [18;26;30;35;36].
Studies in breast cancer patients show that single and combined deletions of GSTM1 and GSTT1 are linked to a better clinical outcome [18;26;35;37] (Table 2). Ambrosone et al [35] genotyped 251 women with primary breast cancer treated with cyclophosphamide, doxorubicin, 5-fluorouracil and radiotherapy. After adjustment for age, race and stage, GSTM1- and GSTT1-null genotypes predicted significantly better disease-free survival and overall survival. More studies in breast cancer patients showed similar results [18;26;37]. These chemotherapeutic agents as well as radiotherapy generate reactive oxygen species. GSTM1 null and GSTT1 null genotypes have reduced ability to detoxify those species, resulting in improved treatment response.
In contrast, Kristensen et al [38] reported a significantly shorter overall survival in breast cancer patients homozygous for the GSTM1 null allele. However, treatment was not reported and no adjustment for other prognostic factors was performed. Therefore, the results of this study should be interpreted with caution.
The less active variants GSTP1 Val105 and GSTA1*B have been associated with an increased survival in patients receiving a cyclophosphamide-based chemotherapy regimen [39-41]. Patients with the GSTP1 Val105 variant or the GSTA1*B variant have a reduced ability to detoxify the active cyclophosphamide metabolites and would therefore receive a higher effective dose. Thus, the improved survival for these patients may be attributable to improved treatment efficacy.
It can be concluded that for patients with breast cancer, treated with cyclophosphamide and anthracyclines, the GST genotypes with less or no activity may result in improved survival, possibly due to a better response to chemotherapy.

Ovarian Cancer

Mounting evidence suggests that polymorphisms in GST genes may affect survival in ovarian cancer patients. GSTP1 expression is common in ovarian tumours and high levels of GSTP1 expression have been associated with drug resistance and poor survival in ovarian cancer patients [42-44]. Therapy for ovarian cancer commonly includes platinum compounds, which are substrates for the GST enzymes [44-47].

Therefore, it is plausible that ovarian cancer patients with GST genotypes with low activity (GSTP1 Ile105/Val105 or Val105/Val105) or deleted GST genotypes (GSTM1 null or GSTT1 null) would have a reduced intracellular metabolism. This could lead to a better response to treatment and a longer overall survival.

Beeghly et al [44] studied disease progression and survival in 215 women with primary invasive epithelial ovarian cancer, who were treated with platinum-based chemotherapy. They reported that women with GSTM1 null and GSTP1 Ile105/Val105 or Val105/Val105 genotype had better progression-free survival and overall survival. This was confirmed by other studies [46-48] (Table 2).

Some studies, however, have reported no relation or opposite associations between GST polymorphisms and survival. Howells et al. [45] concluded that patients with both GSTM1 null and GSTT1 null genotypes had a significantly shorter overall survival (P=0.001). However, the results were not adjusted for potential confounders. The association became insignificant when either residual disease or tumour grade was included in a subgroup analysis performed later [49].

Overall, it can be concluded that less active GSTP1 variants and the null genotypes of GSTM1 and GSTT1 may result in better progression-free survival and longer overall survival in patients with ovarian cancer, possibly due to decreased detoxification of platinum compounds.

Colorectal cancer

The platinum compound oxaliplatin is an important drug in the treatment of colorectal cancer [50]. Inconclusive results have been found in studies investigating the influence of GST polymorphisms in relation to survival in colorectal cancer patients. Stoehlmacher et al [50] demonstrated that the GSTP1 Ile105Val polymorphism was associated with increased survival in colorectal cancer patients who received oxaliplatin/5-fluorouracil chemotherapy. The survival increased with the number of GSTP1 Val105 alleles. Sun et al [51] showed the opposite (Table 2). These conflicting results were considered by the authors to be related to the different characteristics of patients included in the two studies. The patient groups differed in ethnicity, age and treatment. Furthermore, follow-up periods were different.

Polymorphisms in GSTM1 and GSTT1 were not associated with survival or clinical response in 107 patients with colorectal cancer treated with oxaliplatin/5-fluorouracil chemotherapy [50].

Lung cancer

Platinum-based treatment is also common for patients with non-small cell lung carcinoma (NSCLC). Lu et al [52] concluded that the GSTP1 Ala114Val polymorphism, which leads to a less active enzyme, significantly increased overall survival (P=0.037) in lung cancer patients

treated with platinum-based chemotherapy (Table 2). The GSTP1 Ile105Val polymorphism was not associated with a better response to treatment or an increase in survival [52-54].
The relation between GSTM1 null and overall survival has also been investigated. Sweeney et al studied 274 men with lung cancer (both non-small cell and small cell histologies) treated with radiotherapy and chemotherapy (not further specified) and found that those who had the GSTM1 null genotype had shorter overall survival. This association was independent of stage at diagnosis and histology, which were strong predictors of survival in this study [54].

Other malignancies
Also in gastric and pancreatic cancer, multiple myeloma or Hodgkin's lymphoma it has been demonstrated that patients harbouring the GSTP1 Val105 allele have a better clinical outcome [29;55-57] (Table 2). Hohaus et al [57] showed that the GSTP1 Ile105Val polymorphism was associated with an improved failure-free survival in patients with Hodgkin's lymphoma receiving combination chemotherapy consisting of alkylating agents and anthracyclines, known substrates for GSTP1. This was also seen in multiple myeloma patients treated with conventional chemotherapy, however, not in those treated with high-dose chemotherapy [55]. The reasons for the differences between conventional and high-dose chemotherapy in effect of GSTP1 polymorphisms on outcome are unknown but may be due to the capacity for high-dose chemotherapy to overcome functional differences between the genotypes. High-dose chemotherapy may lead to cellular depletion of glutathione [58;59]. This may obscure any effect of polymorphisms in the GSTP1 gene. Furthermore, differences in GSTP1 specificity for the chemotherapeutic agents used in each arm could have contributed to the differences in effect seen.
In contrast, Lee et al [60] found a poorer prognosis of carriers of the GSTP1 Val105 allele in oesophageal cancer patients treated with platinum-based chemotherapy. The increase in hazard ratio of death with the allelic number of GSTP1 Val105 supported a gene-dose effect on patient survival.
Deletions in GSTM1 and/or GSTT1 have been associated with a longer overall survival and a longer disease-free survival in patients with Hodgkin's Lymphoma or glioma treated with anthracyclines and/or alkylating agents [61;62].

Table 2. Association studies of polymorphisms in glutathione-S-transferase (GST) genes and survival.

Genes	SNP	Subjects	Treatment	Effect on end point	Risk measure (95% CI)	Ref
AML						
GSTM1/T1	GSTM1/T1 null	106	Anthracycline-based	OS↓	GSTM1/T1 null HR=2.4 (1.2-4.9)	[31]
GSTM1/T1	GSTM1/T1 null	254	Anthracycline-based	OS↓	GSTM1/T1 null P=0.03	[32]
GSTM1	GSTM1 null	83	Anthracycline-based	DFS↓	GSTM1 null RR=2.43 (1.05-5.58)	[33]
GSTT1	GSTT1 null	306	Anthracycline-based	OS↓	GSTT1 null RR=1.6 (P=0.02)	[20]
GSTT1	GSTT1 null	193	Anthracycline-based	OS↓	GSTT1 null RR=1.53 (1.05-2.18)	[34]
Breast cancer						
GSTM1	GSTM1 null	85	CP and cisplatin	OS↑	GSTM1 null P=0.041	[18]
GSTM1	GSTM1 null	239	Not reported	OS↓	GSTM1 null P=0.036	[38]
GSTM1/T1	GSTM1/T1 null	251	CAF	OS↑	GSTM1/T1 both null HR=0.3 (0.11-0.70)	[35]
GSTM1/T1	GSTM1/T1 null	53	CAF	DFS↑	GSTM1/T1 both null P=0.01	[26]
GSTT1	GSTT1 null	79	Not reported	OS↑	GSTT1 null HR=0.2 (0.0-0.9)	[37]
GSTP1	GSTP1 Ile^{105}Val	1034	CP-based	OS↑	GSTP1 Val^{105}/Val^{105} HR=0.4 (0.2-0.8)	[39]
GSTP1	GSTP1 Ile^{105}Val	240	CP-based	OS↑	GSTP1 Val^{105}/Val^{105} HR=0.3 (0.1-1.0)	[40]
GSTA1	GSTA1*B	245	CP-based	5-year survival↑	GSTA1*B/*B HR=0.3 (0.1-0.8)	[41]
Ovarian cancer						
GSTM1/T1	GSTM1/T1 null	148	Platinum-based	OS↓	GSTM1/T1 both null HR=3.44 (1.67-7.09)	[45]
GSTM1	GSTM1 null	24	Platinum-based	OS↑	GSTM1 null P=0.013	[46]
GSTM1/P1	GSTM1 null / GSTP1 Ile^{105}Val	215	Platinum-based	PFS↑	GSTM1 null and GSTP1 Val^{105} HR=0.42 (0.24-0.75)	[44]
GSTP1	GSTP1 Ile^{105}Val	266	Platinum-based	5-year survival↑	GSTP1 Val^{105} HR=0.77 (0.61-0.99)	[47]
GSTP1	GSTP1 Ile^{105}Val	81	Platinum-based	OS↑	GSTP1 heterozygous Ile^{105}Val HR=0.34 (0.12-0.98)	[48]
Colorectal cancer						
GSTP1	GSTP1 Ile^{105}Val	107	Platinum-based	OS↑	GSTP1 Val^{105} P=0.042	[50]
GSTP1	GSTP1 Ile^{105}Val	125	Chemotherapy/radio-therapy	OS↓	GSTP1 Val^{105}/Val^{105} and Ile^{105}/Val^{105} HR=5.3 (1.26-19.53)	[51]

Table 2. Continued

Genes	SNP	Subjects	Treatment	Effect on end point	Risk measure (95% CI)	Ref
Lung cancer						
GSTP1	GSTP1 Ala^{114}Val	425	Platinum-based	OS↑	GSTP1 Ala^{114}/Val^{114} or Val^{114}/Val^{114} HR=0.75 (0.54-1.05)	[52]
GSTM1	GSTM1 null	274	Chemotherapy/radiotherapy	OS↓	GSTM1 null RR=1.36 (1.04-1.80)	[54]
Other malignancies						
Gastric cancer						
GSTP1	GSTP1 Ile^{105}Val	52	Platinum-based	OS↑	GSTP1 Val^{105}/Val^{105} P=0.037	[56]
Pancreatic cancer						
GSTP1	GSTP1*C Ile^{105}Val-Ala^{114}Val	138	5-FU	OS↑	GSTP1*C Val^{105}-Val^{114} HR=0.45 (0.22-0.94)	[29]
Hodgkin's Lymphoma						
GSTM1/T1	GSTM1/T1 null	90	Alkylating agents and anthracyclines	DFS↑	GSTM1 null or GSTT1 null HR=0.29 (0.10-0.86)	[61]
GSTP1	GSTP1 Ile^{105}Val	97	Alkylating agents and anthracyclines	Failure-free survival↑	GSTP1 Val^{105} HR=0.42 (0.21-0.85)	[57]
Multiple Myeloma						
GSTP1	GSTP1 Ile^{105}Val	101	Alkylating agents and anthracyclines	PFS↑	GSTP1 Val^{105}/Val^{105} HR=0.52 (0.28-0.96) GSTP1 Ile^{105}/Val^{105} HR=0.55 (0.32-0.96)	[55]
Oesophageal cancer						
GSTP1	GSTP1 Ile^{105}Val	233	Platinum-based	OS↓	GSTP1 Val^{105} HR=1.36 (1.01-1.84)	[60]
GSTP1	GSTP1 Ala^{114}Val	210	Platinum-based	OS↓	GSTP1 Ala^{114}/Val^{114} HR=2.10 (1.14-3.89)	[78]
Glioma						
GSTP1 and GSTM1	GSTP1 Ile^{105}Val , Ala^{114}Val+ GSTM1 null	278	Alkylating agent-based	OS↑	GSTP1 Ile^{105}/Ala^{114} + GSTM1 null P=0.06	[62]

CP, cyclophosphamide; CAF, cyclophosphamide, doxorubicin, 5-fluorouracil; 5-FU, 5-fluorouracil; DFS, disease-free survival; PFS, progression-free survival; OS, overall survival; HR, hazard ratio; RR, relative risk

Uridine diphosphate glucuronosyltransferase

Glucuronidation is a major pathway for the elimination of hydrophobic endogenous substrates such as steroids and bile acids and numerous xenobiotics including environmental carcinogens and cytotoxics. The enzymes that catalyse these reactions, UDP-glucuronosyltransferases (UGTs), are endoplasmic reticulum-bound transmembrane proteins that display tissue-specific expression patterns, including the gut, skin, placenta, breast and prostate gland [4;30]. Most of the UGTs are expressed in the liver as well as other extrahepatic tissues; however, some are exclusively extrahepatic [63].

Thus far, 17 functional UGTs encoded by the UGT gene family have been identified in humans, with some substrate specificity but also significant overlap in their ability to detoxify

substrates. Human UGT isoforms have been classified into two families of proteins, UGT1 and UGT2 [4;30].
UGT has several functional genetic polymorphisms. The UGT1A1*6 variant is associated with defective glucuronidating function [64], while the UGT2B15*2 variant is located within the putative substrate recognition site of the enzyme and is associated with increased catalytic activity [65].
UGT1A1 is responsible for the inactivation of SN-38 (the active metabolite of irinotecan) to SN-38 glucuronide [66]. UGT2B15 catalyzes the biotransformation of a number of steroid substrates, including 4-hydroxytamoxifen, thereby facilitating their excretion [67]. Table 3 gives an overview of studies describing a relation between polymorphisms in UGT genes and survival.
Han et al [63] studied a group of 81 patients with non-small cell lung cancer, who were treated with irinotecan. The patients who had the UGT1A1*6/*6 genotype had significantly shorter overall survival compared to patients with other genotypes (Table 3). This finding is difficult to explain, since decreased inactivation of SN-38 in tumour cells, as a result of the presence of the defective glucuronidation, should be associated with better survival. These data, however, were not controlled for other genetic and environmental factors, patient demographics and tumour histology, which can result in the identification of false positive results. UGT1A1*6 homozygous patients also had more toxicity. Therefore, another explanation for the negative effect of UGT1A1*6 on efficacy is that irinotecan dose-intensity/density or cycle number might have been lower in *6 carriers because of toxicity observed during the first cycle of chemotherapy. However, dose delays and number of cycles were not reported [68].
The UGT2B15*2 variant appears to be a risk factor for the recurrence and poorer survival of breast cancer patients treated with tamoxifen who also have the variant SULT1A1*2 allele. A study by Nowell et al [65] in breast cancer patients treated with tamoxifen showed, that patients with at least one UGT2B15*2 allele and also the SULT1A1*2 allele had significantly reduced 5-year survival rates (P=0.003).

NAD(P)H:quinine oxidoreductase 1

NAD(P)H:quinine oxidoreductase 1 (NQO1, EC 1.6.99.2) is a flavoprotein that has a wide variety of substrates, including quinones and their derivatives. NQO1 catalyzes the two-electron reduction of substrates by using either NADH or NADPH as an electron donor. NQO1 is thought to be of importance in the activation of mitomycin C. Furthermore, NQO1 exerts a detoxifying role through protection from free radicals generated by both drug and carcinogen metabolism [69-71].
A genetic polymorphism has been characterized in NQO1 (C609T), which results in the conversion of proline to serine in the NQO1 protein. This SNP is named NQO1*2 and results in almost no active enzyme in persons with homozygous variant alleles and reduced activity in persons with heterozygous alleles. Approximately 5% of the Caucasian population are homozygous variant and nearly 40% of Caucasian individuals are heterozygous for the substitution [69;70]. Table 3 gives an overview of studies describing a relation between polymorphisms in the NQO1 gene and survival.

Overall, it can be stated that patients treated with substrates for NQO1 and having the NQO1*2 variant have a worse therapeutic outcome than patients with wild-type NQO1 [69-71] (Table 3).
Krajinovic et al [71] showed that children with ALL, who were treated with a combination of chemotherapeutic agents, those individuals with the NQO1*2 variant had a significantly shorter event-free survival (P=0.003) compared to individuals with wild-type NQO1. It can be hypothesized that impaired cell protection from free radicals that have arisen through drug and carcinogen metabolism led to the development of recurrent malignancies.
Other studies have shown that this effect was also seen in patients who received mitomycin C therapy and had the NQO1*2 variant. Patients with the NQO1*2 variant had a poorer survival than patients with wild-type NQO1 [69;70]. The lower NQO1 activity, caused by the NQO1*2 variant, results in reduced activation of mitomycin C and diminished cytotoxic activity.

Sulfotransferase
Cytosolic sulfotransferases (SULTs) transfer sulfate-moieties to nucleophilic groups of xenobiotics and small endogenous compounds, thereby increasing water solubility and decreasing passive penetration of cell membranes. This enhances the urinary and biliary excretion.
All known cytosolic sulfotransferases are members of a single superfamily, named SULT. In humans, 10 SULT genes are known [72]. Genetic polymorphisms have been described for three human SULTs, namely SULT1A1, SULT1A2 and SULT2A1. SULT1A1 is present in various human tissues such as the liver, brain and platelets. A wide racial variation in SULT1A1 polymorphisms is reported. The allele frequency for SULT1A1*2 in Caucasian subjects is 33.2%, while Chinese subjects have an allele frequency of 8.0% and African-American subjects have an allele frequency of 29.4% [73]. The SULT1A1*2 variant allele is associated with decreased catalytic activity [74]. 4-Hydroxytamoxifen and endoxifen are subject to conjugation leading to excretion by sulfation, via SULT1A1.
Table 3 gives an overview of studies describing a relation between polymorphisms in the SULT1A1 gene and survival.
Two studies have demonstrated an association between the presence of SULT1A1*2 and shorter survival (Table 3). In 160 breast cancer patients who were treated with tamoxifen, those patients homozygous for the low-activity SULT1A1*2 allele had a threefold increase in risk of death as those with one or more common alleles. This association persisted even when adjustments were made for age, race, clinical stage of tumour at diagnosis and presence or absence of progesterone receptor [75]. Similar associations were reported by Wegman et al [13]. They showed that in 112 breast cancer patients who received tamoxifen, SULT1A1*2 was associated with an increased risk of recurrence.
Although the results of these studies are in contrast to what was expected (that lower SULT1A1 activity would theoretically result in reduced elimination of active metabolites), sulfation of 4-hydroxytamoxifen may modify the pharmacokinetics of tamoxifen therapy or beneficially alter the receptor-binding properties of 4-hydroxytamoxifen.

Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism pathway and regulates the intracellular folate pool for synthesis and methylation of DNA. Two common allelic variants of the MTHFR gene have been described, C677T and A1298C, that lead to amino acid substitutions, Ala222Val and Glu428Ala, and to decreased enzyme activity. Heterozygous and homozygous carriers of the 677T allele variant have a 30-40% and 60-70% reduced enzyme activity, respectively, as determined by in vitro analysis of the MTHFR activity. The effect of the 1298C allele variant is less pronounced and homozygous carriers of this allele have a more moderate 30-40% reduction of the enzyme activity, yet its function remains controversial. Furthermore, individuals who are heterozygous at both loci, C677T and A1298C, experience an intermediate activity loss of 40-50% [76].

The frequency of the homozygous variant 677TT genotype is approximately 10-16% in Caucasians and only a few percent in African-Americans. The C677T mutation enhances the thermolability of the enzyme. The A1298C polymorphism has been studied less extensively and the frequency of the homozygous variant 1298CC subjects is approximately 12% in Caucasians and 3% in Japanese [77]. Table 3 gives an overview of studies describing a relation between polymorphisms in the MTHFR gene and survival.

The MTHFR variant genotypes are expected to result in improved clinical outcomes when patients are treated with 5-fluorouracil. Both MTHFR C677T and A1298C SNPs result in reduced MTHFR enzymatic activity. Reduced MTHFR activity increases the amount of folate, specifically 5,10-methylenetetrahydrofolate and this enhances the action of 5-FU [78]. Indeed, a study by Wu et al [78] found protective roles of the variant alleles of both MTHFR C677T and A1298C SNPs in survival in oesophageal cancer patients treated with 5-fluorouracil. However, others found a reduced survival in breast cancer patients [76] and colorectal cancer patients [77] with the MTHFR A1298C SNP (Table 3).

Methotrexate cytotoxicity will be compromised by the MTHFR C677T and A1298C SNPs because of the increased intracellular concentration of 5,10-methylenetetrahydrofolate that ensures the flow of one-carbon units into thymidylate and purine biosynthesis [79]. Therefore, variant MTHFR genotypes are expected to result in worse clinical outcome when patients are treated with methotrexate. This was confirmed in a study of Krajinovic et al who reported a decreased survival in children with the T677/A1298 MTHFR haplotype, with acute lymphoblastic leukaemia treated with methotrexate [80]. But keeping in mind the small sample size of the study, this finding should be replicated in independent and larger patient cohorts.

Table 3. Association studies of polymorphisms in other phase II enzyme genes and survival.

Genes	SNP	Sub-jects	Disease	Treatment	Effect on end point	Risk measure (95% CI)	Ref
UGT							
UGT1A1	UGT1A1*6	81	NSCLC	Irinotecan	OS↓	UGT1A1*6/*6 P=0.017	[63]
UGT2B15	UGT2B15*2	160	Breast cancer	TAM	OS↓	UGT2B15*2 and SULT1A1*2 HR=4.40 (1.17-16.55)	[65]
NQO1							
NQO1	NQO1*2	109	Peritoneal cancer	Mitomycin C	OS↓	NQO1*2 P=0.037	[69]
NQO1	NQO1*2	27	NSCLC	Mitomycin C	OS↓	NQO1*2/*2 P=0.007	[70]
NQO1	NQO1*2	320	ALL	Vincristine, CP, doxorubicin, methotrexate	EFS↓	NQO1*2 HR=3.6 (1.7-7.4)	[71]
SULT1A1							
SULT1A1	SULT1A1*2	160	Breast cancer	TAM	OS↓	SULT1A1*2/*2 HR=2.9 (1.1-7.6)	[75]
SULT1A1	SULT1A1*1	112	Breast cancer	TAM	RFS↑	SULT1A1*1/*1 and/or CYP2D6*4 RR=0.38 (0.19-0.74)	[13]
MTHFR							
MTHFR	MTHFR A1298C	101	Breast cancer	CP/doxorubi-cin or 5-FU/ methotrexate	OS↓	MTHFR AC/CC HR=2.05 (1.05-4.00)	[76]
MTHFR	MTHFR A1298C	98	Colorectal cancer	5-FU	OS↓	MTHFR CC HR=2.05 (1.06-3.98)	[77]
MTHFR	MTHFR C677T/ A1298C	30	ALL	Methotrexate	DFS↓	MTHFR T677/A1298 HR=3.3 (1.1-9.9)	[80]
MTHFR	MTHFR A1298C/ C677T	179	Oesopha-geal cancer	5-FU	OS↑	MTHFR AC/CC, CT/TT HR=0.34 (0.15-0.75)	[78]

CP, cyclophosphamide; TAM, tamoxifen; 5-FU, 5-fluorouracil; DFS, disease-free survival; EFS, event-free survival; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; RR, relative risk

Drug transporters

Multidrug resistance 1

The multidrug resistance 1 (MDR1) gene (ABCB1) in humans is responsible for multidrug resistance. The protein product of this gene is a 170-kDa transmembrane glycoprotein referred to as P-glycoprotein (P-gp), which is a membrane protein. P-gp is a member of the adenosine triphosphate-binding cassette (ABC) superfamily of membrane transporters and is involved in the active transport of a great number of amphipathic molecules through lipid membranes. P-gp is expressed in tumour cells, but also in normal tissues with an excretory function (intestine, liver, kidney) [81].

The most important physiological role of P-gp is the protection against toxic xenobiotics. Moreover, a wide range of anticancer agents can be actively extruded by P-gp, which can lead to chemoresistance.

To date, at least 50 SNPs and 3 insertion/deletion polymorphisms have been reported in the MDR1 gene, some of which appear to be associated with altered transporter function and expression, thereby affecting the metabolism and disposition of drugs [82;83]. The G2677T SNP at exon 21 leads to a change in the amino acid sequence from Ala to Ser (G2677T) or Thr (G2677A) in the second transmembrane domain of P-gp. The functional role for this SNP still remains controversial. A SNP in exon 26 position 3435 (C3435T) has been found to be associated with the expression and function of P-gp [84]. The variant TT genotype of the C3435T SNP is associated with lower MDR1 expression and higher plasma drug concentration [78]. Table 4 gives an overview of studies describing a relation between polymorphisms in the MDR1 gene and survival.

Anthracyclines, vinca alkaloids and epipodophyllotoxins, which are drugs commonly used in the treatment of AML and ALL, belong to the P-gp substrates [85]. Functional polymorphisms of MDR1 may, therefore, be involved in the outcome of AML and ALL by two mechanisms. The first involves the multi-drug resistance related to P-gp expression in AML/ALL blasts and the other involves the influence of P-gp on the pharmacokinetics of anti-cancer drugs. Carriers of low P-gp expression-coding genotype may show higher exposure to chemotherapy considering the role of P-gp in multi-drug resistance and in the pharmacokinetics of anticancer agents, resulting in a better survival.

A study by Illmer et al [86] that investigated three MDR1 SNPs at exons 12, 21 and 26 revealed that the genotype at exon 26 (position 3435) was associated with overall survival and the probability of relapse in the Caucasian AML population (Table 4). They found that AML patients who were wild-type for the polymorphism of the MDR1 gene at position 3435 were likely to have decreased survival because of an increased relapse risk. They were not able to determine whether this phenomenon was attributable to characteristics of the AML blast population or attributable to altered P-gp-mediated drug pharmacokinetics. This finding is in accordance with the finding of Jamroziak et al [83] who found a worse prognosis in children with ALL with the wild-type genotype.

In contrast, Kim et al [87] reported a better event-free survival in AML patients with the wild-type genotype at exon 26. This could be due to ethnic differences in the patient groups in those studies (Caucasians and Koreans) as regards MDR1 processing, including transcriptional initiation and RNA maturation [88].

Studies evaluating the G2677T polymorphisms showed that the GG-genotype of G2677T/A of the MDR1 gene had an adverse impact on survival after allogeneic stem cell transplantation in patients receiving cyclosporine [84;84]. However, the study failed to provide clear evidence of the impact of the MDR1 SNP on the pharmacokinetics of cyclosporine. Van den Heuvel-Eibrink et al [89] found that patients with homozygous alleles of G2677T (GG or TT) had a shorter overall survival from relapse/refractory AML. However, they also found that P-gp function and expression were similar in any one of the specific allelic variants.

Therefore, further studies including patients with different ethnicities are warranted, to assess the role of genetic polymorphisms of MDR1 in AML.

ATP-binding cassette transporter G2

The ATP-binding cassette transporter G2 gene (ABCG2) is a member of the G subfamily of ABC transporters and encodes breast cancer resistance protein (BCRP), which is also called mitoxantrone resistant protein (MXR) or placenta-specific ATP binding cassette transporter (ABCP). The protein with six transmembrane segments and one ATP-binding cassette is located in the plasma membrane. ABCG2 protects tissues by actively transporting toxic substances and xenobiotics out of the cells. SNPs have been reported in the ABCG2 gene [90;91]. The C421A polymorphism is associated with decreased protein expression [92].

Hahn et al [93] showed that a greater proportion of patients survived beyond 15 months with docetaxel-based therapy in the presence of the ABCG2 C421A polymorphism (Table 4). The effect of ABCG2 polymorphisms on docetaxel disposition is unknown. The increased survival observed in individuals with an ABCG2 C421A polymorphism may suggest a less functional drug efflux pump, leading to increased intracellular docetaxel concentrations and improved cytotoxic activity. However, this hypothesis should be interpreted cautiously due to the small patient sample size and potential confounding variables. No multivariate analysis and no adjustment for multiple testing were performed.

Table 4. Association studies of polymorphisms in drug transporter genes and survival.

Genes	SNP	Sub-jects	Disease	Treatment	Effect on end point	Risk measure (95% CI)	Ref
MDR1							
MDR1	MDR1 G2677A	99	Hepatocellular carcinoma	Prednisone, tacrolimus, mycophenolate	RFS↑	MDR1 GA/AA P=0.015	[82]
MDR1	MDR1 G2677A	82	AML, CML, ALL	Cyclosporine	OS↓	MDR1 GG HR=2.651 (1.386-5.070)	[84]
MDR1	MDR1 G2677T	30	AML	Etoposide, mitoxantrone, idarubicin	OS↓	MDR1 GG or TT P=0.02	[89]
MDR1	MDR1 G2677T/A + C3435T	81	AML	Idarubicin, cytarabine	EFS↓	MDR1 without homozygous GC haplotype HR=2.455 (1.088-5.539)	[87]
MDR1	MDR1 C3435T	405	AML	Etoposide, mitoxantrone, daunorubicin	OS↓	MDR1 CC P<0.01	[86]
MDR1	MDR1 C3435T	111	ALL	Etoposide, daunorubicin, vincristine	OS↓	MDR1 CC HR=3.3 (P=0.02)	[83]
MDR1	MDR1 C3435T	134	Oesophageal cancer	Platinum-based	OS↑	MDR1 CT/TT HR=0.44 (0.23-0.85)	[78]
ABCG2							
ABCG2	ABCG2 C421A	51	Prostate cancer	Docetaxel	OS↑	ABCG2 CA/AA P=0.05	[93]

EFS, event-free survival; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio

Discussion

A number of polymorphisms in genes encoding drug metabolising enzymes and drug transporters have been described in relation to cancer treatment response.
Concerning polymorphisms in genes encoding CYP enzymes, the clearest relation is found for CYP2D6 and tamoxifen treatment. Several studies have found that patients with breast cancer and CYP2D6 non-functional alleles treated with tamoxifen have shorter relapse-free periods, worse event-free survival and lower overall survival. Even though the studies differed in number of patients and patient groups with different ethnicity were included, these studies have identified consistent results. Based on these results it can, however, not be concluded yet that genotyping for the treatment of tamoxifen is needed. The data available at present represent retrospective analyses of banked samples. Therefore, the association between polymorphisms in the CYP2D6 gene and survival needs further evaluation in prospective studies in a large population of breast cancer patients treated with tamoxifen.
The GST genes are the most extensively studied genes of the phase II enzymes. Several associations have been found between polymorphisms in the various GST genes and cancer treatment response. Polymorphisms in GSTP1 and GSTA1, leading to a less active enzyme, have been associated with improved survival. Deletions in GSTM1 and GSTT1, leading to reduced or no GST activity, resulted in a significantly better prognosis in most malignancies due to a better response to chemotherapy. However, in AML patients with GSTM1 null and/or GSTT1 null genotypes, a worse prognosis was seen due to an increase in toxic deaths. Therefore, the significance of GST enzymes may be different for each malignancy and therapy.
Pharmacogenetic studies in cancer patients have several limitations. Firstly, the small number of patients and the lack of control for other genetic and environmental factors, patient demographics and tumour histology, can result in the identification of false positive results. Secondly, the use of different genotyping techniques and patients of different ethnic origin, that often show different frequencies for mutant alleles, make it difficult to compare the different studies.
Most pharmacogenetic studies investigate the effect of only one or a few single nucleotide polymorphisms in a specific gene at a time, the candidate gene approach. Obviously, this mechanistic approach seems logical. This approach is hypothesis driven, uses a priori knowledge of single nucleotide polymorphisms and gene functions and has produced informative data. The disadvantage of this approach is that it is limited by the present knowledge of pathophysiology and the mechanism of action of a drug. Therefore, future research will also use hypothesis-free whole genome approaches such as SNP arrays. The genome wide approach can discover previously unknown associations of factors as well as identify potential multigenetic associations. A drawback is that the results can be affected by false positives, associated with unimportant genes identified by chance.
It is evident that large studies based upon combined analysis of multiple genes within the metabolic pathway are needed for unravelling prognostically important individual polymorphism profiles. Furthermore, prospective trials are required to establish clinical value and cost-effectiveness of pharmacogenetic testing in oncology.

Conclusion

A single nucleotide polymorphism is not likely to afford a straightforward risk factor for analysis of survival. Population diversities and pharmacokinetic/pharmacodynamic polymorphisms must also be taken into account. The studies presented here suggest that genetic polymorphisms may provide useful prognostic markers in some situations. More insight into the mechanism of action of these markers, the biological determinants of response to treatment and prognosis in cancer will ultimately lead to individualised cancer treatment based on a combination of genotype and tumour characteristics of a patient.

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Chapter 2

Bioanalysis

Chapter 2.1

Simultaneous quantification of cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-MS/MS)

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Abstract

Cyclophosphamide is a cytotoxic prodrug with a very narrow therapeutic index. To study the clinical pharmacology of cyclophosphamide in a large cohort of patients a previously published method for the simultaneous quantitative determination of cyclophosphamide and 4-hydroxycyclophosphamide in human plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS) was optimized. Addition of an isotopically labelled internal standard and adaptation of the gradient resulted in a fast, robust and sensitive assay. Because 4-hydroxycyclophosphamide is not stable in plasma, the compound is derivatized with semicarbazide immediately after sample collection. Sample preparation was carried out by protein precipitation with methanol-acetonitrile (1:1, v/v), containing isotopically labelled cyclophosphamide and hexamethylphosphoramide as internal standards. The LC separation was performed on a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 µm) with 1 mM ammonium hydroxide in water – acetonitrile (90:10, v/v) as the starting gradient, at a flow-rate of 0.40 mL/min with a total run time of 6 min. The lower limit of quantification (LLQ, using a 100 µL sample volume) was 200 ng/mL and the linear dynamic range extended to 40,000 ng/mL for cyclophosphamide and 50 to 5,000 ng/mL for 4-hydroxycyclophosphamide. Accuracies as well as precisions were lower than 20% at the LLQ concentration and lower than 15% for all other concentrations. This method has been successfully applied in our institute to support ongoing studies into the pharmacokinetics and pharmacogenetics of cyclophosphamide.

Introduction

Cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide; CP) is a cytotoxic drug widely used in the treatment of various malignancies. It is a prodrug that requires activation by the cytochrome P450 enzyme system (CYP) to form its pharmacologically active metabolite 4-hydroxycyclophosphamide (4OHCP) [1].
Assays quantifying CP and/or 4OHCP using high-performance liquid chromatography (HPLC) with mass spectrometry (MS) detection have been described previously [2-6]. However, these methods either described the quantification of CP and other metabolites in urine [4;6] or quantification of 4OHCP without CP in human plasma [3]. Sadagopan et al described the simultaneous quantification of CP and 4OHCP in mouse plasma [5]. Baumann et al described the simultaneous quantification of CP and 4OHCP in human plasma albeit with a run time of 20 min [2]. In an earlier publication we described the development and validation of a liquid chromatography tandem mass spectrometric assay for the simultaneous quantification of CP, 4OHCP, thiotepa and tepa in human plasma [7]. This assay has been used successfully for the therapeutic drug monitoring (TDM) of CP and thiotepa [8]. However, application of this assay in a large scale pharmacokinetic study of cyclophosphamide was limited by the run-time and the absence of a good internal standard for cyclophosphamide.
In this paper, we present an optimized method for the simultaneous determination of CP and 4OHCP in human plasma using HPLC coupled with electrospray ionization (ESI) MS/MS with a run time of 6 min, using a stable isotope for the quantification of CP to improve the accuracy and precision of the method. A partial validation of the adapted method was performed based on the international FDA guidelines for bioanalytical validation [9]. Stability data have been reported before [7].

Experimental

Chemicals

CP and 4-hydroperoxycyclophosphamide (4OOHCP) and d4-cyclophosphamide (d4-CP) were purchased from Niomech, Bielefeld, Germany (purity >95%). Hexamethylphosphoramide (HMP) originated from Sigma (Zwijndrecht, The Netherlands).
Acetonitrile and methanol were HPLC-grade reagents and were obtained from Biosolve BV (Valkenswaard, The Netherlands). Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). Distilled water (B. Braun Medical, Emmenbrücke, Switzerland) was used throughout the analysis and all other chemicals used were of analytical grade and used without further purification. Drug free human heparinized plasma originated from Bioreclamations, Hicksville, New York, USA.

Instrumentation

The LC system consisted of an Agilent 1100 series pump, degasser and cooled autosampler (10°C) (Agilent Technologies). A stepwise gradient was used to elute the compounds from a Zorbax Extend C18 analytical column (150 x 2.1 mm ID, particle size 5 µm; Agilent Technologies, Palo Alto, CA, USA) protected with an Agilent Extend C18 Narrow-Bore

Guard Column (12.5 x 2.1 mm ID, particle size 5 µm; Agilent Technologies). At time zero, 90% eluent A (1 mM ammonium hydroxide in water, pH=10) and 10% eluent B (100% acetonitrile) was flushed through the column. After 0.5 minute, the fraction of acetonitrile was increased to 35% in 0.01 minute time. This mobile phase composition was maintained for 1.5 minutes. Subsequently, in 0.01 minute time the mobile phase composition was set back at 90% eluent A, remaining as such for the final 4 minutes of the run. The flow rate was 0.4 mL/min. The eluate (first two minutes of run discarded) entered into an API 3000 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) controlled by Analyst™ software (Applied Biosystems/ MDS Sciex, Analyst software version 1.2). The source temperature was set at 400°C. The curtain gas (1.1 ml/min) and the collision-induced dissociation gas ($342*10^{15}$ molecules/cm^2) consisted of nitrogen (grade 5.0) and the nebulizer and turbo gases (1.6 l/min and 7.0 l/min, respectively) were zero air. The electrospray source was operated in the positive ion mode. The electrospray voltage was +2.5 kV and the dwell time was 150 ms for HMP, 350 ms for CP and d4-CP and 400 ms for 4OHCP-semicarbazide with a 5 ms pause between scans. Mass transitions monitored were *m/z* 261→140 for CP, *m/z* 334→221 for 4OHCP-semicarbazide, *m/z* 267→146 for d4-CP and *m/z* 180→135 for HMP and were monitored in the positive multiple reaction monitoring (MRM) mode.

Sample preparation

Since 4OHCP is not stable in plasma, but degrades rapidly into phosphoramide mustard, semicarbazide was used for stabilization of 4OHCP, and quantified as the 4OHCP-semicarbazide derivative, a reaction optimised previously [10]. To 100 µL plasma sample derivatized with semicarbazide, 25 µL of IS solution I (20 ng/mL d4-CP) for the quantification of CP and 25 µl of IS solution II (100 ng/mL HMP) for the quantification of 4OHCP-semicarbazide were added. Protein precipitation was performed by addition of 300 µL methanol-acetonitrile (1:1, v/v). The samples were mixed and centrifuged for 15 minutes at 23,100 g. After dilution of 50 µL of supernatant with 400 µL eluent A (1mM ammonium hydroxide in water), 10 µL aliquots were injected onto the analytical column.

Validation procedures

A partial validation based on the FDA guidelines was performed by means of linearity, accuracy and precision, selectivity and specificity, cross-analyte/IS interference and carry-over. Furthermore, clinical application of the assay was demonstrated.

Results

Chromatography

LC separation of the two analytes was performed under basic conditions using an eluent composed of aqueous 1 mM ammonium hydroxide and acetonitrile together with a column containing a base-stable stationary phase (pH of the aqueous component was 10). The capacity factors for CP and 4OHCP-semicarbazide were 2.1 and 1.5 respectively. The run time could be limited to 6 minutes. For the quantification of CP the deuterated IS d4-CP resulted in intra-assay imprecisions of less than 2.77% versus 26.3% when HMP was used

as IS. Furthermore, the calibration curve of CP was linear over the complete validated range in contrast to the curve of CP with HMP as internal standard (Figure 1). 4OHCP-semicarbazide could be best quantified using HMP as IS, probably due to the same retention time of 4OHCP-semicarbazide and HMP. Furthermore, when d4-CP was used as IS for the quantification of 4OHCP-semicarbazide the LLQ of 50 ng/mL could not be quantified with sufficient accuracy and precision and needed to be increased to 100 ng/mL. The calibration curve of 4OHCP with HMP and d4-CP as internal standards is shown in Figure 2.

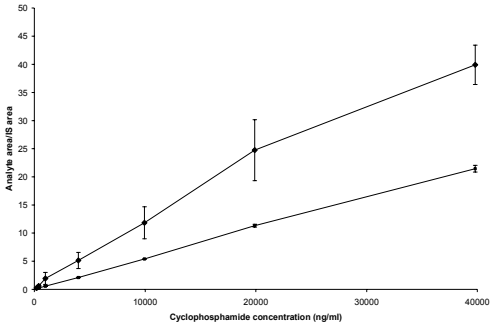

Figure 1. Calibration curve of cyclophosphamide using hexamethylphosphoramide(♦) and d4-cyclophosphamide(■) as internal standard.

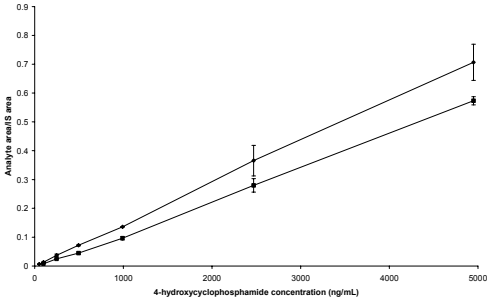

Figure 2. Calibration curve of 4- hydroxycyclophosphamide using hexamethylphosphoramide(♦) and d4-cyclophosphamide(■) as internal standard.

Validation procedures

Linearity

For the validation, calibration standards (7 non-zero standards) were prepared in control human plasma and analyzed in duplicate in three analytical runs. The assay was linear over the validated concentration ranges of 200 to 40,000 ng/mL for CP and 50 to 5,000 ng/mL for 4OHCP.The best fit for the calibration curves was obtained by using a weighting factor of 1/concentration^2 for CP and 1/concentration for 4-OHCP. Correlation coefficients were > 0.995. At all concentration levels deviations of measured concentrations from nominal concentrations were between -3.27 and 4.02% with CV values less than 4.46% for CP. For 4OHCP deviations of measured concentrations from nominal concentrations were between -3.13 and 5.10% with CV values less than 9.65%.

Accuracy and precision

Five replicates of independently prepared quality control samples in human plasma (200, 400, 4,000 and 20,000 ng/mL of CP and 50, 100, 500 and 2,500 ng/mL of 4OHCP) were analyzed in three analytical runs together with calibration standards. Intra-assay inaccuracy was determined as the percent difference between the mean concentration in one analytical run and the nominal concentration, inter-assay inaccuracy as the percent difference between the mean concentration after three analytical runs and the nominal concentration. Intra-assay imprecision was determined as the standard deviation in an analytical run divided by the mean of the run. Inter-assay imprecision was determined as the standard deviation of three analytical runs divided by the overall mean. Assay performance data for CP and

4OHCP are summarized in Table 1 and 2. The assay performance data of the previous method [7] for CP are also included in Table 1 and 2.

Table 1. Inter-assay performance data for cyclophosphamide and 4-hydroxycyclophosphamide.

Compound	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Inter-assay inaccuracy (%)	Inter-assay inaccuracy (HMP as IS)[a] (%)	Inter-assay imprecision (%)	Inter-assay imprecision (HMP as IS)[a] (%)	Number of replicates
cyclophosphamide	199	199	-0.067	-3.74	0.907	9.79	15
	398	390	-2.08	-0.79	2.16	3.64	15
	3980	3971	-0.235	7.15	1.26	3.22	15
	19900	20093	0.972	3.84	1.55	2.13	15
4-hydroxy-cyclophosphamide	50	54	7.91		8.43		15
	102	96	-5.53		3.69		15
	508	501	-1.34		7.08		15
	2540	2525	-0.577		2.06		15

[a]assay performance data of previous method [7]

Table 2. Intra-assay performance data for cyclophosphamide and 4-hydroxycyclophosphamide.

Compound	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	Intra-assay inaccuracy (%)	Intra-assay imprecision (%)	Intra-assay imprecision (HMP as IS)[a] (%)	Number of replicates
cyclophosphamide	199	197	-1.01	2.38	6.32	5
	398	398	0.00	1.38	2.73	5
	3980	3958	-0.553	1.42	2.75	5
	19900	19840	-0.302	2.77	1.94	5
4-hydroxy-cyclophosphamide	50	56	13.7	7.08		5
	102	94	-7.73	5.59		5
	508	504	-0.787	3.17		5
	2540	2514	-1.02	3.18		5

[a]assay performance data of previous method [7]

Samples originally above the upper limit of quantification (ULQ) could be quantified with acceptable accuracy and precision after dilution with drug free human plasma. Measured inaccuracies ranged from 1.85% to 3.87% for CP and 0.921% to 5.39% for 4OHCP. Imprecisions ranged from 1.00% to 2.90% for CP and 2.45% to 6.34% for 4OHCP.

As defined by the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range of this method, based on 100 μL of derivatized human plasma, is 200 to 40,000 ng/mL for CP and 50 to 5,000 ng/mL for 4OHCP (measured as 4OHCP-semicarbazide). This range was suitable for use in our clinical studies, as demonstrated by quantification of plasma samples obtained from a patient treated with CP (Figure 3).

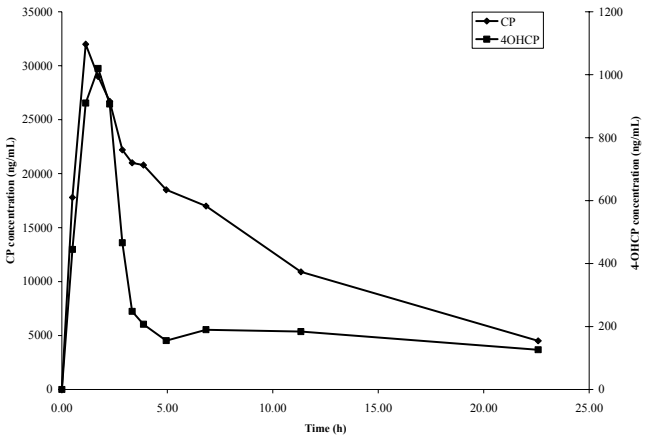

Figure 3. Concentration-time curves of cyclophosphamide and 4-hydroxycyclophosphamide of a patient receiving a 1-hour infusion of 1500 mg cyclophosphamide.

Selectivity and specificity

MRM chromatograms of six batches of control human plasma contained no co-eluting peaks >20% of the CP and 4OHCP-semicarbazide areas at the LLQ level, and no co-eluting peaks >5% of the area of the IS. Figure 4 shows MRM chromatograms of the analytes and IS. Deviations from the nominal concentrations at the LLQ level were between -9.55% and 0.00% for CP and between -20.0% and 3.03% for 4OHCP and thus acceptable.

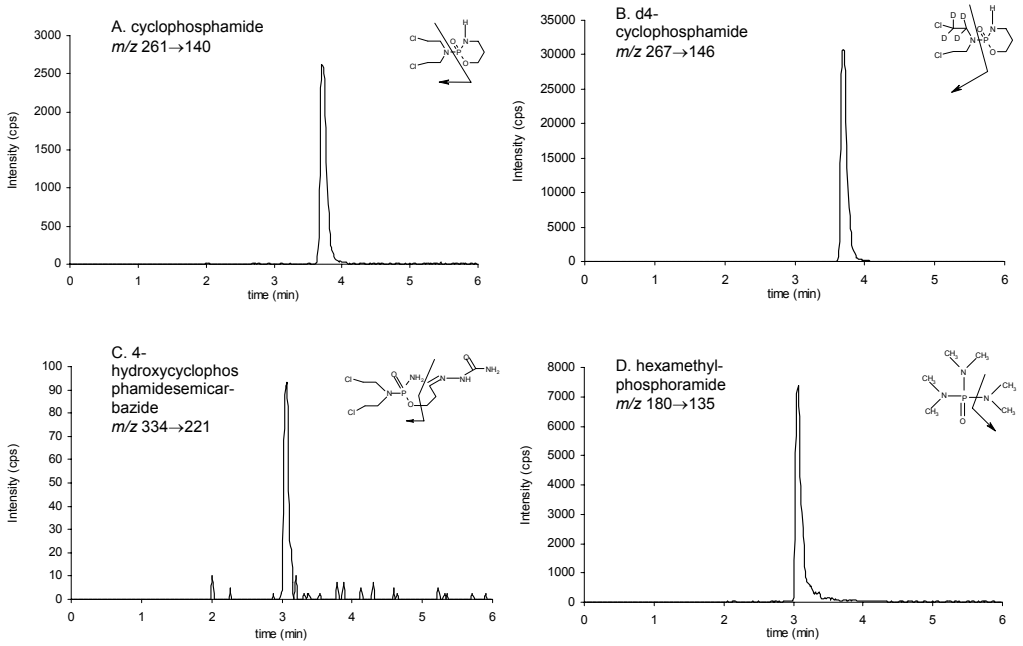

Figure 4. MRM chromatograms (A to D) for a processed sample at the lower limit of quantification level. Cyclophosphamide (A, 200 ng/mL); d4-cyclophosphamide (B, 5 ng/mL); 4-hydroxycyclophosphamide as a semicarbazone derivative (C, 50 ng/mL); hexamethylphosphoramide (D, 25 ng/mL).

Cross-analyte/Internal standard interference

Four separate blank plasma samples were spiked with CP at the ULQ level, 4OHCP at the ULQ level, d4-CP and HMP to determine whether these compounds show interference with each other. No interference from cross-analytes and IS at the retention time of CP and 4OHCP-semicarbazide with areas >20% of the LLQ area, nor interference with areas >5% of the IS area were observed.

Carry-over

To determine the carry-over, 2 blank samples were injected after a sample at the ULQ level (containing CP, 4OHCP-semicarbazide, d4-CP and HMP). No carry-over from the analytes and IS in the first blank sample after the sample at the ULQ level with an area >20% of the corresponding peak area at the LLQ level was observed.

Conclusion

For the simultaneous quantification of CP and its active metabolite 4OHCP (measured as 4OHCP-semicarbazide) in human plasma, a fast, accurate, reproducible and selective LC-MS/MS assay has been developed. The assay quantifies a range for CP from 200 to 40,000 ng/mL and for 4OHCP from 50 to 5,000 ng/mL, sufficient for pharmacokinetic studies with cyclophosphamide in both low and high-dose regimens, using 100 μL human plasma aliquots. Validation results demonstrate that CP can be accurately quantified using d4-CP as IS and 4OHCP concentrations (as 4OHCP-semicarbazide) can be accurately quantified using HMP as IS. Imprecision was markedly reduced by using a deuterated IS. This assay with a runtime of 6 min is now used to support ongoing studies into the pharmacokinetics and pharmacogenetics of CP and TDM programs with CP in our institute.

Acknowledgement

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Chapter 3

Pharmacogenetics

Chapter 3.1

Polymorphisms of drug metabolising enzymes (GST, CYP2B6 and CYP3A) affect the pharmacokinetics of thiotepa and tepa

Submitted for publication

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Abstract

Purpose: Thiotepa is widely used in high-dose chemotherapy. Previous studies show relations between exposure and severe organ toxicity. Thiotepa is metabolised by cytochrome P450 and glutathione S-transferase enzymes. Polymorphisms of these enzymes may affect elimination of thiotepa and tepa, its main metabolite. The purpose of this study was to evaluate effects of known allelic variants in CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 genes on pharmacokinetics of thiotepa and tepa.

Experimental design: 124 Caucasian patients received a high-dose regimen consisting of cyclophosphamide, thiotepa and carboplatin as IV infusions. Genomic DNA was analysed using PCR and sequencing. Plasma concentrations of thiotepa and tepa were determined using validated GC and LC-MS/MS methods. Relations between allelic variants and elimination pharmacokinetic parameters were evaluated using nonlinear mixed effects modelling (NONMEM).

Results: Allele frequencies for CYP2B6 C1459T variant, CYP3A4*1B, CYP3A5*3, GSTA1 (C-69T, G-52A) and GSTP1 C341T were 20.9%, 4.9%, 92.8%, 43.3% and 9.3%, respectively. These polymorphisms had a significant effect on clearance of thiotepa or tepa. Clearance of thiotepa and tepa was predominantly affected by GSTP1 C341T polymorphism. This polymorphism increased noninducible thiotepa clearance by 52% ($p<0.001$) and decreased tepa clearance by 32% ($p<0.001$) in heterozygous patients, which resulted in an increase in combined exposure to thiotepa and tepa of 45% in homozygous patients.

Conclusions: This study indicates that the presently evaluated variant alleles only explain a small part of the substantial interindividual variability in thiotepa and tepa pharmacokinetics. Patients homozygous for the GSTP1 C341T allele may have enhanced exposure to thiotepa and tepa.

Introduction

Interindividual variability in pharmacokinetics may lead to unpredictable toxicity and efficacy. This variability is particularly important in cancer therapy because of the narrow therapeutic window of anticancer agents. Factors contributing to this interpatient variability are environmental, physiologic as well as genetic factors. Polymorphisms in genes encoding drug metabolising enzymes, drug transporters and/or drug targets may explain some of the interindividual variation [1].

Thiotepa is an alkylating anticancer agent often co-administered with other anticancer agents in high-dose chemotherapy regimens for the treatment of advanced or metastatic breast, ovarian and testis tumours. With the use of peripheral blood progenitor cell support, bone-marrow toxicity is not dose limiting in these high-dose regimens. However, other toxicities can be severe and sometimes life-threatening such as mucositis, veno-occlusive disease, oto- and cardiotoxicity [2].

Thiotepa is metabolised to its main metabolite tepa, which shows comparable alkylating activity [3]. Over a broad range of doses in adults, thiotepa has been reported to have an elimination half-life of 1.3-5.2 h, a clearance of 10-28 L/h/m^2, and a volume of distribution of 27-65 L/m^2 [4]. Tepa is detectable in plasma within a few minutes after start of thiotepa infusion and persists longer with a half-life of 3-21 h. Interpatient variability in clearance of thiotepa has been reported to be between 28-90%. Variability in tepa exposure in high-dose thiotepa was 15-50% [5].

Relations between pharmacokinetics and toxicity have been described in several studies. It has been shown that an increased systemic exposure to thiotepa and tepa (as a measure of total alkylating activity) leads to an increase in mucositis severity [6]. Przepiorka et al described a relation between the exposure to thiotepa and tepa and tepa peak levels with the occurrence of major nonhematopoietic toxicity in a high-dose regimen [4]. Hence, identification of factors that are associated with the clearance of thiotepa and tepa could aid in individual dose selection.

Thiotepa is metabolised by oxidative desulfuration by the cytochrome P450 (CYP) enzymes CYP2B6 and CYP3A4 to tepa [7]. Furthermore, thiotepa and tepa are conjugated to glutathione which is catalyzed by glutathione S-transferase (GST) isoenzymes A1-1 and P1-1 [8].

It is known that polymorphisms in the CYP2B6 gene cause functional alterations and decreased protein expression levels in human liver, although the role of the different CYP2B6 alleles for the *in vivo* metabolism of drugs remains largely unknown [9]. A single-nucleotide polymorphism in the 5'-regulatory region of the CYP3A4 gene (CYP3A4*1B) has been associated *in vitro* with enhanced CYP3A4 expression [10;11], and the CYP3A5*3 polymorphism leads to an inactive truncated protein [12]. GST has an important role in the inactivation of alkylating agents [8]. Allelic variants of GSTs show different catalytic efficiency in conjugating glutathione to thiotepa [13].

In the present study, it was evaluated whether known variants of CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 genes are associated with the pharmacokinetic profile of thiotepa and its metabolite tepa in adult Caucasian cancer patients.

Materials and methods

Patient selection

124 patients were included in a number of clinical studies that employed the CTC (cyclophosphamide, thiotepa, carboplatin) chemotherapy regimen with peripheral blood progenitor cell transplantation [14-16]. Patients had either high-risk primary breast cancer and received high-dose chemotherapy as part of their adjuvant treatment, or had advanced breast, germ-cell or ovarian cancer. All protocols were approved by the Committee of Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from all patients.

Drug administration

Patients received either the full-dose CTC regimen (n=67) or the "tiny"CTC (tCTC) regimen (n=57). The full-dose regimen consisted of four days of chemotherapy with cyclophosphamide (1500 mg/m^2/day) as a 1-h infusion, immediately followed by carboplatin (AUC 5 mg*min/mL/day) as a daily 1-h infusion and thiotepa (120 mg/m^2/day) divided over two 30-min infusions (the second daily dose of thiotepa was administered 12 h after the first dose). The tCTC regimen was identical to the CTC regimen except that it incorporated 2/3rd of the dose of each agent. Patients received either one or two courses of CTC or two or three courses of tCTC, when possible every four weeks. Details of the CTC and tCTC regimens have been published previously [15;16].
A part of the patients (n=62) was included in a protocol in which pharmacokinetically guided dosing was applied. Doses on the third and fourth day of a course were adapted based on concentrations in samples collected at day one using defined targets. Doses in subsequent courses were calculated by also using data of previous courses [14].

Sampling design

During the four-day CTC course, blood samples were collected in polypropylene tubes containing lithium heparin as anticoagulant and immediately centrifuged (3,500 g for 3 min at 4ºC) to separate plasma. Complete pharmacokinetic profiles were assessed on two separate days, always including day 1 and days 3 or 4. Blood samples were obtained prior to the start of the infusions on all four days of chemotherapy and at the following time points: 30 (end of thiotepa infusion), 60, 90, 165, 270, 540 min after the start of thiotepa infusion. On day 5 an additional sample was collected approximately 8 h after the last thiotepa infusion. A total of 15 samples were available per patient per course.

Bioanalysis

Thiotepa and tepa plasma concentrations were measured using a validated high-performance liquid chromatography (HPLC) method coupled with electrospray ionization tandem mass spectrometry [17] or a validated gas chromatographic assay with selective nitrogen/phosphorous detection [18]. Both methods have been extensively validated and cross-validated according to FDA guidelines*. Therefore, no discrimination between these methods during data analysis was employed. The lower limit of quantification using 100 μL

plasma samples was 5 ng/mL for thiotepa and tepa. Accuracies and precisions were within ±12% and less than 11%, respectively.

*http://www.fda.gov/cder/guidance/4252fnl.htm

Pharmacogenetic analysis

Genomic DNA was extracted from whole blood using the method of Boom [19] and plasma using the QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA), following the manufacturer's instructions. The methods used for the amplification of the CYP genes have been described by Lang et al [20] for CYP2B6 and Sata et al [21] for CYP3A4 and van Schaik et al [22] for CYP3A5. Genetic polymorphisms in GSTA1 and GSTP1 were analysed according to slightly modified methods previously described by Coles et al [23], Jeronimo et al [24] and Wang et al [25]. Variations in CYP2B6 (C64T, A785G, C1459T, G516T), CYP3A4 (*1B (A-392G), *3 (T1334C)), CYP3A5 (*2 (C27289A), *3 (A6986G)), GSTA1(C-69T, G-52A), GSTP1 (A313G, C341T) were analysed using PCR and sequencing.

In brief, PCR amplification reactions were performed in a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The results of the PCR reactions were analysed by gel electrophoresis. DNA cycle sequencing was carried out essentially as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) on a PTC-200 thermocycler using the Big Dye Terminator Cycle sequencing mix v3.1. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems) was used. Hardy-Weinberg equilibrium was evaluated using the χ^2-test.

Population data analysis

Pharmacokinetic data of thiotepa and tepa were analysed with a previously developed and evaluated population pharmacokinetic model [26] using the non-linear mixed effect modelling program NONMEM (version V, level 1.1) (Globomax LLC, Hanover, MD, USA). The analysis was performed using the first-order estimation method in NONMEM. The use of the first-order conditional estimation method (FOCE) was not feasible due to computational intensity.

In this model (Figure 1), pharmacokinetics of thiotepa and tepa were both described with a two-compartment model. Thiotepa was eliminated by a non-inducible route (CL_{nonind}) and an inducible route (CL_{ind}), the latter leading to formation of tepa. Tepa elimination was described by a first-order elimination clearance (CL_{tepa}). Metabolism of thiotepa to its metabolite tepa was induced in the presence of cyclophosphamide. This process was modelled using an indirect effect model in which the indirect effect increased in the presence of cyclophosphamide. The apparent thiotepa clearance of the inducible route was directly proportional to this indirect effect. This indirect effect model was an "on-off" model (in which the induction is switched 'on' after start of first administration of cyclophosphamide and 'off' in the absence of cyclophosphamide), since cyclophosphamide concentration did not influence the rate of enzyme formation or elimination.

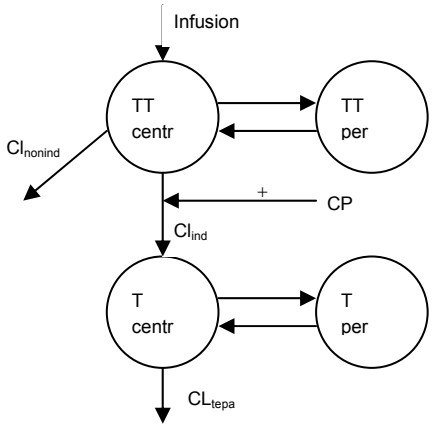

Figure 1. Population pharmacokinetic model of thiotepa and tepa

TT centr = central thiotepa compartment; TT per = peripheral thiotepa compartment; T centr = central tepa compartment; T per = peripheral tepa compartment; CL_{nonind} = non-inducible elimination route of thiotepa; CL_{ind} = inducible elimination route of thiotepa; CL_{tepa} = clearance of tepa; CP=cyclophosphamide

In the current study, the elimination model was extended to evaluate the impact of CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 polymorphisms on the pharmacokinetics of thiotepa and tepa. Genetic polymorphisms in the CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 genes were tested on their association with the elimination pharmacokinetic parameters CL_{nonind} and $CL_{tepa,}$ as were genetic polymorphisms in CYP2B6, CYP3A4 and CYP3A5 on the elimination parameter CL_{ind}. In stage I of the pharmacogenetic analysis 3 separate pharmacogenetic models were evaluated; one model evaluating the effects of polymorphisms in CYP2B6, one model evaluating the effects of polymorphisms in CYP3A4 and CYP3A5 and one model evaluating the effects of polymorphisms in GSTA1 and GSTP1. All polymorphisms per enzyme were included simultaneously on the elimination parameters. Polymorphisms of CYP3A4 and CYP3A5 and also polymorphisms of GSTA1 and GSTP1 could have a combined effect and were therefore included simultaneously. The statistical significance of these polymorphisms was tested in backward elimination procedures. For comparison of models with and without a pharmacogenetic effect the minimal value of objective function (OFV), which is proportional to negative twice the log likelihood, was used. A difference in OFV of 13.8 (df=2, corresponding to a p-value of 0.001, which includes a correction for multiple testing) was considered statistically significant.

In stage II the remaining significant relations of the three models of stage I were combined and the statistical significance was tested in a second backward elimination procedure. Only effects of allelic variants that were significant in this analysis ($p<0.001$), were retained in the final pharmacogenetic model. The 95% confidence intervals for the covariate effects were assessed by log-likelihood profiling.

Allelic variants were incorporated in the population model as covariate relations, according to the following equation:

$$CL=CL_{pop}*(1-(\theta_1*\text{heterozygous}+2*\theta_1*\text{homozygous}))*(1-\theta_2*\text{genotype unknown})$$

Clearance had a typical value of CL_{pop} in wild-type patients. The typical value of heterozygous patients was equal to CL_{pop} reduced by θ_1*100%. Homozygous mutations were assumed to have twice the impact of heterozygous mutations, therefore, the typical value of homozygous patients was equal to CL_{pop} reduced by 2*θ_1*100%. A separate effect was estimated if the genotype was unknown to avoid bias caused by missing data.
In order to investigate whether the relations between genotype and clearance remained significant in the presence of the demographic factors age, weight and sex, these relations were simultaneously included in the final pharmacogenetic model according to the following equations:

$CL=CL_{pop}*(1-\theta*age/median)$
in which age is the individual age, and median is the median age of the population

$CL= CL_{pop}*(weight/median)^{0.75}$
in which weight is the individual weight, and median is the median weight of the population

$CL=CL_{pop}*\theta^{sex}$
in which CL_{pop} is the population value for female patients (sex=0) and θ is the fractional change in CL_{pop} for male patients (sex=1)

Significance of the pharmacogenetic effects was tested in a backwards elimination procedure.

Assessment of impact on exposure
Since patients were included in several protocols including a protocol in which pharmacokinetically guided dosing was applied, the effect of the polymorphisms on exposure could not directly be estimated from the available data. Therefore, the effect on the AUC of thiotepa and tepa of allelic variants that remained in the final pharmacogenetic model were evaluated using a simulation study.
In this simulation study, a large cohort of patients with the same genetic variants as our own patient group was simulated to receive a standard CTC dose of thiotepa of 108 mg (60 mg/m^2, BSA 1.8 m^2) twice daily for 4 consecutive days. For each patient, the AUC of thiotepa and tepa and the sum AUC was generated using the parameters of the final pharmacogenetic model. Wild-type, heterozygous and homozygous patient groups were simulated for each polymorphism, to estimate the effect of that polymorphism in the presence of the other polymorphisms. Each simulated group consisted of 12,400 patients (100 times the original dataset). Median AUCs and quartile values were calculated for wild-type patients, heterozygous patients and homozygous patients.
Furthermore, to determine which combinations of polymorphisms present in our population result in low or high exposure to thiotepa and tepa, 1,000 new values of the AUC were simulated for each individual in the original dataset. Median values of these 1,000 simulated individuals were ranged in an increasing order to detect specific combinations of polymorphisms that result in extremely low or high exposures to thiotepa and tepa.

Results

Patients

Complete pharmacokinetic data were available for a total of 124 adult Caucasian patients with cancer (26 males and 98 females) with a median age of 40 years (range, 16-59; Table 1). The most frequent primary tumour types were breast cancer (n=93) and germ cell cancer (n=26).

Table 1. Baseline patient characteristics.

	Number	Median (range)
Patients	124	
Male	26	
Female	98	
Site of disease		
Breast cancer stage II or III	46	
Breast cancer stage IV	47	
Ovarian cancer	5	
Germ cell cancer	26	
Courses with pharmacokinetic data of thiotepa and tepa available	214	
1st course CTC	67	
2nd course CTC	16	
1st course tCTC	57	
2nd course tCTC	43	
3rd course tCTC	31	
Age (years)		40 (16-59)
Body surface area (m^2)		1.8 (1.5-2.9)
Weight (kg)		69 (50-170)
Height (cm)		172 (153-210)

Population pharmacogenetic data analysis

Eleven single nucleotide polymorphisms (SNPs) were analysed in five genes (CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1) of putative relevance for thiotepa and tepa disposition (Table 2).

Table 2. Genotype and allele frequencies for the studied variant genes.

Polymorphism	Nucleotide change	Effect	rs number	Genotype[1]				Allele frequency[2] (%)
				Wt	Het	Hom	Unknown	
CYP2B6 C64T	C64T	R22C	rs8192709	105	6	2	11	4.4
CYP2B6 A785G	A785G	K262R	rs2279343	82	23	6	13	15.8
CYP2B6 C1459T	C1459T	R487C	rs3211371	72	30	8	14	20.9
CYP2B6 G516T	G516T	Q172H	rs3745274	88	18	7	11	14.2
CYP3A4*1B	A-392G	Promoter	rs2740574	103	9	1	11	4.9
CYP3A4*3	T1334C	M445T	rs4986910	109	3	0	12	1.3
CYP3A5*2	C27289A	T398N	rs28365083	109	3	0	12	1.3
CYP3A5*3	A6986G	Splicing defect	rs776746	2	10	85	27	92.8
GSTA1 (C-69T, G-52A)	C-69T, G-52A	Promoter	rs3957356, rs3957357	37	53	22	12	43.3
GSTP1 A313G	A313G	I105V	rs1695	52	50	11	11	31.9
GSTP1 C341T	C341T	A114V	rs1138272	93	19	1	11	9.3

[1]Wt=wild-type; Het=heterozygous mutant; Hom=homozygous mutant

[2]Total number of mutated alleles/total number of alleles

Observed allele frequencies of CYP3A4 and CYP3A5 in the current study were consistent with previously reported data in Caucasian populations [22;27]. Frequencies of GSTA1 and GSTP1 were similar to those reported by Sweeney et al and Dasgupta et al [28-30]. All polymorphisms were in Hardy-Weinberg equilibrium, except CYP2B6 C64T and G516T.
For CYP3A4*3 and CYP3A5*2 no homozygous variants and three heterozygotes were observed. These polymorphisms were not linked. Because of this low frequency, these SNPs were excluded from further analysis. The clearances of thiotepa or tepa in patients with a CYP3A4*3 or CYP3A5*2 variant were not different from the patients wild-type for these enzymes.
The CYP2B6 (C64T, A785G, C1459T, G516T), CYP3A4*1B, CYP3A5*3, GSTA1 (C-69T, G-52A), GSTP1 (A313G, C341T) variants were included in the statistical analysis to evaluate their effect on thiotepa and tepa pharmacokinetics. Since CYP2B6 A785G and G516T variants were closely linked (except for 4 patients who were heterozygous for the A785G variant and wild-type for the G516T variant and 1 patient who was homozygous for the G516T variant and heterozygous for the A785G variant), these were simultaneously included as single parameter in the analysis.
Upon inclusion of all significant effects from the stage I backwards elimination procedure, several relations between the allelic variants and elimination parameters of thiotepa and tepa remained statistically significant in stage II. Results are shown in Table 3. These effects remained significant when corrected for known and possible factors involved in the pharmacokinetics of thiotepa, such as age, weight and sex.

Table 3. Effect of heterozygous mutations on the pharmacokinetic parameters CL_{nonind}, CL_{ind} and CL_{tepa}.

Polymorphism	Effect	Effect size[1]	95% CI[2]	Effect size genotype unknown	ΔOFV	P-value[3]
CYP2B6 C1459T	increase CL_{ind}	5%	0-9%	46%	16	<0.001
CYP2B6 C1459T	increase CL_{tepa}	21%	17-26%	26%	70	<0.001
CYP3A4*1B	decrease CL_{ind}	21%	11-32%	32%	21	<0.001
CYP3A5*3	increase CL_{nonind}	3%	1-5%	29%	21	<0.001
GSTA1 (C-69T, G-52A)	decrease CL_{nonind}	13%	11-16%	15%	39	<0.001
GSTP1 C341T	increase CL_{nonind}	52%	41-64%	1%	78	<0.001
GSTP1 C341T	decrease CL_{tepa}	32%	29-35%	13%	168	<0.001

[1]Effect size=relative change in pharmacokinetic parameter.
[2]The 95% confidence interval as established by likelihood profiling.
[3]The log-likelihood ratio test was used to calculate the P-value.

Pharmacokinetic parameter estimates for the final pharmacogenetic model are given in Table 4 together with the interindividual, interoccasion and residual variability. All parameters were estimated with an acceptable precision (coefficient of variation 6.1-36.7%). Interoccasion variability could not be estimated for tepa elimination. Residual variability for thiotepa and tepa was rather small. Inclusion of pharmacogenetic effects resulted in a reduction of the interindividual variability in non-inducible thiotepa clearance (46% vs 55% in basic population model).

Table 4. Population variable estimates for thiotepa (TT) and tepa (T) in final pharmacogenetic population model.

	Estimate (RSE %)	% IIV (RSE %)	% IOV (RSE %)
Non-inducible clearance of TT (L/h)	20.0 (36.7)	46.2 (14.7)	25.7 (14.2)
Initial inducible clearance of TT (L/h)	13.0 (16.2)	30.8 (11.4)	17.1 (9.3)
Clearance of T (L/h)	7.02 (13.1)	31.8 (10.1)	
Proportional error of TT (%)	27.9 (9.3)		
Additive error of TT (μM)	0.0673 (22.3)		
Proportional error of T (%)	16.5 (6.1)		

RSE = relative standard error; IIV = interindividual variability; IOV = interoccasion variability

Impact on exposure of pharmacogenetic effects

Figure 2 shows the influence of the various allelic variants on the exposure to thiotepa and tepa. Increases or decreases in exposure to thiotepa, tepa and the combination of thiotepa and tepa in heterozygous and homozygous patients are shown relative to the exposure in wild-type patients. The increase in thiotepa and tepa clearance caused by the CYP2B6 C1459T variant resulted in a reduction of the combined exposure to thiotepa and tepa of 20% in homozygous patients. Thiotepa exposure decreased 4%, while tepa exposure decreased 26% in homozygous patients. The CYP3A4*1B variant reduced CL_{ind}, which resulted in an increased exposure to thiotepa of 26% and a decreased exposure to tepa of 27% in homozygous patients. The combined exposure in homozygous patients was reduced by 12%. Since the effect of CYP3A5*3 on thiotepa clearance was so small, we did not further evaluate the impact of this effect. CL_{nonind} was reduced in patients with the GSTA1 (C-69T, G-52A) variant. This resulted in an increased exposure to the alkylating agents in homozygous patients of 17%. The GSTP1 C341T variant caused an increase in CL_{nonind} and a decrease in CL_{tepa}. The simulation study showed that this resulted in a decrease in thiotepa exposure of 34% and an increase in tepa exposure of 79% in homozygous individuals. The combined exposure of thiotepa and tepa in homozygous individuals increased with 45%.

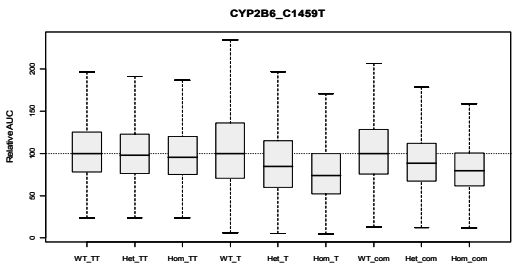

Figure 2A. Effect of CYP2B6_C1459T on exposure to thiotepa and tepa

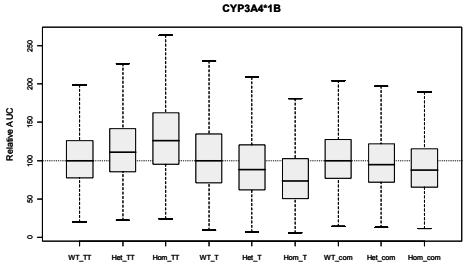

Figure 2B. Effect of CYP3A4*1B on exposure to thiotepa and tepa

wt_tt: exposure to thiotepa in wild-type patients; het_tt: exposure to thiotepa in heterozygous patients; hom_tt: exposure to thiotepa in homozygous patients; wt_t: exposure to tepa in wild-type patients; het_t: exposure to tepa in heterozygous patients; hom_t: exposure to tepa in homozygous patients; wt_com: combined exposure to thiotepa and tepa in wild-type patients; het_com: combined exposure to thiotepa and tepa in heterozygous patients; hom_com: combined exposure to thiotepa and tepa in homozygous patients; AUC values of wild-type patients were set at 100% (dashed line)

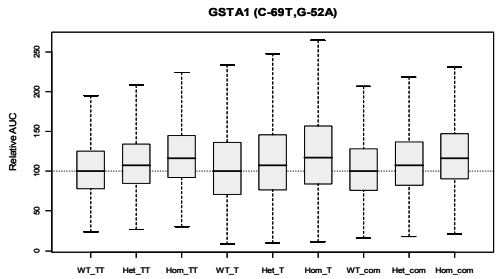

Figure 2C. Effect of GSTA1 (C-69T, G-52A) on exposure to thiotepa and tepa

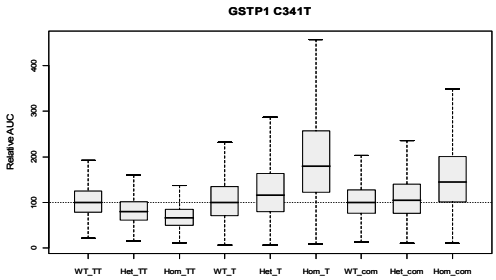

Figure 2D. Effect of GSTP1 C341T on exposure to thiotepa and tepa

The simulation study of the combination of polymorphisms confirmed this finding. The 124 individuals could be divided into 26 groups with the same combination of polymorphisms (based on the genotypes that were included in the final pharmacogenetic model). In this simulation, the group that contained patients with the homozygote variant GSTP1 C341T (along with homozygote variant CYP3A5*3 and GSTA (C-69T, G-52A) and wild-type CYP3A4*1B and CYP2B6 C1459T) showed the highest exposure to thiotepa and tepa (median combined exposure 687 h*uM) while the median exposure to thiotepa and tepa for the other combination of polymorphisms ranged from 336-519 h*uM. No other combination of polymorphisms resulted in extremely low or high exposures to thiotepa and tepa.

Discussion

In this study, we tested the hypothesis that the disposition of thiotepa and tepa is dependent on genetic variability in the CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 genes. We demonstrated that the non-inducible thiotepa clearance was significantly increased by CYP3A5*3 and GSTP1 C341T polymorphisms and decreased by the GSTA1 (C-69T, G-52A) polymorphism. The inducible clearance of thiotepa was significantly increased by the CYP2B6 C1459T variant, and decreased by CYP3A4*1B. CYP2B6 C1459T significantly increased clearance of tepa and GSTP1 C341T decreased clearance of tepa.

The CYP2B6 gene has been mapped to chromosome 19 between 19q12 and 19q13.2, and is composed of nine exons [31]. Lang et al [20] identified nine point mutations of the CYP2B6 gene in a Caucasian population. Five of these polymorphisms cause amino acid substitutions in exons 1, 4, 5, and 9. Polymorphisms in the CYP2B6 gene have been shown to result in altered protein expression and activity [31].

In vitro studies have shown that protein levels of variant CYP2B6 were less than that of wild-type CYP2B6 [20;31]. The activity of the CYP2B6 variants A785G and G516T was significantly higher than that of wild-type CYP2B6 [31;32]. In contrast, the activity of the C64T variant was not different from that of wild-type [31] and the activity of the C1459T variant was reduced [20]. In the current pharmacogenetic study, the CYP2B6 C1459T variant increased the clearance of thiotepa and tepa. This resulted in a reduction of combined exposure to thiotepa and tepa of 20% in homozygous patients. While Lang et al [20] showed a reduced enzyme activity in carriers of the C1459T mutation, in this study the C1459T variant increased clearance of thiotepa and tepa. This might be due to the different

substrates used and furthermore, we determined the effects in patients while Lang et al used human liver samples.

The CYP3A locus comprises four genes that code for the functional enzymes CYP3A4, CYP3A5, CYP3A7 and CYP3A43, of which CYP3A4 and CYP3A5 are the most important [33]. Because of polymorphisms in the genes encoding these proteins [34], it has been proposed that genotyping for CYP3A4 and CYP3A5 variants may be useful for prediction of total CYP3A activity. Many SNPs have been identified in the CYP3A4 gene, however, most are unlikely to affect CYP3A4 activity *in vivo* [34;35]. In the present study CYP3A4*1B decreased the inducible thiotepa clearance. Other authors showed that CYP3A4*1B caused a modest decrease in systemic midazolam [36] and cyclosporine clearance [37;38], whereas others did not find an effect [39;40].

In contrast to CYP3A4, the CYP3A5 protein isoform is known to be expressed in only a small percentage of Caucasian individuals, and this has been linked to a common transition in intron 3 of the CYP3A5 gene (CYP3A5*3) which results in a splicing defect [12;41]. Approximately 85% to 95% of Caucasian subjects are homozygous variant for CYP3A5*3 and thus are deficient of functionally active CYP3A5 [42], which is consistent with the currently observed genotype frequency of 92.8%. In this study, CYP3A5*3 resulted in a minor increase of the noninducible thiotepa clearance. Although CYP3A5*3 results in a deficiency in active CYP3A5, the observed increase in clearance could be due to linkage of CYP3A5*3 to other not determined polymorphisms that result in increased activity. This is similar to findings obtained in healthy subjects and cancer patients using CYP3A phenotyping probes [43;44] or paclitaxel [45], in which no major influence of the CYP3A5 polymorphism could be demonstrated.

Conjugation of glutathione by glutathione S-transferase has been postulated as a mechanism by which alkylating cytostatic drugs can be inactivated intracellularly. It has been shown that both GSTA1 and GSTP1 catalyze the formation of the monoglutathionyl conjugate of thiotepa and tepa [8].

In the present study, the GSTA1 (C-69T, G-52A) variant resulted in a lower thiotepa clearance compared to wild-type GSTA1. Coles et al [23] have shown that GST expression of the GSTA1 variant was reduced in 55 normal human liver samples. Kusama et al [46] also showed that busulfan clearance was reduced in patients with the heterozygous variant of GSTA1. On the other hand, Bredschneider et al [47] investigated GSTA1 expression and GST activity in 48 normal human liver samples and reported no relation with the GSTA1 SNPs.

In the GSTP1 gene, 2 functional polymorphisms (A313G and C341T) have been identified, which resulted in decreased enzyme activity [48]. Srivastava et al [13] showed that the catalytic efficiency of GSH conjugation to thiotepa was lower in the A313G and C341T variants compared to wild-type. We showed that the GSTP1 C341T variant resulted in an increase in noninducible thiotepa clearance and a decrease in tepa clearance. This caused an increase in combined exposure to thiotepa and tepa of 45% in homozygous individuals. It should be noted however, that there were only one homozygous and 19 heterozygous GSTP1 C341T individuals. The homozygous individual in our dataset did not have an extremely high exposure to thiotepa and tepa and did not experience severe toxicity. Other unidentified polymorphisms could have compensated for the increased exposure caused by

GSTP1 C341T in this individual. Furthermore, all patients received a chemotherapy regimen consisting of cyclophosphamide, thiotepa and carboplatin. Therefore, it is difficult to attribute the occurrence or absence of toxicity to thiotepa alone.

In this study, it was assumed that homozygous mutations had twice the impact of heterozygous mutations. This does not take into account possible up-regulation of the functional allele in heterozygous patients. However, frequencies of homozygous mutations were too low to precisely estimate both effects.

Although various significant relations between polymorphisms and clearance of thiotepa and tepa have been established, their effect on combined exposure was generally less than 20% and the effects were small compared to the interindividual variability. In a previous study, we have shown that the mean exposure to thiotepa and tepa in patients with grade 2/3 hepatic toxicity was 39% higher than in patients with grade ≤ 1 toxicity (395 vs 284 μM*h) [6]. Thus, the increase in combined exposure caused by the GSTA1 (C-69T, G-52A) variant alone (17% in homozygous individuals) will probably not have a large impact on treatment related toxicity. However, an increased combined exposure of thiotepa and tepa of 45% in homozygous individuals, caused by GSTP1 C341T variant, could be of clinical relevance.

In conclusion, the effect of CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 polymorphisms on thiotepa and tepa pharmacokinetics was investigated for the first time. This study indicates that the presently evaluated variant alleles only explain a small part of the substantial interindividual variability in thiotepa and tepa pharmacokinetics. Patients homozygous for the GSTP1 C341T allele may have enhanced exposure to thiotepa and tepa.

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Chapter 3.2

Influence of polymorphisms of drug metabolising enzymes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) on the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide

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Abstract

Purpose: The anticancer agent cyclophosphamide is metabolised by cytochrome P450 (CYP), glutathione S-transferase (GST) and aldehyde dehydrogenase (ALDH) enzymes. Polymorphisms of these enzymes may affect the pharmacokinetics of cyclophosphamide and thereby its toxicity and efficacy. The purpose of this study was to evaluate the effects of known allelic variants in the CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes on the pharmacokinetics of the anticancer agent cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide.

Experimental design: A cohort of 124 Caucasian patients received a high dose chemotherapy combination consisting of cyclophosphamide (4-6 g/m^2), thiotepa (320-480 mg/m^2) and carboplatin (area under the curve (AUC) 13-20 mg*min/mL) as IV infusions over four consecutive days. Genomic DNA was analysed using PCR and sequencing. LC-MS/MS was used to measure plasma concentrations of cyclophosphamide and 4-hydroxycyclophosphamide. The relations between allelic variants and the elimination pharmacokinetic parameters (CL_{nonind}, CL_{ind}, k_{4OHCP}) were evaluated using nonlinear mixed effects modelling (NONMEM).

Results: The interindividual variability in the noninducible cyclophosphamide clearance, inducible cyclophosphamide clearance and 4-hydroxycyclophosphamide clearance was 23%, 27% and 31%, respectively. No effect of the allelic variants investigated could be demonstrated on the clearance of cyclophosphamide or 4-hydroxycyclophosphamide.

Conclusions: This study indicates that the presently evaluated variant alleles in the CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes do not explain the interindividual variability in cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics and are, probably, not the cause of the observed variability in toxicity.

Introduction

Cyclophosphamide is a cytotoxic drug widely used in the treatment of various malignancies. It is a prodrug that undergoes complex metabolic activation and inactivation reactions (Figure 1).

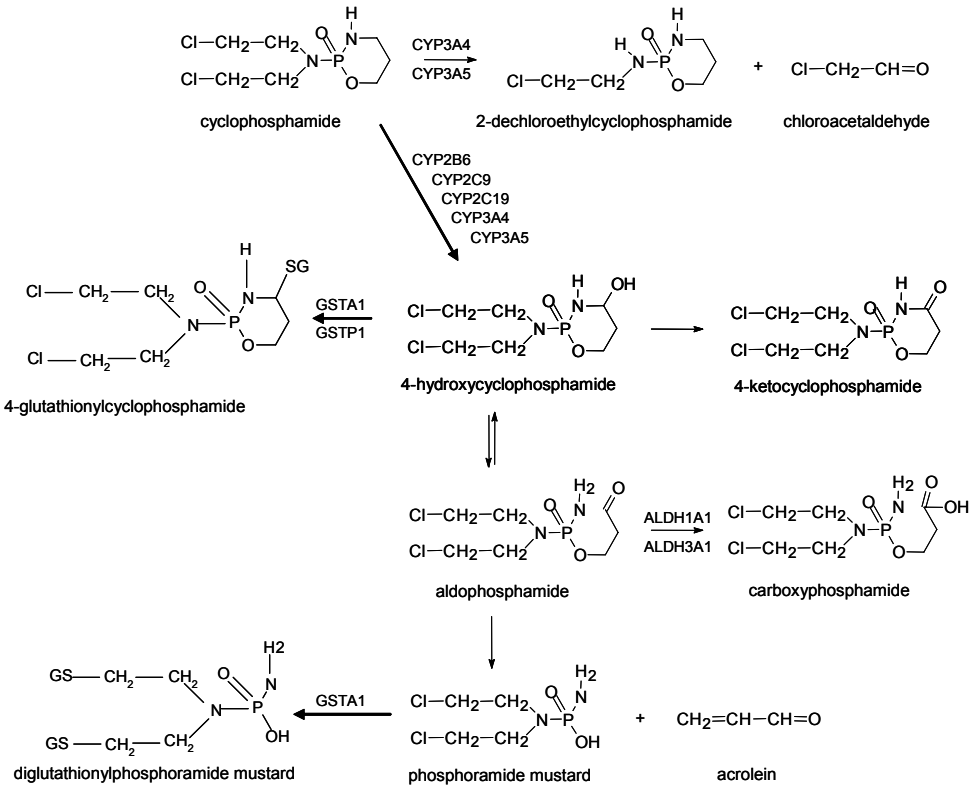

Figure 1. Metabolism of cyclophosphamide. Horizontally, the inactivation pathways are depicted while vertically cyclophosphamide activation is shown.

Cyclophosphamide shows profound autoinduction resulting in an increased clearance of the parent compound and increased formation of its metabolites over time. After cyclophosphamide administration approximately 70-80% of the dose is converted into 4-hydroxycyclophosphamide. Various CYP enzymes have been demonstrated to be involved in the 4-hydroxylation of cyclophosphamide in humans, including CYP2B6, 2C9, 2C19, 3A4 and 3A5, with 2B6 displaying the highest 4-hydroxylase activity [1]. 4-Hydroxycyclophosphamide exists in equilibrium with its ring-opened aldehyde tautomer aldophosphamide, which undergoes spontaneous β-elimination to form phosphoramide mustard and acrolein. Phosphoramide mustard is considered to be the ultimate alkylating, cytotoxic metabolite. 4-Hydroxycyclophosphamide and phosphoramide mustard are detoxified by glutathione S-transferase to form 4-glutathionylcyclophosphamide and diglutathionylphosphoramide mustard, respectively. The isoforms GSTA1 and GSTP1 are

mainly involved in the metabolism of cyclophosphamide [2]. Aldophosphamide is metabolised by the aldehyde dehydrogenase enzymes ALDH1A1 and ALDH3A1 to carboxyphosphamide [3]. Direct detoxification of cyclophosphamide occurs via side chain oxidation resulting in the formation of 2-dechloroethylcyclophosphamide. This reaction is predominantly mediated by CYP3A4 and CYP3A5 and accounts for less than 5% of the total elimination of cyclophosphamide [4].

It has been shown that large interindividual variations in response to cyclophosphamide treatment exist (both efficacy and toxicity), which may reflect interpatient differences in metabolism and distribution of the drug [5]. Several studies describe a relation between lower cyclophosphamide clearance and reduced efficacy [4]. Furthermore, an inverse correlation between cyclophosphamide exposure and treatment-related cardiotoxicity was found [6] and our group has reported strong indications for a relation between exposure to 4-hydroxycyclophosphamide and the occurrence of veno-occlusive disease of the liver [5]. Increased exposures to carboxyphosphamide have been associated with increased liver toxicity and mortality [7].

Strategies for a priori identification of patients at risk for severe toxicity or undertreatment would help in optimising treatment with cyclophosphamide. The drug-metabolising enzymes involved in cyclophosphamide metabolism are known to be highly polymorphic and have variant alleles with decreased or absent metabolic activity, which may influence the clearance of cyclophosphamide and metabolites. Therefore, the genotype of these enzymes is expected to be a major determinant of pharmacokinetic variability.

A few studies have assessed the role of genetic polymorphisms of drug-metabolising enzymes on the pharmacokinetics of cyclophosphamide in patients. Nakajima et al [8] have shown that polymorphisms in the promoter region or introns in the CYP2B6 gene decreased cyclophosphamide 4-hydroxylation in 103 Japanese cancer patients, while the genotypes of CYP2C19, CYP3A4, CYP3A5, ALDH1A1 and GST showed no effect.

Xie et al [9] have demonstrated that the CYP2B6 G516T mutation increased the rate of 4-hydroxycyclophosphamide formation in 29 patients with haematological malignancies, to whom a conventional cyclophosphamide dose was administered. Mutations in CYP2C9 and CYP2C19 showed no effect. In contrast, Timm et al [10] have reported that carriers of CYP2C19*2 had lowered elimination constants of cyclophosphamide at doses ≤ 1000 mg/m^2, whereas no evidence of an association of polymorphisms of CYP2B6, CYP2C9, CYP3A5 and GSTA1 and cyclophosphamide elimination could be found in 60 Caucasian cancer patients.

These varying results led us to investigate whether known variants of CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes are associated with the pharmacokinetics of cyclophosphamide and its metabolite 4-hydroxycyclophosphamide in 124 adult Caucasian cancer patients.

Materials and methods

Patient selection

124 patients were included in a number of clinical studies that employed the CTC (cyclophosphamide, thiotepa, carboplatin) chemotherapy regimen with peripheral blood

progenitor cell transplantation [11;12]. Patients had either high-risk primary breast cancer and received high-dose chemotherapy as part of their adjuvant treatment, or had advanced breast, germ-cell or ovarian cancer. All protocols were approved by the Committee of Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from all patients.

Drug administration

Patients received either the full-dose CTC regimen (n=67) or the "tiny" CTC (tCTC) regimen (n=57). The full-dose regimen consisted of 4 days of chemotherapy with cyclophosphamide (1500 mg/m^2/day) as a 1h infusion, immediately followed by carboplatin (AUC 5 mg*min/mL/day) as a daily 1h infusion and thiotepa (120 mg/m^2/day) divided over two 30 min infusions (the second daily dose of thiotepa was administered 12 h after the first dose). The tCTC regimen was identical to the CTC regimen except that it incorporated 2/3rd of the dose of each agent. Patients received either one or two courses of CTC or two or three courses of tCTC, when possible every 4 weeks.

Mesna (500 mg) was administered 6 times daily for a total of 36 doses, beginning 1h prior to the first cyclophosphamide infusion. All patients received anti-emetics both prophylactically and as indicated, which usually included dexamethasone and granisetron. A subgroup of patients (n=31) received aprepitant as part of their anti-emetic treatment. Patients received prophylactic antibiotics, including ciprofloxacin and amphotericin B orally, starting 4 days before chemotherapy. Details of the CTC and tCTC regimens have been published previously [11;12].

Sampling design

During the 4-day CTC course, blood samples were collected in polypropylene tubes containing lithium heparin as anticoagulant and immediately centrifuged (3,500 g for 3 min at 4ºC) to separate plasma. A 500 μL volume of plasma was immediately added to 50 μL of 2M semicarbazide solution for the derivatization of 4-hydroxycyclophosphamide. 4-Hydroxycyclophosphamide is an unstable compound and requires immediate derivatization to a more stable derivative. Complete pharmacokinetic profiles were assessed on two separate days, always including day 1 and either day 3 or day 4. Blood samples were obtained prior to the start of the infusions on all 4 days of chemotherapy and at the following time points: 30, 60, 90, 120, 150, 180, 210, 285, 390, 660 min after the start of cyclophosphamide infusion. On day 5, an additional sample was collected approximately 22 h after the last cyclophosphamide infusion. A total of 20 samples were available per patient per course.

Bioanalysis

Cyclophosphamide and 4-hydroxycyclophosphamide plasma concentrations were measured using a validated high-performance liquid chromatography (HPLC) method coupled with electrospray ionization tandem mass spectrometry [13;14] The lower limit of quantification, using 100 μL plasma sample, was 200 ng/mL for cyclophosphamide and 50 ng/mL for 4-hydroxycyclophosphamide. Accuracies and precisions were within ±14% and less than 8%, respectively.

Pharmacogenetic analysis

Genomic DNA was extracted from whole blood using the method of Boom et al [15] or plasma using the QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA), following the manufacturer's instructions. The methods used for the amplification of the CYP genes have been described by Lang et al [16] for CYP2B6 , Sullivan-Klose et al [17] for CYP2C9, Goldstein et al [18] for CYP2C19, Sata et al [19] for CYP3A4 and van Schaik et al [20] for CYP3A5. Genetic polymorphisms in GSTA1 and GSTP1 were analysed according to slightly modified methods previously described by Coles et al [21], Jeronimo et al [22] and Wang et al [23]. ALDH1A1 was analysed using as forward primer GGTCTACTTACCCAGCACTGAAA and reverse primer ACAAAGCCGAAACCTGTGAT.

ALDH3A1 was analysed using as forward primer TCCAACTCTGGCTTGTTTCC and reverse primer CTTCTCACGCTGGTTGATGA. Variations in CYP2B6 (C64T, A785G, C1459T, G516T), CYP2C9 (*2 (C430T), *3 (A1075C)), CYP2C19 *2 (G681A), CYP3A4 (*1B (A-392G), *3 (T1334C)), CYP3A5 (*2 (C27289A), *3 (A6986G)), GSTA1(C-69T, G-52A), GSTP1 (A313G, C341T), ALDH1A1*2 (17 base pair deletion -416/-432) and ALDH3A1*2 (C985G) were analysed using PCR and sequencing. In brief, PCR amplification reactions were performed in a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The results of the PCR reactions were analysed by gel electrophoresis. DNA cycle sequencing was carried out essentially as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) on a PTC-200 thermocycler using the Big Dye Terminator Cycle sequencing mix v3.1. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems) was used. Hardy-Weinberg equilibrium was evaluated using the χ^2-test.

Population data analysis

Pharmacokinetic data of cyclophosphamide and 4-hydroxycyclophosphamide were analysed with a previously developed population pharmacokinetic model [24] using the non-linear mixed effect modelling program NONMEM (version VI) (Globomax LLC, Hanover, MD, USA). The analysis was performed using the hybrid estimation method in NONMEM after logarithmic data transformation. With this method, variability in the noninducible cyclophosphamide clearance was estimated using the first-order conditional estimation (FOCE) method, while the other random effects were estimated using the first-order (FO) method. In this model (Figure 2), pharmacokinetics of cyclophosphamide (CP) and 4-hydroxycyclophosphamide (4OHCP) were described with a one-compartment model. Cyclophosphamide was eliminated by a non-inducible route (CL_{nonind}) and an inducible route (CL_{ind}). The non-inducible route represents the renal elimination and hepatic elimination of cyclophosphamide metabolites other than 4-hydroxycyclophosphamide (e.g. 2-dechloroethylcyclophosphamide). The inducible route represents the elimination of cyclophosphamide that leads to the formation of 4-hydroxycyclophosphamide, which is mediated by inducible enzymes. The inducible clearance was modelled using an indirect effect model in which the indirect effect increased in the presence of cyclophosphamide. Since these patients received cyclophosphamide and thiotepa sequentially and it has been shown that thiotepa inhibits the conversion of cyclophosphamide to 4-hydroxycyclophosphamide, this influence was modelled as a thiotepa concentration-

dependent reversible decrease in the indirect effect. Elimination of 4-hydroxycyclophosphamide was modelled as a first-order process with rate constant k_{4OHCP}.

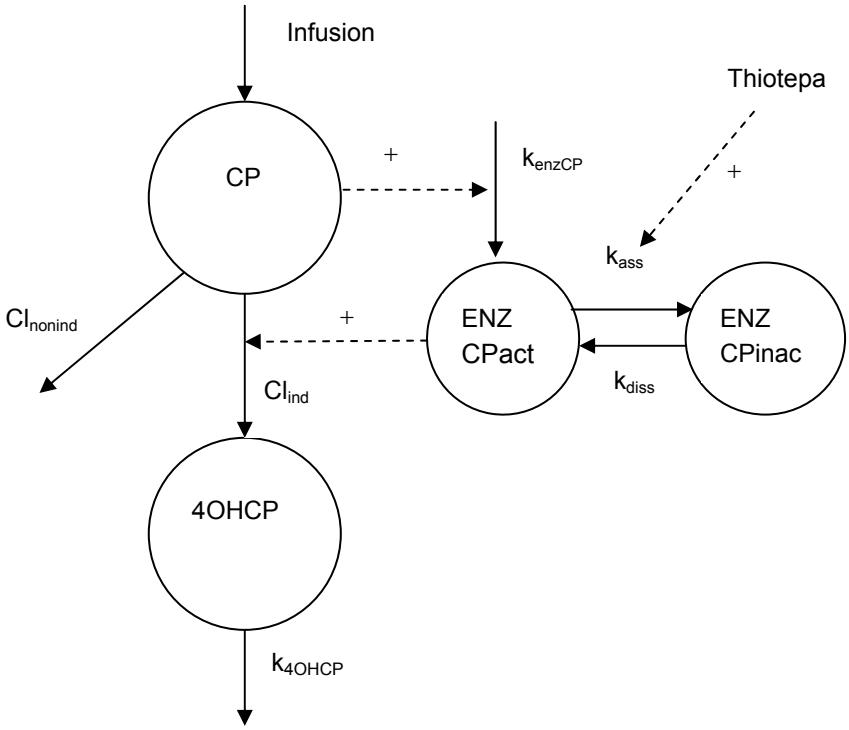

Figure 2. Population pharmacokinetic model of cyclophosphamide and 4-hydroxycyclophosphamide
ENZ_{CPact} = active enzyme pool involved in cyclophosphamide metabolism; $ENZ_{CPinact}$ = inactive enzyme pool involved in cyclophosphamide metabolism [24]

In the current study, the elimination model was extended to evaluate the impact of CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 polymorphisms on the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide. Genetic polymorphisms in the CYP2B6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 genes were tested on their association with the elimination pharmacokinetic parameters CL_{nonind} and CL_{ind}. In addition, the relations between polymorphisms in the GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes and k_{4OHCP} were tested. Polymorphisms in the CYP2B6, CYP2C9 and CYP2C19, CYP3A4 and CYP3A5, GSTA1 and GSTP1 and also ALDH1A1 and ALDH3A1 genes were included simultaneously to take into account combined effects of these polymorphisms. For comparison of models with and without a pharmacogenetic effect, the minimal objective function value (OFV), which is proportional to negative twice the log likelihood, was used. A difference in OFV corresponding to a P-value of 0.001 was considered statistically significant. Allelic variants were incorporated in the population model as covariate relationships, according to the following equation:

$CL=CL_{pop}*(1-(\theta_1*heterozygous+2*\theta_1*homozygous))*(1-\theta_2*genotype\ unknown)$

Clearance had a typical value of CL_{pop} in wild-type patients. The typical value of heterozygous patients was equal to CL_{pop} reduced by θ_1*100%. Homozygous mutations were assumed to have twice the impact of heterozygous mutations, therefore, the typical value of homozygous patients was equal to CL_{pop} reduced by $2*\theta_1$*100%. A separate effect was estimated if the genotype was unknown. Polymorphisms which were observed in the study population at an allele frequency >4%, were tested for their effect on CL_{nonind} and CL_{ind} of cyclophosphamide and k_{4OHCP}.
To test for possible confounding factors influencing the pharmacokinetics of cyclophosphamide, several known and plausible covariates were included in the pharmacokinetic model. Aprepitant has been shown to inhibit cyclophosphamide metabolism [25] and therefore, the effect of aprepitant on CL_{ind} was estimated. Correlations of the pharmacokinetic parameters with weight, sex, creatinine clearance and aprepitant were investigated as follows:

$CL= CL_{pop}*(weight/median)^{0.75}$
in which weight is the individual weight, and median is the median weight of the population

$CL=CL_{pop}*\theta^{sex}$
in which CL_{pop} is the population value for female patients (sex=0) en θ is the fractional change in CL_{pop} for male patients (sex=1)

$CL=\theta_1*CL_{creat} + \theta_2$
in which CL_{creat} is the creatinine clearance estimated using the Cockcroft-Gault equation

$CL=CL_{pop}*\theta^{aprepitant}$
in which CL_{pop} is the population value for patients not using aprepitant (aprepitant=0) en θ is the fractional change in CL_{pop} for patients using aprepitant (aprepitant=1)

Results

Patients

Complete pharmacokinetic data were available for a total of 124 adult Caucasian patients with cancer (26 males and 98 females) with a median age of 40 years (range, 16-59; Table 1). The most frequent primary tumour types were breast cancer (n=93) and germ cell cancer (n=26).

Table 1. Baseline patient characteristics

	Number	Median (range)
Patients	124	
Male	26	
Female	98	
Site of disease		
Breast cancer stage II or III	46	
Breast cancer stage IV	47	
Ovarian cancer	5	
Germ cell cancer	26	
Courses with pharmacokinetic data of cyclophosphamide and 4-hydroxycyclophosphamide available	214	
1st course CTC	67	
2nd course CTC	17	
1st course tCTC	57	
2nd course tCTC	44	
3rd course tCTC	29	
Age (years)		40 (16-59)
Body surface area (m2)		1.8 (1.5-2.9)
Weight (kg)		69 (50-170)
Height (cm)		172 (153-210)
Serum creatinine (uM)		60 (33-259)

Cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics

Plasma concentration-time profiles of cyclophosphamide and 4-hydroxycyclophosphamide were adequately described by the model. The population variables are presented in Table 2.

Table 2. Population variable estimates for cyclophosphamide (CP) and 4-hydroxycyclophosphamide (4OHCP)

Variable	Notation	Estimate (RSE %)	% IIV (RSE %)	% IOV (RSE %)
Non-inducible clearance of CP (L/h)	CL_{nonind}	3.04 (3.2)	23 (26)	21 (23)
Inducible clearance of CP (L/h)	CL_{ind}	2.66 (4.4)	27 (33)	16 (19)
Volume of distribution of CP (L)	V	45.6 (2.8)	19 (24)	12 (25)
Zero-order formation rate constant of the enzyme involved in CP metabolism (h^{-1})	k_{enzCP}	0.0291 (5.5)	37 (19)	
First-order elimination rate constant of 4OHCP (h^{-1})	k_{4OHCP}	149 (4.1)	31 (20)	
Rate constant of reversible enzyme inactivation ($h^{-1}*\mu M^{-1}$)	k_{ass}	0.189 (3.9)	32 (21)	
Rate constant of reversible enzyme activation (h^{-1})	k_{diss}	0.174 (5.2)		
Proportional error CP (%)		20.8 (2.5)		
Proportional error 4OHCP (%)		25.2 (3.6)		

RSE = relative standard error; IIV = interindividual variability; IOV = interoccasion variability.

The typical values of noninducible cyclophosphamide clearance (3.04 L/h), inducible cyclophosphamide clearance (2.66 L/h) and elimination rate constant of 4-hydroxycyclophosphamide (149 h^{-1}) were consistent with earlier data [24;26].

Interindividual variability of the noninducible cyclophosphamide clearance, inducible cyclophosphamide clearance and elimination of 4-hydroxycyclophosphamide was 23%, 27% and 31%, respectively, with individual values ranging from 0.90 to 6.66 L/h for the noninducible cyclophosphamide clearance, 1.24 to 6.97 L/h for the inducible cyclophosphamide clearance and 65-304 h^{-1} for the elimination rate constant of 4-hydroxycyclophosphamide.

Population pharmacogenetic data analysis

Sixteen polymorphisms were analysed in nine genes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) of putative relevance for cyclophosphamide and 4-hydroxycyclophosphamide disposition (Table 3).

Table 3. Genotype and allele frequencies for the studied variant genes (n=124)

Polymorphism	Nucleotide change	Effect	Genotype[1]				Allele frequency[2] (%)
			Wt	Het	Hom	Unknown	
CYP2B6 C64T	C64T	R22C	106	6	2	10	4.4
CYP2B6 A785G	A785G	K262R	85	23	6	10	15.4
CYP2B6 C1459T	C1459T	R487C	76	30	8	10	20.2
CYP2B6 G516T	G516T	Q172H	89	18	7	10	14.0
CYP2C9*2	C430T	R144C	85	19	8	12	15.6
CYP2C9*3	A1075C	I359L	99	15	0	10	6.6
CYP2C19*2	G681A	Splicing defect	81	25	8	10	18.0
CYP3A4*1B	A-392G	Promoter	104	9	1	10	4.8
CYP3A4*3	T1334C	M445T	110	3	0	11	1.3
CYP3A5*2	C27289A	T398N	111	3	0	10	1.3
CYP3A5*3	A6986G	Splicing defect	4	13	96	11	90.7
GSTA1 (C-69T, G-52A)	C-69T, G-52A	Promoter	37	54	22	11	43.4
GSTP1 A313G	A313G	I105V	53	50	11	10	31.6
GSTP1 C341T	C341T	A114V	94	19	1	10	9.2
ALDH1A1*2	17 base pair deletion -416/-432	Promoter	100	13	0	11	5.8
ALDH3A1*2	C985G	P329A	71	38	5	10	21.1

[1]Wt=wild-type; Het=heterozygous mutant; Hom=homozygous mutant
[2]Total number of mutated alleles/total number of alleles

Observed allele frequencies of CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 variants in the current study were consistent with previously reported data in Caucasian populations [27-30]. All polymorphisms were in Hardy-Weinberg equilibrium, except CYP2B6 C64T, G516T, CYP2C9*2 and CYP3A5*3.

For CYP3A4*3 and CYP3A5*2 no homozygous variants and three heterozygous variants were observed. These polymorphisms were not linked to each other. Because of this low frequency these SNPs were excluded from further analysis. The clearances of cyclophosphamide or 4-hydroxycyclophosphamide in patients with a CYP3A4*3 or CYP3A5*2 variant were not different from the clearances in patients wild-type for these enzymes. The CYP2B6 C64T, A785G, C1459T, G516T, CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP3A4*1B, CYP3A5*3, GSTA1 (C-69T, G-52A), GSTP1 A313G, C341T, ALDH1A1*2 and ALDH3A1*2 variants occurred at a frequency >4% and were therefore included in the statistical analysis to evaluate their effect on cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics. Since CYP2B6 A785G and G516T variants were closely linked (except for 4 patients who were heterozygous for the A785G variant and wild-type for the G516T variant and 1 patient who was homozygous for the G516T variant and heterozygous for the A785G variant), these were simultaneously included as single parameter in the analysis.

No statistically significant relations were found in the likelihood ratio test for any of the tested genotype-pharmacokinetic parameter relations. The estimated covariate effects are shown in Table 4.

Table 4. Effect of heterozygous mutations on the pharmacokinetic parameters CL_{nonind}, CL_{ind} and k_{4OHCP}.

Polymorphism	Effect	Effect size[1]
CYP2B6		
CYP2B6 C64T	increase CL_{nonind}	5%
CYP2B6 C64T	decrease CL_{ind}	11%
CYP2B6 A785G/G516T	decrease CL_{nonind}	9%
CYP2B6 A785G/G516T	decrease CL_{ind}	2%
CYP2B6 C1459T	increase CL_{nonind}	3%
CYP2B6 C1459T	decrease CL_{ind}	5%
CYP2C9 and CYP2C19		
CYP2C9*2	increase CL_{nonind}	2%
CYP2C9*2	decrease CL_{ind}	5%
CYP2C9*3	decrease CL_{nonind}	8%
CYP2C9*3	decrease CL_{ind}	4%
CYP2C19*2	decrease CL_{nonind}	7%
CYP2C19*2	decrease CL_{ind}	14%
CYP3A4 and CYP3A5		
CYP3A4*1B	increase CL_{nonind}	2%
CYP3A4*1B	increase CL_{ind}	0.3%
CYP3A5*3	increase CL_{nonind}	7%
CYP3A5*3	decrease CL_{ind}	12%
GSTA1 and GSTP1		
GSTA1 (C-69T, G-52A)	decrease k_{4OHCP}	4%
GSTP1 A313G	decrease k_{4OHCP}	5%
GSTP1 C341T	decrease k_{4OHCP}	7%
ALDH1A1 and ALDH3A1		
ALDH1A1*2	decrease k_{4OHCP}	10%
ALDH3A1*2	decrease k_{4OHCP}	6%

[1]Effects were not significant (P>0.05)

The covariate effects were low, ranging from 0.3 to 14% in effect size for heterozygous individuals. Individual inducible clearance values of cyclophosphamide as a function of the variant genotypes of CYP2C19*2 are shown in Figure 3. This figure shows that there is a trend in reduced inducible clearance of cyclophosphamide in patients with the variant CYP2C19*2 allele. Individuals heterozygous and homozygous for variant CYP2C19*2 had a mean clearance of 2.46 L/h and 2.17 L/h, respectively, compared with 2.88 L/h for wild-type patients.

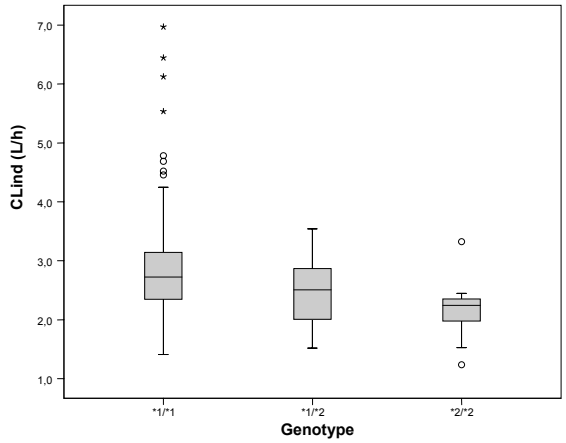

Figure 3. Individual inducible clearance values of cyclophosphamide as a function of variant CYP2C19*2 genotypes

CLind: inducible cyclophosphamide clearance; *1/*1, wild-type patients; *1/*2, heterozygous patients; *2/*2, homozygous variant patients; ○, "mild" outlier (between 1.5 and 3 times the interquartile range); *, "extreme" outlier (more than 3 times the interquartile range)

Influence of weight, sex, creatinine clearance and aprepitant
The other covariates tested (weight, sex, creatinine clearance and aprepitant) did not significantly influence the pharmacokinetic parameters. Inclusion of weight, sex, creatinine clearance and aprepitant resulted in reductions in the OFV of less than 1.5. The estimated effect of aprepitant was a reduction in inducible cyclophosphamide clearance of 6% in patients receiving aprepitant.

Discussion

In this study, we tested the hypothesis that the disposition of cyclophosphamide and 4-hydroxycyclophosphamide is dependent on genetic variability in the CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes. We demonstrated that carriers of at least one CYP2C19*2 allele had a trend towards lower inducible cyclophosphamide clearance, which results in lower exposure to the activated metabolite.
Previous in-vitro studies demonstrated that several cytochrome P450 enzymes are able to catalyze cyclophosphamide 4-hydroxylation, however, these results are partly conflicting. Some investigators [1;31] identified CYP2B6 as the major cyclophosphamide 4-hydroxylase, others found that CYP2C9 [32;33], CYP2C19 [32;34] and CYP3A4/5 [33] were important enzymes for cyclophosphamide activation. The polymorphic GSTA1 and GSTP1 contribute to the detoxification of reactive intermediates [2]. ALDH1A1 and ALDH3A1 have been reported to be involved in the formation of carboxyphosphamide [3].
The determination of well-characterized pharmacogenetic traits could help to understand the different enzymes involved in cyclophosphamide activation and detoxification. The CYP2C19*2 allele is associated with defective enzyme function [35]. Timm et al [10] reported that CYP2C19*2 carriers had lowered elimination constants of cyclophosphamide in patients receiving doses ≤1000 mg/m^2. This effect could not be demonstrated in patients receiving doses >1000 mg/m^2. Increased induction caused by higher doses of cyclophosphamide possibly superimposes any effect of CYP2C19 variants. However, only 11 patients received doses ≥1000 mg/m^2. In two studies on the toxicity of pulse cyclophosphamide treatment, CYP2C19*2 carriers had a significantly lower risk of developing ovarian toxicity [36;37], indicating less activation of cyclophosphamide. However, others could not demonstrate an effect of CYP2C19 on cyclophosphamide pharmacokinetics [8;9].
Although the involvement of CYP2C9 in cyclophosphamide metabolism has been demonstrated *in vitro*, its role *in vivo* seems minor. Our study, as well as the study by Timm et al [10] and Xie et al [9] could not demonstrate an effect of polymorphisms of CYP2C9 on cyclophosphamide pharmacokinetics.
Polymorphisms in the CYP2B6 gene have been shown to result in altered protein expression and activity [38]. In-vitro studies have shown that protein levels of variant CYP2B6 were less than that of wild-type CYP2B6 [16;38]. The activity of the CYP2B6 variants A785G and G516T was significantly higher than that of wild-type CYP2B6 [38;39]. In contrast, the activity of the C64T variant did not differ from that of wild-type CYP2B6 [38], whereas the activity of the C1459T variant was increased [16]. In an in-vivo study it has been shown that

the clearance of bupropion in CYP2B6*4 carriers was higher than in wild-type participants, whereas CYP2B6*6 had no effect [40]. Our study could not demonstrate an effect of the different CYP2B6 variants in cyclophosphamide pharmacokinetics. This is supported by the findings of Timm et al [10]. Nakajima et al [8], however, found that polymorphisms in the promoter region and introns of CYP2B6 caused a decreased cyclophosphamide 4-hydroxylation. Xie et al [9] demonstrated that the CYP2B6 G516T mutation increased the rate of 4-hydroxycyclophosphamide formation in patients with haematological malignancies. In the study of Takada et al [36] patients with lupus nephritis homozygous for CYP2B6*5 had a higher probability of reaching end-stage renal disease and exhibited higher serum creatinine levels, indicating a reduced metabolic activation of cyclophosphamide in these patients. Due to these discrepancies in reported associations, the role of CYP2B6 genotypes needs further clarification.

Many SNPs have been identified in the CYP3A4 gene, however, most are unlikely to affect CYP3A4 activity *in vivo* [41;42]. The CYP3A4*1B variant has been shown to result in increased transcriptional activity [43]. In the present study no effect of this polymorphism on cyclophosphamide pharmacokinetics was found. This could be because of the minor contribution of CYP3A4 to the 4-hydroxylation of cyclophosphamide (12-18% in human liver microsomes) [1]. In contrast to CYP3A4, the CYP3A5 protein isoform is known to be expressed in only a small percentage of Caucasian individuals, and this has been linked to a common transition in intron 3 of the CYP3A5 gene (CYP3A5*3) which results in alternative splicing and protein truncation [44;45]. In this study, CYP3A5*3 did not influence cyclophosphamide pharmacokinetics. The study of Nakajima et al [8] also showed no association between the CYP3A5*3 genotype and cyclophosphamide metabolism. Interestingly, Petros et al [46] demonstrated that patients with CYP3A5*1 had higher cyclophosphamide plasma levels compared with patients with the CYP3A5*3 genotype.

Conjugation of glutathione by glutathione S-transferase has been postulated as a mechanism by which alkylating cytotoxic drugs can be inactivated intracellularly. It has been shown that both GSTA1 and GSTP1 are involved in cyclophosphamide metabolism [2]. In the present study, no effect of polymorphisms in GSTA1 or GSTP1 could be demonstrated. This may be due to the fact that glutathione S-transferases function primarily intracellularly, so the effect of polymorphisms in these enzymes may not be reflected by changes in the pharmacokinetics but rather in susceptibility to toxicity. Indeed, Nakajima et al [8] and Timm et al [10] did not find an effect of the GST enzymes on the pharmacokinetics of 4-hydroxycyclophosphamide, while Zhong et al [47] showed that the GSTP1 A313G polymorphism significantly increased the risks of short-term side-effects of pulsed high-dose cyclophosphamide in SLE patients.

Although ALDH1A1 and ALDH3A1 have been reported to be involved in cyclophosphamide metabolism, we could not demonstrate an effect of polymorphisms in these enzymes on the pharmacokinetics of 4-hydroxycyclophosphamide. The ALDH1A1*2 polymorphism eliminates a c-myb binding site from the promoter region of ALDH1A1. Although in-vitro expression analysis did not show altered activity [28], this may not fully reflect the regulatory mechanism underlying gene expression *in vivo*. We, however, could not demonstrate an effect of ALDH1A1*2. Increased expression of ALDH3A1 may play a role in detoxification and development of tumour resistance to cyclophosphamide. Transfection of ALDH3A1

expression vectors into human MCF-7 cell lines decreased sensitivity to oxazaphosphorines [3]. Although a variant allele (ALDH3A1*2) has been observed in the ALDH3A1 gene, the effect is unknown [48]. We could not demonstrate an effect of the ALDH3A1*2 variant on 4-hydroxycyclophosphamide pharmacokinetics.

Various studies determined the influence of genetic factors on cyclophosphamide pharmacokinetics with varying results. Factors including ethnic differences, the number of participants, dose of cyclophosphamide or comedication might be responsible for the discrepancy between the different effects seen in these studies.

The interindividual variation in cyclophosphamide metabolism observed in this study was modest. Interindividual variation in noninducible cyclophosphamide clearance, inducible cyclophosphamide clearance and 4-hydroxycyclophosphamide clearance were 23%, 27% and 31%, respectively. This is probably due to stringent in- and exclusion criteria applied in these studies. Patients had to have normal cardiac, renal, hepatic, haematopoietic and pulmonary function. Furthermore, comedication and hydration was regulated according to study-protocol.

In contrast to a previous report, we could not demonstrate a significant effect of aprepitant on cyclophosphamide pharmacokinetics. This is most likely due to the larger dataset used in this study, 31 patients received aprepitant versus 6 patients in the study of de Jonge et al [25].

In this study it was assumed that homozygous mutations had twice the impact of heterozygous mutations. This does not take into account possible up-regulation of the functional allele in heterozygous patients. However, frequencies of homozygous mutations were too low to precisely estimate both effects.

In conclusion, the studied variants in the CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes do not cause a substantial interindividual difference in cyclophosphamide and 4-hydroxycyclophosphamide clearance. Additional genetic variants or haplotypes of importance to cyclophosphamide pharmacokinetics may yet be discovered.

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Chapter 3.3

Relations between polymorphisms in drug metabolising enzymes and toxicity of chemotherapy with cyclophosphamide, thiotepa and carboplatin

Submitted for publication

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Abstract

Purpose: High-dose chemotherapy with cyclophosphamide, thiotepa and carboplatin has been developed as a possible curative treatment modality in several solid tumours. However, a large interindividual variability in toxicity is encountered in high-dose chemotherapy. A priori identification of patients at risk for toxicity could be an attractive prospect. Genotyping of genes encoding drug metabolising enzymes might provide such a tool.

Experimental design: We assessed sixteen selected polymorphisms in nine genes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) of putative relevance in cyclophosphamide, thiotepa and carboplatin metabolism using polymerase chain reaction and DNA sequencing in 113 patients who were treated with high-dose chemotherapy regimens based on cyclophosphamide, thiotepa and carboplatin.

Results: Patients heterozygous for the ALDH3A1*2 allele (allelic frequency 21.2%) had an increased risk of haemorrhagic cystitis when compared to patients with wild-type alleles (5/38 vs. 1/70; OR 11.95, 95% CI 1.18-120.56; P=0.04). Furthermore, patients heterozygous for the ALDH1A1*2 allele (allelic frequency 5.8%) had an increased risk of liver-toxicity when compared to patients with wild-type alleles (6/13 vs. 19/99; OR 5.13, 95% CI 1.30-20.30; P=0.02). No other relations reached significance.

Conclusion: Patients heterozygous for the ALDH3A1*2 and ALDH1A1*2 allele have an increased risk of haemorrhagic cystitis and liver-toxicity, respectively, compared to patients with wild-type alleles when treated with a high-dose chemotherapy combination of cyclophosphamide, thiotepa and carboplatin. Pharmacogenetic approaches can identify patients who are at risk of experiencing toxic side-effects in high-dose chemotherapy.

Introduction

High-dose chemotherapy in combination with peripheral blood progenitor cell transplantation is used in the treatment of several malignancies. Two common regimens used in the Netherlands are the CTC regimen, which consists of cyclophosphamide (6000 mg/m^2), thiotepa (480 mg/m^2) and carboplatin (1600 mg/m^2) given as short infusions divided over four days and the “tiny”-CTC (tCTC) regimen which is identical to the CTC regimen but incorporates 2/3rd of the dose of each agent [1-3].
Single courses of CTC chemotherapy are generally well tolerated. However, severe and sometimes irreversible organ toxicity (such as mucositis, ototoxicity, sensory neuropathy, haemorrhagic cystitis and cardiotoxicity) may occur. The tCTC regimen is better tolerated than the full-dose CTC regimen, but when multiple courses are given, it may also cause severe toxicity such as veno-occlusive disease of the liver and haemorrhagic cystitis [4;5]. Toxicity is highly variable and currently unpredictable. However, the occurrence and severity of toxicity are clearly related to the pharmacokinetics and metabolism of the cytotoxic agents [6;7].
Cyclophosphamide is a prodrug that needs to be activated by cytochrome P450 to 4-hydroxycyclophosphamide. CYP2B6, 2C9, 2C19, 3A4 and 3A5 have been reported to be involved in this activation step, of which CYP2B6 has the highest activity [8]. Detoxification of activated cyclophosphamide metabolites is mediated by aldehyde dehydrogenase (ALDH) enzymes and the glutathione S-transferase (GST) enzyme system. The isoforms ALDH1A1, ALDH3A1, GSTP1 and GSTA1 are the main actors involved in the detoxification of cyclophosphamide [8]. Thiotepa is metabolised by CYP2B6 and CYP3A4 to its main and active metabolite tepa. Detoxification of thiotepa and tepa is mediated by the GST isoforms GSTA1 and GSTP1 [9]. Carboplatin is mainly excreted by renal filtration with limited tubular reabsorption and secretion [10]. Intracellular detoxification of carboplatin can occur by GSTs. The isoforms GSTP1 and possibly GSTA1 are the dominant forms of GSTs involved [11;12].
The genes encoding the enzymes involved in the metabolism of the compounds of the CTC regimen are genetically polymorphic and are known to have variant alleles which result in diminished or absent metabolic activity of the expressed proteins. This may result in a wide interpatient variability in exposure to the different compounds. Therefore, inherited genetic variations in genes related to drug metabolism may influence the toxicity of chemotherapy in cancer patients. Especially, since the enzymes are also important in the intracellular detoxification.
Since the therapeutic window is narrow in the setting of high-dose chemotherapy, it is important to determine factors that could aid in predicting the occurrence of toxic effects. In this study, we therefore tested the hypothesis that genetic polymorphisms of the enzymes involved in the metabolism of the compounds of the CTC regimen influence the risk of severe toxicity associated with the CTC regimen.

Patients and methods

Patients

Patients were entered in a number of clinical studies that employed the CTC or tCTC high-dose chemotherapy regimens with peripheral blood progenitor cell transplantation as described previously [2;13-15]. Patients either had high-risk primary breast cancer and received high-dose chemotherapy as part of their adjuvant treatment, or had advanced breast, germ-cell or ovarian cancer. All patients had to be below 60 years of age and had to have a good performance status (WHO 0 or 1). For patients with breast cancer, no previous chemotherapy was allowed, unless it had been limited to non-anthracycline-based adjuvant therapy at least one year before relapse. Patients with germ cell cancer had received prior chemotherapy, which usually included cisplatin. Before the first course, patients had to have adequate renal function (creatinine clearance >60 mL/min) and hepatic function (bilirubin <20 umol/L, ALAT and ASAT <1.5 times the upper limit of normal). All patients received induction and peripheral blood progenitor cell mobilization chemotherapy followed by G-CSF (filgrastim) prior to high-dose chemotherapy.
Written informed consent was obtained from all patients and the Committee on the Medical Ethics of The Netherlands Cancer Institute had approved all protocols.

Treatment

Two different high-dose chemotherapy regimens, consisting of cyclophosphamide, thiotepa and carboplatin (CTC), were administered. The full dose CTC regimen consisted of cyclophosphamide 1500 mg/m^2 as a daily one-h infusion, carboplatin 400 mg/m^2 as a daily one-h infusion and thiotepa 60 mg/m^2 as twice daily 30-min infusions during four consecutive days. The tCTC regimen was identical to the CTC regimen except that it incorporates 2/3rd of the dose of each agent. Patients received either one (high-risk primary breast cancer) or two (refractory germ cell cancer) courses of CTC or two (high-risk primary breast or metastatic ovarian cancer) or three (metastatic breast or germ cell cancer) courses of tCTC, when possible every four weeks. Before a second or third course was started, creatinine clearance had to exceed 40 mL/min and renal function loss as determined by the 24-hour creatinine clearance had to be less than 20% of the baseline estimate. Furthermore, the hepatic function had to be recovered (bilirubin <20 umol/L, ALAT and ASAT <2 times the upper limit of normal). Mesna (500 mg) was administered 6 times daily for a total of 36 doses, starting one hour prior to the first cyclophosphamide infusion. All patients received antiemetics both prophylactically and as indicated, which usually included dexamethasone and granisetron. Antibiotic prophylaxis consisted of ciprofloxacin and amphotericin B orally, starting four days before chemotherapy. Approximately 60 hours after the last thiotepa infusion the peripheral blood progenitor cells were reinfused. A part of the patients (70%) was included in a protocol in which pharmacokinetically guided dosing was applied. Doses on the third and fourth day of a course were adapted based on concentrations in samples collected at day one using defined targets. Doses in subsequent courses were calculated by also using data of previous courses [16].

Toxicity

The toxicity of the CTC chemotherapy was scored during and after each course. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria [17]. The clinical diagnosis veno-occlusive disease (VOD) was based on elevation of transaminases, hepatomegaly and ascites. Cardiotoxicity, haemorrhagic cystitis and VOD were scored in a dichotomous manner (e.g. no toxicity vs. toxicity of any grade). Mucositis, diarrhoea and liver-toxicity (based on ALAT, ASAT and total bilirubin) were transformed to dichotomous variables (e.g. grade 0-1-2 toxicity vs. grade 3-4 toxicity). Weight loss, ototoxicity and sensory neuropathy were transformed to dichotomous variables according to grade 0-1 toxicity vs. grade 2-3 toxicity.

Pharmacogenetic analysis

Genomic DNA was extracted from whole blood using the method of Boom et al [18] or plasma using the QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA), following the manufacturer's instructions. The methods used for the amplification of the CYP genes have been described by Lang et al [19] for CYP2B6, Sullivan-Klose et al [20] for CYP2C9, Goldstein et al [21] for CYP2C19, Sata et al [22] for CYP3A4 and van Schaik et al [23] for CYP3A5. Genetic polymorphisms in GSTA1 and GSTP1 were analysed according to slightly modified methods previously described by Coles et al [24], Jeronimo et al [25] and Wang et al [26].

ALDH1A1 was analysed using as forward primer 5'-GGTCTACTTACCCAGCACTGAAA-3' and reverse primer 5'-ACAAAGCCGAAACCTGTGAT-3'. ALDH3A1 was analysed using as forward primer 5'-TCCAACTCTGGCTTGTTTCC-3' and reverse primer 5'-CTTCTCACGCTGGTTGATGA-3'. Variations were analysed using PCR and sequencing. In brief, PCR amplification reactions were performed in a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The results of the PCR reactions were analysed by gel electrophoresis. DNA cycle sequencing was carried out essentially as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) on a PTC-200 thermocycler using the Big Dye Terminator Cycle sequencing mix v3.1. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems) was used. Hardy-Weinberg equilibrium was evaluated using the χ^2-test.

Statistical analysis

This was an exploratory study in which an a priori defined analysis plan was used. To reduce the probability of finding false-positive relations, only relations that were considered plausible were evaluated. Cyclophosphamide is mainly related to VOD, haemorrhagic cystitis and cardiotoxicity. Mucositis and weight loss are mainly associated with cyclophosphamide and thiotepa administration. Diarrhoea and liver-toxicity are associated with all three compounds, while carboplatin is mainly related to sensory neuropathy and ototoxicity. Therefore, VOD, haemorrhagic cystitis, cardiotoxicity, mucositis, diarrhoea, weight loss and liver-toxicity were related to the variants CYP2B6 (C64T, A785G, C1459T, G516T), CYP2C9 (*2, *3), CYP2C19*2, CYP3A4 (*1B, *3), CYP3A5 (*2, *3), GSTA1 (C-69T, G-52A), GSTP1 (A313G, C341T), ALDH1A1*2 and ALDH3A1*2. Sensory neuropathy and ototoxicity were related to

the variants GSTA1 (C-69T, G-52A) and GSTP1 (A313G, C341T). The relations between genotypes of the drug-metabolising enzymes and toxicity were tested with binary logistic regression to be able to correct for confounding variables (number of courses administered, cisplatin pre-treatment and type of high-dose chemotherapy regimen), by including these variables in the logistic regression equation. The analyses were performed by comparing those patients with side effects greater than or equal to grade 2 or 3 (depending on type of toxicity) to those with no or less severe side effects. Patients with VOD, haemorrhagic cystitis and cardiotoxicity of any grade were compared to those with no side effects.
Analyses were performed with SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). A significance level of 0.05 was used for all tests.

Results

Patient characteristics

Data were available of 113 Caucasian patients (90 women, 23 men) with a mean age of 41 years (range 16-59 years) who received 206 courses of high-dose chemotherapy. Demographic data are shown in Table 1. The most frequent diagnosis was breast cancer (n=86), followed by germ cell cancer (n=19). All patients with germ cell cancer had been pre-treated with cisplatin.

Table 1. Patient characteristics.

	Number	**Median (range)**
Patients	113	
Male	23	
Female	90	
Site of disease		
Breast cancer stage II or III	53	
Breast cancer stage IV	33	
Ovarian cancer	4	
Germ cell cancer	19	
Ewing's sarcoma	4	
Total number of courses	206	
1st course CTC	62	
2nd course CTC	17	
1st course tCTC	51	
2nd course tCTC	43	
3rd course tCTC	33	
Age (years)		41 (16-59)
Body surface area (m^2)		1.8 (1.5-2.9)
Weight (kg)		70 (52-170)
Height (cm)		171 (153-210)

Toxicity

The number of patients experiencing toxicity after chemotherapy with cyclophosphamide, thiotepa and carboplatin is shown in Table 2. In general, few severe toxic events occurred. VOD was seen after a second CTC course in 3 patients and after a second tCTC course in 1 patient. Haemorrhagic cystitis was seen after a single course of CTC, after a second course of CTC, twice after a third course of tCTC and twice after a second course of tCTC. The latter two patients received their third course of tCTC without cyclophosphamide without further severe complications. Some degree of cardiac failure was observed in 4 patients and was completely reversible in all patients. Symptoms consisted of tachycardia (n=3) and pericardial effusion (n=1). Mucositis was usually mild to moderate, but grade 3-4 toxicity was

seen in 13 patients. Mostly this occurred after single courses of CTC or after multiple courses of tCTC. Diarrhoea grade 3-4 was found in 17 patients, mostly after single courses of CTC. In general, peak levels of ALAT, ASAT and total bilirubin were detected 2 days after the chemotherapy (the day of peripheral blood progenitor cell transplantation). In most patients the liver functions normalised in 1-2 weeks. Grade 2-3 tinnitus and hearing loss were noted in 10 patients. Four of these patients had previously received cisplatin. Neuropathy was more common in patients with germ cell tumours pre-treated with cisplatin than in breast cancer patients (47 vs. 6%). In eight patients sensory neuropathy grade 2-3 was observed.

Table 2. Summary of first events in patients receiving high-dose chemotherapy. Number of patients experiencing toxicity is shown.

Toxicity	1st course (n=113)	2nd course (n=60)	3rd course (n=33)
Veno-occlusive disease	0	4	0
Haemorrhagic cystitis	1	3	2
Cardiotoxicity	3	0	1
Mucositis grade 3-4	8	3	2
Diarrhoea grade 3-4	12	4	1
Liver-toxicity grade 3-4	15	8	2
Weight loss grade 2-3	6	3	1
Sensory neuropathy grade 2-3	4	3	1
Ototoxicity grade 2-3	3	6	1

Pharmacogenetic analysis

Genotype and allele frequencies of the studied variant genes are shown in Table 3.

Table 3. Genotype and allele frequencies for the studied variant genes (n=113).

Polymorphism	Nucleotide change	Effect	Genotype§				Allele frequency* (%)
			Wt	Het	Hom	Unknown	
CYP2B6 C64T	C64T	R22C	105	6	2	0	4.4
CYP2B6 A785G	A785G	K262R	85	23	5	0	14.6
CYP2B6 C1459T	C1459T	R487C	75	30	8	0	20.4
CYP2B6 G516T	G516T	Q172H	89	18	6	0	13.3
CYP2C9*2	C430T	R144C	84	19	8	2	15.8
CYP2C9*3	A1075C	I359L	98	15	0	0	6.6
CYP2C19*2	G681A	Splicing defect	81	24	8	0	17.7
CYP3A4*1B	A-392G	Promoter	104	8	1	0	4.4
CYP3A4*3	T1334C	M445T	109	3	0	1	1.3
CYP3A5*2	C27289A	T398N	110	3	0	0	1.3
CYP3A5*3	A6986G	Splicing defect	3	13	96	1	91.5
GSTA1 (C-69T, G52A)	C-69T, G-52A	Promoter	37	53	22	1	43.3
GSTP1 A313G	A313G	I105V	53	49	11	0	31.4
GSTP1 C341T	C341T	A114V	93	19	1	0	9.3
ALDH1A1*2	17 base pair deletion -416/-432	Promoter	99	13	0	1	5.8
ALDH3A1*2	C985G	P329A	70	38	5	0	21.2

§Wt=wild-type; Het=heterozygous mutant; Hom=homozygous mutant
*Total number of mutated alleles/total number of alleles

Sixteen polymorphisms were analysed in nine genes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) of putative relevance for cyclophosphamide, thiotepa and carboplatin metabolism. Observed allele frequencies of CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 variants in the current study were consistent with previously reported data in Caucasian populations [27-30]. All polymorphisms were in Hardy-Weinberg equilibrium, except CYP2B6 C64T and

CYP2C9*2. The small number of patients combined with the low allele frequency of CYP2B6 C64T and CYP2C9*2 might cause this deviation from Hardy-Weinberg equilibrium.

Association between the genetic polymorphisms and toxicity

Significant associations between genetic polymorphisms and toxicity are given in Table 4.

Table 4. Significant associations between genotypes, pre-treatment and toxicity.

Toxicity	Variable	Odds ratio	95% confidence interval	P-value
Haemorrhagic cystitis	ALDH3A1*1/*2	11.95	1.18-120.56	0.04
Haemorrhagic cystitis	ALDH3A1*1/*2, ALDH3A1*2/*2	9.08	1.02-80.58	0.05
Liver-toxicity grade 3-4	ALDH1A1*1/*2	5.13	1.30-20.30	0.02
Sensory neuropathy grade 2-3	Cisplatin pre-treatment	6.00	1.35-26.62	0.02
Ototoxicity grade 2-3	Cisplatin pre-treatment	3.91	0.99-15.52	0.05

Heterozygosity for the ALDH3A1*2 allele was significantly associated with haemorrhagic cystitis. Individuals who had the ALDH3A1*1/*2 genotype had a 11.95-fold (95% CI 1.18-120.56) increased risk of haemorrhagic cystitis as those who were wild-type for ALDH3A1, after adjustment for number of courses administered and type of high-dose chemotherapy regimen received. Homozygosity for the ALDH3A1*2 allele was not significantly associated with haemorrhagic cystitis. Combination of heterozygous and homozygous individuals with the ALDH3A1*2 allele resulted in a 9.08-fold (95% CI 1.02-80.58) increased risk of haemorrhagic cystitis.

Patients heterozygous for the ALDH1A1*2 allele showed a 5.13-fold (95% CI 1.30-20.30) increased risk of liver-toxicity compared to those wild-type for this allele. None of the other studied polymorphisms added any prognostic value for toxicity to the model.

After adjustment for number of courses administered and type of high-dose chemotherapy regimen received, cisplatin pre-treatment was predictive for occurrence of sensory neuropathy and ototoxicity (P=0.02 and 0.05, respectively, Table 4).

Discussion

In high-dose chemotherapy with peripheral blood progenitor cell transplantation haematological toxicity is not dose limiting. However, other severe and unpredictable toxicities may occur. Therefore, it is important to identify factors that could help in predicting the occurrence of toxicity after high-dose chemotherapy.

Cyclophosphamide is a prodrug that needs to be activated by several cytochrome P450 enzymes. Furthermore, (intracellular) detoxification of cyclophosphamide is mediated by GST and ALDH enzymes. Most of the genes encoding these enzymes are genetically polymorphic, leading to altered metabolic activity of the expressed proteins or varied levels of protein expression [19;31;32]. Reduced metabolic activation of cyclophosphamide, due to genetic polymorphisms in genes encoding the cytochrome P450 enzymes, might lower the risk of toxicity. Reduced detoxification, due to polymorphisms in GST and ALDH genes, might increase the risk of toxicity. In this study we could not demonstrate an association between the various cytochrome P450 enzymes or GST enzymes and toxicity. However,

patients heterozygous for the ALDH3A1*2 allele and ALDH1A1*2 allele had an increased risk of haemorrhagic cystitis and liver-toxicity, respectively. Detoxification of cyclophosphamide by ALDH enzymes is the most important metabolic detoxifying pathway of cyclophosphamide [8]. ALDH enzymes are expressed in various tissues. ALDH3A1 is constitutively expressed in cornea, stomach, oesophagus, urinary bladder and lung, but is undetectable in liver [33]. ALDH1A1 is constitutively expressed in eye lens, brain and red blood cells, but highest levels occur in the liver [34]. ALDH1A1 and ALDH3A1 have been characterised as determinants of cellular sensitivity to cyclophosphamide. Introduction of ALDH1A1 and ALDH3A1 vectors in human and hamster cell lines decreased sensitivity of these cell lines to cyclophosphamide [35]. Furthermore, cyclophosphamide-resistant cancer cell lines exhibit ALDH up-regulation, which occurs at the transcriptional level [35]. Human ALDH1A1 and ALDH3A1 genetic variants have been reported [28;34]. The ALDH1A1*2 polymorphism eliminates a c-myb binding site from the promoter region of ALDH1A1. Although the in-vitro expression analysis showed no significant change in expression between ALDH1A1*2 and wild-type, this may not fully reflect the regulatory mechanisms underlying gene expression *in vivo* [28]. The ALDH3A1*2 variant allele has been observed with a high frequency in Caucasian individuals [34]. The C985G nucleotide change leads to amino acid change P329A. Although this amino acid substitution may affect the ALDH3A1 enzyme, the functional changes remain to be elucidated. It could be that the ALDH3A1*2 and ALDH1A1*2 variants result in decreased activity of the ALDH enzyme. This will lead to decreased detoxification of 4-hydroxycyclophosphamide and could, therefore, explain the increased risk of haemorrhagic cystitis and liver-toxicity observed in patients with the ALDH3A1*2 and ALDH1A1*2 variants. Homozygosity for the ALDH3A1*2 allele was not significantly associated with haemorrhagic cystitis. However, the number of ALDH3A1*2 homozygous patients was probably too low to determine this effect.

In contrast to our study, other studies have shown an effect of polymorphisms in cytochrome P450 and GST enzymes on the risk of toxicity in patients treated with cyclophosphamide. The presence of at least one CYP2C19*2 allele was associated with a significantly lower risk of developing ovarian toxicity in systemic lupus erythematosus (SLE) patients treated with pulsed cyclophosphamide therapy. No association was seen for the CYP2B6, CYP2C9 and CYP3A5 polymorphisms studied [36;37]. The SLE patients received doses ≤1000 mg/m^2, while the patients in the present study received doses ≥1000 mg/m^2. Timm et al [38] have shown that CYP2C19*2 carriers had lowered elimination constants of cyclophosphamide in patients receiving doses ≤1000 mg/m^2. This effect could not be demonstrated in patients receiving doses >1000 mg/m^2. Increased induction caused by higher doses of cyclophosphamide possibly superimposes any effect of CYP2C19 variants. This could explain the discrepancy between the observed effects of CYP2C19*2 variants on toxicity.

Furthermore, Zhong et al showed that the GSTP1 A313G polymorphism, but not GSTM1 or GSTT1 null mutations, significantly increased the risk of myelotoxicity and gastrointestinal toxicity in patients with systemic lupus erythematosus treated with pulsed cyclophosphamide therapy [39]. No significant associations between GSTP1, GSTA1, GSTM1 and GSTT1 polymorphisms and liver-toxicity were observed in patients after busulfan/cyclophosphamide-based allogeneic haematopoietic stem cell transplantation [40].

An explanation for the absence of an effect of the GSTA1 or GSTP1 polymorphisms on toxicity in our study might be the fact that these patients received high doses of cyclophosphamide, thiotepa and carboplatin. High doses of these chemotherapeutic agents may lead to cellular depletion of glutathione [41-43]. This may obscure any effect of polymorphisms in the GST genes.
In a previous study we have shown that polymorphisms in CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 did not influence the pharmacokinetics of cyclophosphamide or 4-hydroxycyclophosphamide in this patient population [44]. However, GST and ALDH enzymes function primarily intracellularly. Therefore, although we could not demonstrate an effect of polymorphisms in these enzymes on the plasma-pharmacokinetics of cyclophosphamide, differences in susceptibility to toxicity might occur due to differences in intracellular detoxification of the active cyclophosphamide metabolites.

Intracellular detoxification of carboplatin can occur by conjugation to glutathione mediated by the GST enzymes [11;12]. In this study, no association was found between polymorphisms in GSTA1 and GSTP1 genes and carboplatin toxicity. Scarce information is available about pharmacogenetic markers for carboplatin toxicity. A large study in 914 ovarian cancer patients treated with carboplatin also showed no association between toxicity and GSTP1 polymorphisms [45]. On the other hand, significantly less neutropenia was demonstrated for non-small cell lung cancer patients possessing the GSTP1 105 Val allele treated with carboplatin or cisplatin [46]. Jatoi et al showed that the GSTM1 null genotype did not predict toxicity in locally advanced oesophageal cancer patients treated with carboplatin [47].

When testing multiple polymorphisms in multiple genes in relation to toxicity there is a risk of identifying false genotype-toxicity relations. We tried to minimize the chance of finding false genotype-toxicity relations by using a pre-specified analysis plan, correcting for confounding variables and evaluating only plausible relations.

To our knowledge this is the largest pharmacogenetic study of toxicity after therapy with high-dose alkylating agents. Although the role of high-dose chemotherapy in breast cancer remains controversial, therapy with alkylating agents may be beneficial for a subgroup of patients. Particularly patients with ER-, PR- and HER2/neu-negative breast tumours may benefit substantially from alkylating chemotherapy, both in terms of recurrence-free and overall survival [1;3]. We have demonstrated that these patients are at increased risk of developing liver-toxicity and haemorrhagic cystitis, despite adequate treatment with mesna, when polymorphisms in ALDH genes are present.

In conclusion, the ALDH3A1*2 and ALDH1A1*2 mutations conferred increased susceptibility to haemorrhagic cystitis and liver-toxicity, respectively, in patients receiving high-dose chemotherapy with cyclophosphamide, thiotepa and carboplatin. Pharmacogenetic approaches have potential for identifying patients who are at a higher risk of experiencing toxic side-effects in high-dose chemotherapy.

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Chapter 4

Pharmacokinetics

Chapter 4.1

Flat dosing of carboplatin is justified in adult patients with normal renal function

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Abstract

Purpose: The Calvert formula is a widely applied algorithm for the a priori dosing of carboplatin based on patients glomerular filtration rate (GFR) as accurately measured using the ^{51}Cr-EDTA clearance. Substitution of the GFR in this formula by an estimate of creatinine clearance (Cl_{CR}) or GFR, as calculated by formulae using serum creatinine (Cockcroft-Gault, Jelliffe, Wright) is, however, routine clinical practice in many hospitals. The goal of this study was to validate this practice retrospectively in a large heterogeneous adult patient population.

Experimental Design: Concentration-time data of ultrafilterable platinum of 178 patients (280 courses, 3119 samples) with different types of cancer receiving carboplatin-based chemotherapy in conventional and high doses were available. Data were described with a linear two-compartment population pharmacokinetic model. Relations between serum creatinine based formulae for estimating renal function and carboplatin clearance were investigated.

Results: None of the tested serum creatinine based estimates of renal function were relevantly related to the pharmacokinetic parameters of carboplatin. Neither serum creatinine (median 51, range 18-124 μM), nor the estimated GFR using the three different formulae were related to carboplatin clearance.

Conclusion: Our data do not support the application of modifications of the Calvert formula, by estimating GFR from serum creatinine in the a priori dosing of carboplatin in patients with relatively normal renal function (Cl_{CR} >50 mL/min). For targeted carboplatin exposures, the original Calvert formula, measuring GFR using the ^{51}Cr-EDTA clearance, remains the method of choice. Alternatively, in patients with normal renal function, a flat dose based on the mean population carboplatin clearance should be administered.

Introduction

The platinum compound carboplatin is used in the treatment of a number of malignancies, often in combination chemotherapy regimens [1]. Carboplatin is mainly eliminated by the kidneys. In patients with normal renal function, between 60 and 70% of an administered carboplatin dose is excreted into the urine within the first 24 h of administration. The remainder of the drug binds irreversibly to proteins and tissue [1,2]. The free, ultrafilterable carboplatin fraction is considered pharmacologically active [3].
The use of carboplatin is mainly limited by myelosuppression. Carboplatin exposure, expressed as area under the plasma concentration versus time curve (AUC), has been related both to severity of thrombocytopenia and leucopenia as well as to therapeutic outcome [reviewed by 4,5]. In high-dose chemotherapy regimens with stem cell support, toxicities such as nephro-, oto-, central nervous system-, and peripheral nervous system toxicities have been associated with a higher carboplatin exposure [6-12]. By controlling carboplatin exposure in both conventional as well as high-dose regimens, the incidence of side effects might be reduced.
Carboplatin clearance appears to be poorly correlated to body surface area (BSA) but it is linearly related to the glomerular filtration rate (GFR) [13-16]. Therefore, various dosing equations have been proposed to calculate *a priori* an appropriate dose for a target exposure of carboplatin in a patient with a known GFR [13,17]. The most simple and widely used formula to calculate the carboplatin dose was proposed by Calvert et al, relating the ultrafilterable AUC to the GFR by Dose=AUC*(GFR+25) [13], with dose in mg, target AUC in mg/mL*min (usually 5-7), and GFR in mL/min. The 25 mL/min is a constant included to account for nonrenal clearance (drug binding irreversibly to tissue or proteins). This formula has been developed using the clearance of chromium 51-ethylenediaminetetraacetic acid (^{51}Cr-EDTA) as an accurate measure of the GFR [4]. This method is, however, expensive, invasive, involves radioactive compounds, requires multiple blood sampling at exact time-points and is not available in most treatment centres. Therefore, in many instances the GFR is substituted by the estimated creatinine clearance (Cl_{CR}) as calculated with the Cockcroft-Gault formula [18] or the Jelliffe formula [19]. These formulae are based on measured serum creatinine (S_{CR}) in µmol/L, age in years, gender (1 if female, 0 if male) and BSA in m^2 or weight in kg.

Cockcroft-Gault (1976) [18]:

$$Cl_{CR} = \frac{(140 - Age) * Weight * 1.23 * (1 - 0.15 * Gender)}{S_{CR}}$$

Jelliffe (1973) [19]:

$$Cl_{CR} = \frac{(98 - 0.8 * (Age - 20)) * (1 - 0.1 * Gender) * (BSA/1.73)}{S_{CR} * 0.0113}$$

Recently, Wright et al [20] developed a method for estimating GFR based on the same parameters.

Wright (2001) [20]:

$$GFR = \frac{(6580 - 38.8 * Age) * BSA * (1 - 0.168 * Gender)}{S_{CR}}$$

To evaluate the dosing-accuracy of the Calvert formula using the above described substitutions of the GFR, and to define patient characteristics influencing carboplatin pharmacokinetics, we performed a large population pharmacokinetic study. We pooled pharmacokinetic data of carboplatin obtained from several studies, including both conventional as well as high-dose chemotherapy regimens. The intention was to use as much data available of many different populations with various types of cancer treated with different schedules of carboplatin. The performance of a formula calculating carboplatin clearance based on S_{CR}, weight, age and sex, as developed by Chatelut et al [14] was also evaluated with our dataset:

$$CLcarbo = \frac{0.134 * weight + [218 * weight * (1 - 0.00457 * age) * (1 - 0.314 * sex)]}{S_{CR}}$$

Patients and methods

Patients

Pharmacokinetic data of ultrafilterable platinum were used as obtained in several previously published studies in which patients received carboplatin both in high-dose as well as in conventional-dose regimens in combination with other chemotherapeutic agents [6,21-24]. Data were available of 178 patients (280 courses) of carboplatin (in total 3119 samples). Of all patients in the dataset baseline patient characteristics and biochemical parameters were available as summarized in Table 1. All protocols were approved by the Committee of Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from all patients.

Pretreatment S_{CR} levels were estimated by the kinetic Jaffé method (Hitachi systems, Roche Diagnostics, The Netherlands) in three studies [22-24] and in the first 32 patients of one study [6], while in the resulting patients [6,21] the *compensated* Jaffé method was used. While the Jaffé method is non-specific, the S_{CR} measurements in the *compensated* Jaffé method are corrected for measured pseudo-creatinine chromogens by subtracting exactly 26 μM from each S_{CR} concentration. Since every individual sample contains different amounts of pseudo-creatinine chromogens, this is a non-specific correction. However, it has been shown that results obtained with the *compensated* Jaffé method were comparable to those obtained with reference methods using HPLC [25]. Therefore, the non-compensated S_{CR} values in our dataset were retrospectively adjusted by subtracting 26 μM from the initial values.

Table 1. Baseline patient characteristics and biochemical parameters of a historical patient population.

	Number	Median	Range
Patient characteristics			
Female/male	107/71		
Age (years)		48	16-75
BSA (m^2)		1.80	1.49-2.94
Weight (kg)		70	46-170
Length (cm)		171	153-210
Protocol			
Non-small cell lung cancer [21] *2-6 x PC[a] (dose carboplatin AUC= 6 mg/mL*min administered in 30 min)*	21 (21 courses) *4 plasma samples per patient per course* *t=0.5, 4.5, 8.5, 23.5 h*		
Non-small cell lung cancer [22,23] *PC (dose carboplatin 300-400 mg/m^2/30 min)*	56 (95 courses) *6-10 plasma samples per patient per course* *t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 24.5, 48.5 h*		
Ovarian [22,24] *PC (dose carboplatin 300-600 mg/m^2/30 min)*	25(25 courses) *8-12 plasma samples per patient per course* *t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 18.5, 24.5, 48.5 h*		
High risk primary breast cancer [6] *CTC[b] (dose carboplatin 400 mg/m^2/day or AUC20 mg*min/mL administered in 1 h for 4 days)*	20 (20 courses) *15-20 plasma samples per patient per course* *t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 18.5, 24.5, 48.5 h* *Day 1 and 3*		
Metastatic breast cancer [6, and more pat's] *tCTC (dose carboplatin 267 mg/m^2/day or AUC13.3 mg*min/mL administered in 1 h for 4 days)*	33 (78 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h, Day 1 and 3*		
Refractory germ cell cancer [6, and more pat's] *CTC (dose carboplatin 400 mg/m^2/day or AUC20 mg*min/mL administered in 1 h for 4 days)*	11 (21 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h, Day 1 and 3*		
Refractory germ cell cancer [6, and more pat's] *tCTC (dose carboplatin 267 mg/m^2/day or AUC13.3 mg*min/mL administered in 1 h for 4 days)*	5 (14 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h, Day 1 and 3*		
Metastatic ovarian cancer [6] *tCTC (dose carboplatin 267 mg/m^2/day or AUC13.3 mg*min/mL administered in 1 h for 4 days)*	5 (6 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h, Day 1 and 3*		
Biochemical parameters			
Serum creatinine (μM)		51	18-124
Creatinine clearance (calculated with the Cockcroft-Gault formula (mL/min) [18])		141	55-451
Albumin (g/L)		40	18-52

[a] PC, paclitaxel and carboplatin

[b] CTC, high-dose cyclophosphamide (1 hour infusion), carboplatin (1 hour infusion) and thiotepa (2x 0.5 hour infusion) every day during 4 days

Sampling and analyses

In all studies, the ultrafilterable plasma fraction was prepared immediately after blood sampling, using the Amicon micropartition system with an YMT-14 membrane (30 kD, Millipore Corporation, Bedford, MA, USA). A volume of 0.5 mL plasma was transferred in the micropartition system and centrifuged at 2,500 *g* for 20 min. Ultrafiltrate was stored at -20°C until analyses. Analyses of platinum in ultrafiltrate were performed using flameless atomic absorption spectrometry as previously described [26]. Accuracy and day-to-day precision of this method were 93.9-103.3% and 1.5-10.2%, respectively. The number and time-points of samples withdrawn in each study protocol are depicted in Table 1.

Population pharmacokinetics

A population pharmacokinetic model of carboplatin (measured as free platinum) was developed using the non-linear mixed effect modelling program NONMEM (version V 1.1) (GloboMax LLC, Hanover, USA) [27]. The first order conditional estimate method (FOCE) with INTERACTION was used after log-transformation of the data [28].

Both interindividual variability (IIV) and interoccasion variability (IOV) were modelled with an exponential function. For example, variability in clearance Cl was estimated using:

$Cl_{ij} = Cl_{pop} * \exp(\eta_i+\kappa_j)$, where Cl_{ij} represents Cl of the i^{th} individual on the j^{th} occasion, Cl_{pop} is the population value of Cl, η is the interindividual random effect with mean 0 and variance ω^2 and κ is the interoccasion random effect with mean 0 and variance π^2 [29].

The difference between observed concentrations and their respective predictions, resulting from measurement error and model misspecification (i.e. the residual or unexplained variability) was modelled with an exponential error model:

$\ln(C_{obs\ ij}) = \ln(C_{pred\ ij}) + \varepsilon_{ij}$, where ε_{ij} is the residual error with mean 0 and variance σ^2, representing the difference between the natural logarithm of the j^{th} observed concentration in the i^{th} individual ($\ln(C_{obs\ ij})$) and its respective prediction ($\ln(C_{pred\ ij})$).

Four different models describing the possible relation between carboplatin clearance and serum creatinine were tested:

1. Cl_{pop} =theta(1)
2. Cl_{pop} =GFR+25
3. Cl_{pop} =GFR+theta(1)
4. Cl_{pop}=theta(1)+(140-age)*theta(2)*weight*theta(3)^{gender}/S_{CR}

(in which GFR was calculated using the Wright formula or replaced by Cl_{CR} using the Cockcroft-Gault and the Jelliffe formula).

In model 1 no relation between serum creatinine and carboplatin clearance was assumed and this model was used as reference for the other models. Model 2 is the regularly applied Calvert formula in which the GFR is approximated by different serum creatinine based formulae. In model 3 the parameter from the Calvert formula was re-estimated on our dataset. Model 4 was used to further analyze the possible relation between serum creatinine, determinants of serum creatinine (i.e. age, gender and body size) and carboplatin pharmacokinetics in our dataset. In this model some of the parameters of the Cockcroft-Gault formula were re-estimated. This model was used to test whether any relation between serum creatinine and carboplatin clearance existed in our dataset after compensation for known determinants of creatinine production (age, body size and gender).

Estimated values of Cl_{CR} >150 mL/min based on the different formulae used are physiologically unlikely. Therefore, we tested the unadjusted Cl_{CR} as covariate but also values >150 mL/min and >250 mL/min truncated to these values [30]. We also tested for possible other determinants of carboplatin pharmacokinetics in our dataset as pretreatment regimen (cisplatin or non-cisplatin containing regimen), study protocol and the administration of multiple doses. A covariate was considered significantly associated with the pharmacokinetic parameter of interest when the OFV decreased > 7.8 ($p<0.005$).

Results

Data were best described with a two compartment model estimating first order elimination (Cl), volume of distribution (Vd) and the distribution rate constants k_{12} and k_{21}. IIV was estimated for Cl, Vd, k_{12} and k_{21}, while IOV was estimated for Cl and k_{12}.
The observed carboplatin concentration was accurately predicted by the model (Figure 1).

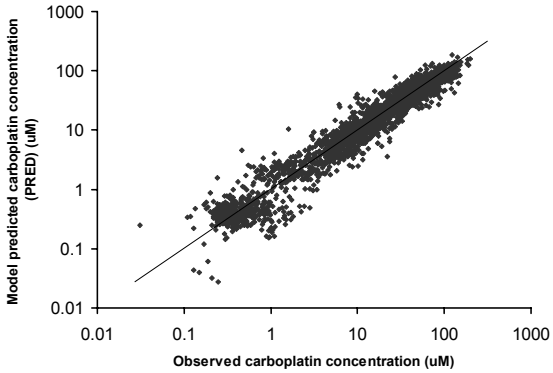

Figure 1. Model predicted versus observed concentrations for carboplatin.

Furthermore, the weighted residuals did not vary according to the predicted concentration or time (Figures 2 and 3) indicating that the model accurately described the data.

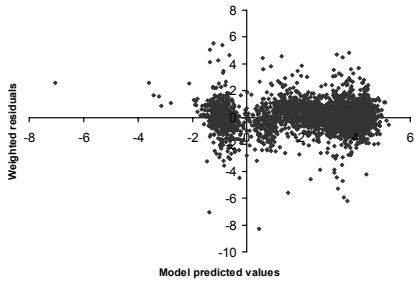

Figure 2. Weighted residuals versus model predicted values

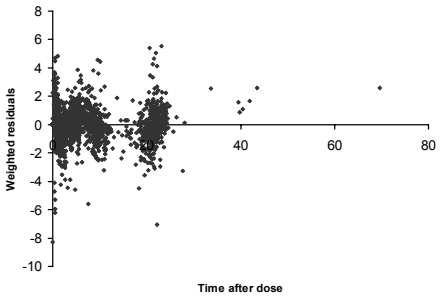

Figure 3. Weighted residuals versus time after dose

Covariance between Cl and Vd was estimated. Population pharmacokinetic parameters obtained from the total carboplatin dataset are summarized in Table 2.

Table 2. Population pharmacokinetic parameters of carboplatin using the full dataset.

Parameter	Estimate (% RSE[a])	IIV[a] (%) (% RSE[a])	IOV[a] (%) (% RSE[a])
Clearance (L/h)	8.33 (1.66)	19.1 (19.3)	9.52 (20.1)
Volume of distribution (L)	16.3 (2.18)	17.0 (17.0)	nd
Distribution microconstant k_{12} (h^{-1})	0.104 (10.0)	37.8 (45.5)	33.8 (38.2)
Distribution microconstant k_{21} (h^{-1})	0.171 (8.42)	49.2 (35.6)	nd
Correlation coefficient (ρ) of Cl and Vd	0.594		
Covariance of Cl and Vd	0.0193 (23.5)		
Proportional residual error (%)	21.8 (5.00)		

[a] RSE, relative standard error of estimate; IIV, interindividual variability; IOV, interoccasion variability; nd= not determined.

Of the four models tested, model 1 in which no relation between serum creatinine and carboplatin clearance was assumed showed a better fit than model 2 and 3 (the original Calvert formula in which GFR is estimated by serum creatinine based formulae and a model in which the parameter of the Calvert formula was re-estimated on our dataset) (Table 3).

Table 3. Alternative models tested for the clearance of carboplatin.

Model	Model description	OFV[a]
Cl_{pop} =theta(1)	No relation between creatinine and Cl	-4764.722
Cl_{pop} =GFR+25	Calvert formula	-4457.087
Cl_{pop} =GFR+theta(1)	Calvert formula, parameter re-estimated	-4460.216
Cl_{pop}=theta(1)+(140-age)*theta(2)*weight*theta(3)^{gender}/S_{CR}	Full model for relation between Scr and Cl	-4789.303

[a] OFV, Objective Function Value; Cl_{pop}, population value of clearance; GFR, glomerular filtration rate; S_{CR}, serum creatinine.

Model 4 (the Calvert formula with GFR estimated by the Cockcroft-Gault formula and all parameters re-estimated on our dataset) showed a significant improvement of fit (ΔOFV = -24.6, df=2, p<0.001). This model yielded the following equation:

Cl_{carbo} (mL/min) =115.5+0.212*(140-age)*weight*0.736^{gender}/S_{CR},

whereas the original Calvert formula (using Cockcroft-Gault to calculate Cl_{CR}) was:

Cl_{carbo} (mL/min) =25+1.23*(140-age)*weight*0.85^{gender}/S_{CR}.

This shows that in the re-estimated equation the intercept is 4.6 times higher and the slope is 6 times smaller than in the Calvert formula (using Cockcroft-Gault to calculate Cl_{CR}) indicating that the relation between carboplatin pharmacokinetics and serum creatinine and determinants of serum creatinine is much weaker than suggested by substituting the Cockcroft-Gault estimate of Cl_{CR} in the original Calvert formula. This is also shown in figures 4A-C.

No differences in pharmacokinetics could be demonstrated between the different study protocols and treatment regimens. A total of 16 patients with refractory germ cell cancer (35 courses) had received cisplatin pretreatment of daily 20 mg/m^2 cisplatin during 5 days for four courses [6]. Also one patient with metastatic ovarian cancer [6] had been pretreated with cisplatin. Pretreatment with platinum-based therapy, however, had no significant effect on carboplatin clearance. Also no difference in carboplatin clearance was found after multiple courses of carboplatin.

Evaluation of relations between pretreatment regimen, protocol and multiple courses and pharmacokinetic parameters, using a univariate procedure, did not result in a significant correlation.

Figures 4A-C show that the expected relation between carboplatin clearance and Cl_{CR} (as calculated using the Cockcroft-Gault or the Jelliffe formula) or GFR (as calculated using the Wright formula), using truncation to 250 mL/min, was not significant. Similar results were obtained when the upper limits were not truncated or truncated to 150 mL/min. Also no relation was found between carboplatin clearance and S_{CR} (Figure 5) not even when only the patients with physiological values of Cl_{CR} were included in the analyses. The performance of the Chatelut formula in predicting carboplatin clearance in our population was also poor, as shown in Figure 6.

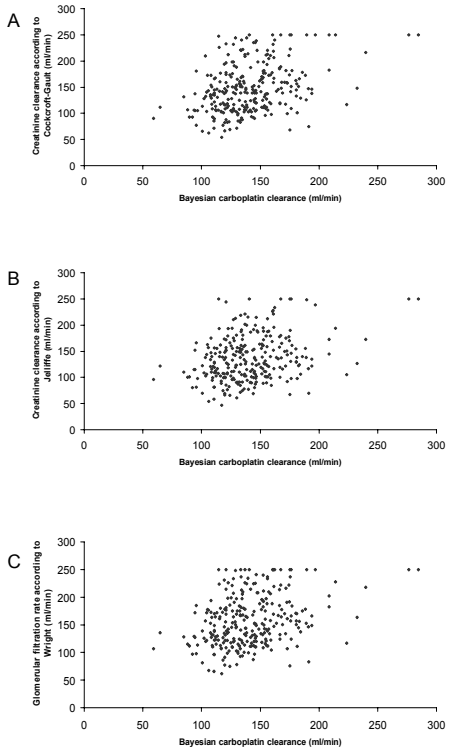

Figure 4. Relation between carboplatin clearance and creatinine clearance / glomerular filtration rate as calculated with **A**) the Cockcroft-Gault formula [18], **B**) the Jelliffe formula [19] or **C**) the Wright formula [20]. The creatinine clearance / glomerular filtration rate values >250 mL/min were truncated to this value.

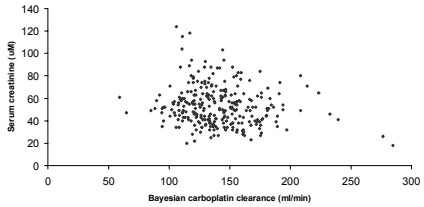

Figure 5. Relation between carboplatin clearance and serum creatinine.

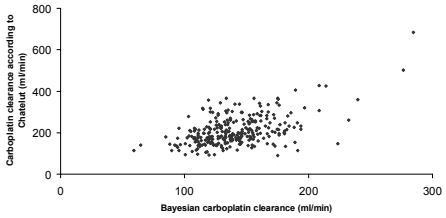

Figure 6. Performance of the Chatelut formula [14] in predicting the carboplatin clearance.

These results question the value of the variables S_{CR}, weight, BSA, gender and age in predicting individual pharmacokinetics of carboplatin in our population. Carboplatin dosing formulae based on these patient characteristics are thus not predictable of carboplatin exposure in this population.

Discussion

In our study no significant relation between carboplatin pharmacokinetics and patient characteristics could be detected with a population pharmacokinetic analysis in a large patient population. Since carboplatin dosing based on patients´ S_{CR}, age, gender, weight and height is widely applied in clinical oncology, the accuracy of this practice should be questioned.

The predominant determinant of carboplatin clearance is the GFR. It has been shown that when GFR was estimated using radioisotopes, a strong relation with carboplatin clearance exist [13]. However, these methods are expensive, inconvenient and not universally available. Cl_{CR} is widely accepted as a simple measure of GFR, although it systematically overestimates GFR because creatinine is not solely filtered by the glomerulus but is also secreted by the proximal tubule. Cl_{CR} can be measured by collection of timed urine, which is also labour intensive and often prone to error. Therefore, simple formulae have been introduced to estimate Cl_{CR} based on S_{CR}, age, gender, weight and length [18,19]. It has, however, also been shown that S_{CR}, as used in these formulae, is a poor indicator of glomerular function, and thus carboplatin clearance. It is an insensitive measure of early glomerular impairment and is also dependent on non-renal factors, especially creatinine production, which itself is dependent on muscle mass. Fluctuations in the endogenous creatinine production may therefore cause erroneous results in estimation of renal function.

In addition, the methodological difficulties inherent in the measurement of S_{CR} reduce the method's accuracy [31]. The enzymatic methods of S_{CR} have been shown to give more reliable results than the widely used Jaffé alkaline picrate colorimetric methods which are complicated by the measurement of non-creatinine chromogens [20]. The use of different assays for S_{CR} may therefore result in systematically different results between studies.
In contrast to our results, many authors have reported relations between S_{CR}, age, gender, weight, height and carboplatin pharmacokinetics [12,14,32-34]. In the studies in which a correlation was found between S_{CR} and carboplatin clearance [14,32], also patients with (moderate) renal insufficiency were included while in our study, renal function was adequate in all patients (Cl_{CR} >50 mL/min, as calculated using the Cockcroft-Gault formula), which is the result of the inclusion criteria of the different protocols. This is probably an important explanation for our different results. Variability in carboplatin clearance in our population was small (Table 2; IIV 19.1% and IOV 9.52%), which may also be caused by the inclusion of patients with relatively normal renal function.
Without a clear correlation between S_{CR} and carboplatin clearance, substituting GFR in the Calvert formula with an estimation of Cl_{CR} or GFR based on S_{CR} measurements (using the Cockcroft-Gault, the Jelliffe or the Wright equations), is therefore also prone to errors. This may explain the lack of relation between Cl_{CR} or GFR and carboplatin clearance in our study. Moreover, it should be questioned whether these equations should be applied in our patient population regarding the large number of patients with very low measured S_{CR} values (Figure 5) and concomitant extremely high physiological unlikely estimated Cl_{CR} or GFR values (Figure 4A-C). In the work of Kirkpatrick et al [30], S_{CR} values less than 60 µM were set at 60 µM in patients receiving gentamicin because it was thought that low values of S_{CR} may reflect decreased production rather than enhanced clearance. In our population, S_{CR} values were <60 µM in more than half the patients, probably due to the disease condition of these patients. In these patients, estimated Cl_{CR} or GFR values were therefore above the physiological meaningful upper limit of 150 mL/min. Applying the Calvert formula with these estimates of Cl_{CR} or GFR may result in inaccurate predictions of the carboplatin exposure.
These low serum creatinine values were not only the result of the subtraction of 26 µM, but were also directly observed. Moreover, these low serum creatinine values were seen in samples from all the different study protocols. Therefore, the observed low serum creatinine values are not due to the different protocols used to measure Scr.
Substitution of the CL_{CR} estimates of GFR from the Cockcroft-Gault formula and the Jelliffe formula into the Calvert formula has become routine practice in many centres. Although the Calvert formula, with GFR calculated using the ^{51}Cr-EDTA clearance, has been shown to be a superior method of dosing carboplatin than the traditional BSA method [35], the use of this formula with inaccurate substitutions of the GFR is not. The same is true for the application of the Chatelut formula. Studies evaluating the performance of the Chatelut formula [36,37] or the Calvert formula using the Cockcroft-Gault [14,36-40], Jelliffe [36,38] or Wright [38] equations in predicting carboplatin exposures indeed reported poor precisions (% root mean squared error 17-43%). The authors, however, did not establish the dosing precision if doses would have been based on BSA or if a flat dose would have been administered. Regarding the observed variation in carboplatin clearance within our patient population (Table 2: IIV 19.1% and IOV 9.52%), the variation in carboplatin exposure in our population after

administration of a flat dose would be approximately 21% ($\sqrt{[19.1]2 + [9.52]2}$). Therefore, in our population, the performance of the dosing formulae in approaching a fixed carboplatin exposure is not better than of a flat dose based on the mean carboplatin population clearance.

In summary, the current data do not support the general use of estimates of renal function by the Cockcroft-Gault, Jelliffe or Wright formulae, based on S_{CR} measurements, to select the dose of carboplatin using the Calvert formula for patients with relatively normal renal function. The original Calvert formula, using the clearance of ^{51}Cr-EDTA as an accurate measure of the GFR, remains the method of choice. When targeted carboplatin exposures are desired and radioisotope methods are not available, we propose to base the carboplatin dose in patients with a Cl_{CR} >50 mL/min (as calculated with the Cockcroft-Gault formula, with S_{CR} measured using the compensated Jaffé method) on the mean carboplatin population clearance of this study (8.33 L/h=138.8 mL/min) using the general formula:

carboplatin dose = desired carboplatin AUC * carboplatin population clearance.

Thus, in case an AUC of 5 mg/mL*min is desired, the appropriate dose for carboplatin would be 5 mg/mL*min * 138.8 mL/min = 694.2 mg (=695 mg). In the future, alternative approaches for estimating renal function, using promising markers of GFR like cystatin C, may prove suitable and applicable in routine clinical practice for estimating accurate *a priori* carboplatin doses [41].

Acknowledgement

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Chapter 4.2

Carboplatin dosing in overweight and obese patients with normal renal function, does weight matter?

Submitted for publication

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Abstract

Background/Purpose: Since the mid-seventies, the prevalence of overweight and obesity has increased sharply. Unfortunately, there is a lack of data on how to dose anticancer agents in overweight and obese patients. The purpose of this study was to determine the potential utility of alternative weight descriptors in the Cockcroft-Gault equation to more accurately predict carboplatin clearance in underweight, normal weight, overweight and obese patients.

Patients and methods: 240 patients were evaluated of whom 7 (3%) were defined as underweight (body mass index (BMI) <18.5 kg/m^2), 146 (61%) were defined as normal weight (BMI ≥18.5-<25 kg/m^2), 72 (30%) were defined as overweight (BMI ≥25-<30 kg/m^2) and 15 (6%) were defined as obese (BMI ≥30 kg/m^2). Clearance values obtained from individual fits using NONMEM were compared to predicted carboplatin clearances calculated using the modified Calvert formula in which creatinine clearance was calculated with the Cockcroft-Gault equation using diverse weight descriptors. Predictive performance of the diverse weight descriptors was assessed using mean prediction errors and mean absolute prediction errors.

Results: Evaluation of diverse weight descriptors, including actual body weight, (adjusted) ideal body weight, mean of ideal and actual body weight, fat-free mass and lean body mass indicated that lean body mass was the best weight descriptor in underweight and normal weight patients, while adjusted ideal body weight was the best weight descriptor in overweight and obese patients. However, a flat dose based on the population carboplatin clearance performed better in all weight categories than the use of the Cockcroft-Gault equation with diverse weight descriptors.

Conclusion: These results suggest that in overweight and obese patients, with a normal renal function, a flat carboplatin dose should be administered, based on the population carboplatin clearance (8.38 L/h = 140 mL/min). Thus, in case an AUC of 5 mg*min/mL is desired, the appropriate dose for carboplatin would be 5 * 140 = 700 mg.

Introduction

Carboplatin is a widely used platinum compound in combination chemotherapy regimens for the treatment of a number of malignancies. Carboplatin is mainly eliminated by the kidneys, as indicated by the fact that about 65% of the administered dose is excreted into the urine within the first 24 hours after administration [1]. A small fraction of the drug binds irreversibly to plasma proteins and the free, ultrafilterable platinum fraction is considered pharmacologically active [2]. Carboplatin clearance appears to be directly related to the glomerular filtration rate (GFR) and several dosing formulae have been suggested to calculate an a priori carboplatin dose based upon renal function. The Calvert formula (dose = target AUC * (GFR + 25)) is the most widely used formula. It does, however, have limitations in clinical practice. Clearance of chromium 51-ethylenediaminetetraacetic acid was used for the determination of the GFR [3]. This method is costly, involves a radioactive compound and is not readily available in most treatment centres. Therefore, for clinical purposes, GFR is usually estimated from a single measurement of serum creatinine by renal function equations that include age, weight and sex to account for interindividual differences in muscle mass and the consequent differences in creatinine generation. The GFR is often substituted by the creatinine clearance (CL_{cr}) calculated using the Cockcroft-Gault equation [4]:

CL_{cr} = 1.23 * (140-age) * weight * 0.85 (if female) / serum creatinine

In a previous study, with approximately the same dataset as this study, no relation between creatinine clearance estimators (using the Cockcroft-Gault, Jelliffe and Wright formulae) and carboplatin clearance was found [5]. However, that study did not take diverse weight measures into account. In the Cockcroft-Gault equation body weight is one of the variables required to calculate the creatinine clearance. This can lead to bias in overweight and obese patients, since in obesity a higher body weight is mainly due to a higher fat mass, whereas the factor body weight in the equation is assumed to reflect muscle mass.
Indeed, it has been shown that creatinine clearance estimates in the obese are inaccurate using either actual body weight (overprediction) or ideal body weight (underprediction) [6]. Clinical evidence showed that using actual body weight in the Cockcroft-Gault equation led to an overprediction of the creatinine clearance and, by applying a modified Calvert formula with creatinine clearance as GFR to calculate the dose, produced a higher than expected carboplatin area under the concentration-time curve [7;8]. Calculation of carboplatin clearance may be more accurate by using other weight descriptors such as fat-free mass or adjusted ideal body weight [8;9]. Since the Cockcroft-Gault equation is widely used in clinical practice for creatinine clearance calculation to be applied in the Calvert formula, there is a risk of significant overdosing of carboplatin in overweight and obese patients. The purpose of this study was to determine which weight descriptor could best be used in the Cockcroft-Gault equation to accurately predict the carboplatin clearance in overweight and obese patients.

Patients and methods

Patients

Pharmacokinetic data of ultrafilterable platinum were available of 240 patients (380 courses, in total 4478 samples). The data were obtained from several previously published studies in which patients received carboplatin both in high-dose as well as in conventional-dose regimens in combination with other chemotherapeutic agents [10-14]. All protocols were approved by the Committee of Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from all patients.

Sampling and analyses

The number and time-points of samples withdrawn in each study protocol are depicted in Table 1. In all studies, plasma ultrafiltrate was prepared immediately after blood sampling, using the Amicon micropartition system with an YMT-14 membrane (30 kD, Millipore Corporation, Bedford, MA, USA). A volume of 0.5 mL plasma was transferred in the micropartition system and centrifuged at 2,500 *g* for 20 min. Ultrafiltrate was stored at -20°C until analysis. Analysis of platinum in ultrafiltrate was performed using flameless atomic absorption spectrometry as previously described [15]. Accuracy and day-to-day imprecision of this method were 93.9-103.3% and 1.5-10.2%, respectively.

Pretreatment serum creatinine levels were estimated by the kinetic Jaffé method (Hitachi systems, Roche Diagnostics, The Netherlands) in three studies [12-14] and in the first 32 patients of one study [11], while in the remaining patients [10;11] the compensated Jaffé method was used. To correct for the different methods used, the serum creatinine values obtained with the kinetic Jaffé method were retrospectively adjusted by subtracting 26 µM from the initial values as proposed and validated by the manufacturer (Roche Diagnostics, The Netherlands).

Table 1. Baseline patient characteristics

	Number	**Median (range)**
Patient characteristics		
Female/male	161/79	
Age (years)		47 (16-75)
BSA (m^2)		1.81 (1.49-2.94)
BMI (kg/m^2)		24 (16-46)
Weight (kg)		70 (46-170)
Height (cm)		171 (153-210)
Protocol		
Non-small cell lung cancer [10]	21 (21 courses)	
*2-6 x PC[a] (dose carboplatin AUC 6 mg*min/mL administered in 30 min)*	*4 plasma samples per patient per course t=0.5, 4.5, 8.5, 23.5 h*	
Non-small cell lung cancer [13;14]	58 (95 courses)	
PC (dose carboplatin 300-400 mg/m^2/30 min)	*6-10 plasma samples per patient per course t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 24.5, 48.5 h*	
Ovarian cancer [12;14]	25 (25 courses)	
PC (dose carboplatin 300-600 mg/m^2/30 min)	*8-12 plasma samples per patient per course t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 18.5, 24.5, 48.5 h*	
High-risk primary breast cancer [11]	44 (44 courses)	
*CTC[b] (dose carboplatin 400 mg/m^2/day or AUC 20 mg*min/mL administered in 1 h for 4 days)*	*15-20 plasma samples per patient per course t=0.5, 0.75, 1, 1.5, 2.5, 4.5, 8.5, 12.5, 18.5, 24.5, 48.5 h, Day 1 and 3*	

	Number	Median (range)
Metastatic breast cancer [11][and additional pat's] *tCTC (dose carboplatin 267 mg/m^2/day or AUC 13.3 mg*min/mL administered in 1 h for 4 days)*	47 (113 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h Day 1 and 3*	
Refractory germ cell cancer [11][and additional pat's] *CTC (dose carboplatin 400 mg/m^2/day or AUC 20 mg*min/mL administered in 1 h for 4 days)*	19 (35 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h Day 1 and 3*	
Refractory germ cell cancer [11][and additional pat's] *tCTC (dose carboplatin 267 mg/m^2/day or AUC 13.3 mg*min/mL administered in 1 h for 4 days)*	5 (14 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h Day 1 and 3*	
Metastatic ovarian cancer [11] *tCTC (dose carboplatin 267 mg/m^2/day or AUC 13.3 mg*min/mL administered in 1 h for 4 days)*	5 (6 courses) *15-20 plasma samples per patient per course* *t=0.5, 1, 1.5, 1.75, 2, 2.5, 3.75, 5.5, 10, 24 h Day 1 and 3*	
Epithelial breast cancer *miniCTC[c] (dose carboplatin 400 mg/m^2/day or AUC 10 mg*min/mL administered in 1 h for 2 days)*	16 (27 courses) *12-16 plasma samples per patient per course* *t= 0.5, 1, 1.5, 2, 2.5, 3.75, 5.5, 10 h, Day 1 and 2*	
Biochemical parameters		
Serum creatinine (μM)		57 (18-124)
Creatinine clearance (calculated with the Cockcroft-Gault formula (mL/min) [4]		126 (55-451)
Albumin (g/L)		42 (18-52)

[a] PC, paclitaxel and carboplatin

[b] (t)CTC, high-dose cyclophosphamide (1 hour infusion), carboplatin (1 hour infusion) and thiotepa (2x 0.5 hour infusion) every day during 4 days

[c] miniCTC, day 1 cyclophosphamide (1 hour infusion) and carboplatin (1 hour infusion), day 2 thiotepa (1 hour infusion) and carboplatin (1 hour infusion)

Pharmacokinetics and data analysis

For the evaluation of the bias and imprecision of the diverse weight descriptors in the Cockcroft-Gault equation in predicting individual carboplatin clearances, individual fits of the observed carboplatin-time data were used. Individual fits were obtained by fitting a two-compartment model to the carboplatin-time data using the non-linear mixed effect modelling program NONMEM (version VI) (GloboMax LLC, Hanover, USA) [16]. Clearance-values obtained from these individual fits were compared to the predicted carboplatin clearances. Predicted carboplatin clearances (CL_{est}) were calculated using the modified Calvert formula ($CL_{carbo}=CL_{cr}+25$), in which the creatinine clearance was calculated with the Cockcroft-Gault equation using diverse weight descriptors (Table 2). Bias and imprecision of the diverse weight descriptors were evaluated using the percentage mean prediction error (MPE%) and the percentage mean absolute prediction error (MAPE%). The MPE% is a measure of bias and MAPE% is a measure of imprecision and were defined as:

$$MPE\% = \sum(pe)/N * 100\%$$
$$MAPE\% = \sum|pe|/N * 100\%$$

with pe = prediction error defined as:

$$(CL_{est}-CL_{ind})/CL_{ind}$$

Table 2. Weight descriptors used in the Cockcroft-Gault equation

Weight descriptor	
ABW	
IBW	49.9 + 0.89 * (height(cm)-152.4) for men 45.4 + 0.89 * (height(cm)-152.4) for women
AIBW	IBW + 0.4 * (ABW-IBW)
Benezet [30]	(IBW+ABW) / 2
FFM	ABW * (1-0.715) + 12.1 * height(m)^2 for men ABW * (1-0.713) + 9.74 * height(m)^2 for women
LBM	1.1 * ABW – 0.0128 * BMI * ABW for men 1.07 * ABW – 0.0148 * BMI * ABW for women

ABW, actual body weight; IBW, ideal body weight; AIBW, adjusted ideal body weight; FFM, fat-free mass according to Salazar and Corcoran[9]; LBM, lean body mass; BMI, body mass index

In the second part of the analysis, the relation between carboplatin clearance and weight was determined using a population pharmacokinetic model. Pharmacokinetic data of carboplatin (measured as free platinum) were analysed with a population pharmacokinetic model developed using NONMEM (version VI). The first order conditional estimation method (FOCE) with INTERACTION was used after logarithmic data transformation [17]. Pharmacokinetics of carboplatin were described with a two-compartment model with first-order elimination from the central compartment. Interindividual variability (IIV), interoccasion variability (IOV) and residual variability were modelled using a proportional error model.
Correlation of carboplatin clearance with the diverse weight descriptors was investigated by estimating the allometric coefficient for carboplatin clearance, according to the following equation:

$$CL_i = CL_{pop} * (Wt_i/Wt)^x$$

where CL_i is the carboplatin clearance in individual i with weight Wt_i, CL_{pop} is the population carboplatin clearance standardized to an individual with median weight Wt and x is the allometric coefficient, which marks the exponential decrease or increase in clearance. In this equation, Wt_i and Wt were substituted by several weight descriptors (Table 2). To correct for possible confounding variables, sex was also incorporated in this equation. Significance of incorporation of an allometric coefficient using diverse weight descriptors was evaluated with the objective function value (OFV), which is proportional to negative twice the log likelihood. A difference in OFV of 6.63 between two nested models (corresponding to a p-value of 0.01) was considered statistically significant.

Results

Patients

A total of 240 cancer patients were studied of whom 7 (3%) were defined as underweight (body mass index (BMI) <18.5 kg/m^2), 146 (61%) were defined as normal weight (BMI ≥18.5-<25 kg/m^2), 72 (30%) were defined as overweight (BMI ≥25-<30 kg/m^2) and 15 (6%) were defined as obese (BMI ≥30 kg/m^2). Of all patients in the dataset baseline patient characteristics and biochemical parameters were available as summarized in Table 1.

Pharmacokinetics and data analysis

Bias and imprecision of estimates of carboplatin clearance based on diverse weight descriptors (Table 2) are listed in Table 3.

Table 3. Bias and imprecision of estimates of carboplatin clearance based on diverse weight descriptors or using a flat dose as assessed by percentage mean errors

Weight descriptor	Underweight (n=7)		Normal weight (n=146)		Overweight (n=72)		Obese (n=15)	
	MPE% (95%CI)	MAPE% (95%CI)	MPE% (95%CI)	MAPE% (95%CI)	MPE% (95%CI)	MAPE% (95%CI)	MPE% (95%CI)	MAPE% (95%CI)
ABW	13.9 (-7.06-34.8)	27.5 (18.6-36.4)	15.4 (9.41-21.3)	26.6 (21.8-31.4)	13.9 (6.97-20.7)	23.7 (18.5-29.0)	35.4 (15.0-55.8)	36.8 (17.1-56.5)
IBW	40.2 (7.82-72.6)	45.7 (18.5-73.0)	12.9 (6.60-19.3)	26.9 (21.8-31.9)	-5.22 (-10.6-0.21)	20.1 (17.0-23.1)	-11.4 (-21.9--0.93)	20.4 (14.8-26.0)
AIBW	29.7 (1.92-57.4)	38.1 (17.9-58.2)	13.9 (7.76-20.1)	26.5 (21.6-31.5)	2.41 (-3.55-8.37)	20.0 (16.2-23.8)	7.34 (-6.77-21.4)	21.0 (11.4-30.6)
Benezet [30]	27.0 (0.43-53.6)	36.3 (18.1-54.5)	14.2 (8.04-20.3)	26.5 (21.6-31.4)	4.32 (-1.78-10.4)	20.4 (16.4-24.3)	12.0 (-3.09-27.1)	22.6 (11.3-33.9)
FFM	6.14 (-18.0-30.3)	30.1 (25.1-35.2)	-7.61 (-12.6--2.63)	25.2 (22.1-28.3)	-17.3 (-21.9--12.8)	22.6 (19.5-25.7)	-14.0 (-24.7--3.40)	22.1 (16.3-27.8)
LBM	-0.90 (-21.0-19.2)	24.3 (19.3-29.3)	-7.26 (-12.1--2.41)	24.6 (21.7-27.6)	-15.4 (-20.0--10.7)	21.9 (19.0-24.9)	-15.9 (-25.3--6.45)	21.0 (15.0-27.1)
Flat dose	-2.06 (-12.3-8.13)	8.96 (1.52-16.4)	3.96 (-0.86-8.77)	18.1 (14.2-22.0)	0.91 (-3.99-5.81)	16.4 (13.3-19.5)	-0.59 (-13.1-11.9)	19.1 (11.6-26.7)

ABW, actual body weight; IBW, ideal body weight; AIBW, adjusted ideal body weight; FFM, fat-free mass according to Salazar and Corcoran[9]; LBM, lean body mass; underweight, BMI<18.5 kg/m^2; normal weight, BMI ≥18.5-<25 kg/m^2; overweight, BMI ≥25-<30 kg/m^2; obese, BMI≥30 kg/m^2

In underweight patients the use of lean body mass (LBM) in the Cockcroft-Gault equation was the predictor with the lowest bias and imprecision for carboplatin clearance calculated using the modified Calvert formula. The other weight descriptors resulted in overprediction of the carboplatin clearance. In normal weight patients, actual body weight (ABW), ideal body weight (IBW), adjusted ideal body weight (AIBW) and the Benezet equation resulted in overprediction of the carboplatin clearance, while fat-free mass (FFM) and LBM resulted in slight underprediction of the carboplatin clearance. LBM was the weight descriptor that resulted in the lowest bias and imprecision in normal weight patients. In overweight and obese patients, AIBW, the Benezet equation and ABW resulted in overprediction of the carboplatin clearance, more so in obese patients compared to overweight patients. IBW resulted in underprediction of the carboplatin clearance also to a greater extent in obese patients compared to overweight patients. LBM and FFM resulted in comparable underpredictions of the carboplatin clearance in overweight and obese patients. The weight descriptor that resulted in the lowest bias and imprecision in overweight and obese patients is AIBW.

Flat dosing based on the population carboplatin clearance resulted in the lowest bias and imprecision in all the weight categories (Table 3), indicating that the relation between carboplatin clearance and weight is much weaker in this patient population than the Cockcroft-Gault equation would imply. This is also shown in Figure 1 in which the relation between carboplatin clearance and weight of the patients included in this analysis is depicted. Neither actual body weight nor lean body mass showed a strong relation with carboplatin clearance.

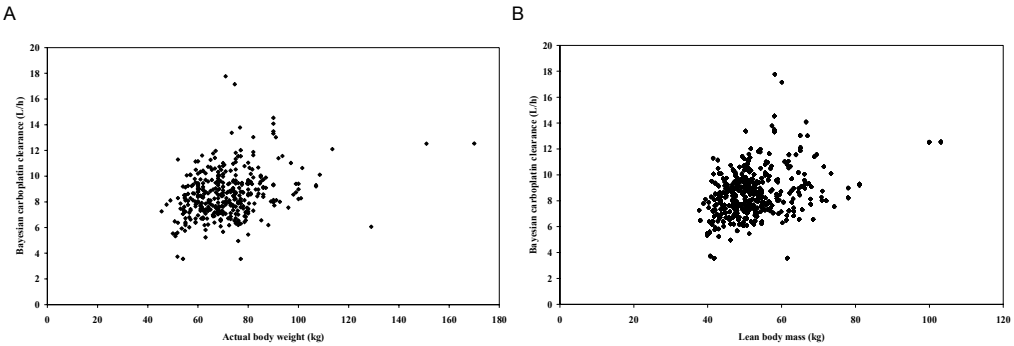

Figure 1. Relation between carboplatin clearance and actual body weight (A) and lean body mass (B).

Table 4 summarizes the population pharmacokinetic parameters of carboplatin of the basic model, together with the IIV, IOV and residual variability. IIV was estimated for clearance (CL), volume of distribution (V) and the distribution parameters k_{12} and k_{21}, whereas IOV was estimated for CL and V. All parameters were estimated with an acceptable precision (coefficient of variation 1.41%-30.8%).

Table 4. Population pharmacokinetic parameters of carboplatin of the basic model

Parameter	Estimate (RSE %)	% IIV (RSE %)	% IOV (RSE %)
Clearance (L/h)	8.38 (1.41)	19.4 (8.34)	9.14 (9.15)
Volume of distribution (L)	15.4 (1.79)	14.5 (11.7)	10.8 (14.9)
Distribution microconstant k_{12} (h^{-1})	0.135 (7.85)	48.2 (18.9)	nd
Distribution microconstant k_{21} (h^{-1})	0.215 (5.91)	43.6 (30.8)	nd
Proportional residual error (%)	19.7 (5.69)		

RSE, relative standard error of estimate; IIV, interindividual variability; IOV, interoccasion variability; nd= not determined

Estimation of the allometric coefficient for carboplatin clearance resulted in values ranging from 0.136 to 0.220 for the diverse weight descriptors used (Table 5), indicating that the relation between carboplatin clearance and weight is much weaker than the theoretical allometric coefficient of 0.75. This is also shown graphically in Figure 2, which shows the relation between weight and carboplatin clearance.

Table 5. Allometric coefficients for carboplatin clearance using diverse weight descriptors

Weight descriptor	Allometric coefficient (RSE %)	% IIV (RSE %)	ΔOFV
Basic model		19.4 (8.34)	
ABW	0.175 (46.2)	18.7 (8.52)	-6.06
IBW	0.136 (72.9)	18.8 (8.91)	-2.22
AIBW	0.218 (48.6)	18.5 (8.84)	-5.03
Benezet [30]	0.220 (46.8)	18.5 (8.79)	-5.49
FFM	0.138 (72.4)	18.7 (8.73)	-2.57
LBM	0.207 (46.7)	18.5 (8.71)	-5.71

ABW, actual body weight; IBW, ideal body weight; AIBW, adjusted ideal body weight; FFM, fat-free mass according to Salazar and Corcoran[9]; LBM, lean body mass; IIV interindividual variability; RSE, relative standard error of estimate; ΔOFV, difference in objective function value of the model incorporating an allometric coefficient compared to the basic model

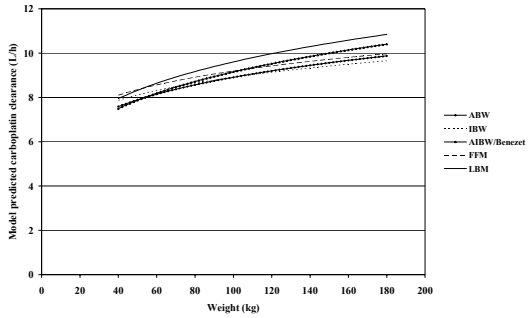

Figure 2. Model predicted carboplatin clearance versus weight calculated using diverse weight descriptors

Incorporation of an allometric coefficient did not significantly improve the fit of the model (differences in OFV were less than 6.63) compared to the basic model in which no relation between weight and carboplatin clearance was assumed (Table 5). Nor did it explain a significant part of the interindividual variability in carboplatin clearance. Interindividual variability values of the models incorporating an allometric coefficient (18.5-18.8%) were all in the same range as the basic model (19.4%, Table 5).

Discussion

The proper dosage in overweight and obese individuals is an important subject in clinical practice. With chemotherapeutic agents being dosed by equations incorporating weight, there are concerns about the safety and efficacy in overweight and obese patients [18]. Unfortunately, there is a lack of data that addresses this issue. In the current study, we sought to identify which weight descriptor should be used in the Cockcroft-Gault equation to accurately predict the carboplatin clearance in overweight and obese patients. We have demonstrated that adjusted ideal body weight was the best weight descriptor to be used in the Cockcroft-Gault equation in overweight and obese patients, however, the use of weight descriptors in carboplatin dosing did not improve the dosing accuracy compared to flat dosing.

Carboplatin is eliminated through the kidney by glomerular filtration and tubular secretion. There are several discrepancies regarding the influence of obesity on these functions. While some studies have shown an increase in glomerular filtration, measured using creatinine clearance, in obese women as compared to normal weight women [19-21], others have shown decreased glomerular filtration [22] or no significant difference between creatinine clearance in obese versus nonobese individuals [23;24]. These discrepancies might be due to the difference in extent of obesity and/or associated renal pathology. Tubular secretion is difficult to ascertain, therefore, conclusions regarding tubular secretion are often indirect. An increase in renal clearance of ciprofloxacin [23], cimetidine [25] and procainamide [26] accompanied by a disproportionate increase in glomerular filtration was seen in obese individuals. Since the renal excretion of these compounds primarily involves glomerular filtration and tubular secretion, these findings support increased tubular secretion in obese individuals.

The Cockcroft-Gault equation has been shown to be biased and inaccurate in some specific patient groups, such as the obese [6;27]. Van de Ree et al compared creatinine clearance as calculated by Cockcroft-Gault to creatinine clearance as determined by 24-hour urine collection in obese patients and stated that in view of the influence of body weight on the Cockcroft-Gault equation, this equation should not be used to estimate the GFR in patients with extreme obesity [28]. Spinler et al showed that the Cockcroft-Gault equation using actual body weight tended to overpredict the creatinine clearance in obese patients, while the modified Cockcroft-Gault equation using ideal body weight tended to underpredict creatinine clearance. The use of adjusted ideal body weight may be more accurate [6].
Several studies have evaluated the performance of different weight descriptors in dosing formulae to predict carboplatin exposure. Herrington et al showed in 19 patients with a body mass index ≥27 kg/m^2 that the use of the adjusted ideal body weight in the Cockcroft-Gault equation led to less bias and more precision than using actual weight [8]. The use of actual weight in obese patients led to an overestimation of the carboplatin clearance and thus carboplatin exposure. Furthermore, Sparreboom et al showed that when calculating carboplatin doses on the basis of body surface area, either predicted normal weight or the mean of ideal and actual weight, resulted in the best prediction of systemic carboplatin exposure in both obese men and women [29]. Benezet et al studied the accuracy of the Chatelut formula to predict the carboplatin clearance in a subpopulation of obese patients. They showed that the average of actual body weight and ideal body weight was the best predictor of carboplatin clearance within the formula, integrating body weight, plasma creatinine level, age and sex [30].
In conclusion, these studies showed that neither actual body weight, nor ideal body weight, but an average of both, results in the best prediction of carboplatin clearance in obese patients. These results are in accordance with our study.

In our patient population no strong relation between weight and carboplatin clearance could be demonstrated. This was also seen in a covariate analysis determining the effect of patient-specific factors on carboplatin clearance [31]. It was shown that creatinine clearance determined by the 24 h urine collection method explained almost two-thirds of the interindividual variability of carboplatin clearance. Height was identified as the second significant covariate of clearance, but accounted for only approximately 10%, while weight was not selected at all.
The extent to which compounds are affected by obesity depends on the lipophilicity of the drug. In general, more lipophilic compounds are affected to a greater extent by obesity than hydrophilic compounds [18]. The excess of adipose tissue in obese patients has a smaller proportion of water compared to muscle tissue. Carboplatin is hydrophilic in nature and would, therefore, not distribute well through adipose tissue. Thus, carboplatin would not be expected to be influenced by obesity to a great extent. In addition, only lean mass is responsible for production of creatinine. Therefore, weight descriptors that correct for the excess of adipose tissue would be expected to be better predictors of carboplatin clearance than actual body weight.

In a previous study, using approximately the same dataset as this study, we have demonstrated that modification of the Calvert formula by estimating GFR from serum creatinine to calculate an a priori carboplatin dose is not justified in adult patients with normal renal function. No relation between creatinine clearance estimators and carboplatin clearance could be demonstrated in that study [5].
The predominant determinant of carboplatin clearance is the GFR. Although creatinine clearance is widely accepted as a simple measurement of the GFR, it systematically overestimates GFR, owing to creatinine not being solely filtered by the glomerulus but also actively secreted by the proximal tubule. This overestimation of the GFR can also be seen in this study. In normal weight patients ABW, IBW, AIBW or weight calculated with the Benezet equation are all roughly the same. Table 4 shows that the use of the Cockcroft-Gault equation incorporating the aforementioned weight descriptors systematically overestimates carboplatin clearance by around 14% in normal weight patients, due to the active secretion of creatinine.

The current study showed that when using the Cockcroft-Gault equation to calculate an a priori dose of carboplatin, the use of adjusted ideal body weight results in the best prediction in overweight and obese patients. However, without a clear correlation of weight with carboplatin clearance, the use of the Cockcroft-Gault equation to estimate an a priori dose of carboplatin in overweight or obese patients with adequate renal function should be questioned. Our results suggest that a flat dose (carboplatin dose=target AUC*carboplatin clearance), based on the population carboplatin clearance (8.38 L/h = 140 mL/min), will result in less bias in overweight and obese patients with adequate renal function.

Acknowledgement
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Chapter 4.3

Pharmacokinetics of cyclophosphamide and thiotepa in a conventional fractionated high-dose regimen compared to a novel simplified unfractionated regimen

Submitted for publication

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Abstract

Purpose: High-dose alkylating chemotherapy with cyclophosphamide (4000 or 6000 mg/m^2) and thiotepa (320 or 480 mg/m^2) has commonly been administered in a fractionated regimen over 4 days. A simplified unfractionated regimen would be preferable especially since cyclophosphamide and thiotepa have been shown to influence each others metabolism. Alteration of administration regimens, however, can have a profound effect on the pharmacokinetics of the compounds involved. The aim of this study was to investigate the effect of alteration of the fractionated administration schedule of the CTC regimen on cyclophosphamide and thiotepa pharmacokinetics.

Patients and methods: Plasma samples were collected from 124 patients who received a fractionated tiny-CTC or CTC regimen of cyclophosphamide (1000 or 1500 mg/m^2/day), thiotepa (40 or 60 mg/m^2, twice daily) and carboplatin (267 or 400 mg/m^2/day) for 4 days and 16 patients who received an unfractionated mini-CTC regimen of cyclophosphamide (3000 mg/m^2, at day 1), carboplatin (400 mg/m^2, at day 1 and 2) and thiotepa (250 mg/m^2, at day 2). Plasma concentrations of cyclophosphamide and 4-hydroxycyclophosphamide were determined using high-performance liquid chromatography coupled with tandem mass spectrometry, plasma concentrations of thiotepa and tepa were determined using gas-chromatography. Pharmacokinetics of cyclophosphamide and thiotepa were assessed using non-linear mixed effect modelling.

Results: This study showed that alteration of a fractionated high-dose regimen into a simplified unfractionated regimen results in saturation of thiotepa elimination with a Vmax of 212 (± 58) μmol/h and a Km of 13.7 (± 5.9) μM. This resulted in an increased dose corrected exposure to thiotepa (13%) and decreased dose corrected exposure to its metabolite tepa (21%). Elimination of cyclophosphamide was not shown to be saturable. Dose corrected exposures to cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide were comparable in both regimens.

Conclusion: Because the simplified unfractionated mini-CTC regimen is more patient-friendly and because overall dose corrected exposure to cyclophosphamide and thiotepa was not affected to a relevant extent, this unfractionated regimen can be safely used in future studies.

Introduction

High-dose chemotherapy has been developed as a possible curative treatment modality in several solid tumours. Although the role of high-dose chemotherapy regimens is still undefined, it has been shown that patients with HER2/neu-negative breast tumours may benefit from high-dose alkylating chemotherapy, both in terms of recurrence-free and overall survival [1;2].

Frequently used high-dose regimens include: the CTC regimen, in which cyclophosphamide (1500 mg/m^2/day), thiotepa (120 mg/m^2/day) and carboplatin (400 mg/m^2/day) are given as short iv infusions during 4 subsequent days [1], or the CTCb or STAMP V regimen, in which cyclophosphamide (6000 mg/m^2), thiotepa (500 mg/m^2) and carboplatin (800 mg/m^2) are given as 96h continuous infusions [3].

Cyclophosphamide is an inactive prodrug, requiring enzymatic bioactivation to form its pharmacologically active metabolite 4-hydroxycyclophosphamide [4]. Thiotepa is rapidly metabolised by oxidative desulfuration to its metabolite tepa [5]. Thiotepa and tepa have similar alkylating activities and exposure to both compounds is a measure of total alkylating activity.

For cyclophosphamide, saturation of bioactivating enzymes, autoinduction and inhibition of bioactivation by thiotepa have been shown [4;6;7]. Thiotepa clearance is increased in the presence of cyclophosphamide [8]. Furthermore, saturable elimination of thiotepa has been described, both in conventional dose and in high-dose regimens [9].

Much work has been done to define the maximum tolerated dose and optimal treatment schedule to improve the safety and effectiveness of high-dose chemotherapy. Fractionated regimens such as the CTC and tiny-CTC regimen (which is identical to the CTC regimen except that it incorporates only 2/3rd of the dose of each agent) have been shown to be feasible and relatively well tolerated, although, patients had to be hospitalised for at least 2 weeks [10]. The continued study of high-dose chemotherapy requires the availability of a regimen that is safe, well-tolerated and can be managed on an out-patient basis. For this purpose, the mini-CTC regimen was developed in which the dose of a CTC cycle is given in two separate cycles of two days, three weeks apart. This regimen allowed intensive treatment with alkylating chemotherapy and supportive care and reduced the hospital stay to 2-4 days compared to 21 days in the CTC regimen. Out-patient care has multiple advantages, such as patient preference, diminished exposure to hospital micro-organisms, better use of available hospital beds and lower costs [11].

A clinical feasibility study has shown that the mini-CTC regimen was feasible and well-tolerated (unpublished).

Alteration of administration regimens, however, can have a profound effect on the pharmacokinetics of the compounds involved, such as saturable elimination and different auto-induction profiles. The aim of this study was to investigate whether modulation of the CTC regimen alters the pharmacokinetics of cyclophosphamide and thiotepa and thereby influences the exposure to these agents.

Methods

Patients and treatment

Details of the patients who received CTC and tiny-CTC have been described previously [12-14]. In brief, the CTC regimen consisted of 4 days of chemotherapy with cyclophosphamide (1500 mg/m^2/day) as a 1-h infusion, immediately followed by carboplatin (400 mg/m^2/day) as a 1-h infusion and thiotepa (60 mg/m^2, twice daily 12 h apart) as a 30-min infusion. The tiny-CTC regimen was identical to the CTC regimen except that it incorporated 2/3rd of the dose of each agent. Patients received either 1 or 2 courses of CTC or 2 or 3 courses of tiny-CTC, when possible every 4 weeks.

Patients who received mini-CTC were entered in a high-dose chemotherapy protocol with peripheral blood progenitor cell transplantation. Patients had high-risk breast cancer and had to be below 51 years of age. Further eligibility criteria included HER2/neu-negative tumours and adequate organ functions (creatinine clearance ≥ 60 mL/min, serum bilirubin ≤ 25 μmol/L). Treatment consisted of 4 cycles of paclitaxel (175 mg/m^2 every 2 weeks) with PEG-filgrastim support. Peripheral blood progenitor cells were harvested following the second chemotherapy cycle. Two to three weeks after the last paclitaxel treatment the first cycle of high-dose chemotherapy was given. The mini-CTC cycle consisted of cyclophosphamide (3000 mg/m^2) as a 1-h infusion immediately followed by carboplatin (400 mg/m^2) as a 1-h infusion. The next day thiotepa (250 mg/m^2) as a 1-h infusion followed by carboplatin (400 mg/m^2) as a 1-h infusion were given. Approximately 48 hours after the thiotepa infusion the peripheral blood progenitor cells were reinfused. The second cycle started on day 22.

Mesna was administered as push (500 mg) before start of cyclophosphamide infusion and as continuous infusion (2000 mg) for 24 hours. All patients received anti-emetics both prophylactically and as indicated, which included aprepitant, dexamethasone and granisetron. Patients received prophylactic antibiotics, including ciprofloxacin and fluconazol.

This study was approved by the Committee on the Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from all patients.

Sampling and analysis

For the tiny-CTC and CTC regimens complete pharmacokinetic profiles were assessed on 2 separate days, always including day 1 and either day 3 or day 4. Blood samples were obtained prior to the start of the infusions and at the following time points: 30 min after the start of cyclophosphamide infusion, 60 (end of cyclophosphamide infusion), 90, 120 (end of carboplatin infusion), 150 (end of thiotepa infusion), 180, 210, 285, 390, 660 min. On day 5, an additional sample was collected approximately 22 h after the last cyclophosphamide infusion. A total of 20 samples were available per patient per cycle.

Blood samples for the mini-CTC regimen were collected prior to the start of the infusions, at t=30, 60, 90, 120, 150, 180, 210, 285 and 390 min on both days of chemotherapy. On day 1 an additional sample was taken 660 min after start of chemotherapy. A total of approximately 17 samples were available per patient per cycle.

After blood sampling, samples were immediately placed on ice. Plasma was separated by centrifuging the sample at 3,500 g for 3 min at 4°C. A 500 μL volume of plasma was immediately added to 50 μL of 2M semicarbazide solution for the derivatization of 4-

hydroxycyclophosphamide. The remaining plasma layer was collected for the quantification of cyclophosphamide, thiotepa and tepa. All samples were stored at -70°C until analysis. Cyclophosphamide and 4-hydroxycyclophosphamide were determined with a previously described and validated high-performance liquid chromatography method coupled with electrospray ionization tandem mass spectrometry. The lower limit of quantification using 100 μL plasma sample was 200 ng/mL for cyclophosphamide and 50 ng/mL for 4-hydroxycyclophosphamide. Accuracies and precisions were within ±14% and less than 8%, respectively [15]. Thiotepa and tepa were quantified with a validated gas chromatographic assay as previously described [16]. The lower limit of quantification using 100 μL plasma samples was 5 ng/mL for thiotepa and tepa. Accuracies and precisions were within ±12% and less than 11% respectively.

Population pharmacokinetic analysis

For the pharmacokinetic analyses the non-linear mixed effect modelling program NONMEM (version VI) (Globomax Icon LLC, Hanover, MD, USA) was used. As a starting point a previously published integrated population pharmacokinetic model of both cyclophosphamide and thiotepa, including the autoinduction process of cyclophosphamide and the mutual drug-drug interaction of cyclophosphamide and thiotepa, was applied [8]. This model was developed on datasets of patients treated with the fractionated CTC and tiny-CTC regimen.

In this model (Figure 1), cyclophosphamide pharmacokinetics were described with a two-compartment model and 4-hydroxycyclophosphamide pharmacokinetics were described with a one-compartment model. Cyclophosphamide was eliminated by a non-inducible route ($CL_{CPnonind}$) and an inducible route (CL_{CPind}) leading to formation of 4-hydroxycyclophosphamide. The inducible clearance was modelled using an indirect effect model in which the indirect effect increased in the presence of cyclophosphamide. Thiotepa inhibits the inducible clearance of cyclophosphamide and this was modelled as a concentration-dependent, reversible decrease of the indirect effect. Elimination of 4-hydroxycyclophosphamide was described by a first-order elimination rate constant (k_{4OHCP}). The analysis was performed using the hybrid estimation method in NONMEM after logarithmic data transformation. With this method the variability in the noninducible cyclophosphamide clearance was estimated using the first-order conditional estimation (FOCE) method, while the other variability terms were estimated using FO.

Pharmacokinetics of thiotepa and tepa were described with two-compartment models. Thiotepa was eliminated by a non-inducible route ($CL_{TTnonind}$) and an inducible route leading to formation of tepa. Tepa elimination was described by a first-order elimination rate constant (k_{tepa}). Metabolism of thiotepa to its metabolite tepa was induced in the presence of cyclophosphamide. This process was modelled using an indirect effect model. The presence of cyclophosphamide resulted in an increase in indirect effect, which was directly proportional to the inducible thiotepa clearance. This indirect effect model was an “on-off” model (in which the induction was switched ‘on’ after start of first administration of cyclophosphamide and ‘off’ in the absence of cyclophosphamide), since cyclophosphamide concentration did not influence the rate of enzyme formation or elimination. The analysis for thiotepa and tepa was performed using the first-order (FO) method in NONMEM.

Interindividual variability (IIV) and interoccasion variability (IOV) in the pharmacokinetic parameters were estimated using a proportional model. For thiotepa, residual variability was estimated with a combined proportional and additive error model. For tepa, cyclophosphamide and 4-hydroxycyclophosphamide, residual variability was modelled using a proportional error model.

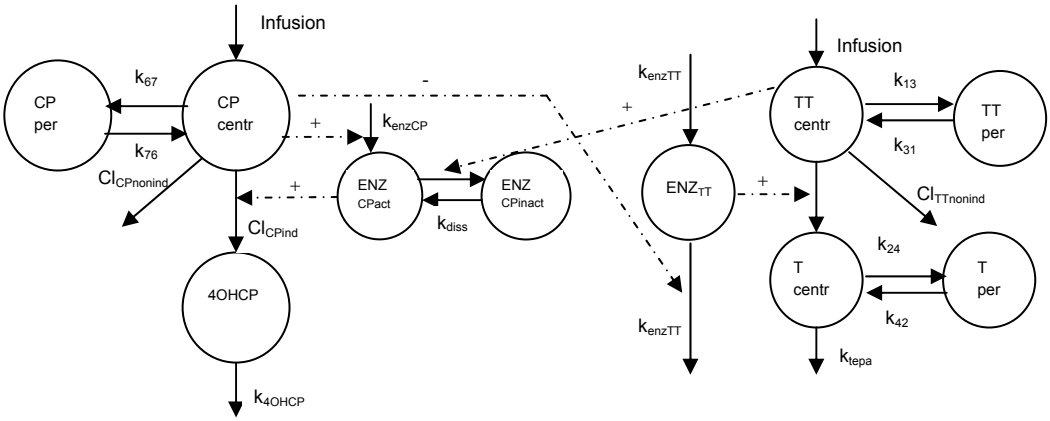

Figure 1. Graphical representation of the model

This model was applied to the dataset including data of the mini-CTC regimen. When indicated by graphical and/or statistical model diagnostics the model was refined. Effects as saturation of metabolism, non-linear inhibition of cyclophosphamide metabolism or different time courses of (auto-)induction were considered. The model for thiotepa and tepa was refined first. The individual empirical Bayesian estimates generated for thiotepa and tepa (using the POSTHOC option in NONMEM) were used as input for subsequent modelling of the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide. Model selection between hierarchical models was performed by the likelihood ratio test, using the objective function value (OFV), which is proportional to negative twice the log-likelihood. A significance level of 0.001, corresponding to a difference in OFV of 10.8, was used to distinguish between nested models. The adequacy of the developed structural models was evaluated according to precision of parameter estimates (standard errors, as calculated using the COVARIANCE option of NONMEM) and using goodness-of-fit plots, such as observed versus model predicted values, observed versus individual predicted values and weighted residual error versus time plots obtained using Xpose [17] and R (http://cran.r-project.org/).

Assessment of impact of regimens on exposure

Since patients were included in several protocols including a protocol in which pharmacokinetically guided dosing was applied, the impact of the different regimens on exposure could not directly be estimated from the available data. Therefore, the impact of the different regimens on the exposure to thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide was evaluated with a simulation study using the final pharmacokinetic model. In this simulation study, groups of 10 000 patients were simulated to

receive either a CTC, tiny-CTC, mini-CTC or Stamp V regimen. For each regimen, median AUCs of thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide were calculated.

Results

Plasma concentration-time data of thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide were available of CTC (67 patients, 84 cycles), tiny-CTC (57 patients, 130 cycles) and mini-CTC (16 patients, 27 cycles) regimens. Patient characteristics are summarized in Table 1.

Table 1. Baseline patient characteristics.

	Number	Median (range)
Patients	140	
Male	26	
Female	114	
Site of disease		
Breast cancer stage II or III	62	
Breast cancer stage IV	47	
Ovarian cancer	5	
Germ cell cancer	26	
Cycles with pharmacokinetic data available	241	
1st cycle CTC	67	
2nd cycle CTC	17	
1st cycle tiny-CTC	57	
2nd cycle tiny-CTC	44	
3rd cycle tiny-CTC	29	
1st cycle mini-CTC	16	
2nd cycle mini-CTC	11	
Age (years)		42 (16-59)
Body surface area (m^2)		1.8 (1.5-2.9)
Weight (kg)		69 (50-170)
Height (cm)		172 (153-210)

Thiotepa pharmacokinetics

Bias was observed when applying the population pharmacokinetic model that was developed previously to the thiotepa data. Observed thiotepa concentrations were higher than the model predicted concentrations (Figure 2). This might indicate that the elimination of thiotepa was saturated. Incorporation of a saturable inducible clearance of thiotepa resulted in an improvement of the model (ΔOFV=-119). Observed thiotepa concentrations and model predicted concentrations were more scattered around the line of identity after incorporation of a saturable inducible thiotepa clearance (Figure 3A and B). Vmax was estimated to be 212 μmol/h and Km 13.7 μM. Saturable non-inducible clearance of thiotepa or saturable elimination of tepa did not improve the fit.

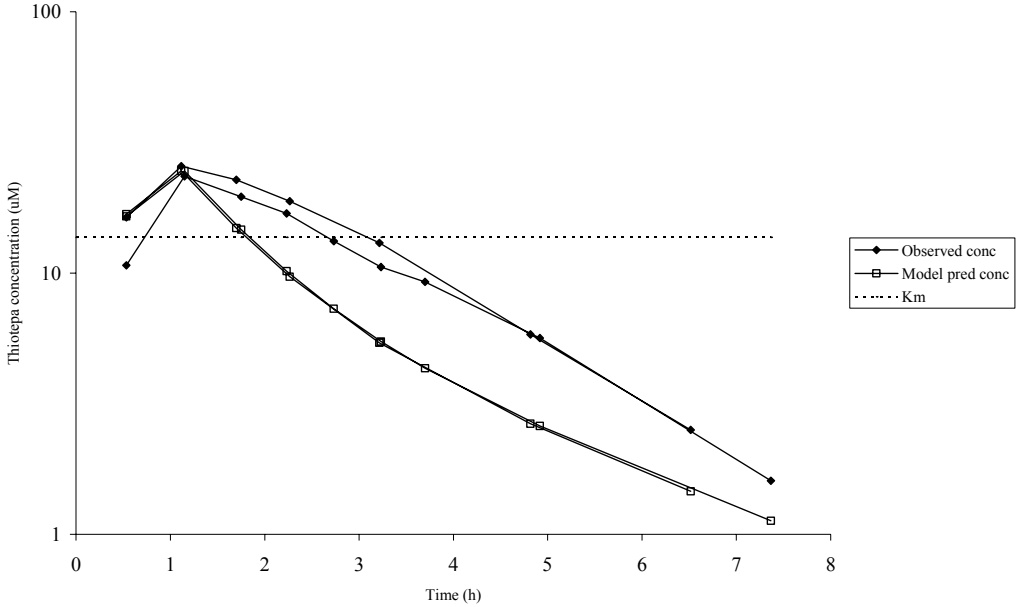

Figure 2. Observed and model predicted (model with no saturable elimination) thiotepa concentration versus time curves of 2 patients receiving the mini-CTC regimen, indicating saturable elimination.

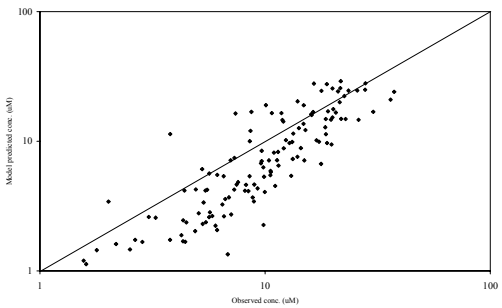

Figure 3A. Model predicted versus observed concentrations of the mini-CTC regimen for thiotepa in model without saturable inducible clearance of thiotepa.

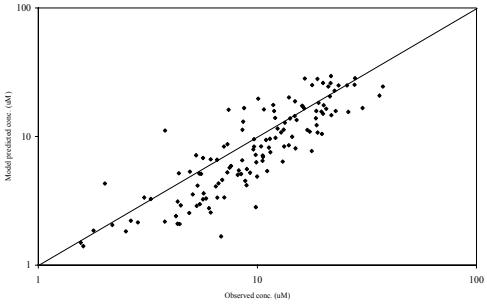

Figure 3B. Model predicted versus observed concentrations of the mini-CTC regimen for thiotepa in model with saturable inducible clearance of thiotepa.

Cyclophosphamide pharmacokinetics

Thereafter, the individual Bayesian estimates of the pharmacokinetic parameters of the thiotepa model were incorporated in the model describing cyclophosphamide pharmacokinetics. Bias was observed. Observed cyclophosphamide concentrations on the second day of the mini-CTC cycle were higher than the model predicted cyclophosphamide concentrations. Therefore, different models to account for the auto-induction of cyclophosphamide were tested. It was hypothesized that the auto-induction process, which was modelled as a continuous process starting immediately after administration of the first dose, actually had a slow onset, hereby explaining the higher observed cyclophosphamide

concentrations. However, different models incorporating a different time course of auto-induction did not improve the fit. The observed bias could also be due to saturable elimination. However, incorporation of this effect also did not improve the model fit.
In the previously developed model that was used as a starting point, it was assumed that the inhibition of the inducible clearance of cyclophosphamide by thiotepa was linearly dependent on thiotepa concentration. Therefore, higher thiotepa concentrations as present in the mini-CTC regimen would result in a higher extent of inhibition of inducible cyclophosphamide clearance. Since we observed higher cyclophosphamide concentrations on the second day of the mini-CTC cycle than model predicted cyclophosphamide concentrations, we assumed that this inhibition by thiotepa might not be a linear process. Indeed, incorporation of an E-max type inhibition of the inducible clearance of cyclophosphamide by thiotepa resulted in an improvement of the model (ΔOFV = -2092). The parameter estimates of the final model are listed in Tables 2 and 3, including interindividual variability, interoccasion variability and residual variability. All parameters of the thiotepa model were estimated with acceptable precision (coefficient of variation 4.1-49.5%). As the covariance step for the final cyclophosphamide model failed in NONMEM, no relative standard errors are given in Table 3. Interindividual variability was considerable for k_{ENZTT}, for the other parameters interindividual variability was moderate. Interoccasion variability could be estimated for only a few parameters. Residual variability was small for all compounds, indicating that the model gave an adequate description of the data.

Table 2. Parameter estimates of the final population pharmacokinetic model for thiotepa (TT) and tepa (T).

Parameter			Estimate (RSE %)[a]	% IIV (RSE %)[a]	% IOV (RSE %)[a]
$CL_{TTnonind}$	non-inducible clearance of TT	($L*h^{-1}$)	25.3 (7.9)	51.3 (35.4)	24.2 (30.3)
V_{max}	maximal elimination rate	($\mu mol*h^{-1}$)	212 (27.3)	37.7 (18.5)	17.8 (20.7)
Km	Michaelis Menten constant	(μM)	13.7 (42.9)		
V_{TT}	volume of distribution of TT	(L)	53.7 (4.1)	29.0 (23.6)	17.5 (20.8)
k_{13}	rate constant distribution TT from central to peripheral compartment	(h^{-1})	0.370 (23.9)	59.6 (18.5)	
k_{31}	rate constant distribution TT from peripheral to central compartment	(h^{-1})	0.571 (10.5)		
k_{tepa}	first-order elimination rate constant of T	(h^{-1})	0.380 (9.3)	44.4 (34.5)	
k_{24}	rate constant distribution T from central to peripheral compartment	(h^{-1})	1.41 (33.8)	41.2 (31.2)	
k_{42}	rate constant distribution T from peripheral to central compartment	(h^{-1})	0.469 (33.1)		
k_{enzTT}	zero-order formation and first-order elimination rate constant of the enzyme involved in TT metabolism	(h^{-1})	0.0344 (10.2)	330 (49.5)	
E_{max}	maximal value of enzyme induction		0.309 (10.7)		
V_T	volume of distribution of T	(L)	16.3 (20.7)		
Proportional error of TT		(%)	24.9 (6.2)		
Additive error of TT		(μM)	0.0683 (12.7)		
Proportional error of T		(%)	16.5 (7.6)		

[a]RSE= relative standard error, obtained with the COVARIANCE option of NONMEM;
IIV= inter individual variability; IOV= inter occasion variability

Table 3. Parameter estimates of the final population pharmacokinetic model for cyclophosphamide (CP) and 4-hydroxycyclophosphamide (4OHCP).

Parameter			Estimate	% IIV	% IOV
$CL_{CPnonind}$	non-inducible clearance of CP	($L*h^{-1}$)	2.61	21.8	28.1
CL_{CPind}	initial inducible clearance of CP	($L*h^{-1}$)	2.84	28.0	18.1
V_{CP}	volume of distribution of CP	(L)	37.8	19.5	11.7
k_{enzCP}	zero-order formation rate constant of the enzyme involved in CP metabolism	(h^{-1})	0.0312	38.5	
k_{4OHCP}	first-order elimination rate constant of 4OHCP	(h^{-1})	173	28.5	
E_{max9}	maximal value of enzyme inactivation	(h^{-1})	1.30	29.3	
Km9	Michaelis Menten constant	(μM)	0.965		
k_{diss}	rate constant of reversible enzyme activation	(h^{-1})	0.518		
k_{67}	rate constant distribution CP from central to peripheral compartment	(h^{-1})	0.0897	23.0	42.9
k_{76}	rate constant distribution CP from peripheral to central compartment	(h^{-1})	0.333		
Proportional error of CP		(%)	17.5		
Proportional error of 4OHCP		(%)	21.5		

IIV= inter individual variability; IOV= inter occasion variability
No RSEs available due to failed covariance step

Impact of regimens on exposure

Table 4 lists the impact of the different regimens on exposure to thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide. The percentage dose corrected AUC of thiotepa increased 13% in the mini-CTC regimen compared to the CTC regimen due to saturable elimination, however, dose corrected exposure to tepa decreased 21%. This resulted in an overall reduction in dose corrected exposure to thiotepa and tepa of 11%. The Stamp V regimen caused a decrease in the percentage dose corrected AUC of thiotepa of 7% compared to the CTC regimen, while dose corrected exposure to tepa increased 10%. This resulted in an overall increase in dose corrected exposure to thiotepa and tepa of 5%.

The impact of the different regimens on exposure to cyclophosphamide and 4-hydroxycyclophosphamide was marginal. The percentage dose corrected AUCs were all around 100%. In the fractionated regimen, the exposure to 4-hydroxycyclophosphamide increases during treatment due to auto-induction. However, 4-hydroxycyclophosphamide formation is inhibited by thiotepa during this regimen. In the mini-CTC regimen auto-induction is negligible, while thiotepa is only administered after 24 hours. As a result, overall dose corrected exposure is almost identical. Moreover, when only the first 24 hours of the different regimens are taken into account, the exposure to 4-hydroxycyclophosphamide in the mini-CTC regimen is substantially higher (31% dose corrected) due to the absence of the interaction with thiotepa (Table 4).

Table 4. Impact of the different regimens on exposure to thiotepa (TT), tepa (T), cyclophosphamide (CP) and 4-hydroxycyclophosphamide (4OHCP).

	Regimen			
	CTC	**tiny-CTC**	**mini-CTC**	**Stamp V**
TT dose (mg/m^2)	480	320	250	500
Median AUC TT (90% PI[1]) (uM*h)	109 (54-206)	70 (36-132)	64 (31-124)	105 (54-199)
Percentage dose corrected AUC TT (%)	100	96	113	93
Median AUC T (90% PI) (uM*h)	267 (92-681)	185 (64-467)	110 (34-284)	307 (105-759)
Percentage dose corrected AUC T (%)	100	104	79	110
Median AUC TT+T (90% PI) (uM*h)	388 (170-817)	263 (116-554)	180 (76-375)	426 (186-888)
Percentage dose corrected AUC TT+T (%)	100	102	89	105
CP dose (mg/m^2)	6000	4000	3000	6000
Median AUC CP (90% PI) (uM*h)	6392 (4037-9917)	4101 (2605-6344)	3339 (2261-4940)	6808 (4238-10520)
Percentage dose corrected AUC CP (%)	100	96	104	107
Median AUC 4OHCP (90% PI) (uM*h)	137 (72-243)	94 (50-164)	67 (36-116)	132 (68-233)
Percentage dose corrected AUC 4OHCP (%)	100	103	98	96
CP dose (mg/m^2) in 24 h	1500	1000	3000	
Median AUC CP 24 h (90% PI) (uM*h)	1858 (1308-2564)	1216 (857-1677)	3119 (2200-4319)	nd[2]
Percentage dose corrected AUC CP 24 h (%)	100	98	84	
Median AUC 4OHCP 24 h (90% PI) (uM*h)	24 (12-45)	16 (8-31)	63 (34-110)	nd
Percentage dose corrected AUC 4OHCP 24 h (%)	100	100	131	

[1]90% PI, 90% prediction interval
[2]nd, not determined

Discussion

Alteration of administration regimens can have a profound effect on the pharmacokinetics of the compounds involved. We demonstrated that administration of thiotepa (250 mg/m^2/day, as one-hour infusion) as in the mini-CTC regimen compared to the CTC regimen (60 mg/m^2, twice daily, as 30-min infusion) leads to saturation of the metabolising enzymes involved in thiotepa metabolism.

Earlier studies of the pharmacokinetics of thiotepa show conflicting results with regard to dose dependent clearance. Both in conventional dose and in high-dose regimens saturable elimination of thiotepa has been described [18-21], while others report no saturable elimination [5;22-24]. Two studies in which patients were treated with thiotepa bolus doses ranging from 25-75 mg/m^2 show an inverse correlation of clearance with the dose, indicating dose-dependent elimination [19;20]. Hussein et al reported a lower clearance in patients treated with doses ranging from 750-900 mg/m^2 in a 1h infusion [18]. A decrease in

clearance at higher doses was also seen in the study of Henner et al in which patients were treated with doses ranging from 180-900 mg/m^2 in a 96h infusion [21]. On the other hand, Lazarus et al and Ackland et al, showed a linear increase in AUC in patients receiving doses ranging from 45-405 mg/m^2/day or 1.8-7.0 mg/kg/day in short infusions, respectively [22;24], although there was a trend toward reduced plasma clearance in the three patients treated at the highest dose level in the study of Ackland et al [22]. These studies suggest that the metabolising enzymes may be saturated at higher doses of thiotepa. The conflicting results found with respect to non-linearity may be due to differences in infusion duration and dose.

For cyclophosphamide we found no evidence for dose dependent clearance. These results are in agreement with other studies in which also no dose dependency of cyclophosphamide pharmacokinetics over a dose range of 6-80 mg/kg/10 min [25], 1500-3500 mg/m^2/30 min [26], 0.02-10 mg/kg/bolus [27], 30-60 mg/kg/bolus [28] and 5000 mg/0.5h [29] was found. However, Busse et al found that, although overall pharmacokinetics of cyclophosphamide were not affected during eightfold dose escalation (500 mg/m^2/1h compared with 100 mg/kg/1h), the fraction of the parent compound cleared by 4-hydroxylation was significantly reduced in favour of renal clearance and formation of 2-dechloroethylcyclophosphamide, indicating saturation of the metabolising enzymes responsible for 4-hydroxylation [6]. Non-linear elimination of cyclophosphamide has also been reported in 9 of 15 patients [30] and 7 of 12 patients [31] treated with 4000 mg/m^2/90 min. However, when the same patients were treated with 6000 mg/m^2/96h saturable elimination was not observed, probably because plasma concentrations of cyclophosphamide were much lower during the 96 h infusion [30;31]. Yule et al reported a prolonged half-life of cyclophosphamide at dose levels up to 2500 mg/m^2/1h [32].

The dose corrected exposures to thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide of the Stamp V regimen and the CTC regimen were comparable. The continuous inhibition of thiotepa on cyclophosphamide activation and the continuous auto-induction of cyclophosphamide in the Stamp V regimen (96-hour infusion) result in the same exposure as when cyclophosphamide and thiotepa are given in short infusions during 4 days. However, pharmacokinetic data of the Stamp V regimen were not available, therefore, these results should be interpreted with caution.

Although saturable elimination of thiotepa and a non-linear inhibition of cyclophosphamide clearance by thiotepa were observed in this study, the overall impact on dose corrected exposure to thiotepa, tepa, cyclophosphamide and 4-hydroxycyclophosphamide after the complete cycle was small. The unfractionated administration of cyclophosphamide in the mini-CTC regimen abolishes the favourable effect of autoinduction, which leads to increased metabolic activation of cyclophosphamide. However, this is compensated for by separating the administration of cyclophosphamide and thiotepa. This eliminates the inhibition of cyclophosphamide clearance by thiotepa. As a result, the overall dose corrected exposure to the active metabolite 4-hydroxycyclophosphamide is similar after the fractionated and unfractionated regimens. Thiotepa strongly inhibits cyclophosphamide activation, as can be seen in the percentage dose corrected AUC of 4-hydroxycyclophosphamide after 24 hours

(Table 4). In the mini-CTC regimen, where no thiotepa is present during the first 24 hours, the percentage dose corrected AUC of 4-hydroxycyclophosphamide is 31% higher than in the CTC regimen.

In conclusion, the simplified unfractionated mini-CTC regimen can be safely given to patients instead of the conventional fractionated CTC or tiny-CTC regimen. Percentage dose corrected exposure to the alkylating agents is comparable, but the mini-CTC regimen is more patient-friendly and lacks the pharmacokinetic interactions observed in the CTC regimen which makes it easier to apply. Moreover, toxicity observed during mini-CTC cycles was shown to be less than during CTC cycles, due to the reduced intensity of the chemotherapy agents in the mini-CTC regimen (unpublished).

Acknowledgement

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Chapter 4.4

Carbamazepine induces bioactivation of cyclophosphamide and thiotepa

Cancer Chemother Pharmacol. 2008

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Abstract

Purpose: We report a patient with metastatic breast cancer who received three cycles of high-dose chemotherapy with cyclophosphamide (1000 mg/m^2/day), thiotepa (80 mg/m^2/day) and carboplatin (dose calculated based on modified Calvert formula with 3.25 mg*min/mL as daily target AUC) over four days, followed by peripheral blood progenitor cell support. During the first two cycles the patient concomitantly used carbamazepine for treatment of epilepsy. Due to severe nausea and vomiting the patient was unable to ingest carbamazepine, therefore, this was discontinued after the second cycle.

Methods: Blood samples were drawn on two days (day 1 and 2, 3 or 4) of each cycle and plasma levels of cyclophosphamide, its active metabolite 4-hydroxycyclophosphamide, thiotepa, its main, active metabolite tepa and carboplatin were determined.

Results: Exposure to 4-hydroxycyclophosphamide and tepa on day one was increased in the presence of carbamazepine (58% and 75%, respectively), while exposure to cyclophosphamide and thiotepa was reduced (40% and 43%, respectively).

Conclusions: Since increased exposure to the active metabolites is associated with an increased risk of toxicity, it is important to be aware of this drug-drug interaction.

Introduction

High-dose chemotherapy with autologous peripheral blood progenitor cell support is used in the treatment of several haematological malignancies and solid tumours. Although haematological toxicity is manageable, significant end-organ toxicity may occur [1]. Interpatient variability in drug exposure has been shown to correlate with both toxicity and effectiveness of high-dose chemotherapy [2]. Since pharmacokinetics may correlate with outcome, drug-drug interactions between the cytotoxic agents and concomitant medication may be of clinical importance.
Cyclophosphamide is an inactive prodrug and is activated by cytochrome P450 to 4-hydroxycyclophosphamide. CYP2B6, 2C9, 2C19, 3A4 and 3A5 have been reported to be involved in the 4-hydroxylation, of which CYP2B6 is the most important. Furthermore, cyclophosphamide shows strong autoinduction, resulting in increased clearance of the parent compound and increased formation of its metabolites [3;4]. Thiotepa is metabolised by CYP2B6 and CYP3A4 to its main metabolite tepa [5]. Thiotepa and tepa have comparable alkylating activity [6].
Here we report the induction of cyclophosphamide and thiotepa metabolism due to carbamazepine, resulting in increased formation of the active metabolites, 4-hydroxycyclophosphamide and tepa, respectively.

Patient and method

Case

A 52-year-old female patient was diagnosed with metastatic breast cancer. As part of her treatment she received three cycles of high-dose chemotherapy with autologous peripheral blood progenitor cell transplantation. This regimen consisted of four days of chemotherapy with cyclophosphamide (1000 mg/m^2/day) as one-hour infusion, immediately followed by carboplatin (dose calculated based on modified Calvert formula with 3.25 mg*min/mL as daily target AUC) as one-hour infusion and thiotepa (80 mg/m^2/day) divided over two 30-min infusions twelve hours apart. Mesna (500 mg) was administered six times daily for a total of 36 doses, beginning one hour prior to the first cyclophosphamide infusion. The three cycles were given with four weeks intervals. The patient prophylactically received antibiotics (ciprofloxacin and fluconazole orally), starting four days before chemotherapy. Furthermore, during the chemotherapy cycles the patient received dexamethasone and granisetron. Full details of the tCTC regimen have been published previously [1].
During the first and second cycle the patient received carbamazepine (twice daily 200 mg slow release) and vigabatrin (twice daily 500 mg) orally for treatment of epilepsy. Due to severe nausea and vomiting, the patient was unable to ingest carbamazepine. Therefore, after the second tCTC cycle anti-epileptic treatment was stopped and not restarted until after the third tCTC cycle.

Pharmacokinetic analysis

For pharmacokinetic analyses, blood samples were collected from a double lumen intravenous catheter inserted in a subclavian vein. Samples were collected on day one and

two, three or four prior to the start of the infusions, at 30 min after the start of cyclophosphamide infusion (t=30) and t=60 (end of cyclophosphamide infusion), 90, 120 (end of carboplatin infusion), 150 (end of thiotepa infusion), 180, 210, 285, 390, 660 min and when possible at 24 h. Thiotepa, tepa and cyclophosphamide were quantified with a validated gas chromatographic assay as described previously. The lower limits of quantitation for thiotepa, tepa and cyclophosphamide were 5, 5 and 50 ng/mL, respectively [7]. 4-Hydroxycyclophosphamide was determined as a semicarbazone derivative with a previously described and validated high-performance liquid chromatography assay. The lower limit of quantitation was 50 ng/mL [8].
A previously published population pharmacokinetic model of thiotepa (and its metabolite tepa) and cyclophosphamide (and its metabolite 4-hydroxycyclophosphamide) was used for estimating the AUC of all compounds using Bayesian analysis [9]. The Committee on the Medical Ethics of the Netherlands Cancer Institute had approved the protocol and written informed consent was obtained from the patient.

Results

Plasma concentration versus time curves of cyclophosphamide and 4-hydroxycyclophosphamide of all three cycles are shown in Figure 1 in combination with the model fitted curves. As can be seen in Figure 1A, cyclophosphamide clearance on day 1 was increased in the presence of carbamazepine, resulting in a lower cyclophosphamide AUC with a concomitant increase in 4-hydroxycyclophosphamide AUC (Table 1). Furthermore, in the absence of carbamazepine, a more pronounced autoinduction of cyclophosphamide was observed. Cyclophosphamide clearance increased during the four days of cycle 3 (Figure 1A) and the C_{max} of 4-hydroxycyclophosphamide was increased on day 4 compared to day 1 (1.9 μg/mL vs 0.99 μg/mL) of cycle 3 (Figure 1B). In the presence of carbamazepine, no autoinduction could be identified.
Figure 2 shows the plasma concentration versus time curves of thiotepa and tepa. Thiotepa clearance was increased in the presence of carbamazepine (Figure 2A), resulting in a lower plasma exposure of thiotepa, with a concomitant increase in tepa AUC (Table 1).
Tables 1 and 2 show the effects of carbamazepine on the AUCs of cyclophosphamide, 4-hydroxycyclophosphamide, thiotepa and tepa on day one and during the whole course, respectively. From these tables it can be seen that the effects of carbamazepine on cyclophosphamide and thiotepa pharmacokinetics are more pronounced during the first day of the course.
The pharmacokinetics of carboplatin were similar during the three cycles. Besides severe nausea and vomiting during cycle 2 and 3, no other toxicity was observed during the three cycles.

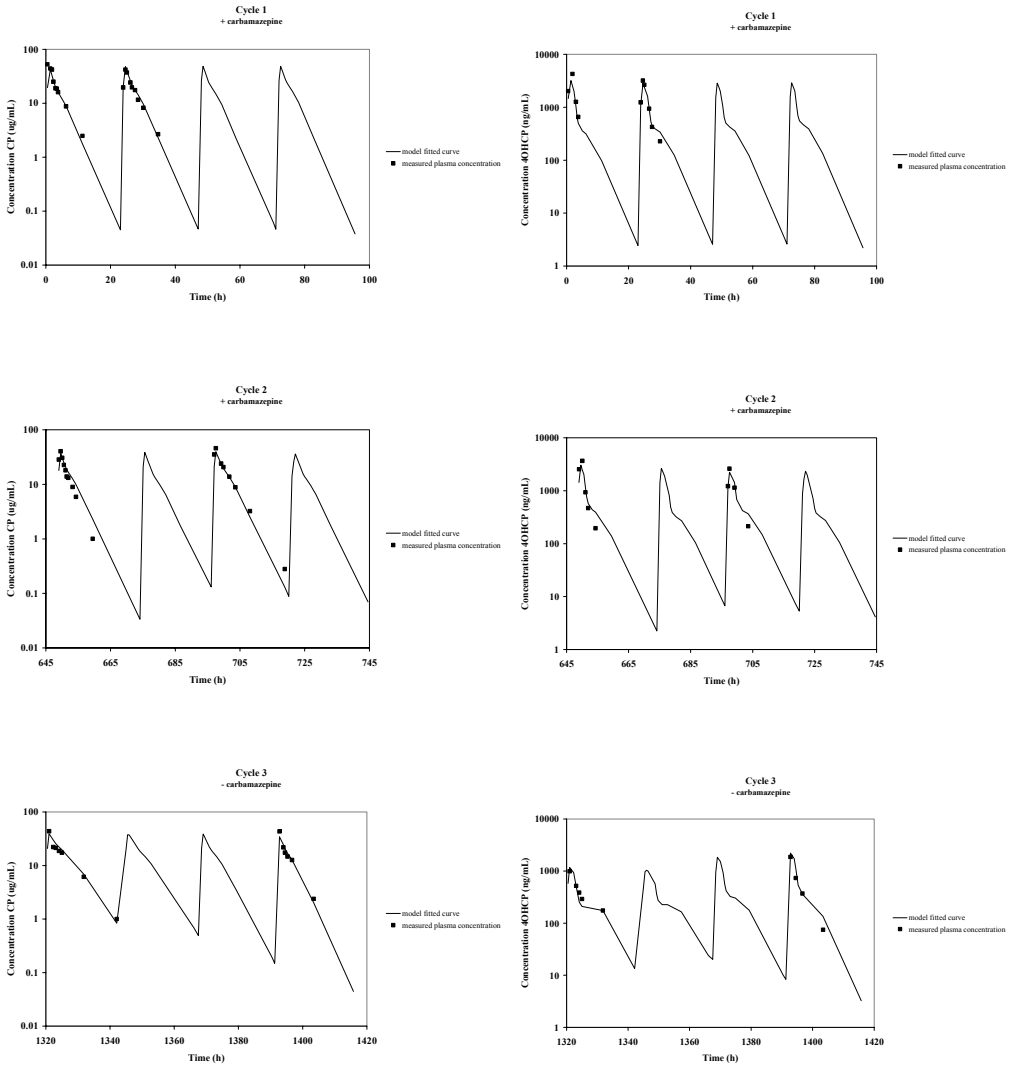

Figure 1. Plasma concentration versus time curves of **A**) cyclophosphamide (CP, 1780 mg) and **B**) 4-hydroxycyclophosphamide (4OHCP) in three high-dose tCTC chemotherapy cycles, the first and second cycle with carbamazepine and the third cycle without carbamazepine.

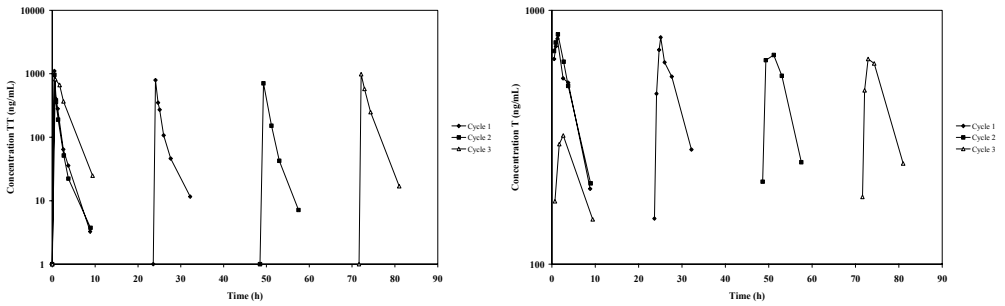

Figure 2. Plasma concentration versus time curves of **A**) thiotepa (TT, 70 mg) and **B**) tepa (T), in three high-dose tCTC chemotherapy cycles, with -♦- the first cycle (with carbamazepine), -■- the second cycle , (with carbamazepine) and -Δ- the third cycle (without carbamazepine).

Table 1. Effect of carbamazepine on the AUC of day 1 of cyclophosphamide, thiotepa and metabolites.

Compound	Cycle 1 and 2 with carbamazepine Mean AUC day 1 (AUC cycle 1-AUC cycle 2)	Cycle 3 without carbamazepine AUC day 1	Difference (%)
Cyclophosphamide	0.57 (0.59-0.56) mM*h	0.95 mM*h	-40
4-Hydroxycyclophosphamide	31.2 (31.8-30.5) uM*h	19.7 uM*h	58
Thiotepa	11.8 (12.3-11.4) uM*h	20.6 uM*h	-43
Tepa	50.3 (50.7-50.0) uM*h	28.8 uM*h	75

Table 2. Effect of carbamazepine on the overall AUC of cyclophosphamide, thiotepa and metabolites.

Compound	Cycle 1 and 2 with carbamazepine Mean AUC (AUC cycle 1-AUC cycle 2)	Cycle 3 without carbamazepine AUC	Difference (%)
Cyclophosphamide	2.5 (2.7-2.3) mM*h	3.1 mM*h	-19
4-Hydroxycyclophosphamide	122 (123-120) uM*h	95 uM*h	28
Thiotepa	46.4 (48.0-44.8) uM*h	94.0 uM*h	-51
Tepa	233 (228-239) uM*h	191 uM*h	22

Discussion

Here we report an increase in cyclophosphamide and thiotepa metabolism caused by co-administration of carbamazepine. Furthermore, in the presence of carbamazepine, autoinduction of cyclophosphamide is not detectable.

Carbamazepine acts as an anti-convulsant for partial and grand mal seizures. The activity of a variety of cytochrome P450 enzymes, including CYP1A2, CYP2B, CYP2C9, CYP2C19 and CYP3A4 is induced by carbamazepine [10-12].

Co-administration with carbamazepine is known to decrease values of mean peak plasma concentration and area under the plasma concentration-time curve of many drugs, resulting in a need to increase the dose of these agents. Carbamazepine and other anticonvulsant drugs have also shown to increase the systemic clearance of chemotherapy, resulting in lower efficacy [13].

The increase in cyclophosphamide and thiotepa clearance observed, may be caused by induction of the CYP enzymes 2B6 and 3A4 which are mainly involved in cyclophosphamide

and thiotepa metabolism. The absence of autoinduction of cyclophosphamide in the presence of carbamazepine is probably due to the metabolising enzymes already being induced by carbamazepine.

The effect of carbamazepine was more pronounced during the first day of the course. The overall AUC of the several compounds was only moderately affected by carbamazepine. This could be due to cyclophosphamide also being an inducer of CYP2B6 and CYP3A4, thereby increasing its own metabolism and the metabolism of thiotepa, if carbamazepine is absent, and therefore producing the same effect as carbamazepine [9].

For both cyclophosphamide and thiotepa, studies have demonstrated that alterations in liver CYP enzyme composition and activity have a major impact on the pharmacokinetics of these agents [14-17]. Xie et al found in their study into the pharmacogenetics of cyclophosphamide in patients with haematological malignancies one patient with a very high 4-hydroxylation activity. Different from the other patients, this patient was treated with carbamazepine [18]. No other reports have been published, to our knowledge, on the induction of cyclophosphamide and thiotepa metabolism by carbamazepine.

The patient in our report also received vigabatrin. It is unlikely that vigabatrin caused this interaction since vigabatrin does not induce the hepatic cytochrome P450 enzyme system [19].

This report shows that carbamazepine induces the metabolism of cyclophosphamide and thiotepa. This interaction will predominantly be important in single-dose administrations. Increases in exposure to the active metabolites could lead to increased toxicity.

Since the effect of changes in enzyme activity can vary substantially between patients and because of the unpredictability of the magnitude of this effect, it is recommended that carbamazepine is used with precaution with cyclophosphamide and thiotepa.

In a previous report a significant induction of cyclophosphamide and thiotepa metabolism by phenytoin was described [20]. When seizure control is necessary in patients receiving concomitant chemotherapy, drugs that do not influence the hepatic cytochrome P450 enzyme system (gabapentin, levetiracetam) are to be preferred [21].

Acknowledgement

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Abstract

Purpose: We report a patient with renal insufficiency (creatinine clearance (CL_{cr}) 38 mL/min) who received high-dose chemotherapy with cyclophosphamide (1500 mg/m^2/day), thiotepa (120 mg/m^2/day) and carboplatin (AUC=5 mg*min/mL/day) for four consecutive days.

Methods: Blood samples were collected on day one and three and plasma levels of cyclophosphamide, its active metabolite 4-hydroxycyclophosphamide, thiotepa, its main metabolite tepa and carboplatin were determined.

Results: Pharmacokinetic analyses indicated that the elimination of cyclophosphamide, thiotepa, carboplatin, but especially tepa was strongly reduced in this patient, resulting in increased exposures to these compounds of 67%, 43%, 30% and 157%, respectively, compared to a reference population (n=24) receiving similar doses. Exposure to 4-hydroxycyclophosphamide increased 11%.

Conclusion: These results suggest that it may not be necessary to alter the dose of cyclophosphamide in patients with moderate renal impairment. However, because high exposures to thiotepa and tepa have been correlated with increased toxicity, caution should be applied when administering thiotepa to patients with renal insufficiency.

Introduction

Cyclophosphamide and thiotepa are alkylating agents that belong to the most frequently used cytotoxic agents in high-dose chemotherapy regimens. A regimen that is commonly used in the Netherlands is the CTC regimen, which consists of cyclophosphamide (6000 mg/m^2), thiotepa (480 mg/m^2) and carboplatin (1600 mg/m^2) divided over four days administered in short infusions [1].
When using chemotherapeutic agents at the limit of non-haematological toxicity, there is a substantial risk of adverse outcome. A wide interpatient variability in toxicity of cyclophosphamide, thiotepa and carboplatin has been described, which can be explained in part by the interpatient variability in pharmacokinetics of the respective compounds. Various relations between exposure and toxicity or even efficacy have been identified [2].
Cyclophosphamide is a prodrug that needs to be activated by cytochrome P450 to 4-hydroxycyclophosphamide, which is the main route of metabolism. Both the metabolites and 12-30% of unchanged parent compound are eliminated in the urine [3]. Thiotepa is rapidly metabolised by cytochrome P450 to tepa, its main and active metabolite. Thiotepa and tepa have similar alkylating properties. Less than 2% of the administered dose of thiotepa is eliminated unchanged in the urine. Renal elimination of tepa accounts for approximately 11% of the administered dose [4;5]. Carboplatin is a platinum compound, with renal elimination accounting for almost all drug elimination. In patients with normal renal function, between 60 and 70% of an administered dose is excreted in the urine within the first 24 h of administration [6].
Renal insufficiency is a factor that may complicate drug dosing. When renal function is compromised, drug and metabolites eliminated through the kidneys will retain in the body and may accumulate to toxic levels with repeated dosing. Scarce information is available about the pharmacokinetics of (high-dose) cyclophosphamide and thiotepa in patients with renal insufficiency. Earlier studies have described the pharmacokinetics of cyclophosphamide in adults with renal dysfunction receiving either standard dose [7-11] or myeloablative doses of cyclophosphamide [12;13]. However, data are controversial. Minimal effects of renal dysfunction upon cyclophosphamide AUC have been reported [8;11;12], while others have shown an elevated AUC in those with impaired renal function [9;10;13]. A report in an anephric child indicated that off haemodialysis clearance of cyclophosphamide was similar to cyclophosphamide clearance in children with normal renal function [14]. Studies reporting the impact of renal dysfunction upon the metabolites of cyclophosphamide show that plasma alkylating activity was increased in patients with renal failure [7;10;11].
It has been shown that carboplatin clearance is decreased in patients with decreased renal function, resulting in increased toxicity [15]. Dose adjustment of carboplatin is required in these patients. The Calvert formula is widely used for carboplatin dosing based on the glomerular filtration rate [16].
In this report we describe the pharmacokinetics of cyclophosphamide, its active metabolite 4-hydroxycyclophosphamide, thiotepa, its active metabolite tepa and carboplatin in a patient with renal insufficiency.

Patient and method

Case

A 40-year-old male patient was diagnosed with Ewing sarcoma. As part of his treatment he received one course of high-dose chemotherapy with autologous peripheral blood progenitor cell transplantation, consisting of cyclophosphamide (1500 mg/m^2/day) as one-hour infusion, immediately followed by carboplatin (dose calculated based on modified Calvert formula (AUC (mg*min/mL) = Dose (mg) / (CL_{cr} (mL/min) + 25)) with 5 mg*min/mL as daily target AUC) as one-hour infusion and thiotepa (120mg/m^2/day) divided over two 30-min infusions twelve hours apart, for four consecutive days. Full details of the CTC regimen have been published previously [17].

The patient had normal cardiac, hepatic, haematopoietic and pulmonary function. Renal function was, however, impaired (calculated creatinine clearance 38 mL/min, using the Cockcroft-Gault equation [18]).

Pharmacokinetic analysis

For pharmacokinetic analyses, blood samples were collected from a double lumen intravenous catheter inserted in a subclavian vein. Samples were collected on day one and three prior to the start of the infusions, at 30 min after the start of cyclophosphamide infusion (t=30) and t=60 (end of cyclophosphamide infusion), 90, 120 (end of carboplatin infusion), 150 (end of thiotepa infusion), 180, 210, 285, 390 and 660 min. Samples were processed as described previously [19-21]. Analytical methods used for the determination of plasma concentrations of cyclophosphamide, 4-hydroxycyclophosphamide, thiotepa, tepa and carboplatin have been reported previously [19-21].

Population pharmacokinetic models of carboplatin, cyclophosphamide (and its metabolite 4-hydroxycyclophosphamide) and thiotepa (and its metabolite tepa) were used for calculating the pharmacokinetic parameters of all compounds using Bayesian analysis [2;22]. The pharmacokinetic parameters of the different compounds in this patient were compared with the respective median values in a reference population of patients (n=24) who also received CTC and were dosed (except for carboplatin, reference population received 400 mg/m^2/day) and sampled as described above. Complete pharmacokinetic profiles of the reference population were available of day one and day three or four. Exposure was defined as the cumulative exposure of the four-day course extrapolated to infinity. The protocol was approved by the Committee on the Medical Ethics of the Netherlands Cancer Institute and written informed consent was obtained from the patient.

Results

Administration of cyclophosphamide, thiotepa and carboplatin in this patient led to increased exposures to all compounds. Table 1 shows the median cumulative exposures following one course in both the population and the patient, the latter based on the pharmacokinetics obtained on the first day of the four-day course. Figure 1 shows the plasma concentration-time data of the patient versus the typical plasma concentration-time curve of the reference population [2;22].

Table 1. Overall exposure (expressed as AUC) to the different compounds and their metabolites during the course, if no dose adjustment would have been done.

Compound	AUC units	AUC patient	Median AUC reference population	2.5-97.5% range	Deviation (%)
Cyclophosphamide	uM*h	11467	6871	4355-10664	67
4-Hydroxycyclophosphamide	uM*h	157	142	105-186	11
Thiotepa	uM*h	185	129	81-201	43
Tepa	uM*h	648	252	108-427	157
Thiotepa and tepa	uM*h	833	384	222-584	117
Carboplatin	mg*min/mL	26	20[a]		30

[a]Target AUC value, reference population received carboplatin dose of 400 mg/m^2/day

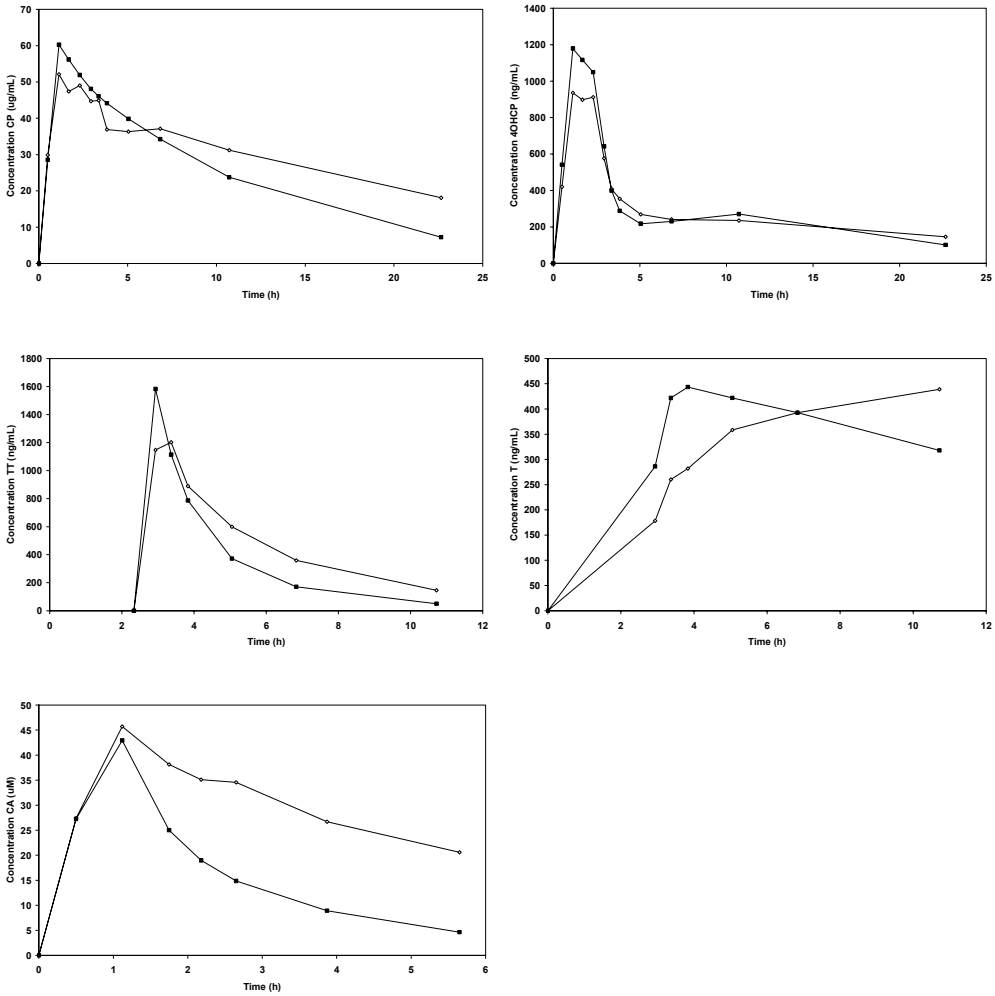

Figure 1. Concentration-time curves of **A**) cyclophosphamide (CP), **B**) 4-hydroxycyclophosphamide (4OHCP), **C**) thiotepa (TT), **D**) tepa (T) and **E**) carboplatin (CA), with -Δ- representing the patient and -■- the typical plasma concentration-time curve of the reference population.

Comparing pharmacokinetic parameters of cyclophosphamide, thiotepa and carboplatin in this patient with those in the reference population, it appeared that the renal clearance of cyclophosphamide and thiotepa decreased in the patient compared to the reference population with 28% and 36%, respectively (2.1 vs. 2.9 L/h and 15.9 vs. 24.9 L/h). This resulted in an increased exposure to cyclophosphamide and thiotepa of 67% and 43%, respectively. Exposure to 4-hydroxycyclophosphamide was only moderately increased, due to a minor decrease in elimination of 4-hydroxycyclophosphamide (7%) and a minor increase in metabolic fraction (11%), compared to the reference population. Exposure to tepa, however, increased dramatically (157%), mainly due to a decreased elimination rate constant of tepa (0.23 h^{-1} in the patient vs. 0.47 h^{-1} in the reference population). Although carboplatin was dosed based on creatinine clearance the exposure was still 30% above the target value (26 vs. 20 mg*min/mL).
Based on the pharmacokinetic analyses of day one of the course, the doses of cyclophosphamide, thiotepa and carboplatin were reduced on days three and four to reach a predefined target exposure (the median exposure in a reference population receiving similar doses) [23]. The cyclophosphamide dose was reduced from 2955 mg/day to 2550 mg/day, the thiotepa dose from 236 mg/day to 162 mg/day and the carboplatin dose from 315 mg/day to 275 mg/day. This resulted in an exposure closer to the target exposure. Combined exposure to thiotepa and tepa, however, was still 75% above the target. Renal function remained the same during the four days of chemotherapy.
The patient experienced grade 3 mucositis and diarrhoea. Furthermore, grade 2 aspartate aminotransferase (ASAT) and bilirubin toxicity was observed. In the reference population, three patients experienced grade 3 mucositis (12.5%), no patients had grade 3 diarrhoea, six patients experienced grade 2 ASAT toxicity (25%) and one patient had grade 2 bilirubin toxicity (4.2%). On day 43 post-transplantation the patient died due to Acute Respiratory Distress Syndrome.

Discussion

Renal insufficiency alters the pharmacokinetics of drugs. If drugs or their active metabolites are excreted by the kidneys they can accumulate in patients with renal insufficiency. In the patient reported here, increased exposures to cyclophosphamide, 4-hydroxycyclophosphamide, thiotepa, tepa and carboplatin were observed.
The patient described here experienced excessive exposure to tepa. Renal elimination of thiotepa accounts for only a small part of its elimination, for tepa, apparently, renal elimination is an important route. Toxicities associated with thiotepa treatment are mainly mucositis and central nervous system toxicity. The most frequently affected organs being the liver and gastrointestinal tract, resulting in nausea, vomiting and diarrhoea [4]. Moreover, a relation between ASAT toxicity and thiotepa AUC has been demonstrated [2]. Indeed, this patient experienced severe mucositis and diarrhoea and grade 2 ASAT toxicity, probably due to excessive exposure to thiotepa and tepa.
Clearance of cyclophosphamide was reduced, which resulted in an increase in cyclophosphamide exposure. Since cyclophosphamide itself is not active, increases in exposure to the active metabolites are more important for the interpretation of the clinical

relevance of this effect. The exposure to 4-hydroxycylophosphamide was only moderately increased, indicating no need to adjust the dose of cyclophosphamide in patients with moderate renal insufficiency. Our data are in agreement with the data of Juma et al who reported a lower clearance (-17%) and an increase in half-life (+24%) of cyclophosphamide in five patients with moderate to severe renal insufficiency (creatinine clearances 18-51 mL/min) compared to eight matched controls with normal renal function [10]. Similar results were reported by Haubitz et al who found a lower clearance (-28%) of cyclophosphamide in patients with creatinine clearances of 25 to 50 mL/min, which resulted in an increase in systemic drug exposure of 38% [9]. Mouridsen et al showed that the biotransformation rate is unaffected in patients with renal impairment [11]. Bramwell at el could not demonstrate a correlation between renal function and clearance of cyclophosphamide or its alkylating metabolites due to the large inter-individual variability in cyclophosphamide break-down seen in that study [8]. Further data regarding the impact of renal dysfunction upon the metabolites of cyclophosphamide are scarce. One study showed that total alkylating activity as measured by the nitrobenzylpyridine (NBP) reaction was significantly increased in renal failure [10]. However, this NBP reaction is highly non-specific and variable [24]. Therefore, no firm conclusions could be attributed to this result. A report about cyclophosphamide disposition in an anephric child showed that the exposure to 4-hydroxycylophosphamide off haemodialysis was in the same range as our results [14]. Clinically relevant changes in cyclophosphamide pharmacology due to alterations in renal function have not been demonstrated.

For carboplatin it is known that renal insufficiency causes increased exposure to carboplatin. Therefore a priori dose adjustments of carboplatin are performed. Although this patient had an adjusted dose of carboplatin based on his creatinine clearance, exposure to carboplatin was still 30% above the target exposure. Substitution of the glomerular filtration rate in the Calvert formula by an estimate of creatinine clearance calculated using the Cockcroft-Gault equation led to an overestimation of the carboplatin clearance in this patient. Froissart et al have shown that the use of the Cockcroft-Gault equation can lead to overestimation of the glomerular filtration rate in patients with GFR <60mL/min [25].

In conclusion, this case report demonstrates the pharmacokinetic disposition of cyclophosphamide, 4-hydroxycyclophosphamide, thiotepa, tepa and carboplatin in a patient with reduced renal function. Renal insufficiency in this patient resulted in a high exposure to thiotepa and especially tepa and is therefore of potential clinical importance. Additional pharmacokinetic and pharmacodynamic data are warranted in future studies to provide more accurate dosage recommendations for thiotepa in these patients.

Acknowledgement

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Chapter 4.5

Altered cyclophosphamide and thiotepa pharmacokinetics in a patient with moderate renal insufficiency

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Conclusions & perspectives

Conclusions and perspectives

High-dose chemotherapy in combination with peripheral blood progenitor cell transplantation has been developed as a potentially curative treatment modality in several solid tumours. The combination of cyclophosphamide, thiotepa and carboplatin (CTC) is a frequently employed high-dose regimen. This regimen has been used in the treatment of high-risk and metastatic breast cancer, metastatic ovarian cancer and germ cell cancer.
Although single and multiple courses of CTC chemotherapy are generally well tolerated, severe and sometimes life-threatening organ toxicity (e.g. veno-occlusive disease of the liver, hemorrhagic cystitis, mucositis, cardiotoxicity, ototoxicity and neuropathy), secondary tumours and long-term effects such as cognitive functioning impairment may occur. Large interpatient variability in toxicity has been observed and the occurrence of toxicity is largely unpredictable. Therefore, in order to reduce the risk for toxicity, several strategies for dose-individualisation have been explored. In previous studies, the pharmacokinetics of cyclophosphamide, thiotepa and carboplatin and their metabolites in the CTC regimen have been established and relations between toxicity and pharmacokinetics have been identified. This has resulted in a pharmacokinetically guided dosing strategy that reduced the variability in pharmacokinetics. However, this dosing strategy is very labour-intensive and difficult to apply in most hospital settings. Furthermore, all patients start with an unadjusted standard dose. Strategies for a priori identification of patients at risk for severe toxicity would therefore be preferable. Since cyclophosphamide and thiotepa are metabolized and detoxified by several enzymes, the genotype of these enzymes was expected to be a major determinant of pharmacokinetic variability and toxicity. In this thesis, we aimed to identify relations between the genotype of the metabolizing enzymes, pharmacokinetics and toxicity. It was demonstrated that the genotypes of drug metabolising enzymes involved in cyclophosphamide metabolism do not explain the interindividual variability observed in cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics. However, two variant alleles observed in the genes encoding enzymes involved in intracellular cyclophosphamide detoxification (ALDH1A1*2 and ALDH3A1*2) appeared to influence the occurrence of toxicity associated with cyclophosphamide administration. Patients heterozygous for these variant alleles had an increased risk of liver-toxicity and haemorrhagic cystitis compared to patients with wild-type alleles. Patients with these variant genotypes might benefit from a dose reduction or substitution of cyclophosphamide by another alkylating agent.
Thiotepa and tepa pharmacokinetics were only moderately influenced by the different genotypes of enzymes involved in their metabolism. The effect of the different genotypes on combined exposure to thiotepa and tepa (as a measure of total alkylating activity) was generally less than 20%. Patients homozygous for the GSTP1 C341T allele may have enhanced exposure to thiotepa and tepa. However, this needs to be confirmed in a larger cohort of patients. The evaluated variant alleles only explained a small part of the large interindividual variability in thiotepa and tepa pharmacokinetics.

Pharmacogenetics is a field in which the genetics of the individual patients is taken into consideration for individualized therapy. The aim is to improve the number of responders

and decrease the number of patients experiencing adverse drug reactions. Although the pharmacokinetics of the compounds of the CTC regimen are not affected by the variability in the presently evaluated genes to a large extent, it is apparent that variability in genes encoding drug metabolizing enzymes does affect outcome in drug treatment. In this respect, it is important not only to focus on the effect of polymorphisms on the plasma pharmacokinetics of the compounds involved, but also to investigate the intracellular processes since some enzymes are predominantly involved in the intracellular detoxification. If multiple enzymes are involved in the metabolism of a compound, however, it is hard to demonstrate an effect of a single polymorphism in one of these enzymes, because the reduced enzyme activity caused by a polymorphism can be compensated for by the other enzymes.

In clinical practice, carboplatin is dosed based on estimates of renal function as calculated using individual measured serum creatinine levels. We found no correlations between pharmacokinetic parameters of carboplatin and serum creatinine or serum creatinine derived measures of renal function. Furthermore, no strong relation between pharmacokinetic parameters of carboplatin and weight could be demonstrated. From our studies it is evident that when a targeted carboplatin exposure is desired, an a priori carboplatin dose in all adult patients (whether overweight/obese or not) with normal renal function can be calculated based on the mean population carboplatin clearance.

Although the role of high-dose chemotherapy in the adjuvant treatment of high-risk breast cancer is still undefined, a subgroup of patients exists, with HER-2/neu negative tumours, that show a survival benefit. Recent evidence suggests that tumour cells lacking a DNA-repair mechanism called homologous recombination are highly sensitive to this approach. High-dose chemotherapy is expensive and demanding for both the patient and the physicians and nurses. If one could identify patients whose tumours are extremely sensitive to this approach and if one could also develop a regimen with alkylating agents that is less toxic than the previously used high-dose chemotherapy regimens, alkylating agents might become again important for some breast cancer patients. For this purpose the mini-CTC regimen was developed. In a feasibility study it was demonstrated that the mini-CTC regimen can be administered without lengthy hospital stays and that the toxicity, apart from bone marrow depression and fatigue, was similar to that of frequently employed standard regimens in breast cancer. We have demonstrated that this unfractionated regimen results in similar exposures to the alkylating agents employed, compared to the conventionally used fractionated regimen and that it is easier to apply since pharmacokinetic interactions between the compounds are negligible in this unfractionated regimen.
Cyclophosphamide, thiotepa and the non-classical alkylator carboplatin are so-called bi-functional alkylating agents. They form inter- and intrastrand DNA cross links that can be repaired by homologous recombination. This is the only reliable repair mechanism that can repair the consequences of DNA adducts caused by bifunctional alkylating agents. Deficiency of homologous recombination is present in breast cancer cells but not in healthy cells of BRCA-1 or BRCA-2 mutation carriers, and also in up to 30% of sporadic breast cancers. It is assumed that deficiency of homologous recombination renders tumour cells

highly sensitive to alkylating agents. Several studies of high-dose chemotherapy suggest that there exists a group of breast cancers, possibly 20 to 30% of all cases, that are exquisitely sensitive to alkylating agents. Further studies are ongoing to investigate the assumption that this group of breast cancers possesses the homologous recombination deficiency, which would then especially benefit from the mini-CTC regimen. By determining tumour characteristics and polymorphisms in ALDH genes and possibly GST genes, this regimen can be safely administered to patients most likely to benefit.
Though certainly not the treatment of choice for most patients, high-dose chemotherapy with CTC might still be beneficial for a select group of patients.

Summary

Samenvatting

Summary

High-dose chemotherapy in combination with peripheral blood progenitor cell transplantation has been developed as a possible curative treatment modality in several solid tumours. Although the role of high-dose chemotherapy in the adjuvant treatment of high-risk breast cancer is still undefined, a subgroup of patients exists, with HER-2/neu negative tumours, that show a survival benefit. Recent evidence suggests that tumour cells lacking a DNA-repair mechanism called homologous recombination are highly sensitive to this approach.
A frequently used high-dose regimen in solid tumours in the Netherlands is the CTC regimen, which is a 4-day course of cyclophosphamide, thiotepa and carboplatin. High-dose chemotherapy is demanding for the patient. Severe and sometimes life-threatening toxicities such as severe mucositis, veno-occlusive disease of the liver, oto-, neuro-, cardio-, hepato-, renal and pulmonary toxicity may occur after high-dose chemotherapy. Therefore, treatment optimization in order to prevent or reduce toxicity is necessary. Relations between systemic exposure and toxic effects have been demonstrated for the components of the CTC regimen. On the basis of these relations a strategy for rapid dose adjustments based on the individual pharmacokinetic parameters has been developed. This strategy reduced the variability in exposure to the cytotoxic agents and a reduction in the occurrence of severe liver toxicity was suggested. This dosing strategy, however, proved to be very labour-intensive and difficult to apply in most hospital settings. Strategies for *a priori* identification of patients at risk for severe toxicity would, therefore, be preferable. Pharmacogenetics focuses on the question to what extent variability in genetic background is responsible for the observed interindividual variability in pharmacokinetics and toxicity and might provide a tool for the *a priori* identification of patients at risk.
The objectives of this thesis were to identify relations between the genotype of the metabolising enzymes involved in cyclophosphamide, thiotepa and carboplatin metabolism, pharmacokinetics and toxicity in order to identify patients at risk for severe toxicity or under-treatment and, when possible, to develop a strategy for safe dosing of the agents included in the high-dose CTC regimen.
In **Chapter 1.1**, an overview of studies describing relations between polymorphisms in drug metabolising enzymes and drug transporters and survival after cancer drug treatment is provided. Variability in drug response among patients after cancer treatment are common and this is attributable to many factors, including genetic polymorphisms in drug metabolising enzymes and drug transporters. The most important polymorphisms shown to influence survival after cancer treatment are polymorphisms in the genes encoding the phase II detoxification enzymes glutathione S-transferases (GSTs). An association between GSTM1 null and GSTT1 null and longer overall survival in patients with different malignancies who are treated with substrates for these GSTs has been shown. The studies presented in Chapter 1 suggest that genetic polymorphisms may provide useful prognostic markers in some situations. More insight into the mechanism of action of these markers, the biological determinants of response to treatment and prognosis in cancer may ultimately lead to individualised cancer treatment based on a combination of genotype and tumour characteristics of a patient.

To study the clinical pharmacology of cyclophosphamide in a large cohort of patients a fast and robust method quantifying all necessary compounds was required. Since cyclophosphamide is a prodrug, it is also essential to monitor the concentrations of its active metabolite 4-hydroxycyclophosphamide. In **Chapter 2.1**, the development and validation of a method for the simultaneous determination of cyclophosphamide and 4-hydroxycyclophosphamide concentrations in plasma samples is described. Liquid chromatography with tandem mass spectrometry was used. This highly selective and specific method allowed accurate and precise quantification of cyclophosphamide and 4-hydroxycyclophosphamide with a high sample throughput and minimal sample clean-up procedures. The developed assay was validated according to the FDA guidelines for bioanalytical validation and was used to support the pharmacogenetic and pharmacokinetic studies described in the subsequent chapters.

Pharmacogenetics

Thiotepa and cyclophosphamide are alkylating anticancer agents often co-administered in high-dose chemotherapy regimens for the treatment of advanced or metastatic breast, ovarian and testis tumours. Previous studies have shown relations between the pharmacokinetics of thiotepa and cyclophosphamide and severe organ toxicity. Hence, identification of factors that influence the pharmacokinetics of these compounds could aid in individual dose selection. Thiotepa is metabolised by cytochrome P450 and glutathione S-transferase enzymes. Polymorphisms of these enzymes may affect elimination of thiotepa and tepa, its main metabolite. The influence of polymorphisms in the CYP2B6, CYP3A4, CYP3A5, GSTA1 and GSTP1 genes on the pharmacokinetics of thiotepa and tepa is described in **Chapter 3.1**. Clearance of thiotepa and tepa was predominantly affected by the GSTP1 C341T polymorphism. Patients homozygous for the variant allele had an increased exposure to thiotepa and tepa of 45% compared to patients with the wild-type genotype. The presently evaluated variant alleles, however, only explained a small part of the substantial interindividual variability in thiotepa and tepa pharmacokinetics.

Cyclophosphamide is metabolised by cytochrome P450, glutathione S-transferase and aldehyde dehydrogenase enzymes. The influence of polymorphisms in the CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1 genes on the pharmacokinetics of cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide is described in **Chapter 3.2.** The presently evaluated variant alleles in the aforementioned genes did not explain the interindividual variability in cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics.

To obtain more insight into the interindividual variability in toxicity observed after treatment with CTC chemotherapy, relations between polymorphisms in drug metabolising enzymes and toxicity were studied. Sixteen selected polymorphisms in nine genes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) of putative relevance in cyclophosphamide, thiotepa and carboplatin metabolism were assessed. Patients heterozygous for the ALDH3A1*2 and ALDH1A1*2 allele had an increased risk of haemorrhagic cystitis and liver-toxicity, respectively, compared to patients with wild-type alleles when treated with a high-dose chemotherapy combination of cyclophosphamide, thiotepa and carboplatin (**Chapter 3.3**). The ALDH enzymes are

important in the intracellular detoxification of cyclophosphamide. Therefore, although no effect of polymorphisms in these enzymes on the plasmapharmacokinetics of cyclophosphamide could be demonstrated, differences in susceptibility to toxicity might occur due to differences in intracellular detoxification of the active cyclophosphamide metabolites. This study suggests that pharmacogenetic approaches can identify patients who are at risk of experiencing toxic side-effects in high-dose chemotherapy.

Pharmacokinetics

Besides genetic polymorphisms, other factors may also influence the disposition of a compound. Carboplatin clearance has been shown to be linearly related to the glomerular filtration rate. Therefore, various dosing formulae have been proposed to calculate *a priori* an appropriate dose for a target exposure of carboplatin based on estimates of renal function as calculated using individual measured serum creatinine levels. This practice, which is routinely used in many hospitals, was retrospectively validated in a large heterogeneous adult patient population (**Chapter 4.1**). No correlations could be found between pharmacokinetic parameters of carboplatin and serum creatinine or from serum creatinine derived measures of renal function. Therefore, it is proposed to calculate the *a priori* carboplatin dose based on the population carboplatin clearance in patients with normal renal function.

In **Chapter 4.2**, the potential utility of alternative weight descriptors in the Cockcroft-Gault equation to more accurately predict carboplatin clearance in underweight, normal weight, overweight and obese patients was determined. Evaluation of diverse weight descriptors indicated that lean body mass was the best weight descriptor in underweight and normal weight patients, while adjusted ideal body weight was the best weight descriptor in overweight and obese patients. However, a flat dose based on the population carboplatin clearance performed better in all weight categories than the use of the Cockcroft-Gault equation with diverse weight descriptors.

A new simplified 2-day unfractionated regimen (mini-CTC) was proposed as alternative for the conventional 4-day fractionated high-dose CTC regimen. Alteration of administration regimens can have a profound effect on the pharmacokinetics of the compounds involved. It was shown that in the unfractionated regimen saturation of thiotepa elimination occurred. Elimination of cyclophosphamide was not shown to be saturable. Furthermore, a non-linear inhibition of cyclophosphamide clearance by thiotepa was observed. Since overall dose corrected exposure to thiotepa and cyclophosphamide was not affected to a relevant extent and since this regimen is more patient-friendly the simplified unfractionated mini-CTC regimen can be safely used in future studies (**Chapter 4.3**).

Both cyclophosphamide and thiotepa are metabolised by cytochrome P450 enzymes. Since patients treated with cyclophosphamide and thiotepa often receive co-medicated agents which may inhibit or induce the activity of CYP enzymes, it is important to be aware of pharmacokinetic interactions. **Chapter 4.4** describes a case report of a patient who received three cycles of chemotherapy with cyclophosphamide, thiotepa and carboplatin. During the first two cycles the patient concomitantly used the CYP-inducer carbamazepine. It was observed that exposure to the active metabolites 4-hydroxycyclophosphamide and tepa was increased in the presence of carbamazepine. Increases in exposure to the active

metabolites could lead to increased toxicity. Since the effect of changes in enzyme activity can vary substantially between patients and because of the unpredictability of the magnitude of this effect, it is recommended that carbamazepine is used with precaution with cyclophosphamide and thiotepa.
In **Chapter 4.5**, a case report is presented of a patient with moderate renal insufficiency who received CTC chemotherapy. Pharmacokinetic analyses indicated that the elimination of cyclophosphamide, thiotepa, carboplatin, but especially tepa was strongly reduced in this patient, resulting in increased exposures to these compounds, compared to a reference population receiving similar doses. Exposure to 4-hydroxycyclophosphamide was only moderately increased. Therefore, it may not be necessary to alter the dose of cyclophosphamide in patients with moderate renal impairment. However, because high exposures to thiotepa and tepa have been correlated with increased toxicity, caution should be applied when administering thiotepa to patients with renal insufficiency.

In conclusion, the major aim of this thesis was to identify relations between the genotype of the metabolising enzymes, pharmacokinetics and toxicity of the components of the CTC regimen. Although the pharmacokinetics of the components of the CTC regimen are not affected by the variability in the presently evaluated genes to a large extent, it is apparent that variability in genes encoding drug metabolising enzymes does affect the occurrence of toxicity. Pharmacogenetic approaches have potential for identifying patients who are at a higher risk of experiencing toxic side-effects in high-dose chemotherapy.

Samenvatting

Hogedosis chemotherapie met ondersteuning van stamceltransplantatie is ontwikkeld als mogelijk curatieve behandelingsstrategie voor bepaalde tumoren. Hoewel de exacte plaats van hogedosis chemotherapie in de adjuvante behandeling van borstkanker nog niet is vastgesteld, bestaat er een subgroep van patiënten, met HER-2/neu negatieve tumoren, die mogelijk baat hebben bij deze intensieve vorm van chemotherapie. Recente studies laten zien dat tumorcellen zonder een bepaald DNA reparatiemechanisme (homologe recombinatie) gevoelig zijn voor alkylerende middelen. Of patiënten met defecten in dit DNA reparatiemechanisme de groep is die baat heeft bij hogedosis chemotherapie wordt momenteel onderzocht.
Een veel gebruikte combinatie in de behandeling van solide tumoren in Nederland is het CTC schema. Dit is een 4-daags schema met cyclofosfamide, thiotepa en carboplatine. Hogedosis chemotherapie vraagt veel van patiënten, het kan ernstige en soms zelfs levensbedreigende toxiciteit veroorzaken, zoals ernstige beschadiging van de lever, nieren, longen, gehoor en blaas. Optimalisatie van de behandeling is nodig om toxiciteit te voorkomen of te verminderen.
In eerdere studies zijn relaties aangetoond tussen de blootstelling aan de middelen in het CTC schema en het optreden van toxiciteit. Op basis van deze relaties is een doseerstrategie ontwikkeld gebaseerd op de individuele blootstelling (farmacokinetiek). Hierdoor is de variatie in blootstelling aan de middelen in het CTC schema verminderd en is mogelijk ook het optreden van ernstige schade aan de lever afgenomen. Deze doseerstrategie is echter erg arbeidsintensief en moeilijk uit te voeren in de meeste ziekenhuizen. Strategieën die van te voren kunnen voorspellen of een patiënt een hoog risico op toxiciteit heeft, hebben deze nadelen mogelijk niet.
Farmacogenetica is het vakgebied dat zich bezig houdt met de vraag in hoeverre variaties in het erfelijk materiaal (polymorfismen) verantwoordelijk zijn voor variatie in de blootstelling aan geneesmiddelen en het optreden van toxiciteit, en zou een belangrijk hulpmiddel kunnen zijn voor het *a priori* identificeren van patiënten met een hoog risico op toxiciteit.
Het doel van dit proefschrift was het onderzoeken van relaties tussen polymorfismen in de enzymen die betrokken zijn bij het metabolisme van cyclofosfamide, thiotepa en carboplatine, de farmacokinetiek en toxiciteit van deze middelen om op voorhand patiënten te identificeren met een verhoogd risico op toxiciteit of onderbehandeling.
Hoofdstuk 1.1 geeft een literatuuroverzicht van studies die relaties tussen polymorfismen in metaboliserende enzymen en geneesmiddeltransporters en overleving hebben onderzocht. Patiënten reageren verschillend op behandeling met chemotherapie. Dit heeft verschillende oorzaken, waaronder polymorfismen in metaboliserende enzymen en geneesmiddeltransporters. Voornamelijk polymorfismen in de fase II detoxificerende glutathion S-transferase enzymen blijken een verschil in overleving te veroorzaken na behandeling met chemotherapie. Zo laten patiënten met deleties in GSTM1 en GSTT1 een langere overleving zien na behandeling met substraten voor deze enzymen. De studies beschreven in hoofdstuk 1 suggereren dat genetische variatie in sommige situaties bruikbare prognostische markers kan opleveren. Meer inzicht in het werkingsmechanisme van deze markers, de determinanten van behandelrespons en prognose van kanker zou

uiteindelijk kunnen leiden tot individuele behandeling van patiënten gebaseerd op genotype en tumor karakteristieken van de patiënt.
Om de farmacokinetiek van cyclofosfamide in een grote groep patiënten te kunnen bestuderen is een snelle en robuuste analysemethode nodig. Omdat cyclofosfamide zelf niet actief is, is het van belang ook de plasmaconcentraties van de geactiveerde metaboliet 4-hydroxycyclofosfamide te bepalen. In **hoofdstuk 2.1** wordt de ontwikkeling en validatie van een methode voor de gelijktijdige bepaling van cyclofosfamide en 4-hydroxycyclofosfamide in plasma beschreven. Er werd gebruik gemaakt van vloeistofchromatografie met massaspectrometrische detectie. Op deze manier konden cyclofosfamide en 4-hydroxycyclofosfamide snel, accuraat en met minimale monstervoorbewerking bepaald worden. Deze methode werd gevalideerd volgens FDA richtlijnen en werd vervolgens gebruikt om de farmacogenetica en farmacokinetiek studies beschreven in de volgende hoofdstukken te ondersteunen.

Farmacogenetica
Thiotepa en cyclofosfamide zijn alkylerende chemotherapeutica die vaak samen worden toegediend in hogedosis chemotherapie schema's voor de behandeling van borst-, eierstok- of zaadbalkanker. In eerdere studies zijn relaties aangetoond tussen de farmacokinetiek van thiotepa en cyclofosfamide en ernstige orgaantoxiciteit. Het bepalen van factoren die de farmacokinetiek van deze middelen beïnvloeden, zou een belangrijke bijdrage kunnen leveren aan verbetering van de behandeling. Thiotepa wordt gemetaboliseerd door cytochroom P450 (CYP) en glutathion S-transferase (GST) enzymen. Polymorfismen in deze enzymen kunnen de uitscheiding van thiotepa en tepa, de belangrijkste en actieve metaboliet, beïnvloeden. **Hoofdstuk 3.1** beschrijft de invloed van polymorfismen in CYP2B6, CYP3A4, CYP3A5, GSTA1 en GSTP1 enzymen op de farmacokinetiek van thiotepa en tepa. Uitscheiding van thiotepa en tepa wordt voornamelijk beïnvloed door het GSTP1 C341T polymorfisme. Patiënten die homozygoot zijn voor deze variant hadden een 45% hogere blootstelling aan thiotepa en tepa dan patiënten met het wildtype genotype. De bestudeerde polymorfismen verklaarden echter maar een klein deel van de grote interindividuele variatie in thiotepa en tepa farmacokinetiek.
Cyclofosfamide wordt gemetaboliseerd door cytochroom P450, glutathion S-transferase en aldehyde dehydrogenase enzymen. De invloed van polymorfismen in CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 en ALDH3A1 enzymen op de farmacokinetiek van cyclofosfamide en 4-hydroxycyclofosfamide wordt beschreven in **hoofdstuk 3.2**. De bestudeerde polymorfismen bleken de interindividuele variabiliteit in cyclofosfamide en 4-hydroxycyclofosfamide farmacokinetiek niet te kunnen verklaren.
Om meer inzicht te krijgen in het optreden van toxiciteit tussen patiënten behandeld met CTC chemotherapie, werden relaties tussen polymorfismen in metaboliserende enzymen en het optreden van toxiciteit onderzocht. Zestien polymorfismen in negen genen (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 en ALDH3A1) van belang voor het metabolisme van cyclofosfamide, thiotepa en carboplatine werden bestudeerd. Patiënten met het heterozygote ALDH3A1*2 en ALDH1A1*2 genotype hadden, respectievelijk, een verhoogd risico op blaastoxiciteit en levertoxiciteit vergeleken met patiënten met het wildtype genotype na behandeling met cyclofosfamide, thiotepa en

carboplatine (**Hoofdstuk 3.3**). De ALDH enzymen zijn betrokken bij de intracellulaire afbraak van cyclofosfamide. Verschillen in intracellulaire afbraak van actieve cyclofosfamide metabolieten kunnen verschillen in het optreden van toxiciteit veroorzaken. Dus hoewel er geen effect van polymorfismen op de plasma-farmacokinetiek van cyclofosfamide te zien was, was er wel een effect op het optreden van toxiciteit. Deze studie liet zien dat farmacogenetica van belang kan zijn voor het identificeren van patiënten met een verhoogd risico op toxiciteit na CTC chemotherapie.

Farmacokinetiek

Naast genetische variatie zijn ook andere factoren van invloed op de farmacokinetiek van chemotherapeutica. Zo is de uitscheiding van carboplatine gerelateerd aan de nierfunctie. Op basis hiervan zijn verschillende doseerformules opgesteld om vooraf een carboplatine dosis te berekenen die een bepaalde blootstelling aan carboplatine geeft. Deze doseerformules zijn gebaseerd op schattingen van de nierfunctie berekend aan de hand van serum creatinine spiegels. Deze doseerstrategie, die routinematig wordt toegepast in veel ziekenhuizen, wordt besproken in **hoofdstuk 4.1**. Er kon geen relatie worden aangetoond tussen farmacokinetische parameters van carboplatine enerzijds en serum creatinine of van serum creatinine afgeleide schattingen van de nierfunctie anderzijds. Wanneer een bepaalde carboplatine blootstelling in een patiënt met normale nierfunctie gewenst is, wordt voorgesteld om de *a priori* carboplatine dosis te berekenen aan de hand van de populatieklaring van carboplatine.

In **hoofdstuk 4.2** wordt gekeken of het gebruik van alternatieve gewichtsmaten in de Cockcroft-Gault vergelijking de schatting van carboplatine-uitscheiding in patiënten met ondergewicht, normaal gewicht, overgewicht en obesitas kan verbeteren. Hieruit kwam naar voren dat in patiënten met ondergewicht en normaal gewicht, “lean body mass” de beste gewichtsmaat was en in patiënten met overgewicht en obesitas, “adjusted ideal body weight”. Dit nam echter niet weg dat de individuele carboplatine-uitscheiding een betere relatie vertoonde met de populatieklaring dan met de carboplatine-uitscheiding berekend aan de hand van de Cockcroft-Gault formule met diverse gewichtsmaten.

Om de toediening van de CTC chemotherapie patiëntvriendelijker te maken en de behandeling verder te optimaliseren was het gebruikelijke 4-daagse gefractioneerde CTC schema versimpeld tot een 2-daags ongefractioneerd schema (mini-CTC). Deze verandering van toedieningsschema kan echter van invloed zijn op de farmacokinetiek van chemotherapeutica. Het bleek dat het nieuwe ongefractioneerde schema leidde tot verzadiging van de thiotepa uitscheiding. Cyclofosfamide uitscheiding bleek met dit nieuwe schema niet verzadigbaar. Verder was er een niet-lineaire inhibitie van cyclofosfamide uitscheiding door thiotepa te zien. Aangezien de blootstelling aan thiotepa en cyclofosfamide in het nieuwe mini-CTC schema vergelijkbaar was met het standaard gefractioneerde CTC schema en aangezien het nieuwe schema patiëntvriendelijker is, kan dit nieuwe schema in toekomstige studies naar hogedosis chemotherapie gebruikt worden (**Hoofdstuk 4.3**).

Zowel cyclofosfamide als thiotepa worden gemetaboliseerd door cytochroom P450 enzymen. Omdat patiënten die met cyclofosfamide en thiotepa behandeld worden vaak co-medicatie krijgen die de activiteit van deze cytochroom P450 enzymen kunnen stimuleren of remmen, is het belangrijk op de hoogte te zijn van mogelijke farmacokinetische interacties.

In **hoofdstuk 4.4** wordt een patiënt beschreven die behandeld werd met 3 cycli chemotherapie met cyclofosfamide, thiotepa en carboplatine. Gedurende de eerste 2 cycli gebruikte de patiënt het cytochroom P450 inducerende anti-epilepticum carbamazepine. Dit leidde tot verhoogde blootstelling aan de actieve metabolieten 4-hydroxycyclofosfamide en tepa. Verhoogde blootstelling aan de actieve metabolieten kan leiden tot een verhoogd risico op toxiciteit. Omdat het effect van veranderde enzymactiviteit tussen mensen een groot verschil vertoont en omdat de grootte van dit effect moeilijk voorspelbaar is, wordt aanbevolen voorzichtig te zijn met het gebruik van carbamazepine tijdens chemotherapie met cyclofosfamide en thiotepa.
Hoofdstuk 4.5 beschrijft een patiënt met verminderde nierfunctie die behandeld werd met CTC chemotherapie. De uitscheiding van cyclofosfamide, thiotepa, carboplatine, maar vooral tepa, was sterk verminderd in deze patiënt, wat resulteerde in een verhoogde blootstelling aan deze componenten vergeleken met een referentie populatie die dezelfde doseringen kreeg. Blootstelling aan 4-hydroxycyclofosfamide was beperkt veranderd. Hierdoor is het niet noodzakelijk de dosering van cyclofosfamide in patiënten met verminderde nierfunctie aan te passen. Echter, daar verhoogde blootstelling aan thiotepa en tepa in verband is gebracht met een verhoogd risico op toxiciteit zou men voorzichtig moeten zijn met de toediening van thiotepa aan patiënten met verminderde nierfunctie.

Concluderend, het doel van dit proefschrift was relaties tussen polymorfismen in metaboliserende enzymen, de farmacokinetiek en toxiciteit van CTC chemotherapie te onderzoeken. Hoewel de farmacokinetiek van deze chemotherapeutica niet zozeer door polymorfismen in metaboliserende enzymen beïnvloed wordt, zorgen deze polymorfismen wel voor een verschil in het optreden van toxiciteit. Voor patiënten die hogedosis chemotherapie krijgen, kan het bepalen van polymorfismen in metaboliserende enzymen dus van belang zijn om patiënten te identificeren met een verhoogd risico op toxiciteit.

Dankwoord

Curriculum Vitae

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Dankwoord

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Corine

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Curriculum Vitae

Corine Ekhart werd op 8 juli 1976 geboren te Bovensmilde. In 1994 behaalde zij het VWO diploma aan het Han Fortmann College in Heerhugowaard. Vervolgens studeerde zij farmacie aan de Universiteit Utrecht. De doctoraalopleiding werd afgerond met een wetenschappelijke stage in het Western General Hospital te Edinburgh. In 2004 behaalde zij het apothekersdiploma. In datzelfde jaar begon zij aan het promotieonderzoek dat is beschreven in dit proefschrift, onder begeleiding van Prof. Dr J.H. Beijnen, Prof. Dr S. Rodenhuis en Dr A.D.R. Huitema.

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