Pharmacokinetics of novel anticancer drugs and dynamics of circulating tumor cells in early clinical studies

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Pharmacokinetics of novel anticancer drugs and dynamics of circulating tumor cells in early clinical studies

Farmacokinetiek van nieuwe anti-kanker middelen en dynamiek van circulerende tumor cellen in vroeg-klinische studies (met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 30 november 2011 des middags te 2.30 uur

door

Lotje Antonetta Devriese

geboren op 20 september 1977 te Velsen

Promotoren: Prof.dr. J.H.M. Schellens
Prof.dr. E.E. Voest

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“If the history of medicine is told through stories of doctors, it is because their contributions stand in place of the more substantive heroism of their patients”

by Siddhartha Mukherjee
in “The emperor of all maladies: a biography of cancer”

CONTENTS

Chapter 1

General Introduction

GENERAL INTRODUCTION

Nearly 12.7 million persons per year are confronted worldwide with the diagnosis of cancer. The impact cancer has on the lives of cancer patients and on society is enormous. Within the European Union, 2.4 million inhabitants per year are diagnosed with cancer and 1.2 million people die as a result of cancer every year. It has been estimated that, currently, the risk of getting cancer before the age of 75 years is 18.7% [1]. These numbers illustrate the imperative to improve therapies for the treatment of cancer.

Anti-cancer treatment is based on combinations of surgery, radiotherapy and drug therapy. Previously, systemic oncolytic therapy used to consist only of chemotherapy with cytotoxic drugs and/or hormonal treatment. In the past decades, the insight in the pathobiology of cancer initiation and its progression has increased largely. Such has resulted in the identification of several proteins and intracellular signal transduction pathways that have been shown to be essential for cancer cell survival. These molecules have therefore become exposed as a specific target for anti-cancer therapy [2]. Nowadays, many new drugs are being developed as “targeted” therapy and these drugs are currently being used in the clinic next to conventional chemotherapy.

This thesis is focused on early clinical studies in the development anti-cancer drugs and reports on two aspects of drug development. The first part of the thesis encompasses phase I pharmacokinetic trials of novel anti-cancer drugs (chapters 2-5). Clinically relevant questions were studied and recommended doses of specific anti-cancer drugs for patients with renal- (chapter 2.2) and hepatic impairment (chapter 3.1) were established. Also, potential drug-drug interactions in oral (chapter 2.1) and intravenous anti-cancer drugs (chapters 3.2 and 3.3) were examined. Next to this, relevance of a food-drug interaction with another oral anti-cancer drug was investigated (chapter 4.1). The recommendations that resulted from these studies can be applied directly into the clinic.

The second part of the thesis reports on dynamics of circulating tumor cells in the peripheral blood of cancer patients (CTCs; chapter 5). Techniques for the detection of CTCs in breast- (chapter 5.2) and non-small cell lung cancer (chapter 5.3) were optimized and applied in exploratory clinical studies. Next to this, the utility of these CTCs to be used as pharmacodynamic biomarkers in the development of anti-cancer drugs was reviewed (chapter 5.1). Finally, an assay was developed and subsequently validated that demonstrated actual intracellular pharmacodynamic changes in activation of specific proteins in CTCs (chapter 5.4). This “proof-of-principle” study

showed that CTCs are amenable for pharmacodynamic measurements of drugs that target a specific signaling cascade or protein within CTCs. Further research should be pursued in order to fully exploit the potential of CTCs in this context.

In summary, this thesis describes both pharmacokinetic trials and exploratory studies with CTCs as pharmacodynamic biomarkers. Some results are available directly for application in the clinic and other results will need to be further investigated before applying them in drug development of new drugs. Both contribute to further development of current and future pharmacologically based anti-cancer treatments, which will hopefully result in an improved outcome for cancer patients.

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Chapter 2

Pharmacokinetics of pazopanib and topotecan

Chapter 2.1

Phase I study of safety, tolerability and pharmacokinetics of pazopanib in combination with oral topotecan in patients with advanced solid tumors

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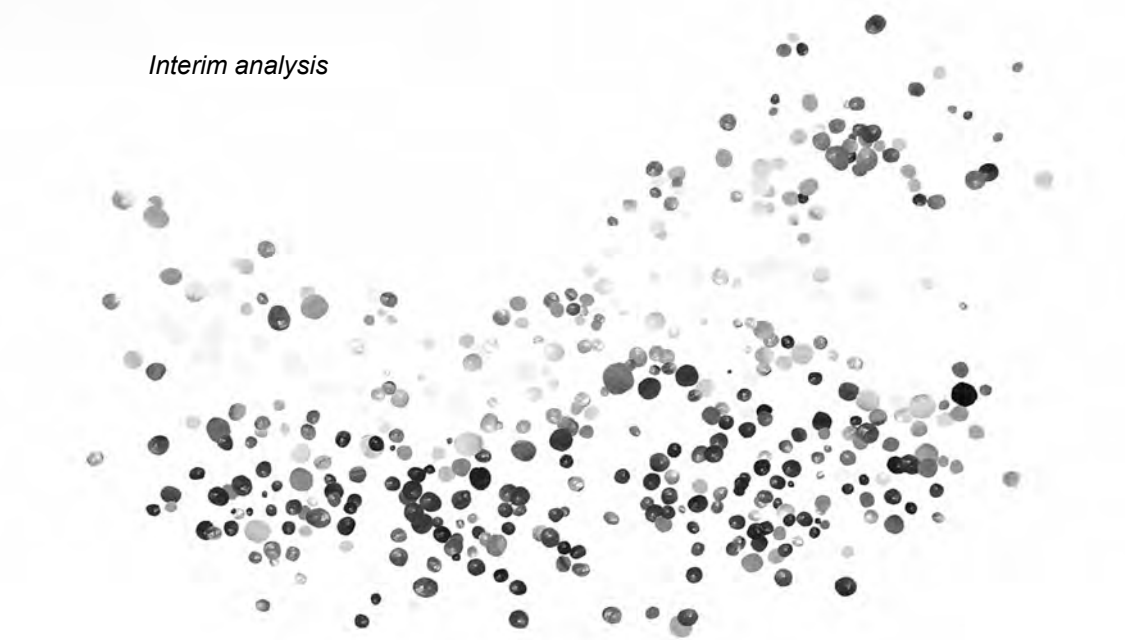
Interim analysis



ABSTRACT

Purpose

To determine the maximum-tolerated dose (MTD), safety, tolerability and pharmacokinetics of pazopanib in combination with oral topotecan.

Experimental design

Two-stage, two-arm, dose escalation and pharmacokinetic phase I study of pazopanib and oral topotecan in patients with advanced solid tumors. This interim report describes the results of the bioavailability part (1) and the dose escalation part (2A) of continuous pazopanib therapy combined with weekly topotecan in a 28-day cycle.

Results

At time of interim analysis, 32 patients were included of which 28 were evaluable. Three dose-limiting toxicities (DLTs) occurred that included grade 3 hand-foot-syndrome, diarrhea and neutropenia. The highest dose in dose-escalation (continuous pazopanib 800 mg in combination with topotecan 10 mg on day 1, 8, 15 in a 28-cycle), was found to exceed the MTD because two DLTs occurred in six patients. Therefore, the MTD is expected to be at the next-lower dose level (pazopanib 800 mg with topotecan 8 mg) and this dose level is currently being explored in an expansion cohort. The most frequently reported treatment-related toxicities were grade 3 anemia (3/28, 11%), leukocytopenia, neutropenia and fatigue (all 2/28, 7.1%). Pharmacokinetic analysis of the bioavailability part (n=7) showed an increase in topotecan exposure when co-administered with pazopanib compared to single administration. Topotecan $AUC_{0-\infty}$ was increased 1.58-fold (90%CI: 1.09–1.29) and C_{max} 1.78-fold (90%CI: 1.08-2.92) when co-administered with pazopanib. Pazopanib AUC_{0-24} and C_{max} ratios were not increased when co-administered with topotecan and were 0.98 (90%CI: 0.95-1.02) and 0.96 (90%CI: 0.92-1.01), respectively. Twenty-three patients were evaluable for anti-tumor activity and two patients (7%) had a partial response as best overall response.

Conclusions

Enrolment in an expansion cohort at the expected MTD (continuous pazopanib 800 mg combined with oral topotecan 8.0 mg on days 1, 8, 15 in a 28-day cycle) is currently ongoing. Pazopanib increased topotecan exposure considerably by approximate 1.58-fold ($AUC_{0-\infty}$) and 1.78-fold (C_{max}).

INTRODUCTION

The oral anti-angiogenic drug pazopanib (GW786034; Votrient; GlaxoSmithKline) is a potent and selective small molecule inhibitor of vascular endothelial growth factor receptor (VEGFR)-1, -2, and -3, platelet-derived growth factor (PDGFR)-α and –β, and stem cell factor receptor (c-kit) [1;2]. Pazopanib is approved for treatment of metastatic renal cell cancer, after showing prolongation of progression-free survival in a phase III trial [3], and has shown clinical benefit in several other tumor types, including soft tissue sarcoma [4], ovarian- [5], non-small cell lung- [6] and thyroid cancer [7]. The most common side effects observed in phase I-III trials were hypertension, diarrhea, hair depigmentation, nausea, vomiting, anorexia, fatigue and aspartate- and alanine aminotransferase (AST and ALT) increase [3-10]. Clinically relevant pharmacokinetic interactions have been observed in combination therapies of pazopanib with lapatinib [11;12], paclitaxel [13] and with food [14].

Topotecan (SK&F-104864; Hycamtin; GlaxoSmithKline) is a semi-synthetic analog of the alkaloid camptothecin and is a specific inhibitor of topoisomerase-I [15], leading to lethal deoxyribonucleic acid (DNA) damage during the course of DNA replication [16]. Intravenous topotecan is approved for the treatment of patients with metastatic ovarian carcinoma and relapsed small cell lung cancer (SCLC). Similar response rates were observed in comparisons of intravenous and oral topotecan regimens in phase II and III studies in SCLC patients [17;18]. Well known side effects include neutropenia, fatigue, nausea, vomiting and diarrhea [19]. Various phase I and II trials have explored safety and efficacy of oral topotecan in combination with other oral [20;21] or intravenous chemotherapy [21-27].

Some combinations of cytotoxic drugs with an anti-angiogenic drug have shown to provide clinical benefit in cancer patients. One example is the addition of bevacizumab, a monoclonal antibody against VEGF-A, to 5-fluorouracil-based chemotherapy [28]. In a landmark phase III trial, the combination with irinotecan and leucovorin, although this combination of cytotoxic drugs is not being used anymore, was shown to increase progression-free survival in metastatic colorectal patients [29]. Although several explanations exist for the synergistic effect observed of anti-angiogenic drugs and some types of chemotherapy, the exact mechanism is currently unknown. It is thought that anti-angiogenic drugs act by normalizing tumor vasculature leading to improved delivery of cytotoxic drugs to the tumor [30]. Other theories are based on the timing of anti-angiogenic drugs during chemotherapy-free periods and on counteracting mobilisation of any bone-marrow derived endothelial progenitor cells upon stress

signals such as chemotherapy [31;32]. The development of oral combination therapies, consisting of cytotoxic drugs and a VEGFR-targeting tyrosine kinase inhibitor, could provide more convenience to the patient than treatment with intravenous drugs. Pre-clinical data in cell lines and mouse models have shown that the combination of pazopanib with metronomic topotecan has enhanced anti-tumor activity in ovarian cancer compared to topotecan monotherapy alone [33;34]. However, present data on the clinical benefit of the combination of VEGFR-TKI and chemotherapy are not promising. Among other studies, the CONFIRM and HORIZON trials, studying the combination of tyrosine kinase inhibitors vatalanib (Novartis) and cediranib (Recentin; AstraZeneca), respectively, with cytotoxic drugs, failed to demonstrate improved survival in colorectal cancer patients [35;36]. Furthermore, the clinical development of such type of combinations has been hampered by the observation of synergy in toxicity, leading to a reduced maximum-tolerated dose (MTD) and exposure to the drugs, for example in the case of sunitinib (Sutent; Pfizer) [37;38] . The present study was employed to clinically evaluate safety and pharmacokinetics of the combination of two oral agents: pazopanib with topotecan.

Currently, the recommended oral topotecan dosing schedule is daily times five (day 1-5) in a 21-day cycle. Alternatively, weekly dosing of the intravenous formulation in a 28-day cycle has been shown to lead to decreased hematologic toxicity [39]. In a phase II trial in patients with ovarian cancer comparing the two schedules, the weekly schedule showed less hematological toxicity with similar overall survival, although progression-free survival was slightly worse than in the daily times five schedule [40]. Therefore, the weekly schedule may offer an alternative treatment option in patients with increased susceptibility to hematological toxicity or indeed in potentially occurring synergistic toxicity of the pazopanib and topotecan combination.

The objectives of this study were to determine the MTD, safety, tolerability and pharmacokinetics of pazopanib administered in combination with oral topotecan in patients with solid tumors. Pazopanib was dosed continuously and two different topotecan regimens were studied (weekly or daily times five). At time of interim analysis, only the weekly topotecan dosing schedule had been studied. Preliminary anti-tumor activity of the combination of pazopanib with oral topotecan was also explored.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed diagnosis of a progressive advanced solid tumor, that is refractory to standard therapy or for which there is no established therapy, were eligible. Other inclusion criteria were: written informed consent; ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) [41] performance status of ≤ 1; able to swallow and retain oral medications; adequate hematological- (neutrophils ≥ 1.5 x 10^9/litre (l); hemoglobin ≥ 6.2 mmol/l; platelets ≥ 100 x 10^9/l) , hepatic- (bilirubin ≤ 1.5 x upper limit of normal (ULN); AST and ALT ≤3 x ULN or ≤ 5x ULN in case of liver metastases) and renal functions (Cockroft-Gault [42] creatinine clearance ≥ 50 ml/min and urine protein creatinine ratio of < 1); normal coagulation tests (prothrombin time, international normalized ratio and partial thromboplastin time ≤ 1.2 x ULN). Exclusion criteria were: less than four weeks since last chemo-, radio-, biologic therapy or surgery or less than 6 weeks since last prior nitrosurea or mitomycin C chemotherapy; administration of investigational drugs within 30 days or 5 half-lives; prior treatment with pazopanib or investigational anti-angiogenic compounds; uncontrolled infection; pregnancy or lactating (all patients had to use adequate contraceptive protection); poorly controlled hypertension (systolic ≥ 140 of diastolic blood pressure ≥ 90 mm Hg); prolonged QTc interval; class III of IV heart failure; vascular events within 6 months; therapeutic heparin or warfarin use; leptomeningeal- or brain metastases; malabsorption syndrome or other disease affecting gastrointestinal function; unresolved bowel obstruction or diarrhea grade ≥ 1; any other condition that would interfere with the patient's ability to comply with the dosing schedule and protocol-specified evaluations. The study was conducted in accordance with the guidelines for good clinical practice (GCP) and was approved by local Medical Ethics committees.

Study design, study procedures and treatment administration.

The primary objectives of this study were to determine the MTD, safety and tolerability of once daily pazopanib administered in combination with oral topotecan in patients with solid tumors. Secondary objectives were to determine the pharmacokinetics of pazopanib and orally administered topotecan alone and in combination, to assess anti-tumor activity of the combination of drugs and to compare dose-intensities between the two topotecan regimens.

This was a two-stage, two-arm, open-label, dose escalation phase I study (NCT00732420, www.clinicaltrials.gov) [43]. The start date was September 2008 and the cut-off date for interim-analysis was March 1st 2011. In total, 3 centers participated in the study. These included the Abramson Cancer Center of the University of Pennsylvania in the United States of America, University Medical Center Utrecht and the Netherlands Cancer Institute, Amsterdam, both in the Netherlands.

In Part 1 the impact of pazopanib on the oral bioavailability of topotecan was investigated. In Part 2, the combination regimens were explored in a dose- escalation phase and a dose expansion phase. There were two different combination regimens for topotecan (treatment arm 2A: topotecan once weekly in a 28-day cycle; arm 2B: topotecan daily times five in a 21-day cycle), while pazopanib was continuously administered once daily. Patients were enrolled in sequential cohorts of three to six patients and the MTD was defined as the highest dose level at which no more than one out of six patients experienced a dose-limiting toxicity (DLT) after completing one treatment cycle. At time of interim analysis, the bioavailability part had been completed and enrollment in part 2A expanded cohort was ongoing.

Study procedures.

Written informed consent was obtained prior to study specific assessments. Demographic data, concomitant medications and medical history were recorded. Complete physical examinations, including ECG, and clinical laboratory tests were performed at screening and at regular intervals during cycle 1, in any following cycles and at study termination. Patients remained on treatment until disease progression, unmanageable toxicity had developed or withdrawal of consent.

Dosing.

Pazopanib monohydrochloride was provided as 200 and 400 mg tablets. Oral topotecan was provided as capsules containing topotecan HCl, equivalent to 0.25 mg or 1.00 mg. Different dose levels of pazopanib and topotecan are summarized in Table 2. Dose reductions following each cycle were allowed twice. Specific guidelines were prescribed for management of hypertension and diarrhea.

In the bioavailability phase (Part 1), pazopanib was dosed continuously from day 2 at 800 mg, which is the recommended dose for monotherapy. Topotecan 4 mg was administered only on day 1 and 15 of cycle 1. This sequence was chosen in order to enable pharmacokinetic sampling of both dugs as monotherapy and in combination therapy. In the dose escalation part (Part 2A), patients started with

continuous pazopanib monotherapy at day -14, which was prior to administration of topotecan on day 1, 8 and 15 every cycle of 28 days (weekly administration). In the dose expansion part (Part 2A) continuous pazopanib dosing was started on day 2 and topotecan was dosed on day 1, 8 and 15 of a 28-day cycle. Pazopanib and topotecan were administered with water on an empty stomach either 1 hour before a meal or 2 hours after a meal.

A DLT was defined as: any grade 3 or 4 clinically significant non-hematological toxicity (excluding grade ≥ 3 nausea and vomiting without maximal anti-emetic prophylaxis); grade 4 neutropenia with fever or infection or grade 4 neutropenia ≥ 5 days or grade ≥ 3 neutropenia requiring delay in the next cycle; grade 4 thrombocytopenia; inadequately controlled grade 3 hypertension in spite of maximal 2 antihypertensive drugs; grade 4 hypertension; grade 3 proteinuria during uncontrolled hypertension and/or renal impairment or lack of improvement to grade ≤ 2 upon interruption of pazopanib; grade 4 proteinuria; delay of next cycle of ≥ 2 weeks due to unresolved toxicity; grade ≥ 2 non-hematological toxicity beyond cycle 1 and any grade ≥ 2 toxicity that was considered a DLT.

Safety assessment.

Adverse events (AE), serious adverse events (SAE) and their relation to the study drug were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE) version 3.0 [44].

Anti-tumor activity assessment.

Radiologic tumor assessments according to RECIST [45] were performed at baseline and at regular intervals. A best response was documented for each patient.

Evaluability.

Patients were considered evaluable for safety and pharmacokinetics when they had completed cycle 1. If a patient vomited between dosing and 6 hours later on the days that pharmacokinetic sampling was performed, pharmacokinetic data of that patient were considered not evaluable.

Pharmacokinetic sampling and analysis.

Whole blood samples for the determination of pazopanib on day 14 and 15 were taken at baseline and scheduled at 1, 2, 3, 4, 6, 8, 10 and 24 hours following administration.

Pharmacokinetic sampling was also performed for total topotecan at day 1 and day 15 at baseline and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 hours following administration. Unscheduled sampling was performed in the case of unexpected toxicity.

At each collection time point, for pazopanib, 2 ml of whole blood was withdrawn into pre-chilled tubes containing potassium ethylenediaminetetra-acetic acid (EDTA; Vacutainer™). Blood and anti-coagulant were mixed by inverting the tube 8-10 times, stored on ice immediately and centrifuged at 2000 g at 4ºC for 10 minutes. Plasma was stored and frozen at -20ºC within 15 minutes of collection. For total topotecan, 3 ml of whole blood was withdrawn into pre-chilled lithium heparinised collection tubes (Vacutainer™). After collection, blood and anti-coagulant were mixed by inverting the tube 8-10 times. Blood samples were immediately stored on ice and centrifuged within 5 minutes at 4000 g at 4ºC for 5 minutes. Plasma was stored and frozen at ≤ -30ºC within 15 minutes of collection.

Plasma concentrations of pazopanib and total topotecan (both carboxylate and lactone form) were quantified using a validated high-performance liquid chromatography (HPLC) method [8;46].

Pharmacokinetic parameters (area under the curve extrapolated to infinity: $AUC_{0-\infty}$, area under the curve up to 24 hours: AUC_{0-24} and elimination half-life: $t_{1/2}$) were derived from plasma concentrations by non-compartmental analysis using the log-linear trapezoidal rule using WinNonLin (Pharsight Corporation, Mountain View, California, USA). Plasma concentration at 24 hours and (time at) maximal concentration ($C_{24,}$ C_{max} and T_{max}) were derived directly from the plasma concentration-time data.

Pharmacogenetic analysis.

Separate written informed consent was required for pharmacogenetic sampling. A whole blood sample (10 ml) was collected in a tube containing EDTA. Several genes involved in safety and efficacy of the study drugs were investigated for single nucleotide polymorphisms (SNP). These included genes coding for drug targets topoisomerase I, VEGF, VEGFR, hypoxia-inducible factor-1 (HIF-1), PDGFR and c-kit; genes involved in cytochrome P450 (CYP) 3A4, 3A5, 1A2, 2C8-mediated metabolism and UDP-glucoronyltransferase (UGT1A1); and drug transporters such as breast cancer resistance protein (BCRP; *ABCG2*) and P-glycoprotein (P-gp; *ABCB1*, MDR1). Additional analysis was included for polymorphisms in the hemochromatosis (*HFE)* gene, which was recently shown to be associated with ALT elevation in renal cell cancer patients treated with pazopanib [47].

Statistical analysis.

No sample size calculations were made. Calculated pharmacokinetic parameters were summarized and tabulated for pazopanib and total topotecan. Coefficient of variation (CV%) was calculated as (SD/mean) x 100%. The geometric least square means ratio for test [e.g. topotecan during pazopanib] over reference [topotecan alone] and associated 90% Confidence Interval (CI) were calculated. Natural logarithm (LN)-transformed $AUC_{0-\infty}$ and C_{max} were subject to analysis of variance (ANOVA), performed in R [48] (version 2.12.0, http://www.r-project.org), and results were presented in terms of geometric means ratio with associated 90% confidence intervals (CI). The estimated means were back-transformed to obtain an estimate for the ratio. In order to test for drug interaction, $AUC_{0-\infty}$, AUC_{0-24} and C_{max} of topotecan or pazopanib monotherapy and combination therapy were also subjected to the Wilcoxon signed ranks test (SPSS version 17.0; SPSS Inc. Chicago, USA) and $P < 0.05$ was considered statistically significant.

RESULTS

Patient inclusion and demographics.

In total, at the cut-off date for interim analysis, 32 patients had been included of whom 28 were evaluable for safety and pharmacokinetics. Seven patients were treated in the bioavailability part and had participated in pharmacokinetic sampling. Twenty-one patients had been enrolled in dose-escalation with weekly topotecan administration (Part 2A). Four patients discontinued before the end of cycle 1. These included two patients in Part 1: one patient with endometrial cancer withdrew informed consent at day 7 and one patient with pancreatic cancer experienced morphine-induced delirium on day 2. The other two non-evaluable patients had been included in Part 2A dose level 4: one patient with pancreatic cancer suffered from clinical progression before day 1 and one patient with vulvar carcinoma experienced grade 3 tumor hemorrhaging after the first topotecan dose.

Patient demographics and characteristics of the evaluable patients are shown in Table 1. The median (range) age was 59 (37-78) years in Part 1 and 51 (21-69) years in Part 2A. Median weight, height and gender was comparable between groups. Most patients were Caucasian (86% in part 1 and 95% in part 2A). The most frequent primary tumor types were soft tissue sarcoma (6 patients in total, 21%), ovarian cancer (5 patients in total, 18%) and colorectal cancer (4 patients in total, 14%). Overall, patients had good performance (57% in both groups had an ECOG score of 0).

Table 1: Patient demographics and characteristics of the evaluable population

	Part 1 Bioavailability	Part 2 Arm A dose escalation
Number of patients	7	21
Age (years)		
Median (range)	59 (37 - 78)	51 (21 -69)
Weight (kg)		
Median (range)	72 (58 - 104)	77.4 (49 – 119)
Height (m)		
Median (range)	1.67 (1.57 – 1.80)	1.71 (1.50 – 1.98)*
Gender		
Male (%)	3 (43)	6 (29)
Female (%)	4 (57)	15 (71)
Race		
African American	1 (14)	1 (5)
Caucasian	7 (86)	20 (95)
Primary tumor, n (%)		
Soft tissue sarcoma	2 (29)	4 (19)
Colon/rectum	2 (29)	2 (10)
Breast cancer	1 (14)	2 (10)
Renal cell	1 (14)	1 (5)
NSCLC, type adenocarcinoma	1 (14)	0
Ovary	0	5 (24)
Osteosarcoma	0	2 (10)
Endometrium or uterus	0	1 (5)
Melanoma (choroidea)	0	1 (5)
Pancreas	0	1 (5)
Peritoneal	0	1 (5)
Bladder	0	1 (5)
ECOG at baseline, n (%)		
0	4 (57)	12 (57)
1	3 (43)	9 (43)

* height of one patient was missing. Abbreviations: ECOG= Eastern Cooperative Oncology Group (ECOG)[41]; NSCLC = non-small cell lung cancer.

Dose escalation and dose expansion.

In Part 2A, dosing started at dose level 0 (dose levels are summarized in Table 2) and was escalated up to dose level 4. In total, three DLTs were observed. The first DLT was grade 3 hand-foot-syndrome (HFS) occurring in dose level 1. Pazopanib

was withheld and toxicity resolved. Re-challenge with a reduced dose (200 mg) did not elicit recurrence. Additionally, three patients were enrolled in this dose level but no DLT was observed. Therefore, dose escalation was further pursued and no DLTs were observed until dose level 4. In this dose level, two DLTs were observed in two out of six patients: grade 3 diarrhea and grade 3 neutropenia leading to omission of topotecan dose on day 15. Therefore, the MTD was expected to be at the next-lower level, which was dose level 3 with topotecan 8.0 mg weekly and pazopanib 800 mg daily in a 28-day cycle. This dose level was further explored with additional patients in an expansion cohort that was enrolling at time of interim analysis. The first two patients (with cholangeal- and ovarian cancer) only experienced grade ≤ 2 toxicities.

Table 2: Dose levels and the number of dosed patients in Part 1 and 2A

Part 1 Bioavailability				
Cohort	**Pazopanib (days 2-28)**	**Topotecan (days 1 and 15)**	**Number of patients**	
1	800 mg	4.0 mg	7	
Part 2 Treatment arm A (weekly topotecan in a 28-day cycle)				
Dose level	**Pazopanib (days 1-28)**	**Topotecan (days 1, 8 and 15)**	**Number of patients**	**DLT**
0	400 mg	4.0 mg	3	0
1	400 mg	6.0 mg	6	grade 3 HFS
2	800 mg	6.0 mg	3	0
3	800 mg	8.0 mg	3	0
4	800 mg	10.0 mg	6	grade 3 diarrhea; grade 3 neutropenia[a]

[a] Neutropenia leading to omission of next dose. Abbreviations: DLT=dose-limiting toxicity; HFS= Hand-foot-syndrome.

Safety.

In total, 28 patients were evaluable for safety. All treatment-related CTCAE grade ≥ 3 toxicities are summarized in Table 3. The most frequently occurring treatment-related hematological toxicities were grade 3 anemia (3/28 11%), grade 3 leukocytopenia and neutropenia (2/28, 7.1% both). Neutropenia was also observed as grade 1

(1/28, 3.6%), grade 2 (4/28, 14%) and grade 4 (1/28, 3.6%). The most frequently occurring treatment-related non-hematological toxicity was grade 3 fatigue (2/28, 7.1%). Fatigue was also frequently found as grade 1 (12/28, 43%) and grade 2 (11/28, 40%) and once as grade 4 (1/28, 3.6%).

Several treatment-related adverse events occurred frequently as grade ≤ 2 but not as grade ≥ 3. This included hypertension (grade 1: 4/28, 14% and grade 2: 5/28, 18%), nausea (grade 1: 21/28, 75% and grade 2: 3/28, 11%) and vomiting (grade 1: 13/28, 46% and grade 2: 3/28, 11%). HFS was reported in one other patient (grade 1, 1/28, 3.6%). Also hypopigmentation of hair was observed once (grade 1, 1/28, 3.6%). Rash was observed infrequently (grade 1: 3/28, 11% and grade 2: 1/28, 3.6%).

There were two deaths during the study that were considered related to the study drug. Both occurred during pazopanib monotherapy (800 mg) in the bioavailability part. One patient with lung adenocarcinoma developed fatal pulmonary hemorrhage at the end of cycle 1. Another patient with synovial sarcoma, without history of hepatic disease, developed elevated ALT and AST in the beginning of cycle 2, leading to hepatic failure and ultimately death 7 days later. Autopsy showed extensive liver necrosis and congestion, ascites and sub-acute heart congestion. Pharmacokinetic results obtained for pazopanib exposure, including sampling at time of the liver event, showed concentration values within the normal range of exposure when comparing to other trials. In this patient, pazopanib AUC_{0-24} was 1246 and 1384 µg*h/ml, for day 14 and 15, respectively. Pazopanib plasma concentration at 4 hours after dosing at time of the liver event on day 33 ($C_{t=4\ hour}$) was 41.9 µg/ml, which did not exceed the plasma concentration obtained at 4 hours after dosing on day 14 ($C_{t=4\ hours}$ = 61.1 µg/ml). Several other causes, besides pazopanib, were assessed for their contribution to the observed hepatic toxicity, but none were identified. Relevant concomitant medication was paracetamol (=acetaminophen; 1 g/day). Low-normal plasma concentrations of paracetamol were found at time of liver event, consistent with therapeutic levels, but an effect of paracetamol on the liver toxicity could not be excluded. No other toxicological- or infectious causes were identified. Pharmacogenetic analysis of germ-line DNA derived from peripheral blood of this patient was performed and the results are discussed separately.

Table 3: All treatment-related CTCAE grade ≥ 3 toxicity observed in the study

		Treatment group						
	CTCAE Grade	Part 1 N=7 (%)	Part 2A DL0 N=3 (%)	Part 2A DL1 N=6 (%)	Part 2A DL2 N=3 (%)	Part 2A DL3 N=3 (%)	Part 2A DL4 N=6 (%)	Total N=28 (%)
Hematological toxicity								
Anemia	3	0	0	1 (17)	0	1 (33)	1 (17)	3 (11)
Leucocytopenia	3	0	0	0	0	1 (33)	1 (17)	2 (7.1)
Neutropenia	3	0	0	0	0	1 (33)	1 (17)	2 (7.1)
	4	0	0	0	0	0	1 (17)	1 (3.6)
Thrombocyto-penia	3	0	0	0	0	0	1 (17)	1 (3.6)
	4	0	0	0	0	1 (33)	0	1 (3.6)
Biochemical laboratory toxicity								
Elevated ALT	4	1 (14)	0	0	0	0	0	1 (3.6)
Elevated AST	4	1 (14)	0	0	0	0	0	1 (3.6)
Hyperglycemia	3	0	0	0	0	0	1 (17)	1 (3.6)
Other toxicity								
Fatigue	3	1 (14)	0	1 (17)	0	0	0	2 (7.1)
	4	1 (14)	0	0	0	0	0	1 (3.6)
Diarrhea	3	0	0	0	0	0	1 (17)	1 (3.6)
HFS	3	0	0	1 (17)	0	0	0	1 (3.6)
Toxic hepatitis	3	1 (14)	0	0	0	0	0	1 (3.6)
Liver failure	5	1 (14)	0	0	0	0	0	1 (3.6)
Pulmonary hemorrhage	5	1 (14)	0	0	0	0	0	1 (3.6)

Abbreviations: CTCAE= common terminology criteria for adverse events; DL=dose level; HFS=hand-foot-syndrome; GGT=gamma-glutamyl transferase. ALT and AST=alanine and aspartate aminotransferase.

Non-fatal treatment-related liver toxicity was observed in three other patients with grade 1, 2 and 4 elevated ALT and AST (1/28, 3.6% each). The patient with grade 4 liver toxicity suffered from colorectal carcinoma with liver metastases. Plasma pazopanib concentration obtained at time of the liver event was not elevated (32.6 µg/ml) and toxicity recovered only partially after discontinuation of study medication and administration of oral steroids. While the use of pazopanib led to an elevation in ALT and AST in this patient, it is possible that the observed hepatic toxicity was only

partially due to pazopanib and that the patient's liver metastasis also contributed to this event. In this patient, also pharmacogenetic analysis was performed.

Pharmacokinetic analysis.

Mean (standard deviation, SD) plasma concentration-time curves after administration of pazopanib (800 mg; day 14 and 15) and total topotecan (4.0 mg; day 1 and 15) are presented in Figure 1. Pharmacokinetic sampling was obtained from 7 patients, but one patient used co-medication (triamterene) that interfered with the bioanalytical analysis of topotecan. Therefore, data were available of 7 patients for pazopanib and of 6 patients for topotecan. Mean curves for single pazopanib (day 14) and combined with topotecan (day 15) are similar (Fig. 1A). The curves for total topotecan, however, show a marked increase in mean exposure for topotecan when co-administered with pazopanib (day 15), compared to single exposure (day 1; Fig. 1B).

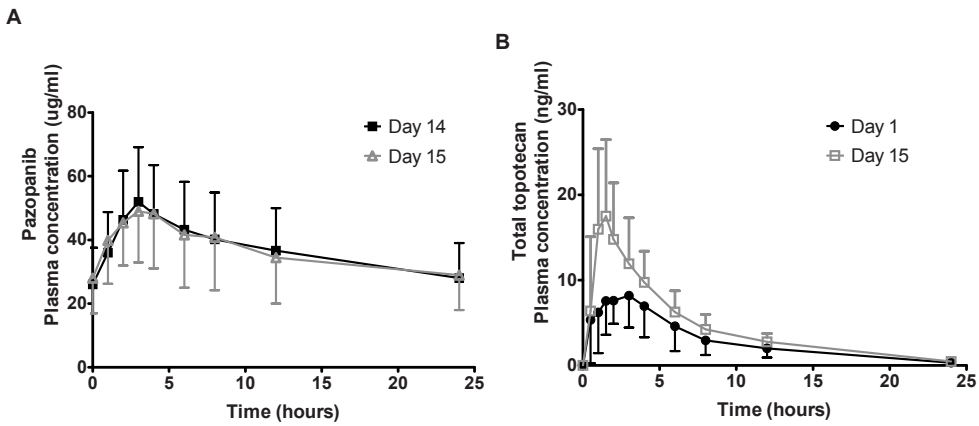


Figure 1: Mean (SD) plasma concentration-time curves of pazopanib (left panel, A, µg/ml) and total topotecan (right panel, B, ng/ml) following single administration of pazopanib (day 14, *n* =7), single administration of topotecan (day 1, *n* =6) and concomitant administration of pazopanib and topotecan (day 15).

Pharmacokinetic data are summarized in Table 4. All parameters for pazopanib, including $AUC_{0\text{-}24,}$ C_{24}, C_{max}, T_{max} and $T_{1/2\ ,}$ were comparable. Administration of pazopanib 800 mg led to mean C_{24} plasma concentration of > 15 ug/ml, which is a known threshold above which concentration-effect relationship can be observed [8]. Individual C_{24} values were > 15 ug/ml for all patients except one (data not shown). Relevant increases were observed in mean topotecan $AUC_{0\text{-}\infty}$, $AUC_{0\text{-}24}$ and C_{max}

when co-administered with pazopanib. Mean (SD) $AUC_{0-\infty}$ was increased from 68.1 (27.6) to 106.6 (42.4) ng*h/ml and C_{max} from 10.0 (3.78) to 18.8 (9.23) ng/ml. T_{max} and $T_{1/2}$ were similar for both administrations. AUC and C_{max} CV% were similar for monotherapy and combination therapy for both drugs (Table 4). Inter-individual changes in pazopanib AUC_{0-24} and C_{max} and topotecan $AUC_{0-\infty}$ and C_{max} after single and concomitant administration are shown in Figure 2.

Table 4: Pharmacokinetic parameters of pazopanib (n=7) and total topotecan (n=6) after single- and concomitant exposure

Parameter	Analyte	CV%	Pazopanib single dose on Day 14	CV%	Pazopanib with topotecan co-administration on Day 15
AUC_{0-24} (µg*h/ml)	GW786034	35	884 (308)	38	873 (330)
C_{24} (µg/ml)	GW786034		28.0 (11.0)		28.9 (10.9)
C_{max} (µg/ml)	GW786034	32	52.4 (16.6)	33	50.6 (16.5)
T_{max} (hours)	GW786034		3.0 (1.0-3.0)		3.0 (2.0-4.0)
$T_{1/2}$ (hours)	GW786034		33.8 (13.9)		28.3 (6.20)
Parameter	**Analyte**	**CV%**	**Topotecan single dose on Day 1**	**CV%**	**Topotecan with pazopanib co-administration on Day 15**
$AUC_{0-\infty}$ (ng*h/ml)	Total Topotecan	41	68.1 (27.6)	40	106.6 (42.4)
AUC_{0-24} (ng*h/ml)	Total Topotecan		65.7 (27.5)		103.2 (41.0)
C_{max} (ng/ml)	Total Topotecan	38	10.0 (3.78)	49	18.8 (9.23)
T_{max} (hours)	Total Topotecan		1.8 (0.5-3.0)		1.3 (1.0-4.0)
$T_{1/2}$ (hours)	Total Topotecan		5.01 (1.30)		4.89 (0.32)

Values are mean (SD), except for T_{max} which is median (range). Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-24} = Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximal observed plasma concentration; CV%= coefficient of variation; GW786034= pazopanib; T_{max} = time to maximal observed plasma concentration; $t_{1/2}$ = terminal half-life.

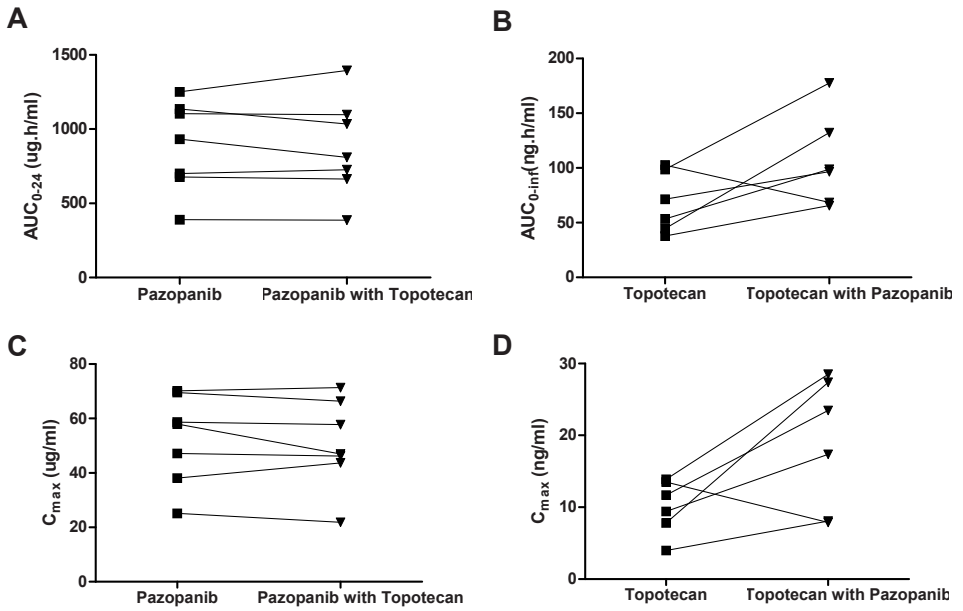


Figure 2: Scatterplots representing AUC_{0-24} and C_{max} for pazopanib (Fig. 3A and C; n=7) and $AUC_{0-\infty}$ and C_{max} for total topotecan (Fig. 3B and D; n=6). Each line connects the calculated exposure of an individual patient to a single drug or in combination therapy. $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-24} = Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximal observed plasma concentration.

Ratios of geometric means (90% CI) for AUC_{0-24}, $AUC_{0-\infty}$ and C_{max} of test groups (concomitant dosing) compared to reference groups (single dosing) were calculated by ANOVA and are summarized in Table 5 (pazopanib) and Table 6 (topotecan). Pazopanib AUC_{0-24} and C_{max} ratios were not increased and were 0.98 (90%CI: 0.95-1.02; *P* = 0.612) and 0.96 (90%CI: 0.92-1.01; *P* = 0.398), respectively, when comparing single drug with co-administration of topotecan. Topotecan $AUC_{0-\infty}$ was increased 1.58-fold (90%CI: 1.09 – 2.29; *P* =0.116), AUC_{0-24} 1.59-fold (90%CI: 1.09 – 2.34; *P* =0.116) and C_{max} 1.78-fold (90%CI: 1.08-2.92; *P* = 0.075) in combination with pazopanib, although these increases were not statistically significant.

Pharmacogenetic analysis.

This analysis had not yet been completed at time of interim analysis, except for the two patients with severe hepatotoxicity that were mentioned previously. Both the patient with fatal hepatotoxicity and the patient with grade 4 ALT and AST elevation were found to be heterozygous for the *ABCB1* gene (*6 and *7, rs1045642 and rs2032582) which has been associated with decreased P-gp activity [49]. Next to this,

the latter patient also had one copy of the *UGT1A6**3A allele (S7A, rs6759892) which has been associated with lower *UGT1A6* expression [50], but whose clinical relevance is unknown currently. Both patients were wild-type for the other *UGT1A6* polymorphisms (*5 and *9, rs2070959 and rs1105879), the *UGT1A1* (*28, rs8175347), the *HFE* (rs1800562, rs2858996 and rs707889) and the *ABCG2* gene polyporphisms (rs2231137 and polyID59476002).

Table 5: Statistical analysis of pharmacokinetic parameters of pazopanib

Parameter	Geometric mean		Ratio of means ($P_{day\ 15}$:P_{day14})	90% confidence interval	*P* value
	Pazopanib Day 14	Pazopanib Day 15			
AUC_{0-24} (µg.h/ml)	829.5	815.0	0.98	0.95-1.02	0.612
C_{max} (µg/ml)	49.71	47.77	0.96	0.92-1.01	0.398

Abbreviations: AUC_{0-24}= Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximal observed plasma concentration.

Table 6: Statistical analysis of pharmacokinetic parameters of topotecan

Parameter	Geometric mean		Ratio of means ($T_{day\ 15}$:$T_{day\ 1}$)	90% confidence interval	*P* value
	Topotecan Day 1	Topotecan Day 15			
$AUC_{0-\infty}$ (ng.h/ml)	63.42	100.1	1.58	1.09-2.29	0.116
AUC_{0-24} (ng.h/ml)	60.87	96.97	1.59	1.09-2.34	0.116
C_{max} (ng/ml)	9.29	16.51	1.78	1.08-2.92	0.075

Abbreviations: $AUC_{0-\infty}$= Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-24}= Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximal observed plasma concentration.

Anti-tumor activity.

Data from 23 patients (23/28, 82%) were available at time of interim analysis. Two patients (2/23, 7%), both with ovarian cancer, had a partial response as best overall response. Stable disease was achieved in 13 (13/28, 46%) and progressive disease was found in 8 (8/28, 29%).

DISCUSSION

This interim report describes the results of an ongoing phase I study of combination therapy of the anti-angiogenic drug pazopanib and oral topotecan in patients with advanced solid tumors. At the cut-off date for interim analysis, the bioavailability during co-administration of these drugs had been studied and dose escalation of a weekly topotecan schedule had been performed leading to enrollment in an expansion cohort at the expected MTD.

Doses were escalated and the highest dose level (continuous pazopanib 800 mg in combination with topotecan 10 mg on day 1, 8, 15 in a 28-cycle) was found too toxic. Therefore, the MTD is expected to be at the next-lower dose level (pazopanib 800 mg with topotecan 8 mg) and this dose level is currently being explored in an expansion cohort. One DLT was observed at a lower dose level (grade 3 HFS) and two occurred at the highest dose level (grade 3 diarrhea and neutropenia). The most frequently reported treatment-related toxicities were grade 3 anemia, leukocytopenia, neutropenia and fatigue.

Pharmacokinetic analysis of the bioavailability part showed an increase in topotecan exposure when co-administered with pazopanib compared to single administration ($AUC_{0-\infty}$ 1.58-fold [90%CI: 1.09 – 2.29], AUC_{0-24} 1.59-fold [90%CI: 1.09 – 2.34] and C_{max} 1.78-fold [90%CI: 1.08-2.92]), although statistical significance was not reached. This observation could be due to the relatively small sample size in the topotecan group (n=6). Further statistical analysis will be performed and will be included in the final report that will include pharmacokinetic data of the patients in the expansion cohort (6 to 9 additional patients).

Unexpected fatal toxicity was observed during the study. Fatal liver necrosis occurred in one patient and another patient with liver metastases developed grade 4 toxic hepatitis after pazopanib exposure, which was only partially reversible. Hepatotoxicity is not likely to be related to topotecan therapy based on experience from previous phase I – III trials [17-19;40;51] and clinical experience. Grade ≥ 3 AST and ALT elevations have indeed been observed previously for single-agent pazopanib with an incidence of 2-8% and 0-19%, respectively, in phase I-III studies in patients with solid tumors [3-10;52]. Grade 3 ALT (8%) and grade 3 AST elevation (8%) had been the most common adverse event leading to discontinuation of study drug in a phase II study in patients with recurrent ovarian cancer [5]. Elevations in ALT and AST (all grades) during pazopanib monotherapy (800 mg) were the most common laboratory abnormalities (54% and 53% respectively) in a phase II trial in patients with advanced renal cell

cancer [9] and in a phase III trial (ALT and AST 53% each), patients with advanced or metastatic renal cell carcinoma [3]. In the latter study, 1 out of 290 patients (< 1%) had died due to hepatotoxicity and extensive liver metastases. In general, the etiology is thought to be a hypersensitivity reaction [53] to pazopanib exposure with a possible role for concomitant paracetamol exposure. In the present study, another possible cause could be the polymorphism that was observed in both patients with hepatotoxicity (*ABCB1**6 and *7) potentially leading to reduced P-gp expression in the liver. Pazopanib is a substrate for P-gp [54] and its reduced expression might have led to reduced hepatic clearance of pazopanib into the bile. Although pazopanib concentration values of this patient were within the normal range of exposure when comparing to other trials, pazopanib exposure in this patient was the highest of all patients in the pharmacokinetic analysis group in the present study.

Hepatotoxicity is not restricted to pazopanib therapy but has also been observed in other VEGFR-targeting tyrosine kinase inhibitors such as sunitinib. ALT and AST elevation were observed in a phase III trial with sunitinib in 56% and 51%, respectively, of 375 patients with metastatic renal cell carcinoma [55] and sunitinib-related hepatic failure has been reported [56]. Hepatotoxicity was also reported in long-term use of the PDGFR and c-kit targeting drug imatinib (Glivec/Gleevec; Novartis) [57]. It is therefore recommended that liver function is closely monitored during pazopanib therapy. The prescribing information for pazopanib therefore includes a special warning for severe hepatotoxicity [58]. It is advised to interrupt pazopanib administration when transaminases increase > 8 X ULN and pazopanib should be discontinued if transaminase elevations > 3 X ULN occur concurrently with bilirubin elevations > 2 X ULN (direct bilirubin > 35% of total bilirubin). In patients with pre-existing moderate hepatic impairment, a reduced pazopanib dose of 200 mg once daily is recommended. Pazopanib is contra-indicated in patients with severe hepatic impairment [58].

Also hemorrhage was a fatal adverse event in one patient with NSCLC and occurred as grade 3 in a patient with vulvar cancer. This is a known, but unfortunate, side effect of anti-cancer therapy in general and occurs in approximately 1-6% of patients receiving VEGF/VEGFR inhibition therapy [59]. The pharmacological activity of this type of therapy is thought to result in endothelial dysfunction and impaired homeostasis, thereby intervening in the balance of prothrombotic and antitrombotic activities. However, it is thought that the over-all effect of this intervention only marginally increases the already existing risk of hemorrhage [59].

A relatively low incidence of hypertension (grade ≤ 2: 32% and no grade ≥3) was found in the present study, compared to other studies (grade ≤ 2: 32-36% and grade 3: 1-9%)[3-5;9]. In the initial phase 1 study of single agent pazopanib, grade ≥ 3 hypertension was observed in even 25% [8]. This discrepancy might be due to the fact that 9/28 patients in the study reported here were exposed to daily pazopanib doses of < 800 mg because of the dose-escalation study design in co-administration with topotecan. It has been shown that a daily dose of 800 mg, the recommended dose, is required to reach a C_{24} at steady state of ≥ 15 µg/ml and a plasma concentration above this threshold has been correlated with the pharmacodynamic effect of hypertension [8]. Another reason that grade ≥ 3 hypertension was not observed as frequently as expected might be that patients were carefully monitored for early signs of hypertension and anti-hypertensive therapy was initiated promptly. These actions may have prevented progression from grade ≤ 2 to grade ≥3.

HFS grade 3 was a DLT for one patient and was observed as grade 1 in another patient (2/28, 7.1%). This is another side-effect that is also known of other VEGFR-targeting tyrosine kinase inhibitors such as sorafenib (Nexavar; Bayer) and sunitinib [57;59-61]. A meta-analysis showed that incidence of high-grade HFS during pazopanib treatment was estimated to be 1.8% [61]. The underlying mechanism is unclear, but is thought to be due to VEGFR and PDGFR targeting endothelial cells [60] in the skin and the skin itself leading to impaired wound healing [61].

Topotecan was administered as a flat (4, 6, 8 or 10 mg/day) dose. Oral topotecan has relatively high intra- and inter-patient variability [62;63] and it has been shown that dosing according to body surface area is not able to improve this [64]. Therefore, fixed dosing was considered appropriate and more convenient for the comparison of different dose levels in dose escalation.

A substantial increase in topotecan exposure was found when co-administered with pazopanib. $AUC_{0-\infty}$ and C_{max} were both increased and $t_{1/2}$ was unaffected. This is most likely the result of increased intestinal absorption of topotecan. Both pazopanib and topotecan are substrates for BCRP and P-gp efflux (data on file at GSK) drug transporters. Topotecan was found to be a good substrate for BCRP [65] and a weak substrate for P-gp[66]. These efflux transporters are located in the intestine where they act to limit drug absorption from the lumen [67]. BCRP and P-gp are also expressed in the liver and the kidney where they promote drug excretion into bile and urine [68;69]. It has been shown previously that inhibition of these two drug transporters by elacridar (GF120918) increased topotecan absorption and exposure significantly [70], which was even higher than in the present study (approximate 2.77-fold for AUC

and 2.43-fold for C_{max}). We therefore hypothesized that topotecan absorption could have been affected in our study by the inhibition of BCRP and/or P-gp by pazopanib. Apparently, in the present study pazopanib is a better substrate for these drug transporters than topotecan, which may be explained by a higher concentration (800 mg pazopanib versus 4 mg topotecan), leading to reduced efflux and increased absorption of topotecan from the gut. This clinically relevant interaction should be taken into account when treating patients with combination therapies consisting of pazopanib.

In conclusion, the results of this interim report demonstrated that the expected MTD for a weekly topotecan schedule was oral topotecan 8.0 mg on days 1, 8, 15 combined with continuous pazopanib 800 mg in a 28-day cycle. This dose level is currently being explored in an expansion cohort. Severe and fatal hepatotoxicity and haemorrhaging was observed. Pazopanib increased topotecan exposure considerably by approximate 1.58-fold ($AUC_{0-\infty}$) and 1.78-fold (C_{max}).

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Chapter 2.2

Phase I study of oral topotecan in patients with cancer and impaired renal function

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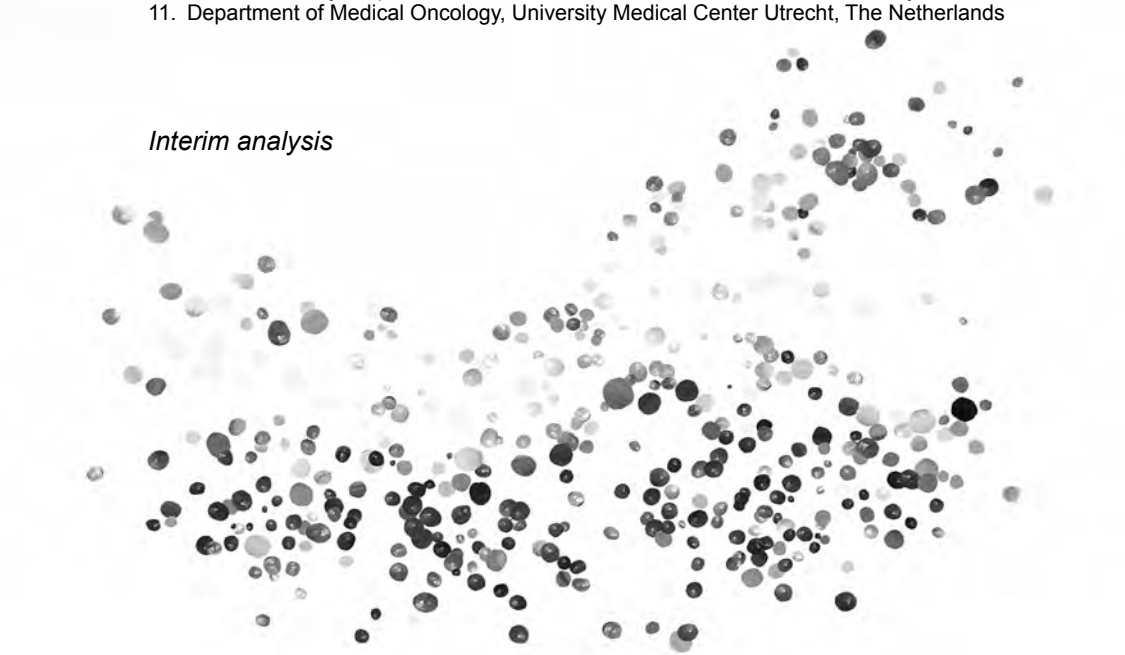
Interim analysis



ABSTRACT

Purpose

The aim of this study was to determine the effect of renal impairment and prior platinum-based chemotherapy on the safety and pharmacokinetics of oral topotecan and to identify recommended doses for patients with renal impairment or prior platinum-based chemotherapy.

Experimental design

A multi-center, phase I, multiple dose, open-label, safety and pharmacokinetic study of oral topotecan in patients with advanced solid tumors was performed. Patients were grouped by normal renal function with limited- (defined as: <3 courses of cisplatin or carboplatin chemotherapy without significant change in creatinin clearance) or prior platinum-based chemotherapy or impaired renal function (mild, moderate, severe). Pharmacokinetic sampling was performed up to 24 hours following administration for the quantitative determination of total topotecan and topotecan lactone concentrations in plasma and total topotecan in urine.

Results

At time of interim analysis, 53 patients were included of which 47 were evaluable for safety and pharmacokinetics. Nine dose-limiting toxicities (DLTs) occurred and the most frequently observed DLTs were grade 3 neutropenia leading to treatment delay (4/9), combined grade 4 thrombocyto- and neutropenia (2/8), grade 3 fatigue (2/8) and grade 4 (1/8) thrombocytopenia. The maximum-tolerated dose (MTD) was determined at 2.3 mg/m^2/day for patients with normal renal function and prior platinum-based chemotherapy and mild impaired renal function. No MTDs were determined for patients with moderate or severe renal impairment and enrolment is currently ongoing at 1.5 and 0.8 mg/m^2/day, respectively. Pharmacokinetic and statistical analysis showed that exposure to total topotecan and topotecan lactone after oral administration of topotecan was increased in patients with moderate and severe renal impairment.

Conclusions

Dose adjustments are not required in patients with normal renal function and prior platinum-based chemotherapy or mild impaired renal function. Dose adjustments are required for patients with moderate and severe renal impairment, but the degree of dose reduction has not been determined yet.

INTRODUCTION

Topotecan (SK&F-104864; Hycamtin; GlaxoSmithKline) is a semi-synthetic analog of the alkaloid camptothecin and is a specific inhibitor of topoisomerase-I [1]. Inhibition of this enzyme results in lethal deoxyribonucleic acid (DNA) damage during the course of DNA replication [2]. Intravenous (i.v.) topotecan monotherapy is approved for the treatment of patients with metastatic ovarian carcinoma as second-line therapy and for patients with relapsed small cell lung cancer (SCLC) for whom re-treatment with the first-line regimen is not considered appropriate. Topotecan in combination with cisplatin is indicated for patients with cervical cancer with recurrence after radiotherapy and for patients with stage IV-B disease. Safety and efficacy of oral topotecan alone [3-7] and in combination with other chemotherapeutic agents [8-15] has been explored in various phase I and II trials. In phase II and III studies in SCLC patients, comparing intravenous (1.5 mg/m^2/day) and oral (2.3 mg/m^2/day) topotecan regimens, similar response rates were observed [16;17]. Oral topotecan has shown improved benefit for survival and quality of life compared to supportive care alone in a phase III trial in SCLC patients [18]. The maximum tolerated dose (MTD) for oral topotecan in a regimen of 5 days every 21 days was determined at 2.3 mg/m^2/day [19].

Excretion of oral topotecan was studied in a human mass-balance study and the principal routes of excretion were renal and biliary. Approximately 20% was recovered as parent drug in urine and 33% of the oral dose was collected unchanged in feces [20]. The contribution of metabolism to topotecan elimination is limited (<10%). Next to this, reversible pH-dependent hydrolysis occurs, yielding topotecan carboxylate. It is thought that the lactone form of the drug, which is the predominant form in an acidic environment (pH ≤ 4) is pharmacologically more active [21;22]. Topotecan has a relatively low plasma protein binding of approximately 30% in humans (data on file at GSK) and the apparent bioavailability of oral topotecan was found to be 30 – 40 % [6;23].

The disposition and toxicological profile of intravenous topotecan was studied by O'Reilly et al. in patients with renal dysfunction. This study showed marked reduction in plasma clearance of topotecan in patients with moderate renal impairment (creatinine clearance (Cl_{cr}) 20-39 ml/min) [24]. The current labelling for i.v. topotecan recommends a dose reduction of 50% for patients with a moderate renal impairment (Cl_{cr}= 20-39 ml/min). It is unknown whether the dose adjustments for i.v. topotecan directly apply to oral topotecan administration. Next to this, in general, there is an increased likelihood for toxicity in patients who have received prior platinum-based chemotherapy [25]. Evidence indicates that cisplatin has a direct toxic effect on the

proximal tubule [26] that could change tubular secretion of topotecan and could increase exposure to topotecan without affecting Cl_{cr}. As a result, prior platinum-based chemotherapy may affect the safety and pharmacokinetics of oral topotecan. The objectives of this study were to determine the effect of renal impairment and prior platinum-based chemotherapy on the safety and pharmacokinetics of oral topotecan and to identify the appropriate dose adjustments for patients with mild, moderate or severe renal impairment and for patients with normal renal function who have received prior platinum-based chemotherapy.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed progressive advanced solid tumors, for whom no standard treatment was available or for whom single-agent topotecan therapy was considered suitable, were eligible. Other inclusion criteria were: written informed consent; ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) [27] performance status ≤ 2; stable renal function, defined as < 10% change in creatinine clearance for more than four weeks prior to start. Exclusion criteria were: current dialysis; hepatitis B or C infection; participation in a clinical study and receiving a drug or new chemical entity within 30 days or 5 half-lives, or twice the duration of the biological effect of any drug prior to the first dose of current study medication; pregnancy or breast-feeding (all patients had to use adequate contraceptive protection); uncontrolled emesis; bilirubin > 1.5 times the upper limit of normal (ULN); alanine aminotransferase, aspartate aminotransferase or alkaline phosphatase > 2 times ULN, in case of liver metastases < 5 times ULN; hemoglobin < 5.6 mmol/l; white blood cell count < 3.5 x 10^9/l, absolute neutrophil count < 1.5 x 10^9/l or platelets < 100 x 10^9/l; active infection; less than four weeks since last chemo-, radio-, biologic therapy or surgery; less than 6 weeks since last prior nitrosurea or mitomycin C chemotherapy; failure to recover from any prior therapy at baseline with the exception of grade 1 neuropathy or any grade alopecia; impaired gastro-intestinal absorption or motility; cyclosporin A treatment; concurrent severe medical problems unrelated to the malignancy significantly limiting compliance; history of allergic reactions to topotecan or chemically related compounds; history of sensitivity to heparin or heparin-induced thrombocytopenia. Patients were not allowed to receive drugs known to inhibit or induce human breast cancer resistance protein (BCRP,

ABCG1), P-glycoprotein (P-gp, *ABCB1*) and multi-drug resistance protein (MRP1, *ABCC1*) during pharmacokinetic sampling. The study protocol was approved by the Medical Ethics committee and all patients had to give written informed consent. The study was conducted in accordance with the guidelines for good clinical practice (GCP).

Study design, study procedures and treatment administration.

The primary objective was to determine the safety profile of oral topotecan in patients with cancer and impaired renal function, compared to patients with normal renal function and limited prior platinum-based chemotherapy. The secondary objectives were: to determine the safety profile of oral topotecan in patients with cancer and normal renal function with prior platinum-based chemotherapy compared to patients with limited prior platinum-based chemotherapy; to evaluate the pharmacokinetics of oral topotecan (total topotecan and topotecan lactone) and to explore the correlation between pharmacokinetic parameters and renal impairment. This was a multi-center, phase I, multiple dose, open-label, safety and pharmacokinetic study of oral topotecan in patients with advanced solid tumors (NCT00483860, www.clinicaltrials.gov) [28]. In total, 6 centers participated in the study. The start date was June 2007 and a cut-off date for interim-analysis was 1 November 2010.

Creatinine clearance (Cl_{cr}) estimate was calculated using the Cockroft-Gault formula [29]. At baseline, Cl_{cr} was calculated based on creatinin excretion obtained from 24-hour urine collection. Prior platinum-based chemotherapy was defined as: ≥3 courses of cisplatin or carboplatin chemotherapy documented with normal Cl_{cr} at the time of screening; or <3 courses of cisplatin or carboplatin chemotherapy associated with a significant change in Cl_{cr} (documented >20% decrease from baseline Cl_{cr}) but documented with normal Cl_{cr} at the time of screening; or documented decreased Cl_{cr}, resulting in cisplatin or carboplatin chemotherapy being withheld, but documented with normal Cl_{cr} at the time of screening. The definition of limited prior platinum-based chemotherapy was applicable in patients in whom the definition of prior platinum-based chemotherapy was not met or if the patient had not received any platinum–based chemotherapy. Patients were assigned to one of five groups (Table 1) according to Cl_{cr} at baseline and, in the case of normal renal function, prior platinum treatments: Cl_{cr} > 80 ml/min and limited prior platinum-based chemotherapy (group A), Cl_{cr} > 80 ml/min and prior platinum-based chemotherapy (group B), mild renal impairment (Cl_{cr} =50-79 ml/min; group C), moderate renal impairment (Cl_{cr} =30-49 ml/min, group D) and severe renal impairment (Cl_{cr} <30 ml/min; group E).

Table 1: Patient demographics and characteristics of the PK evaluable population

	Treatment group					
Group	**A**	**B**	**C**	**D**	**E**	**Total**
Baseline creatinin clearance (ml/min)	**>80**	**>80**	**50-79**	**30-49**	**< 30**	
Prior platinum therapy[a]	**< 3 cycles**	**≥ 3 cycles**	**N.A**	**N.A.**	**N.A.**	**N.A.**
Number (n)	**6**	**12**	**17**	**9**	**3**	**47**
Age (years)						
Median (range)	64 (54–74)	54 (39–68)	65 (40-78)	60 (44-77)	65 (63-75)	62 (39-78)
Weight (kg)						
Median (range)	75 (64-90)	81 (43-92)	74 (41-115)	72 (53-117)	62 (60-68)	75 (41-117)
Height (m)						
Median (range)	1.71 (1.51-1.86)	1.69 (1.49-1.82)	1.65 (1.46-1.78)	1.70 (1.50-1.83)	1.64 (1.55-1.73)	1.67 (1.46-1.86)
Body surface area (m^2)						
Median (range)	1.88 (1.71-2.17)	1.92 (1.40-2.12)	1.84 (1.37-2.30)	1.84 (1.50-2.28)	1.68 (1.60-1.81)	1.84 (1.37-2.30)
Cl_{cr} [a] (ml/min)						
Median (range)	103 (90-119)	92 (83-156)	64 (51-79)	38 (32-47)	20 (10-26)	N.A.
Gender						
Male (%)	5 (83)	9 (75)	10 (59)	4 (44)	2 (67)	30(64)
Female (%)	1 (17)	3 (25)	7 (41)	5 (56)	1 (33)	17 (36)
Race, n (%)						
Caucasian	5 (83)	6 (50)	11 (65)	6 (67)	0	28 (60)
Asian	1 (17)	6 (50)	6 (35)	3 (33)	3 (100)	19 (40)
Primary tumor, n (%)						
Bile duct	0	2 (17)	0	1 (11)	0	3 (6)
Breast	0	0	2 (12)	0	1 (33)	3 (6)
Colorectal	1 (17)	0	1 (6)	0	1 (33)	3 (6)
Head & Neck	0	1 (8)	2 (12)	1 (11)	0	4 (9)
NSCLC	0	3 (25)	1 (6)	0	1 (33)	5 (11)
Ovarian cancer	0	2 (17)	3 (18)	1 (11)	0	6 (13)
Prostate cancer	3 (50)	0	3 (18)	0	0	6 (13)
Renal cell	0	0	2 (12)	1 (11)	0	3 (6)
Urothelial cell	0	1 (8)	0	2 (22)	0	3 (6)
Other	2 (33)	3 (25)	3 (18)	3 (33)	0	11 (23)
ECOG at screening, n (%)						
0	4 (67)	6 (50)	5 (29)	2 (22)	0	17 (36)
1	2 (33)	4 (33)	10 (59)	6 (67)	1 (33)	23 (49)
2	0	2 (17)	2 (11)	1 (11)	2 (67)	7 (15)

[a] Both group A and B have Cl_{cr} > 80 ml/min (according calculation from 24-hour urine collection at baseline), but group A had received "limited platinum-based therapy", defined as: <3 courses of cisplatin or carboplatin chemotherapy without significant change in Cl_{cr}. Abbreviations: Cl_{cr}= creatinine clearance; ECOG= Eastern Cooperative Oncology Group (ECOG) [27]; N.A.=not applicable.

Study procedures.

Complete physical examinations, ECG (including QT interval) and clinical laboratory tests were performed at screening and at regular intervals during cycle 1 and at study termination. Patients remained on study until occurrence of unacceptable toxicities, disease progression, withdrawal of consent, investigator discretion or treatment delay for more than 2 months.

Dosing.

Oral topotecan was provided as capsules containing topotecan HCl, equivalent to 0.25 mg or 1 mg of the anhydrous free base. Topotecan was administered on days 1-5 of a 21-day cycle. On day 1 of course 1, topotecan was administered with water after an overnight fast and a standardized lunch was scheduled two hours after dosing. Upon completion of cycle 1, patients were allowed to continue in the continuation phase during which topotecan could be administered in either the oral or the i.v. form.

The MTD and recommended dose for patients without renal impairment has been established on 2.3 mg/m^2/day [19]. The initial starting doses were: 2.3 mg/m^2/day (groups A-C), 1.2 mg/m^2/day (group D) and 0.6 mg/m^2/day (group E). In the control group (group A) twelve patients were enrolled and in the four test groups (groups B-E) patients were enrolled in cohorts of three. The maximum tolerated dose (MTD) was exceeded if two or more patients per six patients experienced a dose limiting toxicity (DLT). Dose reductions were allowed in all groups. Dose escalations were only allowed in group D and E.

A DLT was defined as: any grade 3 or 4 non-hematological toxicity (except alopecia, liver toxicity based in tumor progression or grade ≥ 3 nausea, vomiting or diarrhea without adequate supportive therapy); grade ≥ 4 neutropenia with fever or infection or > 7 days; dose delay due to grade ≥ 3 neutropenia; grade 4 thrombocytopenia; dose delay of > 28 days or dose reduction of > 75%; grade ≥ 2 toxicity for > 28 days or grade ≥ 2 (renal, neurological or cardiovascular) considered a DLT.

Safety assessment.

Adverse events (AE) and their relation to the study drug were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE) version 3.0 [30].

Evaluability.

Patients were considered evaluable it they received one dose of oral topotecan and provided pharmacokinetic sampling through the 24-hour collection period. If a patient vomited between dosing and 6.25 hours on day 1 cycle 1, dosing and sampling were repeated on day 2.

Pharmacokinetic sampling and analysis.

Blood samples for the determination of total topotecan and topotecan lactone plasma concentrations were taken at day 1 at baseline, and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours following administration. At each time point, 5 ml of whole blood was withdrawn into pre-chilled lithium heparinised collection tubes (Vacutainer™). Blood samples were immediately mixed, stored on ice and centrifuged within 5 minutes at 4000 g 4ºC for 5 minutes. For determination of total topotecan concentrations, plasma was stored and frozen at ≤ -30ºC within 15 minutes of collection. For determination of topotecan lactone concentrations, plasma protein precipitation was performed by adding 1.0 ml of the freshly separated plasma to a cold polypropylene tube containing 2.0 ml of ice-cold (≤ -30ºC) methanol. Tubes were vortex mixed for 15 seconds and immediately centrifuged again at 4000 g 4ºC for 2 minutes. The clear supernatant was transferred immediately to a polypropylene storage tube, tightly capped and stored upright at ≤ -30ºC. For total topotecan, the remaining plasma from the initial centrifugation process was stored and frozen at ≤ -30ºC within 15 minutes of collection. Urine samples were kept cool and protected from light during the collection period. Collection intervals consisted of 0-6 h, 6-12h and 12-24h after administration and total volumes were recorded. Collected urine was mixed before obtaining a 15 ml aliquot that was stored at ≤ -20ºC.

Plasma concentrations of total topotecan (both carboxylate and lactone form) and topotecan lactone and urine concentrations of total topotecan were quantified using a validated high-performance liquid chromatography (HPLC) method [31].

Total topotecan and topotecan lactone pharmacokinetic parameters including dose-normalized area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), area under the concentration-time curve extrapolated to time of last quantifiable concentration (AUC_{0-t}),terminal half-life ($t_{1/2}$), apparent clearance (Cl_{ss}/F) and volume of distribution at steady state divided by bioavailability (V_{ss}/F) were derived from plasma concentrations on day 1 to 2 by non-compartmental analysis using the log-linear trapezoidal rule employing a validated script in R [32] (version 2.12.0, http://www.r-project.org). The maximum observed plasma concentration (C_{max}) and time

to maximum observed plasma concentration (T_{max}) were directly derived from the plasma-concentration data. A statistical analysis plan was composed in advance. If calculation of $AUC_{0-\infty}$ of an individual patient resulted in a ≥ 20% difference with its AUC_{0-24}, the $AUC_{0-\infty}$ was not used in further pharmacokinetic analysis. For $AUC_{0-\infty}$, AUC_{0-t} and C_{max} , different doses were normalized to 1 mg/m^2/day and under the assumption of dose-proportional pharmacokinetics. Individual topotecan elimination constants in urine were determined by linear regression analysis of topotecan concentrations in urine at different time points. Elimination constants were calculated using the slope of the fitted linear regression lines**.**

Statistical analysis.

Differences between groups in continuous variables were tested using non-parametric Mann-Whitney *U* test (SPSS version 17.0; SPSS Inc., Chicago, USA). All significance tests were two-sided and *P*-values < 0.05 were considered statistically significant.

Statistical analysis of dose-normalized $AUC_{0-\infty}$ and C_{max} used an estimation approach based on mean ratio (i.e. the ratio of the pharmacokinetic parameters for test [groups B-E] over reference [group A]) and was performed in R [32]. LN-transformed $AUC_{0-\infty}$ and C_{max} were subjected to analysis of variance (ANOVA) and results were presented in terms of geometric means ratio with associated 90% confidence intervals (CI), no p-values were presented. In order to control type I error rate, a 'multiple comparison procedure' was used. The estimated means were back-transformed to obtain an estimate for the ratio. Potential age, gender, weight and ethnicity effects were explored. Correlations between pharmacokinetic parameters and Cl_{cr} were explored graphically.

RESULTS

Patient inclusion and demographics.

In total, at the cut-off date for interim analysis, 75 patients had been screened and 53 patients were included in the study, of which 47 were evaluable. Patient demographics and characteristics are summarized in Table 1. The median age was 62 years (range: 39 – 78). Median baseline Cl_{cr} was lower in group B (92 ml/min) compared to group A (103 ml/min) but this was not a statistically significant difference (Mann-Whitney *U* test: *P* =0.291). The majority of patients was male (64%), Caucasian (60%) and had an ECOG score of 1 (49%). The most frequently observed primary tumor sites were: ovarian- and prostate cancer (both 13%) and non-small lung cancer (NSCLC; 11%).

Safety.

In total, 47 patients were evaluable for safety: 6 in group A, 12 in group B, 17 in group C, 9 in group D and 3 in group E. No unexpected toxicity was observed and there were no obvious differences in type and severity of toxicity across the groups. All CTCAE grade ≥ 3 toxicities occurring > 1 occasion or Serious Adverse Events reported as treatment-related are summarized in Table 2. The most frequently occurring toxicities were grade 3 neutropenia and thrombocytopenia (12/47, 26% both), grade 4 neutropenia (9/47, 19%), grade 3 fatigue (5/47, 11%), nausea and anemia (6/47, 13%, both). Also grade 3 neutropenia with fever (2/47, 4.3%), with infection (1/47, 4.3%) and with sepsis (grade 5, 1/53, 1.9%) were reported. When comparing safety between patients with prior platinum-based chemotherapy and limited platinum-based therapy, primarily grade 3 and 4 neutropenia (17 and 25%), grade 3 thrombocytopenia (42%) and fatigue (25%) toxicities were found more often in the prior platinum-based chemotherapy group compared to the limited platinum-based therapy patients (grade 3 neutropenia, thrombocytopenia, leucopenia, nausea and vomiting, 17% each).There were two deaths during the study. One patient with renal cancer in group C (dose 2.3 mg/m^2/day) died during cycle 2 with neutropenic sepsis, that was considered related to the study drug. One patient in group D (dose 1.5 mg/m^2/day) died due to disease progression of vaginal carcinoma.

Nine dose-limiting toxicities (DLTs) occurred. The number of patients included in each dose level and all DLTs are summarized in Table 3. The most frequently observed DLTs were grade 3 neutropenia leading to treatment delay (4/9), combined grade 4 thrombocyto- and neutropenia (2/8), grade 3 fatigue (2/8) and grade 4 (1/8) thrombocytopenia. A detailed description of safety, dose escalation and types of DLTs occurring in all groups following oral topotecan administration is presented in the Appendix. In summary, the MTD was determined at 2.3 mg/m^2/day for groups B and C. No DLTs have been determined yet in groups D and E and further enrollment continues at present at 1.5 mg/m^2/day (group D) and 0.8 mg/m^2/day (group E).

Pharmacokinetic analysis.

Mean (standard deviation, SD) plasma concentration-time curves are presented in Figures 1 (total topotecan) and 2 (topotecan lactone). The curves show higher plasma concentrations for group D (dosed at 1.8 mg/m^2/day) and slightly higher plasma concentrations for group B, compared to group A, for both total topotecan and topotecan lactone. For total topotecan in group E, the shape of the curve appears to be different compared to the reference group (A), with a less steep rise in plasma concentration and a slower decline after T_{max} up to 24 hours.

Table 2: All CTCAE grade ≥ 3 occurring > 1 occasion or Serious Adverse Events (SAEs) reported as treatment-related

	Grade	Treatment group					Total
Group		**A N=6 (%)**	**B N=12 (%)**	**C N=17 (%)**	**D N=9 (%)**	**E N=3 (%)**	**Total N=47 (%)**
Neutropenia	3	1 (17)	2 (17)	5 (29)	1 (11)	3 (100)	12 (26)
	4	0	3 (25)	5 (29)	1 (11)	0	9 (19)
Thrombocytopenia	3	1 (17)	5 (42)	6 (35)	0	0	12 (26)
	4	0	0	1 (5.9)	2 (22)	0	3 (6.4)
Anemia	3	0	0	1 (5.9)	3 (33)	2 (67)	6 (13)
Leukopenia	3	1 (17)	1 (8.3)	2 (12)	0	0	4 (8.5)
	4	0	0	0	1 (11)	0	1 (2.1)
Elevated GGT	3	1 (17)	1 (8.3)	1 (5.9)	0	0	3 (6.4)
Hypokalemia	3	1 (17)	1 (8.3)	1 (5.9)	0	0	3 (6.4)
Fatigue	3	0	3 (25)	1 (5.9)	1 (11)	0	5 (11)
Nausea	3	1 (17)	0	1 (5.9)	2 (22)	0	4 (8.5)
Vomiting	3	1 (17)	0	1 (5.9)	1 (11)	0	3 (6.4)
Diarrhea	3	0	0	1 (5.9)	0	0	1 (2.1)
Neutropenic fever	3	0	0	1 (5.9)	1 (11)	0	2 (4.3)
Infection with neutropenia	3	0	0	1 (5.9)	0	0	1 (2.1)
Neutropenic sepsis	5	0	0	1 (5.9)	0	0	1 (2.1)
Dehydration	3	0	0	1 (5.9)	0	0	1 (2.1)
Constipation	3	0	0	0	1 (11)	0	1 (2.1)

Abbreviations: CTCAE= common terminology criteria for adverse events; GGT= gamma-glutamyl transferase.

Table 3: Number of patients included at each dose level per group, types of DLTs and declared MTDs per group

Group	Dose topotecan (mg/m^2/day)	Number of evaluable patients enrolled	DLT (type)	MTD (mg/m^2/day)
A	2.3	6	Grade 3 neutropenia[a]	2.3
B	2.3	12	Grade 3 neutropenia (2x)[a] Grade 3 fatigue	2.3
C	2.3	12	Grade 3 fatigue Grade 3 neutropenia[a] Grade 4 thrombocytopenia	2.3
	1.9	5	none	
D	1.2	3	None	
	1.5	3 (+ currently enrolling)	None	
	1.8	3	Combined grade 4 thrombocytopenia and grade 4 neutropenia (2x)[a]	
E	0.6	3 (+ currently enrolling)	None	

[a] Neutropenia leading to dose delay. Abbreviations: DLT= dose-limiting toxicity; MTD=maximum tolerated dose.

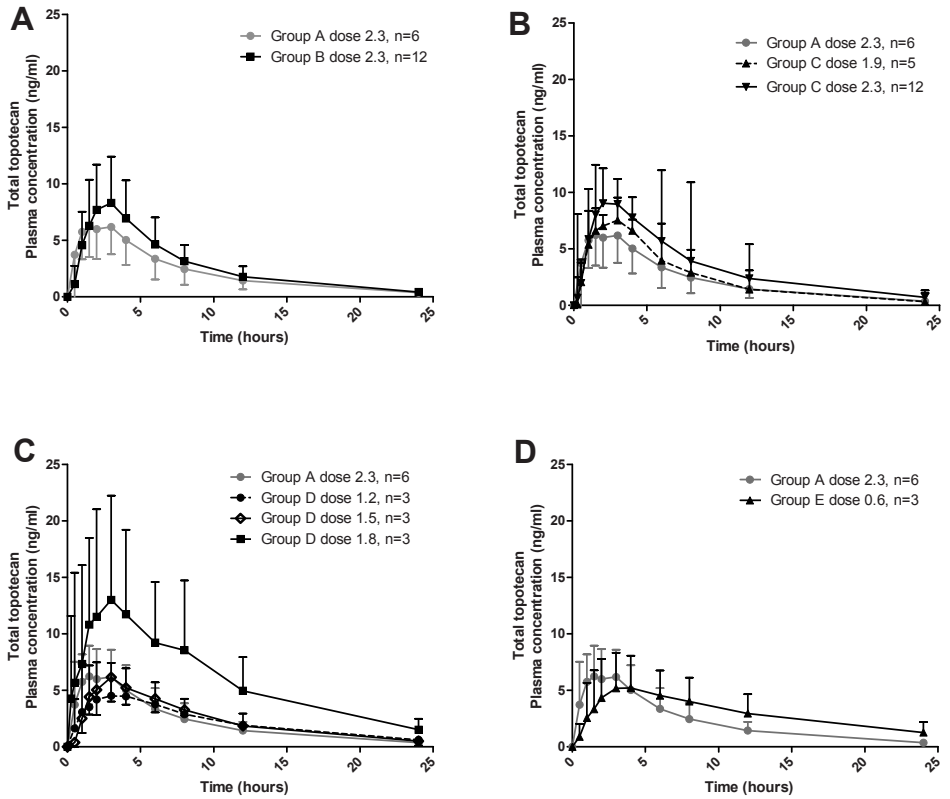


Figure 1: Mean (SD) plasma concentration (ng/ml)-time curve of total topotecan following oral administration. Groups A-E represent different groups with normal renal function, with limited previous platinum-based chemotherapy (group A) and with platinum-based chemotherapy (group B, Fig 1A), or impaired renal function (group C: mild, group D: moderate and group E: severe, Fig. 1-B-D). Group A is depicted in all graphs as reference (normal renal function). Oral topotecan doses per group are indicated in mg/m2/day. N=number of patients.

Pharmacokinetic data are summarized in Table 4. In two patients $AUC_{0-\infty}$ could not be estimated correctly because the percentage extrapolated from $T_{24\ hours}$ to infinity was > 20%. This concerned a patient in group D and in group E. In group D, for another patient, sampling was not completed up to 24 hours for total topotecan, and therefore, also in this patient $AUC_{0-\infty}$ could not be estimated. These $AUC_{0-\infty}$ values were excluded from further analysis. Generally, pharmacokinetic parameters appeared to change incrementally with increasing renal impairment. Slight changes could also be seen in group B, with patients who had normal renal function but had received prior platinum-based chemotherapy, compared to reference. Mean dose-normalized $AUC_{0-\infty}$, AUC_{0-t} and C_{max} and also $T_{1/2}$ appeared to increase with increasing renal impairment (from group B to E) compared to reference for both total

topotecan and topotecan lactone. Median T_{max} was similar for groups B through E for total topotecan and differed slightly from group A (reference). Cl_{ss}/F and V_{ss}/F were both reduced with increasing renal impairment. Unfortunately, because of exclusion of two patients in the essential groups D and E due to inability of estimating $AUC_{0-\infty}$, the effect of reduced renal function could not be estimated more accurately.

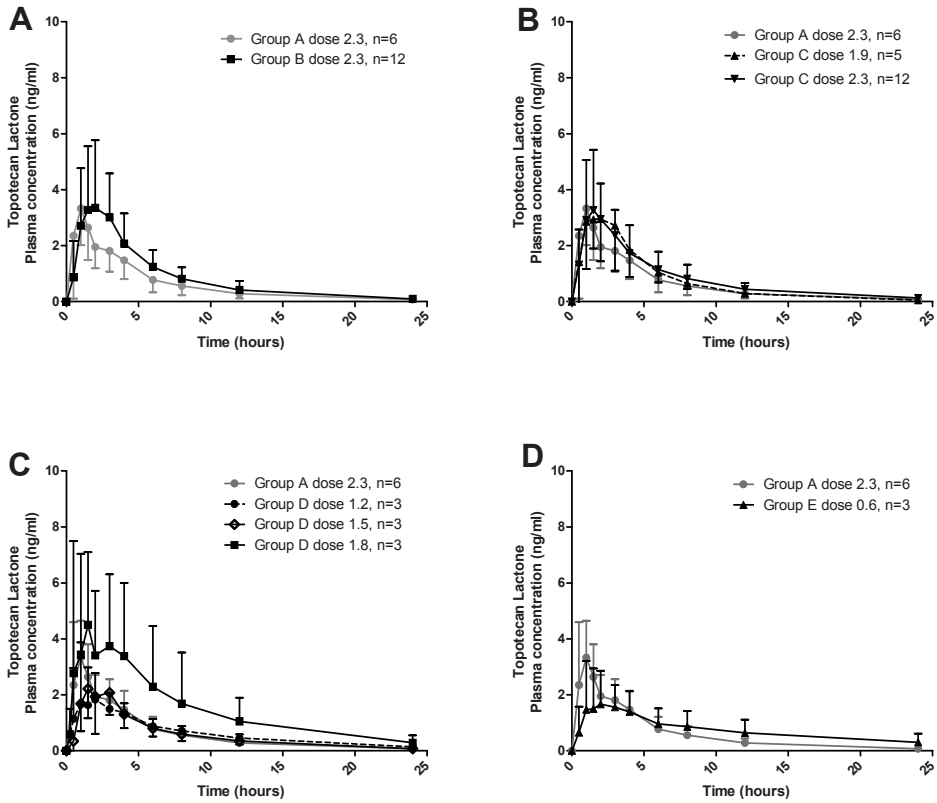


Figure 2: Mean (SD) plasma concentration (ng/ml)-time curve of topotecan lactone following oral administration. Groups A-E represent different groups with normal renal function, with limited previous platinum-based chemotherapy (group A) and with platinum-based chemotherapy (group B, Fig 1A), or impaired renal function (group C: mild, group D: moderate and group E: severe, Fig. 1-B-D). Group A is depicted in all graphs as reference (normal renal function). Oral topotecan doses per group are indicated in mg/m2/day. N=number of patients.

Table 4: Pharmacokinetic parameters of total topotecan and topotecan lactone, after oral administration of topotecan (per group)

	Treatment group				
Group	**A**	**B**	**C**	**D**	**E**
Cl_{cr} (ml/min)	**>80**	**>80**	**50-79**	**30-49**	**< 30**
N	**6**	**12**	**17**	**9**	**3**
Dose-normalized					
$AUC_{0-\infty}$ (h*ng/ml*m^2 per unit of dose)[a]					
Total topotecan	23.8 (11.2)	29.0 (12.6)	35.8 (6.18)	60.2 (42.5)[b]	119 (101)[c]
Topotecan lactone	6.78 (2.83)	8.98 (4.62)	9.09 (2.77)	15.4 (11.1)[c]	26.0 (23.8)[c]
AUC_{0-t} (h*ng/ml*m^2 per unit of dose)[a]					
Total topotecan	22.4 (10.5)	27.5 (12.1)	33.2 (5.17)	51.5 (35.2)	117 (65.1)
Topotecan lactone	6.49 (2.72)	8.64 (4.43)	8.64 (2.69)	13.6 (9.89)	29.6 (18.4)
C_{max} (ng/ml*m^2 per unit of dose)[a]					
Total topotecan	2.87 (1.19)	3.80 (1.83)	4.45 (1.51)	5.11 (3.33)	8.98 (5.19)
Topotecan lactone	1.61 (0.71)	1.84 (1.08)	1.88 (0.83)	2.26 (1.29)	3.34 (2.29)
Actual values					
T_{max} (h)					
Total topotecan	2.45 (1.00-3.05)	3.00 (1.50-3.03)	2.50 (1.05 -4.00)	3.00 (1.00-6.07)	3.00 (3.00-3.97)
Topotecan lactone	1.00 (0.35-1.55)	1.51 (1.00-3.00)	1.05 (0.25-3.02)	1.50 (0.57-4.00)	1.00 (1.00-3.12)
Cl_{ss}/F (ml/min*m^2)					
Total topotecan	898 (525)	707 (387)	480 (101)	372 (186)[b]	219 (186)[c]
Topotecan lactone	2890 (1363)	2526 (1727)	1973 (531)	1426 (621)[c]	1105 (1012)[c]
V_{ss}/F (l/m^2)					
Total topotecan	419 (219)	330 (170)	245 (69.3)	205 (122)[b]	129 (97)[b]
Topotecan lactone	1528 (988)	1320 (1638)	936 (368)	730 (468)[c]	679 (626)[b]
$T_{1/2}$ (h)					
Total topotecan	5.63 (1.29)	5.45 (0.91)	6.01 (1.75)	6.74 (1.87)	9.45 (3.87)
Topotecan lactone	5.80 (1.09)	5.32 (1.77)	5.51 (1.91)	6.87 (3.51)	10.44 (5.85)
T_{last} (h)					
Total topotecan	24.0 (24.0-24.17)	24.0 (23.9-24.3)	24.0 (23.9-24.3)	24.0 (11.9-24.1)	24.0 (24.0-24.1)
Topotecan lactone	24.0 (24.0-24.2)	24.0 (23.9-24.3)	24.0 (23.9-24.3)	24.0 (24.0-24.1)	24.0 (24.0-24.1)

All data are shown as mean (SD), except T_{max} and T_{last} which are median (range). [a] Doses are normalized to 1 mg/m^2/day, actual values were not normalized for different doses. One([c]) or two ([b]) patients were excluded because of invalid estimation of $AUC_{0-\infty}$. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; C_{max} = maximum observed plasma concentration; T_{max} = time to maximum observed plasma concentration; Cl_{ss}/F = apparent clearance; F=bioavailability; $t_{1/2}$ = terminal half-life; T_{last} = time to last quantifiable concentration V_{ss}/F= volume of distribution at steady state divided by F.

Table 5: Pharmacokinetic parameters of total topotecan and topotecan lactone after oral administration of topotecan (per dose level within each group)

	Treatment group							
Group	**A**	**B**	**C**	**C**	**D**	**D**	**D**	**E**
Cl_{cr} (ml/min)	**>80**	**>80**	**50-79**	**50-79**	**30-49**	**30-49**	**30-49**	**< 30**
Dose (mg/m²/day)	**2.3**	**2.3**	**1.9**	**2.3**	**1.2**	**1.5**	**1.8**	**0.6**
N	**6**	**12**	**5**	**12**	**3**	**3**	**3**	**3**
$AUC_{0-\infty}$ (h*ng/ml*m²)								
Total topotecan	54.8 (25.7)	66.7 (29.0)	59.8 (9.13)	86.7 (13.6)	57.9 (33.4)	61.8 (1.19)[a]	174 (133)[a]	71.4 (60.6)[b]
Topotecan lactone	15.6 (6.50)	20.7 (10.6)	17.5 (4.76)	20.8 (6.86)	16.4 (6.93)	14.2 (3.30)	48.0 (36.7)[a]	15.6 (14.3)[b]
AUC_{0-t} (h*ng/ml*m²)								
Total topotecan	51.6 (24.2)	63.1 (27.9)	57.3 (4.76)	79.2 (11.6)	51.7 (30.6)	51.2 (10.3)	139 (93.7)	70.3 (39.1)
Topotecan lactone	14.9 (6.26)	19.9 (10.2)	17.2 (4.78)	19.5 (6.55)	14.9 (6.32)	13.6 (3.89)	35.0 (29.7)	17.7 (11.1)
C_{max} (ng/ml*m²)								
Total topotecan	6.61 (2.73)	8.73 (4.22)	8.82 (2.47)	10.1 (3.77)	4.73 (2.88)	6.16 (2.14)	13.1 (9.16)	5.39 (3.11)
Topotecan lactone	3.72 (1.63)	4.24 (2.48)	4.01 (1.62)	4.10 (1.93)	2.48 (1.57)	2.71 (0.89)	5.21 (3.41)	2.00 (1.38)
Cl_{ss}/F (ml/min*m²)								
Total topotecan	898 (25)	707 (387)	541 (98.3)	455 (94.6)	436 (250)	404 (7.77)[a]	242 (184)[a]	219 (186)[b]
Topotecan lactone	2890 (1363)	2526 (1727)	1904 (432)	2001 (582)	1388 (617)	1826 (453)	882 (674)[a]	1105 (1012)[b]
$t_{1/2}$ (h)								
Total topotecan	5.63 (1.29)	5.45 (0.91)	5.06 (1.00)	6.41 (1.87)	6.99 (0.24)	6.15 (2.18)	7.10 (2.90)	9.45 (3.87)
Topotecan lactone	5.80 (1.09)	5.32 (1.77)	4.30 (0.42)	6.01 (2.08)	6.76 (2.11)	5.71 (2.52)	8.14 (5.83)	10.44 (5.85)

All data are shown as mean (SD). One ([a]) or two ([b]) patients were excluded because of invalid estimation of $AUC_{0-\infty}$. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; C_{max} = maximum observed plasma concentration; Cl_{ss}/F = apparent clearance; F= bioavailabilty; $t_{1/2}$ = terminal half-life.

In order to better compare exposure to topotecan between dose levels, also actual $AUC_{0-\infty}$, AUC_{0-t}, C_{max}, Cl_{cc}/F and $t_{1/2}$ values were calculated per group and dose level (Table 5). Although the results of these calculations are affected by the small sample size and therefore show large variability, a rough estimate of mean and SD could be calculated. The highest mean $AUC_{0-\infty}$ and AUC_{0-t} were observed in group D dose 1.8

mg/m^2/day, followed by group C dose 2.3 mg/m^2/day and group E dose 0.6 mg/m^2/day. Mean AUC $_{0-\infty}$ for group D dose 1.8 mg/m^2/day, a dose that had exceeded the MTD, was approximately 3-fold higher compared to reference in group A. C_{max} was high in group D dose 1.8 mg/m^2/day and group C 2.3 mg/m^2/day, but relatively low in group E compared to reference.

Table 6: Statistical analysis of the primary pharmacokinetic parameters

Parameter	Geometric mean		Comparison (Group names)	Ratio of means (impaired: normal function)	90% confidence interval
	Impaired	Normal renal function			
$AUC_{0-\infty}$ (ng.h/ml*m^2; per unit of dose)[a]					
Total topotecan	26.36	21.24	B : A	1.24	(0.98 – 1.57)
Topotecan lactone	7.81	6.28	B : A	1.24	(0.96 – 1.61)
Total topotecan	35.35	21.24	C : A	1.66	(1.46 – 1.90)
Topotecan lactone	8.75	6.28	C : A	1.39	(1.21 – 1.61)
Total topotecan	51.00	21.24	D : A	2.40	(1.45 – 3.99)
Topotecan lactone	13.08	6.28	D : A	2.09	(1.33 – 3.28)
Total topotecan	95.15	21.24	E : A	4.48	(2.53 – 7.95)
Topotecan lactone	19.80	6.28	E : A	3.16	(1.85 – 5.37)
C_{max} (ng/ml*m^2; per unit of dose)[a]					
Total topotecan	3.42	2.65	B : A	1.29	(1.11 – 1.49)
Topotecan lactone	1.58	1.47	B : A	1.08	(0.90 – 1.28)
Total topotecan	4.26	2.65	C : A	1.60	(1.01 – 2.54)
Topotecan lactone	1.72	1.47	C : A	1.18	(0.64 – 2.15)
Total topotecan	4.42	2.65	D : A	1.67	(0.97 – 2.87)
Topotecan lactone	1.93	1.47	D : A	1.32	(0.72 – 2.41)
Total topotecan	7.85	2.65	E : A	2.96	(1.97 – 4.44)
Topotecan lactone	2.77	1.47	E : A	1.89	(1.18 – 3.02)

[a] Doses are normalized to 1 mg/m^2/day. Abbreviations: $AUC_{0-\infty}$= Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration.

Ratios of geometric means (90% CI) for dose-normalized $AUC_{0-\infty}$ and C_{max} of test groups compared to reference (normal renal function, group A) are summarized in Table 6. Geometric means are visualized in Figure 3 (3A and B for total topotecan and 3C and D for topotecan lactone). ANOVA analysis was performed and no effect of weight, age and gender was found. A markedly increased ratio was found for $AUC_{0-\infty}$ for groups D and E compared to and for C_{max} for group E compared to reference.

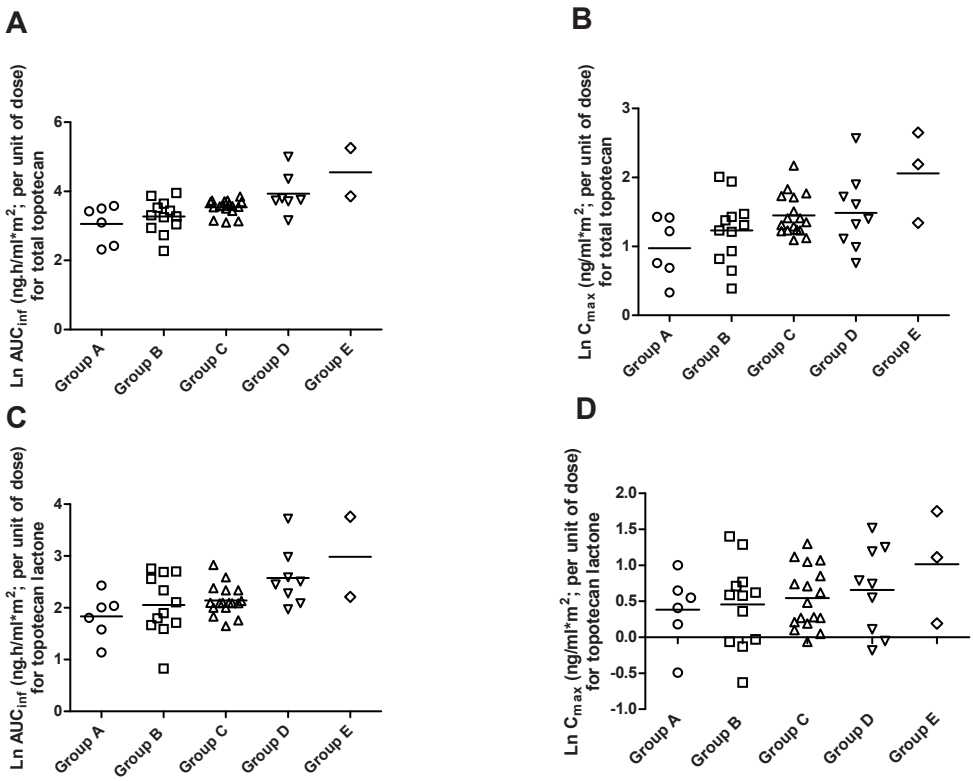


Figure 3: Scatterplots representing natural logarithm (Ln)-transformed values of dose-normalized $AUC_{0-\infty}$ and C_{max} for total topotecan (Fig. 3A and B) and topotecan lactone (Fig. 3C and D), according to groups with normal and impaired renal function. Horizontal lines represent the mean. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration and C_{max}.

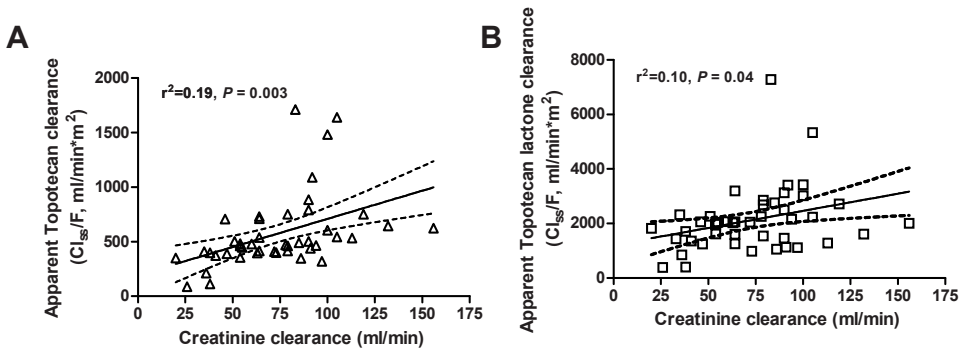


Figure 4: Linear regression analysis (95% confidence interval) between individual creatinine clearance (according to calculation from 24-hour urine collection at baseline) and apparent clearance (Cl_{ss}/F) of total topotecan (Fig. 4A) and topotecan lactone (Fig. 4B).

Correlations between Cl_{cr} and topotecan apparent clearance, $AUC_{0-\infty}$, AUC_{0-t} and C_{max} were explored graphically and by linear regression. A significant, but weak, correlation was found between individual Cl_{cr} and Cl/F (for total topotecan: coefficient of determination (r^2)=0.19, *P* = 0.003, Figure 4A; for topotecan lactone: r^2=0.09; *P* = 0.04, Figure 4B).The influence of ethnicity on topotecan exposure was assessed by comparing dose-normalized AUC_{0-t} between Asians (n=19, 40%) and Caucasians (n=28, 60%). Dose-normalized AUC_{0-t} (SD) was significantly higher in Asians for topotecan lactone (Asians: 15.0 (11.7); Caucasians: 7.74 (2.26); *P* = 0.013), but not for total topotecan (Asians: 52.2 (45.2); Caucasians: 30.4 (9.60); *P* = 0.165).

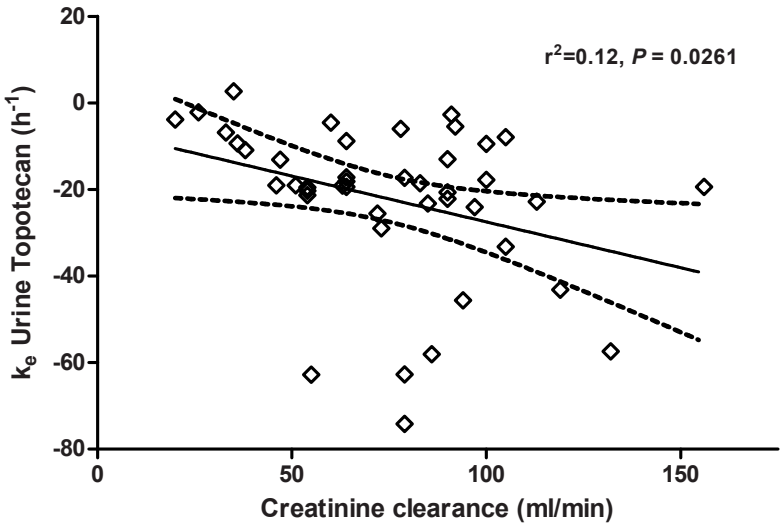


Figure 5: Linear regression analysis (95% confidence interval) between individual creatinine clearance (according to calculation from 24-hour urine collection at baseline) and elimination constant (k_e) of total topotecan in urine.

Pharmacokinetic data in urine were available from 42 patients. Because oral bioavailability was unknown, no calculations of renal clearance could be made. The fraction excreted of total dose was calculated. The mean (SD) percentage of oral dose (per m^2) excreted in urine within 24 hours was comparable between all groups with 24 (11), 19 (13), 23 (11), 18 (5.5) and 24 (20) % for total topotecan, for groups A through E, respectively. Correlation between individual k_e of topotecan in urine and creatinine clearance at baseline were also explored graphically and in linear regression analysis (Figure 5). Here, also a weak but significant correlation was found (r^2=0.12, *P* = 0.0261).

DISCUSSION

In this study, the effect of renal impairment and prior platinum-based chemotherapy was determined on the safety and pharmacokinetics of oral topotecan in patients with solid tumors. The most frequently occurring DLT was neutropenia, which was also observed as most common DLT together with thrombocytopenia in patients with impaired renal function treated with i.v. topotecan [24]. These are commonly known side effects of both i.v and oral formulation as observed in phase I-III trials [3;16-19;33]. In total, 7 out of 9 DLTs were hematological toxicities due to myelosuppression and the importance of this type of toxicity was underscored by one death as a result of neutropenic sepsis. The only non-hematological DLT was grade 3 fatigue, which occurred twice, in patients with prior platinum-based chemotherapy and in patients with mild renal impairment. Because of incomplete enrollment at time of writing of this interim report in the moderate and severe renal impairment cohorts, more DLTs are expected to occur in the latter groups. Therefore, no mature evaluation of the pattern of DLTs between different treatment groups could be made hitherto.

The most frequent non-hematologic toxicities were fatigue, nausea and vomiting. These side effects were also well known from experience in previous phase I to III trials [3;16-19;33]. It had been observed in patients treated with i.v. topotecan that fatigue was reported significantly more frequently in patients with renal impairment [24] but this was not observed in the present study. Fatigue grade 3 was predominantly experienced by patients with normal renal function and prior platinum-based chemotherapy (25%) and this was less frequently observed in the mild (6%), moderate (11%) and severe (0%) renal impairment groups. This could be due to the incomplete enrollment in the latter group resulting in a small sample size.

It was hypothesized that prior cisplatin or carboplatin therapy could lead to permanent renal tubular dysfunction without affecting Cl_{cr} and that this could interfere with renal secretion of topotecan. This, in turn, could lead to increased drug exposure, increased toxicity and reduced safety. O'Reilly et al. had observed that DLTs were more frequently reported in extensively pre-treated patients when treated with intravenous topotecan and that further dose-reductions were required [24]. Indeed, in the present study, a minimal increase in total topotecan and topotecan lactone $AUC_{0-\infty}$ and C_{max} was observed in the ratios of geometric means compared to reference, (group B: group A, Table 6) but this was not considered statistically relevant. Safety was also directly compared between these groups. Although the incidence of toxic events was higher in patients that had received prior platinum-based chemotherapy

compared to patients that had received limited platinum-based therapy (Table 2), the MTD was the same in both groups (2.3 mg/m^2/day day 1-5 in a 21-day cycle). These results showed that there was no clinically relevant difference in drug exposure that required a reduced dose for this group of patients.

A significant, but weak, correlation (r^2 = 0.19 and *P* = 0.003; r^2 = 0.09 and *P* = 0.04; for total topotecan and topotecan lactone, respectively) was found between Cl_{cr} and Cl_{ss}/F after administration of oral topotecan. The correlation was weaker than the correlation observed between the same parameters after i.v. administration (r^2=0.67, *P* = 0.00001 and r^2=0.65, *P* = 0.00003, for total topotecan and topotecan lactone) [24]. This difference may be due to the low and variable bioavailability of oral topotecan compared to that of the intravenous formulation [6;23]. Cross-study analysis in patients receiving oral topotecan (*N*= 217, of whom 99 (46%) with Cl_{cr} < 80 ml/min) revealed that <20% of variability in topotecan exposure was due to Cl_{cr}. Next to Cl_{cr}, other variables may also affect the exposure of oral topotecan. In the present analysis, an effect of ethnicity on topotecan exposure was observed, which was statistically significant for topotecan lactone. Others have reported that a specific polymorphism in *ABCB2* (421 C>A) led to increased oral bioavailability of topotecan compared to that in patients with wild-type alleles [34]. The frequency of this polymorphism was estimated much higher in Japanese, Koreans and Chinese (26-35%) compared to Caucasians (11%) [35], and could therefore explain the observed difference in exposure between Asians and Caucasians in the present study by variability in drug absorption and/or excretion irrespective of renal impairment. Pharmacogenetic analysis of germ-line DNA of patients participating in the study will be pursued in the future.

Renal drug clearance is achieved by glomerular filtration, tubular secretion and/or tubular reabsorption. Active secretion of drugs or metabolites from the blood to the lumen takes place in the proximal tubule, that is equipped with multiple transporters including P-gp, BCRP, MRPs and organic anion transporters (OATs) [36;37]. Topotecan is a substrate for P-gp [38] and BCRP [39], that are both also located in the intestine where they have a major influence in limiting oral bioavailability [23;33]. Co-administration of the P-gp and BCRP inhibitor elacridar has been shown to increase topotecan exposure significantly [23;33]. In order to reduce inter-patient variation, patients were therefore not allowed to receive drugs known to inhibit or induce BCRP, P-gp or MRP during pharmacokinetic sampling. Involvement of OAT3 and MRP in topotecan transport has also been described *in vitro* [40;41], but its clinical relevance is currently unknown.

Chronic renal disease might not only have an effect on clearance but also on the absorption of oral topotecan by delaying gastric emptying in patients that have high

plasma urea levels. In the present study, indeed increased mean dose-normalized C_{max} values were observed in the renal impairment groups but no effect was observed on median T_{max} values. Also, in these patients, there appears to be a plateau phase for both total topotecan and topotecan lactone in the concentration-time curve following T_{max} for group E. This pattern has been described previously by O'Reilly et al. in a patient with severe renal dysfunction (Cl_{cr}=32 ml/min) treated with i.v. topotecan and this was thought to be due to enterohepatic recirculation [24].

The results of this interim analysis indicate that a dose reduction below 1.8 mg/m^2/day will be necessary in order to administer oral topotecan safely in patients with moderate and severe renal impairment. Population pharmacokinetic/pharmacodynamic models have been reported that related pharmacokinetics to probability of experiencing severe toxicity (neutropenia) after i.v. administration of topotecan [42]. Inter-patient variability in clearance could be reduced by taking individual characteristics into account, such as performance status and body weight, resulting in better prediction of toxicity. Such model has also been described for predicting pharmacokinetics following different doses of oral topotecan in individual patients [43], although the influence of ethnicity was not reported in the latter study. These tools may become valuable in translating the results of the present study into relevant recommendations for the appropriate dose for an individual patient, based on its characteristics such as creatinine clearance.

In conclusion, no dose adjustments of oral topotecan are required in patients with normal renal function and prior platinum-based chemotherapy or mild impaired renal function (Cl_{cr} > 50 ml/min). Dose adjustments are probably required for patients with moderate and severe renal impairment, but more pharmacokinetic and safety data are awaited before definitive conclusions can be drawn.

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APPENDIX

Description of safety, dose escalation and DLTs occurring in all groups following oral topotecan administration

In group A, three patients were treated with the recommended dose of 2.3 mg/m^2/day and one patient experienced a DLT (treatment delay due to grade 3 neutropenia). Three additional patients were entered and none experienced a DLT, resulting in total in one DLT in six patients at this dose level.

In group B, three patients started at 2.3 mg/m^2/day without experiencing DLTs. Additional three patients were entered of whom one experienced a DLT (treatment delay due to grade 3 neutropenia). Another three patients were entered (no dose escalation was allowed) of whom also one patient experienced a DLT (treatment delay due to grade 3 neutropenia). This was followed by inclusion of three additional patients without DLTs. Another DLT in this group during cycle 1 was recognized in retrospect (grade 3 fatigue), resulting in total in three DLTs in twelve patients. The MTD was determined at 2.3 mg/m^2/day.

In group C, three patients started at 2.3 mg/m^2/day in whom one patient developed a DLT (grade 3 fatigue). Another patient in this group had developed grade 3 thrombocytopenia, which was initially considered a DLT, but this judgment was later revoked. Six other patients were entered at 1.9 mg/m^2/day, of whom one patient was not evaluable, resulting in total in no DLTs in five patients at this dose level. Next, three patients were entered in the 2.3 mg/m^2/day cohort, without DLTs and three additional patients, of whom one patient experienced a DLT (treatment delay due to grade 3 neutropenia). Three other patients were entered resulting in one DLT grade 4 thrombocytopenia. In total, three DLTs were observed in twelve patients. The MTD in group C was therefore also determined at 2.3 mg/m^2/day.

In group D, five patients were entered at 1.2 mg/m^2/day of whom three were evaluable and none experienced a DLT. Subsequently, three other patients started at 1.5 mg/m^2/day with no DLTs, followed by three other patients being entered at 1.8 mg/m^2/day of whom 2 experienced DLTs (both: combined grade 4 thrombocytopenia and neutropenia, resulting in treatment delay). Further enrollment continues at present with three additional patients at 1.5 mg/m^2/day. No MTD has been determined yet.

In group E, in total five patients were entered at 0.6 mg/m^2/day, of whom three were evaluable, and none experienced a DLT. Further enrollment continues at present at 0.8 mg/m^2/day. No MTD has been determined yet.

Chapter 3

Pharmacokinetics of eribulin mesylate

Chapter 3.1

Eribulin mesylate pharmacokinetics in patients with solid tumors and hepatic impairment

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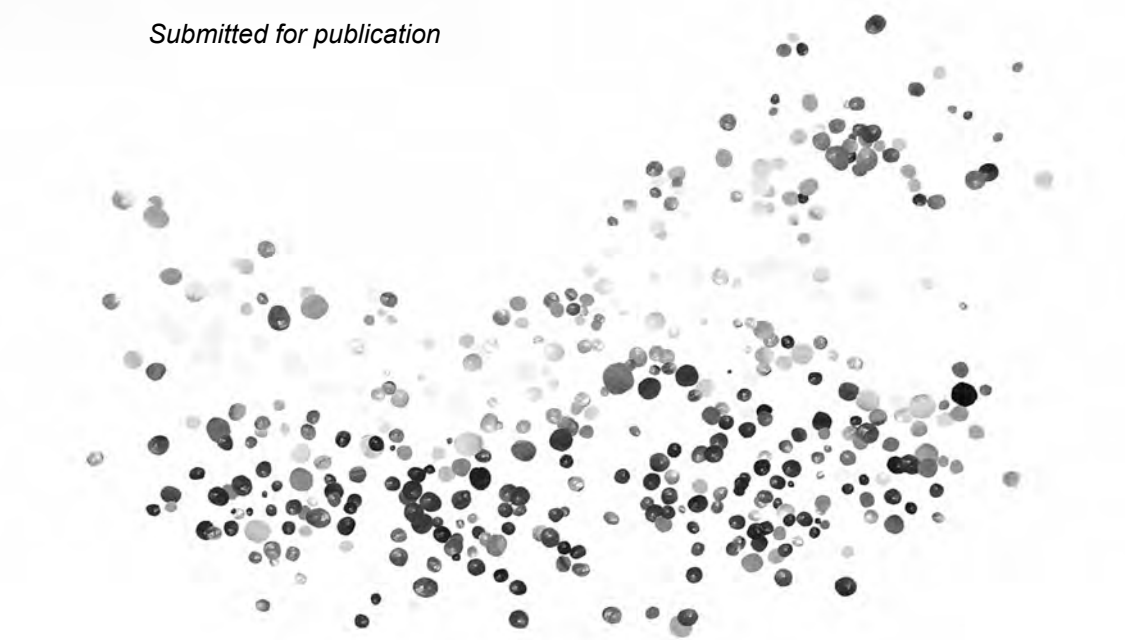


ABSTRACT

Background

The aim of this study was to determine the effect of mild and moderate hepatic impairment on the plasma pharmacokinetics of eribulin mesylate in patients with solid tumors. Eribulin is a non-taxane microtubule dynamics inhibitor.

Patients and methods

A phase I, pharmacokinetic study was performed in patients with advanced solid tumors and normal hepatic function or mild (Child-Pugh A) or moderate (Child-Pugh B) hepatic impairment. Treatments were given on day 1 and 8 of a 21-day cycle and consisted of 1.4 mg/m^2, 1.1 mg/m^2 and 0.7 mg/m^2 eribulin mesylate, for normal hepatic function, mild and moderate hepatic impairment, respectively. Also safety and anti-tumor activity were determined.

Results

Hepatic impairment increased exposure to eribulin. In patients with Child-Pugh A (*N*=7) and Child-Pugh B (*N*=5), mean dose-normalized $AUC_{0-\infty}$ was 1.75-fold (90% Confidence intervals (CI): 1.16-2.65) and 2.48-fold (90% CI: 1.57-3.92) increased, respectively compared with patients who have normal function (*N*=6). The most frequently reported treatment-related events were alopecia (12/18) and fatigue (7/18). Nine patients (50%) had stable disease as best response.

Conclusions

A reduced dose of 1.1 mg/m^2 and 0.7 mg/m^2, of eribulin mesylate is recommended for patients with mild or moderate hepatic impairment, respectively.

INTRODUCTION

Liver disease is a common cause of altered disposition of drugs and can impact efficacy and safety [1;2].The liver has a pivotal role in the clearance of many drugs through metabolism and/or biliary excretion of unchanged drug or metabolites [1]. Alterations of these excretory and metabolic activities by hepatic impairment can lead to drug accumulation and can increase toxicity of drugs that are detoxified in the liver. This may necessitate dose adjustments in patients with an impaired liver function [3]. Tubulin-acting agents, such as vinca-alkaloids and taxanes, are clear examples of agents that require dose adjustments in hepatic impaired patients in order to avoid over-exposure to the drug, potentially leading to increased toxicity [2;4]. Eribulin mesylate (E7389; Halaven™) is a non-taxane microtubule dynamics inhibitor with a novel and distinct mechanism of action. It is a synthetic analogue of halichondrin B, a product isolated from the marine sponge *Halichondria okadai.* Eribulin prevents microtubule growth by binding directly to microtubule ends. This results in formation of abnormal mitotic spindles leading to G2-M arrest and ultimately cell death [5-8].

Eribulin mesylate demonstrated efficacy and a manageable side-effect profile in patients with solid tumors including in heavily pre-treated metastatic breast cancer patients [9-11]. Results of a phase III trial (EMBRACE) demonstrated that eribulin mesylate treatment significantly improved median overall survival of metastatic breast cancer patients that were previously treated with an anthracycline and a taxane compared to 'treatment of physician's choice' [11]. Following the results of this trial, eribulin mesylate was recently approved in the United States of America (USA), Singapore, Europe and Japan for this indication.

Eribulin pharmacokinetics are dose-proportional with a rapid distribution phase, extensive volume of distribution, low to moderate clearance and a relatively long elimination half-life ($t_{½}$=40hr) [12;13]. Human plasma protein binding investigated *in vitro* showed that eribulin was not strongly bound to plasma protein (49%-65%; data on file at Eisai). Animal studies showed that eribulin was mainly eliminated by excretion. Unchanged drug was the major component found excreted in bile (36%), feces (2%) and urine (21%) of bile-canulated rats that had received radiolabelled eribulin intravenously (data on file at Eisai).

Eribulin metabolism was studied in human liver microsomes. The results indicated that eribulin is predominantly metabolized by cytochrome P450 (CYP) 3A4 [14]. CYP3A4 content is highest in the liver and its quantity and activity are known to be affected by liver disease [15]. Therefore, the aim of the present study was to determine the effect of

hepatic impairment on the pharmacokinetics of eribulin in patients with solid tumors. The primary objective of the study was to define dosing recommendations for the safe use of eribulin mesylate in patients with hepatic impairment. This was achieved by comparison of eribulin mesylate single dose pharmacokinetic parameters between groups of patients with mild or moderate hepatic impairment, according to the Child-Pugh score system [16], and a control group of patients with normal hepatic function. Safety and anti-tumor efficacy in patients with impaired hepatic function were also explored.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed progressive advanced solid tumors, for whom no standard treatment was available, were eligible. Other inclusion criteria were: age ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) [17] performance status ≤ 2; serum creatinine ≤ 176 µmol/l or calculated creatinine clearance ≥ 40 millilitre (mL)/minute (min) (Cockroft-Gault formula [18]); absolute neutrophil count ≥ 1.5 x 10^9/l, hemoglobin ≥ 6.2 mmol/l; platelet count ≥ 100 x 10^9/l. Additional inclusion criteria for the group of patients without hepatic impairment were: International Normalized Ratio (INR), albumin, bilirubin, alanine aminotransferase and aspartate aminotransferase (AST) ≤ the upper limit of normal (ULN); alkaline phosphatase ≤ 2.5 times ULN without clinical signs of ascites. Exclusion criteria included: chemotherapy, radiation or biological therapy within 3 weeks, hormonal therapy within 1 week or any investigational drug within 4 weeks; severe (Child-Pugh C) hepatic dysfunction; encephalopathy ≥ grade 1; anti-coagulant therapy with warfarin or related compounds; treatment with any medication, dietary supplements or other substances known to induce or inhibit CYP3A4 activity; pre-existing neuropathy > grade 2; significant cardiovascular impairment; meningeal carcinomatosis; brain or subdural metastases, unless stable after completed local therapy and discontinuation of corticosteroids for at least 4 weeks; patients who had participated in a clinical trial with E7389. The study protocol was approved by the Medical Ethics committee of both hospitals and all patients had to give written informed consent. The study was conducted in accordance with the requirements of the world medical association declaration of Helsinki in its revised edition (Tokyo, 2004), the guidelines of International Conference on Harmonization (ICH) and good clinical practice (GCP).

Study design, study procedures and treatment administration.

This was a 2-center, phase I, open-label, three parallel group study to determine the influence of hepatic impairment on plasma pharmacokinetic parameters of eribulin mesylate following a single intravenous infusion (www.ClinicalTrials.gov, NCT00706095) [19]. Also safety and anti-tumor efficacy of eribulin among patients with reduced hepatic function were explored.

Up to a maximum of 18 patients (6 patients per group) were enrolled in two centers: the University Medical Center Utrecht and the Netherlands Cancer Institute, Amsterdam, both in The Netherlands.

Patients were assigned to one of three groups: normal hepatic function, mild hepatic impairment (Child-Pugh A) or moderate hepatic impairment (Child-Pugh B) according to the Child-Pugh system for classifying hepatic impairment [16]. Patients with normal hepatic function received 1.4 mg/m^2 eribulin mesylate and patients with Child-Pugh A or B hepatic impairment received a reduced starting dose of 1.1 and 0.7 mg/m^2, respectively, for safety reasons. After the first dose on day 1, patients could continue to receive eribulin treatment on day 8 and subsequently on days 1 and 8 of each 21-day cycle. Patients with normal hepatic function continued to receive the same dose of 1.4 mg/m^2, and patients with hepatic impairment were allowed to continue to receive eribulin in escalating doses up to 1.4 mg/m^2. Eribulin was provided as a sterile solution in vials containing 1.0 mg eribulin mesylate in 2 mL solution (0.5 mg/mL) containing ethanol/water (5:95) and was administered as a 2-5 minutes intravenous bolus injection.

Complete physical examinations, including vital signs, height, weight, ECOG performance status and ECG, and clinical laboratory tests (hematology, clinical chemistry and urinalysis) were performed at screening and at regular intervals.

Safety assessment.

Adverse events (AE) and concomitant medications were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE) version 3.0 [20]. Where a CTCAE grade did not exist for the AE, the event was graded on a three point scale (mild, moderate or severe). Treatment emergent AEs (TEAEs) were defined as any AE with a start date/time beyond or equal to the day of initial dosing (cycle 1 day 1), up to 30 days after the last dose of study drug, and any AE that increased in severity during the study. Patients were discontinued from the study in case of progressive disease (clinical evaluation or as documented by RECIST1.0 [21]), patient refusal of further therapy or withdrawal of consent.

Anti-tumor activity assessment.

Radiologic tumor assessments according to RECIST 1.0 [21] were performed at baseline and at regular intervals. Best response was documented for each patient.

Evaluability.

Patients who received at least one dose of the study treatment were included in the safety evaluation and patients who also had evaluable pharmacokinetic data were included in the pharmacokinetic evaluation. Patients with baseline measurable disease as per RECIST and who also received at least one dose of the study treatment were included in the evaluation of anti-tumor activity.

Pharmacokinetic sampling and analysis.

All patients entering the study provided blood samples for pharmacokinetic analysis. Blood samples for the determination of free-base eribulin plasma concentrations were taken at day 1 at baseline, and at 0.25, 0.5, 1, 2, 4, 6, 10, 24, 48, 72, 96, 120 and 144 hours following administration. Whole blood samples were collected from the contra-lateral arm to the infusion. At each time point, 7 mL of venous blood was withdrawn into sodium heparinised collection tubes (Vacutainer™). After collection, blood and anti-coagulant were mixed by inverting the tube 8-10 times. Blood samples were stored on ice and centrifuged within 60 minutes at 1500 g/3000 rpm 4ºC for 10 minutes. Plasma was transferred into polypropylene tubes and stored at -70ºC until analysis. Plasma concentrations of free-base eribulin were quantified using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method. The lower limit of quantitation (LLOQ) was 0.2 ng/mL. The inter-assay accuracy ranged from -3.18 to 2.77% with an inter-assay precision of ≤ 13.0% [22].

Statistical analysis.

No sample size calculations were made. Eribulin pharmacokinetic parameters were derived from plasma concentrations on day 1 to 7 by non-compartmental analysis using WinNonlin Professional® (version 5.1.1, Pharsight Corp, CA, USA). Statistical analysis was performed using SAS® (version 9.1).

Dose was normalized to 1 mg free-base eribulin and calculations were conducted using the assumption of a linear dose relationship as both concentrations were within the linear range of eribulin (0.25–4.0 mg/m^2) [12;13].

$AUC_{0-\infty}$ and C_{max} were considered the primary endpoints. Analysis of the primary PK parameters used an estimation approach based on mean ratio ((hepatic impairment)/

normal hepatic function) and 90% confidence intervals (CI). Log-transformed, dose normalized AUC and C_{max} were analyzed using analysis of variance (ANOVA). A large difference (i.e. two-fold [3] difference shown from CIs and least square means) was considered to show evidence that hepatic impairment affected eribulin pharmacokinetics. No specific CI boundaries were specified to show evidence.

RESULTS

Patient inclusion and demographics.

In total 18 patients were enrolled in the study, six in the normal hepatic function group, seven in the Child-Pugh A and five in the Child-Pugh B group. Patient demographics and characteristics are summarized in Table 1. All patients were Caucasian and aged between 50 and 70 years. Median body surface area (BSA) was the highest in the normal liver function group, followed by the Child-Pugh B group and was the lowest in the Child-Pugh A group. The majority of patients suffered from colorectal cancer (*N*=6, 33.3%).

Liver metastases were present in 2 (33%) patients of the normal hepatic function group, in all (100%) patients in the Child-Pugh A group and in 3 (60%) patients in the Child-Pugh B group. Three patients in the Child-Pugh A group suffered from the following liver conditions: one patient had hepatic steatosis, one patient had hepatitis with unknown cause and one patient had portal hypertension and esophageal varices grade III. In the Child-Pugh B group, no additional symptoms of liver disease were noted. A summary of the Child-Pugh scores per hepatic impairment group is shown in Table 2.

Pharmacokinetic analysis.

Pharmacokinetic samples were obtained from all 18 patients up to 144 hours (=T_{last} for all patients). The actual mean eribulin plasma concentration-time curves of the three groups (normal hepatic function, Child-Pugh A and B) following normal dose for patients without- or reduced dose for patients with hepatic impairment are presented in Figure 1 and the data are summarized in Table 3. Mean plasma concentrations of eribulin over time appear to be similar across all doses.

Table 1: Patient demographics and characteristics of the study population

Category	Normal liver function	Child-Pugh A	Child-Pugh B	Total
Number (*N*)	6	7	5	18
Age (years)				
Median (range)	60.0 (52 – 70)	60.0 (50 – 65)	64.0 (62 – 68)	61.5 (50 – 70)
Gender, *N* (%)				
Male	5 (83.3)	2 (28.6)	4 (80)	11 (61.1)
Female	1 (16.7)	5 (71.4)	1 (20)	7 (38.9)
Race, *N* (%)				
Caucasian	6 (100)	7 (100)	5 (100)	18 (100)
Weight (kg)				
Median (range)	77.5 (59.3-131)	62.0 (56.5-98.0)	65.0 (62.0-103.7)	67.4 (56.5-131.0)
Height (cm)				
Median (range)	179.5 (163–197)	168.0 (163–186)	173.0 (162–186)	172.5 (162–197)
Body surface area (m^2)				
Median (range)	1.99 (1.65–2.70)	1.70 (1.60–2.20)	1.78 (1.70–2.16)	1.80 (1.60–2.70)
Tumor type				
Urothelial cancer	1 (16.7)	0	0	1 (5.6)
Prostate cancer	1 (16.7)	0	1 (20.0)	2 (11.1)
Chordoma	1 (16.7)	0	0	1 (5.6)
Esophageal cancer	1 (16.7)	0	0	1 (5.6)
ACUP[a]	1 (16.7)	0	0	1 (5.6)
Colorectal cancer	1 (16.7)	5 (71.4)	0	6 (33.3)
Lung cancer (anaplastic)	0	1 (14.3)	0	1 (5.6)
Breast cancer	0	1 (14.3)	1 (20.0)	2 (11.1)
Pancreatic cancer	0	0	2 (40.0)	2 (11.1)
Gastric cancer	0	0	1 (20.0)	1 (5.6)

Abbreviations: ACUP=adenocarcinoma of unknown primary.[a]=patient with lung metastases, probably cholangiocarcinoma.

In order to compare eribulin exposure in patients with normal and impaired hepatic function, $AUC_{0-\infty}$, AUC_{0-t} and C_{max} were dose-normalized to account for the different doses tested in the treatment groups. Hepatic impairment resulted in increased mean dose-normalized $AUC_{0-\infty}$, AUC_{0-t} , C_{max} of eribulin (Figure 2 and Table 3). Hepatic impairment decreased clearance and prolonged elimination half-life of eribulin (Figure 3, Table 3).

Table 2: Scores for the Child-Pugh A and B groups according to the Child-Pugh score classification

Measure	Score (points)	Child-Pugh A (*N*=7, %)	Child-Pugh B (*N*=5, %)
Encephalopathy (grade)			
Absent	1	7 (100)	5 (100)
Moderate	2	0	0
Severe	3	0	0
Ascites			
Absent	1	7 (100)	2 (20)
Slight	2	0	3 (60)
Moderate	3	0	0
Serum bilirubin (µmol/l)			
<34	1	6 (86)	3 (60)
34-50	2	1 (14)	0
>50	3	0	2 (40)
Serum albumin (g/l)			
> 35	1	7 (100)	0
28 – 35	2	0	2 (40)
< 28	3	0	3 (60)
INR			
< 1.7	1	7 (100)	5 (100)
1.71 – 2.20	2	0	0
> 2.20	3	0	0

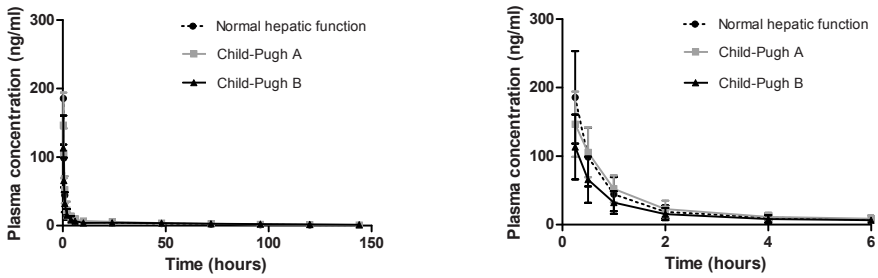
A total of 5-6 points scores for Child-Pugh A (mild hepatic impairment) and 7-9 points scores for Child-Pugh B (moderate hepatic impairment). Abbreviations: INR= International Normalized Ratio.

Table 3: Pharmacokinetic parameters for eribulin in patients with normal hepatic function and Child-Pugh A and B impaired hepatic function

Parameter	Normal liver function (*N*=6)	Child-Pugh A (*N*=7)	Child-Pugh B (*N*=5)
Dose administered (mg/m^2) i.v.[a]	1.4	1.1	0.7
Dose-normalized[b]			
$AUC_{0\text{-}t}$ (ng.h/mL; per unit of dose)	218 (53.3)	386 (161.5)	518 (312.0)
$AUC_{0\text{-}\infty}$ (ng.h/mL; per unit of dose)	229 (58.3)	420 (175.4)	646 (412.6)
C_{max} (ng/mL; per unit of dose)	72.0 (20.2)	83.9 (28.5)	100 (46.2)
Actual values			
$AUC_{0\text{-}t}$ (ng.h/mL)	571 (243.1)	671 (258.6)	579 (306.5)
$AUC_{0\text{-}\infty}$ (ng.h/mL)	600 (267.1)	731 (288.3)	720 (407.4)
C_{max} (ng/mL)	186 (67.4)	147 (47.6)	113 (47.2)
T_{max} (hours)	0.330 (0.03-0.37)	0.350 (0.33-0.47)	0.330 (0.25-0.35)
$t_{1/2}$ (hours)	36.1 (8.65)	41.1 (12.73)	65.9 (18.50)
CL (l/h)	4.57 (0.959)	2.75 (1.094)	2.06 (1.028)
V_{ss} (l)	166 (50.1)	113 (29.1)	149 (81.5)

Data are shown in mean (SD), except for t_{max} which is median (range). [a] Doses are for eribulin mesylate. The equivalent doses for eribulin as 'ready to use solution' (0.44 mg/mL) are: 1.23 mg/m^2 eribulin (normal liver function), 0.97 mg/m^2 eribulin (Child-Pugh A) and 0.62 mg/m^2 eribulin (Child-Pugh B). [b] Data are dose-normalized to 1 mg of free-base eribulin. Abbreviations: $AUC_{0\text{-}\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; $AUC_{0\text{-}t}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; BSA= body surface area; C_{max} = maximum observed plasma concentration; CL = systemic clearance; h= hour; mL= milliliter; T_{max} = time to maximum observed plasma concentration; $t_{1/2}$ = terminal half-life; V_{ss}= volume of distribution at steady state.

Linear scale



Log-linear scale

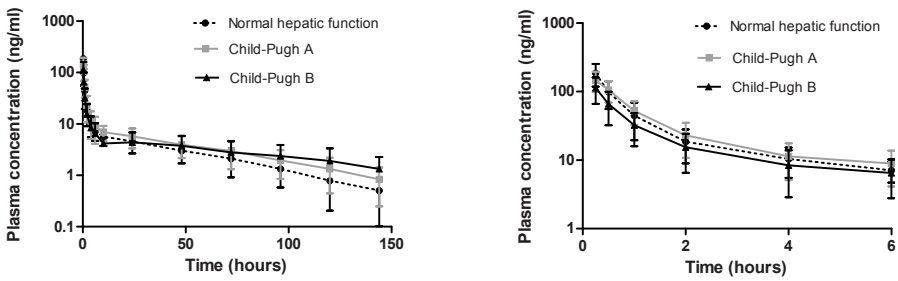


Figure 1: Mean (SD) plasma concentration (ng/mL)-time (h) curve (upper panel: linear scale, lower panel: log-linear scale) following intravenous administration of eribulin mesylate in normal hepatic function (1.4 mg/m^2; *N*=6; dotted line), in mild hepatic impairment (Child-Pugh A; 1.1 mg/m^2; *N*=7; grey line) and in moderate hepatic impairment (Child-Pugh B; 0.7 mg/m^2; *N*=5; black line). Left: time scale up to 150 hours. Right: time scale up to 6 hours.

The primary pharmacokinetic endpoints of dose-normalized $AUC_{0-\infty}$ and C_{max} and also AUC_{0-t} for eribulin exposure in normal and impaired hepatic function were analyzed by ANOVA (Table 4). Hepatic impairment increased exposure to eribulin. Eribulin mean dose-normalized $AUC_{0-\infty}$ was 1.75-fold (90%CI: 1.15-2.66) increased in the Child-Pugh A group and 2.48-fold (90%CI: 1.57-3.92) increased in the Child-Pugh B group, when compared to the normal hepatic function group. Eribulin mean dose-normalized AUC_{0-t} was (1.70-fold (90%CI: 1.15-2.51) increased in the Child-Pugh A group and 2.13-fold (90%CI: 1.39-3.26) increased in the Child-Pugh B group, when compared to the normal hepatic function group. Eribulin mean dose-normalized C_{max} was similar in the Child-Pugh A group (1.15-fold; 90%CI: 0.81-1.63) and the Child-Pugh B group (1.29-fold; 90%CI: 0.89-1.89) compared to normal hepatic function.

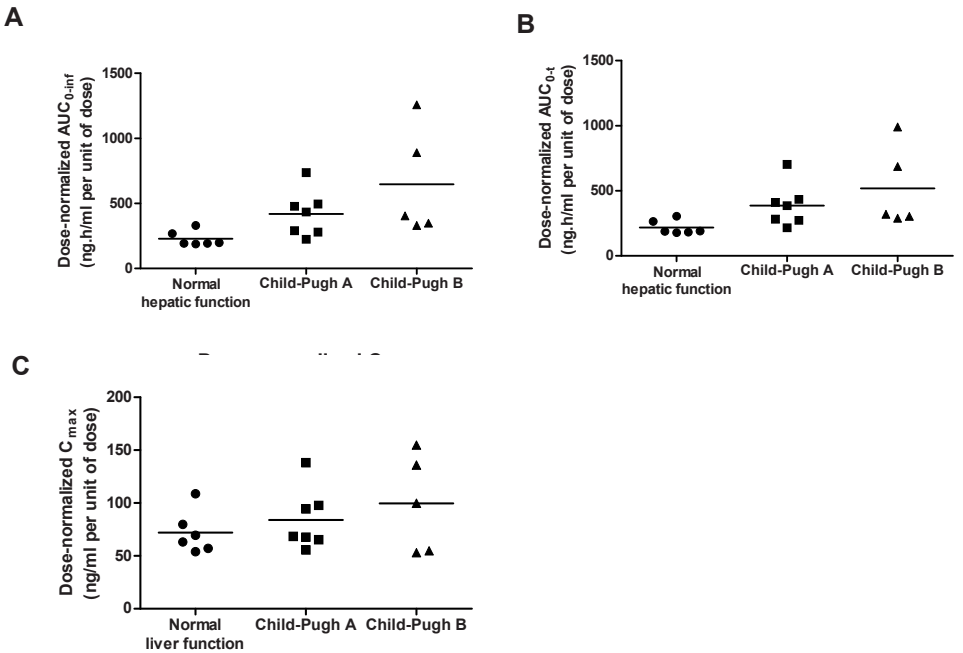


Figure 2: Effect of hepatic impairment on dose-normalized $AUC_{0-\infty}$, AUC_{0-t} and C_{max} of eribulin. Horizontal bars represent the mean (also summarized in table 3). Data are dose-normalized to 1 mg of free-base eribulin. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; C_{max} = maximum observed plasma concentration.

Table 4: Statistical analysis of the primary pharmacokinetic parameters

Parameter	Geometric least square means		Comparison	Ratio of means (impaired: normal hepatic function)	90% confidence interval
	Impaired	Normal hepatic function			
$AUC_{0-\infty}$ (ng.h/mL; per unit of dose)	390	223	Child-Pugh A : Normal	1.75	(1.15 - 2.66)
	554		Child-Pugh B: Normal	2.48	(1.57 - 3.92)
AUC_{0-t} (ng.h/mL; per unit of dose)	361	213	Child-Pugh A : Normal	1.70	(1.15 - 2.51)
	453		Child-Pugh B: Normal	2.13	(1.39 - 3.26)
C_{max} (ng/mL; per unit of dose)	80.3	69.9	Child-Pugh A : Normal	1.15	(0.81 - 1.63)
	90.5		Child-Pugh B: Normal	1.29	(0.89 - 1.89)

$AUC_{0-\infty}$, AUC_{0-t} and C_{max} are dose-normalized to 1 mg of free-base eribulin. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; C_{max} = maximum observed plasma concentration.

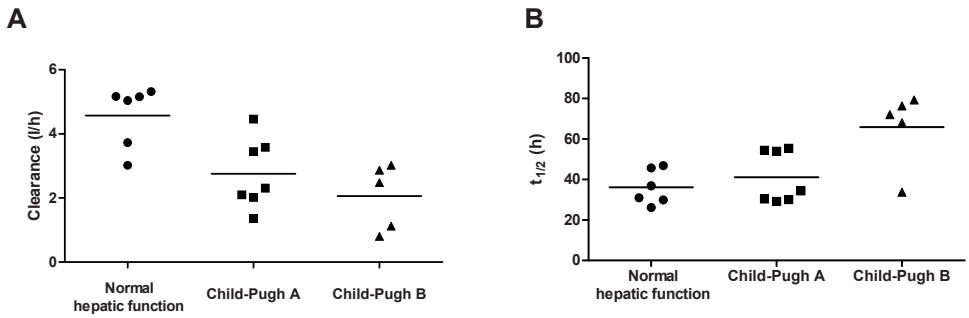


Figure 3: Effect of hepatic impairment on clearance (CL) and elimination half-life ($t_{1/2}$) of eribulin. Horizontal bars represent the mean (also summarized in Table 3).

Safety.

All 18 patients were evaluable for safety and no patients withdrew during the study phase (cycle 1). Overall, patients commenced one to seven additional cycles of study drug (median of two cycles in each group). Two patients required dose reduction due to TEAEs and this also led to dose interruption in one patient and dose delay in five patients. Twelve patients (12/18; 66.7%) discontinued because of progressive disease (according to RECIST), 5/18 (27.8%) patients discontinued because of clinical progression and one patient withdrew informed consent after cycle 2.

All patients experienced at least one TEAE and the majority of patients (16/18) experienced at least one AE reported to be treatment-related ("probably" or "possibly"). The most frequently reported TEAEs reported to be treatment-related were alopecia (12/18), fatigue (7/18), neutropenia (6/18), nausea (5/18) and vomiting (4/18). All CTCAE grade ≥ 3 or SAEs reported as treatment-related are summarized in Table 5. There were no apparent differences in distribution of treatment-related TEAEs across all groups.

There were no life-threatening SAEs or SAEs reported as treatment related. Overall, eight patients experienced SAEs which required hospitalization in all cases. None were reported as related to study drug. Death occurred in two patients during the study or within 30 days after study drug administration, both due to disease progression. Both events were not considered related to the study drug or to complications related to deterioration of hepatic function. There were no clinically significant ECG abnormalities or obvious changes in vital signs noted. There were no obvious laboratory abnormalities between the different hepatic function groups.

Anti-tumor activity.

Overall, half of the patients (9/18, 50%) had stable disease as best tumor response. These included 4/6 (66.7%), 1/7 (14.3%) and 4/5 (80%) patients in the normal hepatic function, the Child-Pugh A and B groups, respectively. Progressive disease was reported in 8/18 (44.4%) patients. Data were missing in one patient (1/18; 5.6%).

Table 5: Incidence of all CTCAE grade ≥3 or Severe Adverse Event (SAE) reported as treatment-related

		Number of patients (%)			
Term	Grading CTCAE	Normal liver function (N=6)	Child-Pugh A (N=7)	Child-Pugh B (N=5)	Total (N=18)
Any TEAE	3	1 (16.7)	0	2 (40.0)	3 (16.7)
	4	0	2 (28.6)	1 (20.0)	3 (16.7)
Fatigue	3	1 (16.7)			1 (5.6)
Malaise	3	1 (16.7)	0	0	1 (5.6)
Nausea	3	1 (16.7)	0	1 (20.0)	2 (11.1)
Neutropenia	3	0	0	2 (40.0)	2 (11.1)
	4	0	2 (28.6)	1 (20.0)	3 (16.7)
Anemia	3	1 (16.7)	0	0	1 (5.6)
Leukocytopenia	3	0	1 (14.3)	0	1 (5.6)
Peripheral motor neuropathy	3	0	0	1 (20.0)	1 (5.6)
AST increase	3	0	1 (14.3)	1 (20.0)	2 (11.1)

Abbreviations: AST= aspartate aminotransferase; CTCAE= common terminology criteria for adverse events; TEAE=treatment emergent adverse event.

DISCUSSION

Liver disease is a common cause of altered pharmacokinetics with clinical relevance [1;2]. Pharmacokinetics can be affected by i) altered intrinsic hepatic clearance, ii) reduced metabolic capacity, iii) changed excretion into the bile, iv) reduced production of albumin thus decreasing serum protein binding, and v) altered absorption from the gastrointestinal tract in case of portal hypertension [2]. The results of a human metabolism and excretion study of radioactively-labeled eribulin were recently reported and showed that the contribution of metabolism to eribulin elimination was limited. The majority of eribulin was excreted unchanged in feces (61.3%) except for a minor part in urine (8.1%). Metabolite profiling of the plasma samples showed that metabolites accounted for ≤ 0.6% of unchanged eribulin in

plasma [23]. Because eribulin was found to be primarily metabolized by CYP3A4 *in vitro*, another phase I study recently investigated the effect of CYP3A4 inhibition by ketoconazole on eribulin exposure. The results showed that CYP3A4 inhibition did not significantly affect eribulin exposure [24]. It can be concluded from both studies, that it was considered unlikely that the increased exposure in patients with impaired hepatic function observed in the present study was caused by reduced CYP3A4 content and/or activity as a result of hepatic impairment in these patients. It was also considered unlikely that reduced albumin synthesis would have affected disposition of eribulin, since eribulin is not strongly bound to plasma proteins (49%-65%; data on file at Eisai). Because eribulin is intravenously administered, pharmacokinetics could not have been affected by reduced absorption. It was therefore hypothesized that reduced hepatic elimination could be the result of reduced biliary excretion of unchanged eribulin in patients with hepatic impairment. Eribulin was shown to be a substrate for P-glycoprotein (P-gp, MDR1) [25], an ATP-binding cassette transporter located in the biliary canalicular surface of hepatocytes and on the apical surface of epithelial cells in small biliary ducts [26]. Reduced biliary excretion could therefore be the result of a combination of reduced biliary flow and reduced transporter-mediated excretion into the bile.

Clearance and elimination half life were affected by hepatic impairment. We hypothesized that clearance would be reduced and elimination half life would be prolonged according to severity of hepatic impairment in the Child-Pugh score and this appears to be the case. Ascites was present in 3/5 (60%) patients in the Child-Pugh B group at the start of treatment and one patient in the Child-Pugh A group developed pleural effusion on day 8 of cycle 1. Eribulin could be detected at low concentrations (range: 1.0 and 2.1 ng/mL) in ascites and pleural fluids of two patients after therapeutic drainage between 1 and 5 days after i.v. drug administration. These results suggest that eribulin is also distributed in the third-space where it may exert pharmacological activity, although this was not further investigated in the present study. This observation could be clinically relevant in patients with metastases in the pleural and/or abdominal cavity.

The Child-Pugh scoring system is a classification system used to define severity of hepatic impairment and was originally developed for surgical evaluation of alcoholic cirrhotic liver disease [16]. Although the Child-Pugh scoring system is currently widely used, there are no well-established, adequate markers available for prediction of pharmacokinetics in patients with impaired hepatic function. Others have used measurements of endogenous markers [27] or elimination rates of exogenous markers

[28], but it has not been clearly demonstrated that one of these tests outperforms the Child-Pugh classification [1;28].

In conclusion, statistical analysis of dose-normalized $AUC_{0-\infty}$ and AUC_{0-t} indicated that hepatic impairment had a significant effect on eribulin exposure. Hepatic impairment resulted in decreased clearance and prolonged elimination half-life of eribulin. A reduced dose of eribulin (Table 6) is recommended in patients with mild or moderate hepatic impairment.

Table 6: Recommended dose for administration of eribulin in patients with mild and moderate hepatic impairment or normal hepatic function

Liver impairment	Normal	Child-Pugh A (mild)	Child-Pugh B (moderate)
Eribulin mesylate dose (i.v)	1.4 mg/m^2	1.1 mg/m^2	0.7 mg/m^2

[a] Doses are for eribulin mesylate on day 1 and 8 of a 21-day cycle. The equivalent doses for eribulin as 'ready to use solution' (0.44 mg/mL) are: 1.23 mg/m^2 eribulin (normal liver function), 0.97 mg/m^2 eribulin (Child-Pugh A) and 0.62 mg/m^2 eribulin (Child-Pugh B). Abbreviation: i.v.= intravenous.

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Chapter 3.2

Eribulin mesylate pharmacokinetics in patients with solid tumors receiving repeated oral ketoconazole

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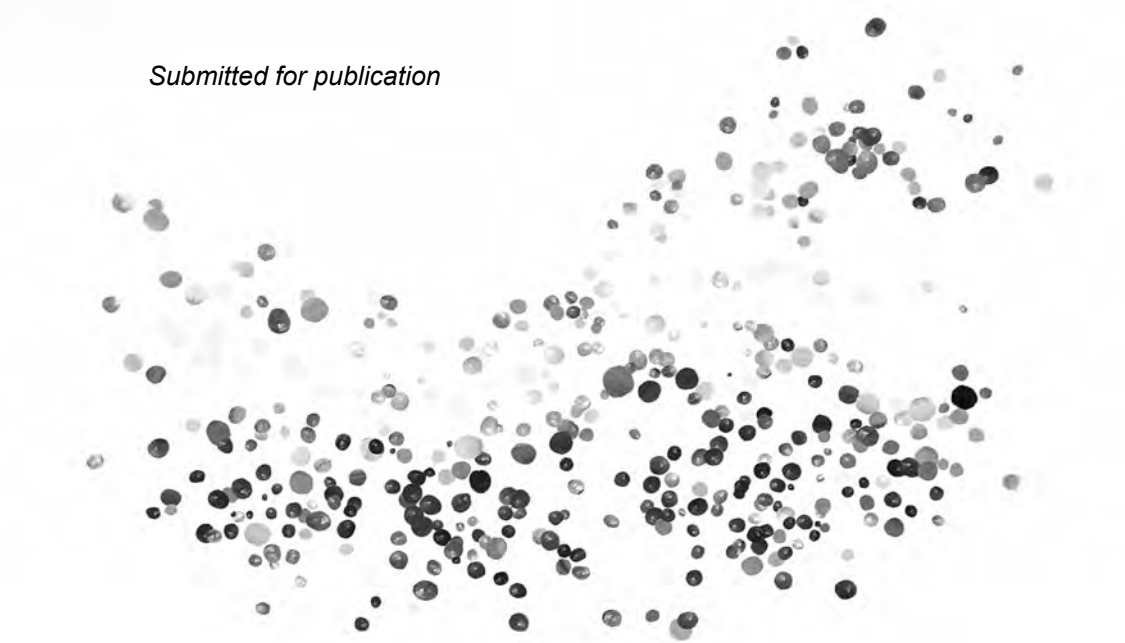

ABSTRACT

Purpose

To study the influence of repeated oral administration of ketoconazole, a potent CYP3A4 inhibitor, on the plasma pharmacokinetics of eribulin mesylate administered by single-dose intravenous infusion. Eribulin mesylate is a non-taxane microtubule dynamics inhibitor that is currently under development in phase I-III trials for the treatment of solid tumors.

Experimental design

A randomized, open-label, two treatments, two sequences, crossover phase I study was performed in patients with advanced solid tumors. Treatments were given on day 1 and day 15 and consisted of 1.4 mg/m^2 eribulin mesylate alone or 0.7 mg/m^2 eribulin mesylate plus 200 mg ketoconazole on the day of eribulin mesylate administration and the following day. Pharmacokinetic sampling for determination of eribulin plasma concentration was performed up to 144 hours following administration of eribulin mesylate. Also safety and anti-tumor activity were determined.

Results

Pharmacokinetic sampling and analysis was completed in ten patients. Statistical analysis of dose-normalized log-transformed $AUC_{0-\infty}$ and C_{max} indicated that single-dose exposure of eribulin was not statistically different when co-administered with ketoconazole (ratio of geometric least square means: 0.95 (90%CI: 0.80–1.12) and 0.97 (90%CI: 0.83-1.12), respectively) in patients with solid tumors. Ketoconazole had no effect on eribulin clearance and elimination half-life. The most frequently reported treatment related adverse events were fatigue and nausea, each reported in 8/12 patients. Seven patients (58.5%) achieved stable disease as best overall response.

Conclusions

The results indicate that eribulin mesylate can be safely co-administered with ketoconazole. Drug-drug interactions are not expected with other CYP3A4 inhibitors.

INTRODUCTION

Drug-drug interactions are a major cause of morbidity and death in clinical practice. Many anticancer drugs have a narrow therapeutic index and are administered to patients who are also taking numerous concomitant medications. Human cytochrome P450 (CYP) 3A4, part of the CYP3A subfamily, is involved in the metabolism of approximately 60% of currently prescribed drugs and plays a dominant role in many clinically relevant drug interactions [1].

Eribulin mesylate (E7389; Halaven™; Eisai Inc) is a non-taxane microtubule dynamics inhibitor that is a synthetic analogue of halichondrin B, a product isolated from the marine sponge *Halichondria okadai.* Eribulin has a novel and distinct mechanism of action. It prevents microtubule growth without affecting shortening, and also sequesters tubulin into non-functional aggregates. The overall effects lead to G2-M arrest and ultimately cell death [2-5].

Eribulin mesylate is currently under clinical development in phase I-III trials for the treatment of metastatic breast cancer and other solid tumors, both as monotherapy as well as in combination therapy with capecitabine, trastuzumab, pemetrexed, erlotinib, gemcitabine, cisplatin and carboplatin [6;7]. In previous trials, eribulin mesylate demonstrated efficacy and a manageable toxicity profile in patients with solid tumors, including in heavily pre-treated metastatic breast cancer patients [8;9]. Recently, a phase III trial (EMBRACE) demonstrated that eribulin mesylate treatment significantly improved median overall survival of metastatic breast cancer patients that were previously treated with an anthracycline and a taxane compared to 'treatment of physician's choice' [10].

Eribulin pharmacokinetics is dose-proportional with a rapid distribution phase, extensive volume of distribution, low to moderate clearance and a relatively long elimination half life [11;12].

Preclinical data in human liver microsomes indicated that eribulin is predominantly cleared by hepatic metabolism and particularly by CYP3A4 [13]. Eribulin is thought to be a competitive inhibitor of CYP3A4, although only high-concentration eribulin (10 µmol/l; 7300 ng/ml) minimally inhibited the CYP3A4 metabolism of several therapeutic agents, including carbamazepine, diazepam, paclitaxel, midazolam, tamoxifen and terfenadine, in primary cultures of human hepatocytes *in vitro* [14]. However, drug-drug interactions could not be excluded in cancer patients, who are likely to be exposed to many other drugs for the treatment of concurrent diseases or treatment-derived complications.

The aim of this study was to assess the influence of repeated oral administration of therapeutic doses of ketoconazole, a potent CYP3A4 inhibitor and a model recommended in regulatory guidelines [15;16], on the plasma pharmacokinetics of eribulin administered by intravenous infusion. Secondary objectives were to explore the safety of eribulin with and without co-administration of ketoconazole and to determine the anti-tumor activity.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed progressive advanced solid tumors, for whom no standard treatment was available, were eligible. Other inclusion criteria were: age ≥ 18 years, Eastern Cooperative Oncology Group (ECOG) [17] performance status ≤ 2, resolution of all chemotherapy- or radiation-related toxicities to grade ≤ 1 severity (except for stable sensory neuropathy ≤ grade 2 and alopecia); serum creatinine ≤ 176 µmol/l or calculated creatinine clearance ≥ 40 milliliter (ml)/minute (min) (Cockroft-Gault formula [18]), bilirubin ≤ 1.5 times the upper limit of normal (ULN), and alkaline phosphatase (AF), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ≤ 3 times ULN and ≤ 5 times ULN in case of liver metastasis; absolute neutrophil count (ANC) ≥ 1.5 x 10^9/l, hemoglobin ≥ 6.2 mmol/l, and platelet counts ≥ 100 x 10^9/l. Exclusion criteria included: anti-coagulant therapy with warfarin or related compounds; any medication, dietary supplements or other substances known to induce or inhibit CYP3A4 activity; patients for whom ketoconazole was contraindicated or who were receiving drugs that might influence ketoconazole metabolism; pregnancy or breast-feeding; significant cardiovascular impairment; presence of meningeal carcinomatosis or of brain or subdural metastases, unless stable after completed local therapy and discontinuation of corticosteroids for at least 4 weeks; pre-existing neuropathy > grade 2; impaired intestinal absorption. The study protocol was approved by the Medical Ethics committee of the hospital and all patients had to give written informed consent. The study was conducted in accordance with the requirements of the world medical association declaration of Helsinki in its revised edition (Tokyo, 2004), the guidelines of International Conference on Harmonization (ICH) and good clinical practice (GCP).

Study design, study procedures and treatment administration.

This phase I study was a randomized, open-label, two treatments, two sequences, crossover study conducted in patients with solid tumors to study the influence of repeated oral administration of ketoconazole, a potent CYP3A4 inhibitor, at a therapeutic dose on the plasma pharmacokinetics of eribulin administered by intravenous infusion (www.ClinicalTrials.gov, NCT01000376) [7]. Safety and tolerability of eribulin when co-administered with oral ketoconazole and/or when given alone on days 1 and 8 of a 21-day schedule in patients with solid tumors were also explored. A total of 12 patients were to be recruited. The cross-over approach was used to adjust for a possible period effect. A 15-day washout period was introduced to prevent the effects of the first treatment carrying over to the second treatment.

Patients were randomly allocated to one of two treatment sequences. Group one received 1.4 mg/m^2 eribulin mesylate on day 1, followed by 0.7 mg/m^2 eribulin mesylate plus 200 mg ketoconazole (Janssen-Cilag Ltd, UK; Nizoral™) [19] on day 15, and 200 mg ketoconazole alone on day 16 of a 28-day cycle. Group two received 0.7 mg/m^2 eribulin mesylate plus 200 mg ketoconazole on day 1 and 200 mg ketoconazole alone on day 2, followed by 1.4 mg/m^2 eribulin mesylate on day 15 of a 28-day cycle. A 50% lower dose of eribulin mesylate was given in combination with ketoconazole for safety reasons. Eribulin mesylate was provided as a sterile injectable solution in vials containing 1.0 mg eribulin mesylate in 2 ml (0.5 mg/ml) solution containing ethanol/water (5:95). A dose of 1.4 mg/m^2 eribulin mesylate is equivalent to 1.23 mg/m^2 eribulin as the 'ready to use solution' (0.44 mg/ml) and a dose of 0.7 mg/m^2 eribulin mesylate is equivalent to 0.62 mg/m^2 eribulin [20]. Eribulin mesylate was administered as a 2-5 minutes intravenous bolus injection. Oral 200 mg ketoconazole was given one hour before and 23 hours after eribulin mesylate administration. Study medications were administered to patients directly by study personnel. After the first cycle, patients were allowed to continue to receive eribulin mesylate at a dose of 1.4 mg/m^2 on day 1 and day 8 of a 21-day cycle.

Complete physical examinations, including vital signs, height, weight, ECOG performance status and ECG, and clinical laboratory tests were performed at screening and at regular intervals.

Safety assessment.

Adverse events (AE) and concomitant medications were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE)

version 3.0 [21]. Treatment emergent AEs (TEAEs) were defined as any AE that occurred after administration of the first dose of study drug (cycle 1 day 1) up to 30 days after the last dose of study drug, and any AE which increased in severity during the study. Patients were discontinued from the study in case of disease progression (symptomatic or according to RECIST [22]), unacceptable toxicity, patient refusal of further therapy or withdrawal of consent.

Anti-tumor activity assessment.

Radiologic tumor assessments according to RECIST 1.0 [22] were performed at baseline and at regular intervals. A best response was documented for each patient.

Evaluability.

All patients who completed PK evaluations of eribulin on days 1-7 and 15-21 were included in the PK evaluable population. Patients who received at least one dose of the study treatment were included in the analysis set for safety. Patients with baseline measurable disease as per RECIST and who also received at least one dose of the study treatment were included in the analysis set for response.

Pharmacokinetic sampling and analysis.

All patients entering the study provided blood samples for pharmacokinetic analysis. Blood samples for the determination of free-base eribulin plasma concentrations were taken at day 1 and day 15 at baseline, and at 0.25, 0.5, 1, 2, 4, 6, 10, 24, 48, 72, 96, 120 and 144 hours following the end of administration. Whole blood samples were collected preferably from the contra-lateral arm to the infusion. At each time point, 7 ml of venous blood was withdrawn into sodium heparinised collection tubes (Vacutainer™). After collection, blood and anti-coagulant were mixed by inverting the tube 8-10 times. Blood samples were stored on ice and centrifuged within 60 minutes at 1500 g/3000 rpm at 4ºC for 10 minutes. Plasma was transferred into polypropylene tubes and stored at -70ºC until analysis. Plasma concentrations of free-base eribulin were quantified using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method. The lower limit of quantitation (LLOQ) was 0.2 ng/ml. The inter-assay accuracy ranged from -3.18 to 2.77% with an inter-assay precision of ≤13.0% [23].

Pharmacogenetic analysis.

Of each participating patient, 5 ml of venous blood was collected using an EDTA-coated tube and stored at -20ºC until analysis. Pharmacogenetic analysis was performed to assess CYP3A4 and CYP3A5 genotype for each individual using a haplotype approach. Patients were classified as extensive, intermediate or poor metabolizers according to haplotype.

Statistical analysis.

Sample size calculations were made. The number of patients recruited was based on the number estimated to provide at least 90% power to detect a clinically significant increase of 30% in $AUC_{0-\infty}$ and C_{max} between eribulin exposure in the presence and absence of ketoconazole. The estimates of the within-subject standard deviation for $AUC_{0-\infty}$ and C_{max} for the 1.4 mg/m^2 dose ranged from 0.16 to 0.20, based on models with all data that was available at the time of calculation, including subjects dosed at 1.4 mg/m^2 at two time-points as well as across all doses (0.25 mg/m^2 to 1.4 mg/m^2). Assuming a within-subject standard deviation of 0.20 and a one-sided alpha level of 5%, 8 patients would provide at least 90% power to detect a 30% increase between eribulin and eribulin plus ketoconazole in the $AUC_{0-\infty}$ and C_{max} of eribulin.

Eribulin pharmacokinetic parameters ($AUC_{0-\infty}$, AUC_{0-t}, C_{max}, T_{max}, $t_{1/2}$, Cl and V_{ss}) were derived from plasma concentrations on day 1 to 7 and 15 to 21 by non-compartmental analysis using WinNonlin Professional® (version 5.1.1, Pharsight Corp, CA, USA). Statistical analysis was performed using SAS® (version 9.1). In order to validate the calculation of $AUC_{0-\infty}$, there were pre-specified conditions to which the calculated terminal phase λ_z (the apparent terminal disposition rate constant) was supposed to adhere. If these conditions were not met, the $AUC_{0-\infty}$ was not calculated for that patient. Firstly, three data points in the terminal phase had to be available in calculating λ_z, with the line of regression starting at any post- C_{max} data point. Secondly, the adjusted regression coefficient (R^2_{adj}) in general was to be > 0.90.

Dose was normalized to 1 mg free-base eribulin and calculations were conducted using the assumption of a linear dose relationship as both concentrations were within the linear range of eribulin (0.25–4.0 mg/m^2) [11;12]. Carry-over (plasma concentration pre-dose at day 15) was tested and appropriate measures were used if found > 5% of observed C_{max} in cycle 1.

Analysis of PK parameters used an estimation approach based on mean ratio (i.e. the magnitude of the interaction) and confidence intervals (CI). Log-transformed, dose normalized primary endpoints, AUC and C_{max}, were analyzed using analysis of variance (ANOVA). These endpoints were compared between eribulin alone

(reference) and eribulin and ketoconazole (test) treatment groups. The ANOVA model included terms for treatment, period (day) and patient. Patient was included as a random effect. Results were presented in terms of the least squares means ratio with associated 90% confidence intervals (CI), no p-values were presented. The estimated treatment effect was back transformed to obtain an estimate for the ratio (eribulin + ketoconazole)/ eribulin.

RESULTS

Patient inclusion and demographics.

In total 12 patients were enrolled in the study, six in group one and six in group two. All 12 patients were included in the safety population. Patient demographics and characteristics are summarized in Table 1. The median age was 63.5 years (range: 45.0-73.0), the median weight was 74.5 kg (range: 65.0 – 112.0) and the majority were male (66.7%). All patients were Caucasian (100.0%).

Table 1: Patient demographics and characteristics of the safety population

Category	Group 1	Group 2	Total
Number (n)	6	6	12
Age (years)			
Median (range)	59.0 (45.0-73.0)	64.0 (52.0-68.0)	63.5 (45.0-73.0)
Gender, n (%)			
Male	3 (50.0)	5 (83.3)	8 (66.7)
Female	3 (50.0)	1 (16.7)	4 (33.3)
Race, n (%)			
Caucasian	6 (100.0)	6 (100.0)	12 (100.0)
Weight (kg)			
Median (range)	83.0 (70.0-112.0)	71.0 (65.0-100.0)	74.5 (65.0-112.0)
Height (cm)			
Median (range)	170.5 (165.0-185.0)	179.0 (165.0-184.0)	178.0 (165.0-185.0)
Body surface area (m^2)			
Median (range)	2.00 (1.8-2.2)	1.90 (1.8-2.3)	1.95 (1.8-2.3)
Tumor type			
Adenocarcinoma	2 (33.3)	0	2 (16.7)
Sigmoid carcinoma	1 (16.7)	0	1 (8.3)
Breast carcinoma	2 (33.3)	1 (16.7).	3 (25.0)
Melanoma (choroid)	1 (16.7)	0	1 (8.3)
Lung carcinoma (large cell)	0	1 (16.7)	1 (8.3)
Urothelial cell carcinoma	0	2 (33.3)	2 (16.7)
Mesothelioma	0	2 (33.3)	2 (16.7)

Pharmacokinetic analysis.

In total 10 patients completed the first cycle, four patients in group one and six in group two. Of all 10 patients that completed cycle 1, pharmacokinetic sampling was obtained and pharmacokinetic analysis was performed. Therefore, the pharmacokinetics evaluable group consisted of 10 patients. No carry-over was found. Four $AUC_{0-\infty}$ calculations (one for eribulin alone and three for eribulin with ketoconazole) were excluded because of invalidity of λ_z : one because of insufficient data points (< 3), and three because the adjusted regression coefficient (R^2_{adj}) could not be estimated sufficiently. In both groups, for all patients T_{last} was 144 hours, except for one patient per group in which T_{last} was 120 hours.

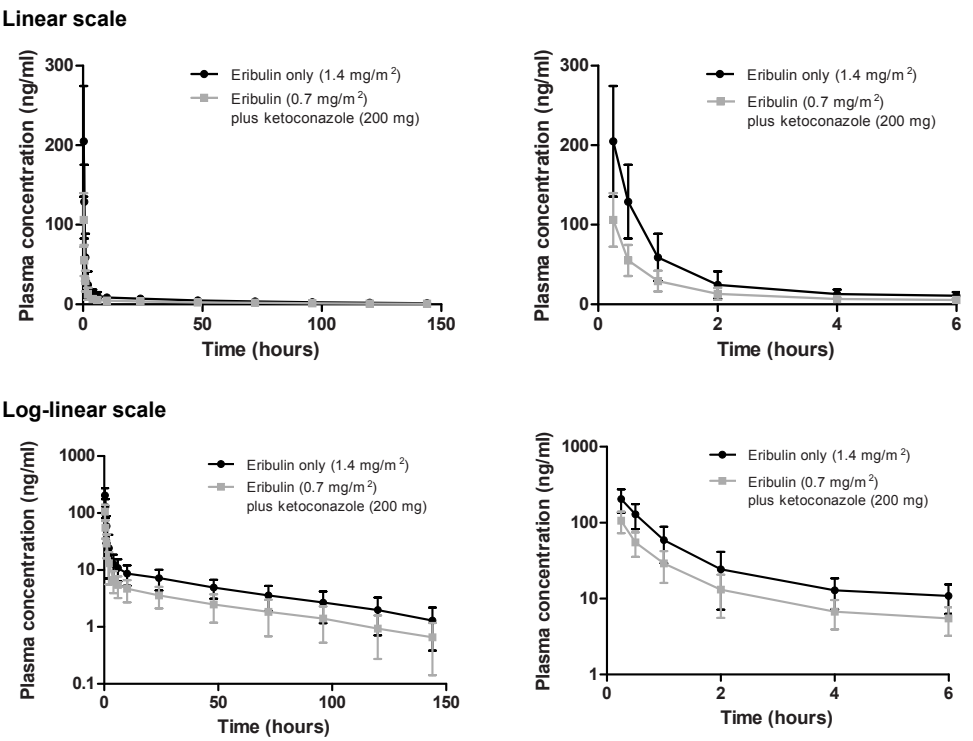

Figure 1: Mean (SD) plasma concentration (ng/ml)-time (h) curve (upper panel: linear scale, lower panel: log-linear scale) following intravenous administration of eribulin mesylate alone (1.4 mg/m²; n=10) or eribulin mesylate (0.7 mg/m²) with ketoconazole (200 mg; n=10 both groups). Left: time scale up to 150 hours. Right: time scale up to 6 hours.

The actual mean plasma eribulin concentration-time curves are presented in Figure 1 and the data are summarized in Table 2. In order to compare eribulin exposure in patients when co-administered with ketoconazole, $AUC_{0-\infty}$, AUC_{0-t} and C_{max} were dose-normalized to account for the different doses tested in the treatment groups. Ketoconazole had no statistically significant effect on mean $AUC_{0-\infty}$, AUC_{0-t} and C_{max} of eribulin (Figure 2 and Table 2). Mean dose-normalized $AUC_{0-\infty}$, AUC_{0-t} and C_{max} for eribulin exposure were comparable with or without ketoconazole. Ketoconazole had no effect on clearance or elimination half-life of eribulin (Table 2). The mean clearance of eribulin was 3.10 l/h in absence of ketoconazole and 3.37 l/h with ketoconazole co-administration.

Table 2: Pharmacokinetic parameters for eribulin mesylate alone or co-administered with ketoconazole: pharmacokinetic population

Parameter	n	Eribulin mesylate	n	Eribulin mesylate with ketoconazole
Dose administered (mg/m²) IV		1.4		0.7
Dose-normalized				
AUC_{0-t} (ng.h/ml; per unit of dose)	10	313 (116.6)	10	326 (133.2)
$AUC_{0-\infty}$ (ng.h/ml; per unit of dose)	9	406 (159.3)	7	410 (204.9)
C_{max} (ng/ml; per unit of dose)	10	86.4 (33.23)	10	89.2 (31.89)
Actual values				
AUC_{0-t} (ng.h/ml)	10	846 (301.2)	10	441 (177.9)
$AUC_{0-\infty}$ (ng.h/ml)	9	971 (371.9)	7	482 (241.5)
C_{max} (ng/ml)	10	207 (73.9)	10	106 (33.7)
T_{max} (hours)	10	0.360 (0.23-24.45)	10	0.365 (0.27-0.42)
$t_{1/2}$ (hours)	9	45.6 (13.62)	7	40.5 (7.69)
Cl (l/h)	9	3.10 (1.903)	7	3.37 (2.507)
V_{ss} (l)	9	153 (63.4)	7	141 (83.7)

Data are shown as mean (SD) except for t_{max} which are median (range) values. AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration; Cl = systemic clearance; h= hour; ml= milliliter; SD= standard deviation; T_{max} = time to maximum observed plasma concentration; $t_{1/2}$ = terminal half-life; V_{ss}= volume of distribution at steady state.

The primary pharmacokinetic endpoints of dose-normalized $AUC_{0-\infty}$ and C_{max} for eribulin exposure with or without co-administration of ketoconazole were also analyzed using analysis of variance (ANOVA; Table 3). Eribulin $AUC_{0-\infty}$ when administered alone was similar to that when co-administered with ketoconazole (geometric least

square means ratio: 0.95; 90%CI 0.80-1.12). Eribulin C_{max} when administered alone was similar to that when co-administered with ketoconazole (geometric least square means ratio: 0.95; 90%CI 0.83-1.12).

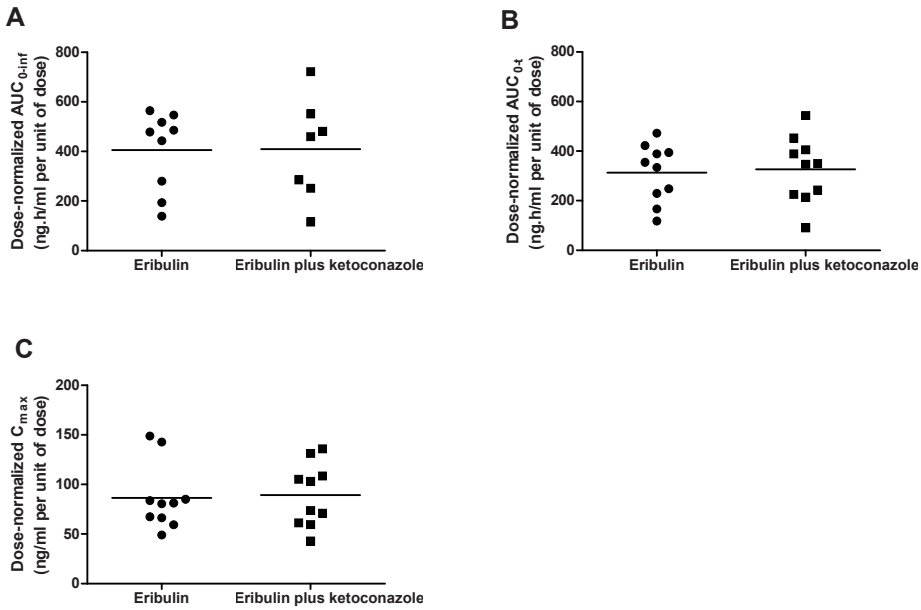

Figure 2: Effect of ketoconazole co-administration on dose-normalized exposure (A: $AUC_{0-\infty}$, B: AUC_{0-t} and C: C_{max}) of eribulin. Horizontal bars represent the mean (also summarized in Table 2). Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; AUC_{0-t} = Area under the concentration-time curve from zero (pre-dose) extrapolated to time of last quantifiable concentration; C_{max} = maximum observed plasma concentration.

Table 3: Statistical analysis of the primary pharmacokinetic parameters

Parameter	Geometric least square means		Ratio of treatment means (eribulin plus ketoconazole: eribulin)	90% confidence interval
	Eribulin plus ketoconazole	Eribulin		
$AUC_{0-\infty}$ (ng.h/ml; per unit of dose)	379	400	0.95	(0.80 to 1.12)
C_{max} (ng/ml; per unit of dose)	81.0	83.9	0.97	(0.83 to 1.12)

$AUC_{0-\infty}$ and C_{max} are normalized to 1 mg of free-base eribulin. Model includes terms for treatment, period (day) and patient. Patient is included as random effect. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve rom zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration.

Pharmacogenetic analysis.

All ten patients had a CYP3A4 *1/*1 haplotype, classifying them as extensive metabolizers for CYP3A4, and a CYP3A5 *3/*3 haplotype, classifying them as poor metabolizers for CYP3A5.

Safety.

All 12 patients were included in the safety population. Three patients discontinued prematurely during cycle 1. This included one patient with disease progression, one patient with AEs that were probably related to eribulin exposure (grade 2 increased ALT and AST), and one patient with an AE and a SAE that were not considered related (grade 1 fatigue and ileus). Overall, nine (75.0%) patients commenced the 2nd cycle of treatment. The mean time on trial was 111 days (range: 21 – 329 days). Four patients required a dose reduction or dose omission. Eight patients discontinued due to disease progression and four patients due to an AE. All patients experienced at least one TEAE during the study. Treatment-related TEAEs occurred in 11/12 patients during exposure to eribulin alone and in 7/10 patients when exposed to eribulin with ketoconazole. Fourteen different treatment-related TEAEs were reported in ≥ 2 patients (Table 4). The most frequently reported treatment related TEAEs were fatigue and nausea, each reported in 8/12 patients, next to alopecia (6/12 subjects) and neutropenia (5/12 patients). Other treatment related TEAEs occurring in ≥ 2 patients included increased lacrimation, stomatitis, anorexia, arthralgia, constipation, dry mouth, myalgia, sensory neuropathy, rash and vomiting. Overall, ten patients had TEAEs CTCAE grade ≥ 3, of which six were reported as treatment related. These included events of fatigue, neutropenia, leukopenia, decreased neutrophil count and peripheral sensory neuropathy. There were no obvious changes in vital signs and no clinically significant ECG abnormalities were noted.

There were no life threatening SAEs or SAEs reported as treatment related. Five patients experienced SAEs which required hospitalization or prolongation of existing hospitalization. Neither one of these events was considered related to study medication. There was one death that was not considered related to the study drug.

Anti-tumor activity.

A total of 10 (83.3%) patients had at least 1 response evaluation in the course of treatment. The majority of patients (seven, 58.3%) achieved stable disease as best overall response. Progressive disease was reported in three patients (25.0%).

Table 4: Incidence of treatment-related adverse events, occurring in at least 2 patients

Term	Number of patients (%)		
	Eribulin only (n=12)	Eribulin plus ketoconazole (n=10)	Total (n=12)
Any TEAE	11 (91.7)	7 (70.0)	12 (100)
Fatigue	7 (58.3)	3 (30.0)	8 (66.7)
Nausea	8 (66.7)	2 (20.0)	8 (66.7)
Alopecia	6 (50.0)	0	6 (50.0)
Neutropenia	5 (41.7)	0	5 (41.7)
Lacrimation increased	4 (33.3)	0	4 (33.3)
Stomatitis	4 (33.3)	1 (10.0)	4 (33.3)
Anorexia	2 (16.7)	0	2 (16.7)
Arthralgia	2 (16.7)	0	2 (16.7)
Constipation	1 (8.3)	1 (10.0)	2 (16.7)
Dry mouth	2 (16.7)	0	2 (16.7)
Myalgia	2 (16.7)	0	2 (16.7)
Peripheral sensory neuropathy	2 (16.7)	0	2 (16.7)
Rash	2 (16.7)	0	2 (16.7)
Vomiting	2 (16.7)	0	2 (16.7)

Abbreviations: TEAE=treatment emergent adverse event.

DISCUSSION

In this study, the effect of repeated administration of ketoconazole, a potent CYP3A4 inhibitor, on the pharmacokinetics of single dose intravenous eribulin mesylate in patients with solid tumors was determined. Co-administration of ketoconazole had no statistically significant effect on eribulin exposure as demonstrated by dose-normalized $AUC_{0-\infty}$ and C_{max} values. Patients were administered a 1.4 mg/m^2 dose when eribulin mesylate was administered alone. Anticipating possible effects of CYP3A4 inhibition on eribulin exposure, a 50% reduced eribulin mesylate dose of 0.7 mg/m^2 was administered in combination with ketoconazole 200 mg daily.

Ketoconazole is a reversible, competitive inhibitor of CYP3A4 [1]. Ketoconazole T_{max} is 1-2 hours after oral administration [19]. The first dose of ketoconazole was timed one hour before eribulin mesylate infusion and its T_{max}, so as to achieve maximal competition with eribulin at the level of CYP3A4 while these were both at C_{max}.

The ketoconazole dose was 200 mg, as recommended for clinical application. Others have shown clinically significant inhibition of CYP3A4 using the same ketoconazole dose (200 mg daily for 3 days) on the pharmacokinetics of docetaxel [24] and the same schedule (200 mg daily for 2 days) on the pharmacokinetics of irinotecan [25]. *In vitro* data had shown that ketoconazole (5 µmol/l; approx. 2.7 µg/ml) suppressed the metabolism of eribulin (1-10 µmol/l) [14] . The dose used in this study (200 mg) has a mean peak plasma concentration of 3.5 µg/ml [19] (approx. 6.6 µmol/l, molecular weight: 531.4 g/mol) and *in vivo* eribulin exposure (estimated as < 1 µmol/l) to ketoconazole in this schedule was considered sufficient since the plasma concentrations *in vivo* exceeded those that had led to inhibition *in vitro*.

Eribulin was shown to be predominantly metabolized by CYP3A4 *in vitro* [13] and it was hypothesized that ketoconazole could inhibit CYP3A4-mediated metabolism of eribulin in humans. The results of a human metabolism and excretion study of radioactively-labeled eribulin recently showed that metabolism represents a minor component for eribulin elimination. Metabolite profiling of the plasma samples showed that metabolites accounted for ≤ 0.6% of unchanged eribulin in plasma. The majority of eribulin was excreted unchanged in feces (61.3%), except for a minor part in urine (8.1%), indicating a major contribution of the liver in eribulin clearance [26]. With this knowledge available, inhibition of CYP3A4 activity, as assessed in this study with the model compound ketoconazole, would not be expected to have a significant effect on the disposition of eribulin, and this is in concordance with the results of the present study. The results of another study investigating the effect of rifampicin, a CYP3A4 inducer, on single eribulin exposure are awaited.

All patients were extensive metabolizers for CYP3A4 and poor metabolizers for CYP3A5. The lack of effect of CYP3A4 inhibition by ketoconazole could therefore not be attributed to endogenous lower levels of CYP3A4 activity.

The effect of co-administration of CYP3A4 inhibitors and inducers on eribulin clearance was also studied in a population pharmacokinetic model that was developed based on the data from seven phase I and one phase II studies with 2729 observations in 269 patients. The population pharmacokinetic analysis demonstrated that the concomitant administration of CYP3A4 inhibitors and inducers did not alter eribulin clearance [14]. These results suggest that other CYP3A4 inhibitors also do not increase eribulin exposure and that co-administration of CYP3A4 inhibitors with eribulin mesylate probably is safe in clinical practice. This is in contrast to other microtubule inhibitors such as docetaxel [24] and ixabepilone [27], that were found to have significantly increased $AUC_{0-\infty}$ with clinical relevance after ketoconazole exposure.

There are other drug-drug interactions that may influence drug exposure, such as inhibition of the drug transporter P-glycoprotein (P-gp, multidrug resistant (MDR) 1, gene: *ABCB1*). P-gp serves as an efflux transporter to form a permeation barrier in the gastrointestinal tract and brain and to increase drug elimination in the liver and kidneys [28]. CYP3A4 and P-gp have a considerable overlap in substrate specificity [28]. It was shown that eribulin is a P-gp substrate *in vitro* and that plasma exposure to eribulin was higher in *abcb1a*-deficient mice than in wild type mice. Eribulin oral bioavailability was shown to be 62.3% in *abcb1a*-deficient mice compared to 7.6% in wild-type mice. Eribulin brain penetration was found to be 30-fold greater in *abcb1a*-deficient mice compared to wild type mice. Eribulin inhibited P-gp-mediated efflux of digoxin *in vitro* but this only occurred at concentrations that were much higher than clinically relevant [29]. Ketoconazole was also found to be a moderate inhibitor of P-gp *in vitro* [30] and could theoretically further increase eribulin exposure after intravenous administration by inhibition of its efflux activity into the bile, but, as mentioned earlier, such effect was not observed.

In conclusion, statistical analysis of $AUC_{0-\infty}$ and C_{max} indicated that co-administration of a potent CYP3A4 inhibitor, ketoconazole, had no statistically significant effect on single-dose exposure to eribulin mesylate when administered to patients with solid tumors. Eribulin mesylate was generally safe and no new safety concerns were identified during this study. The results indicate that eribulin mesylate can be safely co-administered with ketoconazole. Drug-drug interactions are not expected with other CYP3A4 inhibitors in patients with up to moderately deviated liver function parameters, as defined in this study.

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Chapter 3.3

Eribulin mesylate pharmacokinetics in patients with solid tumors receiving repeated oral rifampicin

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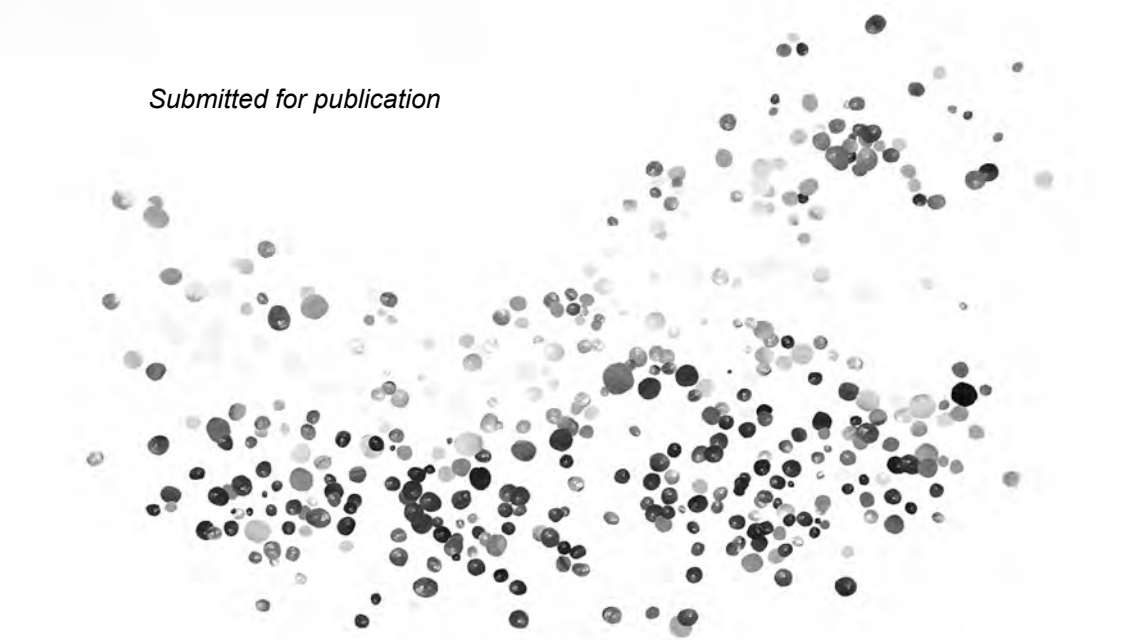


ABSTRACT

Purpose

Eribulin mesylate is a non-taxane microtubule inhibitor that was recently approved for treatment of metastatic breast cancer. The aim of this study was to determine the effect of rifampicin, a CYP3A4 inducer, on the plasma pharmacokinetics of eribulin in patients with solid tumors.

Experimental design

An open-label, non-randomized, cross-over phase I study. In the first cycle, patients received intravenous 1.4 mg/m^2 eribulin mesylate on day 1 and 15 and oral rifampicin 600 mg on days 9 to 20 of a 28-day cycle. Pharmacokinetic sampling for determination of eribulin plasma concentrations was performed up to 144 hours following administration of eribulin mesylate on day 1 and 15. $AUC_{0-\infty}$ and C_{max} for eribulin exposure without or with co-administration of rifampicin were subjected to an analysis of variance (ANOVA) and corresponding 90% confidence intervals (CI) were calculated. Subsequently, patients were allowed to continue eribulin mesylate treatment with 1.4 mg/m^2 eribulin mesylate on days 1 and 8 of a 21-day cycle. Also safety and anti-tumor activity were assessed.

Results

Fourteen patients were included and eleven patients were evaluable for pharmacokinetic analysis. Co-administration of rifampicin had no effect on single-dose exposure to eribulin (geometric least square means ratio: $AUC_{0-\infty}$ =1.10 [90% CI 0.91 – 1.34] and C_{max} = 0.97 [90% 0.81 – 1.17]). The most common treatment-related grade ≥ 3 adverse events were grade 4 leukocytopenia, neutropenia and decreased neutrophil count (all 2/14, 14%), grade 3 fatigue (3/14, 21%) and grade 3 decreased leukocyte and neutrophil count and neutropenia (both 2/14, 14%).

Conclusions

The results indicate that eribulin mesylate can be safely co-administered with rifampicin, a potent model CYP3A4 inducer, and drug-drug interactions with other compounds that are CYP3A4 inducers are not expected.

INTRODUCTION

Drug-drug interactions are a major cause of morbidity and death in clinical practice. Drug-drug interactions are estimated to be responsible for 20-30% of all adverse drug reactions and the risk of clinically significant drug interactions increases with the number of concomitantly prescribed drugs [1]. These concerns are especially relevant in anti-cancer therapy, where patients are often exposed to many other drugs for treatment-derived complications or concurrent diseases. Furthermore, anti-cancer drugs are usually dosed at the maximum-tolerated dose. Next to this, patients may use over-the-counter drugs, or 'complementary and alternative medicines' such as St. John's wort that may interact with anti-cancer therapy and treating physicians may not be aware of this [2]. Therefore, it is important to gain insight into potentially clinically relevant drug-drug interactions already in the early development of new drugs.

Eribulin mesylate (E7389; Halaven™; Eisai Inc) is a non-taxane microtubule dynamics inhibitor with a distinct mechanism of action. Eribulin prevents microtubule growth and sequesters tubulin into non-functional aggregates. The overall effects lead to G2-M arrest and ultimately cell death. Eribulin is a synthetic analogue of halichondrin B, a product isolated from the marine sponge *Halichondria okadai* [3-6]. Eribulin pharmacokinetics exhibit a rapid distribution phase followed by a slow elimination phase [7;8].

Eribulin mesylate is in development in phase I-III trials for the treatment of solid tumors, both as monotherapy as well as in combination therapy with capecitabine, trastuzumab, pemetrexed, erlotinib, gemcitabin, cisplatin and carboplatin [9;10]. Recently, a phase III trial (EMBRACE) demonstrated that eribulin mesylate treatment significantly improved median overall survival of metastatic breast cancer patients that were previously treated with an anthracycline and a taxane compared to 'treatment of physician's choice' [11]. Following the results of this trial, eribulin mesylate was approved as 3rd line of treatment in metastatic breast cancer patients.

Human cytochrome P450 (CYP) 3A4 is involved in the metabolism of approximately 60% of currently prescribed drugs and plays a dominant role in many clinically relevant drug-drug interactions [12;13]. Preclinical data in human liver microsomes indicated that eribulin is predominantly cleared by hepatic metabolism *in vitro* and that CYP3A4 is the major enzyme involved [14]. Animal studies showed though that metabolism was limited. After intravenous administration of radiolabelled eribulin mesylate in bile-duct canulated rats and dogs, unchanged drug was the major component found excreted

in bile, feces and urine. Because it is thought that eribulin is primarily metabolized by CYP3A4 in humans, it is potentially subject to interaction with compounds that are known to either inhibit or induce CYP3A4.

The aim of this study was to explore the effect of repeated oral administration of rifampicin, a potent model CYP3A4 inducer recommended in regulatory guidelines [15;16], on the plasma pharmacokinetics of eribulin mesylate administered by intravenous infusion. The secondary objectives were to determine the safety and tolerability of eribulin mesylate with and without co-administration of rifampicin.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed progressive advanced solid tumors, for whom no standard treatment was available, were eligible. Other inclusion criteria were: age ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) [17] performance status ≤ 2; life expectancy of ≥ 3 months; resolution of all chemotherapy- or radiation-related toxicities to grade 1 severity or lower, except for stable sensory neuropathy ≤ grade 2 and alopecia; adequate renal- and liver function (defined as: serum creatinine ≤ 176 µmol/l or calculated creatinine clearance ≥ 40 millilitre/minute (ml/min; Cockroft-Gault formula) [18], bilirubin ≤ 1.5 times the upper limit of normal (ULN), alkaline phosphatase (AP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ≤ 3 times ULN, in the case of liver metastasis ≤ 5 times ULN, liver specific AP ≤ 3 times ULN); adequate bone marrow function (defined as: neutrophil count (ANC) ≥ 1.5 x 10^9/l, hemoglobin ≥ 6.2 mmol/l and platelets ≥ 100 x 10^9/l); adequate contraceptive protection. Exclusion criteria included: hypersensitivity to halichondrin B and/or –like compounds or rifampicin; subjects with prior participation in an eribulin clinical trial; chemotherapy, radiation or biological therapy within 2 weeks, hormonal therapy within 1 week or any investigational drug within 4 weeks; any medication, dietary supplements or other compounds or substances known to induce or inhibit CYP3A4 activity at the time the study started; impaired intestinal absorption; significant cardiovascular impairment; clinically significant electrocardiogram (ECG) abnormality; brain or subdural metastases, unless stable and completed local therapy and discontinuation of corticosteroids for at least 4 weeks; meningeal carcinomatosis; any other significant disease or disorders that would exclude the patient from the study. The study protocol was approved by the

Medical Ethics committee of participating hospitals and all patients had to give written informed consent. The study was conducted in accordance with the requirements of the world medical association declaration of Helsinki (2008), international conference of harmonization (ICH) and good clinical practice (GCP) guidelines.

Study design, study procedures and treatment administration.

This phase I study was a 2-center, open label, non-randomized, crossover study in patients with solid tumors to investigate the effect of oral administration of rifampicin on the plasma pharmacokinetics of eribulin administered by intravenous infusion. Also safety and tolerability of eribulin mesylate alone or when co-administered with rifampicin were assessed during cycle 1 (study treatment phase). Another objective was to further explore safety and tolerability of eribulin when given alone on days 1 and 8 of a 21-day schedule in patients with solid tumors (study extension phase).

Up to a maximum of 14 patients were recruited in two centers: the University Medical Center Utrecht (UMCU), Utrecht and the Netherlands Cancer Institute (NKI), Amsterdam, both in The Netherlands. The date of interim analysis was 1 May 2011.

In cycle 1, patients received intravenously 1.4 mg/m^2 eribulin mesylate on day 1 and 15 and rifampicin 600 mg on days 9 to 20 of a 28-day cycle. A 15-day washout period was introduced to prevent the effects of the first treatment carrying over to the second treatment. Eribulin mesylate was provided in vials containing 1.0 mg eribulin mesylate in 2 ml solution (0.5 mg/ml) with ethanol/water (5:95). A dose of 1.4 mg/m^2 eribulin mesylate is equivalent to 1.23 mg/m^2 eribulin in the 'ready to use solution' (0.44 mg/ml) [19]. Eribulin was administered as a 2-5 minutes intravenous bolus injection. Rifampicin (Sanofi-Aventis, UK; Rifadin™) [20] was orally administered once daily in two 300 mg capsules (total dose per day: 600 mg). Rifampicin was taken at least 30 minutes before a meal or 2 hours after a meal to ensure rapid and complete absorption. Patients were instructed to record a diary of rifampicin dosings, concomitant medication and side-effects from day 9 to 14. Patients were allowed to continue treatment with eribulin mesylate at a dose of 1.4 mg/m^2 on day 1 and day 8 of a 21-day cycle.

Complete physical examinations, including vital signs, height, weight, ECOG performance status and ECG, and clinical laboratory tests (hematology, clinical chemistry and urinalysis) were performed at screening and at regular intervals during cycle 1, in any following cycles and at study termination.

Safety assessment.

Adverse events (AE) and concomitant medications were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE) version 3.0 [21]. Treatment emergent AEs (TEAEs) were defined as any AE with a start date/time beyond or equal to the day of initial dosing (cycle 1 day 1), up to 30 days after the last dose of study drug, and any AE which increased in severity during the study. Patients were discontinued from the study for any of the following reasons: progressive disease by clinical evaluation or as documented by RECIST 1.0 [22]; loss of clinical benefit because of undue toxicity; patient refusal of further therapy or withdrawal of consent.

Anti-tumor activity assessment.

Radiologic tumor assessments according to RECIST 1.0 were performed at baseline and at regular intervals. Best response was documented for each patient.

Pharmacokinetic sampling and analysis.

All patients entering the study provided blood samples for pharmacokinetic analysis. Whole blood samples for the determination of free-base eribulin plasma concentrations were taken at day 1 and day 15 at pre-dose, at end of infusion, and at 0.25, 0.5, 1, 2, 4, 6, 10, 24, 48, 72, 96, 120 and 144 hours following the end of the administration. Whole blood samples were collected from the contra-lateral arm to the infusion. At each time point, 6 ml of whole blood was withdrawn into sodium heparinized collecting tubes (Vacutainer™) and blood and anti-coagulant were mixed, stored on ice and centrifuged within 60 minutes at 1500 g/3000 rpm at 4ºC for 10 minutes. Plasma was transferred into polypropylene tubes and stored at -70ºC until analysis. Plasma concentrations of free-base eribulin were quantified using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method. The lower limit of quantitation (LLOQ) was 0.2 ng/ml. The inter-assay accuracy ranged from -3.18 to 2.77% with an inter-assay precision of ≤ 13.0%[23] . Carry-over was defined as $C_{eribulin\ pre\text{-}dose,day\ 15}$ of > 5% of $C_{max,\ day\ 1}$.

Eribulin pharmacokinetic parameters were derived from plasma concentrations on day 1 to 7 and 15 to 21 by non-compartmental analysis using WinNonlin Professional® (version 5.1.1, Pharsight Corp, CA, USA). These included: area under the curve extrapolated to infinity ($AUC_{0-\infty}$), terminal half-life ($t_{1/2}$), clearance (Cl) and volume of distribution at steady state (V_{ss}). Maximum observed plasma concentration (C_{max})

and time to C_{max} (T_{max}) were derived from the data directly. Statistical analysis was performed using SAS® (version 9.1).

Statistical analysis.

Sample size calculations were made. The number of patients recruited was based on the number estimated to provide at least 90% power to detect a clinically significant decrease of 30% in $AUC_{0-\infty}$ and C_{max} between eribulin exposure in the presence and absence of rifampicin. The estimates of the within-subject standard deviation for $AUC_{0-\infty}$ and C_{max} for the 1.4 mg/m^2 dose ranged from 0.16 to 0.20, based on models with all data that was available at the time of study design, including subjects dosed at 1.4 mg/m^2 at two time-points as well as across all doses (0.25 mg/m^2 to 1.4 mg/m^2). Assuming a within-subject standard deviation of 0.20 and a one-sided alpha level of 5%, 10 patients would provide at least 90% power to detect a 30% decrease in the $AUC_{0-\infty}$ and C_{max} by rifampicin.

Statistical analysis of $AUC_{0-\infty}$ and C_{max} used an estimation approach based on mean ratio (i.e. the ratio of the pharmacokinetic parameters for test [eribulin with rifampicin] over reference [eribulin alone] defining the magnitude of the interaction). Natural logarithm (ln)-transformed $AUC_{0-\infty}$ and C_{max} were subjected to an analysis of variance (ANOVA) and results were presented in terms of geometric means ratio with associated 90% confidence intervals (CI) and no p-values. If the upper and lower bounds were within 0.7 to 1.43 for both $AUC_{0-\infty}$ and C_{max}, then the interaction is considered to have no effect. The estimated means were back-transformed to obtain an estimate for the ratio (eribulin + rifampicin)/ eribulin. The ANOVA model included terms for treatment, investigational center and patient.

RESULTS

Patient inclusion and demographics.

A total of 14 patients were enrolled in the study. Patient demographics and characteristics are summarized in Table 1. The median age was 58.5 years (range: 40.0 – 79.0) and the median weight was 70 kg (range: 49 - 105). The majority was male (57%) and Caucasian (93%). The most frequent primary tumor types were colorectal (29%) and ovarian cancer (21%).

Table 1: Patient demographics and characteristics of the safety population

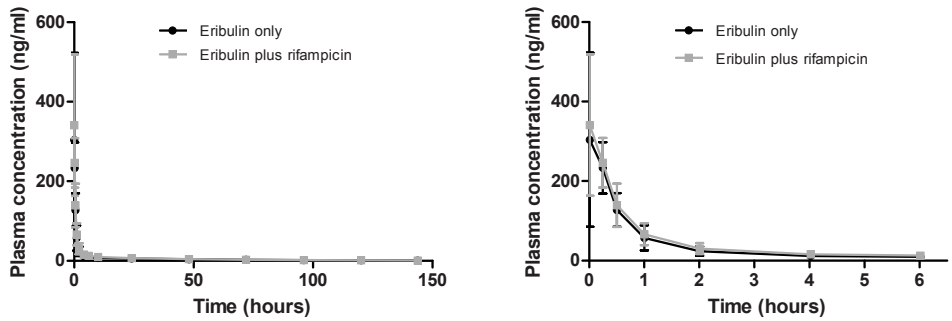
Number (n)	14
Age (years)	
Median (range)	58.5 (40.0 – 79.0)
Gender, n (%)	
Male	8 (57)
Female	6 (43)
Race, n (%)	
Caucasian	13 (93)
Asian	1 (7)
Weight (kg)	
Median (range)	70 (49 – 105)
Height (cm)	
Median (range)	172 (158 – 189)
Body surface area (m^2)	
Median (range)	1.83 (1.5 – 2.3)
Tumor type, n (%)	
Colorectal	4 (29)
Ovary	3 (21)
Lung	2 (14)
Breast	1 (7.1)
Other	4 (29)

Pharmacokinetic and statistical analysis.

Eleven patients were evaluable for pharmacokinetics. Two patients discontinued before day 15 and one patient was excluded because of non-compliance in rifampicin intake. For all patients, $AUC_{0-\infty}$ was evaluable for both day 1 and 15. No carry-over was observed.

Mean plasma eribulin concentration-time curves are presented in Figure 1. Pharmacokinetic data are summarized in Table 2 and shown in Figure 2 (for $AUC_{0-\infty}$ and C_{max}). Data of all calculated pharmacokinetic parameters that had been determined were comparable for eribulin without and with rifampicin administration. Mean $AUC_{0-\infty}$ (SD) for eribulin exposure without and with rifampicin was 757 (264) ng.h/ml and 846 (326) ng.h/ml, respectively (Figure 2B). Mean C_{max} (SD) for eribulin exposure without and with rifampicin were 371 (164) ng/ml and 360 (168) ng/ml, respectively (Figure 2D). Eribulin $AUC_{0-\infty}$ and C_{max} had moderate inter-individual variation, with CVs of 35% and 39%, and 44% and 47%, respectively, without and with rifampicin exposure (Figure 2A and C). Rifampicin had no effect on eribulin Cl and $t_{1/2}$.

Linear scale



Log-linear scale

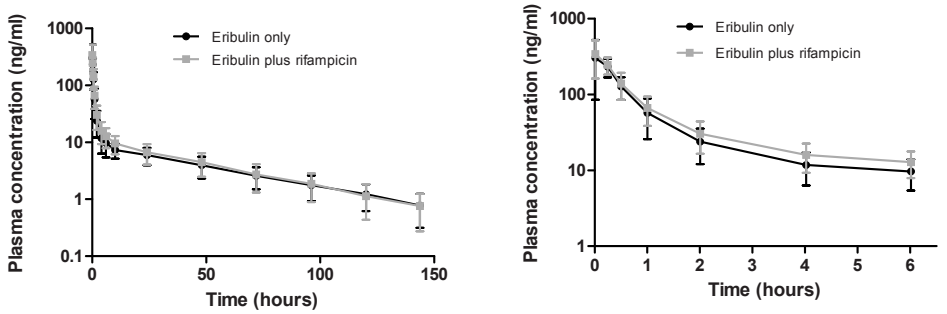


Figure 1: Mean (SD) plasma concentration (ng/ml)-time (h) curve (upper panel: linear scale, lower panel: log-linear scale) following intravenous administration of eribulin mesylate alone (1.4 mg/m^2; n=11) or eribulin mesylate (1.4 mg/m^2) with rifampicin (600 mg; n=11 both groups). Left: time scale up to 150 hours, right: time scale up to 6 hours.

Table 2: Pharmacokinetic parameters for eribulin mesylate alone or co-administered with rifampicin: pharmacokinetic population

Parameter Actual values	Eribulin mesylate	Eribulin mesylate with rifampicin
$AUC_{0-\infty}$ (ng.h/ml)	757 (264)	846 (326)
C_{max} (ng/ml)	371 (164)	360 (168)
T_{max} (hours)	0.17 (0.08-0.32)	0.10 (0.05-0.37)
$t_{1/2}$ (hours)	40.4 (9.67)	36.6 (8.25)
Cl (l/h)	3.41 (1.31)	3.18 (1.38)
V_{ss} (l)	134 (42.7)	109 (38.9)

Eribulin mesylate dose was 1.4 mg/m^2 (n=11 both). Data are shown as mean (SD) except for t_{max} which are median (range) values. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration; Cl = systemic clearance; T_{max} = time to maximum observed plasma concentration; $t_{1/2}$ = terminal half-life; V_{ss} = volume of distribution at steady state.

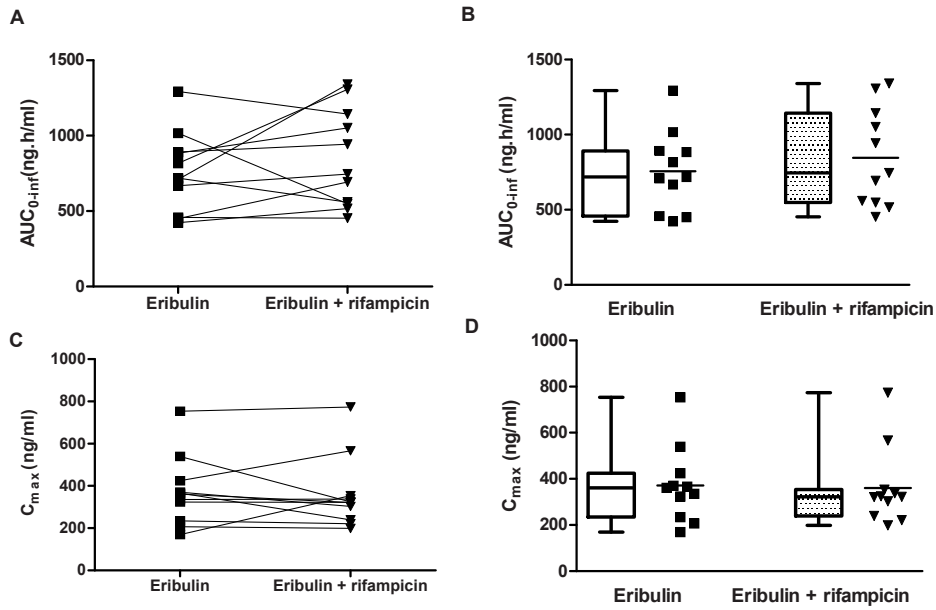


Figure 2: Effect of rifampicin co-administration on $AUC_{0-\infty}$ (Fig. 2A and B) and C_{max} (Fig. 2C and D) of single dose intravenous administration of eribulin mesylate (1.4 mg/m^2). Squares (without rifampicin) and triangles (with rifampicin) represent eribulin values for individual subjects. Left: inter-individual change in $AUC_{0-\infty}$ (Fig.2A) and C_{max} (Fig. 2C; n=11). Right: Comparative box-plots of eribulin $AUC_{0-\infty}$ and C_{max} (Fig. 2B and D; also summarized in table 2) in the absence and presence of rifampicin. Box plot represents the 25th -75th percentiles, whiskers extend to 5th and 95th percentiles and median is indicated by a line within the box. Box-plots are flanked by scatter plots of individual data points, horizontal line represents the mean. Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration.

For the primary pharmacokinetic endpoints of $AUC_{0-\infty}$ and C_{max} without or with co-administration of rifampicin, the geometric means, ratio and confidence intervals were calculated (Table 3). The confidence intervals of the ratios were within the pre-defined range of 0.7 to 1.43 ($AUC_{0-\infty}$: geometric least square means ratio= 1.10; 90% CI 0.91 – 1.34, and C_{max}: geometric least square means ratio= 0.97; 90% 0.81 – 1.17). Therefore, no difference was determined for $AUC_{0-\infty}$ and C_{max} of eribulin when administered alone or in co-administration with rifampicin.

Safety.

All 14 patients were evaluable for safety and tolerability of eribulin alone and when co-administered with rifampicin. Two patients discontinued during the first cycle, one due to an AE (leukocytopenia and febrile neutropenia, both grade 3) and one due to patient's choice. Ten patients (10/14, 71%) commenced the second cycle and the reason for discontinuation for these patients was progressive disease. The median number of treatment cycles was 2 (range: 0 - 8).

Table 3: Statistical analysis of the primary pharmacokinetic parameters

Parameter	Geometric mean		Ratio of treatment means (eribulin plus rifampicin: eribulin)	90% confidence interval
	Eribulin plus rifampicin	Eribulin		
$AUC_{0-\infty}$ (ng.h/ml)	789	716	1.10	(0.91 to 1.34)
C_{max} (ng/ml)	333	342	0.97	(0.81 to 1.17)

Model includes terms for treatment, investigational center and patient (n=11 patients). Abbreviations: $AUC_{0-\infty}$ = Area under the concentration-time curve from zero (pre-dose) extrapolated to infinity; C_{max} = maximum observed plasma concentration.

All patients experienced at least one treatment-related AE. The most common AEs were fatigue (10/14, 71%), alopecia (7/14, 50%) and nausea (6/14, 43%). The majority (13/14, 93%) of patients experienced a treatment-related AE. The incidence of treatment-related AEs that occurred in at least 2 patients during cycle 1 is summarized in Table 4. The most common treatment-related AEs during the whole study were fatigue (9/14, 64%), nausea (6/14, 43%), alopecia (7/14, 50%) and pyrexia (5/14, 36%). In total, 10/14 (71%) experienced any treatment-related AE grade 3 or 4 during the study. Two patients (2/14, 14%) experienced toxicity grade 4 (leukocytopenia, neutropenia and decreased neutrophil count). Other treatment related grade 3 AEs that were observed in more than 1 patients included fatigue (3/14, 21%) and decreased leukocyte and neutrophil count and neutropenia (both 2/14, 14%).

Table 4: Incidence of treatment-related adverse events occurring in at least 2 patients during cycle 1

Term	Number of patients (%)		
	Eribulin only (n=14)	Eribulin plus rifampicin (n=12)	Total (n=14)
Any TEAE	8 (57)	11 (92)	13 (93)
Fatigue	3 (21)	2 (17)	5 (36)
Pyrexia	3 (21)	1 (8.3)	4 (29)
Nausea	2 (14)	2 (17)	4 (29)
Alopecia	0	4 (33)	4 (29)
Leukocytes decreased	0	3 (25)	3 (21)
Leukopenia	2 (14)	1 (8.3)	2 (14)
Neutropenia	1 (7.1)	2 (17)	2 (14)
Neutrophil count decreased	1 (7.1)	2 (17)	2 (14)
Chromaturia	0	2 (17)	2 (14)

Abbreviations: TEAE= treatment-emergent adverse event.

The following six SAEs were reported to be related to the study drug: leukocytopenia, neutropenia, decreased leukocyte- and neutrophil count, febrile neutropenia, pyrexia, abdominal pain and gastric hemorrhage. There were no deaths during the study or in the 30 days of follow-up after study discontinuation.

Anti-tumor activity.

One patient (1/11, 9.1%) with breast carcinoma had a partial response and four patients had stable disease as best response (4/11, 36%). Four patients (4/11, 36%) had progressive disease and data were missing in two patients (2/22, 18%).

DISCUSSION

In this study, the effect of rifampicin, a potent CYP3A4 inducer, was determined on the pharmacokinetics of single dose intravenous eribulin mesylate in patients with solid tumors. Rifampicin is a derivative of rifamycin B and is currently used as an oral antimicrobiotic drug for treatment of tuberculosis and bacterial infections. Rifampicin is eliminated in the bile and has enterohepatic circulation [20;23]. Rifampicin is a potent inducer of the expression of CYP3A4 which is located in the liver and the epithelial layer of the intestine. In general, CYP3A4 induction by rifampicin can lead to clinically significant drug interactions by reducing the plasma concentrations and the effects of drugs that are metabolised by CYP3A4 [13]. Cancer patients may very well use co-medication, including a CYP3A4-inducer during treatment with eribulin. Examples of CYP3A4 substrates and inducers used in anti-cancer therapy include paclitaxel, cyclophosphamide and dexamethasone [24]. Another example of a CYP3A4 inducer is hyperforin, which is the active constituent of the herbal antidepressant St. John's wort, a drug that is popular among cancer patients [25]. This could result in lower eribulin exposure, which in turn could possibly lead to reduced anti-tumor activity. Therefore, rifampicin was chosen as a model compound to determine if such unfavorable drug-drug interaction was present.

The study design was a non-randomized, one-way crossover study. In order to avoid any long-term effects of enzyme induction by rifampicin, a randomized two-way crossover design was not employed, but a one-way design was chosen in which all patients first received eribulin mesylate monotherapy followed by eribulin mesylate co-administration with rifampicin. Carry-over effects of eribulin exposure from the first dose to day 15 were investigated, but were not observed, indicating that the

wash-out period of 15 days was sufficient for the elimination of eribulin.

Rifampicin was administered at a dose of 600 mg daily during 12 days (days 9 - 20) according to FDA guidance [16]. Eribulin mesylate was dosed on the 7th day of rifampicin exposure (day 15) and rifampicin was continued 5 additional days (up to day 20). Patient compliance was checked by a diary. Clinically relevant interactions have been reported using rifampicin 600 mg daily for at least 4 days [13]. In one study, actual hepatic CYP3A4 protein content was determined in liver biopsies and a 2.4 to 4.7 fold increase was found in patients exposed to rifampicin (600 mg daily for four days) compared to patients that were not exposed to rifampicin [26]. Thus, the rifampicin dosing schedule used was considered long enough and high enough to reach sufficient CYP3A4 induction in order to determine any potential clinical effect on eribulin exposure in this study.

Enzyme induction by rifampicin is mediated by the pregnane X receptor (PXR). Rifampicin binds and activates the PXR, leading to increased DNA transcription and subsequent protein synthesis of specific enzymes [27]. Next to CYP3A4, also other proteins are thought to be induced by rifampicin by modulation of the PXR receptor, such as the ATP-binding cassette drug transporter P-glycoprotein (P-gp, multidrug resistant (MDR) 1, gene: *ABCB1*) [28] . P-gp serves as an efflux transporter to form a permeation barrier in the gastrointestinal tract and the brain and to increase drug elimination in the liver and kidneys[29] and eribulin was shown to be a P-gp substrate [30]. Induction of P-gp by rifampicin could theoretically have resulted in increased elimination of eribulin, but such effect was not observed in the present study.

Co-administration of rifampicin had no effect on eribulin exposure as demonstrated by $AUC_{0-\infty}$ and C_{max} values. These results are consistent with the results of the eribulin-ketoconazole interaction study [31], in which inhibition of CYP3A4 was studied. Here, also no effect on eribulin exposure was found for dose-normalized $AUC_{0-\infty}$ and C_{max} values. Apart from these two clinical studies using model compounds to study CYP3A4 induction and –inhibition, also the effect of these types of drugs on eribulin clearance was assessed in a population pharmacokinetic model that was developed based on the data from seven phase I and one phase II studies with 2729 observations in 269 patients. This analysis demonstrated that the concomitant administration of CYP3A4 inhibitors and –inducers did not alter eribulin clearance [32]. It can be concluded that 2 clinical studies did not demonstrate an effect of CYP3A4 induction and inhibition and that these results were supported by the population pharmacokinetic model. An explanation for the lack of observation of an interaction for eribulin at the level of CYP3A4 may be found in the results of a recent human

metabolism and excretion study of radioactively-labelled eribulin [33]. The majority of eribulin was excreted unchanged in feces (61.3%) except for a minor part in urine (8.1%). Plasma metabolite profiling showed that metabolites accounted for ≤ 0.6% of unchanged eribulin in plasma. These results indicate that, while CYP3A4 probably is the main enzyme for eribulin metabolism based on *in vitro* data, the contribution of metabolism to the elimination of eribulin is limited, and that elimination is mostly achieved by biliary excretion of unchanged drug. Therefore, the effects of CYP3A4 inducing and –inhibiting drugs on eribulin exposure are not likely to have clinical significance in the treatment of cancer patients.

In conclusion, analysis of $AUC_{0-\infty}$ and C_{max} indicated that co-administration of a potent CYP3A4 inducer, rifampicin, had no effect on single-dose exposure to eribulin when administered to patients with solid tumors. These results indicate that eribulin mesylate may be safely co-administered with compounds that are CYP3A4 inducers.

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Chapter 4

Pharmacokinetics of lapatinib

Chapter 4.1

Effects of low-fat and high-fat meals on steady-state pharmacokinetics of lapatinib in patients with advanced solid tumors

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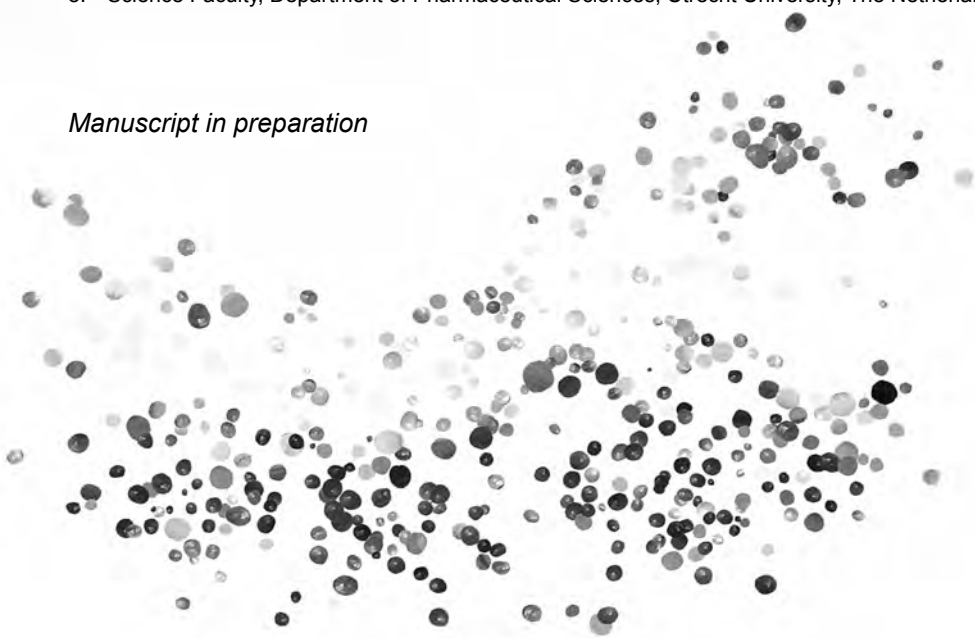

ABSTRACT

Purpose

To determine the effect of food on the pharmacokinetics, safety and tolerability of a single-dose of lapatinib taken at steady state in patients with advanced solid tumors.

Experimental design

This study was a three-treatment, randomized, three sequence cross-over study. The primary objective was to determine the effect of food, ingested one hour before oral dosing of lapatinib, on systemic exposure of lapatinib. The secondary objectives were to assess safety and tolerability of lapatinib. Lapatinib was administered one hour after a low- [B] or a high-fat [C] breakfast and pharmacokinetics were compared to those obtained following lapatinib administration one hour before a low-fat breakfast [A].

Results

In total, 25 patients were included of whom 12 were evaluable for pharmacokinetics. Both low-fat and high-fat food affected lapatinib exposure. Lapatinib AUC_{0-24} increased following lapatinib administration 1 hour after a low-fat meal by 1.80-fold (90%CI: 1.37 – 2.37; $P = 0.005$) and after a high-fat meal by 2.61-fold (90%CI: 1.98 – 3.43; $P = 0.002$) compared to administration one hour before a low-fat meal. Lapatinib C_{max} increased following lapatinib administration 1 hour after a low-fat meal by 1.90-fold (90%CI: 1.49 – 2.43; $P = 0.004$) and after a high-fat meal by 2.66-fold (90%CI: 2.08 – 3.41; $P = 0.002$) compared to administration one hour before a low-fat meal. The most commonly occurring treatment-related toxicity was diarrhea (8/25, 32% grade 1 and 2/25, 8% grade 2). Other treatment-related toxicity included nausea, vomiting, rash, stomatitis and fatigue. One patient experienced a treatment-related CTCAE grade ≥ 3 event (fatigue grade 3, 4%). No serious adverse events or deaths were reported during this three-week study of lapatinib monotherapy.

Conclusions

Both low-fat and high-fat food consumed one hour before lapatinib administration increased lapatinib exposure, compared to administration one hour before a low-fat meal. In order to administer lapatinib in a fasted state, it is advised to administer the drug one hour before a meal.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer type among women. The estimated incidence of breast cancer worldwide was 1.38 million in 2008, thereby accounting for 23% of all diagnosed cancers. Breast cancer is also the most frequent cause of cancer death in women (12.7% of total) [1]. Expression of the epidermal growth factor receptors (ErbB1, EGFR) and ErbB2 (HER2) has been shown to promote tumor growth and survival in a variety of epithelial tumors, including breast cancer [2;3]. Overexpression of HER2 is observed in approximately 25-30 % of primary breast cancers and is correlated with poor clinical outcome [2].

Lapatinib ditosylate (GW572016; Tyverb/Tykerb™; GlaxoSmithKline), a 4-quinazolamine, is an orally administered reversible tyrosine kinase inhibitor of the intracellular domains of EGFR and HER2 [4;5]. Lapatinib was shown to inhibit phosphorylation and activation of EGFR and HER2 and of proteins in the signal transduction cascade downstream of the receptors in tumor cell lines and xenografts [5]. In humans, the biologic effects of lapatinib were demonstrated in sequential tumor biopsies showing variable levels of inhibition of EGFR, HER2 and of phosphorylated downstream proteins [6].

Safety and efficacy of lapatinib monotherapy has been studied in several phase I-II trials in a variety of advanced solid tumors [7;8], including breast cancer [9] and gastric cancer [10]. The most frequently reported drug-related side effects include diarrhea, rash, nausea anorexia, pruritus and fatigue [7-11]. Asymptomatic and symptomatic decreases in ardiac left ventricular ejection fraction (LVEF) [12] and hepatotoxicity were also reported [13].

Following the results of a phase III trial, lapatinib was approved by the U.S. Food and Drug Administration (FDA) in combination with capecitabine (Xeloda™; Roche) for treatment of patients with advanced or metastatic ErbB2-overexpressing breast cancer after anthracycline, taxane and trastuzumab (Herceptin™; Genentech/Roche) therapy [14]. The combination of lapatinib and capecitabine was conditionally approved by the European Medicines Agency (EMA). The recommended dose of lapatinib once daily continuously is 1500 mg in monotherapy and 1250 mg in combination with capecitabine. In addition, results from a subgroup analysis in HER2-positive metastatic breast cancer patients in another phase III trial led to approval by the FDA and conditional approval by the EMA of the combination of lapatinib with the aromatase inhibitor letrozole (Femara™, Novartis) in postmenopausal patients with hormone receptor positive, HER2-positive metastatic disease [15].

Lapatinib is a lipophilic drug with low, pH dependant solubility. Oral bioavailability has considerable inter-patient variability with a coefficient of variation (CV%) of 43% [8]. In plasma, lapatinib is highly bound to protein (>99%; data on file at GSK). The primary route of excretion for lapatinib and its metabolites is in feces. Steady-state pharmacokinetics are achieved in 6-7 days [8].

Lapatinib is predominantly metabolized by cytochrome P450 (CYP) 3A4 and 3A5 with minor contributions of CYP2C19 and CYP2C8. Co-administration of ketoconazole, a CYP3A inhibitor, and carbamazepine, a CYP3A inducer, affected lapatinib exposure significantly in healthy volunteers [16]. Lapatinib is a substrate for the efflux transporters P-glycoprotein (P-gp, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*) [17] that are both located in the intestine, liver, kidney and at the blood-brain barrier [18;19]. Bioavailability of lapatinib is significantly affected by food [8;20;21]. Currently, it is advised to administer lapatinib in a fasted condition, being at least one hour before or at least one hour after food intake [22]. The first study to investigate the effect of food on lapatinib pharmacokinetics in humans was performed in 19 healthy volunteers receiving a low dose (100 mg) of lapatinib and a high-fat meal. Lapatinib exposure (area under the plasma concentration-time curve extrapolated to infinity , $AUC_{0-\infty}$) and maximal plasma concentration, C_{max}) was 1.6-fold higher in the fed state compared to the fasted state [20]. Next, the effect of a low-fat meal on pharmacokinetics of single-dose lapatinib was investigated in six patients who were participating in a dose-finding phase I study [8]. After receiving a single dose of 1250 mg lapatinib after an overnight fast, either fasting was continued for 4 h (fasted state) or a low-fat breakfast was consumed (fed state). Administration of lapatinib with a low-fat breakfast resulted in approximately 3.0-fold increase in AUC_{0-24} and 3.2-fold increase in C_{max}. In this small group, the CV% in AUC was lower in the fed (43%) than in the fasted state (59%). A larger phase I study, that was designed specifically to study food-effects on lapatinib, was reported by Koch et al. in 27 patients with advanced solid tumors. This study evaluated the effect of overnight fasting and simultaneous administration of high-fat and low-fat meals on single-dose 1500 mg lapatinib pharmacokinetics [21]. The low-fat meal increased $AUC_{0-\infty}$ 2.7-fold and C_{max} 2.4-fold compared to the fasted state. The high-fat meal increased $AUC_{0-\infty}$ 4.3-fold and C_{max} 3.0-fold compared to the fasted state. The CV% for AUC was larger for the fasted state (68%) than for the low-fat and the high-fat states (52%, both). However, these differences were not found to be significantly different which was most likely due to the limited sampling size. These results underscored the clinically relevant interaction with food and it was subsequently advised to dose in the fasting state in order to achieve a consistent exposure.

The objectives of the present study were to evaluate the pharmacokinetic effect of consumption of low-fat and high-fat meals on the pharmacokinetics of lapatinib, when meals were consumed one hour before a dose of 1250 mg lapatinib, compared to consuming a low-fat meal an hour after dosing, in patients with solid tumors. Also safety and tolerability of lapatinib in combination with these meals was explored.

MATERIALS AND METHODS

Patient selection.

Patients with histologically or cytologically confirmed diagnosis of ErbB2 overexpressing metastatic breast cancer or other progressive advanced solid tumor regardless of ErbB2-status that was refractory to standard therapy or for which there was no established therapy, were eligible. Other inclusion criteria were: written informed consent; age between 18-75 years; of non-child-bearing potential or with a negative pregnancy test; Eastern Cooperative Oncology Group (ECOG) [23] performance status of ≤ 2; able to swallow and retain oral medications; adequate hematological- (neutrophils ≥ 1.5 x 10^9/l; hemoglobin ≥ 5.6 mmol/l; platelets ≥ 75 x 10^9/l) , hepatic- (bilirubin ≤ 1.5 x upper limit of normal (ULN); AST and ALT ≤3 x ULN or ≤ 5x ULN in case of liver metastases) and renal functions (Cockroft-Gault [24] creatinine clearance ≥ 50 ml/min; normal coagulation test (international normalized ratio ≤ 1.5 x ULN); left ventricular ejection fraction (LVEF) within the normal institutional range based on echocardiography or MUGA (multi gated acquisition scan); life expectancy of ≥ 12 weeks. Exclusion criteria were: any investigational drug in the previous four weeks; less than 14 days since last chemo-, immuno-, biologic- or hormonal therapy or less than 6 weeks since last mitomycin C chemotherapy; any ongoing potentially reversible toxicity from prior anti-cancer therapy that is > grade 1 or progressing in severity; pregnancy or lactating (all patients had to use adequate contraceptive protection); receiving any of the prohibited medications or consuming any of the prohibited fruits or fruit-juices within 7 days prior; clinically significant electrocardiogram (ECG) abnormality including baseline QTc prolongation; sensitivity to heparin or heparin-induced thrombocytopenia; uncontrolled leptomeningeal- or brain metastases; malabsorption syndrome or other disease affecting gastrointestinal function; resection of the colon, stomach or bowel; active hepatitis or biliary disease; hypersensitivity to drugs chemically related to the study drug; any other condition that would interfere with the patient's ability to comply with the dosing schedule and

protocol-specified evaluations. The study was conducted in accordance with the guidelines for good clinical practice (GCP) and was approved by local Medical Ethics committees.

Study design.

This was a three treatment, randomized, three sequence cross-over, Latin-square design, phase I study (EGF111582, NCT00821054 www.clinicaltrials.gov) [25]. The primary objective was to determine the effect of food on the steady-state pharmacokinetics of lapatinib, dosed with an hour interval between meal and drug administration. Secondary objectives were to determine safety and tolerability of lapatinib. Patients were recruited at the following hospitals: Centre Hospitalier de l'Université de Montréal-Hotel-Dieu de Montréal, Quebec, and Cross Cancer Institute, Edmonton, both in Canada; Roswell Park Cancer Institute, Buffalo, and Greenville Hospital system Institute for translational Oncology Research, Greenville, both in the United States of America (USA); The Netherlands Cancer Institute, Amsterdam, The Netherlands.

From day one, each patient was treated as an outpatient with lapatinib 1250 mg daily, to be taken in a fasted state (fasting for at least 8 hours prior to dosing and for at least one hour after dosing), except on the days that pharmacokinetic sampling was performed. These were: at the end of week 1 (between days 7 and 9), week 2 (7 days after week 1) and week 3 (7 days after week 2). On these days, patients stayed in the research hospitals and received a standardized breakfast, as described below and in accordance with regulatory guidelines [21;26].

The different treatments are summarized in Table 1. Patients were randomly assigned to one of three sequences (treatment order: ABC, BCA or CAB). The low-fat breakfast contained approximately 5% fat (500 calories) and the high-fat meal contained approximately 50% fat (800-1000 calories). Patients were not allowed to take fruits or juices known to inhibit CYP3A or P-gp. Patients were also not allowed to use tobacco, nicotine or alcohol during pharmacokinetic sampling.

Study procedures.

Written informed consent was obtained prior to study specific assessments. Standard physical examinations, ECG (including QT interval) and clinical laboratory tests were performed at regular intervals. Patients were asked to record daily drug intake in a diary. Patients remained on treatment until disease progression, unmanageable toxicity or withdrawal of consent. After completion of pharmacokinetic sampling in

week 3, patients were allowed to transition to a continuation study receiving lapatinib, alone or in combination with other chemotherapy (EGF111767).

Table 1: Study schedule for different treatments randomly assigned on day 7, 14 or 21 for every patient

Treatment	A	B	C
Prior to pharmacokinetic sampling	> 6 days lapatinib 1250 mg daily	6 days lapatinib 1250 mg daily	6 days lapatinib 1250 mg daily
Day of pharmacokinetic sampling	> 8 h fasting	> 8 h fasting	> 8 h fasting
	lapatinib 1250 mg + 240 ml water	Low-fat breakfast	High-fat breakfast
	1 h fasting	1 h fasting	1 h fasting
	Low-fat breakfast	lapatinib 1250 mg + 240 ml water	lapatinib 1250 mg + 240 ml water
	4 h fasting	4 h fasting	4 h fasting
	Standardized lunch	Standardized lunch	Standardized lunch

Note: Lunch was identical for all patients during all weeks. Abbreviations: h=hour

Dosing.

Oral lapatinib was provided as 5 tablets each containing 250 mg of lapatinib and were taken with 240 mL of water. Dose adjustments were not allowed in this study and patients that required a dose modification were withdrawn from the study.

Evaluability.

If a dose was missed or lost due to vomiting, the patient was considered unevaluable for pharmacokinetic analysis. If a patient was unable to consume ≥ 75% of calories within 30 minutes at each of the different breakfasts, the patient was also considered unevaluable for pharmacokinetic analysis. All patients included in the study were evaluable for safety evaluation.

Safety assessment.

Adverse events (AE), serious adverse events (SAE) and their relation to the study drug were assessed throughout the study. The incidence and severity of AEs were evaluated and coded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (NCI-CTCAE) version 3.0 [27].

Pharmacokinetic sampling and analysis.

Whole blood samples for the determination of lapatinib were taken at the visits at the end of week 1, 2 and 3 at pre-dose and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 24 hours following administration. At each collection time point 4 ml of whole blood was withdrawn into pre-chilled potassium ethylenediaminetetra-acetic acid (EDTA; Vacutainer™) tubes. After collection, blood and anti-coagulant were mixed by inverting the tube 8-10 times. Blood samples were immediately stored on ice and centrifuged at 1500g 4ºC for 10 to 15 minutes. Plasma was stored and frozen at -20ºC within 45 minutes of collection. Plasma concentrations of lapatinib were quantified using a validated high-performance liquid chromatography (HPLC) method [28].

Standard non-compartmental methods were used to calculate area under the serum concentration time curve up to 24 hours (AUC_{0-24}) and elimination half-life ($t_{1/2}$) were derived from plasma concentrations by non-compartmental analysis using the log-linear trapezoidal rule using WinNonLin (Pharsight Corporation, Mountain View, California, USA). Plasma peak concentration (C_{max}), time to C_{max} (t_{max}) and time to first quantifiable plasma concentration (t_{lag}) were derived directly from the plasma concentration-time data.

Statistical analysis.

An estimation approach was used to assess pharmacokinetic parameter differences between treatments using SAS. Following log_e-transformation, geometric last square means of AUC_{0-24} and C_{max} were analyzed using analysis of variance (ANOVA). The two test treatments were lapatinib dosing one hour after a low-fat meals or after a high-fat meal. The reference treatment was lapatinib dosing one hour prior to a low-fat breakfast. Point estimates and corresponding 90% confidence intervals (CI) were constructed for the following differences: ([after a high-fat meal]/ [before a low-fat meal]) and ([after a low-fat meal/before a low-fat meal]). The point estimates and 90%CI were back-transformed to provide point estimates and 90%CI for the ratios. In order to test for a food-drug interaction, AUC_{0-24} and C_{max} of lapatinib under the previously mentioned circumstances were also subjected to the Wilcoxon signed ranks test.

Inter-patient variability of AUC_{0-24} and C_{max} per treatment was expressed as coefficient of variation (CV%= SD/mean * 100%). Equality of variances between treatment groups (also: “sphericity”) were assessed using Mauchly’s sphericity test in one-way repeated measures ANOVA with the null-hypothesis that variances were equal. Analysis was performed using SPSS (version 17.0, Chicago, USA). All significance tests were two-sided and *P*-values < 0.05 were considered statistically significant.

RESULTS

Patient inclusion and demographics.

In total, 25 patients were included in the study and were evaluable for safety. Four (4/25, 16%) patients discontinued prior to completing sampling in week 3 and nine patients (9/25, 36%) were considered non-evaluable for pharmacokinetics due to the following reasons: four patients did not meet the consumption requirements for all three breakfasts within time (30 minutes); one patient missed a single dose in week 3; two patients could not account for drug compliance because of an incomplete diary; one patient missed two doses due to vomiting in week 1 and 2; one patient was enrolled in violation to the protocol (resection of the colon was prohibited). Therefore, 12 patients (12/25, 48%) were considered evaluable for pharmacokinetics.

Table 2: Patient demographics and characteristics of the population evaluable for pharmacokinetics and the total population

	Pharmacokinetic population	Safety population (=total)
Number of patients	12	25
Age (years)		
Median (range)	50.5 (34 - 72)	51 (34 – 73)
Weight (kg)		
Median (range)	68 (48 – 104)	70 (48 – 104)
Height (m)		
Median (range)	1.68 (1.55 – 1.87)	1.68 (1.50 – 1.87)
Gender (%)		
Female	10 (83)	21 (84)
Male	2 (17)	4 (16)
Race (%)		
Caucasian	11 (92)	22 (88)
African American	1 (8.3)	2 (8.0)
Asian	0	1 (4.0)
Prior treatment		
Chemotherapy	12 (100)	25 (100)
Biologic therapy	10 (83)	22 (88)
Radiotherapy	9 (75)	19 (76)
Hormonal therapy	2 (17)	6 (24)
Primary tumor, n (%)		
Breast	7 (58)	17 (68)
Gastric	2 (17)	3 (12)
Esophageal	1 (8.3)	1 (4.0)
NSCLC	1 (8.3)	1 (4.0)
Mesothelioma	1 (8.3)	1 (4.0)
Colorectal	0	1 (4.0)
Other, coecum	0	1 (4.0)
ECOG at baseline, n (%)		
0	7 (58)	11 (44)
1	4 (16)	10 (40)
2	1 (8.3)	4 (16)

Abbreviations: ECOG= Eastern Cooperative Oncology Group (ECOG)[23]; NSCLC = non-small cell lung cancer.

Patient demographics and characteristics are shown in Table 2. The median (range) age was 51 (34-73) years and median weight (range) was 70 (48 – 104) kg. The majority was female (21/25, 84%) and Caucasian (22/25, 88%). The most frequently observed primary tumor types were breast cancer (17/25, 68%) and gastric cancer (3/25, 12%). All patients had received prior therapy for their disease that consisted of chemotherapy (25/25, 100%), biologic therapy (22/25, 88%), radiotherapy (19/25, 76%) or hormonal therapy (6/25, 24%).

Pharmacokinetic analysis.

Mean (standard deviation, SD) plasma concentration-time curves (N=12) following administration of a dose of 1250 mg lapatinib one hour before a low-fat meal [A], one hour after a low-fat meal [B] and one hour after a high-fat meal [C] are presented in Figure 1. The curves show a substantial increase in lapatinib exposure when administered one hour after a breakfast. The largest effect is seen after the high-fat meal [C]. As pharmacokinetic sampling was performed at steady state (after at least 7 consecutive days of lapatinib administration), plasma concentrations of lapatinib are detectable at baseline and up to 24 hours of sampling.

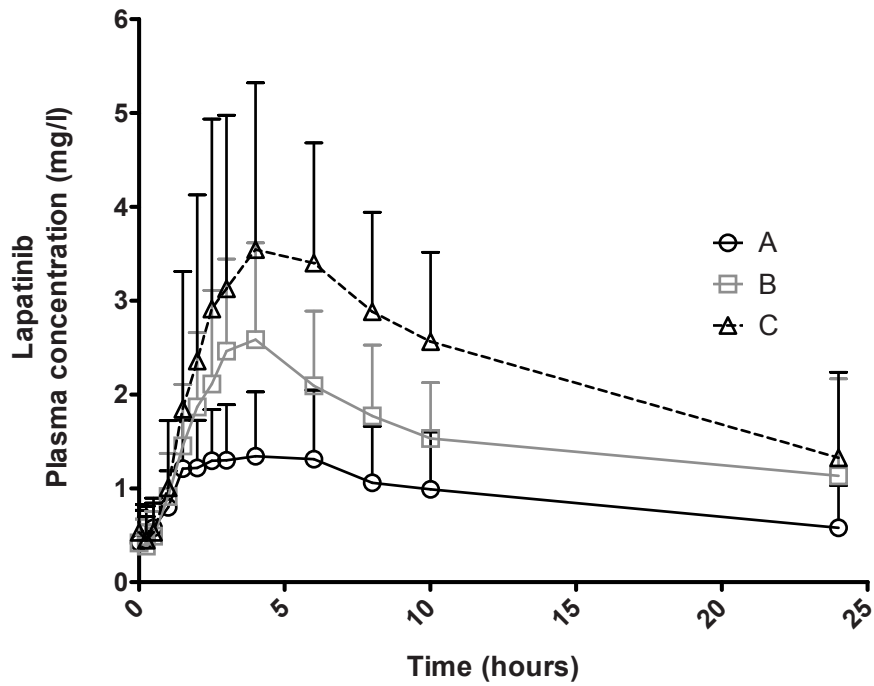

Figure 1: Mean (SD) plasma concentration (mg/l)-time curves of lapatinib following administration of a dose of 1250 mg one hour before a low-fat meal [A, black circles], one hour after a low-fat meal [B, grey squares] and one hour after a high-fat meal [C, black triangles].

Pharmacokinetic data are summarized in Table 3. Relevant increases were observed in lapatinib bioavailability when taken one hour after food, compared to dosing one hour before food. Effects were larger for the high-fat meal than for the low-fat meal. Mean (SD) AUC_{0-24} for lapatinib was increased from 21.7 (11.7) mg*h/ml when administered before a low-fat breakfast, to 36.5 (17.1) mg*h/ml and 53.0 (22.7) mg*h/ml, after a low-fat and high-fat breakfast, respectively. Similar increases were seen for mean (SD) C_{max}, being 1.50 (0.68) mg/l, 2.70 (1.02) mg/l and 3.96 (1.77) mg/l, when lapatinib was taken before or after a low-fat meal or after a high-fat meal, respectively. Also increases were observed in t_{max} and t_{lag} , with the largest increase after a high-fat meal (median (range) t_{max} = 5.0 (2.5-10.0) and t_{lag} = 0.5 (0.3-2.6) hours after a high-fat meal, compared to median (range) t_{max} = 3.0 (1.5-8.0) and t_{lag} = 0.0 (0.0-0.5) hours before a low-fat meal).

Table 3: Pharmacokinetic parameters of lapatinib (n=12) in each prandial state

Parameter	A Before low-fat meal	CV%	B After low-fat meal	CV%	C After high-fat meal	CV%
	Mean (SD)		Mean (SD)		Mean (SD)	
AUC_{0-24} (mg*h/l)	21.7 (11.7)	54	36.5 (17.1)	47	53.0 (22.7)	43
C_{max} (mg/l)	1.50 (0.68)	45	2.70 (1.02)	38	3.96 (1.77)	45
	Median (range)		Median (range)		Median (range)	
t_{max} (hours)	3.0 (1.5-8.0)		3.9 (3.0-6.0)		5.0 (2.5-10.0)	
t_{lag} (hours)	0.0 (0.0-0.5)		0.3 (0.0-1.0)		0.5 (0.3-2.6)	

Abbreviations: AUC_{0-24} = Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximum observed plasma concentration; CV%= coefficient of variation; t_{lag} = absorption lag-time; t_{max} = time to maximum observed plasma concentration.

Ratios of least square means (90% CI) for AUC_{0-24} and C_{max} after low-fat [B] and high-fat breakfast [C] compared to the reference group (before a low-fat meal, [A]) were subjected to ANOVA and are summarized in Table 4. Both low-fat and high-fat food affected lapatinib exposure considerably. Lapatinib AUC_{0-24} after a low-fat meal was 1.80-fold (90%CI: 1.37 – 2.37; *P* = 0.005) higher and after a high-fat meal 2.61-fold (90%CI: 1.98 – 3.43; *P* = 0.002) higher compared to before a low-fat meal. Lapatinib C_{max} after a low-fat meal was 1.90-fold (90%CI: 1.49 – 2.43; *P* = 0.004) higher and after a high-fat meal 2.66-fold (90%CI: 2.08 – 3.41; *P* = 0.002) higher compared to before a low-fat meal.

Table 4: Comparison of the log_e-transformed pharmacokinetic parameters of the prandial states (lapatinib dosed after low-fat [B] and after high-fat [C] breakfast) compared to lapatinib dosing before a low-fat breakfast

Parameter	Comparison	Least Square Means [Fed : Fasted [A])		Ratio of means	90% confidence interval	*P* value
AUC_{0-24} (mg.h/l)						
	B : A	34.8	19.3	1.80	(1.37 – 2.37)	**0.005**
	C : A	50.4	19.3	2.61	(1.98 – 3.43)	**0.002**
C_{max} (mg/l)						
	B : A	2.67	1.41	1.90	(1.49 – 2.43)	**0.004**
	C : A	3.75	1.41	2.66	(2.08 – 3.41)	**0.002**

Abbreviations: AUC_{0-24} = Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximum observed plasma concentration.

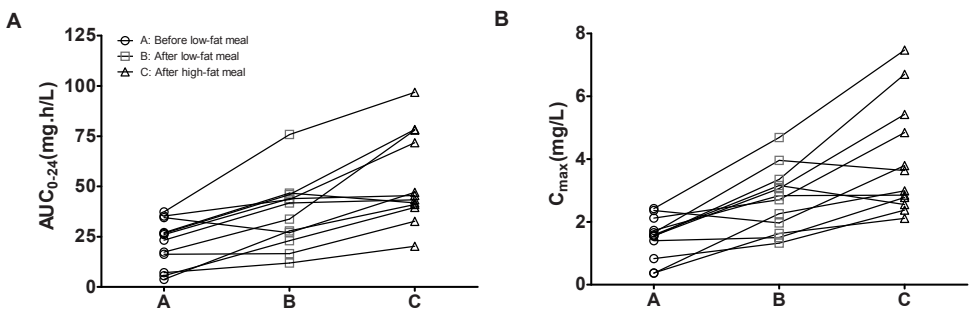

Figure 2: Inter-individual change in lapatinib AUC_{0-24} (mg*h/l) and C_{max} (mg/l) following administration of a dose of 1250 mg one hour before a low-fat meal [A], one hour after a low-fat meal [B] and one hour after a high-fat meal [C]. Abbreviations: AUC_{0-24} = Area under the concentration-time curve from zero (pre-dose) to 24 hours; C_{max} = maximum observed plasma concentration.

Inter-individual changes in lapatinib AUC_{0-24} and C_{max} are shown in Figure 2. The CV% for AUC_{0-24} (Table 3) was 54% before a low-fat meal [A], 47% after a low-fat meal [B] and 43% after the high-fat meal [C]. For $C_{max,}$ CV% (Table 3) was 45% for both before the low-fat meal [A] and after the high-fat meal [C] and was 38% after a low-fat meal [B]. There was no statistically significant difference between the variances of AUC_{0-24} and C_{max} (Mauchly's test, $P = 0.26$ and $P = 0.156$, respectively).

Safety.

All 25 patients were evaluable for safety. No unexpected toxicity was observed. All treatment-related toxicities observed during the study, which spanned three weeks of lapatinib monotherapy (1250 mg daily) are summarized in Table 5. Overall, diarrhea

was the most commonly occurring treatment-related toxicity (8/25, 32% grade 1 and 2/25, 8% grade 2), followed by nausea (8/25, 32% grade 1 and 1/25, 4% grade 2). Other drug-related AEs were vomiting, rash, stomatitis and fatigue. There was one patient that experienced a CTCAE ≥ grade 3 event considered related to the study drug which was fatigue grade 3 (1/25, 4%). No SAEs related to the study drug or deaths occurred during the study.

Table 5: All treatment-related toxicity observed during the study in more than 1 patient of the safety population (N=25)

	CTCAE grade (N; %)					
Toxicity	**1**	**2**	**3**	**4**	**5**	**Total**
Diarrhea	8 (32)	2 (8)	0	0	0	10 (40)
Nausea	8 (32)	1 (4)	0	0	0	9 (36)
Vomiting	3 (12)	3 (12)	0	0	0	6 (24)
Rash	5 (20)	1 (4)	0	0	0	6 (24)
Fatigue	3 (12)	1 (4)	1 (4)	0	0	5 (20)
Stomatitis	2 (8)	0	0	0	0	2 (8)

Abbreviations: CTCAE= common terminology criteria for adverse events.

DISCUSSION

Oral anti-cancer therapy can provide convenience to the patient by bypassing intravenous administration and by enabling therapy at home in an out-patient setting. From a pharmacological perspective, oral anti- cancer therapy can facilitate daily dosing resulting in prolonged and continuous exposure to a drug. Moreover, oral anti-cancer therapy potentially reduces costs of anti-cancer therapy by reducing the need for hospital admissions for the administration of therapy [29]. However, there are important pharmacokinetic properties of oral anti-cancer therapy that should be addressed in order to successfully develop a drug for oral dosing. Firstly, sufficient bioavailability of the pharmacologically active drug should be reached. Secondly, inter- and intra-patient variability should be limited because anti-cancer drugs frequently have a narrow therapeutic window. Lastly, the potential for drug-drug or food-drug interactions should be evaluated and any disadvantageous interactions should be avoided. The present study was undertaken in patients taking lapatinib once daily

for seven consecutive days to evaluate the pharmacokinetic effect of consumption of low-fat and high-fat meals on the pharmacokinetics of lapatinib, when meals were consumed one hour before a dose of 1250 mg lapatinib, compared to consuming a low-fat meal an hour after dosing.

Food can cause clinically relevant interactions with drugs. The interaction occurs mainly at the level of drug absorption in the intestine and in its metabolism [30]. In general, food slows gastric emptying, raises the pH of the proximal small bowel, increases hepatic blood flow and prolongs the gastrointestinal transit time. High-fat meals in particular can lead to increased bile output, leading to increased micelle solubility, and can further delay gastric emptying. Fatty acids present in the intestine are thought to transiently increase the permeability of epithelial membrane [30], thereby leading to increased absorption.

It had already been concluded earlier that lapatinib has a clinically relevant interaction with food [8;20;21]. This interaction has been attributed to enhanced permeability of the drug into enterocytes as a result of bile salt solubilisation. Next to this, it was postulated that increased substrate availability could lead to saturation of efflux transporters such as P-gp and BCRP and metabolizing enzymes such as CYP3A4 in the intestinal wall, resulting in a reduced first-pass effect and increased drug exposure.

The results of the present study show that exposure to lapatinib was increased when dosed an hour after a low-fat or a high-fat meal compared to dosing one hour before a low-fat meal. The effect was most pronounced when dosed an hour after a high-fat meal. A 1.80-fold and 2.61-fold difference for $AUC_{0-\infty}$ and a 1.90-fold and 2.66-fold difference for C_{max} for the low-fat and the high-fat meal, respectively, were observed. It can be concluded from the present study that an hour interval after consuming a meal until drug dosing did not abolish the food-drug interaction. Therefore, in order to administer lapatinib in a fasted state, it is advised to only administer the drug one hour before a meal and not one hour after a meal. Currently, either option is recommended [22], but following the results of the present study, the current advice should be amended accordingly.

Following consumption of meals one hour before dosing, and especially the high-fat meal, t_{lag} and t_{max} of lapatinib were increased. These results indicated that intestinal absorption of lapatinib was delayed and increased by food, which can be attributed to delayed gastric emptying by fat.

Although a direct comparison cannot be made, the increases in AUC and C_{max} observed in the present study appeared to be less pronounced than those reported

by Koch et al. [21]. In the present study, there was a delay of one hour between consumption and dosing and exposure to lapatinib was lower than with concomitant consumption, as reported by Koch et al. This observation suggests that the extent of lapatinib absorption is mostly influenced by actual presence of fat and solubilised fat particles together with the drug in the intestinal lumen as a result of concomitant consumption of a meal.

Another difference between the study reported by Koch et al. and the study reported here was that the present study investigated lapatinib pharmacokinetics at steady state in a chronic dosing schedule (after seven consecutive days) compared to single-dose by Koch et al. The effect of chronic dosing on lapatinib exposure is unclear. From one perspective, since lapatinib was found to also be an inhibitor of CYP3A4, P-gp and BCRP *in vitro* [17], a chronic dosing schedule theoretically could lead to increased absorption and exposure by a reduced first-pass effect. Such relatively high exposure could potentially lead to a reduction of the effect of food. From another perspective, chronic dosing may have a direct toxic effect on enterocytes resulting in local mucositis. In turn, such could lead to a reduced mucosal surface area and reduced absorption of the drug.

Inter-patient variability (CV%) for AUC was lowest in the high-fat meal treatment (43%). CV% in previous studies also had lowest CV% in the fed states (43% and 52%) and highest in the fasted states (59% and 68%) [8;21]. Such inverse relationship between bioavailability of a drug and CV (the lower bioavailability, the higher CV) is well known and has been described for other oral drugs [31;32]. Although variances were not significantly different across different treatments in this study with a limited number of patients, this observation could have additional clinical relevance. Especially in oral anti-cancer drugs, there is a narrow therapeutic window and inter-patient variability should be minimized as much as possible. Dosing of anti-cancer drugs should be aimed at achieving exposures within the therapeutic dosing range that are not too high (leading to increased toxicity) or too low (leading to reduced efficacy). As food increases lapatinib bioavailability, administration of lapatinib with food could provide a means for reducing inter-patient variability. Others have speculated that the administration of lapatinib with food could reduce the number of pills that have to be taken (five in the present study), lower costs and reduce in the occurrence of diarrhea as side effect [33]. In clinical practice though, it can be difficult to consequently consume standardized meals, especially when the drug itself can cause nausea and vomiting as side effects. In turn, variability in actual fat intake could increase variability in exposure. It is currently advised to administer lapatinib

fasted [22] and further development, including two phase III trials [14;15], was executed with this recommendation. The risk of introducing day-to-day intra-patient variability, caused by an inconsequent diet, is thought to outweigh the benefit of reaching higher exposures. However, if bioavailability could be increased in a more reliable manner, and consequently variability reduced, this could serve as an alternative for food. One way to achieve this could be by advising patients to only administer lapatinib one hour before a meal, and not one hour after a meal.

In conclusion, a clinically relevant interaction of high-fat and low-fat meals consumed with an interval of an hour before lapatinib administration compared to dosing one hour before a low-fat meal, was observed. In order to administer lapatinib in a fasted state, it is advised to administer the drug one hour before a meal and not one hour after a meal. This food-drug interaction warrants further investigation for treatment optimization.

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Chapter 5

Circulating tumor cells

Chapter 5.1

Circulating tumor cells as pharmacodynamic biomarker in early clinical oncological trials

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ABSTRACT

Circulating tumor cells (CTCs) have received a lot of attention from both researchers and clinicians because of their prognostic value for progression-free and overall survival in selected tumor types. CTCs are readily available by single venipuncture, thereby posing little burden on the patient and allowing for repeated, sequential sampling during therapy. Nowadays, the sensitivity of several CTC detection and capture techniques allow for further characterization and analysis of specific targets of interest on the CTC itself. These techniques have given CTCs the potential to be used as a pharmacodynamic read-out in drug development. In this review, we explore the utility of CTCs as a pharmacodynamic biomarker in early clinical oncological trials. We present an overview of current literature on assays for CTCs as pharmacodynamic biomarker, their different targets of interest and their level of validation, followed by discussion of their limitations.

INTRODUCTION

Today anti-cancer therapies are increasingly focusing on specific molecular targets underlying malignant growth. This “targeted therapy” aims to interfere at the molecular level of the malignant process by blocking a specific protein or group of proteins, such as a growth factor receptors, signal transduction pathways, DNA repair mechanisms or the cell cycle. This is in contrast to conventional chemotherapy, where the patient is exposed to cytotoxic drugs that are usually based upon histology and tumor type regardless of any molecular characteristics. This paradigm shift from classical to targeted therapy has substantial consequences for the design of early clinical trials, in terms of efficacy and toxicity evaluation and the use of biomarkers [1-6].

In drug development of “targeted therapy”, the proportional development of suitable biological markers will become increasingly important [5]. Ideally, in the lengthy and costly development of a targeting drug, biomarkers could serve to early predict success or failure of drug development [7].

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention” [8]. In cancer, several types of biomarkers are of importance: prognostic, predictive and pharmacodynamic biomarkers. Prognostic biomarkers assess the natural course of the disease and predictive biomarkers assess the probability that a patient will benefit from a specific treatment. In early clinical trials aimed at pharmacokinetics and safety, the pharmacodynamic biomarker is the most useful one, potentially providing a crucial link between pharmacokinetics and response markers [7].

Commonly the following definition of a pharmacodynamic biomarker is used: a biomarker “that provides evidence that there is a direct pharmacological effect of a drug” [9]. Information from a pharmacodynamic biomarker can be combined with pharmacokinetics, and can include a molecular, cellular, histopathologic and imaging parameter [7]. The biological effects that are used as pharmacodynamic endpoints are often measures of altered activity or expression of a molecular target in response to therapy [6]. Potential applications of pharmacodynamic biomarkers are: (i) to provide proof of mechanism of action of a drug, (ii) to select optimal dose and schedule of administration of the drug, when combined with pharmacokinetics and toxicity,(iii) to increase understanding of response and resistance mechanisms and (iv) to design rational combination therapies. They may also help in predicting outcome [6].

In targeted therapy, frequently the biological action is cytostatic rather than cytotoxic

and tumor size may not be altered although there is a biological effect. Therefore, in targeted therapy, tumor size may sometimes be unsuitable as a biomarker for the evaluation of anti-tumor activity and a pharmacodynamic biomarker could serve as an alternative [1]. Monitoring a pharmacodynamic biomarker may also help in evaluating toxicity when establishing the right dose for further development in subsequent phase II trials. Targeted therapy usually has a different toxicity profile than cytotoxic chemotherapy. Also, the maximum tolerated dose (MTD) may be higher than the dose that gives maximal biologic inhibition [6]. A pharmacodynamic biomarker may therefore help selecting the optimal biological dose for phase II studies.

In order to study the pharmacological effect of the drug on cancer tissue, there is a need to obtain actual tumor tissue, or a suitable surrogate, that is available on repeated occasions. For patients with solid tumors this is a heavy burden because biopsies often have to be obtained with an invasive technique. Therefore, there is a need for a suitable surrogate tumor tissue that is easily accessible and preferably non-invasive. Currently, plucked hair follicles [10,11], skin biopsies [12], buccal mucosa scrapings [13] are often being used. Also venipuncture is used, not only to obtain peripheral blood mononuclear cells (PBMCs), but also to collect other circulating serum biomarkers: free nucleic acids, proteomics and circulating tumor cells (CTCs) [5,7].

Of all surrogate tumor tissues, CTCs have probably received the greatest attention the last years [14-16]. It is becoming increasingly clear that the number, and the change in number, of CTCs is prognostic for progression-free and overall survival in several types of cancer, including breast, colorectal and prostate cancer [17-20]. Now that CTC detection techniques have significantly improved, a feasible, new goal is to characterize CTCs and to study specific molecular targets on CTCs [15,16,21]. In this review, we aim to explore the utility of CTCs as a pharmacodynamic biomarker in early clinical oncological trials. We will review the current status of development of assays for CTCs as pharmacodynamic biomarkers, their targets and their level of validation, followed by discussion of their limitations.

METHODS

Study design

A systematic review was conducted to find literature reporting on the development and implementation of CTCs as pharmacodynamic marker in early oncology trials. The search was focused on reports on assays measuring a pharmacological effect

of a targeting drug on a target in CTCs. Some studies however reported only on identification of a specific target and not on a pharmacological intervention at the target. These studies were included only when the target was considered relevant in current targeted therapy development. Reports on CTCs as a prognostic or predictive marker, based on changing CTC numbers, were beyond the scope of this review and were therefore not included.

Search strategy

To identify all studies on the development of assays on targets on CTCs and the utility of CTCs as pharmacodynamic biomarkers in early clinical oncological trials, we performed a search in PubMed, NHSEED and MEDLINE, using the following search string ("circulating" [All Fields] AND ("tumor" [All Fields] OR "neoplasms" [MeSH Terms] OR "neoplasms" [All Fields] OR "tumor" [All Fields] OR "epithelial" [All Fields]) AND ("cells" [MeSH Terms] OR "cells" [All Fields])). Search strategy was then further specialized by adding AND ("pharmacodynamic" [All Fields] OR ("biological markers" [MeSH Terms] OR ("biological" [All Fields] AND "markers" [All Fields]) OR "biological markers" [All Fields] OR "biomarker" [All Fields])) and limiting the query to "Clinical trial", "Phase I" or "Phase II". References of retrieved publications and of relevant overview publications were checked to identify additional studies. A cut-off date of 1 December 2010 was used.

Data extraction

Of each study, information on target, targeting drug, research population, technique, level of validation, sensitivity and intended clinical application were retrieved. For each report, the level of validation was assessed according to US Food and Drug Administration (FDA), the National Cancer Institute (NCI) and European Medicines Agency (EMA) guidelines [9,22,23].

RESULTS

Assays for CTCs as pharmacodynamic biomarker

Twenty-five literature reports were identified. An overview of the literature found on assays for CTCs applied as pharmacodynamic biomarker is shown in Table 1. The different types of pharmacodynamic targets on CTCs that were explored consisted of membrane-bound growth factor receptors, a nuclear receptor, intracellular proteins

as part of a signal transduction cascade, mRNA transcripts and DNA mutational analysis. Additional targets included markers for double-strand DNA break damage, apoptosis, mitotic arrest and tumor aggressiveness. CTCs in patients with early breast cancer and the following types of metastatic cancer were studied: breast-, colorectal-, castration-resistant prostate and non-small cell lung cancer (NSCLC) and “a phase I population”.

In 5 out of 25 studies (20%), actual pharmacodynamic interaction at the target level on the CTCs was studied during treatment with the targeting agent [24-28]. These targeting drugs were 4 antibodies directed at a growth hormone receptor and one tyrosine kinase inhibitor. Next to this, pharmacodynamic markers for DNA damage and apoptosis in CTCs were reported in response to chemotherapy [29,30].

Techniques for CTC detection

CTC enumeration and identification techniques and the clinical applicability of CTCs as a prognostic marker were recently reviewed [15,16,31]. In brief, enrichment techniques are frequently based on a separation of rare CTCs from peripheral blood cells by density, size or enrichment by specific protein expression. Usually an enrichment of epithelial cell adhesion molecule (EpCAM; CD326)-positive cells is used. Enrichment is followed by identification of the CTCs by either immunocytochemistry or by reverse-transcriptase polymerase chain reaction (RT-PCR) of different epithelial antigens for different tumors, for example cytokeratins (CK), mammaglobin for breast cancer and carcinoembryonic antigen (CEA) for colorectal cancer. One example is the AdnaTest® BreastCancer test (AdnaGen AG, Langenhagen, Germany) which is based on immunomagnetic cell selection followed by multiplex RT-PCR of tumor associated transcripts [32]. The only assay that has been approved by the FDA is the CellSearch® Circulating Tumor Cell assay (Veridex, Warren, New Jersey, USA). Immunomagnetic enrichment with ferrofluids conjugated to antibodies directed at EpCAM is used in this semi-automated assay. The next step is automated fluorescence detection using specific fluorescent labels and final assessment is performed by an operator based on an image gallery [33,34]. Next to these techniques, there are new developments in the detection of CTCs without an enrichment step that generally have a higher recovery rate, using microfluidic platforms, such as the “CTC-chip”, with an array of microposts coated with anti-EpCAM [24,35-37], OncoCEE Enumeration™ (Biocept Inc., San Diego, USA) and On-Q-ity™ (On-Q-ity, Boston, USA) [38], filter-based microfluidic device [39,40], scanning microscopy [41-44] and fiber-optic array scanning techniques [45].

The techniques that were used to study targets on CTCs were additional

immunofluorescence- [26,29,30,32,46,47,47-52] and nuclear acid [24,28,32,53,54] evaluation techniques, fluorescent *in situ* hybridization (FISH) [25,32,38,41,47,47,51,52,55], high-performance liquid chromatography (HPLC) [56], Papanicolau staining [47], confocal laser scanning microscopy [41,42] and flow cytometry [27,28].

Targets on CTCs

EGFR expression and phosphorylation of associated signaling pathway proteins

The epidermal growth factor receptor (EGFR) is currently a target in treatment of metastatic colorectal, pancreatic, squamous type head-and-neck cancer and locally advanced and metastatic NSCLC. Drugs that interfere with the EGFR and its signaling cascade include monoclonal antibodies cetuximab (Erbitux®) and panitumumab (Vectibix®) and oral tyrosine kinase inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®). Their anti-tumor activity may depend on activating- and resistance conferring mutations in the *EGFR* gene, or mutations down-stream of the signaling cascade, such as *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), and possibly *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) and *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide) [57].

Several studies focused on detecting EGFR on CTCs after their identification by the CellSearch® assay. Weak but consistent EGFR expression was observed in repeated sampling over time on CTCs in 44 out of 51 (86%) CTC-positive samples obtained from 33 metastatic breast cancer patients [46]. In CTC-positive metastatic prostate cancer patients, a variety in EGFR expression was found between patients in 18 out of 20 (90%) samples [47]. Heterogeneity in EGFR expression was also observed in NSCLC patients as part of an amenability study for biomarker analysis using CTCs [38].

Actual *EGFR* gene mutational analysis was performed in 20 CTC-positive patients with metastatic NSCLC. The CTC-chip was used in combination with an allele-specific PCR amplification technique and a *EGFR* mutation was found in 19 (95%) [24]. Results were also compared to mutational analysis of the original tumor-biopsy specimens. The expected *EGFR* activating mutations were identified in CTCs from 11 out of 12 (92%) patients. Four patients were serially tested while exposed to gefitinib. This study demonstrated that the genotypes of circulating tumor cells evolved during treatment, for example with emergence of the resistance-conferring mutation T790M.

In colorectal cancer patients, EGFR expression in CTCs was observed in two studies using immunomagnetic enrichment techniques in combination with multigene RT-PCR techniques. In the first study, feasibility of CTC enrichment and subsequent

RNA extraction was shown. In 4 out of 19 (21%) patients that had ≥ 2 CTCs/7.5 ml peripheral blood, EGFR expression was found. Also flow cytometry was used to study EGFR and CK protein expression on CTCs, which was found in 8 out of 49 (16%) of sampled patients. Sequential sampling of one patient during treatment with panitumumab showed a decrease in the number of EGFR-positive CTCs [28]. The aim of the second study was to compare EGFR-expression of primary tumor, related metastases and CTCs. Twenty patients were studied and EGFR expression was observed in 2 out of 11 (18%) CTC-positive patients. One patient with EGFR-positive CTCs also had EGFR expression in its metastatic tissue, as determined by immunohistochemistry, but the primary tumor tissue was not available for comparison. The other patient with EGFR-positive CTCs had no EGFR expression in either primary tumor or metastases. Of the 9 patients that had EGFR-negative CTCs, six also had EGFR-positive metastases and all the primary tumor tissues that were available in this group (three) were also EGFR-positive. Additional PCRs were used for expression profiling of EGFR variants [53].

Also, signaling proteins downstream of EGFR have been studied in CTCs of early and metastatic breast cancer patients using peripheral blood mononuclear cell (PBMC) cytospins and confocal laser scanning microscopy. EGFR and phospho-EGFR expression were observed in CTCs of 7 out of 16 (44%) and 6 out of 7 (86%) metastatic breast cancer patients, respectively, that were pre-selected for having CK-19 mRNA-positive peripheral blood cells. Subsequent double staining experiments following immunomagnetic enrichment showed co-expression of down-stream molecules phospho-PI3K (phosphoinositide-3 kinase) and phospho-Akt (v-akt murine thymoma viral oncogene homolog 1), both in 13 out of 16 (88%) patients. This indicated activation of the EGFR-pathway in CTCs [42].

HER2

Overexpression of the HER2 (ErbB2, *neu*) receptor is a target in early and advanced breast- and gastric cancer. Drugs that target this receptor include the monoclonal antibody trastuzumab (Herceptin®) and the oral tyrosine kinase inhibitor lapatinib (Tyverb/Tykerb®). Treatment with these drugs is based on the prognostic significance of the expression level of HER2 and/or *HER2* gene amplification in the tumor tissue. CTCs and their HER2 expression have been studied using a variety of techniques in several studies. The sensitivity for detecting CTC numbers in blood samples of advanced breast cancer patients was approximately 30-60%[25,27,32,48,50,54,55]. It has become clear that while HER2 overexpression on CTCs can be demonstrated,

the primary tumor tissue may be HER2-negative [25,27,32,48-50,54,55]. This might have consequences for treatment of patients that were originally considered HER2-negative, based on analysis of the primary tumor, but show HER2 overexpression on CTCs.

The first studies on HER2-status on CTCs used immunophenotyping and FISH to show feasibility of quantifying HER2 on CTCs. In 2002, serial peripheral blood samples of 19 metastatic breast cancer patients were studied and HER2 expression was observed in 7 out of 10 (70%) women that had circulating epithelial cells. Expression levels of HER2 were calibrated using HER2-expressing cell-lines. During the course of different treatments, including one with trastuzumab, varying levels of expression and conversion from HER2-negative to positive were found [27]. Next, concordance was studied in *HER2*-gene amplification status between primary tissue, as determined by a pathologist, and CTCs in 42 CTC-positive breast cancer patients using FISH. HER2-positivity was defined as *HER2* copy number of ≥ 4. In 29 patients (69%) the *HER2*-gene status was concordant, showing both primary tumor tissue and CTCs either HER2-positive (11 patients) or HER2-negative (18 patients). In addition to this, in 9 patients (21%), the primary tumor was HER2-negative, but the CTCs had acquired *HER2* gene amplification. This resulted in treatment with trastuzumab in four patients, giving benefit to three [25]. Nucleic acid and FISH CTC studies were combined with serum HER2 measurements to study concordance in HER2 status at diagnosis and in metastatic disease. In total, 67 metastatic breast cancer patients were studied of whom 21 had CTCs and 8 (38%) were found *HER2*-amplified. Non-concordance regarding HER2-status was found in 6 out of 21 (29%) [32], and this was also found by others, using a cut-off for HER2-positivity when ≥ 50% of CTCs were HER2-positive by immunofluorescence, in 32% of cases [50]. In prospective clinical trial that incorporated three chemotherapy arms and trastuzumab treatment for HER2-positive early breast cancer patients, GeparQuattro, CTCs were also detected and characterized for HER2 expression before and after neo-adjuvant therapy using CellSearch®. Validation experiments of the immunocytochemical method for HER2 determination on CTCs were performed using breast cancer cell lines with known *HER2* gene amplification status. By comparing HER2 staining intensities of CTCs to cell lines, intensities of HER2-specific immunofluorescence could be categorized into negative (0), weak (1+), moderate (2+), and strong (3+). Cases were categorized HER2-overexpressing if at least one CTC showed strong (3+) HER2 immunofluorescence. HER2-overexpressing CTCs were observed in 14 (24%) of 58 CTC-positive patients, including 8 (14%) patients with a HER2-negative

primary tumor and 3 (5%) after trastuzumab therapy [49]. Recently, CellSearch® and AdnaTest® BreastCancer test were prospectively compared for detection of HER2 expression in metastatic breast cancer patients in a prospective, multicenter study. CTCs were considered HER2-positive when either 3+ staining was observed in the CellSearch® method [49] or when a PCR fragment of the *HER2* transcript could be detected (peak concentration of > 15 ng/µl) in the AdnaTest® BreastCancer test. Both methods were performed in 221 cases. Only 62 (28%) patients were CTC-positive in both tests and 13 (21%) had HER2-positive CTCs in both assays. Concordance in HER2 status was observed in only 31 (50%) of these patients [48]. Sensitivity of the CellSearch® system was improved using a different CTC identification method (CellSearch® Profile kit) combined with FISH [55]. In breast cancer patients with HER2-negative primary and metastatic tissue, *HER2*-amplified CTCs (defined as: *HER2* copy number ≥ 4 [25]) were observed in 10 out of 30 (33%). Also, because of a relatively high yield, further evaluation of expression of EGFR, phospho-EGFR, HER2 and phospho-HER2 was done in breast and lung cancer patients [55]. In a feasibility study that was also comparing CellSearch® and OncoCEE™, *HER2* amplification (also: copy number ≥ 4) was studied in metastatic breast cancer patients. CTC-positivity was found in 1/13 (8%) patients for the CellSearch® method and in 1/12 (8%) patients for the CTC-chip technology. Using CellSearch®, HER2 expression on CTCs was found concordantly in 12/29 patients (41%) whose primary tumor was considered HER2-positive. Also, HER2-positivity was found in CTCs of 3/18 (17%) of patients whose primary tumor was considered negative [38].

For *HER2* transcript quantity analysis after identification with the CellSearch® system, also nucleic acid techniques [54] and confocal laser scanning microscopy [41] have been applied. The first study also showed a discrepancy of HER2-positive CTCs in 29% of patients that had a HER2-negative primary tumor [54]. The second study showed concordance in all 10 patients [41].

No *HER2* amplification was observed by FISH in CTCs of 9 metastatic prostate cancer patients with unknown *HER2* status [47].In addition to HER2, feasibility of detecting amplification of another marker that is associated with increased tumor aggressiveness, the urokinase plasminogen activator receptor (*uPAR*) gene, was demonstrated in CTCs of breast cancer patients [51].

IGF-IR

The insulin-like growth factor type I receptor (IGF-IR) is a tyrosine kinase that plays a role in cell differentiation and proliferation [58]. Alterations in expression of its signaling

pathway are involved in the development of different types of malignancies [59].
In phase I trials with the IGF-IR inhibiting antibody CP-751,871 (figitumumab), expression of IGF-IR on CTCs was investigated using the CellSearch® system. This way, presence of the IGF-IR on CTCs could be assessed and followed during treatment with CP-751,871, either alone in dose-escalation or in combination with cytotoxic chemotherapy. Before treatment, in 26 (33%) out of 80 patients CTCs were found, and IGF-IR-positive CTCs could be demonstrated in 23 (29%) of these patients. Treatment reduced the number of CTCs, including the IGF-IR-positive ones [26].

Androgen receptor

Progression of castration-resistant prostate cancer (CRPC) is believed to be driven by activation of the androgen receptor (AR), caused by incomplete blockade of the receptor or acquired mutations in the receptor and/or associated signaling pathways. Strategies to target this receptor include direct inhibition of the receptor itself, for example by MDV3100, and blockade of endogenous androgen synthesis by the CYP17A1 inhibitor abiraterone acetate [60].
Molecular profiling of this target in CTCs of metastatic prostate cancer patients was reported in several studies. In a first study, FISH was applied to analyze *AR* gene copy numbers. *AR* gene amplification was shown in 5 out of 9 (56%) patients, in which the CellSearch® test had identified ≥ 5 CTCs/7.5 ml of peripheral blood. Results of Papanicolau staining were reported in 24 CRPC patients with ≥ 2 CTCs/7.5 ml peripheral blood, in which 21/24 (88%) were found positive [47]. *AR* amplification was also found in 17 out of 49 (35%) analyzes performed in patients with more than 10 CTCs. In addition to this, relative gain in the oncogene *MYC* was found in 24 out of 43 (56%) samples analyzed [61]. CTCs were also characterized for *ERG*, *AR* and *PTEN* gene loci in 51 CTC-positive patients, while on treatment with abiraterone acetate in phase 1 and 2 trials. All 33 (100%) CTC-positive patients were found to have CTCs with at least > 1 *AR* copy number. It was not reported which patients were considered to have *AR* amplified CTCs. *ERG* was found to be rearranged in 23 out of 49 (47%) patients and these were all found to match the gene status in archival tumor tissue from the same patient. Thirteen out of 49 (27%) had *PTEN* (phosphatase and tensin homolog) loss. *AR* gene copy number was also assessed but found to be heterogeneous between CTCs [52].
Recently, actual mutational analysis of the *AR* gene was performed in CTCs of 35 patients with CRPC. *AR* gene exons were amplified by PCR, digested with endonucleases, fractionated and analyzed using specific denaturing high-

performance liquid chromatography (DHPLC) followed by sequencing. *AR* mutations were detected in 20 of 35 (57%) CRPC patients. Paired sampling in one patient before and after chemotherapy showed consistency in these samples, while CTC numbers had dropped. Also, new *AR* gene mutations were found. This method could enable monitoring of the *AR* gene mutational status during therapy, thereby detecting acquired mutations that could confer resistance to therapy [56].

Angiogenesis

Vascular endothelial growth factor (VEGF) and its receptors are targets for anti-angiogenic therapy in advanced and metastatic renal cell carcinoma and some types of NSCLC, metastatic colorectal, breast, hepatocellular cancer and gastointestinal stromal tumor (GIST). Drugs that target the ligand VEGF include the monoclonal antibody bevacizumab (Avastin®) and drugs that target the receptor include the tyrosine kinase inhibitors sunitinib (Sutent®), sorafenib (Nexavar®) and pazopanib (Votrient®).

One report studied targets for anti-angiogenic therapy in CTCs of patients with metastatic breast cancer using PBMC cytospin double staining and confocal laser scanning microscopy. VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) expression were observed in 21 (62%) and 16 (47%) out of 34 patients, respectively, that were pre-selected for having CK-19 mRNA-positive peripheral blood cells. Also hypoxia-inducible factor-1α (HIF-1α), a transcription factor that, when activated, increases transcription of *VEGF*, and phosphorylation of focal adhesion kinase (pFAK), a non-receptor tyrosine kinase involved in cell adhesion and mobility, expression were observed in 26 out of 34 (76%) and 11 out of 12 (92%) patients, respectively. Additional co-staining experiments with immunomagnetic bead selected CTCs showed co-expression of VEGF with HIF-1α and VEGFR2 [43].

DNA damage and apoptosis

In classical cytotoxic chemotherapy, DNA damaging drugs such as alkylating agents and topoisomerase I inhibitors are widely used. Recently, inhibition of DNA damage repair by poly(ADP-ribose) polymerase (PARP) inhibitors has become a target in patients with BRCA1 and BRCA2 deficient tumors. In a phase I study with olaparib [10], both inhibition of PARP in tumor-biopsied cell extracts and formation of γH2AX foci, a marker for double-strand DNA damage, in plucked eyebrow-hair follicles were studied as pharmacodynamic biomarkers.

Pharmacodynamic changes in the number of CTCs expressing nuclear γH2AX

were reported [29]. CTCs were first identified with the CellSearch® system and then analyzed using fluorescent antibodies for HER2 and γH2AX. First, a dose-dependent relationship was found between the γH2AX-signal in spiked cells exposed to topotecan (Hycamtin®). After several analytical validation tests, the assay was used in 15 patients that were participating in a phase I trial before starting treatment. In 11 of these patients CTCs could be identified and in 6 γH2AX-positive CTCs were found. The percentage of γH2AX-positive cells among CTCs varied from 1.6% to 31%. Next, drug-induced changes over time of the number of CTCs and the percentage of CTCs expressing γH2AX was determined in 5 patients treated with topotecan alone or in combination with a PARP inhibitor, and cyclophosphamide in combination with a PARP inhibitor. Increased numbers of γH2AX-positive CTCs were found in all post-treatment samples, with an increase from a mean of 2% (range, 0-6%) at baseline to a mean of 38% (range, 22-64%) after a single day of drug administration. This study showed the feasibility of rapidly assessing nuclear markers in CTCs and monitoring of DNA damaging activity of a drug during treatment[29].

Expression of M30, a caspase-cleaved fragment of cytokeratin 18 and a marker for apoptosis, was observed in CTCs of metastatic CRPC patients [62]. Feasibility of measuring M30 in CTCs was also reported in another study in CTC-positive patients with different types of epithelial cancer before starting therapy. M30-positive CTCs were found in 15 out of 19 (79%) breast cancer patients, in 24 out of 26 (92%) colorectal patients and in 17 (90%) of 19 metastatic renal cancer patients. Next, feasibility of monitoring a pharmacodynamic change after starting therapy was shown. An increase of M30-positive CTCs was found in 6 (75%) of 8 breast cancer patients [30].

The availability of these markers (γH2AX, M30 and phospho-histone H3) on CTCs could enable monitoring of DNA damaging and apoptosis-inducing activity of drugs, such as PARP inhibitors, during treatment.

Validation of assays for CTCs as pharmacodynamic biomarker in early clinical trials of targeted therapy

The level of validation was assessed for each report according to guidelines [9,22,23] (Table 1). Typical parameters for analytical validation would include: sensitivity, specificity, intra-patient and inter-patient accuracy, precision, robustness and stability [23]. In most studies, partial validation with archival tumor tissue and *in vitro* experiments of sensitivity, recovery and stability has performed but no full analytical validation. In fact, only the CellSearch® technique has been fully analytically validated

in a multicenter setting and is therefore approved by the FDA for the enumeration of CTCs [34]. The CellSearch assay is currently not cleared for the assessment of additional markers, such as HER2 (Table 1). All assays reported here are therefore to be classified as “exploratory” and its assessments in early clinical trials, for now, can only be used for further development of the test itself and not in medical decision making in drug development.

However, some reports have highlighted the work performed in validation of the markers and its assays are therefore more likely to be used in an integrated role in pharmacodynamic studies in the future. For IGF-IR [26] expression on CTCs, lack of analytic interference of diagnostic and targeting antibodies was confirmed. The recovery when using spiked and fixed MCF-7 breast cancer cell line cells was 95% and the analytic imprecision was ≤10%. For test periods up to 96 h, the recovery rate remained ≥80%. The lower limit of quantitation (LLOQ) was established with negative controls, e.g. a hematopoietic cell line and cells treated with an excess of targeting antibody. Deviation in precision and stability testing was within ± 10% and ± 15%, respectively, which was according to the bioanalytical guideline [23]. For γH2AX [29], the precision and accuracy of γH2AX-positive CTC recovery was estimated using the PC-3 prostate cancer cell line exposed to different concentrations of topotecan. The measured recovery was 84%. The intra-day and inter-day variation in γH2AX-positive cell recovery both had a coefficient of variation (CV) of <10%, except for the lowest topotecan concentration in the inter-day variability. Here, the CV was 38.4%. There was a dose-dependent γH2AX response to topotecan. In both assays, exploratory assessments were performed in serial sampling during a phase I trial. Another study was aimed at comparing a different CTC identification method with the CellSearch® method and also method optimization was included [55]. Intra-patient variability in CTC number was determined using triplicate blood samples of 7 NSCLC patients, and a low CV was found (9.7%). Also, stability of CTC number recovery over time was assessed in triplicate in blood samples of these patients in 2 different fixatives (CellSave®; Veridex; or EDTA). Up to 72 h, no decline in the recovery of the number of CTCs was found for both fixatives (CV 9.1% and 6.5%, respectively) and this extended up to 144 h for the CellSave® fixative.

The report on molecular characterization of CTCs in CRPC patients demonstrated the feasibility of the assay in a hospital-based clinical laboratory setting, during routine patient management, using a semi-automated method for immunocytochemistry [47].

DISCUSSION

Nowadays, new anti-tumor therapies are often targeted therapies. Targeted drug development may be improved by simultaneous development of biomarkers specific for these targets. The types of targets on CTCs presented in this review were in concordance with the types of targeting drugs that are currently under development. Good examples of how CTCs have shown utility as a pharmacodynamic biomarker in development of its targeting drug, were IGF-IR for an IGF-IR-inhibiting antibody and γH2AX for DNA damage. The latter was studied in classical chemotherapy, but may also be used in development of PARP inhibitors.

Level of validation

The pharmacodynamic assays described here were in the exploratory- and feasibility phase and not yet fully validated. Therefore, it would be premature to use them for actual decision making in a clinical trial, e.g. dose selection. The assays should first be applied in clinical trials in order to gain insight into the assay itself and to further develop and validate the assay. A uniformly accepted scoring system or definition should be formulated to determine overexpression of a specific protein, such as HER2. Major issues that should be addressed in assay validation before its incorporation into early clinical oncology trials include robustness and sensitivity of the assay and logistic issues for multi-sites international trial settings such as sample stability during shipment and storage. Some valuable experience has been gained in determining sample stability during transport and implementing these assays in a multi-center setting [47,55], but so far insufficient validation has been performed to incorporate CTCs as pharmacodynamic biomarkers in early clinical oncological trials. On the other hand, it is being recognized that validation of a biomarker assay has different demands than validation of a typical analytical method, for example for drug analysis by liquid chromatography, and this observation could be taken into account when designing validation studies for pharmacodynamic biomarkers. The main reasons why biomarker assays are considered to be different than typical analytical assays are that they use a range of different methodologies and that often the target molecule is not available to act as a certified calibration standard. Analytical methods for biomarkers frequently lack sensitivity and a dynamic range and the procedures are often labor intensive and prone to variability. Furthermore, in contrast to stable chemical compounds in biological matrices, the biological sample is often unstable and variable [63]. Therefore, it has been proposed to adapt the validation

plan of biomarker assays to a “fit-for-purpose” method. In this method, it has been suggested that method validation should proceed down two parallel tracks which converge eventually. The first should establish the scientific goals of the studied biomarker assay, in order to define the purpose of the assay in terms of outcome in a trial. The second should characterize the technical performance of the assay. For the quasi-quantitative biomarker assays that were described here, precision, specificity, sensitivity and the dynamic range of the assay should form the core of the performance parameters during pre-study validation, in addition to sample- and reagent stability validation. The key stage is the evaluation of whether the technical performance of the assay is deemed fit for demonstrating the intended goal [63]. Although this seems to be a sensible way of approaching validation of these assays, currently there is lack of guidelines in which this approach is formulated.

Limitations of CTCs as pharmacodynamic biomarker

Obviously, CTCs may serve as tissue to study pharmacodynamic effects but may not represent real tumor tissue. Evidence suggests that considerable phenotypic heterogeneity exists in intratumoral cancer cells, including the presence of cancer stem cells. CTCs most likely represent a subset of cells derived from this heterogeneous group of primary tumor cells that survived selection by somehow managing to stay in circulation [64]. The concept of heterogeneity between CTCs has been underscored by the observation of considerable pleomorphism in DNA content, chromosomal abnormalities and apoptosis [62,65], and it is thought that these differences are reflected in biological behavior of CTCs leading to different degree of aggressiveness in different CTC subtypes. It is furthermore believed that CTCs loose epithelial markers, such as EpCAM, in the process of Epithelial-to-Mesenchymal transition (EMT), in order to intravasate into circulation [66]. EpCAM-based CTC detection may therefore very well cause a bias for cells that have a low or no EpCAM expression. In fact, it has been shown that a subtype of breast cancer cells is not detected in the EpCAM-based CellSearch® assay [67] and that detection can be improved by using additional anti-CD146 antibodies [68]. Lack of EpCAM expression also limits the detection of CTCs derived from non-epithelial tumors, for example sarcoma. As a consequence, only a subset of patients that participate in early clinical trials is amenable for this type of pharmacodynamic biomarker testing. One way to circumvent this is to base the capture of CTCs on their size and not on EpCAM expression [69]. Using this approach, substantial heterogeneity in expression of EMT markers were recently demonstrated within and on the outside of circulating tumor microemboli (CTM) of patients with

advanced lung cancer [70]. Lastly, a new immunofluorescence methodology combined with confocal microscopy was developed for the detection of CTCs with a putative stem cell phenotype expressing $CD44^{high}/CD24^{low/-}$ or aldehyde dehydrogenase 1 $(ALDH)^{+}/CD24^{-}$. These cells are thought to have a higher tumorigenic potential and to play a role in the development of resistance to therapy [71]. Studying a pharmacodynamic effect in such subpopulation of CTCs might prove to be essential for monitoring response or development of resistance to therapy in a patient.

Another limitation of using CTCs as biomarker is that the assay depends on the number of observed CTCs. CTC frequency and abundance may vary greatly between patients [16,31], cancer types [16,31], and type of metastatic sites [72]. Even though early clinical oncological trials are likely to be performed with patients with metastatic cancer, who generally have a higher number of CTCs compared to patients with early stage cancer [31], CTCs may remain undetected in a considerable proportion of patients thereby excluding them from pharmacodynamic evaluation. For example, only 20.1% of advanced NSCLC patients had positive CTC counts in the CellSearch® assay, defined as ≥ 2 CTCs/7.5 ml of peripheral blood and individual CTC numbers ranged from 0 to 146 [73]. Such variability has a direct impact on clinical trial design, for example by necessitating inclusion of large groups of patients to reach sufficient sample size of CTC-positive patients. High inter-patient variability may also hamper statistical analysis of comparisons between patients. One way to circumvent this is to compare relative changes in individual CTC numbers with each other [18]. Another issue that was raised in this context is the question how many CTCs are required to be evaluated in order to accurately determine (over)expression of a target on CTCs in a patient. For example in HER2-status determination on CTCs, a cut-off of ≥ 10 cells [25] was previously used, but others have been able to determine CTC-HER2-status using 1 HER2-positive CTC [49]. These important issues should be taken into consideration when designing early clinical oncological trials in which incorporation of CTCs as pharmacodynamic biomarkers eventually is pursued.

Although the CellSearch® assay is the only FDA-approved assay for CTC enumeration, high inter-laboratory variability has recently been shown in a feasibility study of external quality assurance in 14 independent laboratories employing this assay. This variability was probably due to inter-operator inconsistencies on image interpretation, leading to misclassification of CTC-status in 7.5% of all samples [74]. It is likely that this inter-operator variability may also influence the interpretation of all other CTC assays that are based on target visualization, such as immunofluorescence. This variability should therefore be addressed in further validation of these assays.

HER2 was the first target to be demonstrated on CTCs in a proof-of-principle study [25]. Determination of presence or absence of HER2-overexpression on CTCs may have consequences for initiating treatment with HER2 targeting therapy in the future and consequently studying drug effects on CTCs. Currently there is no guideline, such as for HER2 determination in tissue samples[75], or uniformly accepted and validated scoring system for the definition of HER2 overexpression on CTCs. Different techniques and definitions for determining HER2 overexpression on CTCs are being used, including FISH, immunofluorescence, nucleic acid techniques and confocal laser scanning microscopy [25,32,48-50,54,55]. Ideally, validation should take place with a uniformly accepted method in an adequately powered, prospective study.
Several studies explored concordance of HER2 between a primary tumor and CTCs in order to assess phenotypic correlations. Discordance was found between HER2 expression in primary tumor and CTCs in several studies [25,27,32,48,50,54,55]. One study also reported on the relationship between HER2 expression in CTCs, primary tumor- and metastatic biopsy tissue. In 6 out of 7 (86%) of patients with HER2-negative primary cancer, but with HER2-positive CTCs, the metastatic tissue biopsy matched the primary tumor tissue and was also HER2-negative [55]. It is currently unknown if patients with a HER2-negative primary tumor, but with HER2-positive CTCs, might benefit from HER2 targeting therapy, and this question should be addressed in prospective clinical trials. For example, a phase II trial that is currently ongoing will evaluate the effects trastuzumab in combination with vinorelbine has in patients with metastatic breast cancer with HER2-negative primary tumors and HER2-positive CTCs (NCT01185509)[76]. Another phase II trial will assess the anti-tumor activity and safety of lapatinib in advanced breast cancer patients with HER2-positive or EGFR-positive CTCs while having HER2-negative primary tumors (NCT00820924) [76]. The results may be informative on the clinical relevance of identifying HER2 and possibly other such markers on CTCs with respect to clinical outcome.

Pharmacodynamic biomarkers in drug development

The usefulness of biomarkers in drug development in general is debated [1,77,78]. The rationale for studying pharmacodynamic biomarkers and their incorporation in early clinical trials is that they are expected to accelerate the lengthy and costly process of drug development. Ideally, biomarkers should be able to predict early success or failure of a drug in development. In order to achieve this, biomarkers should be employed early in development, or even in the pre-clinical phase [1]. The question remains if spending considerable time and effort of patients and researchers in

the development of biomarkers is worthwhile. In our opinion, biomarkers have the potential to generate essential pharmacodynamic knowledge for the development of targeted therapies, since they can give insight into the actual pharmacological effect of a targeted drug on the target. The application of CTCs as pharmacodynamic biomarker may improve our knowledge of the actual effect on tumor tissue, although this remains a surrogate for real tumor tissue. Considering this limitation, we believe that CTCs may well serve as pharmacodynamic marker in targeted therapy and that its further development is justified.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, literature was presented that reported on CTCs as pharmacodynamic biomarker in early oncology trials. We concluded that most reports only showed feasibility of identification of a specific target on CTCs. Few studies (20%) reported on actually measuring a pharmacological effect of a targeting drug on the target in CTCs. Potential applications of measuring actual target modulation are, for example, to provide proof of mechanism of action of the drug and to study the biologically active dose range. Validation of these pharmacodynamic assays has currently been insufficiently performed for incorporation of these assays as pharmacodynamic biomarkers into early clinical oncological trials. We concluded that there are many promising exploratory assays under development, but none are fully available for clinical application at this moment. Results of prospective clinical trials will be needed to fully understand the relevance of the expression of such markers on CTCs.

As a result of the development of pharmacodynamic assays for growth hormone receptors on CTCs, opportunities arise in monitoring of activating- or resistance-conferring mutations [24,56] and measuring change in intracellular activity of down-stream signaling molecules that can indicate the level of inhibitory activity of the drug [42]. Here, EGFR, HER2, IGF-IR, AR and VEGFR2 were studied, but other hormone receptors with their signaling pathways may be used in future research.

The development of new techniques that improve CTC detection sensitivity [24,37,39,45,55] allows for increase sensitivity in subsequent characterization, e.g. studying specific RNA transcripts, gene expression profiling [79], oncogene expression such as *TMPRSS2-ERG* in prostate cancer [36,37,52], cytogenetic changes and other cytoplasmatic and nuclear markers. These techniques may also enable studying CTC subpopulations, for example CTC that are stem-cell like and may have a more

aggressive biological behavior [71] which could lead to a more personalized therapy for the patient in the future.

In summary, there are many interesting and encouraging developments in the field of CTC detection and characterization that may lead to further development and incorporation of CTCs as pharmacodynamic biomarker in early clinical trials of targeted anti-cancer therapy. In order to maximally exploit the potential of CTCs as pharmacodynamic biomarker in early clinical trials, first further technical validation of the assays should be pursued before applying in drug development.

Table 1: Summary of literature on assays for CTCs as pharmacodynamic biomarker.

Target on CTC	Drug	Research population	Technique	Level of validation	Sensitivity	Intended clinical application	Ref
EGFR receptor and signalling cascade							
EGFR expression		33 metastatic BC patients	CellSearch® and immunofluorescence	Exploratory	51/74 samples were CTC-positive, of which 44/51 (86%) was EGFR positive	For patient selection	46
EGFR and ErbB2 expression		20 CTC-positive CRPC patients	CellSearch® with immunofluorescence, FISH	Exploratory, feasibility study;	18/20 (90%) with EGFR expression > 0 % 0/9 (0%) for ErbB2	For patient selection	47
EGFR expression		20 EGFR expressing, CTC-positive, metastatic NSCLC patients	CellSearch® with FISH	Exploratory, feasibility study	Heterogeneous expression within patients	Biomarker development	38
EGFR mutational analysis	4 Patients exposed to gefitinib	20 CTC-positive NSCLC patients	CTC-Chip and allele-specific PCR technique	Exploratory	19/20 (95%) had an EGFR mutation	For treatment selection; Monitoring of drug resistance mutations (T790M)	24
EGFR expression	1 patient exposed to panitumumab	50 metastatic CRC patients (19 patients had ≥ 2 CTCs)	Flow cytometry and RT-PCR	Exploratory	4/19 (21%) expressed EGFR; flow cytometry: 8/49 (16%) had ≥ 1 cell with CK & EGFR expression	CTC phenotypic characterization, Pharmacodynamic monitoring	28
EGFR		20 metastatic CRC patients	AdnaTest ® and multiplex RT-PCR	Exploratory	11/20 showed CTCs and 2/11 (18%) showed EGFR expression	To monitor drug effects	53
EGFR/pPI3K /pAKT and pEGFR		38 CK19-mRNA positive BC patients (16 metastatic patients)	PBMC cytospin and confocal laser scanning microscopy, immunomagnetic enrichment	Exploratory	7/16 (44%) were EGFR-positive,13/16 (88%) were pPI3K and pAKT positive; 6/7 (86%) were pEGFR positive	For treatment selection	42

ErbB2/HER2							
ErbB2/HER2 expression	Different therapies, including 1 with trastuzumab	10 circulating epithelial cells-positive metastatic BC patients	Automated immunomagnetic and fluorescent system (CellTracks), flow cytometry	Exploratory	7/10 circulating epithelial cells-positive patients	To predict therapy outcome, for disease monitoring	27
ErbB2/HER2 amplification	Trastuzumab	42 advanced BC patients (15 patients with HER2-positive primary tumors and 27 patients with HER2-negative primary tumors)	Immunomagnetic enrichment with ferrofluids conjugated to EpCAM and FISH	Exploratory proof-of-principle	20/42 (48%) showed *HER2*-amplification, of which 11/20 patients with HER2-positive primary tumor and 9/20 patients with a HER2-negative primary tumor.	For treatment selection	25
ErbB2/HER2 amplification		67 metastatic (HER2 negative or unknown) BC patients, of which 21 CTC-positive	Immunomagnetic enrichment for EpCAM; AdnaTest ® BreastCancer Select/Detect test; immunofluorescence and FISH	Exploratory	8/21 (38%) of CTC-positive patients had HER2-positive CTCs	For treatment selection	32
ErbB2/HER2 expression and amplification		66 advanced BC patients (40 patients were CTC-positive)	CellSearch® HER2 Tumor Phenotyping Reagent	Exploratory	15/40 (37%) HER2-positive CTCs.	For treatment selection	50
ErbB2/HER2 expression		62 CTC-positive metastatic BC patients in both CellSearch® and AdnaTest® BreastCancer	CellSearch® HER2 Tumor Phenotyping Reagent and AdnaTest ® BreastCancer	Exploratory, prospective, multicenter trial	13/62 (21%) HER2-positive CTCs in both assays. Concordance in HER2 status in 31/62 (50%) patients	For treatment selection	48
ErbB2/HER2 expression		58 CTC-positive early-stage BC patients	CellSearch® HER2 Tumor Phenotyping Reagent	Exploratory, prospective, multicenter trial (GeparQuattro)	14/58 (24%) HER2-positive	For treatment selection	49

ErbB2/HER2 amplification, other markers *EGFR* and *MET*, pEGFR and pHER2		75 metastatic BC patients	CellSearch® Profile Kit (CPK) and FISH	Exploratory	54/75 (72%)	To identify predictive biomarkers, mechanisms of resistance and facilitation of pharmacodynamic studies	55
ErbB2/HER2 expression and amplification		29 and 13 metastatic BC patients, with at least 1 CTC	CellSearch®, OncoCEE™ microchannel platform, immunofluorescence and FISH	Exploratory	IF: 12/29 (41%) were positive; FISH CellSearch: 1/13 (8%) were positive FISH OncoCEE™: 1/12 (8%) were positive	For biomarker development	38
ErbB2/HER2 transcripts		42 metastatic BC patients (22 patients were CTC-positive)	AdnaTest ® BreastCancer	Exploratory	7/22 (32%) was HER2-positive	Developed as a predictive marker	54
ErbB2/HER2 expression and amplification		30 stage II and III BC patients (10 patients were amenable for HER2 testing; 5 patients with either HER2-positive- or negative primary tumor)	Immunomagnetic enrichment with ferrofluids conjugated to EpCAM, FISH and confocal microscopy	Exploratory	5/5 (100%) had HER2-positive and –negative CTCs for patients with HER2-positive and –negative primary tumors, respectively.	For disease monitoring	41
ErbB2/HER2 and *uPAR* amplification		25 advanced BC patients with *HER2* amplified primary tumor	CellSearch® immunofluorescence and FISH	Exploratory, feasibility study	23/25 (92% had *uPAR* amplification	Therapy selection	51
IGF-IR receptor							
IGF-IR	CP-751,871 (IgG2 antibody with affinity for the IGR-IR)	80 metastatic carcinoma and sarcoma patients (26 were CTC-positive)	Immunomagnetic EpCAM enrichment (CellTracks) and immunofluorescence	Exploratory in phase I	23/26 (88%) of CTC-positive patients was IGF-IR positive	Pharmacodynamic study in drug development, patient selection	26

AR receptor amplification and expression							
AR amplification		9 CTC-positive CRPC patients; for Papanicolau staining: 24 CRPC patients with ≥ 2 CTCs/7.5 ml peripheral blood	CellSearch®, Immunofluorescence, FISH and Papanicolau staining	Exploratory, feasibility study	5/9 (56%) positive for AR; 21/24 (88%) positive for Papanicolau staining	For patient selection	47
AR amplification or relative gain of *MYC*		77 metastatic CRPC patients (49 for *AR* and 43 for *MYC* analysis)	CellSearch® and FISH	Exploratory, feasibility study	Amplification *AR* in 17/49 (35%) and relative gain in MYC in 24/43 (56%) samples analyzed	Individual patient management, tumor profiling	61
AR, ERG and *PTEN* gene	Abiraterone acetate	51 CTC-positive CRPC patients (33 amenable for AR analysis, and 49 for FISH)	CellSearch® and FISH	Exploratory in phase I and II	33/33 (100%) > 1AR copy number, ERG in 23/49 (47%), 13/49 (27%) PTEN loss,	For treatment selection; Assessment of concordance of ERG gene status in tumor biopsies and CTCs	52
AR gene mutations		40 metastatic CRPC patients	CellSearch®, RNA isolation, PCR denaturing HPLC technology and sequencing	Exploratory	35/40 could be evaluated for mutational analysis of the AR gene; 20/35 had AR gene mutations	Treatment selection and direction	56
Angiogenesis							
VEGF, VEGFR2, HIF-1α and pFAK		34 CK-19 mRNA positive metastatic BC patients	PBMC cytospin and confocal laser scanning microscopy	Exploratory	VEGF 21/34 (62%), VEGFR2 16/34 (47%), HIF-1α 26/31 (76%) pFAK 11/12 available patients (92%)	For treatment selection	43

DNA damage and apoptosis							
γH2AX	Topotecan and cyclophosphamide, PARP inhibitor	15 advanced metastatic cancer patients participating in phase I trial, with 5 patients during chemotherapy	CellSearch® and immunofluorescence	Exploratory	At baseline, a mean of 2% of CTCs were positive for γH2AX	Pharmacodynamic study in phase I	29
M30 and chromosomal abnomalities		CRPC patients	CellSearch® and FISH	Exploratory	Additional study in selected group showed 175/765 CTCs (23%) M30-posiitve	Patient selection	62
M30	8 BC patients exposed to chemotherapy	Before therapy: 19 and 26 CTC-positive BC and CRC patients respectively, 19 CTC-positive metastatic RCC patients	CellSearch® and immunofluorescence	Exploratory	Before therapy: 15/19 (79%) breast cancer-, 24/26 (92%) colorectal- and 17/19 (90%) renal cell carcinoma patients During therapy: 6/8 (75%) showed increase and 2/9 (25%) showed decrease in M30+CTCs	Treatment response	30

Abbreviations: (p)AKT= (phosphorylated) v-akt murine thymoma viral oncogene homolog 1, AR=androgen receptor, BC=breast cancer; CK19=cytokeratin-19; CRC=colorectal cancer; CRPC=castration-resistant prostate cancer; CTC(s)=circulating tumor cell(s); DHPLC=denaturing high-performance liquid chromatography; (p)EGFR=(phosphorylated) epidermal growth factor receptor; EpCAM=epithelial cell adhesion molecule; (p)ErbB2/HER2=(phosphorylated) epidermal growth factor receptor 2; ERG=oncogene; (p)FAK=(phosphorylated)-focal adhesion kinase; FISH=fluorescence *in situ* hybridization; γH2AX=phosphorylated H2AX; HIF-1α= hypoxia-inducible factor-1α; IGF-IR=insulin-like growth factor type 1 receptor; M30= caspase-cleaved fragment of cytokeratin 18; MET=met proto-oncogene (hepatocyte growth factor receptor); MYC=oncogene; NSCLC= non-small cell lung cancer; PARP=poly(ADP-ribose) polymerase; PBMCs= peripheral blood mononuclear cells; (p)PI-3K =phosphorylated phosphoinositide-3 kinase; PTEN=phosphatase and tensin homolog; (m)RNA=(messenger) ribonucleic acid; RCC=renal cell carcinoma; RT-PCR=reverse-transcriptase polymerase chain reaction; uPAR=urokinase plasminogen activator receptor; VEGF=vascular endothelial growth factor; VEGFR2=vascular endothelial growth factor receptor 2.

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Chapter 5.2

A multi-marker QPCR-based platform for the detection of circulating tumour cells in patients with early-stage breast cancer

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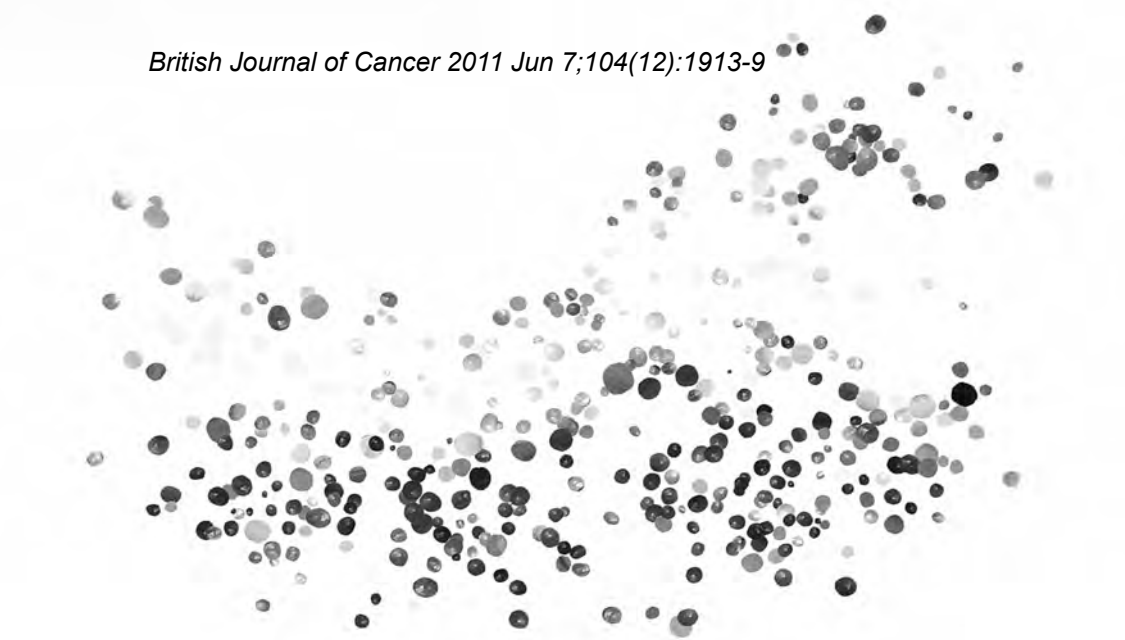

ABSTRACT

Background

The detection of circulating tumour cells (CTCs) has been linked with poor prognosis in advanced breast cancer. Relatively few studies have been undertaken to study the clinical relevance of CTCs in early-stage breast cancer.

Methods

In a prospective study, we evaluated CTCs in the peripheral blood of 82 early-stage breast cancer patients. Control groups consisted of 16 advanced breast cancer patients and 45 healthy volunteers. The CTC detection was performed using ErbB2/EpCAM immunomagnetic tumour cell enrichment followed by multi-marker quantitative PCR. The CTC status and common clinicopathological factors were correlated to relapse-free-, breast cancer-related and overall survival.

Results

CTCs were detected in 16 of 82 (20%) patients with early-stage- and in 13 out of 16 (81%) with advanced breast cancer. The specificity was 100%. The median follow-up time was 51 months (range: 17 – 60). CTC positivity in early-stage breast cancer patients resulted in significantly poorer relapse-free survival (log rank test: $P = 0.003$) and was an independent predictor of relapse-free survival (multivariate hazard ratio = 5.13, $P = 0.006$, [95% CI: 1.62-16.31]).

Conclusions

The detection of CTCs in peripheral blood of early-stage breast cancer patients provided prognostic information for relapse-free survival.

INTRODUCTION

Breast cancer mortality rates have declined over the last decade because of better screening and improved diagnostic techniques and treatments, however, it still remains the main cause of cancer-related deaths in women worldwide [1]. Approximately one third of all women with primary breast cancer will develop metastatic disease [2;3] whereby the risk of relapse is strongly related to lymph node involvement, tumour size, grade at diagnosis [4], lymphatic and/or vascular invasion, hormone receptor status and presence of HER2 over-expression [5]. More recently, risk of metastasis has also been shown to correlate well with prognostic gene expression profiles [6-8].

The detection of circulating tumour cells (CTCs) has been correlated to poor progression-free survival in patients with metastatic breast cancer [9;10]. For CTCs, starting with peripheral blood as sampling material is advantageous for the patient, as it is much less invasive to obtain than a tumor biopsy. Moreover, the accessibility enables sequential sampling during therapy. In patients with early-stage breast cancer, the detection of CTCs or disseminated tumour cells (DTC) in the blood and/or bone marrow has also been found to be an independent negative prognostic factor for disease recurrence and overall survival [11-13]. Relatively few studies have been undertaken to investigate the prognostic significance of CTCs in non-metastatic breast cancer patients [14-16] however, perhaps because of the high assay sensitivity and specificity required for such studies as a result of the relative rarity of circulating tumour cells.

Previously, we designed a quantitative PCR (QPCR)-based assay that utilizes a panel of four tumour marker genes for the detection of occult tumour cells in the peripheral blood of metastatic breast cancer patients. The tumour marker genes had been selected after a systematic search for genes that are highly expressed in breast cancer, but not in the cellular constituents of peripheral blood [17]. Our test showed a sensitivity of 31% and a specificity of 100% in metastatic breast cancer patients and predicted for a worse progression-free and overall survival [18]. Next, we optimized the assay's sensitivity by introducing a dual-antigen immunomagnetic tumour cell enrichment procedure prior to marker gene quantitation and by refining the panel of marker genes as follows: *cytokeratin 19* (*CK19*), *human secretory protein p1.B* (*p1B*), *human epithelial glycoprotein* (*EpCAM*; here: *EGP*), and *mammaglobin* (*MmGl*) [19]. In spiking experiments, we showed that our assay has the sensitivity of detecting as few as 10 tumour cells from a background of 10^6 peripheral blood mononuclear cells [19].

In this study, we used our improved platform for CTC detection in a prospective cohort

of patients with early-stage breast cancer, in an effort to detect the presence of CTCs at diagnosis. These data were then correlated to disease outcome. In addition, we used our platform in two control groups, being advanced breast cancer patients and healthy volunteers.

MATERIAL AND METHODS

The methods and data described herein adhere to the REMARK criteria for the reporting of tumour marker prognostic studies [20].

Patient selection and peripheral blood sampling.

Written informed consent was obtained from all participants and the study was approved by the Medical Ethical Committee of the Netherlands Cancer Institute. Women presenting at the outpatient clinic of The Netherlands Cancer Institute with clinically stage I-III breast cancer were invited to participate between May 2005 and May 2006. Patients with a history of previous malignancy and patients with disseminated breast cancer or active infection were excluded. Type of surgery, loco-regional radiotherapy and adjuvant systemic therapy was left to the treating physician, following nationwide standardized protocols.

From all patients, 8.0 millilitre (ml) whole blood samples were collected during routine preoperative blood sampling in tubes containing a Ficoll-Hypaque density fluid separated by a polyester gel barrier from a sodium citrate anticoagulant (Vacutainer® CPT™, Beckton Dickinson, Breda, The Netherlands). Mononuclear cells, including any tumour cells present, were isolated from blood samples within 24 hours of collection.

Selection of advanced breast cancer patients and healthy volunteer control subjects.

Patients with advanced breast cancer (M1 disease, according to the Union Internationale Contre le Cancer criteria) were included as “positive controls” as the majority were expected to have circulating tumour cells. They were invited to participate if they were between treatments or soon to start subsequent palliative treatment modality. Also, a third group of healthy, female anonymous control subjects, who were randomly selected from hospital staff, were asked to participate. Blood sample collection and preparation were performed as described above.

Additional analyses: effect of frozen storage and the value of repeated sampling after therapy.

In order to assess the effect of frozen storage, samples of a subgroup of patients and healthy volunteers were collected in duplicate. Mononuclear fractions were isolated and one sample was analysed directly and the other was supplemented with “freezing medium” (RPMI 1640 medium with L-glutamine (Gibco, Breda, The Netherlands) containing 10% dimethylsulfoxide, (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (Gibco)) and stored in liquid nitrogen. After 3 months, the frozen pellets were thawed and enriched as described above.

In order to gain insight into the additional value of our assay after therapy, peripheral blood samples were collected between October 2008 and February 2009, after written informed consent, from a subset of patients that continued to visit the out-patient clinic and had remained disease free.

Tumour cell enrichment.

Tumour cells were separated from peripheral blood mononuclear cells (PBMCs) using anti-EpCAM (CD326) (clone HEA-125) and anti-ErbB2 (HER2) Micro Beads (MACS®, Miltenyi Biotec, Utrecht, The Netherlands) according to the manufacturers instructions. In brief, beads were incubated with the PBMCs for 30 minutes at 4°C, after which labelled cells were collected on a magnetic separation column. After removal of the column from the magnetic field, the retained $EpCAM^+$ and/or $ErbB2^+$ cells were eluted, and stored at –70°C in lysis buffer (5M Guanidine thiocyanate (Merck, Darmstadt, Germany), pH 6.8, 0.05M Tris (Roche, Mannheim, Germany), 0.02M EDTA, 1.3% Triton X-100 (Sigma, Steinheim, Germany)) until mRNA isolation and cDNA synthesis.

mRNA isolation and cDNA synthesis.

mRNA was precipitated from the cell lysate and dissolved in lysis buffer from the μMACS™ One-step cDNA kit (Miltenyi Biotec). Oligo(dT) Micro Beads were added and the mixture placed onto the μMACS column in the thermo MACS™ Separator. cDNA synthesis was carried out as per the manufacturers instructions, with an additional elution with 20 μl of elution buffer, resulting in a total volume of 70 μl.

Quantitative real-time PCR.

Quantitative real-time PCR primers (Sigma Genosys, Cambridge, UK) and 5'-fluorescently FAM-labeled TaqMan® probes (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) were designed using the Perkin Elmer Primer Express® software (PE, Foster City, USA) based on the published sequences of CK19, p1B, EGP, and MmGl as previously described [19] (Table 1). All primers were designed to be intron-spanning to preclude amplification of genomic DNA. Commercially available primers and probes for the "housekeeping" genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) were also used.

Serially diluted cDNA synthesized from the amplified RNA of 82 snap frozen breast cancer tissues was used to generate standard curves for control and marker gene expression. For all cDNA dilutions, fluorescence was detected from 0 to 50 PCR cycles for the control and marker genes in singleplex reactions, which allowed the deduction of the C_T-value for each product. The C_T-value (threshold cycle) is the PCR cycle at which a significant increase in fluorescence is detected due to the exponential accumulation of PCR products and is represented in arbitrary units (TaqMan® Universal PCR Master Mix Protocol, Applied Biosystems) [21]. The expression of each tumour marker gene was calculated relative to β-actin in each sample, and the second "housekeeping" gene, GAPDH, was used only to confirm reaction efficiency. Each experiment was performed in triplicate. Quality control measures for the PCR reactions included the addition of a genomic DNA control and a non-template control.

Clinical follow-up.

Of every patient, up to February 1st 2010, regular clinical follow-up was recorded in the patient file and the Institute's Medical Registry. This included evaluation of relapse of disease, breast cancer-related death and death by other causes. Relapse of disease was defined as the development of either local or distant breast cancer metastases. In case of relapse, the date of diagnosis was recorded. In the absence of relapse, the date of the last visit to the outpatient clinic within a year before February 2010 was recorded as last follow-up date. When needed, for example when the last follow-up was longer than a year before or further treatment took place in another hospital, information was verified with the general physician and the last visit there was recorded as last follow-up. One patient without relapse refused further participation after 44 months of follow-up, being longer than a year before February 2010. This date was recorded as last follow-up and censored for further analysis.

Table 1: Primer sequences

Gene	GenBank accession	Sequence	Probe (5'FAM-3'TAMRA)
p1B	L15203	Sense: CTGAGGAGTACGTGGGCCTG Antisense: AGTCCACCCTGTCCTTG-GC	CTGCAAACCAGTGTGCCGT-GCC
CK19	M002276	Sense: CTACAGCCACTACTACACGAC Antisense: CAGAGCCTGTTCCGTCT-CAAA	CACCATTGAGAACTCCAG-GATTGTCCTGC
EGP	M32306	Sense: CAGTTGGTGCACAAAATACT-GTCA Antisense: CCATTCATTTCTGC-CTTCATCA	TTGCTCAAAGCTGGCTGC-CAAATGTT
MmGl	AF015224	Sense: TTCTTAACCAAACGGAT-GAAACTCT Antisense: GGTCTTGCAGAAAGT-TAAAATAAATCAC	TGCTGTCATATATTAATTGCAT-AAACACCTCAACATTG

Abbreviations: p1B=human secretory protein p1.B; CK19=cytokeratin 19; EGP=human epithelial glycoprotein; MmGl= mammaglobin.

Statistics.

Analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL). All statistical tests were performed at the 5% level of significant difference. Differences of rates between groups were compared with either the two-sided Fisher's exact test or Pearson's χ^2- test or, for ordinal variables, χ^2- test for trend. Differences between groups with continuous variables were tested by the non-parametric Mann-Whitney *U* test. The quadratic discriminant analysis (QDA) score function was calculated from the expression data of four marker genes (CK19, p1B, EGP and MmGl) as previously described [17;22]. QDA is a statistical approach to find the combination of quadratic and linear functions of variables (in this case marker genes), which leads to the optimal separation between groups (in this case advanced breast cancer patients and healthy volunteers). It is a generalization of the more familiar Fisher's Linear Discrimination Analysis (LDA), which allows only linear functions [23]. The highest value of the healthy control group was set as threshold-value for positivity (QDA value > than threshold: "QDA-positive") and negativity (for QDA value ≤ threshold: "QDA-negative") of other samples. This threshold was fixed for any future study. QDA-positivity indicated the presence of tumour cells in a sample and conversely QDA-negativity indicated their absence. Patients with a QDA-positive or QDA-negative test from their blood sample were defined as having a positive or negative CTC-status, respectively. QPCR

measurement and QDA data analysis in this way offers a simple and objective estimate of tumour cell presence in a given sample. Survival was illustrated by Kaplan-Meier plots and compared between groups by the log rank test. Clinicopathologic factors known to be associated with prognosis (age ≤ 45 years versus > 45 years), tumour size (T2 and T3 versus T1), lymph node involvement (yes versus no), both following TNM 6 classification according to Union Internationale Contre le Cancer criteria[24], histological grade (3 versus 1 or 2), estrogen receptor (ER) and/or progesterone receptor (PR) hormone receptor (both negative versus either or both positive) and HER2 (positive = 3+ in immunohistochemistry or in fluorescent *in situ* hybridization versus negative) were tested in univariate analysis to calculate hazard ratios (HRs), their 95% confidence intervals (CIs) and *P*-value. Variables that were found to be significant or with a HR > 2.0 or < 0.5 in the univariate analysis were included in a multivariate Cox regression model to identify those with independent prognostic information and furthermore to calculate HR and their 95% confidence intervals CIs.

RESULTS

Study inclusion and patient characteristics.

A total of 82 women with early-stage breast cancer were included and a peripheral blood sample was obtained before surgery and before initiating adjuvant therapy. The clinical characteristics are described in Table 2. The median age was 56 years (range: 34 – 86 years), 70 (85%) were older than 45 years, 12 (15%) had stage III disease at diagnosis, 20 (24%) had tumours > 2 cm, 22 (27%) had infiltrated axillary lymph nodes, 18 (22%) had tumours with histological grade 3, 9 (11%) had tumours that were ER and PR negative and 28 (22%) had tumours that were HER2 positive. In total, 68 (83%) of the patients received any type of adjuvant therapy and 14 (17%) received trastuzumab therapy. The median time from diagnosis to blood collection was 14 days (range: 0.0 - 61 days) and the median follow-up time from sampling to “relapse of disease” or “last follow-up” was 51 months (range: 17 – 60). The median follow-up time for patients that did not experience an event was 51 months (range: 40 – 60).

Table 2: Clinical characteristics of stage I-III breast cancer patients (*n* = 82), according to CTC status.

Patients (n)		Total (%) n= 82	CTC- negative (within group %) n=66	CTC- positive (within group %) n=16	*P*-value
Age (years)	≤ 45	12 (15)	9 (14)	3 (19)	0.694
	> 45	70 (85)	57 (86)	13 (81)	
Stage of disease[a]	I	47 (57)	39 (59)	8 (50)	0.285
	II	23 (28)	19 (29)	4 (25)	
	III	12 (15)	8 (12)	4 (25)	
Tumour size[a]	T1	62 (76)	51 (77)	11 (69)	0.200
	T2	14 (17)	12 (18)	2 (13)	
	T3	3 (4)	1 (2)	2 (13)	
	T4	3 (4)	2 (3)	1 (6)	
Node stage[a]	N0	60 (73)	48 (73)	12 (75)	0.785
	N1	17 (21)	14 (21)	3 (19)	
	N2	3 (4)	2 (3)	1 (6)	
	N3	2 (2)	2 (3)	0 (0)	
Histological grade	Grade 1	26 (32)	22 (33)	4 (25)	0.329
	Grade 2	38 (46)	31 (47)	7 (44)	
	Grade 3	18 (22)	13 (20)	5 (31)	
Hormone receptor	Negative	9 (11)	7 (11)	2 (13)	1.00
	ER and/or PR positive	72 (88)	58 (88)	14 (88)	
	Unknown [c]	1 (1)	1 (2)	0 (0)	
HER2 status[b]	Negative	63 (77)	52 (79)	11 (69)	0.332
	Positive	18 (22)	13 (20)	5 (31)	
	Unknown [c]	1 (1)	1 (2)	0 (0)	
Adjuvant therapy	None	14 (17)	9 (14)	5 (31)	0.581
	CT	1 (1)	1 (2)	0 (0)	
	RT	25 (31)	22 (33)	3 (19)	
	HT	3 (4)	2 (3)	1 (6)	
	CT + RT	13 (16)	10 (15)	3 (19)	
	RT + HT	11 (13)	10 (15)	1 (6)	
	CT + RT+ HT	15 (18)	12 (18)	3 (19)	
Any adjuvant therapy	No	14 (17)	9 (14)	5 (31)	0.134
	Yes	68 (83)	57 (86)	11 (69)	
Trastuzumab therapy	No	68 (83)	54 (82	14 (88)	0.726
	Yes	14 (17)	12 (18)	2 (12)	

Abbreviations: CTC-positive or negative= positive or negative circulating tumour cell status according to quadratic discriminant analysis (QDA) score; ER= estrogen receptor; PR= progesterone receptor; CT= chemotherapy; RT= radiotherapy; HT= hormonal therapy; [a] TNM 6 classification according to the Union Internationale Contre le Cancer criteria. [b]HER2 positivity= 3+ in immunohistochemistry or positive fluorescent in situ hybridization test (FISH). [c] One patient with unknown hormone receptor- and HER2 status was excluded from Fisher's exact test.

Circulating tumour cell detection in patient groups.

In this prospective study, 16 (20%) of the 82 primary breast cancer patients that were included had a positive QDA score (Figure 1). The majority of patients with a QDA-positive score (12 out of 16; 75%) had a blood sample that was positive for the marker gene and at least one other marker gene (5/16, 31% was *EGP* and *p1B* positive; 4/16, 25% was *EGP* and *CK19* positive and 3/16, 19% was *EGP*, *p1B* and *CK19* positive). The four other QDA-positive patients (4/16, 25%) had a blood sample that was *EGP* negative but positive for both *p1B* and *MmGl*. The sensitivity and specificity of our test was 20% [95% CI: 12 – 30] and 100% [95% CI: 92 – 100], respectively.

The distribution of patient- and primary tumour tissue characteristics were not significantly different between the CTC-positive and -negative patients (Table 2). The median age at diagnosis was 55 (range: 36 -84) and 57 (range: 34 – 86) for the CTC-positive and –negative patients, respectively. In addition to this, a female healthy volunteer control group (n=45, none positive) and advanced breast cancer patients group (n = 16; 13 (81%) positive) were tested (Figure 1). QDA values among advanced breast cancer patients were higher compared with early breast cancer patients as well as with healthy controls (Mann-Whitney U test: both P< 0.001), although three was no significant difference between early stage patients and healthy controls (P = 0.123). Median QDA values (range) were -1.16 (-9.78 to 0.00), -1.16 (-6.25 to 1.99) and 2.39 (-1.16 to 3.69) for the healthy controls group, early-stage breast cancer patients and advanced breast cancer patients, respectively. For further analysis, patients with a zero or negative QDA were considered “CTC-negative” and patients with a positive QDA were considered “CTC-positive”.

CTC status at time of diagnosis and clinical outcome.

During follow-up period of the early breast cancer patients, 12 patients (14%) experienced clinical relapse: 6 of whom were CTC-positive (38% of all CTC-positive patients), and 6 of whom were CTC-negative (9% of all CTC-negative patients; Fisher’s exact test: P = 0.010; Table 3). One CTC-positive patient had a local relapse, followed by distant metastasis one month later. The other eleven patients had distant metastases in pleura, liver, brain, bone or in multiple sites.

Despite the relatively short follow-up period of this prospective study, CTC status at time of diagnosis as determined by our assay was a significant predictor of relapse-free survival, with a hazard ratio of 4.72 ([95% CI: 1.52-14.66]; *P* = 0.003) (Figure 2, Table 4). Importantly, multivariate analysis demonstrated that CTC status at time of diagnosis was a significant and independent predictor of relapse-free survival (multivariate Cox regression, multivariate hazard ratio = 5.13, *P* = 0.006, [95% CI: 1.62-16.31]) (Table 5). The 4-year relapse-free survival rates were 92% and 69% for CTC-negative and CTC-positive patients, respectively (Figure 2).

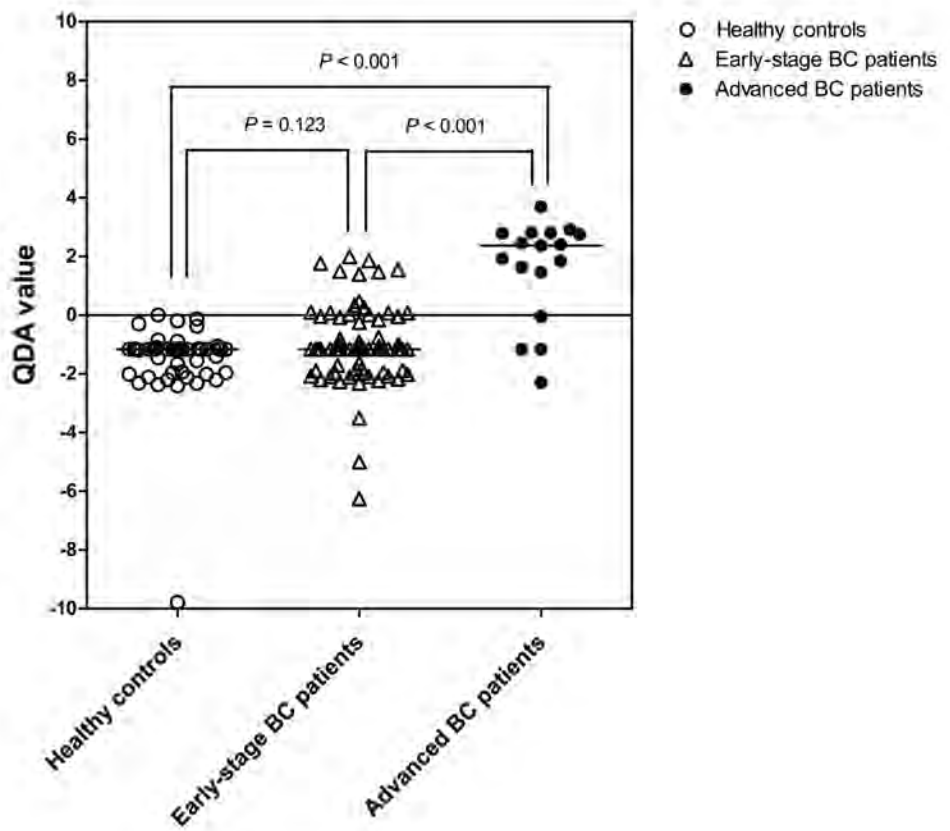

Figure 1: Quadratic discriminant analysis (QDA) values incorporating the expression of the four marker genes *CK19*, *p1B*, *EGP* and *MmGI* measured in the peripheral blood of healthy controls (*n*=45; open circles), early-stage breast cancer (BC) patients (*n* = 82; triangles) and advanced BC patients (*n*=16; closed circles). The median expression levels for the QDA are indicated by a horizontal line (healthy controls = -1.16, early-stage BC patients = -1.16, advanced breast cancer patients = 2.39).

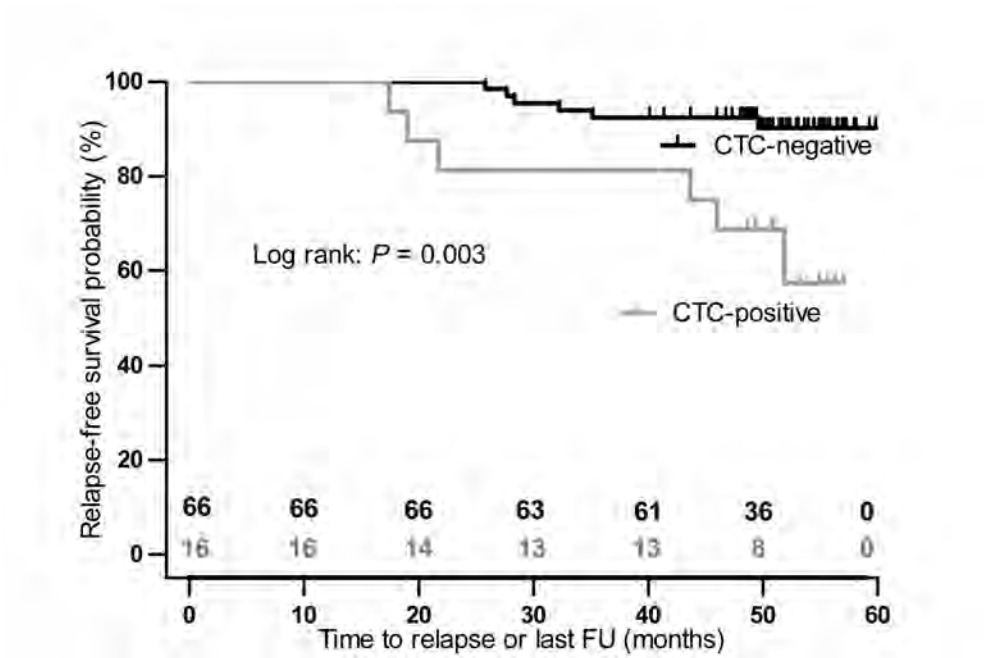

Figure 2: Kaplan-Meier survival curve for relapse-free survival of early-stage breast cancer patients (*n*=82) that are CTC-negative (*n*= 66) or positive (*n*= 16) at diagnosis. CTC-positive patients had a significantly poorer relapse-free survival than CTC-negative patients (univariate hazard ratio = 4.72 [95% CI: 1.52-14.66]; Log rank test *P* = 0.003). The number of patients at risk at each time point (months) are indicated for the CTC-negative (black) and CTC-positive (grey) groups.
Abbreviations: CTC-positive or -negative= positive or negative circulating tumour cell status according to quadratic discriminant analysis (QDA) score; FU= follow-up.

Table 3: Incidence of relapses and overall and breast cancer-related deaths in early breast cancer patients, in total and according to CTC status at diagnosis

Patients (n)		Total (%) n= 82	CTC- negative (within group%) n=66	CTC- positive (within group %) n=16	P-value
Relapse of disease	No	70 (85)	60 (91)	10 (63)	**0.010**
	Yes	12 (15)	6 (9)	6 (38)	
Overall survival	Alive	74 (90)	59 (89)	15 (94)	1.000
	Death	8 (10)	7 (11)	1 (6)	
Breast cancer-related survival	Alive	74 (90)	59 (89)	15 (94)	0.686
	Death, breast cancer related	5 (6)	4 (6)	1 (6)	
	Death, other cause	3 (4)	3 (5)	0 (0)	

Abbreviations: CTC-positive or -negative= positive or negative circulating tumour cell status according to quadratic discriminant analysis (QDA) score. Significant *P*-values are shown in bold.

Table 4: Univariate analysis of relapse-free survival by CTC positivity and common clinical variables.

Parameter	Hazard ratio	95% CI	*P*-value	Cox regression: *P*-value
CTC (positive vs negative) (n=82)	4.72	1.52 – 14.66	**0.007**	**0.003**
Age (< 45 years vs > 45 years), (n=82)	1.22	0.27 – 5.58	0.797	0.797
Tumour size[a] (≥T2 vs T1) (n=82)	3.40	1.10 – 10.55	**0.034**	**0.025**
Node stage[a] (N+ vs N0) (n=82)	1.40	0.42 – 4.65	0.586	0.584
Histological grade (grade 3 vs 1 and 2), (n=82)	1.19	0.32 – 4.39	0.798	0.798
Hormone receptor negative vs positive, (n=81)	3.04	0.82 – 11.25	0.096	0.080
HER2 positive vs negative, (n=81)	1.17	0.32 – 4.31	0.818	0.817

Abbreviations: CTC-positive= positive circulating tumour cell status according to quadratic discriminant analysis (QDA) score. [a] TNM 6 classification according to the Union Internationale Contre le Cancer criteria. Significant *P*-values are shown in bold.

Table 5: Multivariate analysis for relapse-free survival.

Parameter	Hazard ratio	95% CI	P-value
CTC-positive	5.13	1.62 – 16.31	**0.006**
Tumour size[a] (≥T2)	3.11	0.99 – 9.72	0.051
Hormone receptor (negative)	2.93	0.77-11.14	0.116

Abbreviations: CTC-positive= positive circulating tumour cell status according to quadratic discriminant analysis (QDA) score. [a] TNM 6 classification according to the Union Internationale Contre le Cancer criteria. Significant P-values are shown in bold.

During the follow-up period, death occurred in total in 8 patients, of which 5 were breast cancer- related and 3 were by other cause (Table 3). One death occurred in the CTC-positive group and this was a breast-cancer related death (6% of all CTC-positive patients) with pleural metastases. In the CTC-negative group, 4 breast cancer-related deaths and 3 deaths by other causes occurred (6% and 5%, respectively, of all CTC-negative patients). The patients with breast cancer related death had pleural, skin, bone and liver metastases. There was no significant difference in overall- and breast cancer-related survival between the CTC-positive and negative group (Fisher's exact test and Pearson's χ^2- test: $P = 1.000$ and $P = 0.686$, respectively; Table 3). CTC positivity was not associated with breast cancer-related or overall-survival with a hazard ratio of 1.02 ([95% CI: 0.11-9.09]; $P = 0.989$)

and 0.57 ([95% CI: 0.07 – 4.63]; *P* = 0.594), respectively. The 4-year overall survival rates were 91% and 94% for CTC-negative and CTC-positive patients, respectively.

Additional analyses: effect of frozen storage and the value of repeated sampling after therapy.

We assessed the effect of frozen storage in 8 early-stage- and one advanced breast cancer patients and 5 healthy controls. All samples had a concordant CTC status in both the fresh and stored sample.

We also used the assay in peripheral blood samples of a subset of patients that were CTC-negative at diagnosis and had remained disease-free. Of the 45 women that participated, 40 women tested CTC-negative and 5 women (5 out of 45 (11%)) tested CTC-positive, currently without experiencing a diagnosed relapse of disease. During follow-up (median time from 2nd blood collection to last follow-up: 6.2 months; range: 0.0- 13) no relapses or deaths occurred.

DISCUSSION

An optimized CTC assay was used to estimate circulating tumour cell load in prospectively-collected peripheral blood samples of 82 early-stage-, 16 advanced breast cancer patients and 45 healthy female controls. In early-stage breast cancer patients, the sensitivity of our assay was 20%. Based on the data from several published studies with early-stage breast cancer patients [15;16;25-28] the assay's sensitivity was found to be comparable to other assays with high specificity, except for Daskalaki et al.[16] who found a higher sensitivity of 52.4% with a specificity of 97.8% in a study that had only included stage I and II breast cancer patients. The CellSearch® system (Veridex, Warren, New Jersey, USA) is one of the most-used and validated commercial CTC detection platforms currently available [28-32]. This method was used in the recent GeparQuattro clinical trial where CTCs were prospectively monitored in neo-adjuvant therapy. Here, at baseline a sensitivity of 21.6% was found for CTC-positivity, using a cut-off of ≥ 1 CTC/7.5 ml peripheral blood [27]. While generally demonstrating a similar sensitivity and specificity to the CellSearch system, our assay offers several other advantages: first, our assay is objective, as an algorithm generates a single score automatically for determining CTC-positivity based on tumour marker gene expression levels detected by QPCR. This is in contrast to the CellSearch system, in which images of cells are determined

to be CTCs by software and need to be manually confirmed by an operator. This step can introduce subjectivity in scoring which is significant when as few as one or two cells in a sample are sufficient to class the patient as “CTC-positive”. Recently, Kraan et al. observed that image interpretation was the main contributor to between-laboratory variation [33]. In addition, our assay is considerably less expensive, costing less than US$25 per sample, compared to approximately US$600 per sample for the CellSearch system [34]. Finally, there already exists equipment for the automation of the enrichment, cDNA preparation and QPCR steps in our assay, and hence it could potentially require little manual work or technical knowledge. Although this is a preliminary study and further validation involving additional patients would be required to confirm that an automated system could provide equivalent prognostic data, there is a potential for the use of such system in a clinical setting.

The threshold for QDA positivity was set at the highest QDA value in the healthy controls group, resulting in 100% specificity, as previously described [17;18]. Although our assay’s sensitivity could have been augmented by lowering the threshold, priority was given to avoid false-positives.

In total, 81% of the advanced patients assayed were CTC-positive versus 0% of the healthy controls (Figure 1). We hypothesized that enriching a sample for tumour cells prior to assessing tumour marker gene expression would be beneficial for assay sensitivity, and this appears to be the case. We had previously demonstrated that when tumour cell enrichment was not performed, 30% of advanced patients assayed from a similarly-selected patient group had a positive QDA score indicating CTC positivity (versus 0% of healthy controls) [18]. We also had previously shown that using a positive enrichment strategy for cells expressing both EpCam and ErbB2 antigens resulted in the detection of higher levels of tumour marker gene expression than enriching for cells expressing just one of the antigens [19]. Also, the multi-marker gene expression panel used has obvious benefits over the use of a single marker for the detection of tumour cells. Finally, the use of the quadratic discriminant score function is not prone to subjectivity in scoring or inaccuracies in quantitation as immunohistochemical staining or densitometry of an electrophoresed nucleic acid band can be.

Our platform also has limitations using immunomagnetic bead selection with antibodies directed at EpCam and ErbB2. This may very well also have caused a bias by not selecting any potential EpCam and ErbB2 non- or low-expressing cells. It is believed that CTCs may loose EpCam in order to intravasate and to reach circulation, in a process called Epithelial-to-Mesenchymal transition (EMT) [35]. Indeed,

Sieuwerts et al showed that a subtype of breast cancer cells is not detected in the EpCam-based CellSearch assay [36]. Consistent with this hypothesis, in the present study, blood samples of 17 early-stage *EGP*-negative patients were found positive for one or more of the other 3 marker genes (data not shown). These studies and our data confirm that indeed there may be phenotypic differences between CTCs.

Our previous study using non tumour-cell-enriched blood samples from advanced patients demonstrated that CTC status was an independent predictor of both progression-free and overall survival [18]. CTC positivity as determined by our assay was a significant predictor of relapse-free survival in the primary breast cancer patient cohort (multivariate HR= 5.13, P = 0.006, [95% CI: 1.62-16.31]; Table 5). Importantly, multivariate analyses demonstrated that CTC status provided significant prognostic information that was independent of other commonly-used clinical variables, including age at diagnosis, lymph node status, histological grade, hormone receptor status and HER2 status (Table 4 and 5). Tumour size, which is known for its prognostic value, did not reach statistical significance in our analysis. In contrast to predicting for relapse, CTC positivity was not a predictor for breast cancer-related- or overall survival. Others have demonstrated a significantly reduced survival for CTC-positive early-stage breast cancer patients [15;16;26]. The observation that CTC-positivity did not predict for survival in the present study may be because of the combination of a follow-up time that was relatively short for the observation of deaths and the occurrence of a limited number of events, being eight deaths in total.

Additional exploratory analyses of our assay on the effect of frozen storage, which found concordant results in all samples, indicated the possibility to store freshly collected samples for a longer period and to process them later. If confirmed on a larger scale, this method would greatly facilitate sample collection and would present another advantage to other methods described earlier [16], including the CellSearch system in which samples are required to be processed within 72 hours [32]. The analysis on the value of repeated sampling after therapy did not show relevance during the relatively short follow-up, and the prospective value of this 2nd sampling will be studied in the future in ongoing follow-up.

In conclusion, we have demonstrated a sensitive and specific platform for the detection of circulating tumour cells in both advanced and early-stage breast cancer patients. In this study with early-stage breast cancer patients, we found that our assay was prognostic for relapse-free survival. Further work will be required in prospective trials to fully determine whether our assay can be used to improve disease outcome in patients that are CTC-positive.

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Chapter 5.3

Circulating tumor cell detection in advanced non-small cell lung cancer patients by multi-marker QPCR analysis

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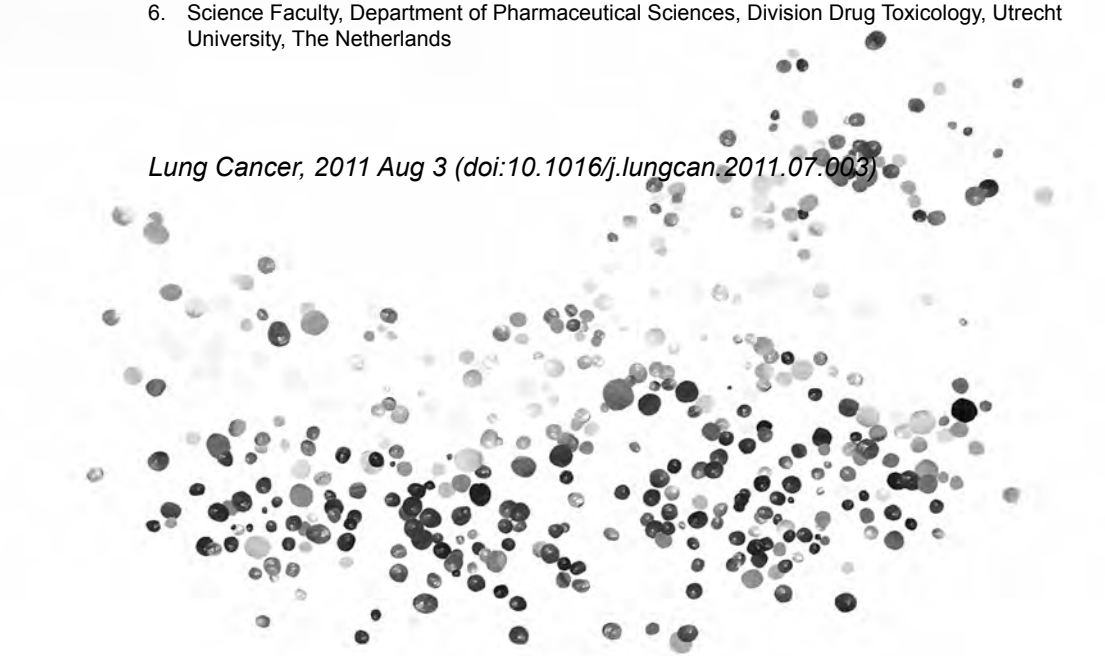

ABSTRACT

Background

The aim of this study was to explore circulating tumor cell (CTC) detection in advanced non-small cell lung cancer (NSCLC). CTCs may not only serve as a prognostic marker in selected tumor types, but may also be useful as pharmacodynamic marker in drug development.

Methods

Fourty-six advanced NSCLC patients and fourty-six healthy controls were included in the study and 8.0 ml of peripheral blood was obtained from each of the participants. Immunomagnetic bead enrichment for cells expressing epithelial cell adhesion molecule (EpCAM) was performed, followed by multi-marker quantitative real-time PCR of a panel of marker genes: cytokeratin 7 (CK7), cytokeratin 19 (CK19), human epithelial glycoprotein (EGP) and fibronectin 1 (FN1). Using quadratic discriminant analysis (QDA), expression values were combined into a single score, which indicated CTC-positivity or –negativity. Test characteristics were assessed using receiver operating characteristic (ROC) curve analysis.

Results

ROC-curve analysis showed capability of discrimination between advanced NSCLC patients and healthy controls (area = 0.712; 95% CI 0.606-0.819; $P < 0.001$). A cut-off minimizing overall misclassification for QDA-positivity reached a sensitivity of 46% (95% CI 31 – 61) and a specificity of 93% (95% CI 82 – 99).

Conclusions

In this exploratory study, an assay was developed for discriminating CTCs in peripheral blood samples of advanced NSCLC patients from healthy controls. The assay demonstrated an acceptable sensitivity in combination with good specificity. Further validation studies should take place in NSCLC patients and a matched control group.

INTRODUCTION

Lung cancer is the most frequently diagnosed type of cancer worldwide and accounts for approximately 18% of all cancer-related deaths [1]. About 75% of all lung malignancies are non-small cell lung cancers (NSCLC). Despite advances in prevention and treatment, NSCLC is often diagnosed at an advanced stage and has a poor prognosis [2].

An important stage in the process of metastasis is the intravasation of tumor cells into the blood stream, which gives rise to circulating tumor cells (CTCs). Although CTCs are rare, CTCs can be detected using a variety of techniques [3]. It has been shown that the number of CTCs before treatment, and the change in number during treatment, is prognostic for several types of solid tumors, including breast-, colorectal- and prostate cancer [4-7]. In contrast to tumor tissue or metastases, CTCs are easily obtained by venipuncture thereby enabling repeated sampling over time. As the sensitivity of techniques for CTC detection continues to improve, it is has become possible to study specific characteristics or "targets" on or in CTCs, such as expression of a growth factor [8], mutational status [9] or specific gene amplifications [10]. Therefore, CTCs do not only serve as a prognostic marker, but may also be used as a pharmacodynamic marker to study the actual pharmacological effect of a drug on a target in CTCs. Such could be achieved by measuring changes in expression of specific genes or proteins in CTCs targeted by a drug.

We previously developed a method to detect CTCs in peripheral blood samples of breast cancer patients [11,12], based on immunomagnetic tumor cell enrichment using antibodies directed at EpCAM and ErbB2 (HER2), followed by multi-marker QPCR [13]. Because of the high incidence and the clinical importance of NSCLC, the aim of our current work was to explore CTC detection in advanced NSCLC patients. Given the heterogeneity of NSCLC, a literature search was performed for additional candidate marker genes. The previously validated assay for breast cancer was adapted for NSCLC by using immunomagnetic beads with an antibody directed at EpCAM only and by incorporating the following marker genes into the assay: cytokeratin 7 (CK7) [14], cytokeratin 19 (CK19) [11,14-20], human epithelial glycoprotein/EpCAM (here: EGP) [11,17] and fibronectin 1 (FN1) [20].

In this exploratory study, the value of the assay using these four selected marker genes was assessed in peripheral blood samples of NSCLC patients and healthy volunteers.

MATERIALS AND METHODS

Patient selection and peripheral blood sampling.

Written informed consent was obtained from all participants and the study was approved by the Medical Ethics Committee of the Netherlands Cancer Institute. Patients presenting at the outpatient clinic of The Netherlands Cancer Institute with clinical stage IIIB or IV non-small cell lung cancer were invited to participate.

From all patients, a single 8.0 millilitre (ml) whole blood sample was collected during routine blood sampling in tubes containing a Ficoll-Hypaque density fluid separated by a polyester gel barrier from a sodium citrate anticoagulant (Vacutainer® CPT™, Beckton Dickinson, Breda, The Netherlands). Mononuclear cells, including any tumor cells present, were isolated from blood samples immediately and stored at room temperature. Age, gender, smoking habits, histological type of the primary tumor, stage of disease[21] and time of sampling (before or during chemotherapy or therapy with a tyrosine kinase inhibitor) were recorded. For smoking habits, patients were categorized as “no smoking”, “previous smoking” (defined as: last smoking at least >1 month before sampling), “current smoking” and “smoking unknown”.

Selection of healthy control subjects.

Anonymous control subjects, randomly selected from hospital staff, were asked to participate. Subjects with a history of any type of cancer were excluded. After informed consent, blood sample collection and preparation were performed as described above. Also age, gender and smoking habits were recorded.

Tumor cell enrichment.

Within 24 hours after collection, tumor cells were separated from peripheral blood mononuclear cells (PBMCs) using anti-EpCAM (CD326) (clone HEA-125) Micro Beads (MACS®, Miltenyi Biotec, Utrecht, The Netherlands) according to the manufacturers instructions. In brief, beads were incubated with the PBMCs for 30 minutes at 4°C, after which labelled cells were collected on a magnetic separation column. After removal of the column from the magnetic field, the retained EpCAM+ cells were eluted, and stored at –70°C in lysis buffer (5M Guanidine thiocyanate (Merck, Darmstadt, Germany), pH 6.8, 0.05M Tris (Roche, Mannheim, Germany), 0.02M EDTA, 1.3% Triton X-100 (Sigma, Steinheim, Germany) until mRNA isolation and cDNA synthesis.

mRNA isolation and cDNA synthesis.

mRNA was precipitated from the cell lysate and dissolved in lysis buffer from the μMACS™ One-step cDNA kit (Miltenyi Biotec). Oligo(dT) Micro Beads were added and the mixture was placed onto the μMACS column in the thermo MACS™ Separator. cDNA synthesis was carried out as per the manufacturers instructions, with an additional elution with 20 μl of elution buffer, resulting in a total volume of 70 μl. mRNA isolation and cDNA synthesis was carried out in batches of samples with approximately 16 samples at the same time, but was performed in separate occasions, due to logistical limitations.

Quantitative real-time PCR.

Quantitative real-time PCR primers (Sigma Genosys, Cambridge, UK) and 5'-fluorescently FAM-labeled TaqMan® probes (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) were designed using the Perkin Elmer Primer Express® software (PE, Foster City, USA) for CK7 and FN1. CK19 and EGP were used as described previously [13] (Table 1). All primers were designed to be intron-spanning to preclude amplification of genomic DNA. Commercially available primers and probes for the "housekeeping" genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) were also used.

Table 1: Primer sequences used to amplify each of the 4 marker genes used to identify CTCs in peripheral blood.

Gene	GenBank accession	Sequence	Probe (5'FAM-3'TAMRA)
CK7	M148059	Sense: GAGGTCAAGGCGCAGTATGAG Antisense: GGAGGTCGTCCCCATGCT	ACCAAGTTTGAGACCCTC-CAGGCCC
CK19	M002276	Sense: CTACAGCCACTACTACACGAC Antisense: CAGAGCCTGTTCCGTCTCAAA	CACCATTGAGAACTCCAG-GATTGTCCTGC
EGP	M32306	Sense: CAGTTGGTGCACAAAATACTGTCA Antisense: CCATTCATTTCTGCCTTCATCA	TTGCTCAAAGCTGGCTGC-CAAATGTT
FN1	M135600	Sense: GATGCCGACCAGAAGTTTGG Antisense: TGACCCATGTCATGCTGCTT	CCCATGGCTGCCCACGAGGA

CK7= cytokeratin 7; CK19= cytokeratin 19; EGP= human epithelial glycoprotein; FN1 = fibronectin-1.

Serially diluted cDNA synthesized from RNA extracted from 4 frozen lung tissues (one adenocarcinoma- , one squamous cell- and one undifferentiated carcinoma tissue, all with at least 80% tumor tissue as determined by pathologic determination, and one normal lung tissue) were used to generate standard curves for control and marker gene expression. For all cDNA dilutions, fluorescence was detected from 0 to 50 PCR cycles for the control and marker genes in singleplex reactions, which allowed the deduction of the C_t-value for each product. The C_t-value (threshold cycle) is the PCR cycle at which a significant increase in fluorescence is detected due to the exponential accumulation of PCR products and is represented in arbitrary units (TaqMan® Universal PCR Master Mix Protocol, Applied Biosystems) [22]. The expression of each tumor marker gene was calculated relative to β-actin in each sample, and the second "housekeeping" gene, GAPDH, was used only to confirm reaction efficiency. Each experiment was performed in duplicate. Quality control measures for the PCR reactions included the addition of a genomic DNA control and a non-template control.

Statistics.

Analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL). All statistical tests were performed two-sided and at the 5% level of significant difference. Differences of rates between groups were compared with χ^2- test. Differences between groups with continuous variables were tested by Student's *t*-test or by non-parametric Mann-Whitney *U* test. The number of positive samples per single marker gene, defined as any expression > 0, was summarized for both groups. The quadratic discriminant analysis (QDA) score function was calculated from the expression data of four marker genes (CK7, CK19, EGP and FN1) as previously described [11,23]. QDA is a statistical approach to find the combination of quadratic and linear functions of variables (in this case marker genes), which leads to the optimal separation between groups (in this case advanced NSCLC patients and healthy controls) [11,23]. A positive QDA value indicates the presence of tumor cells in a sample (here referred to as "QDA-positive"), and conversely a negative or zero discriminant value indicates their absence (here referred to as "QDA-negative"). Patients with a QDA-positive or QDA-negative test from their blood sample were defined as having a positive or negative CTC-status, respectively. Test characteristics were assessed by calculating area under the receiver operating characteristic (ROC)-curve and this was compared to the reference (area = 0.5). A cut-off was set to obtain optimal sensitivity and specificity.

RESULTS

Study inclusion and patient characteristics.

In total, fourty-six patients with advanced NSCLC and fourty-six healthy volunteers were included. The clinical characteristics are described in Table 2. The mean age (SD) was 36 (11.1) years and 58 (10.2) years for healthy controls and advanced NSCLC patients, respectively. The majority was male (52% of healthy controls and 63% of advanced NSCLC patients). Most patients (91%) had stage IV disease [21]. In the NSCLC patient group, the majority smoked (previous smoking: 37% and current smoking: 35%) and this was in contrast to the healthy volunteer group (previous smoking: 9% and current smoking: 24%). There was a significant difference in age and smoking habits between healthy volunteers and NSCLC patients ($P < 0.001$ for both), but not in gender ($P = 0.291$). Peripheral blood sampling was performed before start of therapy in 24 out of 46 (52%) and during therapy in 21 out of 46 (46%) of patients. Time of sampling was unknown in one patient (2%; data not shown).

Table 2: Clinical characteristics of healthy controls and advanced NSCLC patients (n = 46 per group).

Patients (*n*)		**Healthy controls (within group %) *n* = 46**	**NSCLC patients (within group%) *n* = 46**	***P*-value**
Age (years)	Mean (SD)	36 (11.1)	58 (10.2)	**< 0.001**
Gender	Male	24 (52%)	29 (63%)	0.291
	Female	22 (48%)	17 (37%)	
Stage of disease [21]	Stage IIIB	n.a.	4 (9%)	n.a.
	Stage IV	n.a.	42 (91%)	
Smoking	No smoking	23 (50%)	10 (22%)	**< 0.001**
	Previous smoking	4 (9%)	17 (37%)	
	Current smoking	11 (24%)	16 (35%)	
	Smoking unknown	8 (17%)	3 (7%)	

Significant *P*-values are shown in bold. NSCLC= non-small cell lung cancer; SD=standard deviation; n.a.= not applicable.

Single marker gene expression in advanced NSCLC patients and healthy controls.

The number of subjects that were found to have positive expression for a marker gene (CK7, CK19, EGP or FN1), defined as any expression > 0, were summarized for healthy controls and advanced NSCLC patients (Table 3). All four markers had a higher positivity rate in advanced NSCLC patients compared to healthy controls. Two markers (CK7 and EGP) had higher sensitivity (both 30%). The other two markers (CK19 and FN1) had lower sensitivity (8% and 9%) but high specificity (both 100%). These four marker genes were subsequently used in quadratic discriminant analysis (QDA).

Table 3: Summary of the number of subjects that were found to have positive expression for a marker gene, defined as any expression > 0, in a peripheral blood sample.

Marker gene		Healthy controls (within group %) *n* = 46	NSCLC patients (within group %) *n* = 46	Total (within group %) *n* = 92
CK7	Negative	40 (87%)	32 (70%)	72 (78%)
	Positive	6 (9%)	14 (30%)	20 (22%)
CK19	Negative	46 (100%)	42 (91%)	88 (96%)
	Positive	0	4 (9%)	4 (4%)
EGP	Negative	44 (96%)	32 (70%)	76 (83%)
	Positive	2 (4%)	14 (30%)	16 (17%)
FN1	Negative	46 (100%)	39 (85%)	85 (92%)
	Positive	0	7 (8%)	7 (8%)

CK7= cytokeratin 7; CK19= cytokeratin 19; EGP= human epithelial glycoprotein; FN1 = fibronectin-1; NSCLC= non-small cell lung cancer.

Effect of age and smoking on marker gene expression.

Because there was a significant difference in age and smoking behaviour between healthy volunteers and NSCLC patients, any potential correlation between these two characteristics and the expression of a single marker gene was explored within both groups. No differences were found in mean age for CK7, CK19, EGP and FN1 expressing subjects compared to non-expressing subjects, for both the patients and healthy volunteers. Next, “current smoking” was compared to non- and “previous smoking” for the expression of individual marker genes (CK7, CK19, EGP and FN1) in both patients and healthy volunteers. The only significant difference was seen in the proportion of smokers in CK7-expressing healthy volunteers (4 out of 6; 67%)

versus the proportion of smokers in non-CK7-expressing healthy volunteers (7 out of 32; 22%; *P* = 0.047). None of the other markers, including CK7 expression in patients, were significantly different for smoking.

Quadratic discriminant analysis (QDA) for healthy controls and advanced NSCLC patients and test characteristics.

Quadratic discriminant analysis in peripheral blood samples of 46 healthy volunteers and 46 advanced NSCLC patients resulted in the calculation of QDA values. In order to determine the value of the assay in discriminating NSCLC patients from healthy controls, the area under the receiver operating curve (ROC)-curve was calculated. There was a statistical difference for the assay obtained in patients (area = 0.712; 95% CI 0.606 – 0.819; *P* < 0.001; Figure 1) compared to reference (area=0.5). QDA values were significantly different (*P* < 0.001; Figure 2) with medians (range) of -0.59 (-1.27 to 1.02) for the healthy controls and -0.59 (-1.32 to 2.94) for the patients.

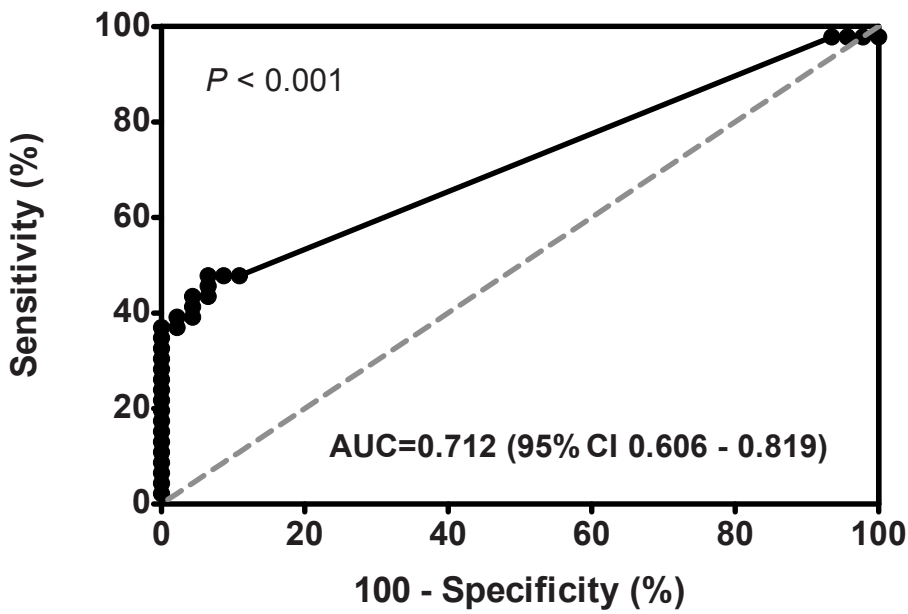

Figure 1: Area under receiver operating characteristic curve (ROC-curve) of the QDA-values (black line) compared to reference line (dashed line; area = 0.5) for discriminating healthy controls from patients with NSCLC (*n* = 46 both). QDA value= quadratic discriminant analysis value incorporating the expression of the four marker genes CK7, CK19, EGP and FN1 measured in peripheral blood.

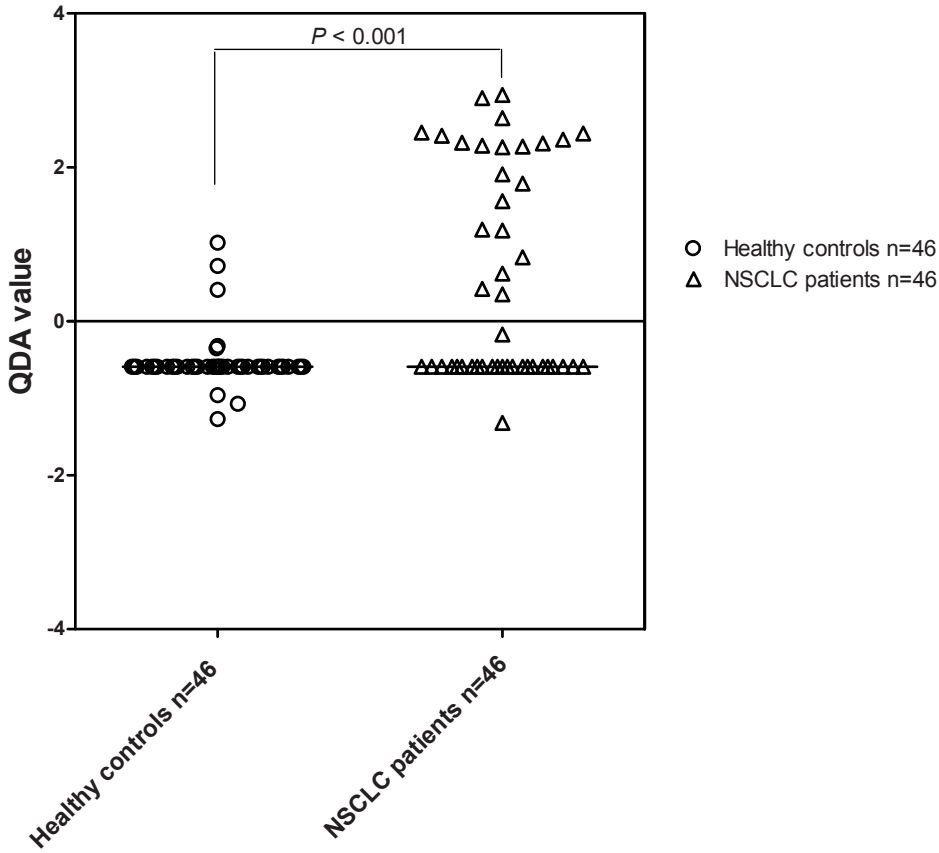

Figure 2: Quadratic discriminant analysis (QDA) values incorporating the expression of the four marker genes CK7, CK19, EGP and FN1 measured in the peripheral blood of healthy controls (circles) and advanced NSCLC patients (triangles; *n* = 46 both). Horizontal lines indicate median values. NSCLC=non-small cell lung cancer.

Circulating tumor cell detection in healthy controls and advanced NSCLC patients.

As QDA-positive samples were considered to contain CTCs, patients with a QDA-positive value were considered CTC-positive, and patients with a QDA negative or zero value were considered CTC-negative (Table 4). Based on this assumption, a cut-off minimizing overall misclassification for QDA-positivity (at QDA=0) reached a sensitivity of 46% (95% CI: 31– 61) and a specificity of 93% (95% CI: 82–99). There was a significant difference in CTC-positivity between healthy volunteers and advanced NSCLC patients ($P < 0.001$).

Table 4: Summary of the number of CTC-negative and –positive healthy controls and advanced NSCLC patients.

	CTC negative *n* = 68	CTC positive *n* = 24	*P*-value
Healthy controls (within group %, n=46)	43 (93%)	3 (7%)	**< 0.001**
Advanced NSCLC patients (within group %, n=46)	25 (54%)	21 (46%)	

Significant *P*-value is shown in bold. NSCLC= non-small cell lung cancer; CTC positive or negative = positive or negative tumor cell status according to quadratic discriminant analysis (QDA) score.

Table 5: Summary of the number of CTC-negative and –positive advanced NSCLC patients according to primary tumor type and time of sampling.

		CTC negative (within group %) (*n*=25)	CTC positive (within group %) (*n*=21)	*P*-value*
Histological subtype	Adenocarcinoma	17 (68%)	13 (62%)	0.665
	Other types, o*f which:*	8 (32%)	8 (38%)	
	Undifferentiated carcinoma	1 (4%)	1 (5%)	
	Squamous cell carcinoma	4 (16%)	4 (19%)	
	NSCLC not specified	3 (12%)	3 (14%)	
Time of sampling	Before therapy	12 (48%)	12 (57%)	0.632
	During therapy	12 (48%)	9 (36%)	
	Unknown	1 (4%)	0	

* *P*-value calculated for 2x2 tables (comparing “adenocarcinoma” versus “other types” and “before therapy” versus “during therapy”). NSCLC= non-small cell lung cancer; CTC positive or negative = positive or negative tumor cell status according to quadratic discriminant analysis (QDA) score.

CTC positivity was compared for different histological subtypes (Table 5). In the NSCLC patient group, adenocarcinoma was most frequently found (30 out of 46; 65%) and this was equally reflected in the CTC-positive (13 out of 21; 62%) and –negative (17 out of 25; 68%) group. Other types included undifferentiated- , squamous cell carcinoma and “NSCLC not specified”, and these were equally found in the CTC-positive and –negative groups. There was no significant difference in the proportion of adenocarcinoma versus “other types” in the CTC-positive and –negative groups (*P* = 0.665).

CTC positivity was also compared for time of peripheral blood sampling in relation to therapy (Table 5). A larger proportion of CTC-positive patients had been sampled before the start of therapy (12 out of 21; 57%) compared to the CTC-negative group (12 out of 25; 48%), but this difference was not statistically significant (P = 0.632).

DISCUSSION

In this pilot study, we explored the value of immunomagnetic bead enrichment followed by QPCR analyses of four selected marker genes in discriminating CTCs in peripheral blood samples of advanced NSCLC patients from healthy controls. As there is no single marker gene that is expressed in all NSCLC tumors and not in peripheral blood cells, a multi-marker gene approach was employed, as described by others [14-17, 19,20,24]. In this study, CK7 [14], CK19 [11,14-20], EGP [11,17] and FN1 [20] were selected.

The keratins (CK7 and CK19) are intermediate filament proteins responsible for the structural integrity of epithelial cells. CK7 is expressed in the simple epithelia lining the cavities of the internal organs and is frequently expressed in lung adenocarcinoma [25]. CK19 is frequently used as marker gene in CTC detection of several epithelial types of cancer[3] and whole blood CK19 mRNA expression has been correlated to a poor prognosis after chemo-radiation in NSCLC [18]. EGP (EpCAM), a cell adhesion molecule, is used as an epithelial marker for enrichment strategies[3] and is under development as a target for immunotherapy [26]. Fibronectin, an extracellular matrix protein, is involved in cell adhesion and migration processes, including metastases [20].

We demonstrated that the outcome of the assay (QDA value) was able to discriminate between advanced NSCLC patients and healthy volunteers with a sensitivity of 46% and a specificity of 93%. There was a significant difference in CTC-positivity between healthy volunteers and advanced NSCLC patients.

Xi et al. had found significant difference in expression for CK7 in 68% of primary lung cancer tissues compared to normal blood samples [14]. These results suggested that CK7 could be a useful marker for a maximum of approximately 2/3rd of NSCLC types. In the present study, a sensitivity of 30% (14/46, Table 3) was reported for CK7-positive expression in CTCs in peripheral blood of NSCLC patients. This result indeed showed that CK7 could be a useful marker in CTC detection in NSCLC, but should be used in a panel with other markers [14].

For the other selected marker genes, lower sensitivity was found compared to literature reports based on QPCR methods. Both Sher et al. and Liu et al. found a sensitivity of 58% and 41%, respectively, for CK19 in lung cancer patients [19,20], which was higher than the results presented here (9%, 4/46, Table 3). FN1 was detected in 39% (21/54) by Sher et al., compared to 8% (7/46, Table 3) here. Both used a more sensitive nested-PCR technique with 65 cycles in total, compared to 50 cycles here, which might explain the higher sensitivity. For EGP, a sensitivity of 30% (14/46) was found in the present study, which was unexpectedly low. Following the results of studies performed in advanced breast cancer patients, where the addition of immunomagnetic enrichment with an antibody directed at EpCAM to PBMC isolation [13,27] led to increased sensitivity, we had expected to also find higher sensitivity in the present assay. We had assumed that high EpCAM protein expression would correlate with high mRNA expression and consequently high expression values in our assay. Immunomagnetic enrichment is performed on fresh material and is a lengthy procedure, compared to other methods, which may have reduced mRNA expression and PCR expression values.

The QDA score function was calculated from the combined expression of four marker genes in advanced NSCLC patients and healthy volunteers. ROC-curve analysis showed capability of discrimination between advanced NSCLC patients and healthy controls value. A cut-off was set to obtain optimal sensitivity and specificity, and this was found to be 46% and 93%, respectively. Sher et al. established a positive detection rate for circulating cancer cells using four marker genes (CK19, ubiquitin thiolesterase (UCHL1), FN1 and tripartite motif-containing 28 (TRIM28)) of 67% (14 of 21) in stage III and 79% (11 of 14) in stage IV NSCLC patients [20]. Liu et al. used the combination of CK19, tumor specific antigen 9 (TSA-9) and pre-progastrin-releasing peptide (Pre-proGRP) in 134 lung cancer patients, and established a sensitivity of 90% in stage III and IV NSCLC patients and a specificity of 93% [19]. The assay reported here had acceptable sensitivity and good specificity compared to other PCR-based assays in the same patient group. Because this exploratory study was conducted in a small group, preferably the assay should be validated in a study with a larger sample size before reaching conclusions.

The only FDA-approved method for quantitative CTC evaluation, CellSearch®, was initially studied by Allard et al. More than ≥ 2 CTCs per 7.5 ml of peripheral blood were found in 34 (20%) of 168 samples of 99 metastatic lung cancer patients [28]. The CellSearch® technique was also prospectively evaluated in a population of 150 patients with a suspicion or diagnosis of lung cancer. Using a cut-off point of ≥ 1

CTC/7.5 ml peripheral blood, the sensitivity and specificity for discriminating any stage of primary lung cancer (*n* =125) from non-malignant disease (*n* =25*)* were 30% and 88%, respectively. In metastatic patients, the sensitivity was 71% (22 out of 31) [29]. The CellSearch® assay performed better for metastatic lung cancer than the assay described here in sensitivity, but with lower specificity. When specificity was increased to 96%, by setting the cut-off at ≥ 2 CTCs/7.5 ml peripheral blood, the sensitivity became 42% (13 out of 31), which is comparable to the assay reported here. Recently, Krebs et al. reported the results of a prospective study using CellSearch® to study the prognostic value of CTCs in 101 untreated stage III or IV NSCLC patients [30]. Here, a cut-off of at ≥ 2 CTCs/7.5 ml of peripheral blood as criterion for CTC-positivity resulted in sensitivity of 7% (2 out of 27) and 32% (19 out of 60) at baseline, in stage IIIB and IV patients, respectively, which is also in line with the sensitivity reported in the present study.

QDA values were calculated for optimal discrimination between advanced NSCLC patients and healthy volunteers. However, the healthy volunteers had a significantly different age distribution and smoking characteristics than the NSCLC patients. In order to explore an effect of these parameters on the outcome of our assay, relationships between single marker gene expression and age or smoking were explored within both groups. No effect of age was found. Only a significant difference was found for current smoking in CK7 expression in healthy controls (Fisher's exact test: P = 0.047). This effect was not found in patients, but an effect of smoking on QDA value could not be excluded. None of the other markers were significantly different. The effect of smoking on the outcome of the assay should therefore be determined in future validation studies, preferably in a matched case-control study.

NSCLC is a heterogeneous disease with a variety of histopathological subtypes. This may affect the recovery of the immunomagnetic bead enrichment which is based on EpCAM-expression. When EpCAM-expression was studied in tumor biopsies, high-level expression was found in 64% of all lung cancers, 81% of adenocarcinoma and 54% of squamous cell carcinoma, and no expression in 14%, 5% and 17%, respectively [31]. This indicates that there might be a considerable subset of EpCAM-low or –none expressing CTCs, derived from EpCAM-low or –none expressing primary tumors, which were not detected in this assay. The role of any EpCAM-negative CTCs remains unclear. It is expected that further research on the characterization of CTCs will increase knowledge on the potential clinical relevance and will yield additional markers that can be used for improving CTC sensitivity, as for example CD146 in breast cancer [32].

CONCLUSION

An assay was developed for detecting CTCs in peripheral blood of advanced NSCLC patients in an exploratory study. The assay showed the combination of sufficient sensitivity and good specificity, but an effect of smoking on the outcome of the assay could not be excluded. In order to determine the value of the assay in practice, further validation studies should be performed in a matched NSCLC patient and control group.

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Chapter 5.4

Validation of a multi-parameter flow cytometry method for the determination of phosphorylated extracellular-signal-regulated kinase and DNA in circulating tumor cells

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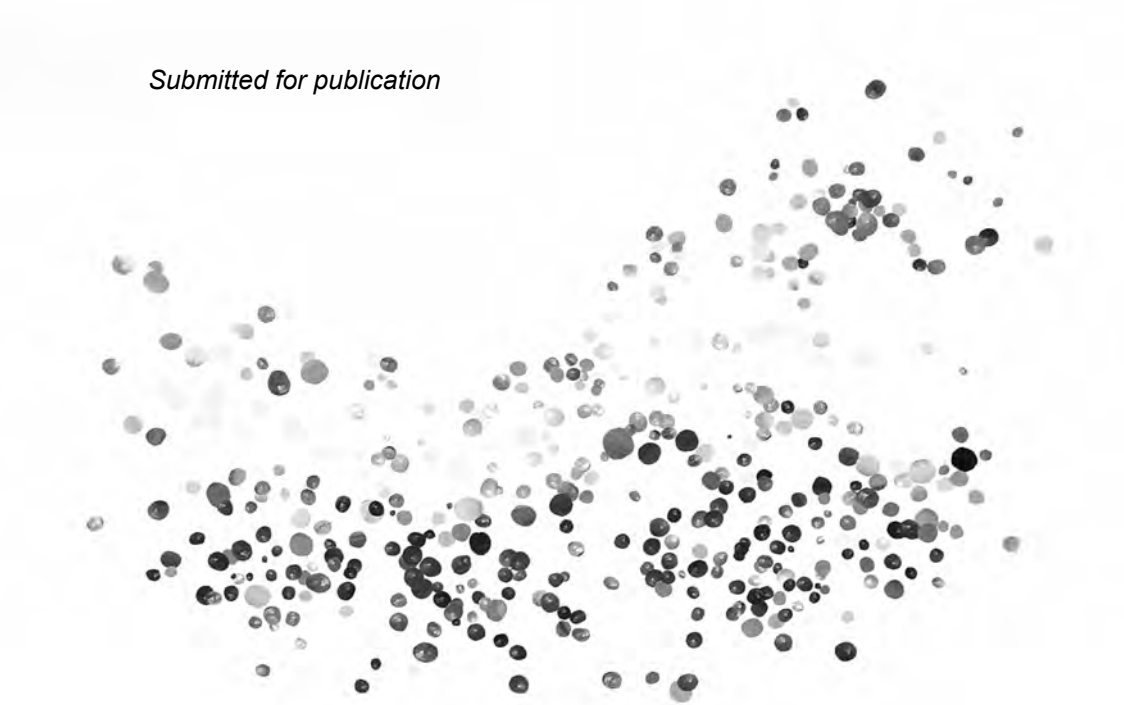

ABSTRACT

Background

There is a need for a reliable method for monitoring the pharmacodynamic effects of molecular targeted anticancer agents in circulating tumor cells (CTCs).

Methods

Cell preparation tubes (CPT) tubes were used for peripheral blood collection and density gradient centrifugation, followed by phosphorylation of extracellular-signal-regulated kinase (ERK) with epidermal growth factor (EGF). After fixation with formaldehyde and methanol magnetic anti-epithelial cell adhesion molecule (anti-EpCAM) Micro Beads were used for the selective isolation of circulating tumor cells (CTCs) from the background, consisting of peripheral blood mononuclear cells (PBMCs) and platelets. Subsequently, samples were stained with Hoechst 33342 and fluorescent antibodies against EpCAM, CD45, and pERK. Flow cytometry was used for identification and enumeration of CTCs and determination of their pERK and DNA content. The validation parameters included specificity, recovery, linearity, precision, sensitivity and stability.

Results

The lower limit of quantification was 2 CTCs per 8 ml peripheral blood. Samples were stable for 4 months in storage at -80°C. The applicability of the method was demonstrated by successful enumeration of CTCs and the determination of DNA and pERK, before and after stimulation with EGF, in 8 ml peripheral blood samples from patients with metastatic cancer.

Conclusions

A simple, selective and sensitive multi-parameter fluorescence activated cell sorting (FACS) method, utilizing density gradient centrifugation and magnetic antibody cell sorting, was developed and validated for the determination of pERK and DNA in CTCs.

INTRODUCTION

Metastatic disease is the main reason for mortality in patients with solid tumors. Metastases are caused by shedding of an estimated one million tumor cells into the bloodstream per gram of tumor tissue per day [1;2]. Circulating tumor cell (CTC) quantity is a proven valuable prognostic marker in metastatic breast, colorectal and prostate cancer [3]. The detection of CTCs before adjuvant chemotherapy as well as the persistence of CTCs after the completion of systemic adjuvant treatment has been correlated with an unfavorable clinical outcome [4]. However, there is evidence that a significant percentage of CTCs is apoptotic and therefore is unable to settle in secondary organs [5].

For many epithelial cancers, minimally invasive biopsies provide insufficient material for molecular analysis at diagnosis and tumors are typically not sampled repeatedly during treatment to monitor changes in genetic abnormalities. The molecular profile of CTCs possibly better resembles that of metastatic cells than of the primary tumor, which can be very different from the tumor of origin. Therefore, the pharmacodynamic effects of cytotoxic drugs and molecular targeted anticancer agents might better be tested in CTCs than in the primary tumor [6]. However, the use of CTCs for molecular profiling has been limited by relatively insensitive detection strategies [7;8].

Patients with non small cell lung cancer (NSCLC) that are identified with activating mutations in the epidermal growth factor receptor (*EGFR*) gene in tumor tissue can respond to EGFR tyrosine kinase inhibitors, including gefitinib (Iressa®) and erlotinib (Tarceva®) [9-11]. Recent studies have reported expression of growth factor receptors on CTCs of patients with breast and prostate cancer [12-14] and the expression of activated EGFR in CTCs has also been reported [4;15]. The cellular effect of activated EGFR is exerted via the extracellular-signal-regulated kinase (ERK) signaling pathway which ultimately leads to phosphorylation of ERK. However, the ERK activation status in CTCs in response to targeted drugs remains unknown [4].

In this paper, we present the development and validation of a multi-parameter fluorescence activated cell sorting (FACS) method for the quantification of pERK and DNA in CTCs isolated from peripheral human blood. Phosphorylation state-specific antibodies [16-21] have recently become available and were used for the quantification of pERK. The applicability of the method was demonstrated in peripheral blood from patients with advanced metastatic breast-, colon-, lung-, ovarian- and urothelial cancer. Although we report results for ERK phosphorylation, we believe that this procedure will be more widely applicable to the study of phospho-epitope expression in peripheral blood samples.

MATERIALS AND METHODS

Reagents and chemicals.

Erlotinib hydrochloride and methanol originated from Roche. Paraformaldehyde (PFA) was purchased from Merck. PBS and RPMI medium were purchased from GIBCO BRL. Epidermal growth factor (EGF), Hoechst33342 and pERK ELISA kit were purchased from Sigma. Anti-human EpCAM-Micro-Beads, magnetic antibody cell sorting (MACS®) columns, Fc-Receptor block (FcR), mouse clone 5B1 IgG2a anti-human CD45-fluorescein isothiocyanate (FITC) and mouse clone HEA-125 IgG1 anti-human EpCAM-phycoerythrin (PE) were purchased from Miltenyi. Rabbit clone D13.14.4E IgG anti-human pERK1/2-Alexa Fluor®647 and isotype rabbit clone DA1E IgG1-Alexa Fluor®647 were purchased from Cell Signalling. Perm/Wash™ was purchased from Becton Dickinson. Beads buffer (BB) was PBS containing 0.5% fetal calf serum and 2 mM EDTA degassed by sonication for 10 min. All buffers were filtered through 0.2 µm filters before use.

Patients and whole blood collection.

For each CTC enrichment procedure, three 8 ml collection tubes were used containing a Ficoll- Hypaque density fluid separated by a polyester gel barrier from a sodium citrate anticoagulant (CPT™, BD Vacutainer®). In order to avoid possible contamination by epithelial cells during skin puncture, the first 4 ml of blood were collected and disposed in a separate tube. CTCs were isolated from 8 ml peripheral blood samples of 25 metastatic cancer patients, of which two patients were being treated with an EGFR tyrosine kinase inhibitor.

Study participants were informed of the investigational nature of this analysis and had given written informed consent in accordance with institutional and national guidelines. The study protocol was approved by the ethical committee of the Netherlands Cancer Institute.

Cell culture and spiking experiments.

The two human lung cancer cell lines MOR/P and A549, as well as the breast cancer cell line MCF-7 (ATCC), were cultured as monolayer in RPMI medium supplemented with 10% fetal calf serum. For spiking experiments cells were counted and sorted by a FACSaria™ cell sorter (Beckman Coulter).

Centrifugation and sample washing.
All centrifugations were performed in 2 ml eps (Eppendorf), in a centrifuge equipped with a swing-out rotor at 1.000 x *g* for 4 min at 4°C. After centrifugation, the supernatant was removed with a 1 ml pipet leaving 100 µl on the pellet. Samples were washed with 1 ml Perm/Wash™ buffer, followed by vortex mixing at half speed.

Sample pre-processing.
CPT tubes containing 8 ml of peripheral blood were centrifuged in a swing-out rotor at 1500 x *g* for 25 min at room temperature (RT). Next, EGFR was stimulated under the following conditions: after centrifugation the CPT tubes were inverted three times and acclimatized in a water bath for 15 min at 37°C. The upper CPT layer is incubated with 100 ng/ml EGF for 5 min at 37°C. Next, the upper CPT layer is transferred to a 50 ml Falcon® tube (Becton Dickinson) containing 1 ml 40% formaldehyde. The CPT tubes were washed with 3 ml physiologic salt, which was pooled with the rest of the sample. After vortex mixing at half speed for 10 sec, incubation for 15 min at RT and addition of 40 ml physiologic salt, samples were centrifuged at 1000 x *g* for 10 min at 4°C. The supernatant was decanted, followed by placing the tubes upside down on filter paper for 5 sec, after which the samples were chilled on ice. Next, the pellets were resuspended in 1 ml of ice-cold 50% (v/v) methanol/PBS by vortex mixing for 10 sec at the highest setting. After incubation on ice for exactly 10 min in pre-chilled 2 ml eps, the samples were stored at -80°C for future analysis.

Cell recovery optimization by anti-EpCAM Micro Beads MACS®.
A549 cells, expressing approximately 50 times less EpCAM than MOR/P, were used for spiking 15 CPT tubes each containing 8 ml peripheral blood from a healthy volunteer, with 1000 cells each. Samples were blocked with FcR for 60 min at RT, next, 10 µl anti-EpCAM-Micro-Beads were added for 30 min at 0°C, and for 30, 60, 120 and 180 min at RT. After tumor cell enrichment recovery was determined by FACS analysis.

Tumor cell enrichment.
CTCs underwent an immunomagnetic enrichment using anti-EpCAM Micro Beads and FcR, with the following modifications to the manufacturer's instructions: samples stored at –80°C were defrosted on ice, and after centrifugation supernatant was removed. The pellets were washed twice with ice-cold BB. After centrifugation, the pellets were resuspended in the remaining 100 µl BB, and 40 µl of FcR was added

for 60 min at RT. Next, 10 µl anti-EpCAM-Micro-Beads were added for 1 hour at RT. Samples were washed twice with 1 ml of BB, followed by centrifugation. After resuspending the pellets in 500 µl of BB, labeled cells were collected on a MACS® column. After removal of the column from the magnetic field, the retained EpCAM+ cells were eluted in two separate elution steps using 1 ml BB each, followed by centrifugation.

(Immuno)fluorescence staining.

The tumor cell enriched samples were stained for intracellular DNA at a final concentration of 5 µM Hoechst33342 for 15 min at RT. Next, samples were washed twice with 1ml Perm/Wash™, centrifuged, followed by staining with 5 µl of anti-CD45-FITC for 15 min at RT in the dark. Subsequently, samples were stained with 5 µl of anti-EpCAM-PE and 10 µl anti-pERK 1/2-Alexa Fluor® 647 for 1 hour at RT in the dark. Next, unbound antibodies were removed with two 1 ml washes of Perm/Wash™ followed by centrifugation.

Staining linearity was measured in triplicate in samples spiked with 10, 30, 100, 300, 1000, 3000 and 10000 MOR/P cells in CPT tubes containing 8 ml peripheral blood from a healthy volunteer. Samples were stained with Hoechst33342, anti-EpCAM-PE and anti-pERK-Alexa647 according to the conditions described above. After removal of unbound antibodies and Hoechst33342, mean fluorescence intensity (MFI) was measured by flow cytometry.

Flow Cytometry.

Flow cytometry was performed using a CyAn ADP™ (Beckman Coulter). Hoechst33342, FITC, PE and Alexa Fluor® 647 were collected through 450 ± 25 nm, 530 ± 40 nm, 575 ± 25 nm, and 665 ± 25 nm band pass filters, respectively. Data analysis was performed with Summit v4.3.01 software (Dako Cytomation).

Specificity.

In order to assess the amount of background interference, three CPT tubes containing 8 ml peripheral blood were drawn from 18 different healthy volunteers. Furthermore, we assessed in triplicate, using 8 ml peripheral blood from a healthy volunteer, the influence of different FcR blocking conditions on the amount of background by using 20 µl FcR for 15 min at 0°C and RT, 40 µl FcR at RT for 15, 30, and 60 min, respectively.

Within-day (WDP) and between-day (BDP) precision and recovery.
A549 and MOR/P cells were spiked at 10, 100, 1000 and 10000 cells per 8 ml of peripheral blood in CPT tubes which were immediately pre-processed as described above. Samples were measured in triplicate on three consecutive days. The within-day and between-day precision were calculated by one-way analysis of variance (ANOVA). Recovery was measured as: (mean observed cell concentration / nominal cell concentration) x 100%

Lower limit of quantification (LLOQ).
The LLOQ was determined by spiking in six fold 1, 2, 3, 4, 5, and 10 MOR/P cells in CPT tubes containing 8 ml of peripheral blood from six different healthy volunteers. The LLOQ was defined as the cell concentration that can be determined with a precision of better than 20%, and a recovery between 80 – 120% of the nominal value.

Sample stability.
Long term storage stability was assessed by spiking 45 CPT tubes, containing 8 ml peripheral blood from a healthy volunteer, with 1.400 MOR/P cells each. Three samples per time point were processed and stored in 50% MeOH at -80°C for 0, 1, 7, 14, 30, 60, 120, and 180 days until analysis. We also assessed the stability of stained samples in triplicate after storage at 4 to 7°C in the refrigerator for 0, 0.25, 0.5, 1, 4, 24 and 48 hours until analysis.

CTC morphology.
Stained and processed CTC samples from two coloncarcinoma and one NSCLC patient were sorted using a FACSaria™. After staining with 0.6 µg/ml Cellmask Orange for 10 min at RT in the dark, samples were washed and centrifuged five times with 1 ml of deionized water (Milli-Q, Millipore), supplemented with 3% FCS. The pellet was resuspended in the remaining 50 µl washbuffer, transferred to a microscope slide and dried by vacuum concentration (SpeedVac). After applying 3 µl of Vectashield H-1000 (Vector) a round object glass of 1 cm in diameter was put on top and sealed with nail polish. CTCs were identified and photographed using an Axiovert 200M epifluorescence microscope (Carl Zeiss).

Statistical Analysis.

Statistical evaluation was performed using either Student's *t*-test or, where indicated, one way analysis of variance (ANOVA) using the software package SPSS (version 15.0; SPSS). *P*-values < 0.05 were considered to be significant.

RESULTS

Method development.

Discrimination of CTCs from the bulk of blood cells was achieved by density gradient centrifugation using CPT tubes. The gel barrier in these tubes separates the blood cells over two compartments. The lower compartment contains red blood cells, granulocytes and the majority of monocytes. The upper compartment consists of plasma, CTCs, PBMCs and platelets. At this stage we exposed the CTCs to a 5 min pulse of EGF, which induced a twofold increase in pERK levels in MCF-7 cells that could be inhibited with erlotinib in a concentration dependent manner (data not shown). Next, cells were treated with 50% methanol for protein denaturation to facilitate detection of pERK with antibodies. Fixation also enabled long-term storage of samples at -80 °C.

Further CTC enrichment from the remaining background was achieved using magnetic anti-human EpCAM-Micro-Beads. In the following steps we deviated from the manufacturer's instructions: for more specific EpCAM binding and higher recovery of samples with low EpCAM levels, samples were first incubated with 40 µl FcR at RT for 1 hour, followed by incubation with 10 µl Micro-Beads for 1 hour at RT. A further 15.7% ± 1.1% increase in recovery was achieved by flushing the CTCs from the MACS® columns with an additional 1 ml of BB. With these modifications the recovery of MOR/P cells was ≥ 75% (Table 1) in a remaining background of 15000 PBMCs and platelets.

Next, the DNA in the cells was stained with Hoechst33342. Unbound stain was removed by washing the cells with Perm/Wash buffer. This buffer contained saponin, which permeabilizes the cell membrane for intracellular pERK staining with antibodies. In order to remove protein aggregates that can bind aspecifically to antibodies, which can result in a false positive CTC count, EpCAM, CD45 and pERK antibody solutions were centrifuged at 10000 x *g* for 10 min at 4°C and all buffers were filtered through 200 nm syringe filters. FcR and EpCAM-Micro-Beads were not centrifuged, because it resulted in a considerably lower antibody concentration and reduced CTC recovery.

Table 1: Recovery, within-day and between-day precision

Nominal spiked cells	A549				MOR/P			
	Measured spiked cells	Recovery (%)	WDP (%)	BDP (%)	Measured spiked cells	Recovery (%)	WDP (%)	BDP (%)
10	5	50.0	11.1	8.9	10	100	12.6	5.0
100	37	37.0	6.9	5.1	75	75.0	6.3	4.1
1000	342	34.2	4.2	5.7	752	75.2	5.5	3.3
10000	4015	40.1	7.1	6.6	7732	77.3	3.1	5.6

Determination of the number of A549 and MOR/P cells spiked in CPT tubes containing 8 ml peripheral blood of a healthy volunteer. Three replicate measurements were performed at each spike level. Within-day (WDP) and between-day (BDP) precision were calculated using one-way analysis of variance (ANOVA)

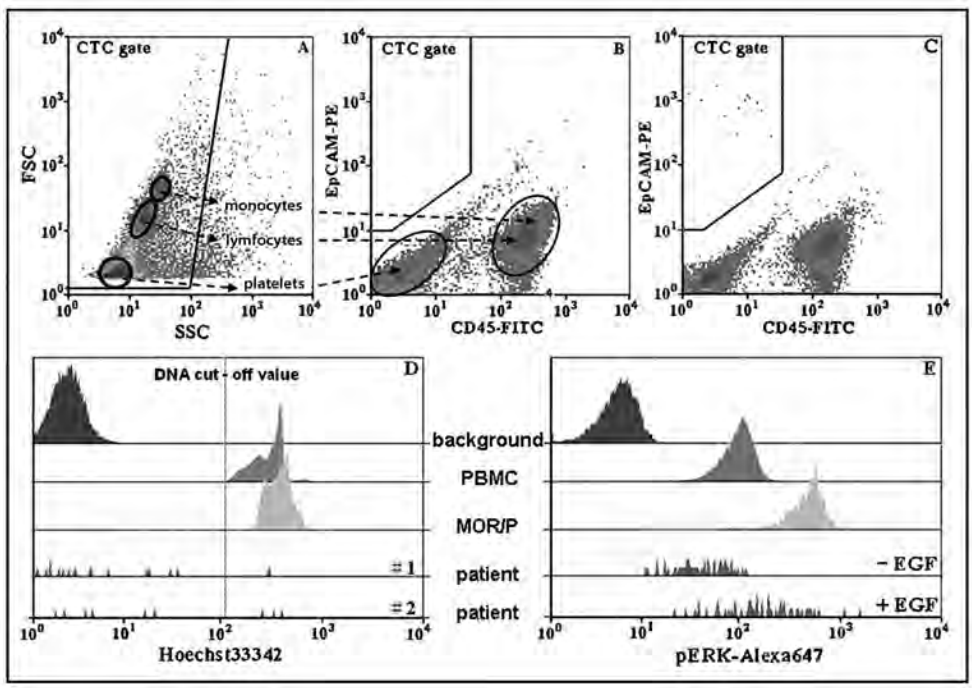

Figure 1: Representative flow cytometry (FACS) density plots with indicated gate settings used for circulating tumor cell (CTC) enumeration and histograms showing biomarker expression levels in CTCs. The shown samples are from a healthy volunteer (A) and different patients (B; D #1 and #2; E +/- EGF). In step 1 (A), CTCs are gated by forward- and side-scatter characteristics for size and cell complexity to eliminate debris. In step 2 (B or C), CTCs are gated by CD45-FITC$^+$ / EpCAM-PE$^+$ double positivity to eliminate platelets and peripheral blood mononuclear cells (PBMCs). In step 3 (D), the DNA content of CTCs is shown for two different patient samples. The phosphorylated- extracellular-signal- regulated-kinase (pERK) levels in CTCs are shown (E) for two samples from a patient treated *ex vivo* with and without EGF. Furthermore, the DNA (D) and pERK (E) content of PBMCs and MOR/P are shown. DNA background staining is shown for platelets and pERK background staining is shown for MOR/P incubated with isotype IgG1-Alexa647.

Identification of the CTCs by FACS using PE against FITC density plots (Figure 1) was achieved after staining CTCs with anti-EpCAM-PE, anti-CD45-FITC and anti-pERK-Alexa647. DNA levels in the CTCs were determined as: (MFI of Hoechst33342 in CTC) – (MFI of Hoechst33342 in platelets). The pERK levels were determined as: (MFI of pERK-Alexa Fluor®647 in CTC) – (MFI of isotype-Alexa Fluor®647 in MOR/P). We determined with a pERK ELISA kit that MOR/P cells with an average MFI of 341 ± 11 contained 50.4 ± 4.6 fg pERK, which enables better inter-laboratory comparison of results by expression of pERK levels in fg/CTC. Results were normalized against two positive control samples that originated from a single large batch of samples each containing 1000 fixed MOR/P cells that were stored at -80°C until analysis. Furthermore, two negative control samples from 8 ml of whole blood from a healthy volunteer were included during each batch analysis.

Specificity.

Total background levels were highest at 0°C using 20 µl FcR for 15 min consisting of a total of $3.7 \times 10^5 \pm 7.6 \times 10^4$ PBMCs and platelets, but decreased significantly ($P < 0.001$) to $1.4 \times 10^4 \pm 6.9 \times 10^3$ when 40 µl FcR was used at RT for 1 hour (Figure 2). We determined that the amount of background counts in the CTC gate during FACS analysis was 0.26 ± 0.29 false positive CTCs per 8 ml peripheral blood (n = 18 healthy volunteers in triplicate).

Cell recovery optimization by anti- human EpCAM–Micro-Beads.

The temperature and incubation time used for Micro-Beads binding had a significant effect on the recovery of A549 cells (Figure3). A recovery 34% of spiked A549 cells was reached after 1 hour incubation with Micro-Beads at RT. No significant increase in recovery was achieved after longer incubation times. Under these conditions, the recovery of MOR/P cells expressing about 50 times more EpCAM than A549 cells was 75 ± 6%.

Within- and between-day precision and recovery.

The precision and recovery of the method were determined by analyses of samples spiked with MOR/P at three different cell concentrations in triplicate in three separate analytical runs. From these results we calculated the within-day and between-day precision and recovery (Table 1). In all cases, the precision and recoveries were well within the limits that are considered acceptable for bio-analytical methods (> 75%). However, the recovery of A549 cells was much lower (> 34%).

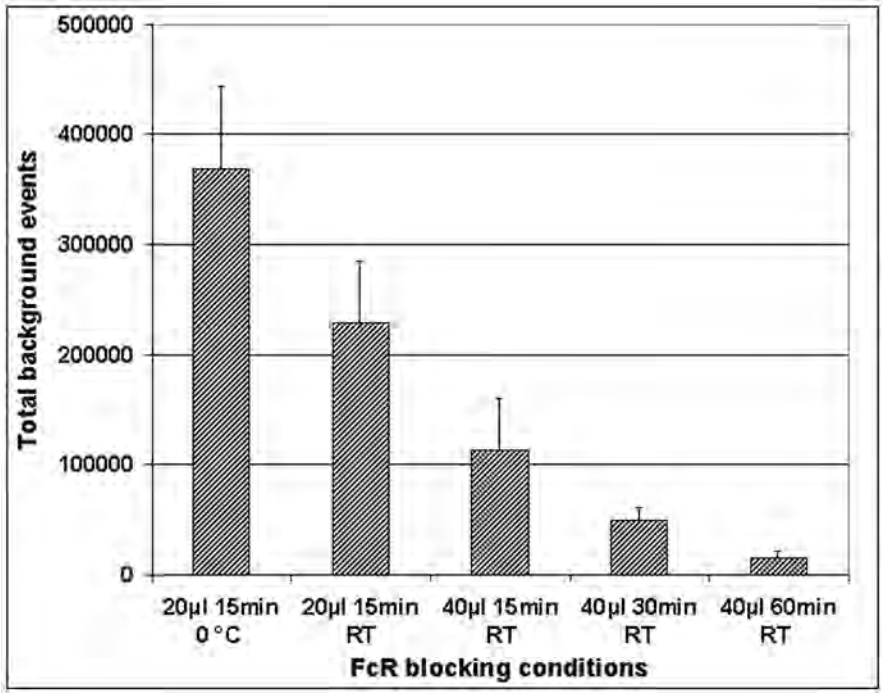

Figure 2: Influence of indicated Fc-receptor blocking conditions at room temperature (RT) on the total number of background events from 8 ml peripheral blood from a healthy volunteer measured in triplicate. Data is expressed as mean ± standard deviation of three different samples.

Lower limit of quantification.

The LLOQ was determined in peripheral blood samples from six different volunteers spiked with a nominal MOR/P cell concentration. The determined LLOQ of the method was 2 cells in 8 ml of whole blood. At this LLOQ the recovery was 108% and the precision 18.8%.

Staining linearity.

Staining linearity was determined in triplicate in samples containing incremental amounts of MOR/P cells spiked in CPT tubes containing 8 ml of peripheral blood of a healthy volunteer. The measured Hoechst33342 staining was not significantly different over the tested 10 – 10000 MOR/P spike range with an MFI of 409 ± 30. Staining of EpCAM and pERK was not significantly effected up to 1000 cells per sample, with an MFI at spike level 10 of $3.5 \times 10^3 \pm 179$ for EpCAM and $3.2 \times 10^2 \pm 11$ for pERK. However, above 1000 cells per sample, staining of EpCAM and pERK was significantly reduced with an MFI at spike level 10000 of $2.6 \times 10^3 \pm 66$ ($P = 0.002$) for EpCAM, and $2.5 \times 10^2 \pm 6$ ($P = 0.003$) for pERK.

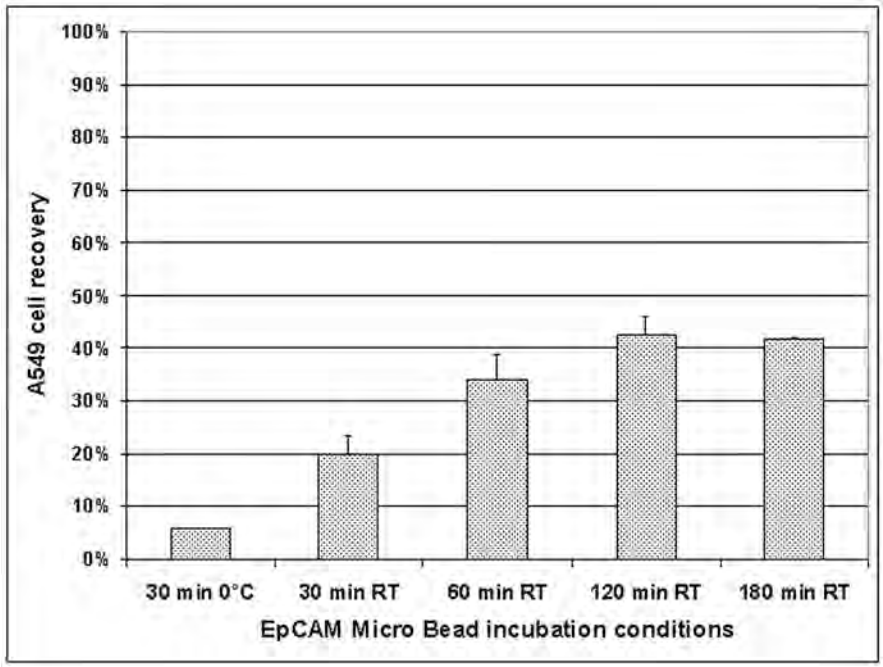

Figure 3: Recovery of A549 cells spiked in 8 ml peripheral blood from a healthy volunteer using incubation with anti-EpCAM Micro-Beads. Data is expressed as mean ± standard deviation of three different samples. RT=room temperature.

Sample stability.

We determined the stability of EpCAM, CD45, DNA and cell counts for MOR/P cells spiked in 8 ml peripheral blood samples after storage at –80°C for incremental time periods. All parameters were stable for at least 6 months, except pERK levels which were stable for 4 months. After 6 months pERK levels were significantly decreased by 10.4% (P = 0.032). Similar results were obtained for the co-isolated lymfocytes and monocytes.

We also assessed the short term stability of stained samples stored at 4°C during incremental time periods. The measured MOR/P cell numbers, levels of Hoechst33342, pERK, EpCAM and CD45 were stable during the tested 48 hour storage period.

CTC morphology.

CTCs from three different patients were processed and sorted by FACS. DNA stained with Hoechst33342 and cell membranes stained with Cellmask Orange were visible by epifluorescence microscopy as blue and orange colors, respectively. The

diameter of the CTCs varied between 4 and 12 µm. In about 50% of the CTCs DNA was detected (Figure 4A), although mostly less and fragmented compared to vital MOR/P cells. In some CTCs membrane blisters were present (Figure 4B), whereas others resembled apoptotic bodies (Figure 4C). However, some CTCs (Figure 4D) had a similar appearance and DNA content as the vital MOR/P control cells.

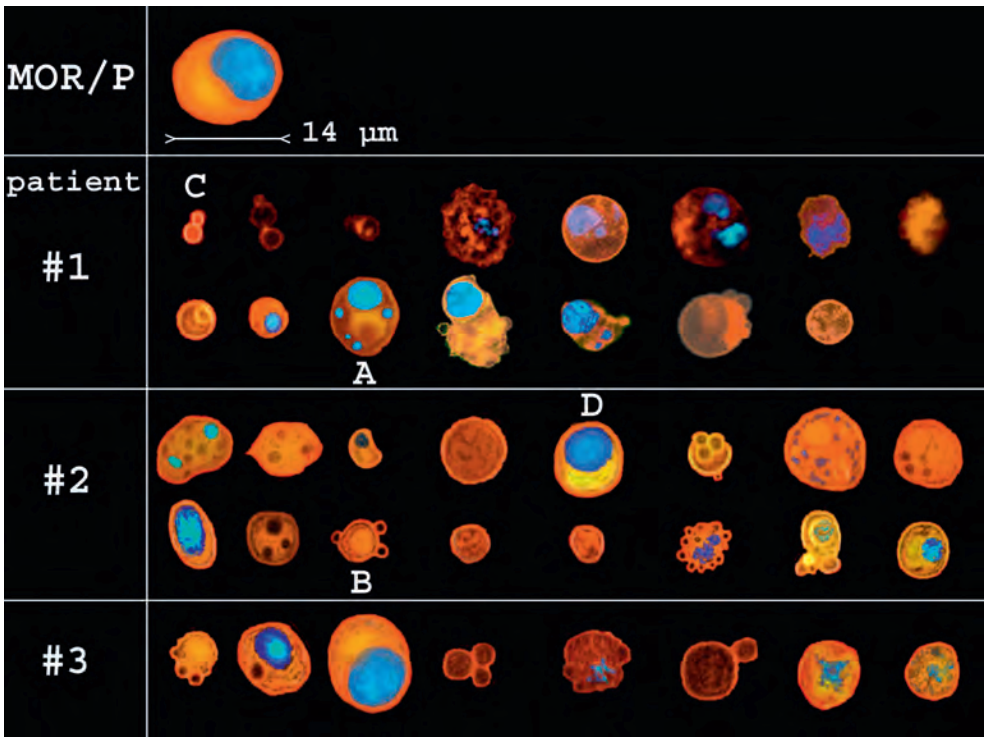

Figure 4: Morphology of circulating tumor cells (CTCs) from two metastatic colon carcinoma patients (# 1 and # 2), and one metastatic non-small cell lung cancer patient (# 3). DNA and cell membranes are visible in blue and orange colors, respectively. An example of a typical vital MOR/P cell is included (top). Imaging was performed with a 100 times magnification factor.

Applicability of method for determination of CTCs and pERK in patient samples. We determined the number of CTCs in 84 samples from 25 metastatic cancer patients consisting of 11 non-small cell lung cancer (NSCLC), 4 colon-, 4 breast-, 3 urothelial-, 2 ovarian cancer and 1 osteosarcoma patient. In 64% of the patients the CTC numbers were above LLOQ with a mean of 12.4 CTCs per sample. All three samples from the osteosarcoma patient tested negative for CTCs. The correlation between the measured CTC numbers in two subsequent CPT tubes was significant (P = 0.02) with a Pearson's correlation coefficient of 0.948 (Figure 5).

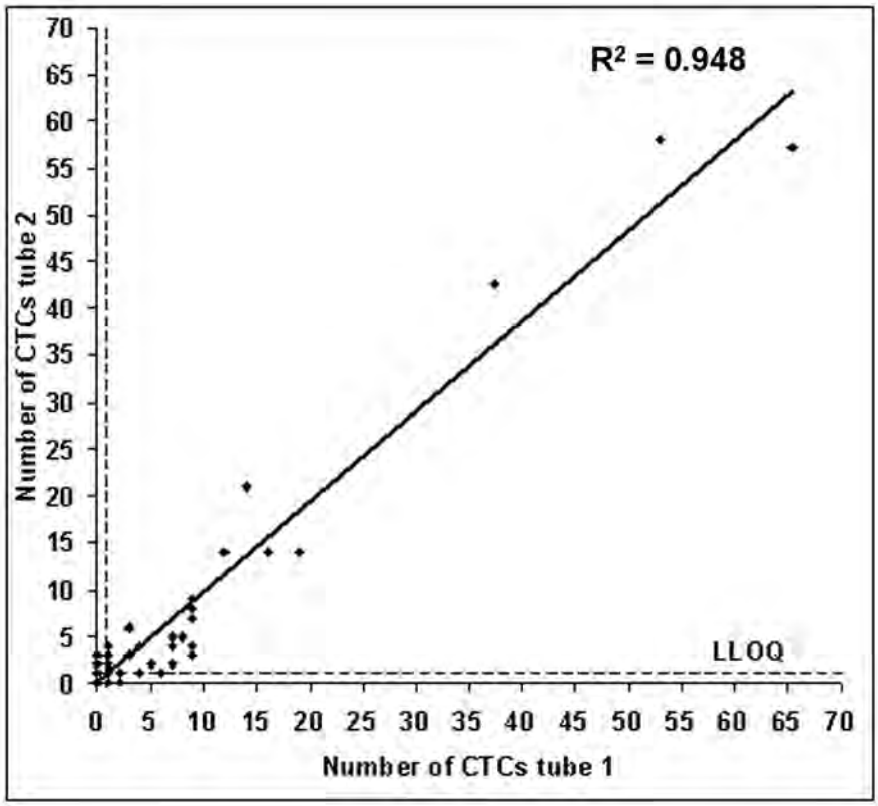

Figure 5: Tube-to-tube variation of circulating tumor cell (CTC) counts in 84 peripheral blood samples from 25 metastatic cancer patients. The lower limit of quantitation (LLOQ) of 2 CTCs per sample is indicated by the dashed lines.

Furthermore, we determined the DNA content and effect of *ex vivo* EGF stimulation on pERK levels in CTCs in triplicate from eight different advanced metastatic patients with the following tumor types: NSCLC (patients 1 and 5), breast cancer (patient 3), bladder cancer (patient 4) and colon cancer (patient 2, 6, 7 and 8). Primary tumor biopsies from all eight patients were screened for mutations in *EGFR* and *K-RAS*. In tumor tissue of patients 1- 5, no mutations were found, but in tumor tissue of patients 6 – 8, activating mutations in *K-RAS* (exon 2) were found. After *ex vivo* EGF stimulation, a significant up-regulation of pERK was observed (Fig. 6) in patients 1-4 with a factor 1.9 (P = 0.011; patient 1), 2.5 (P= 0.024; patient 2), 2.1 (P= 0.035; patient 3) and 3.7 (P=0.010; patient 4). CTCs from patients 5 and an additional sample of patient 8 were obtained while receiving treatment with the EGFR tyrosine kinase inhibitor lapatinib. CTCs from these patients and patients 6 and 7 did not

show a significant increase of pERK following *ex vivo* EGF stimulation (Figure 6). The percentage of "viable" CTCs, with a DNA content in the same range as PBMCs, varied between 7 ± 0.7% (patient 5) and 50 ± 8.5% (patient 2), with an average of 21.9 ± 4.5%.

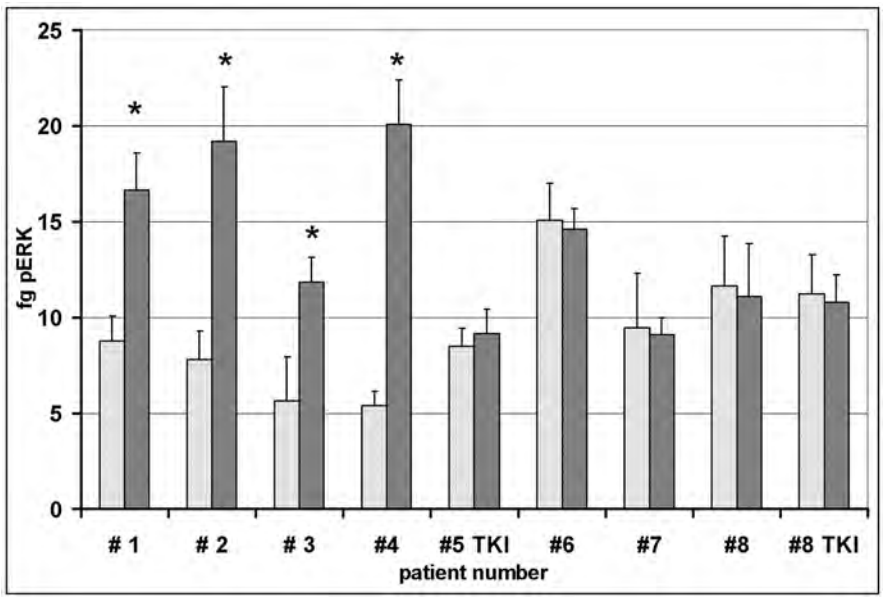

Figure 6: Levels of phosphorylated- extracellular-signal- regulated-kinase (pERK) in circulating tumor cells (CTCs) from eight different advanced metastatic cancer patients with the following tumor types: non-small cell lung cancer (patients 1 and 5), breast cancer (patient 3), bladder cancer (patient 4) and colon cancer (patients 2, 6, 7 and 8). Patients were treated *ex vivo* without (□) or with (▨) 100 ng/ml epidermal growth factor (EGF) for 5 min at 37°C. Patients 1 – 5 screened negative for mutations in *EGFR* and *K-RAS*. Patients 6 – 8 had an activating mutation in *K-RAS*. Additional samples from patients 5 and 8 were from patients that received the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor lapatinib. Samples indicated with an asterix showed a significant increase of pERK after EGF stimulation. Data is expressed as mean ± standard deviation of three different samples.

DISCUSSION

This is the first report to demonstrate *ex vivo* EGFR stimulation and subsequent activation of ERK in CTCs. Furthermore, this is the first immunocytochemical FACS method for the quantification of DNA and pERK in CTCs.

Rapid and routine analysis of large batches of formaldehyde fixed samples was feasible after storage in 50% methanol at -80°C. Fixation is necessary because the time interval and manipulations used to eliminate blood cells have the potential to introduce artifacts [22], as pERK is part of a signaling network that responds to the environment and turns over rapidly.

In order to minimize the number of background events after the MACS® enrichment step, we reduced aspecific binding of the magnetic anti-EpCAM antibodies by increasing the incubation time with a larger amount of Fc-receptor block.

We increased the recovery of cells with low EpCam expression by increasing the incubation time with the magnetic MACS® antibodies at room temperature, by the addition of a second MACS® elution step and by performing all centrifugations in a swing-out rotor. This is important, because cells of high metastatic potential may lose expression of EpCAM during the course of metastatic disease [23-26].

The method was validated using batch-stored fixed MOR/P cells spiked in blood from healthy volunteers. Control MOR/P cells were used for assuring proper performance on a daily and run-to-run basis. We showed that the method was able to recover 75% of MOR/P cells, which was considered acceptable considering the complexity of the assay. We also used A549 cells as a model for low-EpCAM- expressing-cells, considering the 50 times lower EpCAM expression in A549 as compared to MOR/P and still found a reasonable recovery of 34%. In our experience, the EpCAM expression in MOR/P is more representative for the average EpCAM expression in human epithelial tumor cells. The method is accurate and reproducible up to 2 cells per 8 ml peripheral blood (LLOQ). By analyzing different spike levels, we were able to show that the enumeration of CTCs was linear over the whole tested spike range of 10 to 10000 MOR/P cells. The within-run precision was 12.6% and the between-run precision was 5.6%. Furthermore, we showed that the detection of the biomarkers EpCAM, DNA and pERK was linear over a spike range of 10 to 1000 MOR/P cells, which is probably enough for the quantification of biomarkers in CTCs from almost every patient sample.

Long-term stability of samples stored at -80°C was good with no detectable degradation of cell numbers, DNA and pERK after 4 and 6 months, respectively. Furthermore, cell numbers, Hoechst33342 and pERK were shown to be stable in samples stored in the refrigerator for up to 48 hours.

Our results show that the method can be applied for the enumeration of CTCs and the determination of DNA and pERK in CTCs from patient samples. We observed CTCs in 64% of patients, which is in line with other published papers [27;28]. As expected, we did not observe CTCs in the patient with osteosarcoma since EpCAM is not expressed on tumors of mesodermal origin [29].

Our results confirm other reports about CTCs exhibiting a high degree of pleomorphism i.e. high and low nuclear-to-cytoplasmic ratios, and early and late apoptotic changes [30;31]. These apoptotic characteristics might explain why the presence of CTCs is necessary

but not sufficient for the metastatic process to occur. Since there is mounting evidence for the importance of DNA content for the clonogenity and metastatic potential of CTCs [2], we belief that monitoring of this biomarker can improve prediction of clinical outcome for patients. Furthermore, we were able to measure pERK in CTCs isolated from patient samples. We found that the expression of pERK was significantly increased in CTCs after *ex vivo* stimulation of patient samples with EGF. As a proof of principle, no increase of pERK was seen in CTCs isolated from samples of a patient with NSCLC that was being treated with an EGFR tyrosine kinase inhibitor and thus counteracts the stimulatory effect of EGF. The same lack of stimulation with EGF was seen in three patients with a *K-RAS* mutation. Due to this mutation, K-RAS is constitutively active and irresponsive to signals originating from EGFR.

In conclusion, a method has been developed and validated for the enumeration of CTCs and the determination of DNA and pERK in CTCs in peripheral blood. The method is straightforward with long-term stability, using standard laboratory equipment and techniques and is suitable for application in clinical trials. We have shown that the method enables reproducible isolation of CTCs in sufficient quantity and with sufficient purity to allow molecular intracellular analyses for pharmacodynamic measurements. Further research will now be pursued with additional patients.

ACKNOWLEDGEMENTS

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Chapter 6

Conclusions and perspectives

CONCLUSIONS AND PERSPECTIVES

This thesis describes several phase I and translational studies in the development of anti-cancer drugs. The pharmacokinetic studies addressed clinically relevant questions regarding treatment of cancer patients with novel anti-cancer drugs. The translational studies aimed to improve techniques for the detection of circulating tumor cells (CTCs) and explored the utility of these CTCs to be used in drug development. Pharmacokinetics of oral anti-cancer drugs were studied in three phase I trials, of which two were performed with the oral formulation of topotecan. The first study described the safety and pharmacokinetics of co-administration of the oral anti-angiogenic inhibitor pazopanib with oral topotecan. Pharmacokinetic analysis showed that pazopanib increased exposure of topotecan considerably, but, in contrast, topotecan did not increase pazopanib exposure in reverse. This effect was most likely due to the inhibition of breast cancer resistance protein (BCRP) and/or P-glycoprotein (P-gp) mediated intestinal absorption of topotecan by pazopanib. Following the results of the dose-escalation part of the study, the maximum tolerated dose is expected to be daily, continuous pazopanib 800 mg and oral topotecan 8.0 mg on days 1, 8 and 15 in a 28-day cycle and this dose is currently being explored in an expansion cohort. There were two serious adverse events (SAEs) related to the study drug that both resulted in death. This concerned one death due to hemorrhaging and one due to liver failure. Although hemorrhaging is a very unfortunate side effect of anti-angiogenic therapy, the benefits of pazopanib monotherapy as demonstrated in phase III trials, combined with the preliminary anti-tumor efficacy observed in the present interim analysis of combination therapy with oral topotecan, was thought to outweigh the side effects. Regarding hepatotoxicity, advances were made recently in identifying single nucleotide polymorphisms (SNPs) in the hemochromatosis (*HFE*) gene that was found to be associated with reversible aspirate aminotransferase (ALT) elevation in renal cell cancer patients treated with pazopanib [1]. The clinical value of such SNP and other SNPs, that are associated with the occurrence of hepatotoxicity in patients receiving VEGFR-targeting tyrosine kinase inhibitors, is currently unknown and further research could reveal if identification of such SNP in a patient before the start of pazopanib therapy is able to prevent hepatotoxicity by reducing or omitting doses.

The second study described the effect or renal impairment on topotecan exposure after oral administration. We demonstrated that no dose adjustments of oral topotecan were required in patients with both normal renal function (creatinine clearance

(Cl_{cr}) > 50 ml/min) and prior platinum-based chemotherapy or mildly impaired renal function (Cl_{cr} 50- 79 ml/min). Dose adjustments were probably required for patients with moderate and severe renal impairment, but more pharmacokinetic and safety data are awaited before definitive conclusions can be drawn. This study will result in determining the recommended dose of oral topotecan in these patients and this advice can subsequently be used in clinical practice. Still, this does not mean that the perfect dose for every individual with impaired renal function will be known. Bioavailability of oral topotecan has moderate inter-patient variability. This means that the same dose, that may be appropriate for one patient, could cause much higher exposure in another patient, potentially leading to increased toxicity, and a much lower exposure in yet another patient, potentially resulting in decreased therapeutic effect. Only part of this variability can now be explained by the patients' renal function. It is therefore important that further research is pursued in order to identify additional patient characteristics that might explain the remaining variability in exposure in these patients. For example, in the present study, also an effect of ethnicity on topotecan exposure was observed. Other biological factors, such as BCRP and P-gp expression levels may also influence variability in bioavailability. Incorporation of parameters, which influence the variability in exposure, into a dosing advice for topotecan could further improve its safety and efficacy.

Another clinically relevant interaction was demonstrated between the oral tyrosine kinase inhibitor lapatinib and food. Exposure to lapatinib was significantly increased when adminstered 1 hour after a meal, compared to administration of lapatinib 1 hour before a meal. Currently, it is advised to administer lapatinib fasted, being either 1 hour before or after a meal, and this advice should therefore be adjusted according to the results of the present study. Although this seems a simple and logical advice to give patients, it should not be underestimated how difficult compliance can be, for example, due to nausea and vomiting as toxic side effects. Food has the ability to directly affect drug toxicity and efficacy by influencing pharmacokinetics and patients are often not aware of that. In that respect, the clinical relevance of food-lapatinib interaction described here is probably larger than the drug-drug interaction that was observed with pazopanib and topotecan or any other interaction, for example at the level of CYP3A, because the latter ones can mostly be avoided while prescribing the drug. Therefore, as an increasing number of anti-cancer therapies are based on oral and not intravenous administration, which are frequently administered continuously at home, potential food-drug interactions of a new drug should be carefully explored. In this thesis, three phase I pharmacokinetic studies were conducted with

intravenously administered eribulin mesylate, a non-taxane microtubule dynamics inhibitor. Firstly, we demonstrated that hepatic impairment had a significant effect on eribulin exposure. We recommended a dose reduction when treating patients with mild or moderate hepatic impairment with eribulin (1.1 mg/m^2 or 0.7 mg/m^2, respectively, on days 1 and 8 of a 21-day cycle). This advice can be put into clinical practice directly because eribulin was recently approved by the European Medicines Agency and the U.S. Food and Drug administration for treatment of metastatic breast cancer patients following the results of a phase III trial. Hepatic function was estimated using the Child-Pugh classification which is the method that is currently recommended in regulatory guidelines. Others have used measurements of endogenous substances affected by the liver, including bilirubin, albumin and prothrombin time, or elimination rates of exogenous markers, including galactose, monoethylglycine-xylide, antipyrine, indocyanine green and dextromethorphan in attempts to develop a model that improves prediction of pharmacokinetics in patients with liver impairment compared to the Child-Pugh score system. However it has not been clearly demonstrated that the use of one of these tests outperformes the Child-Pugh classification. Next to traditional non-compartmental pharmacokinetic analysis, population pharmacokinetic-pharmacodynamic (PK-PD) models may also improve further development of anti-cancer drugs in this type of patients. Preferably, once a PK-PD model is constructed, it should be explored in a direct comparison if such PK-PD model is able to better predict drug exposure in a given patient with an impaired hepatic function than the Child-Pugh classification.

Next, two studies focused on drug-drug interactions with eribulin at the level of cytochrome P450 (CYP) 3A4. Eribulin was co-administered with either oral ketoconazole, a CYP3A4 inhibitor, or rifampicin, a CYP3A4 inducer. We demonstrated that exposure of eribulin was not different when co-administered with ketoconazole or rifampicin. The results indicated that eribulin can be safely co-administered with ketoconazole or rifampicin. In clinical practice, concomitant use with other CYP3A4 inhibitors or inducers is probably also justified but should be administered with care. Other potentially clinically relevant drug-drug interactions that should be further explored are at the level of BCRP and P-gp, both efflux transporters that are also located at the blood-brain barrier. It is currently unknown if eribulin is a substrate for BCRP and this should be further explored. It is known that eribulin is a substrate of P-gp. The interaction of eribulin with P-gp could have clinical relevance in reducing anti-tumor efficacy in the brain because penetration across the blood-brain barrier is limited. Theoretically, this issue could be overcome by co-administration of a P-gp

and/or BCRP inhibitor, or by altering the molecular structure of eribulin resulting in decreased susceptibility for P-gp [2], but safety should be carefully monitored when investigating such combinations or compounds.

Drug development is a lengthy and costly process. After pre-clinical development of a new drug using *in vitro*- testing and animal studies, further development can be pursued in human volunteers or patients in phase I trials in which safety, tolerability and pharmacokinetics are evaluated. If safety in humans is confirmed, the drug can be investigated in phase II in order to observe signs of anti-tumor activity. Ultimately, anti-tumor activity should be demonstrated in a phase III trial.
It has been estimated that a novel anti-cancer drug entering phase I has a 5% chance of reaching registration, making the drug available to patients by prescription. Of all oncology drugs that currently enter phase II, approximately 70% undergo attrition, which is most frequently due to lack of efficacy (30%) [3]. Because of the need for additional drugs in order to improve cancer treatment and the high costs involved in drug development, it is necessary to improve drug development.
In order to achieve a higher success rate for registration of new drugs, demonstration of actual pharmacological activity and signs of anti-tumor activity should already be pursued in early clinical trials by means of pharmacodynamic biomarkers. Pharmacodynamic biomarkers measure a pharmacological effect of a drug on its target, for example by measuring altered activity or expression of a protein in the target tissue. In order to early identify anti-tumor efficacy, such biomarkers should be developed in parallel with the preclinical development and, once validated, be applied as early as in phase I [4]. During phase I, the pharmacodynamic biomarker should confirm a pharmacological effect of the drug on the target tissue, in order to subsequently proceed with development in phase II. Implementation of pharmacodynamic biomarkers in early clinical studies, with a requirement for demonstrating anti-tumor efficacy, should improve further development and should ultimately increase the success rate for registration of new drugs.
Several translational studies described in this thesis reported on detection of CTCs. An immunomagnetic bead enrichment technique, combined with multi-marker QPCR was developed for detection of CTCs in peripheral blood of non-small cell lung cancer (NSCLC) and breast cancer patients. Assay sensitivity and specificity were demonstrated. Next to this, we showed that detection of CTCs in peripheral blood of early-stage breast cancer patients predicted for a worse progression-free survival.
The advantage of using QPCR in these assays was the high sensitivity in detecting

the rare CTCs in a background of peripheral blood cells (approximately 1 CTC in 1.0 x 10^6). The disadvantage of QPCR is that cells have to be lysed in order to obtain mRNA for analysis. This way, specific cellular characteristics are lost, such as the localisation of a specific protein of interest on the membrane or the phosphorylation (or: "activation") state of a protein. These characteristics are specifically interesting when developing a pharmacodynamic biomarker. With the help of new techniques in flow cytometry and the development of new anti-bodies, in another study, further characterization of CTCs was achieved in intact, fixed cells. The goal of this study was to measure actual pharmacological effects of a drug on CTCs and to determine if this could be used as a pharmacodynamic biomarker. The phosphorylation state of the intracellular protein ERK (extracellular-signal-regulated kinase) was measured as a marker for activation of the epidermal growth factor receptor (EGFR) signalling transduction cascade in CTCs and the method was successfully validated. ERK is a protein located "down-stream" of the EGFR signalling transduction cascade and its activation promotes cell growth and survival. This proof-of-principle study demonstrated that pharmacodynamic changes in activation of EGFR after stimulation or inhibition of the pathway could be measured in CTCs. Further research will now first be performed to determine pharmacodynamic changes in pERK levels in CTCs of additional cancer patients obtained before and during EGFR-targeting therapy. Such study will have to confirm if activation or inhibition of ERK measured by flow cytometry in CTCs indeed reflects activation or inhibition of the whole EGFR-pathway. It can be hypothesized that, if a patient develops resistance to EGFR-tyrosine kinase inhibitors in a later phase of treatment, activation of other proteins that are possibly located "parallel" of ERK could promote cellular survival irrespective of ERK. In that case, the pharmacodynamic assay described here would show inhibitin of ERK by a drug, but the CTCs would actually have become resistant to the drug. If the protein responsible for the development of resistance becomes known, the assay should be adapted for measurement of activity of that specific protein.

Next to these aspects, it should be established if detection of a pharmacodynamic effect in CTCs serves as a surrogate for pharmacodynamic effect in actual tumor tissue and its metastases. Ideally, in order to address this question, sampling for CTCs and tumor biopsies in patients should be performed simultaneously and the results of pharmacodynamic measurements should be correlated to each other.

Both the QPCR assay and the flow cytometry assay are based on positive selection of EpCAM-positive cells. It has been shown recently that some CTCs posses a different, more stem-cell like and EpCAM-low or –negative phenotype. These cells

are assumed to have a different, more aggressive biological behaviour. These CTCs are also thought to be responsible for development of resistance to anti-cancer therapy. This finding implicates that some CTCs may not be suitable as a surrogate marker for tumor tissue, while others may, and that CTC detection techniques such as the techniques demonstrated in this thesis may require further refinement with other markers in order to enable detection of these specific CTCs and subsequent pharmacodynamic measurements. Ultimately, it should be investigated which level of pharmacodynamic drug effect (for example, inhibition of ERK phosphorylation), achieved by what dose of drug, is required to establish actual anti-tumor efficacy. Such finding could enable dose recommendations for further research in phase II.

In conclusion, several phase I and exploratory studies were reported in this thesis. The results obtained from the pharmacokinetic studies directly contribute to the pharmacotherapeutic treatment of patients with advanced cancer, including patients with advanced cancer and impaired renal- or hepatic function. While some drug-drug interactions could be excluded, other drug-drug or drug-food interactions were demonstrated. This knowledge can be used in clinical practice in order to improve safety and anti-tumor efficacy of anti-cancer therapies. Next to this, exploratory technical work was performed to improve CTC detection in peripheral blood of breast cancer and NSCLC patients. The utility of these CTCs to be used as actual pharmacodynamic biomarkers was also investigated. A pharmacodynamic assay in CTCs in peripheral blood of cancer patients measuring levels of activation of intracellular proteins in CTCs was developed and validated. Further research and validation will reveal if pharmacodynamic measurements in CTCs will indeed contribute to drug development by implementation of these techniques in early clinical oncological trials.

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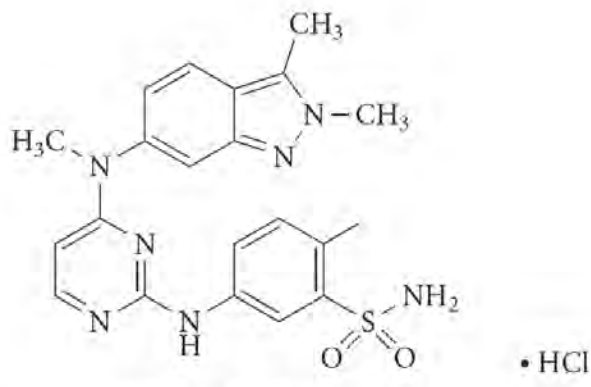
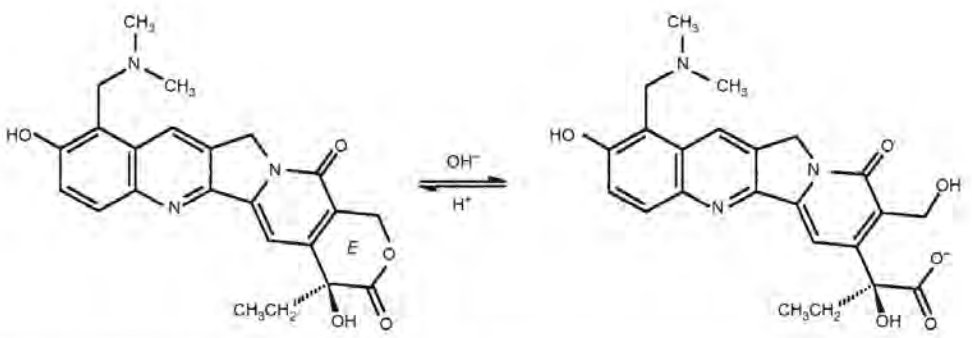
Appendix

Chemical structures of anti-cancer drugs investigated in this thesis

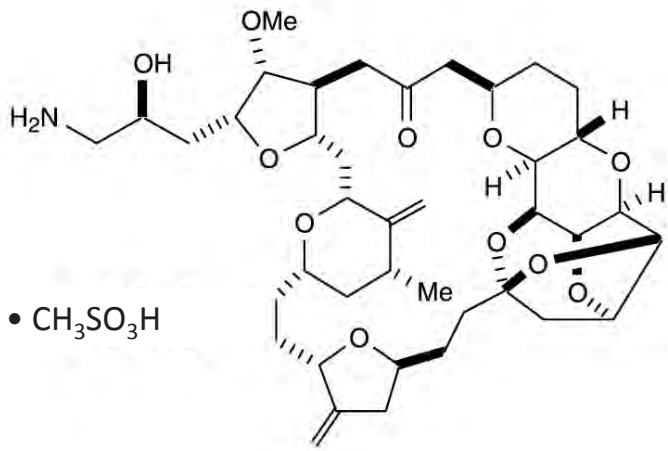
Pazopanib (chapter 2.1)



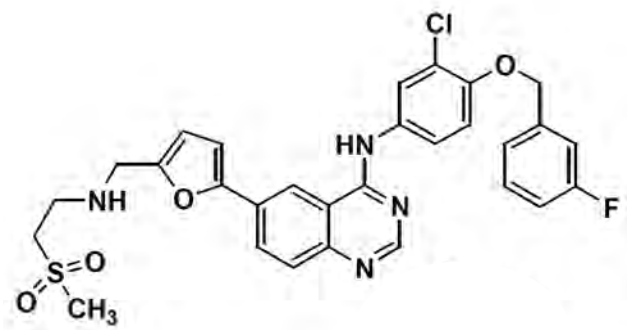
Topotecan (chapters 2.1 and 2.2)



Eribulin mesylate (chapters 3.1 - 3.3)



Lapatinib (chapter 4.1)



Summary

SUMMARY

Cancer has an enormous impact on the lives of patients and on society. Despite recent advances in the treatment of cancer, 1.2 million people still die as a result of cancer within the European Union annually. Therefore, there is an urgent need for further improvements in therapy. This thesis is focused on early clinical studies and examines pharmacokinetics of novel anti-cancer drugs and the utility of circulating tumor cells to be used in drug development.

In the first part of the thesis, several phase I studies evaluating novel anti-cancer drugs are described (chapters 2-4). In these studies, small groups of patients with advanced solid tumors received a specific dose of an anti-cancer drug in order to investigate safety and to evaluate the effects the body has on the drug ("pharmacokinetics") by studying drug concentrations in blood and urine samples. In some studies, specific patient categories were studied, such as those with impaired renal (chapter 2.2) and hepatic function (chapter 3.1). In other studies, the investigational drug was combined with other drugs (chapters 2.1, 3.2 and 3.3) or with food (chapter 4.1) in order to establish the effect of these combinations on safety and pharmacokinetics. The results of these studies led to dosing recommendations that can be translated into clinical practice directly.

Chapter 2 describes two studies evaluating the oral formulation of topotecan. Topotecan is a drug that interferes with cellular DNA replication, leading to cell death. This drug was combined with pazopanib, a novel anti-cancer drug that targets new blood-vessel formation (chapter 2.1). The combination of pazopanib and topotecan was evaluated because preclinical data indicate that this combination may result in more effective anti-cancer treatment than either formulation alone. Our pharmacokinetic analysis showed that pazopanib increased the exposure to topotecan considerably, whereas topotecan did not increase exposure to pazopanib. This effect is most likely caused by the fact that pazopanib inhibits Breast Cancer Resistance Protein and/or P-glycoprotein. These two proteins are located in the intestinal mucosa where they act to limit the uptake of drugs like topotecan and pazopanib under physiological conditions. We hypothesize that limiting the uptake of topotecan into the bloodstream is hampered by pazopanib, thereby leading to increased exposure to topotecan. Following analysis of the safety results, the maximum-tolerated dose is predicted to be daily continuous pazopanib 800 mg and topotecan 8.0 mg on days 1, 8 and 15 in a 28-day cycle and this schedule is currently under further investigation. Dose-limiting

toxicities were hand-foot-syndrome, diarrhea and neutropenia. The most frequently reported treatment-related side-effects were anemia, leukocytopenia, neutropenia and fatigue. Next, chapter 2.2 describes a study with oral topotecan in patients with renal impairment. The aims of this study were to study safety of oral topotecan and to establish dosing recommendations in patients with mild, moderate and severe renal impairment. Because prior platinum-based chemotherapy may also affect topotecan pharmacokinetics in patients with normal renal function, safety and pharmacokinetics are also investigated in these patients. As this study is currently still ongoing, the results for patients with moderate and severe renal impairment are awaited, but the data currently available demonstrate that no dose adjustments are required in patients with prior platinum-based chemotherapy or mildly impaired renal function (creatinine clearance > 50 ml/min, dose for both: 2.3 mg/m^2/day on days 1 to 5 in a 21-day cycle). Dose-limiting toxicities consisted of neutropenia, leukocytopenia, thrombocytopenia and fatigue.

Chapter 3 describes three pharmacokinetic studies with eribulin mesylate, an intravenous drug derived from a marine sponge that interferes with cellular replication and division, leading to cell death. Previous preclinical studies in animals have demonstrated that eribulin is mainly eliminated by the liver via excretion into bile. The registered dose for patients without liver impairment is 1.4 mg/m^2. In chapter 3.1, eribulin dosing was investigated in patients with an impaired liver function, as classified by the Child-Pugh classification. The following dose recommendations were established: 1.1 mg/m^2 for mild (Child-Pugh A) and 0.7 mg/m^2 for moderate (Child-Pugh B) liver impairment on days 1 and 8 of a 21-day cycle. In chapters 3.2 and 3.3, the effects of co-administration of ketoconazole and rifampicin, respectively, on eribulin pharmacokinetics were investigated. These drugs were studied as model inhibitor and inducer of the metabolizing enzyme cytochrome P450 (CYP) 3A4, which is thought to be the main enzyme responsible for eribulin metabolism. The results indicated that eribulin can be safely co-administrated with these drugs and that drug-drug interactions with other CYP3A4 inhibitors or inducers are not expected. The most frequently reported treatment-related side-effects in these three studies with eribulin were alopecia, fatigue, nausea, leukocytopenia and neutropenia.

In chapter 4.1, we investigated the pharmacokinetic interaction between food and lapatinib, an oral tyrosine kinase inhibitor targeting the Epidermal Growth Factor Receptor 1 and 2 (EGFR and HER2). We observed that food, consumed one hour before lapatinib administration, increased lapatinib exposure (area under the curve, AUC_{0-24}: 1.80-fold [90% confidence interval (CI): 1.37 – 2.37; P = 0.005] for a low-fat

meal and 2.61-fold [90% CI: 1.98 – 3.43; P = 0.002] for a high fat meal), compared to consumption of a low-fat meal one hour after lapatinib dosing. When aiming to reduce day-to-day intra-patient variability in lapatinib exposure, it is advised to administer the drug one hour before a meal and not one hour after a meal.

The second part of this thesis (chapter 5) focused on circulating tumor cells (CTCs). Recent developments have led to techniques that can not only quantify the number of CTCs in peripheral blood samples from cancer patients, but are also able to characterize specific proteins on CTCs. This approach facilitates the evaluation of pharmacodynamics of an anti-cancer drug targeting these specific proteins on CTCs. In future drug development, this method could be used to confirm a specific mechanism-of-action of a drug and could perhaps also guide the selection of optimal dosing schedules of a drug under development. In chapter 5.1, the utility of CTCs as pharmacodynamic biomarker in the development of anti-cancer drugs was reviewed. We concluded that there are many interesting and encouraging developments for CTC characterization that may lead to incorporation of CTCs as pharmacodynamic biomarkers in anti-cancer drug development. Standardization and prospective validation of these assays is essential before they can be used in this field. In chapters 5.2 and 5.3 CTC detection was explored in early-stage breast cancer patients and advanced non-small cell lung cancer (NSCLC) patients, respectively. CTCs were detected by multi-marker quantitative polymerase chain reaction in peripheral blood samples from 20% of 82 early-stage breast cancer patients. Presence of CTCs predicted a significantly worse relapse-free survival (multivariate hazard ratio = 5.13 [95% CI: 1.62 – 16.31, P = 0.006]), compared to absence of CTCs. An exploratory study on the detection of CTCs in advanced NSCLC patients and healthy controls resulted in the development of an assay with a sensitivity of 46% (95% CI: 31 – 61) and a specificity of 93% (95% CI: 82 – 99) to detect the presence of CTCs. This assay requires further validation in a patient series with controls matched for smoking. In chapter 5.4, another assay for CTC detection was developed and validated using fluorescence activated cell sorting (FACS). This technique enabled measurements of specific proteins in CTCs and their level of activation. CTC morphology and DNA content were also studied. Pharmacodynamics of activation of a specific protein (extracellular-signal-regulated kinase; ERK) down-stream in the EGFR signaling transduction cascade, as a marker for EGFR pathway inhibition, was also studied in CTCs of patients who were receiving an anti-cancer drug inhibiting activation of EGFR. This proof-of-principle demonstrated that CTCs can be used for pharmacodynamic analyses which can be applied in early-stage drug development.

In summary, both phase I trials with novel anti-cancer drugs as well as exploratory studies into dynamics of CTCs are reported in this thesis. Dosing recommendations were established for oral topotecan in patients with renal impairment and for eribulin in patients with hepatic impairment. Clinically relevant drug-drug interactions were observed between pazopanib and oral topotecan and also between lapatinib and food, when consumed an hour after lapatinib dosing. Drug-drug interactions were not observed between eribulin and the CYP3A4 inhibitor ketoconazole or the inducer rifampicin. These observations can be put to use in dosing recommendations for use in clinical practice, which should lead to reduced drug toxicity. Technical advancements in the detection of CTC in peripheral blood samples of cancer patients were achieved and we also provided proof for the concept that CTCs can be used as pharmacodynamic biomarkers. The implementation of such biomarker may facilitate the future development and evaluation of novel anti-cancer drugs, which will hopefully will lead to improved outcome for cancer patients.

Samenvatting

SAMENVATTING

Kanker heeft enorme impact op het leven van patiënten en op de maatschappij in het algemeen. Ondanks recente vooruitgang in de behandeling van kanker, overlijden in de Europese Unie nog steeds 1.2 miljoen mensen per jaar aan de gevolgen van kanker. Daarom blijft het belangrijk om de bestaande therapieën verder te verbeteren en nieuwe therapieën te ontwikkelen. Dit proefschrift beschrijft vroeg-klinische studies en onderzoekt de veiligheid en famacokinetiek van nieuwe anti-kanker geneesmiddelen en de bruikbaarheid van circulerende tumor cellen om gebruikt te worden in de ontwikkeling van geneesmiddelen.

Het eerste deel van het proefschrift bevat meerdere "fase I" studies waarin nieuwe anti-kanker geneesmiddelen worden geëvalueerd (**hoofdstukken 2-4**). In deze studies kregen kleine groepen patiënten met gevorderde solide tumoren een specifieke dosis van een anti-kanker middel om de veiligheid te onderzoeken en worden concentraties van het middel bepaald in bloed- en urine monsters om de effecten te kunnen beschrijven die het lichaam heeft op het medicijn ("farmacokinetiek"). In twee studies werden patiënten met nierinsufficiëntie (**hoofdstuk 2.2**) en patiënten met leverlijden (**hoofdstuk 3.1**) bestudeerd om te kijken wat het effect van deze comorbiditeit is op de farmacokinetiek van de geneesmiddelen. In andere studies werden het effect van combinaties van anti-kanker geneesmiddelen (**hoofdstukken 2.1, 3.2** en **3.3**) en het effect van voedsel (**hoofdstuk 4.1**) onderzocht op de veiligheid en farmacokinetiek van deze middelen. De resultaten van deze studies hebben geleid tot doseringsadviezen die direct in de klinische praktijk toegepast kunnen worden.
Hoofdstuk 2 beschrijft twee studies naar het anti-kanker medicijn topotecan voor orale inname. Topotecan is een medicijn dat de cellulaire DNA replicatie beïnvloedt hetgeen leidt tot celdood. Dit medicijn werd gecombineerd met pazopanib, een nieuw anti-kanker middel dat gericht is tegen bloedvat nieuwvorming (**hoofdstuk 2.1**). De combinatie van pazopanib en topotecan werd onderzocht omdat pre-klinisch onderzoek aangaf dat deze combinatie mogelijk resulteert in een effectievere anti-kanker behandeling dan een van deze middelen apart. Onze farmacokinetische analyse toonde aan dat pazopanib de blootstelling aan topotecan aanzienlijk verhoogde, terwijl topotecan dat niet deed bij pazopanib. Dit effect wordt waarschijnlijk veroorzaakt doordat pazopanib twee eiwitten kan remmen: "Breast Cancer Resistance Protein" en/of "P-glycoprotein". Deze twee eiwitten zijn gelokaliseerd in het darmslijmvlies, waar zij onder fysiologische omstandigheden de opname van stoffen

als topotecan en pazopanib kunnen remmen. Wij veronderstellen dat de opname van topotecan in de bloedstroom minder wordt tegengegaan door de remmende werking van pazopanib op deze eiwitten. Dit leidt daardoor tot een grotere blootstelling aan topotecan. Naar aanleiding van de veiligheidsresultaten wordt verondersteld dat de maximaal tolereerbare dosis als volgt zal zijn: dagelijks pazopanib 800 mg en op dag 1,8 en 15 topotecan 8.0 mg in een 28-dagen durende cyclus. Dit schema wordt op dit moment nog verder onderzocht. Dosislimiterende bijwerkingen waren hand-voet-syndroom, diarrhee en neutropenie. De meest frequent gerapporteerde bijwerkingen gerelateerd aan de behandeling waren anemie, leukopenie, neutropenie en vermoeidheid. Vervolgens beschrijft **hoofdstuk 2.2** een studie met oraal topotecan voor patiënten met nierinsufficiëntie. De doelen van deze studie waren om de bijwerkingen te beschrijven en om doseringsadviezen voor topotecan te kunnen geven voor patiënten met een milde, matige en ernstige nierinsufficiëntie. Omdat de farmacokinetiek van topotecan mogelijk ook beïnvloed wordt door behandeling met platinum-bevattende geneesmiddelen in het verleden, zelfs bij patiënten met een normale nierfunctie, werden veiligheid en farmacokinetiek van topotecan ook bij deze patiënten onderzocht. Omdat deze studie op dit moment nog niet afgerond is, zijn de resultaten voor patiënten met matige en ernstige nierinsufficiëntie nog niet bekend. Wel werd aangetoond dat er geen dosisreductie nodig is voor patiënten die eerder zijn behandeld met platinum-bevattende therapie of voor patiënten met een milde nierinsufficiëntie (creatinine klaring > 50 ml/min; dosering voor beide: 2.3 mg/m^2/dag op dag 1 tot en met 5 in een cyclus van 21 dagen). Dosislimiterende bijwerkingen bestonden uit neutropenie, leukopenie, trombopenie en vermoeidheid. **Hoofdstuk 3** beschrijft drie farmacokinetische studies met eribuline mesylate, een intraveneus medicijn afgeleid van een zeespons, dat interfereert met celreplicatie en celdeling hetgeen leidt tot celdood. Eerdere preklinische studies hebben laten zien dat de eliminatie van eribuline voornamelijk plaatsvindt via de lever door middel van galuitscheiding. De geregistreerde dosis voor patiënten zonder leverlijden is 1.4 mg/m^2. In **hoofdstuk 3.1** werd de dosering van eribuline onderzocht in patiënten met leverlijden, geclassificeerd volgens de Child-Pugh classificatie. Op basis van de resultaten kunnen de volgende doseringen worden geadviseerd: 1.1 mg/m^2 voor patiënten met mild (Child-Pugh A) en 0.7 mg/m^2 voor patiënten met matig (Child-Pugh B) lever lijden op dagen 1 en 8 in een cyclus van 21 dagen. In **hoofdstukken 3.2** en **3.3** werden de effecten onderzocht van gelijktijdige toediening van eribuline en respectievelijk ketoconazole en rifampicine op de farmacokinetiek van eribuline. Deze medicijnen werden gebruikt als model om remming en inductie van het

metaboliserende enzym cytochroom P450 (CYP) 3A4 te bestuderen, omdat dit waarschijnlijk het belangrijkste enzym is dat verantwoordelijk is voor het metabolisme van eribuline. De resultaten wijzen erop dat eribuline veilig kan worden toegediend in combinatie met deze medicijnen en dat interacties met ander medicijnen die CYP3A4 remmen of induceren, niet worden verwacht. De bijwerkingen die het vaakst werden gerapporteerd als gerelateerd aan de behandeling waren haaruitval, vermoeidheid, misselijkheid, leukopenie en neutropenie.

In **hoofdstuk 4.1** werd de farmacokinetische interactie onderzocht tussen voedselinname en lapatinib, een orale tyrosine kinase remmer gericht tegen de Epidermale Groei Factor Receptor 1 en 2 (EGFR en HER2). De blootstelling aan lapatinib was hoger wanneer lapatinib werd ingenomen een uur na voedselinname dan een uur voor een maaltijd ("oppervlakte onder de curve", AUC_{0-24}: 1.80-maal [90% confidentie interval (CI): 1.37 – 2.37; P = 0.005] voor een maaltijd met weinig vet en 2.61-maal [90% CI: 1.98 – 3.43; P = 0.002] voor een maaltijd met veel vet). Wanneer het doel is om de dag-tot-dag variatie in lapatinib blootstelling te beperken voor de individuele patiënt, dan wordt geadviseerd om het medicijn een uur voor de maaltijd in te nemen en niet een uur na de maaltijd.

Het tweede deel van het proefschrift (**hoofdstuk 5**) gaat over circulerende tumor cellen (CTCs). Recente ontwikkelingen hebben geleid tot nieuwe technieken die het niet alleen mogelijk maken om het aantal CTCs in perifeer bloed van patiënten met kanker te kwantificeren, maar ook om specifieke eiwitten op CTCs the karakteriseren. Op deze manier wordt het wellicht mogelijk om de farmacodynamiek te evalueren van anti-kanker middelen die gericht tegen zulke specifieke eiwitten op CTCs. Deze methode zou in de toekomst gebruikt kunnen gaan worden bij de ontwikkeling van nieuwe medicijnen om een specifiek werkingsmechanisme van een middel te bevestigen en zou mogelijk ook kunnen dienen om een optimaal doseringsschema te bepalen van een middel in ontwikkeling. In **hoofdstuk 5.1** werd de bruikbaarheid van CTCs als farmacodynamische biomarker in de ontwikkeling van nieuwe anti-kanker middelen in kaart gebracht. We concluderen dat er veel interessante en bemoedigende ontwikkelingen zijn op het gebied van CTC kwantificering en karakterisering, die mogelijk kunnen gaan leiden tot de daadwerkelijke toepassing van CTCs als farmacodynamische biomarkers in de ontwikkeling van anti-kanker medicijnen. Standaardisatie en prospectieve validatie van deze assays zijn essentieel voordat deze toegepast kunnen worden in dit veld. In **hoofdstukken 5.2** en **5.3** werd de detectie van CTCs onderzocht in respectievelijk patiënten met vroeg-stadium

borstkanker en in patiënten met gevorderd niet-kleincellig long kanker (NSCLC). CTCs werden aangetoond in het perifere bloed van 20% van 82 patiënten met vroeg-stadium borstkanker. Dit werd gedaan met behulp van een multi-marker kwantitatieve "polymerase chain reaction" techniek. De aanwezigheid van CTCs was voorspellend voor een significant slechtere progressie-vrije overleving (multivariate "hazard ratio" = 5.13 [95% CI: 1.62 – 16.31, *P* = 0.006]), vergeleken met afwezigheid van CTCs. Een studie bij patiënten met gevorderd NSCLC en gezonde vrijwilligers als controlegroep resulteerde in de ontwikkeling van een assay voor het detecteren van CTCs met een gevoeligheid van 46% (95% CI: 31 – 61) en een specificiteit van 93% (95% CI: 82 – 99). Voor verdere ontwikkeling van deze assay is aanvullend validatie onderzoek vereist in patiënten en controles gematcht voor roken. In **hoofdstuk 5.4** werd een andere assay ontwikkeld en gevalideerd voor de detectie van CTCs met behulp van "fluorescence activated cell sorting" (FACS). Deze techniek maakte het mogelijk om specifieke eiwitten in CTCs te meten inclusief het niveau van activatie van deze eiwitten. CTC morfologie en DNA inhoud werden ook bestudeerd. Farmacodynamiek in activatie van een specifiek eiwit "lager" gelegen in de EGFR-signaal transductie cascade ("extracellular-signal-regulated kinase"; ERK), als marker voor remming van EGFR-signalering", werd bestudeerd in CTCs van patiënten die behandeld werden met een anti-kanker middel dat EGFR remt. Deze studie bevestigde het principe dat CTCs inderdaad gebruikt kunnen worden voor farmacodynamische analyses die toegepast zouden kunnen worden in de vroege ontwikkeling van medicijnen.

Samengevat worden in dit proefschrift zowel fase I studies met nieuwe anti-kanker middelen als exploratieve studies naar de dynamiek van CTCs beschreven. Doseringsadviezen werden opgesteld voor de orale vorm van topotecan bij patiënten met nierinsufficiëntie en voor eribuline bij patiënten met leverlijden. Er werden klinisch relevante interacties werden aangetoond tussen pazopanib en orale topotecan en tussen lapatinib en voedsel, wanneer dit een uur na de lapatinib werd geconsumeerd. Er werden geen interacties aangetoond tussen eribuline en de CYP3A4-remmer ketoconazol en de inductor rifampicine. Deze observaties kunnen direct worden toegepast in de klinische praktijk als doseringsadviezen, hetgeen zal leiden tot minder toxiciteit. Ook werden enkele studies verricht waarin de technieken voor de detectie van CTCs in perifeer bloed van patiënten met kanker werden verbeterd. Verder toonden we ook aan dat CTCs kunnen worden toegepast als farmacodynamische biomarkers. De implementatie van dergelijke biomarkers zou de ontwikkeling van toekomstige anti-kanker middelen kunnen vergemakkelijken,

wat er hopelijk toe zal leiden dat behandelingen en prognose voor patiënten met kanker in de toekomst zullen verbeteren.

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DANKWOORD

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lot

Publications

PUBLICATIONS

T.J. Molloy*, **L.A. Devriese***, H.H. Helgason, A.J. Bosma, M. Hauptmann, E.E. Voest, J.H.M. Schellens, L.J. van't Veer. A multimarker QPCR-based platform for the detection of circulating tumour cells in patients with early-stage breast cancer. *Br J Cancer* 2011 Jun 7;104(12):1913-9. *These authors contributed equally

L.A. Devriese, E.E. Voest, J.H. Beijnen, J.H.M. Schellens. Circulating tumor cells as pharmacodynamic biomarker in early clinical oncological trials. *Cancer Treat Rev* 2011 Epub 16 May 2011.

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ABSTRACTS

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Curriculum Vitae

CURRICULUM VITAE

Lotje Antonetta Devriese was born on September 20th 1977 and grew up in Amsterdam, the Netherlands. In 1995 she graduated from the St. Ignatius Gymnasium in Amsterdam, after which she spent a year in Granada, Spain, to study Spanish. Starting in 1996, she studied Medical Biology at Utrecht University. From 1998, she also studied Medicine at the same university. In 2001 en 2002 she performed two research projects at the department of Vascular Medicine at the University Medical Center Utrecht under supervision of prof.dr. T.J. Rabelink. At the end of 2004, she obtained both her Master of Science in Medical Biology and her medical degree. Subsequently, she started a residency in Internal Medicine under supervision of prof. dr. E. van der Wall. Between 2005 and 2008, she worked as a resident at the Meander Medical Center in Amersfoort, initially under supervision of dr. A. van de Wiel and later dr. C.A.J.M. Gaillard. In 2008, she suspended her residency for a position as PhD student at the departments of Experimental Therapy and Clinical Pharmacology at the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital in Amsterdam under supervision of prof.dr. J.H.M. Schellens and prof.dr. E.E. Voest. The results of this research are described in this thesis. In 2011, she resumed her residency in Internal Medicine at the University Medical Center Utrecht under supervision of prof. dr. M.M.E. Schneider. In 2012, she will start a fellowship in Medical Oncology under supervision of prof.dr. E.E. Voest.
Lot and her partner Matthijs Boekholdt have a daughter Merle.

Lotje Antonetta Devriese werd op 20 september 1977 geboren en groeide op in Amsterdam. In 1995 deed ze eindexamen op het St. Ignatius Gymnasium te Amsterdam, waarna zij een jaar Spaans studeerde in Granada, Spanje. In 1996 startte zij met de studie Medische Biologie aan de Universiteit Utrecht. Vanaf 1998 studeerde zij daarnaast ook Geneeskunde aan dezelfde universiteit. In 2001 en 2002 deed zij twee wetenschappelijke stages bij de vakgroep Vasculaire Geneeskunde in het Universitair Medisch Centrum Utrecht onder supervisie van prof.dr. T.J. Rabelink. Eind 2004 behaalde zij zowel haar doctoraal examen Medische Biologie als haar artsexamen. Aansluitend startte zij met de opleiding tot internist onder supervisie van opleider prof.dr. E. van der Wall. Tussen 2005 en 2008 werkte zij als arts-assistent in het Meander Medisch Centrum te Amersfoort, eerst onder supervisie van opleider dr. A. van de Wiel en later dr. C.A.J.M. Gaillard. In 2008 onderbrak zij de opleiding om promotieonderzoek te doen op de afdelingen Experimentele Therapie en Klinische Farmacologie in het Nederlands Kanker Instituut- Antoni van Leeuwenhoek Ziekenhuis te Amsterdam onder begeleiding van prof.dr. J.H.M. Schellens en prof.dr. E.E. Voest. De resultaten van het onderzoek staan beschreven in dit proefschrift. In 2011 hervatte zij de opleiding tot internist in het Universitair Medisch Centrum Utrecht onder supervisie van opleider prof.dr. M.M.E. Schneider. In 2012 zal zij starten met het aandachtsgebied Medische Oncologie onder supervisie van prof.dr. E.E. Voest. Lot woont samen met Matthijs Boekholdt en hun dochter Merle.

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