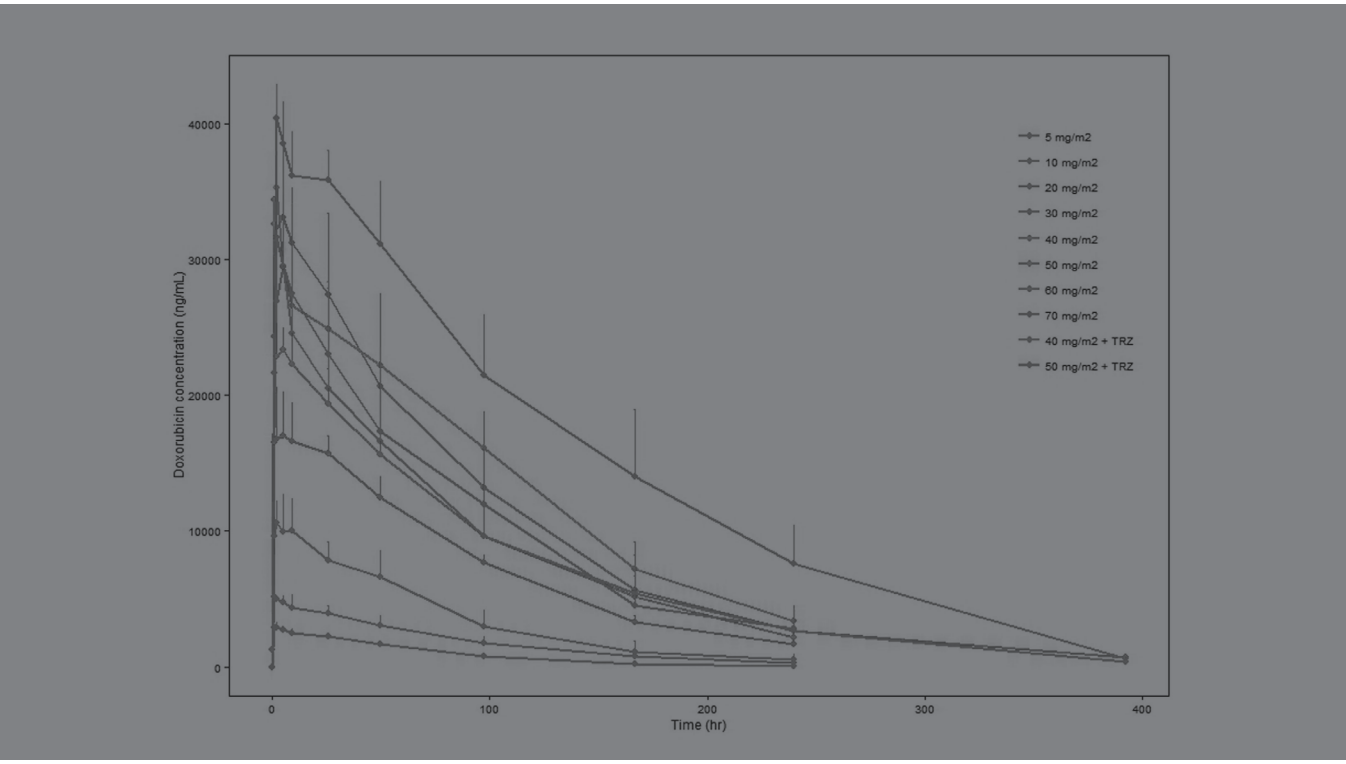
NOVEL SYSTEMIC TREATMENT OPTIONS FOR ADVANCED SOLID TUMORS WITH OR WITHOUT CENTRAL NERVOUS SYSTEM METASTASES OR MALIGNANT GLIOMA



Bojana Milojkovic Kerklaan

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Novel systemic treatment options for advanced solid tumors with or without central nervous system metastases or malignant glioma

Nieuwe systemische behandelmogelijkheden voor patiënten
met gevorderde tumoren met of zonder
metastasen in het centrale zenuwstelsel of kwaadaardige gliomen
(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 verdediging
op woensdag 25 maart 2015 des middag te 12.45 uur

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Bojana Milojkovic Kerklaan
geboren op 1 juni 1979 te Belgrado, Joegoslavië

Promotoren:	Prof.dr. J.H.M. Schellens
	Prof.dr. J.H. Beijnen
Copromotor	Dr. D. Brandsma

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Once we accept our limits, we go beyond them. Albert Einstein

Voor alle patienten en hun families

Contents

Introduction/Preface

Preface

There is a substantial unmet need for better therapeutic options in patients with cancer. Increasing knowledge about the pharmacology, safety and efficacy of oral drugs as a single treatment modality, or in combination with two or more drugs is an essential process in drug development in oncology. This thesis analyses the safety, dosing, pharmacokinetics, pharmacodynamics, drug-drug or food-drug interactions and preliminary antitumor activity of some new (oral) treatment options, or new oral drug combinations. Oral anticancer treatment is more practical and convenient for patients, and may therefore increase the quality of life. One of the features is that treatment can be taken at home, which may also lower costs of drug therapy. However, not all drugs have characteristics required for oral administration, due to poor solubility, affinity for ABC-drug transporters, or drug metabolizing enzymes like CYP3A4 highly expressed in the gut wall and liver. Better understanding of oral anti-cancer therapy is one of the aims of this thesis.

Another aim is to try to improve the safety of trastuzumab therapy. Trastuzumab targets HER2 and is one of the breakthroughs in breast cancer therapy. However, cardiotoxicity especially when co-administered with anthracyclines is limiting drug safety. With the aim to prevent this drug adverse event we performed a randomized study with the ATII antagonist candesartan in combination with trastuzumab, and monitored cardiac events outlined in this thesis.

Although drug development for early and advanced cancer of various tumor types is showing a remarkable progress for some tumor types and results in increased survival, unfortunately the number of patients that eventually develop brain metastases is increasing. Brain metastases and primary brain tumors are difficult to treat because of the presence of the blood-brain barrier. The function of this barrier is to protect the brain from possible intoxications from compounds in the systemic circulation. In case of cancer treatment however, this barrier prevents entrance of efficient systemic chemotherapy. The complex structure of the barrier in the highly vascularized brain tissue is discussed in the following chapters together with new strategies for systemic drug delivery in patients with brain tumors and central nervous system metastases.
During the early development of new brain anti-cancer treatments pharmacodynamic assays to monitor drug effects could guide drug development. Therefore we developed and validated a novel assay quantitating circulating tumor cells in cerebrospinal fluid. In addition, we monitored drug concentrations in cerebrospinal fluid in patients with leptomeningeal carcinomatosis.

CHAPTER 1: PHARMACOKINETICS OF ORAL ANTI-CANCER DRUGS

1.1 Phase I and pharmacological study of pazopanib in combination with oral topotecan in patients with advanced solid tumors

B. Milojkovic Kerklaan[1,2], M.P.J. Lolkema[3], L.A. Devriese[1] E.E. Voest[3], A. Nol-Boekel[1], M. Mergui-Roelvink[1], M. Langenberg[3], K. Mykulowycz[4], J. Stoebenau[5], S. Lane[5], P. Legenne[5], P. Wissel[5], D.A. Smith[5], B.J. Giantonio[4], J.H.M. Schellens[1,2,6] and P.O. Witteveen[3]

1. Department of Clinical Pharmacology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
2. Division of Molecular Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
3. Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
4. Abramson Cancer Center, University of Pennsylvania, Philadelphia, U.S.A.
5. GlaxoSmithKline, Research Triangle Park, North Carolina, U.S.A.
6. Science Faculty, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands

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ABSTRACT

Purpose: This phase I study evaluated the safety, tolerability, maximum-tolerated dose and pharmacokinetics of two dosing schedules of oral topotecan in combination with pazopanib in patients with advanced solid tumors.
Design: Stage I of this study was to determine if there was an impact of pazopanib on topotecan exposure. In stage II, the MTD and safety profile of oral topotecan given weekly on days 1, 8 and 15 in a 28-day cycle; or daily times-five on days 1-5 in a 21-day cycle, both in combination with daily pazopanib, were explored.
Results: Nine patients were enrolled in stage I and 58 patients in stage II. Pazopanib co-administration caused a substantial increase in exposure of total topotecan (1.7-fold) compared to topotecan alone, which is considered clinically relevant. Topotecan had no effect on pazopanib concentrations. Safety findings were consistent with the known profile of both agents. There were three drug-related deaths, liver failure, tumor hemorrhage, and myelosuppression. Two patients experienced DLTs (hand-foot syndrome, myelosuppression and diarrhea) on the weekly topotecan schedule and four patients experienced DLTs (myelosuppression) on the daily-times-five topotecan schedule. When combined with pazopanib, 800 mg daily, the recommended doses for oral topotecan are: 8 mg weekly; and 2.5 mg daily times-five.
Seven of eight patients with partial response had ovarian cancer. Additionally, 54% of patients had stable disease as the best response with 22% stable for 6 months.
Conclusion: Total topotecan exposure is 1.7-fold higher when co-administered with pazopanib. Both schedules of administration were tolerated and merit further evaluation.
Key words: pazopanib, topotecan, ovarian cancer

INTRODUCTION:

Pazopanib (GW786034; Votrient; GlaxoSmithKline) is an oral multi-target tyrosine kinase inhibitor (TKI) targeting receptors of vascular endothelial growth factor (VEGF-1, -2 and -3), platelet-derived growth factor (PDGF- α and β), and stem cell factor (c-kit).[1] Pazopanib is FDA an EMA approved for the treatment of advanced renal cell carcinoma (RCC) and advanced soft tissue sarcoma (STS).[1,2] It has demonstrated activity in the preoperative setting for NSCLC and advanced epithelial ovarian cancer, fallopian tube or primary peritoneal cancer.[3,4] The recommended dose (RD) of oral pazopanib is 800 mg once daily (QD). The most common side effects (>30%) included: diarrhea, hair and skin hypopigmentation, hypertension, nausea, fatigue, anorexia, vomiting, and elevated alanine aminotransferase (ALT) and elevated aspartate aminotransferase (AST).[1] Peak concentrations are achieved within 2-4 hours (h) with a mean elimination half-life ($t_{1/2}$) of approximately 30h in human plasma. Pazopanib absorption is increased by food and therefore was administered on an empty stomach. Pazopanib is not extensively metabolized and major route

of elimination of pazopanib is excretion of parent compound in feces.[1] Metabolism of pazopanib is primarily by cytochrome P-4503A4 (CYP3A). Pazopanib is a substrate with a moderate affinity for the drug efflux transporters P-glycoprotein-1 (P-gp/ABCB1) and with a high affinity for Breast Cancer Resistance Protein (BCRP/ABCG2).[1,5–7]

Topotecan (Hycamtin®, GlaxoSmithKline), a semisynthetic analogue of camptothecin, inhibits DNA topoisomerase I in dividing cells. By binding to the cleavable complex, topotecan blocks further replication, which leads to cell death.[8,9] Intravenous (i.v.) topotecan is approved for the treatment of small-cell lung cancer (SCLC), cervical cancer and metastatic ovarian carcinoma, while oral topotecan is approved for SCLC only. [8,10] The oral formulation enables more convenient dosing than i.v. administration, especially in combination regimens with other oral anti-cancer agents [11] and has similar activity to i.v. topotecan, with less grade 4 neutropenia and greater convenience of administration. [12] In patients with platinum-resistant recurrent ovarian cancer the five-day schedule had better progression-free survival than the weekly schedule, but the weekly schedule had comparable overall survival and a favorable toxicity profile. [13] However, the 5-day schedule is limited by the occurrence of hematological toxicity. [14,15]

The time to reach peak plasma concentration ($T_{max)}$ for oral topotecan is 2 h. Following oral administration approximately 20% was recovered as parent (total topotecan) drug in urine and 33% of the oral dose was found to be unchanged (total topotecan) in feces. [16] The contribution of metabolism to topotecan total body clearance is limited (<10%). Topotecan undergoes reversible pH-dependent hydrolysis, yielding topotecan carboxylate. Elimination $t_{1/2}$ for oral topotecan is between 4 and 6 h.[16] The absorption of oral topotecan is limited largely due to BCRP- and Pgp-mediated efflux of oral topotecan in the intestinal epithelium that varies among subjects. [17–19]

Various preclinical and clinical studies of anti-angiogenic agents in combination with chemotherapy showed mild toxicity and improved anti-tumor activity. [20] The observed additive effect could be attributed to the anti-angiogenic drugs that act by normalizing tumor vasculature, which can then lead to improved delivery of cytotoxic drugs to the tumor. [21] Other theories are based on timing of anti-angiogenic drugs during chemotherapy-free periods. [22,23] Preclinical models of pazopanib and topotecan co-administration showed significantly improved anti-tumor activity compared with the respective single agents. [24,25] Prolonged combination therapy with low dose topotecan and pazopanib in mouse models demonstrated sustained anti-angiogenic activity.[24] A study in patients with gynecologic tumors showed the lack of a statistically significant drug-drug interaction between pazopanib and low dose topotecan. [26]

The aim of this study was to evaluate the safety, tolerability, maximum-tolerated dose and pharmacokinetics of two topotecan dosing schedules in patients with advanced solid tumors.

PATIENTS AND METHODS:

Patient selection.

Eligible patients were those with histologically or cytologically confirmed diagnosis of a progressive advanced solid tumor that was refractory to standard therapy or for whom there was no established therapy. Other inclusion criteria were: written informed consent; ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) performance status of ≤ 1; able to swallow and retain oral medications; adequate hematological (neutrophils ≥ 1.5 x 10^9/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 ≤ 5 x ULN in case of liver metastases) and renal function (Cockroft-Gault creatinine clearance ≥ 50 ml/min and urine protein creatinine ratio and partial thromboplastin time ≤ 1.2 x ULN). Exclusion criteria were: less than four weeks since last chemo-, radio- or 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 elimination half-lives; prior treatment with pazopanib or investigational anti-angiogenic compounds; uncontrolled infection; pregnancy or lactating (all patients with child bearing potential had to use adequate contraceptive protection); poorly controlled hypertension (systolic ≥ 140 or diastolic blood pressure ≥ 90 mm Hg); prolonged QTc interval; class III or IV heart failure; vascular events within 6 months; therapeutic heparin or warfarin use; leptomeningeal- or brain metastases; 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 and treatment administration

This was a two-stage, two arm, open-label, dose escalation phase I study (NCT00732420, ***www.clinicaltrials.gov***). From September 2008 to September 2013 three 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 Antoni van Leeuwenhoek Hospital, Amsterdam, both in the Netherlands. In the drug-drug interaction study portion (P1), the impact of pazopanib on the exposure of oral topotecan was investigated. In P2, the combination regimens were explored in a dose-escalation phase and a dose expansion phase. There were two different combination regimens for oral topotecan: P2A: topotecan once weekly (Day 1, 8 and 15) in a 28-day cycle; P2B: topotecan on days 1-5 in a 21-day cycle, while oral pazopanib was taken daily (QD) throughout the cycle. Patients were enrolled in sequential cohorts of three to six patients and the maximal-tolerated dose (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.

Study procedures, safety and efficacy assessments

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, during any following cycles and at study termination. Toxicities were graded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (CTCAE) version 3.0. Radiologic tumor assessments were performed at baseline and every two cycles. Tumor measurements were done according to RECIST 1.0. Patients remained on treatment until disease progression, unmanageable toxicity had developed or withdrawal of consent. Patients were considered to be evaluable for safety when they completed cycle 1.

Dosing

Pazopanib monohydrochloride was provided as 200 mg 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 1. Pazopanib and topotecan were administered with water on an empty stomach either 1-hour before a meal or 2 hours after a meal. Dose reductions following each cycle were allowed twice. Specific guidelines were prescribed for management of hypertension and diarrhea. Oral topotecan was administered as a flat dose, in this study, to facilitate the interpretation of the pharmacokinetic data and decrease medication errors.
In P1, the interaction portion of the study, pazopanib was dosed continuously from day 2 at 800 mg. Topotecan 4 mg was administered only on day 1 and 15 of cycle 1. This order was chosen with the aim to enable pharmacokinetic sampling of both drugs as monotherapy and in combination therapy. On completion of the P1 (Days 1-15), subjects continued pazopanib monotherapy (continuation phase) in 28-day cycles.
In the P2A of the dose escalation component, patients started with continuous pazopanib monotherapy at day -14, which was prior to administration of topotecan once weekly on days 1, 8 and 15 in 28-day cycles. The presence of steady-state levels of pazopanib ensured accurate determination of Cycle 1 DLTs during the oral topotecan dosing.
In the P2A dose expansion, continuous pazopanib dosing started on day 2 and topotecan was dosed on days 1, 8 and 15 of a 28-day cycle.
In the P2B dose escalation, patients started with continuous pazopanib monotherapy at day -14, which was prior to administration of topotecan on a daily-times five-consecutive days (on Days 1, 2, 3, 4, and 5) of a 21–day cycle.
In the P2B dose expansion oral topotecan was given continuous on Days 1- 5 every cycle of 21 days (daily–times-five). Dosing with pazopanib began on Day 6 of Cycle 1.

Dose limiting toxicities (DLT)

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 (less then <25,000/mm^3 / <25.0 x 10^9 /L; inadequately controlled grade 3 hypertension in spite of maximal two 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.

Pharmacokinetic sampling and analysis

Blood samples for the determination of pazopanib in P1 and P2A, were obtained on days 14 and 15 at baseline and at 1, 2, 3, 4, 6, 8, 10-12 and 24 hours following administration of pazopanib. 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, 10-12 and 24 hours following administration of topotecan.

In P2B dose expansion, the pharmacokinetic sampling was performed according the same schedules but on Day 5 of Cycle 1 for topotecan alone, Day 21 of Cycle 1 for pazopanib alone, and Day 5 of Cycle 2 (Day 26) for both topotecan and pazopanib.

At each collection point, for pazopanib, 2 ml of whole blood was withdrawn into a tube containing potassium ethylenediaminetetra-acetic acid (EDTA). For total topotecan, 3 ml of whole blood was withdrawn into lithium heparinised collection tube. After separation, plasma was stored frozen at -30°C. Plasma concentrations of pazopanib and total topotecan (both carboxylate and lactone form) were quantified using a validated high performance liquid chromatography (HPLC) method. (Rosing H 1995, Hurwitz 2009). Total topotecan and pazopanib concentrations and actual sample collections times were used to carry out non-comparmental analysis using WinNonLin (v 6.2). The following pharmacokinetic parameters were determined: area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), terminal half-life ($t_{1/2}$) for total topotecan and only $AUC_{(0-24)}$ for pazopanib. The maximum observed plasma concentration (C_{max}), time to maximum observed plasma concentration (t_{max}), and concentration at 24 hours (C_{24}) was directly obtained from the plasma-concentration data.

Pharmacogenetics (Pg)

Pg and biomarker analyses in blood and on archive tumor samples were collected. Separate written informed consent was required for Pg sampling. Several genes involved in safety and efficacy of the study drugs were planned to be in-

vestigated for single nucleotide polymorphisms (SNP). These included genes coding for drug targets such as UDP-glucoronyltransferase (UGT1A1) and drug transporters such as P-gp. 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. [6]

Statistical Methods

Safety and preliminary anti-tumor activity data, as well as calculated pharmacokinetic parameters were summarized and tabulated using descriptive statistics. The effect of pazopanib on total topotecan and of total topotecan on pazopanib was assessed. Pharmacokinetic parameters including $AUC_{0-\infty}$, C_{max} and $t_{1/2}$ for total topotecan, and $AUC_{(024)}$, C_{max}, and C_{24} for pazopanib, were analyzed using a mixed effects model on log-transformed data with treatment as a fixed effect and subject as a random effect. The geometric last squares (LS) mean ratio and associated 90% confidence interval (CI) were calculated after transforming the log-transformed results back to the original scale. For paired data, t_{max} was compared between groups using the Wilcoxon matched pairs method.[27] With point estimates and 90% CIs for median differenced were calculated.

RESULTS

In total, 9 patients with mean age 59 (range 37 - 78) were treated in the interaction study (P1). In dose-escalation study (P2) 58 patients with mean age of 52.3 (range 18-72) years were treated. The patient characteristics are listed in Table 2.

Dose Escalation

Planned dose escalation is summarized in Table 1. The weekly dose of 10 mg topotecan and continuous 800 mg pazopanib (P2A) was determined to be above the maximal tolerated dose as two out of six patients experienced a DLT (one grade 3 diarrhea and one grade 3 neutropenia). The next lower dose-level (8 mg topotecan and 800 mg pazopanib) was taken forward into the expansion cohort. The expansion cohort consisted of additional 11 patients who did not experience any DLT, as well as the initial three patients during the dose-escalation part.

A second dosing schedule (P2B) was explored based on daily–times-five oral topotecan dose every 3 weeks. Dosing started at 1.75 mg topotecan daily-times-five in combination with 400 mg pazopanib continuously. The combination of 3 mg topotecan daily-times-five with 800 mg pazopanib continuously resulted in a cohort of three patients that all experienced at least one DLT: grade 4 neutropenia lasting > 5 days; grade 3 thrombocytopenia, anemia, leucocytopenia and bleeding; and grade 4 thrombocytopenia in combination with grade 3 neutropenia. The next lower dose level, 2.5 mg topotecan daily-times-five with 800 mg pazopanib daily in 21-day cycle was expanded, where one out of six enrolled patients experienced DLT (grade 4 neutropenia lasting > 5 days in combination with grade 4 thrombo-

cytopenia). Therefore, this dose was taken forward into the expansion cohort. Eight additional patients were enrolled in the expansion cohort, in which no further DLT was observed.

Safety

The Summary of Drug-Related Adverse Events (grade 3, 4 and 5) in dose-escalation study (P2) is listed in Table 3. The most frequently occurring treatment-related hematological toxicities grades ≥ 3 were neutropenia (15, 25.8%), thrombocytopenia (12, 20.6%), leucocytopenia (6, 10%) and anemia (3, 9%). The daily-times-five topotecan regimen had higher rate of hematologic toxicities than the weekly schedule. In both drug-combination regimens the most frequently occurring treatment-related non-hematological toxicities grades ≥ 3 were fatigue (5,13%) and hypertension (4, 11%). Gastro-intestinal side effects such as nausea, vomiting and diarrhea, all grades, occurred with comparable percentage in both schedules. Deaths related to study drug occurred in three patients. One patient with lung adenocarcinoma treated in P1 developed a fatal pulmonary hemorrhage while on pazopanib, 3 days following the last topotecan dose. The second death occurred also in P1 in a patient with synovial sarcoma, without history of hepatic disease, who developed hepatic failure while on pazopanib, 21 days following the last topotecan dose. At autopsy there was extensive liver necrosis and congestion, ascites and sub-acute heart congestion. Drug concentration values were consistent with therapeutic exposure. Several other causes, sub-clinical heart failure and paracetamol toxicity, were evaluated for their contribution to the observed hepatic toxicity, but none were confirmed; therefore this event was classified as a pazopanib related liver failure. One patient in the 3 mg topotecan daily-times-five and daily 800 mg pazopanib experienced grade 3 neutropenia and grade 4 thrombocytopenia on day 22 and treatment was interrupted. However the patient died on 35th day of drug-related pancytopenia resulting in pneumonia and septic shock. This side effect has been reported in literature.[28] Non-fatal reversible treatment-related liver toxicity (elevated ALT, AST) occurred in 13 patients (22%) during the whole study. The only other grade 4 elevated ALT and AST (1/67, 1.5% each) was also observed in the P1 while on pazopanib, 15 days post last topotecan dose. This patient had colorectal carcinoma with liver metastases and experienced partial recovery after discontinuation of study medication and administration of oral steroids. Therefore this hepatic toxicity event was considered to be only partially due to pazopanib.

Pharmacokinetics (PK)

The pharmacokinetic population included data from patients who received all doses in P1 and received all MTD doses in P2 (Table 4). Figure 1 shows the mean plasma concentration-time curves of pazopanib (A) and total topotecan (B) of patients treated in the drug-drug interaction study (P1) and individual total topotecan concentration-time curves in the MTD part of the study (P2) for weekly (C) and

daily-times-five (D) schedules. No differences in pazopanib plasma concentration can be seen following pazopanib dose alone versus co-administration with topotecan (Figure 1 A). However, a marked increase in mean total topotecan exposure was observed on the sampling day when topotecan was co-administered with pazopanib, compared to dosing alone (Figure 1 B, C and D). Pazopanib exposure on both schedules was similar with or without co-administration of topotecan (figures not shown). Detailed pharmacokinetic data are summarized in Table 4 and Table 5. Total topotecan exposure was increased when co-administered with pazopanib. Total topotecan Cmax increased approximately 1.9-fold in P1 and both schedules in P2, while $AUC_{(0-\infty)}$ increased between 1.5- and 1.7-fold depending on study part and schedule. Individual increases in ratios ranged from 0.58 to 3.5 and 0.66 to 2.9 for C_{max} and $AUC_{(0-\infty),}$ respectively, for the topotecan 4 mg single dose in P1, 1.0 to 3.0 and 1.5 to 2.1 for C_{max} and $AUC_{(0-\infty),}$ respectively, for 8.0 mg on weekly schedule, and 1.0 to 3.6 and 1.1 to 1.9 for C_{max} and $AUC_{(0-\infty),}$ respectively, for the topotecan 2.5 mg daily-times-five schedule. The minor differences between the three doses and schedules were likely due to the small sample sizes. Total topotecan mean $t_{½}$ values were approximately 4 to 5 hours and were similar whether topotecan was dosed alone or with pazopanib.
Pazopanib mean C24h plasma concentrations were > 25 µg/ml in P1 and > 35 µg/ml in P2 both schedules.

Pharmacogenetic analysis

Pharmacogenetic analysis was performed in only two patients with severe hepatotoxicity in the P1. 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. [29] The latter patient also had one copy of the UGT1A6*3A allele (S7A, rs6759892), which has been associated with lower UGT1A6 expression and possibly higher likelihood of paracetamol induced hepatotoxicity. [30]

Preliminary anti-tumor activity

Eight out of 50 patients evaluable in combination therapy (P2) showed partial tumor response (PR) (8/50, 16%). Seven patients with PR had ovarian cancer (47%, 7 of 15 evaluable ovarian cancer) and one cervical cancer. Five patients with PR were observed in P2A and 3 in P2B. Duration of response (PR) was median 24 weeks (range 16-63). Twenty-seven patients showed stable disease SD as best response (54%). Seventeen were enrolled in P2A and 10 in P2B. SD > 6 months were observed in 11 (22%) patients, in patients with ovarian (3) and colon-rectum cancer (2) and other tumor types (6). The most frequent tumors that showed SD were soft tissue sarcoma and ovarian. Early progression of disease was seen in 30% of the patients.

Pharmacokinetics and tolerance of the combination of oral topotecan and pazopanib treatment were studied in this phase I study. MTD of weekly oral topotecan was 8 mg on day 1, 8 and 15 in combination with 800 mg pazopanib daily in a 28-day cycle. For the daily-times-five regimen, MTD is 2.5 mg oral topotecan on days 1 through 5 with 800 mg pazopanib daily in the 21-day cycle. Both schedules could be administered for longer periods and showed initial signs of anti-tumor activity. Pazopanib substantially increased exposure of total topotecan by 1.8-fold for C_{max} and 1.7-fold for $AUC_{(0-\infty)}$, which is considered clinically relevant, but did not increase $t_{½}$ values, when compared with topotecan alone. This suggests that the effects of pazopanib on topotecan pharmacokinetics were pre-systemic (increase in oral bioavailability) and not related to changes in elimination. These findings can be compared with an effect of concomitant administration of oral topotecan and elacridar, the known potent inhibitor of BCRP and Pgp that resulted in a 2.8- and 2.4-fold increase in total topotecan Cmax and AUC, respectively, and confirmed the effects of transporter modulation on the pharmacokinetics of topotecan. In contrast, there was only a 10% decrease in topotecan clearance after intravenous administration and co-administration with elacridar. [31] These results indicate that the effects of elacridar on orally administered topotecan pharmacokinetics were also primarily pre-systemic and likely due to inhibition of transporters in the gut. These efflux transporters are located in the intestine where they act to limit drug absorption from the lumen.[17,18] BCRP and P-gp are also expressed in the liver and the kidney where they promote drug excretion into bile and urine, respectively.[17–19] However, topotecan is primarily renally excreted and undergoes little metabolism. [16] While both topotecan and pazopanib are found to be high affinity substrates for BCRP, topotecan is a weak substrate for P-gp and pazopanib moderate, [1,5,8,17] which means that pazopanib binds has a higher affinity for P-gp.

Previously published total topotecan exposure when a 14 mg weekly was dosed without pazopanib (published data, von Gruenigen 2012) was similar to total topotecan plasma exposure following a weekly 8 mg oral topotecan dose in combination with daily pazopanib 800 mg. [32] This is not unexpected as pazopanib increased $AUC_{(0-\infty)}$ approximately 1.7 fold and the difference between 8 mg and 14 mg dose is approximately 1.7.

The previous study with low dose oral topotecan and pazopanib indicated there was no statistically significant drug-drug interaction, which might be because the model did not adequately describe the data, the study was not powered to see an effect, or the low-dose topotecan shows different results than standard doses. Their finding was based on a population pharmacokinetic analysis that included oral clearance (CL/F), central volume (Vc/F), absorption rate constant (ka) and lag time. The CL/F estimate with 95% confidence intervals was 11.5 l/h (5.9-17.1). But this value is greater than 2-fold lower the median post-hoc estimation quoted in their study (26.7 l/h) and the absolute clearance (CL) for total topotecan 24.8 (L/h).[26]

Pazopanib plasma concentrations were similar when pazopanib was dosed alone or in combination with topotecan in both topotecan regimens and were near or above the values resulted in a longer PFS (> 20.6 μg/ml).[33] The severe hematological toxicities (grade ≥ 3) were not more frequently reported in the present study with topotecan and pazopanib co-administration, than in the study with single weekly topotecan administration.[32] This is likely due similar total topotecan concentrations in both studies.

In present study, the daily-times-five regimen resulted in more severe hematologic toxicity in comparison with the weekly topotecan regimen, whereas the dose density of the recommended topotecan dose in the daily-times-five regimen was lower (4.16 mg/wk vs 6 mg/wk).

The frequency of the gastrointestinal side effects (nausea/vomiting and diarrhea) showed an additive effect in the combination treatment, when compared with safety information of single agent treatment with both pazopanib and topotecan from the literature.[34] No congestive heart failure (CHF) was reported in our study, despite the fact that the recently published review with VEGFR-TKIs showed all-grade pazopanib-related CHF in 6.1% of patients.[35]

Elevated transaminases were seen in 22% of the patients which corresponds to previously reported frequencies for single-agent pazopanib (0-35%).[2,3,34,36] Grade 3 and 4 liver enzyme increase were seen in < 1% of patients treated with pazopanib.[1] Rare but potentially severe and fatal hepatotoxicity has been observed with pazopanib treatment. There was one occurrence of fatal liver necrosis and another patient with liver metastases who developed grade 4 toxic hepatitis after pazopanib exposure. The liver necrosis could have been affected by the ABCB gene polymorphism, concomitant administration of topotecan and pazopanib and concomitant paracetamol treatment. Hepatotoxicity is not likely to be related to topotecan therapy based on experience from previous phase I-III trials.[8]

In the present study stomatitis of all grades was observed two times more frequently (22% vs 11%) than in the pazopanib literature. [1] The reason for this could be underreporting of oral adverse events secondary to TKIs, as they more closely resemble aphthous stomatitis than oral mucositis or stomatitis caused by conventional agents. [37]

In total, 8 patients out of evaluable 50 (16%) had partial response (PR) and 27 patients (54%) had stabile disease (SD). Seven out of 15 (47%) heavily pretreated patients with ovarian cancer evaluable for anti-tumor activity showed PR as their best response. In the literature, topotecan alone in patients with recurrent ovarian cancer, in the daily-times-five oral regimen yielded objective response ranging from 13-16.3% when administered as second line and/or later lines of therapy, [38–41] and 30% (6/20) as a salvage i.v. topotecan single therapy.[38] Seven of the total number of patients had soft tissue sarcoma and all of them had stable disease, as their best response with duration of 15.5 weeks (STDev=4.9).

CONCLUSION:

Pazopanib substantially increased exposure of total topotecan by 1.8-fold for Cmax and 1.7-fold for AUC $_{(0-\infty)}$, which is considered clinically relevant, but did not increase $t_{1/2}$ values, while topotecan had no effect on the exposure of pazopanib The combination of both oral topotecan chemotherapy and angiogenesis inhibitor pazopanib was found to be active and moderate to well tolerated by patients with solid tumors in two administration regimens: oral topotecan with 8 mg weekly in a 28-day cycle and 2.5 mg five-times weekly in a 21-day cycle, both in combination with pazopanib 800mg daily.
Preliminary anti-tumor activity was observed in patients with advanced ovarian cancer, who had all been pretreated with carboplatin.

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Part 1 (P1)				
	Pazopanib (mg) (days 2-28)	Topotecan (days 1 and 15)	Number of evaluable patients	DLT (number of DLT events)
	800 mg	4 mg	9	1 DLT liver failure gr 5
Part 2 Treatment arm A (P2A) - weekly topotecan + daily pazopanib in a 28-day cycle				
Dose level	Pazopanib (days 1-28)	Topotecan (days 1, 8 and 15)	All treated population	
0	400 mg	4 mg	3	
1	400 mg	6 mg	6	1 DLT HFS* gr 3
2	800 mg	6 mg	3	
3	**800 mg**	**8 mg**	**14**	
4	800 mg	10 mg	7	2 DLTs diarrhea gr 3 neutropenia gr 3
Part 2 Treatment arm B (P2B) - topotecan daily times five + daily pazopanib in a 21-day cycle				
Dose level	Pazopanib (days 1-21)	Topotecan (days 1-5)	All treated population	
0	400 mg	1.75 mg	3	
1	800 mg	1.75 mg	3	
1a	800 mg	0	1	
2	800 mg	2.5 mg	14	1 DLT thrombocytopenia gr 4 and neutropenia gr 4
2a	0	2.5 mg	1	
3	800 mg	3 mg	3	3 DLTs thrombocytopenia gr 3 in combination with anemia, leucocytopnia and bleeding; thrombocytopenia gr 4 and neutropenia gr 3 ; neutropenia gr 4
			Total P2A + P2B treated population	**Total DLTs**
			58	7
* HFS - hand-foot syndrome				
MTD - maximal tolerated dose				
MTD P2A				
MTD P2B				

Table 1. Dose levels and Dose limiting Toxicities (DLTs)

	P1	P2A (DL0-DL4)	P2B (DL0-DL3)	TOTAL P2A + P2B
Number of patients	9	33	25	58
Gender (n (%))				
Male	4 (44%)	14 (42%)	11 (44%)	37 (64%)
Female	5 (56%)	19 (57%)	14 (56%)	21 (36%)
Age (mean (range))	59 (37 - 78)	50.4 (18-69)	49.9 (32-72)	52.3 (18-72)
ECOG performance status (n (%))				
0	5 (56%)	19 (57%)	8 (32%)	27 (47%)
1	4 (44%)	14 (42%)	17 (68%)	31 (53%)
Primary site of disease (n (%))			0	
ovary	0	10 (30%)	6 (24%)	16 (28%)
pancreas	1 (11%)	2 (6%)	5 (20%)	7 (12%)
sarcoma (STS)	1 (11%)	2 (6%)	3 (12%)	4 (7%)
colon/rectum	2 (22%)	2 (6%)	3 (12%)	4 (7%)
breast	0	2 (6%)	0	2 (3%)
oesophagus	0	1 (3%)	1 (4%)	2 (3%)
gastric	1 (11%)	2 (6%)	0	2 (3%)
NSCLC	1 (11%)	0	1 (4%)	1 (2%)
endometrium or uterus	0	1 (3%)	1 (4%)	1 (2%)
cervix	0	0	1 (4%)	1 (2%)
ampulla	0	1 (3%)	0	1 (2%)
peritoneal	0	1 (3%)	0	1 (2%)
bladder	0	1 (3%)	0	1 (2%)
kidney	0	1 (3%)	0	1 (2%)
neuroendocrine	0	0	1 (4%)	1 (2%)
vulva	0	1 (3%)	0	1 (2%)
bone	0	1 (3%)	0	1 (2%)
GIST	0	2 (6%)	0	2 (3%)
leiomyosarcoma	0	3	1 (4%)	4 (7%)
angiosarcoma	0		1 (4%)	1 (2%)
osteosarcoma	1 (11%)	2 (6%)	0	2 (3%)
synovial sarcoma	0	1 (3%)	0	1 (2%)
epitheloid hemangio-epithelioma	0	0	1 (4%)	1 (2%)
melanoma choroidea	1 (11%)	1 (3%)	0	1 (2%)
renal cell		0		
Duration of disease, days (median (range))	788 (147-4307)	1000 (151-4415)	540 (33-4192)	718 (70-4415)
Prior therapies (n (%))				
Any Therapy	9 (100%)	33 (100%)	24 (96%)	57 (98%)
Immunotherapy	0	30 (90%)	22 (88%)	52 (90%)
Chemotherapy 1 line	1 (11%)	8 (24%)	1 (4%)	10 (17%)
Chemotherapy 2 lines	3 (33%)	4 (12%)	3 (12%)	7 (12%)
Chemotherapy 3 lines	1 (11%)	5 (15%)	8 (32%)	13 (22%)
Chemotherapy 4 lines	3 (33%)	5 (15%)	7 (28%)	12 (21%)
Chemotherapy 5 and + lines	1 (11%)	8 (24%)	2 (%)	10 (17%)
Hormonal Therapy	2 (22%)	1 (3%)	1 (4%)	2 (3%)
Biologic Therapy	3 (33%)	7 (21%)	5 (20%)	12 (21%)
Surgery	8 (89%)	30 (90%)	19 (76%)	49 (84%)
Radiotherapy	3 (33%)	12 (36%)	12 (48%)	24 (41%)
Unknown	1 (11%)	2 (6%)	1 (4%)	3 (5%)

P1 - Part 1; P2 - Part 2: cohort A - weekly topotecan + daily pazopanib in a 28-day cycle, cohort B - topotecan daily times five + daily pazopanib in a 21-day cycle

Table 2: Demographics

	P2A DL0	P2A DL1	P2A DL2	P2A DL3	P2A DL4	P2B DL0	P2B DL1	P2B DL1A	P2B DL2	P2B DL2A	P2B DL3	P2A+ P2B TOTAL
No.patients	3	6	3	14	7	3	3	7	14	1	3	58
Hematological toxicity												
Neutropenia gr 3				2 (14%)	1 (14%)	1 (33%)	1 (33%)		3 (21%)		2 (67%)	10 (17%)
Neutropenia gr 4					1 (14%)				3 (21%)		1 (33%)	5 (9%)
Leucocytopenia gr 3					2 (29%)	1 (33%)	1 (33%)		1 (7%)		1 (33%)	6 (10%)
Thrombocytopenia gr 3				2 (14%)	1 (14%)		2 (67%)		3 (21%)		2 (67%)	10 (17%)
Thrombocytopeniagr 4				1 (7%)							1 (33%)	2 (3%)
Anemia gr 3		1 (14%)					1 (33%)		1 (7%)	1 (100%)	1 (33%)	5 (9%)
Hemolitic uremic syndrome									1 (7%)			1 (2%)
Lymphopenia gr 3				1 (7%)								1 (2%)
Lymphopenia gr 4				1 (7%)								1 (2%)
Pancitopenia gr 5											1 (33%)	1 (2%)
Biochemical laboratory toxicity												
Elevated ALT gr 3				1 (7%)					1 (7%)			2 (3%)
Elevated AST gr 3				1 (7%)								1 (2%)
Hyperbilirubinemia gr 3									1 (7%)			1 (2%)
Hyperglycemia gr 3					1 (14%)							1 (2%)
Hypokalemia gr 3						1 (33%)						1 (2%)
Increased lipase gr 4							1 (33%)					1 (2%)
Metabolic acidosis gr 3											1 (33%)	1 (2%)
Gastrointestinal disorders												
Diarrhoea gr 3				1 (7%)	1 (14%)				1 (7%)			3 (5%)
Abdominal pain gr 3				1 (7%)								1 (2%)
Other toxicity												
Fatigue gr 3		1 (14%)		1 (14%)	2 (29%)				2 (14%)		1 (33%)	7 (12%)
Hypertension gr 3				2 (14%)		1 (33%)	1 (33%)		2 (14%)			6 (10%)
Pneumothorax gr 3									1 (7%)			1 (2%)
Tumor haemorrage gr 3				1 (7%)	1 (14%)							2 (3%)
Insomnia gr 3				1 (7%)								1 (2%)
Urinary infection gr 3									1 (7%)			1 (2%)
Hand-foot syndrome gr 3		1 (14%)										1 (2%)
P2B DL1a and DL2a no related AE ≥ grade 3 reported				MTD P2A					MTD P2B			

Table 3. Summary of possibly, probably or definitely Drug-Related Adverse Events (CTC grade 3, 4 and 5)

PK PARAMETERS	N	CMAX	TMAX	AUC(0-∞) OR AUC(0-24	T½	C24
Part 1 Drug-Drug Interaction						
Total Topotecan						
Units		ng/mL	h	hr*ng/mL	hr	ng/mL
Alone (D1)	6	9.29 (5.68, 15.2)	1.75[0.50-3.07]	61.0 [36.7-97.2]	5.23 [3.90-7.17]	-
Co-administered with Pazopanib (D15)	6	16.5(8.89, 30.7)	1.25[0.93-3.88]	102 (73.0, 142)	4.87 (4.62, 5.14)	-
Pazopanib						µg/mL
Units		µg/mL	h	hr*ng/mL	hr	25.9 (17.0, 39.5)
Alone (D14	7	49.7 (35.3, 70.0)	2.98[0.97-3.05]	818 (560, 1195)	-	26.9 (18.0, 40.2)
Co-administered with Topotecan (D15)	7	47.8 (33.2, 63.7)	3.00[2.00-4.00]	807 (548, 1188)	-	ng/mL
Part 2 – Maximum Tolerated Dose						
Arm A - 8 mg topotecan weekly and 800 mg pazopanib daily						
Total topotecan/Total Topotecan Units		ng/mL	hr	hr*ng/mL	hr	ng/mL
Alone (D1)	10	24.8 (17.6, 34.9)	1.78[0.98-3.30]	157 (120, 205)	5.49 (4.76, 6.32)	-
Co-administered with Pazopanib (D15)	4	43.6 (24.7, 77.2)	1.28[0.98-3.00]	237 (145, 386)	4.70 (3.40, 6.50)	-
Pazopanib/Pazopanib Units		µg/mL	hr	hr*µg/mL	hr	µg/mL
Alone (D14)	8	51.1 (39.8-65.6)	4.49[2.00-10.06]	968 (744-1260)	-	35.0 (25.4-48.3)
Co-administered with Total Topotecan (D15)	5	51.8 (37.8-71.1)	3.00[2.02-8.00]	10671 (886-1286)	-	36.0 (25.9-50.0)
Arm B - 2.5 mg topotecan daily times 5 and 800 mg pazopanib daily						
Total Topotecan/ Total Topotecan Units		ng/mL	hr	hr*ng/mL	hr	ng/mL
Alone (D5C1)	7	7.52 (4.32, 13.1)	1.97[1.50-6.00]	48.71 (23.8, 99.8)	4.551 (3.83, 5.42)	-
Co-administered with Pazopanib (D5C2))	5	11.1 (5.75, 21.3)	2.00[1.00-3.90]	53.2 (38.1, 74.2)	3.77 (2.09, 6.78)	-
Pazopanib/Pazopanib Units		µg/mL	hr	hr*µg/mL	Hr	µg/mL
Alone (D21C1)	5	58.8 (33.6-103)	2.00[1.00-4.02]	1034 (544-1967)	-	31.91 (13.1-77.5)
Co-administered with Total Topotecan (D5C2)	5	54.7 (35.6-83.9)	2.05[2.00-8.02]	882 (508-1532)	-	28.7 (17.4-47.4)

Abbreviations Cmax= maximum concentration, Tmax = time to maximum concentration, AUC(0-∞) = area under concentration time curve from time 0 to infinity (total topotecan only); AUC(0-24) =area under concentration time curve from time 0 to 24 hours and C24 = concentration at 24 hours (pazopanib only).

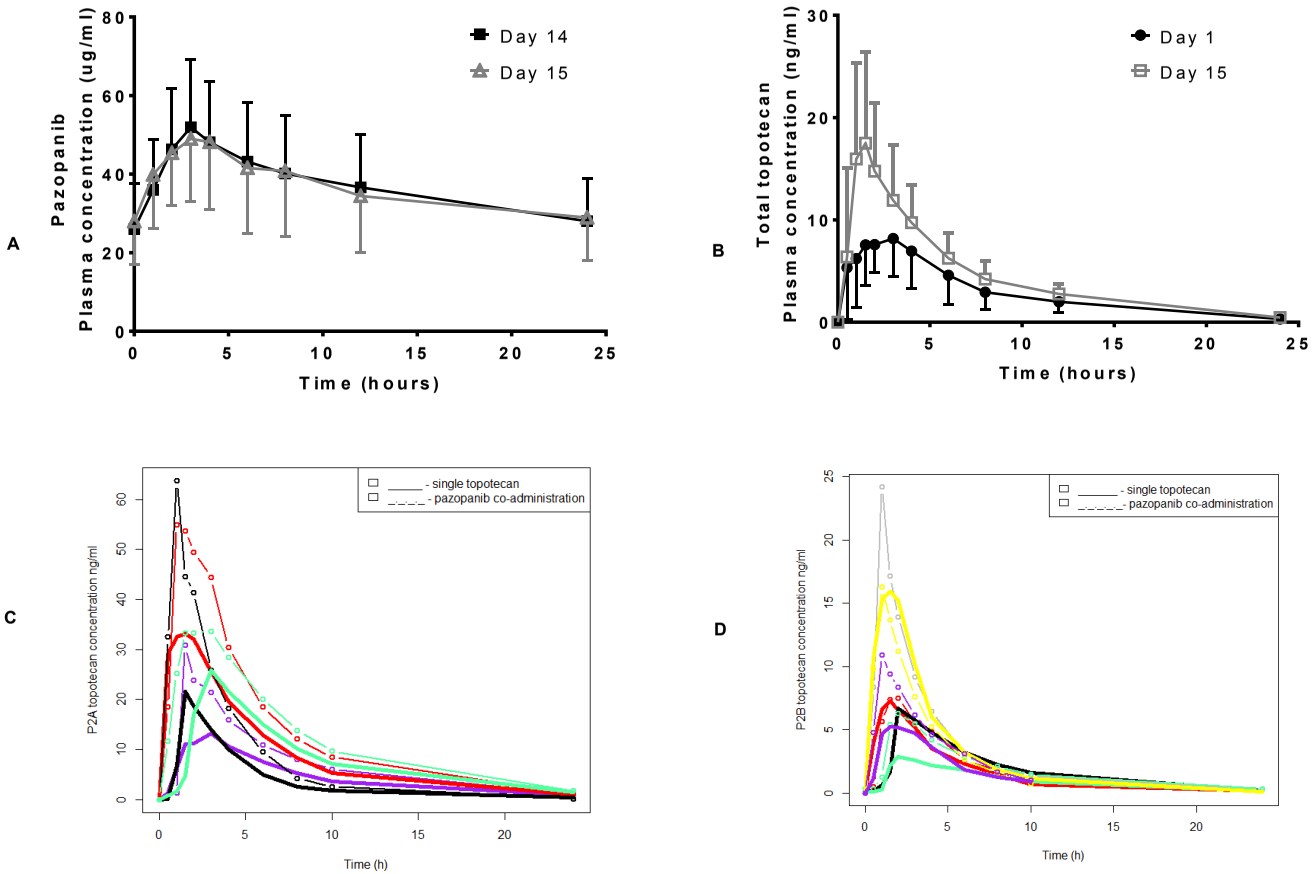
Note: PK parameters reported as geometric mean, 95% confidence interval and ranges except Tmax reported as median (minimum –maximum).

1. n-2
2. n-3
3. n-2

Table 4. Pharmacokinetic parameters of oral topotecan and pazopanib during single administration or in co-administration during P1 (Part 1: drug-drug Interaction study portion, P2A (Part 2-Arm A-MTD level) and P2B (Part 2-Arm B-MTD level) study parts

PARAMETER	GEOMETRIC MEAN OR MEDIAN		COMPARISON (GROUP NAMES)	RATIO OF MEANS OR DIFFERENCEOF MEDIANSB	90% CONFIDENCE INTERVAL
	Alone (A)	Co-administration(B)			
Part 1 Drug-Drug Interaction					
Total topotecan					
Cmax (ng/mL)a	16.51	9.29	B : A	1.78	(1.09 , 2.89)
AUC0-∞ (h*ng/mL)a	101.83	60.96	B : A	1.67	(1.14 , 2.44)
t½ (hr)	4.95	5.23	B : A	0.95	(0.73 , 1.22)
Tmaxb (hr)	1.25	1.75	B : A	-0.5	(-1.57 , 0.88)
Pazopanib					
Cmax (µg/mL)a	47.77	49.71	B : A	0.96	(0.89 , 1.04)
AUC0-24 (h*µg/mL)a	806.45	817.95	B : A	0.99	(0.93 , 1.04)
C24 (	26.89	25.87	B : A	1.04	(0.99 , 1.09)
Tmaxb (hr)	3.00	2.98	B : A	0.92	(-0.95 , 1.02)
Part 2 Maximum Tolerated Dose					
Arm A - 8 mg topotecan weekly and 800 mg pazopanib daily					
Total topotecan					
Cmax (ng/mL)a	46.79	24.81	B : A	1.89	(1.27,2.81)
AUC0-∞ (h*ng/mL)a	254.09	156.95	B : A	1.62	1.29,2.03
t½ (hr)	4.59	5.49	B : A	0.84	(0.62,1.13)
Tmaxb (hr)	1.28	1.78	B : A	-0.49	(-1.70,0.52)
Pazopanib					
Cmax (µg/mL)a	53.89	50.87	B : A	1.06	(0.82,1.37)
AUC0-24 (h*µg/mL)a	1109.28	965.31	B : A	1.15	(0.89,1.49)
C24 (	38.97	34.73	B : A	1.12	(0.86,1.47)
Tmaxb (hr)	2.53	3.00	B : A	-0.465	(-3.95,1.05)
Arm B - 2.5 mg topotecan daily times 5 and 800 mg pazopanib daily					
Total topotecan					
Cmax (ng/mL)a	12.36	6.64	B : A	1.86	(1.29,2.69)
AUC0-∞ (h*ng/mL)a	66.57	45.01	B : A	1.48	(1.18,1.86)
t½ (hr)	3.81	4.59	B : A	0.83	(0.61,1.13)
Tmaxb (hr)	2.00	1.99	B : A	-0.485	(-2.00,0.63)
Pazopanib					
Cmax (µg/mL)a	55.42	59.42	B : A	0.93	(0.66,1.31)
AUC0-24 (h*µg/mL)a	920.11	1034.4	B : A	0.89	(0.64,1.24)
C24 (	28.71	30.45	B : A	0.94	(0.66,1.34)
Tmaxb (hr)	2.05	2.01	B : A	-0.94	(-5.17,1.97)

Table 5. Summary of statistical comparisons of selected topotecan and pazopanib pharmacokinetic parameters between treatments – P1 (Part 1: drug-drug interaction study part), P2A (Part 2 - Arm A - MTD) and P2B (Part 2 - Arm B - MTD): Expanded Cohort B



C – P2A: 4 out of 10 sampled patients had evaluable topotecan PK sampling on Day 1 and Day 15

D- P2B : 5 out of 10 sampled patients had evaluable topotecan PK sampling on day C1D5 and on C2D5

Figure 1. Mean (SD) plasma concentration-time curves of pazopanib - A and total topotecan - B during Part 1 (P1) following single administration of pazopanib (day 14, n=7), single administration of topotecan (day 1, n=6) and concomitant administration of pazopanib and topotecan (day15); C - Plasma concentration-time curves per patient treated with 8 mg topotecan weekly and 800 mg pazopanib daily in P2A; and D – treated with 2.5 mg topotecan daily times five and 800 mg pazopanib daily in P2B

1.2 An Open-label, Dose Escalation, Pharmacodynamic, Pharmacokinetic, and Effect of Food Phase 1 Study of Twice Daily Oral Administration of E7820 in Subjects with Unresectable Solid Tumors

B. Milojkovic Kerklaan[1], M. Roelvink-Mergui[1], P.O.Witteveen[2], M.P.J. Lolkema[2], F.de Vos[2], T.R.J. Evans[3], R. Morrison[3], R Plummer[4], M. Ranson[5], A. Greystoke[5]; E. Dean[5], M. Flynn[6,] L. Reyderman [7], J.H.M. Schellens[1,8]

1. Clinical Pharmacology Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands
2. Department of Medical Oncology, University Medical Centre Utrecht, Utrecht, The Netherlands
3. Cancer Research UK Clinical Trials Unit (CTU), The Beatson West of Scotland Cancer Centre, Glasgow, United Kingdom
4. Sir Bobby Robson Cancer Trials Research Centre Newcastle, United Kingdom
5. Department of Medical Oncology, University of Manchester, Christie Hospital NHS Trust, Manchester, UK.
6. University College London Hospital, London, United Kingdom
7. Eisai Inc, New York, USA
8. Utrecht University, Utrecht Institute of Pharmaceutical Sciences (UIPS),

Manuscript in preparation

ABSTRACT

Introduction: E7820 is an orally administered sulfonamide that inhibits alfa-2-integrin mRNA expression. Preclinically E7820 showed tumor anti-angiogenic effect in various tumor cell lines and mice models. Human daily dosing of 100 mg showed to be safe and tolerable.

Methods: the study consisted of two parts, Part A (food effect) and Part B (determination of MTD for BID Dosing). E7820 dose-levels started from 50 mg/BID with planned escalation to 60, 80 and 100 mg/BID with duration of 28 per cycle.

Results: Fifteen patients were enrolled in Part A and 26 in Part B. The most frequent adverse events all grades were constipation, diarrhea, nausea, and fatigue while anemia, neutropenia, and fatigue were most frequent grade ≥ 3.

At dose-level 60 mg BID two patients experienced dose-limiting toxicities (grade 3 neutropenic sepsis and grade 4 neutropenia). Therefore the recommended dose (RD) was 50 mg BID.

Food does not have an effect on E7820 exposure. E7820 exposure following twice daily administration was dose-related. Expression of platelet integrin-α_2 generally decreased in average 8.9% from baseline following treatment with E7820, where reduction was most pronounced immediately after treatment. At the RD 66.7% patients showed stable disease as their best response.

Conclusions: Food had no effect on E7820 exposure. A dose of 50 mg BID was considered the MTD. Treatment with E7820 is safe and tolerable with 2/3 of patients having stable disease as their best response.

INTRODUCTION

Integrins are transmembrane receptors important for tumor angiogenesis as they mediate migration, proliferation and differentiation of human endothelial precursor cells. By blocking the apoptosis they interfere the survival of endothelial cells.[1,2] Inhibition of integrins results in tumor remission.[3] E7820 is an orally administered, small aromatic sulfonamide that inhibits alfa-2(α_2)-integrin mRNA expression. In vitro, E7820 inhibits tube formation and endothelial cell growth, as demonstrated on human umbilical vein endothelial cells (HUVEC) by vascular endothelial growth factor (VEGF).[4] In vivo, twice-daily oral treatment with E7820 clearly inhibited tumor growth and tumor induced angiogenesis in mice of subcutaneously implanted tumor lines derived from human colon, breast, pancreas, kidney and completely suppressed the growth of human pancreatic and colon cell lines. [4,5]

The antitumor effect of E7820 was associated with inhibition of integrin- α_2 expression on epithelial cells and on platelets.[5] E7820 reduced integrin-α_2 expression level on platelets at a dosage close to that affording antitumor activity and showed that platelet integrin-α_2 expression level is a favorable biomarker for E7820 anti-tumor activity. [5]In the first-in-man, dose-escalation study with E7820 (study 102), 37 patients with advanced or refractory malignancies were enrolled. The treatment included daily (QD) dosing for 28 days in cycle 1, followed by a 7-day no-treatment

rest period and thereafter continuous daily dosing. [6] Two out of six enrolled patients at the highest dose-level of 200 mg/day experienced dose limiting toxicities (DLT) and the maximal tolerated dose (MTD) and the recommended dose (RD) for further testing of E7820 was 100 mg QD based on a fasting schedule. Most grade 3 and 4 adverse events were hematological at 100 and 200 mg/day, such as thrombocytopenia grade 4 with neutropenia and renal and liver dysfunction, pancytopenia with fatal bleeding secondary to advanced cervical cancer and thrombocytopenia grade 4. The appearance of decreased platelets count was not seen in the preclinical studies during a dose-dependent reduction in platelet integrin-α_2 expression. E7820 administration with food resulted in delayed absorption (median time to reach maximum concentration t_{max} - 6 hours fed vs. 2.5 hours fasted) and increased exposure by 58% at the 100 mg dose-level. Study 102 showed in 1/3 of patients stable disease as their best response.
A pharmacokinetic/pharmacodynamic (PK/PD) modeling and simulation analysis integrating data from preclinical experiments and Study 102 showed that daily 200 mg dosing would result in a reduction in integrin-α_2 expression accompanied by 90% tumor stasis in more than 95% of subjects, while bi-daily 50 mg dosing was predicted to result in even greater and more sustained inhibition than was predicted for 200 mg QD dosing.[7]
Therefore this phase I dose-escalation study was planned to further investigate the (hematological) safety and confirm predicted food effect, pharmacokinetics and preliminary anti-tumor activity of bi-daily (BID) E7820 in patients with unresected solid tumors.

PATIENTS AND METHODS

Study design

This open-label, multi-center study was conducted at 6 sites in Europe (4 sites in the UK and 2 sites in The Netherlands) and consisted of 2 parts: Part A (food effect study) and Part B (determination of MTD for BID Dosing). E7820 treatment cycles were 28 days long.
Part A was 2-way cross-over study with the aim to determine the effect of a high fat meal on oral bioavailability E7820 in comparison with fasting conditions. In the treatment phase each subject received a single 50 mg dose of E7820 on Day 1, either after fasting for 10 hours, or immediately after consuming a high fat breakfast. Following a 7-day washout period, the subjects crossed-over and a second 50 mg dose of E7820 was administered on Day 8. At this time, patient's transition into the extension phase could be done. All patients, if still on treatment, would start receiving E7820 100 mg once daily (QD) in the fasted state until disease progression.
Part B was a multiple dose study designed to establish the MTD of E7820 given by BID dosing, starting with 50 mg BID and escalating the dose to 60 mg BID, 80 mg BID, and 100 mg BID. The results from Part A were evaluated to determine if E7820 was to be administered with or without food in Part B.

Subjects were enrolled into Part B using a conventional algorithm (3+3 subjects per dose level) to identify the MTD. The dose of E7820 was to be escalated if 0 of 3 or ≤1 of 6 subjects experienced dose-limiting toxicity (DLT). The MTD was defined as the highest dose-level at which no more than 1 of 6 subjects experienced a DLT. At the time that the last enrolled subject completed 6 cycles of treatment or discontinued early, all subjects could transition into the Extension Phase of Part B, where subjects were to continue to receive the same treatment until disease progression.
The explorative objective of this study was to explore the effect of E7820 on changes in tumor volume, platelet integrin-α_2 expression and changes in circulating endothelial precursor cells.

Dose-limiting toxicities (DLTs)

DLTs were defines as neutropenia < 0.5 x 10^9/L for > 5 days or neutropenia < 1 x 10^9 /L with fever, thrombocytopenia < 25 x 10^9/L accompanied by bleeding or thrombocytopenia < 10 x 10^9/L, any grade 3 or 4 non-hematological toxicity for which the study drug could not be excluded as a cause (other than nausea, vomiting or diarrhea in the absence of appropriate prophylaxis); treatment delay of greater than 14 days required to recover from E7820-related toxicities.

Inclusion/Exclusion Criteria

Patients ≥ 18 years with histological or cytological evidence of an unresectable or refractory solid tumor, ECOG performance status ≤ 2 and stabile or asymptomatic brain metastases were eligible. Patients needed to have adequate liver, bone marrow and renal function as well as provided written informed consent. Females (also partners) that were lactating or pregnant were non-eligible. Exclusion criteria were also leptomeningeal metastases and unstable brain metastases, active hemoptysis within 3 weeks prior to the first dose of study drug, hypersensitivity to sulfonamide derivatives, subjects who had radiation to ≥30% of their bone marrow, subjects who required therapeutic anti-coagulant therapy with warfarin or related vitamin K antagonists. Prophylactic doses of heparin or low molecular weight heparin or thrombin inhibitors could be used in place of warfarin.
Other important exclusion criteria were left ventricular ejection fraction (LVEF) < 50% on echocardiography or multiple-gated acquisition (MUGA) scanning, anticancer therapies that had not been completed at least 28 days (42 days in the case of mitomycin C or nitrosoureas) prior to treatment with E7820 (other than surgery or treatment with a protein kinase inhibitor which had to have been completed no less than 1 week prior to treatment with E7820), incomplete recovery from previous radiotherapy, chemotherapy, or surgery other than residual cutaneous effects or stable < grade 2 gastrointestinal toxicity, history of an ischemic cardiac event, myocardial infarction or unstable cardiac disease within 3 months before study entry, a clinically significant electrocardiogram (ECG) abnormality, including a

marked baseline prolongation of QTc interval > 480 ms, concurrent treatment with cytochrome P450 (CYP3A4) inhibitors such as verapamil, cyclosporin, quinidine, erythromycin, mibefradil, clarithramycin and azoles and concurrent treatment with drugs known to be extensively metabolized by CYP2C9 and/or CYP2C19. Chronic treatment with known inducers of CYP3A4 within 4 weeks of receiving treatment with E7820 other than corticosteroids.

Pharmacokinetics

Blood samples for PK analyses were collected at the following time points: for Part 1: day 1 and 8 at predose, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 30 and 48 h postdose; for Part B: cycle 1 Day 1 and 8 at predose, 0.5, 1, 1.5, 2, 3, 4, 5, 8 and 12h postdose, cycle 1 day 15 at predose, cycle 1 day 22 at predose, 1 and 4h postdose.
E7820 concentration in plasma samples was measured using a validated liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) method. Part A was a comparative bioavailability study to estimate the effect of food on the primary E7820 PK parameters ($AUC_{(0\text{-}inf)}$, $AUC_{(0\text{-}t)}$, C_{max}). The effect of food was estimated using a mixed linear model of logarithmically transformed values of the primary PK parameters with fixed effects for treatment, period and sequence and a random effect of subject. Ratios of geometric means and associated two-sided 90% confidence intervals were presented. If the 90% confidence interval (CI) of the model-based geometric mean ratio of fed to fasted were to fall within 70% to 143% for clinical significance, then the fasted state could be declared to have similar bioavailability to the fed state.

Pharmacodynamics

Platelet integrin alpha2 expression was collected predose on day 1 of cycle 1, then weekly during cycle 1 and on day 1 of every subsequent cycle in Part B. measurements were performed using fluorescence activated cell-sorting (FACS) analysis.

Pharmacogenomics/Pharmacogenetics

An archived tumor sample was collected. The tumors were genotyped for Kirsten-ras oncogene (KRAS) mutation.

Safety and anti-tumor activity

Safety assessments consisted of clinical laboratory parameters, vital signs, 12-lead ECG results, physical examinations, and MUGA scans or echocardiograms. Safety variables were shown as treatment-emergent adverse events (TEAEs), AEs absent before the pre-treatment state, graded by Common Terminology Criteria for Adverse Events (CTCAE) version 4.03.

Tumor assessments were performed and assessed by each site investigator using RECIST 1.1 (with the modification that chest disease could not be followed using chest x-ray alone) at Baseline and every 8 weeks of treatment using CT scans and brain MRI.

RESULTS

In Part A, 15 patients were randomized into the study, completed the treatment phase and after day 8 entered the extension phase. In Part B, 26 patients were treated, 18 completed the treatment phase from whom three patients treated with 50 mg BID E7820 continued and after 6 cycles and entered the extension phase (Table 1 and 2). At the time of data cutoff (30 Apr 2014), one subject was ongoing in Part B of the study. The majority of patients were male 53.3% (Part A) and 69.2 % (Part B). Median age was 58.0 (range: 41 to 77) years in Part A and 59.0 (range: 38 to 77) in Part B. Almost all subjects had a baseline ECOG performance status ≤ 1.

Safety

The most frequent treatment-emergent (TE) adverse events (>50% in any treatment group) were constipation, diarrhea, nausea, and fatigue (Table 2). Grade ≥ 3 TE adverse events were experienced by 7/15 (46.7%) of patients in Part A and 19/26 (73.1%) in Part B. The most frequent grade ≥ 3 TE adverse event (>15% in any treatment group) were anemia, neutropenia, and fatigue. There was one grade 5 (fatal) bronchitis, not related to treatment (100 mg QD). Adverse events of special interest were present in the following percentages: anemia (6.7% in the extension phase of Part A and 30.8% in Part B), leukopenia (6.7% and 3.8%), neutropenia (6.7% and 7.7%), thrombocytopenia (6.7% and 11.6%), ALT increased (26.7% and 19.2%), AST increased (13.3% and 11.6%), blood creatinine increased (6.7% and 0), dry skin (20.0% and 11.6%), pruritus (20.0% and 13.8%), rash (0 and 15.4%), and rash pustular (0 and 3.8%). The most frequent events, across all treatment groups, were ALT increase and anemia. Most events were treatment-related. Thirty-three percent of patients in the extension phase of Part A (100 mg QD) withdrawn the study because of adverse events, 36.8% in the 50 mg BID group in Part B, and 42.9% in the 60 mg BID group. Neutropenia resulted in the withdrawal of 3 subjects (1 in Part A Extension and 2 in Part B 60 mg BID); 2 of these 3 subjects were also withdrawn due to leukopenia and thrombocytopenia. All other events resulted in withdrawal of only 1 subject each. Dose was interrupted in 13.3% subjects in the Extension Phase of Part A (100 mg QD), 36.8% in the 50 mg BID group in Part B, and 28.6% in the 60 mg BID group.

Determination of MTD (Part B)

Sequential cohorts of subjects were enrolled to determine the MTD during the treatment phase of Part B (dose escalation portion of the study). In cohort 1 (50 mg BID), 3 subjects were treated without experiencing DLTs. In cohort 2 (60 mg BID), 2 out of 3 treated patients experienced DLT. One patient experienced grade

3 neutropenic sepsis considered related to treatment with E7820, starting on cycle 1, day 23 and lasting 5 days. The second patient experienced grade 4 neutropenia considered related to treatment with E7820, starting on cycle 1, day 19 and lasting > 5 days (27 days). The 60-mg dose level (cohort 2) was then expanded to a total of 7 subjects (including 1 replacement) without further DLT. Following de-escalation to 50 mg BID, 4 additional subjects (including 1 replacement) were enrolled at the 50 mg BID dose-level to a total of 7 patients without reporting DLT. The study investigators and Eisai team agreed that 50 mg BID should be considered the MTD for this treatment. Twelve additional subjects were treated at the 50 mg BID dose-level to confirm the MTD and further establish the safety of this dose.

Pharmacokinetics: Food Effect Study (Part A) and Part B

Administration of E7820 immediately after the high fat breakfast resulted in a slight delay in absorption (time to reach maximum concentration [t_{max}]: fed 4 hours vs. fasted 3 hours) and a similar maximum concentration (mean C_{max}: fed 1030 ng/mL vs. fasted 901 ng/mL) and exposure (mean $AUC_{(0\text{-}inf)}$: fed 12500 ng•h/mL vs. fasted 11500 ng•h/mL). Mean elimination half-life ($t_{1/2}$) was comparable in both treatment arms ($t_{1/2}$: fed 10.2 hours vs. fasted 11.7 hours) (Table 3).
In Part 2, E7820 exposure following twice daily administration was dose-related. E7820 accumulation (~2.5-fold) following twice-daily administration is consistent with ($t_{1/2}$). (Table 3)
The ratio of geometric means (fed/fasted) for $AUC_{(0\text{-}inf)}$, $AUC_{(0\text{-}t)}$, and C_{max} were 1.06, 1.11, and 1.13, respectively, and the corresponding 90% confidence intervals (CI) all fell within 70% to 143%, the specified criteria for concluding no clinically significant effect of food on E7820 exposure. Thus, subjects receiving E7820 can be dosed with or without food (Figure 1).

Pharmacodynamics: Platelet Integrin Alpha2 Expression (Part B)

The reduction of expression of platelet integrin-α_2 was most pronounced immediately after treatment. Measured only in Part B as a response biomarker, it decreased in average 8.9% (range 72% reduction till 17.2% increase) from baseline till cycle 1 day 8 following treatment with E7820. (Figure 2).

Pharmacogenomic/Pharmacogenetic Results

Mutations in the KRAS gene were identified in 3/12 subjects (1 subject each with G12S, G12V, and G13D) in Part A and 4/19 subjects (3 subjects with G12D and 1 subject with G12V) in Part B.

Anti-tumor activity

The best overall response in any treatment group was stable disease: 23.1% overall in Part A; 66.7% in the 50 mg BID group in Part B, and 40.0% in the 60 mg BID group in Part B (Table 4).

The median duration of treatment was 54 to 60 days. Treatment duration lasted longer than 6 cycles in two patients (13.3%) in Part A and in two patients (10.5%) in Part B 50 mg BID. One of the latter patient had adenoid cystic carcinoma and is still on study currently completing his 26 cycle with E7820 (as per October 2014).

DISCUSSION

This phase I study in patients with unresectable solid tumors treated with bi-daily E7829 showed that the treatment with E7820 is safe and well tolerated while food does not have a significant effect on the oral pharmacokinetics of E7820. The maximal tolerated dose (MTD) was established at 50 mg BID. In total 66.7% of patients treated at MTD showed stable disease as their best response.

The most frequent adverse events all grades were constipation, diarrhea, nausea, and fatigue, while anemia, neutropenia, and fatigue were adverse events with grade ≥ 3. No treatment related adverse events leading to death occurred. Thrombocytopenia grade 4, reported in the previous phase I study with E7820 (Study 102) were not observed. [6] Two DLTs were observed at 60 mg BID E7820, such as grade 3 neutropenic sepsis lasting 5 days considered related to treatment with E7820 and grade 4 neutropenia considered related to treatment with E7820 lasting 27 days. Therefore this dose level was found to be too toxic and one dose-level below, 50 mg BID, was found to be MTD and therefore the recommended dose for phase II, which is comparable with the recommended dose from Study 102 (100 mg E7820 QD). [6] The effect of food on E7820 exposure that was seen as a trend in the Study 102 could not be confirmed in this study. The effect of food in 15 patients dosed both in fed and fasted state with one week in between showed no statistically significant differences in exposure of E7820. The elimination half-life ($t_{1/2}$) is found to be around the time for the second E7820 dose (between 10.2 and 11.7 h) and accumulation of approximately 2.5-fold was found to be consistent with the bi-daily dosing and $t_{1/2}$.

No partial responses were observed but 2/3 (66.7%) of patients treated with 50 mg BID showed to have stable disease as their best response. In total 10% of the patients showed to be stable for more than 6 months and one patient at the time of this manuscript is completing his 27th cycle at 50 mg E7820 BID.

This study confirmed the findings from the clinical study 102 and PK/PD modeling and simulation analysis that E7820 down-regulates integrin α-2 expression in surrogate tissues platelets. They predicted greater than 50% decrease beyond day 28 of platelet integrin-α_2 expression in 3 of 4 patients at 200 mg, whereas moderate (<30%) decreases at 70 - and 100-mg dose levels. In our study with 50 mg BID E7820 reduction of platelet integrin-α_2 expression was mostly pronounced immediately after treatment average reduction of 8.9%.Unfortunately, due to hematological toxicity 200 mg/day, the dose predicted to target and inhibit enough mRNA integrin expression, could not be achieved in our study.

Integrin inhibitors with diverse molecular structures are safe and potentially active. Interestingly, just few studies showed objective responses. Mainly integrin inhibitors provided prolonged stable disease, such as E7820 in our study.

As a single agent a humanized monoclonal immunoglobulin G2 antibody against the αv subunit of human integrins in patients with progressive castration-resistant prostate cancer with bone metastases was investigated. Septicemia and an increase in ʸ-glutamyl-transferase (GGT) grade ≥ 3 were drug-related toxicities while two out of 26 enrolled patients showed clinically significant PSA reductions and pain relief with one confirmed partial response (PR).[9] Another single agent, β integrin inhibitor, the antiangiogenic peptide, ATN-161 (Ac-PHSCN-NH(2)), administered in patients with solid tumors, showed few side effects ≤ grade 2 and manifested prolonged SD in 1/3 of enrolled patients.[11] In combination with dacarbazine in stage IV melanoma, intetumumab (CNTO 95), a fully human anti-αv integrin monoclonal antibody showed trend towards improved overall survival although still not significant.[8] The same drug, intetumumab, combined with docetaxel in patients with castrate-resistant metastatic prostate cancer with main drug-related toxicity neutropenia and febrile neutropenia, showed in four of ten enrolled patients a serum PSA response and one PR response for 11 weeks. A promising preliminary anti-tumor activity showed volociximab, an anti-α5β1 integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer (NSCLC). It showed neutropenia in 24% of patients. Eight (24%) out of 33 patients enrolled patients achieved PR and 17 (52%) had SD. The median progression-free survival was 6.3 and levels of potential biomarkers of angiogenesis or metastasis were reduced following six cycles of treatment. [10] However, the furthest investigated integrin inhibitor, cilengitide, did not show better overall survival than temozolomide alone in a phase III study in patients with glioblastoma. [12,13] and in recurrent/metastatic squamous cell carcinoma of the head and neck in combination with cisplatin, 5-fluorouracil, and cetuximab and its further development was stopped.[14]

Therefore, a careful choice of the chemotherapeutic drug combination(s) and trial design are of importance in the development of anti-integrin cancer treatment development. Based on the preclinical knowledge and trends shown with the clinical trials this treatment combination could prevent drug resistance and tumor relapse.[2]

CONCLUSIONS

A dose of 50 mg BID was considered the MTD. Tolerance of E7820 was acceptable and no significant safety concerns were identified. The best overall response in any treatment group was stable disease, with two third of patients showing stable disease (66.7%) at MTD. Food had no effect on E7820 exposure.

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Category	Part A	Part B
	Overall (N=15)	E7820 Total(N=26)
Age (year)a		
Median	58.0	59.0
Min, Max	41, 77	38, 77
Sex, n (%)		
Male	8 (53.3)	18 (69.2)
Female	7 (46.7)	8 (30.8)
ECOG Status, n (%)		
0	5 (33.3)	8 (30.8)
1	9 (60.0)	17 (65.4)
2	1 (6.7)	1 (3.8)
Tumor type		
colon/rectum	5 (33.3)	15 (57.7)
lung	3 (20)	1 (57.7)
sarcoma	3 (20)	2 (3.8)
other	4 (26.7)	8 (30.7)
Target Lesions, n (%)		
Lymph Node Target Lesions	2 (13.3)	9 (34.6)
Non-Lymph Node Target Lesions	14 (93.3)	26 (100)
Non-Target Lesions, n (%)		
No	2 (13.3)	3 (11.5)
Yes	13 (86.7)	23 (88.5)
Metastatic Disease and/or locally advanced disease, n (%)		
Both	3 (20.0)	5 (19.2)
Locally advanced	1 (6.7)	2 (7.7)
Metastatic	11 (73.3)	19 (73.1)
Time from original histological/cytological diagnosis to first dose (months)		
Mean (SD)	35.0 (20.34)	44.3 (28.41)
Median	30.5	35.0
Min, Max	9.8, 69.4	14.6, 126.4
Time from last progression to first dose (months)		
Mean (SD)	3.6 (1.90)	9.6 (18.97)b
Median	3.4	3.0b
Min, Max	1.3, 8.3	1.4, 92.4b

Table 1. Parts A and B: Demography and Baseline Characteristics

MEDRA SYSTEM ORGAN CLASS	PART A			PART B	
Preferred Term	**Treatment Phase**		**Extension Phase**	**Treatment and Extension Phases**	
	E7820 50 mg Fed	**E7820 50 mg Fasted**	**E7820 100 mg QD**	**E7820 50 mg BID**	**E7820 60 mg BID**
	(N=15)	**(N=15)**	**(N=15)**	**(N=19)**	**(N=7)**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Anaemia	1 (6.7)	0	1 (6.7)	8 (42.1)	0
Neutropenia	0	0	1 (6.7)	0	2 (28.6)
Thrombocytopenia	0	0	1 (6.7)	1 (5.3)	2 (28.6)
Abdominal pain	0	1 (6.7)	3 (20.0)	6 (31.6)	2 (28.6)
Ascites	0	0	0	2 (10.5)	0
Constipation	0	1 (6.7)	4 (26.7)	8 (42.1)	4 (57.1)
Diarrhoea	0	0	2 (13.3)	10 (52.6)	1 (14.3)
Dyspepsia	1 (6.7)	0	3 (20.0)	2 (10.5)	2 (28.6)
Nausea	2 (13.3)	1 (6.7)	2 (13.3)	5 (26.3)	4 (57.1)
Vomiting	0	0	3 (20.0)	5 (26.3)	3 (42.9)
Fatigue	0	1 (6.7)	5 (33.3)	10 (52.6)	4 (57.1)
Oedema peripheral	0	0	2 (13.3)	3 (15.8)	1 (14.3)
Infections and infestations	0	1 (6.7)	7 (46.7)	8 (42.1)	4 (57.1)
Alanine aminotransferase increased	0	0	4 (26.7)	3 (15.8)	2 (28.6)
Aspartate aminotransferase increased	0	0	2 (13.3)	2 (10.5)	1 (14.3)
Blood albumin decreased	0	0	0	2 (10.5)	0
Blood alkaline phosphatase increased	0	0	2 (13.3)	3 (15.8)	2 (28.6)
Blood bilirubin increased	0	1 (6.7)	5 (33.3)	1 (5.3)	0
Decreased appetite	0	0	4 (26.7)	7 (36.8)	1 (14.3)
Hyperglycaemia	1 (6.7)	0	2 (13.3)	3 (15.8)	1 (14.3)
Back pain	1 (6.7)	0	2 (13.3)	4 (21.1)	1 (14.3)
Headache	0	0	1 (6.7)	3 (15.8)	1 (14.3)
Lethargy	1 (6.7)	0	2 (13.3)	7 (36.8)	1 (14.3)
Dry skin	0	0	3 (20.0)	3 (15.8)	0
Pruritus	0	0	3 (20.0)	1 (5.3)	0
Rash	0	0	0	3 (15.8)	1 (14.3)
Hypertension	1 (6.7)	0	0	2 (10.5)	2 (28.6)

Table 2: Part A and B - overview of treatment-emergent adverse events (TEAE) during the treatment and extension phases

PARAMETER	PART A E7820 50 MG	
	Fed (N=14)a	Fasted (N=15)
Cmax (ng/mL)	1030 (311)	901 (242)
tmax (h)b	4.05 (1.02, 10.05)	3.07 (0.50, 6.03)
AUC(0-t) (ng•h/mL)	11200 (3680)	10500 (3460)
AUC(0-inf) (ng•h/mL)	12500 (3510)c	11500 (3490)d
t½ (h)	10.2 (2.77)c	11.7 (4.81)d
CL/F (L/h)	4.29 (1.21)c	4.89 (2.19)d
Vz/F (L)	63.6 (27.9)c	76.8 (31.5)d

TREATMENT CONTRAST (TEST : REFERENCE)	PARAMETER	TESTN	REFERENCEN	GEOMETRIC LS MEANSTEST	GEOMETRIC LS MEANS REFERENCE	GEOMETRIC LS MEAN RATIO (90% CI)
Fed: Fasted	AUC(0-inf) (h*ng/mL)	12	14	11373	10733	1.06 (0.97, 1.15)
Fed: Fasted	AUC(0-t) (h*ng/mL)	14	15	10741	9701	1.11 (1.02, 1.20)
Fed: Fasted	Cmax (ng/mL)	14	15	966	858	1.13 (1.00, 1.27)

PHARMACOKINETIC PARAMETER	PART B E7820 50 MG BID		PART B E7820 60 MG BID	
	Cycle 1 Day 1 n=19	Cycle 1 Day 8 n=18a	Cycle 1 Day 1 n=7	Cycle 1 Day 8 n=7
Cmax (ng/mL)	1150 (338)	1880 (591)	1310 (424)	2950 (1530)
DNCmax (ng/mL)	23.0 (6.76)	37.6 (11.8)	21.8 (7.05)	49.1 (25.5)
tmax (h)bMedian	2.00 (0.50, 5.00)	1.75 (1.00, 8.08)	3.05 (1.55, 5.08)	2.02 (0.50, 4.08)
AUC(0-t) (ng•h/mL)	6780 (2070)	14500 (4850)	9620 (3500)	26500 (14500)
DNAUC(0-tau) (ng•h/mL)	136 (41.2)	289 (96.8)	160 (58.3)	442 (242)
RacCmax	NA	1.70 (0.445)	NA	2.19 (0.643)
RacAUC(0-tau)	NA	2.19 (0.597)	NA	2.73 (0.805)

Table 3: Part A and B - Mean (SD) pharmacokinetic parameters of E7820 following twice daily administrationLast sampling time point was 12 hours after administration. AUC(0-t) = area under the concentration × time curve from time zero to time of last measurable concentration, Cmax = maximum drug concentration, DNAUC(0-tau) = dose normalized AUC(0-tau), DNCmax = dose-normalized Cmax, NA = not applicable, tmax = time to reach maximum (peak) concentration after drug administration, RacAUC(0-tau) = accumulation index based on AUC(0-tau), RacCmax = accumulation index based on Cmax.

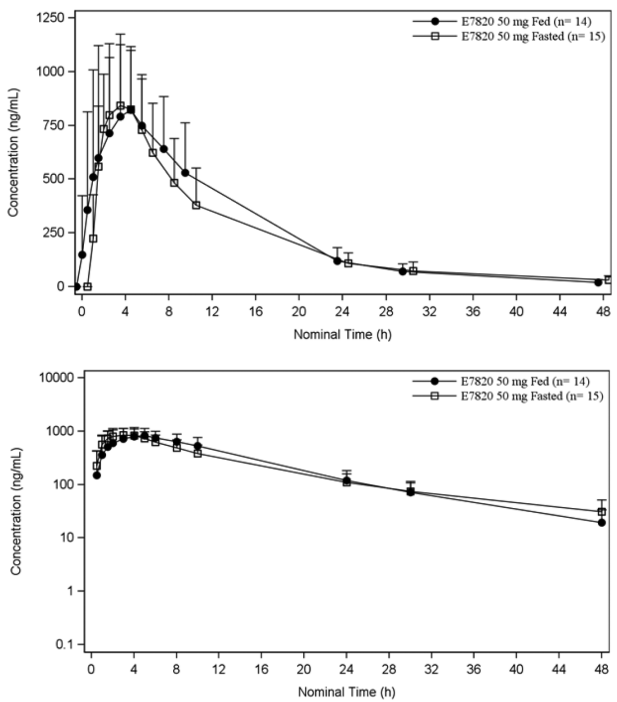


Figure 1: Part A - Mean plasma concentration-time curve of E7820 following administration of a single dose of 50 mg E7820 to subjects with solid tumors on linear (upper panel) and semilog (lower panel) scales – pharmacokinetic analysis set

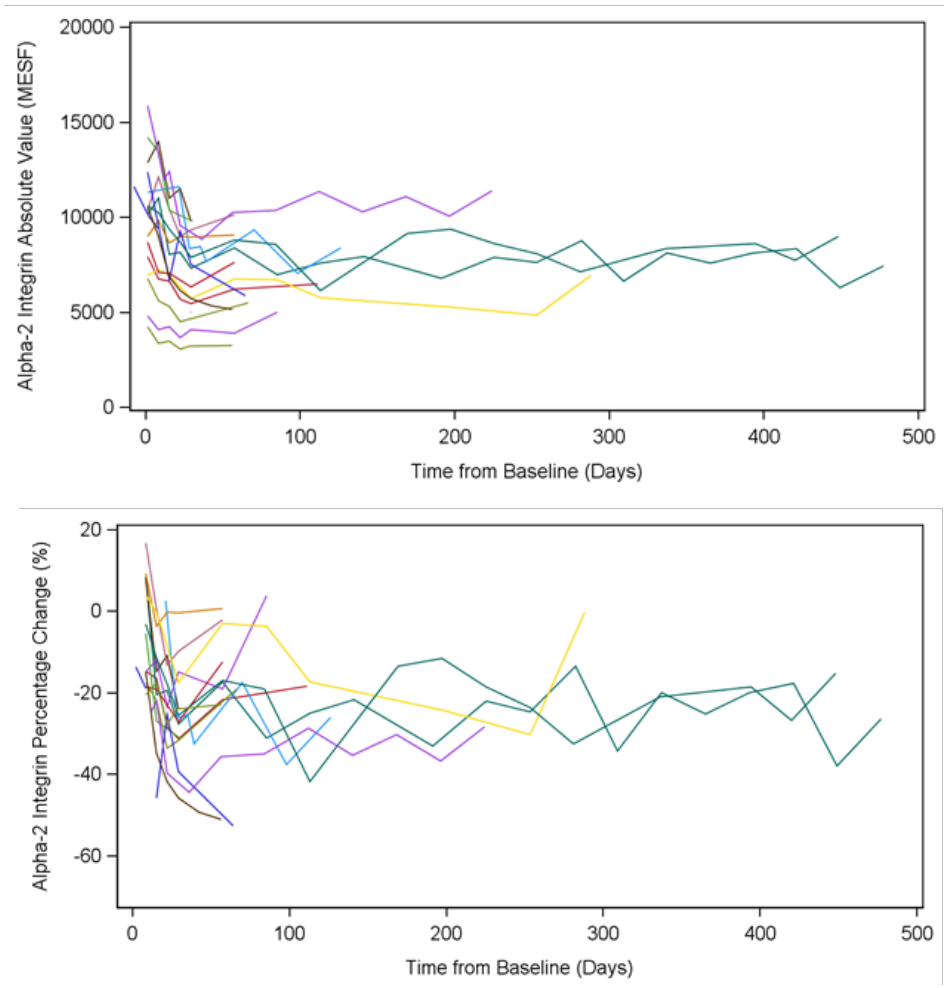
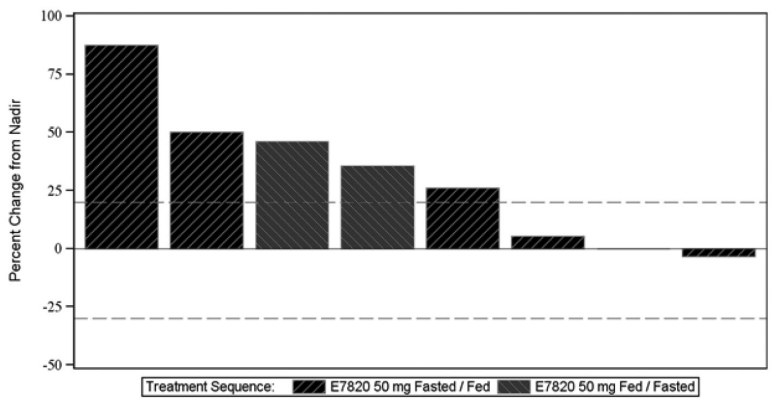


Figure 2. Part B: Individual absolute values (upper panel) and percentage change from baseline (lower panel) of platelet alpha-2 integrin in the 50 mg BID group

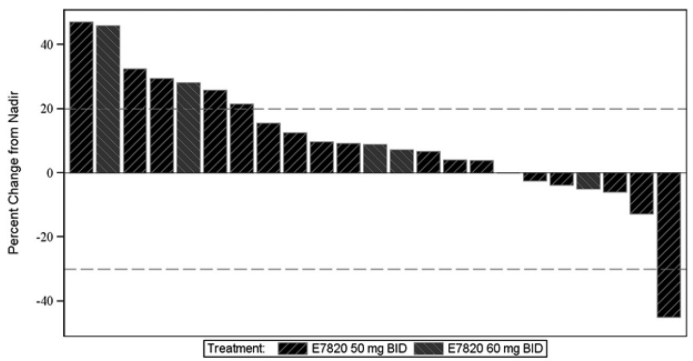
RESPONSE CATEGORY	PART A	PART B	
	E7820 Overall(N=13) n (%)	E7820 50 mg BID(N=18) n (%)	E7820 60 mg BID(N=5) n (%)
Complete Response (CR), n (%)	0	0	0
Partial Response (PR), n (%)	0	0	0
Stable Disease (SD), n (%)	3 (23.1)	12 (66.7)	2 (40.0)
Progressive Disease (PD), n (%)	9 (69.2)	6 (33.3)	2 (40.0)
Not Evaluable (NE), n (%)	1 (7.7)	0	1 (20.0)
Objective Response Rate (CR + PR), n (%)	0	0	0
95% CI of Objective Response Ratea	(0.0, 24.7)	(0.0, 18.5)	(0.0, 52.2)
Disease Control Rate (CR + PR + SD), n (%)	3 (23.1)	12 (66.7)	2 (40.0)
95% CI of Disease Control Ratea	(5.0, 53.8)	(41.0, 86.7)	(5.3, 85.3)

Table 4: Parts A and B - Best overall response, extension phase

Part A: Waterfall plot of percentage change from baseline in target lesions



Part B: Waterfall plot of percentage change from baseline in target lesions



1.3 A multicentre, open label, Phase I trial of the MEK inhibitor MSC1936369B (pimasertib) given orally to Subjects with Solid Tumors

B. Milojkovic Kerklaan[1], N. Houede[2], J.P. Delord[3], A. Awada[4], C. Lebbe5, T. Lesimple[6], S. Rottey[7], R. Kefford[8], M. Roelvink-Mergui[1],O. von Richter[9], E. Raymond[10]. J.H.M. Schellens[1,11]

1. The Netherlands Cancer Institute –Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands,
2. Institut Bergonie, Bordeaux, France;
3. Institut Claudius Regaud, Toulouse, France;
4. Jules Bordet Institute, Brussels, Belgium;
5. APHP Hopital Saint-Louis, Paris, France;
6. Centre Eugene Marquis,Rennes, France;
7. Heymans Institute of Pharmacology Ghent University Hospital, Ghent, Belgium;
8 Westmead Hospital and Melanoma Institute, University of Sydney, Sydney NSW, Australia;
9. Merck KGaA, Darmstadt,Germany;
10. Beaujon University Hospital, Clichy, France;
11. Utrecht University, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands

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ABSTRACT

Introduction: Pimasertib (MSC1936369B) is a highly selective, uncompetitive oral MEK1/2 inhibitor demonstrating robust anti-tumor activity in vitro and xenograft models.

Methods: In this phase, first-in-man, open-label study with four dosing regimens, the recommended dose (RD) for phase II was investigated along with the safety, pharmacokinetics, pharmacodynamics (PD) profile, food-effect and preliminary anti-tumor activity.

Results: A total of 180 patients were included in four regimens. The dose of 120 mg/day (60 mg BID) was defined as Maximal Tolerated Dose and RD on the basis of the number of patients with DLTs (3/17 patients) in cycle 1 (regimen 3) with dermatitis acneiform grade 3 and popular rash grade 3. Pimasertib was rapidly absorbed reaching maximal drug concentrations in plasma within T_{max}=1h. No food effect could be seen. Terminal half-life was $T_{1/2}$=5.1h with negligible renal elimination of unchanged pimasertib. At doses ≥ 28 mg/day PD marker, pERK inhibition in a peripheral blood mononuclear cell (PBMC) was fully inhibited at 2h and returned to baseline at 24h. Patients with melanoma and mutated NRAS (NRASmut) showed 1 complete response (CR), 2 partial responses PR (12%), 7 stable diseases SD (44%), which in total showed 62% of disease control (CR+PR+ SD). Patients with BRAFmut status showed 4 PR (21%) and 6 SD (31%) 53% disease control.

Conclusions: Oral pimasertib as a single agent is relatively safe with mostly dermatological, ocular and gastrointestinal toxicities. The RD for phase II is 120 mg/day (60 mg BID) pimasertib. Pimasertib exhibits a favorable PK profile in patients with solid tumors.

INTRODUCTION

Signals for cell proliferation and survival are transmitted from the cell surface to the nucleus mainly by the mitogen-activated protein kinase (MAPK) pathway. [1] Dysregulation of these functions are hallmarks of cancer. [2] Extracellular signals (including growth factors that activate cell surface receptors (e.g. Her2, EGFR) can activate the Rat sarcoma protein (RAS) family of proteins that further relay signals to the downstream intracellular RAF/MEK/ERK or MAP-kinase pathway. In cancer cells in case of ras mutations this pathway is continuously activated due to extracellular signals or hyperactive downstream components. Mutated KRAS is identified in approximately 30% of colon, 25% of non-small cell lung cancer (NSCLC) and 90% of pancreas adenocarcinoma, while BRAF gene mutations are found in about 60% of malignant melanomas. [3–5]

MEKs, members of the MAPK signaling cascade, play a crucial role in transmitting signal inputs from a variety of protein kinases. [6]

In addition, MEKs are restricted in their substrate specificity, with ERK being the sole known substrate. Activation of this MEK/ERK pathway alone is sufficient for tumor progression together with a malignant phenotype. [7,8] As previously reported, se-

lective pharmacological inhibition of MEK by other MEK inhibitors inhibits the proliferation of melanoma cells with NRAS mutations.[9] Based on all these data, MEKs are the targets of great interest for the development of cancer therapeutics. [10]

MSC1936369B (Pimasertib), a biaryl amine derivative with a molecular weight of 467.8 Da is a highly selective, uncompetitive oral MEK1/2 inhibitor demonstrating robust anti-tumor activity in cell proliferation in vitro. [11] (Figure 1) Pimasertib affects mainly cancer cells with activated MAPK signaling, exerting its action via G1 arrest resulting in apoptosis. Tumor cell lines with BRAF and KRAS mutations were more sensitive to pimasertib. In the resistant human lung adenocarcinoma cell line continuously treated with tyrosin kinase inhibitors (TKIs – erlotinib, gefitinib, vandetanib and sorafenib), pimasertib caused inhibition of cell proliferation, invasion and tumor growth. [11]

When treating tumor-bearing mice with pimasertib using different dosing schedules, highest tumor regression was achieved with 14 days continuous BID treatment, compared to a once daily dosing. Including a 2-day dosing holiday per week resulted in similar tumor growth inhibition as was seen with every other day dosing BID and with once daily continuous dosing. Based on efficacy, PK/PD and scheduling studies, the greatest tumor growth inhibition in mouse xenograft models was achieved when MEK was continuously and maximally inhibited. (Merck Serono data on file)
This is the first-in-man study with pimasertib with the aim to determine the maximal tolerated dose (MTD) for several regimens administered orally, to provide preliminary safety information on the safety profile, to assess the pharmacokinetics (PK) and pharmacodynamic (PD) markers in blood and in tumor biopsies, to explore the effect of food on PK and to assess the preliminary anti-tumor activity of pimasertib.

PATIENTS AND METHODS

Design

In this phase I first in man, open-label, dose-escalation trial, cohorts of 3 subjects with solid tumor patients were sequentially assigned to regimen 1 or regimen 2 and received pimasertib (MSC1936369B) at escalating dose levels. At each dose level subjects received pimasertib orally once daily on: days 1 to 5, 8 to 12 and 15 to 19 of a 21-day cycle (regimen 1, once daily dosing including a 2-day dosing holiday per week), or days 1 to 15 of a 21-day cycle (regimen 2). (Table 1)
After determination of the maximum tolerated dose (MTD) for dosing regimen 2, 12 additional patients with melanoma were enrolled in the food-effect (FE) cohort, part of regimen 2. The FE evaluation followed a cross-over design with alternating assignment to two sequences. Half of the subjects in the expanded cohort

received a pre-defined and standardized breakfast prior to drug administration on day 1 of cycle 1, and had the drug administration after an overnight fasting on day 1 of cycle 2. The second half of the subjects in this cohort fasted on cycle 1 day 1, and received breakfast on day 1 of cycle 2.
A third dosing regimen (regimen 3) was investigated separately after completion of dose escalation of regimens 1 and 2. Only patients with melanoma were enrolled in cohorts of 3 and received pimasertib at escalating dose levels.

Main eligibility criteria

Eligible patients were ≥ 18 years old with pathologically-confirmed solid tumor which is locally advanced or metastatic, and either refractory after standard therapy for the disease or for which no effective standard therapy is available. In regimen 3 (QD and BID cohorts) and regimen 2 food-effect, the tumor type was restricted to melanoma. Patients with stable brain metastases or previously treated with no evidence of cerebral edema, and no requirements for corticosteroids or anticonvulsants could enter the study. Patients with impaired bone marrow, renal and liver function were excluded. Other major exclusion criteria were INR > 1.5 x ULN, serum calcium > 1 x ULN, Eastern Cooperative Oncology Group Performance status (ECOG PS) > 1, known chronic infection, previous chemotherapy, immunotherapy, hormonal therapy, biologic therapy, or any other anticancer therapy or surgical intervention within 28 days or 5 half-lives for non - cytotoxic treatment. Extensive prior radiotherapy on more than 30% of bone marrow reserves was an exclusion criteria, as well as prior bone marrow/stem cell transplantation, significant cardiac conduction defects and/or pacemaker, uncontrolled hypertension by medication, pregnant or nursing female, retinal degenerative disease (hereditary retinal degeneration, or age-related macular degeneration), history of uveitis or history of retinal vein occlusion.

Pimasertib and dose escalation rules (DLT, MTD)

Pimasertib (MSC1936369B, formerly known as AS703026) was supplied as 0.5, 4, 15, and 30 mg hard gelatine capsules, size 0. Pimasertib capsules were taken orally. The starting dose was taken at 1/10th of the human equivalent dose HED (0.014 mg/kg) found in experiments in dogs. Therefore, the first cohort of subjects in regimens 1 and 2 were treated at a dose-level of 1.0 mg/day of pimasertib orally. Dose escalation proceeded separately for both regimens according to an accelerated dose - escalation schedule (100% dose increment) until observation of grade 2 drug-related toxicities. After that a modified Fibonacci scheme was be used (approximately 66%, 50%, 40% then 33% dose increments). The trial will follow a sequential dose-escalation cohort design ("3+3" design), with 3 or up to 6 subjects in each cohort and up to a maximum of 12 at the MTD.

The MTD is defined as the dose level below the one at which dose limiting toxicity (DLT) occurs in > 1 out of 3 or > 1 out of 6 subjects. If > 1/3 or > 2/6 subjects experience DLT, additional subjects will be treated at the previous dose, which will be considered as MTD, to have a total number of 12 at that level.
Each cycle consisted of 21 days. The treatment could be continued until the disease progression. Patients presenting ≥ grade 3 ophthalmologic changes had to discontinue the treatments.

Safety and preliminary antitumor activity assessments

At screening, during the study and at the end of the study the following will be performed: obtain informed consent, physical examination (ECOG PS, vital signs, medical history, concomitant medication history), laboratory samples analyses (for hematology, serum chemistry, parathyroid hormone, serum pregnancy test, urinalysis), cardiac monitoring by echocardiogram (ECG) and ECHO or MUGA scan, chest x-ray, ophthalmologic assessment, tumor assessment with CT or MRI. During the whole study duration adverse events will be followed, during the first cycle, DLT assessment will be done.

Pharmacokinetics (PK)

Plasma and urine were collected for the assessment of pharmacokinetics. Concentrations of pimasertib in plasma and in urine were determined by a LC-MS/MS method. Intensive blood sampling for pharmacokinetics was performed for all regimens during cycle 1 at day 1, day 4, day 12 (regimen 1) and day 15 (regimen 2 and 3), at cycle 2 day 1 and cycle 3 day 1 at pre-dose and at 0.5, 1, 1.5, 2, 2.5, 4, 6, 8, 12 and 24 hours after drug administration; at day 3 and 8 of cycle 1 blood sampling was performed pre-dose and at 1 and 4 hours after drug administration. In the subsequent cycles and at the end of treatment one (pre-dose) blood sample was collected. Urine was collected in the collection periods 0-4, 4-8, and 8-24 hours, except for the BID cohort.

Pharmacodynamics (PD)

Blood samples for PD marker p-ERK activity in a peripheral blood mononuclear cells (PBMC) were collected during cycle 1 and 3 at day 1 pre-dose and 2 hours, 8 and 24 hours after drug administration and blood circulating markers (IL-8 and other potential cytokines) collected during cycle 1 and 3 pre-dose and at 4, 6, 8 and 24 hours after drug administration. At the subsequent cycles and at end of treatment PD markers were collected at pre-dose. At cycle 1 day 12 (regimen 1) or day 15 (regimen 2 and regimen 3) p-ERK activity in PBMC was collected pre-dose, 2 hours, 8 hours and 24 hours after drug administration. Cells from the blood sample were fixed with BD Phosflow fixation buffer for 10 min, after which the cells were permeabilized with BD Phosflow permeabiliza-

tion buffer for 30 min and thereafter stained by BD Phosflow antibodies in BD Pharmingen Stain Buffer. The prepared samples were analyzed by BD FACS™ flow cytometry.

Tumor tissue biomarkers

Pre- and on-treatment mandatory tumor biopsies were collected at screening and at day 3: Tumor biopsy was performed for analysis of p-ERK and p-MEK activity, marker of proliferation, apoptosis, gene expression profile and known mutations in candidate genes (mutation analysis can be performed on archived tumor tissue as needed). In sequential tumor biopsies the following PD markers were followed pERK, pS6, Cyclin D1 and Ki-67.

Pharmacogenetics (optional)

Desoxyribonucleic acid (DNA) from PBMCs was analyzed for polymorphisms (i.e. variations) in genes that could be involved in pharmacokinetics and might influence adverse events in response to treatment with pimasertib. One blood sample was collected for ADME/PK pharmacogenetics at day 1 of cycle 1.

Food-effect, regimen 2 cohort

Patients participating in this part of the study were requested to fast for at least 10 hours prior to drug administration. The PK profiles obtained in fasted and fed conditions were compared. PK sampling including a time point at 12 hours after dosing on day 1 of cycles 1 and 2 were obtained in order to characterize the PK of pimasertib in fasted and fed state. Fluid and food intake was controlled in the following 24 hours as subjects were hospitalized on day 1 of cycle 1 and day 1 of cycle 2 for full PK profiling. Except on pre-specified days, the administration of pimasertib was in the same fasted condition as defined for the other cohorts. The breakfast for this food-effect investigation was pre-defined and standardized across all trial centers. The caloric content of the breakfast was in line with the recommendation for a light meal in the draft EMEA Guideline on the Investigation of Drug Interactions – CPMP/EWP/560/95/Rev. 1 – April 2010. Subjects discontinuing the trial before the end of cycle 2 were replaced in this cohort.

Statistical analyses

Descriptive statistics and graphical representations were the main analysis tools. For all analyses, results and graphical representation of individual subjects data were presented separately for each regimen.

RESULTS

Patient characteristics

Patients median age 60, 60, 57 and 60 respectively were enrolled in regimen 1, 2, 3 QD or 3 BID. Except in regimen 3, they were mainly female patients with ECOG PS status ≤ 1. Main tumor type in regimen 1 was colorectal carcinoma and in other regimens melanoma. The median (range) number of prior systemic anti-cancer therapies was respectively 4 (1-14), 3 (0-16), 2 (0-6) and 2 (0-6).

DLT/MTD

In total 15 DLTs were reported during the study. In regimen 1, one DLT – grade 3 hepatotoxicity at dose-level 28 mg/day pimasertib at day 11 and at day 52 one Retinal Vein Occlusion (RVO) grade 2 occurred at 120 mg/day. (Table 3) The maximum administered dose (MAD) for regimen 1 was 120 mg/day; this dose was considered the MTD. One DLT of retinal vein thrombosis in cycle 3 was experienced at 120 mg/day dose-level out of 11 evaluable subjects. Further dose-escalation was stopped, as this regimen appeared inferior in terms of safety compared to regimen 2. In regimen 2 six DLTs occurred. The MAD for regimen 2 was 255 mg/day. At this dose level 2 out of 4 patients experienced DLT: dermatitis acneiform grade 3 and macular degeneration grade 3 with macular edema at day 2 after start with pimasertib. Expansion of the next lower dose-level (195 mg/day) brought 2 DLTs in 12 enrolled patients: rash grade 3 at day 8 and stomatitis grade 3 at day 7. One of the patients at that dose-level experienced RVO grade 2 in cycle 7, thus the next lower dose-level (150 mg/day) was explored and expanded with in total 13 patients who experienced 1 DLT acne grade 3 at day 14 and 1 DLT dermatitis acneiform grade 3 at day 15. The MTD for regimen 2 was considered to be 195 mg/day. The starting dose for regimen 3 was based on the data from regimen 1 and the fact that the median half-life observed for pimasertib in humans is 5 hours without observed accumulation of the compound after administration of multiple doses. Therefore, the proposed starting dose was 60 mg daily, which corresponds to a 30% lower dose than the weekly dose intensity of the R1 MAD (120 mg/day). The dose was escalated to MAD 150 mg/day at which level in 3 out of 13 patients DLTs were recorded during cycle 1. They experienced skin toxicity grade 3, hand-foot syndrome grade 3, macular degeneration grade 3 and creatinine phosphokinase increase grade 4. One lower dose-level was explored for safety and expanded with in total 17 patients of whom 3 patients experienced during cycle 1 dermatitis acneiform grade 3 and papular rash grade 3. The MTD for regimen 3 BID is considered to be 150 mg/day pimasertib. No DLTs were observed at the dose of 90 mg/day (45 mg BID or 90 mg QD).

Safety

The most common related treatment emerged adverse events (TEAE) were affecting gastrointestinal tract (diarrhea, nausea, vomiting and stomatitis), skin (dermatitis acneiform and rash), eye (macular degeneration and retinal detachment) and cardiovascular system (peripheral edema). They all showed early occurrence (from cycle 1) and in most regimens dose dependence, except for diarrhea, ocular events and peripheral edema in regimen 3.

Diarrhea was observed in 32% of patients treated in regimen 1, 48% in regimen 2, 93% in regimen 3 QD and 79% in regimen 3 BID regimens. In 15% of patient treated in regimen 3 diarrhea was grade ≥ 3 which led 7 % of patients to discontinue the treatment.

Dermatitis acneiform were observed in 14%, 23%, 47% and 38% respectively in all 4 regimens. Rash grade ≥3 was observed in 21% of patients treated in regimen 3. No Serious Cutaneous Adverse Reactions (SCARs) were observed, such as Stevens-Johnson syndrome, Lyell Syndrome, Drug Rash Eosinophilia Systemic symptoms (DRESS).

Macular degeneration was observed in 8%, 18%, 60% and 82% of patients respectively in all four regimens. Visual disturbance with grade ≥ 3 was observed in 3% of patients treated in regimen 3. Ocular events, such as retinal vein occlusion, visual disturbances, scotoma, blurred vision, visual acuity reduced or serous retinal detachment showed early occurrence (from cycle 1) and dose dependence in all regimens. Those adverse events, all grades were the reason for treatment discontinuation in 17% in regimen 1 and overall in 8% of patients. However, no permanent impairment of vision was observed.

Peripheral edema was present in 2%, 16%, 40% and 56% of patients respectively in four regimens. It showed a moderate increase over time at lower doses in all four regimens.

Concerning the cardiotoxicity the number of affected patients with left ventricular ejection fraction (LVEF) was too low to draw any definitive conclusions.

There were 1(at 120 mg/day), 10 (90, 150, 195 and 255 mg/day), 1 (60 mg/day) and 15 (90, 120 and 150 mg/day) dose reductions respectively per regimen, while dose omissions were present in 15, 22, 5 and 23 cases respectively, at the similar dose-levels.

Pharmacokinetics

Regimen 1 and 2 yielded similar results. At the recommended dose for regimen 2 (150 mg daily) the maximal peak pimasertib plasma concentration C_{max} was 605.1 ng/ml (range 366.2-1085 ng/ml) and time needed to reach maximal concentrations T_{max} 1 h (0.3-2.0h). Area under the curve AUC_{0-t} was 2287 h*ng/ml and elimination half-time 5.1 h (3.7-9.2) with 95% wash-out in about 1 day. The dose proportionality was observed without considerable deviation at doses ranging from approx. 30 to 255 mg pimasertib per day. Upon multiple dosing no considerable

accumulation with QD or BID dosing regimen could be observed. Concerning the fluctuations of plasma concentrations of pimasertib BID dosing showed marked reduction in Peak-to Trough (PTT) compared with QD dosing.
Concerning the Food effect (FE) study of 11 PK evaluable patients from regimen 2 the median $AUC_{0\text{-inf}}$ during the fed status was 1434 h*ng/ml while $AUC_{0\text{-t}}$ during the fasted status was 1424 h*ng/ml (Ratio 100.7%, 90%CI of range 77.3-131.2) No FE in $AUC_{0\text{-t}}$, $AUC_{0\text{-last}}$ and C_{max} could be seen at the dose-level of 90 mg pimasertib single dose, after intake of a light meal. There is insignificant renal elimination of unchanged drug.

Pharmacodynamics

-Effect of Pimasertib on p-ERK induction by PMA in PBMCs.

ERK phosphorylation was fully inhibited at pimasertib doses ≥ 28mg/day at 2 hours post-dose. QD administration results in short-lived p-ERK inhibition and returned to baseline levels in 24 hours. BID administration reduced p-ERK levels over 24h. Inhibition of ERK phosphorylation gradually disappeared over time and was reduced at 8 h post-dose.

-Effect of marker in tumor biopsy.

In figure 3 markers pERK, pS6, Cyclin D1 and Ki-67 in biopsies pre- and post-treatment are shown in one of the patients with partial remission (PR), a female patient 41 years old that showed 52% tumor reduction and time to progression of 26.4 weeks (figure 3). A visible loss of all four tissue tumor markers is evident after treatment with pimasertib.

Preliminary antitumor activity

In regimen 1 in all dose-levels, 19 patients had stable disease (SD) as their best response, of whom 7 were with colorectal cancer and 5 with melanoma. There were 20 patients with progressive disease (PD) while 10 patients were non-evaluable (NE). In regimen 2 at all dose-levels, 3 patients, all with melanoma had partial response (PR) as their best response, 35 had SD (7 colorectal, 20 melanoma), 33 progressive disease (PD) and 11 NE.
Sixty-three patients with melanoma were enrolled in either the food effect study from regimen 2 or regimen 3 (QD or BID). Sixteen of them had NRAS mutation (mut), 19 BRAFmut, 22 were NRAS/BRAF wild-type (wt) and 6 had unknown mutation status. The NRASmut group one patient (6%) showed complete response (CR), 2 PR (12%) , 6 SD (44%), which in total shows 62% of disease control (CR+PR+SD). Patients with BRAFmut status showed 4 PR (21%) and 6 SD (31%) and 53% disease control . Other two groups with melanoma patients showed 59% and 83% disease control and 1 PR per group. Tumor responses (PR and SD) were mainly observed in melanoma in the cohort receiving 90 mg /day.

The patient aged 66 years with melanoma and NRAS mut, enrolled in the regimen 3, 90 mg BID pimasertib, showed CR as her best response and had time to progression of 71.4 weeks. CR and PR were observed at doses of 90 - 120mg / day. The mean response durations were 23.1 weeks at 120mg/d and 39.6 weeks at 90mg/day. The response durations ranged between 12.6 - 68.1 weeks.

DISCUSSION

In this phase I, first-in-man study with pimasertib (MSC1936369B) in regimen 3 three out of 17 patients experienced DLTs in cycle 1 at the dose of 120 mg/day (60 mg BID). Three out of 13 patients experienced DLTs at the dose of 150 mg/day (75 mg BID), while no DLTs were observed at the dose of 90 mg/day (45 mg BID or 90 mg QD).
The dose of 120 mg/day (60 mg BID) was defined as Maximal Tolerated Dose and Recommended Phase 2 Dose on the basis of the number of patients with DLTs (3/17) in cycle 1 (regimen 3).
The most common drug-related treatment emerged adverse events (TEAE) were diarrhea, nausea, vomiting, stomatitis, dermatitis acneiform, rash, macular degeneration, retinal detachment and peripheral edema. Except for less digestive tract toxicitites, trametinib, the registered MEK inhibitor, shows similar safety profile to pimasertib. Some other MEK inhibitors still in development seem to show less eye toxicities , such as selumetinib, MEK162, RO4987655, GDC-0973, AZD8330 and RO5126766.[12]
The number of subjects affected by skin toxicities after pimasertib treatment stayed unchanged after the first cycle. After cycle one the number of patients affected by ocular toxicities grade ≥ 2 increased from 4% in cycle 1 to 28% from cycle 5 and further at dose-levels 45-120 mg/day.

Pharmacokinetic assessment showed a dose-dependent increase in drug exposure. T_{max} is found to be 1.5 hours, while $t_{1/2}$ was approximately 5 hours. BID dosing reduces peak/trough fluctuations and food did not have an effect on pimasertib pharmacokinetics. There is a negligible renal elimination of unchanged drug.

CR and PR were observed at doses of 90 – 120 mg/day. The mean response durations were 23.1 weeks at 120 mg/day and 39.6 weeks at 90 mg/day. The response durations ranged between 12.6 - 68.1 weeks.

In the cohorts of NRAS-mutant treatment-resistant melanoma 8 patients were treated with pimasertib doses of 90-120 mg/day. There was 1 CR (12.5%), 2 PR (25%) and 4 SD (50%), with 37.5 % response rate and 87.5% clinical benefit. In the cohort of BRAF mutant treatment-resistant melanoma 9 patients were treated with pimasertib doses of 90-120 mg/d, of whom 2 showed PR (22.2%), 2 SD (22.2%). From the cohort of patients with BRAF/NRAS wild-type treatment-resistant melanoma 13 patients were treated and 1/13 responded (7.7%) to pimasertib. Therefore, genetic screening for

NRAS mutations appears to be crucial. Selective treatment of NRAS mutant melanoma patients is currently planned in the phase II study EMR200066-007/ EMR200066-007 (pimasertib 60mg BID).

Currently, there are several MEK inhibitors in clinical trials.[12] The first generation of MEK inhibitors was designed for treatment of tumors with RAS mutations. During the development melanoma cells with BRAF mutations showed higher sensitivity to MEK inhibition than NRAS or KRAS mutated tumors. [13] This was explained by the fact that BRAF mutant cells are more dependent on MEK activity, while RAS mutations are able to activate additional signalling pathways bypassing MEK. [6] Therefore, most studies on MEK inhibitors focus on BRAF mutant cancers. However, a recent study combining selumetinib with docetaxel showed promising efficacy in phase II study in patients with KRAS-mutant NSCLC compared with docetaxel alone. [5]

Trametinib, MEK1 / 2 inhibitor, improved rates of progression-free (PFS) and overall survival (OS) in patients with metastatic melanoma with a BRAF V600E or V600K mutation with rash, diarrhea, and peripheral edema as the most common toxic effects. The trametinib group showed PFS of 4.8 months and OS at 6 months 81% as compared with chemotherapy with PFS of 1.5 months and OS at 6 months of 67%. [9,14] Unfortunately, within 6-8 months these patients progress after commencing therapy, including those who experienced initial, profound tumor regression.[15] The reason is resistance development due to activation of alternative signaling pathways or alternative reactivation of the MAP kinase pathway.

To overcome the resistance to single agent MEK inhibition dual blockade of MAPK with MEK/BRAF inhibitors or MAPK blockage by either BRAF or MEK inhibitor in combination with PI3K pathways inhibitor in tumors with PTEN loss or in combination with mTOR inhibitors or in combination with immune agents, such as ipilimumab were suggested. [15] The argumentation for combining PI3K pathway inhibitors with MAPK pathway inhibitors is based on the possibility of activated PI3K–AKT pathway signal cascades, while MAPK pathway is suppressed and possible crosstalk with the RAS–RAF–MEK1/2–ERK1/2 pathway.

In pimasertib-resistant human colon carcinoma (HCT15) and lung adenocarcinoma (H1975) cells a combination of pimasertib and PI3-kinase inhibitors, or everolimus, sorafenib, or regorafenib demonstrated a synergistic effect in cell growth inhibition and induction of apoptosis with sustained blockade in MAPK- and AKT-dependent signaling pathways. [12] In vivo, the combined treatment with pimasertib and BEZ235 (a dual PI3K/mTOR inhibitor) or with sorafenib caused significant tumor growth delays and increase in mice survival as compared to single agent treatment. [12] The PI3K–AKT pathway signal cascades may be activated and crosstalk with the RAS–RAF–MEK1/2–ERK1/2 pathway may take place. Co-inhibition of these pathways might be required to optimize treatment efficacy. [17]

Combining pimasertib with cytotoxic chemotherapy, and/or other targeted agents could also increase the efficacy of the single agent, however biomarker driven studies need to be executed. Pimasertib is currently further developed in several indications in in combination with a range of different anticancer agents.

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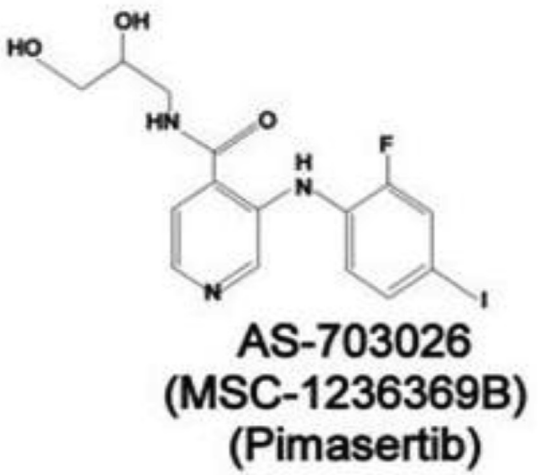
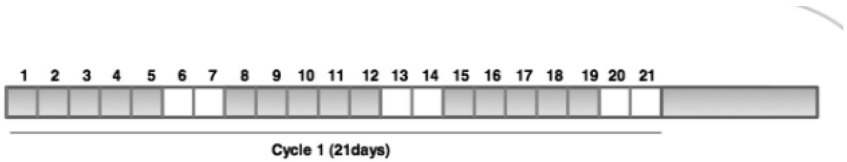


Figure 1. Pimasertib structural formule

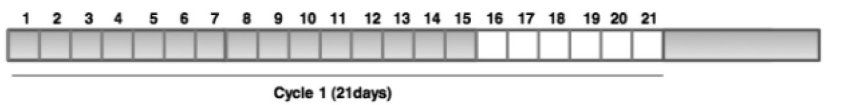
Regimen 1

- Dose range : 1-120mg
- Solid Tumors
- Dose Escalation 1mg – 120mg
- 5d *on* / 2d *off* – QD



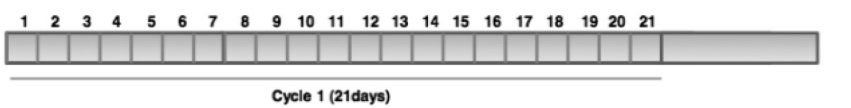
Regimen 2

- Dose range: 1-255mg
- Solid Tumors
- Dose Escalation 1mg – 255mg
- 15d *on* / 6d *off* – QD



Regimen 3QD

- Dose range: 60-90mg
- Melanoma
- Continuous, QD



Regimen 3BID

- Dose range: 90-150mg
- Melanoma
- Continuous, BID

Table 1. Regimens of MSC1936369B given orally to subjects with solid tumor

Patients (N = 180)	Regimen 1 (N = 49)	Rgimen 2 (N = 82)	Regimen 3 QD (N = 15)	Regimen 3 BID (N = 34)
Age	60	60	57	60
Age range	34 - 83	30 -78	30 -77	32 -78
Gender				
Men	29 (59%)	52 (63%)	6 (40%)	23 (68%)
Women	20 (41%)	30 (37%)	9 (60%)	11 (32%)
Performance Status				
ECOG 0	28 (57%)	44 (54%)	4 (27%)	17 (50%)
ECOG 1	21 (43%)	38 (46%)	10 (67%)	17 (50%)
Tumor types (≥ 5% patients)				
Melanoma *	7 (14%)	18 (22%)	12 (80%)	29 (85%)
Uveal Melanoma	1 (2%)	4 (5%)	3 (20%)	5 (15%)
CRC	21 (43%)	16 (20%)		
Breast	1 (2%)	7 (9%)		
Prostate	4 (8%)	4 (5%)		
Mesothelioma	3 (6%)	1 (1%)		
NSCLC	3 (6%)	1 (1%)		
Others	10 (20%)	16 (20%)		
* Cutaneous, mucosal or unknown primary				

Table 2. Patients characteristics

Dose Limiting Toxicities (DLTs) and other related Adverse Events	Regimen 1 (N = 44)		Regimen 2 (N = 74)			Regimen 3 BID (N = 33)	
	28 mg/day (N = 6)	120 mg/day (N = 10)	150 mg/day (N = 13)	195 mg/day (N = 12)	255 mg/day (N = 4)	120 mg/day (N = 17)	150 mg/day (N = 13)
DLTs - N (%)	1 (17%)	1 (10%)	2 (15%)	2 (16%)	2 (50%)	3 (18%)	3 (23%)
Hepatotoxicity (ALT, AST, GGT - G3)	1 (day 11)						
Dermatitis Acneiform - G3			1 (d 15)		1 (d 9)	2 (d 15, d 16)	
Acne - G3			1 (d 14)				
Rash - G3				1 (d 8)			
Rash papular - G3						1 (d 19)	
Skin toxicity - G3							1 (d 14)
Retinal Vein Occlusion (RVO) - G2		*1 (day 52)*		*1 (cycle 7)*			
Macular Degeneration - G3 (LLT: SRD)					1 (d 2)		1 (d 2)
Macular oedema - G3					1 (d 2)		
Creatine Phosphokinase increase G4							1 (d 15)
Palmar-plantar erythrodysesthesia G3							1 (d 10)
Stomatitis - G3				1 (d 7)			
MTD / MAD, mg / day	120 mg/day		195 mg/day			150mg /day	

Table 3. Summary of DLTs in all regimens (hepatotoxicity was considered drug-related)

Overall Incidence* Related TEAEs in all Cycles	**Regimen 1 (N = 49) N (%)**	**Regimen 2 (N = 82) N (%)**	**Regimen 3 QD (N = 15) N (%)**	**Regimen 3 BID (N = 34) N (%)**
Daily Dose Range	1-120mg	1-255mg	60-90mg	90-150mg
Subjects with at least 1 event	**40 (82)**	**72 (88)**	**14 (93)**	**34 (100)**
Gastrointestinal disorders	**28 (57)**	**51 (62)**	**14 (93)**	**30 (88)**
Diarrhea	**16 (32)**	**39 (48)**	**14 (93)**	**27 (79)**
Nausea	13 (26)	13 (16)	4 (17)	9 (27)
Vomiting	7 (14)	13 (16)	2 (13)	11 (32)
Stomatitis	6 (12)	10 (12)	1 (7)	7 (21)
Skin and subcutaneous Disorders	**23 (47)**	**55 (67)**	**12 (80)**	**30 (88)**
Dermatitis Acneiform	**7 (14)**	**19 (23)**	**7 (47)**	**13 (38)**
Acne	9 (18)	13 (16)	0	2 (6)
Rash	**7 (14)**	**18 (22)**	**6 (40)**	**12 (35)**
Dry Skin	6 (12)	6 (7)	4 (27)	5 (17)
Pruritus	4 (8)	12 (15)	6 (40)	5 (15)
Ocular Disorders	**19 (39)**	**44 (54)**	**13 (87)**	**26 (77)**
Vision blurred	6 (12)	18 (22)	2 (13)	6 (18)
Retinal Detachment	5 (10)	11 (13)	3 (20)	6 (18)
Macular Degeneration (LLT: Serous Retinal Detachment)	**4 (8)**	**15 (18)**	**9 (60)**	**28 (82)**
General Disorders	**19 (39)**	**37 (45)**	**9 (60)**	**28 (82)**
Asthenia	13 (27)	20 (24)	2 (13)	10 (29)
Peripheral Oedema	**1 (2)**	**13 (16)**	**6 (40)**	**19 (56)**
Face Oedema	0	3 (4)	1 (7)	7 (21)
*Incidence of TEAEs (Worst Grade) in ≥ 20% of subjects at least in one of the Regimens				

Table 4. Most common related treatment emerged adverse event (TEAE)

Regimen 2 Day 1

Dose (mg/QD)	N of patients	C_{max} (ng/ml)	T_{max} (hr)	AUC_{0-t} (hr*ng/ml)	$t_{1/2}$ (hr)
1	3	1.7 (1.2-3.1)	1.6 (1.5-1.9)	1.9 (0-5)	/
2	3	2.9 (1.9-5.2)	1.1 (0.5-2.5)	8.2 (5-14)	/
3,5	3	4.6 (1.6-8.4)	1.4 (1.0-2.0)	6.7 (1-17)	/
5	3	17.3 (12.6-20.5)	2.3 (0.5-25)	67.9 (43-170)	/
7	3	30.9 (24.1-44.2)	0.8 (0.5-2.0)	74.4 (60-87)	2.6 (2.5-2.7)
14	5	39.2 (18.2-82)	1.6 (0.5-4.0)	188.4 (117-436)	5.1 (3.8-7.9)
28	3	188 (128.2-275.3)	1.1 (0.6-2.0)	625.7 (497-734)	5.1 (4.8-5.5)
45	3	321.9 (257.9-463.5)	0.6 (0.5-1.0)	808 (680-921)	4.2 (3.8-4.7)
68	3	306.6 (113.7-641.6)	0.9 (0.5-1.5)	861.1 (305-1602)	3.3 (1.4-5.4)
94	3	373.6 (317.4-495.1)	1.1 (0.5-1.6)	1484.6 (1364-1723)	4.8 (4.4-5.7)
120	4	605.1 (366.2-1085)	1.0 (0.3-2.0)	2287 (1102-7163)	5.1 (3.7-9.2)
150	14	539 (171.5-1416.8)	1.5 (0.6-4.0)	2216.5 (746-9088)	4.9 (3.8-7.0)
195	12	680.9 (196-1005.6)	1.1 (0.5-2.5)	3415.6 (1437-5496)	5.6 (3.6-7.1)
255	5	990.9 (625-1978.7)	1.4 (0.5-2.5)	4041.3 (1798-7068)	4.3 (3-6.6)

Values in geomean (range)

Table 5. Pharmacokinetic (PK) Analysis. Dose Proportionality, Tmax, elimination half-life. PK analysis from Regimen 1 yielded very similar results

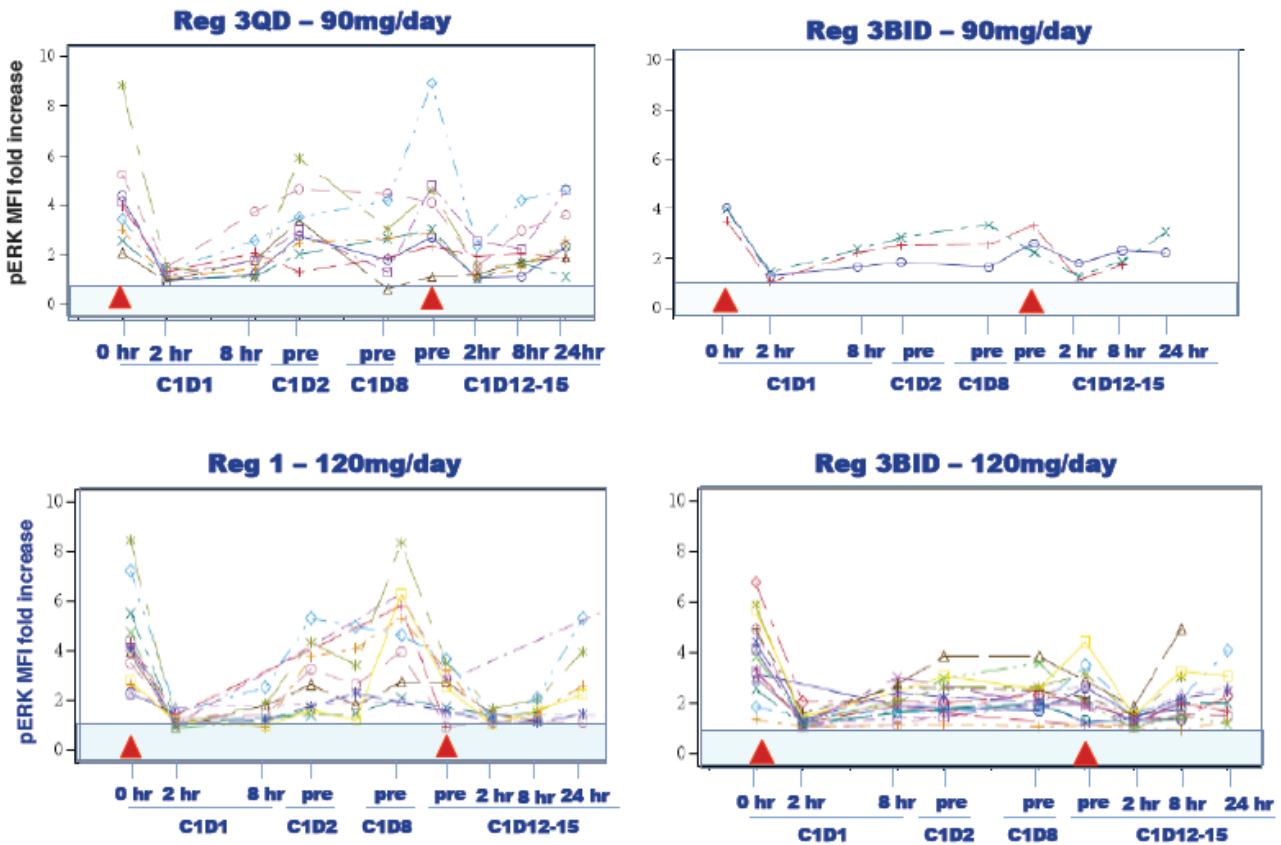
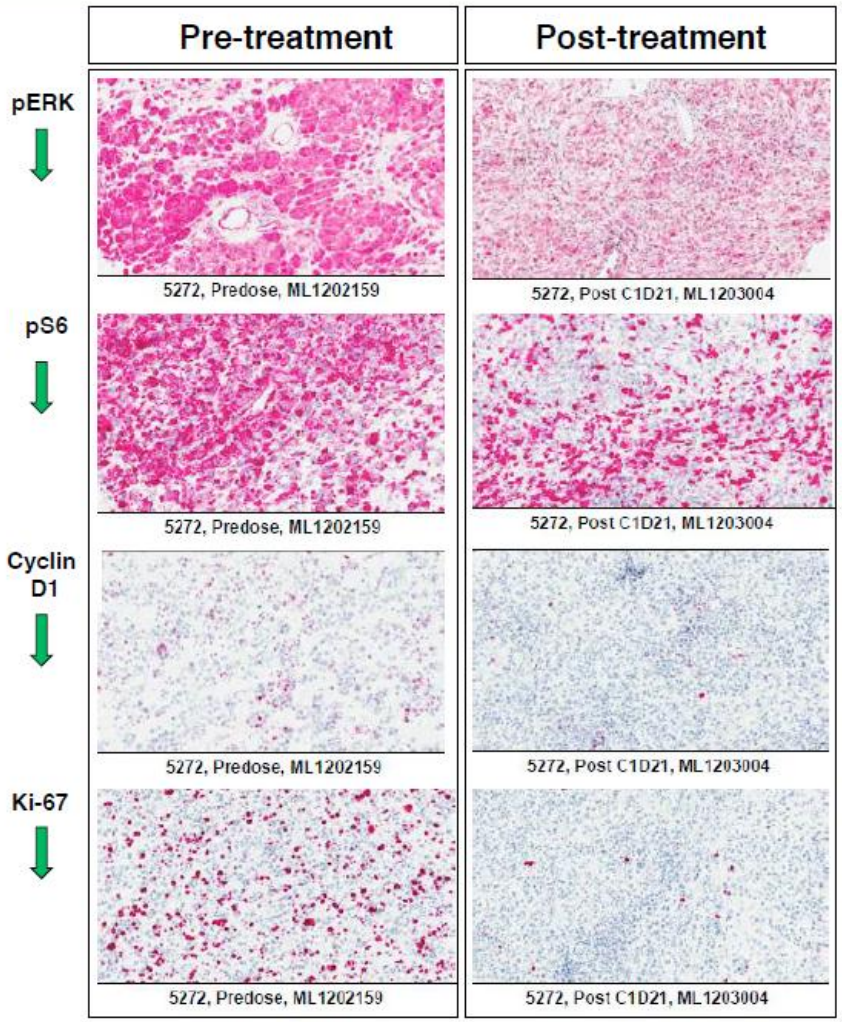


Figure 2. Effect of Pimasertib of pERK induction by PMA in PBMCs. QD vs BID



Pt. # 5272	Pre-treatment H-Score	Post-treatment H-Score	Change (%)
pERK	200	153	-24
pS6	140	95	-33
Cyclin D1	40	6	-85
Ki-67	53	4	-92

Patient ID	5272
Age/Gender	41/F
Regimen - Dose/day	3BID -120mg
Best Overall Response	PR
Mutational status	NRAS mut
Staging	M1c
LDH (IU/L)	344
Time to Progression (wks)	26,4
% Tumor Change	-52%
Previous Immunotherapy	Ipi

Figure 3. Case report- patient with NRAS mut with 52% reduction of tumor and 26.4 weeks of partial response

1.4 A dose escalating phase I study of GLPG0187, a broad spectrum integrin receptor antagonist, in adult patients with advanced high-grade glioma and other solid malignancies.

Geert A. Cirkel[1], Bojana Milojkovic Kerklaan[2], Frédéric Vanhoutte[3], Annegret Van der Aa[3], Giocondo Lorenzon[4], Florence Namour[4], Philippe Pujuguet[4], Sophie Darquenne[4], Filip Y.F. de Vos[1], Tom J. Snijders[5], Emile E. Voest[1,7], Jan H.M. Schellens[2], Martijn P. Lolkema[1,6]

1. Department of Medical Oncology, University Medical Center Utrecht, The Netherlands
2. Department of Clinical Pharmacology, Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands
3. Galapagos NV, Mechelen, Belgium
4. Galapagos SASU, Romainville, France
5. Brain Center Rudolf Magnus, Department of Neurology and Neurosurgery, University Medical Center Utrecht, Utrecht, The Netherlands
6. Present address: Erasmus MC Cancer Institute, Rotterdam, The Netherlands,
7. Present address: Netherlands Cancer Institute -Antoni van Leeuwenhoek Hospital - Board of Directors, Amsterdam, The Netherlands

Submitted for publication

ABSTRACT

Background: Integrin signaling is an attractive target for anti-cancer treatment. GLPG0187 is a broad spectrum integrin receptor antagonist (IRA). GLPG0187 inhibited tumor growth and metastasis in mouse models. The mechanism of action of GLPG0187 especially suggests activity in patients with high-grade glioma.

Methods: We aimed to determine the Recommended Phase II Dose (RP2D) and to assess safety and tolerability in patients with advanced malignant solid tumors. Anticipated dose levels were 20, 40, 80, 160, 320, and 400 mg/day in a modified 3+3 design. Plasma concentrations of GLPG0187 were assessed to characterize the pharmacokinetics (PK) after a single 1h i.v. administration and during continuous i.v. infusion. C-terminal telopeptide of type I collagen (CTX) was used as pharmacodynamics marker.

Results: Twenty patients received GLPG0187. No dose limiting toxicities (DLTs) were observed. The highest possible and tested dose (400 mg/day) is the RP2D. Fatigue was the most frequently reported side effect (25%). Recurrent Port-A-Cath-related infections and skin toxicity suggest cutaneous integrin inhibition. No dose-dependent toxicity could be established. PK analysis after a 1h i.v. administration of each dose showed a short average distribution (0.16h) and elimination (3.8h) half-life. Continuous infusion resulted in dose proportional PK profiles. Serum CTX levels were decreased independent of the dose. Three patients experienced stable disease including two patients with glioblastoma multiforme.

Conclusions: GLPG0187 was well tolerated with a dose-proportional PK profile upon continuous infusion. The RP2D is 400mg/day. GLPG0187 is considered a strong IRA with a favourable toxicity profile suitable for future combination therapy regimens.

Keywords

integrin, antagonist, glioma, phase 1, GLPG0187

INTRODUCTION

Integrin receptors play an important role in cancer biology providing a strong rationale to pursue integrin receptor antagonists (IRA) as therapeutic agents in cancer patients[1]. Integrin receptors are heterodimeric cell surface molecules that act as adhesion molecules connecting the cytoskeleton to the extracellular matrix. Moreover they are involved in activating intracellular signalling pathways involved in actin cytoskeleton remodelling and three-dimensional cell growth. Integrin-mediated signalling is implicated in modulation of well-known cancer-related pathways such as the TGF-beta pathway in glioblastoma and the Rho-Rac pathway[1, 2].

Currently, cilengitide is the most advanced IRA in clinical development. Cilengitide showed signs of efficacy without significant additive toxicity both as single agent and combined with radiation and temozolomide in patients with glioblastoma multiforme (GBM)[3-6]. When compared to cilengitide, GLPG0187 is an IRA with a stronger

nanomolar affinity for a broader panel of RGD (Arg-Gly-Asp) integrin receptors (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1; supplementary table S1)[7, 8]. In preclinical models GLPG0187 significantly inhibited angiogenesis both in vivo and in vitro, osteoclastogenesis in vitro, and bone loss in vivo[9]. In mouse cancer models GLPG0187 inhibited de novo formation and progression of bone and visceral metastases in prostate cancer and breast cancer[7, 8, 10, 11]. We hypothesize that GLPG0187, a more potent and broader spectrum IRA when compared to cilengitide, may improve the anti-tumor efficacy of IRA therapy. Therefore a phase I dose escalation study was initiated to investigate the safety and tolerability of GLPG0187 when administered intravenously in end-stage cancer patients. In healthy volunteers, GLPG0187 was rapidly eliminated after oral administration with a terminal half-life of about 5-6 hours[12]. To ensure continuous target inhibition despite its relatively short half-life GLPG0187 was administered as a continuous i.v. infusion in this study. We aimed to determine a safe dose in cancer patients and to determine the pharmacokinetics (PK), pharmacodynamics (PD) and evaluate preliminary signs of efficacy.

MATERIALS AND METHODS

Patient selection

Patients, aged over 18 years, with pathologically confirmed advanced or metastatic malignant solid tumors refractory to standard therapy or for whom no standard treatment options were available were eligible for participation. Additional inclusion criteria included: written informed consent, measurable disease according the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1[13], Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, estimated life expectancy of at least 12 weeks and no previously incurred anticancer therapy related toxicities higher than grade 2. Patients were considered ineligible if there was less than 4 weeks since their last anticancer therapy (less than 6 weeks for nitrosoureas and mitomycin C) or they were previously treated with IRAs. Additional exclusion criteria were: chronic treatment with corticosteroids equivalent to 10 mg methylprednisolone per day or more, current or recent (within 30 days) treatment with another investigational drug or participation in another investigational study, clinically symptomatic or progressive brain or leptomeningeal metastases, major surgical procedure within 28 days before first dose, congestive heart failure (NYHA class 3 or 4), clinical significant cardiac arrhythmias, signs and symptoms of relevant cardiovascular disease, known hypersensitivity to any of the study drugs and significant medical conditions possibly interfering with patient compliance or safe study participation.
Female patients with reproductive potential were only eligible with a negative pregnancy test obtained less than 7 days before first dose and if an adequate contraceptive method was used while on study.

The study was centrally approved by the ethics committee of the University Medical Center Utrecht and was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines. Patients were accrued in the University Medical Center Utrecht and the Netherlands Cancer Institute. The study was registered on clinicaltrials.gov (NCT01313598).

Investigational agent

GLPG0187 supply was controlled by Galapagos SASU (Romainville, France) and was delivered to participating sites as a 35 mg/ml injectable solution in type 1 clear glass vials. A 40% HP-β-CD (Kleptose®) injectable solution manufactured by Roquette pharma (Lestrem, France) was used to improve solubility of GLPG0187. Depending on dose level, various amounts of GLPG0187 were solved in HP-β-CD injectable solution and saline and administered by continuous infusion to patients at the recommended infusion rate. The amount of HP-β-CD needed to prepare a 400 mg/day GLPG0187 solution is considered the maximum acceptable dose in humans. Therefore no dose escalation beyond 400 mg/day was planned within this study. After preparation, the solution was stored at room temperature protected from daylight for a maximum of 7 days.

Study design

This study was performed as a multicenter, open-label, dose-escalation, phase I study.
Decisions regarding dose escalation were made by using a modified 3+3 dose escalation design. To reduce the number of patients treated at possible suboptimal dose concentrations, only 2 evaluable patients were assigned in the first two cohorts.
Patients in the first cohort received a starting dose of 20 mg/day which was chosen based on results from a preceding healthy volunteer study[12]. The anticipated sequential dose escalations were 20, 40, 80, 160, 320, 400 mg/day. Intrapatient dose-escalations were not allowed. Dose reductions or interruptions were allowed after cycle 1 which equals the dose limiting toxicity (DLT) window.
On day 1 of cycle 1, a single daily dose of GLPG0187 was administered at a constant infusion rate for a period of one hour after which the patient was followed for 24-hours to assess the PK profile. On day 8 of cycle 1 continuous infusion was initiated at the assigned dose level for 21 days. Thereafter, treatment was continued in 21-day cycles until disease progression, occurrence of a DLT, unacceptable toxicity, death, poor study compliance or withdrawal of informed consent.
A DLT was defined as one of the following adverse events (AEs) considered related to GLPG0187 occurring within the first cycle of 28 days: grade 4 neutropenia lasting ≥ 7 consecutive days, febrile neutropenia (defined as absolute neutrophil count (ANC) ≤ 1000 cells per μL and fever ≥ 38.5°C) or documented infection ≥ grade 3 with ANC ≤ 1000 cells/μL, grade 4 thrombocytopenia, thrombocytopenia

requiring platelet transfusion, or bleeding requiring medical intervention, alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 5 × upper limit of normal (ULN) (> 7.5 x ULN in case of liver metastases) for greater than 14 days, ALT or AST > 5 × ULN (> 7.5 x ULN in case of liver metastases) co-occurring with a total bilirubin of > 2.5 × ULN (not explained by obstruction) regardless of duration, non-hematological toxicity ≥ grade 3. GLPG0187-related nausea, vomiting, and diarrhoea were only considered DLTs if they persisted at ≥ grade 3 for > 3 days despite adequate supportive care measures.
The Maximum Tolerated Dose (MTD) for this study was defined as the dose level below the dose level at which ≥ 2 patients in a dose cohort experienced a DLT within the DLT observation period. The resulting recommended phase II dose (RP2D) was defined as the MTD or the highest tested dose which is tolerable and safe.

Safety and efficacy assessments

After signing informed consent, patients were screened for eligibility. Screening assessments were performed within 14 days of the first dose.
Safety was assessed weekly by means of physical examination, weight, vital signs, ECOG performance status, laboratory evaluations (hematology, biochemistry and urinalysis), electrocardiograms, and recording of concurrent illness/therapy and AEs throughout the study course. An AE was defined as appearance of any (or worsening of any preexisting) undesirable sign, symptom or medical condition occurring after signing the informed consent, whether related to treatment or not. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03. For each AE an absent, unlikely, possible, probable or certain relationship with GLPG0187 was assessed by the local investigator.
Preliminary efficacy was measured bi-cyclic and at end of treatment by CT scan, MRI, or a bone scan following RECIST 1.1 guidelines[13]. Recent literature has highlighted the need for better criteria for response assessment in high-grade gliomas, and the Response Assessment in Neuro-oncology (RANO) group has published new MRI-based response criteria[14]. For this reason, we evaluated all high-grade glioma patients according to RANO criteria. Concordance between RANO and RECIST evaluation of all gliomas was 100%.

Pharmacokinetic and pharmacodynamic methods

PK blood samples were collected at baseline and on day 1 of cycle 1 at 0.5, 1, 1.5, 2, 4, 6, 8 and 24 hours after start of the single first dose. Additional samples were obtained on day 8, 15, 22 and 29 of cycle 1. PK samples were analyzed for determination of GLPG0187 plasma levels by a validated liquid chromatography-mass spectrometry methods at AtlanBio (Saint-Nazaire, France). PK parameters in plasma such as maximum concentration (C_{max}), Area Under the Curve (AUC), total plasma

clearance, steady state volume of distribution (V_{ss}) and distribution and elimination half-lives ($t_{1/2lbd1}$ and $t_{1/2lbdz}$) were calculated, as well as dose standardized parameters (C_{max}/dose and AUC/dose).
In a previously performed phase I healthy volunteer study GLPG0187 was shown to reduce CTX (C-terminal telopeptide of type I collagen) levels significantly when compared with placebo[12]. In addition, GLPG0187 was found to inhibit osteoclastic bone resorption in mice significantly[9]. Therefore CTX serum levels were adopted as a PD marker and measured by ELISA, according to the manufacturer instructions (CrossLaps, Immuno Diagnostic Systems, ref. AC-02F1).
CTX levels were measured in blood samples collected at baseline and on day 1 of cycle 1 at 1, 2, 4, 6, 8 and 24 hours after start of the first single dose. Additional samples were obtained on day 8, 15, 22 and 29 of cycle 1.

Statistical methods

Study results were obtained by analyzing the safety population which contains all patients who received at least 1 dose of GLPG0187. Results were summarized descriptively and if applicable plotted by dose level over time. CTX levels at different time points were compared by using a Wilcoxon Signed Rank Test.

RESULTS

Patients

Twenty patients received GLPG0187, between 22nd of March 2011 and 10th of April 2013. Patient characteristics are depicted in Table 1. Fifteen patients completed cycle 1 and were considered evaluable for DLT assessment. High-grade glioma was the most commonly included tumor type (40%, mostly GBMs).

Dose escalation and safety

No DLTs were observed in any cohort. The absence of DLTs resulted in an undisturbed dose escalation scheme towards the final planned cohort of 400 mg/day. GLPG0187 dosed at 400mg/day equals the highest possible dose based on the maximal dose of HP-β-CD, the solvent. Therefore, the highest tested dose (400 mg/day) is the RP2D.

GLPG0187 showed a relatively safe and tolerable toxicity profile in this study. The incidence of at least possibly related AEs per cohort is summarized in Table 2. Most frequently observed toxicities were fatigue (5 patients, 25%) and skin related adverse events (5 patients, 25%). Twenty-three AEs were considered possibly related and 6 probably related to GLPG0187. All but two AEs are reported only once. During the study, 14 (70%) patients experienced a total of 23 serious adverse events (SAE). Only one SAE was considered possibly related (fatigue). All other SAEs were assessed as unlikely or not-related.

All toxicity seemed manageable and did not lead to significant dose reductions or dose interruptions. No clear relationship was observed between GLPG0187 dose level and the occurrence of AEs or laboratory abnormalities.

Pharmacokinetic data

After intravenous infusion, GLPG0187 was rapidly distributed and eliminated as illustrated in figure 1A. The PK profile was dose proportional over the 20 to 400mg/day dose range when infused continuously (Figure 1B). PK parameters per cohort are displayed in supplementary table S2. GLPG0187 showed a moderate total plasma clearance (average: 40.1 L/h) and short distribution and elimination half-lives of on average 0.16 and 3.8 hours, respectively. GLPG0187 plasma concentration was maintained during the PK sampling period of 21 days while receiving continuous i.v. infusion (Figure 1B). PK in patients was predictable from healthy subjects' PK suggesting that concomitant therapies do not impact the PK of GLPG0187.

Effects on bone resorption marker CTX

The effect of GLPG0187 treatment on bone resorption marker CTX was measured in serum during the first cycle (Figure 2A/B). High interpatient variability in the CTX concentration measurements was observed. A Wilcoxon Signed Rank Test was conducted to compare CTX levels at baseline to 2 hours post infusion on cycle 1 day 1. Additionally, the effect of continuous infusion was analyzed by comparing mean CTX levels at day 15 to day 8. A significant change in CTX level was observed 2 hours after the single dose infusion on day 1. The mean CTX level was higher at baseline: 0.58 (SD 0.39) versus 0.42 (SD 0.32), $p<0.0001$. At day 15 CTX levels were lower compared to day 8 ($p= 0.007$). No clear relationship between GLPG0187 dose and CTX concentration could be established within cycle 1 (Figure 2A/B and 3).

Efficacy

The best overall tumor response was stable disease was achieved in 3 (15%) out of 20 patients. These 3 patients were treated at 20 mg/day (patient with non-small cell lung cancer, stable disease during 14 weeks), 80 mg/day (patient with GBM, stable disease during 19 weeks) and 160 mg/day (patient with GBM, stable disease during 8 weeks). No tumor responses were observed.

DISCUSSION

We performed a phase I, open-label, dose escalation study using GLPG0187 in patients with solid tumors. GLPG0187 was well-tolerated and displayed a predictable and dose proportional PK profile. The toxicity profile of GLPG0187 is very mild and we did not identify a true maximal tolerated dose.
Measurement of CTX levels provided the proof-of-mechanism that GLPG0187 inhibits integrin function. Despite the variation in baseline CTX levels we found a statistically significant decrease in CTX levels after treatment.

Patients in the first two cohorts received GLPG0187 through a Port-A-Cath (PAC) system. Three out of six patients had a PAC related infection classified as a SAE unrelated to GLPG0187. The median reported infection rate of totally implantable intravenous catheter devices within an immunosuppressed population approximates 0.2 per 1000 catheter days[15]. Therefore, we probably should consider these events retrospectively as at least possibly being related to GLPG0187. For all three patients the infection was evident at the skin location where the needle enters the skin.

From cohort 3 onwards we switched to a peripherally inserted central catheter (PICC), where access to the system does not require repeated skin punctures. No catheter related infections were observed after the switch from PAC to PICC.

In a GBM patient in the 80 mg/day cohort, epidermolysis induced by removal of a common skin patch from his forearm followed by a significantly delayed healing process was observed (Figure 4). Moreover, skin toxicity was among the most common findings in these 20 patients (Table 2). An explanation could be that continuous IRA exposure leads to altered adhesion and homing of various inflammatory cells. Although speculative, these findings suggest that skin immunity and possibly skin integrity were affected while treated with GLPG0187.

The kinetics of our PD marker, the infectious problems and the skin problems occurred independent of dose. Together these data suggest that we started with dose levels that already represented a biologically active dose.

GLPG0187 failed to show clear signs of clinical efficacy in this study. Moreover, cilengitide statistically failed to live up to its promise in a phase III clinical trial when combined with standard treatment in GBM[16].

However, 9% of patients with recurrent GBM did show a neuroradiological partial response on cilengitide monotherapy in phase II and remained progression free for at least 10 months[5]. GLPG0187 has, compared to cilengitide, a broader profile of inhibition for multiple integrins that would specifically be present in GBM (supplementary table S2)[2]. It could be that the failure of cilengitide in phase III and the observed efficacy of GLPG0187 in this study might be caused by poor patient selection rather than inactivity of the compound itself.

Integrin-PET-based probes could be used to assess target engagement directly as was done in preclinical models[7]. In mice harboring melanoma xenografts, GLPG0187 displaced up to 70% of an αvβ3 integrin-targeted molecular imaging agent. Additionally a significant decrease in standardized uptake values for fluorodeoxyglucose (FDG) was observed after exposure to GLPG0187. PET may provide a tool to test a tumor for integrin expression and thereby facilitate patient selection.

Literature showing a role for integrins in cancer is overwhelming. Mechanisms of integrin-mediated evasion of apoptosis by either drugs or radiotherapy have been described in several hematological and non-hematological malignancies including small cell lung cancer, gliomas, and breast cancer lines[17-20]. These findings provide a rationale to further explore the potential synergistic effects between cytotoxic treatments and IRAs.

We feel that the quest towards a validated biomarker for response on integrin inhibition should be continued and subsequently GLPG0187 deserves further exploration within a selected patient population. Additionally, treatment of a selected population with a combinatory treatment regimen might lead to an even larger proportion of patients experiencing clinical benefit.

Acknowledgements

Cilengitide, used for a head-to-head comparison with GLPG0187 of relative binding affinities to RGD integrin receptors, was kindly provided to us by prof D. Cheresh (Moores, UCSD Cancer Center, LaJolla CA 92093).
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	TOTAL N = 20, N (%)
Age (years) mean (SD)	56.4 (11.9)
Male	14 (70.0)
Female	6 (30.0)
ECOG 0	4 (20.0)
1	11 (55.0)
2	5 (25.0)
Primary tumor	
Adenocarcinoma of Unknown Primary	1 (5)
Adenoid cystic carcinoma	1 (5)
Cholangiocarcinoma	1 (5)
Colorectal carcinoma	3 (15)
Endometrial cancer	1 (5)
Glioblastoma multiforme	5 (25)
High grade astrocytomaa	1 (5)
Anaplastic , oligodendroglioma	2 (10)
Nasopharynxcarcinoma	1 (5)
Non-small cell lung cancer	1 (5)
Ocular Melanoma	1 (5)
Osteosarcoma	1 (5)
Urothelial cell carcinoma	1 (5)

Table 1: Patient demographics, N = number of patients; ECOG = Eastern Cooperative Oncology Group performance status; asecondary form, from low grade astrocytoma

	20 MG/DAY		40 MG/DAY		80 MG/DAY		160 MG/DAY		320 MG/DAY		400 MG/DAY		TOTAL	
	(N = 2)		(N = 5)		(N = 4)		(N = 3)		(N = 3)		(N = 3)		(N = 20)	
Adverse event description a	All	≥Gr 3	All	≥Gr 3	All	≥Gr 3	All	≥Gr 3	All	≥Gr 3	All	≥Gr 3	All	≥Gr 3
Dry mouth	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Epidermolysis	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Fungal skin infection	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Herpes zoster	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Mucosal inflammation	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Rash	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Rash Maculo-papular	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Skin hyperpigmentation	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Arthralgia	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Conjunctival haemorrhage	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Diarrhoea	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Dysgeusia	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Fatigue	0	0	0	0	2	0	1	0	1	1	1	0	5	1
Headache	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Oedema peripheral	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Peripheral sensory neuropathy	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Pleural effusion	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Thrombosis	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Vasculitis	0	0	1	0	0	0	0	0	0	0	0	0	1	
ALT increased	0	0	0	0	1	1	0	0	0	0	0	0	1	1
Anaemia	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Blood albumin decreased	0	0	2	0	0	0	0	0	0	0	0	0	2	0
Blood bilirubin increased	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Blood creatinine increased	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Total	7	0	7	0	7	1	1	0	4	1	3	0	29	2

Table 2: All and ≥ Grade 3 at least possibly GLPG0187-related AEs per dose cohort. a Adverse events were evaluated using the National Cancer Institute Common Toxicity Criteria for Adverse Events, version 4.03, ALT = Alanine aminotransferase, N = number of patients;

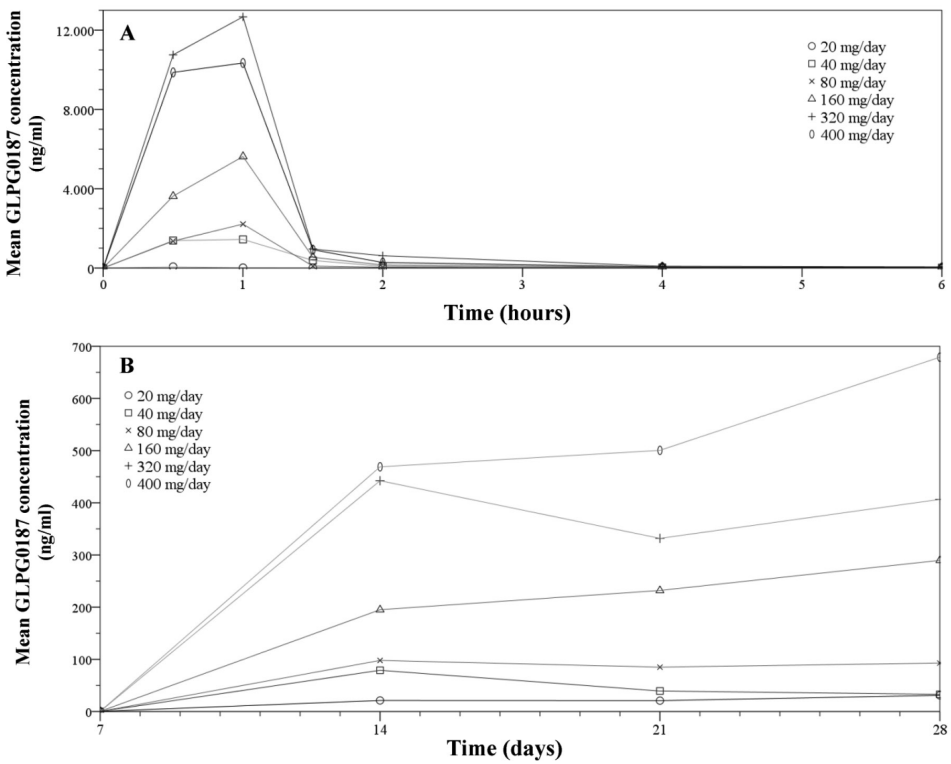

Figure 1: (A) The graph depicts the mean plasma concentration of GLPG0187 over the first 6 hours after GLPG0187 treatment start. GLPG0187 concentration observed at 8 and 24 hours post-infusion was below the limit of quantification. (B) The graph depicts the mean plasma concentration of GLPG0187 over 21 days during continuous GLPG0187 i.v. infusion.

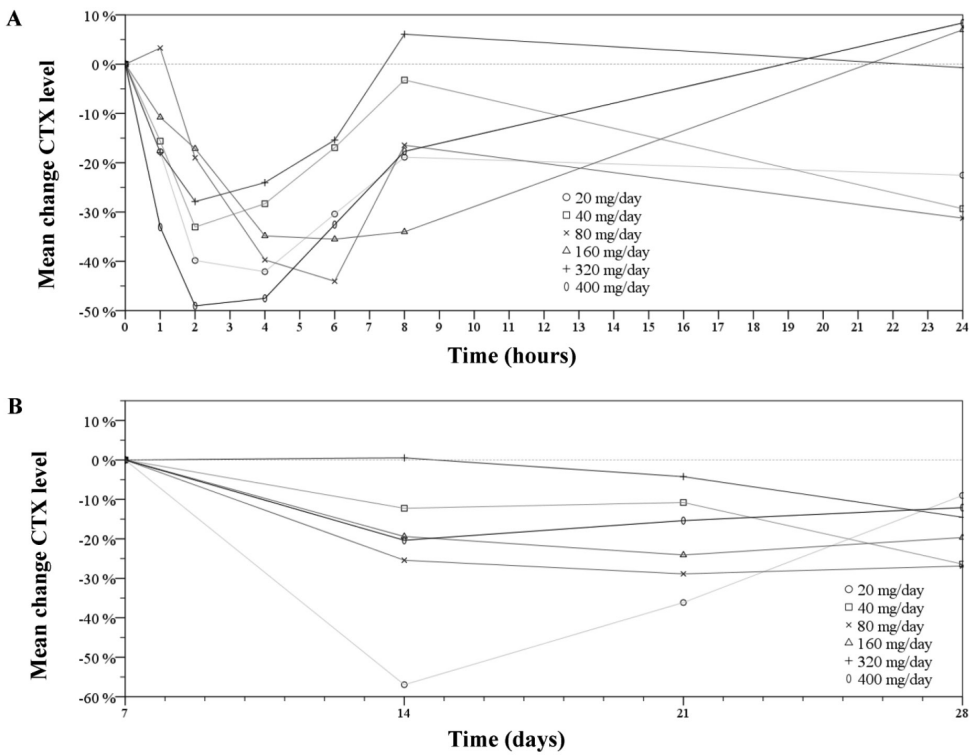

Figure 2: (A) The graph depicts the mean percentage change compared to baseline of bone resorption marker CTX over the first 24 hours after a single i.v. dose of GLPG0187. (B) The graph depicts the mean percentage change of CTX concentration compared to the baseline level measured on day 8 (7 days after cycle 1 day 1) over 21 days. Continuous infusion of GLPG0187 was initiated on day 8.

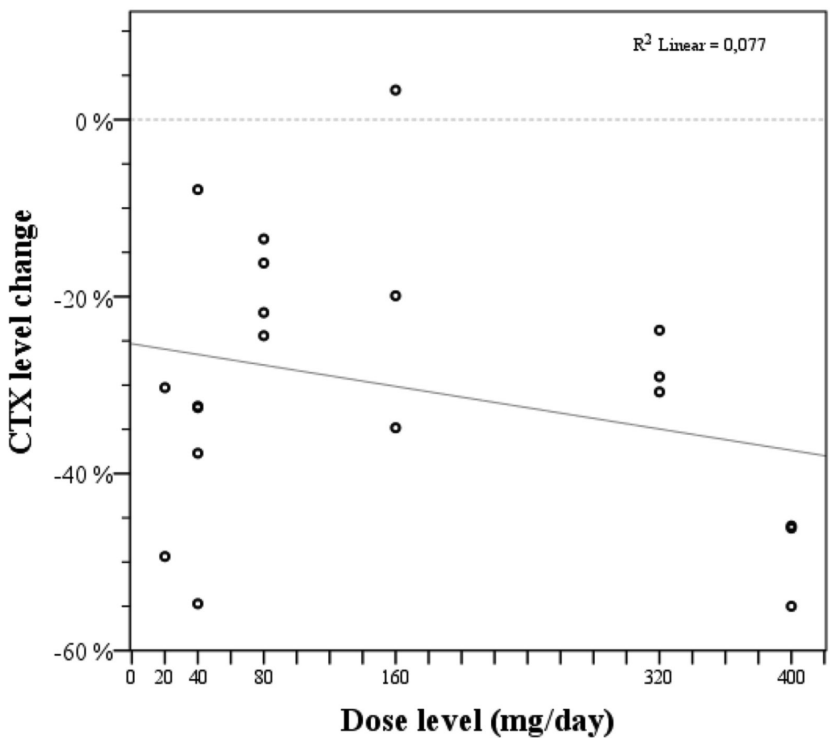

Figure 3: Percentage change in individual CTX levels obtained 2 hours after a single i.v. dose of GLPG0187 compared to baseline. No dose-dependent effect on CTX levels was observed.

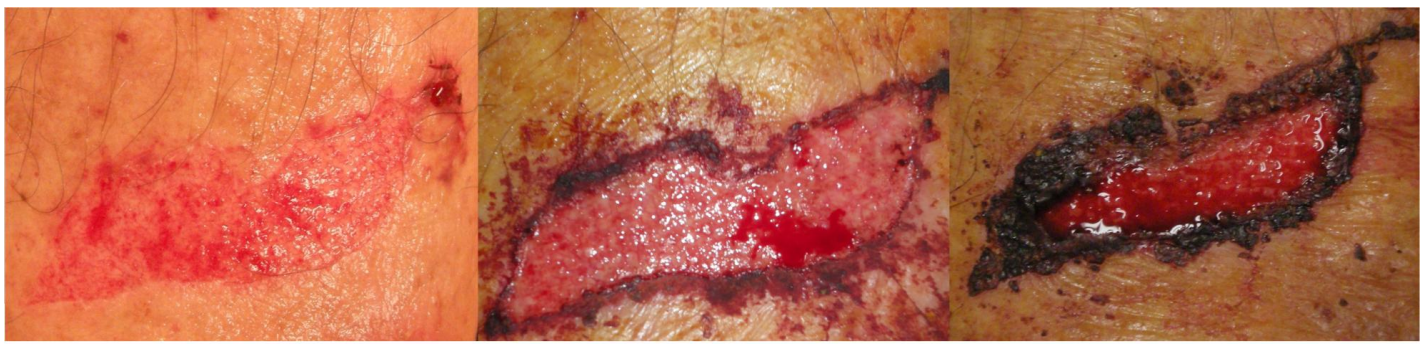

Figure 4: Epidermolysis and delayed healing after removal of plaster in patient with GBM in 80 mg/day cohort

	αVβ1	αVβ3	αVβ5	αVβ6	avβ8	α5β1
Cilengitide	11.2 ± 3.7	6.5 ± 2.3	5.2 ± 1.3	122 ± 14	436 ± 85	30.5 ± 3.3
GLPG0187	1.3 ± 0.4	3.7 ± 0.6	2.0 ± 0.6	1.4 ± 0.3	1.2 ± 0.3	7.7 ± 4.0
a: Competitive binding (in a solid phase assay)						

Supplementary Table S1: Comparative affinities for different integrin receptorsa (IC50, as nanomoles/L)

PARAMETER	20 MG/DAY (N = 2)	40 MG/DAY (N = 5)	80 MG/DAY (N = 4)	160 MG/DAY (N = 3)	320 MG/DAY (N = 3)	400 MG/DAY (N = 3)
AUC(0-inf) ng.h/mL)	422	1689 (822)	1866 (387)	4357 (1764)	12386 (3951)	10114 (6693)
AUC (0-inf)/dose	21.1	47.3 (18.4)	23.3 (4.8)	27.2 (11.0)	38.7 (12.3)	25.3 (16.7)
t1/2,lbd1 (h)	0.182	0.180 (0.097)	0.117 (0.045)	0.165 (0.060)	0.148 (0.022)	0.140 (0.030)
t1/2,lbdz (h)	2.41	2.98 (1.01)	3.92 (1.27)	4.68 (0.35)	5.07 (0.26)	3.44 (1.11)
Cmax (ng/mL)	391	1460 (677)	1751 (396)	3969 (1511)	11490 (3821)	9592 (6340)
Cmax/dose(ng/mL.mg)	19.5	41.1 (15.5)	21.9 (5.0)	24.8 (9.4)	35.9 (11.9)	24.0 (15.9)
CL (L/h)	48.7	24.3 (10.3)	44.3 (9.2)	42.5 (21.6)	27.6 (8.2)	53.2 (33.3)
Vss (L)	25.6	15.4 (13.0)	25.7 (17.3)	26.1 (2.2)	20.6 (7.1)	23.0 (14.5)

Supplementary table S2: Mean PK (SD) parameters per dose cohort, N = number of patients; AUC = area under the curve;t1/2,lbd1 = distribution half- life;t1/2,lbdz = terminal elimination half- life; Cmax = maximum concentration; CL = clearance; Vss = steady state volume of distribution

1.5. Phase I study of lonafarnib (SCH66336) in combination with trastuzumab plus paclitaxel in Her2/neu overexpressing breast cancer. EORTC study 16023

Bojana Milojkovic Kerklaan[1], Veronique Diéras [2], Christophe Le Tourneau [2], Marja Mergui - Roelvink[1], Alwin D. R. Huitema[3], Hilde Rosing[3], Jos H. Beijnen[3,] Sandrine Marreaud4, Anne-Sophie Govaerts [4] , Martine J. Piccart-Gebhart[5], Jan H.M. Schellens , Ahmad Awada [4,5]

1. Department of Clinical Pharmacology, the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands
2. Department of Medical Oncology, Institute Curie, Paris, France
3. Slotervaart Hospital, Amsterdam, the Netherlands
4. NDDG/BCG group - European Organization for Research and Treatment of Cancer (EORTC)
5. Institute Jules Bordet, Brussels, Université Libre de Bruxelles, Belgium
6. Science Faculty, Department of Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands

Cancer Chemother Pharmacol (2013) 71:53–62

ABSTRACT:

PURPOSE: This phase I study was performed to determine the maximum tolerated dose (MTD), dose limiting toxicities (DLT), safety profile, recommended dose for phase II studies, the pharmacokinetics and antitumor activity of the combination of lonafarnib (farnesyl transferase inhibitor), trastuzumab and paclitaxel in Her2-positive advanced breast cancer.

METHODS: Twenty-three patients with Her2 overexpressing breast cancer received in the first cycle paclitaxel and trastuzumab and from cycle two onwards lonafarnib which was added to the combination. Dose-limiting toxicity (DLT) was determined during the second cycle.

RESULTS: The MTD and recommended dose for phase II trials is lonafarnib: 250 mg/day (125 mg / bi-daily (BID)) continuously, paclitaxel: 175 mg/m^2 3h infusion every 3 weeks, and trastuzumab: 4 mg/kg loading dose and 2 mg/kg/week thereafter. The most frequently observed adverse events starting from cycle one onwards were alopecia, myalgia, sensory neuropathy, fatigue, arthralgia, leucocytopenia and neutropenia. From cycle two onwards additional adverse events appeared, such as diarrhea, nausea, dyspepsia, vomiting and allergy. The mean systemic exposures of both lonafarnib and paclitaxel through all dose levels were higher in the regimen with all three study medications but with no statistically significant difference. Preliminary antitumor activity (CR+PR) was observed in 58 % of all patients.

CONCLUSION: Lonafarnib can be safely combined and tolerated with full doses of paclitaxel and trastuzumab in Her2-positive advanced breast cancer patients. Promising preliminary anti-tumor activity warrants further evaluation of lonafarnib in combination with paclitaxel and trastuzumab in Her2-positive breast cancer.

Keywords: Her2-positive breast cancer, chemotherapy, activated MAPK pathway, farnesyl transferase inhibitor, lonafarnib, trastuzumab, paclitaxel

INTRODUCTION

Breast cancer is a significant global health problem. One third of women who are diagnosed with breast cancer will ultimately die of the disease. Metastatic breast cancer (MBC) is currently incurable. The human epidermal growth factor receptor, encoded by the Her2 proto-oncogene is upregulated in 15-25% of breast cancers and is an indicator of more aggressive clinical behavior and poor prognosis.[1] Trastuzumab combined with a taxane as first-line therapy is now the standard of care for patients with Her2-positive metastatic breast cancer (MBC). However, most of patients ultimately develop resistance to this combination. Therefore, the development of novel strategies to improve the taxane plus trastuzumab in Her2-positive MBC is of major therapeutic interest.

For this study lonafarnib (SCH 66336) was selected as a novel agent with a specific mechanism of action. It is a potent and selective farnesyl transferase inhibitor (FTI) that inhibits Ras function by farnesylation as has been shown in vitro[2]. It also blocks the transformed growth properties of fibroblasts and human cell lines expressing activated H-Ras, N-Ras or K-Ras proteins.

Ras proteins are present in all human cells[2]. When activated by extracellular signals (including growth factors that activate cell surface receptors such as Her2), Ras proteins play a critical role in intra-cellular transduction of cell growth signal via multiple cell signaling pathways, such as mitogen-activated protein kinase cascades (MAPK) through RAF, MEK and ERK mitogen-activated kinases. This pathway also overlaps and cross talks with other signaling cascades that regulate the balance of cell survival.[3] In mutated ras, Ras protein becomes constantly activated by several post-translational modifications, including the farnesylation (i.e. addition of a C15-prenyl moiety to a cystein residue). This results in uncontrolled cell growth and proliferation. This ras mutation occurs in 20-30% of all malignancies[4]. The cascade of uncontrolled proliferation can theoretically be stopped by inhibiting the farnesylation of the Ras protein by FTI, which should prevent proper membrane anchoring of the Ras protein and thereby stop cell growth.[2, 5]

Although the frequency of ras mutations in breast cancer is low (<2%), hyperactivation of Ras protein and its downstream effectors is common as a result of overexpression of upstream components, such as the epidermal growth factor and Her2.[6] Non mutated Ras is also sensitive to FTIs[2], which may be explained by the presence of transforming events upstream of Ras that require Ras function to induce cellular transformation. Alternatively, this might also be due to a role of other farnesylated proteins in cell growth and transformation (e.g. CENP proteins, RhoB [7], Rheb and components that are essential for separation of spindle poles). [8] [3, 9] Although the pharmacological effects of FTIs at the cellular level remain unclear, several trials have been testing FTIs potential to enhance the activity of current therapies in breast cancer. [10, 11]. Lonafarnib is active in vitro against a broad spectrum of tumor cell lines and primary human tumors.[12] The drug has shown significant antitumor activity in a variety of human tumor xenograft models in mice at different schedules of administration.[13]

Lonafarnib alone or in combination has undergone various clinical studies for the treatment of solid tumors. Lonafarnib as a single agent is well tolerated with reversible and manageable GI toxicity (diarrhea, vomiting and nausea) possibly due to the fact that it is highly selective for farnesyl transferase. [14]

Other studies show myelosuppression as a common feature[15]. As a single agent lonafarnib can be safely administered using a continuous oral bi-daily (BID) dosing regimen. Bi-daily dosing is chosen because of the pharmacokinetic profile of lonafarnib and because the compound is a competitive inhibitor, and the schedule should result in continuous inhibition of farnesyl transferase. The recommended dose as single agent is 200 mg BID.

Numerous preclinical studies have demonstrated a synergistic interaction between lonafarnib and taxanes, anticancer drugs that both target mitotic apparatus. [7, 16, 17] In addition, lonafarnib has been shown to be able to reverse resistance to taxanes.[18] Inhibition of farnesyl protein transferase might enhance the mitotic block induced by paclitaxel.[19]

Promising antitumor activity has been reported with the lonafarnib – paclitaxel combination and their recommended dose for phase II trials is lonafarnib 125 mg orally twice daily in combination with weekly paclitaxel 80 mg/m2.15
In combination therapy, an effect of trastuzumab and paclitaxel on the pharmacokinetics of lonafarnib could be anticipated due to the long terminal half-life of trastuzumab (25 +/- 5 days) and of paclitaxel (ranged from 3 to 52.7 hours).
This phase I study aimed at evaluating the safety of the combination of lonafarnib, trastuzumab and paclitaxel in Her2-positive advanced breast cancer.

PATIENTS AND METHODS:

Main eligibility criteria:

Patients with histologically/cytologically confirmed diagnosis of Her2 -positive (IHC 3+ or ICH2+ with positive FISH) metastatic breast cancer were eligible. Other important eligibility criteria were as follows: patients for whom paclitaxel/trastuzumab might be an appropriate treatment and for whom an anthracycline was not suitable; prior treatment with chemotherapy and/or radiation completed for at least 4 weeks prior to study enrolment and hormonal therapy discontinued at least one day prior to treatment start; no previous therapy with trastuzumab and/or paclitaxel within the last year and no clinical signs of central nervous system (CNS) involvement; normal cardiac ejection fraction assessed by MUGA scan and QTc interval ≤ 440 msec; effective contraception.
The study was approved by the local Ethics Committees of the participating institutions.
Before patient registration, written informed consent was signed according to ICH/GCP, and national/local regulations.

Study design:

The primary study objectives of this open label, non-randomized, multi-center, phase I dose escalation trial, were to establish the maximum tolerated dose (MTD), dose limiting toxicities (DLT), safety profile, recommended dose for phase II trials, and pharmacokinetic parameters of the triple combination of lonafarnib, paclitaxel and trastuzumab in patients with Her2-positive advanced breast cancer administered in first or second line therapy. The secondary study objective was to document antitumor activity of the combination.

To determine the MTD trastuzumab was administrated at its full dose (4 mg/kg loading dose and 2 mg/kg weekly) as a single agent while doses of lonafarnib and paclitaxel were escalated starting with a dose of 75 mg twice daily for lonafarnib and 135 mg/m^2 every three weeks for paclitaxel. One cycle was defined as a time-period of three weeks. Dose-levels (DL) were allocated according to a 3+3 scheme (3 patients/dose-level, up to 6 in case of a DLT). The dose-escalation proceeded in a stepwise

manner (Table 2). The schedule of administration of lonafarnib was changed and applied from cohort five onwards into a "one-week on, one-week off, one-week on schedule" instead of the originally applied 3 weeks schedule. The idea was to allow a one-week recovery period from toxicities.

During the first cycle, patients received only trastuzumab and paclitaxel (both obtained commercially) in order to assess the tolerability of this combination and PK profile which will be compared with the PK profile from the cycle two, as from cycle two onwards, the administration of lonafarnib started (supplied by Schering-Plough Research Institute, Kenilworth, New Jersey, United States). The order of administration was lonafarnib first followed by trastuzumab (90 min i.v. infusion) and then paclitaxel (3 hours i.v. infusion). Lonafarnib capsules were taken orally bi-daily approximately 12 hours apart, with the morning and evening meals. Patients were advised not to drink grapefruit juice while taking lonafarnib.

Toxicity, MTD, DLT, patient replacement and reasons to stop treatment:
Toxicity was evaluated in all patients who started treatment and graded according to the National Cancer Institute criteria (CTCAE version 2.0). At each cycle, the worst grade of toxicity was recorded.

The Maximum Tolerated Dose (MTD) was defined as the dose associated with a probability of DLT during cycle 2 closest to 20% of the patients who will suffer from severe toxic side effects (DLT).
The MTD was assessed on the basis of DLTs observed during cycle 2, after lonafarnib was introduced.

The evaluable patient population was used to decide on the dose escalation for lonafarnib. It consisted of all patients who started cycle 2 and either completed cycle 2 or stopped treatment during cycle 2 due to toxicity related to any of the three drugs. Patients withdrawn from the study for any reason, except for toxicity related to any of the drugs at cycle 2 before they have been appropriately evaluated, were replaced by new patients.

The Dose Limiting Toxicity (DLT) was defined as: any non hematological grade 3/4 toxicity with the exclusion of alopecia, nausea, vomiting, diarrhea and fever controlled after 48 hours of maximal anti-emetic, anti-diarrheal or anti-pyretic treatment, respectively; an absolute neutrophil count (ANC) < 0.5x109/L lasting for > 7 days; febrile neutropenia defined as ANC < 1.0x109/L and fever at least 38.5°C; grade 4 thrombocytopenia; treatment delay for toxicity lasting more than four weeks. These acute toxicities must be thought to be related to study treatment (i.e. to one or several drugs in the combination) by the clinical investigator to be considered dose-limiting.

Patient replacement: If the following toxicities had occurred during cycle 1, cycle 2 would not be administered and patients would stop the study and be replaced: - grade 4 anemia or thrombocytopenia, - grade 4 neutropenia lasting at least 7 days, - febrile neutropenia or grade 3-4 non-hematological toxicity.

Reasons to stop treatment: Treatment was given as indicated per protocol unless an early withdrawal criterion would occur: i.e. patient refusal, unacceptable toxicity or investigator decision.

Treatment assessment:

Before initiating therapy, a complete medical history was recorded and a physical examination was performed. Complete blood counts including hemoglobin, total white blood cells (WBC),
neutrophils, platelets, and hematocrit) and serum chemistry was performed (including creatinine, electrolytes, total bilirubin, AST, ALT, ALP, albumin, glucose, urea, LDH, and GGT). Vital signs and evaluation of all clinical symptoms as well as WHO/ECOG performance status, ECG and MUGA scan were performed. A serum pregnancy test was required in women of reproductive potential. Tumor evaluation (RECIST criteria version 1.0) within 21 days prior to treatment start was assessed by standard methodology, using X-rays, CT scans and/or MRIs. The same method was then used for repeated measurements throughout the study.
Clinical, cardiac and biological evaluations included: a clinical examination at the end of each cycle of therapy, i.e. immediately before the administration of the next cycle, and three weeks after the last drug administration. ECG was repeated every cycle.
MUGA was to be repeated if clinically relevant. Serum chemistry, including creatinine, electrolytes, total bilirubin, AST, ALT, ALP, albumin, glucose, urea, LDH, GGT was performed at the end of each cycle and complete blood counts, including hemoglobin, total WBC, neutrophils, platelets; hematocrit every week.

Sample collection and drug analysis

Pharmacokinetics (PK):
Pharmacokinetics data of lonafarnib and paclitaxel of 12 patients treated in the Netherlands Cancer Institute were analyzed at the Department of Pharmacy and Pharmacology of the Slotervaart Hospital/The Netherlands Cancer Institute in Amsterdam.
Patients received trastuzumab on day 1 of each cycle. Paclitaxel was given on day 1 of cycle 1, day 2 of cycle 2 and day 1 of every following cycle. Lonafarnib continuous BID administration started at day 3 of cycle 2. Pharmacokinetics of paclitaxel alone was performed on cycle 2 day 2. For this aim whole blood samples were drawn on day 2 before paclitaxel administration and at 1 hour after start of infusion, 10 minutes before the end of infusion, 15 minutes, 2 and 4.5 h after the end of

infusion of paclitaxel. Pharmacokinetics of lonafarnib alone was performed at cycle 2 day 3. In cycle 2 lonafarnib administration on day 3 followed the administration of paclitaxel day 2 and trastuzumab day 1. For this aim whole blood samples were taken on day 3 before lonafarnib intake and at 1, 2, 3, 3.45, 5.30 and 8 h after lonafarnib intake and just before the next lonafarnib intake and on day 8 before and 15 minutes after trastuzumab administration. During the first three days of cycle 2 patients were hospitalized. The three drug interactions were assessed during cycle 3 day 1. For this aim the whole blood samples were drawn on day 1 before lonafarnib intake, 15 and 30 minutes, 1 and 1.5 h after start of paclitaxel administration, 10 min before the end of paclitaxel administration, 15 minutes, 2, 4.5 and 8.5 h after the end of infusion of paclitaxel and a morning sample on day 2 approximately 20 h after the end of infusion.

To determine the PK of paclitaxel, whole blood samples of 10 mL were collected using heparin tubes. Whole blood samples were centrifuged at 3000 rpm to obtain plasma within 5 minutes, which was stored at –80°C until shipment on dry ice.
To determine the PK of lonafarnib, whole blood samples of 3 mL were collected using chilled sodium heparin tubes. The blood samples were gently mixed and centrifuged at 3000 rpm for 15 minutes at 4°C and frozen immediately at –80°C.
During cycle 3 patients were hospitalized on Day 1.
Lonafarnib and paclitaxel were analyzed employing validated HPLC-MS/MS (high-performance liquid chromatography coupled with tandem mass spectrometry detection) analytical methodologies. An isocratic HPLC method has been developed and validated for the quantitative determination of paclitaxel. The PK parameters (Cmax, Tmax, AUC and CL) were determined by non-compartmental analysis using validated R script.

RESULTS:

3.1. Patient characteristics

From August 2003 to November 2007 twenty-three patients were enrolled in this phase I dose escalation study in three centers: the Netherlands Cancer Institute, Amsterdam, the Netherlands, Institute Curie Hospital, Paris, France and Institut Jules Bordet, Brussels, Belgium. Patient characteristics are listed in Table 1. Safety information was collected from all 23 patients, 22 patients were evaluable. Two patients out of 23 enrolled never received lonafarnib. One ineligible patient started cycle one of trastuzumab plus paclitaxel, but she stopped protocol treatment because of development of neutropenia grade 4 with infection. She did not start cycle 2 and thus did not receive lonafarnib. One patient never received lonafarnib but was not excluded from the group of evaluable patients because she completed cycle 1 and discontinued during cycle 2 of trastuzumab plus paclitaxel without receiving lonafarnib. Treatment was stopped because of treatment-related toxicity

consisting of ischemia and QT prolongation. Patients received on average 6 cycles in the range of 1-75 cycles of study combination (Table 2). Paclitaxel was stopped in 11 patients on the half treatment duration (44% of number of received cycles), while lonafarnib and trastuzumab were continued.

Toxicity:

The study showed that the combination was generally well tolerated.
The most common related non hematological toxicities - AE during the treatment of combination trastuzumab and paclitaxel in the first cycle reported were alopecia (15 patients, with any grade), myalgia (13 patients), sensory neuropathy (10 patients), fatigue (9 patients) and arthralgia (9 patients).

Adding lonafarnib from cycle 2 onwards to trastuzumab and paclitaxel combination revealed new drug-related toxicities. Reported drug-related toxicities in cycle 2 were diarrhea (15 patients, with any grade), nausea (14 patients), dyspepsia (6 patients), vomiting (4 patients) and allergy (3 patients). In addition, the similar related adverse event trend appearance as in treatment cycle 1 (without lonafarnib), such as alopecia (18 patients), fatigue (16 patients), myalgia (12 patients), sensory neuropathy (11 patients), rash (7 patients) and stomatitis (5 patients) continued.

During the whole treatment period the most common hematological toxicities were neutropenia and leucocytopenia reported in almost all patients (22 patients) and anemia (20 patients). (Table 3 presents grade 3 and 4 toxicities). Hepatic toxicities included increased alkaline phosphates (9 patients), increase in liver enzymes ALT (12 patients) and AST (7 patients) and GGT (9 patients). Almost all patients in the study developed hyperglycemia grade 1-3 (21 patients). This is most likely due to dexamethason premedication.
Lonafarnib was frequently interrupted; in 13 patients for more than two days (59 % of patients who started lonafarnib). The most frequent reasons for dose interruptions were neutropenia and leucocytopenia, thrombocytopenia, diarrhea, nausea, anorexia and abdominal pain.

At dose-level one, 3 patients presented hematological toxicities leading to interruption of lonafarnib as per protocol and in some cases for several cycles. Therefore, the dose-intensity of lonafarnib ranged from 29 % to 84 % with a mean per cycle of 66 %. At dose-level two, 2 patients experienced hematological toxicities that led to treatment interruption of lonafarnib. The dose-intensity of lonafarnib ranged from 69 % to 100 % with a mean per cycle of 80 %.

Given the interruption of lonafarnib at dose-levels one and two, it was decided to modify the schedule of administration of lonafarnib into a "one-week on, one-week off, one-week on schedule" instead of the originally applied 3 weeks schedule (see 2.2 for study design). This amendment was applied only during the dose-level 5.

The first dose-reduction was made in dose-level four by the patient herself – she was forgetting to take the evening dose of lonafarnib. This patient had also an active inflammation of the breast, pain and mood alteration-depression with no evidence of brain metastasis. A second patient at this dose-level had interruption and eventually had to stop lonafarnib due to diarrhea grade 3. At dose-level five in five out of six enrolled patients the dose of lonafarnib needed to be ≥ 10 % reduced due to various toxicities: ANC grade 4 in two patients, nausea grade 2, vomiting grade 2 and diarrhea grade 2, which in one patient led to definitely stop the study. Prolonged thrombocytopenia grade 2 and sensory neuropathy grade 2 were reasons to stop the study in two patients, and allergy grade 3 in one patient. Dosing of trastuzumab proceeded without development of major toxicities; one patient experienced decreased LVEF starting from cycle 8. Dose of paclitaxel was only once interrupted due to hematological toxicity (ANC grade 2). Reduction of the dose of paclitaxel was reported in five patients, which was due to cardiac ischemia and increased QTc in one patient, extravasation in the infusion arm in the second patient and neuropathy in three other patients.

Treatment discontinuations were due to disease progression - 8 patients and non - tolerable toxicity in 12 patients, while the three other patients discontinued therapy due to investigator or patient decision or due to social circumstances.
Cardiac toxicities related to the drug combination were observed in dose-level two in two patients. The first patient experienced hypertension, hypoxia and tachycardia 10 minutes after the infusion of paclitaxel. The ECG revealed a prolonged QT interval and ischemia. However, during that time-period lonafarnib was not yet introduced. The patient had a medical history of hypertension since more than 10 years, for which she received first bisoprolol and later verapamil. She was a smoker and had a positive family history. Oxygen therapy was initiated as well as potassium supplementation. Tachycardia recovered within four hours and within six hours the QTc interval returned to the baseline value. The second patient had chest pain grade 3 and typical trastuzumab related toxicity of decreased LVEF and this was seen in cycle 7 (42%) and after the recovery period and the trastuzumab rechallenge in cycle 20 (45%).

Determination of the maximum tolerated dose, dose limiting toxicities and the recommended dose for Phase II trials

No DLTs were observed up to dose level 4. Two patients from dose-level one and one patient from dose-level two were replaced, as defined in the replacement rules for evaluable patients. The dose was escalated until dose-level five (Table 2).
Adverse events grade 3 and 4 were mainly hematological (ANC and WBC) (Table 3), which would improve after dose interruption. At dose-level five the schedule of lonafarnib was changed from continuous to one week on - one week off – one week on. During this dose schedule and dose level 5 one patient experienced an allergic reaction grade 3 related AE, which was seen as a DLT. Therefore, dose-level

was expanded with 3 additional patients (3+3). One of these additional patient experienced DLT consisting of a grade 3 increase of GGT. This dose-level was considered not tolerable also due to the high toxicity profile in this cohort of patients. This concerned appearance of infections with neutropenia, fatigue, gastrointestinal events: nausea, diarrhea, distension/bloating, taste disturbance, disphagia; dermatology: alopecia, rash, flushing and hand foot skin reactions, acne; hemorrhage: epistaxis with normal platelet count; neurology: neuropathic pain, myalgia, muscle pain, dizziness, mood alterations. As the Maximum Tolerated Dose MTD had been reached at dose-level five, the recommended dose for phase II was determined to be dose-level four: lonafarnib: 250 mg/day (125 mg/BID) continuously, paclitaxel: 175 mg/m^2/cycle and trastuzumab: 4 mg/kg loading dose followed by the 2 mg/kg given weekly thereafter.

Pharmacokinetics

A total of 12 patients recruited at The Netherlands Cancer Institute treated with lonafarnib plus trastuzumab and paclitaxel provided blood samples for pharmacokinetic analyses after lonafarnib dose was given alone (during the cycle 2, day 3). Ten patients continued to cycle 3 and provided blood samples on cycle 3, day 1 during the administration of the combination of the three study drugs – lonafarnib plus trastuzumab and paclitaxel. Data from 9 patients were evaluable.

Figure 1 represents the mean plasma concentrations – time curves for lonafarnib for all five dose-levels. It can be seen that lonafarnib was slowly absorbed and eliminated in both dosing treatments.
The pharmacokinetic data of lonafarnib are presented in table 4
The maximum concentrations of lonafarnib when given alone were reached at 3.35 h (SD: 1.6) after drug intake and when given in the three drug combination at 3.1 h (SD: 0.7) after for all dose-levels. The plasma concentration-time profile of lonafarnib alone or in combination supports the bi-daily dosing, as the plasma concentrations had significantly decreased 12 hours after lonafarnib intake, as also previously reported. [20]
Increases in lonafarnib Cmax and AUC values were dose-related following oral administration of 75, 125 and 150 mg BID for both lonafarnib alone and in combination with two other drugs, except in the dose-level 3 with 100 mg BID where lonafarnib plasma levels were with slightly lower Cmax and AUC values. This can be explained with an interindividual pharmacokinetic variability which can be avoided with evaluating more patients per dose-level. In this case only one patient had evaluable PK data in this dose level. The pharmacokinetics of lonafarnib when given alone was compared with those in the combination for both analyzed study medications (paclitaxel and lonafarnib). To be able to compare the data from different dose-levels, the ratio AUC/D (AUC area under the curve, D – Dose – dose normalized or corrected AUC) was used as projected in figure

2 where the difference in AUC/D ratio between lonafarnib when given alone or in combination with paclitaxel and trastuzumab. The difference between two regimes was not statistically significant (t test, p value 0.79).
Paclitaxel pharmacokinetics was similar to the data extensively described in the literature[21, 22]. When given in combination with lonafarnib and trastuzumab paclitaxel pharmacokinetic values resulted also in a higher systemic exposure compared with paclitaxel given alone. However, the difference between the PK data for both was not statistically significant.

Preliminary antitumor activity:

In total 17 patients were evaluable for response evaluation. Six patients were not evaluable.
One patient achieved a complete radiological response lasting for more than four years. Nine patients had a partial response lasting for more than four cycles, and six patients had stable disease as their best response.
Therefore as the preliminary antitumor activity the total response rate of complete and partial response (CR+PR) was 10/17 = 58%. Mean progression-free survival of evaluable patients was 21 months with CI 8.1-34.5.

DISCUSSION:

This phase I study aimed to determine the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), safety profile, recommended dose for phase II trials, the pharmacokinetics and antitumor activity of the combination of lonafarnib, trastuzumab and paclitaxel in Her2-positive advanced breast cancer was performed. Results indicate that the recommended dose for Phase II is lonafarnib 250 mg/day (125 mg/BID) continuously in combination with paclitaxel 175 mg/m^2/cycle 3h infusion every 3 weeks and trastuzumab 4 mg/kg loading dose followed by 2 mg/kg given weekly thereafter. The dose escalation was precluded in cycle 2 at dose-level five with lonafarnib at 300 mg due to two reported DLTs, which were allergy and increase of GGT in combination with high, albeit formally non dose-limiting, toxicity among almost all enrolled patients at the highest dose-level explored.
Toxicity observed during the cycle one with the combination of trastuzumab and paclitaxel, was consistent with previous publications.[20] The severity of these toxicities was mainly CTC grade 1 to 2. Related non-hematological adverse events already observed in treatment cycle one (without lonafarnib), such as alopecia, myalgia, sensory neuropathy, fatigue, arthralgia, varied from CTC grade 1 to 2 continued also in cycle two onwards. Adding lonafarnib from the second cycle onwards to the combination resulted in frequent diarrhea, nausea, dyspepsia, vomiting, and allergic reactions that are common side effects observed with lonafarnib when administrated as single agent.

Myelosuppression (neutropenia and leucocytopenia) and gastrointestinal symptoms were expected as observed in many previous studies with single agent bi-daily continuous dosing of lonafarnib.[23] The studies with a wash-out period of one week within each 21 day-cycle or two weeks off within every 28 days-cycle showed less hematological toxicities. In our study high hematological toxicity was seen during the two first dose-levels of continuous dosing of lonafarnib as well as non-hematological adverse event such as infections and hemorrhages, dizziness, fatigue, diarrhea, dyspepsia, neuropathy. The modification of the schedule of administrationinto a "one-week on, one-week off, one-week on schedule" instead of the applied 3 weeks schedule could be unfortunately applied only during the dose level 5, which was too toxic.

Despite that we reported two cases with cadiotoxicity, it seems that lonafarnib did not contributed to its development. One patient who experienced prolonged QT interval did not receive lonafarnib and the other patient who experienced LVEF decrease recovered after trastuzumab interruption. Typical toxicities for paclitaxel were seen starting from the first cycle, such as hematological (neutropenia, leucocytopenia), hypersensitivity, neuropathy, myalgia and arthralgia, gastrointestinal symptoms and alopecia. Those toxicities continued also in later cycles but did not seem to be increased after adding lonafarnib.

Because paclitaxel is eliminated by the enzymes CYP2C8 and CYP3A4, and lonafarnib is a substrate primarily for CYP3A4 and its minor metabolites for CYP2C8,[24] the pharmacokinetics for both agents were evaluated in this study and possible interaction.

As reported also in the literature,[25] this study shows that lonafarnib had no statistically significant effect on the pharmacokinetics of paclitaxel or trastuzumab (for trastuzumab: no data reported here) or vice versa. Comparing the dose-corrected AUCs between two drug regimes no significant difference in the pharmacokinetics between the days when lonafarnib was given alone or in combination with paclitaxel and trastuzumab was found.

In combination with paclitaxel on day 8 in the 21 cycle, BID continuous treatment with lonafarnib, Khuri et al 2004 showed in a phase one study for patients with solid tumors (mainly NSCLC) 7 partial responses (PR) and 10 stable diseases (SD) out of 21 evaluable patients.[26] With the same treatment regime Kim et al 2005 showed in a phase II trial in NSCLC 14 patients with PR+SD out of 29 enrolled.[27] Kauh et al 2011 (mainly head and neck, lung, colorectal and neuroendocrine malignancies) showed 7 patients out of 36 clinically benefiting (SD+CR, no PR reported) from treatment with lonafarnib and docetaxel. Remarkably, 6 of these patients had previously failed taxane based therapy, thus the lonafarnib/docetaxel combination seems to be able to overcome this resistance at least temporarily. The phase III study with advanced pancreatic adenocarcinoma with gemcitabine and tipifarnib showed acceptable toxicity profile but did not show prolong overall survival in advanced pancreatic cancer compared with single-agent gemcitabine. [28] First, the ras mutation status does not predict sensitivity of human tumors to FTIs. [9] Sec-

ond, K-Ras, the most prevalent mutated form of Ras in human tumors, becomes geranylgeranylated in the presence of FTIs. Blockage of farnesylation of mutated and therefore continuously activated K-Ras does not result in deactivated (unprenylated) K-Ras. Instead, geranylgeranyl transferase (GGTase-I) takes over the post-translational modification of K-Ras, thereby preserving its function in cell growth process.[29, 30] Possibly, both FTase and GGTase-I should have been assigned as "drugable" targets for cancers with mutated ras. Further, PI3K can also be mutated in pancreatic cancer in 9%[31]. The cross-talk of PI3K and RAS-MAPK pathway and Ras activation by PI3K has been well characterized.[32] In this case screening of the patient for possible ras or PI3K mutations would be of great importance. Those independent activations downstream from the HER2 receptor are in favor of adding targeted therapies to the standard combination of trastuzumab and paclitaxel for HER2-positive metastatic breast cancer. We believe that FTIs have greater effect in combinational therapy in tumors with the activated RAS from the upstream signals, such as HER2-positive overexpressing breast cancer than cancers with mutated ras, such as pancreatic adenocarcinoma (56%-90% K-ras mutations[28, 31]).

Various studies showed effect of only paclitaxel and trastuzumab combination[33]. However it is difficult to compare progression free survival of standard therapy (paclitaxel and trastuzumab) with many phase III studies with this active combination (lonafarnib, paclitaxel and trastuzumab).

Authors would like to draw attention of those designing future studies with this study combination to the fact that paclitaxel has greater efficacy in management of metastatic breast cancer when administrated weekly rather than 3 weekly and the weekly administration of paclitaxel is associated with less toxicity, mainly of neutropenia and neuropathy. [34] No studies were performed till now with the combination of lonafarnib and trastuzumab, without paclitaxel.

Trastuzumab and paclitaxel combination are a standard of care in the treatment of Her2-positive breast cancer. However the clinical development of the FTIs alone or in various combinations is taking a lot of time, in view of the reported safety, tolerability and preliminary activity of the combination of lonafarnib and paclitaxel and trastuzumab in patients with Her2-positive breast cancer, this combination is worth exploring further in this disease.

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No. of patients (female)	23
Age (median) (range)	49 34-68
Median Time since first diagnosis (years)	2.8
Prior surgery	18
Prior radiotherapy	15
Prior chemotherapy for metastatic disease	15
Prior anthracycline/taxane	14
Prior endocrine therapy	10
Number of prior chemotherapy regiments 1 2 3 missing	 11 3 1 8
Time since most recent chemotherapy (months) median	24

Table 1. Patient characteristics (whole population)

DOSE-LEVELS	LONAFARNIB mg/DAY (12 HOURS APART)	PACLITAXEL	NO. OF PATIENTS (FEMALE)	NUMBER OF CYCLES (MEDIAN, RANGE)	DLT
1	150 mg	135 mg/m^2	5	2 1.0-6.0	
2	150 mg	175 mg/m^2	4	5 2-38.0	
3	200 mg	175 mg/m^2	3	22 8.0-75.0	
4	250 mg	175 mg/m^2	3	6 5.0-16.0	
5	300 mg**	175 mg/m^2	8	7 2.0-32.0	- GGT increase grade 3 - allergy grade 3 (+ high toxicity profile observed in this cohort: see paragraph 3.3)

Table 2. Dose escalation levels:

*Trastuzumab is given at the standard dose of 4mg/kg IV followed by the weekly maintenance dose of 2 mg/kg
** Amendment 5: From level 5: schedule of lonafarnib changed from 3 weeks continuously into a "1-week on, 1-week off, 1-week on" schedule

DOSE-LEVEL LONAFARNIB DOSE PACLITAXEL DOSE CYCLE	DOSE-LEVEL 1 150mg /DAY 135 mg/m^2 C1 C2		DOSE-LEVEL 2 150mg /DAY 175 mg/m^2 C1 C2		DOSE-LEVEL 3 200mg /DAY 175 mg/m^2 C1 C2		DOSE-LEVEL 4 250mg /DAY 175 mg/m^2 C1 C2		DOSE-LEVEL 5 300mg /DAY 175 mg/m^2 C1 C2	
CTC grade	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**	**3-4**
Leucopenia	1	-	-	-	1	1	1	-	-	-
Neutropenia	2	1	2	1	2	1	2	-	6	3
Hyperglycemia				1						4
GGT	-	-	2	-	-	-	-	-	-	**1 DLT**
Allergy	-	-		-		-		-		**1 DLT**
Tumor pain	-	-	1	1	-	-	-	-	-	-
Diarrhea	-	-	-	-	-	1	-	-	-	-

Table 3: Grade 3-4 toxicities reported during cycle one (trastuzumab + paclitaxel) and cycle two (trastuzumab + paclitaxel + lonafarnib) per dose-level

*Trastuzumab is given at the standard dose of 4mg/kg IV followed by the maintenance dose of 2 mg/kg
**GGT grade 3 and allergy grade 3 in dose-level 5 were both related to study medications and therefore DLTs, as per study protocol

	CYCLE	N	CMAX MEAN SD (ng/ml)	TMAX MEAN SD (h)	AUC 0-12H MEAN SD (ng/ml*h)	AUCINF MEAN SD (ng/ml*h)	V (CL/K) SD	AUC/D MEAN SD (ng*h/ml/mg)
DL1-75 mg LNF	2	3	199 65	4.2 1.2	1140 624	1409 989	267 31	15.2 8.3
DL2-75 mg LNF	2	1	209 -	1.0 -	811 -	828 -	248.5 -	10.8 -
DL2-75 mg LNF + 4 mg/kg TRZ + 175 mg/m2 PCT	**3**	**1**	**250 -**	**2.3 -**	**1533 -**	**1671 -**	**163.8 -**	**20.44 -**
DL3-100 mg LNF + 4 mg/kg TRZ + 175 mg/m2 PCT	**3**	**1**	**180 -**	**3.0 -**	**990 -**	**1224 -**	**427.3 -**	**9.9 -**
DL4-125 mg LNF (mean)	2	2	416 -	4.4 -	2131 -	2446 -	263.1 -	17 -
DL4-125 mg LNF + 4 mg/kg TRZ + 175 mg/m2 PCT (mean)	**3**	**2**	**313 -**	**3.25 -**	**1767.5 -**	**1949.5 -**	**215.5 -**	**14.1 -**
DL5-150 mg LNF (mean)	2	2	549.5 -	3.8 -	3871.5 -	4731.5 -	189.7 -	25.8 -
DL5-150 mg LNF + 4 mg/kg TRZ + 175 mg/m2 PCT (mean)	**3**	**2**	**700.5 -**	**3.9 -**	**4529.5 -**	**5822.5 -**	**195.95 -**	**30.2 -**

Table 4. Pharmacokinetic parameters of lonafarnib alone (cycle 2, D3) and in combination with trastuzumab and paclitaxel (cycle 3 D1, marked with grey). (abbreviations: LNF – lonafarnib; TRZ – trastuzumab; PCT – paclitaxel).

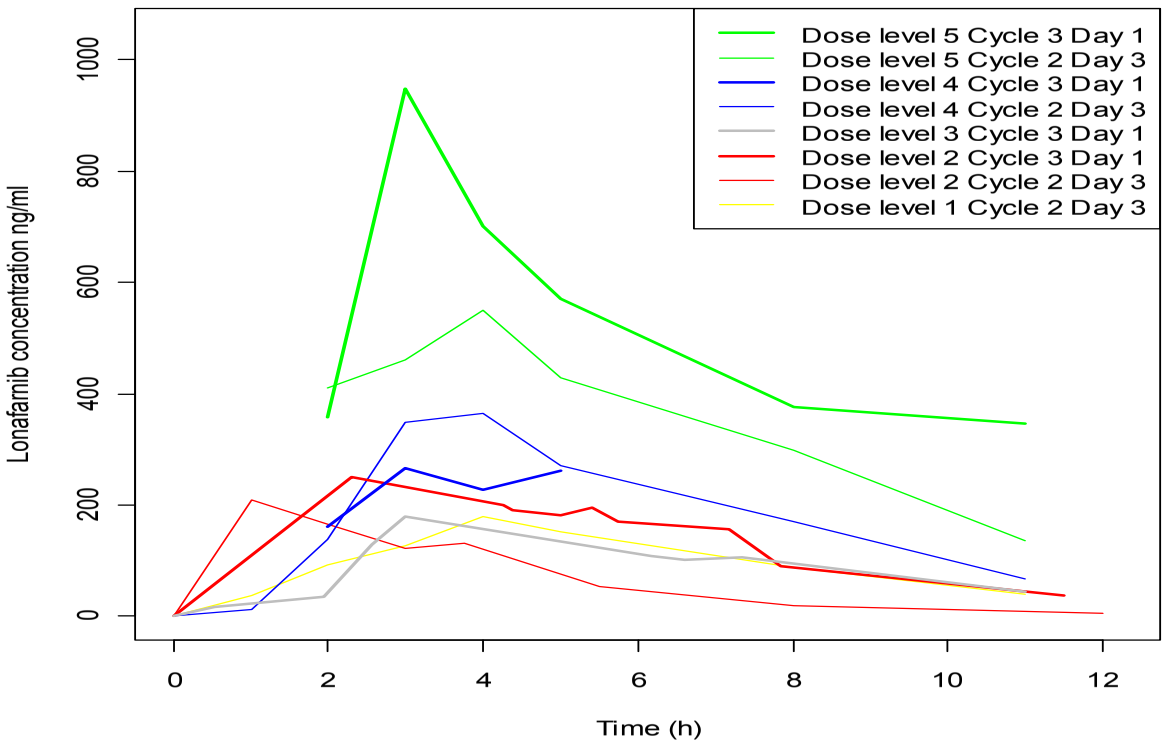

Figure 1. Plasma concentration vs. Time curves for lonafarnib from 9 patients in 5 dose-levels treated with (BID) orally administrated lonafarnib with trastuzumab and paclitaxel (Thin colored lines represent concentration of lonafarnib given alone (in cycle 2, day 3). Bolded lines represent lonafarnib given in combination (in cycle 3, day 1). l

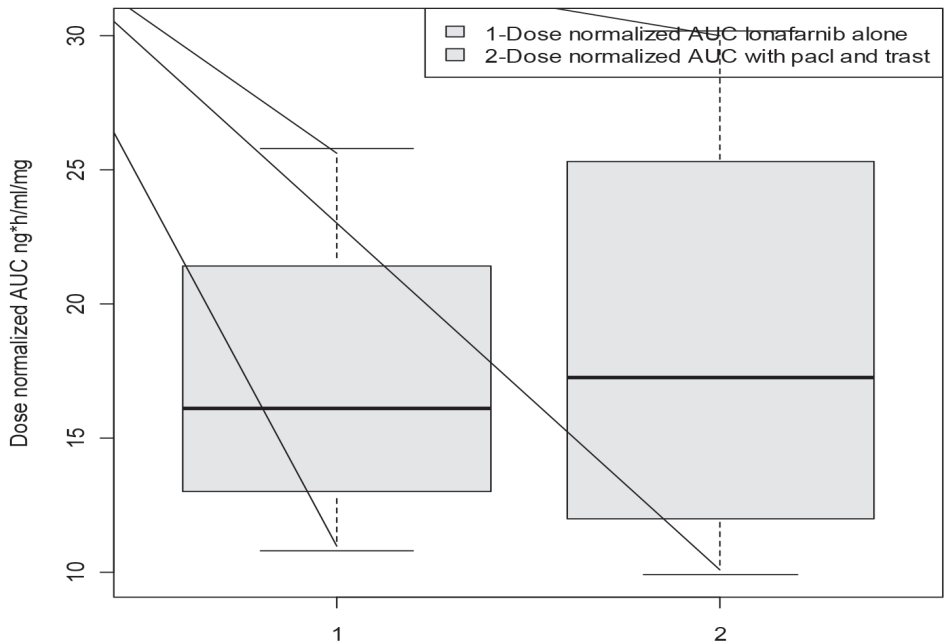

Figure 2. Dose normalized AUC of lonafarnib when given alone or in combination with trastuzumab and paclitaxel

CHAPTER 2: TRASTUZUMAB-ASSOCIATED CARDIOTOXICITY PREVENTION WITH PHARMACEUTICAL INTERVENTION

2.1 Prevention study of trastuzumab-associated cardiotoxicity in early breast cancer patinets by angiotensin II-receptor inhibition: a randomized, duoble-blind, placebo-controled trial

Boekhout, AH [1], Milojkovic Kerklaan, B [1], Gietema, JA [2], van Werkhoven, ED [3], Altena, R [2], Honkoop, A [4], Maartje Los, M [5], Smit, W [6], Nieboer, P [7], Smorenburg, C [8], Mandigers, C [9], van der Wouw, AJ [10], Kessels, L [11], van der Velden, AWG [12], Ottevanger, PB [13], Smilde, T [14], de Boer, J [15], Fiets, WE [16], Soesan, M [17], Schiphorst, PPJ [18], de Valk, B, [19], Dalesio, O [2], de Vries, EGE [3], Schellens, JHM [1,20]

1 Division of Clinical Pharmacology, Netherlands Cancer Institute, Amsterdam, The Netherlands; 2 Department of Medical Oncology University Medical Center Groningen, Groningen, The Netherlands; 3 Biometrics Department, Netherlands Cancer Institute, Amsterdam, The Netherlands; 4 Isala klinieken, Zwolle, The Netherlands; 5 Antonius hospital, Nieuwegein, The Netherlands 6 Medisch Spectrum Twente, Enschede, The Netherlands; 7 Wilhelmina hospital, Assen, The Netherlands; 8 Medisch Center Alkmaar, Alkmaar, The Netherlands; 9 Canisius Wilhemina Hospital, Nijmegen, The Netherlands; 10 VieCuri Medisch Center Noord-Limburg, Venlo, The Netherlands; 11 Deventer hospital, Deventer, The Netherlands; 12 Martini Ziekenhuis, Groningen, The Netherlands; 13 University Medical Center St. Radboud, Nijmegen, The Netherlands; 14 Jeroen Bosch Hospital, 's Hertogenbosch, The Netherlands; 15 Ziekenhuis de Tjongerschans, Heerenveen, The Netherlands; 16 Medical Center Leeuwarden, The Netherlands; 17 Slotervaart hospital, The Netherlands; 18 Beatrix hospital Winterswijk, The Netherlands; 19 Onze Lieve Vrouwe Gasthuis Amsterdam, The Netherlands; 20 Department of Pharmaceutical Sciences, Science Faculty Utrecht University, Utrecht, The Netherlands;

Manuscript in preparation

ABSTRACT

Importance: To the best of our knowledge, this is the first randomized placebo-controlled evaluation of a pharmaceutical intervention in the prevention of trastuzumab related cardiotoxicity.

Objective: To determine whether concurrent ATII-antagonist treatment can prevent trastuzumab-related cardiotoxicity, defined as a decline in left ventricular ejection fraction (LVEF) of more than 15% or a decrease to an absolute value <45%; whether N-terminal pro-B-type natriuretic peptide (NT-proBNP) and troponin T (cTnThs) can be used as surrogate marker in the monitoring of trastuzumab related cardiotoxicity; and whether genetic variability in HER2 correlates with trastuzumab related cardiotoxicity.

Design: Randomized, placebo-controlled trial. Patients were enrolled into the study between October 2007 and October 2010.

Participants: 210 women with primary HER2 positive breast cancer who were considered for adjuvant systemic treatment with anthracycline containing chemotherapy and trastuzumab.

Setting: 19 Dutch hospitals

Intervention: 78 weeks of candesartan (dose of 16 mg BID) or placebo treatment (1:1). Patients started at the same day as the first trastuzumab administration with the study treatment until 26 weeks after completion of trastuzumab treatment.

Main Outcome Measures: LVEF, NT-proBNP, cTnThs and HER2-genotyping.

Results: 206 patients were evaluable for the primary end point. Overall, 36 cardiac failures were observed in 206 evaluable patients: 20 (19%) in the candesartan and 16 (16%) in the placebo group (p=0.58). The baseline LVEF value (≥ 55 versus < 55%) was a prominent prognostic factor for the occurrence of a cardiac failure (p=0.0004). The NT-proBNP and cTnThs values were not statistically significantly associated with the occurrence of cardiac failures. The Ala1170Pro homozygous genotype was associated with a lower likelihood of the occurrence of a cardiac failure in comparison to Val/Val + Ile/Val (OR=0.15, 95% CI, 0.03 – 0.63, p=0.01).

Conclusions and relevance: There is no evidence that prophylactic use of candesartan treatment may protect the myocardium against trastuzumab in breast cancer patients. Baseline LVEF value is a prominent predicator for the occurrence of a cardiac failure. The HER2-germline Ala1170Pro SNP might contribute to the development of trastuzumab related cardiotoxicity. To date, the effectiveness of ARBs in the prevention of trastuzumab related cardiotoxicity remains uncertain and needs further investigation.

Clinical trial gov number: NCT00459771

INTRODUCTION AND BACKGROUND

Trastuzumab, a humanized monoclonal antibody against the extracellular domain of HER2, has been shown to benefit patients with HER2 positive metastatic breast cancer and improve disease-free and overall survival in HER2 positive early stage breast cancer [1-4]. Although trastuzumab is generally well tolerated, cardiac

dysfunction is an important side effect, especially in women who are previously treated with anthracycline-based chemotherapy[5-7]. The most frequent clinical manifestation of trastuzumab-associated cardiotoxicity is decline in left ventricular ejection fraction (LVEF). The presence of cardiac dysfunction due to cancer treatment, negatively affects patients' cardiologic outcome and also seriously limits their therapeutic opportunities. It limits the feasibility of continuation or re-introduction of trastuzumab treatment. Therefore, effective interventions to protect or limit the development of cancer treatment related cardiotoxicity are warranted. Pharmaceutical interventions have been shown to attenuate or reverse left ventricular remodeling in patients with heart failure. Several approaches to protect or limit the development of anthracycline related cardiotoxicity have been proposed. Angiotensin-converting enzyme inhibitors (ACEs), β-blockers and angiotensin II-receptor blockers (ARBs), may be effective, in preventing anthracycline related cardiotoxicity[8-11]. To date, there are no published prospective clinical data on the protective properties of ACEs, ARBs or β-blockers against trastuzumab related cardiotoxicity.

Preliminary data suggest that cardiac markers such as N-terminal pro-B-type natriuretic peptide (NT-proBNP) and troponins are sensitive and specific markers to detect myocardial injury and to predict the development of future LVEF dysfunction [12-14]. Moreover, several single nucleotide polymorphisms (SNPs) in the extracellular, transmembrane and intracellular region of HER2, have been studied to examine the impact of these polymorphisms on trastuzumab related cardiotoxicity [15,16]. Currently, however, results of clinical trials are contradictory.

Therefore, we have conducted a double-blind multicenter trial of the ARB candesartan and placebo treatment in early stage breast cancer patients who were treated with anthracycline- based chemotherapy and trastuzumab. The objective of this trial was to determine if an ARB prevents or limits trastuzumab related cardiotoxicity and if cardiac markers and SNPs detect and predict trastuzumab-related cardiotoxicity. We hypothesized that concurrent use of ARBs in patients treated with trastuzumab can prevent or limit trastuzumab related cardiotoxicity.

PATIENTS AND METHODS

Patients

This multicenter study was performed at 19 Dutch hospitals. Women eligible for this trial had early-stage HER2-positive breast cancer; were aged ≥18 years; completed at least an approved anthracycline-based chemotherapy regimen; had a performance status ≤ 2; LVEF ≥ 50 percent as measured on echocardiography or multiple gate acquisition (MUGA); creatinine clearance > 50 ml/min (by Cockcroft-Gault formula); thyroid stimulating hormone (TSH) between 0.5 - 3.9 MU/l or thyroid hormone FT4 between 8 – 26 pmol/l; blood pressure systolic ≥ 100 mmHg

and ≤ 180 mmHg and diastolic ≥ 60 mmHg and ≤ 100 mmHg; received the first trastuzumab infusion at least 3 weeks after day 1 of the last anthracycline infusion. Patients were excluded if they had a history of hypersensitivity to the study medication; previous malignancy requiring anthracycline-based chemotherapy, prior biologic or immunotherapy or mediastinal radiotherapy; uncontrolled serious concurrent illness; New York Heart Association (NYHA) class II/III/IV congestive heart failure; myocardial infarction < 6 months before registration; treatment with an ACE- inhibitor, ARB or lithium; pregnancy or breast feeding. The study protocol was approved by the Medical Ethics Committees of all participating centers and all patients gave written informed consent.

Design and procedures

Eligible patients were randomly assigned (1:1) to candesartan 32 mg or placebo daily. The pharmacy supplied identical tablets and labeled the study medication for patients enrolled on this trial. Each bottle of investigational product included an investigational-use label, box-code and space for patient name. Treatment allocations were kept in sealed envelopes to be opened only at an imperative need to identify the actual treatment given to a certain patient such as in medical emergencies. During the treatment period patients received study medication three-monthly and were instructed to return unused study medication to the hospital at each next follow-up visit. The pharmacy maintained a complete drug accountability record, including the number of tablets dispended to each patient. The Data Center of the Netherlands Cancer Institute (DC-NKI) conducted the registration and randomization procedure and assigned box-numbers to individual patients.

Blood samples for hematological and serum biochemical monitoring were analyzed by the local laboratories. Analyses of the cardiac markers, troponin T high sensitivity (cTnThs), N-terminal pro-B-type natriuretic peptide (NT-proBNP) and the single nucleotide polymorphisms (SNP) genotyping were performed centrally. The cardiac markers were measured in plasma samples using a sandwich immunoassay (Modular E system, Roche Diagnostics, Mannheim, Germany). In this study we determined HER2-genetic variability in the extracellular domain; FcγRIIIa-158 valine (V)/pheylaline (F), FcγRIIa-131histidie (H)/arginine (R) and FcγRIIIa-232 isoleucine (I)threonine (T), in the transmembrane domain; Val654Ille, Val655Ile and in the intracellular domain; P1170A. The used assay details are outlined in the supplements (Appendix A and B).

Cardiac monitoring

Cardiac monitoring included recording of symptoms, findings on clinical examination, side effects (graded according to the National Cancer Institute Common Terminology Criteria of Adverse Events version 3.0 [CTCAE]), an assessment of the New York Heart Association classification (NYHA) and LVEF, with hematologic

and chemistry studies at baseline, in week 12, 24, 36, 52, 78 and 92. At each study assessment, cardiac questionnaires were used to estimate presence of signs or symptoms of CHF. An electrocardiogram (ECG) was performed at baseline, in week 52 and 78.
Cardiac failures were defined as a decrease in LVEF of more than 15 percentage points compared to baseline or an absolute value of LVEF < 45%. The decision to (dis)continue trastuzumab and/or study treatment was based on an algorithm, as depicted in eFigure 1. All cardiac failures, Suspected Unexpected Serious Adverse Reactions (SUSAR) and Serious Adverse Events (SAE) were reported from start of the study treatment until the end of the study period at the DC-NKI. A data and safety monitoring committee (DSMC), consisting of independent physicians and statisticians with access to unblended data, monitored the safety of the study.

Study drug and treatments

Approved anthracycline-based (neo)adjuvant chemotherapy was completed before randomization. Trastuzumab treatment was given (tri-)weekly. Trastuzumab treatment was commonly used in combination with taxane-based chemotherapy followed by trastuzumab as single agent. Adjuvant endocrine therapy was given after chemotherapy to women with hormone receptor positive disease. Patients started at the same day as the first trastuzumab administration with candesartan 16 mg daily for one week. From week two until 26 weeks after completion of trastuzumab treatment, patients took 32 mg candesartan daily. If CTCAE grade 1 toxic effects occurred, the dose was reduced to 16 mg and it was discontinued if the patient developed CTCAE grade 2, 3 or 4 toxicity.

Outcomes

The primary end point of the study was the occurrence of cardiac failure (defined as decline in LVEF of > 15 percentage points or an absolute value < 45%) during trastuzumab treatment and 40 weeks after discontinuation of trastuzumab. Secondary endpoints were safety of the combination of trastuzumab and candesartan treatment; an evaluation of the cardiac markers, NT-proBNP and cTnThs; and SNPs in the HER2 gene as detective and predictive parameters in trastuzumab related cardiotoxicity.

Statistical considerations

The sample size was calculated to detect a decrease in the proportion of patients with cardiac failures from 30% in the placebo group to 13% in the candesartan group (ref 2 en 24 toevoegen). To obtain 80% power, 100 patients in each treatment group were required at significance level $\alpha = 0.05$. Three prespecified interim analyses were performed after 10, 20 and 30 cardiac failures, with the final analysis at 200 evaluable patients. Error spending functions resembling the O'Brien-Flemming and the Pocock boundaries were used for finding the efficacy and safety

stopping boundaries respectively. At the interim analysis the logrank test was used, patients without complete follow-up were taken into account. All analyses were conducted according to the intention-to-treat principle, but patients with no LVEF measurement after randomization were inevaluable for the primary endpoint. Safety analysis was performed on all patients who received treatment.
Prognostic factors for the occurrence of cardiac failures (age, baseline LVEF, NT-proBNP, cTnThs, performance status and family and medical history) were investigated by using univariable logistic regression models. Associations between LVEF and NT-proBNP and cTnThs marker values were assessed with linear mixed-effects models with LVEF as outcome. Randomized treatment, time since randomization and log marker values were included as fixed effect explanatory variables.
All statistical analyses were performed with SAS software version 9.2 and R 3.1.1.

RESULTS

Patients

At baseline, the patient characteristics were balanced across treatment groups (Table 1). A total of 210 patients were enrolled into the study between October 2007 and October 2010. Of these, 206 women were evaluable for the primary endpoint, 103 women were treated with candesartan and 103 women with placebo (Figure 2). In total 48 patients went off study treatment prematurely (23.3%). The median time from randomization to the last LVEF measurement was 21 months in the candesartan (range 5.4 - 24.9) and 21 months in the placebo (range 5.5 – 26.8) group. Median duration of trastuzumab treatment was 52 weeks in the candesartan (range 19 -53) and 52 weeks in the placebo (range 6 – 53) group. Median time of study treatment was 78 weeks in the candesartan (25 – 87) and 78 weeks in the placebo (7 – 92) group.

Cardiac outcome and adverse events

Overall, 36 cardiac failures were observed in 206 evaluable patients: 20 (19%) in the candesartan and 16 (16%) in the placebo group (p=0.58). The 2-year cumulative incidence of cardiac failures was 0.72 (95% CI, 0.6 – 0.87) in the candesartan and 0.84 (95% CI, 0.78 – 0.92) in the placebo group (p=0.56) (Figure 2). Changes in LVEF values from baseline to week 92 in both treatment groups are shown in eFigure 2 and Figure 3. There was a significant association between the baseline LVEF (≥ 55 vs. < 55%) value as prognostic factor for the occurrence of a cardiac failure (p=0.0004) (eTable 1).
The incidence of adverse events was generally similar across treatment groups. Table 2 shows the overall incidence of any grade 3 or higher adverse event. AEs leading to withdrawal of study treatment were noted in 6 patients (6%) in the candesartan group and 6 patients (6%) in the placebo group including dizziness (n=1, 1% vs. n=1, 1%), hypotension (n=3, 3% vs. n=0, 0%), headache (n=0, 0% vs. n=1,

1%), myalgia (n=0, 0% vs. n=1, 1%), fever (n=0, 0% vs. n=1, 1%) thrombosis (n=0, 0% vs. n=1, 1%), psychological stress (n=0, 0% vs. n=1, 1%) and dyspnea (n=2, 2% vs. n=0, 0%). After trastuzumab treatment, 13 patients developed NYHA class ≥ II in the placebo group in comparison to 8 patients in the candesartan group (p=0.36) (eTable 2). A greater proportion of patients in the candesartan group had serious adverse events; 24.8% (26 of 105) versus 15.5% (16 of 103) in the placebo group (p=0.12). There was no SUSAR reported. The interim safety analyses did not show any significant difference between groups (not presented).

HER2-genotyping

The Ala1170Pro homozygous genotype was associated with a lower likelihood of the occurrence of a cardiac failure in comparison to Val/Val + Ile/Val (OR=0.15, 95% CI, 0.03 – 0.63, p=0.01). The other SNPs were not associated with cardiac failures (eTable 3).

Cardiac markers

No statistically significant association was observed between LVEF and NT-proBNP (p=0.51) or cTnThs (p=0.78) values (eFigure 3 and 4). The risk of cardiac failures was not significantly associated with baseline NT-proBNP (p=0.34) and cTnThs (p=0.54) values. Also no correlation was found between changes in LVEF from baseline and changes in NT-proBNP (Spearman's ρ=-0.24, p=0.47) and cTnThs (Spearman's ρ=-0.12, p=0.74) values during anthracycline treatment.

DISCUSSION

The results of this trial do not support the hypothesis that the concurrent use of candesartan in patients treated with trastuzumab prevents or ameliorates trastuzumab related cardiotoxicity. We found no benefit of candesartan over placebo in either LVEF changes or in the defined primary outcome parameter. Alternative explanations for why we found no statistically significant difference in asymptomatic decline in LVEF need to be considered. Angiontensin II, an effector peptide of the renin-angiotensin system, plays a significant role in the pathophysiology of hypertension, in mediating the development of cardiac hypertrophy and left ventricular remodeling [17,18]. A protective effect of an ARB telmisartan against anthracycline induced cardiotoxicity was demonstrated in (pre) clinical trials [11,19]. Anthracyclines can induce myocardial oxidative stress, which can lead to myocyte cell death and HER2 upregulation. Trastuzumab binds to the extracellular domain of HER2 expressed on the myocardium, which appears to play an important role in compensatory cardiac hypertrophy, leading to an insufficient compensatory mechanism. Although, trastuzumab related cardiotoxicity is dissimilar to anthracycline-induced cardiotoxicity we hypothesized that AT_1 blockade by candesartan, was able to reduce the cardiac pre- and afterload and to reverse left ventricular remodeling in trastuzumab treated patients. It may be that our study results could have been dif-

ferent by starting candesartan treatment during anthracyclines and before trastuzumab. Hence, restoring both the depressed antioxidant defense capacity and the myocardial dysfunction could have been occurred before trastuzumab treatment. Furthermore, patients were excluded with a pre-existing history of CHF or baseline LVEF below 50%. AT_1 blockade as preventive strategy of trastuzumab related cardiotoxicity might be effective in high risk patient populations.

An additional consideration is the choice of the measurement of LVEF by MUGA or echocardiography which may not have been sensitive enough to capture some intervention effects. In current clinical practice, MUGA or echocardiography measurements are the most commonly used modalities for assessing the LVEF. Several studies examining real-time three-dimensional echocardiography and strain rate imaging appear promising in detecting early sub-clinical changes in cardiac performance [20-22]. Moreover, significant functional changes were seen in left and right ventricular function in anthracycline and trastuzumab treated patient by cardiovascular magnetic resonance (CMR) imaging[23]. However, no instruments validated for trastuzumab treated patients were available at the time of trial implementation.

The sample size was calculated based on initial published data which suggested a high incidence of trastuzumab related cardiotoxicity[2,24], but later reports have suggested a much lower incidence[6]. Although, the incidence of cardiac failures was higher in this study compared with large adjuvant trials,

in fact, the sample size and the number of cardiac failures means that we had power to detect only large associations. We acknowledge that larger sample sizes may be needed to determine the preventive effect of candesartan in trastuzumab treated patients. In the present study, NT-proBNP and cTnThs values were not associated with cardiac failures and did not predict or detect cardiotoxicity. Clinical data suggest that the increase in NT-proBNP levels is predictive for the development of chemotherapy related cardiotoxicity. Moreover, pre trastuzumab treatment NT-proBNP levels might predict trastuzumab related cardiotoxicity [13,25]. However, to the best of our knowledge, no association between changes in NT-proBNP and LVEF values during trastuzumab treatment has been reported to date [26,27]. Several clinical studies, demonstrated an association between changes in troponin I levels and the risk of trastuzumab related cardiotoxicity [12,27,28]. In contrast, neither Morris[29] nor Fallah-Rad et al.[30] detected an association between changes in troponin and LVEF values, consistent with our study results. Currently, cardiac biomarkers cannot replace conventional LVEF measurements and more clinical data is needed whether these tests offer a significant advantage in the detection or prediction of trastuzumab related cardiotoxicity.

In this study, we observed a possible association of the valine allele of the Ala-1170Pro SNP and lower risk of a cardiac failure. In contrast, a retrospective cohort study of 73 breast cancer patients by Lemioux et al., found no association of the germline Ala1170Pro SNP and trastuzumab related cardiotoxicity[16]. A major limitation to the study by Lemioux et al. was the small sample size and the study design. Furthermore, baseline LVEF values were not available in 35.9% of the total study

population. In the present study, the consistent measurement of study related assessments provides a reliable relation between trastuzumab treatment, predictive and detective measurements and cardiac failures.
Although a role of germ-line genetic variants could be demonstrated to the Ala-1170Pro SNP, we found no association between the Ile655Val, Ile654Val, FCGR2A 166His, FcgR2B Ile232Thr and FcgR3A Phe158Val SNPs and trastuzumab related cardiotoxicity. Two studies reported an association between Ile655Val polymorphism [15,16] and two published abstracts reported no association between Ile655Val polymorphism and trastuzumab related cardiotoxicity [31,32]. The results of gene polymorphisms of HER2 and their impact on trastuzumab-related cardiotoxicity are contradictory. Therefore, future trials are warranted to explore relationships between these SNPs and trastuzumab related cardiotoxicity.
In conclusion, in comparison to placebo, there was no difference in the number of cardiac events in patients who were treated with candesartan during trastuzumab treatment. Therefore, to date, the effectiveness of ARBs in the prevention of trastuzumab related cardiotoxicity remains uncertain and warrants further investigation.

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Characteristic	CANDESARTAN N=106	Placebo N=104	TOTAL N=210
Age at randomization (years; median; range)	50 (25-69)	51 (30-67)	50 (25-69)
Performance status: n (%) WHO 0 WHO 2 NA	 85 (84) 16 (16) 5 (5)	 86 (86) 14 (14) 4 (4)	 171 (85) 30 (15) 9 (4)
NYHA classification 1 2 3	 99 (96) 4 (4)* 0 (0)	 97 (95) 5 (5)* 0 (0)	 196 (93) 9 (4) 0 (0)
LVEF: median, range	60 (50-74)	61 (50-82)	61 (50-82)
Family history of cardiovascular disease No Yes NA	 79 (78) 22 (22) 5 (5)	 71 (68) 33 (32) 0 (0)	 150 (73) 55 (27) 5 (2)
Medical History: Hypertension No Yes NA Hyperlipidemia No Yes NA Diabetes mellitus No Yes NA Any cardiac history No Yes NA	 91 (87) 14 (13) 1 (1) 101 (96) 4 (4) 1 (1) 102 (97) 3 (3) 1 (1) 103 (98) 2 (2) 1 (1)	 93 (89) 11 (11) 0 (0) 99 (95) 5 (5) 0 (0) 101(97) 3 (3) 0 (0) 101 (97) 3 (3) 0 (0)	 184 (88) 25 (12) 1 (1) 200 (96) 9 (4) 1 (1) 203 (97) 6 (3) 1 (1) 204 (98) 5 (2) 1 (1)
Cumulative dose anthracyclines (mg/m2; median, IQR)	440 (408 – 475.5)	440 (423 - 480)	440 (413 - 480)

Table 1. Patient baseline characteristics
Abbreviations: IQR, interquartile range; LVEF, left ventricular ejection fraction; NA, not applicable; WHO, world health organization

	CANDESARTAN N=105			PLACEBO N=103		
Adverse event	Grade 3 N(%)	Grade 4 N(%)	Grade 5 N(%)	Grade 3 N(%)	Grade 4 N(%)	Grade 5 N(%)
Neutropenia	1 (1)	3 (3)	0 (0)	0 (0)	2 (2)	0 (0)
Hypotension	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fatigue	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Fever	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Dermatology	0 (0)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)
Hot flashes	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gastrointestinal disorder	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Neutropenia	4 (4)	0 (0)	0 (0)	3 (3)	0 (0)	0 (0)
Infection	5 (5)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Metabolic disorder	0 (0)	3 (3)	0 (0)	2 (2)	0 (0)	0 (0)
Musculoskeletal disorder	2 (2)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Dizziness	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Neurology	3 (3)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)
Peripheral neuropathy	3 (3)	0 (0)	0 (0)	3 (3)	0 (0)	0 (0)
Pain	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dyspnea	5 (5)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Secondary malignancy	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Any surgery	3 (3)	0 (0)	0 (0)	4 (4)	0 (0)	0 (0)
Vascular	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)

Table 2. Adverse events according to the National Cancer Institute's Common Terminology Criteria for Adverse Events ≥ grade 3

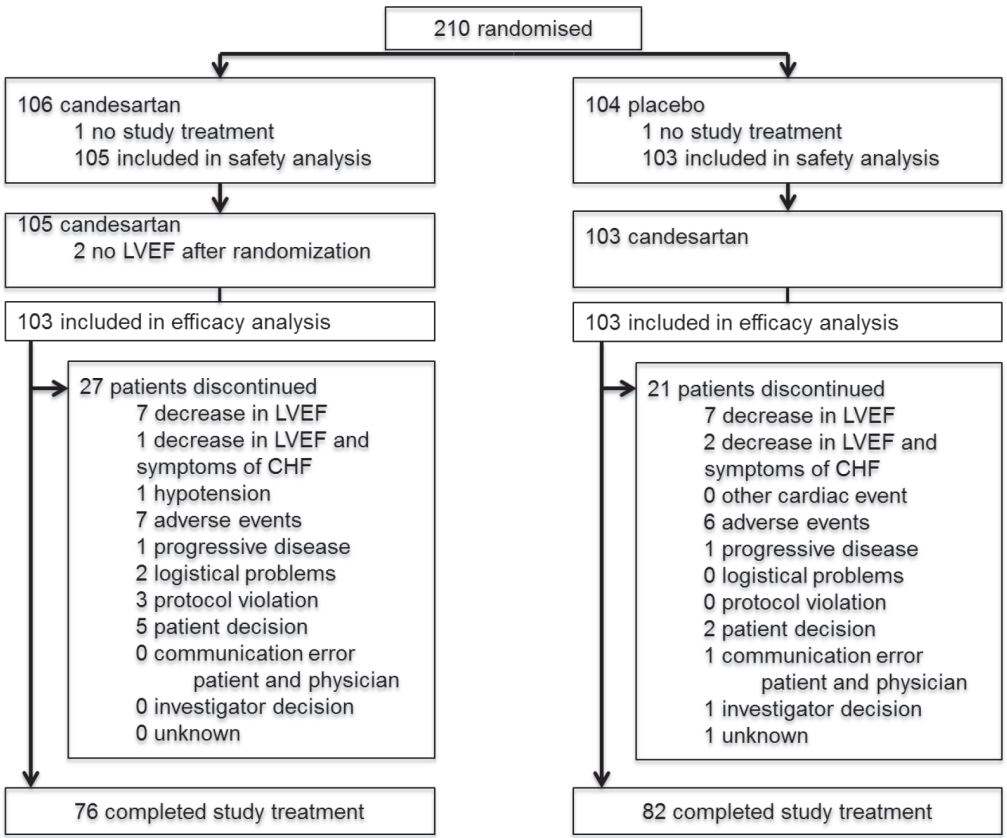


Figure 1. Trial Profile
Abbreviations: CHF, congestive heart failure; LVEF, left ventricular ejection fraction

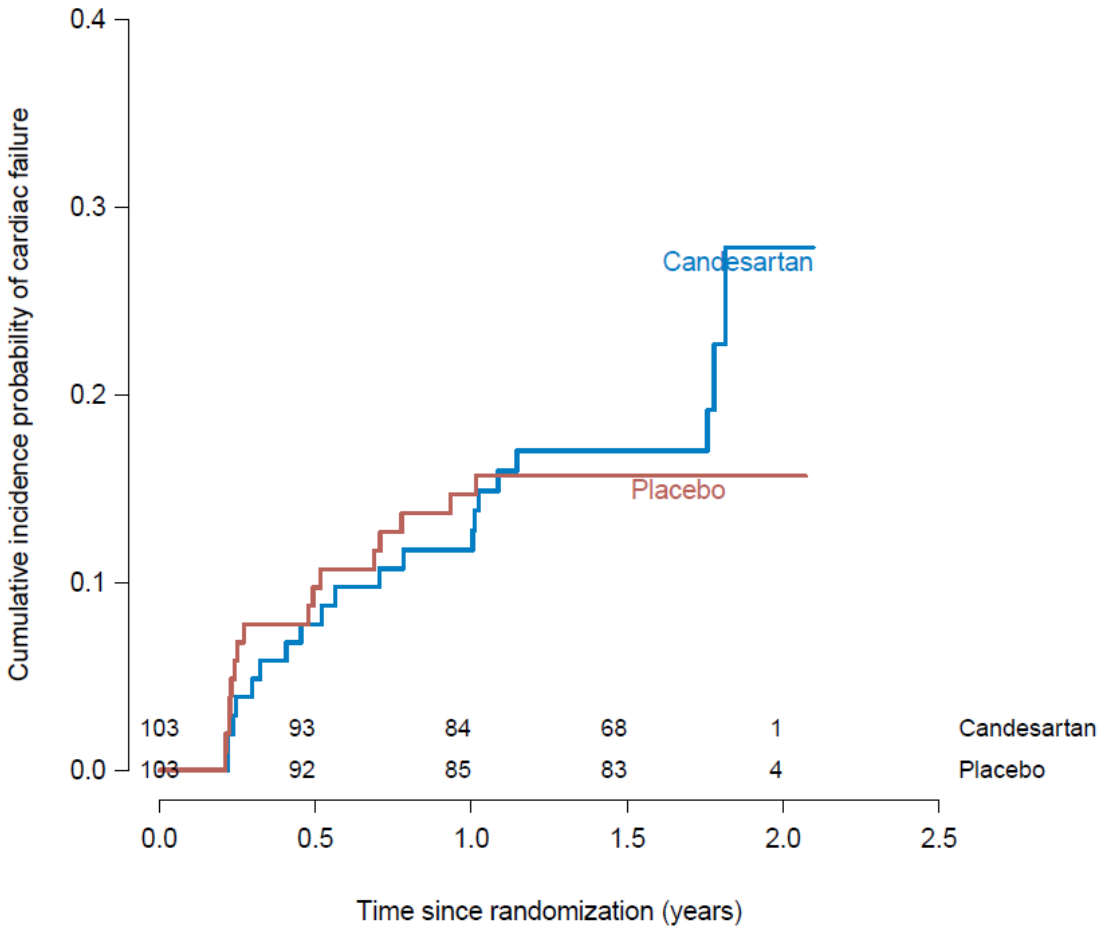


Figure 2. Incidence of cardiac failures

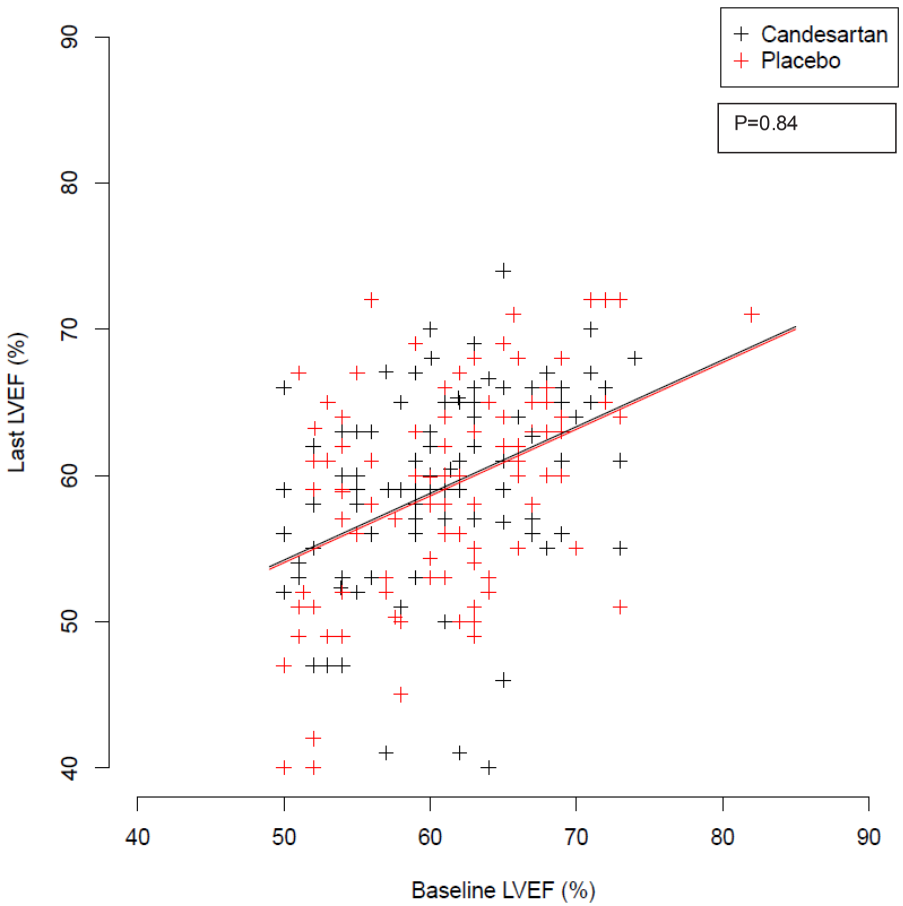


Figure 3. Scatter plot showing LVEF values at baseline and last measurement

Abbreviation: LVEF, left ventricular ejection fraction

Univariable Logistic Models for Cardiac Failure*				
Covariate/Factor		Units/Comparison	OR (95% CI)	p-value
Randomization group		Candesartan vs. placebo	0.76 (0.37 - 1.57)	0.46
Age at randomization		Per year	1.01 (0.97 - 1.05)	0.72
Baseline LVEF value		≥ 55 vs. < 55%	0.24 (0.11 – 0.53)	0.0004
Performance status		WHO 0 vs. WHO 1	0.76 (0.32 – 1.80)	0.54
Family history of cardiac disease		Yes vs. no	0.76 (0.32 – 1.80)	0.54
Medical history:	Hypertension	Yes vs. no	0.94 (0.30 – 2.93)	0.91
	Diabetes Mellitus	Yes vs. no	1.19 (0.13 – 10. 93)	0.88
Baseline NT-proBNP value (µg/L)		≥ 68 vs. < 68 unit	1.57 (0.62–3.93)	0.34
Baseline cTnThs value (µg/L)		≥ 14 vs. < 14	0.75 (0.30–1.89)	0.54

Online only-tables

eTable 1. Potential Prognostic Factors for occurrence of Cardiac Failure

Abbreviations: CI, confidence interval; cTnThs, troponin T; NT-proBNP, N-terminal pro-B-type natriuretic peptide; OR, odds ratio; vs., versus; WHO, World Health Organization

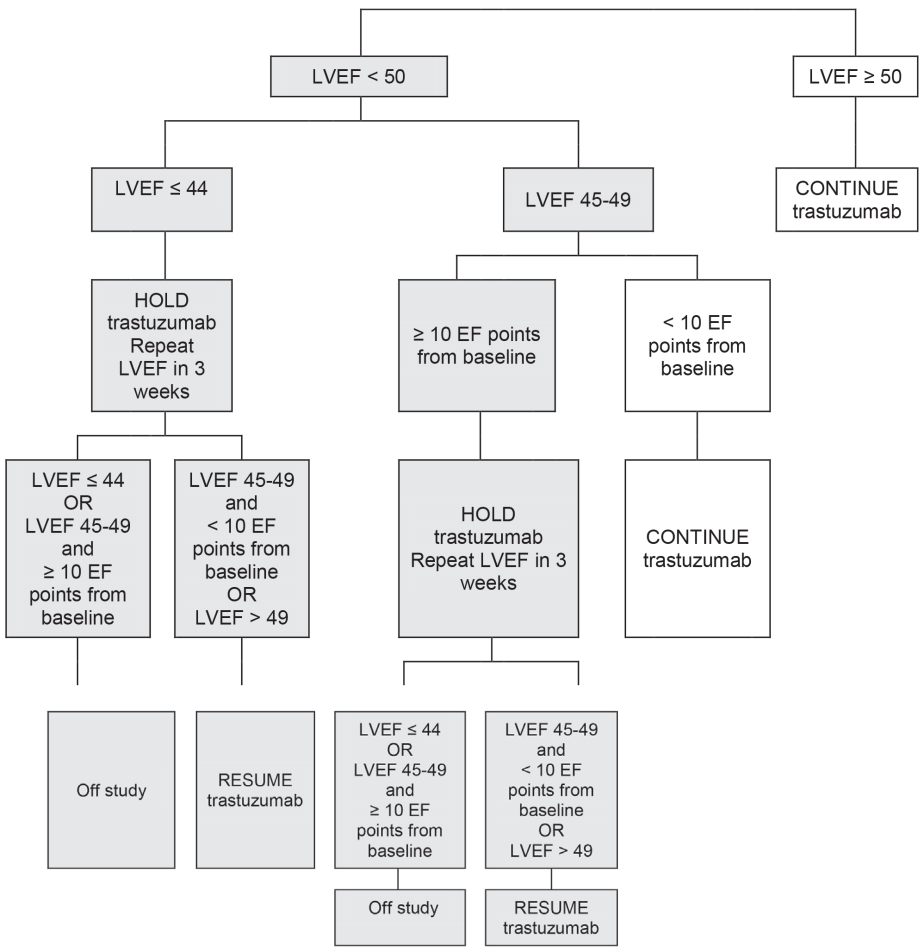
	CANDESARTAN	Placebo	TOTAL
NYHA classification* During trastuzumab	N=102	N=103	N=205
1 2 3	75 (74%) 26 (25%) 1 (1%)	74 (72%) 29 (28%) 0	149 (73%) 55 (27%) 1 (1%)
NYHA classification* After trastuzumab	N=96	N=98	N=194
1 2 3	88 (92%) 8 (8%) 0 (0%)	85 (87%) 12 (12%) 1 (1%)	173 (89%) 20 (10%) 1 (1%)

eTable 2. Number of patients with worst NYHA score by time

* number of patients with worst cardiac symptoms by time
Abbreviations: NYHA, New York Heart Association

Recessive model for Cardiac Failure* (Val/Val versus Ile/Ile and Ile/Val)									
	Subjects	Genotype distribution			Missing	Cox PH model		Logistic model	
		Ile/Ile	Ile/Val	Val/Val		HR (95%CI)	P-value	OR (95%)	P-value
Ile654Val									
No cardiac failure	170	141	4	0	24	Not fitted because of no homozygote subjects			
Cardiac failure	36	30	1	0	5				
Ile655Val						1.23 (0.29 – 5.14)	0.78	1.18 (0.24 –5.85)	0.84
No cardiac failure	170	91	46	8	25				
Cardiac failure	36	16	13	2	5				
Ala1170Pro						0.16 (0.04 – 0.68)	0.01	0.15 (0.03 – 0.63)	0.01
No cardiac failure	170	22	77	47	24				
Cardiac failure	36	9	20	2	5				
FCGR2A 166His						1.81 (0.85 – 3.84)	0.12	1.92 (0.82 – 4.52)	0.13
No cardiac failure	170	36	81	29	24				
Cardiac failure	36	9	12	10	5				
FcgR2B Ile232Thr						1.47 (0.20 –10.85)	0.7	1.30 (0.14 – 12.05)	0.82
No cardiac failure	170	110	30	4	26				
Cardiac failure	36	21	6	1	8				
FcgR3A 158Phe/Val						1.70 (0.84 – 3.44)	0.14	1.76 (0.81 – 3.85)	0.15
No cardiac failure	170	18	73	55	24				
Cardiac failure	36	4	11	16	5				

eTable 3. HER2-genetic variability among the study population
Abbreviations: HR, hazard ratio; OR, odds ratio; PH, proportion hazard



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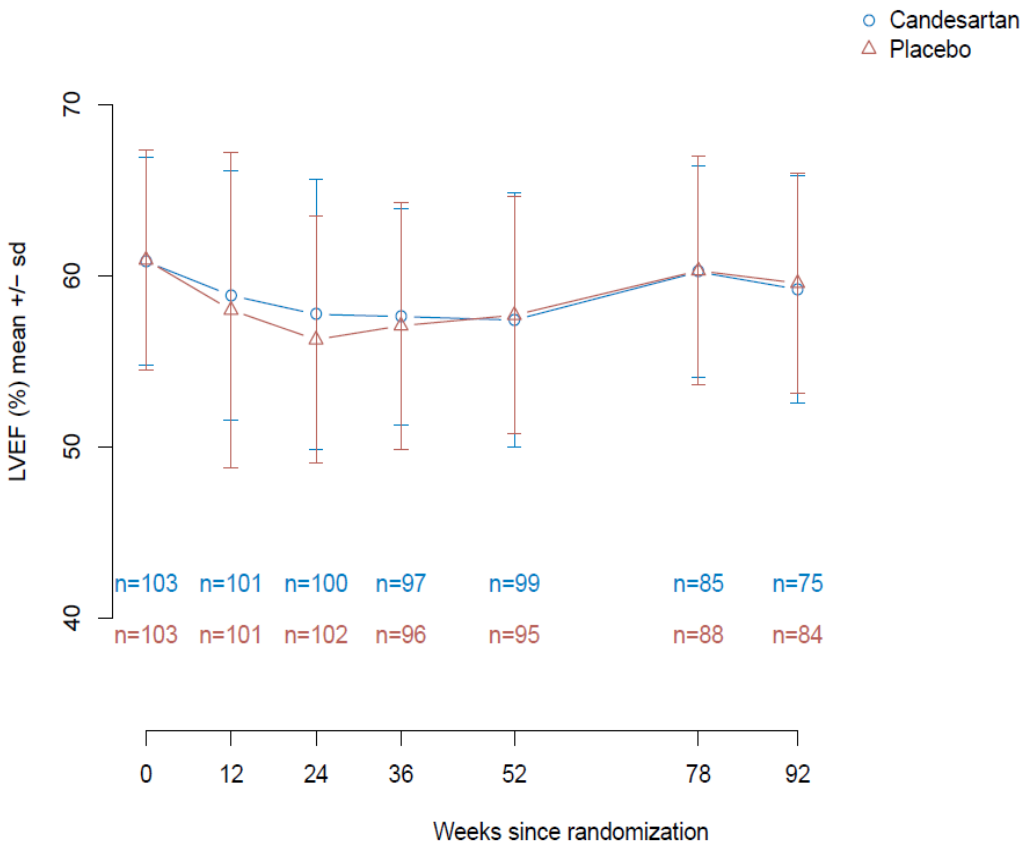
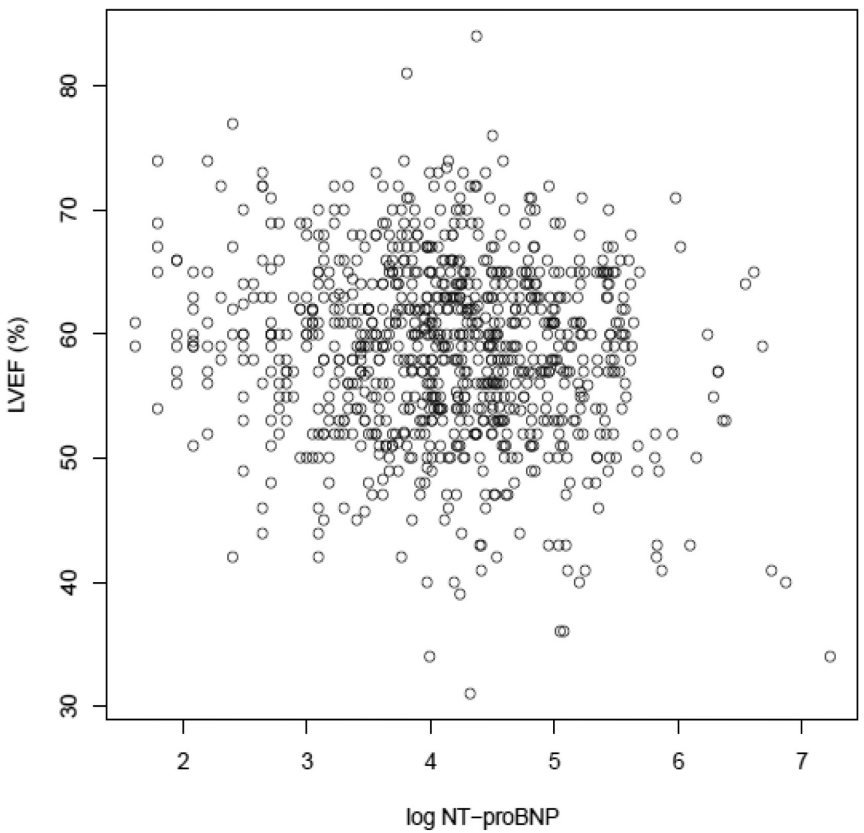
eFigure 1. Algorithm (dis)continuation trastuzumab treatment
Abbreviations: EF, ejection fraction; LVEF, left ventricular ejection fraction

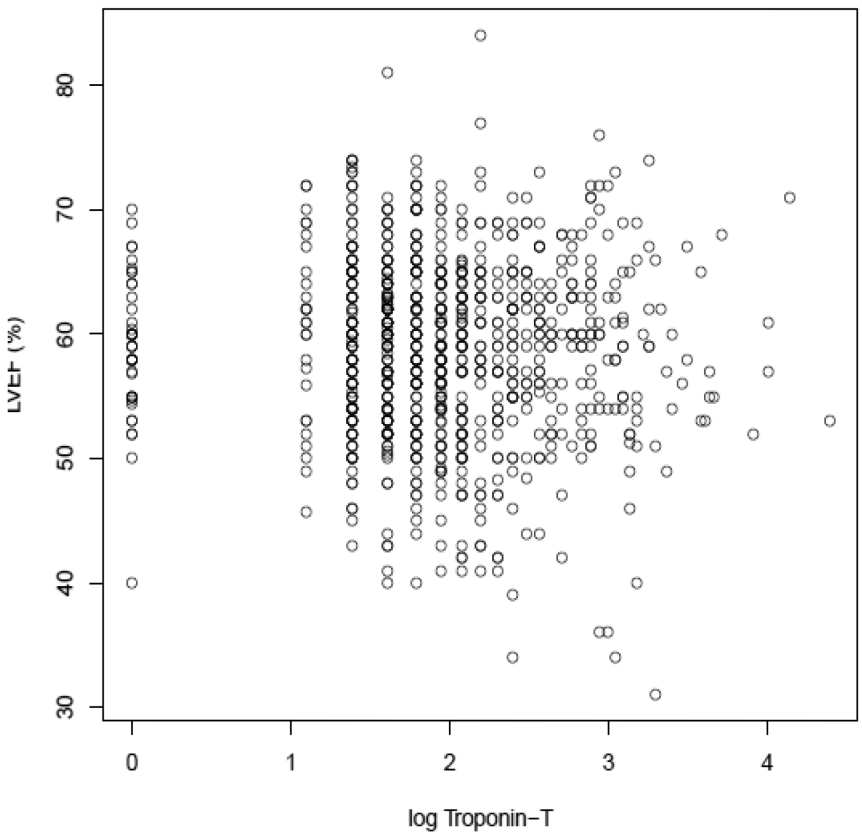


eFigure 2. Mean and standard deviation of LVEF over time

Abbreviation: LVEF, left ventricular ejection fraction



eFigure 3. LVEF values at baseline by log NT-proBNP values
Abbreviation: LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide



eFigure 4. LVEF by log cTnThs values
Abbreviations: LVEF, left ventricular ejection fraction; cTnThs, troponin T

3.1 Strategies to target drugs to gliomas and CNS metastases of solid tumors.

B. Milojkovic Kerklaan [1, 2], O. van Tellingen,[3] A.D.R. Huitema,[4] J.H.Beijnen,[4,5] , W. Boogerd [6], J.H.M. Schellens [1,2,5], D. Brandsma[6]

1 Department of Molecular Pathology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
2 Department of Clinical Pharmacology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
3 Department of Preclinical Pharmacology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
4 Department of Pharmacy & Pharmacology , The Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
5 Utrecht Institute of pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands,
6 Department of Neuro-oncology , Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands.

Manuscript submitted

INTRODUCTION

The treatment for brain tumors, malignant glioma and central nervous system (CNS) metastases is limited. The drug development in this area brought only modest improvements in the last decades.[1]

The treatment of glioblastoma, the most malignant type of glioma, is since 2005 intensified with surgical debulking, radiotherapy and chemoradiation with temozolomide. However the mean overall survival is only 12-15 months. [2] Once the glioblastoma reoccurs, the median overall survival decreases to less than 6 months despite many different treatment modalities.[3–5]

In addition to glioma, 20-40% of patients with tumors of other origin (mostly lung, breast, renal cancer and melanoma) tend to develop metastases in the CNS. [6,7] The current treatment for brain metastases (BM) is surgical resection in case of a single metastasis, (stereotactic or whole brain) radiotherapy and/or systemic therapy. Survival of patients with BM from solid tumors ranges from 5-19 months depending on more factors such as performance status, the number of brain metastases, the primary tumor type and progression of systemic metastases.[8–10] Patients with leptomeningeal metastases have a survival of only 4-6 weeks if not treated.[11] Several months can be gained with treatment of symptomatic sites by radio – or systemic therapy.[12–14] Prognosis depends on the patient's neurological status, tumor type and progression of systemic metastases. [14] More effective treatment modalities for both malignant gliomas and CNS metastases from solid tumors are urgently needed. Unfortunately, promising concepts from preclinical experiments often do not translate into similar results in the clinic.[15–17] One of the main causes for this might be the difficulty in designing the preclinical brain tumor models, which poorly reflect the complexity of human brain tumors. Furthermore, phase I/II clinical studies may suffer from biased patient selection, in which patients with a favorable prognosis achieve a longer progression free survival (PFS). Another major contributing factor is the poor penetration of the drugs across the blood-brain barrier (BBB). Some agents can cross the BBB, but cause considerable neurotoxicity. The vinca-alkaloids, cisplatin and the taxanes can cause peripheral neurotoxicity, while methotrexate, cytarabine and ifosfamide can lead to central neurotoxicity with cognitive deficits, hemiparesis, aphasia and progressive dementia. [18] The aim of the brain tumor drug development is to achieve the sufficient concentrations of drug in the brain. Further, the drug needs to be specifically chosen to target tumor mutation/expression status or multiple targets simultaneously, with a limiting (neuro)toxicity. In this review we focus on current and new strategies to transport anti-cancer drugs across the BBB or/and blood-CSF barrier (BCSFB) or bypass these barriers.

METHODS

The heading search terms for this review in the PubMed/Medline and ClinicalTrials.gov databases were: blood-brain barrier, brain tumors, glioma, glioblastoma, brain metastases, leptomeningeal metastases or carcinomatosis, targeted therapies, new strategies, nanocarriers, immunotherapy, viral gene therapy, toxin, siRNA, intrathecal therapy, blood-brain or blood CSF barrier. Articles published in English starting from 2000 till 2014 were included.

Blood brain barrier (BBB) and drug characteristics requirements

The presence of BBB is the reason why only few systemically administered drugs can reach it, despite the fact that the brain is one of the highly perfused organs. [19] The barrier is formed by the monolayer of tightly sealed brain endothelial cells that are closely connected by the tight junctions. The tight junctions limit the paracellular transport of (hydrophilic) molecules. (Figure 1A) This monolayer of endothelial cells is encircled by a basement membrane, pericytes and astrocytic endfeet. [20,21] Paucity of endocytosis and presence of specific efflux pump proteins in the endothelial cells further contribute to the barrier function of the cerebral vessels.

Small lipophilic molecules with a molecular weight less than 400 Da can penetrate in a trans-cellular way via passive lipid-mediated diffusion, such as alkylating agents, temozolomide (194 Da), nitrosoureas, eg, BCNU (carmustine, 214 Da), CCNU (lomustine, 233 Da) and procarbazine (221 Da), drugs already used for malignant glioma treatment. [22] (Table 1)

Larger and protein bound lipophilic drugs or hydrophilic molecules of any size cannot passively cross the BBB. Thus, an alternative way should be found to open the BBB for these types of drugs. (Table 2)

Pharmacological, biological or physical ways to open/cross the BBB.

The BBB can be opened by chemical, biological or physical stimuli. For instance, mannitol can lead to endothelial cell shrinkage and opening of the tight junctions. Biological stimuli such as bradykinin agonists can open the BBB selectively triggering calcium Ca(2+) and clauding-5 at BBB. [23,24] Bacteria and bacterial toxins can penetrate and disrupt BBB, such as in case of meningitis.[25]

The representatives of the physical stimuli that can open BBB are ultrasound and electromagnetic fields. [26–28]

Transcellular transport mechanisms over the BBB.

Nutrients and some drugs/toxins are using the mechanisms of transcellular transport over an intact BBB. Mostly nutrients utilize facilitated diffusion using carrier-mediated transport (CMT). Glucose can cross the BBB using CMT via glucose carrier. The transport of larger molecules, such as peptides and proteins, can be

performed using receptor-mediated transcytosis (RMT). Example of RMT are transport of insulin and transferrin.[29,30] Once the protein is bound to its specific receptor on the BBB the internalization of the protein into a vesicle starts. The vesicle crosses the whole brain endothelial cell and fuses with the membrane on the parenchymal side, after which the protein can be released in a process called transcytosis.[31] RMT is generally used for liposomes and other nanotechnology-based systems to cross the BBB. Using RMT a toxic anti-cancer drug packed inside a liposome and coupled to a molecule that is recognized by the receptor facilitates shuttling of the toxin across the BBB. This process is called the molecular Trojan horse. [32] Enhanced brain penetration employing CMT requires a close structural analogy to endogenous carrier substrates. [33,34]
Brain-targeted drugs that are currently in (clinical) use and utilize CMT are levodopa for Parkinson disease, donepezil and tacrine for Alzheimer disease and gabapentin, pregabalin, valproate for epilepsy and baclofen for multiple sclerosis. [29] Drugs currently used in drug development programs, that cross the BBB as mediated through RMT, employ the following receptors: low density lipoproteins (LDL), glutathione receptor; insulin-receptor, insulin-like growth factor receptor, transferrin receptor, or diphtheria toxin receptor.[19,32,35–38] RMT is generally used for liposomes and other nanotechnology-based systems to cross the BBB.
Another mechanism used in drug development to target the brain is the Adsorptive-Mediated Transport (AMT) mechanism. In ATM cationic molecules bind to anionic sites on the BBB and induce vesicle formation and endocytosis. The example of ATM is the uptake by BBB cells of chemically conjugated iRNA or paclitaxel by a cell penetrating peptides. [39] ATM is not as specific as RMT and CMT. It has a risk of BBB disruption and toxicity. The cationic molecules have a potentially high adsorptive property towards anionic sites on many cell surfaces in different tissues and organs. ATM can be a disadvantage for specific targeting to a desired organ, particularly when the protein is administered intravenously.[39] Another limitation of this strategy is compliment activation. Therefore human proteins, recombinant humanized proteins or conjugates of cationic proteins to polyethylene glycol are now being used. [29,39]

Blood-CSF barrier (BCSFB)

Another important barrier of the CNS is the Blood-Cerebrospinal fluid Barrier (BCSFB).[40,41] Although 500-fold less extensive in surface than the BBB, the BCSFB protects the CSF from the systemic circulation. Transport between blood and CSF, necessary for maintaining the equilibrium in the physiological status of the CNS occurs in the choroid plexus of the ventricles. It is formed by fenestrated capillaries and epithelial cells. The epithelial cells are, as well as in the BBB, tightly sealed with tight junctions. [46,47] In the leptomeninges that cover the CNS, the barrier function is exerted by the arachnoid cells and (to a lesser extent) by the pia mater. Similar transporters exist at the BCSFB as at the BBB, with some exceptions. (Figure 1B) Glutamate and some Na+ dependent transporters are present on the BCS-

FB at the increased number, such as bicarbonate transporters. Those transporters are required for CSF production and secretion. Heme-, sugar efflux- and several amino-acid transporters are present with a fewer number than at BBB. [44] (www.bioparadigms.org)
Between the CSF and the interstitial brain fluid (ISF) a dynamic exchange of nutrients and water is present. [45] It was demonstrated in an in vivo imaging study of Liff et al (2012) that CSF enters the brain ISF space following paravascular spaces that surround penetrating arteries. The ISF is than cleared again following the paravenous drainage pathways. [46] The BCSFB removes brain metabolites and toxins, as brain lacks lymphatic vessels.[45,47]

Drug efflux transporters on the blood-brain and blood-CSF barrier and their inhibitors

Except for being able to penetrate BBB, effective drugs in neuro-oncologyneed to avoid drug efflux transporters that tend to pump them out of the brain.[48]
P-glycoprotein (P-gp or ABCB1) and breast cancer resistance protein (BCRP or ABCG2), ATP-binding cassette (ABC) drug efflux transporters, are highly expressed in the brain endothelial cells. [53] Concentrated mainly at the luminal side of BBB, P-gp and BCRP are limiting the penetration of their substrates across the BBB. [51–53] At the BCSFB, the expression of P-gp and BCRP might be very low or functionally not important, as their substrates can more easily penetrate the blood-CSF membrane. [54–56]
Various preclinical studies suggest that efflux transporter inhibitors can increase drug accumulation in the brain .[57] An 11- fold higher brain uptake of paclitaxel was observed in P-gp knockout mice compared to wild-type mice, suggesting that paclitaxel penetration can be facilitated with efficient blocking of those transporters. However, co-administration of paclitaxel with P-gp inhibitors (cyclosporin A, valspodar, elacridar or Cremophor EL) in mice resulted in only 3-6.5 fold higher paclitaxel brain concentrations as compared to paclitaxel treatment without adding inhibitor. [58] This can be explained by the fact that available efflux P-gp inhibitors van only partially inhibit P-gp function depending strongly on the P-gp inhibitor of choice and their dose level. [58,59] [60]

Damaged barriers in brain or leptomeningeal tumors as a potential entrance for systemic drugs

In the presence of tumor the paracellular transport over the BBB increases because of disruption between the endothelial cells, changes in the tight junction adhesive properties or an increased number of vasogenic molecules that can all contribute to edema formation.[23] The damage to the BBB can be demonstrated by gadolinium (gdDTPA) enhanced MRI T1 scans. The molecular weight of gdDTPA is +/- 550 Da. [61] The extent of the disruption of the BBB in high-grade glioma is not uniform. As demonstrated by gdDTPA-MRI, contrast enhancement is mainly observed

in the area where vascular proliferation is evident or so- called leading/growing edge of the bulk of the tumor. [62,63] However, in the infiltrative areas of the tumor visualized on T2 or FLAIR (Fluid-attenuated Inversion Recovery) MRI, contrast enhancement is much less prominent, indicating a more intact BBB function or leakage that could not be assessed by gdDTPA MRI.
In case of BM, it is known that macroscopic BM (> 2-3 mm) develop new tumor vessels that resemble the vessels of the primary tumor and lack BBB characteristics. Lockman et al. 2010 analyzed over 2,000 BM and demonstrated that blood-tumor barrier (BTB) permeability was damaged in over 89%. However, brain uptake of 14C-paclitaxel and 14C-doxorubicin, although generally greater than in normal brain, only reached cytotoxic concentrations in about 10% of BM.[64]
Characteristics of specific tumors in the brain may determine the extent of BBB disruption. For example, in preclinical experiments BM of triple negative or basal-type breast cancers showed different BBB leakage patterns when compared to HER2-positive breast cancer. [65]
A recent preclinical study showed that invading glioma cells could displace the astrocytic endfeet of the BBB. This could cause loss of endothelial cell tight junctions and an increase of the permeability of the BBB. One single invading glioma cell can cause local BBB damage.[20]

In contrast, other studies suggest that the BBB is continuously being restored. During restoration the BBB is being protected by paracellular adherens junctional proteins, a remodeled basement membrane and perivascular leukocytes.[66]

Strategies to cross the BBB and target brain tumors

Several strategies have been developed to increase drug brain concentrations. These strategies can be grouped in three categories.

1) Brain targeting receptor- or carrier mediated transport strategies (Table 3 and 4)

a. (pro-)Drug conjugates

The drug is being kept inactive in the blood till the release at the site of action. The inactive drug uses a specific receptor-mediated transcytosis (RMT) or carrier-mediated transport (CMT) to target and to cross the BBB.

b. nanocarriers

Nanocarriers carrying drugs but also other active pharmaceutical ingredients (API's), e.g. siRNA, enzymes are important for metabolic diseases. The drug is being inactive as being inside a carrier (drug conjugates or nanoparticles, such as liposomes or polymer-based nanocarriers). Via specific RMT the liposome targets and crosses BBB and releases the active drug at the site of action.

2) Immunotherapeutic strategies.

The systemically administered drug recruits and stimulates cells of the immune system. The activated immune cells (usually T-lymphocytes) can then pass the BBB and target the brain tumor. Not the drug itself but the immune cells activated by the drug need to cross the BBB or BCSFB. (Table 5)

3) Gene transfer strategy mediated by neural stem cells.

Gene transfer strategy is systemic administration of genetically modified stem cells that can cross the BBB. Stem cells are loaded with enzymes that can activate drugs administered systemically as prodrugs.
These strategies are being discussed in more detail below.

1) Brain targeting receptor- or carrier mediated transport strategies

Prodrug strategies

The prodrug is a bioreversible derivative of an active drug compound. By increasing the lipid solubility of the drug or by conjugation of a drug into a carrier (brain-targeting drug conjugates), the drug is being transformed in a prodrug. [67,68] The active drug is kept inactive in the systemic circulation. Via CMT or RMT it crosses the BBB. Because of the presence of elevated levels of certain proteolytic enzymes in tumors, the cleavage of conjugate can take place at the site of action in the brain tumor cells where the active drug becomes released. [31] Examples of prodrug stategy are drug conjugates and nanoparticles. (Table 3)

Drug conjugates

Paclitaxel-Angiopep-2 conjugate named ANG1005 or GRN1005 is the RMT-based vector utilizing the low-density lipoprotein receptor-related protein-1 (LRP-1), highly expressed on the BBB. LRP-1 mediates transcytosis of normal ligands across the BBB, such as tissue-type plasminogen activator, thyroglobulin, and lactoferrin. [69] Preclinically GRN1005 showed a broad brain parenchymal distribution in comparison with conventional paclitaxel, while avoiding P-gp efflux transporters. It showed anti-tumor activity in mice with BM of lung cancer and glioblastoma. [70,71]

In a phase I trial GRN1005 showed one complete response (CR) and two partial responses (PR) out of 63 enrolled patients with recurrent malignant glioma. There were 5 intracranial responses in 41 patients with solid tumors and BM.[72,73] In the substudy of 9 patients with malignant glioma, GRN1005 (>200 mg/m^2 iv) was administered 3.5-6 hours before resection. In the resected tissue the cytotoxic levels of paclitaxel (>0.3µmol/L) were found in all 9 patients. Further in phase II study because of hematological toxicity, the dose needed to be reduced from the one that was recommended in the phase I study (650 mg/m^2).[74] At a lower dose-lev-

el (550 mg/m^2) no more intracranial responses could be seen and the study was terminated after the first 30 enrolled patients with breast cancer and BM were enrolled. No further development of this component was performed.[75]

A non-toxic mutant of diphtheria toxin, the cross-reacting material 197 (CRM197), is another example of receptor-specific carrier protein for small molecules. Diphtheria toxin receptor (DTR) is expressed on the cells of BBB, neurons, and glial cells. Preclinical experiments using CRM197 are ongoing. [38,76,77] Unfortunately those receptors are also expressed on myocardial cells. Therefore, the use of CRM197 carrier may induce cardiotoxicity, which will probably depend on the carried drug by CRM197.

Nanoparticles

Nanoparticles with their small molecules size of 1-100 nanometer and are used as a carrier of an active drug to the place of action where it releases the drug.[78] While circulating in blood the drug is trapped inside the liposome and only upon the release, a controlled amount of drug is being available. There are 250 nanoparticle products in various stages of clinical trials.[79] There are oral, topical, inhaled or parenteral nanoparticle formulations. They can be divided into two groups: nanovectors, (liposomes and nanoparticulate drug carriers) and polymer-based nanocarriers, (dendrimers and polymeric micelles). [29] In case of nanoparticles RMT is facilitating drug delivery across the BBB. Liposomes are spherical phospholipid bilayer vesicles with a hydrophilic inner space for the carried drugs. By encapsulating drugs in stealth liposomes the volume of drug distribution can be reduced and the drug circulation time can be prolonged. The stability is one of the main concerns during the liposomal development. Liposomes consist of (semi) natural, biodegradable lipids. It is protected by nonionic hydrophilic polymers, for example polyethylene glycol (PEG), with the aim to avoid interaction with immune cells and normal tissue.[78,80] It is being hypothesized that liposomes extravasate from the leaky tumor vasculature once they reach the tumor area and accumulate in the tumor. This is so-called process of enhanced permeability and retention (EPR) effect.[81] Unfortunately, only a few liposomal drugs are approved for CNS indications. One of them are intravenous amphotericin B for cryptococcal meningitis and intrathecal liposomal cytarabine (Depocyte®) for leptomeningeal metastases.

Liposomal amphotericin B showed a decreased fungal burden with a significantly higher brain tissue concentrations than other lipid amphotericin formulations. [82] One of the representatives of liposomal drugs that specifically targets drugs to brain tumors is 2B3-101, a glutathione PEGylated liposomal formulation of doxorubicin. In this formulation glutathione (GSH) is attached to the PEG chains on the surface of the liposome, which targets the liposome to the active GSH transporters on the BBB. [35] (Table 3)

In mice, a 5-fold enhanced delivery of doxorubicin to the brain was seen after intravenous treatment with 2B3-101 as compared to PEGylated liposomal doxorubicin (PLD)[83] Furthermore, as compared to PLD survival of mice with glioblastoma improved strongly after intravenous treatment with 2B3-101. [84]
A phase I/IIa clinical study 2B3-101 showed a moderate safety profile (i.e. hematological and mucocutaneous toxicity and infusion reactions) with preliminary anti-tumor activity in patients with recurrent high grade glioma and patients with BM from solid tumors, warranting further clinical development. (Milojkovic-Kerklaan et al., submitted for publication). Other brain-targeting drugs that are currently in development are shown in Table 3.

Polymer-based nanocarriers

A single structural units, such as synthetic plastics, DNA and/or proteins with multiple repetitions are called polymer-based nanocarriers. They can be divided into dendrimers and polymeric micelles.[29,85] A dual targeting of the BBB with transferrin (Tf) and tamoxifen (TAM) is designed with a PEGylated doxorubicin dendrimer carrier (G4-DOX-PEG-Tf-TAM). The transferrin facilitates the penetration of the doxorubicin conjugate across BBB and inside glioma cells, while tamoxifen inhibits drug efflux transporters, such as P-gp, BCRP and MDR4 [85]. Doxorubicin showed accumulation in C6 glioma cells only and not in in vitro murine BMVEC (brain microvascular endothelial cells) after incubated with G4-DOX-PEG-Tf-TAM. Another brain targeting polymer-based nanocarrier is the polymeric micelle Pluronic P105 that uses also dual targeting of glucose via CMT and folic acid receptors via RMT. Internalization of doxorubicin into C6 glioma cells was seen after crossing the in vitro BBB model.[86] Mice could be safely i.v. treated with Pluronic P105 showing intracranial C6 glioma cell growth suppression. [86] However, it is unclear how two different mechanisms CMT (using glucose) and RMT (using folic acid receptors) can transfer one drug i.e. doxorubicin across the BBB.

Nanocarrier siRNA delivery strategies

RNA interference (RNAi) is a natural process of gene expression inhibition. This post-transcriptional gene expression silencing can be triggered also by synthetic short interfering RNA (siRNA). [87] Because of its instability, large size and its negative charge, siRNA needs to be delivered by liposomes.[88] (Table 4) The well-known O-6-methylguanine-DNA methyltransferase MGMT gene in gliomas is responsible for DNA repair of DNA lesions that are naturally occurring or are induced by drug. In 45% of patients with glioblastoma, MGMT status is methylated (inactive) which is associated with a better prognosis and better response to temozolomide therapy. [89] The response to temozolomide in patients with non-methylated MGMT may be improved when MGMT could be silenced or suppressed by sIRNA. [90] A preclinical study with siRNA silencing MGMT in a locally applied cationic liposomal formulation was however disappointing, as insufficient distribution of cationic li-

posomes in either the rat or the porcine brain tissue was achieved. [90] Better in vivo results in mouse brain and in an EGFR-driven mouse model of glioblastoma were found after treatment with siRNA against the epidermal growth factor receptor (EGFR) using a different nanocarrier, viz the dendrimer-conjugated magneto-fluorescent nanoparticle (nanoworm). In mice, internalization of the siRNA against EGFR was shown along with 70-80% reduction of EGFR protein level in intracranial glioma cells. [91]
Finally a strategy with promising in vivo results is the use of docetaxel and a liposomal formulation of siRNA silencing vascular endothelial growth factor the VEGFR gene and targeting the LDL-1 receptor on the BBB (Angiopep 2). [92]

Strategy to deliver antibody therapy across the BBB

The monoclonal antibody trastuzumab targeting HER2 in HER2+ breast cancer inhibits overexpressed receptors on tumor cells. Antibodies cannot cross an intact BBB or BCSFB but can have (limited) activity on intracranial tumors when the BBB and/or BCSFB are partly being disturbed.
Bispecific antibodies that can bind to the transferrin receptor (TfR) and at the same time target β-secretase (BACE1), were investigated in preclinical models of Alzheimer's disease. The data showed that anti-TfR/BACE1 antibodies can cross the BBB and reduce brain amyloid-β (Aβ) in mice.[93]

Immunotherapeutic strategies

Tumor-associated genes are found specifically in tumor cells and in their environment. The tumor-proteins derived from these genes can have the potency to activate immune cells.
The aim of immunotherapy in neuro-oncology is to enhance the immune process against tumors in the brain and/or CSF by activating immune cells. These activated immune cells can cross BBB and BCSFB and target tumor cells. [94] Immunotherapy can be divided into passive, active and immunomodulatory immunotherapy.

Passive immunotherapy

Adoptive cellular therapy is a passive form of immunotherapy, a transfer of immune cells. In this method patient's T-lymphocytes are being ex-vivo stimulated with autologous inactivated tumor cells. [95] Thus, under laboratory conditions the surgically removed brain tumor is being used to isolate the patient's T lymphocytes. Once those pretreated and expanded T-lymphocytes are administered to the patient intravenously, they can migrate to the antigen-expressing tumor cells.[96] An example of this immunotherapy is tumor-infiltrating lymphocyte (TIL) therapy in metastatic melanoma.[97] For treatment of patients with malignant glioma T-cell receptor (TCR) gene therapy was used to redirect the patient's T-lymphocytes to the glioma. To be able to specifically target glioma two highly expressed EGFRvIII and viral derived CMV antigens are used. [98] Administration of CMV-specific cy-

totoxic T lymphocytes (CTL) expressing chimeric antigen receptor (CAR) targeting HER2 shows 1 PR, 4 SD and 8 PD in 13 patients with glioblastoma in a phase I trial. (NCT01109095)

Cancer vaccines – an active type of immunotherapy

In contrast to passive immunization, active immunotherapy uses a natural system of immune defense in which the immune reaction is stimulated inside the body. The immunogenic particles (resected tumor tissue) are being purified and injected intradermally. Consequently, antigens are further delivered to presenting cells (APC) or dendritic cells in the systemic circulation. Now stimulated immune system of the patient can recognize the tumor and target the specific brain tumor antigens after crossing BBB. The vaccine targeting Epidermal Growth Factor Receptor variant 3 (EGFRvIII, specific receptors at the surface of the glioma cells) (Rindopepimut) [99] showed in the Phase II study with newly diagnosed patients with glioblastoma a PFS of 10-15 months and an overall survival (OS) from 22.8-24.6 months. [99–101] A phase III study in newly diagnosed glioblastoma patients with this EGFRvIII vaccine is ongoing (NCT01480479).
Promising results in a phase I and a phase II study were found when targeting multiple agents (radiotherapy and chemotherapy) with a tumor lysate-dendritic vaccine (ICT-107) in patients with newly diagnosed glioblastoma.[102,103] Wheeler et al. demonstrated a correlation between immune and clinical responses in patients with glioblastoma. They found that 53% of GBM patients treated with dendritic cell vaccine exhibited ≥1.5 fold cytokine responses. A statistically significant better PFS and OS occurred in 22% of patients (responders) [104]

Immunomodulation

The administration of agents that regulate lymphocyte turnover, differentiation and activation is called immunomodulation. (Table 5) Ipilimumab, immunomodulator used in metastasized melanoma, is a monoclonal antibody targeting cytotoxic T-lymphocyte antigene-4 (CTLA-4), which leads to an increased T-cell response directed against the tumor. Disease control rate of patients with melanoma and asymptomatic BM at 12 weeks was seen in 18- 26% (median survival = 7 months) and 5% - 10% in patients with symptomatic BM on a stable steroid dose (median survival = 4 months). [105] It is presumed that ipilimumab does not have to penetrate the brain, but that activated T-lymphocytes can reach the intracranial target.
Other immune checkpoint inhibitors that are currently under investigation in clinical trials are anti-PD1 agents such as nivolumab and pembrolizumab that target the programmed death 1 (PD-1) molecule and its ligand PD-L1. PD-1 is expressed in 88% of newly diagnosed glioblastoma and 72% of recurrent glioblastomas. [106] Currently a study combining CTLA-4 and PD-1 antibodies in recurrent glioblastoma is in the clinical phase III investigation.(NCT02017717)

Gene transfer therapy mediated by neural stem cells

Genetically-modified neural stem cells have the ability to cross the BBB, and migrate through the brain parenchyma. [107] In the experiment with nude mice neural stem cells were injected into systemic circulation and showed that they can cross the BBB and reach intracranial glioma.[108] These neural stem cells can be used as a delivery system for gene transfer, which express enzymes, and can activate pro-drugs into active drugs. [109] There is an ongoing phase I study with capecitabine prodrug and genetically-modified human neural stem cells expressing cytosine deaminase in patients with recurrent high-grade glioma (NCT01172964). In this study, genetically-modified neural stem cells convert 5-fluorocytosine (5-FC) into the chemotherapy agent 5-FU (5-fluorouracil) in the brain tumors. Despite the fact that this type of treatment is designed for systemic administration, neural stem cells will firstly be investigated by a local application in the tumor cavity after tumor resection.

CONCLUSION

To achieve future effective therapies for brain tumors, sustained drug concentrations are needed in the brain to reach cytotoxic dose-levels in the tumor or specifically target tumor-specific proteins or RNA. This review gives a concise overview of the therapeutic strategies to transfer drugs o to cross the BBB and/or BCSFB or bypass these drug-barriers by inducing the immune system or using neural stem cells. Some treatments are currently only in preclinical investigation, while others are already showing promising clinical results. Further research on drug-strategies to efficiently cross the BBB is warranted, as novel drugs and treatment strategies are needed in the treatment of brain tumors.

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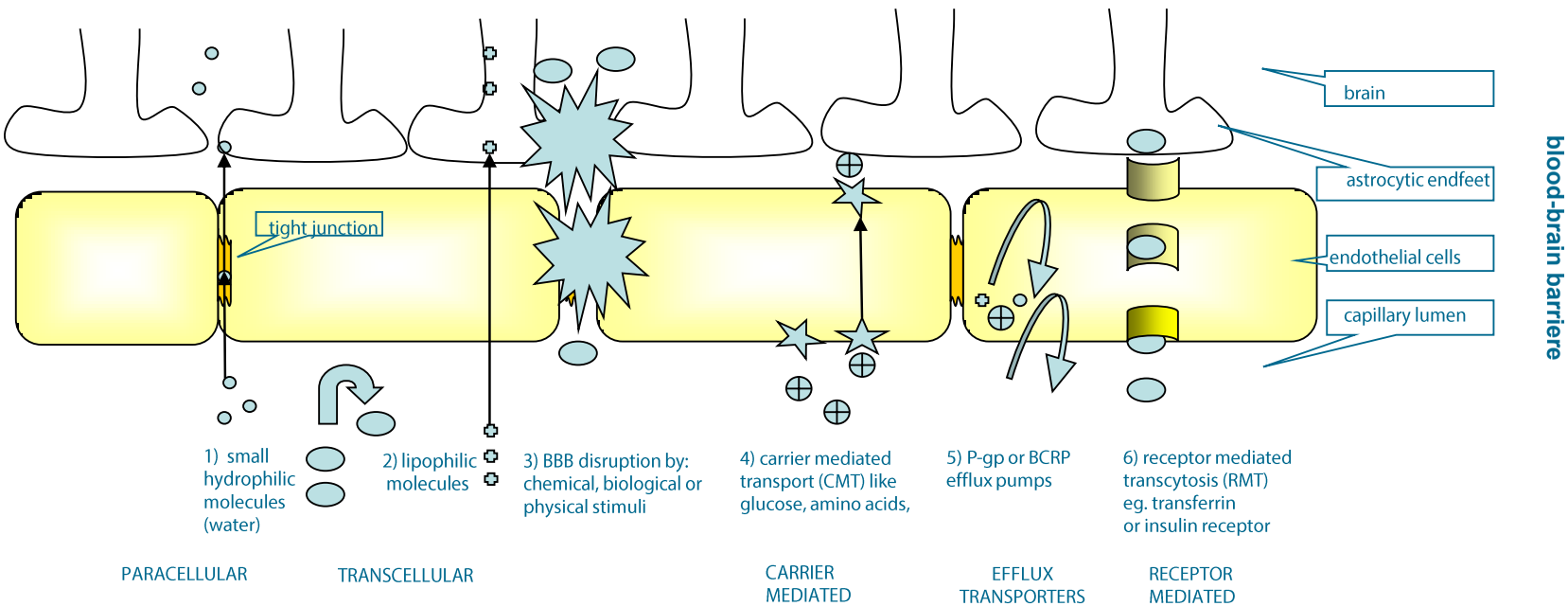

Figure 1. A blood-brain barrier (BBB)

name	indication	route	mechanism	results	references
temozolomide	newly diagnosed GBM	oral	small lipid -soluble alkylating agents	RT with TMZ during 6 weeks + 6 adjuvant TMZ cycles: newly diagnosed GBM - 2 years OS 26 % vs 10 % radiotherapy alone	Stupp et al. 2005
nitrosourea (CCNU; lomustine)	recurrent GBM, anaplastic oligodendroglioma with 1p19qloss	oral	small lipid -soluble alkylating agents	PCV (procarbazine, CCNU and vincristine): recurrent GBM - 3 PR in 86 pts, PFS > 6 months in 38.4% pts. (EORTC brain tumor group study 26951.)	van den Bent 2013, Schmidt 2006
procarbazin	recurrent GBM, anaplastic oligodendroglioma	oral	small lipid -soluble alkylating agents	see also 'nitrosurea'	see also 'nitrosurea'

Table 1. BBB penetrating cytotoxic therapies GBM – glioblastoma, RT - radiotherapy, PFS – progression free survival, OS – overall survival

different transport mechanisms across the BBB	drug examples
passive lipid-mediated diffusion	small lipophilic molecules
chemical, biological or physical stimuli opening the BBB for drugs	mannitol, bradykinin agonists, chemokines and cytokines, bacterial, viral components, ultrasound and electromagnetic fields
facilitated diffusion using carrier-mediated transport (CMT)	nutrients, levodopa, gabapentin
receptor-mediated transcytosis (RMT) - molecular Trojan horse approach	for larger (liposomal) molecules (peptides and proteins) using receptors: low density lipoproteins (LDL), glutathione, insulin-receptor; transferrin receptor, insulin-like growth factor receptor, or diphtheria toxin receptor)
Adsorptive-mediated transport (AMT) mechanism	cationic liposomes
co-administration P-gp and BCRP inhibitor	elacridar co-administration
Indirect drug targeting to CNS	via immunotherapy

Table 2. Different transport mechanisms penetrating blood-brain barrier (BBB)

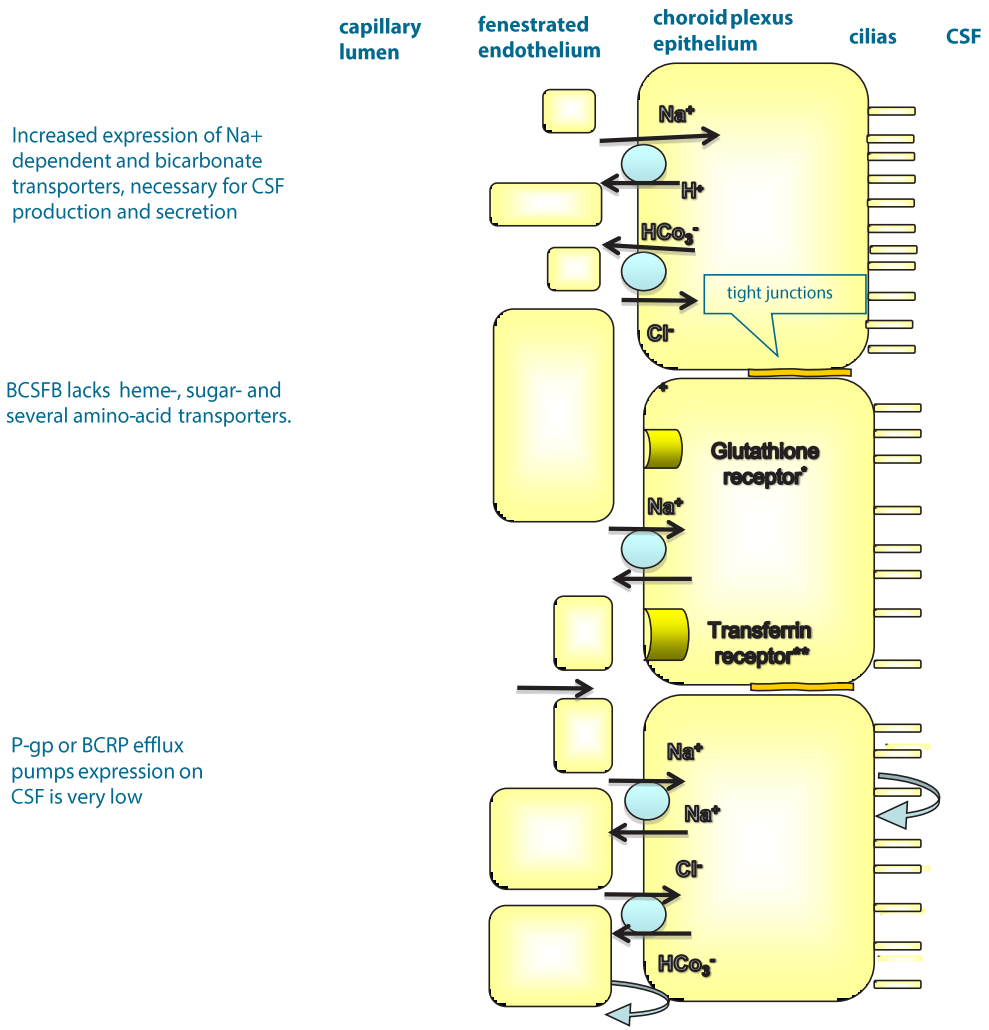

Figure 1B. Blood-cerebrospinal fluid barrier (BCSFB) in the choroid plexus restricts and regulates para- and transcellular transport. P-gp= P-glycoprotein, BCRP= breast cancer resistance protein, * Otieno 1997, **(Connor 2001a)

name	phase	route	mechanism	results	references
2B3-101	phase I/II	iv	doxorubicin in glutathion pegylated liposomes probably binding glutathion receptors on BBB	Mice: a 5-fold higher doxorubicin concentration in brain compared to Caelyx®; improved anti-tumor activity and survival of mice with GBM, phase I/II clinical studies ongoing.	Gaillard 2014 et al
GRN1005	phase I/II	iv	paclitaxel-Angiopep-2 conjugate via low-density lipoprotein receptor-related protein-1 (LRP-1) transcytosis	1 CR and 2 PRs in 63 patients with recurrent malignant glioma and 5 IC PR in 41 patients with solid tumors and brain metastasesy. Phase II study stopped as anti-tumor efficacy could not be confirmed.	Regina A 2008, Demeuele 2008
Tf-PO-DOX	in vivo	iv	biodegradable polymersomes (PO) loaded with doxorubicin, transport via trans ferrin receptor-mediated	in rats: increased median OS of brain tumor, 70% longer than standard doxorubicin	Pang Z 2011 1171-80,
Paclitaxel poliglumex	phase I/II	iv	BBB not known, presumed via endocytosis due to ncrease in vascular leakiness by means of the enhanced permeability and retention	Median PFS: 13.5 months in 17 HGG pts. OS: > 22 months	Jeyapalan S, Am J Clin Oncol 2013
K16ApoE	in vivo	iv	A synthetic peptide carrier of non-covalently binded cisplatin and methotrexate. K16ApoE generates transient BBB permeability between cells that express Low-density Lipoprotein R eceptor (LDLR).	34-50-fold and 54-92 fold higher brain uptake for cisplatin and methotrexate, respectively, with K16ApoE than without	Sarkar G PLoS One. 2014 May 21;9(5):
CPP-Dox/NGR-TSL	in vivo	iv	a thermosensitive liposome containing cell penetrating peptide as the targeting moiety-doxorubicin conjugate	specifical targeting shown in tumor lines, significantly inhibited tumor growth in nude mice, 95%, good stability	Yang Y 4368-81
GPNMB conjugated Pac-MNPs	in vivo	iv	paclitaxel loaded magnetic nanoparticle manipulated by magnetic fieldheading for glycoprotein non-metastatic melanoma protein B (GPNMB) overexpressed by glioblastoma cells	prolonged blood circulation in vivo, significant accumulation of drug in rat brain tissues as compared to native paclitaxel.	Dilnawaz 2936-51
CRM197	in vivo	iv	a non-toxin mutant of diphtheria toxin (DT)acts as a DT receptor-specific carrier protein serves as carrier protein for vaccine and other therapeutic agents	increased BBB permeability,pinocytotic vesicles number and redistribution of tight junction-associated proteins in brain microvessels	Wang P 2011

Table 3. Agents that can cross the blood-brain barrier via carrier-mediated or receptor–mediated transport in (pre) clinical studies. BBB = blood-brain barrier RD - recommended dose, HGG - high grade glioma, PR-partial responce, SD - stable disease, BM - brain metastases, GBM - glioblastoma

name	current phase	route	mechanism	results/conclusion	references
SiRNA - gene therapy without viral vectors					
CALAA-01	phase I	i.v.	siRNA in liposome targeting transferrin	terminated, no data available	NCT00689065
Atu027 + gemcitabine	phase I/II	i.v.	siRNA in liposome targeting protein kinase N3	recruiting patients with metastatic pancreatic carcinoma	NCT01808638
siRNA for EphA2 gene	phase I	I.v.	siRNA in liposome targeting over-expressed receptor tyrosine kinase EphA2 on glioma cells	reduction of tumor growth in preclinical models; recruiting patients with ovarian cancer	NCT01591356
microRNA and siRNA	in vitro	i.v.	Si RNA + miRNA in liposome;	siRNA - mediates silencing of specific gene that results in reduction of tumor growth, while miRNA further boosts its anti-tumor effect	Nishimura 2013
Gene therapy applications using viral vectors					
TK-GCV (G2017)	phase I/II	local treatment of postoperativesite	Adenovirus mediated herpes simplex virus-thymidine kinase/ganciclovir gene therapy	a safe, multiple dose delivery. suggestive of antitumor activity in rat glioma model	Gunther 2014 Markert 199-207

Table 4. Nanocarrier siRNA gene therapy (i.v. – intravenous, siRNA – short interfering RNA)

name	current phase	route	mechanism	results/conclusion	ref.
Immunotherapy					
nivolumab	phase IIb	iv	human mAb directed against the programmed death-1 receptor (PD-1R), the ligand of which (PD-1L) can be directly expressed on melanoma cells	clinical data pending, preclinical studies demonstrate benefit of nivolumab with radiation in a mouse glioma model	Sampson 2014
ipilimumab	phase II	iv	human monoclonal antibody (IgG1) that inhibits the function of cytotoxic T cell associated antigen 4 (CTLA-4) enhancing immune response against melanoma	18% asymptomatic and 5% of symptomatic patients with BM requiring steroids achieved disease control after 12 weeks treatment.	Margolin, 2012
HSPPC-96vaccin	phase II	intra-dermal	patient's own GBM antigenes delivered to presenting cells for immune stimulation	OS > 6 months in 90.2% and >1 year in 29.3% of pts with reccurent GBM. OS 10.5 months	Bloch 2014
PEP-3-KLH/CDX-110 in comb with TMZ	phase I/II	intradermal	peptide that spans the EGFRvIII fusion junction; molecule is taken up by Ag-presenting cells, stimulates Ag-specific effector, regulatory T lymphocytes and B lymphocytes	Increased median PFS and OS in pts with GBM, narrow specificity of a vaccine likely brings transient benefit	Del Vechio 2010
macrophage-LP-Dox	in vivo	peritoneal	doxorubicin in liposome carried by macrophage	in mice: macrophages containing iron oxides could migrate from systemic circulation through BBB and infiltrate into brain tumors effectively; effective inhibition of in vivo	Choi 2012

Table 5. Immunotherapy (iv – intravenous, BM – brain metastases, GBM – glioblastoma, OS – overall survival, EGFRvIII – epidermal growth factor survival specifically expressed in GBM tissue, PFS – progression three survival

3.2 Phase I and pharmacological study of 2B3-101, glutathione PEGylated liposomal doxorubicin as a single agent or in combination with trastuzumab in patients with solid tumors and brain metastases or recurrent malignant glioma

B. Milojkovic Kerklaan[1], P. Aftimos[2], S. Altintas[3], A. Jager[4], W. Gladdines[5], P. Gaillard[5], P. Soetekouw[6], V. Dieras[7], M. van Linde[8], A. Awada[2], A.D.R Huitema[1,9], J.H.M. Schellens[1,10], D. Brandsma[11].

1 Netherlands Cancer Institute - Antoni van Leeuwenhoek, Department of Clinical Pharmacology, Amsterdam, The Netherlands.
2 Institut Jules Bordet, Université Libre de Bruxelles, Medical Oncology Clinic, Brussels, Belgium.
3 Antwerp University Hospital, Department of Medical Oncology, Antwerp, Belgium.
4 Erasmus MC Cancer Institute, Department of Medical Oncology, Rotterdam, The Netherlands.
5 BBB Therapeutics B.V., Leiden, The Netherlands.
6 Maastricht University Medical Center, Department of Medical Oncology, Maastricht, The Netherlands.
7 Institut Curie, Department of Medical Oncology, Paris, France
8 VU Medical Center, Department of Medical Oncology, Amsterdam, The Netherlands,
9 Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, Department of Pharmacy and Pharmacology , The Netherlands
10 Utrecht University, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands
11 Netherlands Cancer Institute - Antoni van Leeuwenhoek, Department of Neuro-oncology, Amsterdam, The Netherlands.

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ABSTRACT

This phase I dose-escalation study evaluated safety, tolerability, maximal tolerated dose and pharmacokinetics (PK) of glutathione PEGylated liposomal doxorubicin (2B3-101) as a single agent in patients with solid tumors and brain metastases (BM) or recurrent malignant glioma, and in combination with trastuzumab in patients with HER2+ breast cancer and BM. Twenty-four patients with BM from solid tumors and 13 patients with recurrent WHO grade III or IV gliomas were included. One dose-limiting toxicity in the first cycle, febrile neutropenia, was observed at 50 mg/m^2 2B3-101 in the combination arm, while one patient developed leucocytopenia grade 4 and pneumonia with fatal outcome during the third cycle of single agent 2B3-101 at 60 mg/m^2. No study drug-related cardiac or CNS toxicity was observed. The most frequently observed 2B3-101-related adverse events were fatigue, hand-foot syndrome (HFS), neutropenia, infusion reaction and stomatitis, being mild to moderate and manageable with standard treatments. PK data showed a dose-proportional increase of exposure to 2B3-101 without accumulation in this 3-weekly schedule. Stable disease was observed in 43% and 67% of patients in the single and combination arm, respectively. Two intracranial and two extracranial partial responses (reduction >50%) were observed.

As dose reductions of 2B3-101 were needed at multiple cycles due to hematological and mucocutaneous toxicity, dose-intensity of 2B3-101 that could be sustained for a long-term duration period was 15 mg/m^2/week. This corresponds with 50 mg/m^2 3-weekly or 60mg/m2 4-weekly as recommended doses for a phase II study.

Conclusions: 2B3-101 is safe and relatively well-tolerated in patients with BM from solid tumors or recurrent malignant gliomas. Further clinical evaluation of 2B3-101 as a single agent or in combination is warranted.

Keywords: blood-brain barrier, glutathione PEGylated liposomal doxorubicin, phase I clinical trial, malignant glioma, brain metastases, trastuzumab

INTRODUCTION

Malignant gliomas. Malignant (WHO grade III and IV) gliomas are the most common primary brain tumors with an incidence of 5-7 per 100,000. [1,2] Median overall survival for patients with WHO grade III glioma is 3-5 years and 12-15 months for patients with WHO IV glioma (glioblastoma). Treatment options for recurrent malignant gliomas are limited with a median survival for recurrent glioblastoma patients of 7 months.[3] Lomustine monotherapy or in combination with procarbazine and vincristine (PCV) can be administered as second-line chemotherapy after failure of radiation therapy and temozolomide, but objective response rates do not exceed 11%. [4]

Brain metastases. The incidence of brain metastases (BM) is about 10-fold higher than that of malignant gliomas. [5,6] The most frequent tumor types that metastasize to the central nervous system (CNS) are non-small cell (NSCLC), small

cell lung cancer (SCLC) (40-50%), breast cancer (20-30%) and melanoma (5-10%). [7–9] With current standard therapeutic options (resection, stereotactic radiotherapy and/or whole brain radiotherapy (WBRT)), median overall survival ranges from 6 – 15 months. [10–13] Systemic chemotherapy can affect BM, particularly the chemosensitive tumor types like breast cancer and SCLC. [14–17] Unfortunately, most of the active chemotherapeutic agents, such as doxorubicin, poorly penetrate the BBB. [14,18] To improve the prognosis of patients with BM from solid tumors and recurrent malignant gliomas, new effective strategies to deliver systemic therapeutic agents in the brain are needed. [19–21]

2B3-101 development

Doxorubicin is an anthracycline that exerts its antineoplastic effect via a cytotoxic mechanism of action. [22,23] In vitro, glioblastoma cell lines are sensitive to doxorubicin. [22,24] Doxorubicin is approved for use in the treatment of different tumor types, such as SCLC, breast and ovarian carcinoma. [23] A relatively new formulation, PEGylated liposomal doxorubicin (PLD, Doxil®/Caelyx®), is associated with less cardiotoxicity than doxorubicin as it achieves lower plasma concentrations of free doxorubicin. As polyethylene glycol polymer (PEG) molecules are being attached to the lipid bilayer of PLD, the mononuclear phagocyte system (MPS) of the liver, spleen and bone marrow does not recognize the doxorubicin-loaded liposome. Therefore, PLD has a reduced clearance [25] and prolonged terminal half-life ($t_{1/2}$) of 73.9 h compared to the triphasic elimination of the conventional doxorubicin with mean half-lives of 12 min, 3.3 hours and about 30 hours. [23,26] At the same time higher concentrations of liposomal doxorubicin target the tumor, possibly due to the Enhanced Permeability and Retention Effect (EPR) of PLD.[27–29]

In a mouse model of breast cancer BM, PLD achieved a 1500-fold higher exposure in plasma and 20-fold higher exposure in BM compared to non-liposomal doxorubicin. [30] The recommended dosing of PLD in patients is 50 mg/m^2 every 4 weeks. However, due to mucocutaneus toxicity (hand-foot syndrome, stomatitis), optimal dosing of PLD is still a matter of discussion. [31–34]
2B3-101, a glutathione (GSH) PEGylated liposomal doxorubicin hydrochloride has been developed by BBB Therapeutics B.V., Leiden, The Netherlands in order to cross the BBB and target brain tumors. 2B3-101 is identical to PLD except for extra attached molecules of GSH, an endogenous anti-oxidant, found at high levels in the brain with their active (sodium-dependent) receptor/transporter abundantly expressed at the BBB. [35,36] Glutathione targets the GSH-loaded liposome towards the active GSH transporters on the BBB to enhance delivery of doxorubicin to the brain. [37–40] In preclinical studies, 2B3-101 showed a 5-fold enhanced delivery of doxorubicin to the brain compared to PLD (Caelyx®) and improved survival of mice with glioblastoma. [37][41,42]

2B3-101 in combination with trastuzumab

Despite its effectiveness in the treatment of HER2+ breast cancer, combining conventional anthracyclines with trastuzumab, a recombinant humanized monoclonal antibody directed against the human epidermal growth factor receptor 2 (HER2), is not recommended because of the risk for cardiotoxicity. However, PLD combined with trastuzumab, was shown to be a well-tolerated and active therapy, with cardiotoxicity limited to asymptomatic declines in LVEF. [43]

To evaluate the safety, tolerability and determine the maximal tolerated dose (MTD) and pharmacokinetics of 2B3-101 as a single agent or in combination with trastuzumab, we performed a multicenter phase I trial in patients with solid tumors and BM or recurrent malignant glioma.

PATIENTS AND METHODS

Patient eligibility. Eligible patients were older than 18 years with a pathologically confirmed diagnosis of either: a) solid tumors and unequivocal evidence of BM that were refractory to standard therapy or for whom no standard therapy exists or solid tumors with unequivocal evidence of untreated BM and controlled extracranial disease or b) histologically-confirmed HER2+ adenocarcinoma of the breast and BM who will start or continue trastuzumab treatment or c) recurrent malignant (grade III and IV) glioma refractory to standard therapy or for whom no standard therapy exists.
Patients should have intracranial measurable disease on a brain MRI. For malignant gliomas, this was defined as bidimensionally contrast enhancing lesions, with two perpendicular diameters of at least 10 mm, visible on two or more axial slices that were preferably, at most, 5 mm apart with 0-mm skip. For BM from solid tumors, measurable disease was defined as BM with ≥ 10 mm in the longest diameter determined on gadolinium enhanced T1-weighted MRI. In patients with untreated BM a longest diameter of ≥ 5 mm on gadolinium enhanced T1-weighted MRI was allowed. Other inclusion criteria were: ECOG Performance Status score of ≤ 2, an estimated life expectancy of at least 8 weeks, no evidence of (cortical) cognitive impairment as defined by a Mini-Mental Status Exam (MMSE) score of ≥ 25/30, use of stable or decreasing dosages of steroids for more than 7 days prior to baseline MRI and/or use of non-enzyme inducing anti-epileptic drugs and no cranial radiotherapy during the last 8 weeks.

Exclusion criteria were: a cumulative dose of > 360 mg/m^2 free (non-liposomal) or liposomal doxorubicin or >600 mg/m^2 of epirubicine; an inadequate bone marrow, liver or renal function; leptomeningeal carcinomatosis as the only site of central nervous system (CNS) metastases; clinically significant (i.e. active) cardiovascular disease and left ventricular ejection fraction (LVEF) < 50% for single agent 2B3-101 or < 55% for 2B3-101 in combination with trastuzumab.

The institutional review board of each participating center approved the study protocol before initial patient enrollment. Written informed consent according to local institutional guidelines was obtained.

Drug administration and study design

2B3-101 was administered as a single-use intravenous infusion on day 1 of a 21-day cycle. In first infusion protocol the drug was administered with a constant infusion rate not greater than 1 mg/min. After 9 enrollments, and in order to minimize the risk of infusion reactions (IR), an amendment on the infusion protocol was approved, i.e. the first 5% of total 2B3-101 dose (in mg) was infused slowly over 30 min and if tolerated, infusion was completed over the next 60 min for a total infusion time of 90 min. In case of an IR, the infusion rate was restarted with 10 ml/h rate for the first 30 min and increased every 30 min as follows: 20 ml/h, 50 ml/h, 100 ml/h, and 200 ml/h. (Pre) medication such as hydrocortisone, ranitidine, cimetidine, anti-emetics, and diphenhydramine was allowed according to local institutional guidelines.
The starting dose was 5 mg/m^2, which is equal to 1/10th of the human equivalent dose of the LD10 of 2B3-101 in rats. The following dose-levels for subsequent cohorts were 10 and increments of 10 mg/m^2 thereafter. In case one of the three patients experienced dose-limiting toxicity (DLT) during the first cycle of 2B3-101 (i.e. three weeks), three additional patients would be enrolled at the same dose-level. The maximum tolerated dose (MTD) was defined as the dose-level at which two patients experience DLT. Definitions of the DLT are being described in Table 1.
Patients would be treated with 2B3-101 until disease progression, unacceptable toxicity or discontinuation for any reason. Inclusion of patients in the combination arm with trastuzumab started after the first five cohorts of 2B3-101 single agent were evaluated, as well as upon previous treatment with PEGylated liposomal doxorubicin in combinations trastuzumab. [43]
Therefore, the starting dose of 2B3-101 was 40 mg/m^2 every 3 weeks with increments of 10 mg/m^2 for the subsequent dose-levels in combination with standard trastuzumab dose (8 mg/kg loading dose, followed by 6 mg/kg intravenously every 3 weeks). Trastuzumab was administered 30 min after 2B3-101 infusion was completed.

Patient assessments

Screening and safety evaluations included medical history, physical and neurological examination, MMSE, HIV-dementia score, ECOG performance status, electrocardiograms (ECG), LVEF (MUGA/cardiac ultrasound), laboratory evaluation and recording of concurrent illnesses/therapies and adverse events (as per Common Terminology Criteria for Adverse Events (CTCAE) 4.0). Patients receiving trastuzumab entered an intensified cardiac evaluation program including ECG, LVEF, Troponin-T and NT-proBNP measurements before the start of every treatment cycle.

Preliminary anti-tumor activity of 2B3-101 as a single agent or in combination with trastuzumab, was determined on the last day of every even cycle (cycle 2, 4, etc.) by brain MRI in all patients and for patients with solid tumors and BM also by CT thorax/abdomen.

Pharmacokinetics

Pharmacokinetic (PK) evaluation was performed in all patients during cycle 1 of 2B3-101 as a single agent or in combination with trastuzumab, and for 19 enrolled patients during cycle 1 and 2. Blood samples were drawn at day 1 predose, 5 min, 1h, 4h, and 8h after the end of infusion, at days 2, 3, 5, 8 and 11 of cycle 1, on day 1 before the second 2B3-101 infusion, and at cycle 2 at day 8 and day 15. Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) was used to determine total (free and encapsulated) and encapsulated doxorubicin. The assay was validated as per the current guidelines for bioanalytical method validation. [44,45] Encapsulated doxorubicin was isolated by solid phase extraction (SPE) after dilution of plasma samples using an anti-oxidant solution (1:2 v/v) containing: 1.25 g EDTA, 0.3 g pyrogallol, 0.3 g ascorbic acid, 0.3 g sodium disulfite, 250 mL Milli-Q water, 50.0 mL and MeOH. After protein precipitation, doxorubicin was quantified using LC-MS/MS. The analytical range of both assays was 50.0 – 50,000 ng/mL for total and encapsulated doxorubicin.
Total doxorubicin pharmacokinetic parameters included dose-normalized area under the concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$), area under the concentration-time curve from time zero to time of last quantifiable concentration ($AUC_{0\text{-last}}$), terminal half-life ($t_{1/2}$), total plasma clearance (CL) and volume of distribution (V). These were derived from plasma concentrations by non-compartmental analysis using the log-linear trapezoidal rule employing a validated script in R (version 3.1.1.0, http://www.r-project.org). The maximum observed plasma concentrations (C_{max}) and time to maximum observed plasma concentration (T_{max}) were directly derived from the plasma concentration-time data.

Statistical analysis

Descriptive statistics were applied for safety analysis and preliminary anti-tumor activity. Calculated pharmacokinetic parameters were summarized and tabulated for doxorubicin. Data are presented as median and range. Coefficient of variation (CV%) was calculated as (SD/mean) x 100%.

RESULTS

Patient characteristics

Twenty-eight patients were enrolled in the single agent arm and nine patients in the trastuzumab-combination arm of this phase I dose-escalation study from August 2011 till June 2013. Patient characteristics are listed in Table 2.

In the single agent arm, 12 patients were male and 16 female with a median age of 54 years (range 31-73 years). In the combination arm all patients were female with a median age 42 years (range 33-61 years). Thirteen patients had a malignant glioma. All of them had been treated with cranial radiotherapy. Twelve patients had been treated with temozolomide. Seven patients with malignant glioma had received two previous lines of chemotherapy and two patients had received more than two lines. Twenty-four

patients had solid tumors with BM; the most common tumor types were breast cancer patients (n = 13, of whom 9 were HER2+), NSCLC (n = 3) and SCLC (n = 2) and other (n = 6). In the single agent arm and in combination arm 21% and 78% of patients respectively had received prior anthracyclines. A total of 83 cycles of 2B3-101 single-agent were administered, 1-8 cycles per patient. In the trastuzumab-combination arm 38 cycles were administered, 1-10 cycles per patient.

Dose-limiting toxicity, maximal tolerated dose and recommended dose

Patients were treated in cohorts of three, starting in the single agent 2B3-101 arm with dose-level 1 (DL 1 = 5 mg/m^2) through to dose-level 8 (DL 8 = 70 mg/m^2). No DLTs were recorded in any of the tested dose-levels. In the first cycle at DL7 (60 mg/m^2) 2B3-101, one of the first three enrolled patients developed thrombocytopenia grade 3 (25 x10^9/l), leucocytopenia grade 4 (0.8 x10^9/l) and neutropenia grade 4 (0.38 x10^9/l) lasting <7 days. Cycle 2 for this patient was delayed for three weeks and the dose was reduced to 50 mg/m^2. The grade 3 thrombocytopenia during cycle 1 was not a DLT, but the investigator's board decided to expand the cohort with four more patients because of severe thrombocytopenia in this patient and as one patient was erroneously dosed with 50mg/m^2. One patient from the expanded group at DL7 developed leucocytopenia grade 4 (0.8 x10^9/l) and neutropenia grade 4 (0.3 x10^9/l) with pneumonia during cycle 3. She received palliative-symptomatic treatment because of her dismal prognosis and she died four days later.
At DL8 (70 mg/m^2), three patients experienced a persistent combination of mucocutaneous (stomatitis grade 2-3 and HFS grade 3) and hematological toxicity (neutropenia and leucocytopenia grade 2-3) starting from cycle 1 and intensifying in subsequent cycles causing numerous dose delays and two dose reductions.
Although no DLT was observed at dose-levels of 5 mg/m^2 to 70 mg/m^2 2B3-101 in the first cycle, it was concluded by the investigator's board that doses higher than 70 mg/m^2 were not sustainable for multiple cycles. Furthermore, repeated dosing at 60 mg/m^2 every 3 weeks resulted in 3 dose delays and 3 dose reductions because of hematological and mucocutaneous toxicity.

In the combination arm with trastuzumab, three patients with HER2+ breast cancer and BM were enrolled at the 40 mg/m^2 cohort. As no DLT was seen in this cohort, the dose was escalated to 50 mg/m^2, with one patient experiencing a DLT, febrile neutropenia grade 3, that recovered in two days and 2B3-101 dose was re-

duced to 40 mg/m^2. The cohort was expanded with three more patients who did not experience DLTs. However, in total 5 dose delays and 3 dose reductions were reported in this dose cohort.

Based on the safety, tolerability and dose intensity over a long-term duration period, it was concluded that the average 2B3-101 dose as a single agent or in combination with trastuzumab that can be sustained for several treatment cycles is 15 mg/m^2/week. Based on this dose-intensity and (future) possibilities of combination therapies in solid tumor and BM and malignant gliomas, two alternative dosing schedules were proposed for the phase II clinical study:
- 50 mg/m^2 2B3-101 as a single agent or in combination with trastuzumab every 3 weeks in patients with solid tumors and BM.
- 60 mg/m^2 2B3-101 every 4 weeks in patients with recurrent malignant glioma.

Safety

The most frequent study related AEs (all grades) in the single agent arm were fatigue (13/28, 46%), HFS (11/28, 39%), neutropenia (10/28, 36%), infusion reaction (9/28, 32%), stomatitis (8/28, 29%) and nausea (8/28, 29%). In the combination arm, similar study related AEs were found with higher incidences: HFS (8/9, 89%), fatigue (7/9,78%), stomatitis (4/9, 44%) and neutropenia (4/9, 44%). (Table 3)

Hand-foot syndrome

Eleven of 28 patients (39%) in the single agent arm and eight of nine patients (89%) in the combination arm experienced HFS (all grades). Hand-foot syndrome grade 3 causing dose reductions occurred in six patients in the single agent arm (21%) and in two patients in the combination arm (22%). Two patients treated with 40 and 50 mg/m^2 2B3-101single agent experienced HFS grade 3 in cycle 2 and 3 respectively. Four patients treated with 60 and 70 mg/m^2 2B3-101 single agent experienced HFS grade 3 in cycle 3 and 4. Two patients treated with 50 mg/m2 2B3-101 combined with trastuzumab experienced HFS grade 3 in cycle 3. Affected areas were treated locally with hydrophilic and indifferent ointments, disinfectants and corticosteroids with concomitant use of oral analgesics. Due to HFS, ten dose delays and seven dose reductions in the single and combination arm were performed at dose-levels from 40 mg/m^2 to70 mg/m^2.

Infusion reactions

Nine of 28 enrolled patients in the single agent arm and two in the combination arm experienced infusion reactions (IR) (9/28, 32%), all grade 1-2. All IRs occurred during the first administration of 2B3-101, except for two patients in the combination arm that experienced IR two and three times, respectively. Infusion reactions were characterized by one or more of the following symp-

toms: chest pain, (lower) back pain, tightness in the chest and throat, shortness of breath, tachycardia, flushing, headache, chills, fever, rash, fatigue and hypotension.
In the first three dose-levels, IRs were reported in four of 9 patients (44%). In these dose-levels 2B3-101 infusion time was 60 min at a rate of 1 mg/min. At the start of DL 4 (30 mg/m^2) the infusion protocol was modified as previously described. Five of the 19 enrolled patients (26%) in the single agent arm and two out of nine patients (22%) in the combination arm, treated according to the new infusion scheme developed an IR. Nine patients (4/19 in the single agent arm and 5/9 in the combination arm; 24%) received prophylactic premedication consisting of corticosteroid (dexamethasone or methylprednisolone) and antihistamines (promethazine, ranitidine or clemastine). Four of these nine patients receiving prophylactic premedication developed an IR (44%). One patient discontinued 2B3-101 treatment due to repeated infusion-related reactions.

Hematological toxicity

Neutropenia was the most frequently observed hematological toxicity of 2B3-101. It was dose related and increased in frequency and grading at higher dose-levels. In the single agent arm, it was observed in one patient per cohort starting from DL 4 (30 mg/m^2) through to DL 6 (50 mg/m^2) (33% per cohort). At DL 7 (60 mg/m^2) 71% of patients developed hematological toxicity, all grades. In patients treated with ≥ 40 mg/m^2 hematological toxicity grade ≥ 3 occurred, such as neutropenia (7/16, 44%), leucocytopenia (5/16, 31%) thrombocytopenia (1/16, 6%). The nadir of bone marrow depression (based upon neutrophil count) was usually found in the third week after 2B3-101 infusion (75% of patients) and recovered to a lower grade within one week (100%). Dose reduction was needed in two patients, one at DL 7 and the other at DL8 because of neutropenia grade 4 and leucocytopenia grade 3 in one patient and neutropenia grade 2 and mucocutaneous toxicity grade 2 in the other patient. In the combination arm hematological toxicity was not seen at dose-level 40 mg/m^2, while neutropenia grades 3 or 4 occurred at dose-level 50 mg/m^2 in four of six (66%) patients. In one patient the 2B3-101 dose was reduced because of febrile neutropenia grade 3 (DLT). (Table 3)

Cardiotoxicity

No clinically significant cardiotoxicity of single agent 2B3-101 was observed at all dose levels. Non-significant decreases in LVEF (< 10%) were seen in four out of 28 (14%) patients. At DL 4 (30 mg/m^2) one patient had a clinically non-significant increase of Troponine T and CPK-MB grade 1, but without a change in LVEF and ECG as compared to baseline. In the combination arm one patient with a history of trastuzumab-related cardiotoxicity experienced a grade 2 decrease in LVEF that recovered after trastuzumab treatment was stopped.

Neurotoxicity

No clinically significant neurotoxicity of 2B3-101 was observed at all dose-levels. Three out of 28 patients (10%) in the single agent arm had a 7-13 points decline in MMSE score all due to intracranial tumor progression. In the combination arm one patient had a decrease of 12 points at cycle 2 due to disease progression.

Pharmacokinetics (PK)

The PK parameters of doxorubicin applied in the liposomal formulation 2B3-101 as a single agent or in combination with trastuzumab are summarized in Table 4. In the single agent arm, doxorubicin showed dose-proportional PK in plasma with linear exposure without signs of accumulation in the 3-weekly schedule (data of second cycle not shown). Plasma concentration-time curves of measured total doxorubicin after single agent administration or in combination with trastuzumab are shown in Figure 1. In the single agent arm the terminal half-life ($T_{1/2}$) was 71 h for 2B3-101 at a 50 mg/m^2 with $V_{(d)}$ of 1.42 l/ m^2, while $T_{1/2}$ was 61 h at 60 mg/ m^2 with a volume of distribution ($V_{(d)}$) of 1.53 l/m^2. The difference in $T_{1/2}$ between these two groups is explained by one patient in DL5, 60 mg/m^2 with a high clearance of 0.035 l/h/m^2 of 2B3-101 and low doxorubicin exposure (1663 µg*h/ml). This correlates with values measured in DL4 (30 mg/m^2) and a consequently lower exposure of the total DL5 patient group. Clearance at this dose-level excluding the value of this patient ranged from 0.013-0.028 l/h/m^2. In the last four enrolled patients, both the total and encapsulated fraction of doxorubicin were measured. The encapsulated fraction of the total measured doxorubicin plasma levels was higher than 85%. The exposure of 2B3-101 at doses of 40 mg/m^2 and 50 mg/m^2 administered in combination with trastuzumab corresponded to 2B3-101 single agent exposure at the same dose-levels. (Figure 1)

Preliminary anti-tumor activity

Twenty-seven patients in the single agent arm and eight in the combination arm had at least one post-treatment anti-tumor evaluation after two cycles of 2B3-101. Two non-evaluable patients went off study early because of clinical deterioration. In Table 5 the preliminary anti-tumor activity is shown. Fifteen patients (57%) in the single agent arm and two (22%) in the combination arm had progressive disease (PD) after 2 cycles of 2B3-101. Twelve patients in the single agent (43%) and six (75%) in the combination arm had stable disease (SD) as their best response, both intracranial and extracranial. Eight of 13 patients with recurrent malignant glioma (62%) had SD as their best response during 2-6 cycles. Seven patients with recurrent malignant glioma were stable for at least 4 and three for 6 cycles. At the presumed therapeutic dose-levels (≥40 mg/m^2 2B3-101) 11 out of 16 (69%) patients with solid tumors and BM or malignant glioma treated with single agent 2B3-101 showed SD. Two intracranial and two extracranial PRs (reductions of >50%) were observed during the treatment with 2B3-101 in patients with solid tumors with BM

and patients with recurrent malignant glioma. Sixty-seven percent of patients with HER2+ breast cancer treated in the combination arm showed SD or PR. Five out of nine patients (56%) in the combination arm had a progression free survival (PFS) > 3 months, while PFS was >6 months in 2 out of 9 patients (22%).

DISCUSSION

Treatment with 2B3-101 (glutathione PEGylated liposomal doxorubicin) as a single agent or in combination with trastuzumab is safe and relatively well-tolerated with preliminary anti-tumor activity in patients with solid tumors and BM or recurrent malignant gliomas. The most frequent toxicities were fatigue, hand-foot syndrome, neutropenia, infusion reaction, stomatitis and nausea with incidences comparable with these described for patients treated with PLD, except for infusion reactions (10.8 % in PLD versus 32% in 2B3-101).[26]

Hand-foot syndrome, a painful, macular reddening skin eruption typical PLD-induced toxicity, was observed in all grades after two or more cycles of 2B3-101 in 39% of patients in the single agent arm and in 89% in the combination arm, probably because the latter patient group received more cycles than the single agent group. Grade 3 HFS was present in approximately 20% in both arms. No HFS grade 4 was reported. There was one permanent treatment discontinuation due to HFS. In comparison, HFS of all grades was reported in a phase III breast/ovarian clinical trials using PLD 50 mg/m^2 in a 4-weeks cycle, in 44-46%; HFS grade 3 was seen in 17-20% and life-threatening HFS (grade 4) in less than 1%. Permanent treatment discontinuation due to HFS occurred in 4-7% of these patients. [26] Some studies suggest that HFS appearance correlates with a longer $T_{1/2}$ of PLD in plasma and in the eccrine sweat glands of hand and feet. [16,46–48] This could not be confirmed in our study, as patients experiencing HFS did not have longer $T_{1/2}$. Although recovery of HFS caused by 2B3-101 was commonly seen within one to two weeks, it caused considerable discomfort and frequently led to dose reductions and dose delays with a potential negative anti-tumor effect. Patient education, supportive measures and reduced dose intensity are current expert recommendations to diminish the HFS due to PLD. [34]

Another frequently occurring toxicity using 2B3-101 was IR, reported in 32% of patients in the single-agent and in 22% in the combination arm. The symptoms typically recovered within several minutes after temporary interruption of the infusion or lowering of the infusion rate. This type of non IgE-mediated hypersensitivity infusion reaction of PLD is named CARPA (complement-activation related pseudoallergy). [49–52] All IRs that were observed in this study with 2B3-101 were mild to moderate, but life-threatening reactions from liposomal drugs have been documented in literature. [53] The new infusion protocol (5% of total dose 2B3-101 in the first 30 min and if tolerated, the remaining dose of 2B3-101 in the next 60 min) reduced the IR incidence from 44% to 26% in the single agent arm and to

22% in the combination arm. In comparison, the reported incidence of infusion reactions for PLD was 10.8 % . [26] The use of premedication like corticosteroids and antihistamines was allowed and performed in eleven patients in total, of whom four (44%) experienced IRs.
Based on the pathophysiology of the allergic reaction (non-IgE mediated reaction), pre-medication is assumed not to be effective in PLD-induced IRs. Slow infusion rechallenge was successful in all patients except for one patient who experienced repeated IRs in all cycles and therefore discontinued study. Infusion reactions have not been reported with conventional doxorubicin and they presumably represent a reaction to the liposomes or one of its surface components in PLD.[26]

With respect to hematological toxicity neutropenia was observed in 36%, leucocytopenia in 18% and thrombocytopenia in 18% of patients in the single agent arm. In the combination arm neutropenia occurred in 44%, leucocytopenia in 33% and thrombocytopenia in 22% of patients. Dose and C_{max} of 2B3-101 strongly correlated with myelosuppression (in particular neutropenia and leucocytopenia), as has been previously reported for PLD. [32] The measured 2B3-101 $t_{1/2}$, mean intrinsic clearance and the mean V_d are within range of the reported pharmacokinetic data for PLDs.[26]

Based on the dose reductions needed for HFS (37%, 7/19 patients) and hematological toxicity (32%, 6/19 patients), a dose-intensity of 15 mg/m^2/week was found to be sustainable for consecutive cycles of 2B3-101. The recommended dose (RD) of 2B3-101 as a single agent or in combination with trastuzumab for the phase II study in the solid tumors and BM was chosen to be 50 mg/m^2 every 3 weeks and for the recurrent malignant gliomas 60 mg/m^2 every 4 weeks.

It was decided to implement a different dose scheduling in patients with solid tumors and BM and malignant gliomas, because of possible future drug combinations (e.g. trastuzumab 3-weekly in HER2+ breast cancer with BM; possibly in future bevacizumab 2-weekly in recurrent malignant glioma. Furthermore, it creates the possibility to compare the incidence of HFS in different dosing schedules, as an extended interval between PLD doses may possible lead to reduced toxicity, in particular HFS. [54]

Treatment with 2B3-101 showed preliminary anti-tumor activity in both patients with solid tumors with BM and patients with recurrent malignant glioma as two intracranial and two extracranial PRs were seen in both arms. Sixty-two percent of patients with recurrent malignant glioma had SD as their best response during 2-6 cycles. Sixty-seven percent of patients with HER2+ breast cancer showed SD or PR, five of them (56%) for at least > 3 months and in 2/9 (22%) PFS was >6 months. The last-mentioned results with 2B3-101 in HER-2+ breast cancer and BM are in line with the data of the LANDSCAPE phase II trial, in which patients with untreated

BM received lapatinib and capecitabine with an objective response rate of 66% (95% CI: 50-80%).[55] These intracranial tumor responses on systemic chemotherapy support the future use of effective chemotherapeutic agents in BM from breast cancer to delay neurotoxicity of whole-brain radiation therapy.

In conclusion, 2B3-101 is safe and relatively well tolerated in both patients with BM from solid tumors and recurrent malignant gliomas. These findings together with preliminary intra- and extracranial anti-tumor activity of 2B3-101 warrant a phase II clinical study.

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Definition of dose-limiting related toxicities		
Toxicity	**Grade**	**Value**
Hematological		
Thrombocytopenia	grade 4, or requiring platelet tranfusion, or medical intervention	< 25 x 109/L
Neutropenia	grade 4 lasting ≥ 7 days	< 0.5 x 109/L
Febrile neutropenia	with ANC < 1.0 x 10^9 cells/L and temperature ≥ 38.5 ° C or documented infection ≥ grade 3	
Serum (total) bilirubin	if no liver metastases with liver metastases	> 1.5 x ULN > 3 x ULN
Serum ALAT or ASAT lasting ≥ 14 days	if no liver metastases with liver metastases	> 3 x ULN > 5 x ULN
Non-hematological ≥ 3 grade (excluding nausea, vomiting, diarrhea and fatigue lasting ≤ 7 days)	grade 3	
neurologic deficit	leading to WHO >2, unrelated to brain tumor progression	
HFS	grade ≥2	
Cardiac toxicity: 1) Signs and symptoms associated with CHF per the Framingham clinical criteria* 2) Clinical signs and symptoms of CHF (dyspnea, orthopnea, S3 gallop, tachycardia, inspiratory crackles) in association with a 10% decline in LVEF from baseline and a value below the lower limit of normal; 3) 15% decline from baseline in LVEF in an asymptomatic patient regardless of the absolute value; 4) Less than 10% decline from baseline in LVEF in an asymptomatic patient and an absolute value less than 45% on MUGA scan.		

Table 1. Dose limiting criteria (DLT) ULN= upper limit of normal; ANC = absolute neutrophile count, ASAT = aspartate aminotransferase ALAT = alanine aminotransferase, HFS = hand-foot syndrome. * the Framingham clinical criteria* (McKee et al, 1971, Maestre at al, 2009);

2B3-101 dose (mg/m²)	5 - 70	40-50 + TRZ
number of patients	28	9
male	12	0
female	16	9
median age in years (range)	54 (31-73)	42 (33-61)
ECOG performance status (n)		
0-1	25	9
2	3	0
primary tumor type (n)		
breast cancer	4	9
NSCLC	3	
SCLC	2	
neuroendocrine lung cancer	1	
ovarium	1	
endometrium	1	
esophagus	2	
melanoma	1	
glioma grade III	3	
glioma grade IV	10	
prior anti-cancer therapies (n (%))		
any therapy	28 (100%)	9 (100%)
cranial surgery	15 (54%)	1 (11%)
cranial radiotherapy	28 (100%)	9 (100%)
extracranial radiotherapy	8 (29%)	9 (100%)
chemotherapy	27 (96%)	9 (100%)
anthracycline	6 (21%)	7 (78%)
hormonal therapy	3 (11%)	4 (44%)
immunotherapy	6 (21%)	9 (100%)
extracranial surgery	8 (26%)	7 (78%)

TTable 2. Patient characteristics SCLC = small cell lung cancer, NSCLC = non-small cell lung cancer, TRZ = trastuzumab

2B3-101 dose (mg/m2)	40		50		60		70		5-70	40		50		40-50 + TRZ
Number of patients	3		3		7		3		28	3		6		9
grade	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-4 (%)	1-2	3-4	1-2	3-4	1-4 (%)
Hematological toxicity														
neutropenia	1	0	0	1	2	3	2	0	10 (36%)			0	4	4 (44%)
leucocytopenia					2	2	2	0	6 (21%)			2	1	3 (33%)
thrombocytopenia					1	1	1	0	4 (14%)			2	0	2 (22%)
anemia					1	0			1 (4%)			1	0	1 (11%)
lymphocytopenia					0	1			1 (4%)					0
Biochemical laboratory toxicity														
elevated ALAT			0	1					2 (7%)					0
elevated ASAT			1	0					1 (4%)					0
elevated Gamma-GT			1	0					1 (4%)					0
elevated alkaline phosphatase			1	0					1 (4%)					0
elevated CPK-MB									1 (4%)					0
elevated troponin									1 (4%)					0
gastrointestinal disorders														
nausea	2	0	1	0	2	0			8 (29%)			2	0	2 (22%)
decreased apetite and dysguesia			1	0	3	0	2	0	8 (29%)	1	0	1	1	3 (33%)
vomiting					2	0			3 (10%)	1	0	1	0	2 (22%)
diarrhea					1	0			1 (4%)	1	0	3	0	4 (44%)
constipation									1 (4%)			1	0	1 (11%)
dyspepsia	1	0							1 (4%)					0
Other toxicity														
fatigue	1	0	2	1	2	1	3	0	14 (50%)	3	0	2	2	7 (78%)
HFS*****	1	1	1	0	2	2	2	0	11 (39%)	3	0	3	2	8 (88%)
infusion reaction (IR)	1	0	2	0	1	0	1	0	9 (32%)			2	0	2 (22%)
stomatitis ***	2	0	1	0	0	2	0	1	9 (32%)	1	0	2	1	4 (44%)
headache									4 (14%)			2	0	2 (22%)
bacterial/viral infections**	1	0			2	1			4 (14%)	1	0	1	0	2 (22%)
dizziness									3 (10%)	1	0	1	0	2 (22%)
myalgia									1 (4%)					0
upper abdominal pain (not IR related)	1	0							2 (7%)			1	0	1 (11%)
fungal infections*					1	0	1	0	2 (7%)					0
hiccup (not IR related)									1 (4%)					0
oedema									0	1	0			1 (11%)
cough (not IR related)	1	0							1 (4%)					0
pulmonar sepsis					0	1^			1 (4%)					0
hypertension									1 (4%)					0
thrombosis					1	0			1 (4%)					0
night sweat									1 (4%)					0
pain	1	0							1 (4%)	1	0	3	0	4 (44%)
lethargy					1	0			1 (4%)					0
dysphonia									0	1	0			1 (11%)
dyspnea(not IR related)												1	0	1 (11%)
hyperkeratosis and dry skin			1	0			1	0	2 (7%)	1	0			1 (11%)
onychomadesis and nail loss									1 (4%)					0
hairloss					1	0			1 (4%)	1	0	2	0	3 (33%)
neuropathy									0	1	0			1 (11%)

Table 3. Summary of all drug related adverse events (starting from 40 mg/m2 2B3-101) pulmonar sepsis was fatal,* fungal infection includes tinea pedis and oral candidiasis, ** bacteral/viral infection includes bacterial conjuctivitis, and erythrasma, ***mucositis term includes stomatitis and oropharyngeal pain, **** hand-foot syndrome (HFS) includes rash and erythema of hands and feet.

dose-level	dose	number of patients	Cmax (µg/ml)		AUC (µg*h/ml)		AUC 0-inf (µg*h/ml)		t1/2 (h)		V (l/m²)		CL (l/h/m²)	
			mean	CV%	mean	CV%	mean	CV%	median	CV%	median	CV%	median	CV%
DL1	5 mg/m²	3	3	10	202	16	209	17	45	18	1.40	19	0.026	14
DL2	10 mg/m²	3	5.1	12	413	25	446	28	49	23	1.70	16	0.023	30
DL3	20 mg/m²	3	11	18	814	32	857	36	43	32	1.62	26	0.024	41
DL4	30 mg/m²	3	18.1	15	1753	8	1925	7	62	25	1.31	26	0.016	7
DL5	40 mg/m²	3	25.9	22	2286	11	2502	11	67	7	1.54	11	0.015	12
40 mg/m² + TRZ		3	29.8	40	2395	29	2646	30	73	22	1.50	37	0.018	23
DL6	50 mg/m²	3	31.6	10	3199	1	3539	4	71	9	1.42	5.48	0.014	4
50 mg/m² + TRZ		6	29.8*	21	2345	35	2569	36	53	18	1.68	21	0.020	34
DL7	60 mg/m²	7	34.4	15	2996	30	3225	33	61	18	1.53	24	0.017	49
DL8	70 mg/m²	3	41.5	4	5106	15	5989	26	87	17	1.62	11	0.013	22

Table 4. Pharmacokinetic parameters TRZ = trastuzumab, T1/2=terminal half-life, CL= total plasma clearance of total drug, V (Vd) =volume of distribution, CV% = coefficient of variation, calculated as (SD/mean) x 100%. * including patients with infusion reactions, with one patient receiving 50% of the dose at day 1 and 50% at day 2.

		SD/PR			PD	
dosel-level (2B3-101 dose)	**n**	**n**	**tumor type**	**SD duration**	**n**	**tumor type**
DL 1 - 5 mg/m2	3				3	BC oesophagus SCLC
DL 2 - 10 mg/m2	3	1	ovarian (IC*)	C8=168d	2	NSCLC oesophagus
DL 3 - 20 mg/m2	3				2	NSCLC BC (**IC****)
DL 4 - 30 mg/m2	3				3	glioma gr IV glioma gr IV BC
DL 5 - 40 mg/m2	3	2	glioma gr III glioma gr IV	C6=126d C4=84d	1	neuro- endocrine lung
DL 6 - 50 mg/m2	3	2	glioma gr IV SCLC (**EC*** PR**)	C4=84d C4=84d	1	glioma gr IV
DL 7 - 60 mg/m2	7	5	glioma gr III glioma gr IV glioma gr III NSCLC melanoma (IC****)	C4=84d C2=42d C4=84d C4=84d C4=84d	2	endometrial BC
DL 8 - 70 mg/m2	3	2	glioma gr IV glioma gr IV	C6=126d C6=126d	1	glioma gr IV
Total 2B3-101 single agent	28	12			15	15
DL 1 TRZ - 40 mg/m2 + TRZ	3	2	HER2+ BC HER2+ BC	C10=210d C4=84d	1	HER2+ BC
DL 2 TRZ - 50 mg/m2 + TRZ	6	4	HER2+ BC **(IC PR)***** HER2+ BC **(EC PR)****** HER2+ BC HER2+ BC	C8=168d C5=105d C2=42d C6=126d	1	HER2+ BC
Total 2B3-101 + TRZ	9	6			2	

Table 5. Preliminary anti-tumor activity C = cycle (21 days), d = days; n.e. lung – neuroendocrine lung cancer, IC = intracranial; EC = extracranial; BC breast cancer; PR – partial response *IC reduction of non-target lesions ** IC target lesion reduction of 25%; *** EC PR 66%; **** intracranial 70% reduction (mostly blood); ***** IC PR and overall reduction of 50% (HER2+ BC) EC PR****** - EC reduction of 75% (HER2+ BC). 2 patients non-evaluable , one in single agent and one in combination cohort

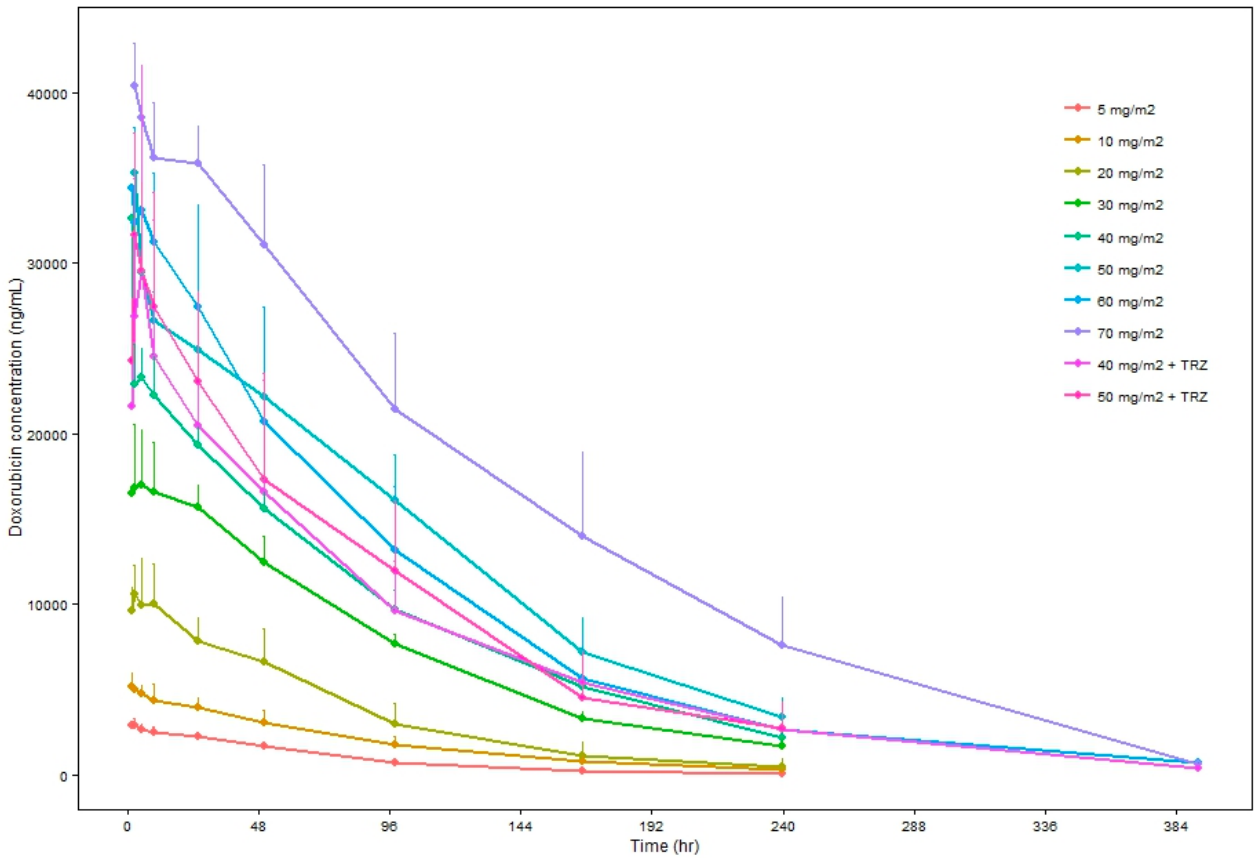

Figure 1. Plasma concentration-time curves of doxorubicin as a single agent and in trastuzumab (TRZ)-combination arm. Standard deviations are only single-side presented because of better visualization of those lines.

3.3 Clinical and pharmacological feasibility phase II study with 2B3-101 in patients with breast cancer and leptomeningeal metastases

B. Milojkovic Kerklaan[1,2], , D. Pluim[2], M. Bol[3], I. Hofland[4], S. Schagen[5], H. van Tinteren[6], G.S. Sonke[7], J.H. Beijnen[8,9], W. Boogerd[10], J.H.M. Schellens [1,2,9], D. Brandsma[10]

1 Department of Clinical Pharmacology, Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
2 Molecular Pathology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
3 Department of Pathology, Netherlands Cancer Instituite - Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
4 Core Facility Molecular Pathology & Biobanking (CFMPB), Department of Molecular Pathology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
5 Department of Psychosocial Research and Epidemiology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
6 Department of Biometrics, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands,
7 Department of Medical Oncology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands.
8 Department of Pharmacy and Pharmacology, Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands.
9 Utrecht University, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands
10 Department of Neuro-Oncology , Netherlands Cancer Institute - Antoni van Leeuwenhoek, Amsterdam, The Netherlands.

Interim analysis

ABSTRACT

Introduction Leptomeningeal metastases (LM) occur in about 5% of patients with metastastic cancer. The prognosis of these patients is poor. Therapeutic agents that are effective for systemic metastases, are often ineffective for LM as they do not cross the blood-cerebrospinal fluid barrier.

Purpose: The aim of this clinical and pharmacological feasibility study is to determine the response of LM from breast cancer on glutathione PEGylated liposomal doxorubicin (2B3-101) treatment.

Patients and methods: Six patients are planned to receive 2B3-101 treatment (50mg/m^2, q3weeks). Response of LM is assessed using the LM response score based on neurological symptoms, MRI of the brains/spinal cord and CSF cytology at every uneven cycle. Whole blood and CSF doxorubicin concentrations are measured by LC-MS/MS to determine the penetration of doxorubicin into the CSF. Circulating tumor cells (CTCs) measured by flow cytometry in the CSF were explored as an additive tool for LM response monitoring.

Results: Three patients with a median age of 64 years (range 46-65 years) were enrolled in the study from September 2013 to October 2014. Two patients stopped 2B3-101 treatment because of neurological progression due to LM (one patient after 1 cycle and one patient after 2 cycles of 2B3-101). The third patient has ongoing stable disease (SD) after 9 cycles of 2B3-101. Doxorubicin concentrations in the CSF of the three patients at day 2 after 2B3-101 treatment ranged from <0.5-18.2 ng/ml. CTC enumeration in the CSF showed an increase in one patient with progressive disease (PD), was incongruent in another patient, and showed a decrease in the patient with SD.

Conclusions: At interim analysis, one of three patients with progressive LM from breast cancer showed SD during more than 6 months treatment with 2B3-101. In two of three patients doxorubicin reaches CSF concentrations that can inhibit breast cancer growth based on in vitro IC50 levels. Additional patients should be included in this feasibility study to conclude on further phase II studies with 2B3-101 in LM patients and to evaluate whether CTC enumeration in the CSF can be used for response-monitoring of LM.

INTRODUCTION

Leptomeningeal metastases (LM) develop when tumor cells reach the cerebrospinal fluid (CSF) and infiltrate the leptomeninges. Clinically symptomatic LM develop in about five percent of patients with metastatic cancer.[1] The most common tumor types among patients with LM from solid tumors are breast cancer (12-35%) and lung cancer (10-26%), melanoma (5-25%) and gastrointestinal malignancies (4-14%).[2] The median survival of untreated patients with LM derived from solid tumors is only 6-8 weeks. Chemotherapy and radiotherapy of symptomatic central nervous system (CNS) sites can extend median survival up to 2-4 months.[3] Median survival of patients with breast cancer and LM is somewhat longer (4-6 months)

with up to 25% 1-year survivors.[4,5] Many potentially efficacious intravenous chemotherapies are currently not effective for LM as they do not adequately cross the blood-CSF barrier. The effectiveness of intrathecal (IT) chemotherapy is thought to be limited due to rapid cerebrospinal fluid (CSF) clearance of the drug and/or insufficient penetration into larger (>1mm) tumor deposits in the subarachnoid space.[6] Moreover, only a few cytostatic drugs can be administered intrathecally because of neurotoxicity.

Doxorubicin, an anthracycline based chemotherapeutic agent, has well-established antineoplastic activity in breast cancer. It has multiple action mechanisms, viz. binding to DNA strands by intercalation, blocking the enzyme topoisomerase II, necessary for DNA replication, formation of free radicals and disintegration of DNA-histone integrity.[7] The treatment of breast cancer patients with anthracycline-containing adjuvant chemotherapy reduces the relative risk (RR) of mortality in breast cancer patients by ± 38%/year in patients < 50 years and by ± 20%/year in patients aged 50 to 69 years.[8] To optimally enhance the delivery of doxorubicin to the brain a glutathione (GSH) PEGylated liposomal doxorubicin hydrochloride formulation (2B3-101) was designed by BBB Therapeutics (Leiden, The Netherlands).

A liposomal formulation that is already in clinical use for metastasized breast cancer is PEGylated liposomal doxorubicin (Caelyx®, Doxil®, PLD). Pegylated liposomal doxorubicin has a prolonged circulation time in plasma and less (cardio)toxicity compared to conventional doxorubicin. It is presumed that the conjugation of GSH to the tips of the PEG molecules in 2B3-101 is targeting the liposomes towards the active GSH transporters on the blood-brain barrier (BBB) to enhance the delivery of doxorubicin to the brain. In preclinical experiments, 2B3-101 showed 5-fold higher brain concentrations of doxorubicin in rats, a stronger inhibition of in vivo glioma growth and increased median survival of mice with intracranial glioma treated with 2B3-101 as compared to PLD. [9,10]

In a clinical phase I/IIa study, 2B3-101 was administered as a single agent and in combination with trastuzumab to patients with brain metastases (BM) from solid tumors or recurrent malignant gliomas. In both groups, 2B3-101 treatment was found to be safe and relatively well-tolerated with hematological toxicity, hand-foot syndrome, stomatitis and infusion reactions as most frequently occurring adverse events. (Milojkovic Kerklaan et al, submitted for publication). The elimination half-time ($t_{1/2}$) of 2B3-101 dosed at 50 mg/m^2 was found to be 71 h with a volume of distribution ($V_{(d)}$) of 1.42 l/m^2. The encapsulated fraction of the total measured doxorubicin plasma levels was higher than 85%. The exposure of 2B3-101 at 50 mg/m^2 administered in combination with trastuzumab in patients with HER2+ breast cancer corresponded to 2B3-101 single agent exposure at the same dose-level. Preliminary anti-tumor activity of 2B3-101 was shown in patients with breast cancer, small cell lung cancer (SCLC) and recurrent malignant glioma.

The purpose of the current study is to determine the preliminary response on 2B3-101 treatment as a single agent or in combination with trastuzumab on LM in breast cancer patients using the LM response score. Whole blood and CSF doxorubicin concentrations are measured to determine the penetration of doxorubicin into the CSF. The use of circulating tumor cells (CTCs) counts in CSF was explored as an additive tool for LM response monitoring.

PATIENTS AND METHODS

Inclusion- and exclusion criteria

Eligible patients are selected on radiological and/or cytological evidence of clinically symptomatic LM of pathologically confirmed breast cancer with or without BM. Inclusion criteria were: written informed consent, a life expectancy of at least 8 weeks, age ≥18 years, ECOG Performance Status ≤ 2, stable or decreasing dosage of steroids (e.g. dexamethasone) for 7 days prior to baseline MRI with allowed use of non-enzyme inducing anti-epileptic drugs and adequate bone marrow, liver and renal function. All toxicities incurred as a result of previous anticancer therapy had to be resolved to ≤ grade 2 (as defined by CTCAE version 4.0). Local radiation of CNS symptomatic sites more than four weeks prior to start of the study was allowed. Radiotherapy of symptomatic bone metastases is allowed before or during 2B3-101 treatment both as single agent and in combination with trastuzumab, but radiated localizations of the nervous system were not used for response evaluation. Previous trastuzumab treatment was allowed to continue without interruption in patients with HER2+ breast cancer. Patients who have received a maximum cumulative dose of free (i.e., non-liposomal) or liposomal doxorubicin > 360mg/m2 or free epirubicin > 600mg/m2 were not eligible. Further exclusion criteria were: pregnancy and lactation, a major surgical procedure or use of other investigational drugs within 4 weeks prior to the first study treatment, anticipation of the need for major surgery during the course of the study treatment; active systemic or CNS infection, uncontrolled hypertension (systolic pressure > 150 mmHg and/or diastolic pressure >100mmHg), clinically significant (i.e. active) cardiovascular disease, Left Ventricle Ejection Fraction (LVEF) determined by MUGA or ECHO of < 50% for patients treated with 2B3-101 as a single agent and < 55% for patients treated with 2B3-101 and trastuzumab. Contra-indications for lumbar punctures were uncorrected blood clotting disorders (INR>1.5, platelets <20x10^9/l, aPTT > 1.5 ULN) and cerebral or spinal space-occupying masses.

Study design

This pilot study will be performed in 6 patients with LM from breast cancer in The Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands. In case at least one of these six planned patients will have a partial response or two patients will have stable disease (SD) as best LM response, a

total of 28 patients will be enrolled. 2B3-101 is given in a dose of 50 mg/m^2 every three weeks either as a single agent or in combination with trastuzumab in HER2+ breast cancer patients.

Safety assessments

Safety was assessed by means of physical and neurological examination, neurocognitive testing and laboratory evaluations, including cardiac enzymes, electrocardiograms (ECG) and LVEF (MUGA/ECHO). Concurrent illnesses/therapies and adverse events were recorded according to The National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE version 4.0). Patients that received 2B3-101 in combination with trastuzumab were required to enter an intensified cardiac program including ECG and LVEF measurements before the start of every treatment cycle.

Anti-tumor activity

Preliminary anti-tumor activity of 2B3-101 as a single agent or in combination with trastuzumab is determined using the LM response score based on neurological signs and symptoms, MRI-scans of brains and spinal cord, and CSF semi-quantitative cytology scoring every six weeks (2 cycles of 2B3-101). (Table 1) To measure the anti-tumor response of 2B3-101 on LM, enumeration of CTCs in the CSF and blood was performed prior to and during 2B3-101 therapy before every uneven cycle. A CT chest/abdomen was also performed before every uneven cycle to determine the response of lung and abdominal metastases, if present.
Patients remained on treatment until disease progression, unmanageable toxicity or in case of withdrawal of consent. Patients were given follow-up until death.

Study medication

A single dose of 50 mg/m^2 2B3-101 was administered IV on day 1 of a 21-day cycle. To minimize the risk of infusion reactions, 5% of the total dose of 2B3-101 (in mg) was given over the first 30 min. If tolerated, the infusion was completed over the next 60 min for a total infusion time of 90 min. In case of a mild infusion reaction (with symptoms like flushing, dyspnea, facial edema, chest- or, backpain) infusion of 2B3-101 was stopped. After recovery of the symptoms, the infusion was restarted with 10 ml/h rate for the first 30 min and increased every 30 min as follows: 20 ml/h, 50 ml/h, 100 ml/h, and 200 ml/h. (Pre) medication such as hydrocortisone, ranitidine, cimetidine, anti-emetics, and diphenhydramine was allowed according to local institutional guidelines.

Patients with HER2+ breast cancer were receiving the standard maintenance dose of 6 mg/kg trastuzumab intravenously every 3 weeks (or 8 mg/kg as a loading dose, in case of the first trastuzumab treatment). Trastuzumab was administered 30 min after the 2B3-101 infusion was completed.

Pharmacokinetics

Plasma and CSF samples for study (non-safety) purposes were drawn at Cycle 1 predose (within 1 week prior to start 2B3-101), Cycle 1 day 2 and day 8 and thereafter every 6 weeks, within one week prior to the start of uneven cycles on day 1.
CSF samples were drawn via a lumbar puncture for determination of total doxorubicin (2.5 ml), cytology (5 ml), cell counts and biochemical parameters (leukocytes, erythrocytes, total protein, glucose and LDH) (2 ml) and enumeration of CTCs (5 ml). Furthermore, blood samples were drawn for CSF/plasma ratio for glucose, LDH and total protein (8 ml), total doxorubicin (4 ml) and for CTC enumeration in blood (3 x 8 ml).
Liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) was used to determine total (free and encapsulated) doxorubicin in CSF and plasma. Doxorubicin in CSF was stabilized directly after collection by 2-fold dilution of CSF sample with antioxidant solution (1.25 g EDTA, 0.3 g pyrogallol, 0.3 g ascorbic acid, 0.3 g sodium disulfite, 250 mL Milli-Q water, 50.0 mL and MeOH) and stored at ≤ -70°C until analysis. The assay was validated in accordance with current guidelines for bioanalytical method validation.[11,12] The analytical quantification range is 0.5 – 50,000 ng/mL for total doxorubicin.

CSF cytology

CSF cytology was performed as a two-step cytocentrifuge method (1600 rpm, 1min and 10 min resp.), which deposits cells from a fluid sample directly onto slides (cytospin). Giemsa staining was performed of two cytospins. The Giemsa stainings of the two cytospins of the CSF were viewed by the pathologist and scored as negative (no tumor cells), positive (tumor cells) or inconclusive (atypical cells).

CSF semiquantitative score and immunostaining

Two additional cytospins were used for immunohistochemical (IHC) staining with an antibody against EpCAM on a BenchMark Ultra autostainer (Ventana Medical Systems, Inc.). Briefly, cytospins were washed in Reaction Buffer (Ventana Medical Systems) and loaded in the autostainer. Epithelial antigen expression was determined by incubating the cytospins with the antibody clone Ber-EP4/EpCAM (M0804; DAKO), The antibody was used at 1:20 dilution and incubated for 32 min at 37^0C. After incubation with the primary antibodies, amplification (Ventana Medical Systems) was selected. The amplification step was used to increase the signal intensity of weak staining (mouse and rabbit) primary antibodies. Specific reactions were detected using UltraView Universal DAB Detection Kit (Ventana Medical Systems). Cytospins were counterstained with Hematoxylin.
Tumor cells on these immunohistochemically stained cytospins were semiquantitativly scored by the pathologist, using "score 0" for 0 tumor cells / cytospin area, "score 1" for 1-10 tumor cells/cytospin area, "score 2" for 11-100

tumor cells/cytospin area, "score 3" for 101-500 tumor cells/cytospin area, and "score 4" for > 500 tumor cells/cytospin area. The results of the semiquantitative method were used for the LM response score.
Archived tumor tissues were collected and will be analyzed for EPCAM expression of the primary tumor by immunohistochemistry (IHC), using EpCam (Ber-EP4) antibody at the end of the study.

CTC flow cytometry assay

Blood was drawn in Cell Preparation Tubes CPT™ (BD Biosciences, USA) tubes and centrifuged for 25 min at 1500g at room temperature (RT) within 1h after collection. During centrifugation the gel portion of the medium moves to form a barrier separating the mononuclear cells with the relative low density from the denser blood components. The upper separated phase from 3 CPT tubes was transferred into three 50 ml centrifuge tubes. CSF was initially collected in the same type of tubes. After fixation with 4% formaldehyde, all four samples were washed twice with up to 50 ml physiologic saline and centrifuged at 1000g for 7 min at 4°C. The pellets were resuspended in 50% methanol/PBS (phosphate buffered saline) and stored at -80C for a maximum of 6 months. Technical details on the EpCam-based CTC assay can be found in Pluim et al (2012).[13] CTC measurements were performed in batches. After defrosting on ice, samples were washed twice with 1 ml of ice-cold Beads Buffer (BB = PBS with 2 mM of EDTA and 0.5% w/v of bovine serum albumin). After each wash, samples were centrifuged at 1000g for 4 min at 4°C. Next, supernatant was removed and the pellets were resuspended in the remaining 100 µl of BB. Subsequently, CTCs were immunomagnetically enriched from the samples using anti-human EpCam- Micro-Beads (Miltenyi, Bergisch Gladbach, Germany). Next, CTCs were fluorescently labeled using anti-EpCam-PE. Hoechst 33258 was used for nuclear staining, and anti-CD45-FITC for leucocyte labeling. After removal of unreacted antibodies, CTCs were quantified by fluorescent activated cell sorting (FACS). Using this method both CTCs with a normal amount of DNA and CTCs with less DNA could be quantified.[14] The flow cytometric EpCam assay has a lower limit of quantification (LLQ) of two CTCs per 8 ml of whole blood. Therefore, CSF samples with < 2 CTCs/8 ml were considered negative. The results of the CTC assays in CSF and blood were performed in batches and were not used in clinical decision-making.

RESULTS

In this interim analysis a total 3 out of 6 planned patients aged 65 years (patient 1), 46 years (patient 2) and 64 years (patient 3) were enrolled from August 2013 till October 2014. Patient 1 and 2 had ER+PR+HER2- breast cancer and patient 3 had a triple negative breast cancer. Patient 1 and 3 both had cerebral and cranial symptoms, patient 2 had spinal symptoms. Patient 2 had been treated with radiotherapy of different spinal cord localizations and two lines of systemic chemother-

apy (capecitabine; cyclophosphamide/methotrexate) for symptomatic LM, before inclusion in the study while the other two patients had not received any prior therapies for LM. (Table 2) Patient 1 received two and patient 3 one cycle of 2B3-101 before they both went off-study because of LM progression. Patient 2 has ongoing SD for at least nine cycles of 2B3-101 (> 6 months).

Adverse events

The monitored safety data were collected at August 1st, 2014. All three patients experienced at least one infusion reaction, maximal grade 2 during 2B3-101 infusion. Patient 1 experienced neutropenia, anemia and leucocytopenia grade 2 after the first cycle of 2B3-101. Patient 2 received 2B3-101 at a very slow infusion rate (27 ml/h), with approximately 24 h duration of infusion because of repeated infusion reactions. After cycle 3, she experienced grade 4 neutropenia, leucocytopenia and anemia and a febrile neutropenia grade 3 due to an urinary tract infection in the presence of a permanent urine-catheter. She was treated with antibiotics and received two packed cells infusion. Febrile neutropenia recovered within 3 days and a dose reduction to 40mg/m^2 2B3-101 from cycle 4 on was performed. Patient 3 experienced a leucocytopenia and neutropenia grade 2 after the first cycle of 2B3-101. Mucositis grade 1 and 2 were present in patient 2 and 3 in the second and first cycle of 2B3-101 respectively. Hand-foot syndrome (HFS) grade I was present in patient 1 during cycle 2. No cardiotoxicity or neurotoxicity was observed. (Table 3)

Pharmacokinetics

Table 5 shows the total doxorubicin concentrations in blood and CSF. The median total doxorubicin concentrations in plasma at Cycle 1, day 2 and day 8 a were 19300 ng/ml and 4850 ng/ml, which were in the range of the total doxorubicin plasma levels measured in the phase I study on 2B3-101 (Milojkovic Kerklaan et al., submitted for publication). In two patients (patient 2 and 3) CSF total doxorubicin concentration levels were 2.3 nM (1.25 ng/ml) and 33 nM (18.2 ng/ml) at Cycle 1 Day 2 and 12.3 nM (6.66 ng/ml) and 13.6 nM (7.37 ng/ml) at Cycle 1 Day 8. Patient 2 received half of the 2B3-101 dose at day 1 and the other half at day two, because of a repeated infusion reaction. The total doxorubicin plasma level in this patient at Cycle 1 day 2 was 13900 ng/ml and at Cycle 1 day 8 below the limit of quantification. However, the CSF doxorubicin concentration at Cycle 1 day 2 and day 8 were 1.25 ng/ml respectively 6.66 ng/ml.

LM response score and anti-tumor activity of 2B3-101

For determining the response of LM to 2B3-101, the LM response score was used. (Table 1) Patient 1 had recently diagnosed LM, without any earlier LM treatment, when entering the study. She was progressive after two 2B3-101 cycles based on neurological deterioration but stable MRI spinal cord and CSF cytology. She was alive after 6 months from the start of the study without any further treatment.

Patient 2 had clinically and radiologically progressive LM after earlier treatment with capecitabine, radiotherapy of several symptomatic spinal LM localizations and cyclophosphamide/methotrexate treatment. She had LM since 2 years and 4 months when entering the study. She showed stable LM for at least nine cycles of 2B3-101, based on stable neurological symptoms, unchanged radiological features and unchanged CSF cytology.
Patient 3 was recently diagnosed with LM and had not received any earlier LM treatment. She had progressive LM based on neurological deterioration and CSF cytology after only one cycle of 2B3-101.This patient died within three months from start of study.

CTC enumeration
As an exploratory method we monitored the number of CTCs in CSF and blood for response-assessment to 2B3-101 therapy. (Table 4) Patient 1 having bone metastases had a 10-fold decrease of CTCs in plasma before cycle 3 as compared to baseline. CTCs in the CSF in this patient were high (>900 CTCs/ml) with a slight increase of CTCs at cycle 3 predose, which is in agreement with progressive disease (PD) based on the LM score. Patient 2 had no detectable CTCs in blood and absence of systemic metastases. CSF cytology showed no or atypical cells, while 18 CTCs/ml were detected in the CSF by CTC assay at baseline. After two cycles CTCs increased slightly (46 CTCs/ml) and subsequently decreased after four (12 CTCs/ml) and six cycles (6 CTCs/ml) with again an increase after 8 cycles (28 CTCs/ml).

Patient 3 discontinued study after 2 cycles of 2B3-101 because of neurological deterioration with unchanged CSF cytology findings. CTC numbers at baseline, cycle 1 day 2 and 8 varied between 135-177 CTCs/ml and were somewhat lower (108 CTCs/ml) at cycle day 28 when a lumbar puncture was performed because of neurological progressive symptoms due to LM.

DISCUSSION

This small feasibility and pharmacological phase II study showed a long-duration SD in one of three patients with LM from breast cancer, treated with three weekly 2B3-101. In all three enrolled patients toxicities of grade ≤ 2 (e.g. hematological toxicity, stomatitis, hand-foot syndrome) were seen. In one patient febrile neutropenia grade 3 with pancytopenia grade 4 was seen after three cycles and a dose-reduction of 2B3-101 to 40mg/m2 was performed. All three patients experienced infusion reactions during infusion administration, a well-known PLD toxicity, reported in 10.8 % of patients.[15] No cardiotoxicity and neurotoxicity was observed in any of the three patients.
Two out of three patients achieved levels of total doxorubicin in CSF that are considered sufficient for 50% inhibition of breast cancer cell line growth in vitro. [16] One of those two patients had a long-duration SD (> 9 cycles 2B3-101).

The half maximum inhibitory concentrations (IC_{50}) for doxorubicin were determined in eight HER2+ breast cancer cell lines ranged from 2.7-121.1 nM.[16] The IC_{50} in another study with breast cancer cell lines was reported to be 25-35 nM. [17]
The measured concentrations of doxorubicin in CSF after intravenous administration of 2B3-101 in our study were in two patients at Cycle 1 Day 1 respectively 2.3 nM and 33.5 nM and at Cycle 1 Day 8 respectively 12.3 nM and 13.6 nM. This means that by treating patients with 50 mg/m^2 2B3-101, doxorubicin CSF concentrations can be reached that can potentially inhibit tumor growth in the CSF. In one patient no doxorubicin concentrations could be detected in CSF. However plasma levels after 2B3-101 treatment were as expected based on the pharmacokinetic data obtained in the phase I study on brain metastases from solid tumors or recurrent malignant gliomas (Milojkovic Kerklaan et al., submitted for publication). The high body mass index (BMI) of this patient may have influenced the low drug penetration in the CSF as liposomal doxorubicin could have been distributed to the fat tissue, as has been formerly suggested for liposomal amphotericin. [18]
Baseline (or cycle 1 day 2) CTC numbers in the CSF for the two patients with rapid LM progression were 913 CTCs/ml and 161 CTCs/ml. In contrast, the patient with long-duration SD had a relatively low number of CTCs in the CSF (18/ml), that could not be detected by standard CSF cytology. Therefore, the number of CTCs measured in the CSF may be a prognostic factor for LM.
The trend of CTCs increase during LM progression and a CTC decrease during SD was contradicted by the patient with clinical progressive LM but a decrease in CTCs in the CSF. Therefore, further evaluation of the use of CTCs as possible tool for LM response monitoring is needed.

CONCLUSION

Treatment with 2B3-101 in three patients with breast cancer and LM was safe and tolerable, with one patient showing durable SD (>6 months). Measured doxorubicin concentrations in the CSF were within the range of known in vitro cytotoxic levels for breast cancer cell lines in two of three patients. The value of enumeration of CTCs in CSF for LM response monitoring is unknown and needs further evaluation. Further inclusion of patients in this phase II clinical and pharmacological feasibility study is awaited.

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RESPONSE	NEUROLOGICAL SIGNS AND SYMPTOMS	RADIOLOGICAL FEATURES	CSF CYTOLOGY SEMIQUANTITATIVE SCORING
response (PR)	improved*	no change or improvement of disease on MRI	no, unchanged or decreased number of tumor cells+
stable (SD)	stable	no change of disease on MRI	unchanged number of tumor cells
progressive disease (PD)	worse #	no change or increase of disease on MRI	unchanged or increased number of tumor cells ^

Table 1. Leptomeningeal metastases (LM) response score* :distinct improvement of at least one neurological sign without worsening of other signs during at least two weeks. # :worsening of neurological signs due to LM for at least two weeks, interfering with daily activities. + :decrease of tumor cells: decrease more than 1 point on semi-quantitative scoring method, except for score 1, in which a decrease of 1 point (to no tumor cells) suffices. ^ increase of tumor cells: increase > 1 point on semi-quantitative scoring method except for score 3 in which 1 point increase suffices.
CSF semiquantitative scoring by pathologist: score 0 = 0 tumor cells / cytospin area, score 1 = 1-10 tumor cells/cytospin area, score 2 = 11-100 tumor cells/cytospin area, score 3 = 101-500 tumor cells/cytospin area, and score 4 for > 500 tumor cells/cytospin area.

	PATIENT 1	PATIENT 2	PATIENT 3
breast cancer type	ductal; ER+, PR+, HER2-	ductal; ER+, PR+, HER2-	large-cell ER-, PR-, HER2-
age in years	65	46	64
symptoms	headache, left visual field defect, dizziness, tinnitus, nausea, vomiting	paresis left arm and leg, hypaesthesia left arm (C6-C7 dermatome), sensory ataxia right arm, impaired micturition	headache, visual loss, dizziness, face tingling, unstable gait, impaired micturition
radiotherapy for CNS symptomatic sites	none	yes - different spinal cord localizations, in total 4x	none
chemotherapy for LM	none	capecitabine cyclophosphamide/metho-trexate	none

Table 2. Patient characteristics.

ADVERSE EVENT	NUMBER (%)
infusion reaction	3 (100%)
leucocytopenia	3 (100%)
neutropenia	3 (100%)
mucositis/oral pain	2 (67%)
lymphocytopenia	2 (67%)
anemia	2 (67%)
monocytopenia	1 (33%)
thrombocytopenia	1 (33%)
febrile neutropenia	1 (33%)
hematuria	1 (33%)
ALAT increase	1 (33%)
HFS	1 (33%)
urinary tract infection	1 (33%)
fatigue	1 (33%)
nausea	1 (33%)

Table 3. Treatment related adverse events (all grades) in patients with leptomeningeal metastases treated with 50 mg/m2 2B3-101 3-weekly. AE = adverse event; HFS = hand-foot syndrome.

PATIENT NUMBER	TIME OF CSF ANALYSIS	LM RESPONSE SCORE	SYSTEMIC LESIONS	CSF CYTOLOGY REPORT	SEMIQUANTITATIVE CSF CYTOLOGY SCORE	CTC/ML CSF	MEDIAN NUMBER OF CTC/ 8 ML BLOOD
1	C1 predose			tumor cells	4	NA	3213
	C1D2			tumor cells	4	913	3173
	C1D8			tumor cells	3	806	3063
	C3 predose	PD	SD	tumor cells	4	1147	348
2	C1 predose			no tumor cells	1	18	0
	C1D2			atypical cells	1	40	0
	C1D8			no tumor cells	0	51	ND
	C3 predose	SD	not present	no tumor cells	0	46	ND
	C5 predose			no tumor cells	NA	12	NA
	C7 predose			no tumor cells	NA	6	NA
	C9 predose			no tumor cells	NA	28	NA
3	C1 predose			tumor cells	3	161	3
	C1D2			tumor cells	3	177	3
	C1D8			tumor cells	2	135	2
	C1D28*	PD	not present	tumor cells	2	108	0

Table 4. LM response score, CSF cytology and CTC in three patients treated with 2B3-101. IC=intracranial, ND= not done, NA =not available. * a lumbar puncture was performed on C1D28 because of the neurological deteroriation

"patient study number"	DOXORUBICIN CONCENTRATION IN PLASMA		DOXORUBICIN CONCENTRATION IN CSF	
	LP visit/number	(ng/mL)	(ng/mL)	(nmol/L) = nM
1	C1 predose	< LLOQ (50.0)	< LLOQ (0.500)	< LLOQ (0.920)
	C1D2	23100	< LLOQ (0.500)	< LLOQ (0.920)
	C1D8	5630	< LLOQ (0.500)	< LLOQ (0.920)
	C3 predose	1560	< LLOQ (0.500)	< LLOQ (0.920)
2	C1 predose	< LLOQ (50.0)	< LLOQ (0.500)	< LLOQ (0.920)
	C1D2	13900	1.25	2.3
	C1D8	< LLOQ (50.0)	6.66	12.3
	C3 predose	< LLOQ (50.0)	< LLOQ (0.500)	< LLOQ (0.920)
3	C1 predose	< LLOQ (50.0)	< LLOQ (0.500)	< LLOQ (0.920)
	C1D2	19400	18.2	33.5
	C1D8	4850	7.37	13.6
	C1D28	< LLOQ (50.0)	< LLOQ (0.500)	< LLOQ (0.920)

Table 5. Doxorubicin concentrations in plasma and CSF. LLOQ= lower limit of quantification.

3.4 Sensitivity and specificity of circulating tumor cell detection by flow cytometry versus cytology in cerebrospinal fluid for the diagnosis leptomeningeal metastases

B. Milojkovic Kerklaan, [1,2] D. Pluim, [1] M. Bol, [3] I. Hofland, [1,4] J. Westerga[5], H. van Tinteren, [6] J. H. Beijnen, [7,8] W. Boogerd, [9] J.H.M. Schellens, [1,2,8], D. Brandsma[9]

1 Department of Molecular Pathology, Netherlands Cancer Institute – Antoni van Leeuwenhoek (NKI-AVL), Amsterdam, The Netherlands
2 Department of Clinical Pharmacology, NKI-AVL, Amsterdam, The Netherlands
3 Department of Pathology, NKI-AVL, Amsterdam, The Netherlands
4 Core Facility Molecular Pathology & Biobanking (CFMPB), Departement of Molecular Pathology, NKI-AVL, Amsterdam, The Netherlands
5 Department of Pathology, Slotervaart Hospital, Amsterdam, The Netherlands
6 Biometric Department, NKI-AVL, Amsterdam, The Netherlands
7 Department of Pharmacy and Pharmacology, NKI-AVL, Amsterdam, The Netherlands
8 Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands
9 Department of Neuro-oncology , NKI-AVL, Amsterdam, The Netherlands

Manuscript submitted

ABSTRACT

Introduction: Low sensitivity of the standard methods of MRI and cerebrospinal fluid (CSF) cytology results in at least 25 % of false negative diagnoses of leptomeningeal metastases (LM) and postponing the start of therapy.
The aim of this prospective clinical study is to determine the diagnostic value of cytology versus flow cytometry of circulatory tumor cells (CTC) of cerebrospinal fluid (CSF) in patients with solid tumors suspected of having LM.
Methods: During a diagnostic lumbar puncture at least 5 ml of CSF was obtained for cytology, the same volume for the CTC assay and 2 ml for biochemistry. Furthermore, almost simultaneously whole blood samples were drawn for the CTC assay and biochemistry. CTCs were detected by multi parameter flow cytometry using antibodies against epithelial cell adhesion molecule (EpCam) and melanoma chondroitin sulfate proteoglycan (MCSP).
Results: In total 47 CSF samples from 34 patients with LM, or clinical suspicion of LM, were obtained of whom 18 patients were ultimately diagnosed with LM. The EpCam CTC assay showed 100% sensitivity and 100% specificity for diagnosing LM, while sensitivity of CSF cytology was only 71%. The MCSP CTC assay also showed a high sensitivity and specificity, but the confidence interval was wide due to the small sample size. In 11 patients with LM CTCs were found in whole blood. All patients with LM had elevated total protein CSF levels, 83% decreased glucose CSF levels, and 44% elevated leucocyte counts in the CSF
Conclusion: The EpCam-based CTC assay is superior to CSF cytology in patients with epithelial tumors and LM for the diagnosis LM. Therefore, we recommend after confirmation of study results, the use of the CTC assay in CSF next to CSF cytology in patients with a clinical suspicion of LM.

INTRODUCTION

Leptomeningeal metastases (LM), also known as meningeal carcinomatosis or neoplastic meningitis, is a diffuse dissemination of tumor cells into the cerebrospinal fluid (CSF) and leptomeninges. [1] Up to 8% of all patients with cancer ultimately develop LM. [2] The highest incidence of LM is seen in patients with breast carcinoma (10%), small cell and non-small cell lung carcinoma (10%), and melanoma (5%). [3] Due to the spread of tumor cells in the CSF, LM is characterized by multifocal symptomatology (cerebral, cranial nerve and/or spinal nerve dysfunction). Gadolinium enhanced MRI of the symptomatic sites of the nervous system is the radiological method of choice when LM is clinically suspected. In patients with a metastasized tumor, the diagnosis LM can be made when clinical signs are compatible with LM and contrast enhancement of the leptomeninges, pia mater/cortex or cranial or spinal nerves on MRI is evident. The sensitivity of MRI with gadolinium for LM is approximately 75% and the specificity approximately 77%. [4] When MRI does not show equivocal abnormalities, CSF cytology needs to be performed. Ma-

lignant cells are found in the first CSF sample in about 55% of patients with LM from solid tumors, as determined in the pre-MRI era. [5,6] The sensitivity rises to 80-90% after the second CSF sampling. [6] The volume of the CSF sample determines partly the sensitivity of CSF cytology. If possible, it is advised to draw 10 ml of CSF and process the CSF as quickly as possible. [7] Cell count and clinical chemical analysis of the CSF (leukocytes, lactate dehydrogenase, total protein, and glucose) is aberrant in 90% of patients with LM. [6,8] However, abnormal biochemical parameters in the CSF do not prove LM as these can also be found in other neurological diseases, including (infectious) meningitis.[8] Therefore, new tests are needed to provide conclusive LM diagnostics after the first lumbar puncture to expedite treatment.

Isolation and quantification of circulating tumor cells (CTC) in whole blood using flow cytometry is a proven valuable prognostic marker in metastatic breast, prostate and colorectal cancer. [9] Concurrent flow cytometry and polymerase chain reaction (PCR) to standard CSF cytology is already being used in confirming the initial diagnosis and monitoring of leptomeningeal disease from hematologic malignancies. [10–13] Recently few studies suggested that a CTC assay could be used for detection of tumor cells in CSF in patients with breast cancer or other solid tumors. [14–16] Patel et al. found up to 10^4 circulating CTCs in 7.5 ml CSF and showed an association trend between the number of CTCs and response to intrathecal and systemic chemotherapy.[14]

Flow cytometry allows detection of multiple fluorescent markers simultaneously. A fluorescent antibody was used against epithelial cell adhesion molecule (EpCam), which is expressed in various epithelial cancers, such as breast cancer (ductal type more frequently than lobular type), upper digestive and respiratory tract, gastrointestinal cancer, cancers of the genital and urinary tract, skin cancer and neuroendocrine tumors and some types soft tissue sarcoma. [17] Malignant melanoma, glioblastoma and hematolymphoid malignancies do not express EpCam. Therefore, the antibody against melanoma chondroitin sulfate proteoglycan (MCSP), also known as high molecular weight melanoma associated antigen (HMW-MAA), melanoma proteoglycan (MPG) or neuron-glia 2 (NG2), was used for samples from patients with melanoma or glioblastoma. MCSP is expressed in 85% of melanomas and 83% of glioblastomas, with an overexpression correlating with an unfavorable prognosis. [18,19]

The aim of this study was to prospectively evaluate, using equal sample volumes of at least 5 ml of CSF, concomitantly conventional CSF cytology, and the CTC assay using EpCam or MCSP fluorescence antibodies, in patients with clinically suspected LM from solid tumors but a non-confirmatory LM diagnosis on MRI.

PATIENTS AND METHODS

Patients

The study was approved by the Ethics Committee of The Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital (PTC NKI-AVL). Patients ≥ 18 years with solid tumors clinically suspected for leptomeningeal metastases, e.g with central, cranial or spinal neurological symptoms and signs and a non-confirmatory LM diagnosis on MRI, who had to undergo a diagnostic lumbar puncture (LP), were asked to participate. Exclusion criteria were: 1) intracranial or intraspinal tumors with mass effect forming a risk for brain stem herniation during LP and 2) coagulation disorders, either spontaneously or due to oral anti-coagulants.
Furthermore, three patients with breast cancer and a definitive diagnosis of LM (two based on CSF cytology and one on MRI), treated with glutathione–coupled liposomal doxorubicin (2B3-101) in a phase II study were included in the analyses. At least four LPs were performed in this group of patients per study protocol (predose cycle 1, cycle 1 day 2 and day 8, and predose cycle 3).

CSF and blood analysis

During the diagnostic lumbar punctures, standard CSF evaluation consisted of CSF pressure measurements, cell counts and biochemical parameters (leucocyte count, total protein, glucose and LDH analyses, 2 ml), CSF cytology (at least 5 ml), and CTC measurement (at least 5ml, in 50 ml conical-bottom centrifuge tube)(Figure 1). In addition 8 ml of blood was drawn for blood cell count and biochemistry to determine the CSF/plasma ratio for glucose, LDH and total protein. For the CTC assay, blood was drawn in three 8 ml cell preparation tubes (CPT™, BD Biosciences, USA). In patients with a high clinical suspicion on LM and a negative CSF cytology at first CSF examination, a second LP was performed to collect CSF and blood for cytology, biochemistry, and the CTC assay.

CSF cytology

CSF cytology was performed as a two-step cytocentrifuge method (1600 rpm, 1min and 10 min resp.), which deposits cells from a fluid sample directly onto slides (cytospin). Giemsa staining was performed of two cytospins. The Giemsa staining of the CSFs were viewed by the pathologist and scored as negative (no tumor cells), positive (tumor cells) or inconclusive (atypical cells). The results of CSF cytology were used in clinical decision-making.

Immunohistochemistry and CSF semiquantitative score

Two additional cytospins were used for immunohistochemical (IHC) staining with antibodies against EpCAM and MCSP. Immunohistochemistry was performed on a BenchMark Ultra autostainer (Ventana Medical Systems, Inc.) Briefly, cytospins were washed in Reaction Buffer (Ventana Medical Systems) and loaded in autostainer.

Epithelial antigen was detected by incubating slides with the antibody clone Ber-EP4/EpCam(M0804; DAKO). Melan-A/MCSP and MART1 expression were detected using antibody clone A103 (M7196; DAKO) and antibody clone 5A4 (ab78284; AbCam) resp. All three antibodies were used at 1: 20 dilution and incubated for 32 min at 37^{0}C (Melan-A at 36^{0}C). For Melan-A heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 36 min at 95^{0}C. After incubation with the primary antibodies, amplification (Ventana Medical Systems) was selected. The amplification step was used to increase the signal intensity of weak staining (mouse and rabbit) primary antibodies.
Specific reactions were detected using UltraView Universal DAB Detection Kit (Ventana Medical Systems), and slides were counterstained with Hematoxylin.
These specific reactions represent the bound primary antibodies to the 'specific' epitopes in tissues that will be visualized using the DAB Detection Kit.Tumor cells in these EPCAM or MCSP stained cytospins were semiquantitatively scored by the pathologist: "score 0" for 0 tumor cells / cytospin area, score 1 for 1-10 tumor cells/ cytospin area, score 2 for 11-100 tumor cells/cytospin area, score 3 for 101-500 tumor cells/cytospin area, and score 4 for > 500 tumor cells/cytospin area. The results of the semiquantitative method were used for research purposes only.

CTC flow cytometry assay

Whole blood was drawn in CPT tubes and centrifuged for 25 min at 1500 g at room temperature (RT) within 1 h after collection.
The upper phase of the separated plasma from 3 CPT tubes were transferred in three 50 ml centrifuge tubes. CSF was initially collected in the same type of the tube. After fixation with 4% formaldehyde, all four samples were washed twice with up to 50 ml physiologic saline and centrifuged at 1000g for 7 min at 4°C. The pellets were resuspended in 50% methanol/PBS (phosphate buffered saline) and stored at -80C for a maximum of 6 months. The details of the EpCam assay are described in the paper of Pluim et al. 2012 [20] and for the melanoma MCSP assay in Pluim et al. (submitted for publication).
CTC measurements were performed in batches. After defrosting on ice, samples were washed twice with 1 ml of ice-cold Beads Buffer (BB: PBS with 2 mM of EDTA and 0.5% w/v of bovine serum albumin). After each wash, samples were centrifuged at 1000g for 4 min at 4°C. Next, supernatant was removed and the pellets were resupended in the remaining 100 µl of BB. Subsequently, CTCs were immunomagnetically enriched from the samples using anti-human EpCam- or MCSP-Micro-Beads(Miltenyi, Bergisch Gladbach, Germany). Next, CTCs were fluorescently labeled using anti-EpCam-PE for samples from patients with epithelial tumor or anti-MCSP-PE, and anti-CD146-APC for samples from patients with melanoma or glioblastoma. Hoechst 33258 was used for nuclear staining, and anti-CD45-FITC for leucocyte labeling. After removal of unreacted antibodies, CTCs were quantified by fluorescent activated cell sorting (FACS). With this method both CTCs with a normal amount of DNA and CTCs with less DNA can be quantified. [21] The flow

cytometric EpCam assay has a lower limit of quantification (LLQ) of two CTCs per 8 ml of whole blood. Therefore, samples with< 2 CTCs/8 ml were considered negative. For one patient with glioblastoma the MCSP assay was adapted, as anti-CD146 could not be used for glioblastoma cells as they would not be detected, therefore the LLQ might be slightly higher for this patient group. The results of the CTC assay were not used in clinical decision-making.

Results obtained with both methods (flow cytometric CTC assay and cytology) were obtained independently without knowledge of the outcome obtained by the different method.

Clinical follow-up

Clinical signs and symptoms were recorded and neurological examination was done in the week before the lumbar puncture (LP). Three days after the LP, patients were asked by telephone for symptoms of post-lumbar puncture headache (headache, nausea increasing in an upright position). When post-lumbar puncture headache was diagnosed, follow-up was performed until 28 days after the LP. Clinical records were studied for the course of neurological symptoms and subsequent MRIs were evaluated to conclude on the final diagnosis (definitive LM versus no LM).

Diagnosis of LM

The diagnosis of LM was considered definitive in case one of the following conditions was fulfilled:
1. positive CSF cytology in the first or repeated lumbar puncture
and/or
2. a follow-up MRI of the brain or neuraxis with gadolinium performed after the diagnostic LP showing unequivocal evidence of LM
and/or
3. progressive neurological symptoms compatible with LM and exclusion of other causes (complication of treatment , infection).

Statistics

Initially, the prevalence of LM was estimated to be 0.70. Further, 0.67 was the highest reported sensitivity in the literature. Total sample size of patients needed was calculated by targeting sensitivity of the new test (flow cytometric CTC assay) to be 0.95, while allowing the lower limit of the 95% confidence interval of 0.67 as the highest reported sensitivity in literature. [16] At the interim analysis the prevalence was adjusted to 0.48 and it was decided to exclude a lower limit of 0.71, which was determined to be the sensitivity of CSF cytology in our study. Using this parameter, at least 30 patients were required in total. Descriptive demographics were used and sensitivity and specificity of used methods were calculated.

RESULTS

Patient characteristics

Thirty-four patients with melanoma, glioblastoma, or solid tumors from epithelial origin with a clinical suspicion on LM or already proven LM were included in the study. A total of 47 CSF samples were collected. Distribution of different tumor types is presented in Table 1.

Twenty-five patients with epithelial primary solid tumors contributed with 34 CSF samples to the study. Of these, sixteen patients had breast cancer (14 ductal, 2 lobular), five had lung cancer (1 SCLC, 4 NSCLC), two had gastrointestinal cancer, one nasopharyngeal cancer and one ovarian cancer. Patients with melanoma (n=8) and glioblastoma (n=1), contributed with 11 and 2 CSF samples respectively.

Three out of sixteen patients with breast cancer had proven LM. They were included in a phase II study with the experimental drug 2B3-101 (glutathione PEGylated liposomal doxorubicin) and were added to the analysis. They contributed with 12 CSF samples; as per study protocol four LPs were performed per patient, one before and three during 2B3-101 treatment. As their diagnosis was already definitive based on MRI or CSF cytology, these patients were not included in the disease prevalence calculation.

In total 18 out of 34 patients were ultimately diagnosed with definitive LM (counting also 3 patients treated with 2B3-101). Fifteen of the 31 enrolled patients clinically suspected for LM but without equivocal evidence for LM on MRI were ultimately diagnosed with LM. The patients with no LM had varying diagnoses like skull base metastases, choroidal metastases, radiation-induced opticopathy, hepatic encephalopathy, clinically isolated (auto-immune) syndrome or chronic daily headache.

Fifteen of the 18 patients (83%) ultimately diagnosed with LM were female and 3 (17%) male. Primary tumors were breast cancer (n=11, 61%), melanoma (n=3, 17%), NSCLC (n=3, 17%) and colon cancer (n= 1, 6%) (Table 2). Fourteen patients (77%) had systemic metastases. Seven patients had concomitant brain metastases (39%). Neurological symptoms and signs were classified as: cerebral symptoms including headache, vomiting, nausea, encephalopathy, dysphasia, dysarthria, ataxia (n=16, 89%); cranial symptoms including decreased or double vision, facial paresis, tinnitus (n=10, 55%); and spinal symptoms including sensibility disorders and/or paresis of arms and legs, impaired miction and/or defecation (n=6, 33%).

CSF pressure and biochemical parameters

Cerebrospinal fluid pressure was increased (> 20 cmH_20) in 8 patients with definitive LM (8/18, 44%). Median CSF pressure in patients with LM was 20 cmH_20 (range 7-50 cmH_20), while in patients with non-LM this was 16 cmH_20 (range 13-30 cmH_20).

An increased total protein level (>0.45 g/l) was present in all 18 patients with definitive LM (median 1.1 g/l, range 0.47-6 g/l). Leucocyte counts were increased in 13 out of 18 patients with LM (72%, range 3.3-62/mm^3) and lactate dehydrogenase

(LDH) levels were increased in 10 out of 18 patients with LM (56%, median 69 U/l, range 41-1287 U/l). Glucose CSF/serum ratio was decreased in 15 out of 18 patients with LM (83%). None of the 34 patients had post-punctional headache after LP.

CSF cytology versus CTCs: solid tumors from epithelial origin

Thirty-four CSF samples were obtained from 25 patients with epithelial primary tumors. Fifteen of these patients had LM and their 24 CSF samples.
In ten patients with LM (with 17 CSF samples) both CSF cytology and the CTC assay were positive.

Five patients with seven CSF samples showed a discrepancy between CSF cytology and the CTC assay (Table 3). While CSF cytology was negative in these samples, the CTC assay showed median 209 CTCs per sample (39.8 CTCs/ml CSF), range 15-358 CTCs (3-46.6 CTCs/ml). Therefore, CSF cytology had 71% sensitivity (95% CI 48.7 – 86.5), while the sensitivity of the CTC assay was 100% (95% CI: 82.8 – 100). Furthermore, both tests were 100% specific (both 95% CI 65.5-100), showing no false positive results. The semiquantitative cytology method had 83% sensitivity (95% CI: 61.8 – 94.5) and 100% specificity (95% CI: 65.5 – 100), performing better than standard CSF cytology, but less than the CTC assay (Table 4).

CSF cytology versus CTCs: melanoma and glioblastoma

Eleven CSF samples were collected from eight patients with melanoma and two samples from one patient with glioblastoma and clinical suspicion on LM. Three patients with melanoma were found to have LM. In two of those patients, CSF cytology and the CTC assay were positive at first lumbar puncture. In one patient there was a discrepancy between CSF cytology and the CTC assay at the first LP. This patient was a 20 years-old female patient with metastasized melanoma with headache and photo- and phonophobia. The first CSF examination showed a negative CSF cytology but 210 CTCs per sample (42 CTCs/ml); a second CSF sample showed a positive CSF cytology and 243 CTCs per sample (48 CTCs/ml). A third LP was performed because of signs of high intracranial pressure. CSF examination showed again positive cytology and 196 CTCs (39.2 CTCs/ml).

One patient with metastasized melanoma, who was treated with a BRAF-inhibitor showed leptomeningeal contrast enhancement on MRI but no definitive diagnosis of LM. CSF cytology was inconclusive, as the pathologist could not make a distinction between large monocytes and melanocytes at first CSF cytology. The CTC assay did not show any CTCs. A second LP was performed, with again negative results for CTCs and CSF cytology. Sudden death occurred in the patient, possibly due to an intracranial hemorrhage. Autopsy was not performed. This patient had a clinical suspicion on LM, although the diagnosis remained unproven.

In the only enrolled patient with glioblastoma, 2 CTC cells per CSF sample were detected using the MCSP CTC assay during both the first and second LP. The final diagnosis was a non-resorptive hydrocephalus due to tumor compression of the cerebral sinuses but no LM.

Currently, the sample size of patients with MCSP positive tumors is too small to prove any significant difference between CSF cytology and the CTC assay, but a similar trend for increased sensitivity of the CTC assay for the diagnosis of LM is seen, like in the EpCam positive tumors.

CTC flow cytometry assay in blood

Eleven of the 18 patients with LM had > 2 CTCs/8 ml of blood (median 6, range 2-2554). All patients had active systemic metastases. Six patients had no CTCs in the blood, 4 of them had no systemic metastases. In one patient no blood sample for the CTC assay was obtained. Four out of six patients with no LM showed >2 CTCs/8ml blood (median 33.5, range 16-102 CTCs). Two CTCs/8 ml were detected in the blood of the patient with a glioblastoma.

DISCUSSION:

This study shows sensitivity (100%) and specificity (100%) of the CTC flow cytometry assay as compared to CSF cytology (sensitivity: 71% specificity: 100%) for the diagnosis of LM in patients with epithelial primary tumors and clinical suspicion on LM without equivocal evidence on MRI for LM or proven LM. A similar trend of increased sensitivity was seen for the MCSP CTC assay as compared to CSF cytology in patients with melanoma and a clinical suspicion on LM without equivocal evidence on MRI for LM.
Using this dual diagnostic method, i.e. standard CSF cytolog
y with a morphological cell assessment by the pathologist and the flow cytometric CTC assay, patients with a clinical suspicion on LM and a non-confirmatory MRI scan can be diagnosed for LM in a timely manner, with a very low chance on false negative results. This will diminish the diagnostic uncertainty of LM both for the patient and its treating (neuro-)oncologist, while preventing multiple lumbar punctures. Furthermore, treatment for LM such as radiotherapy, chemotherapy or targeted agents can be initiated without further delay to prevent progressive neurological symptoms. Adding EpCam CTC CSF immunostaining to the standard cytology in case of LM detection brings higher sensitivity, especially when the CTC count is under 200 cells per sample (<50 cells/ml), as in our study the standard CSF cytology was able to detect LM when CTC count was >1000 cells, but was less sensitive for the cases below 100 cells per sample or up to 200-300 CTCs. Up to now, it is unknown whether CTC measurements can play a role in LM treatment response monitoring, as this is currently being investigated.

Two earlier studies on CTC measurements in the CSF of patients with LM have been performed by Subira et al.[22] and Nayak et al.[16] They included 78 and 51 patients, respectively, with EpCam positive primary tumors and unequivocal evidence for LM on MRI. EpCam positive CTCs in the CSF were found in 75.5% and 100% of these patients, respectively.

In comparison, we enrolled 34 patients, 25 with (metastasized) EpCam positive solid tumors and 9 with MCSP positive solid primary tumors with a clinically suspicion on LM, but a non-confirmatory MRI or already proven LM. This first cohort represents an important patient group with solid tumors in clinical practice with a diagnostic uncertainty, which was not included in the earlier studies. Furthermore, we achieved a higher sensitivity of the CTC flow cytometry assay for EpCam sensitive tumors as compared to the study Subira et al. and anequivocal sensitivity to the study of Nayak et al. The discrepancy in sensitivity of CTC flow cytometry CSF assay for EpCam sensitive tumors between our study and the study of Subira et al. is probably due to an extra cell fixation step that was performed during sample preparation in our CTC assay. Furthermore, in comparison with the commercial test used by Nayak et al., our non-commercial method using only 5 ml CSF can be easily performed with relatively simple and cheap laboratory techniques.[7,15,16] This is the first report of a CTC assay using MCSP antibodies for patients with melanoma or glioblastoma in a substantial group of 9 patients, although a Cell Search based technique for detection of melanoma cells in CSF has been published in 2013. This assay was used in only two patients with LM from melanoma using CD146+, HMW-MAA+, CD34-, and CD45- cell positivity in the CSF.[24]

The CTC assay in the only patient with s glioblastoma showed 2 CTCs both in the CSF and the whole blood sample. However, definitive diagnosis of LM could not be made, as neurological symptoms were probably due to a non-resorptive hydrocephalus accompanying the bifrontal glioblastoma.

In contrast to melanoma, CTC detection in glioblastoma was based only on MCSP positivity because no CD146 expression has been reported for glioblastoma. Therefore, the CTC assay will probably be less specific for glioblastoma as compared to melanoma. This might be the reason that the LLQ in glioblastoma patients has to be higher than the current LLQ of 2 CTCs and that the 2 CTC in the CSF found in this in glioblastoma patient is eventually a negative result.

In conclusion, in the studied cohort the CTC flow cytometry assay has 100% sensitivity and 100% specificity as compared to CSF cytology (sensitivity: 71% and specificity: 100%) for the diagnosis of LM in patients with primary epithelial tumors and clinical suspicion but a non-confirmatory diagnosis of LM on MRI or already proven LM. If confirmed in another independent and representative cohort, we recommend the use of both cytology and CTC flow cytometry assay in CSF of patients clinically suspected for LM. Ongoing research is being performed onthe diagnostic value of a MCSP- antibody based CTC assay in CSF for the diagnosis of

LM in patients with melanoma or glioblastoma. The use of the CTC assay for monitoring treatment effects is currently studied in a phase II clinical study in patients with LM treated with 2B3-101.

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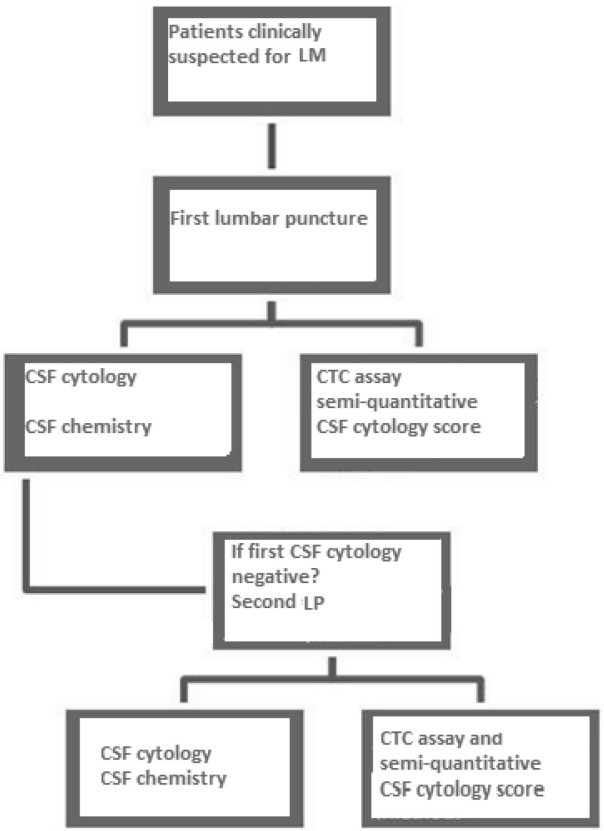

Figure 1. Study design. LM= leptomeningeal metastases, CSF = cerebrospinal fluid. CTC= circulating tumor cells, LP = lumbar puncture

tumor type	no pat. (%)	number of lumbar punctures (LPs)
breast cancer	n=16 (47%)	16 patients with clinical suspicion on LM 3 patients with definitive LM (4 LPs per patient)
melanoma	n=8 (24%)	1 patient : 2 LPs, 1 patient:3 LPs
NSCLC	n=4 (12%)	
neuroendocrine cancer (colon/rectum)	n=2 (6%)	
SCLC	n=1 (3%)	
ovarian cancer	n=1 (3%)	
nasopharyngeal cancer	n=1 (3%)	
glioblastoma	n=1 (3%)	1 patient: 2 LPs
total	n=34	total: 47 LPs

Table 1. Tumor type distribution in included patients. NSCLC – non-small cell lung cancer, SCLC – small cell lung cancer, LM – leptomeningeal metastases, LP – lumbar puncture.

patients with proven LM:	18/34 (52%)
age (years) – mean	53
gender - male	3 (17%)
- female	15 (83%)
WHO - 0	11%
-1	22%
-2	44%
-3	0.5%
primary tumor type: - breast cancer	11 (61%)
- melanoma	3 (17%)
- NSCLC	3 (17%)
- colon	1 (6%)
systemic metastases	14 (77%)
- lymph nodes	10 (55%)
- bone metastases	7 (39%)
- lung metastases	5 (28%)
brain metastases	7 (39%)

Table 2. Characteristics of patient with proven LM. LM - leptomeningeal metastases, WHO – WHO performance status, NSCLC – non-small cell lung cancer.

tumor type	CSF cytology	CTC number per sample/ CTCs per 1 ml CSF	Semi-quantitative CSF cytology score per sample
colon (neuro-endocrine)	negative	24 / 4.8	0
NSCLC	negative	209 / 41.8	2
breast (ductal)	negative	15 / 3	0
breast (ductal)	negative	233 / 46.6	1
breast (ductal) - three consecutive LPs	negative	117 / 18	1
	negative	358 / 39.8	0
	negative	349 / 46.5	0

Table 3. Discrepancies between CSF cytology versus CTC flow cytometry vs semi-quantitative CSF cytology score in 5 patients with EPCAM positive solid tumors and 7 CSF samples in patients with a final diagnosis of proven LM.
NSCLC – non-small cell lung cancer, CSF – cerebrospinal fluid, CTC – circulating tumor cells, LP – lumbar puncture

	SENSITIVITY (%)	SPECIFICITY (%)	PPV (%)	NPV (%)
CSF cytology	71	100	100	59
semi-quantitative CSF cytology score	83	100	100	71
CTC assay	100	100	100	100

Table 4. Sensitivity and specificity of CSF cytology (%), CTC assay and semi-quantitative CSF cytology score for diagnosing leptomeningeal metastases in EPCAM positive tumors. CSF = cerebrospinal fluid, CTC= circulating tumor cells; PPV= positive predictive value; NPV= negative predictive value.

3.5. Quantification of circulating melanoma cells in peripheral blood and cerebrospinal fluid by positive immunomagnetic enrichment and multi-parameter flow cytometry

Dick Pluim[a], Bojana Milojkovic Kerklaan[a,b], Dieta Brandsma[c], Jos H. Beijnen[d,e], Jan H. M. Schellens[a,b,d,e]

a - Department of Molecular Pathology,
b - Department of Clinical Pharmacology,
c - Department of Neuro-oncology,
d - Department of Pharmacy & Pharmacology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands;
e - Science faculty, Dept. Pharmaceutical Sciences, Div. Pharmaco-epidemiology and Clinical Pharmacology, University of Utrecht, Sorbonnelaan 14 – 16, 3584 CA Utrecht, The Netherlands

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ABSTRACT

Background:

Evidence is mounting for the importance of circulating melanoma cells (CMCs) as biomarker for overall survival of melanoma patients. Investigations are hampered by a lack of thoroughly validated protocols that miss sensitivity and specificity, are uneconomical, and lack objectivity due to reliance on CMC identification by human observation.

Methods:

CMCs were enriched by melanoma-associated chondroitin sulfate proteoglycan (MCSP) immunomagnetic cell sorting with subsequent detection by fluorescent-activated cell sorting (FACS) using antibodies against MCSP, CD146, CD45, and Hoechst33258 for DNA staining to distinguish apoptotic cells.

Results:

The method was highly sensitive with only 0.3 ± 0.8 background events, and lower limit of quantification (LLOQ) of 2 melanoma cells per 8 ml of whole blood. We detected CMCs (mean = 9.8, range 2 - 33) in 8 ml of whole blood from 82% (11 stage 3 - 4 metastatic melanoma patients, n = 3 per patient). Two melanoma patients with confirmed leptomeningeal metastasis (LM) had > 10,000 CMCs per 7.5 ml of CSF. The CSF from another 5 patients with definitively no LM contained 0 CMCs.

Conclusions: A FACS method has been developed and validated for the enumeration and classification of CMCs based on DNA content for blood and CSF. The application of standard laboratory equipment and techniques allows wide spread use in clinical trials.

Keywords:
Circulating melanoma cells; CMC; CD146; MCSP; magnetic cell sorting; FACS

INTRODUCTION

Circulating melanoma cells (CMCs) disseminate from solid tumor sites and are seen as a prerequisite for the development of distant metastases. Both the quantity and quality of CMCs determine metastatic success and hence the fate of the individual patient [1,2]. Central nervous system (CNS) metastasis including leptomeningeal metastasis (LM) is frequent in melanoma [3]. Median overall survival (OS) of LM is only 4 – 6 weeks [4-6]. The present techniques of choice for LM diagnosis are magnetic resonance induction with gandolinium enhancement (MRI-Gd) and cytological identification by Wright-Giemsa staining in CSF [7]. Both methods lack the opportunity for CMC quantification, while their sensitivity and specificity for prognostic application are low even after repeated lumbar punctures.

In the past 8 years technologies for the identification of intact circulating tumor cells (CTCs) have emerged [8]. Most cellular approaches for CTC identification are based on immunomagnetic enrichment using specific surface markers, and subsequent detection by flow cytometry or immunohistochemistry (IHC). An example of this approach, using immunomagnetic EpCAM capture beads, is the US Food and Drug Administration approved CellSearch platform, which is the current gold standard for the prognosis and follow-up assessments of patients with epithelial cancer [9-12]. Numerous studies with epithelial cancers have validated CTCs as adverse prognostic markers for disease free and overall survival [13]. However, for melanoma relatively few studies have reported the prognostic value of CMCs in blood due to a lack of thoroughly validated CMC quantification protocols with low variation in sensitivity and specificity [8].

Recently, the CellSearch technology was adapted for the identification of CMCs in blood and CSF, using CD146 for immunomagnetic capture with subsequent detection of CMCs by melanoma-associated chondroitin sulfate proteoglycan (MCSP), CD34 (for detection of contaminating endothelial cells), and CD45 (for detection of contaminating leukocytes) [3]. MCSP and CD146 are expressed on > 85% of melanomas [14,15]. The adapted CellSearch method was used in 2 clinical studies that reported shorter overall survival in patients with 2 or more CMCs per 7.5 ml of whole blood [16,17]. Preliminary data show superiority of this method in terms of quantification, sensitivity and precision when compared to the current gold standard of cytomorphological analysis [3].
However, despite these promising results the adapted CellSearch method has also some drawbacks: necessary expensive special equipment prevents wide-spread adoption; final CMC identification depends on human observation; and the CD146 capture antigen is not specific for CMCs as endothelial cells and a subset of T and B cells also express CD146 [18].

In this paper we present the development and validation of a multi-parameter fluorescent-activated cell sorting (FACS) method for the enumeration of CMCs in peripheral blood and CSF. Our method is based on the immunomagnetic enrichment of cells expressing MCSP. In contrast to CD146, MCSP expression in peripheral blood is highly specific for CMCs. Therefore, no extra selection with the endothelial cell marker CD34 was necessary. CMCs were defined by immunofluorescence detection of MCSP, CD146, and CD45 (for detection of contaminating leukocytes). Finally, Hoechst staining was used to distinguish between CMCs with low (apoptotic cells) and normal-high DNA content (vital cells with high metastatic potential).
The method is based on fully automative CMCs detection using commonly available laboratory equipment and commercially available antibodies, and was thoroughly validated for sensitivity, linearity, reproducibility, and stability. The feasibility of the method was demonstrated in peripheral blood and CSF samples from patients with advanced metastatic melanoma.

MATERIALS AND METHODS

Reagents and chemicals

Milli-Q grade (Millipore, USA) water was used. Phosphate buffered saline (PBS) and RPMI medium were purchased from GIBCO BRL (Gaithersburg, USA). Neutral buffered methanol-free 40% formaldehyde was prepared from paraformaldehyde purchased from Merck (Darmstadt, Germany). Hoechst33258 was purchased from Sigma (St. Louis, USA). Anti-human MCSP-Micro-Beads, MS Magnetic antibody cell sorting (MACS®) columns, Fc-Receptor block (FcR), mouse clone 5B1 IgG2a anti-human CD45 labeled with fluorescein isothiocyanate (FITC) or allophycocyanin (APC), mouse clone EP-1 IgG1 MCSP-phycoerythrin (PE), and mouse clone 541-10B2 IgG1 CD146-allophycocyanin (APC) were purchased from Miltenyi (Bergisch Gladbach, Germany). Perm/Wash™ (P/W) was purchased from Becton Dickinson (Heidelberg, Germany). Beads buffer (BB) was PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA degassed by sonication for 10 min. All buffers and formaldehyde solution were filtered through 0.22 µm filters before use.

Subjects and sample collection

Subjects asked for study participation included 20 healthy volunteers ≥ 21 years of age, not known with cancer, not treated with investigational or other drugs within 30 days before start of the study, and who had not undergone surgery within the past six months.

Blood samples from 11 cancer patients with stage III or IV advanced melanoma were used for determination of the method sensitivity. Patients had not been on treatment for at least 4 weeks before whole blood was drawn for determination of CMC counts. For each subject three 8 ml cell preparation tubes (BD Vacutainer® CPT™) were used containing a Ficoll-Hypaque density fluid separated by a polyester gel barrier from a sodium citrate anticoagulant.

Aliquots of 7.5 ml of CSF were drawn from 7 patients suspected of LM melanoma metastasis within a running diagnostic study at the Netherlands Cancer Institute. Two of these patients were definitively diagnosed with LM metastasis during follow-up appointments based on a combination of neurological symptoms, MRI evaluation, and tumor cell cytology. 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

Human melanoma cell lines A375, M19MEL, MELJUSO, SK-MEL-28 (from ATCC, Rockvile, USA) 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 (BD Biosciences, USA).

Centrifugation and pellet resuspension

Unless stated otherwise, all centrifugations were performed in 2 ml eppendorf tubes in a centrifuge equipped with a swing-out rotor at 1,000g for 4 min at 4°C. After centrifugation the supernatant was removed with a 1 ml pipet leaving 100 µl on the pellet. The pellet was resuspended in the remaining supernatant by vortex mixing at 50% speed setting.

Sample pre-processing

CPT tubes containing 8 ml of peripheral blood were centrifuged in a swing-out rotor at 1,500g for 25 min at ambient temperature (RT). Next, the upper CPT layer was transferred to a 50 ml tube. The CPT tubes were washed with 3 ml physiologic salt, which was pooled with the rest of the sample. CSF was collected in 50 ml tubes. Sample volume was adjusted to 9 ml with physiological salt and 1 ml of 40% formaldehyde was added. After vortex mixing at half speed for 10 s, samples were incubated for 15 min at RT. Next, sample volume was adjusted to 50 ml using physiologic salt, followed by centrifugation at 1,000g for 10 min at 4°C. The supernatant was decanted, followed by placing the tubes upside down on filter paper for 5 s, 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 s at the highest setting. The samples were stored at -80°C for future analysis.

Cell recovery optimization

M19MEL cells were spiked at 10,000 cells in 35 CPT tubes each containing 8 ml of blood from healthy volunteers. Samples were pre-processed as described above and incubated with 40 µl of FcR-block for 1 h at RT. Next, 24 samples were incubated in triplicate at 0 °C and 37 °C for 1 h with, respectively, 10 µl, and 0, 0.25, 0.5, 1, 2.5, 5, 10 µl of anti-MCSP-Micro-Beads. An additional 21 samples were incubated in triplicate at RT with 2.5 µl of anti-MCSP-Micro-Beads for 0, 0.08, 0.25, 0.5, 1, 2, and 4 h. Next, the tumor cells were isolated by tumor cell enrichment. The input control samples consisted of 10,000 M19MEL in 100 µl P/W. After immunofluorescent staining, the cell recovery and total event counts were determined by FACS.

Tumor cell enrichment

CMCs underwent an immuno-magnetic enrichment using anti-MCSP-Micro-Beads, and FcR-block, with the following modifications to the manufacturer's protocol: Samples stored at – 80°C were defrosted on ice. After centrifugation, supernatant was removed and the cell pellets were washed twice with ice-cold BB. After centrifugation, the pellets were resuspended in the remaining 100 µl BB. Next, 40 ul of FcR was added and the samples were incubated for 1 h at RT. Subsequently, a volume of 2.5 µl of anti-MCSP-Micro-Beads was added, and the samples were incubated for an additional 1 h at RT. Next, samples were washed twice with 1 ml of BB, followed by centrifugation. After discarding the superna-

tant, the cell pellets were resuspended in 500 µl of BB. Subsequently, labeled cells were separated using a MS column. After removal of the column from the magnetic field, the retained $MCSP^+$ cells were eluted into 2 ml eppendorf tubes using two volumes of 1 ml BB. After centrifugation, supernatant was removed and the pellet was resuspended.

(Immuno)fluorescence staining

The CMC enriched samples were stained in 100 µl P/W containing 10 µM Hoechst33258, 0.25 µl CD146-APC, 5 µl anti-MCSP-PE, and 5 µl anti-CD45-FITC for 1 h at RT. Next, samples were washed twice with 1 ml of P/W followed by centrifugation.

CD45, MCSP, CD146 and DNA staining linearity were measured in triplicate in samples spiked with 10,000 SK-MEL-28 cells in CPT tubes containing 8 ml peripheral blood from a healthy volunteer. Subsequently, samples were stained with 0, 0.25, 0.5, 1, 2, 5, 10, and 20 µl of anti-CD45-FITC and anti-MCSP-PE, 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 µl of anti-CD146-APC, and 10 µM of Hoechst33258 for 1h at RT. Next, samples were washed twice with 1 ml P/W and mean fluorescence intensity (MFI) was measured by FACS.

Fluorescence-activated cell sorting

FACS analysis was performed using a CyAn ADP™ (Beckman Coulter, Brea, USA). Hoechst33258, FITC, PE, and APC 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, Fort Collins, USA).

Specificity

In order to assess the background level, three CPT tubes containing 8 ml peripheral blood were drawn from 20 healthy volunteers. Background levels in CSF were determined using 7 ml of CSF from 8 patients diagnosed as LM negative based on cytology, and/or MRI. The background was defined as the total number of events in gate 1 of the FSC/SSC density plots (Fig. 1A).

Within- and between-day precision and recovery

CPT tubes containing 8 ml of peripheral blood were spiked with 10, 100, 1,000, and 5,000 SK-MEL-28, and M19MEL cells with 10 times less expression of MCSP. After pre-processing as described, samples were measured in triplicate on three consecutive days. The between-day (BDP) and within-day precision (WDP) were calculated by one-way analysis of variance (ANOVA) for each spike level using the run day as classification variable using the software package SPSS v15.0 for windows (SPSS, Chicago, USA). The day mean square (DayMS), error mean square

(ErrMS) and the grand mean (GM) of the observed cell concentrations across run days were used. The WDP% and BDP% for each spike level was calculated using the formulas:

$WDP\% = (ErrMS)^{0.5} / GM \times 100\%$
$BDP\% = [(DayMS - ErrMS)/n]^{0.5} / GM \times 100\%$
(Where n is the number of replicates within each run).
The accuracy = GM / nominal cell concentration x 100%.
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 SK-MEL-28 cells in CPT tubes containing 8 ml of peripheral blood from six different healthy volunteers. The LLOQ was defined as the cell concentration that could be determined with a precision of 0 - 20%, and a recovery between 80 – 120% of the nominal value.

Sample stability

Long term storage stability was assessed by spiking 21 CPT tubes, containing 8 ml peripheral blood from a healthy volunteer, with 1000 SK-MEL-28 cells each. Three samples per time point were processed and stored in 50% MeOH at -80°C for 0, 1, 14, 30, 60, 180, and 360 days until analysis. We also assessed the stability of stained samples in triplicate after storage at 4 - 7°C in the refrigerator for 0, 4, and 24 until analysis.

CTC morphology

Stained and processed CSF samples from two melanoma patients were sorted using a FACSaria™ cell sorter with gates set for PBMCs and CMCs as shown in Figure 1. After centrifugation the cell pellet was resuspended in 100 µl of BB and stained with 5 ul of anti-CD45-APC for 1 h at RT. After washing twice with 1 ml of MQ, samples were resuspended in 10 µl of MQ and transferred to a microscope slide. The slide was dried for 5 min at 30°C by vacuum concentration in a Speed-Vac (Savant, Rarmindale, USA). After applying 3 µl of Vectashield H-1000 (Vector, Burlingame, USA) a round object glass of 1 cm in diameter was put on top and sealed with nail polish. CTCs were identified and photographed using a SP5 confocal fluorescence microscope (Leica, Rijswijk, The Netherlands).

Statistical Analysis

Statistical evaluation was performed using the Student's t-test unless indicated otherwise. P-values of 0.05 were considered to be significant.

RESULTS

Method development

Discrimination of CTCs from the bulk of blood cells with red blood cell lysis is impossible due to more than 90% reduction of MCSP staining (data not shown). Therefore, density gradient centrifugation with CPT tubes was used. The gel barrier in these tubes separates the blood cells over two compartments. The lower compartment contains red blood cells and granulocytes. The upper compartment consists of CMCs, PBMCs, and platelets. Further, enrichment of CMCs was achieved by MACS using anti-human EpCAM-Micro-Beads.

For removal of protein aggregates that can bind nonspecifically to antibodies, which resulted in false positive CMC counts (data not shown), all antibody solutions except FcR-block and MCSP-Micro Beads were centrifuged at 10,000g for 10 min at 4°C. Furthermore, buffers were filtered through 0.2 µm syringe filters. The use of wing-out rotors for centrifugations prevented the loss of 10% of cells observed with fixed angle rotors. CMCs were identified based on double positivity for MCSP and CD146 in combination with CD45 negativity (Fig. 1). Release of adherent cells from the culture plates by trypsinisation reduced MCSP recognition by more than 90% (data not shown). Therefore, we used 10 mM EDTA for 5 min at 37°C to release adherent cells from the culture plates. Negative and positive quality controls were prepared by spiking, respectively, 0 and 1,000 SK-MEL-28 in the upper layer of centrifuged CPT tubes containing 8 ml of whole blood from a healthy volunteer. QCs were formaldehyde fixed and stably stored for a maximum of 360 days in 50% methanol/PBS at -80°C. The background from negative QCs was always ≤ 1, and the recovery of SK-MEL-28 cells from positive QCs was always ≥ 90 ± 6%. CMCs were considered to be nucleated if the Hoechst33258 MFI was above the indicated DNA cut-off level determined from endogenous PBMCs present in the same sample (Fig. 1I).

Staining Linearity

Staining linearity was determined in triplicate in samples containing 10,000 SK-MEL-28 cells spiked in CPT tubes containing 8 ml of peripheral blood from a healthy volunteer. CD146 and MCSP were maximally stained with, respectively, an MFI of 2545 ± 182 and 771 ± 31 using 0.25 and 5 µl of anti-CD146-APC and anti-MCSP-PE. Staining did not significantly increase at higher antibody concentrations.

Specificity

The amount of background counts in the CMC gate during FACS analysis was 4 ± 0.6 false positive CMCs per 8 ml of peripheral blood (n = 18 healthy volunteers in triplicate) if MCSP positivity in combination with CD45 negativity were used as criteria for CMC identification after the FS/SSC gating (Fig.1A+B). Specificity strongly

improved to 0.3 ± 0.8 false positive CMC counts when CD146 positivity was included as marker for CMC identification. Aliquots of 7.5 ml of CSF from 5 patients who were diagnosed with definitive negative LM contained 0 CMC / 7.5 ml CSF (Fig. 1E).

Cell recovery optimization

The recovery of M19MEL cells after the anti-MCSP-Micro-Beads enrichment step was significantly affected by the amount of anti-MCSP-Micro-Beads and incubation temperature (Fig. 2). The use of an extra volume of 1 ml of BB for elution of CMCs from the MACS® columns resulted in 11% increase of cell recovery (P = 0.002, data not shown). The maximum M19MEL recovery of 28.1% was obtained after 1 h of incubation (Fig. 2A) using 2.5 µl of anti-MCSP-Micro-Beads (Fig. 2B). Under these conditions recovery of SK-MEL-28, with about 10 times higher MCSP levels as compared to M19MEL, was 70 -80% (Table 1). Cell recovery did not significantly increase after prolonged incubation, or with more anti-MCSP-Micro-Beads.

Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was determined in 8 ml peripheral whole blood samples from six different volunteers spiked with 0, 1, 2, 3, 4, and 5 SK-MEL-28 cells per CPT. The determined LLOQ of the method was 2 cells in 8 ml of whole blood. At this LLOQ the recovery was 105% and the precision 16.9%.

Within- and between-day precision and recovery

The precision and recovery of the method were determined by analyses of samples spiked with SK-MEL-28 at four different cell concentrations in triplicate in three consecutive 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 [19]. An exception was the low recovery of M19MEL.

Sample stability

We determined the stability of CD146, MCSP, CD45, DNA, and cell counts for SK-MEL-28 cells spiked in 8 ml peripheral blood samples after storage at – 80°C for incremental time periods. All parameters were stable for at least 12 months. The fluorescent signals from stained CD146, MCSP, CD45, DNA, and SK-MEL-28 cell counts were also stable during the tested 24 h storage period at 4 – 7°C.

CTC morphology

CTCs isolated from 5 ml of CSF from two patients were processed and sorted by FACS. Stained DNA, MCSP, and CD45 were visualized by confocal fluorescence microscopy as blue (Hoechst 33258), green (anti-MCSP-PE), and red (anti-CD45-APC) colours, respectively. The diameter of the CMCs varied between 12 and 22 µm (Fig.

3). Most CMCs had an oval or irregular shape, with lobular nuclei and a fluorescent green membrane due to specific anti-MCSP-PE staining. CMCs were easily distinguished from PBMCs, which were smaller with a mean diameter of 8.0 ± 1.5 µm, had a more round shape and condensed nucleus, and a bright red cell membrane due to specific anti-CD45-APC staining. No PBMCs were detected in the CMC gate and vice versa no CMCs were detected in the PBMC gate.

Applicability of method for determination of CMCs in patient samples

We determined in triplicate the number of CMCs in samples from 11 advanced metastatic melanoma patients. In 82% of patients two or more (mean = 9.8, range 2 - 33) CMCs were detected in 8 ml of whole blood. The correlation between the measured CTC counts in two subsequent CPT tubes was significant (P = 0.012) with Pearson correlation coefficient of 0.94 (Fig. 4). The percentage of nucleated CMCs isolated from blood was 27.5% (range 16.7 – 40.0 %).
Two patients diagnosed positive for LM had 13754 and 40567 CMCs / 7.5 ml of CSF, of which, respectively, 97.8% and 99.8% of CMCs were nucleated.

DISCUSSION

This is the first report to demonstrate the use of immunomagnetic cell enrichment in combination with FACS for the quantification of circulating melanoma cells and their DNA content in both peripheral whole blood and CSF. In contrast to the CellSearch method, we based our method on fixed gate settings and fully automative CMC identification to avoid operator bias. Furthermore, instead of CD146, we used the relatively more specific MCSP marker for immunomagnetic enrichment of CMCs. Therefore we were able to omit the CD34 selection, used in the CellSearch method for identification of contaminating endothelial cells.

We showed that the method was able to recover 70 - 80% of SK-MEL-28 cells, which was considered acceptable considering the complexity of the assay. Furthermore, a reasonable recovery of 28.1% was found of M19MEL cells, which express approximately 10 times less MCSP as compared to SK-MEL-28. In our experience the MCSP expression in SK-MEL-28 is more representative of the average MCSP expression in human CMCs. The method is accurate and reproducible up to 2 cells per 8 ml of whole blood (LLOQ). By analyzing different spike levels, we were able to show that the quantification of CMCs was linear over the whole tested spike range of 10 to 10,000 SK-MEL-28 cells. The mean within-run precision and between-run precision were 11.4% and 7.0%, respectively. Long-term stability of samples stored at -80°C was good with no detectable degradation of cell numbers, MCSP, CD146, CD45, and DNA after 12 months of storage. Furthermore, samples were stable for up to 24 hours of storage in the refrigerator. Method performance was successfully monitored by inclusion of positive and negative control samples in each analytical run.

Our results show that the method can be applied for the quantification of CMCs and their DNA content in CMCs from patient whole blood and CSF. We observed CMCs in 82% of patients in blood (n=11), which is higher than for previously reported methods [8,20]. Both CSF samples from patients with confirmed LM contained CMCs at higher levels than previously reported [3]. Cytomorphological analysis of CMCs isolated from these two CSF samples clearly identified all of the FACS events in the CMC gate as CMCs (Fig. 3). We were not able to perform the same analysis for whole blood due to the relatively low concentration of CMCs in blood as compared to CSF. Employing our method we found an average of 72.5% apoptotic CMCs in whole blood (n = 33). Others, have reported 50% to 80% apoptotic CTCs for tumors of epithelial origin, using the CellSearch® method that doesn't include a density gradient centrifugation CTC enrichment step [21]. Therefore, apoptotic CMCs seem retained in the upper CPT layer despite an increase in buoyant density of apoptotic cells [22]. In contrast, only 0.2% and 2.2% of CMCs were apoptotic in the two CSF samples from different patients. This is the first report showing relatively low apoptotic CMC amounts in CSF as compared to whole blood. Recently, we found similar high vitality of CTCs from epithelial origin in CSF when compared to whole blood (data in submission). Our results confirm other reports that CTCs in whole blood exhibit a high degree of pleomorphism i.e. high and low nuclear-to-cytoplasmic ratios, and early and late apoptotic changes [23,24]. These apoptotic characteristics might explain why the presence of CTCs is necessary but not sufficient for the metastatic process to occur [25]. Evidence for the importance of DNA content for the clonogenity and metastatic potential of CTCs is mounting [26]. Therefore, monitoring of this biomarker might improve prediction of clinical outcome for patients.

In conclusion, a method has been developed and validated for the enumeration and classification of CMCs based on DNA content for blood and CSF. The method is straightforward with long-term stability, and application of standard laboratory equipment and techniques allow wide spread use in clinical trials. The method is currently successfully validated against cytomorphological analysis in a clinical trial with melanoma patients with suspicion of LM.

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Figures and Legends

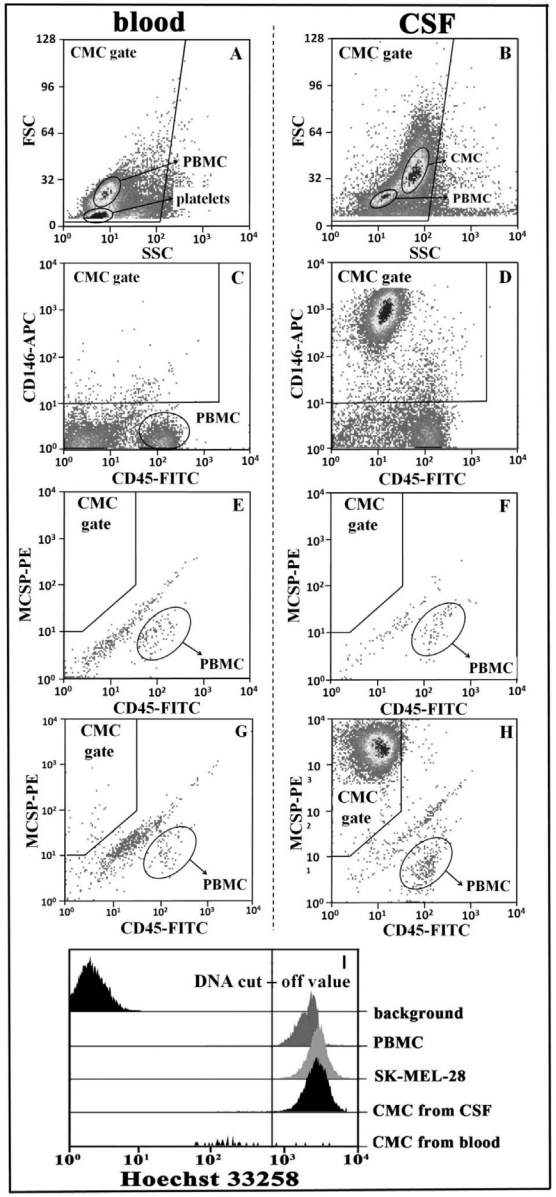


Fig.1. Representative fluorescence activated cell sorting (FACS) plots with indicated gate settings used for circulating melanoma cells (CMC) enumeration and DNA content determination. Samples E and F are negative controls from a healthy volunteer and a melanoma patient with no leptomeningeal metastasis (LM), respectively. All other samples are from melanoma patients with confirmed LM. In step 1 (A and B), CMCs are gated by forward and sideward scatter characteristics for size and cell complexity to eliminate debris. In step 2 (C and D), CMCs are gated for CD146-APC positivity. In step 3 (G and H) CMCs are gated for MCSP-PE and CD45-FITC double positivity to eliminate platelets and peripheral blood mononuclear cells (PBMCs). In step 4 (I), the DNA content of platelets (background), PBMCs, SK-MEL-28, and CMCs isolated from blood and cerebrospinal fluid (CSF) are shown. The DNA cut-off value was used to differentiate between CMCs with low and normal to high DNA content.

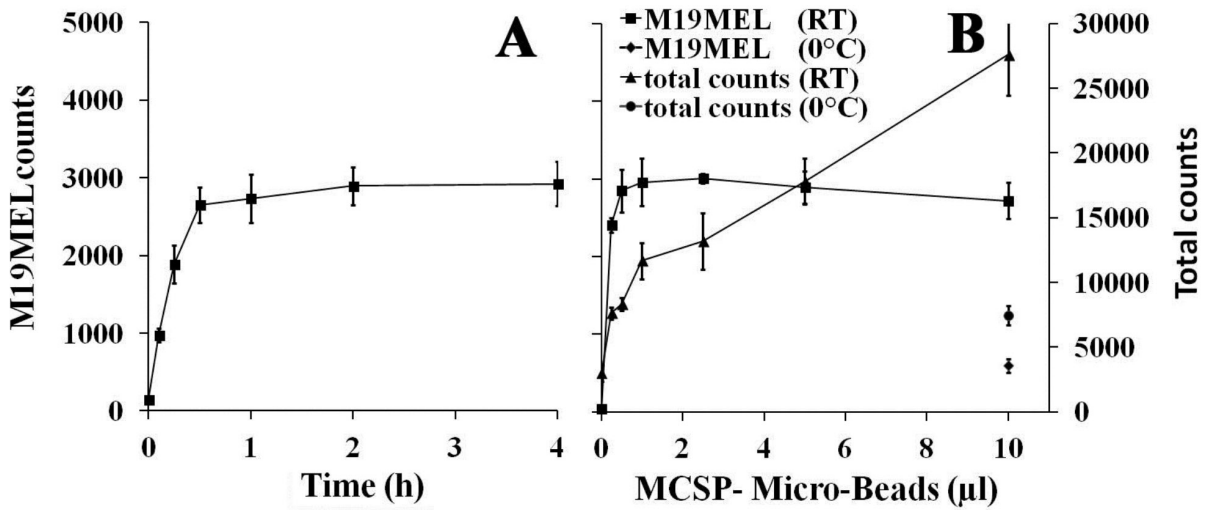
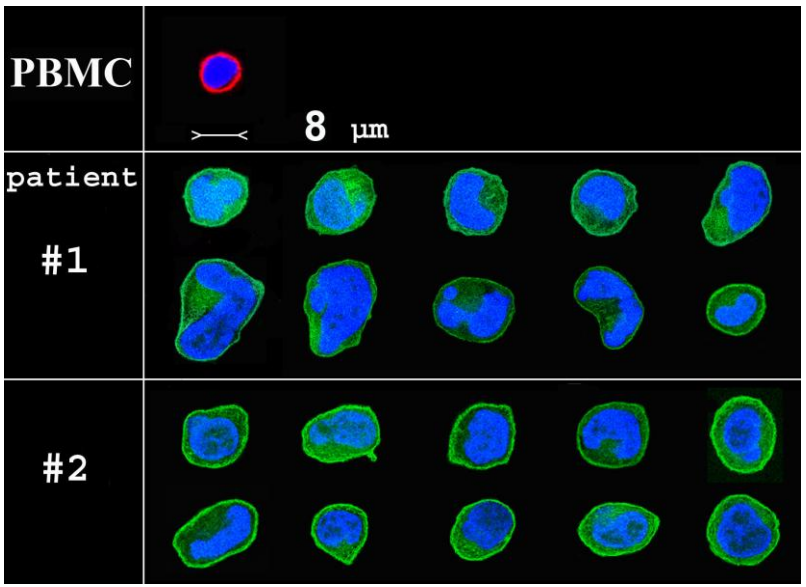


Fig.2. Total background events and recovery of 10,000 M19MEL cells spiked in 8 ml peripheral blood from a healthy volunteer, respectively, indicated incubation time using 4 µl of anti-MCSP-Micro-Beads (Fig. 2A), or different amounts of anti-MCSP-Micro-Beads (Fig. 2B). Data is expressed as means ± S.D. of three different samples.

Fig.3. Morphology of representative circulating melanoma cells (CMCs), isolated from 7.5 ml of cerebrospinal fluid (CSF) from two metastatic melanoma patients (# 1 and # 2), sorted by fluorescence-activated cell sorting (FACS) using the CMC gate setting (Fig.1H). CD45, Hoechst33258, and MCSP are visible as red, blue, and green colors, respectively. An example of a typical PBMC sorted by FACS using the PBMC gate setting (Fig.1H) is included. Imaging was performed at 100 times magnification.



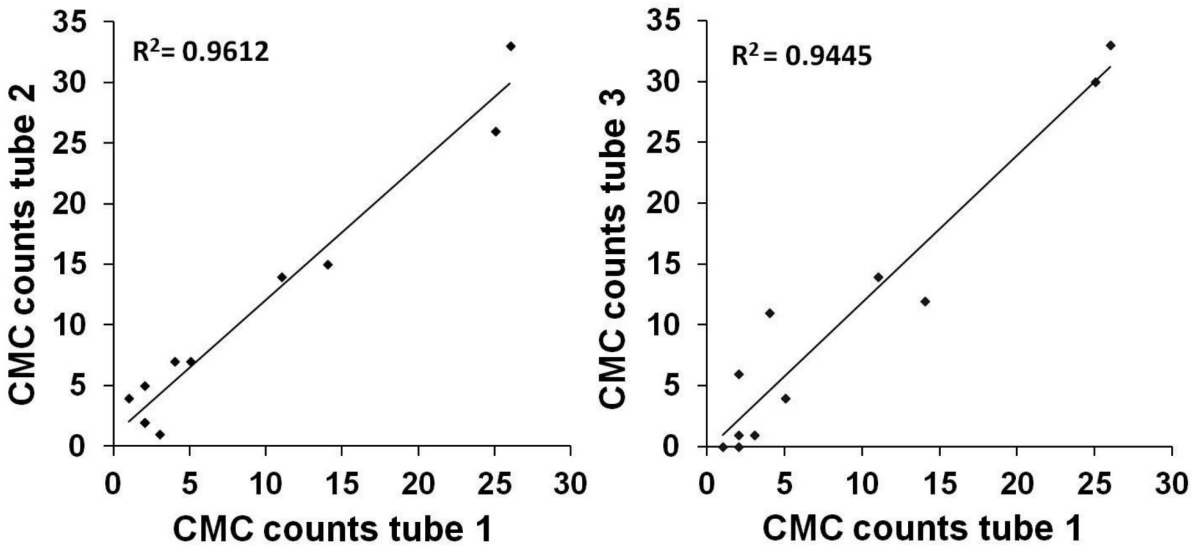


Fig. 4. Tube-to-tube variation of circulating melanoma cells (CMC) counts in 3 sequentially drawn volumes of 8 ml peripheral blood from 11 metastatic melanoma patients. The lower limit of quantification (LLOQ) of 2 CMCs per 8 ml is indicated by the dashed line.

CHAPTER 4: CONCLUSIONS AND PERSPECTIVES

This closing chapter recapitulates the main findings from the presented phase I studies **(chapter one)**, in particular the newly seen interaction between two already in use but now combined oral anti-cancer drugs, topotecan and pazopanib **(1.1)**, other novel agents with oral formulations i.e. α2-integrin inhibitor E7820 , MEK and Ras-inhibitor lonafarnib **(1.2 1.3 and 1.5)** and a proof-of concept study with a continuous intravenous delivery pump of integrin antagonist GLPG0187 **(1.4). In chapter two** a placebo-controlled trial with candesartan to prevent cardiotoxicity in the treatment with trastuzumab is being discussed. A review of the literature in **chapter 3 (3.1)** describes the current (experimental) strategies to bring active drugs over the blood-brain barrier and blood-cerebrospinal fluid barrier. One of the proposed strategies, a new brain-targeted liposomal formulation of doxorubicin, 2B3-101, was investigated in a phase I and II study in patients with brain metastases or recurrent malignant glioma or patients with leptomeningeal metastases from breast cancer **(chapter 3, 3.2 and 3.3)**. Finally, chapter 3.4 and 3.5 focus on the accuracy of EPCAM- and MCSP-based immuno-assays to detect malignant cells in the cerebrospinal fluid (CSF) of patients with a clinical suspicion on leptomeningeal metastases (LM).

Pharmacokinetics of oral anti-cancer drugs were studied in the four presented phase I studies with pazopanib in combination with topotecan, E7820, pimasertib and lonafarnib. Oral formulations have several advantages in comparison to intravenous administrations. Oral treatment is non-invasive, convenient for patients as drugs can be taken at home and it is less expensive. However, several factors affect the absolute bioavailability of drugs, i.e., the amount of drug that passes across the gut into the portal system and from there through the liver into the systemic circulation, drug solubility in gastro-intestinal (GI) fluids, including presystemic intestinal drug degradation, its permeability (via passive and active transport) of biologic membranes, affinity for drug efflux transporters and hepatic first pass metabolism. In the first study **(chapter 1, 1.1)** pazopanib substantially increased exposure of total topotecan by 1.8-fold for Cmax and 1.7-fold for AUC(0-∞), which is considered clinically relevant, but did not increase t½ values, when compared with topotecan alone. This suggests that the effects of pazopanib on topotecan pharmacokinetics were pre-systemic and were not related to changes in systemic elimination. Efflux transporters, BCRP (ABCG2) and P-glycoprotein (P-gp, MDR1; ABCB1) located in the epithelial layer of the intestine play an important role in limiting oral pazopanib and topotecan absorption from the gut.1,2 While both topotecan and pazopanib are found to be high-affinity substrates for BCRP, topotecan is a weak and pazopanib a moderate substrate for P-gp.1–4 Pazopanib plasma concentrations were similar after pazopanib treatment as a single agent as compared to pazopanib treatment in combination with topotecan. The measured pazopanib plasma concentrations were all > 20.6 μg/ml, values that are associated with longer progression free survival (PFS).5 This finding improves our knowledge about the drug-drug interaction between those two drugs and may help to prevent overtreatment with

topotecan when combined with pazopanib. Furthermore, our study showed that the severe hematological toxicities (grade ≥ 3) were not reported more frequently in the weekly schedule of topotecan and pazopanib co-administration, than in a study using weekly topotecan administration.6 This is likely due to overlapping total topotecan concentrations in both studies. The daily-times-five regimen resulted in more severe hematologic toxicity in comparison with the weekly topotecan regimen, whereas the dose density of the recommended topotecan dose in the daily-times-five regimen was lower (4.16 mg/wk vs 6.0 mg/wk). Fatal liver necrosis occurred in one patient while grade 4 toxic hepatitis after pazopanib exposure developed also in another patient with liver metastases. Liver necrosis could have been affected by the ABCB1 gene polymorphism, concomitant administration of topotecan and pazopanib and concomitant paracetamol treatment. However, no in-depth pharmacogenetic investigations have been performed to unravel whether there are specific single nucleotide polymorphisms (SNPs) that might put patients at higher risk of liver toxicity when treated with tyrosine-kinase inhibitors (TKIs). Further pharmacogenetic investigations in patients treated with TKI could provide essential information in preventing liver toxicity in this patient group and such analysis is warranted.

The oral sulfonamide (E7820), inhibiting α2-integrin mRNA expression in patients with unresectable solid tumors was studied in **chapter 1, 1.2.** Firstly, it was shown that bi-daily treatment with E7820 was safe and well tolerated. Secondly, it was demonstrated that food intake did not significantly influence pharmacokinetics of oral E7820. The most effective daily dose of 200 mg E7820 (100 mg bi-daily) based on pharmacokinetic/ pharmacodynamic modeling and simulation analysis integrating data from (pre)clinical studies was unfortunately above the maximal tolerated dose-level (MTD). In line with this, the pharmacodynamic marker of this treatment, the expression of platelet integrin-α2, showed a decrease of only 8.9% from baseline following treatment. However, two thirds of the treated patients achieved stable disease (with a median duration of treatment of 60 days and in four patients (10%) a treatment-duration of more than 6 cycles (168 days) was achieved. Possible future studies with combination treatment with other chemotherapies may have more effect.

The results of another study with GLPG018, an αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α5β1 integrin inhibitor is described in **chapter 1, 1.4.** Due to its low oral bio-availability and short half-life GLPG0187 needs to be administered continuously with a 24h intravenous infusion. GLPG0187 was shown to be well-tolerated and displayed a dose-proportional increase in exposure with a short terminal half-life (T1/2 = 3.75h). The effect of GLPG0187 treatment was monitored with the bone resorption marker, carboxy-terminal collagen crosslinks (CTX), and showed a statistically significant decrease in CTX levels after treatment. Although GLPG0187 administration resulted in in vitro necrosis and autophagy of glioma cells, no anti-tumor effect was seen in patients with glioblastoma, probably due to an insufficient capacity to cross the blood-brain barrier (BBB).

In **chapter one (1.3)** MEK inhibitor pimasertib (MSC1936369B) was given orally to subjects with solid tumors. Four schedules were investigated and 180 patients were enrolled. Pimasertib showed to be a relatively safe single agent treatment with recommended dose for phase II (RDII) of 120 mg/day (60 mg BID) for patients with melanoma and NRAS or BRAF mutations. It was rapidly absorbed reaching maximal drug concentrations in plasma within Tmax=1h. No food effect could be seen as AUC0-inf during the fed status vs AUC0-t during the fasted status showed ratio 100.7%, 90%CI of 77.3-131.2. Terminal half-life was T1/2=5.1h with negligible renal elimination of unchanged pimasertib. At doses ≥ 28 mg/day PD marker, pERK inhibition in a peripheral blood mononuclear cell (PBMC) was fully inhibited at 2h and returned to baseline at 24h.

In **chapter one (1.5)** the results of a phase I study on the oral use of the Ras inhibitor lonafarnib-SCH66336 combined with chemotherapy was described. Lonafarnib is administered in a bi-daily manner in combination with paclitaxel 3-weekly and trastuzumab weekly in patients with HER2+ advanced breast cancer. 7 Pharmacokinetic analysis supported the bi-daily dosing of lonafarnib as the plasma concentrations showed a significant decrease after 12 h after lonafarnib intake. Increases in Cmax and AUC values of lonafarnib were dose-proportional. The difference in pharmacokinetics of lonafarnib as a single agent or in combination with the other two drugs was not significant, despite the fact that the metabolism of paclitaxel and lonafarnib is partially mediated by the same enzymes. While paclitaxel is being metabolized by the enzymes CYP2C8 and CYP3A4, lonafarnib is primarily a substrate for CYP3A4 and its minor metabolites are formed by CYP2C8. 8

Paclitaxel in combination with trastuzumab treatment caused a prolonged QT interval and LVEF decrease, which is a known cardiotoxicity of this combination. Lonafarnib caused mostly hematological and gastrointestinal toxicity. The preliminary anti-tumor activity (CR+PR) for the triple combination in this study was 58%, while combined treatment of paclitaxel and trastuzumab showed a response rate of 50%. 9 Nevertheless, clinical development of lonafarnib in this group of patients did not proceed.

Furthermore, in a phase III study Ras- inhibitor tipifarnib plus gemcitabine failed to show prolonged survival compared to gemcitabine in patients with K-ras mutations, frequently seen in pancreatic cancer, possibly because another enzyme (geranyl-geranyl transferase) takes over the posttranslational modification of K-ras.10 Alternative strategies, a.o. inhibition of mutant K-ras downstream in the MAPK pathway, may be more efficacious.

Interestingly, the concept of Ras inhibition in combination with other chemotherapy was further investigated in patients with gliomas, as lonafarnib crosses the BBB to some extent, despite its molecular weight of 638 Da. This study revealed only modest antitumor effect in pediatric progressive or recurrent primary brain tumors (glioma, medulloblastoma, ependymoma). One PR was observed in a patient with anaplastic astrocytoma for 13 cycles while nine patients (18.8%) demonstrated at best SD for a median of 13 cycles (range 4-20 cycles). 11 Recently, a study combin-

ing both oral lonafarnib 200 mg bi-daily 7-day on, 7-day off and alternating 150 mg/m2 temozolomide (TMZ) on a 7-day on, 7-day off schedule in patients with recurrent or temozolomide refractory glioblastoma showed promising results, with a median survival of 14.9 months (95% CI = 8.9 -23.3 months) and a 6-month PFS of 42.3% (95% CI = 27-66%). This effect may be due to the ability of lonafarnib to cause cell cycle arrest, which leads to an increase in farnesyltransferase activity and subsequently to a high percentage of tumor cells in the S phase, which could maximize the alkylating effect of TMZ.12

In oncology, selected treatments may cause serious cardiotoxicity, such as treatment with anthracyclines or anthracyclines in combination with trastuzumab in HER2+breast cancer patients. In **chapter two** a randomized placebo-controlled trial of candesartan (an angiotensin ATII-antagonist) to prevent trastuzumab-related cardiotoxicity was presented. We hypothesized that ATII inhibition might attenuate or even prevent left ventricular remodeling in patients treated with trastuzumab after anthracycline chemotherapy in adjuvant breast cancer treatment. Preliminary results however showed no evidence that the prophylactic use of candesartan treatment protected the myocardium against toxicity from trastuzumab in combination with anthracycline-containing chemotherapy in HER2+ breast cancer patients. Furthermore, the values of the cardiac markers NT-proBNP and Troponine T values were not statistically significantly associated with the incidence of cardiac failures. However, the baseline LVEF value (≥ 55% versus < 55%) was a prominent prognostic factor for the incidence of a cardiac failure (p=0.0004). Several single nucleotide polymorphisms (SNPs) in the extracellular, transmembrane and intracellular region of HER2 have been studied to examine the impact of these polymorphisms on trastuzumab- related cardiotoxicity. A possible association between the homozygous genotype of the valine Ala1170Pro SNP and a lower risk on cardiac failure was observed.

In **chapter three (3.1)** the strategies to target brain tumors were further discussed. The focus of this review was to describe systemically administered drugs that can either cross the BBB or cause a systemic (I,e. immunological) reaction and effect the brain tumor without causing (neuro)toxicity. We discuss current experimental brain-targeting receptor- or carrier-mediated transport strategies of drug conjugate or liposomal drug formulations, immunotherapeutic strategies and gene transfer strategy mediated by neural stem cells for brain tumors.

Chapter three, 3.2 and 3.3 provide clinical results about phase I and II studies with one of the proposed strategies targeting brain tumors i.e. receptor-mediated transcytosis via glutathione receptors, which are highly expressed on the BBB. Glutathione PEGylated liposomal doxorubicin, 2B3-101, was developed as a systemically long circulating, brain-targeted liposomal formulation of doxorubicin. The treatment was found to be safe and relatively well tolerated without observing cardio-

toxicity and neurotoxicity. The modest preliminary anti-tumor activity, both intra- and extracranially in patients with brain metastases from solid tumors or recurrent high grade glioma warrants further investigation in phase II studies.
In a clinical and pharmacological phase II feasibility study on 2B3-101 in patients with breast cancer and LM one patient had SD for more than nine (three-weekly) cycles. The total doxorubicin concentrations found in CSF at day 2 and day 8 after 2B3-101 in two out of three enrolled patients were within the IC50 of doxorubicin found in the most frequently used breast cancer cell lines in vitro.
Chapter three, 3.4 and 3.5 discusses the implementation of a circulating tumor cells (CTC) flow cytometric assay to quantify tumor cells in the CSF. This method was developed in order to improve the sensitivity of CSF examination for diagnosing LM in patients with solid tumors and clinical suspicion on LM but no confirmatory MRI. The CTC assay in the CSF showed a 100% sensitivity and 100% specificity for the diagnosis LM in patients with epithelial solid tumors as compared to CSF cytology with a 71% sensitivity and 100% specificity. Prospective clinical validation of a flow cytometric assay using another membrane protein (MCSP) in patients with melanoma and suspected LM is needed. However, preliminary data presented in Chapter **3.4** also reveal a high potency of the melanoma CTC assay along with standard CSF cytology to diagnose LM.

Presented studies in this thesis may lead to improved application of the investigated anticancer drugs and contribute to improved diagnosis of LM.

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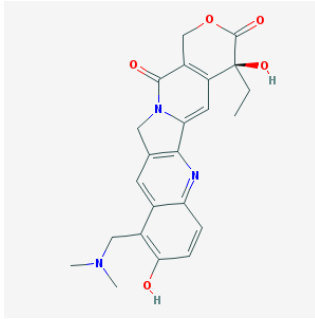
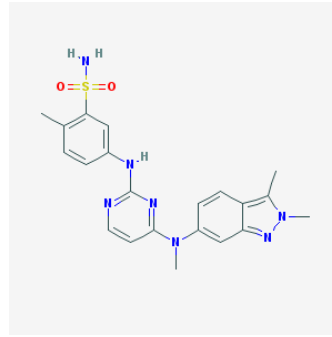
APPENDIX:

Chemical structures
Summary
Samenvatting
Dankwoord
Curriculum Vitae
List of publications

Physico-chemical structures of investigated anticancer agents in this thesis



Chapter 1.1 Topotecan



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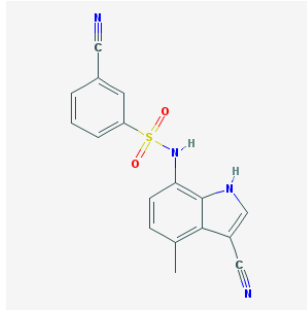

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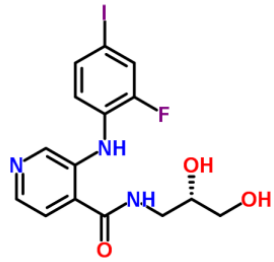
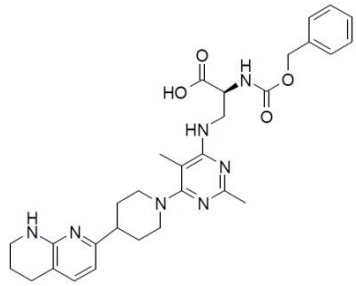
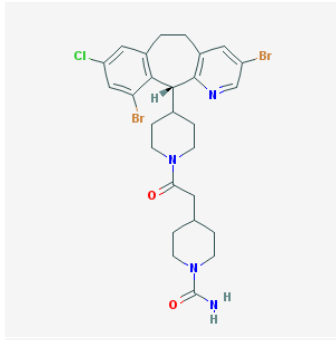


Chapter 1.3. Pimasertib



Chapter 1.4 GLPG0187



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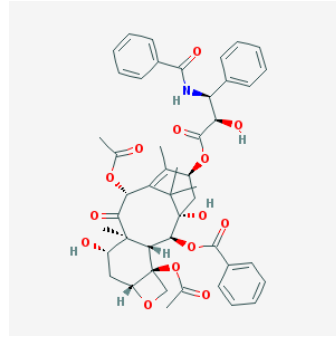
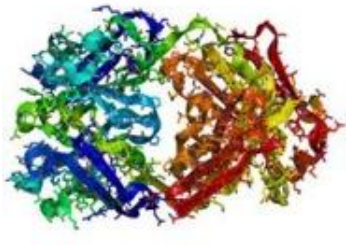
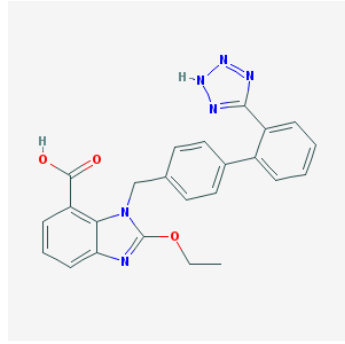


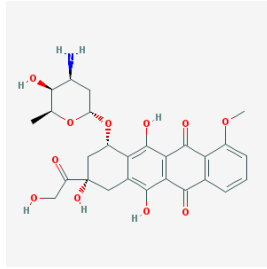
Paclitaxel



Chapter 2. Trastuzumab



Candesartan



Chapter 3.
Doxorubicin

SUMMARY

Chemotherapy is a very frequently used therapy in patients with advanced tumors with or without central nervous system (CNS) metastases or primary brain tumors. Despite the significant progress in drug development, the survival of patients is limited with an unmet need for more effective chemotherapeutics that have an acceptable safety profile. The focus of our research is directed on the development of new drugs or their combinations with an oral route. Oral drugs are convenient for the patient and -extra hospital visits and costs for drug administration are being avoided. With an opportunity to dose the drug more frequently, drug exposure in the circulation can be prolonged. Secondly, we clinically investigated a new drug formulation with an enhanced CNS delivery in patients with brain and leptomeningeal metastases or primary brain tumors.

This thesis starts with an **Introduction** of the performed research. The aim of the shown phase I/II clinical studies was to investigate the safety, tolerability, pharmacokinetics (PK), pharmacodynamics (PD) and the preliminary anti-tumor activity of novel anti-cancer drugs or new combinations of drugs already in use. During the research we were able to recommend dosing schedules and dose-levels of the (combination of) drug(s) for further clinical studies and confirmed drug-drug interactions or food-effects. At the end we showed that the circulating tumor cell (CTC) assay in cerebrospinal fluid (CSF), is a sensitive diagnostic tool for leptomeningeal metastases and a promising pharmacodynamic marker in the treatment of this disease.

Chapter 1 describes the results of five multi-center, open-label phase I clinical studies. The first study **(1.1)** describes two different schedules of pazopanib, an anti-angiogenic drug, combined with topotecan, chemotherapeutic drug that inhibits DNA topoisomerase I in dividing cells. This study reveals that the pharmacokinetics of topotecan is affected when combined with pazopanib and shows differences in safety and tolerability in different schedules. Two other studies with E7820 and GLPG0187 were described. **(1.2 and 1.4)** Both agents are integrin inhibitors with the potential to block tumor angiogenesis, cell migration, proliferation and differentiation of endothelial cells that can lead to tumor remission. In the study with E7820 it was shown that food does not have an effect on drug exposure. Furthermore, the treatment was safe and tolerable with achieved stable disease in two third of patients. The study with GLPG0187 showed a dose-proportional PK profile of the drug and good tolerability upon administration via continuous infusion by an externally carried pump. Studies described in **Chapter 1, 1.3 and 1.5** are describing clinical studies with molecularly targeted agents, such as lonafarnib, ras inhibitor and pimasertib, MEK kinase inhibitor, that both target one of the molecules on the mitogen activated protein kinase (MAPK) pathway, that is responsible for cell proliferation and

survival signaling from the cell surface to the nucleus. The study with lonafarnib was performed in combination with trastuzumab and paclitaxel in patients with advanced HER2+ breast cancer. Despite the safe and tolerable profile of lonafarnib, it did not demonstrate better preliminary anti-tumor as compared to the data available in the literature on the combination of trastuzumab and paclitaxel, which forms the standard therapy for patients with advanced HER2+ breast cancer. The study using pimasertib, an inhibitor of MEK in the MAPK pathway, in 180 enrolled patients showed that the drug was relatively safe and had a favorable PK profile without food-effect. In patients with NRAS and BRAF mutated melanoma it showed promising preliminary anti-tumor activity with a disease control of 62% and 53%, respectively.

Chapter 2 describes the Dutch 20-center, randomized placebo-controlled evaluation of a pharmaceutical intervention in the prevention of trastuzumab-related cardiotoxicity. As clinical evidence supports candesartan, a selective blocker of the angiotensin II receptor sub-type 1, for use in systolic heart failure, up to this article there was no published prospective clinical data of this or other cardiocirculatory drug in the prevention of trastuzumab-related cardiotoxicity. Co-administration of candesartan however did not have a significant beneficial effect in preventing decline in LVEF and cardiac events induced by trastuzumab.

Chapter 3 presents new pharmaceutical/nanomedicine strategies and laboratory assays in the treatment, diagnosis and drug-monitoring of central nervous system (CNS) tumors. **Chapter 3.1** discusses new drug strategies of targeting blood-brain barrier (BBB) by active chemo- and immunotherapies. Due to the presence of the blood-brain barrier (BBB) only few systemic drugs can be used to treat brain tumors. As treatment of systemically metastasized cancer patients becomes more effective and prolongs patient's survival, CNS metastases are more frequently observed. Recently, usingnanotechnologyactive chemotherapeutics can be safely transported across the barriers (BBB and blood-cerebrospinal fluid barrier (BCSFB)) and target brain and CSF, respectively. Further studies on improving the brain-penetration of potentially effective drugs for brain tumors is warranted. **Chapter 3.2 and 3.3** describe two clinical studies with a novel strategy consisting of administration of glutathione liposomal PEGylated doxorubicin (2B3-101) as a treatment of patients with solid tumors and brain metastases or recurrent malignant glioma and leptomeningeal metastases from breast cancer. Doxorubicin is a well- known, frequently used chemotherapeutic agent in various tumor types, such as breast cancer and lung cancer. Glioblastoma cell lines showed to be sensitive to doxorubicin, however, without the carrier, doxorubicin cannot pass the BBB. The first study with 2B3-101 **(Chapter 3.2)** demonstrated that treatment of patients with solid tumors and brain metastases or malignant gliomas with 2B3-101 showed a dose-dependent PK profile and is safe and rel-

atively well tolerated with both as single agent and with trastuzumab co-administration. Intracranial and extracranial preliminary anti-tumor activity was observed in patients with (HER2+) breast cancer with brain metastases and malignant glioma. In the second study using 2B3-101 in patients with leptomeningeal metastases (LM) from breast cancer, doxorubicin concentrations in the CSF were within the reported $IC_{50,}$ a measure of effectiveness in-vitro in two out of three patients. One of the three treated patients with LM showed stable disease for 10 cycles and progression free survival for more than six months. **Chapter 3.4 and 3.5** describe the clinical application of the circulating tumor cell assay (CTC) for EPCAM-positive cells (an epithelial tumor cell marker) and the circulating melanoma cell (CMC) assay and their laboratory validation. The CTC assay for EPCAM-positive cells showed to be more sensitive in diagnosing leptomeningeal metastases in CSF (100%), than the standard CSF cytology method (71%) in patients with clinically suspected LM or with already diagnosed LM, while both methods showed a very high specificity (100%). For the CMC method more patients need to be enrolled to be able to assess whether the FACS based assays is superior.

Finally, in the **Chapter Conclusions and perspectives** we conclude that targeting tumor-affected CNS compartment with new strategies and further development of current oral targeted therapies and chemotherapeutics are attractive approaches to improve the treatment and survival of patients with advanced cancer.

SAMENVATTING

Chemotherapie is een vaak gebruikte therapie bij patiënten met vergevorderde tumoren, al dan niet met metastases in het centrale zenuwstelsel of bij patiënten met primaire hersentumoren. Ondanks substantiële vooruitgang in de medicijnontwikkeling is de overlevingskans van patiënten gering en blijft er vraag naar effectievere chemotherapeutica met een acceptabel veiligheidsprofiel.
Ons onderzoek was allereerst gericht op de ontwikkeling van nieuwe, oraal in te nemen chemotherapeutica of nieuwe combinaties hiervan. Medicijnen die oraal ingenomen kunnen worden zijn praktischer voor de patiënt en maken extra bezoeken aan het ziekenhuis en extra kosten voor medicijnenadministratie overbodig. Bovendien bieden ze de mogelijkheid voor frequentere dosisinname, waardoor de blootstelling aan de tumoren kan worden verlengd.
In de tweede plaats onderzochten we in de kliniek een nieuwe medicijnformulering met een verhoogde afgifte aan het centraal zenuwstelsel bij patiënten met primaire hersentumoren of patiënten met metastasen in de hersenen of hersenvliezen.
In de Introductie wordt het doel van de getoonde fase I/II klinische studies besproken. Het doel was om de veiligheid, verdraagzaamheid, farmacokinetiek, farmacodynamiek en de voorlopige antitumor effecten van nieuwe antikankermiddelen of nieuwe combinaties van bestaande middelen te onderzoeken. Het onderzoek heft geleid tot adviezen over de dosering en doseringsschema's van de (combinaties van) middelen voor toekomstige klinische studies. Tevens toonden we interacties tussen middelen aan en eventuele effecten van voeding op deze middelen. Tot slot lieten we zien dat een test voor circulerende tumorcellen in cerebrospinale vloeistof een gevoelige diagnostische test is voor metastasen in de hersenvliezen en een veelbelovende farmacodynamische test is in de behandeling van deze ziekte.
Hoofdstuk 1 beschrijft de resultaten van vijf multi-center, open-label fase I klinische studies. De eerste studie (1.1) beschrijft twee verschillende doseringsschema's van pazopanib, een angiogeneseremmer, in combinatie met topotecan, een chemotherapeutisch middel dat DNA topoisomerase I in delende cellen remt. De studie toont aan dat de farmacokinetiek van topotecan wordt beïnvloed door de combinatie met pazopanib en laat verschillen zien in veiligheid en verdraagzaamheid tussen de verschillende doseringsschema's. Twee andere studies met E7820 en GLPG0187 worden beschreven in **1.2** en **1.4.** Beide middelen remmen integrine, wat zou kunnen leiden tot blokkering van angiogenese in de tumor, migratie van cellen, celgroei en differentiatie van endotheelcellen, met het uiteindelijke resultaat een afname van de tumor grootte. De studie met E8720 toont aan dat er geen effect was van voedselinname op de blootstelling van het middel. Bovendien is de behandeling veilig en goed te verdragen en werd in twee derde van de patiënten een stabiele ziekte bereikt. De studie met GLPG0187 laat zien dat het farmacokinetisch profiel dosis proportioneel is en dat het middel goed te verdragen is bij toediening door middel van continue infusie door een buiten het lichaam gedragen pomp. De studies in **Hoofdstuk 1, 1.3 en 1.5** beschrijven klinische studies met middelen met

een moleculair doelwit, zoals de ras-remmer lonafarnib en de MEK kinase remmer pimasertib. Beide middelen richten zich op een van de moleculen in de zogenaamde mitogen-activated protein kinase (MAPK) route, die zorgt voor signaaltransductie voor celgroei en overleving van het celoppervlak naar de celkern. De studie met lonafarnib is uitgevoerd in combinatie met trastuzumab en paclitaxel in patiënten met vergevorderde HER2+ borstkanker. Hoewel lonafarnib veilig en verdraagbaar was, waren de voorlopige antitumor effecten niet beter dan beschreven in de literatuur voor de combinatie van trastuzumab en paclitaxel, de standaard behandeling van patiënten met HER2+ borstkanker. De studie waarbij pimasertib, een remmer van MEK in de MAPK route, getest was bij 180 patiënten, laat zien dat het middel relatief veilig was een gunstig farmacokinetisch profiel had zonder dat de voedselinname effect op het middel toonde. Veelbelovende antitumor activiteit is gezien bij patiënten met NRAS en BRAF gemuteerde melanomen, met stabiele ziekte als beste respons bij respectievelijk 62% en 53% van de patienten.

Hoofdstuk 2 beschrijft de in 20 Nederlandse centra uitgevoerde, gerandomiseerde, placebo-gecontroleerde evaluatie van een farmacotherapeutische interventie ter voorkoming van trastuzumab-gerelateerde cardiotoxiciteit. Er zijn aanwijzingen dat candesartan, een selectieve type 1 angiotensine II-receptor-(AT_1-) antagonist, klachten vermindert bij systolisch hartfalen. Op deze manier zou candesartan de bijwerking/cardiotoxiciteit kunnen verminderen. Echter, totdat dit artikel verscheen bestonden er geen gepubliceerde prospectieve klinische data van dit of een ander cardiovasculair geneesmiddel ter voorkoming van trastuzumab-gerelateerde cardiotoxiciteit. De toevoeging van candesartan naast de trastuzumab) leverde geen significant voordelig effect op bij het voorkomen van een LVEF-daling en van cardiale gebeurtenissen als gevolg van trastuzumab.

Hoofdstuk 3 beschrijft nieuwe farmaceutische/nanomedicine strategieën en laboratoriumtesten voor de behandeling, diagnostiek en geneesmiddelenbewaking van tumoren binnen het centraal zenuwstelsel. **Hoofdstuk 3.1** bediscussieert nieuwe strategieën voor geneesmiddelen om de bloed-hersenbarrière te passeren door actieve chemo- en immunotherapieën. Door de aanwezigheid van de bloed-hersenbarrière kunnen slechts enkele systemische antikankermiddelen gebruikt worden voor de behandeling van hersentumoren. Aangezien de behandeling van patiënten met systemisch gemetastaseerde tumoren steeds effectiever wordt, waardoor de levensduur van de patiënt worden verlengd, komen metastasen in het centraal zenuwstelsel steeds vaker voor. Onlangs is aangetoond dat nanotechnologie het mogelijk maakt om chemotherapeutica veilig te transporteren over de barrières (de bloed-hersenbarrière en de barrière tussen bloed en de cerebrospinale vloeistof) en zo respectievelijk bij de hersenen en in de cerebrospinale vloeistof te laten komen. Additionele studies ter verbetering van de penetratie in de hersenen van mogelijk effectieve middelen tegen hersenentumoren blijven nodig. **Hoofdstuk 3.2 en 3.3** beschrijven twee klinische studies met een nieuwe strategie. Deze bestaat uit toediening van glutathion liposomaal gePEGyleerde doxorubicine (2B3-101) als behandeling van patiënten met solide tumoren en hersenmetastasen of met

kwaadaardige gliomen. Doxorubicine is een bekend en vaak gebruikt antikankermiddel voor verschillende typen tumoren, zoals borstkanker en longkanker. Het is aangetoond dat glioblastoomcellen gevoelig zijn voor doxorubicine, echter, zonder een drager kan doxorubicine de bloed-hersenbarrière niet passeren. De eerste studie met 2B3-101 **(Hoofdstuk 3.2)**, laat zien dat behandeling van patiënten met solide tumoren en hersenmetastasen met 2B3-101 een dosis-afhankelijk farmacokinetisch profiel opleverde, veilig is en relatief goed verdragen wordt, zowel alleen als in combinatie met trastuzumab. Bij patiënten met (HER2+) borstkanker met hersenmetastasen en kwaadaardige gliomen werd enige intracraniale en extracraniale anti-tumoractiviteit gezien. In de tweede studie werd 2B3-101 gebruikt bij patiënten met hersenvliesmetastasen van borstkanker. Bij twee van de drie patiënten vielen de doxorubicine concentraties in de cerebrospinale vloeistof binnen de gerapporteerde IC_{50}, een maat voor in vitro effectiviteit. Een van de drie behandelde patiënten met hersenvliesmetastasen had een stabiele ziekte gedurende 10 cycli en een progressievrije overleving van meer dan zes maanden. **Hoofdstuk 3.4 en 3.5** beschrijven de klinische toepassing en laboratorium validatie van een circulerende tumorcellen test (CTC) voor EPCAM positieve cellen (een tumorcelkenmerk in het epitheel) en een test voor circulerende melanoomcellen (CMC). De CTC test voor EPCAM-positieve cellen was gevoeliger voor het diagnosticeren van hersenvliesmetastasen in cerebrospinaal vloeistof (100%) dan de standaard cerebrospinale vloeistof cytologie methode (71%) bij patiënten met gediagnostiseerde of klinische verdenking op hersenvliesmetastasen, terwijl beide methoden zeer specifiek waren (100%). Om te bepalen of de CMC test beter is dan de op FACS gebaseerde testen moeten meer patiënten getest worden.

Tot slot, in het **Hoofdstuk "Conclusions and perspectives"**, concluderen we dat naar aanleiding van onze resultaten verder onderzoek en ontwikkelen van nieuwe strategieën of combinaties van de bestaande orale gerichte behandelingen tegen tumoren van o.a. van het centraal zenuwstelsel nodig zijn om de behandeling en de overlevingskans van patiënten te verbeteren.

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CURRICULUM VITAE

Bojana Milojkovic Kerklaan werd geboren op 1 juni 1979 te Belgrado, Servië. In 1998 behaalde zij het VWO diploma aan het V^{de} Belgrado Gymnasium. In september van datzelfde jaar begon zij aan de studie Geneeskunde aan de Universiteit van Belgrado, Servië. Tijdens deze studie deed zij een onderzoeksstage op de afdeling Fysiologie en Sport Geneeskunde over het aerobische metabolisme van topsporters onder begeleiding van Prof dr S. Mazic. Een internationaal keuze stage deed zij op de afdeling Revalidatie en Sport geneeskunde in Tartu, Estonia. Tevens deed zij een keuzestage bij verschillende farmaceutische organisaties/bedrijven in Servië. In 2006 heeft zij haar artsen titel behaald. Daarna heeft Bojana 4 jaar gewerkt binnen de Clinical Research Organisation Parexel te Belgrado, Berlijn en Amsterdam waar zij de international ervaring heeft opgedaan met klinish trials. Hierna besloot zij in april 2010 om weer binnen het ziekenhuis te gaan werken en de uitdaging van een promotieonderzoek aan te gaan op de afdeling klinische farmacologie aan het Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis te Amsterdam. In dit onderzoek werd zij begeleid door prof. dr. Jan H.M. Schellens, prof. dr. Jos H. Beijnen en dr D. Brandsma.

CURRICULUM VITAE

Bojana Milojkovic Kerklaan was born on the 1th of June 1979 in Belgrade. In 1998 she received her gymnasium diploma from the 5th Belgrade Gymnasium. In September that year she started to study Medical School at Belgrade University. During this study she did a research internships about the role of the aerobic metabolism of top sportsman at the Department of Physiology and Sport Medicine at the University of Belgrade, under the supervision of prof.dr. S. Mazic and at the Department of Rehabilitation and Sport Medicine in Tartu, Estonia. Additionally, she did internships in 2005 at several pharmaceutical organizations in Serbia. After receiving the medical doctor diploma she spent 4 years working in the Clinical Reseach Organisation Parexel in Belgrade, Berlin and Amsterdam. IN April 2010 she decided to take on the challenge of doing a PhD research project at the Clinical Pharmacology Department, Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital in Amsterdam. During her research she was supervised by prof.dr. J.H.M. Schellens, prof.dr. J.H. Beijnen and dr D. Brandsma.

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1. Milojkovic Kerklaan B, Diéras V, Le Tourneau C, Mergui – Roelvink M, Huitema ADR, Rosing H, Beijnen JH, Marreaud S, Govaerts A, Piccart-Gebhart MJ, Schellens JHM, Awada A. Phase I study of lonafarnib (SCH66336) in combination with trastuzumab plus paclitaxel in Her2/neu overexpressing breast cancer. EORTC study 16023. Cancer Chemother Pharmacol (2013) 71:53–62

2. Milojkovic Kerklaan B, Lolkema MPJ, Devriese LA, Voest EE, Nol-Boekel A, Mergui-Roelvink M, Langenberg M, Mykulowycz K, Stoebenau J, Lane S, Legenne P, Wissel P, Smith DA, Giantonio BJ, Schellens JHM, Witteveen PO. A. Phase I and pharmacological study of pazopanib in combination with oral topotecan in patients with advanced solid tumors. Submitted for publication.

3. Milojkovic Kerklaan B. Van Tellingen O, Huitema ADR, Beijnen JH, Boogerd W, Schellens JHM, Brandsma D. Strategies to target drugs to primary brain tumors and CNS metastases (review). Submitted for publication.

4. Milojkovic Kerklaan B, Aftimos P, Altintas S, Jager A, Gladdines W, Gaillard P, Soetekouw P, Dieras V, Van Linde M, Awada A, Huitema ADR, Schellens JHM, Brandsma D. Phase I and pharmacological study of 2B3-101, glutathione PEGylated liposomal doxorubicin, in patients with solid tumors and brain metastases or recurrent malignant glioma. Submitted for publication.

5. Milojkovic Kerklaan B. Pluim D, Bol M, Hofland I, Westerga J, Van Tinteren H, Beijnen JH, Boogerd W, Schellens JHM, Brandsma D. Sensitivity and specificity of circulating tumor cell detection by flow cytometry versus cytology in cerebrospinal fluid for the diagnosis leptomeningeal metastases. Submitted for publication.

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7. Cirkel G.A, Milojkovic Kerklaan B, Vanhoutte F., Van der Aa A., Lorenzon G., Namour F., Pujuguet P., Darquenne S., de Vos F, Snijders T.J., Voest E.E., Schellens J.H.M., Lolkema M.P. A phase I study of GLPG0187, a broad spectrum integrin receptor antagonist in patients with glioblastoma multiforme and other solid malignancies. Submitted for publication.

LIST OF ABSTRACTS

Sensitivity and specificity of circulating tumor cell detection by flow cytometry versus cytology in cerebrospinal fluid for the diagnosis leptomeningeal metastases. Oral/poster presentation tbd **NVKFB mededelingendag,** Amsterdam, the Netherlands, March 2015

Phase I/IIa study of glutathione PEGylated liposomal doxorubicin (2B3-101) as a single agent or in combination with trastuzumab in patients with brain metastases from solid tumors or recurrent high grade gliomas - **SNO Congress** - oral presentation – Miami, USA, Nov 2014and on **ESMO/ECCO/ESTRO Congress** –poster presentation – Amstredam, Sep 2013

Phase I study of the safety, tolerability and pharmacokinetics of pazopanib in combination with oral topotecan in patients with advanced solid tumor - **ASCO Congress** –poster presentation, Chicago, USA, May 2013

The impact of body composition on the success rate of elite soccer players on aerobic tests – **ECSS Congress** - poster presentation, Belgrade, Serbia, Jul 2005

One more reason why talent is not enough **- ECSS Congress – poster presentation, Clermont-Ferrand, France, July 2004**