The cytotoxic drug cyclo-pentenyl cytosine: from manufacturing to anti-tumor activity and (cardio)toxicity

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THE CYTOTOXIC DRUG

CYCLO-PENTENYL CYTOSINE:

FROM MANUFACTURING TO ANTI-TUMOR ACTIVITY AND (CARDIO)TOXICITY

The cytotoxic drug cyclopentenyl cytosine:
From manufacturing to anti-tumor activity and cardiotoxicity

ISBN: 978-90-9021981-3

Graphic design
MEGLA | www.megla.nl

Printing
Littera Picta

The research presented in this thesis was performed at the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Centre, Leiden, The Netherlands.

The printing of this thesis was financially supported by:
AZL Onderzoeks- en Ontwikkelingskrediet Apotheek

2007 Leiden

THE CYTOTOXIC DRUG
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Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 5 september 2007 klokke 13.45 uur
door

KIRSTEN JOHANNA MARIA SCHIMMEL

geboren te Apeldoorn
op 22 september 1973

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voor .....

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CHAPTER 1

AIM AND OUTLINE OF THE THESIS

The cytotoxic drug cyclopentenyl cytosine:
From manufacturing to anti-tumor activity
and *cardio*toxicity

SCOPE OF THE THESIS

The aim of this study is to explore pharmaceutical aspects as well as anti-tumor activity and cardiotoxicity of the cytostatic drug cyclopentenyl cytosine.

OUTLINE

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) is a pyrimidine analogue of cytidine. After transmembrane transport, CPEC is subsequently phosphorylated by the enzymes uridine/cytidine kinase, nucleoside monophosphate-kinase (NMP-kinase) and nucleoside diphosphate-kinase (NDP-kinase) to form CPEC-triphosphate (CPEC-TP), being the major metabolite [1]. CPEC-TP is an inhibitor of cytidine triphosphate synthetase (CTP-synthetase), this enzyme catalyses the synthesis of the ribonucleotide cytidine triphosphate (CTP) [2,3]. Inhibition results in a decrease of RNA and DNA synthesis and S-phase accumulation. Moreover, a high CTP synthetase activity has been found in various malignant and non-malignant tissues in humans and animals [4,5], making this enzyme an attractive target for inhibition. Originally selected for its antiviral activity, most research has been done to investigate the activity of CPEC in several malignancies. In chapter 2 an overview of both the preclinical and early clinical studies with CPEC is given. These studies showed promising results on hematological malignancies and plans for phase I and II clinical trials were initiated.

As only the raw drug substance was available we developed a pharmaceutical formulation of the drug to be used in these clinical trials. A stable sterile infusion concentrate of CPEC was manufactured (chapter 3).

During an early phase I trial with CPEC in solid tumors, serious cardiotoxic side effects were observed [6]. As these side effects seemed to be dose related, future trials would start with low dose CPEC and plasma monitoring of CPEC levels would become necessary. Therefore, we developed a sensitive and rapid HPLC MS/MS method for measuring plasma levels of CPEC and its metabolite cyclopentenyl uridine (CPEU) (chapter 4).

To explore the antitumor potential of CPEC in leukemia, we tested the drug in an *in vivo* animal model for human ALL using NOD/scid (nonobese diabetic/severe combined immunodeficient) mice (chapter 5).

Before initiating clinical trials with the experimental drug, it was necessary to further study and understand the mechanism of the aforementioned cardiotoxic side effects of CPEC. Cardiotoxicity is not uncommon among cytotoxic agents and especially the anthracyclines are well known to cause severe cardiotoxicity. In chapter 6 the cardiotoxicity of several cytotoxic drugs is described including (possible) mechanisms and preventive measures.

The exploration of the cardiotoxic effects of CPEC and the underlying mechanism was studied in both *in vitro* and *in vivo* in animal models (chapter 7).

We further hypothesized that cardiotoxicity of cytotoxic drugs including CPEC, might have a genetic origin. We first performed a retrospective case control analysis in oncology patients having received the anthracycline doxorubicine. We investigated the differences in polymorphisms in several candidate genes between patients with and without anthracycline-induced cardiotoxicity and between cases and healthy control subjects. Furthermore, we tested *in vitro* in a rat cardiomyocyte cell line, whether doxorubicine and CPEC influenced the expression of genes that were suspected to be related with drug induced cardiotoxicity (chapter 8).

In chapter 9 the results of the studies presented in this thesis are interpreted and suggestions for further research are given. Chapter 10 provides a summary of the results.

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CHAPTER 2

CYCLOPENTENYL CYTOSINE (CPEC): AN OVERVIEW OF ITS IN VITRO AND IN VIVO ACTIVITY

Current Cancer Drug Targets 2007;7:325-334 (adapted version)

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ABSTRACT

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) is an analogue of cytidine. Besides its antiviral effect, its potential use in the treatment of cancer has become an important area of research. CPEC is activated by intracellular phosphorylation ultimately forming its metabolite CPEC-TP. CPEC-TP is a non competitive inhibitor of cytidine-5'-triphosphate synthetase (CTP-synthetase), an important enzyme in the formation of CTP. Studies have shown that cancer cells have a high CTP synthetase activity, thus making CTP synthetase an interesting target for chemotherapy. CPEC has been preclinically studied in different malignancy models. *In vitro* results on leukemia show activity in the nanomolar range on several cell lines. However *in vivo* results are conflicting and the findings vary from increase in life span over 100% to only limited effectiveness. Interesting results have been obtained in colorectal and neuroblastoma cells. In several neuroblastoma cell lines incubation with CPEC in combination with cytarabine or gemcitabine has resulted in increased cell death compared to incubation with only one of the agents.

CPEC has been studied in a phase I trial in patients with solid tumors. In five of 26 patients unexplained cardiotoxicity (extreme hypotension) occurred.

In this overview, it is demonstrated that CPEC has an anti-cancer effect in several tumor models and might be a potentially useful drug in anticancer treatment.

Keywords: Cyclopentenyl cytosine, CPEC, cancer, leukemia, cardiotoxicity

INTRODUCTION

Nucleotides are the phosphorylated forms of nucleosides and the mature precursors of DNA and RNA. Because of their important role in DNA and RNA synthesis and thus in cell survival, nucleotides appear to be important targets in anticancer therapy. Indeed, several anticancer drugs, such as methotrexate or 5-fluorouracil, exert their action by interfering with nucleotide biosynthesis. Moreover, as cancer cells show an increased demand for nucleotides when compared to healthy cells, they may preferentially be targeted by these anticancer drugs. Nucleosides contain either a purine (adenosine or guanosine) or a pyrimidine base (cytidine, thymidine or uridine) [1]. The least abundant nucleoside in the cell is cytidine [2]. Cytidine 5'-triphosphate (CTP) can either be formed from cytidine 5'-di- and monophosphate (CDP, CMP) or from UTP (uridine 5'-triphosphate). The formation of CTP from UTP is catalyzed by the enzyme cytidine 5'-triphosphate synthetase (CTP-synthetase). Fig. (***1***)

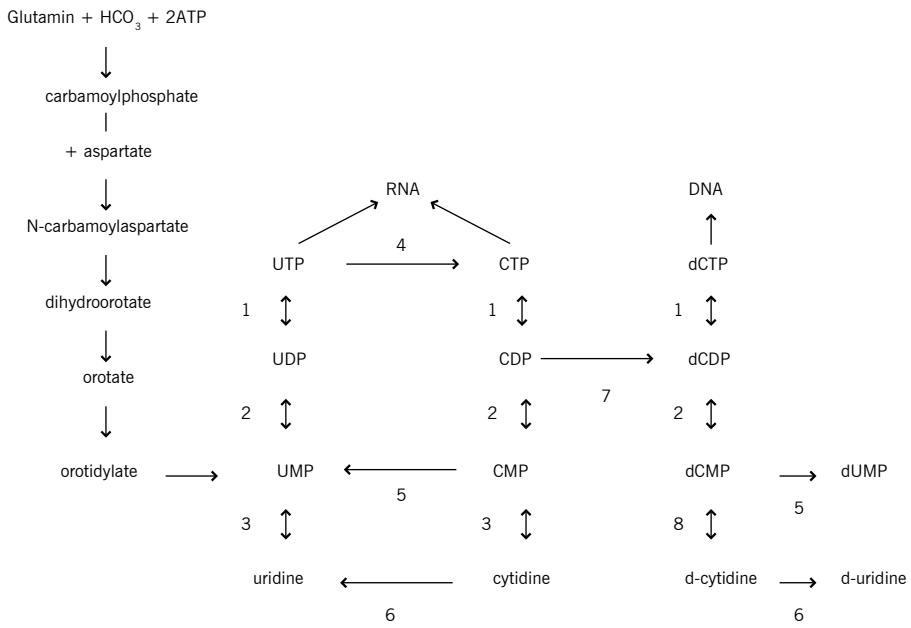

Figure 1 Pyrimidine synthesis

The pyrimidine (deoxy) ribonucleotide synthesis is shown. Pyrimidine nucleotides can either be formed by de novo synthesis starting with glutamine or from uridine and cytidine.
ATP: adenosine 5'-triphosphate; UTP: uridine 5'-triphosphate; UDP: uridine 5'-diphosphate; UMP: uridine 5'-monophosphate; CTP: cytidine 5'-triphosphate, CDP: cytidine 5'-diphosphate, CMP: cytidine 5'-monophosphate; dCDP: 2'-deoxycytidine 5'-diphosphate, dCTP: 2'-deoxycytidine 5'-triphosphate.
The numbers represent the enymes catalyzing the conversions:
1: NDP kinase; 2: NMP kinase; 3: uridine/cytidine kinase; 4: CTP synthetase; 5: (deoxy) CMP deaminase;6: (deoxy) cytidine deaminase; 7: ribonucleotide reductase; 8: deoxy cytidine kinase

Thus, a cell can have two different sources for CTP: CTP can be provided by the salvage pathway by phosphorylation of cytidine or by 'de novo' synthesis out of UTP. An increased activity of CTP-synthetase has been demonstrated in several malignant cell types such as hepatic carcinoma, renal cell carcinoma, acute lymphocytic leukemia and lymphoma [3-6]. CTP synthethase might therefore be an attractive target for growth inhibition of malignant cells by depletion of CTP pools. Depletion of CTP pools will lead to a reduction of proliferation of cells. Furthermore, as depletion of the CTP ribonucleotide pool also leads to a depletion of the cytidine deoxyribonucleotide (dCTP) pool, the balance in the ribonucleotide amount in cells will be disturbed and other deoxyribonucleotides than dCTP can be misincorporated during DNA synthesis, triggering apoptosis [7, 8].

The cytotoxic drug cyclopentenyl cytosine (CPEC) was designed in 1979 based on the biologically active and toxic nucleoside neplanocin A that was found in fermentation broth. Out of several purine and pyrimidine analogues of neplanocin A, CPEC was found to be the most biologically active compound with regard to antiviral activity and activity against murine leukemias and human tumor xenografts [9]. CPEC is an analogue of cytidine in which the ribose moiety is substituted by a carbocyclic sugar. Fig. (**2**)

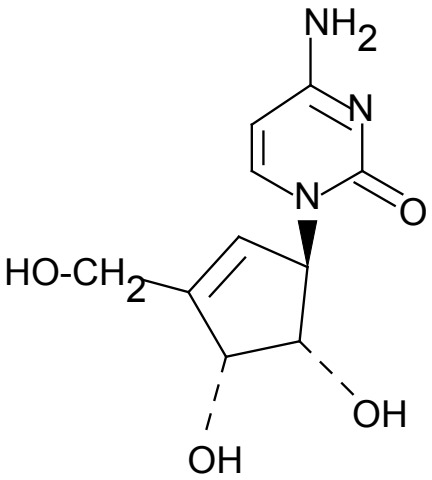

Figure 2 Chemical structure of CPEC

As cytidine is hydrophilic, passive diffusion across cell membranes is not likely and nucleoside transporters are necessary for uptake of CPEC in cells. The equilibrative nucleoside transporters (ENTs) ENT1 and ENT2 both seem to be involved. Whether other nucleoside transporters such as the concentrative nucleoside transporter (CNT) are also involved is not clear [10]. The multidrug resistance proteins 4 and 5 (MRP4 and MRP5) also are suggested to be involved in nucleoside transport [11]. However, in human cells the transport of other nucleoside analogs such as gemcitabine and cytarabine seems to be predominantly regulated by ENT and CNT [12,

13]. After the facilitated diffusion through the cellular membrane, CPEC is phosphorylated [14]. CPEC-monophosphate is formed by uridine cytidine kinase. Nucleoside monophosphate (NMP) and nucleoside diphosphate kinase (NDP) are responsible for the further phosphorylation ultimately leading to CPEC triphosphate (CPEC-TP). Fig. (**3**)

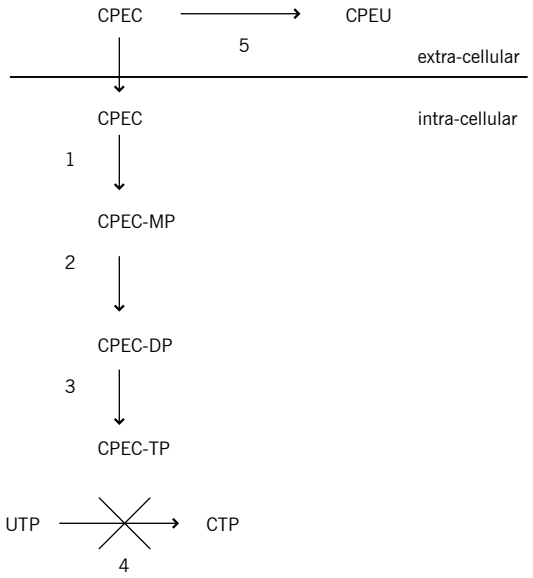

Figure 3 Metabolism and proposed mechanism of action of CPEC

After intra-cellular transport CPEC is phosphorylated to CPEC-TP. CPEC-TP inhibits CTP synthetase (4) resulting in CTP depletion. CPEC can be either cleared as unchanged drug or deaminated to its metabolite CPEU by the enzyme cytidine deaminase (5).
CPEC-MP: CPEC-monophosphate; CPEC-DP: CPEC-diphosphophosphate; CPEC-TP: CPEC-triphosphate
The numbers represent the enymes catalyzing the conversions:
1: uridine/cytidine kinase; 2: NMP kinase; 3: NDP kinase; 4: CTP synthetase; 5:cytidine deaminase

After incubation with CPEC, Moyer *et al* found strong inhibition of the formation of [3H]-CTP from [3H]-uridine in L1210 cells. This suggested inhibition of CTP synthethase by CPEC [15]. In K562 cells CPEC also induced erythroid differentiation in presence of p38 MAP kinase activity [10]. CPEC-TP was found to be mainly responsible for this effect as μM-range concentrations of CPEC-TP were able to inhibit CTP synthetase, whereas CPEC, CPEC-MP and CPEC-DP showed no inhibition at all or only at much higher concentrations [16]. Cyclopentenyl uridine (CPEU), the deamination product of CPEC, seems to be the major metabolite of CPEC with almost no cytotoxic effects [14, 17].

The observed preclinical effects of CPEC suggest a potential use as an anti-cancer agent. In this review an overview of both the preclinical and early clinical studies undertaken with CPEC will be given. Studies were selected by Medline search using the keywords [cyclopentenyl cytosine], [cyclopentenylcytosine] and [CPEC].

PRECLINICAL ACTIVITY OF CPEC

In vitro antiviral activity

Like several other pyrimidine nucleoside analogues, CPEC has both antiviral and anti-tumor effects. The mechanism of action of the antiviral effect is believed to be based on the CTP depletion caused by CPEC. Apparently CTP synthetase interacts as a host cell enzyme that may be used as a target enzyme for antiviral agents. In *in vitro* assays, CPEC showed antiviral activity against a broad range of viruses (e.g. herpes simplex, polio, rhino, influenza, yellow fever, West Nile) at a wide range of concentrations. An IC50 of 0.02 μg/ml (80 nM) was observed for vaccinia viruses [18]. This concentration is comparable to concentrations at which anti-tumor effect is observed. However, most of the viruses were inhibited at concentration of 0.1 μg/ml (400 nM) and higher.

The *in vitro* assays for antiviral activity were conducted on resting confluent cells whereas exponentially growing cells were used for the anti-tumor assays. Exponentially proliferating cells seem to preferentially use the 'de novo' synthesis of CTP (involving CTP synthetase) thereby making them more sensitive to CPEC.

Antiviral activity has not yet been established in animal models. Whether it is possible to create an antiviral effect without toxic effects on rapidly growing cells is therefore not clear yet [18-22].

Activity in malignancies

LEUKEMIA, IN VITRO STUDIES

In MOLT-4 lymphoblasts CPEC concentrations between 20 nM (72 hr incubation) and 75 nM (16 hr incubation) were able to reduce proliferation rates by 50% [14]. In the human promyelocytic leukemia cell line HL-60, DNA synthesis was almost completely inhibited after 24 hrs incubation with 30 nM CPEC. At this concentration RNA synthesis was less reduced (approximately 30% reduction) [23]. In cells from pediatric patients with acute lymphocytic and acute non-lymphocytic leukemia, incubation with CPEC caused a dose dependent depletion of CTP [24, 25]. CPEC was also used in combination with cytarabine and analogues. Cytarabine must be phosphorylated before it can be incorporated into DNA and exert its cytotoxic effect. The rate limiting enzyme in this process is deoxycytidine kinase (dCK). The activity of dCK is regulated and can therefore be inhibited by deoxycytidine-triphosphate (dCTP). *De novo* synthesis of dCTP occurs by reduction of cytidine 5'-diphosphate to 2'-deoxycytidine 5'-diphosphate by ribonucleotide reductase and subsequent phosphorylation to dCTP by nucleoside 5'-diphosphate kinase. Depletion of CTP pools leads to a decrease in dCTP and could have a positive influence on incorporation of cytarabine. Incorporation of cytarabine into DNA was increased with by 41% in a human T-lymphoblastic cell

line (MOLT-3) after preincubation with CPEC (100nM), followed by incubation of cytarabine (2nM) [26]. Similar results were obtained with the deoxycytidine analogue 5-aza-2'-deoxycytidine (DAC) and gemcitabine in combination with CPEC in HL60 cells [27, 28] and MOLT-3 cells [28].

LEUKEMIA, IN VIVO STUDIES

Moyer *et al* inoculated mice with the lymphoid leukemia cell line L1210 ($1x10^5$ cells). Several dose regimens were applied; from 10-50 mg/kg as a single dose to 1-6 mg/kg/day for 5 days and 1 mg/kg/day for 9 days. All mice, including those receiving saline, died within 20 days after inoculation. An increase in life span (ILS) of 111-122% was observed after 9 days of treatment with 1 mg/kg CPEC. The other regimens were either too toxic (> 3 mg/kg for 5 consecutive days) or ineffective (single dose up to 50 mg/kg) [15]. These results correspond with other experiments in L1210 inoculated mice [29]. Although with a broader range in ILS (73-129%), increase in life span was also reported for mice inoculated with P388 lymphocytic leukemia [15]. Combination treatment of the palmitate derivative of cytarabine and CPEC in mice inoculated with L1210 cells (with a subpopulation resistant to cytarabine), resulted in an increase in lifespan when compared to single treatment with the palmitate derivative only. However, since toxicity of the combination was more severe than while using monotherapy, the maximum tolerated dose (MTD) of palmitate cytarabine as a single agent was not achieved. When the MTD in both regimens was compared there were no longer significant differences in survival [30].

NEUROBLASTOMA

At concentrations similar to those at which anti-leukemic activity was observed, CPEC was also active on SK-N-BE(2)-C neuroblastoma cells [7, 31]. Moreover, coincubation of CPEC (50-250 nM) and cytarabine (37.5-500 nM) increased the cytotoxic effects of cytarabine [32]. Preincubation of CPEC (100 nM) followed by the deoxycytidine analogue gemcitabine (50 nM), also resulted in increased cell death for 13 of the 15 neuroblastoma cell lines when compared to a set up in which incubation with only gemcitabine took place [33].

BRAIN TUMORS

CPEC has demonstrated in vitro activity against human glioblastoma cells [34]. However, CPEC shows relatively poor penetration of the blood brain barrier. In mice inoculated intracerebrally with leukemic L1210 cells intraperitoneal administration of CPEC was less effective than in mice inoculated intraperitoneally or subcutaneously with L1210 [18]. It can be concluded that CPEC does not appear to be a suitable agent to be used in brain tumors. However, the poor penetration of CPEC intracerebrally might be overcome by direct intratumoral administration of the drug. In one study CPEC (200 μM by continuous infusion in 4 weeks) was directly infused into brain gliosarcomas in rats [35]. Rats treated with CPEC survived 32 days versus 25 days for rats treated with saline ($p<0.0001$). In tumor tissue CTP was depleted to a much greater extent

than in the adjacent tissues, indicating that exposure to CPEC was restricted to the infused area. The absence of systemic exposure might indicate that intratumoral administration results in less toxic effects. Whether intratumoral administration of CPEC is feasible in humans needs to be investigated further.

COLORECTAL CARCINOMA

Growth inhibitory effects of CPEC have been demonstrated in four different human colorectal cell lines (HCT 116, SNU-C4, NCI-H630 and HT-29) [36, 37]. The IC50 values vary between 10 and 60 nM after 72 hours of incubation (HCT 116, SNU-C4 and NCI-H630) and 460 nM after 24 hours of incubation (HT-29). For an *in vivo* study, mice were inoculated with HT-29 cells. Although CPEC treatment did not fully halt tumor growth, it was shown that the increase was only one third of the growth measured in controls [38]. Combination treatment of CPEC with cisplatin was examined *in vitro* and *in vivo* (athymic mice) using HT-29 cells [39]. Cisplatin treatment alone did not result in significant tumor reduction; this is in line with clinical data indicating limited activity of cisplatin in colorectal carcinoma as a single agent [40]. However, when cisplatin (4 mg/kg Q7Dx3) was combined with CPEC (1.5 mg/kg, QDx9) tumor volume was reduced to 16% of the volume in the control group. Treatment with CPEC alone resulted in a reduction of 40%. However, when treatment was stopped, tumor growth was detected again, indicating a cytostatic and not cytocidal effect [39].

IN VITRO RESISTANCE

Since in clinical oncology drug resistance is a frequent cause of treatment failure, attempts have been made to investigate CPEC resistance *in vitro* in MOLT-4 lymphoblasts and L1210 leukemic cells [41,42]. In the resistant MOLT-4 lymphoblasts, CPEC-TP was formed 10-100 fold lower than in the wild type cell line. Resistance could be partly explained by a decreased activity of the enzyme uridine-cytidine kinase which catalyses the first phosphorylation step of CPEC. However, this was not the only possible explanation as concentrations of CPEC-TP that were cytotoxic in the wild type cell line, were found not cytotoxic in the resistant cell line and high CTP levels were found in the resistant cells. Therefore it could be concluded that there was another mechanism at work and it is believed that this could be a change in CTP-synthetase activity [42, 43]. This second mechanism was confirmed in leukemic cells, where an increased activity of CTP-synthethase was found in the resistant cells without a change in uridine-cytidine kinase [42].

An increased activity of the enzyme that is responsible for the deamination of CPEC (cytidine deaminase, CDD, Fig.**3**) to its metabolite CPEU could be a third cause of resistance. By deaminating cytosine nucleosides and analogs, CDD prevents the accumulation of the intracellular active triphosphates. Overexpression of CDD has been associated with protection of cells from

cytarabine and gemcitabine [44]. A fourth hypothetic mechanism might be found in the transport over the cellular membrane. Huang *et al* showed that CPEC diffusion is facilitated by ENT1 and ENT2 [10]. Changes in these transporters might influence the uptake of CPEC in the cell. Nitric oxide has been able to reduce ENT1 promotor activity in human fetal endothelium [45] and during hypoxia ENT1 function seems to be repressed, making hypoxic tumors potentially susceptible for reducing CPEC uptake [46]. It is not clear whether other transporters involved in nucleoside transport such as CNT and MRP4 and MRP5 are involved in CPEC transport. If CPEC transport would not be mediated by other nucleoside transporters, tumors predominantly expressing CNT might be resistant to CPEC. However, there are little data as yet about nucleoside transporter expression among tumors. What is clear however, is that leukemia cells seem to have both an ENT and a CNT transporter function [47].

Although it is unclear whether *in vitro* created resistance is a good model for future *in vivo* resistance, the results described here might be a useful tool in understanding resistance *in vivo*.

MODULATION OF CYTOTOXIC EFFECT

The deamination product of CPEC, CPEU was found to protect cells against the cytotoxic effects of CPEC [17]. Coincubation of a 100-fold higher concentration of CPEU (50 μM) with CPEC (0.5 μM) resulted in 50% survival of cells, whereas only 10% survived with 0.5 μM CPEC alone. Addition of the CPEU after incubation of CPEC diminished the increase in survival. An inhibition of uridine-cytidine kinase was suggested to be responsible for the 'rescue' by CPEU. This might result in decreased concentrations of CPEC-TP as uridine-cytidine kinase is necessary for the first phophorylation step of CPEC. Clinical implications of this effect are not to be expected as CPEU levels in humans did not exceed those of CPEC [48]. Cytidine might be a more useful modulator of CPEC activity. *In vitro* experiments in leukemic and colorectal cells have shown an increase in survival even after delayed administration of cytidine to CPEC treated cells [14, 37]. Combination treatment of CPEC and cytidine in mice inoculated with L1210 cells resulted in less toxicity without significant changes in increase in life span [29]. Competition for transmembrane transport and phosphorylation might be responsible for the observed effects. It was suggested that by delaying the administration of cytidine a first rapid effect of CPEC could be induced, followed by a rescue of toxic effects of cytidine [14].

In neuroblastoma cells retinoic acid attenuated the effects of CPEC and resulted in a 5 to 20 fold increase of IC50 of CPEC. As both agents show activity against neuroblastoma, this might have consequences for future combined therapy regimens [49].

PHARMACOKINETICS

Animal

Zaharko *et al* studied the pharmacokinetics of CPEC in mice, rats and beagle dogs. The plasma concentration was best described by a three compartment model consisting of a central compartment (extracellular fluid) and two cellular compartments. After distribution a rapid first elimination phase was observed followed by a long terminal half-life which probably is caused by the retention of CPEC as CPEC-TP and subsequent slow release of CPEC from CPEC-TP by phosphatases. CPEC was mainly cleared into urine unchanged for all different species studied [50]. In contrast to these data, clearance of CPEC in nonhuman primates occurred primary by deamination by cytidine deaminase to the inactive metabolite CPEU. Only 20% of the total dose was excreted as an unchanged drug. The deamination resulted in a lower total exposure of CPEC (expressed by a lower AUC) in monkeys compared to an equivalent dose of CPEC in rodents. CPEC was rapidly eliminated with a terminal half life of 20 to 60 minutes. Two hours after a single dose of CPEC, CPEU levels exceeded CPEC levels more than 40 fold. However, after continuous infusion steady state concentrations of CPEU varied no more than 4 times the CPEC levels, suggesting a saturable metabolism. The differences between rodents and nonhuman primates may be explained by the activity of cytidine deaminase. Rats have an almost non-existent cytidine deaminase, whereas high levels can be found in nonhuman primates [51]. Results from these studies have been used to determine the dose of CPEC to be used in clinical trials.

Human

Data from two patients that received an intravenous test dose of 24 mg/m^2 showed two phases of rapid elimination of CPEC from the plasma (half-lives 8 and 100 minutes respectively). CPEC could still be detected 24 hrs after the end of the infusion, suggesting the existence of a third phase resulting in a long terminal elimination half-life. Measurements from 26 patients receiving a 24 hrs continuous infusion of CPEC confirmed these results. During the 24 hrs-infusions the steady state plasma levels increased linearly with increasing doses and steady state was achieved after approximately 12 hrs of infusion. Plasma concentrations of CPEU were below CPEC levels [48]. These pharmacokinetic data are in line with the results from preclinical studies in rodents [50]. Humans are reported to have less cytidine deaminase than nonhuman primates [51] Deamination therefore seems to be not as important in clearance as it is in the case of nonhuman primates and CPEC is mainly eliminated as an unchanged drug in the urine.

TOXICOLOGY

Animal

Toxicity of CPEC seems to be dose and schedule related. Mice can tolerate single doses of CPEC up to 50 mg/kg without showing any signs of toxicity [15]. However, more than 2 mg/kg for at least 9 days results in weight loss [15, 29]. Tolerability of CPEC also seems to differ among the different species. No toxicity of CPEC was detected in rats treated with 2 mg/kg/day for a period of four weeks [35]. The difference in tolerability between species was confirmed by other experiments showing a more toxic effect of CPEC on mice than on rats [52]. The high cytidine levels or almost absent levels of cytidine deaminase in rats are thought to account for these differences. Single doses of CPEC in beagle dogs (3-40 mg/kg) resulted in oral lesions and a decrease in body weight. Bone marrow and gastro-intestinal epithelium were also affected [53].

Human

In a phase I study in 26 patients with solid tumors granulocytopenia and thrombocytopenia were reported as dose limiting toxicities in 2 of 3 patients during the first 3 weeks after a 24-hour infusion of CPEC at a dose of 5.9 mg/m^2 per hr, whereas non dose limiting were vomiting, mucositis and diarrhea. The majority of patients had colorectal cancer and most of them were heavily pretreated with chemo- and/or radiotherapy. All but one patient had documented disease progression prior to entering this study. The median time to treatment failure was > 3 months in 11 patients (42%), which is compatible with an active antitumor agent. However, the most severe adverse effect was a severe hypotension which occurred in 5 patients at the lower dose levels of 3.5 and 4.7 mg/m^2 per hr and resulted in death in two of them. None of the patients experiencing the hypotension was dehydrated. Hypotension occurred 24 to 48 hours after the end of infusion and seemed to be dose related. No hypotensive episodes or other important toxicity occurred at doses equal or below 2.5 mg/m^2 per hr. Laboratory results from the hypotensive patients showed a pattern consistent with hypoperfusion (hypoxemia, increased creatinine and metabolic acidosis). The echocardiograms showed left ventricular contraction but no signs of pericardial effusion. Post mortem examination on one of the two deceased patients revealed subendocardial necrosis and minimal pericardial effusion. From the other patient it was known that there was no prior cardiac history [48]. The mechanism of these hypotensive episodes was not clarified. Between those patients that did experience hypotension and those that did not there were no differences in the CPEC-CPEU ratio. Moreover, inhibition of CTP synthase activity seemed to be similar for all patients. These findings suggested that there were no differences in uptake or excretion between the patients. Influence of CPEC on cardiolipin metabolism (a major phospholipid in the heart) [54, 55] or a preference in the cardiomyocytes for CTP synthesis by the salvage pathway via CTP synthetase, were proposed mechanisms.

CONCLUSION AND PLACE IN THERAPY

The mechanism of action of CPEC is supposed to involve inhibition of the enzyme CTP-synthetase. As a high activity of this enzyme has been observed in several malignancies [3-6], CTP-synthetase seems to be an interesting target for a wide range of tumors.

The effects of CPEC have been studied most extensively in leukemia. Current therapy for leukemia has improved survival, however, e.g. ALL is still associated with a poor prognosis and new agents are warranted. Therapy with CPEC in humans with solid tumors resulted in hematotoxic side effects [48], suggesting that leukemic cells might be sensitive to CPEC. Indeed several preclinical studies show anti-leukemic activity of CPEC. Moreover, it might be worthwhile to investigate the use of CPEC in combination with other drugs for the treatment of ALL, like cytarabine. Other promising areas might be colorectal carcinoma and neuroblastoma. For colorectal carcinoma addition of CPEC to currently used therapy combinations (e.g. oxaliplatin with fluorouracil and the VEGF inhibitor bevacizumab) could be of interest. As the use of CPEC in neuroblastoma has only been studied *in vitro*, testing the drug in an animal model will be necessary to confirm the *in vitro* data. Based on the *in vitro* data it might be interesting to study the effect of CPEC on neuroblastoma in combination with other drugs, like gemcitabine.

The observed cardiotoxic side effects in the Phase I trial remain a point of concern and care should be taken if the drug is to be administrated in future clinical trials. As the toxicity seemed to be dose related, a restriction in the maximum administrated dose will have to be considered in these trials. Moreover, close monitoring of plasma levels will be necessary to check whether the administrated dose does not lead to plasma levels at which cardiotoxicity occurred in the Phase I trial.

The reviewed data in this manuscript illustrate an anti-cancer effect of CPEC in several tumor models and suggest that CPEC might be a potential drug in anticancer treatment. Further study is needed, however, until now only preclinical data on efficacy are available and it is as yet unclear whether the same anti-cancer effect of CPEC can be reached in humans.

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CHAPTER 3

FORMULATION, QUALITY CONTROL AND SHELF LIFE OF THE EXPERIMENTAL CYTOSTATIC DRUG CYCLOPENTENYL CYTOSINE

Drug Development and Industrial Pharmacy 2006;32:497-503

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ABSTRACT

This paper describes the formulation and quality control of an aqueous sterilized formulation of the experimental cytostatic drug cyclopentenyl cytosine (CPEC) to be used in Phase I/II clinical trials. The raw drug substance was extensively tested. A High Performance Liquid Chromotography (HPLC) method was validated for the quality control of the formulated product. The aqueous formulation was found to be stable for at least 2 years at 2-8°C. Sterilization (15 min at 121 °C) showed no influence on drug stability. The results show that CPEC can be formulated in an aqueous solution. The described HPLC method is a useful tool in the pharmaceutical quality control.

Keywords: Cyclopentenyl cytosine, CPEC, Cytostatic drug, Formulation, Quality control

INTRODUCTION

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC, Fig. 1) is a pyrimidine analogue of cytidine, currently entering phase I/II trials in recurrent leukemia. After transmembrane transport, CPEC is subsequently activated by phosphorylation by the enzymes uridine-cytidine kinase, nucleoside monophosphate-kinase (NMP-kinase), and nucleoside diphosphate-kinase (NDP-kinase) respectively to form CPEC-triphosphate (CPEC-TP), the major intracellular pharmacologically active metabolite. Cyclopentenyl cytosine-triphosphate (CPEC-TP) is an inhibitor of cytidine triphosphate-synthetase (CTP-synthetase) resulting in inhibition of RNA and DNA synthesis and leading to S-phase accumulation (Fig. 2). So far, CPEC pharmacokinetics and toxicity have been studied in a single phase I trial in 26 patients with solid tumors (in majority colorectal carcinoma). Dose limiting toxicity was of hematological nature. However, the most severe adverse drug reaction was cardiovascular: six episodes of hypotension occurred in five patients (dose range: 3-4.7 mg/m^2/h) and two patients, treated with the highest applied dose (4.7 mg/m^2/h), had a fatal hypotensic episode [1]. In the early phase I study, a lyophilized formulation of CPEC dispensed by the National Cancer Institute (NCI, Bethesda, Maryland, USA), was applied. In the present article we describe the development of an aqueous drug formulation of CPEC for intravenous use in our phase II trial and methods for the pharmaceutical quality control of the raw drug substance and formulated product.

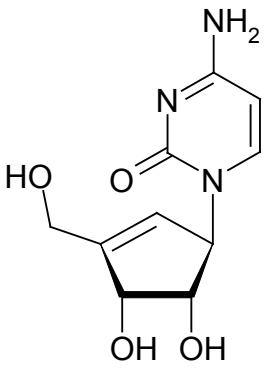

Figure 1 Chemical Structure of CPEC.

The mechanism of the cardiotoxic effects remains yet unclear [2] and has hampered the initiation of phase II studies with CPEC. In various human and animal leukemia models, antitumor activity of CPEC was demonstrated at relatively low drug concentrations [3,4]. Following these observations, we recently have initiated a phase II study of low dose CPEC in adults with hematological malignancies (acute myeloid leukemia, acute lymphocytic leukemia, and myelodysplastic syndrome) under strict cardiac monitoring. Furthermore, a phase I/II trial in pediatric patients with recurrent leukemia is planned.

MATERIAL AND METHODS

Chemicals

Cyclopentenyl cytosine (CPEC) (NSC375575, lotnr. BK-09-142) was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment of the NCI. For the manufacturing, 10 ml glass vials (DIN-I, Aluglas, Uithoorn, Netherlands) were used to contain the product solution and sterile 0.2 μm cellulose acetate filters (Millipore, The Netherlands) for filtration. Water for injections was manufactured in house (conform PhEur) (Department of Pharmacy, Academic Medical Centre, Amsterdam, The Netherlands).

The following analytical grade chemicals were used for quality control: methanol (Labscan Ltd, Ireland); ammoniumacetate (Merck, Darmstadt, Germany), 5- methylcytosine (Sigma-Aldrich, Munich, Germany), and tetrahydrofuran (THF, Merck, Darmstadt, Germany).

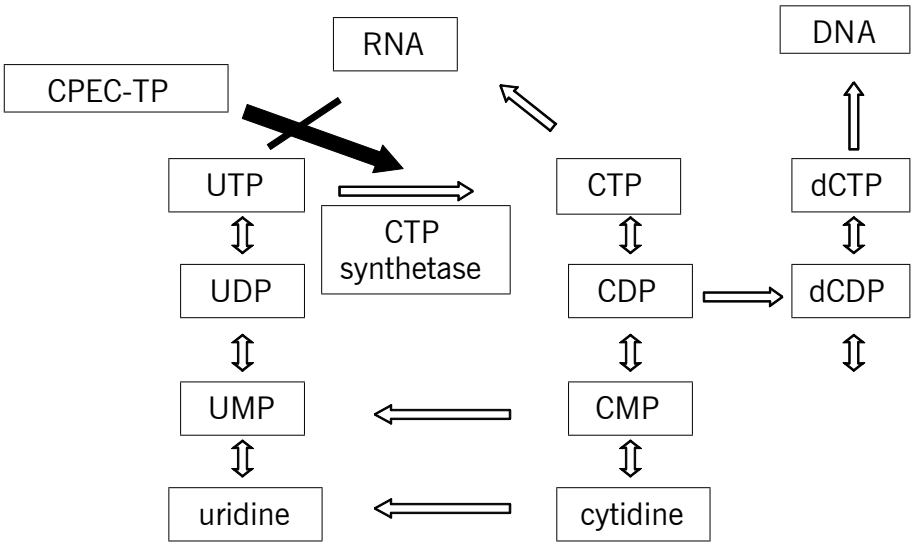

Figure 2 Mechanism of Action of CPEC.

In the figure a part of the pyrimidine (deoxy) ribonucleotide synthesis is shown. The enzyme CTP-synthetase is inhibited by CPEC-TP, leading to depletion of the CTP pool and subsequently inhibtion of DNA and RNA synthesis. CPEC-TP: CPEC-triphosphate; UTP: Uridine-triphosphate; UDP: Uridine-diphosphate; UMP: Uridine-monophosphate; CTP: Cytidine-triphosphate; CDP: Cytidine-diphosphate; CMP: Cytidine-monophosphate; dCDP: Deoxycytidine-diphosphate; dCTP: Deoxycytidine-triphosphate.

Quality Control of Drug Substance

The raw active substance was stored at 20ºC in a glass airtight container. The National Cancer Institute (NCI) initially performed identity and purity tests on each lot of the bulk drug substance (personal communication, Dr. R. Vishnuvajjala). The general tests performed by the NCI consisted of appearance, melting point, optical rotation, and elemental analysis. Conformity was further tested with UV (ultra violet), IR (infrared), MS (mass spectrometry), H-1, and C-14 NMR (nuclear magnetic resonance). Furthermore, the drug substance was chromatographically tested [thin layer and reverse phase high performance liquid chromatography (RP-HPLC)].

Before drug formulation, the following quality control tests on the raw material were performed (Laboratory of Clinical Pharmacy & Toxicology, AMC, Amsterdam).

IDENTITY

Identity was confirmed by infrared spectroscopy (FTIR-8201PC Shimadzu Corp., Japan), LC-MSMS product ion scan, concentration app. 100 μg/ml and UV-VIS spectroscopy (UV-2410 PC, Shimadzu, 8.4 mg/ml in H2O).

LOSS ON DRYING

An amount of approximately 100 mg CPEC (precisely weighed) was dried for 4 h at 100-105ºC in a pre-dried glass vial (1 h at 100-105ºC).

HEAVY METALS

Presence of heavy metals was not tested as no heavy metals that can be detected by the USP method were used in the synthesis of CPEC.

QUALITATIVE DRUG ANALYSIS

Analysis of impurities of the drug substance and content of the product was performed using an RP-HPLC equipped with a UV photo diode array detector (Jasco MD 1510 Multiwavelength detector, Jasco Corp., Japan), an autosampler (Jasco AS 1555 Intelligent sampler), and a pump (Jasco PU 1580 HPLC pump). Chromatograms were processed using Empower software (Waters, Netherlands). Separation was achieved using a Supelcosil LC-18 column (25 cm x 4,6 mm, 5 μm) (Supelco, Sigma-Aldrich, Netherlands). The method was originally developed at the NCI. The mobile phase consisted of 2% methanol in 0.1 M ammoniumacetate at a flow rate of 1.0 ml/min. The detection wavelength was 276 nm. The injection volume was 20 μl and a run time of 20 min was employed. A concentration of 24 mg CPEC/l and 50 mg CPEC/l was used for analysis of impurities of the drug substance. A concentration of 4.5 mg/l was used for the analysis of content of the product. The internal standard used was 5-methylcytosine (5 mg/l).

Validation of HPLC Procedure

Validation of the method of analysis was performed according to good clinical laboratory practice (GCLP) guidelines as follows.

DETERMINATION OF THE SELECTIVITY AND SPECIFICITY

Impurities or degradation products were required to elute separately from CPEC in order to assess possible impurities. Cytosine is used in the synthesis of CPEC, and dideoxycytidine and cytarabine have a strong structural relationship with CPEC. Therefore, cytosine, dideoxycytidine, and cytarabine were examined. In order to test if degradation products could be detected and separated, samples of CPEC were exposed to extreme temperature (48 h at 120ºC), acid and alkaline conditions, respectively. All substances were dissolved in water and injected onto the HPLC system. To evaluate proper separation from CPEC, the resolution factor (R) between CPEC and the nearest peak (internal standard, 5-methylcytosine) was determined. To obtain a separation of peaks of at least 99.7% for quantification, a resolution factor of at least 1.5 was required.

DETERMINATION OF RANGE

The final product (CPEC, 4.5 mg/ml) was diluted to obtain a concentration suitable for quantification with HPLC. A quantification range of 75% and 125% of this concentration was chosen.

ACCURACY AND RECOVERY

As the final product will be dissolved in an aqueous solution, which will only have to be diluted for analysis, no tests on accuracy and recovery were performed.

REPEATABILITY AND INTERMEDIARY PRECISION

Repeatability was tested by analyzing a reference sample within one day ($n = 6$) and on six different days. Repeatability was determined for four different concentrations (90%, 95%, 105%, and 110% of the nominal product concentration). Concentrations were calculated relative to the 100% value (“one point calibration”). The mean, standard deviation, and variation coefficient were determined of each series. Repeatability variation coefficients were considered acceptable below 5%. Intermediary precision was performed in the same way as the repeatability but with varying equipment, technician, and eluent lot.

LINEARITY

Reference samples with 75%, 90%, 95%, 100%, 105%, 110%, and 125% of the declared product concentrations were analyzed on six different days. A calibration line was calculated for each different day. Each reference sample was recalculated on the calibration line obtained with the other reference samples of that day. The obtained individual concentrations were not

allowed to differ more than 5% of the nominal values. Linearity ($y = ax + b$) was tested with the "goodness of fit test" (GOF-test) using SPSS software (version 9.0). An F-value of the test of at least 7.71 and an r^2 value of >0.990 were considered acceptable.

ROBUSTNESS AND SUITABILITY OF THE METHOD

In order to test whether the method could be used for the final (sterilized) product, two reference samples were sterilized (15 min at 121°C) before analysis. The following changes in chromatography conditions were tested in order to investigate whether the separation could be further optimized: change of the MeOH (Methanol) concentration in the mobile phase (1% instead of 2%) and addition of THF.

Drug Formulation

Cyclopentenyl cytosine (CPEC) was dissolved in purified water at a concentration of 4.5 mg/ml (dry weight, determined one day before production). The dissolved product was filtered through a 0.2 μm membrane filter in a class A laminar flow safety cabinet with class D background environment. Ten milliliter sterile vials were filled with 2.0 ml of the product solution. Vials were sterilized (15 min 121°C) in a heat water autoclave. As initially little information was available on the stability of CPEC in an aqueous solution, the vials were stored at 4°C after sterilization.

QUALITY CONTROL OF PRODUCT

Before the product could be used, several analyses were performed. Labeling, volume, pH, and physical appearance had to comply with product specifications. Sterility was assured by parametric release. Content was assessed in duplo by using the above described HPLC method.

SHELF LIFE EXPERIMENTS

In order to test whether the content of the product remained stable until the end of the planned expiration date, two different batches of the product were analyzed on several time points up to 24 months after production.

RESULTS

Quality Control of Drug Substance

IDENTITY

Infrared spectroscopy showed a spectrum consistent with the chemical structure and with results obtained earlier (certificate of analysis, NCI). The UV-VIS spectrum showed a major absorption band with a maximum at 276 nm which corresponded with earlier data. The mass spectrum also complied with the chemical structure of CPEC (Fig. 3).

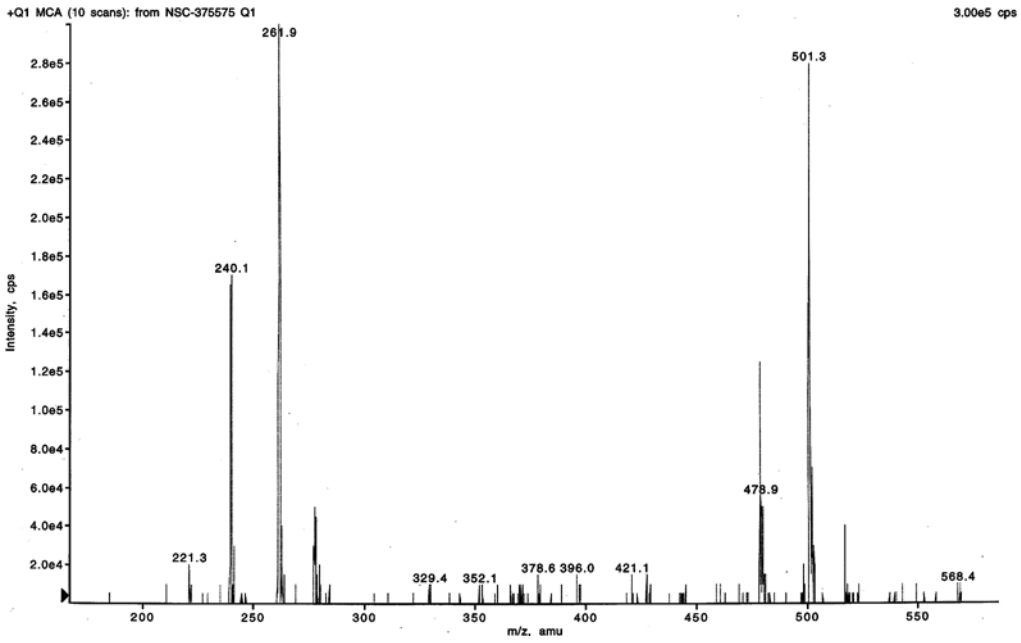

Figure 3 Mass Spectrum of CPEC

LOSS ON DRYING

Loss on drying was determined on two samples; the loss on drying was respectively 9.07% and 9.93%, with a mean loss of 9.5%.

Validation of the HPLC Method

DETERMINATION OF THE SELECTIVITY AND SPECIFICITY

Cyclopentenyl cytosine (CPEC), dideoxycytidine, 5-methylcytosine, and cytarabine eluated well separated from the column. The resolution between CPEC and the internal standard (5-methylcytosine) was 35 which is above the limit of acceptance of 1.5. A representative chromatogram of a reference sample containing CPEC and the internal standard is shown in Fig. 4. The chromatogram of the sample which was kept 48 h at 120ºC, showed a minor unidentified peak next to the CPEC peak, probably indicating a degradation product. The chromatograms of the samples submitted to acid and alkaline conditions were similar to the unexposed reference sample.

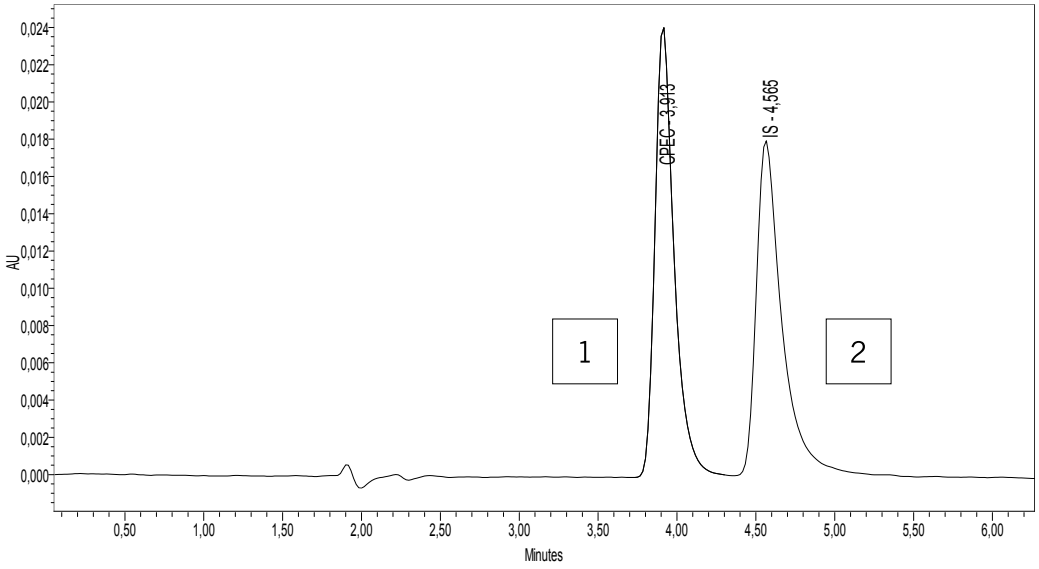

Figure 4 Characteristic HPLC Chromatogram of CPEC. 1: CPEC. (4.5 mg/l); 2: 5-methylcytosine (Internal Standard, 5 mg/l).

DETERMINATION OF RANGE

Dilutions of the final product to 75% (3.375 mg/l) and 125% (5.625 mg/l) of the declared product concentration could be measured and quantified.

REPEATABILITY AND INTERMEDIARY PRECISION

The repeatability and intermediary variation coefficients for all the determined concentrations (90%, 95%, 105%, and 110%) were less than 3%. Mean, standard deviation, and variation coefficients for the intra (six in one day) and inter (six different days) day repeatability are shown in Table 1.

Table 1 Repeatability and intermediary precision of HPLC method

	Mean concentration (%)		Standard deviation		Variation coefficient (%)	
90%	repeatability	intermediary	repeatability	intermediary	repeatability	intermediary
Within 1 day	91.07	89.50	0.58	2.04	0.63	2.28
6 days	90.37	91.41	0.91	1.02	1.01	1.12
95%						
Within 1 day	93.69	94.84	0.49	2.00	0.53	2.07
6 days	95.41	95.54	1.34	0.64	1.40	0.67
105%						
Within 1 day	104.82	105.10	2.12	1.21	2.03	1.14
6 days	103.99	105.66	1.32	1.09	1.27	1.03
110%						
Within 1 day	109.24	111.17	2.31	2.16	2.11	1.94
6 days	109.06	110.74	1.48	1.33	1.36	1.21

LINEARITY

All seven-point calibration curves of CPEC were linear with a mean correlation coefficient of 0.994 (standard deviation 0.004) and F-values above 7.71. The maximum difference of the individual concentrations from the nominal concentrations was 1.31% (see Table 1).

ROBUSTNESS AND SUITABILITY OF THE METHOD

The chromatograms of the sterilized product were not different from the non-sterilized standard solution. Changes in chromatographic condition did not further improve the separation (data not shown).

Formulation of Product

Two lots (batch size: 100 vials) were produced and could be released for clinical use after quality control. Shelf life experiments showed no deterioration in CPEC concentration (Table 2) at 24 months after production. The lowest concentration after two years in the two lots was 97.2 and 99.5%, respectively, of the declared concentration. After 18 months the concentration seemed to increase. A possible cause for the assumed increase could be analytical and probably due to insufficient drying of the standard used in the HPLC method. The results at 20 and 24 months seemed to confirm this assumption as the concentrations remained then within 10% of the declared value.

Table 2 Shelf life experiment

month s of storage	Content CPEC batch 1 (% of declared concentration	Content batch 2 (% of declared concentration, mean of two assays)
0	102.0 [101.66-102.30]	99.3 [99.3-99.3]
2	99.7 [99.6-99.7]	*
9	99.5 [99.0-99.9]	97.2 [96.4-98.0]
11	101.3 [101.32]**	102.4 [102.38]**
18	112.6 [112.2-113.0]	109.4 [109.2-109.6]
20	106.5 [106.1-107.0]	108.1 [107.7-108.4]
24	108.3 [106.2-111.5]	101.2 [98.1-104.3]

The percentages in the table represent the mean of two assays, between brackets the individual values are given.
*Not determined
**Only one assay performed.

DISCUSSION

In an early and single Phase I trial of CPEC in patients with solid tumors, a lyophilized formulation of CPEC was used. A drawback of this approach is that lyophilization facilities are required for drug formulation and once formulated the presentation form needs reconstitution before administration. However, in case of labile compounds, lyophilization permits much longer storage as compared to aqueous solutions. As pilot experiments suggested good stability of CPEC in aqueous solutions, we investigated whether the production of an aqueous CPEC drug formulation was feasible. We started our experiments with testing the raw drug substance. As no Pharmacopoeia monograph is available, quality control criteria were absent and a set of specifications and analytical methods were determined in our laboratory based upon information from the NCI. The chosen HPLC method was developed for pharmaceutical quality control and not for the determination of CPEC concentrations in biological specimens such as serum. This implicates that stability indicating performance and the precision at the declared product concentration were considered more relevant than e.g., the limit of detection. The method was validated and found to be precise, linear, and stability indicating. The HPLC method was accepted for use in pharmaceutical quality control of both the raw active substance and the product. The initial expiration date was set at three years after production and actual parent drug concentration was frequently monitored at a shelf life experiment during storage at 4ºC. Currently, after two years of shelf life, the CPEC concentration remained above 97% of the declared concentration. Measurements will be continued for the remaining storage time. Before administration the product can be further diluted with standard infusion solutions. There is no reason to assume that the product will be less stable after dilution. However, as the product does not contain preservatives, a short period of usage will be advised for microbiological reasons. This study shows that CPEC can be formulated in an aqueous solution and stored for at least two years at 4ºC. The described HPLC method is a useful tool in the pharmaceutical quality control of the drug substance and product.

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CHAPTER 4

QUANTITATIVE ANALYSIS OF THE EXPERIMENTAL CYTOTOXIC DRUG CYCLOPENTENYL CYTOSINE (CPEC) AND ITS METABOLITE IN PLASMA WITH HPLC TANDEM MASS SPECTROMETRY

Submitted

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ABSTRACT

The cytotoxic drug cyclopentenyl cytosine (CPEC) is currently being investigated in early clinical trials. Monitoring of plasma levels is required for pharmacokinetic analysis and management of toxicity. This paper describes the analysis of CPEC and CPEU in plasma by HPLC-electrospray ionization tandem mass spectrometry. The calibration curves for CPEC and the metabolite cyclopentenyl uracil (CPEU) were linear up to 10 μM with correlation coefficients of 0.997 (SD = 0.002, n = 10) and 0.997 (SD = 0.004, n = 10), respectively. The detection limit for CPEC was 0.03 μM and 0.15 μM for CPEU. The intra- and interassay coefficients of variation for CPEC and CPEU were less then 10%. The usefulness of the method was demonstrated by analyzing CPEC and CPEU in plasma of a patient treated with CPEC. HPLC with electrospray ionization tandem mass spectrometry allowed rapid and sensitive determination of CPEC and CPEU levels in plasma.

INTRODUCTION

The experimental drug cyclopentenyl cytosine (CPEC) is a pyrimidine analogue of cytidine (figure 1). The active metabolite CPEC-triphosphate (CPEC-TP) is formed after transmembrane transport and the successive phosphorylation by the enzymes uridine-cytidine kinase, nucleoside monophosphate kinase and nucleoside diphosphate kinase. CPEC-TP inhibits cytidine triphosphate synthetase (CTP synthetase) which is known to catalyse the formation of cytidine triphosphate (CTP) from uridine triphosphate (UTP). Inhibition of CTP synthetase leads to diminished RNA and DNA synthesis and accumulation of cells in the S-phase of the cell cycle. Although CTP can also be synthesized by the salvage of cytidine, it has been shown that several malignant tissues predominantly use the pathway involving CTP synthetase [1,2]. CPEC can be deaminated to its metabolite cyclopentenyl uracil (CPEU). Pharmacokinetic data from animals revealed major interspecies differences. In rodents and dogs renal excretion of the unchanged drug was the primary route of elimination whereas in primates deamination to CPEU was dominant [3,4]. Although deamination to CPEU accounted for a significant part of the elimination in humans, elimination of CPEC occurred mainly by renal excretion of the unchanged drug [5].

CPEC has been studied in humans in a phase I trial involving 26 adults with solid tumors [5]. Hematological toxicity was dose limiting, however, the most serious adverse effect was severe hypotension. Five patients experienced in total 6 episodes of hypotension, which resulted in the death of two patients. As the events occurred in the higher dose groups, a dose-effect relationship was suggested. Animal studies gave no further indication of the underlying mechanism of this adverse effect [6]. Currently, we are initiating phase I/II trials with low dose CPEC in adult and pediatric patients with refractory hematological malignancies (acute myeloid leukemia, acute lymphoid leukemia and myelodysplastic disorders) under strict cardiac monitoring.

In the previous phase I trial, CPEC was measured in serum samples using a HPLC method with UV detection [7]. However, this method had a detection limit of 0.1 μM for CPEC, and was expected not to be sensitive enough when using low dosage regimens. Moreover, the method was laborious requiring ultra filtration and subsequent solid phase extraction of the plasma before it could be subjected to HPLC analysis.

Therefore, we have developed a rapid, specific and more sensitive method, using HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI MS/MS).

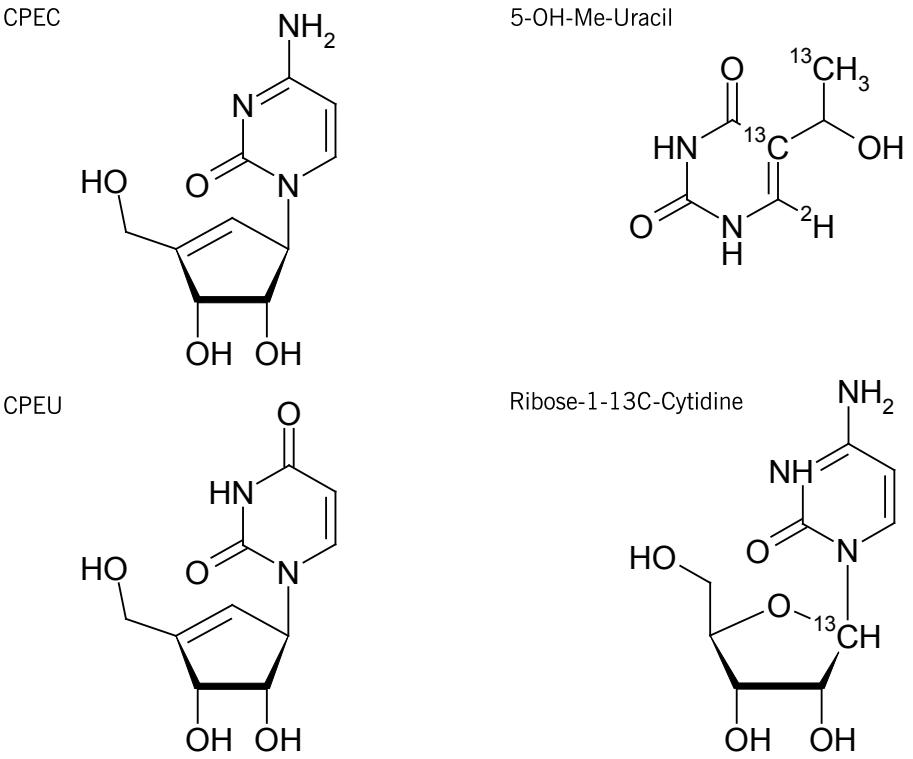

Figure 1

Chemical structure of CPEC, CPEU, 5-OH-Me-Uracil (+4) (internal standard for CPEC) and ribose-1-^{13}C-Cytidine (internal standard for CPEU)

MATERIALS AND METHODS

Chemicals

CPEC (NSC375575) and CPEU (NSC375574) were kindly donated by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute (NCI, Bethesda, Maryland, USA). The internal standards 4,5-$^{13}C_2$-5',5'-D_2-5-Hydroxymethyluracil (5-OH-Me-Uracil(+4)) and ribose-1-^{13}C-Cytidine (Cytidine(+1)) were obtained from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). Methanol, acetic acid and ammonia were obtained from Merck (Amsterdam, The Netherlands) and were of analytical or HPLC grade. Demineralised water from a Milli Q water purification system (Millipore, Amsterdam, The Netherlands) was used for preparation of stock solutions. Pooled plasma (free from CPEC and CPEU) from different individuals was obtained from the hospital laboratory.

Sample preparation

INTERNAL STANDARD

Stock solutions of Cytidine(+1), (99% pure, internal standard for CPEU) and 5-OH-Me-Uracil(+4), (99% pure, internal standard for CPEC) were prepared in demineralised water. A 1 mM solution of both stable isotopes was used as internal standard.

SAMPLES PREPARATION

Stock solutions of CPEC and CPEU consisted of adequately weighed quantities of CPEC and CPEU dissolved in purified water to obtain concentrations of 4.07 mM CPEC and 1.89 mM CPEU. These stock solutions were combined and further diluted in purified water to obtain working solutions of 400 μM CPEC and CPEU. The working solutions were used to spike the plasma in appropriate concentrations. All solutions were stored at -20ºC. For calibration and validation, concentration series of CPEC and CPEU in pooled plasma (0.03 – 10 μM) were prepared.

A volume of 20 μl internal standard was added to 200 μl plasma. Proteins were precipitated by adding 10 μl 11.6 M $HClO_4$ followed by centrifugation (5 minutes 10.000 x g). Subsequently 150 μl of the supernatant was neutralized with 5 μl 5 M K_2CO_3. After 10 minutes the samples were centrifuged in a microcentrifuge (5 minutes 10.000 x g) and an aliquot of 100 μl of the supernatant was injected in the HPLC-MS/MS.

HPLC-electrospray ionization ms/ms

Separation of the components in the samples was performed on a Phenomenex Aqua C18 analytical column (250 x 4.6 mm) (Phenomenex, Torrance, USA). Solvent A consisted of 0.05 M acetic acid in water (pH set at 4.70 with 25% ammonia) and solvent B of methanol. Elution was performed by use of a gradient, at a flow rate of 1 ml/min, as follows: 0-6 min, 100% solvent A to 30% solvent B; 6-6.1 min, 30% solvent B to 80% solvent B; 6.1-9.0 min, 80% solvent B; 9.0-9.1 min, 80% solvent B to 100% solvent A; 9.1-15 min 100% solvent A. A splitter between the HPLC column and the mass spectrometer was used to introduce the eluate between 6.0 and 9.0 minutes at a flow of 50 μl/min into the mass spectrometer. Samples were analyzed on a Waters Micromass Quattro II tandem mass spectrometer (Micromass, Manchester, UK) in the positive ion mode using electrospray ionization (ESI). Nitrogen was used as nebulizing gas and Argon as collision gas (collision cell pressure $2.5*10^{-3}$ mBar). The source temperature was set at 80ºC and the capillary voltage at 3.5kV. Detection took place by multiple reaction monitoring (MRM) with a dwell time of 0.1 second per channel. The following transitions and settings were used: CPEC m/z 240>112 (cone voltage 35V); 5-OH-Me-Ura(+4): 147>86 (cone voltage 27V); CPEU 241>113 (cone voltage 35V), Cytidine(+1) 245>112 (cone voltage 28V). A collision energy of 15eV was used.

Validation

Linearity of CPEC and CPEU was tested by injecting calibration mixtures with different concentrations (10, 8, 6, 2, 0.5, 0.05 and 0.03 μM). The calibration curves were obtained from a least-squares regression for the analyte/IS peak-area ratio versus the concentration of the analyte in the calibration mixture. The correlation coefficients of the calibration curves for CPEC and CPEU were required to be $\geq$ 0.990. The detection limit was established by injection of the calibration samples and defined as a signal to noise ratio of 3.

Intra-assay variation (intra-day precision) and the accuracy of the method was established by measuring plasma enriched with CPEC and CPEU at low (0.1 μM for CPEC and 0.5 μM for CPEU), medium (1μM) and high (4μM) concentrations (10 samples of each concentration in a single run). The inter-assay variation (inter-day precision) and accuracy was established by measuring the low, medium and high concentrations and the calibration line on 10 different days. The concentration of each sample was calculated using the calibration curves. The coefficients of variation were considered acceptable in case they were below 10%. Accuracy was expressed as [(overall mean concentration/nominal concentration) $\times$ 100%], and should be within the range from 85 to 115%. Three samples with 0.1 μM CPEC or 1 μM CPEU were analyzed at different time points after storage at -20^{o}C (0, 1 and 2 months).

The influence of the matrix was tested by determination of the recovery of CPEC and CPEU in plasma samples of 10 different patients spiked with medium and low concentrations of CPEC and CPEU.

Suitability of the method was tested by analyzing plasma samples that were obtained at different time points from a patient treated with CPEC in a phase I/II trial (approved by the Medical Ethical Committee of the Academic Medical Center) at our institute. In addition plasma from a mouse that was treated with CPEC for an *in vivo* study (approved by the Ethical Animal Research Committee of the Leiden University Medical Center) was also analyzed.

RESULTS

The calibration curve for CPEC was best described by a weighted (1/x) quadratic curve (R^2 = 0.997, SD = 0.002, n = 10) and not by a linear curve as non-linear responses at the higher points of the validation curve were observed. The calibration curve for CPEU was linear in the concentration range tested (r^2 = 0.997, SD = 0.004, n = 10). The detection limit (defined as a signal-to-noise ratio of 3) for CPEC proved to be 0.03 μM and for CPEU 0.12 μM. As the detection limit of CPEU was below the lowest validation point, we analyzed a spiked plasma sample with a concentration of 0.15 μM (n=3) and found an accuracy of 90%. Therefore the detection limit of CPEU was set at 0.15 μM. After storage at -20ºC we found an accuracy of 92% (mean 0.09, SD 0.006) for CPEC and 103% for CPEU (mean 1.03 SD 0.06).

Representative chromatograms of the analysis of a plasma sample spiked with CPEC (0.1 μM) and CPEU (0.5μM) are shown in figure 2.

The intra- and inter-assay variations and accuracy for the different concentrations analyzed are represented in tables 1 (CPEC) and 2 (CPEU). The coefficients of variation (CV) were all below 10%. The recovery of CPEC and CPEU in the different patient samples is presented in table 3.

Table 1 Intra- and inter-assay variation and accuracy for CPEC

	Intra-assay			Inter-assay		
	CV, %	Mean (SD)	Accuracy (%)	CV, %	Mean (SD)	Accuracy (%)
0.1 μM	7.5	0.1 (0.007)	92	6.2	0.1 (0.009)	107
1 μM	5.9	1.0 (0.06)	103	5.08	1.1 (0.05)	109
4 μM	1.3	4.1 (0.05)	103	6.6	3.9 (0.3)	98

Table 2 Intra- and inter-assay variation and accuracy for CPEU

	Intra-assay			Inter-assay		
	CV, %	Mean (SD)	Accuracy (%)	CV, %	Mean (SD)	Accuracy (%)
0.5 μM	3.9	0.5 (0.02)	97	3.7	0.5 (0.02)	102
1 μM	4.4	1.0 (0.04)	97	5	1.1 (0.05)	106
4 μM	4.1	3.7 (0.2)	93	8.5	4.0 (0.3)	100

Table 3 Recovery in 10 different plasma samples

	CPEC		CPEU	
	CV, %	Recovery, % (SD)	CV, %	Recovery % (SD)
0.1 μM	10.1	100.0 (1.0)	nd	nd
1 μM	10.0	99.0 (1.0)	4.5	95.0 (4.2)

nd = not determined

In plasma samples from a patient treated with low dose CPEC (1 mg/m^2/h for 24 hours), CPEU could be detected at the end of the infusion period and CPEC could be detected up to 48 hours after the end of the infusion (figure 3). When analyzing the plasma of a mouse treated with CPEC (5 mg/kg on 3 consecutive days) a concentration of 0.24 μM for CPEC was found, whereas no CPEU could be detected in the plasma of a control mouse that was treated with saline.

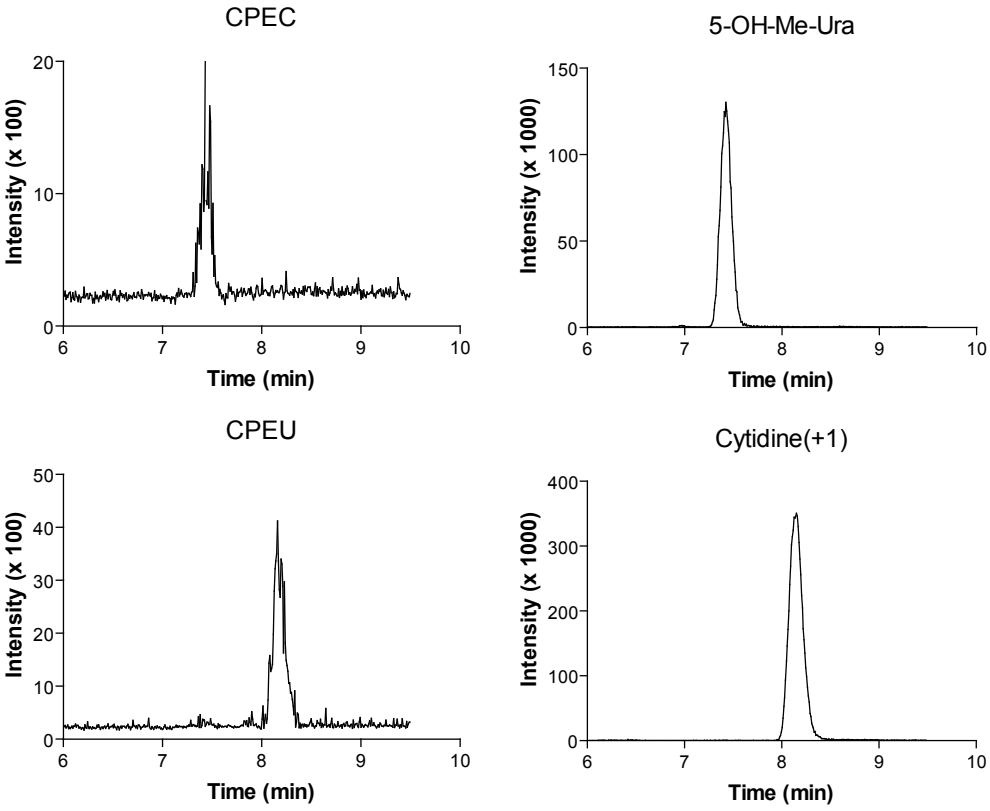

Figure 2

HPLC-ESI MS/MS chromatograms of CPEC and internal standard cytidine(+1) and CPEU and internal standard 5-OH-Me-Uracil(+4).

DISCUSSION

The first Phase I study with CPEC performed by Politi *et al*, showed that plasma levels of CPEC were in the low micromolar range. That study described two rapid phases of elimination after a bolus injection (24 mg/m2) in two patients. A late phase of elimination was also suggested, however, it was not possible to describe this phase of the pharmacokinetics profile as plasma concentrations 24 h after the end of the infusion were below the limit of quantitation of the used HPLC-UV method. Plasma levels in that study increased linearly with CPEC dose. The phase I study was terminated because of severe cardiovascular adverse events in the patients receiving more than 2.5 mg/m^2 CPEC [5]. Due to these adverse effects, new trials with CPEC will apply lower dosages (0.5-1.5 mg/m2/h for 24 hours) and remain under 2.5 mg/m^2/h. Monitoring of the plasma levels will be necessary to detect possible toxic plasma levels and to further explore CPEC pharmacokinetics. With lower dosages of CPEC, plasma levels are expected to be lower than 0.1 μM shortly after the end of the infusion. In order to be able to measure these low plasma CPEC concentrations, the use of a sensitive method allowing the quantification of CPEC at concentrations below 0.1 μM CPEC is necessary. Therefore, we developed a new HPLC-ESI MS/MS method, which proved to have the required sensitivity. With the method described and our instrumentation, CPEC can be detected at concentrations as low as 0.03 μM and CPEU at 0.15 μM. An additional advantage is the high specificity of the method making extraction unnecessary and offering an easy and rapid workup of the plasma samples. We found no signs of degradation of CPEC and CPEU upon storage. Earlier experiments also showed no degradation of CPEC for several years in aqueous solutions [8] and CPEC was found to be enzymatically and hydrolytically stable in plasma [7].

Internal standards for CPEC and CPEU were added to the plasma samples to compensate for losses during sample preparation and sensitivity loss due to quenching of the signal in the mass spectrometer. Cytidine(+1) and 5-OH-ME-Uracil(+4) were chosen as no isotopically labelled CPEC and CPEU were available.

In the phase I study the maximum concentration of CPEC after a bolus injection of 24 mg was approximately 10 μM. This was also the maximum concentration in our calibration line. Steady state plasma concentrations in the study increased from 0.4 to 3.1 μM at dose levels of 1 to 5.9 mg/m^2/h. As in the phase I/II study in our institute dose levels of 0.5-1.5 mg/m^2/h CPEC would be applied, we expected lower steady state plasma concentrations and therefore the lowest concentration for testing the intra- and inter-assay variation was 0.1 μM. The samples of the patient treated with low dose CPEC confirmed that plasma levels remain in the lower range as a maximum concentration of 0.58 μM of CPEC was detected. CPEU levels were lower than CPEC levels and at several time points the concentrations were below the detection limit of 0.15 μM. However, after the end of the infusion a maximum concentration of 0.16 μM was found. The low CPEU concentrations are in line with previous data from the phase I study indicating that

deamination of CPEC is not the primary route of excretion in humans [5]. CPEU has almost no cytotoxic effects [9,10]. Moreover, cardiotoxicity in the phase I study was not associated with CPEU concentrations. Therefore, the inability to quantify very low CPEU concentrations will not hamper the use of our method in clinical practice.

As demonstrated by the plasma levels of CPEC of the mouse, the method is also suitable for use in animal studies. Moreover, analysis of the plasma of an untreated mouse, suggested no interference of the difference in matrix with the method.

In conclusion, with the described HPLC-ESI MS/MS method we show that plasma concentrations of CPEC and CPEU can be measured at low concentrations with a simple and rapid preparation of the samples. The method can be used for monitoring plasma levels of patients treated with CPEC.

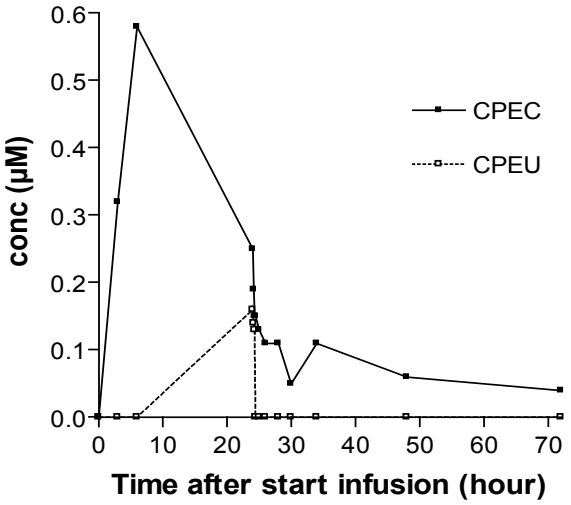

Figure 3

CPEC and CPEU plasma concentrations of a patient treated with CPEC (1 mg/m^2/h for 24 hours).

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CHAPTER 5

LIMITED ANTITUMOR-EFFECT ASSOCIATED WITH TOXICITY OF THE EXPERIMENTAL CYTOTOXIC DRUG CYCLOPENTENYL CYTOSINE IN NOD/SCID MICE WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Leukemia Research 2007; april 23, epub ahead of print

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ABSTRACT

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) is a non-competitive inhibitor of the enzyme cytidine triphosphate (CTP) synthethase. We evaluated the *in vitro* and *in vivo* antitumor activity of CPEC on human ALL cell lines. CPEC displayed anti-leukemic activity with IC50 (after 3 days of incubation) ranging from 6 to 15 nM. Subsequently the *in vivo* activity of CPEC against primary human ALL was evaluated in a xenogeneic model of human ALL using NOD/scid mice inoculated with primary human ALL cells. In the model, only a marginal anti-leukemic activity was observed at 1.5 mg/kg (5 days per week) and 5 mg/kg (2 days per week), however, this activity was associated with severe systemic toxicity. The observed toxicity was not specific for the NOD/scid model, as toxicity at comparable treatment intensity was also observed in Balb/c mice. In conclusion, although CPEC showed antitumor activity against human ALL cells *in vitro*, its activity in the *in vivo* human leukemia model was only marginal and accompanied by severe toxicity.

Keywords: cyclopentenyl cytosine, CPEC, NOD/scid, leukemia, ALL

INTRODUCTION

Acute lymphoblastic leukemia (ALL) generally has a poor prognosis in adults, with an overall leukemia-free survival of 30 to 40%. Current therapy strategies include chemotherapy and allogenic stem cell transplantation [1]. As prognosis decreases after the first relapse, agents improving first line therapy are still warranted.

In leukemic cells of adults with acute lymphoblastic leukemia (ALL), CTP (cytidine triphosphate) synthetase activity was found increased as compared to nonmalignant cells [2,3]. High enzyme activity has also been found in lymphoblasts of pediatric patients with ALL and cells of pediatric patients suffering from acute myeloid leukemia (AML) [4,5]. These findings suggest that CTP synthetase might be an attractive target enzyme for inhibition. Moreover, it has been shown that some malignant tissues synthesize CTP predominantly via the uridine pathway and thus via CTP synthetase [2].

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC), is a non-competitive inhibitor of the enzyme CTP synthetase. CPEC is a pyrimidine analogue of cytidine in which the furan ring of the ribose sugar has been replaced by a carbocyclic cyclopentenyl moiety [6]. CPEC is activated by intracellular phosphorylation ultimately forming its 5'-triphosphate analogue (CPEC-TP) by subsequently uridine-cytidine kinase, nucleoside monophosphate kinase and nucleoside diphosphate kinase [7]. CTP-synthetase catalyzes the conversion of uridine triphosphate (UTP) into cytidine triphosphate (CTP), which is one of the only two cellular pathways for synthesizing CTP. CTP is a precursor for RNA, DNA and phospholipids.

CPEC has shown to have activity in human mammary, colon carcinoma and melanoma xenograft models [6,8]. In humans, CPEC has been studied in a single phase I clinical trial in adults with a variety of solid tumors. Twenty six patients suffering from predominantly colorectal cancer were treated every 3 weeks with increasing doses of CPEC. Treatment was associated with dose-dependent and dose-limiting decreases in granulocyte and platelet counts. Non dose-limiting nausea, vomiting, mucositis and diarrhea were also reported. The most severe toxicity observed was cardiovascular in the form of hypotension. From the 26 patients treated with CPEC, 5 patients experienced a hypotensive episode (dose range: 3-4.7 mg/m^2/h for 24 h) which was in two cases fatal. Toxicity seemed to be dose-related as no hypotension was seen in patients receiving doses equal or below 2.5 mg/m^2/h [9]. However, these cardiotoxic effects could not be reproduced in animal experiments and it remains unclear whether or not the observed hypotension was a true cardiotoxic side effect [10].

In vitro experiments on leukemic cell lines showed reduction of RNA- and DNA-synthesis and growth inhibiting effects of CPEC [7,11,12,13]. Moreover, CPEC also seemed to increase the phosphorylation of the cytostatic drug arabinofuranosyl cytosine (AraC) in a T-lymphoblastic

(MOLT-3) and a myeloid (HL-60) leukemic cell line, enhancing the cytotoxicity of AraC [13]. *In vivo* anti-leukemic activity of CPEC was demonstrated in mice inoculated with murine leukemias by Moyer *et al* [12].

Considering the results of the various *in vitro* and *in vivo* experiments with lymphocytic and myeloid leukemia, and the hematotoxic side effects in humans with solid tumors, CPEC might have potential in the treatment of ALL. However, the *in vivo* experiments providing this evidence were carried out in mice inoculated with murine leukemic cells from leukemia-derived cell lines [12] whereas no data are available with human leukemic cells.

We therefore studied the antitumor effect of CPEC on human acute lymphoblastic leukemia cell lines in vitro, as well as on corresponding human primary acute lymphoblastic leukemia cells in a xenogeneic in vivo model. Although CPEC exerted activity on human leukemic cell lines, CPEC displayed no detectable activity at tolerated (i.e. non-lethal) doses, suggesting a minimal therapeutic window in ALL.

METHODS

Leukemic cells

We established a number of continuously proliferating ALL cell lines. These cell lines were generated from primary cells by culturing these cells in a serum-free medium that was previously developed [14]. The emerging cell lines displayed similar phenotype as compared to the primary cells. Karyotype analysis revealed similarity with primary cells although additional aberrations were observed in some, but not all, cell lines. The leukemic cells were obtained by leukapheresis from 5 patients with ALL after informed consent. Patient and cell line characteristics are presented in Table 1.

The serum-free medium consisted of IMDM (BioWhittaker, Verviers, Belgium), supplemented with 3mM L-glutamine (BioWhittaker), antibiotics, 0.4% human serum albumin (HSA) (wt/vol) (Sanquin, Amsterdam, The Netherlands), 20 μg/ml cholesterol (Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 μg/ml transferrin (Serva, Heidelberg, Germany), $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich).

Table 1 Patient and cell line characteristics

Patient	Diagnosis	Karyotype of primary cells	Cell line	Karyotype of cell line**
VBK	pro-B ALL	46,XY,t(9;22)(q34;q11),der17t (1;17)(q21;p11.2)	LeidenALL-BV	46,XY,t(9;22)(q34;q11),der17t(1;17)(q21;p11.2)
COA	common-ALL	46,XY,del(7)(q22),dup(8)(q24q21), der(9)t(7;9)(?;p1)t(9;22)(q34;q11) , del(20)(q11),der(22)t(9;22)(q34; q11)	LeidenALL-CM*	47,XY,del(7)(q22),dup(8)(q24q21), der(9)t(7;9)(?;p1)t(9;22)(q34;q11), del(20)(q11),der(22)t(9;22)(q34; q11),+der(22)t(9;22)(q34;q11)
MHX	common-ALL	47,XX,+i(21)(q10)	LeidenALL-HP	45,XX, -13,add(16)(q2?4),i(21)(q10)
WKD	common-ALL	48,XY,+5,+8,t(9;22)(q34;q11)	LeidenALL-KW	48,XY,+5,+8,t(9;22)(q34;q11)
MRJ	pre-B ALL	46,XX,i(7)(q10),der(19)t(1;19)(q23;p13)	LeidenALL-RL	46,XX,i(7)(q10),t(9;10)(q10;q10), der(19)t(1;19)(q23;p13)

*CML in lymphoid blast crisis. **Additional abberations as compared to the primary cells are underlined.

In vitro proliferation assay

The effect of CPEC (NCI, Maryland, USA) and cytarabine (AraC) (Pfizer, Capelle a/d IJssel, The Netherlands) on proliferation of leukemic cell lines was tested by [3H]thymidine incorporation assay. Leukemic cell lines were cultured in the absence or presence of the agents in 96-well tissue culture plates (Corning Costar, Schiphol-Rijk, The Netherlands) at 4 x 10^4 cells/well in serum-free medium). After 72 hours of incubation, 1 μCi [^{3}H]thymidine (Amersham, Roosendaal, The Netherlands) was added to each well. After 18 h of further culture [^{3}H]thymidine incorporation was measured as described earlier [15]. By using the isobologram method [16] we analyzed whether the combination of CPEC and AraC was synergistic, additive or antagonistic. We determined the concentration combinations that caused 50% survival for the different cell lines. For each pair of drug concentrations producing 50% survival (IC50), the combination index (CI, based on Loewe additivity [16, 17] was calculated as follows: CI = (CPEC concentration/IC50 CPEC) + (AraC concentration/IC50 AraC). IC50 CPEC and AraC in this formula represent the concentrations of the individual drugs that would result in 50% survival.

In vivo NOD/scid mouse leukemia model

Female NOD/scid mice, aged 5-6 weeks (Charles River, les Oncins, France) were housed in sterile cages supplied with sterile filtered air and were supplied with sterile food and sterile water containing ciprofloxacin. Mice were engrafted with primary human leukemic cells by injecting 10^6 leukemic cells intravenously in a lateral tail vein. Engraftment and progression of leukemia was monitored twice a week by flowcytometric analysis of peripheral blood samples of individual animals as described before [18]. Briefly, blood samples were taken from a lateral tail vein by using capillary blood collection tubes (Sarstedt, Nümbrecht, Germany). Total nucleated cell counts (NCC) were determined on a Sysmex F 820 automated cell counter (Sysmex Corparation, Kobe, Japan). After lysis of red blood cells, murine leukocytes and human ALL cells were stained using PE-conjugated anti-murine CD45 (Ly5; Pharmingen, San Diego, CA) and FITC-conjugated anti-human CD45 (Becton Dickinson, San Jose, CA) respectively, allowing determination of the percentage of murine and human cells (%Hu). Samples were analyzed on a Becton Dickinson FACScan flow cytometer. Leukemic cell counts (LCC, 10^6/ml) were calculated as LCC = NCC (10^6/ml) x %Hu.

Treatment was performed by administrating CPEC (in 200 μl normal saline) or normal saline (control group) intravenously as a bolus injection in the tail vein. Animals were further monitored for response or tumor progression. Toxicity was evaluated by determining weight loss, hemaglobin (Hb) and visual aspects (breathing, activity, shaking). At the end of the experiment, or after observing severe toxicity (>20% weight loss), animals were euthanized by CO_2 inhalation. Blood, bone marrow and spleen were tested for leukemic cells. Heart, spleen, liver and kidney were further processed for histological evaluation. For this purpose paraplast embedded organs were mounted on slides and stained with haematoxylin-azophloxin.

RESULTS

In vitro experiments

To evaluate the activity of CPEC on human leukemic cells, we incubated five different cell lines with CPEC (1-1000 nM) for 72 hours. The mean IC50 of CPEC was 12 nM (range 6-15 nM). Total cell death was observed at 50 nM and higher. By comparison, the IC50 of AraC was 2-4 nM. (Fig. 1).

As earlier studies reported a possible synergistic effect of Ara-C and CPEC, we also studied anti-leukemic activity of combinations of CPEC and AraC. In figure 2 the isobologram of the co-incubation of CPEC and AraC (concentration CPEC 0-63 nM, AraC 0-32 nM) is presented. Most of our data are slightly above the diagonal that defines Loewes additivity. We also calculated the average CI from all the concentration combinations giving 50% survival. The average CI was 1.09 (SD 0.13) and as defined by Chou, CI values between 0.90 and 1.10 may be considered as nearly additive [19].

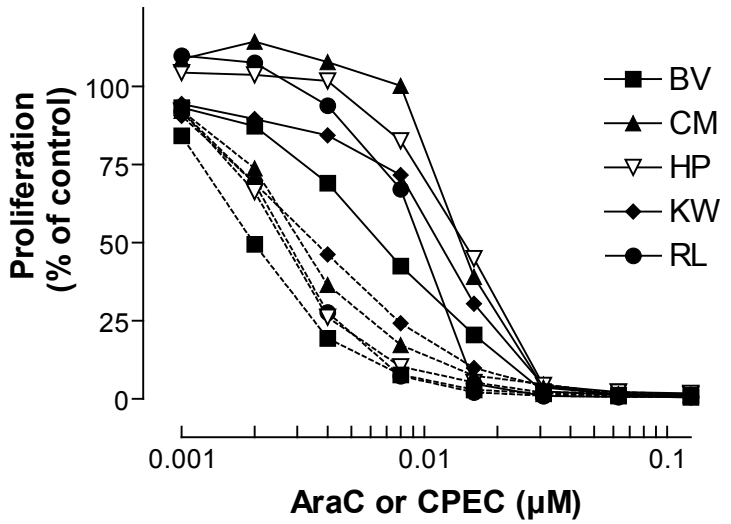

Figure 1 Activity of CPEC on proliferation of ALL cells

To study the *in vitro* activity of CPEC and AraC, five different leukemic cell lines (derived from primary leukemic cells from 5 patients with ALL) were exposed to serial dilutions of CPEC and AraC (1-1000 nM). A thymidine incorporation assay was performed after 3 days of incubation. The results shown in the figure are representative of three separate experiments. The IC50 of AraC was observed at 0.002-0.004 μM, IC50 of CPEC at 0.006-0.015 μM.
Dotted lines (…) represent the AraC curves, CPEC is represented by the solid lines (-).

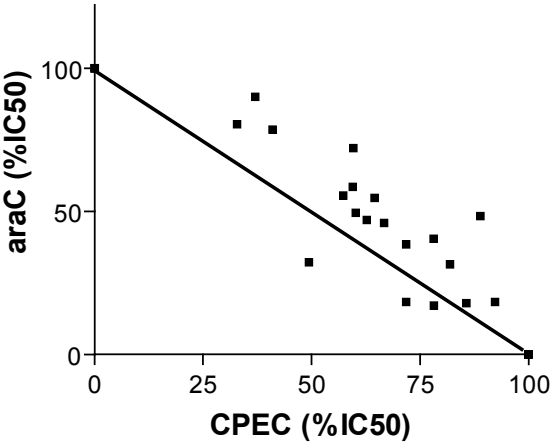

Figure 2 Absence of synergistic activity of coincubation of CPEC and Ara-C in vitro

Isobologram showing absence of synergistic activity of co-incubation of CPEC and AraC in vitro
To study a possible synergistic effect of CPEC and AraC, 4 leukemic cell lines (derived from primary leukemic cells from patients with ALL) were exposed to serial dilutions of CPEC and AraC. The thymidine incorporation assay was performed after 3 days of coincubation. Data points represent the drug combinations leading to 50% survival. The diagonal represents Loewe additivity. On the axis percentages of the IC50 concentrations of the drugs are plotted. The isobologram indicates a nearly additive and no synergistic effect.

In vivo experiments

The five ALL cell lines displayed similar sensitivity to CPEC in vitro. As the in vitro experiments did not show a superior effect of CPEC and AraC co-incubation over CPEC alone, we chose to study CPEC mono-therapy in vivo. Primary leukemic cells from patients MHX and COA, from which the HP and CM cell lines were previously generated, were selected for evaluation in the animal model. These patients were selected in order to include both a Philadelphia (Ph) positive (COA) as well as a Ph negative sample (MHX) in the in vivo studies. Mice inoculated with cells from patient MHX first showed quantifiable leukemic cell counts (LCC) 84 days after inoculation. Mice inoculated with cells from patient COA first showed quantifiable LCC 17 days after inoculation.

In the phase I study CPEC was tested in a dose range from 0.7 – 4 mg/kg/day as a 24 h infusion every three weeks [9]. As in vivo studies on murine leukemia demonstrated activity of CPEC in a more frequent dose schedule [12], we started with administration of CPEC on 2 days/week (2 consecutive days followed by 5 days of rest). Three dosages were tested: 0, 0.5 and 5 mg/kg (3 mice per group). Only the highest dose was associated with marginal activity, however, this was accompanied by severe toxicity. We therefore tested whether a more intense schedule with a lower maximum dose would result in more effect with less toxicity. We administrated respectively 0, 0.5 and 1.5 mg/kg for 5 times a week (4 mice per group). Still neither effect nor toxicity was seen in the lower dose ranges. A marginal effect was associated with 1.5 mg/kg, however, again severe toxicity, expressed by more than 20% decrease in weight or Hb, was observed. The logLCC counts for the two dose schedules tested are represented in figure 3.

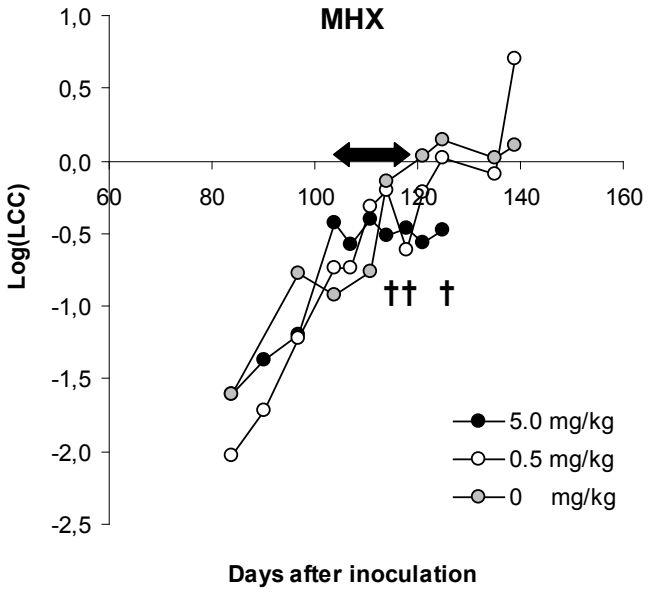

Figure 3a logLCC counts for MHX ALL cells in NOD/scid mice

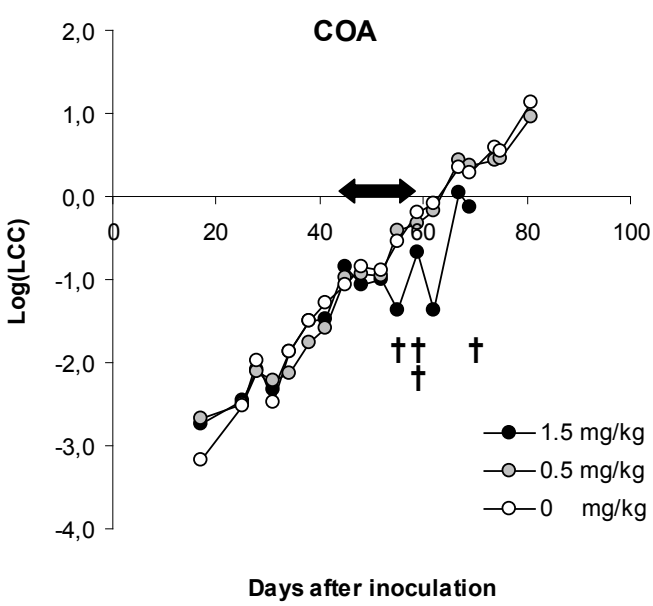

Figure 3b logLCC counts for COA ALL cells in NOD/scid mice

Figure 3 Marginal activity accompanied by severe toxicity of CPEC on primary ALL cells in NOD/scid mice

In figure 3a data from mice inoculated with MHX primary leukemic cells are shown.
CPEC administration was started in this group 104 days after inoculation. Mice received CPEC on two consecutive days with a 5-day interval for a maximum of four weeks (time points of the first and last injection are indicated by the arrow). Each curve represents the mean logLCC counts for each group during the study.
Figure 3b represents data from mice inoculated with COA primary leukemic cells. CPEC administration was started 46 days after inoculation. Mice received CPEC for five days per week for a maximum of four weeks.
In both figures the highest dose group demonstrate a possible treatment effect of CPEC. However, these were the only groups with unintentional death during treatment (indicated by †).

Toxicity

In animals treated with more than 1.5 mg/kg CPEC/day, analysis of blood samples revealed induction of pancytopenia and anemia. After 1.5 mg/kg CPEC for 5 days/week hemoglobin levels decreased to 5.7 mmol/l (SD 0.74) as compared to 8.6 mmol/l (SD 0.49) in control treated animals within 14 days after treatment start. Diarrhoea and significant weight loss (>25%) were observed. Hematological toxicity of CPEC was confirmed microscopically. The bone marrow compartment of treated animals was void of hematopoietic cells (Fig. 4). In the kidneys tubular necrosis, and signs of proteinuria and bleeding were observed (Fig.5). Gut toxicity of CPEC presented as atrophy of ilear epithelium. No signs of cardiotoxicity were found.
No toxicity was observed in the lower dosage regimens (equal or below 1.0 mg/kg CPEC, 5 days/week). To investigate whether susceptibility to CPEC-mediated toxicity was a strain-specific trait of NOD/scid mice, we administrated CPEC to non-leukemic Balb-c mice. Although 1.5 mg/kg (5 days/week) was well tolerated, daily administration of 5 mg/kg CPEC resulted in severe toxicity and death.

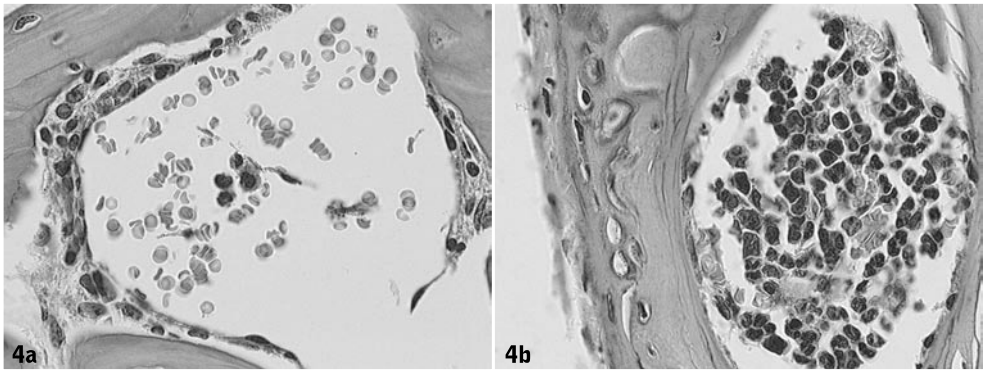

Figure 4 Severe aplasy in the bone marrow compartment after CPEC treatment

Details of the femur of a CPEC treated animal (revealing severe aplasy in the bone marrow) (4a), whereas no abnormalities were observed in the femur of an untreated animal (figure 4b).

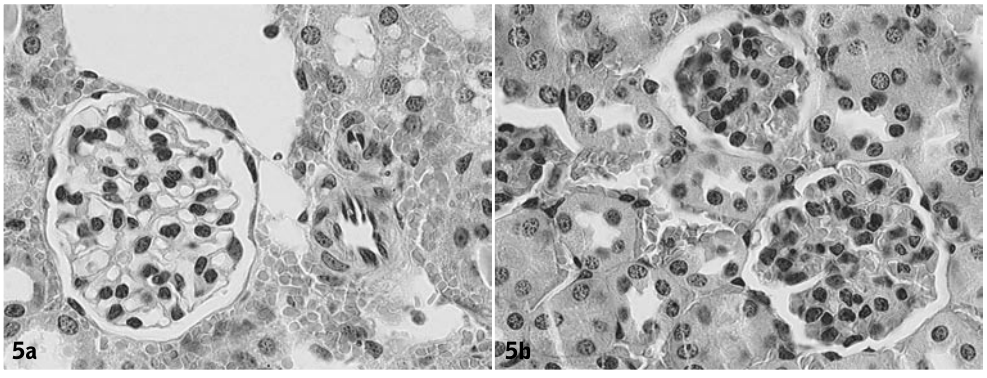

Figure 5 Kidney toxicity after CPEC treatment

Details of the kidney showing tubular necrosis, signs of proteinurea and bleeding after treatment with CPEC (figure 5a). In figure 5b details of the kidney of an untreated animal are shown.

DISCUSSION

With this study we demonstrated that CPEC has *in vitro* activity on human leukemic cells. However, in our NOD/scid model CPEC showed no detectable activity at tolerated doses whereas at higher doses limited activity was associated with severe toxicity. Balb/c mice were also susceptible to CPEC toxicity, suggesting a minimal therapeutic window in ALL.

CPEC has been evaluated clinically in the phase I study by Politi *et al* in patients with solid tumors [9]. In this study hematological toxicity was dose-limiting and the most severe side effect was cardiotoxic. The cardiotoxicity only occurred in the higher dose levels and could not be reproduced in animal experiments [10]. The hematological toxicity however, suggested a potential antitumor effect in leukemia. Various *in vitro* and *in vivo* experiments with CPEC on lymphoid and myeloid leukemia seemed to confirm this hypothesis [7,11,12,13]. These in vivo experiments however, were murine tumor-based models. Therefore, the activity of CPEC against primary human leukemia cells in vivo remained unclear.

In vitro, on previously established LeidenALL acute lymphoblastic leukemia cell lines, CPEC exerted antileukemic activity in the nanomolar range, comparable to other reports. Previously, Verschuur *et al* reported a synergistic effect between AraC and CPEC because co-incubation with AraC and CPEC increased cell death in a T-lymphoblastic cell line (Molt-3) as compared to either agent alone[20]. However, in our B-lineage ALL cell lines, we detected a nearly additive and no synergistic relationship between the activity of CPEC and cytarabine. Therefore, we investigated monotherapy with CPEC in the *in vivo* model for primary human ALL.

Previous experiments in mice bearing murine lymphoid leukemia reported an antitumor effect of CPEC when administrating 1 mg/kg CPEC for a maximum of 9 days [12]. In these experiments CPEC activity was evaluated by measuring the increase in life span, giving no indication on the actual activity of CPEC during treatment. Moreover, since both treated and untreated mice died within 20 days after inoculation, no discrimination between death due to toxicity of CPEC or leukemic progression was made. In our tumor model, leukemic progression is continuously monitored, allowing the identification of the actual response during treatment [17]. Because of the application of this technique, leukemic progression can be excluded as a possible cause of death. This allows precise discrimination between activity and toxicity of therapeutic interventions. Moreover, the model employs a curative setting (i.e. treatment is started after confirmation of leukemic engraftment), whereas in the earlier experiments treatment is applied simultaneously with, or very shortly after tumor inoculation.

In the present studies, CPEC at highest doses, appeared to induce a small decrease in leukemic progression. However, this activity was accompanied by severe anemia. There were no indications of preferential toxicity of leukemic cells. Histological findings confirmed hematological toxicity as

severe aplasy was observed in the bone marrow. No cardiotoxicity was detected upon histological examination. Besides hematological toxicity, renal toxicity was apparent. It remains unclear however, whether renal toxicity was primary and caused anemia through hematuria, or whether renal necrosis was secondary and caused by anemia and the resulting hypoxia.

Non-hematological toxicity consisted of weight loss and diarrhea. Upon histological examination the ileum showed abnormalities that might be related to the diarrhea. Moreover, in the phase I trial patients experienced (non dose-limiting) grade 2 diarrhea, occurring more frequently in the higher dosage levels. It is conceivable that a possible hematological effect in humans will be associated with diarrhea as well.

To exclude the possibility that the small therapeutic window that was observed in our *in vivo* studies was a specific artifact of the NOD/scid mice model, we also administered CPEC to non-leukemic Balb/c mice. Although Balb/c mice tolerated CPEC at a dose of 1.5 mg/kg/day, administration of 5 mg/kg/day induced similar and severe toxicity (both hematological and non-hematological) as was observed in NOD/scid mice. Although these observations suggest that Balb-c mice may be slightly less sensitive to CPEC-mediated toxicity, it has to be noted that the coping ability of the hematopoietic system of leukemic NOD/scid mice may be impaired due to leukemic engraftment. Moreover, the highest dose tolerated by Balb-c mice (1.5 mg/kg 5 days/week) was not associated with a clear anti-leukemic activity in the NOD/scid mice.

Our *in vitro* results with CPEC are in line with previous studies and although in vitro less active that AraC, CPEC showed promising activity. The fact that we were not able to demonstrate this activity *in vivo,* might have been partly caused by CPEC induced toxicity which may have overwhelmed anti-leukemic activity. However, other *in vivo* models with solid and lymphoid tumors showed activity of CPEC before severe toxicity occurred [12]. It is possible that cytidine is involved in the low activity of CPEC in mice. *In vitro* results in leukemic and colorectal cells have shown an increase in survival after administration of cytidine to CPEC treated cells. The mechanism of action is supposed to involve competition for transmembrane transport and phosphorylation [7]. It is conceivable that high endogenous cytidine levels could protect tumor cells against CPEC. However, administration of cytidine has also been shown to protect against toxicity, without changes in activity [11]. The results of our study are not suggestive for an important role of cytidine, as we studied the effects of CPEC on a systemic single cell leukemic tumor and we experienced systemic toxicity. If high endogenous cytidine levels were responsible for the lack of activity in our model, this would most likely also have resulted in lower toxicity, which was not the case.

We conclude that, although CPEC shows antitumor activity against human ALL cells *in vitro*, its antitumor activity in the human *in vivo* leukemia model as a single agent is only marginal and is accompanied by severe toxicity.

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CHAPTER 6

CARDIOTOXICITY OF CYTOTOXIC DRUGS

Cancer Treatment Reviews 2004;30:181–191

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ABSTRACT

Cardiotoxicity is a well-known side effect of several cytotoxic drugs, especially of the anthracyclines and can lead to long term morbidity. The mechanism of anthracycline induced cardiotoxicity seems to involve the formation of free radicals leading to oxidative stress. This may cause apoptosis of cardiac cells or immunologic reactions. However, alternative mechanisms may play a role in anthracycline induced cardiotoxicity. Cardiac protection can be achieved by limitation of the cumulative dose. Furthermore, addition of the antioxidant and iron chelator dexrazoxane to anthracycline therapy has shown to be effective in lowering the incidence of anthracycline induced cardiotoxicity. Other cytotoxic drugs such as 5-fluorouracil, cyclophosphamide and the taxoids are associated with cardiotoxicity as well, although little is known about the possible mechanisms. Recently, it appeared that some novel cytotoxic drugs such as trastuzumab and cyclopentenyl cytosine also show cardiotoxic side effects.

Keywords: Cardiotoxicity; cytotoxic drugs; cancer; chemotherapy

INTRODUCTION

Cardiotoxicity occurs during therapy with several cytotoxic drugs and may be the dose limiting factor in cancer treatment and hence tumour response. Furthermore, cardiotoxicity can also be responsible for long term side effects and may cause severe morbidity in surviving cancer patients [1], which may be relevant especially in pediatric oncology. Cardiotoxicity from cytotoxic treatment is known to have a high prevalence [2]. Cardiotoxicity includes a wide range of cardiac effects from small changes in blood pressure and arrhythmias to cardiomyopathy. In literature different mechanisms of chemotherapy induced cardiotoxicity are postulated including cellular damage due to the formation of free oxygen radicals and the induction of immunogenic reactions with the presence of antigen presenting cells in the heart [3]. Moreover, the influence of the cytotoxic agent on certain phospholipids, especially cardiolipin, may also explain the development of cardiotoxicity [4].

The anthracyclines, such as doxorubicin and epirubicin, are potent cytotoxic drugs but their clinical use is often limited by their cardiotoxic side effects. Other cytotoxic drugs that have reported cardiotoxicity include 5-fluorouracil, capecitabine, mitoxantrone, cisplatin, the taxoids paclitaxel and docetaxel and newer drugs such as the monoclonal antibody trastuzumab [5-8].

In this manuscript the different mechanisms of chemotherapy induced cardiotoxicity and attempts to circumvent this side effect are reviewed.

ANTHRACYCLINES

Cardiotoxicity has been extensively reviewed with the use of anthracyclines [2,5,9,10]. Anthracyclines have been reported to cause cardiomyopathy, congestive heart failure and ECG alterations (e.g. nonspecific ST-T changes, decreased QRS voltage and prolongation of QT interval). Both early and late onset cardiac effects are reported. Early onset effects occur within one year after start of the anthracycline therapy and can be acute, subacute or chronically progressive. In children, early onset cardiotoxicity seems to occur less frequently than late onset clinical cardiotoxicity. Late onset effects can occur up to 20 years after completion of anthracycline therapy [11].

Acute toxicity

Acute or subacute cardiotoxicity with anthracyclines is rare and will occur during or immediately following infusion, is usually transient (e.g. electrocardiographic abnormalities such as nonspecific ST-T changes and QT prolongation, pericarditis–myocarditis syndrome and ventricular dysfunction with congestive heartfailure) and will attenuate after discontinuation of the therapy.

Chronic toxicity

The chronic effects start with early cardiac abnormalities which can progress to overt cardiac disease. Chronic effects persist after discontinuation of the anthracyclines and the clinical symptoms may include all signs of cardiomyopathy such as electrophysiologic changes, decrease of left ventricular function, changes in exercise-stress capacity, and overt signs of congestive heart failure [2,5].

Risk factors

Cumulative dose, age, prior irradiation, concomitant administration of other chemotherapeutics and underlying heart disease are considered as being risk factors for anthracycline cardiotoxicity [12]. Of these the cumulative dose seems to be the most important factor. The usual dosage of doxorubicin is 60–75 mg/m^2, every 3 weeks [13]. above a cumulative dose of 450–550 mg/m^2 doxorubicin, cardiomyopathy and congestive heart failure occur most frequently. However, as of individual variation, signs of cardiotoxicity have also been seen with cumulative dosages below 300 mg/m^2 [2,14]. The cumulative probability of doxorubicin induced cardiotoxicity was estimated by Von Hoff at 0.18 at a cumulative dose of 700 mg/m^2, 0.07 at 550 mg/m^2 and 0.03 or less at 400 mg/m^2 [13]. Recently, higher estimations were reported for the elevated cumulative doses, respectively, 0.48 at a cumulative dose of 700 mg/m^2 and 0.26 at 550 mg/m^2. An explanation for this difference can be the difference in determination of congestive heart failure. The risk estimation for the lower dosage (0.05 at 400 mg/m^2) is comparable with Von Hoff's findings [14]. The maximum cumulative dosage needed to obtain minimal cardiotoxicity varies among the different anthracyclines. With epirubicin a lower frequency of cardiotoxicity at therapeutic dosages is reported in comparison with doxorubicin (i.e. an incidence of 0.03 at 900 mg/m^2 epirubicin is reported, the usual dosage is 50–90 mg/m^2, every 3 weeks). A lower frequency of cardiotoxicity has been reported also for mitoxantrone and a new anthracycline (MEN 10755) [15–18].

Previous radiotherapy may enhance anthracycline induced cardiotoxicity. In a study, left sided irradiation in combination with standard adjuvant chemotherapy (doxorubicin at a cumulative dose of 300 mg/m^2) gave 2.6% of cardiac heart failure versus 0.3% for right-sided or no irradiation [19].

Monitoring and markers of cardiotoxicity

Routine cardiac imaging studies (echocardiogram or multiple gated acquisition scans) can be used to identify (sub)clinical myocardial dysfunction. Endomyocardial biopsy directly measures the presence and extent of fibrosis due to anthracycline cardiotoxicity. However, it is limited by its invasiveness, need for histologic expertise and costs. There is a need for simple methods (like serum or plasma markers) to identify patients at risk.

The cardiac biomarker Troponin T is indicative for myocardiocyte damage and is currently used in the diagnosis and prognosis of myocardial ischemia. In children treated with anthracyclines elevation of Troponin T was found. These elevations were, however, well below those observed in patients with myocardial infarction [20].

Studies in adults have given conflicting results regarding Troponin T elevations. In one study no change in Troponin T could be found, whereas in another an elevated level was associated with a greater decrease in left ventricular ejection fraction (10% vs 2%, p=0.017) [21]. Another possible biochemical marker is BNP (b-natriuretic peptide). ANP (atrial natriuretic peptide) and BNP are hormones that are secreted by the myocytes of the heart. The plasma concentrations are increased in patients with asymptomatic and symptomatic left ventricular dysfunction. BNP seems to be a more sensitive marker of cardiac dysfunction than ANP. A few small studies concerning the relationship between anthracyclines, BNP and cardiotoxicity have been published. BNP measurement in 27 patients, treated with anthracyclines for hematological cancers, showed significant BNP elevations after anthracycline treatment. Due to a short follow-up and the small size of the group this, however, could not be related to existing cardiotoxicity [22]. Another study in 30 patients suggested that the natriuretic peptides cannot predict cardiotoxicity, but can be useful in the detection of subclinical left ventricular dysfunction [23]. Further studies are needed to define the role of circulating markers such as Troponin T and BNP as parameters of chemotherapy induced cardiotoxicity.

Mechanisms

OXIDATIVE STRESS HYPOTHESIS

The most common hypothesis for the mechanism by which anthracyclines cause cardiotoxicity includes the formation of free radicals and superoxides [15;24–26]. this hypothesis is based mainly on in vitro experiments and only a few studies have been performed in humans. Several in vitro and in vivo models have been used to study the cardiotoxic effects of anthracyclines assessing a variety of endpoints. With the free radical theory the reaction starts with a one-electron reduction of doxorubicin to form a doxorubicin semiquinone radical by a reduced flavoenzyme such as NADPH-cytochrome P450 reductase. The semiquinon radical forms a complex with iron leading to an anthracycline-iron ($Fe2^{+}$) free radical complex. This complex reduces oxygen to produce superoxide and to regenerate doxorubicin. The superoxide is dismutated into hydrogen peroxide and oxygen (fig. 1). Vasquez *et al* have shown that doxorubicin binds to the reductase domain of endothelial nitric oxide synthase. This causes an increase in superoxide and a decrease in nitric oxide formation. The consequent formation of peroxynitrite could also play a role in the cardiotoxicity [25].

From the combination of superoxide, hydrogen peroxide and free iron, lipid peroxidation may be initiated.

The specific susceptibility of the cardiac cells to the oxidative stress would be due to relatively low levels of antioxidant enzymes in the heart [27]. Indeed studies in rat hearts suggest that doxorubicin is able to cause a further reduction in the a priori low levels of the antioxidant enzymes in rat hearts [28].

Following the oxidative stress theory, cardiotoxicity can be achieved by different mechanisms. Studies indicate that the myocardial damage caused by doxorubicin involves apoptosis. This programmed cell death process would be initiated by the formation of oxidative free radicals. Apoptotic cell death was indeed found in rat cardiomyocytes and bovine aortic endothelial cells upon exposure to doxorubicin [3,29].

Sawyer *et al* [30] also showed apoptosis at low concentrations (1 μM) of daunorubicin, whereas at higher concentrations (>10 μM) necrosis was observed. This is in agreement with the observation of Guchelaar *et al* [31] who reported that in cell lines for a variety of apoptosis-inducing anticancer drugs a relatively increase of induction of necrosis was observed at high drug concentrations whereas at lower concentrations apoptosis dominates.

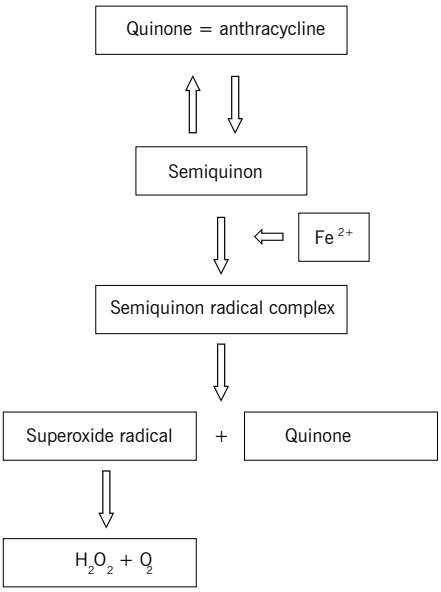

Figure 1

METABOLITE THEORY

Minotti *et al* suggest that cancer patients often have a spontaneous exacerbation of lipid peroxidation and doxorubicin probably inhibits this effect in a paradoxic manner. It is suggested that lipid peroxidation occurs when iron oxidizes incompletely to the ferric form. Doxorubicin and the formed hydrogen peroxide would inhibit cardiac lipid peroxidation by affecting the Fe(II)–Fe(III) equilibrium of iron–oxygen complexes. This would mean that cardiac damage might involve parent doxorubicin or its metabolites other than the semiquinon. This hypothesis is strenghtened by results of a study in which the formation of the metabolite doxorubicinol was demonstrated. This metabolite mediates iron release and negatively affects the function of apoprotein as an iron regulatory protein [32,33].

INFLUENCE ON CALCIUM HOMEOSTASIS

Another possible mechanism involves the influence of anthracyclines on the calcium homeostasis. Before leading to apoptosis, oxidative stress can induce mitochondrial permeability transition with alterations in mitochondrial calcium transport. Changes in calcium transport can lead to tissue injury and cell killing and impaired cardiac contraction. In vitro experiments showed that doxorubicin treatment caused an irreversible decrease in mitochondrial calcium loading capacity [34].

Moreover, anthracyclines could stimulate the release of calcium from isolated cardiac and skeletal muscle sarcoplasmic reticulum vesicles [35]. This theory is strengthened by the observation of Rossi et al. who found a protective effect of the calcium blocking agent verapamil on doxorubicin induced cardiotoxicity in rats. This effect would be due to the calcium blocking capacities of verapamil by inhibiting the intracellular calcium overload and hence antagonizing the effect of doxorubicin on mitochondria [24]. However, others have demonstrated an increase in cardiotoxicity when doxorubicin was given in combination with verapamil and different mechanisms for this effect are postulated. One is based on the capacity of verapamil to inhibit the function of P-glycoprotein and therefore may increase intracellular cytotoxic drug concentrations. This may be useful in overcoming resistance to chemotherapeutic drugs in cancer cells, but the concern is that it could also lead to toxic effects in normal such as cardiac cells. Some in vitro studies indeed showed increased doxorubicin accumulation in rat cardiomyocytes when incubated with a combination of verapamil and doxorubicin [36,37]. Akimoto *et al* [38,39] did not show an increased cellular anthracycline uptake but additive cardiotoxicity by verapamil due to its selective inhibition of cardiac actin gene expression, a similar effect which was demonstrated before with doxorubicin alone. The exact role of the altering capacities of doxorubicin on calcium regulation and its implications for cardiotoxicity remains to be elucidated.

ROLE OF IMMUNE SYSTEM

Involvement of an immunogenic reaction after oxidative stress is an alternative mechanism of anthracycline induced cardiotoxicity. Huber [40] suggested that doxorubicin could lead to a damaged plasma membrane of cardiac myocytes with consequently an enhanced immune response. A study in hypertensive rats showed an increase in antigen presenting dendritic cells after treatment with doxorubicin, indicating a stimulation of expression of antigens. Pretreatment with dexrazoxane attenuated this increase, confirming the suggestion of the involvement of oxidative stress, followed by an immunogenic reaction [41].

Protection

Apart from cumulative dose limitations several attempts have been made to develop chemoprotectants to prevent the cardiotoxicity of anthracyclines without attenuating their anti-tumor effect. Following the free radical hypothesis, antioxidants used as free radical scavengers have been tested in clinical trials but without significant success. Human studies with the scavengers acetylcysteine or tocopherol did not show any cardioprotective effect [42,43]. In one clinical study, administration of melatonin together with different chemotherapeutic regimens was associated with reduced overall toxicity including cardiotoxicity. This effect was thought to be ascribed to its antioxidant capacities [44].

DEXRAZOXANE

Considering the essential role of iron and the doxorubiciniron complex, iron chelators have been developed to circumvent anthracycline induced cardiotoxicity. These agents bind to intracellular iron and remove the iron from the anthracycline–iron complex and are applied aimed at preventing free radical formation [45].

Dexrazoxane (ICRF-187) was found to be the most promising agent. After being tested in animals [46], several clinical trials showed its capacities in reducing doxorubicin induced cardiotoxicity. In two multicenter double blind randomized phase III trials 15% of the patients treated with dexrazoxane experienced a cardiac side effect versus 31% of the patients on placebo (hazard ratio 2.63, $p < 0.001$ with log rank) [47]. Another trial reported 4% in treated versus 24% in nontreated patients ($p= 0.02$) [48]. Reported differences in cardiac side effects may be explained by the different criteria applied for cardiac events and different dosage schedules of doxorubicin. Complete protection, however, could not be achieved in most of the studies [47,48]. Moreover, it is not known if dexrazoxane provides any protection against late cardiovascular effects [49].

In a study in children treated with doxorubicin (38 patients, 18 control and 20 treated with dexrazoxane) a cardioprotective effect of dexrazoxane was found [50]. However, because of the

small number of clinical trials yet performed in pediatric populations there is too little evidence to draw definite conclusions. Indeed the American Society of Clinical Oncology concluded that there is insufficient evidence to recommend the use of dexrazoxane in the treatment of pediatric malignancies [51]. The FDA has approved dexrazoxane for use in adults if cumulative doses of doxorubicin exceed 300 mg/m^2 [5]. Doxorubicin pharmacokinetics seems to be unaffected upon dexrazoxane treatment [52]. A pharmacokinetic study with epirubicin revealed an increased clearance of the anthracycline when dexrazoxane had been administered which theoretically could lead to a decreased epirubicin exposure and hence a possible difference in treatment efficacy of the combined treatment [53]. In a randomized clinical trial however, no statistically significant differences in survival and progression-free survival could be demonstrated between the dexrazoxane and placebo group [54].

Dexrazoxane can be administered intravenously either as a slow injection or fast infusion before doxorubicin is initiated. The dosage to be given is usually a 10-fold of the doxorubicin dose and its dose limiting toxicity appears to be leukopenia [10].

MONOHER

Another radical scavenger that has been studied is the flavonoid monoher. Monoher is able to protect the heart against doxorubicin toxicity without affecting its anti-tumor effect in a mouse model. However, because of its low potency the effective dosage needed in humans would be too high (500 mg/kg in mice) to make it a useful drug. Recently, a derivate of monoher, called frederine was developed which could provide total heart protection in mice with a 5-fold lower dosage as compared to monoher. Clinical trials with this drug will be undertaken [55].

LIPID LOWERING AGENTS

Lipid lowering agents also seem to be able to lower the cardiotoxic effects of anthracyclines [56]. When rats were concomitantly treated with doxorubicin and the lipid lowering and antioxidant agent probucol, an increase in the antioxidant enzymes superoxide dismutase and glutathione peroxidase activities and a decrease in lipid peroxidation were found. According to the oxidative stress theory this improvement of antioxidant state of the heart could possibly lead to a better myocardial structure and function [28,57]. Recently, Feleszko *et al* [58] showed both a potentiation of anti-tumor activity and a cardioprotective effect by the cholesterol lowering HMG coenzyme-A re- ductase inhibitor lovastatin, in mice treated with doxorubicin.

CHANGES IN FORMULATION

Another strategy to achieve reduced cardiotoxicity is the development of liposomal drug formulations of the anthracyclines. Liposomes are preferentially taken up by tissues enriched in phagocytic reticuloendothelial cells and with a sinusoidal capillary system like the liver and spleen.

The continuous capillaries containing tissues like skeletal and cardiac muscles will therefore hardly take up liposomes. Preclinical studies have indeed shown a decreased uptake of doxorubicin in cardiac muscle cells when a liposomal formulation was used. The constituents of the liposomes itself do not seem to have a negative influence on the heart or other tissues. However, there may exist important differences among different liposomal formulations since changes in vesicle size, drug-to- lipid ratio and lipid composition can have great influence on the biodistribution and toxicity of doxorubicin [59,60].

In a retrospective analysis of eight clinical Phase I and phase II trials the safety of more than 500 mg/m^2 pegylated liposomal doxorubicin was studied. The study was performed with a median follow-up of 2.7 years (range 1.2–6.0 years) after the initiation of treatment. None of the evaluable 41 patients experienced clinical congestive heart failure secondary to cardiomyopathy. The left ventricular ejection fraction (LVEF) was reduced with more than 10% in five patients; however, three of these had received conventional doxorubicin before treatment with liposomal doxorubicin. The mean change in LVEF was -2%, which was not considered clinically significant. This study suggests that doxorubicin induced cardiotoxicity can be reduced upon using liposomal formulations of the drug [61].

TAXOIDS

The taxoids paclitaxel and docetaxel are important agents in the treatment of a variety of tumors but have been associated with cardiotoxicity [31]. During administration of paclitaxel, whether or not combined with cisplatin, various cardiac disturbances are reported like brady- and tachyarrhythmias, atrioventricular and bundle branch blocks and cardiac ischemia. Hypotension is also reported, probably as a result of a hypersensitivity reaction [62]. When evaluating three phase I and one phase II studies performed at the John Hopkins institute it appeared that 5% (n = 7) of the patients showed overt cardiac disturbances as ventricular tachycardia and atrioventricular conduction abnormalities. Asymptomatic bradycardia occurred in 29% of patients receiving maximal tolerable doses (110–250 mg/m^2) of paclitaxel in the phase II study. These disturbances, however, did not lead to clinical symptoms. The abnormalities usually started several hours following the initiation of paclitaxel therapy and resolved after discontinuation. This evident time relationship and the fact that most patients had no cardiac risk factors supports the assumption of causality between paclitaxel and the observed cardiac rhythm disturbances [62].

Another concern with the use of taxoids has been the development of congestive heart failure in patients treated with a combination of doxorubicin and taxoids [63–65]. the cardiotoxicity associated with taxoids seems to be mild in most cases. However, in clinical trials patients with prior history of cardiac disturbances were often excluded. Therefore, the rate of cardiotoxicity in

this group of patients is yet difficult to estimate. A study in patients with major cardiac risk factors revealed that paclitaxel could be safely administered as single therapy or in combination with a platinum agent such as cisplatin or carboplatin. Cardiac risk factors included unstable angina, severe coronary artery disease, congestive heart failure and atrial fibrillation [66].

Mechanism

Paclitaxel is formulated in a cremophor EL vehicle to enhance the drug solubility and it is suggested that the vehicle and not the cytotoxic drug itself is responsible for the cardiac disturbances. However, the cardiac rhythm disturbances are not reported with use of other drugs containing cremophor EL such as cyclosporin. The possible mechanism by which cremophor EL would cause cardiotoxicity is massive histamine release. Indeed, stimulation of histamine receptors in cardiac tissue in animal studies has resulted in conduction disturbances and arrhythmias. An alternative explanation for paclitaxel induced cardiotoxicity could be the induction of cardiac muscle damage by affecting subcellular organelles [62,67,68].

Enhanced cardiac toxicity has been found in combined therapy of paclitaxel and doxorubicin. At doses of doxorubicin exceeding 380 mg/m^2, the toxicity increased in combination therapy compared to doxorubicin single therapy. A pharmacokinetic interaction appears to be responsible for this effect as paclitaxel has been found to decrease doxorubicin hepatic elimination and therefore lead to increased plasma concentrations of doxorubicin. This effect depends on the interval and sequence of drug administration as well as the duration of the paclitaxel infusion [69]. A similar effect has been shown for epirubicin. The pharmacokinetics of the active metabolite epirubicinol are changed leading to increased plasma concentrations, whereas epirubicin pharmacokinetics remain unchanged [70]. In a clinical trial, combination therapy with epirubicin doses up to 720 mg/m^2 was associated with a relatively low cumulative risk (7.7%) of congestive heart failure but increased (to 48.7%) at a cumulative dose of 1080 mg/m^2. The study included a group patients with at least one cardiac risk factor and a group without cardiac risk factors (10% and 12% cumulative risk for cardiac heart failure, respectively, at epirubicin doses up to 990 mg/m^2). Cardiac risk factors included were age, hypertension, diabetes and prior radiotherapy to the chest wall [6].

Docetaxel shows no increase in cardiac toxicity when combined with doxorubicin. This is in line with the observation that a pharmacokinetic interaction with doxorubicin as described for paclitaxel has not been observed [71].

Protection

In combination therapy with anthracyclines, the cumulative dose of the anthracycline remains an important risk factor and should be lower as compared to anthracyclines monotherapy. In combination therapy cumulative doxorubicin doses up to 340–380 mg/m^2 are reported to be safe whereas in monotherapy cumulative doses up to 450–550 mg/m^2 can be safely administrated [72,73] As in mono-therapy, epirubicin is associated with less cardiotoxicity compared to doxorubicin in combination therapy with paclitaxel [74]. Sequential administration of doxorubicin and paclitaxel does not seem to increase the risk of cardiotoxicity as compared to doxorubicin alone [72]. However, as shown by Gianni *et al* [69] this seems to depend on interval and sequence.

Taxoid associated cardiotoxicity is limited and therefore no specific agents are developed specifically for taxoid induced cardiotoxicity. The mechanism and clinical relevance of the cardiac rhythm disturbances as observed with paclitaxel therapy is not yet elucidated making it difficult to foresee if and what kind of protection would be rational and successful.

5-FLUOROURACIL

The antimetabolite 5-fluorouracil (5-FU) is associated with myelosuppression, diarrhoea, mucositis and dermatitis. Cardiotoxicity may also occur and estimates of the incidence vary from 1% to 5% to as much as 18% [5,75,76]. Cardiotoxicity with 5-FU is usually described with continuous infusion and less upon bolus injection. Recently, a case report was published reporting acute cardiotoxicity during capecitabine treatment. Capecitabine is an orally administrated 5-FU prodrug and can deliver 5-FU selectively to the tumour although toxicities are reported similar to infused 5-FU [77].

Symptoms of 5-FU cardiotoxicity include cardiac arrhythmias, silent myocardial ischemia, angina, congestive heart failure and even sudden death [7,8,78,79].

Risk factors include preexisting coronary artery disease and concurrent radiotherapy. Measurements to protect against 5-FU induced cardiotoxicity are not available yet, although symptoms (chest pain, nausea, diaphoresis with ECG changes) often disappear upon discontinuation of the infusion [76]. Fatal events, however, have been described. After having experienced cardiac side effects, patients are at increased risk to have a relapse if they are reexposed to 5-FU [75].

Mechanism

Several risk factors seem to be involved in 5-FU related cardiotoxicity like age, high dose chemotherapy, continuous 5-FU infusion and past or concomitant radiation therapy [7].

The pathophysiological mechanism of 5-FU related cardiotoxicity is still unclear and suggested mechanisms cannot be explained by the pharmacological action of 5-FU. Hypotheses postulated are vasospasms leading to ischemia, direct toxicity on the myocardium, activation of coagulation system, coronary artery thrombosis, immunoallergic phenomena and cardiotoxic impurities in the 5-FU formulation [75,76,80,81]. A small study in 11 men treated with 5-FU unexpectedly showed a decrease in blood viscosity instead of an increase. The number of patients, however, was too small to conclude that thrombogenecity is involved in 5-FU cardiotoxicity [8]. Kohne *et al* report two patients with 5-FU cardiotoxicity who were treated successfully with the thymidylate synthase inhibitor raltitrexed without evidence of cardiotoxicity. The authors suggest that as 5-FU also targets thymidilate synthase, its cardiotoxicity is not likely due to its interaction with this enzyme [82].

CYCLOPHOSPHAMIDE AND IFOSFAMIDE

Cyclophosphamide and ifosfamide are alkylating oxazaphosphorine agents that need to be metabolized in vivo in the liver to form the active cytotoxic agent phosphoramide mustard. High dose cyclophosphamide is used in transplant regimens and is associated with acute cardiotoxicity such as cardiac decompensation as well as fatal cardiomyopathy. Acute reversible decrease in systolic function has been described [10,83]. The incidence has been estimated to range from 2% to 10% [5]. Ifosfamide cardiotoxicity is reported in only a single study [84].

Mechanism

The pathogenesis is not fully understood but an increase in free oxygen radicals seems to play a role in oxazaphosphorine induced cardiotoxicity. This increase would be mediated by elevated intracellular levels of the actual cytotoxic metabolite phosphoramide mustard [5].

CISPLATIN

Cisplatin is a platinum substance and used in the treatment of many tumors (i.e. testicular cancer). Several cases of acute myocardial infarction after cisplatin therapy are reported [85,86].

In a retrospective study 87 long term survivors of metastatic testicular cancer treated with cisplatin were evaluated for the occurrence of cardiovascular events. A significantly increase in cardiac events as well as an unfavorable cardiovascular risk profile were observed [87].

Mechanism

Several factors have been suggested to be involved like vascular damage, alterations in platelet aggregation and hypomagnesemia [85,86,88,89]. In experiments on human platelets cisplatin was able to trigger platelet aggregation and/or enhance thromboxane formation by platelets. Activation of an arachidonic pathway in platelets by cisplatin seemed to be involved [89].

TRASTUZUMAB

Trastuzumab is a monoclonal antibody directed against the HER2 receptor protein on breast cancer cells and it has been used alone or in combination with other chemotherapeutic agents. Cardiac toxicity associated with trastuzumab seems to be similar with the congestive heart failure observed with anthracycline therapy. Evaluation of data of 1024 patients who received trastuzumab revealed that combination therapy of trastuzumab with an anthracycline and cyclophosphamide showed 28% of reported cardiac dysfunction against 3–5% with doxorubicin monotherapy (cumulative dosage 400 mg/m^2). Concomitant anthracycline therapy and age appeared to be independent risk factors [90]. When receiving trastuzumab as single therapy in an open label study, 7% of the women developed heart failure [91].

OTHER AGENTS

Cardiotoxicity has been associated incidentically with several other cytotoxic drugs such as cisplatin, melphalan, fludarabin, mitomycin, busulfan, mechlorethamine and dacarbazine. However, the lack of structured data on cardiotoxic side effects of these drugs from clinical studies makes it difficult to assess its importance and evidence.

CONCLUSION

Chemotherapy with certain cytotoxic drugs is associated with severe side effects such as cardiotoxicity. As these side effects can be dose limiting and cause severe morbidity and even mortality. Knowledge about their incidence and mechanism is important.

Cardiotoxicity can occur as acute or as long term side effect. With increasing survival rates interest is also focussed on avoiding the late onset and chronically effects.

Anthracyclines are well known for their cardiotoxic side effects and comprehensive research is done to explore the mechanism of anthracycline induced cardiotoxicity. Until now the most accepted hypothesis is the so-called 'free radical' theory. However, protective measurements following this theory such as administration of free radical scavengers have not appeared to be clinically successful. The antioxidant and iron chelator dexrazoxane has been successfully applied to protect the heart from cardiotoxicity with high dose anthracycline therapy, although complete abolishment of cardiotoxicity cannot not be achieved.

Other cytotoxic drugs like 5-fluorouracil, cisplatin and cyclophosphamide are also associated with cardiotoxicity, but yet little is known about the possible mechanisms and methods of prevention. Paclitaxel enhances the cardiotoxic effects of anthracyclines by inhibiting the latters metabolism and therefore leading to increased anthracycline exposure.

Unfortunately, cardiotoxicity is also associated with newer drugs like trastuzumab and cyclopentenyl cytosine and may hamper the development of potential successful anticancer drugs [92]. Further research is warranted to understand the mechanism of chemotherapy induced cardiotoxicity and to develop strategies to circumvent this side effect.

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CHAPTER 7

ABSENCE OF CARDIOTOXICITY OF THE EXPERIMENTAL CYTOTOXIC DRUG CYCLOPENTENYL CYTOSINE (CPEC) IN RATS

Archives of Toxicology 2005;79:268–276

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ABSTRACT

The experimental anticancer drug cyclopentenyl cytosine (CPEC) was associated with cardiotoxicity in a phase I study. The aim of the present study was twofold; first we investigated whether the observed effects could be reproduced in in vitro and in vivo rat models. Second, we intended to investigate the underlying mechanism of the possible cardiotoxicity of CPEC. Effects on frequency and contractility were studied on the isolated atria of 18 male Wistar rats. Atria were incubated with 0.1 mmol/l (n=6) or 1 mmol/l (n=6) CPEC for 1.5 h and compared with control atria (incubation with buffer solution, n=6). The cardiac apoptosis-inducing potential was studied in vivo on 66 rats by 99mTc-AnnexinV scintigraphy, followed by postmortem determination of radioactivity in tissues, histological confirmation with the TUNEL assay (late-phase apoptosis), and immunohistochemical staining for cleaved caspase-3 and cytochrome C (early-phase apoptosis). Serum levels of the necrotic cardiomyopathy marker troponin T were also determined. No effect on heart frequency was found in the isolated atria after CPEC treatment. A trend towards a decrease of contraction force was observed. However, the differences were not statistically significant. 99mTc-Annexin V scintigraphy showed no increase in cardiac uptake ratio upon CPEC treatment in the in vivo rat model, which was confirmed by determination of radioactivity in heart versus blood ratios. At each section a few individual isolated late apoptotic cells (<5) could be identified by the TUNEL assay in the highest CPEC dose group (90 mg/kg) but not in controls or in rats treated with 60 mg/kg CPEC. Staining for the early apoptosis markers cleaved caspase 3 and cytochrome C did not reveal any significant differences between treated and control rats. Cardiac troponin T levels were not increased after CPEC treatment. CPEC does not affect heart frequency or contraction force in our cardiotoxicity models. Moreover, we did not find an indication of CPEC-induced apoptosis in heart tissue.

Keywords: Cyclopentenyl cytosine; CPEC; 99mTc-Annexine; Rat; Cardiotoxicity; Apoptosis

INTRODUCTION

The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) is a pyrimidine analogue of cytidine and has been studied in a phase-I trial in 26 patients with colon carcinoma. Dose-limiting toxicity was hematological. However, the most severe adverse reaction was cardiovascular: six episodes of hypotension occurred in five patients (dose range: 3-4.7 mg/m^2/h). The highest applied dose was 5.9 mg/m^2/h; no hypotension was seen in patients receiving doses below 2.5 mg/m^2/h. Two patients, treated with 4.7 mg/m^2/h, had a fatal hypotensic episode [1]. Postmortem examination was performed in one patient, revealing subendocardial necrosis. Therefore, the hypotension seemed more likely to be caused by cardiac toxicity than by a deregulation of the vascular tone. These observations hampered the initiation of phase II studies with CPEC. The underlying mechanism of the possible cardiotoxicity of CPEC is yet unclear.

In the study of Politi et al [1], a linear relationship was observed between the administered dose and the steady-state plasma concentration, indicating linear pharmacokinetics. After transmembrane transport CPEC is phosphorylated by the enzymes uridine/cytidine kinase, nucleoside monophosphate-kinase (NMP-kinase), and nucleoside diphosphate-kinase (NDP-kinase) to form CPEC-triphosphate (CPEC-TP), the major metabolite. CPEC-TP is an inhibitor of cytidine triphosphate synthetase (CTP-synthetase); this enzyme catalyses the synthesis of the ribonucleotide CTP. Inhibition results in a decrease of RNA and DNA synthesis and S-phase accumulation. In various human and animal leukemia models, anti-tumor activity of CPEC has been demonstrated [2, 3]. Despite its promising anti-tumor effects in preclinical investigations, the unexpected and unpredictable toxicity limited the drug's further development. However, if more information about the mechanism of the cardiotoxicity can be obtained, it may well appear that this serious side-effect can be circumvented or that the patients at risk can be selected before drug administration.

Cardiotoxicity is a well-known adverse effect of some cytotoxic drugs and varies from small changes in blood pressure and arrhythmias to cardiomyopathy [4]. Cardiotoxicity of anthracyclines is the best studied, and different mechanisms have been postulated, including cell damage as a consequence of the formation of free oxygen radicals, DNA damage as a result of disturbed DNA repair, and the induction of immune reactions with the presence of antigen presenting cells in the heart [5]. Moreover, the cytotoxic drugs may induce apoptosis more or less specifically in cardiac cells. Indeed, chemotherapeutics could possibly not only induce apoptosis in fast-dividing tumor cells, as is currently considered to be the main mechanism of action of this class of drugs [6], but also in healthy tissues like the heart. This hypothesis is strengthened by the observation of dose-dependent induction of apoptosis in cardiomyocytes by doxorubicine in a rat cardiotoxicity model [5]. CPEC has also been associated with apoptosis. Verschuur et al [7] demonstrated apoptotic cell death in a T-lymphoblastic cell-line upon CPEC exposure.

In an early phase of apoptosis the cell membrane phospholipid phosphatidylserine (PS), which is normally confined to the inner leaflet of the membrane, is rapidly exposed on the cell's outer surface. Annexin V is a human protein (36 kDa) with high in vivo and in vitro affnity for PS and can be used as a marker for detection of apoptotic cells [8]. By labelling annexin V with 99mTechnetium (99mTc), apoptotic cell death can be imaged in vivo [9, 10]. Doxorubicin-induced apoptosis in the heart of rats has been imaged with 99mTc-Annexin V [11]. From preclinical studies it appears that CPEC has promising anti-leukemic potential and we have initiated a phase-II trial in adults with ALL and AML and a phase I/II study in pediatric patients with ALL and AML. In the phase-II trial, the CPEC doses remain below the doses at which cardiotoxic effects in the phase I trial were observed, and CPEC serum levels are monitored. Moreover, patients will be cardiovascularly monitored. However, it is necessary to learn more about the clinical toxicity, and in particular the possible cardiotoxicity, associated with the use of CPEC.

The objectives of the present study were twofold. First, we intended to establish the cardiotoxic adverse effect of CPEC in specific pharmacological and toxicological cardiac models to confirm or rebut the observation of drug-induced cardiotoxicity in the above mentioned phase I trial. The second aim was to investigate the underlying mechanism of the possible cardiotoxicity of CPEC. The emphasis was put on cardiac effects, a possible influence of CPEC on vascular tone was not investigated in this study.

Because the observed hypotension might have been caused by a decrease in contraction force or heart rate we studied acute effects of CPEC on these in isolated rat atria. The cardiac apoptosis-inducing potential of CPEC was studied in a rat cardiotoxicity model with post-mortem histological confirmation.

MATERIALS AND METHODS

Materials

CPEC was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD, USA. The raw material was analysed and processed to obtain a sterile injectable solution of 4.5 mg/ml in water. The analytical kit for analysis of troponin T, a marker of myocardial damage, was donated by Roche Diagnostics (Mannheim, Germany). The terminal transferase-mediated DNA nick end labelling (TUNEL) assay was obtained from Roche Diagnostics (Mannheim, Germany), and cleaved caspase 3 polyclonal antibody from Cell Signalling Technology. Cytochrome C polyclonal antibody was donated by Dr Reutelingsperger (Academic Hospital, Maastricht, The Netherlands) and a smooth muscle actin (a-SMA) monoclonal antibody was obtained from Sigma (IMMH-2,

Sigma, St Louis, USA). Paraplast for embedding the hearts was obtained from Tyco Healthcare (The Netherlands). HYNIC-annexin V conjugate was kindly provided by North American Scientific, Theseus Imaging Division, Boston, MA, USA. All other chemicals used were of analytical grade.

Animals

Male Wistar rats (200-250 g) were obtained from Harlan (Zeist, The Netherlands).

Effect on frequency and contractility

The effect of CPEC on the cardiac contractility and frequency was studied in isolated rat atria. Eighteen male Wistar rats were killed by stunning and decapitation. The hearts were removed quickly and placed in a Tyrode's solution of composition (mmol/l): NaCl 119; KCl 4.5; MgCl2 0.5; CaCl2 2.5; glucose 11; Tris 30 at pH 7.5 at room temperature, and the medium was bubbled with 100% oxygen. The isolated atria were suspended in water-jacketed organ baths (kept at 37°C and gassed with 100% oxygen) filled with 5 ml Tyrode's solution (pH adjusted to 7.5 at 37°C) and connected with a silk thread to an isometric force transducer. The left atria were paced with a field stimulator (Hugo Sachs Electronic, Germany) at a frequency of 3 Hz, whereas the right atria were allowed to beat spontaneously. The isometric force of contraction was recorded on a Power Lab/8s data-acquisition system (ADInstruments, Australia). The resting tension was adjusted to 5 mN and the atria were allowed to equilibrate for at least 45 min. At 30-min intervals the medium was changed to fresh Tyrode's buffer. After equilibration, CPEC was added to the buffer to obtain a final concentration of 1 mmol/l (0.239 mg/ml) or 0.1 mmol/l (0.0239 mg/ml); these solutions were refreshed every 30 min. After 1.5 h, atria were taken out of the organ bath and put on ice.

In order to study whether CPEC was phosphorylated to its active metabolite and exerted its biological activity in this *in vitro* model, we determined nucleotide and CPEC-TP concentrations in the atria (n=8). A nucleotide extraction was performed immediately after the atria were taken out of the organ bath, by sonicating 2x2x2 mm^3 atria samples in 300 μl 0.4 mol/l perchloric acid. After 10-min incubation on ice, the samples were centrifuged (5 min, 4°C, 11,000g), and neutralized with 15 μl K_2CO3. The ribonucleotide extracts obtained were kept at 20°C. The nucleotides were analysed by anion-exchange HPLC as described elsewhere [3].

Cardiac apoptosis induction

The effect of CPEC on the cardiac induction of apoptosis was studied in vivo in rats and confirmed by histology. Male Wistar rats (six dose-levels; nine rats per dose-level) were exposed to a single

dose of CPEC by intraperitoneal injection. The tested dosage levels were:0, 20, 40, 50, 60 and 90 mg/kg). To study multidose toxicity, one group of three rats received two cycles of 90 mg/kg on days 0 and 7. To avoid potentially antagonistic concentrations of cyclopentenyl uracil (CPEU) [12], the CPEC dosage was divided into three consecutive intraperitoneal injections administered over 2 h.Twenty-four hours after the last CPEC injection, rats were injected with approximately 75 MBq 99mTc-Annexin V. One hour thereafter pinhole SPECT scintigraphy was performed. After the scintigraphy the rats were killed by cervical dislocation. Serum was taken for analysis of troponin T (serum was stored at 20°C in accordance with the manufacturer's instructions). Hearts, blood, and sections of liver, fat, muscle, kidney, spleen were weighed and radioactivity counted using a multichannel analyser. Hearts were further processed for histological examination. To study whether cardiotoxicity would occur later than 24 h and therefore might not be detected in the first six groups, an extra group of nine rats received 90 mg/kg CPEC and a SPECT scintigram with 99mTc-Annexin V was performed on days 1, 3, 6 and 8 after CPEC administration. Rats were killed after the scintigraphy on day 8 and further processed as described above.

Annexin labelling

Human annexin V was produced by expression in Escherichia coli as described elsewhere [13]. The purified protein was derivatized with hydrazinonicotinamide (Hynic) [14]. To bind 99mTc to the Hynic-annexin V conjugate, 0.8 ml containing approximately 1,000 MBq pertechnetate was added. Subsequently, 0.02 ml of freshly prepared stannous tricine was added to the solution. The reaction vial was incubated for 15 min at room temperature. Radiochemical purity was determined chromatographically using instant thin-layer chromatography. Labelling efficiency was consistently above 92%, providing a specific activity of 7.4 MBq/g protein. Under these conditions, annexin labelling was stable for at least 4 h.

Camera design

For imaging of 99mTc-annexin V uptake in the rat heart, a gamma camera (Philips ARC3000) situated in a dedicated animal care facility was equipped with a small animal SPECT (single photon emission computed tomography) gantry and a pinhole collimator fitted with a 3-mm tungsten aperture [15]. Compared with planar imaging, SPECT gives a higher contrast and better localization. The gamma camera is interfaced to a NUD (Nuclear Diagnostics, Stockholm, Sweden) Hermes acquisition and processing station. SPECT acquisition of the thorax was performed for 30 min at the 140 keV 99mTc-peak with a 20% window in a 64x64 matrix (50 projections, 30 s per projection).

Scintigraphy and interpretation

The animals were sedated with ketamine and xylazine i.m. (40 and 2 mg/kg, respectively). Once sedated, the rats were injected with 75 MBq 99mTc-Annexin (40 μg/kg protein) intravenously in a tail vein (24 h after last CPEC injection). The animals were scanned 1 h after injection of the radiopharmaceutical. A region of interest (ROI) was drawn around the uptake in the heart. A second ROI was drawn in the lung. A cardiac uptake ratio (CUR) representing specific uptake of 99mTc-Annexin in the heart region vs uptake in lung was calculated according to the formula [(mediastinum lung)/lung]. A semi-quantitative assessment was preferred over visual interpretation.

Histology

After removal the hearts were fixed for 4 h in ice-cold freshly dissolved 4% paraformaldehyde in PBS (10 mmol/l NaH_2PO_4/Na_2HPO_4 and 150 mmol/l NaCl, pH 7.4) and subsequently transferred to 70% ethanol. Hearts were dehydrated in a graded ethanol series, and embedded in Paraplast. Hearts were sectioned at 7 μm and mounted on polylysine-coated slides. Slides were histologically stained (haematoxylin-azophloxin) or used for apoptosis detection. Detection of the early phase of apoptosis was performed by immunohistochemical staining using an antibody directed against cleaved caspase 3 (1:100 diluted) or cytochrome C (1:5,000 diluted). Antibody binding was visualized using the unconjugated peroxidase technique previously described [16]. The myofibroblasts marker a-SMA (1:4,000 diluted) was used as a positive control; negative control consisted of replacing the primary antibody with PBS. Detection of the late phase of apoptosis was performed by the TUNEL assay, according to the manufacturer's instructions [17].

Statistical analysis

Differences between several independent groups were analysed by the non-parametric Kruskal-Wallis test. Differences between two independent groups were analysed by the non-parametric Mann-Whitney test. Results are expressed as mean ± SD. All statistical tests were two-tailed and differences were evaluated at the 5% level of significance. Differences were considered significant at P-values <0.05.

RESULTS

Effect on frequency and contractility

No effect on frequency was observed after CPEC incubation of right atria. The frequency was observed on line, but not registered. The profiles of right atria exposed to CPEC were similar to those of untreated atria in this experimental setting (frequency 190-240 beats per minute). Correct measurement of contraction force could be obtained from 17 of the 18 left atria (six control, five exposed to 0.1 mol/l CPEC, six exposed to 1 mol/l CPEC). As shown in Fig. 1a, a trend towards a decrease in contraction force with increasing CPEC dosage was observed. However, the differences were not significant. The decrease in contraction force corresponded to an increase in CPEC-TP concentration and a decrease in CTP levels (Fig. 1b).

1a

Influence on contraction force

1b

CPEC-TP and CTP concentrations in atria

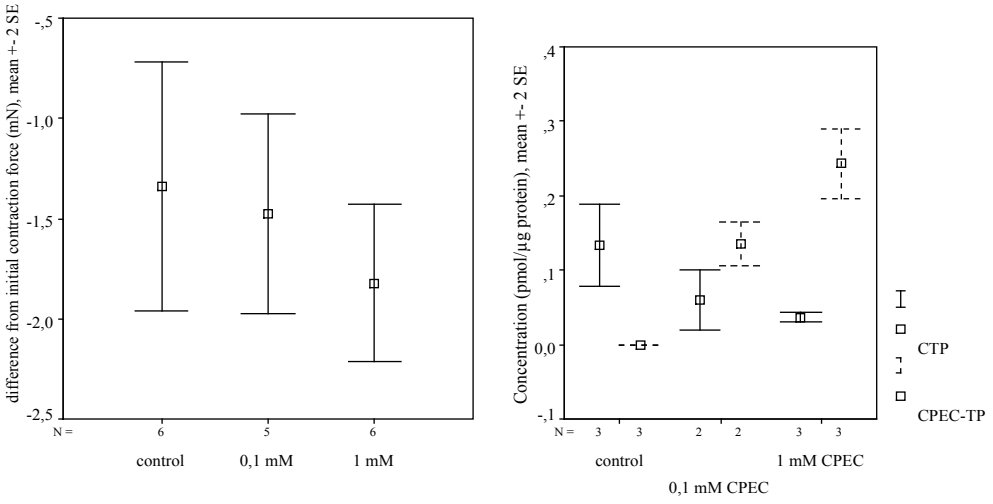


Figure 1a

Influence of CPEC on contraction force in rat atria (difference from initial contraction force in mN) showing a trend in decrease of contraction force after CPEC treatment. b The corresponding CPEC-TP and CTP concentrations in the atria are represented

Apoptosis

Representative scintigraphic images obtained from control and treated rats are shown in Fig. 2.

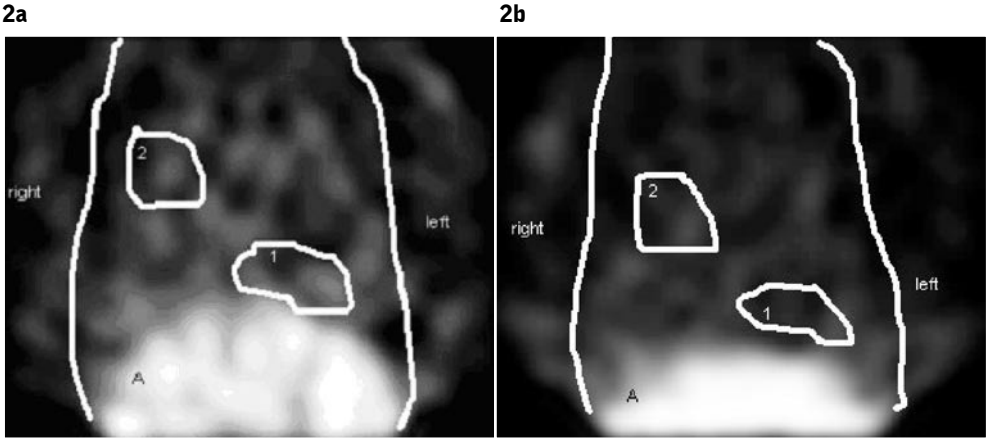


Figure 2 Pinhole SPECT scintigraphy of a CPEC-treated rat (90 mg/kg) (a) and a control rat (b), 1 h after i.v. injection of 75 MBq 99mTc-Annexin V. On the images of the thorax (anterior view, coronal) including a part of the liver (A), the physiological distribution of the radiopharmaceutical, with prominent uptake in the liver and moderate remaining blood pool activity in the mediastinal region, is shown. The white lines indicate the contours of the rat, regions of interest are drawn around the cardiac region (1) and the lung (2) for determination of the cardiac uptake ratio. No significant differences were found between cardiac uptake ratio for control and treated rats

On scintigraphy, rats showed faint mediastinal uptake, representing circulating blood pool and bone marrow uptake. No increase in cardiac uptake ratio was observed (Fig. 3) after CPEC dosage.

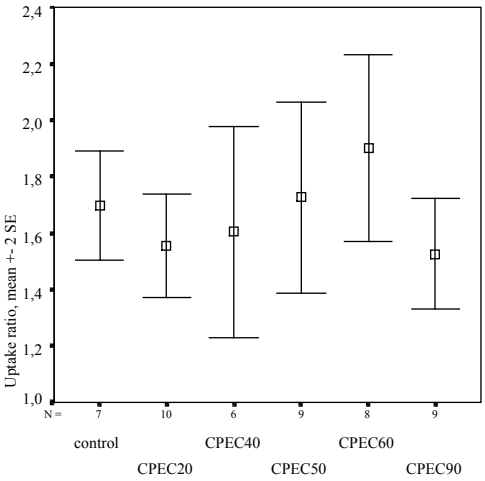


Figure 3 Annexin uptake

Cardiac uptake ratio in control animals versus rats treated with CPEC (mean + 2SD), no significant differences in cardiac uptake ratios were found.

Scanning at later intervals or giving an extra dose of CPEC, did not result in higher uptake ratios. This corresponded with the results obtained after counting of radioactivity in blood and cardiac samples. No significant differences were seen in the uptake ratios of heart vs blood, nor heart vs lung (data not shown).

When correcting the counted activity in blood for the injected dose, higher blood activity was found for the 90 mg/kg dose group (P=0.001), no significant differences between blood activity were found for the other groups (Fig. 4). General histological staining of heart sections using routine histological staining procedures did not reveal any gross morphological abnormalities (haematoxylin-azophloxin staining) (Fig. 5). A few late apoptotic cells could be identified using the TUNEL assay in the highest dose group (Fig. 6). Late apoptotic cells were not identified in controls and rats treated with 60 mg/kg. Staining for the early apoptosis markers caspase 3 and cytochrome C did reveal a few caspase 3 positive cells in controls and in hearts of rats treated with 90 mg/kg. However, the amount of apoptotic cells was very small and not enough to differentiate between control and treated hearts (Fig. 6). Moreover, no increase was observed after two cycles of CPEC. No cytochrome C pattern indicating apoptosis was detected.

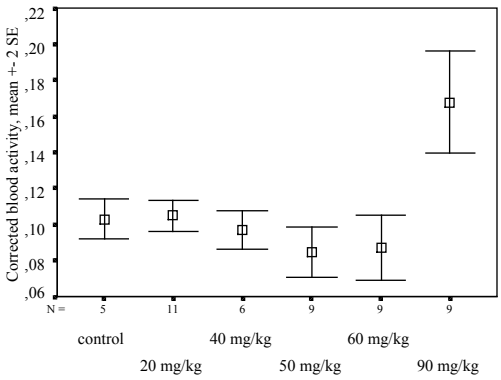


Figure 4 Activity in blood

Radioactivity in the blood of control animals and of rats treated with CPEC. Activity in blood was corrected for sample weight, animal weight and injected dose. Blood from rats treated with 90 mg/kg CPEC showed significantly higher blood activity. This might indicate slower Annexin V clearance in this group

Based on these results it was considered unlikely that the lower dosage groups would show a different pattern, therefore they were not histologically examined. Troponin T concentrations were determined in rat serum. As shown in Fig. 7, no differences in troponin T levels could be observed within the different treatment groups.

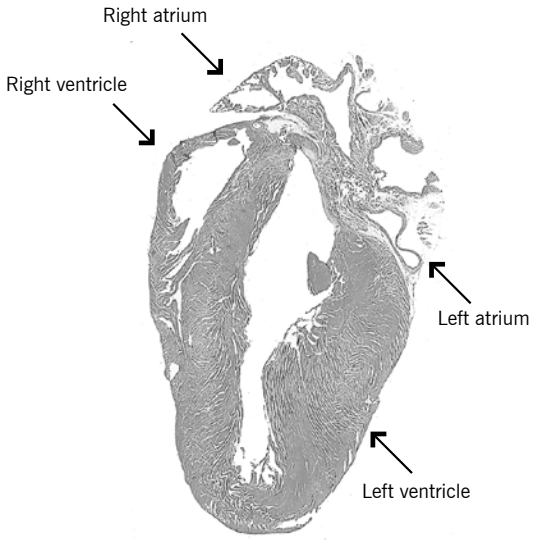
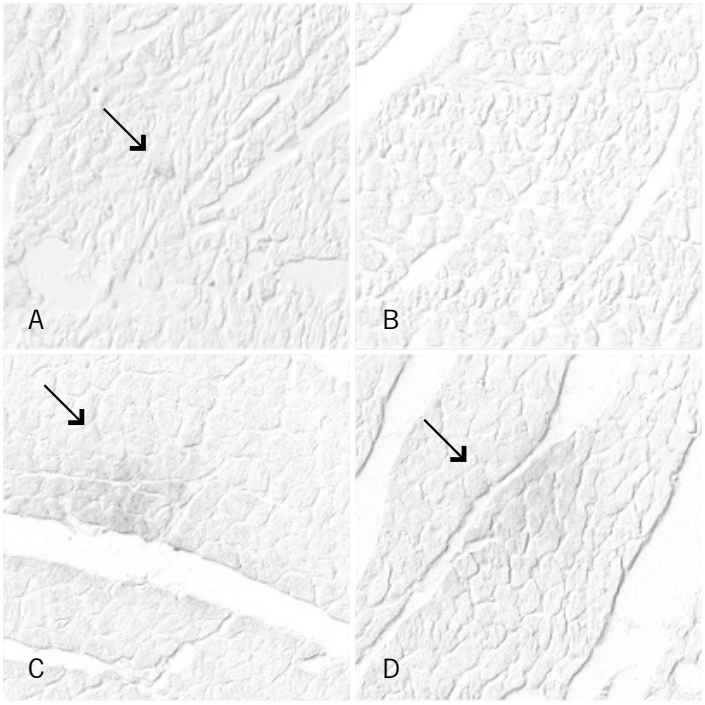


Figure 5

Haematoxylin-azophloxacin staining of the heart (four-chamber view) of a rat treated with 90 mg/kg CPEC, showing no gross abnormalities

Figure 6

A late apoptotic cell, detected by the TUNEL assay, in the septum of a heart treated with 90 mg/kg is shown in A, whereas in the heart of a control rat no apoptotic cells were present. In B, a detail of the septum of a control rat is shown. Early apoptosis was detected by using antibodies against caspase 3. A few early apoptotic cells were found in rats treated with CPEC and in control rats. The number of early apoptotic cells was too small to differentiate between controls and treated rats. C and D show early apoptotic cells in a detail of the septum of a heart treated with 90 mg/kg CPEC (C) and a control heart (D). All images are taken at the same magnification (10x)



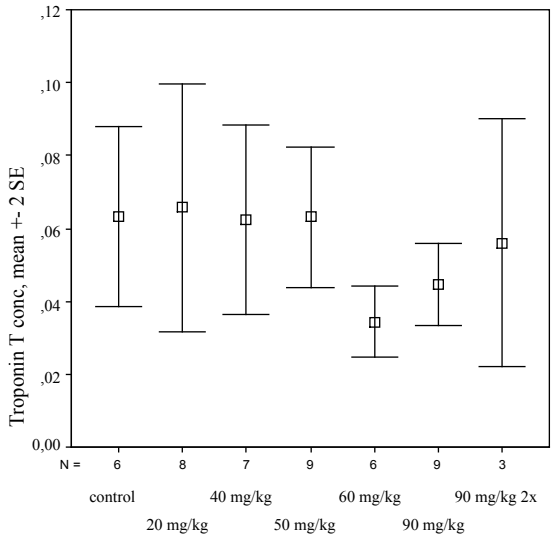


Figure 7 Cardiac Troponin T level

The serum level of the cardiac (necrosis) marker troponin T was measured (μg/l). No significant differences between cardiac troponin T levels were found. The detection limit of the assay was 0.04 μg/l

DISCUSSION

Cardiotoxicity occurs during therapy with several cytotoxic drugs and may be the dose-limiting factor in cancer treatment and hence tumour response. Furthermore, cardiotoxicity can also be responsible for long-term side- effects and may cause severe morbidity in surviving cancer patients [18], which may be relevant, especially in pediatric oncology patients. Cardiotoxicity from anthracycline treatment is known to have high prevalence. The events in the phase I study of Politi et al [1] suggested that new drugs like CPEC may also have cardiotoxic effects. The symptoms were characterized by (irreversible) hypotension. Hypotension can have vascular or myocardial causes. By acting on endothelial receptors, stimulating synthesis and release of EDRF/NO, vasodilatation may occur [19]. This usually is an acute effect and as the hypotension in the clinical trial occurred 24-48 h post-infusion, an acute vasodilative effect of CPEC was considered less probable. Moreover, postmortem examination in one of the patients in the phase-I trial, showed signs of subendocardial necrosis. We therefore choose to investigate possible cardiac effects of CPEC. We hypothesized that the hypotension might be caused by a decrease in contraction force of the heart. Although we observed a trend in decrease of contraction force, the in vitro experiments on rat atria did not show an acute effect on the heart rate. This is especially true when considering that the concentrations used in our in vitro experiments to study the effects

on the contraction force were 0.1 and 1 mmol/l CPEC. The most elevated concentration used in humans in the phase-I trial was 0.01 mmol/l [1]. Even if a clinically relevant effect in our experiments had been found, the effect in humans would probably be more attenuated, because much lower concentrations of CPEC were used. The fact that the active metabolite CPEC-TP could be measured in the heart tissue indicates that the absence of a clinical effect was not caused by inadequate metabolism in the in vitro model used. Furthermore, a decrease in CTP concentrations was observed after increasing CPEC dosages, showing the desired biochemical effect of CPEC. The limitation of in vitro experiments as performed in this study is the relatively short exposure time (1.5 h) of the atria to CPEC. Because the hypotension in the clinical trial was observed after 24-28 h, the exposure might have been too short to obtain toxic effects. However, if cardiac damage was the cause of the hypotension in the clinical study, it is also conceivable that there is a time lag between early cardiac effects and the finally observed hypotension.

Because cardiotoxicity is a well-known side-effect of the anthracyclines, a tremendous amount of research is focussing on the mechanism of this side-effect. Studies indicate that the myocardial damage caused by the anthracycline drug doxorubicin involves apoptosis. This programmed cell death process would, for anthracyclines, be initiated by the formation of oxidative free radicals. Apoptotic cell death was indeed found in rat cardiomyocytes and bovine aortic endothelial cells upon exposure to doxorubicin [5, 20] and could be imaged in vivo in a rat model with 99mTc-Annexin V [11]. Although there is no structural relationship between doxorubicin and CPEC, CPEC is also associated with apoptosis and, therefore, we investigated the apoptosis-inducing potential in the heart. Our results with 99mTc-Annexin V suggest no cardiac involvement of apoptosis after CPEC treatment. Cardiac uptake ratios with scintigraphy were not increased upon CPEC treatment, neither were ratios of heart-to-blood activity. The measured cardiac uptake ratio with scintigraphy was probably due to activity in circulating blood. Ohtsuki et al [21]have reported that residual blood activity in mice 1 h after injection was about 5% of the injected dose. Delaying imaging to reduce blood pool activity was not considered in our study, because the late-phase plasma clearance is slow and image-count statistics would decrease because of radioactive decay. The timing of scintigraphy of 60 min after intravenous injection of 99mTc-annexin was based upon a previous study of a doxorubicin cardiotoxicity model in the rat [11]. Annexin V has a short half-life in the circulation without significant redistribution after 30 min, enabling radionuclide imaging within 1 h of injection [22]. Blood activity was increased in the group treated with the highest CPEC dose (90 mg/kg), and this might indicate slower clearance because of renal toxicity in this group. However, as creatinin levels were not measured in our study we cannot confirm this assumption. In earlier toxicity studies kidney toxicity was observed in mice after CPEC treatment in a single-dose toxicity study (CPEC dose: 0-140 mg/kg). However, no kidney toxicity was observed in rats (maximum dose: 70 mg/kg) (data on file at the National Cancer Institute, Bethesda, MD, USA). The doses we used were based on these previous toxicity studies, where the lowest dose (20 mg/kg) was shown not to be toxic as a bolus. Our highest dose (90 mg/kg) was 20 mg/kg higher than the maximum dose used.

Because results from the group that was scanned on several consecutive days after CPEC treatment were not different from those from other groups, it is unlikely that the absence of increased 99mTc-annexin V uptake in the heart was due to the short interval between injection of CPEC and 99mTc-annexin V (24 h). Cumulative dosage does not seem to be of influence, because administration of two CPEC dosages did not give an increase in 99mTc-annexin V uptake in the heart. In earlier experiments with doxorubicin [11] the detection level of apoptosis with 99mTc-annexin V, seemed to be higher than with the TUNEL assay. In order to investigate whether this might have been of influence in our study, we also performed histological apoptosis assays. Late and early-phase apoptosis was investigated. Our results from histology correspond with the absence of difference among the dose groups in radioactive cardiac uptake ratios. The TUNEL assay was used to detect the late phase of apoptosis. In the clinical phase-I trial, cardiotoxicity was observed 24-48 h after CPEC treatment. Because 99mTc-annexin V visualizes an early (preclinical) phase of apoptosis, we performed scintigraphy 24 h after the last CPEC injection. This might be too early to detect late-phase apoptosis, as is done with the TUNEL assay. Therefore, we also performed histological staining with two early phase markers: cleaved caspase 3 and cytochrome C. Activation of caspase 3 occurs early in the apoptotic pathway and leads, among other effects, to the externalization of PS and fragmentation of several cytoplasmic proteins and nuclear DNA. Caspase 3 can either be activated by cytokines and upstream caspases (e.g. caspases 1 and 8) or by mitochondrial (or oxidative) stress. The stress leads to the release of cytochrome C and processing of caspase 9, which subsequently activates caspase 3 [23]. As well as with the TUNEL assay, no massive apoptosis could be observed in CPEC-treated rats after immunostaining with cleaved caspase 3 or cytochrome C.

Studies with cytotoxic drugs have shown involvement of apoptosis and necrosis [23-25]. Therefore, we also investigated whether necrosis was involved in possible CPEC cardiotoxicity. Serum levels of cardiac troponin T were determined. Cardiac troponin T (cTnT) is a cardiac-specific marker for monitoring necrotic cardiomyopathy. The gene expression is restricted to striated muscle, principally cardiac muscle, and cTNT is released relatively rapidly into the blood. The cTNT immunoassay used for detection of myocardial injury in people is able to detect blood activity in rat after toxic, ischaemic and immunologic injury to myocardium [26]. In our study cTNT levels were not increased after CPEC treatment, suggesting that necrosis does not play a significant role in the possible cardiotoxicity.

Recently, Kostin *et al* [27] have reported autophagy as a third form of cell death in human myocytes. Whether this also occurs in rats treated with CPEC remains unclear, because this form of cell death is not detected by the methods we used.

In the present study we were not able to reproduce significant (semi)-acute cardiotoxicity of CPEC as observed in the phase I clinical trial. The rat model with 99mTc-Annexin V was an adapted version of a model that has been used successfully to detect doxorubicin- induced apoptosis in the heart [11]. Our results seem to suggest that apoptosis does not play a significant role in CPEC-induced cardiotoxicity. It remains unclear what caused the severe hypotension in the phase-I trial and whether or not this was a true cardiotoxic effect.

Acknowledgements Dr M van den Hoff is financially supported by The Netherlands Heart Foundation (grant M96.003). The animal studies were performed after approval of the Ethical Animal Research Committee of the University of Amsterdam and following its guidelines.

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CHAPTER 8

DOXORUBICIN AND CPEC INDUCED CARDIOXICITY: association with the GTPase gene Rac2, and drug transporter genes MRP1 and MRP2

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ABSTRACT

Chemotherapy with anthracyclines is associated with cardiotoxicity. Besides known risk factors such as cumulative dose and mediastinal irradiation, there are indications that genetic variation might also be associated with the development of anthracycline induced cardiotoxicity. In the current study a retrospective case control analysis was performed in oncology patients having received anthracyclines. Allele frequencies of genetic polymorphisms in several candidate genes between patients with and without anthracycline-induced cardiotoxicity and between cases and healthy control subjects were studied. Anthracycline-induced cardiotoxicity was associated with the T7508A variant in the GTPase Rac2, a gene with a known functionality in NAD(P)H oxidase activity. Furthermore, we tested *in vitro* in a rat cardiomyocyte cell line (H9c2), whether the anthracycline doxorubicine and the cytotoxic and cardiotoxic drug cyclopentenyl cytosine (CPEC) influenced the expression of the candidate genes (p22phox, Rac2, MRP1 and MRP2), that were reported earlier to be related with doxorubicin-induced cardiotoxicity. CPEC caused a small decrease in the expression of MRP2 and did not influence the expression of the other genes. The expression of MRP1 was decreased after incubation with doxorubicin whereas no changes were found in the expression of Rac2, p22phox and MRP2.

It is concluded that although the expression of Rac2 did not seem to be influenced by doxorubicin, a SNP in the GTPase Rac2 was associated with anthracycline induced cardiotoxicity. As demonstrated by their influence on MRP1 and MRP2 respectively, doxorubicin and CPEC decreased the expression of genes involved in multi drug resistance.

INTRODUCTION

Cardiotoxicity is associated with several cytotoxic drugs, among which the anthracyclines seem to be the most important group and much research has been done with anthracyclines to unravel the mechanisms behind this severe side effect [1-4]. Oxidative stress caused by reduction of doxorubicin seems to play an important role in the pathogenesis. Several risk factors such as age, mediastinal irradiation and cumulative dose have been associated with anthracycline induced cardiotoxicity. Of these, cumulative dose seems to be the most important risk factor [5,6]. The incidence of doxorubicin induced cardiomyopathy and congestive heart failure increases from approximately 3-5% below 400 mg/m^2 to 20-50% at 700 mg/m^2 [7,8]. A maximum cumulative dose of 450-550 mg/m^2 for doxorubicin is nowadays commonly accepted. Depending on their cardiotoxic potential, maximum cumulative doses are also established for the other anthracyclines [9]. However, as indicated by the 5% of patients with cardiotoxicity having received 400 mg/m^2 doxorubicin or less, there is a category of patients still developing cardiotoxicity upon low to normal cumulative doxorubicin doses.

These inter-individual differences may have a genetic cause and several mechanisms are conceivable that could explain this relationship. One mechanism might be found in the oxidative stress theory. As oxidative stress seems to play an important role in the development of the anthracycline induced cardiotoxicity [1-3], changes in enzymes involved in oxidative stress may result in more or less susceptibility for anthracycline induced cardiotoxicity. The NAD(P)H oxidase multi-enzyme complex is an important source of reactive oxygen species in the myocardium [10]. Indeed, recently Wojnowski *et al* reported associations between polymorphisms in the NAD(P)H oxidase multi-enzyme complex and cardiotoxicity of doxorubicin [11]. They used a candidate gene approach to detect associations between cardiotoxicity and single nucleotide polymorphisms (SNPs). Besides the three SNPs in genes related to the NAD(P)H oxidase complex (p22phox, p40phox and Rac2), two SNPs in genes coding for the drug efflux pumps MRP1 and MRP2 (multidrug resistance protein 1 and 2) were also associated with doxorubicin induced cardiotoxicity. Anthracyclines are known substrates for the efflux transporter MRP1 [12,13], and polymorphisms of this pump are described in other studies [14,15].

Apparently the five genes studied by Wojnowski *et al* are involved in the mechanisms behind doxorubicin induced cardiotoxicity. However, NAD(P)H oxidase and the efflux pumps both are not exclusively related to the function or metabolism of doxorubicin. It is therefore conceivable that changes in these genes are also related to cardiotoxicity induced by other cytotoxic drugs. The experimental cytotoxic drug cyclopentenyl cytosine (CPEC) has also been associated with cardiotoxicity and until now the mechanism has not been clarified [16, 17]. CPEC is a nucleoside analogue structurally related to the cytotoxic drugs cytarabine and gemcitabine. These drugs are not thought to be substrates nor modulators for MRP1. Substrates for MRP2 are supposed to be similar to those for MRP1, however, there is little information available upon modulators for

MRP2 [18]. If CPEC would be able to modulate MRP2 by altering its expression, this might be involved in CPEC induced cardiotoxicity.

In the present study we aim to replicate the results of the study by Wojnowski *et al* in a case control study. Moreover, in order to further investigate the association of the candidate genes and cytotoxic drug induced cardiotoxicity, we have also investigated in a rat cardiomyocyte cell line (H9c2), the expression of the candidate genes upon incubation with the anthracycline doxorubicin and the experimental cytotoxic drug CPEC.

MATERIAL AND METHODS

Study population

We retrospectively selected study subjects by reviewing all patients with cancer (n=1074) that received doxorubicin between january 1999 and april 2005. Patients were included in this study if they were subjected to routine cardiac assessment (either by left ventricular ejection fraction (LVEF) measurement or echocardiography) and if DNA was available. Cases could also be directly selected by the participating physicians if the patients were known for clinically overt anthracycline induced cardiotoxicity. Patient material was obtained from blood samples from routine control and was coded before analysis in order to prevent results from being related to individual patients. The protocol was approved by the institutional Medical Ethical Committee.

Based on the described selection a group of cases (n=11) and the first control group (n=26) were determined. The first control group consisted of patients without reported cardiotoxicity, for criteria see below. Most patients had received doxorubicin, if patients had received another anthracycline (2 of the cases: one received daunorubicin and mitoxantrone, the other received doxorubicin and daunorubicin, 1 control patient received epirubicin and doxorubicin), the received cumulative dose of that anthracycline was converted to the equivalent cumulative doxorubicin dose. The conversions were based on the different maximal cumulative dosages which were respectively 450-550 mg/m^2 for doxorubicin, 500-600 mg/m^2 for daunorubicin, 850-1000 mg/m^2 for epirubicin and 160-200 mg/m^2 for mitoxantrone [9]. For example, 420 mg of epirubicin was converted to 227 mg of doxorubicin (420 * 500/925 = 227). Patients having received more than 550 mg/m^2 of cumulative doxorubicin dose were excluded from the analysis.

The second control group consisted of a panel of 180 healthy volunteers. The DNA of these subjects was previously selected in families of patients who had had meningococcal disease[19] and in subjects who served as healthy controls to study the susceptibility to systemic lupus erythematosus [20].

Cardiotoxicity assessment

Cardiotoxicity was determined for the group of cancer patients and was based on the following criteria: clinically overt cardiotoxicity or a decrease in left ventricular ejection fraction (LVEF) of more than 20% or a decrease of more than 10% resulting in a LVEF below 50% [8]. If assessment of cardiac condition was performed by echocardiography, cardiotoxicity was defined as a decrease of more than 10% or a decrease resulting in a shortening fraction (SF) below 30% [21].

Sample preparation and measurement of SNPs

Six SNPs in five candidate genes were selected that had been associated with doxorubicin induced cardiotoxicity in a previous study [11]. Three selected genes had a relation with the NAD(P)H oxidase multi-enzyme complex: those encoding for the GTPase Rac2, the subunit p40phox (NCF4) and the subunit p22phox (CYBA) from the NAD(P)H oxidase multi-enzyme complex. The other genes were encoding for MRP1 and MRP2. The following SNPs were analyzed: p40phox 212A>G (rs1883112), p22phox His72Tyr (rs4673), Rac2 7508T>A (rs13058338), MRP1 Gly671Val (2012G>T), MRP2 Val1188Glu (rs8187694), MRP2 Cys1515Tyr (rs8187710). The SNPs for p22phox, MRP1 and MRP2 were non-synonymous. The genes encoding for Rac2 and NCF4 were both found on chromosome 22, p22phox and MRP1 on chromosome 16 and MRP2 on chromosome 10.

Table 1 primer sequences used for SNP assay

Name	primer direction	sequence 5'-3'
NADPH-f	forward	GCAAAGGCTTGGCAGTAAGAGAA
NADPH-rBio	reverse	CAAGCCTCAGTTGGGTATCAGAA*
NADPH-fs	forward	GGTCACAAGACA
P22-f	forward	GCAAAGGAGTCCCGAGTG
P22-rBio	reverse	GTAAAGGGCCCGAACAGC
P22-fS	forward	CCCCAGGGGACAG
Rac2-fBio	forward	CCCCAGCACCCAGGTATCA
Rac2-r	reverse	TTGCCCTGAGAACCAAGACCT
Rac2-rS	reverse	TCTGGGTTCCTTGAATGC
MRP2-val1188glu-f	forward	AGCACCAGCAGCGATTTC
MRP2-val1188glu-rBio	reverse	GGAGGTGATCCAGGAAAAGAC
MRP2-val1188glu-fS	forward	GATTTCTGAAACACAATG
MRP2-cys1515tyr-fBio	forward	ATGGTCCTAGACAACGGGAAGAT
MRP2-cys1515tyr-r	reverse	GCCTTCTGCTAGAATTTTGTGC
MRP2-cys1515tyr-rS	reverse	TCTTCAGGGCTGCCG
MRP1-f	forward	GAGCGACCCTCCCACACT
MRP1-rBIO	reverse	ACTTTCCGCAGCCCACCT
MRP1-fs	forward	TCTCCATCCCCGAA

* Primers are Biotine labeled at 5'-end

DNA was isolated from peripheral blood cells by a standard manual salting-out method. Sterile water was used as a negative control. Genotyping was performed using real-time polymerase chain reaction with TaqMan (MRP1) (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) or Pyrosequencer (Isogen Lifesciences, Maarssen, The Netherlands) (other SNPs) according to manufacturer's instructions. In table 1 the sequences of the primers are displayed.

Statistical analysis

Differences in baseline characteristics between the cancer patients were analyzed by Student's t-test (continuous variables) or chi-square test (dichotomous variables). P values less than 0.05 were considered significant. For association with cardiotoxicity, differences in genotype distribution were tested by 3 x 2 cross tabulations for each genotype with analysis by Fisher's exact test, and by 2 x 2 cross-tabulations for allele carriers versus noncarriers with calculation of the odd's ratios (95% confidence interval). Binary logistic regression with mediastinal irradiation as a covariate was used to test whether mediastinal irradiation had to be considered as a possible confounder.

All statistical analysis were performed using SPSS 12.0.1 software (SPSS, Chicago, USA).

Cellular experiments

The rat cardiomyocyte cell line H9c2 (ECACC, Wiltshire, UK) was used for in vitro assays. This cell line has been used before to study anthracycline induced cardiac injury [22-24]. The ability of doxorubicin and CPEC to change the expression of the candidate genes was explored. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Cambrex Corporation, East Rutherford, NJ, USA) containing 2mM L-glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin and 10% fetal calf serum (Invitrogen, Breda, The Netherlands). Cells were not allowed to reach more than 70% confluency.

For determination of cytotoxicity cells were plated in 96-well plates (approximately 20 x 10^3 cells per well). After 24 hours incubation at 37ºC in a humidified environment of 5% CO_2, cells were exposed to different concentrations of doxorubicin (0 – 10 μg/ml) (Pharmachemie, Haarlem, The Netherlands) and CPEC (0-1 μg/ml) (kindly provided by the National Cancer Institute, Maryland, USA) for 24 hours. After incubation with the active ingredient, medium was replaced and cells were further cultured for 72 hours. Cytotoxicity was determined by the sulforhodamine B (SRB) assay as described earlier [25,26]. Briefly, after drug treatment, cells were fixed with 100 μl thrichloroacetic acid (10%) for 60 minutes at 4ºC, washed with water and stained with 100 μl SRB (0.4% in 1% acetic acid) for 30 minutes at room temperature. Then plates were rinsed with acetic acid (1%) and 200 μl Tris base (10mM, pH 10.5) was added. Optical density was determined at 560 nm in each well in a microplate reader (Softmax, Biorad, Veenendaal, The Netherlands).

For the expression assays H9c2 cells were treated with doxorubicin 1 μg/ml or CPEC 1 μg/ml for 4 or 24 hours (in triplo). At these concentrations approximately 90% of the cells were still viable during the incubation time (4 to 24 h). Total RNA was isolated from the cells using the mini RNeasy total RNA isolation kit (Qiagen, Venlo, The Netherlands) and the protocol supplied by the manufacturer. The isolated RNA was converted to cDNA using standard techniques. cDNA of Rac2, p22phox, MRP1 and MPR2 were analyzed by RT-PCR on the Taqman 7500 in duplo using SYBR green (Applied Biosystem, Nieuwerkerk aan de IJssel, The Netherlands). The sequences of the used primers are described in table 2. The expression of β2-microglobuline gene was used as an internal control, which had been proven to be stable expressed in these experiments.

The relative changes in gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method With this method data are presented as the fold change in gene expression normalized to the endogenous control gene (β2-microglobuline) and relative to the untreated control at the same time interval. The range in fold change is determined by calculating respectively the fold change out of the average $\Delta Ct + SD$ and $\Delta Ct - SD$ [27].

Table 2: primer sequences used for expression assay

Name	primer direction	sequence 5'-3'
MRP1-f	forward	GGTCAGCCCGACACTGCTA
MRP1-r	reverse	TCCTTCGCTCAAACTGAATTAAAA
Rac2-f	forward	CAGACGTGTTCCTCATCTGCTT
Rac2-r	reverse	CCACTTGGCTCGGACGTT
MRP2-f	forward	CGTCTCCTACGGTTTCCAGATT
MRP2-r	reverse	GGAGTTTGTGTTGAGTCACTTGGT
P22-f	forward	CTCTATTGTTGCAGGAGTGCTCAT
P22-r	reverse	GGTGGAGCCCTTTTTCCTCTT

RESULTS

Patients

The characteristics of the cases and the first group of control (cancer) patients are represented in table 3. Cases were (non significantly) more frequently irradiated in the mediastinal region. However, when mediastinal irradiation was used as a covariate in the statistical analyses, this was of no relevance for the odd's ratios for the calculated differences between cases and controls for the SNPs that were analyzed. Therefore, we concluded that mediastinal irradiation did not confound our analysis.

Table 3 Patient characteristics

	cases (n=11)	controls (n=26)
Gender, M/F	1/10	13/13
Age in years(SD)	36 (19)	42 (16)
Range in age	13-69	21-73
Cumulative doxorubicin dosein mg/m2 (SD)	399 (93)	332 (113)
Mediastinal irradiation	5	6
Disease		
Sarcoma*	6	13
Mamma carcinoma	2	3
Acute Myeloid Leukemia (AML)	2	0
Non Hodgkin Lymphoma (NHL)	1	3
Hodgkin Lymphoma (HL)	0	6
M Kahler	0	2

*different forms of sarcoma's: ewing, osteo, synovium, bone-leiomyo

There were no statistic differences between the two groups in age, cumulative dose or mediastinal irradiation.

No significant associations with cardiotoxicity were found for the p40phox 212A>G, p22phox His72Tyr, MRP1 Gly671Val, MRP2 Val1188Glu and MRP2 Cys1515Tyr polymorphisms between either cases and control patients or healthy subjects. Combinations of SNPs based on either the location on the same chromosome (Rac 2 with p40phox, p22phox with MRP1 and both SNPs in MRP2) or physiological role (Rac2, p40phox and p22phox) neither showed a significant association with cardiotoxicity.

A significant association with cardiotoxicity was found for the SNP in Rac2. Frequency of the A allele was significantly lower in healthy subjects compared to cardiotoxic cancer patients (35% versus 82%) with an odds ratio of 0.12 (0.03-0.58). Distribution in the control patient group was in line with this observation (57% versus 82%), although it reached not significance (odds ratio 0.29, range 0.05-1.65).

Cells

In table 4 the relative fold expression changes with their ranges are presented for the genes studied. The expression of MRP1 was approximately 3 fold decreased upon 24 hr exposure of doxorubicin (mean fold change 0.32, range 0.18-0.57), see figure 1. No changes in expression were seen after 4 h of incubation. The expression of the other genes remained unchanged after treatment with doxorubicin both after 4 and 24 h of incubation. Although the range in fold change was relatively large, CPEC seemed to decrease the expression of MRP2 after a 24 h incubation period (mean 0.49, range 0.25-0.85), no changes were seen after 4 h of incubation. CPEC did not induce changes in expression of the other genes (4 and 24 h of incubation).

Table 4 Relative fold changes in expression upon exposure to doxorubicin or CPEC

	normalized fold change ($2^{-\Delta\Delta Ct}$) relative to untreated control (range)			
	Doxorubicin 24 hr	Doxorubicin 4 hr	CPEC 24 hr	CPEC 4 hr
MRP1	0.32 (0.18-0.57)	0.79 (0.31-1.45)	0.81 (0.39-1.30)	0.94 (0.34-2.30)
MRP2	0.55 (0.23-1.09)	0.68 (0.28-1.12)	0.49 (0.24-0.85)	0.98 (0.46-2.34)
Rac2	0.61 (0.23-1.14)	0.71 (0.19-1.77)	0.54 (0.21-1.16)	0.88 (0.27-2.93)
P22phox	0.85 (0.28-1.27)	1.14 (0.45-2.37)	1.03 (0.53-1.80)	1.11 (0.56-2.5)

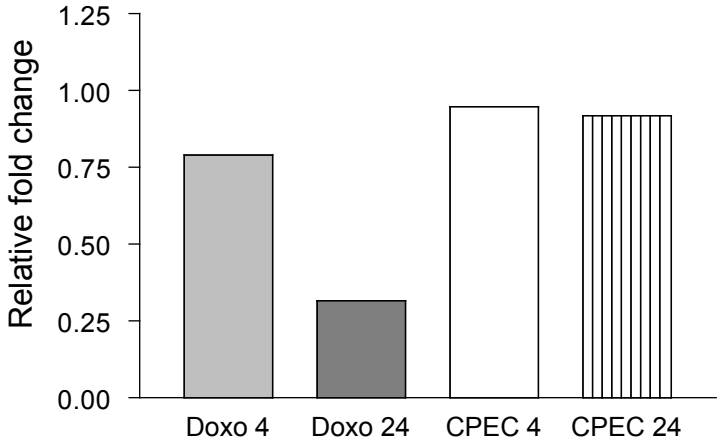

Figure 1 Fold change in expression of MRP1 relative to untreated samples

In the figure the fold change in expression of MRP1 relative to the untreated samples is presented. The four bars indicate the different treatments (e.g. doxo 4 means treatment with doxorubicin for 4 h). After 24 h of incubation with doxorubicin the expression of MRP1 is approximately 3 fold decreased compared to the untreated samples.

DISCUSSION

In the present study we have used two approaches to investigate the association between five candidate genes (p22phox, p40phox, Rac2, MRP1 and MRP2) and cytotoxic drug induced cardiotoxicity. First we studied SNPs in these genes in a retrospective case control study and secondly we investigated whether the expression of these genes changed upon incubation with doxorubicin or CPEC.

The five candidate genes were selected based on a study by Wojnowski *et al.* In a population of non-Hodgkin patients they found an association between 6 SNPs in five genes related with either NAD(P)H oxidase enzyme complex (p22phox, p40phox and Rac2) or multidrug efflux pumps (MRP1 and MRP2) and doxorubicin induced cardiotoxicity [11]. There is very little information on the functional analysis of the SNPs studied. For the SNP in p22phox (CYBA) conflicting results regarding its influence on NAD(P)H oxidase are published [28,29]. The SNP in MRP1 is located near the 'Walker A motif' and studies suggest that mutations near this motif would be able to decrease the activity of the efflux pump [14,15]. For the other SNPs no data in the published literature are available.

In our study we have been able to reproduce the association as reported earlier by Wojnowski *et al* between the SNP in the Rac2 gene in cancer patients experiencing anthracycline induced chronic cardiotoxicity when compared with a healthy population.

No difference between cancer patients with and without cardiotoxicity was found in our study, although the difference in allele frequency observed for Rac2 was in line with the observed statistical difference between cases and the healthy population. There were no statistical differences between the cases and the patient control group in the known risk factors cumulative dose and mediastinal irradiation. Several patients had received cyclophosphamide which has also been associated with cardiotoxicity, but there was no statistical difference between the two groups. Moreover, little information is known about prevalance, risk factors and mechanism of cyclophosphamide induced cardiotoxicity [6]. There were significantly more female patients in the case group, however, gender is not a known risk factor for anthracycline induced cardiotoxicity.

We did not observe associations between the other SNPs and cardiotoxicity. Our patient population probably was too small to detect differences as reported by Wojnowski et al [11]. In that study 83 patients with doxorubicin induced cardiotoxicity were evaluated whereas in the present study 11 cases were included. However, the cases in our study were selected out of a group of 1074 patients treated with anthracyclines in our large academic medical center in a 6.5 years period, indicating the low frequency of overt anthracycline induced cardiotoxicity. Indeed, a crude incidence of approximately 1.1% of anthracycline induced cardiotoxicity can be estimated from our cohort which is in line with published incidence rates being approximately 3-5% [7,8]. This observation also implicates that, when implementing (with the current knowledge) a diagnostic screening aimed at detecting patients at increased risk for anthracycline induced cardiotoxicity, would result in an unrealistically high number needed to genotype.

Besides the analysis of the SNPs, our second approach to investigate the association between the candidate genes and cardiotoxicity consisted of determining the influence of the cytotoxic drugs doxorubicin and CPEC on the expression of the genes. As little information on the functional analysis of the SNPs is yet available, this might help to clarify the role of the genes and the specific SNPs.

The NAD(P)H oxidases are involved in the formation of reactive oxygen species. More NAD(P)H activation leads to more superoxide formation and this may result in increased cardiotoxicity. Rac2 is essential for stimulation of the NAD(P)H oxidase [30] and is mainly expressed on cells from hematopoietic system, however the Rac2 protein has also been detected on rat enterocytes [31] and human aortic smooth muscle cells [32]. A decreased expression of Rac2 may result in a less activated NAD(P)H oxidase. Wojnowski *et al* have shown that mice with reduced NAD(P)H oxidase activity experienced less cardiotoxicity after treatment with doxorubicin [11]. The protein p22phox (CYBA) is necessary in NAD(P)H activation [33,34]. Based on their different roles, an increased expression of Rac2 (SNP rs13058338) and p22phox (SNP 4673) upon incubation with doxorubicin might be expected. However, we did not reveal any significant changes in the expression of both genes upon exposure to doxorubicin and CPEC when compared to the untreated cells. Rac2 was weakly expressed (Ct values between 30 and 34) in the H9c2 cells, which might

explain the relatively large range in fold change. However, p22phox had a higher expression (Ct values between 22 and 24) and the range was still relatively large suggesting that other influences might have played a role as well.

Expression of multidrug resistance proteins in tumor cells is associated with resistance to anthracyclines [18,35]. An impaired function of MRP1 might be associated with higher anthryacycline levels in the heart, rendering it more susceptible to the detrimental effects of anthracyclines. The reduced expression of MRP1 that was detected in our study after 24 hr incubation with doxorubicin seems to confirm this hypothesis. Apparently doxorubicin needs time to exert its influence on the expression of MRP1, as no difference with the untreated samples was detected after 4 h of incubation. Earlier reports on human small cell lung cancer cell lines [36] and heart tissue of mice [37], showed an increase in MRP1 expression after treatment with doxorubicin. In the study with the human small cell lung cancer cell line, c-jun N-terminal kinase activation (JNK) seemed to be essential for the induction of MRP1 by doxorubicin [33]. It is conceivable that JNK acts differently in H9c2 cells. The fact that the results of the two studies were obtained in different tissues or species might therefore have been responsible for the apparently conflicting results on MRP1 expression by doxorubicin.

CPEC did not influence the expression of MRP1, which might be partly explained by its chemical structure. CPEC is a nucleoside analogue and has structural similarities with the cytotoxic drugs cytarabine and gemcitabine and both drugs are not thought to be either substrates nor modulators of MRP1 [18]. MRP2 was slightly downregulated after incubation with CPEC and although substrates for MRP1 also are suggested to be substrates for MRP2, there is no information available upon modulators of MRP2 [18].

In this study we evaluated the abilities of doxorubicin and CPEC to influence the expression of four selected genes. However, doxorubicin has also been reported to induce the expression of several other genes involved in heart function like endothelial nitric-oxide synthese (eNOS), atrial and brain natriuretic peptide (ANP and BNP) and Fas antigen, the latter being involved in apoptosis [38-40]. Based on the structure of CPEC it might have been interesting to investigate the influence on MRP4 and MRP5 as they are believed to be involved in nucleoside transport [18].

Moreover, it remains to be solved whether the induced damage by doxorubicin or CPEC is related to altered gene or whether they directly influence the expression. This illustrates the complexity behind the induced cardiotoxicity and changes in expression of specific genes may only partly explain the mechanism.

In conclusion, in our small patient population, we have been able to detect an association between anthracycline induced cardiotoxicity and a polymorphism in the GTP-ase Rac2 which is associated with the function of the NAD(P)H oxidase complex. This is in line with the hypothesis regarding

the involvement of oxidative stress in the development of anthracycline induced cardiotoxicity. Although we did not find an association between SNPs in the efflux pumps and cardiotoxicity, the results of our expression study show a downregulation of MRP1 in rat cardiomyocytes after incubation with doxorubicin and a small decrease in expression of MRP2 after incubation with CPEC, suggesting that increased drug exposure of heart cells to doxorubicin or CPEC might also be involved in the complex mechanism of this severe side effect.

Overall, these results suggest that genetic analysis of SNPs or genes might clarify some of the issues, but will probably only partly explain cytotoxic drug induced cardiotoxicity.

Acknowledgements We would like to thank C. Zwanenburg for his technical assistance.

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

INTRODUCTION

The agent cyclopentenyl cytosine (CPEC) is a cytidine analogue with potential anti-tumor activity. Contrary to the newest targeted drugs, CPEC is not involved in specific intracellular signalling pathways or angiogenesis, but ultimately leads to inhibition of DNA synthesis and therefore could be classified as a classic cytostatic drug [1]. In this thesis, pharmaceutical aspects as well as anti-tumor activity and mechanisms of cardiotoxicity of CPEC are explored.

CPEC

CPEC can be considered as a prodrug; after transmembrane transport the drug is phosphorylated to form its active metabolite CPEC-TP (cyclopentenyl cytosine triphosphate). CPEC-TP inhibits the enzyme CTP synthetase (cytidine triphosphate synthetase) which is involved in the *de novo* synthesis of CTP out of UTP (uridine triphosphate). CTP can also be formed from the salvage pathway by phosphorylation of cytidine. However, several malignancies seem to have a preference for the formation out of UTP. Inhibition of CTP synthetase may result in depletion of CTP pools leading to a decrease of RNA and DNA synthesis and S-phase accumulation. Several *in vitro* and *in vivo* studies have shown activity of CPEC against leukemia, neuroblastoma and colorectal cancer [2-4]. During a phase I study in patients with solid tumors severe cardiotoxic effects was found [5]. However, based on the studies that showed promising results on hematological malignancies, plans for phase I and II clinical trials (under cardiovascular monitoring) were initiated.

DEVELOPMENT OF CPEC FOR CLINICAL USE

CPEC is only available as a raw substance and was kindly provided by the National Cancer Institute (Bethesda, Maryland) in the U.S.A. Formulation is an important issue during the preclinical development of a drug. Aspects that need to be considered with regard to drug formulation: stability, reconstitution and safety upon handling. These last two characteristics are especially important for cytostatic drugs as they often form a potential risk upon occupational exposure. As described in chapter 3, it is possible to formulate a sterile and stable solution of CPEC which can be easily administered.

Therapeutic drug monitoring (TDM) can be useful when there is a proven relationship between blood levels and either toxicity or efficacy. Whether CPEC levels in humans are associated with efficacy is yet unclear. However, in a phase I study [5] it was shown that high concentrations of CPEC seemed to be associated with cardiotoxic side effects. The consequences of this finding are that future trials will have to start with lower dosages and that monitoring of plasma levels

may be required. This also means that an assay enabling monitoring of relatively low levels of CPEC becomes essential. Using LC-MS/MS we were able to quantitatively determine low levels of CPEC (chapter 4). The deaminated metabolite of CPEC: cyclopentenyl uracil (CPEU) could also be measured, although with a lower sensitivity. This might result in undetectable concentrations of the metabolite in the lower dose levels as is demonstrated by our results of the analysis of plasma levels of the first patient receiving low dose CPEC. However, as CPEU has almost no cytotoxic effects and its concentration was not associated with cardiotoxicity in the phase I study, no consequences are expected for the application of the assay in TDM of CPEC.

EFFICACY

The first studies with CPEC mainly focus on its activities as an antiviral agent. However, only *in vitro* studies on the antiviral activity have been published and no results in animals or humans are as yet known. More research has been performed on the use of CPEC as an anti-tumor agent. Activity against several solid tumors as well as hematological malignancies has been demonstrated in *in vitro* and in animal studies [2,4,6]. Considering the promising results in lymphocytic and myeloid leukemia, and the hematotoxic side effects in humans with solid tumors, CPEC might be of use in the treatment of ALL. As shown by our results using a xenogeneic *in vivo* model for ALL, efficacy was associated with severe toxicity (chapter 5). In earlier studies an increase in life span upon CPEC treatment was demonstrated. There might be several explanations for the different results between our study and the earlier *in vivo* studies. First, as we continuously monitored leukemic progression and toxicity, we were able to determine the actual response during treatment instead of having death as the only evaluation point for efficacy and toxicity. Moreover, in our model leukemic progression could be excluded as a possible cause of death, allowing discrimination between activity and toxicity of therapy. Secondly, we started treatment from the moment that leukemic cells could be detected and not immediately after inoculation. The third explanation might be that we administrated human leukemic cells whereas earlier studies used cells of murine origin which might respond differently to CPEC.

In leukemia combination therapy is frequently used. Reasons for this strategy might be to create synergy between drugs by acting via multiple pathways or reduction of toxicities by lowering the dose of the individual drugs. Regarding CPEC, cytarabine seems to be a logical candidate for combination therapy as the uptake and therefore activity of cytarabine is regulated by feedback inhibition of deoxy-CTP (dCTP). As CPEC can deplete CTP and therefore dCTP, it might be responsible for enhanced activity of cytarabine. Our *in vitro* results suggest an additive but not synergistic effect of CPEC and cytarabine. Therefore we chose not to investigate the combination treatment of CPEC and cytarabine in our *in vivo* animal model for human ALL. However, even if no synergistic effect can be accomplished, it might be interesting to investigate whether a combination of cytarabine and low dose CPEC will reach similar efficacy when compared to the individual agents and their level of toxicity.

*CARDIO*TOXICITY

As survival and cure rates for cancer increase, reduction and management of side effects become more important. Cardiotoxicity is an important problem associated with current chemotherapeutic regimens (chapter 6). Moreover, this is not exclusively a problem of the classic cytotoxic drugs, as several studies report cardiotoxic side effects after treatment with the newer targeted drugs. The monoclonal antibody trastuzumab and recently also imatinib and sunitinib [7-9] are examples of newer cytostatic drugs associated with cardiotoxicity. As shown in the case of CPEC, cardiotoxic side effects might hamper the introduction of potentially interesting drugs. It would be interesting to know why in particular the heart is susceptible to toxic side effects and how these effects can be prevented or predicted.

Mechanism

When taking a better look at anthracycline induced cardiotoxicity, it becomes clear that these answers are not so easily found. Although extensive research has been undertaken to study anthracycline induced cardiotoxicity, the mechanism is not completely clarified. Reactive oxygen radicals are supposed to play an important role and it has been postulated that due to a low level of antioxidant enzymes (e.g. SOD), the heart would be more susceptible to the destructive action of these radicals [10]. Following this theory antioxidant therapy might be useful; however, until now there have been no antioxidants that have been able to yield good results against anthracycline induced cardiotoxicity. In the anti-oxidative theory iron plays an important role as it is believed to form a free radical complex with reduced anthracyclines. The iron chelator dexrazoxane can bind iron and thereby prevent or reduce the formation of the complex. Dexrazoxane has shown promising results in preventing cardiotoxicity and is currently the only agent approved for the prevention of anthracycline induced cardiotoxicity [11,12]. It was also suggested that apoptosis plays a role and indeed doxorubicin treated mice showed an increased apoptotic rate in their hearts [13].

When studying the cardiotoxic effects of CPEC, we used an anthracycline based approach in our attempts to clarify its cardiotoxic effects (chapter 7). There are a few objections to be made against this approach. First, CPEC does not have a structural relationship with anthracyclines and from that point of view a similarity in mechanism might not be logical. Secondly, the observed hypotension after treatment with CPEC is not a common cardiotoxic side effect seen with anthracyclines. However, as anthracycline induced cardiotoxicity has been known for years, many models have been developed and validated to study this phenomenon. These models might therefore also be suitable for studying the side effects of other drugs. Moreover, in our animal model we studied a general phenomenon (apoptosis) that is also associated with CPEC treatment. We have not been able to detect CPEC induced apoptosis in rats. It is possible that some physiological

aspects of rats might have played a role in the absence of cardiotoxicity of CPEC in our study. Rats are reported to have very low levels of cytidine deaminase, the enzyme responsible for the deamination of cytidine to uridine. This might lead to high levels of cytidine which have been suggested to protect against CPEC induced toxicity. However, cytidine deaminase is also responsible for the deamination of CPEC to CPEU, and it is not known what ratio of CPEC-cytidine levels is necessary for efficacy or toxicity. Moreover, although we did not study it as extensively as in rats, we also could not demonstrate signs of cardiotoxicity in mice, which are reported to have cytidine deaminase activities comparable to humans [14,15].

Prevention and prediction

In current practice restricting the total cumulative dose, reducing peak levels, delivering the anthracycline in liposomes or administration of a protective agent (dexrazoxane) are the most effective tools to prevent cardiotoxicity. Although dose restriction might be easy to undertake, it is not always desirable and there are still patients (up to 5%) with cardiotoxicity despite the administration of lower doses, as well as patients that do not encounter any problems even after very high dosage of anthracyclines.

Moreover, the measures currently taken all seem to merely attempt to lower the total exposure of the drug to heart cells without understanding differences in individual sensitivity. It therefore becomes more important to be able to predict which patients are prone to chemotherapy induced cardiotoxicity.

The individual differences might indicate that there is a role for pharmacogenetics in chemotherapy induced cardiotoxicity. The study by Wojnowski *et al* is the first study to report an association between single nucleotide polymorphisms (SNPs) and anthracycline induced cardiotoxicity [16]. In this study a candidate gene approach is used and the investigators report an association with cardiotoxicity for six SNPs in five genes. The associated SNPs are located in genes involved in either NAD(P)H oxidase (p22phox, p40phox, Rac2) or doxorubicin efflux transporters (MRP1, MPR2). As described in chapter 8 we have demonstrated that the expression of MRP1 in rat cardiomyocyte cells decreased by doxorubicine and the expression of MRP2 showed a small decrease after incubation with CPEC. No changes in expression were observed for the other genes studied. Information on the influence of doxorubicin or CPEC on expression of the candidate genes might help in understanding the role of the gene and the specific SNP and their possible association with chemotherapy induced cardiotoxicity. Furthermore we have been able to reproduce the association between a polymorphism in the GTPase Rac2 which is essential for the function of the NAD(P)H oxidase multi-enzyme complex and cardiotoxicity of doxorubicin in a case control study. Sample size might have been an important reason for the absence of an association for the other five SNPs in our study. However, this is also an illustration of an important problem

for the clinical use of screening for these SNPs in anthracycline induced cardiotoxicity. Even if we would have been able to increase our sample size and could have reproduced the findings of the previous study, the frequency of overt anthracycline induced cardiotoxicity would still be low (1.1% in our study). Therefore, in order to identify patients with the associated SNPs, the 'number needed to genotype' would be unrealistically high. Moreover, as demonstrated by Wojnowski *et al* only 7-29% of the cases could be attributed to the carrier status of one of the candidate genes [16]. This implicates that knowledge of a cardiotoxicity associated SNP of a patient will only partly help in predicting whether this patient will ultimately develop anthracycline induced cardiotoxicity. Nevertheless, when combining genetic variants with other known risk factors, such algorithms or predictive models may prove a useful tool in the prediction of chemotherapy induced cardiotoxicity.

CONCLUSION AND FUTURE ASPECTS

In conclusion, the results presented in this thesis show that CPEC might not have fulfilled all of its promises. Single agent therapy in ALL proved to have a therapeutic window too small for further development. However, it cannot be ruled out that combination therapy in other hematological or solid tumors might be an useful addition to current therapeutic regimens. Positive findings are that in the studied animal models no further indications for CPEC induced cardiotoxicity were found. However, the results of the first study in patients experiencing severe hypotension cannot be neglected. This implicates that if CPEC is administrated in experimental protocols to patients, monitoring of cardiac function remains necessary. Unfortunately, cardiotoxicity is common among cytotoxic drugs and the vast amount of studies undertaken with anthracycline induced cardiotoxicity demonstrate that solutions to resolve the problem are not easily found. With the increasing possibilities of pharmacogenetics, a new modality might have become available. Although pharmacogenetics will not provide an absolute answer, combining our existing knowledge of risk factors with new genetic findings might bring us closer to good management of chemotherapy induced cardiotoxicity.

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SUMMARY

In this thesis pharmaceutical aspects as well as anti-tumor activity and cardiotoxicity of the experimental cytostatic drug cyclopentenyl cytosine (CPEC) are explored. CPEC is derived from the nucleoside neplanocin A and structurally related to the clinically used cytostatic drugs cytarabine and gemcitabine.

By inhibiting the enzyme CTP-synthetase (cytidine triphosphate synthetase), CPEC decreases the 'de novo' synthesis of CTP from UTP (uridine triphosphate), resulting in depleted CTP pools. CTP can also be generated from the so called 'salvage pathway' out of cytidine. However, several malignancies have been shown to predominantly use the 'de novo synthesis' involving CTP synthetase. A depletion of CTP pools may ultimately lead to death as a result of impaired RNA and DNA synthesis and S-phase accumulation, resulting in cell death.

In **chapter 2** an overview is given of the studies undertaken with CPEC. Originally the drug was selected out of several analogues of neplanocin A based on its antiviral properties and indeed *in vitro* studies show activity against a wide range of viruses. However, its anti-tumor activity was considered more interesting for further development and no animal or human studies have been undertaken to investigate its antiviral properties.

CPEC has been most extensively studied in hematological malignancies and several *in vitro* and *in vivo* studies show activity of CPEC against leukemia. Other promising results were achieved in models for colorectal carcinoma and neuroblastoma.

There is one clinical trial published using CPEC in a phase I study in patients with solid tumors. Besides dose limiting hematological side effects, the most severe toxicity observed was cardiovascular. This side effect was seen in the higher dose groups.

As new trials with low dose CPEC were planned and only the raw substance was available, there was a need for a pharmaceutical formulation of CPEC. In **chapter 3** we describe the development of a stable sterile infusion concentrate that can be easily administered.

Because the cardiotoxicity as observed in the phase I trial was thought to be related to high CPEC plasma levels, therapeutic drug monitoring was considered to be necessary in future trials. In **chapter 4** a HPLC-MSMS method is described which enabled us to quantitatively determine low levels of CPEC. Although not at levels as low as CPEC, its metabolite CPEU (cyclopentenyl uridine), could also be detected with this method. CPEU is not thought to have anti-tumor activity and its plasma levels did not seem to be related with cardiotoxicity in the phase I study. Therefore, the higher limit of detection for CPEU was not considered as a problem for the use of the method in clinical trials.

Several *in vivo* studies showed promising activity of CPEC in leukemia. However, these studies used murine leukemic cells and no data were available with human leukemic cells. In **chapter 5** we analyzed the activity of CPEC on human acute lymphoblastic leukemia (ALL) cell lines *in vitro,* as well as on corresponding human primary ALL cells in a xenogeneic *in vivo* model using NOD/scid mice. Our *in vitro* results on five different human cell lines show activity of CPEC in the nanomolar range (IC50 6-15nM).

Based on their mechanisms of action and earlier *in vitro* results, combination therapy of CPEC with the cytotoxic drug cytarabine was suggested to have a synergistic effect. However, we detected no such effect on our leukemic cell lines after coincubation with CPEC and cytarabine. Therefore, single agent therapy with CPEC was studied in the NOD/scid mice inoculated with primary human ALL cells. Whereas no activity nor toxicity was seen in the lower dose ranges (0.5 mg/kg for 2 or 5 days per week) a marginal anti-leukemic activity was observed at 1.5 mg/kg (5 days per week) and 5 mg/kg (2 days per week), however, this activity was associated with severe systemic toxicity.

In the phase I study with CPEC the most severe side effect was cardiovascular. Cardiotoxicity induced by cytotoxic drugs is unfortunately not uncommon and in **chapter 6** several classes of cytotoxic drugs that have been associated with cardiotoxicity are reviewed. Anthracyclines are well known for this toxicity and its mechanism has been extensively studied. Oxidative stress seems to play an important role, however, apoptosis, genetic causes and influence on calcium homeostasis might also be involved. Prevention mainly consists of restricting the maximum cumulative dose and avoiding peak levels. Alternatively administration of a liposomal formulation or addition of a protective agent can be applied. However, none of these measures offer full protection.

Although not as frequently as anthracyclines, other cytotoxic drugs associated with cardiotoxicity are 5-fluorouracil, cyclophosphamide, cisplatin and more recently trastuzumab, imatinib and sunitinib.

The mechanism behind the severe hypotension associated with CPEC in the phase I trial had not been clarified. Before initiating clinical studies with CPEC it was necessary to investigate if the cardiotoxicity could be reproduced and what might have been the mechanism. **In chapter 7** we first studied the effects of CPEC on contraction force and frequency in a model using isolated atria of male Wistar rats. No changes in frequency were detected and although a trend in decrease of contraction force was observed, the differences were not significant. Our second hypothesis focused on the possible induction of apoptosis in the heart by CPEC. In an *in vivo* model we administrated CPEC to male Wistar rats and evaluated the presence or absence of apoptosis by 99mTc-AnnexinV scintigraphy, followed by postmortem determination of radioactivity in tissues, and histological confirmation. This model had been used before to demonstrate the apoptosis inducing potential of doxorubicin. We detected no increase in cardiac uptake of 99mTc-AnnexinV after treatment with CPEC, thereby having no indications for increased apoptosis in the heart.

With the models described in chapter 7 we only studied a few aspects that might have an association with cardiotoxicity. In **chapter 8** we investigated whether cardiotoxicity induced by cytotoxic drugs might have a genetic origin. Even in the case of anthracycline induced cardiotoxicity little is known about the role of genetics. We first attempted to reproduce the results of a previous study reporting an association between single nucleotide polymorphisms (SNPs) and doxorubicin induced cardiotoxicity. SNPs in genes related to the NAD(P)H oxidase enzyme complex (p22phox, p40phox and Rac2) and the drug efflux pumps MRP1 and MRP2 were investigated. We were able to reproduce the association between a SNP in Rac2 and anthracycline induced cardiotoxicity. No other associations were observed, which might have been caused by the relatively small size of our group of patients with anthracycline induced cardiotoxicity.

The role of the candidate genes was further explored by studying changes in expression after exposing rat cardiomyocytes to doxorubicin and CPEC. No changes in expression were seen for the genes involved in the NAD(P)H oxidase enzyme complex. However, after 24 hr of incubation doxorubicin decreased the expression of the MRP1 gene and CPEC seemed to induce a small decrease in the expression of MRP2.

Based on the association with the SNP in Rac2 and the decrease in expression in MRP1 and MRP2, we concluded that genetic aspects of the NAD(P)H oxidase enzyme complex and the efflux pumps may be involved in cytostatic drug induced cardiotoxicity.

In **chapter 9** the results from the studies in this thesis are discussed and future aspects are indicated. Although CPEC as a single agent in our leukemic *in vivo* model did not fulfill its promises, we believe that this agent might still have potential in combination with other agents in several malignancies. Its possible cardiotoxic side effects could not be reproduced in an animal model.

As demonstrated by the results of our last study cardiotoxicity of cytotoxic drugs might also have genetic aspects. However, we only studied expression profiles of a few genes and it is not unlikely that a vast amount of other genes might be involved as well. Moreover, the frequency of clinically overt cardiotoxicity is relatively low, indicating that a high number of patients would have to be genotyped in order to identify patients at risk. Therefore it may be concluded that pharmacogenetics will not provide an absolute answer but may be a valuable addition to the existing tools for the management of chemotherapy induced cardiotoxicity.

NEDERLANDSE SAMENVATTING

In dit proefschrift worden zowel farmaceutische aspecten als ook anti-tumor activiteit en cardiotoxiciteit van het cytostaticum cyclopentenyl cytosine (CPEC) onderzocht. CPEC is afgeleid van het nucleoside neplanocine A en lijkt voor wat betreft de chemische structuur op de klinisch gebruikte cytostatica cytarabine en gemcitabine. CPEC remt het enzym CTP-synthetase (cytidine trifosfaat synthetase), hierdoor vermindert de zogenoemde 'de novo synthese' van CTP uit UTP (uridine trifosfaat). Dit leidt tot uitputting van de CTP voorraden in de cel. CTP kan ook uit cytidine worden gemaakt, via de 'salvage pathway'. Het blijkt echter dat verschillende maligniteiten vooral de 'de novo synthese' route volgen waarbij gebruik wordt gemaakt van CTP synthetase. Een uitputting van de CTP voorraden kan uiteindelijk leiden tot celdood door problemen met RNA- en DNA-synthese en stoppen van de celcyclus in de S-fase.

In **hoofdstuk 2** wordt een overzicht gegeven van de verschillende studies die met CPEC hebben plaatsgevonden. Oorspronkelijk was het geneesmiddel geselecteerd uit een groep van analoga van neplanocine A vanwege zijn antivirale eigenschappen zoals ook later is aangetoond in verschillende *in vitro* studies. De mogelijke anti-tumor effecten werden echter als interessanter beschouwd en er zijn geen studies naar het antivirale effect uitgevoerd in dieren of mensen.

Het effect van CPEC in hematologische maligniteiten is het meest onderzocht en verschillende *in vitro* en *in vivo* studies laten een anti-leukemisch effect van CPEC zien. Andere veelbelovende preklinische resultaten zijn behaald op het gebied van darmkanker en neuroblastomen.

Er is één fase I studie gepubliceerd waarin CPEC onderzocht is bij mensen met solide tumoren. Naast dosisbeperkende hematologische bijwerkingen, was de ernstigste bijwerking die optrad cardiovasculair. Deze bijwerking trad op bij patiënten in de hogere doseringsgroepen.

Aangezien nieuwe klinische studies met CPEC (in een lage dosering) zouden worden uitgevoerd en alleen de grondstof beschikbaar was, was er behoefte aan een farmaceutische formulering van CPEC. In **hoofdstuk 3** wordt de ontwikkeling beschreven van een stabiel en steriel infuusconcentraat dat gemakkelijk kan worden toegediend.

De cardiotoxiciteit die in de fase I studie optrad leek een relatie te hebben met hoge plasma spiegels van CPEC en controle van de bloedspiegels werd daarom noodzakelijk geacht. **In hoofdstuk 4** wordt een HPLC-MSMS methode beschreven waarmee lage CPEC spiegels nauwkeurig gemeten konden worden. CPEU (cyclopentenyl uridine), de metaboliet van CPEC, kon ook met deze methode worden bepaald, zij het in hogere concentraties. CPEU heeft waarschijnlijk geen anti-tumor effecten en CPEU spiegels werden in de fase I studie niet in verband gebracht met cardiotoxiciteit. Voor de toepassing van de methode in klinische studies lijkt de hogere bepalingslimiet van CPEU dan ook geen consequenties te hebben.

Op het gebied van leukemie hebben verschillende preklinische studies goed resultaat van CPEC laten zien. Al deze studies zijn echter uitgevoerd met leukemie cellen van muizen oorsprong en er waren geen gegevens beschikbaar met humane leukemie cellen. In **hoofdstuk 5** hebben we de activiteit van CPEC op humane acute lymfoblastische leukemie (ALL) cellijnen *in vitro* bestudeerd. Daarnaast is het effect van behandeling met CPEC bekeken in NOD/scid muizen die humane primaire ALL cellen hadden gekregen.

Op basis van de *in vitro* resultaten leek CPEC activiteit te hebben in het nanomolaire gebied: 50% van de cellen overleed (IC50) bij doseringen tussen de 6 en 15 nM.

Op basis van het werkingsmechanisme en eerdere *in vitro* resultaten, zou combinatie therapie van CPEC en het cytostaticum cytarabine een synergistisch effect kunnen hebben. Een dergelijk effect kon echter niet worden aangetoond in onze leukemie cellen na co-incubatie met CPEC en cytarabine. Er is daarom besloten om in het muizenmodel alleen het effect van CPEC te onderzoeken en geen combinatietherapie. In de lagere dosisgroepen (0.5 mg/kg gedurende 2 of 5 dagen per week) werd noch een anti-leukemisch noch een toxisch effect gezien. Een klein anti-leukemisch effect trad op na 1.5 mg/kg (5 dagen per week) en 5 mg/kg (2 dagen per week), dit effect ging echter gepaard met ernstige systemische toxiciteit.

In de fase I studie met CPEC was cardiotoxiciteit de ernstigste bijwerking. Helaas komt cardiotoxiciteit vaker voor bij de behandeling met chemotherapie en in **hoofdstuk 6** worden verschillende cytostatica besproken waarbij dit effect kan optreden. Anthracyclines zijn hier vooral berucht om en veel studies zijn uitgevoerd om het mechanisme te achterhalen. Hoewel oxidatieve stress een belangrijke rol lijkt te spelen, worden ook apoptose (geprogrammeerde celdood), genetische oorzaken en invloed op de calcium regulering met anthracycline geïnduceerde cardiotoxiciteit in verband gebracht. Preventie bestaat voornamelijk uit het beperken van de cumulatieve dosering en het vermijden van hoge piek spiegels. Andere maatregelen kunnen bestaan uit het toedienen van het anthracycline in een liposomale vorm of het toevoegen van een beschermend middel aan de behandeling. Geen van de genoemde maatregelen biedt echter volledige bescherming.

Hoewel minder vaak voorkomend dan bij anthracyclines, wordt cardiotoxiciteit ook wel gezien bij 5-fluorouracil, cyclofosfamide, cisplatin en de nieuwere middelen trastuzumab, imatinib en sunitinib.

De oorzaak van het optreden van de ernstige hypotensie na behandeling met CPEC in de fase I studie is niet bekend. Voordat nieuwe studies met CPEC zouden kunnen starten, was het noodzakelijk om hier meer onderzoek naar te verrichten. In **hoofdstuk 7** hebben we eerst gekeken naar de effecten van CPEC op contractiekracht en frequentie in een model waarbij geïsoleerde atria (hartboezems) van mannelijke Wistar ratten werden gebruikt. Er traden geen veranderingen in hartfrequentie op en ondanks dat er een trend in daling van de contractiekracht werd gezien,

het verschil in contractiekracht tussen behandelde en onbehandelde atria niet significant. In een *in vivo* model is vervolgens CPEC toegediend aan mannelijke Wistar ratten en de aan- of afwezigheid van apoptose bestudeerd door middel van scintigrafie met 99mTc-Annexine V, gevolgd door postmortem radioactiviteits-telling van de organen en histologisch onderzoek. Dit diermodel was in een eerdere studie toegepast om de apoptose inducerende eigenschappen van doxorubicine te onderzoeken. De resultaten van onze studie lieten geen verhoging van hartopname van Tc99mAnnexine V zien, zodat er geen aanwijzingen waren dat CPEC apoptose in het hart veroorzaakt.

Met de modellen die in hoofdstuk 7 zijn beschreven, zijn slechts een paar aspecten die met cardiotoxiciteit te maken kunnen hebben, bestudeerd. In **hoofdstuk 8** is onderzocht of cytostatica geïnduceerde cardiotoxiciteit ook een genetische oorzaak kan hebben. Zelfs in het geval van de anthracyclines is weinig bekend in hoeverre genetische oorzaken een rol spelen bij de cardiotoxiciteit.

Allereerst is gekeken of de resultaten konden worden herhaald van een studie die een verband had beschreven tussen SNPs[1] en doxorubicine geïnduceerde cardiotoxiciteit. Het betrof SNPs in genen die een relatie hadden met het NAD(P)H-oxidase enzym complex (p22phox, p40phox en Rac2) en de geneesmiddel efflux pompen MRP1 en MRP2. In onze studie hebben we de associatie tussen de SNP in Rac2 en anthracycline geïnduceerde cardiotoxiciteit kunnen bevestigen. De andere associaties konden niet worden aangetoond, vermoedelijk speelde de relatief kleine groepsgrootte daarbij een rol.

De mogelijke invloed van de kandidaat genen is vervolgens verder onderzocht door naar expressieveranderingen te kijken in rat cardiomyocyten na behandeling met doxorubicine of CPEC. De expressie veranderde niet voor de genen die een relatie hadden met het NAD(P)H-oxidase enzym complex. Na 24 uur incubatie met doxorubicine werd echter wel een afname in de expressie van MRP1 gezien. CPEC leek na 24 uur een kleine daling in de expressie van MRP2 te veroorzaken.

Gebaseerd op de associatie met de SNP in Rac2 en de expressie veranderingen in MRP1 en MRP2, is geconcludeerd dat zowel genetische aspecten van het NAD(P)H oxidase enzym complex als van de effluxpompen een rol lijken te spelen bij cytostatica geïnduceerde cardiotoxiciteit.

In **hoofdstuk 9** worden de voorgaande hoofdstukken bediscussieerd en mogelijke toekomstige richtingen voor onderzoek aangegeven. Hoewel wat betreft anti-tumor activiteit CPEC in het *in vivo* leukemie model niet aan de verwachtingen heeft voldaan, is het niet ondenkbaar dat dit middel mogelijk een toepassing kan hebben in de combinatie met andere middelen in verschillende maligniteiten. De mogelijke cardiotoxiciteit is in het diermodel niet bevestigd.

Zoals aangetoond in de laatste studie lijken genetische aspecten een rol te kunnen spelen bij cytostatica geïnduceerde cardiotoxiciteit. In deze studie zijn echter maar enkele genen bestudeerd en het is niet onwaarschijnlijk dat een groot aantal andere genen ook een rol speelt. Daarnaast is de frequentie van klinisch relevante cardiotoxiciteit relatief laag, hetgeen ertoe zou leiden dat het genotype van een groot aantal patiënten bepaald zou moeten worden om risicopatiënten te kunnen identificeren.

Farmacogenetica zal dan ook vermoedelijk niet in staat zijn om de volledige oplossing voor het probleem te kunnen geven, maar kan mogelijk wel een goede aanvulling zijn bij de huidige maatregelen die worden genomen in het voorkomen en behandelen van cytostatica geïnduceerde cardiotoxiciteit.

[1] SNP: single nucleotide polymorphism: een variatie in het DNA (polymorfisme) waarbij één base is veranderd

DANKWOORD

Het is zover, het boekje is klaar. Van vele kanten heb ik hulp gekregen en een woord van dank is dan ook zeker op zijn plaats. Het is onmogelijk om volledig te zijn, maar ik zou er toch graag een aantal personen uit willen lichten.

Het begon allemaal in Amsterdam. Dankzij de inbreng van de medewerkers van de apotheek van het AMC is er een injecteerbare vorm van CPEC gekomen. Erik wil ik in het bijzonder bedanken voor zijn bijdrage hierin. Roel, Formijn en Cora, bedankt voor jullie hulp bij het 'nucleaire' deel van het onderzoek.

De hulp van Andre en René van het laboratorium voor genetisch metabole ziekten, is onmisbaar geweest, bedankt hiervoor.

Na Amsterdam volgde Leiden en ook daar heb ik weer dankbaar gebruik gemaakt van de hulp van velen. Professor Falkenburg, Bart en Marianke wil ik bedanken voor de hulp bij het bestuderen van CPEC in het NOD/scid muizen model van de experimentele hematologie.

Farmacogenetica mocht natuurlijk niet ontbreken en Tahar, Judith en Chris hebben er mede voor gezorgd dat dit mogelijk was, bedankt hiervoor.

Zonder belangstelling en de gelegenheid om me vrij te maken voor onderzoek naast het 'gewone' werk, was promoveren onmogelijk geweest. Alle medewerkers van de apotheek van het LUMC wil ik dan ook bedanken. Erica en Gert in het bijzonder, mede doordat jullie mijn onderzoeksdag soms nog beter bewaakten dan ikzelf, is het gelukt om het af te maken.

Dank ook aan mijn paranimfen. Dinemarie, samen roeien hebben we maar even gedaan, maar samenwerken doen we hopelijk nog lange tijd. Ingrid, mijn 'grote' zus, ook bij deze belangrijke gebeurtenis in mijn leven ben je er gelukkig weer bij.

Alle vrienden, bedankt voor de gezelligheid, het vragen naar de vorderingen, maar ook juist niet als het even 'verboden onderwerp' was.

Pap en mam, bedankt voor jullie steun die er altijd is.

Vincent, de laatste in de rij, maar ook de belangrijkste factor in mijn leven. Zowel aan je motiverende als ook relativerende eigenschappen heb ik de afgelopen onderzoeksjaren veel gehad. Rustige tijden zullen nu vast niet aanbreken, maar genieten zullen we zeker...

CURRICULUM VITAE

Kirsten JM Schimmel is op 22 september 1973 geboren te Apeldoorn. In 1991 haalde zij het VWO diploma aan het Marianum in Groenlo waarna zij 1 jaar in Frankrijk doorbracht op het lycée Jacques Prevert te Taverny en daar het baccalauréat français behaalde. In 1992 begon zij met de studie farmacie aan de Universiteit van Utrecht. Tijdens de studie heeft zij onderzoek verricht naar de hormonale regulatie van de Bcl-2 gen-familie in borstkankercellen aan de Université Paris VII te Parijs. Dit onderzoek is verder voortgezet gedurende een onderzoeksstage op het endocrinologie laboratorium van het toenmalige Academische Ziekenhuis Utrecht.

In 1999 rondde zij de studie farmacie af met het apothekersdiploma waarna zij als projectapotheker begon in de ziekenhuisapotheek van het Academisch Medisch Centrum te Amsterdam. In 2000 startte zij daar met de opleiding tot ziekenhuisapotheker in combinatie met de verantwoordelijkheid voor de radioactieve stoffen apotheek en een promotieonderzoek. De opleiding tot ziekenhuisapotheker rondde zij af in december 2003 waarna zij begon als productieapotheker in de ziekenhuisapotheek van het Leids Universitair Medisch Centrum. Hier heeft zij het onderzoek tevens voortgezet.

Kirsten Schimmel is getrouwd met Vincent Tan.