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

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CHAPTER 7

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

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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 ^{99m}Tc-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. ^{99m}Tc-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; ^{99m}Tc-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/m²/h). The highest applied dose was 5.9 mg/m²/h; no hypotension was seen in patients receiving doses below 2.5 mg/m²/h. Two patients, treated with 4.7 mg/m²/h, had a fatal hypotensive 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* affinity 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 (α -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 mm³ 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 K₂CO₃. 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 ^{99m}Tc -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 ^{99m}Tc -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 ^{99m}Tc 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 tricline 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 ^{99m}Tc -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 ^{99m}Tc -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 ^{99m}Tc-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 ^{99m}Tc-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 NaH₂PO₄/Na₂HPO₄ 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 α-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.

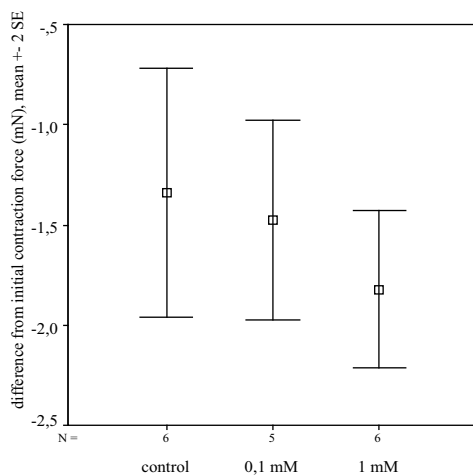
RESULTS

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

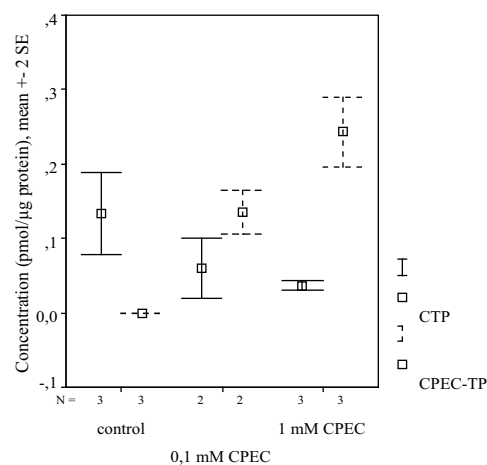


Figure 1a

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.

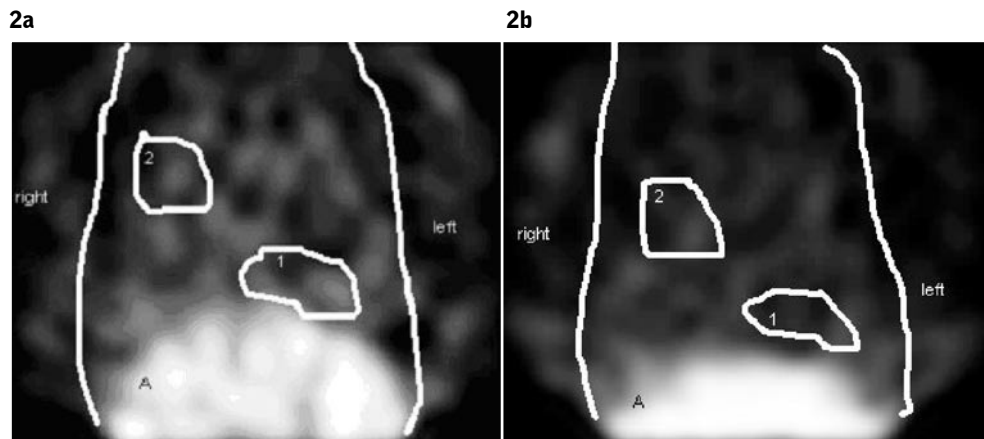


Figure 2 Pinhole SPECT scintigraphy of a CPEC-treated rat (90 mg/kg) (a) and a control rat (b), 1 h after i.v. injection of 75 MBq ^{99m}Tc -Annexin V. On the images of the thorax (anterior view, coronal) including a part of the liver (A), the physiological distribution of the radiopharmaceutical, with 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.

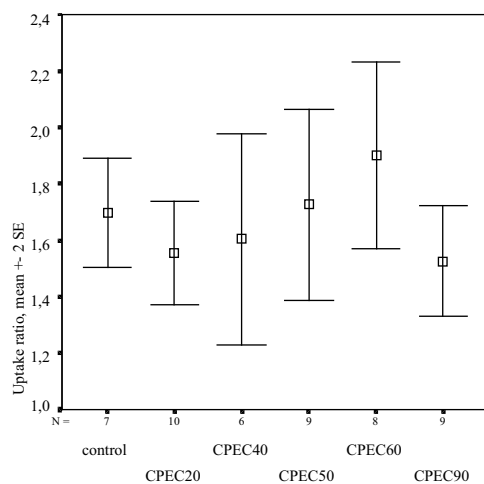


Figure 3 Annexin uptake

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.

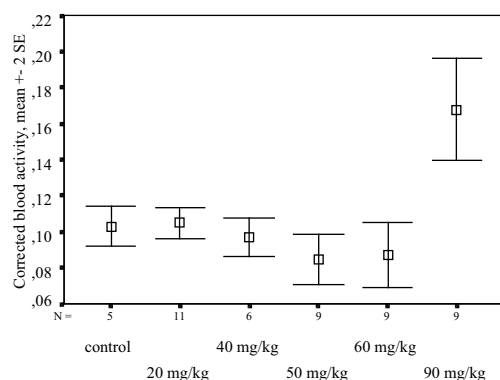


Figure 4 Activity in blood

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.

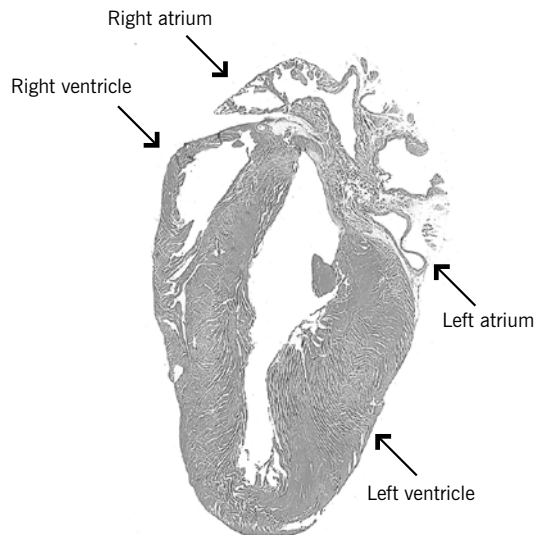
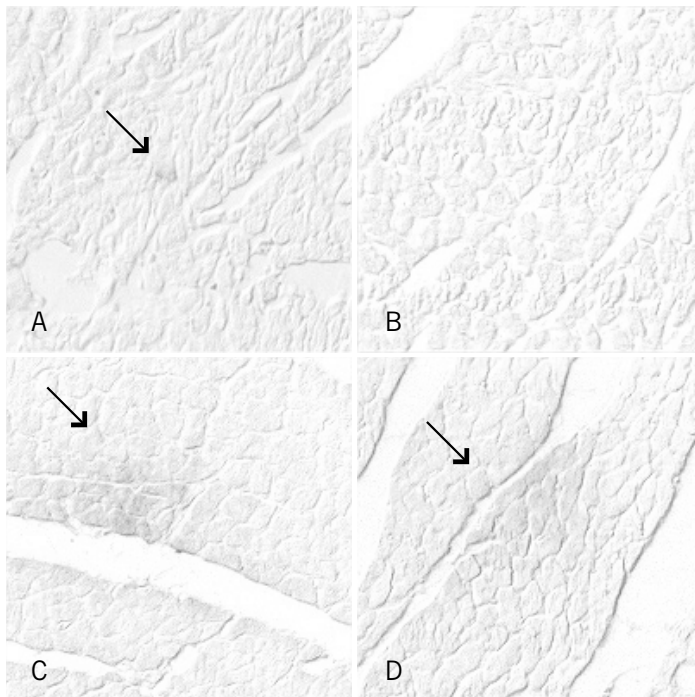


Figure 5

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)



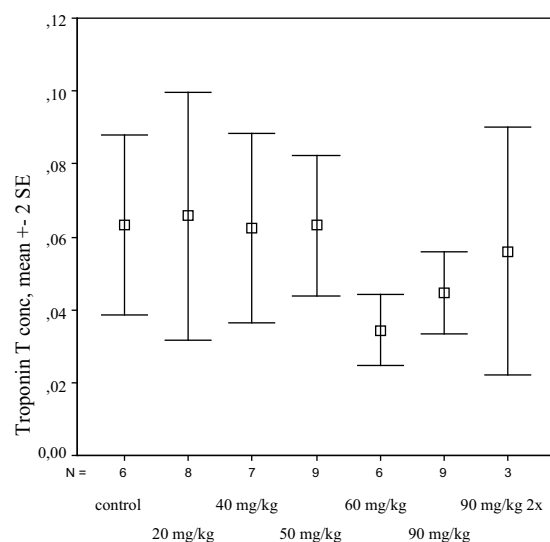


Figure 7 Cardiac Troponin T level

The serum level of the cardiac (necrosis) marker troponin T was measured ($\mu\text{g/l}$). No significant differences between cardiac troponin T levels were found. The detection limit of the assay was $0.04 \mu\text{g/l}$

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 [11]. 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 ^{99m}Tc-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 ^{99m}Tc-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 ^{99m}Tc-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 ^{99m}Tc -annexin V uptake in the heart was due to the short interval between injection of CPEC and ^{99m}Tc -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 ^{99m}Tc -annexin V uptake in the heart. In earlier experiments with doxorubicin [11] the detection level of apoptosis with ^{99m}Tc -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 ^{99m}Tc -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 ^{99m}Tc-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.

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