
Anthracycline-induced cardiotoxicity, a pathophysiology based approach for early detection and protective strategies
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ANTHRACYCLINE-INDUCED CARDIOTOXICITY, *A PATHOPHYSIOLOGY BASED APPROACH FOR EARLY DETECTION AND PROTECTIVE STRATEGIES*

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A PATHOPHYSIOLOGY BASED APPROACH FOR EARLY DETECTION AND PROTECTIVE STRATEGIES

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INHOUDSOPGAVE

CHAPTER 1

Introduction and outline of the thesis

Cardiac effects of anthracyclines

The first anthracycline, daunorubicin, was originally isolated from the *S. peucetius* in 1957.(1) Since then, numerous analogues have been developed, of which doxorubicin (Adriamycin®) is still the most commonly used (figure 1).(2) Doxorubicin is used in the treatment of a wide range of malignancies, including breast cancer, ovarian cancer, leukemia and sarcomas.(3)

Soon after the introduction of doxorubicin, cardiotoxic side-effects were noted.(1;4) These effects are commonly divided into acute, sub-acute and chronic effects.(5) The acute effects consist of rhythm disturbances and myocarditis and occur almost instantaneously after doxorubicin administration. The sub-acute and chronic effects develop after at least 3 months, but can also become apparent years later and may lead to congestive heart failure (CHF). The latter types of toxicity are mainly dose-dependent and vary between < 1% for doses up to 450 mg/m^2 and 47% for doses over 700 mg/m^2.(6) Other risk-factors include age and gender.(4) Commonly used concomitant therapies in the treatment of cancers, like radiation and Herceptin, are also known to increase the occurrence of doxorubicin induced cardiotoxicity.(7)

Molecular mechanisms of cardiac effects of anthracyclines

The cardiotoxic effects of doxorubicin are probably mediated by the induction of apoptosis via free radical mediated mechanisms, but deregulation of intracellular Ca-homeostasis seems to play a role as well.(8) This is different from the anti-tumor effect, which is mainly regulated via interference with DNA replication, alkylation and cross-linking, RNA transcription and inhibition of topoisomerase II.(3)

Oxidative stress and apoptosis

Oxidative stress is defined as 'a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage'.(9) This imbalance can either be caused by an excess of reactive oxygen species (ROS) or a diminished amount of antioxidants such as superoxide dismutase (SOD), catalase, vitamins. Oxidative stress is involved in the pathophysiology of a wide range of diseases and an excess of ROS also plays an important role in anthracycline-induced cardiotoxicity. After administration of doxorubicin, free radicals are formed by one-electron addition to its quinone moiety, which quickly regenerates to its original structure by reducing oxygen to the superoxide anion ($O_2^{\bullet -}$) (figure 2).These reactive oxygen species induce apoptosis, both via the extrinsic (Fas-mediated) and the intrinsic (mitochondrial) pathway.(10-13) Cardiomyocytes are especially vulnerable to doxorubicin-induced apoptosis, as they contain low levels of ROS scavenging enzymes.(14) Although the role of apoptosis in doxorubicin-induced cardiotoxicity is well-established *in vitro*, it remains uncertain whether this mechanism is responsible for the chronic cardiac toxicity.(8)

Secondary alcohol metabolites

Doxorubicin may also cause cardiac myopathy by the formation of its secondary alcohol metabolites such as doxorubicinol (DOXOL). Two mechanisms for its cardiotoxicity have been postulated: interference with intracellular Ca handling, and indirectly by disrupting iron metabolism, mainly by switching off the Iron Regulatory Protein 1 (IRP-1).(15) Direct evidence that the alcohol metabolites of doxorubicin play a relevant role in cardiotoxicity is based upon the observation that animals lacking or overexpressing the gene coding for the enzyme that catalyses the conversion of doxorubicin to DOXOL show decreased and increased cardiotoxicity respectively. The involvement of secondary alcohol metabolites is further supported by the observation that taxanes that stimulate

the formation of doxorubicinol aggravate the cardiotoxicity of doxorubicin.(16;17) In keeping with these findings is the relationship between the extent of formation of secondary alcohol metabolites of anthracycline analogue *in vitro* and the clinically observed cardiotoxicity.(8;18)

Iron-mediated toxicity

Iron can intensify the damage induced by ROS and induce the formation of hydroxyl (.OH) radicals via the Haber-Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$). This reaction can only occur when an intracellular pool of free iron is present.(19) To date, it has not been completely elucidated how such a pool of free iron is formed within the cardiomyocyte. Most of the iron is stored in the iron-storage protein ferritin. Conflicting evidence exists with regard to iron release from ferritin in the presence of doxorubicin. Earlier studies showed that the presence of $O_2^{\bullet -}$ and the semiquinone of doxorubicin enabled Fe^{2+} release from ferritin.(20-22) However, subsequent studies paradoxically demonstrated that doxorubicin favors accumulation of iron in ferritin by causing post-transcriptional changes to ferritin resulting in a decreased ability to release Fe^{2+}.(23;24) It is hypothesized that these mechanisms can be both protective and unfavorable, as iron within ferritin is not available for free radical reactions. However, free iron deficiency hampers several intracellular processes, such as DNA synthesis.(19)

Doxorubicin and its metabolite doxorubicinol influence iron metabolism in a different way as well, as they interfere with the regulation by Iron Regulatory Proteins (IRPs).(15) Cellular iron regulation is partly dependent on regulation by IRPs which can bind to the iron-responsive regions (IRES) present on 5'- or 3' untranslated regions mRNA of, among others, ferritin and the transferrin receptor (TfR). Binding IRP to IRE results in increased intracellular iron uptake and decreased iron storage of iron in ferritin. Two related IRPs have been identified in humans, namely IRP1 and IRP2. IRP1 switches from its active apo form, which is capable to bind IRES, to its inactive holo form in the presence of abundant intracellular iron. This switch is controlled via a 4Fe-S cluster present within IRP1 (figure 3).(25;26)

IRP2 shares extensive sequence homology with IRP1, but lacks the 4Fe-S cluster and its activity is regulated by protosomal degradation in the presence of intracellular iron.(25;26) Doxorubicin and doxorubicinol interfere with this mechanism via several distinct mechanisms. It has been described that doxorubicin (and doxorubicinol) irreversibly inactivated IRP1 (formation of a null protein), resulting in decreased IRP1-RNE binding.(27) Further investigations revealed that low (sub-clinical) concentrations of doxorubicin actually increased IRP1-RNE interaction, while higher concentrations of doxorubicin indeed led to the formation of a null protein.(28;29) In contrast with the "null protein"-theory, a subsequent study showed that complexes of doxorubicin Fe and Cu reversibly decreased IRP1-RNE binding by formation of disulfide complexes.(13) The influence of doxorubicin on IRP2-RNE binding is less extensively investigated. However, doxorubicin also appears to decrease IRP2-RNE binding.(29;30) It thus seems that doxorubicin favors iron sequestration over iron uptake by diminished IRP1- and IRP2-RNE binding.

In summary, the pathophysiology of doxorubicin is an accumulation of several processes, in which the formation of free radicals and the disturbance of iron metabolism are key features.

Endogenous defense mechanisms against cardiac effects of anthracyclines

At present, several mechanisms to protect against free radical induced cardiotoxicity have been identified. These include prooxidant-reducing proteins such as transferrin and haptoglobin, heat-shock proteins and antioxidants, such as vitamin C and E, catalase and superoxide dismutase (SOD). SOD catalyzes ($O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$) the reaction in which $O_2^{\bullet -}$ is converted to hydrogen peroxide, which is further degraded by catalase to water. ($2H_2O_2 \rightarrow 2\ H_2O + O_2$). Three isoforms of SOD have been identified: cytosolic SOD (SOD1), mitochondrial SOD (SOD2) and extracellular SOD (SOD3). The intracellular forms are more abundantly present.

Detection methods for cardiac side effects of anthracyclines

Detection of anthracycline-induced cardiotoxicity is difficult, as a clinically relevant decline in left ventricular function often appears late after the administration of anthracyclines. Because of the impact of the impaired cardiac function, several detection methods have been investigated and evaluated to detect anthracycline-induced cardiotoxicity as early as possible.

ENDOMYOCARDIAL BIOPSY Endomyocardial biopsy was often used until the 1980s for the detection of anthracycline-induced cardiotoxicity.(31-33) After exposure to anthracyclines, highly specific histopathological changes occur in the myocardium, including extensive depletion of myofibrillar bundles, myofibrillar lysis, distortion and disruption of z-lines, mitochondrial swelling and swelling and disruption of the sarcoplasmatic reticulum, leading to intramyocyte vacuolization.(34-36) These changes are dose-dependent and occur scattered throughout the myocardium. (36) Endomyocardial biopsy is the most sensitive and specific method to detect anthracycline-induced cardiotoxicity. However, it is largely abandoned now, because of the lack of experience in obtaining and assessing the biopsies and the fact that it is a highly invasive procedure.(31-33)

MEASUREMENT OF LEFT VENTRICULAR FUNCTION Another commonly used method to assess cardiac function after treatment with anthracyclines is by determination of left ventricular systolic and diastolic function by either multigated radionuclide angiography (MUGA) or cardiac echography. With a MUGA scan, gamma radiation produced by 99Technetium-labeled erythrocytes is measured and used to calculate several cardiac indices for systolic and diastolic function, such as left ventricular ejection fraction (LVEF).(37) Radionuclide assessment of LVEF is widely used to determine left ventricular function in cardiac disease. As several studies have shown that a decline in nuclear LVEF is indeed predictive (sensitivity varies between 55% and 100%) for future congestive heart failure in patients using anthracyclines, it is currently regarded as the gold standard.(38-44)

Also echographic assessment of LVEF has been used to assess anthracycline-induced cardiotoxicity. Literature shows that, when appropriate techniques are used, assessment of LVEF with this method is comparable to LVEF assessment with radionuclides. (45-48)

A disadvantage of both nuclear and echographic determination of the LVEF is that it is unclear if it is feasible for the early detection of cardiotoxicity, as the decline in LVEF commonly occurs late in the pathophysiological process and is often insidious. (49-51) However, other reports show that even small early (a change of 4% in ejection fraction) changes in LVEF measured with echocardiography, as well as with radionuclide methods may be predictive for the occurrence of anthracycline-induced cardiotoxicity.(52-54)

In the past few decades, heart failure with preserved ejection fraction has become increasingly important and has also been associated with significant morbidity and mortality.(55;56) Administration of anthracyclines also impairs diastolic function, even in the absence of a declined left ventricular ejection fraction. (57-63) It has therefore been suggested that diastolic dysfunction could precede an ensuing decline in LVEF and may be useful as a marker for anthracycline-induced cardiotoxicity.

Biochemical markers - cardiac troponins and natriuretic peptides

In cardiovascular disease, including anthracycline-induced heart failure, an extensive amount of biomarkers has been studied for the detection of myocardial injury, including creatine kinases, cardiac troponins and natriuretic peptides.

Troponins are thin-filament associated complexes that are involved in the regulation of the actin-myosin cross-bridges of striated muscles and consist of three subunits: troponin T, C and I.(64) Cardiac troponin T and I are both highly sensitive and specific markers for myocardial injury.(65) Both markers are established as diagnostic and prognostic tools in acute coronary syndromes. (66) Cardiac troponins have also been suggested as early markers for anthracycline-induced cardiotoxicity, albeit with ambivalent

results. (For an excellent review, see Germanakis et al.(67)) These contrasting findings can be related to many factors, including heterogenic study populations, variable cumulative anthracycline doses, and different study protocols with regard to type of assay and sampling time. Nevertheless, most reports show that at least some patients have detectable troponin, suggesting that it might be a prognostic marker in anthracycline-induced cardiotoxicity.

The family of natriuretic peptides consists of 3 distinct types: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). All natriuretic peptides share vasodilative properties and are involved in sodium and water homeostasis.(68) ANP is stored in granules inside cardiomyocytes and released in response to cardiac wall stress. The formation of BNP in response to cardiac wall stress is more complex; pre-proBNP is synthesized in the ventricular wall and subsequently cleaved via proBNP into its active form BNP and the inactive amino-terminal fragment NT-proBNP. As BNP is less sensitive for transient changes in hemodynamics, such as the administration of infusion fluids, it is a better marker for cardiovascular disease, such as heart failure, than ANP.(68) Indeed, both BNP and NT-proBNP are known to be increased in heart failure and both markers are widely used as independent risk factors for cardiovascular events. Most studies have shown that elevated BNP and NT-proBNP levels correlate well with echocardiographic and/or radionuclide parameters of myocardial dysfunction.(69) CNP is mainly produced by the endothelium and its role in the pathophysiology of heart disease is yet to be established.(70) It has been reported that after administration of anthracyclines, concentrations of circulating natriuretic peptides increase. Especially persistently elevated (NT-pro)BNP levels have prognostic value.(71) However, NT-proBNP has also been suggested as possible early marker for the evaluation of anthracycline-induced cardiotoxicity in children,(71) and adults.(this thesis) A disadvantage however is that BNP (and to a lesser extent also NT-proBNP) levels are subject to biological (day-to-day) variation which make them less suitable for monitoring disease progression unless strict protocols are followed.(72)

ELECTROCARDIOGRAPHY Electrocardiography is widely used for the evaluation of cardiac function, and is useful for the diagnosis of cardiac ischemic diseases, congestive heart failure and arrhythmias. Several clinical studies have related prolongation of the QT-interval to anthracycline administration.(73-76) As anthracyclines are not known to influence cardiac ion channels, these prolonged QT-intervals may be related to a disturbed repolarization due to myocardial injury. Although no relation between the degree of QT-prolongation and cardiac disease has been shown in patients treated with anthracyclines, it is known that prolonged QT-intervals are associated with increased mortality in patients with heart failure.(77) Other evidence that QT-prolongation might be an appropriate marker for cardiac injury is suggested by the commonly used preclinical model for anthracycline-induced cardiotoxicity. In mice, adriamycin induces ST-prolongations that can be abolished by concomitant administration of the clinically effective protective compound dexrazoxane.(78) Some investigators also related an increased QT-dispersion, which reflects the regional differences in repolarization, to exposure to anthracyclines.(79-81) Increased QT-dispersion has been related to increased cardiac mortality in various clinical conditions.(82) However, more recent studies indicate that QT-dispersion is an unreliable predictor of cardiac events in general(83;84), making it unlikely that QT-dispersion will prove to be a suitable marker for anthracycline-induced cardiac injury. Finally, changes in heart rate variability (HRV), which reflects changes in autonomic regulation of circulatory function, have also been described after anthracycline administration(85;86), but a subsequent report failed to confirm these results.(49) rendering the value of this measure questionable.

Protective strategies

Several protective strategies have been suggested in order to diminish the cardiotoxicity by anthracyclines, including less toxic compounds, improved dosage schedules and the concomitant administration of protective compounds.

LESS TOXIC ANTHRACYCLINES Numerous presumed less toxic analogues have been developed, of which only epirubicin

and idarubicin are used in clinical practice. Although epirubicin is less cardiotoxic than doxorubicin, this advantage is clinically less significant as higher doses are needed to achieve similar anti-tumor efficacy compared to doxorubicin, thereby offsetting the favorable cardiotoxic profile. The data for idarubicin are contradictory and larger trials are needed to assess if this analogue indeed has a lower incidence of cardiotoxicity.(8) Some advances have been made with the development of liposome-encapsulated doxorubicin, these suggest a favorable cardiotoxic profile. However, only limited efficacy data are available and treatment costs are relatively high. The value of this compound thus remains to be established.(2)

DIFFERENT DOSING STRATEGIES Traditionally, anthracyclines are administered as bolus infusion over a maximum of approximately 60 minutes. Soon after their introduction it was suggested that a prolonged infusion period (up to 96 hours) could reduce cardiotoxicity.(87) Since then, over 30 trials have compared the occurrence of (sub-)clinical cardiotoxicity after bolus injection with prolonged infusions. (For a review, see (88) According to a meta-analysis, the occurrence of clinical heart failure is significantly lower in patients receiving anthracyclines with an infusion duration of six hours or longer as compared to bolus infusion (RR = 0.27; 95%CI 0.09 to 0.81, P = 0.02).(88) Although it seems that the anti-tumor efficacy is not hampered by slow infusion, these trials were mainly performed in patients with metastasized disease and the follow up period was not clearly specified. Should it be proven, however, that modification of dosing schedules does not to impair the intended anti-tumor effects of anthracyclines while avoiding the untoward cardiac effects, this might prove a feasible future strategy.

PROTECTIVE AGENTS Based on the (presumed) pathophysiological mechanism, protective compounds were developed that should be administered concomitantly with the anthracycline-containing chemotherapy. As ROS-overload is a major pathophysiological mechanism it is logical that free radical scavengers like N-acetylcysteine, coenzyme Q10, vitamin E and C were evaluated as protective agents.(89-92) Indeed, animal studies with these compounds showed promising results, but evidence of protective effects in humans could not be not demonstrated.

Blocking the renin-angiotensin system (RAS), either by ACE inhibitors or ATII receptor blockers, improves the outcome of patients with systolic heart failure.(93) In the treatment of anthracycline-induced cardiac failure, treatment with ACE-inhibitors was efficacious, too.(94-97) This suggests that concomitant administration of RAS-inhibiting agents may be beneficial. This hypothesis was supported by animal studies showing that modulation of the RAS could prevent against anthracycline-induced cardiotoxicity.(89;98-103) It appears that in clinical practice, too, ACE inhibitors and ATII-antagonists are beneficial in patients treated with anthracyclines.(104;105) However, these trials were performed in small patient populations and had several methodological shortcomings, so additional research is needed to confirm whether or not ACE-inhibitors/ATII antagonists can be considered as protective.

The only compound that has a proven efficacy against anthracycline-induced cardiac failure is the iron chelator dexrazoxane.(106) This compound is capable to chelate intracellular iron (complexes), thereby preventing the formation of free radicals.(107) However, the possible association of dexrazoxane with a higher risk for secondary malignancies and an increased occurrence of (serious) adverse effects limits its clinical use to patients with advanced (metastasized) tumors.(106)

Aims of the thesis

It is clear that there is a special need for markers that can be used to detect anthracycline-induced cardiotoxicity in an early stage and identify those patients at risk for the development of CHF.

The studies described in this thesis aim to, firstly, identify possible biomarkers and detection methods to identify anthracycline-induced cardiotoxicity early and secondly, identify new possible strategies to prevent anthracycline-induced cardiotoxicity. Chapter 2 comprises a pilot-study to identify biomarkers for course-to-course evaluation of

anthracycline-induced cardiotoxicity. In chapter 3, the effects of doxorubicin on the iron metabolism is discussed. A novel method to assess repolarization disturbances after anthracycline therapy is described in chapter 4. In chapter 5 and 6, the pharmacokinetics of a potentially novel protective compound lecithinized superoxide dismutase (PC-SOD) in healthy subjects are described. These data were used to design the study described in chapter 7. This study investigated the clinical efficacy of PC-SOD against anthracycline-induced cardiotoxicity in female breast cancer patients. It concludes with an overall discussion, conclusions and suggestions for further research (chapter 8).

Figure 1 Chemical structure of Doxorubicin

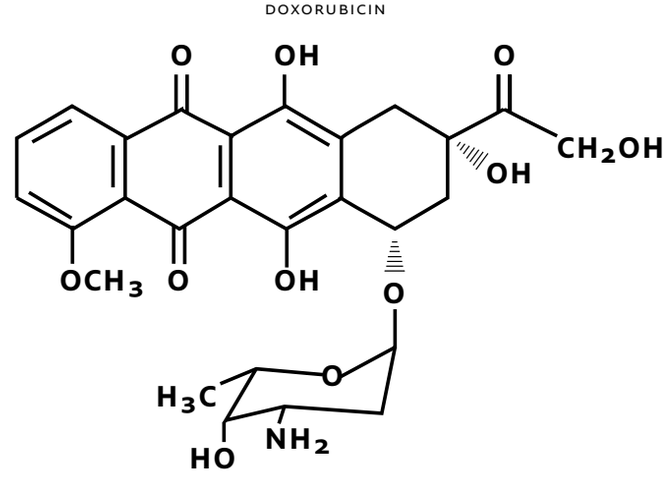


Figure 2 ROS generation after anthracycline administration

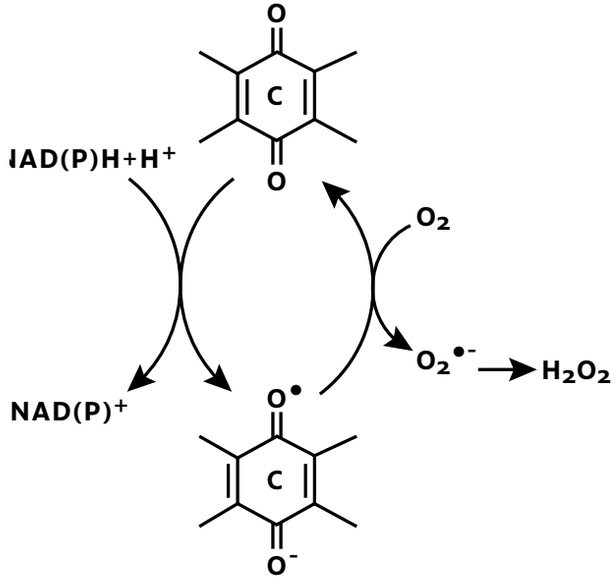
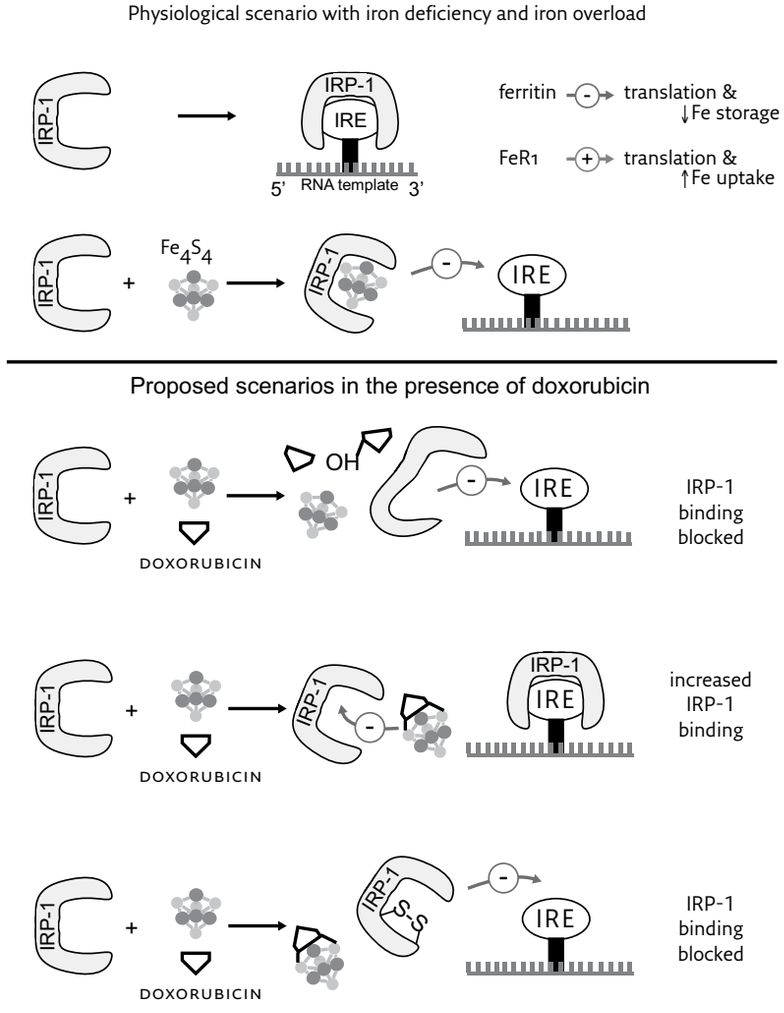


Figure 3 Proposed mechanisms of iron-mediated cardiotoxicity



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CHAPTER 2

Evaluation of biomarkers for cardiotoxicity of anthracycline-based chemotherapy

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ABSTRACT

The clinical assessment of the myocardial damage caused by anthracycline (ANT)-therapy is difficult. Therefore a study was performed to evaluate non-invasive markers of anthracycline-induced cardiac effects, with emphasis on course-to-course variation.Eligible for study participation were patients, without known cardiologic abnormalities who did not use cardiotoxic medication (except for ANT-therapy), who had previously completed at least 3 cycles of anthracycline-containing chemotherapy (n=14) and patients who were ANT-naïve and who were scheduled to receive doxorubicin (DXR)-containing chemotherapy (n=12). Seven patients in this last group also completed at least 3 cycles and were available for follow-up assessments; thus a total population of 21 patients (12F/9M) completed at least 3 courses ANT-chemotherapy. In these patients blood samples and ECG-recordings were taken within 6 months after completion of ANT-therapy. In 12 patients (10F/2M) assessments were also done before, immediately afterwards and at 24hr after each course of ANT.

In the patients who completed chemotherapy, NT-proBNP was 277% (n=21; 95% CI: 86%-661%, p<0.001) higher compared to healthy volunteers.

During the first course NT-proBNP rose 269% (n=12; 167-409%, p<0.0001) at 24hr post-administration.

The linear corrected QT (QTcL) directly after the first administration of ANT increased by 9.56 msec (n=12; 3.85-15.27, p<0.001) and this prolongation was still present at 24 hours, 11.48 msec (n=12; 5.61-17.34, p<0.0001).

Both NT-proBNP and QTcL returned to baseline before the start of the next course and a similar pattern was observed during each course.

NT-proBNP and QTcL may be useful markers for course-to-course evaluation of anthracycline-induced cardiotoxicity.

INTRODUCTION

Anthracyclines, such as Doxorubicin (DXR), cause serious cardiac side-effects.(1) Acute tachy-arrhythmias and acute heart failure may occur after high doses, but these reactions are now rare due to changed dosage-schemes (e.g. slower infusion) with the aim to prevent this. However, the sub-acute or chronic cardiac effects of anthracyclines remain a clinical problem. Clinically, anthracycline-induced cardiotoxicity manifests itself as left ventricular failure which develops insidiously over months to years after completion of the anthracycline-based chemotherapy and may result in congestive heart failure (CHF).(2;3)

Recent studies suggest an incidence of this type of cardiotoxicity of 5% in doses up to 400 mg/m^2 increasing to 48% in subjects receiving 700 mg/m^2.(4) But even at doses up to 150 mg/m^2 CHF was occasionally reported.(4) In addition to the cumulative dose, age, gender and dosing schedule have been reported as independent risk factors.(5)

The mechanism of anthracycline-induced cardiotoxicity is not totally unravelled. It is likely that the decline in myocardial function is related to apoptosis of cardiac myocytes that occurs apparently at random in the myocardium.(6) Anthracycline-induced formation of reactive oxygen species (ROS) in the presence of intracellular iron, impaired homeostasis of intracellular iron and calcium (that may facilitate the apoptosis induced by the ROS) have been put forward as mechanisms. However, other possible mechanisms have been suggested and it is likely that anthracycline-induced cardiotoxicity develops as a result of a large number of different insults.(3)

It is generally acknowledged that anthracycline-induced cardiotoxicity becomes evident after completion of the chemotherapy. The gold standards to detect anthracycline-induced cardiotoxicity are cardiac imaging techniques or myocardial biopsy. However, these methods have either the disadvantage that cardiotoxicity is detected late, namely when decline in left ventricular ejection fraction (LVEF) already has occurred (imaging techniques) or that it is highly invasive and based on the assumption that the damage is equally distributed over the myocardium (biopsy).

Animal studies have shown that anthracycline-induced apoptosis can occur already after a single dose.(7-9) This is line with the finding in humans that even at low cumulative doses cardiotoxicity have been reported.(4) If this could be measured and confirmed in humans, it might be possible to detect anthracycline-induced cardiotoxicity in an early stage. Unfortunately, the assessment of apoptosis itself in humans is difficult, but it can be hypothesised that the cell loss in the heart may be detected indirectly. It is conceivable that cardiac damage results in leakage of cardiac enzymes into the circulation, conduction disturbances and that the loss of function will be compensated by autocrine cardiac mechanisms. Indeed, it has been reported that elevated concentrations of troponin, prolongation of QT-interval and increased levels of natriuretic peptides are associated with anthracycline-induced cardiotoxicity after completion of chemotherapy.(10-13) However, little information is available for the course-to-course effects of anthracyclines on these markers and thus they are not used to assess the early effects of anthracyclines on the heart. Additionally, the evaluation of interventions or treatments designed to prevent the damage requires a robust early and preferably quantitative marker of the damage. Hence, there is a need for biomarkers that can be used to detect early anthracycline-induced cardiotoxicity. Therefore a study was performed to evaluate non-invasive markers of anthracycline-induced cardiotoxicity, with emphasis on course-to-course variation during 4 subsequent chemotherapy courses.

MATERIALS AND METHODS

Study population

The study was carried out in 26 patients with various malignancies who received DXR as chemotherapy, in combination with other chemotherapeutics (etoposide, vincristine, ifosfamide, actinomycine, docetaxel, cyclophosphamide, methotrexate, 5-flurouracil, cisplatin) and a group of healthy controls. One patient received epirubicin; for this patient the epirubicin dose was converted to the equivalent DXR-dose with the commonly used conversion factor of 0.5.(14;15)

Eligible for study participation were patients who had previously (within six months) completed at least 3 cycles of anthracycline-containing chemotherapy (n=14) and patients who were anthracycline-naïve and who were scheduled to receive doxorubicin (DXR)-containing chemotherapy (n=12). Seven patients in this last group also completed at least 3 cycles and were available for follow-up assessments; thus a total population of 21 patients completed at least 3 courses ANT-chemotherapy. Of the anthracycline-naive group the majority (n=9) received DXR combined with cyclophosphamide for breast cancer. Two patients were treated with DXR and cisplatin for a carcinoma of the endometrium and osteosarcoma respectively and one patient received DXR in combination with vincristine, etoposide and ifosfamide for an Ewing sarcoma. The median number of courses of this subpopulation was 4; four patients received more courses, namely 5, 6 and 8 courses respectively.

Patients and volunteers with known cardiac abnormalities, e.g. symptoms of angina pectoris, myocardial infarction or other myocardial abnormalities, or receiving other drugs with a known or suspected cardiotoxic potential and patients with clinically significant abnormalities, other than those related to their malignancy, were excluded from the study. Subjects did not use any QT-prolonging agents, except for 5-HT3-receptorantagonists for the prevention of nausea. Especially patients with an impaired renal function, haemoglobin level below 5 mmol/L or other clinically significant laboratory abnormalities other than those possibly related to their disease were excluded. A summary of the demographics of the patients and the volunteers is given in table 1.

The Medical Ethics Committee of Leiden University Medical Center (LUMC) approved the protocol for this observational study. All patients were included after giving written informed consent to participate.

Study outline

For each patient assessment consisted of blood sampling and ECG-recording within 6 months after completion of the chemotherapy. In the subpopulation of anthracycline-naïve patients (n=12)

measurements were made at each chemotherapy course. For comparability only data from the first 4 courses were used in the analysis. At each of these assessments blood sampling and ECG recording were done before (t=0), at completion of the chemotherapy infusion (t=4) and at 24 hours (t=24) after the drug administration. For the controls a single assessment was done.

Medication

All chemotherapy was prepared by the pharmacy of Leiden University Medical Center according to the applicable guidelines. The median total volume load for the anthracycline-naïve subpopulation during the courses was 250ml over 2 hours.

Sample handling and assays

Blood samples were centrifuged immediately after collection and serum/plasma was stored at -40° until analysis. The samples were analysed for cardiac troponin T (cTnT), the mass concentration of creatine kinase-MB (CK-MB mass), atrial natriuretic peptide (ANP) and the N-terminal propeptide of B-type natriuretic peptide (NT-proBNP). Each individual assay was performed batchwise to avoid interassay and interindividual variability using automated and validated assays at the Central Laboratories for Clinical Chemistry of LUMC. The concentrations of cTnT and NT-proBNP in serum were determined using an automated electrochemiluminescence immunometric assay on a Modular E170 Immunoanalyser (ECLIA, Roche Diagnostics, Mannheim Germany). Lower limits of detection and CV's were 0.01 ng/ml and 2.6%-5.6% for cTnT and 5 ng/L and 2.3%-3.2% for NT-proBNP. CK-MB (mass) was analysed in serum using an automated analyser IMx with the kit provided by the manufacturer (Abbott Diagnostics, Illinois, USA; detection limit 0.7 mg/L). Concentration of ANP was determined using an immunoextraction (RIA) with a C-terminal-specific antiserum (Incstar, Stillwater, MN, USA; lower limit of detection 0.1 pmol/L, CV: 6.8%-8.9%) as previously described.(16) In addition haemoglobin, electrolytes and creatinine concentrations were measured using routine methodology at the central laboratories for clinical chemistry of LUMC.

Renal function

Glomerular filtration rate was estimated using the MDRD formula: $GFR_{estimate}(mL/min) = 32788 \times creatinine\ (\mu mol/L)^{-1,154} \times age^{-0,203} \times constant$ (1 for males, 0.742 for females).(17)

ECG recordings and analysis

For each patient a 5-minute ECG recording was made using the CardioPerfect device (Welch Allyn, Delft, The Netherlands). Heart rate (HR) and QT-interval were measured using the software supplied with the device. Care was taken to optimally assess the duration of the QT-interval as it has been reported that in 10-15% of cases ECG's fiducial points (e.g. P-onset, QRS-onset and end of T-wave) are not measured correctly by automated computer programs. Therefore the ECG recordings were additionally analysed after fiducial segment averaging (FSA) to obtain heart rate and QT-interval. This analysis was done using Intraval (Advanced Medical Systems, Maasdam, the Netherlands).(18)

For both analyses correction of the QT-interval for heart rate was done using Bazett's formula ($QTcB = QT/\sqrt{RR}$) and using the linear correction method according to Framingham ($QTcL = QT + 0.154 * (1 - RR)$).

Statistics

Data from all patients were compared to the data obtained in the group of controls. The analysis of variance was conducted with factors group and gender and BMI and age as covariate, contrasts between the two groups were calculated along with 95% confidence intervals and least square mean estimates.

Data from the subpopulation of anthracycline-naïve patients were analysed for changes occurring during the course

(measurements at t = 0, 4 and 24) and whether there was a difference between courses. Two analyses were performed (1) analysis of variance with (within subject) factors time (0, 4 and 24), course and time by course using the data in original measurement units and (2) analysis of variance on change from baseline with baseline (t=0) as covariate, with (within subject) factors time (4 and 24), course and time by course. Least square means were calculated for the different course/time point combinations along with 95% confidence intervals.

In order to quantify the overall (average) time effect, the average change from baseline averaged over the 4 courses for the t=4 and t=24 time point with 95% confidence interval was calculated within the ANOVA model.

All parameters, except for the ECG measurements, were log transformed prior to analysis to realize a normal distribution of the data and meet the requirements for ANOVA. In case of log transformation results were back-transformed resulting in geometric means and geometric mean ratios (for the contrasts and change from baseline). Geometric mean ratios were further translated into percentage change along with their 95% confidence intervals. Correlation analysis was performed on several biomarkers using regression analysis. All calculations were performed using SAS V9.1.2 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

Biochemical markers

Patients who completed chemotherapy had 277% (n=21; 95% CI: 86%-661%, p<0.001) higher NT-proBNP levels in comparison to healthy volunteers (n=14).
For the subpopulation of patients who were also followed during chemotherapy it was first investigated whether difference in volume loading resulted in different NT-proBNP responses. As this was not the case the data are given for the entire group. In these anthracycline-naïve patients, the estimated increase in NT-proBNP was 269% (n=12; 167-409%, p<0.0001) at 24 hour after the first DXR-course. Directly after completion of the DXR-infusion no increment was observed (figure 1). Similar 2-3 fold increases were found after the 2nd, 3rd and 4th course of DXR-therapy. NT-proBNP levels had returned to baseline before the start of each subsequent DXR course. CK-MB and ANP showed no differences between the groups, although there was a trend towards an increased ANP in the group of patients who already completed chemotherapy (n=21) (table 2). Renal function (estimated using the MDRD formula) was stable during the 4 courses and none of the subjects experienced clinical significant electrolyte abnormalities during the courses.

Anthracycline-naïve patients had higher ANP and NT-proBNP than the healthy controls (n=14) by 140.8% (n=12; 19.4%-385.6%, p<0.02), 113.1% (n=12; 8.0%-320.1%, p<0.04) respectively.

cTnT concentrations were below the limit of detection in most cases and not further analysed for this reason.

In additional analyses the percentage change from baseline was calculated for all courses together (table 3). Rises of 238% (n=12; 149-358%, p<0.0001), 44% (n=12; 4-101%, p<0.04), 26% (n=12; 4-52%, p<0.02) for NT-proBNP, ANP and CK-MB were found at 24 hours. There was no correlation between CK-MB on one hand and ANP and NT-proBNP.

ECG

No differences in HR and (corrected) QT-time could be observed when the patients who had completed the chemotherapy (n=21) were compared with the healthy volunteers (n=14) (table 2). However there was a trend to a slightly prolonged QTc, corrected according to Bazett.

During each chemotherapy course (corrected) QT-time was prolonged immediately after completion of a course and remained so at 24 hours after the DXR-course. As an example: during the first course QTc (using a linear correction method) increased by 9.56 msec (n=12; 3.85-15.27, p = <0.001) at completion and was increased by 11.48 msec (n=12; 5.61-17.34, p = < 0.0001) at 24 hours. Similar prolongations were observed during the subsequent courses. Heart rate did not change during the courses (figure 2). In the additional ECG-analyses using FSA, similar results were found.

DISCUSSION

In the present study several markers that may be indicative for cardiac damage after DXR administration were investigated. The most important findings of this study are the observed change in NT-proBNP and prolonged QTc-intervals. This study indicates that at 24 hours after each course of anthracycline chemotherapy as well as after completion of a full chemotherapy regimen, significant increases in serum NT-proBNP are observed. Furthermore, the (corrected) QT-interval was prolonged with each course (course-to-course prolongation).

NT-proBNP is cleaved from proBNP when it is converted into active BNP upon secretion. It is secreted equimolarly with BNP by the ventricle wall mainly in response to wall stretch and its secretion may be enhanced by catecholamine's, angiotensin II, endothelin and hypoxia.(19) NT-proBNP is a well-established marker for congestive heart failure.(20) Elevated levels of NT-proBNP (and BNP) are most notably associated with CHF, but also with other cardiac conditions.(21) It is also known that concomitant kidney failure results in more pronounced elevations of NT-proBNP, especially in patients with an ejection fraction below 35%.(22;23) As all included patients had a stable renal function during the study period, this could not explain the elevations of NT-proBNP in our study.

Elevation of concentrations of natriuretic peptides associated with previous exposure to anthracyclines has been described before, but this is mostly reported after completion of chemotherapy.(10;11;24) An important additional finding of this study is that NT-proBNP is elevated relatively rapidly after exposure to anthracyclines. This effect was present for each individual subject, during each subsequent chemotherapy cycle. Therefore, we assume that DXR causes sufficient myocardial wall stress or neurohumoral responses on catecholamines or angiotensin to result in acutely elevated concentrations of NT-proBNP. This effect takes some time to develop as the NT-proBNP concentrations were unchanged immediately after the courses (t=4 hrs). However, elevated concentrations were invariably found in each patient at the 24 hours' time point. Although it cannot totally be excluded that the increases in NT-proBNP are (partly) caused by volume loading, this is highly unlikely as the infused volumes were low.

Additionally, the increase in NT-proBNP was transient as levels had returned to baseline at the subsequent course. This temporal relationship suggests that the initial effects of DXR on the heart are at least partly reversible during its first phases or compensated for by other mechanisms. This is plausible as animal studies indicated that the number of apoptotic cardiomyocytes after DXR show a biphasic pattern.(8) It is also in keeping with the notion that in clinical practice overt anthracycline-induced cardiotoxicity develops after repeated exposure and failure of compensatory mechanisms.(3)

When all courses were taken together, an increase in atrial natriuretic peptide (ANP) and CK-MB occurred after DXR. This effect could not be demonstrated for each individual patient-course combination, most likely because the increase in ANP varied between individuals and the study population was small. Furthermore cTnT level stayed below the detection limit in most cases. Both cTnT and CK-MB are widely used to assess myocardial injury in different clinical conditions.(25) There are conflicting data in the literature with regard to cTnT.(12;13;26-28) A possible explanation for this discrepancy may be that many of the studies that failed to show an effect were small.

A surprising finding was that NT-proBNP and ANP were higher in the group of patients prior to anthracycline-therapy when compared to healthy volunteers. Missov et al. reported comparable results for cardiac Troponin I in patients with hematologic malignancies prior to anthracycline-therapy.(29) They suggested that patients with malignancies could experience some myocardial wall stress because of increased catecholamine release and an elevated sympathetic drive associated with anaemia and reflex tachycardia. However, our patients had normal haemoglobin concentrations, were normotensive, and had a normal heart rate, making this explanation for our population unlikely.

We also found significant prolongations of the QT-interval immediately after and at 24 hours after each chemotherapy cycle. As it has been shown that in 10-15% of cases ECG's fiducial points (e.g. P-onset, QRS-onset and end of T-wave) are not measured correctly by automated computer programs, the analysis of

QT-intervals was performed using two methods which basically showed identical results.(18) The mechanism for the prolonged repolarisation of the heart after DXR-administration is unclear. Hypothetically, it may be a direct effect of DXR on cardiac repolarisation or the outcome of the loss of cardiomyocytes. The first explanation is supported by the observation that DXR is associated with acute arrhythmias and sudden cardiac death; however, there are no reports of DXR being a QT-prolonging drug. Although it is true that 5-HT3 receptor antagonists, which were used for the prevention of nausea, can prolong the QT-interval. However, QT-prolongations are present only very shortly after administration (up to 20 min) and QT-intervals even decline thereafter.(30) Therefore 5-HT3 receptor antagonists cannot be responsible for the changes in QT-interval we observed at 4 and 24 hours after the chemotherapy. The second explanation which links QT-interval prolongation to myocardial cell death may be more plausible. Whatever the cause of the QT-interval prolongation, it is certain that prolonged QT-intervals are linked to increased mortality and a variety of cardiac disorders, including heart failure.(31)

Although our exploratory study may have identified promising markers to assess the course-to-course effects of doxorubicin on the heart, it is important to also stress the limitations of this study. Obviously, the findings reported here should not be considered synonymous with anthracycline-induced cardiotoxicity, because no information is available to relate our findings to a measure of left ventricular function. However, it is unlikely that during the observation period of this study signs of ventricular dysfunction would have been detected as it is known that for the majority of the patients this develops over a longer period. Secondly, the patients included in this study received different dosing regimens and cytostatic drug combinations, which may have influenced the findings in this small cohort of patients. At present there is no indication that this occurred, but our group was probably too small to find such differences would they have been present.

In conclusion, the data indicate that NT-proBNP and the QT-interval are sensitive markers for the early detection of the course-to-course cardiac effects of DXR. Further research is needed to prospectively establish how the early changes in these markers relate to left ventricular dysfunction.

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Table 1 Subject characteristics and cumulative doxorubicin dose

	Patients		**Controls**
	Completed >3 course ant-chemotherapy	ant-naive	
	(N=21)	(N=12)	(N=14)
Age (yrs)	46 ± 15	48 ± 13	46 ± 14
Gender (F/M)	12/9	10/2	8/6
BMI (kg/m2)	22.9 ± 2.7	25.3 ± 2.1	23.0 ± 2.1
Cumulative doxorubicin dose (mg)	520 (270)	440 (138)	NA
Estimated Glomerular Filtration Rate (mL/min)	84.5 ± 26.4	86.2 ± 21.2	NA
Haemoglobin(µmol/L)	6.9 ± 1.1	7.8 ± 0.8	NA

BMI, age and the estimated glomerular filtration rate (using the MDRD formula) are given as mean ± SD.
The cumulative dose is given as median with the interquartile range. ANT: anthracycline., BMI: Body Mass Index.

Table 2 (Bio)markers and the differences between patients receiving at least three courses of anthracycline-containing chemotherapy and healthy controls

Variable	**Controls (n=14)**	**Patients (n=21)**	**Patients vs controls**	**95%-confidence interval**		**p-value**
				LOWER	UPPER	
ANP (pmol/L)	11.3	20.7	82.9%	-0.2%	235.0%	0.05
CK-MB mass (mg/L)	1.1	0.9	-15.7%	-48.1%	37.0%	0.48
NT-proBNP (ng/L)	39.5	148.9	276.6%	86.3%	661.4%	<0.001
HR	67	71	4.20	-1.95	10.36	0.17
QT (msec)	400	400	-0.31	-18.1	17.47	0.97
QTc Bazett (msec)	420	433	13.05	-0.36	26.46	0.06
QTc linear (msec)	414	422	8.22	-2.68	19.13	0.13

Average values of biochemical markers and ecg parameters and contrasts between anthracycline-chemotherapy completers and controls, including 95%-confidence intervals and P-values (for the biochemical markers contrasts are represented in percentage difference, for ecg parameters results are shown in absolute differences).
ANP: Atrial Natriuretic Peptide, CK-MB: Creatine Kinase Isoenzyme MB, NT-proBNP: N-terminal pro Brain Natriuretic Peptide, HR: heartrate, QT: qt-interval, QTc Bazett: QT interval corrected according to Bazett, QTc linear: QT interval corrected according to Framingham's linear correction method. Averages are estimated, as mixed model analysis was used to correct for predose and missing values.

Table 3 Summary table change from baseline (average of all 4 courses)

	4H				24H			
	CHANGE	95%-confidence interval		P-VALUE	CHANGE	95%-confidence interval		P-VALUE
		LOWER	UPPER			LOWER	UPPER	
Atrial natriuretic peptide	38.1%	-0.3%	91.3%	0.05	44.3%	3.6%	101.2%	0.03
CK-MB mass (mg/L)	-2.2%	-18.6%	17.6%	0.80	26.1%	4.4%	52.2%	0.02
NT-PROBNP (ng/L)	18.0%	-14.6%	63.0%	0.30	237.9%	149.4%	357.8%	<0.001
HR (BPM)	-0.29	-3.37	2.79	0.85	1.46	-1.67	4.60	0.34
QT (msec)	10.95	3.91	17.98	0.004	10.96	3.77	18.15	0.005
QTC Bazett (msec)	10.79	4.70	16.88	0.002	15.82	9.66	21.97	<0.001
QTC linear (msec)	10.04	5.39	14.69	<0.001	13.13	8.40	17.85	<0.001

Average change (for all four courses) from baseline, 95%-confidence intervals and P-values (for the biochemical markers results represent percentage change, for ECG parameters results are shown in absolute changes).
ANP: Atrial Natriuretic Peptide, CK-MB: Creatine Kinase Isoenzyme MB, NT-PROBNP: N-terminal pro Brain Natriuretic Peptide, HR: heartrate, QT: QT-interval, QTC Bazett: QT interval corrected according to Bazett, QTC linear: QT interval corrected according to Framingham's linear correction method.
Averages are estimated, as MIXED model analysis was used to correct for predose and missing values.

Figure 1 NT-PROBNP, mean serum concentration (standard deviation) at baseline, 4 and 24 hours in 12 patients who were sampled during each course of anthracycline-containing chemotherapy.

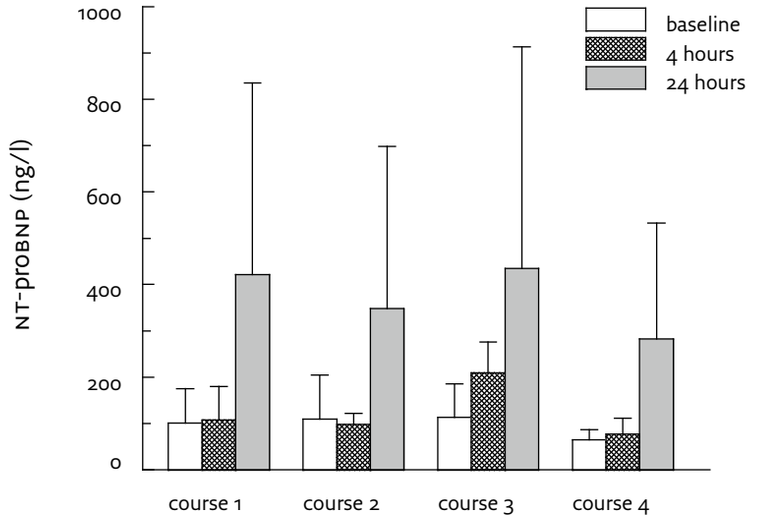
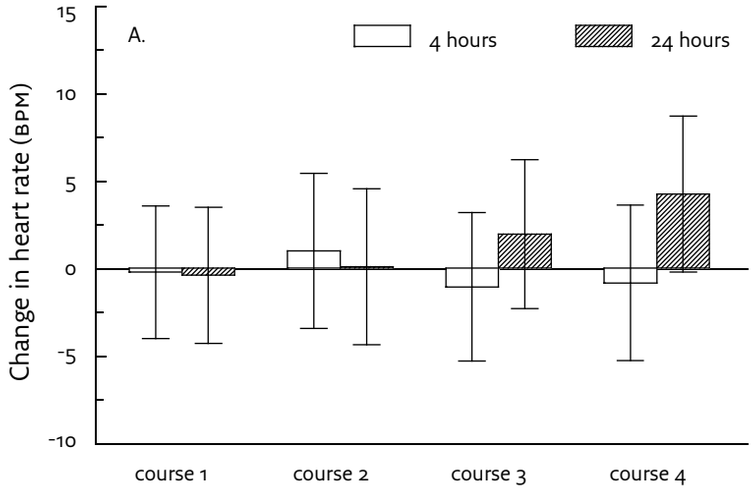
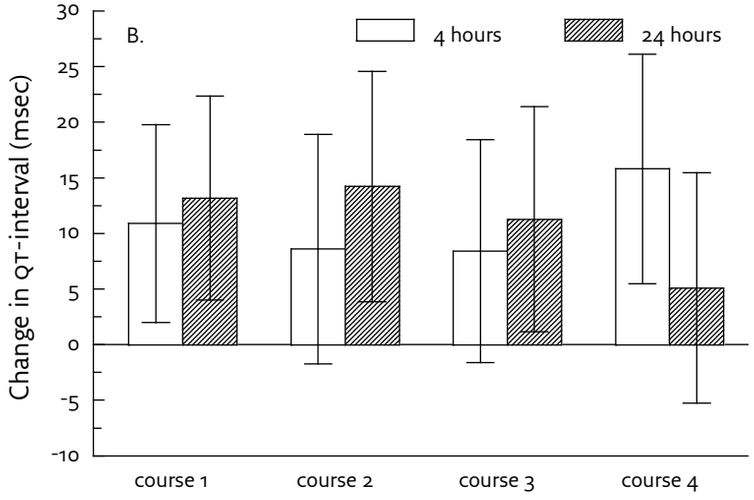
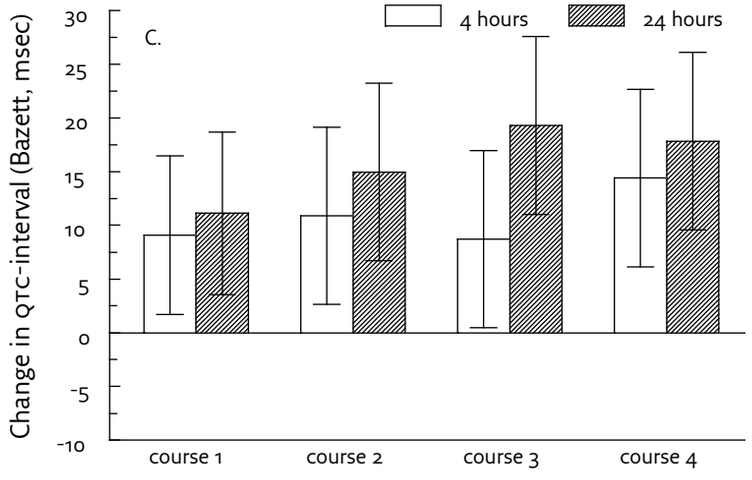
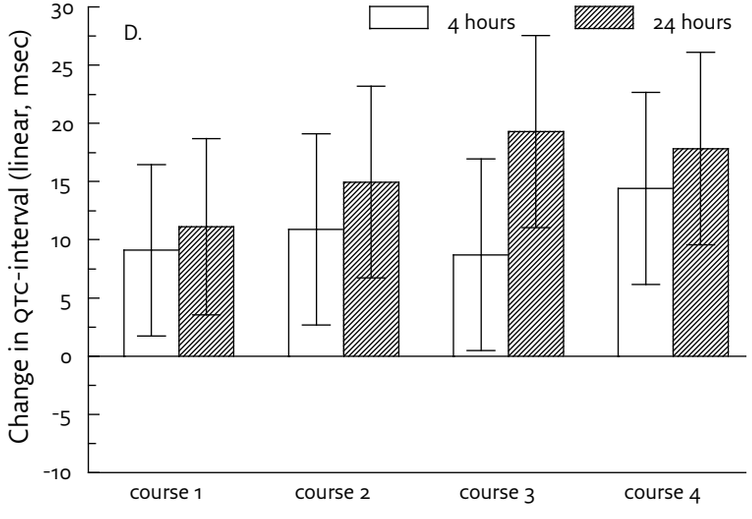


Figure 2 Heart rate (beats per minute) and QT-intervals (milliseconds) change from baseline with 95% CI error bars. A. Heart rate, B. QT-interval, C. QTC-interval (Bazett), D. QTC-interval (linear).









CHAPTER 3

Disturbed iron metabolism due to anthracycline-based chemotherapy in early stage, surgically cured female breast cancer patients

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ABSTRACT

INTRODUCTION Iron-catalyzed free radicals seem to play a role in anthracycline-induced cardiotoxicity and may lead to organ damage and dysfunction. The aim of this study was to evaluate iron metabolism in breast cancer patients who received adjuvant doxorubicin/cyclophosphamide treatment (AC).

PATIENTS AND METHODS We included 39 female breast-cancer patients (median age 47), scheduled to receive intravenous AC-chemotherapy. Iron status [total iron, transferrin, ferritin, latent iron binding capacity (LIBC) and non-protein bound iron (NPBI)] was studied during the first course of chemotherapy. Samples were taken prior to, immediately and 2:30 hrs after the doxorubicin infusion (total iron, LIBC and NPBI) and at 24 hrs after completion of the chemotherapy course. Additional measurements (at baseline and 24 hrs) were done during the subsequent chemotherapy courses and at 1 and 4 months after completion of the entire chemotherapy treatment.

RESULTS Immediately after the first administration of doxorubicin NPBI increased by 65.7% (95%CI: 23.5 to 122.3%) and returned to baseline at 24 hours. In parallel, total iron increased with 187.1% (95%CI: 153.7 to 225.0%) at 24 hours, accompanied by an almost total saturation of transferrin. Ferritin levels increased gradually over baseline, and were 79.1% (95%CI: 37.1 to 33.9%) higher at baseline of the fifth course.

DISCUSSION AND CONCLUSION This study shows that a single intravenous dose of doxorubicin immediately results in an increase of highly toxic NPBI in early stage breast cancer patients and this suggest that that NPBI may be, at least in part, responsible for the toxicity caused by doxorubicin.

INTRODUCTION

Anthracyclines are used in the treatment of several cancers because of their ability to inhibit topoisomerase II.(1) They are also known to facilitate the formation of free radicals in the presence of (non-protein bound) iron, which are supposed to be responsible for the (cardio)toxic side effects of anthracyclines.(1-4)

There seems to be consensus that under physiological conditions non-protein bound iron (NPBI, or sometimes referred to as non-transferrin bound iron) is not present extracellularly as iron is tightly bound to transport- and storage proteins, such as transferrin (serum) and ferritin (intracellular).(5) However, in case of iron overload, which may occur in hemochromatosis, dialysis, hemolytic anemia's and after certain (mostly high-dose) chemotherapy regimens the presence of NPBI has been reported. (6-11) This suggests that the binding capacity of the transport- and storage proteins does not suffice under these pathological conditions. NPBI in its ferrous form (Fe^{2+}) is highly reactive and capable to catalyze the Haber-Weiss reaction in which hydroxyl radicals are formed, resulting in lipid peroxidation, DNA damage, and eventually apoptosis.(5,12-14) It can be envisaged that in the case of anthracycline-induced cardiotoxicity, NPBI can even be more harmful as the heart has relatively low levels of antioxidants and (excess) iron is able to form a stable complex with doxorubicin (DOX), which easily undergoes self-reduction to form a semiquinone free radical of DOX.(3;12) In addition, *in vitro* studies have shown that anthracyclines deregulate intra-cellular iron metabolism and iron trafficking pathways, thereby aggravating the effects of (intracellular) iron overload.(13;14)

Although iron metabolism has been investigated in cancer patients receiving high doses of chemotherapy, effects in (surgically tumor-free) cancer patients receiving lower doses of anthracyclines have not been studied. Therefore, we performed a study to evaluate the effects of the combination of the anthracycline doxorubicin and cyclophosphamide (AC) on iron metabolism in female breast cancer patients who underwent adjuvant treatment with AC chemotherapy for early stage breast cancer.

METHODS

Patient population and study protocol

The patient population consisted of early-stage female breast cancer patients who underwent adjuvant treatment with a combination of doxorubicin (DOX) and cyclophosphamide chemotherapy. Main exclusion criteria included prior or concomitant use of cardiotoxic medication, distant metastases, a history of other malignant disease, a life expectancy of less than one year, pre-existing cardiovascular diseases and elevated transaminases above 3 times the upper limit of normal.

Eligible patients were scheduled for four or five (depending on the institutional guideline) three-weekly courses of intravenous (IV) doxorubicin (60mg/m^2 over 15 min) and cyclophosphamide (600 mg/m^2 over 15 min).

The medical ethical committee of Leiden University Medical Center (LUMC) approved the study protocol before inclusion of the first subject. All subjects gave written informed consent before participation.

Study procedures and measurements

Before the first course of chemotherapy concentrations of total iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), ferritin and transferrin were determined at baseline (t=0). After the patients had received anti-emetic therapy, the IV infusion of doxorubicin was started. Immediately after the doxorubicin infusion was completed (t= 0:15 hours) the first sample was obtained for determination of total iron, LIBC and NPBI, followed by the cyclophosphamide infusion. At t=2:30 hours after the doxorubicin administration a second sample (total iron, LIBC and NPBI) was obtained. The following morning (t=24:00 hours) blood was sampled for determination of iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), ferritin and transferrin. Baseline and 24 hour measurements were repeated during each subsequent chemotherapy course. At approximately 1 month after chemotherapy blood was sampled for iron, LIBC, non-protein bound iron NPBI, ferritin and transferrin. At approximately 4 months after chemotherapy ferritin and hemoglobin were determined.

LABORATORY PROCEDURES

All assays were performed at the Central Laboratories of Leiden University Medical Center (LUMC).

Ferritin, transferrin, LIBC and total iron

Assays for ferritin, transferrin, LIBC and total iron were performed using routine methodology. Lower limits of detection (inter- and intra-assay variability between brackets) were 0.5 µg/L (<5.35%), 13 mg/L (<1.2%), 4.2 µmol/L (<4.3%) and 0.24 µmol/L (<2.8%) for ferritin, transferrin, LIBC and total iron respectively.

NPBI

NPBI concentrations were measured using a colorimetric method as described previously.(15) Briefly, the serum samples were mixed 9:1 with a 40 mM NTA containing buffer of 5 mM Tris-HCl pH 6.5. After filtration and centrifugation thioglycolic acid sodium salt (3 mM) was added. Measurements were done using a Reader Spectra Max 250 plate reader at 537 nm. The (pooled) sera used for repeated experiments and patient sera were stored at -80 ºC until the measurements (no influence of storage on the NPBI results were found). The lower limit of detection (inter- and intra-assay variability between brackets) was 0.01 µmol/L (<9.2%).

Liver chemistry and hemoglobin

Assays for lactate dehydrogenase (LDH), bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and hemoglobin were measured using routine methodology.

STATISTICAL ANALYSES

To assess the changes within the first course (course 1) measurements at baseline, 0:15, 2:30 (only total iron, LIBC, NPBI) and 24:00 were analyzed (after log-transformation) using a mixed model analysis of variance (SAS proc mixed) with visit (occasion) as repeated factor within subject and time as fixed effects, and subject as random effect. To assess long-term treatment effects the course baseline and follow up measurements of all variables were analyzed the same way. Correction for multiple comparisons was not done because of the exploratory nature of the study. All statistical analyses were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Baseline characteristics

We included 39 patients (23 were scheduled for 4 courses and 16 for 5 courses) with a median age of 49 years (range 30-66 years), mean BMI 25.3 kg/m2 (SD 4.4) and a mean cumulative doxorubicin dose 255 mg/m2 (SD 58) (table 1).

Iron metabolism during the first AC course

At baseline, 32 of the 37 (maximal value: 1.26 µmol/L) obtained samples were positive for NPBI, directly and at 2:30 hours after doxorubicin infusion all samples turned positive for NPBI, ranges were 0.14 - 1.51 µmol/L and 0.09 - 1.45 µmol/L respectively. At 24 hours following the chemotherapy course 26 of the 35 (maximal value: 1.18 µmol/L) obtained samples were positive for NPBI.

Mean NPBI concentration increased (percentage change, 95% confidence interval between brackets) directly after the doxorubicin infusion with 65.7% (23.5 to 122.3%). At 2:30 hours post-dose the increase was 47.3% (14.2 to 90.1%) and at 24 hours no differences were observed (table 2, figure 1).

After a small initial decline, iron increased with 187% (154 to 225%) at 24 hours after the first chemotherapy course. LIBC did not change during the first 2:30 hours post-dose but declined with -75.6% (-82.6 to -65.8%) at 24 hours following chemotherapy. Ferritin was increased with 17.2% (8.3 to 26.9%) at 24 hour after AC administration. Hemoglobin and transferrin did not change significantly during the first course.

Additional analyses showed that the changes (for all parameters) at 24 hours were similar during all subsequent chemotherapy courses.

Long-term effects of chemotherapy on iron metabolism

Ferritin increased by 79.1% (37.1 to 133.9%) during the courses (difference between baseline course 5 and 1) and decreased during the follow up period (table 2, figure 2).

LIBC and transferrin changed minimally over course baseline. NPBI did not change significantly over baseline during the courses.

Hemoglobin level declined during each subsequent chemotherapy course, difference (percentage change, 95%-confidence interval between brackets) between the baseline values of course 5 and 1 was -10.2% (-12.9 to -7.4%). After a full chemotherapy cycle hemoglobin levels increased again.(table 2, figure 2).

Liver function and haemolysis parameters

During the first course bilirubin increased from 6.8 U/L baseline to 9.9 U/L at 24 hours, while LDH declined from 359 U/L to 297 U/L and concentrations of AST and ALT did not change markedly during the courses and concentrations remained within the normal limits.

DISCUSSION

During the first course of doxorubicin in early breast cancer patients an increase in NPBI level occurred almost immediately after doxorubicin infusion and at 24 hours total iron concentration

almost tripled, leading to a complete saturation of transferrin. It was also shown that within 24 hours after administration small rises in serum ferritin were observed and that ferritin increased gradually over baseline during the AC courses.

An intriguing finding of this study was that almost directly after DOX infusion NPBI increases, indicating that even relatively low doses of DOX as employed in the adjuvant setting are potentially harmful. The observation that the peaks are lower compared to previous studies could relate to the fact that we used an optimized spectrophotometric method, which allowed us to determine free iron concentrations more realistically.(15) However, it cannot be ruled out that the increase in NPBI is dose-dependent and that the dose of doxorubicin that we studied did not provoke a similar iron overload as in patients receiving high dose chemotherapy. The fact that in other studies NPBI was detectable for a longer period of time also supports this hypothesis.(8-10) The current opinion that NPBI is not detectable in (healthy) subjects without apparent iron overload can be questioned based on the data in this study, as we found NPBI levels prior to chemotherapy in a majority of patients. This is in keeping with other data indicating that under physiological conditions NPBI can be present, although it is important to note that in these papers different assays were used.(8;15) Obviously, our population consisted of patients and it cannot be excluded that the presence of NPBI before dosing with anthracyclines reflects the disease state, but we consider this unlikely in view of the fact that they were treated curatively and did not have macroscopic residual tumor. Thus, it seems that also under conditions without apparent iron overload circulating NPBI can be present, although its role is unclear.

The observation that iron concentrations increase shortly after chemotherapy in patients who are tumor free may help to further understand the possible source of iron that is not immediately apparent from previous experiments. Previous experiments have suggested that the possible sources of iron includes destructed tumor cells, impaired erythropoiesis, damage to the gastrointestinal mucosa, hemolysis and liver injury. (6;8-11;16-18)However, in our surgically tumor free population, tumor lysis, hepatic injury, and also hemolysis does not seem a major contributor for the observed iron bursts, as tumor mass was negligible, and bilirubin, transaminases and LDH did not change considerably during the courses. Although erythropoiesis is affected by chemotherapy, this does not seem to be the cause of the iron peaks as these occurred almost immediately after AC administration, while the effects on erythrocytes occurred later and no signs of hemolysis were present. We hypothesize that AC-chemotherapy provoke diffuse (low-grade) injury to several tissues, such as the gastro-intestinal mucosa, splenic cells, etc., which together cause sufficient cellular damage to provoke the observed iron peaks.

We also found small increases of ferritin within 24 hours after each course which seems to be cumulative and results in a gradual increase over the entire treatment period. The iron-storage protein ferritin, which is abundantly present intracellularly and in small amounts in serum, is associated with total body iron-store in healthy individuals. Elevated ferritin levels have been reported to result from increased synthesis in response to inflammation and because of cellular damage and/or a nonspecific response of the reticuloendothelial system to an increased tumor load in cancer patients.(19-24) Also, it has been suggested that increased ferritin levels could protect against tumor proliferation.(21) However, a response to tumor proliferation is unlikely in our population of early stage breast cancer patients. We cannot exclude that the rise is caused by an acute phase response to a (low-grade) inflammatory response to the administered chemotherapy, but the absence of increases in hsCRP (results not shown) render this explanation less likely. Another possibility is that (at least) some of the observed changes reflect a direct effect of doxorubicin on iron metabolism or a protective response to the chemotherapy-induced iron overload. This explanation would be in keeping with the notion that pre-clinical studies have shown that doxorubicin has marked effects on intra-cellular iron homeostasis by increasing accumulation of iron in ferritin, inducing increased expression of ferritin and inhibiting release of iron from ferritin.(4)

A potential drawback of our study is that we attribute the changes to effects of AC-chemotherapy as we (for obvious reasons) did not include a placebo control. However, we consider it unlikely that the changes that we observed can be attributed to spontaneous time (circadian) effects. Also, it is unlikely that

cyclophosphamide contributed greatly to the increased release of NPBI, as the maximal increase in NPBI was already present immediately after completion of the doxorubicin infusion and before the administration of cyclophosphamide.

In summary, we found that a single IV dose of doxorubicin immediately results in occurrence of highly toxic NPBI in the circulation. This could help to further understand the *in vivo* mechanism of doxorubicin toxicity and may produce leads into protective agents for this.

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Table 1 Baseline characteristics

	Female breast cancer patients (n = 39)	
	MEAN	SD
Iron parameters		
Hemoglobin - mmol/L	7.7	0.7
Total iron - µmol/L	16.5	6.7
NPBI - µmol/L	0.44	0.26
LIBC - µmol/L	43.9	13.6
Ferritin - µg/L	70.7	62.7
Transferrin - g/L	2.61	0.60
Liver chemistry		
ALT - U/L	24.4	24.9
AST - U/L	31.7	16.5
LDH - U/L	393	222
Bilirubin - mg/dL	7.8	4.5

Table 2 Percentage change from course baseline (95% confidence interval) for total iron, latent iron binding capacity (LIBC), non-protein bound iron (NPBI), hemoglobin haemoglobin, ferritin and transferrin during the first chemotherapy course

Time post-dose	**0:15 HR**	**2:30 HR**	**24 HR**
Total iron	-15.4 (-19.8 TO -10.8)	-25.1 (-33.0 TO -16.2)	187.1 (153.7 TO 225.0)
LIBC	-2.1 (-6.8 TO 2.8)	3.1 (-5.0 TO 11.9)	-75.6 (-82.6 TO -65.8)
NPBI	65.7 (23.5 TO 122.3)	47.3 (14.2 TO 90.1)	-10.6 (-44.7 TO 44.5)
Ferritin			17.2 (8.3 TO 26.9)
Transferrin			6.0 (-8.9 TO 23.3)
Hemoglobin			-2.4 (-4.2 TO -0.5)

Table 3 Percentage change from baseline of course 1 (95% confidence interval) for hemoglobin, ferritin and transferring for each chemotherapy course and at follow up.

	Hemoglobin	**Ferritin**	**Transferrin**
Course 2	-4.4% (-6.6 TO -2.1)	19.9 (-1.2 TO 45.4)	-0.8 (-15.1 TO 15.9)
Course 3	-7.8 (-10.5 TO -4.9)	46.9 (14.4 TO 88.7)	-3.8 (-18.2 TO 13.2)
Course 4	-8.3 (-10.5 TO -6.0)	59.3 (29.2 TO 96.3)	2.0 -13.3 TO 19.9)
Course 5	-10.2 (-12.9 TO -7.4)	79.1 (37.1 TO 133.9)	0.2 (-14.7 TO 17.8)
Follow up 1 month	-4.4 (-6.7 TO -2.0)	68.6 (37.9 TO 106.1)	14.9 (-2.0 TO 34.8)
Follow up 4 months	6.6 (4.1 TO 9.1)	25.0 (2.6 TO 52.2)	N/A

Figure 1 Mean (standard deviation as error bars) serum concentrations of non-protein bound iron (a), total iron (b) and latent iron binding capacity (c) during the first course. *significant p < 0.001

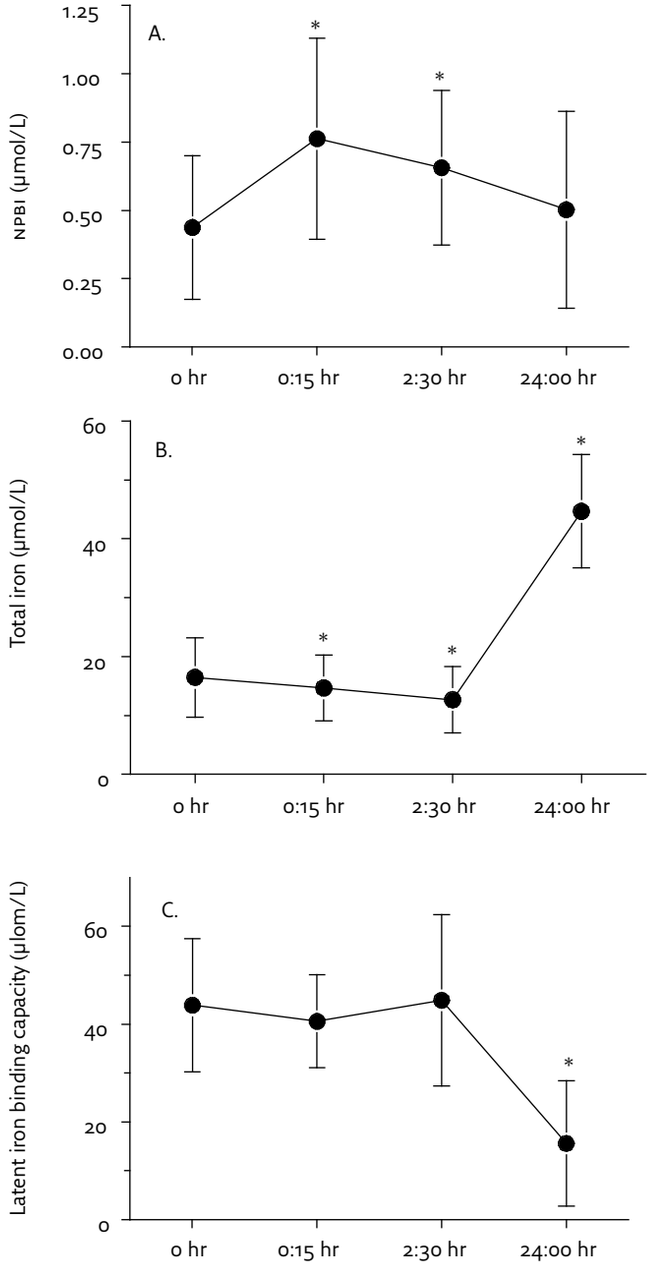
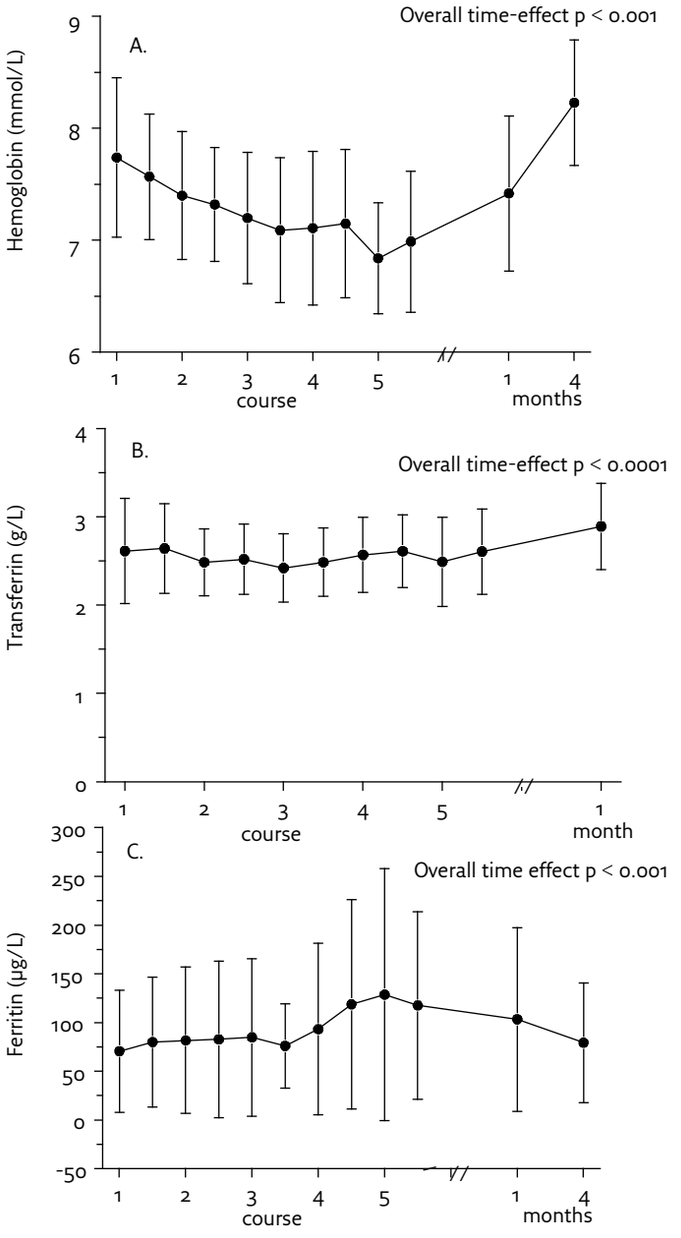


Figure 2 Mean (standard deviation as error bars) serum concentration of hemoglobin (a), transferrin (b) and ferritin (c) at baseline and 24 hours after each chemotherapy course and during the follow-up period.



CHAPTER 4

Increased Beat-to-Beat variation of the QT-interval in early-stage breast cancer patients treated with doxorubicin

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ABSTRACT

INTRODUCTION Diminished repolarization reserve is regarded as predictive for pro-arrhythmic events. Recently a new method for the evaluation of changes in QT-intervals, based on the dimensions of a Poincaré plot, has been developed to assess the repolarisation reserve. Further, it was recently discovered that doxorubicin, an antitumor drug with known cardiotoxic properties, influences cardiac repolarisation in rabbits. The aim of this study was to assess the effect of doxorubicin on cardiac repolarisation in humans using this new method.

PATIENTS AND METHODS In 39 patients treated with doxorubicin for early-stage breast cancer, 5-minute ECG recordings were obtained before, at 3 and 24 hr after the first and the last scheduled doxorubicin infusion. All ECG recordings were analyzed using fiducial fragment averaging, after which beat-to-beat QT variability was calculated. Data are shown as means and 95% confidence intervals (95%CI) and compared using analysis of variance.

RESULTS Mean short term QT-interval variability (STVmean) was 1.25 msec (95%CI: 1.08-1.42) at baseline of the first course and increased to 1.78 msec (1.48-2.08) and 1.81 msec (1.48-2.13) at 3 and 24 hrs after doxorubicin infusion respectively. During the last course a higher pre-dose STVmean of 1.72 msec (1.38-2.06) compared to the first course was observed. Also, the doxorubicin-induced increases were larger; STVmean increased to 2.45 msec (1.69-3.22) and 3.17 msec (2.35-3.99) at 3 and 24 hours post-administration respectively. Comparable changes in the normalized QTVI, as proposed by Berger, were observed.

DISCUSSION AND CONCLUSION We show that after doxorubicin infusion QT-variability increased, suggesting an effect of doxorubicin on the repolarisation reserve in humans. It remains to be elucidated whether these effects actually relate to an increased susceptibility for anthracycline induced cardiac failure.

INTRODUCTION

Prolongation of the QT/QTC interval is considered a risk factor for the development of arrhythmias, in particular Torsade de Pointes (TdP). Especially, drug-induced increases in QT/QTC-interval duration receive substantial scrutiny and has been an important reason for drugs to be taken off the market. To prevent these withdrawals it is obligatory that (almost) all drugs before receiving market authorization have to be evaluated for their potential to prolong the QT-interval.(1)

However, despite the wide-spread use of QT/QTC-prolongation as marker to assess pro-arrhythmic risk, there is increasing evidence that its ability to predict drug-induced arrhythmogenicity is limited.(2) Therefore, several other markers have been suggested, including T-wave morphology changes, increased spatial dispersion of repolarisation, and elevated lability of repolarisation also known as decreased repolarisation reserve.(3)

The latter can be measured using variation in T-wave morphology (eg T-wave alternans) and beat-to-beat QT-interval variations, such is the QT variability index as proposed by Berger.(4) Recently, another method for the assessment of short term beat-to-beat variations (STV) based on the dimensions of a Poincaré plot has been suggested.(5) Indeed, increased STV was predictive for the occurrence of TdP in animals (5-7) and also in humans increased STV was noted after administration of drugs with known arrhythmogenic potential such as sotalol.(8)

The cardiotoxicity of anthracyclines is well known and includes changes in repolarization (prolongation of QT-interval), arrhythmias, and congestive heart failure which develop years after exposure.(9) The mechanism of the arrhythmogenic potential of anthracyclines has never been fully explored. Recently it has been shown in animals that anthracyclines are able to diminish repolarization reserve, but is unclear if this also occurs in humans.(10)

We hypothesized that anthracyclines also reduce repolarisation reserve in humans at clinically employed doses. Therefore, serial 5-min ECG recordings obtained in female breast cancer patients treated with doxorubicin were analyzed for beat-to-beat QT variation and assessment of repolarization reserve.

METHODS

Patient population and study protocol

The patient population consisted of early-stage female breast cancer patients who underwent adjuvant treatment with a combination of cyclophosphamide and doxorubicin chemotherapy. Main exclusion criteria included pre-existing cardiovascular diseases, prior or concomitant use of drugs with known or suspected cardiotoxic effects, distant metastases, a history of other malignant disease, a life expectancy of less than one year, and elevated transaminases above 3 times the upper limit of normal. Eligible patients were scheduled for four or five (depending on the institutional guideline) three-weekly courses of doxorubicin 60mg/m2 and cyclophosphamide 600 mg/m2. Prior to every doxorubicin and cyclophosphamide administration all patients received anti-emetic therapy according to the institutional guideline. The medical ethical committee of Leiden University Medical Center (LUMC) approved the study protocol before inclusion of the first subject. All subjects gave written informed consent before participation.

ECG recordings and analysis

For each patient 5-minute ECG recordings (sampling rate 600/s, without filtering) were made at baseline, and at 4 and 24 hours after the start of the chemotherapy at the first and the last chemotherapy course, and 6 weeks after the last course. Recordings were made using the CardioPerfect device (Welch Allyn, Delft, The Netherlands). ECG recordings were analyzed for heart rate, QT-intervals and beat-to-beat QT-variability after fiducial segment averaging (FSA) (11) with the Intraval software package (Advanced Medical Systems, Maasdam, the Netherlands). FSA is based on the coherence of relative small segments within the QRS-complex from beat-to-beat. Fiducial points of each individual complex are first detected by the analysis software supplied with the ECG-recording device. The individual complexes were then cross-correlated in turn with the average of the remainder complexes until maximum correlation was attained. In a similar way the other trigger points were identified, after which a similar fine-adjustment procedure was followed. Two independent observers assessed whether the endT-segment was correctly cross-correlated, and if necessary correlation was manually adjusted until maximum correlation was attained.

QT variability parameters

Poincaré plots were constructed by plotting each QT value against the preceding value (figure 1). Short term QT variability (STV30) was calculated as proposed by Thomsen, [3] from the mean distance orthogonal to the diagonal between the points of the Poincaré plot in a window of 30 consecutive QT intervals. This window is moved over the total length of the recording, tracking the STV value over the full 5 min data set. This results in the following parameters: mean short-term variability (STVmean) that is defined as the average of all STV values in the 5 minutes, the QT variability index over 30 consecutive QT intervals (QTVI30) as proposed by Berger, the average of all QTVI30 (QTVImean) values, and the normalized overall variability index (QTVIN) over the entire 5 min recording period.

Heart rate variability

Heart rate variability was assessed according to the most recent guidelines using validated HRV-analysis software (The Biomedical Signal Analysis Group, Kuopio, Finland).(12) The analyses were performed for the time domain and included the RR-interval (RR), standard deviation of the RR-interval (RRSD), the root-mean square of the difference of successive R-R intervals (RMSSD) and the percentage of intervals differing more than 50 msec (NN50) were calculated. After Fourier transformation was done analyses in the frequency were performed for the very low frequency power (0-0.04 Hz), low frequency power (0.04-0.15 Hz), high frequency power (0.15-0.4 Hz) and the ratio between LF and HF were calculated.

Statistics

All variables were analyzed using a mixed model analysis of variance with time, group and time by group as fixed factors and subject as random factor. The following contrasts were calculated within the model: for both the first and the last chemotherapy course the value obtained at baseline was compared to the values at 4 and 24 hrs after start of the chemotherapy, comparison of the baselines at the first and last chemotherapy course, and comparison of the baseline at the first course with the value obtained at 6 wks follow-up value. The effects were reported as the estimate of the difference, least square mean estimates, 95% confidence intervals and the p-value. All calculations were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Baseline characteristics

Thirty-nine patients were included in this study. The median age of the patients was 49 years (range 30-66 years) and the mean BMI was 25.3 kg/m2 (SD 4.4). Twenty-three and sixteen patients completed the scheduled 4 or 5 courses of chemotherapy respectively, which translates into a mean cumulative doxorubicin dose of 255 mg/m2 (SD 58).

QT interval and QT variability

Mean corrected (linear) QT interval was 424 msec at baseline and was prolonged at the 4 hrs time point by 13 msec (95%CI: 7-19msec), this prolongation was still present 24 hrs after administration. During the last chemotherapy course similar increments were observed (table 2).

The mean short term QT variability (STVmean) was 1.25 msec at baseline of the first course and increased to 1.78 msec ($p<0.0001$) and 1.81 msec ($p<0.0001$) at 4 and 24 hours after the start of the doxorubicin infusion respectively. The baseline STVmean value before the last chemotherapy course was with 1.72 msec higher than the baseline value before the first course ($p < 0.01$). During the last course larger increases compared to the effects after the first course were observed; STVmean was 2.45 msec ($p <0.02$) and 3.17 msec ($p <0.0001$) at 4 and 24 hours post-administration respectively.

The normalized QTV was -1.73 (-1.83 to -1.63) at baseline and increased to -1.46 (-1.57 to -1.34) and -1.55 (-1.66 to -1.43) at 3 and 24 hours post-chemotherapy. During the last course QTV was -1.47 (-1.61 to -1.34) at baseline and increased to -1.31 (-1.47 to -1.14), -1.18 (-1.37 to -0.99) at 3 and 24 hours post-chemotherapy respectively (figure 2).

Short term RR-variability and QT-dispersion did not change significantly during the courses.

Heart rate variabilty

Mean RR-interval did not change during the courses, but we observed changes in autonomic nervous system mediated regulation of the heart rate variability (table 2). This comprised of changes in the parasympathetic and sympathetic activity in both the time and frequency domain. The change in parasympathetic activity consisted of changes in RMSSD, NN50 and the high frequency component of the spectral analysis. For the sympathetic activity changes in RRSD and the low frequency component of the spectral analyses were noted. Consistent with these changes were the changes in LF/HF ratio, suggesting a shift in sympathetic/parasympathetic balance.

Comparable changes were observed after the first and the last chemotherapy course.

DISCUSSION

The main finding of the present study is that it in humans a combination of doxorubicin and cyclophosphamide at clinically relevant doses diminishes repolarization reserve. This was

evidenced by both an increase in beat-to-beat QT-variation and changes in the normalized QT-variation index. The changes in repolarisation reserve were accompanied by a change in the heart rate variability.

Repolarization reserve is a measure of the ability of the myocardial membrane to maintain its normal repolarisation behavior.(13) Important contributors to a stable repolarisation are the normal function of the delayed rectifier potassium currents Ikr and Iks. Several factors that may influence the repolarization reserve, such as gender, electrolyte imbalances, and congestive heart failure have been described. Importantly, it was recently described that in rabbits repolarisation reserve was reduced after administration of doxorubicin.(10) In these experiments the animals became more susceptible to erythromycin-induced TdP. Interestingly, erythromycin blocks the rapid component of the delayed rectifier potassium current, Ikr. This observation is in keeping with clinical reports showing that patients being treated with anthracyclines are more susceptible to TdP after receiving Ikr-blocking drugs.(9;14-17)

It is already known that anthracyclines can prolong of the QT-interval, but the underlying mechanism is unclear.(18) We suggest that the decreased repolarization reserve may play a role in the pro-arrhythmogenic properties of anthracyclines. Also beat-to-beat QT-variation is increased after exposure to doxorubicin, as shown by the increased short term variability and changes in the normalized QT-variation index.

The molecular or electrophysiological mechanisms underlying the finding are not immediately clear. Doxorubicin is not known to directly block Ikr/Iks channels. However, it may be possible that doxorubicin specific factors, including doxorubicin induced down-regulation of Ikr, increased interventricular and transmural heterogeneity by reduced cell-to-cell coupling, apoptosis of cardiomyocytes and the fact that the secondary alcohol metabolite of doxorubicin, doxorubicinol, influences several ion-pumps play a role.(13) It is also known that repolarisation reserve is influenced by the autonomic nervous system.(12;19) As we showed that both the parasympathetic and sympathetic activity were affected by the chemotherapy, altered nervous system tone could also have contributed to our observations.

There are several limitations in our experiment to unambiguously ascribe the observed effects to doxorubicin. First, the chemotherapy included both anthracyclines and cyclophosphamide rendering it theoretically possible that the observed effects are attributable to the administration of cyclophosphamide. However, we consider this unlikely as there is no evidence that cyclophosphamide has effects on cardiac conduction. Secondly, it might have been that general stress experienced by the patients caused by their clinical condition and the anticipation of treatment altered autonomic nervous system tone. We consider it unlikely that this causes the changes in repolarization reserve during the courses, as a constant "stress-level" can be assumed during a chemotherapy course.

Finally, our data conform with the findings after doxorubicin in animals.

In conclusion, we showed for the first time in humans that assessing beat-to-beat QT-variability in 5 min ECG recordings is a suitable approach to assess changes in repolarisation reserve. The results of the analysis suggest that doxorubicin decreases repolarization reserve in female breast cancer patients and confirms results in animals. We suggest that this method may possibly be suitable as a marker for anthracycline-induced arrhythmogenicity in humans.

It is tempting to speculate that STV could also be of use in drug-development programs to identify agents capable of inducing pro-arrhythmic events, but this has to be investigated further by exploring the effects of QT-prolonging agents with and without known association to TdP.

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Table 1

Baseline characteristics	**n = 39**
	MEAN ± SD
QT variability parameters	
QT (msec)	405 ± 27
QTC (msec)	424 ± 19
QTVI	-1.73 ± 0.29
STVi (msec)	1.24 ± 0.51
HRV analysis	
RR (msec)	881 ± 117
RRSD (msec)	33 ± 13
RMSSD (msec)	29.9 ± 15.3
NN50 (%)	11.0 ± 13.0
LF (n.u.)	53.3 ± 18.0
HF (n.u.)	46.7 ±18.0
LF / HF	1.52 ± 1.11

QTVI: QT-variation index; STVi: short term variation; QTd: QT dispersion; RR: RR-interval; RRSD: RR-interval; RMSSD: root-mean square of the difference of successive R-R intervals; NN50: percentage of intervals differing more the 50 msec; LF: low frequency component of HRV-spectral analysis, normalized units; HF: high frequency component of HRV-spectral analysis, normalized units.

Table 2

	1st course			last course			follow up
	pre-dose	4 hours	24 hours	pre-dose	4 hours	24 hours	
	LSM (95% - CI)	LSM (95% - CI)	LSM (95% - CI)	LSM (95% - CI)	LSM (95% - CI)	LSM (95% - CI)	LSM (95% - CI)
QT variability parameters							
QT (msec)	406 (397 TO 415)	415 (406 TO 423)	420 (412 TO 429)	404 (393 TO 415)	421 (410 TO 432)	427 (417 TO 438)	405 (395 TO 414)
QTC (msec)	424 (418 TO 431)	437 (431 TO 443)	436 (431 TO 441)	429 (422 TO 436)	447 (439 TO 454)	451 (443 TO 458)	427 (420 TO 434)
QTVI	-1.73 (-1.84 TO -1.61)	-1.43 (-1.57 TO -1.29)	-1.50 (-1.64 TO -1.36)	-1.40 (-1.60 TO -1.20)	-1.25 (-1.44 TO -1.06)	-1.08 (-1.30 TO -0.86)	-1.47 (-1.62-1.33)
STV (msec)	1.25 (1.08 TO 1.42)	1.78 (1.48 TO 2.08)	1.81 (1.48 TO 2.13)	1.72 (1.38 TO 2.06)	2.45 (1.69 TO 3.22)	3.17 (2.35 TO 3.99)	1.55 (1.33 TO 1.78)
HRV analysis							
RR (msec)	882 (843 TO 921)	854 (818 TO 891)	899 (857 TO 942)	839 (795 TO 882)	840 (805 TO 876)	848 (810 TO 886)	861 (812 TO 910)
RRSD (msec)	33.5 (29.1 TO 37.9)	28.8 (24.8 TO 32.8)	31.5 (27.4 TO 35.6)	28.1 (24.0 TO 32.2)	26.1 (22.6 TO 29.7)	29.3 (25.1 TO 33.4)	34.7 (27.6 TO 41.7)
RMSSD (msec)	30.1 (24.9 TO 35.3)	25.2 (20.5 TO 29.8)	32.3 (26.5 TO 38.1)	24.8 (20.1 TO 29.4)	21.8 (18.1 TO 25.5)	28.7 (23.2 TO 34.3)	32.3 (24.4 TO 40.2)
NN50 (%)	10.8 (6.4 TO 15.2)	6.1 (2.4 TO 9.8)	12.7 (7.4 TO 18.0)	8.2 (4.7 TO 11.8)	4.4 (1.8 TO 7.0)	8.9 (4.0 TO 13.9)	10.9 (5.5 TO 16.3)
LF (n.u.)	54 (47 TO 60)	56 (50 TO 62)	46 (39 TO 52)	58 (51 TO 66)	58 (52 TO 63)	49 (42 TO 56)	55 (47 TO 62)
HF (n.u.)	46 (40 TO 53)	44 (38 TO 50)	54 (48 TO 61)	42 (34 TO 49)	42 (37 TO 48)	51 (44 TO 58)	45 (38 TO 53)
LF / HF	1.5 (1.1 TO 1.9)	1.7 (1.2 TO 2.2)	1.2 (0.8 TO 1.6)	2.3 (1.3 TO 3.3)	1.8 (1.3 TO 2.4)	1.3 (0.8 TO 1.8)	2.0 (1.0 TO 3.0)

QTVI: QT-variation index; STVi: short term variation; QTd: QT dispersion; RR: RR-interval; RRSD: RR-interval; RMSSD: root-mean aquare of the difference of successive R-R intervals; NN50: percentage of intervals differing more the 50 msec; LF: low frequency component of HRV-spectral analysis, normalized units; HF: high frequency component of HRV-spectral analysis, normalized units.

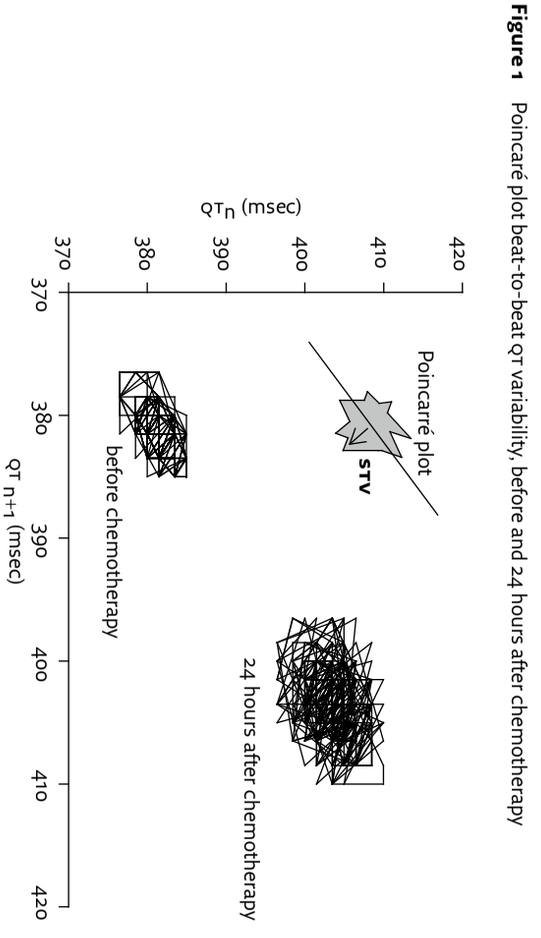

Figure 1 Poincaré plot beat-to-beat QT variability, before and 24 hours after chemotherapy

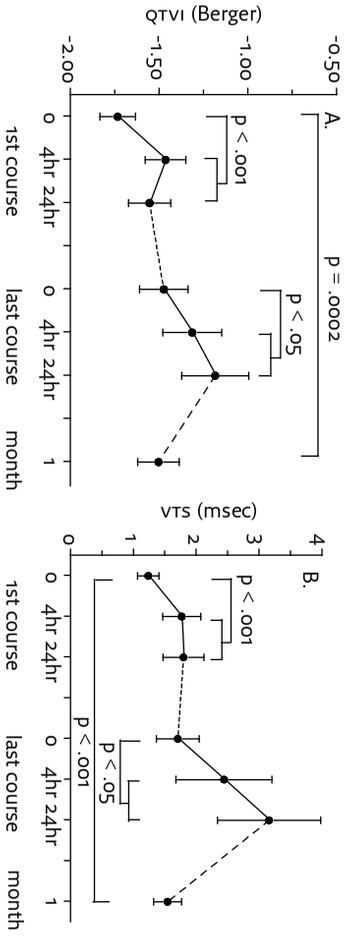

Figure 2 A: QT variation index, B: short term variability

CHAPTER 5

Disturbed iron metabolism due to anthracycline-based chemotherapy in early stage, surgically cured female breast cancer patients

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ABSTRACT

AIM To study the pharmacokinetics, safety and tolerability of single rising doses up to 80mg of PC-SOD in healthy Caucasian volunteers.

METHODS This double blind, placebo controlled, 4-period cross over study was performed in eight healthy volunteers (4 male/4 female). Three doses of PC-SOD (20, 40 and 80 mg) and placebo were administered IV in randomised order. Serum and urinary PC-SOD concentrations were measured pre-dose and up to 96 hours after dosing. In addition to standard safety measurements, the urinary excretion of NAG, a-GST, p-GST was measured to evaluate renal function. The PK of PC-SOD was analysed using non-compartmental and compartmental methods.

RESULTS All treatments were well tolerated, and no obvious relationship between adverse events and treatment was observed. No effects of PC-SOD on renal function could be detected. Dose normalised C_{max} and AUC were not different between the different dosages, indicating linearity of plasma concentrations with dose. Estimated PC-SOD clearance was 2.54 ml/min (95%-CI 2.07-2.83). The terminal half-life was estimated to be 1.54 days (95%-CI: 0.93-2.15). SOD activity was elevated above baseline for 19 ± 6 hours after the 80mg dose.

DISCUSSION Single IV administrations of PC-SOD in doses up to 80 mg were well tolerated in healthy Caucasian male and female volunteers. With the doses used, SOD-activity was linearly related to the dose, after the 80mg dose it was present for an appreciable period. These findings suggest that it is worthwhile to investigate PC-SOD in clinical conditions characterised by a high radical overload.

INTRODUCTION

Reactive oxygen species (ROS), like super oxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), play an important role in health and disease. They have been implicated in the pathophysiology of different disease states, including anthracycline-induced cardiotoxicity (AIC), inflammatory bowel disease, ischemia/reperfusion injury and neurodegenerative conditions.(1-8) The hypothesis is that in these pathologic conditions, relatively large amounts of ROS are produced which cause functional damage to many tissues and even apoptosis.(9) The underlying mechanism for the deleterious effect of ROS on tissues is not totally unravelled, but includes cell membrane damage due to lipid peroxidation, and direct damage to proteins and DNA.(9)

There are different endogenous defence mechanisms against the ROS damage such as superoxide-dismutase (SOD), catalase, peroxidases and vitamin A and E which all share free radical scavenger properties.(10-12)

SOD acts as a free radical scavenger by catalysing the dismutation of superoxide to hydrogen peroxide and oxygen as shown below:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + OO_2^{\bullet -}$$

Three iso-forms of SOD exist in humans: cytosolic Cu,Zn SOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular Cu,Zn SOD (SOD3) of which the intracellular forms are the more abundant. The endothelial cell surface is protected by SOD3, but this protection seems insufficient in many clinical conditions and therefore it has been suggested that additional protection may be of benefit.(13) Indeed, over the last decade therapeutic use of SOD has been explored, but there is consensus that up to now this has been of limited value. (14) Likely explanations for the limited success of exogenously administered SOD are that the intracellular iso-forms of SOD hardly bind to the endothelium and that they are relatively short-lived. In addition, particularly for SOD3, which is an attractive candidate for therapeutic use, the manufacturing process is difficult.(15)

Therefore, there is a need for SOD preparations that are relatively easy to manufacture, show a reasonably long residence time in the body and will be taken up by organs that are relatively poorly protected against free radicals. This has resulted in the development of PC-SOD (recombinant human SOD1 covalently coupled to an average of 4 molecules of lecithin) and a chimeric recombinant superoxide dismutase consisting of SOD2 and SOD3. (13;16;17)

PC-SOD has a higher affinity to the cell membrane, an enhanced distribution to various tissues and a prolonged systemic half-life compared to SOD1 alone. In addition, it has a 4.5-fold increase in oxygen-radical scavenging effects resulting in a 100-fold increase in protective effects against vascular endothelial cell injuries, compared to unmodified SOD.(16) Pre-clinical data showed that PC-SOD is effective in several models including inflammation, chemotherapy-induced cardiotoxicity, ischemia-reperfusion injury, and motor dysfunction after spinal cord injury.(18) The pre-clinical data also indicated that PC-SOD is well tolerated, although multiple doses to monkeys were associated with the presence of lipid inclusion bodies in renal tubular cells. However, this was entirely reversible and not associated with functional impairment or necrosis of cells. Thus, PC-SOD is a potentially protective agent in pathological conditions mediated by free radical overproduction. (19-22)

In a previous study in Japanese volunteers, where doses up to 20mg were investigated, PC-SOD was well tolerated, but the duration of increased elevation of SOD activity was only 3 hrs which is too short to be of likely clinical relevance. The current study was performed to assess the tolerability, pharmacokinetics and effects of single (higher) ascending doses of PC-SOD in healthy Caucasian volunteers. The study was designed such that detectable SOD activity would be present for a period of 12-24 hrs. Furthermore, special attention was given to the effects of the compound on renal function and tubular integrity as this was an issue with very high doses of PC-SOD in pre-clinical experiments. The effects on renal function were assessed by measurement of the urinary excretion of specific markers for tubular damage (N-acetyl-ß-glucosaminidase (NAG), α- and Π-glutathione S-transferase (GST)) and microalbumin.

SUBJECTS AND METHODS

The study protocol was approved by the Medical Ethical Committee of the Leiden University Medical Center and performed according to the principles of the International Conference on Harmonisation and Good Clinical Practise and the Helsinki Declaration. Written informed consent was obtained for all subjects before study entry.

Subjects

Eight healthy subjects (4 female and 4 male) aged between 18-45 years and within 20% of the normal body weight range relative to height and frame size were included in this double blind, placebo-controlled, 4-way cross over study. Subjects were included after a full medical screening showed no clinically significant abnormalities. Subjects were excluded in case of a history of drug allergy or hypersensitivity, or drug, alcohol or nicotine abuse.

Study medication

The subjects were dosed 4 times using an ascending dose schedule with randomised placebo as summarised in table 1. Dose escalation was performed when no significant clinical abnormalities were observed after the previous lower dose. The washout period between doses was at least 1 week.

The PC-SOD preparation consists of an average of 4 molecules lecithin derivative covalently bound to the human derived CuZn-SOD, produced by genetic recombination using *E.coli* as a host cell. The lecithinised product has 3x103 U SOD-activity per mg. For this study, a single batch of the lyophilised formulation also containing sucrose was used. Placebo consisted of sucrose. The final preparation that was administered consisted of PC-SOD or placebo diluted with distilled water and 5% mannitol.

Study days

The subjects were admitted to the research unit after an overnight fast. After preparation and baseline measurements, the study drug was administered intravenously over 60 min. During the study days, frequent measurements of vital signs, 12-lead ECG recording and evaluation of adverse events, blood sampling and fractionated urine collection took place. The subjects remained in the unit for 24 hrs and returned for follow-up assessments and blood sampling at 48 and 96 hours after dosing. During the study day's subjects used standard meals and abstained from using xanthine-containing drinks or food.

Sampling and assays

Serum PC-SOD concentrations and SOD-activity were measured in venous blood samples that were taken pre-dose (twice), at 20, 40, 60, 65, 75, 90 min, and at 2, 3, 4, 8, 12, 24, 48, 96 and 168 hrs after start of the infusion. The last time point coincided with the first pre-dose sample of the subsequent study day. After collection, the tubes were kept at 4°C for and subsequently centrifuged at 2000*g* for 10 minutes at 4°C. The separated serum was stored at -20°C until analysis within 1 month after sampling.

Urine was collected during the study period over the following time spans: 0-4 hr, 4-8 hr, 8-12 hr, 12-24 hr, and 24-48 hr. Urine samples were, immediately after voiding, stored at 4°C and from each collection period, aliquots of 2 ml were taken and stored at -20°C until analysis within 1 month after sampling. Samples to assess antibody formation were taken at completion of the last administration and at 1 and 3 weeks after the last dosing.

Blood samples for routine haematology and biochemistry were taken before and at 24 hrs after each infusion.

Serum and urinary PC-SOD concentrations were measured using an enzyme linked immunosorbent assay (ELISA), consisting of an antibody against human Cu, Zn-SOD, and a second antibody against human Cu, Zn-SOD conjugated with horseradish peroxidase. The assay has a lower limit of quantification 626 ng/mL. The intra-assay variability and inter-assay was investigated at PC-SOD concentrations of 626, 2500 and 10000 ng/ml for serum and 626, 5000 and 20000 ng/ml for urine; each concentration in triplicate. The coefficients of variation for the intra-assay variability for the respective concentrations were 5.6, 3.2 and 1% in serum, and 7.3%, 2.3% and 2.3% in urine. The coefficients of variation for the inter-assay variability in serum and urine were 7.9, 2.7 and 1.3% and 4.9%, 8.2% and 1.2% respectively. Repeated freezing and thawing had no appreciable effects (CV < 10% after 3 freeze/thaw cycles).

PC-SOD activity was measured using a nitrite method previously described.(23)

The test is based upon the principle that when hypoxanthine and xanthine-oxidase are brought together superoxide anion is formed. When superoxide anion reacts with hydroxylamine, nitrite is formed and this can be measured by colour densitometry with the aid of a colouring reagent. SOD present in serum will inhibit the formation of nitrite by reacting with the superoxide anion. Serum SOD activity was quantified using the reduction in superoxide anion generation caused by serum added to the system. The assay had a lower limit of quantification of 3 µg/mL. Intra- and interassay variability was 3.9% and 7.5% for serum and 6.8% and 10.9% for urine respectively. Both assays were performed at Daiichi Pure Chemicals Co. Ltd, Ibaraki (Japan).

Antibody formation against PC-SOD was measured by quantification of specific IgE, IgG and IgM titres. For anti-PC-SOD IgE antibody measurement, anti-human IgE mouse monoclonal antibody (alkaline phosphatase labelled) was used as secondary antibody. The titre was qualitatively judged using the level of the positive control (human anti-perennial rye class IgE antibody) as the reference value and was described as positive if the titre was higher than 0.2 IU/ml. For anti-PC-SOD-IgG+IgM measurements anti-human IgG and IgM mouse monoclonal antibody (alkaline phosphatase labelled) was used as secondary antibody. The titre was qualitatively judged in reference to the antibody level of a pooled normal human serum sample (negative control) and indicated as positive if the value exceeded the average value of 4 normal human serum samples 3.1-fold.

Urinary NAG activity was measured using a commercially available colorimetric assay (Roche Diagnostics, Switzerland,

reference value: 1.39 - 3.23 U/24hr, detection limit 1 U/L). Urinary excretion of alpha-glutathione S-transferase (α-GST) and pi-glutathione S-transferase (π-GST) was determined using validated quantitative enzyme immunoassays (Biotrin, Dublin, Ireland; limit of detection: α-GST 0.09 µg/L and π-GST 1.72 µg/L and both intra- and inter assay variability below 6.9%). Urinary microalbumin and creatinine concentrations were measured using routine methodology at the central laboratories for clinical chemistry of LUMC.

Data analysis

Vital signs, ECG and laboratory parameters were analysed by generating average graphs of parameters over time per treatment. If these graphs suggested possible differences between treatments, areas under the effect curve over the first 12 hours divided by the corresponding time span (AUC) were calculated and compared between treatments using factorial analysis of variance (factors subject and treatment).

The cumulative urinary excretion of NAG, α-GST, π-GST and creatinine over 0-4h and over 0-48h were calculated. For values below the detection limit, the detection limit was used. The cumulative 24 hours microalbumin excretion was evaluated as the microalbumin over creatinine ratio. The values were compared between treatments using factorial analysis of variance (factors subject and treatment).

The pharmacokinetics of PC-SOD was assessed using a non-compartmental PK approach for C_{max}, $AUC_{0\text{-}48hr}$ and $AUC_{0\text{-}7days}$. These parameters were compared between doses after dividing the parameter by the doses using factorial analysis of variance (ANOVA; factors subject and dose) to assess dose-linearity. Within-individual ratios for the different doses were compared using paired Student t-tests.

Compartmental pharmacokinetics (using a two compartment open model) was performed on all of the profiles by analysing the data as arising from a multiple dose sequence. The analyses were performed using non-linear mixed effect modelling, which estimates all curves for all subjects simultaneously. First order conditional error estimation with the 'interaction' option was used and residual error was modelled as the sum of an additive and a constant coefficient of variation component.

Multiplying the urine weights with the associated concentrations and summing over 48 hours calculated the cumulative excretion of PC-SOD. Average renal clearance over this period was calculated by dividing the cumulative renal excretion by the serum AUC over the same time span. Renal clearance was compared between doses using factorial analysis of variance (factors subject and treatment).

The relationship between activity and serum concentration was investigated using graphical and regression techniques. Linear mixed effect modelling was performed to examine the relationship between PC-SOD concentration and SOD activity.

The compartmental pharmacokinetic analyses were performed using NONMEM version V (GloboMax LLC, Hanover, MD). All statistical calculations were performed using SPSS for Windows software (SPSS, Inc., Chicago, IL).

RESULTS

General

Eight subjects (4 female and 4 male; age range: 18-27 years; mean BMI: 23.4 kg/m2) were included. All subjects completed the study and no important drug-related adverse events were noted. No serious adverse events occurred during the study. There was no obvious relationship between the occurrence of any adverse event and one of the treatments. The most frequently observed adverse event was an upper respiratory tract infection, which occurred on placebo (twice) as well as on active drug (twice after 20 and 40 mg and three times after 80 mg). Other common adverse events were headache and haematoma's after blood sampling. One subject experienced multiple premature ventricular complexes, independent of treatment. No clinical significant changes were observed during any treatment in vital signs, ECG-monitoring, and the routine laboratory tests. No antibodies against PC-SOD were found during 2 subsequent follow up visits.

PC-SOD concentrations

For two occasions at which placebo was infused (F3 and F4; both female) concentrations of PC-SOD were found in 5 samples (5/543 = 0.92%). No explanation for this anomaly could be found, and these data were omitted from the analysis.
Mean plasma profiles are given in figure 1, and the non-compartmental parameters (C_{max} and $AUC_{0\text{-}7days}$) are summarised in table 2. No significant changes were observed in the dose normalised C_{max} (p=0.402) and $AUC_{0\text{-}7days}$ (p=0.102) for the different doses given, indicating linear pharmacokinetics. The within individual ratio's (40 vs 80mg) were 1.98 (95%-CI: 1.80-2.14), 1.99 (95%-CI: 1.71-2.27) and 1.88 (95%-CI: 1.60-2.16) for C_{max}, $AUC_{0\text{-}48}$ and $AUC_{0\text{-}7days}$ respectively, which confirmed that no significant dose effect was present. The mean cumulative excretion of PC-SOD over 48 hours increased with higher doses (table 3), but renal clearance was independent of the dose (p=0.154).

When the profiles were modelled using a 2-compartment model and as if originating from a multiple dose regimen, a good fit of the data was obtained (figure 1; table 2). When the model parameters are expressed differently, estimates for the half-lives can be calculated. This showed that the initial half-life ($t_{½\alpha}$) was 11.0 hours (95%-CI: 5.0-17.0) and terminal half-life ($t_{½\beta}$) was 1.54 days (95%-CI: 0.93 – 2.15).

PC-SOD activity

After the 20 mg dose, SOD-activity could not be detected for a number of individuals, which may be attributed to the relatively high limit of quantification. At each higher dose, a higher SOD activity was observed which was present for a longer time-period (figure 2). Mean ± SD maximum SOD activity increased from 10.4 ± 2.8 µg/ml after 40 mg to 18.7 ± 2.0 µg/ml after 80 mg PC-SOD dosing. Analysis after log-transformation revealed a (back-transformed) geometric mean ratio of 1.85 (95%-CI: 1.53 - 2.24) indicating a doubling of activity with a doubling of administered dose. The mean ± SD duration of the period during which SOD activity was above the limit of quantification increased from 8 ± 3 hours after the 40mg dose to 19 ± 6 hours after the 80mg dose.

Relationship between activity and concentration in serum

Individual graphs indicated that a linear model was most suitable to describe the PC-SOD concentration - SOD activity relationship. The average estimated linear relationship between PC-SOD concentration and SOD activity had an intercept of 650 ng/ml (95% CI: -746 - 2046) and a slope of 0.913 (0.790 – 1.036) ng SOD activity per ng PC-SOD.

Effects on renal function

The urinary excretion of NAG, α-GST, π-GST and microalbumin/creatinine ratio over both 4 (not shown) and 48 hours (table 3) after each subsequent dose, did not differ between active drug and placebo.

DISCUSSION

This study showed that single iv administration of PC-SOD in doses up to 80 mg was well tolerated in healthy Caucasian volunteers. For all safety parameters that were assessed, no treatment effect was observed. Particularly, the absence of effect on renal function is important, as there were indications from pre-clinical data that PC-SOD could possibly affect renal function. All markers for evaluation of renal function, including protein and creatinine excretion, did not show differences between the different PC-SOD doses and placebo. In our assessment urinary NAG, a- and p-GST were included, enzymes used to evaluate tubular damage. The first is derived from tubular lysosomes, the latter are cytosolic enzymes that are found in the proximal and distal tubular cells respectively. All these markers are specific for tubular damage and are very sensitive in detecting renal dysfunction in a very early stage.(24)

These findings suggest that single iv doses of PC-SOD up to 80 mg is not associated with untoward effects on renal function in humans.

Non-compartmental pharmacokinetic analyses indicated linearity of serum concentrations with increasing dose. The compartmental pharmacokinetic analysis of the PC-SOD profiles was complicated by the occurrence of detectable PC-SOD concentrations in 5 samples of 2 subjects (<1% of the total amount of samples) during placebo treatment. Sampling and environmental factors were investigated for these samples but no explanation was found for the aberrant results. It may be that an interfering endogenous compound was present in these subjects. The data of these samples were omitted and this resulted in an adequate description of the concentration profiles. It was shown that the compound has a relatively small central volume of distribution (5 L) and a low clearance (2.5 ml/min). As the renal clearance was only approximately 0.05 ml/min, it is concluded that the clearance is predominantly extra-renal. This is in keeping with data in non-human primates using [3H]-labelled PC-SOD showing that only 10% of PC-SOD is excreted unchanged in the urine. Although the exact clearance mechanism of PC-SOD remains to be elucidated, it is likely that the compound is cleared through multiple mechanisms among which utilization in various biochemical processes, hepatic clearance and inactivation by esterase's may play a role.

Previous trials with SOD-preparations failed to show beneficial effects in humans.(25) A cause of this failure could be the short half-life of these compounds. With the doses used in this study, it was shown that SOD-activity was linearly related to the dose, and that is was present for an appreciable period. After the 80 mg dose, the SOD-activity was elevated above baseline for at least 24 hrs. This indicates that PC-SOD could be beneficial in pathological conditions characterised with an acute ROS-overload, like ischemia/reperfusion injury, neurological ischemic disease and AIC.(19;26-28) Another reason why earlier trials with SOD-preparations in humans did not show beneficial effects may be explained by the finding that in these trials the target such as the cytosol and the mitochondria was not reached. This seems necessary as especially the intra-cellular isoforms of SOD play an important role in the protection against myocardial damage after ischemia/reperfusion.(29-31) Due to its increased affinity for the cell membrane it is possible that with PC-SOD this problem can be overcome.(16) Indeed, several in vitro and in vivo studies showed beneficial effects of PC-SOD in various disease models.(19;26-28;32-38)

The study reported here has some shortcomings. First, only serum PC-SOD activity was measured and no information is provided on the presence of PC-SOD intracellularly or at the cell membrane. In this study we found a small volume of distribution of PC-SOD in humans. This suggests that the drug does not have high intracellular penetration and hence its likely therapeutic benefit will only be assessable after demonstration of intracellular activity. However, it may also be that the beneficial effects of PC-SOD are not dependent on the intracellular activity as the volume of distribution (range: 0.05-0.10 L/kg) in animal species in which the compound was tested for efficacy is comparable to the volume of distribution in humans (0.07 L/kg). Second, it seems paradoxical that SOD converts $O_2^{\bullet -}$ in H_2O_2 which is also a ROS, and therefore potentially harmful. However, although the exact mechanism is not elucidated, it is apparent that this does not translate into 'clinical damage'. Indeed, many laboratory models show that administration of exogenous SOD provides protection against damage induced by free radicals.(19;26-28;32-38) Moreover, in the protection against free radical induced damage during the reperfusion phase of ischemia-reperfusion injury, there are strong indications that SOD is of prime importance.(39)

In summary, this study showed that PC-SOD in doses up to 80 mg was well tolerated in healthy Caucasian volunteers. For the 80 mg dose, serum SOD-activity was elevated above baseline for at least 19 $\pm$ 6 hours. These findings suggest that is worthwhile to further investigate PC-SOD as protective agent in patients with clinical conditions associated with a high radical overload.

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Table 1 Administration schedule of PC-SOD

Subject code	Study day 1	Study day 2	Study day 3	Study day 4
F1/M1	20 mg PC-SOD	40 mg PC-SOD	80 mg PC-SOD	Placebo
F2/M2	20 mg PC-SOD	40 mg PC-SOD	Placebo	80 mg PC-SOD
F3/M3	20 mg PC-SOD	Placebo	40 mg PC-SOD	80 mg PC-SOD
F4/M4	Placebo	20 mg PC-SOD	40 mg PC-SOD	80 mg PC-SOD

F = female, M = male

Table 2 Mean (SD; n=8) Pharmacokinetic parameters of PC-SOD administered as iv-infusion over 1 hour. The summary of the non-compartmental analyses is given in the upper part of table and the parameters based upon population pharmacokinetic approach using a 2-compartment pharmacokinetic model are given in the lower part of the table.

Non-compartmental pharmacokinetic parameters for IV SOD			
	DOSE (mg)		
Parameter	20	40	80
C_{max} (µg/ml)	4.95 (0.91)	9.33 (1.12)	18.38 (2.58)
Dose-normalised C_{max} (ng/ml/mg)	247 (46)	233 (28)	230 (32)
$AUC_{0\text{-}7days}$ (µg/ml•day)	6.73 (1.77)	11.63 (2.21)	21.67 (4.64)
Dose-normalised $AUC_{0\text{-}7days}$ (ng/ml•day/mg)	336 (88)	291 (55)	271 (58)
Compartmental pharmacokinetic parameters for IV PC-SOD			
	MEAN	95%-confidence interval	
Clearance(L/day)	3.53	2.98 – 4.08	
Intercompartmental clearance(L/day)	1.17	0.57 – 1.77	
Central volume(L)	4.98	4.37 – 5.59	
Steady State volume(L)	7.44	6.70 – 8.18	
Initial half-life*(days)	0.47	0.21 – 0.72	
Terminal half-life*(days)	1.54	0.93 – 2.15	
Residual error			
Constant CV(%)	42.1		
Additive SD(ng/ml)	20.2		

CV: inter-individual variability in population parameters; *results from alternative parameterisation.

Table 3 Summary of urinary PC-SOD excretion. Urinary PC-SOD excretion in 48 hours (percentage of dose, SD; n=8) and renal clearance of PC SOD over 48 hours. (upper panel) Cumulative urinary excretion of NAG, a-GST and p-GST over 48 hours and the ratio of microalbumin over creatinine 24 hr after IV administration of PC-SOD. (lower panel)

Urinary PC-SOD excretion					
PC-SOD dose	Placebo	20 mg	40 mg	80 mg	P-value
Cumulative PC-SOD excretion (% dose per 48 hours)	NA	1.58 (0.56)	1.03 (0.76)	1.52 (0.54)	NA
Renal clearance PC-SOD over 48 hours† (ml/min)	NA	0.048 (0.015)	0.036 (0.026)	0.057 (0.022)	NA
Renal safety parameters					
PC-SOD dose	Placebo	20 mg	40 mg	80 mg	P-value
NAG (U)*	4.1 (1.8)	4.0 (1.6)	3.7 (1.4)	4.3 (1.4)	0.77
a-GST (µg)*	14.1 (6.5)	18.6 (12.6)	14.5 (8.7)	15.1 (9.1)	0.35
p-GST (µg)*	9.5 (3.2)	10.4 (3.2)	8.9 (2.6)	9.6 (3.1)	0.53
Microalbumin/ creatinine ratio 24 h after dose	0.043 (0.031)	0.039 (0.023)	0.044 (0.024)	0.03 (0.017)	0.53

† Average renal clearance was calculated using the serum AUC over 48 hours: renal clearance$_{0\text{-}48h}$ = cumulative renal excretion$_{0\text{-}48h}$/serum $AUC_{0\text{-}48h}$ No difference in renal clearance between the different doses was observed (p=0.154)
* normal values: NAG-excretion: 2.8 – 6.4U per 48 hours;a-GST: < 22.2 µg per 48 hours; p-GST: < 85.2 µg per 48 hours

Figure 1 Mean (+SD) observed PC-SOD serum concentration-time profiles (symbols) following IV administration of PC-SOD. The lines indicate the predicted profiles based upon the pharmacokinetic modelling.

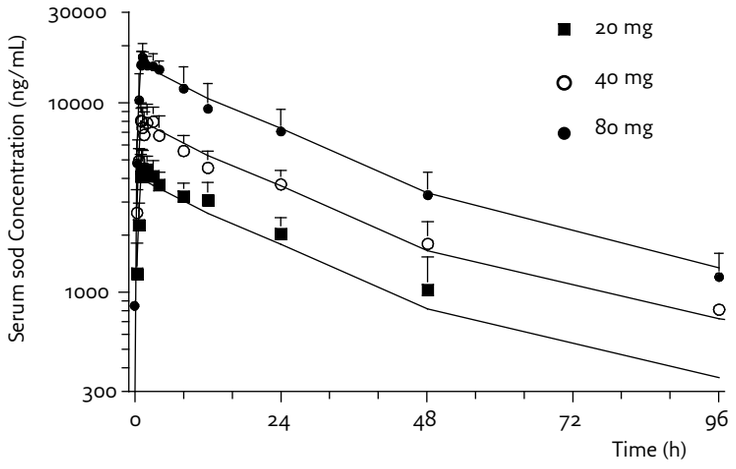
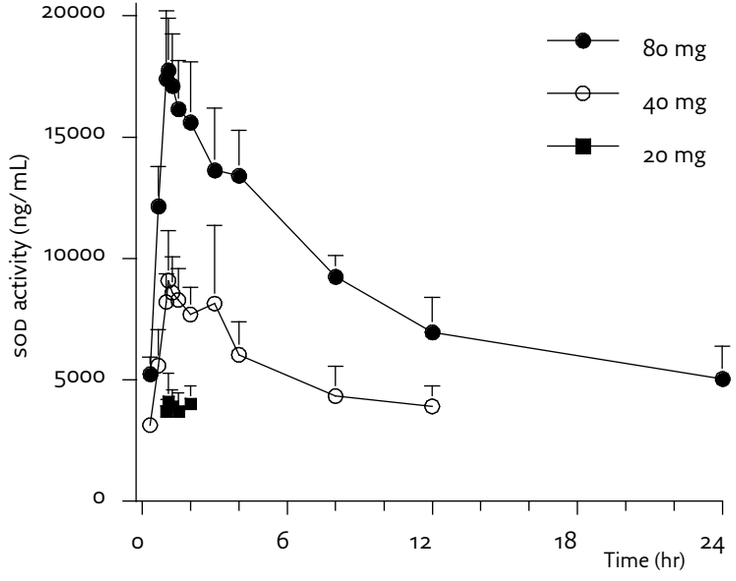


Figure 2 Mean (SD) SOD activity profile after intravenous administration of 20, 40 and 80 mg PC-SOD.



CHAPTER 6

The pharmacokinetics of PC-SOD, a lecithinized recombinant superoxide dismutase, after single- and multiple-dose administration to healthy Japanese and Caucasian volunteers

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ABSTRACT

To study the pharmacokinetics (PK) of single rising intravenous doses (40-160mg) and repeated doses (80mg for 7 days) of lecithinized superoxide dismutase (PC-SOD) in Japanese volunteers and to compare the PK of PC-SOD between Caucasians and Japanese.

The Japanese study consisted of two parts: a single dose, open-label, dose-escalation and a multiple dose, single-blind, placebo-controlled part. The PK of PC-SOD was determined using non-compartmental and compartmental methods. PK-data from a study with PC-SOD in Caucasians was reanalyzed using the same methodology.

The mean (SD) terminal half-life of PC-SOD in Japanese subjects was 25 (4) hours for the 40mg and 80mg and 31 (15) hours for the 160mg dose. There was non-linearity between dose-normalized C_{max} and clearance (p-values 0.002 and 0.022). After multiple dosing, steady state was reached after 5 days. The observed accumulation ratio was 2.6 (0.5).

The PK of the single 80 mg dose was similar for Japanese and Caucasians.

The PK of PC-SOD was shown to be non-linear with dose which may be attributable to a saturable clearing mechanism. The relative long half-life of PC-SOD (>24 hrs) suggests that it is worthwhile to study the compound as protective agent in clinical conditions with free radical overload.

INTRODUCTION

Overproduction of free radicals, such as the superoxide anion, is associated with the pathology of different diseases.(1-3) Superoxide dismutase (SOD), which catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, is important in the defense against free radical overload.(4) It thus seems logical to develop SOD as a potential treatment modality. However, attempts to achieve this have failed mainly because exogenous SOD has a low affinity for the cell-membrane and has unfavorable pharmacokinetics (e.g. a very short half-life).(5) These characteristics limit the clinical use of SOD, as especially the intra-cellular isoforms of SOD play a role in protection against free-radical induced damage and exogenous SOD needs to be active for a certain period of time to exert its potential protective effect.(5-7)

Therefore a recombinant Cu,Zn SOD, covalently bound to on average 4 molecules of lecithin (PC-SOD), have been developed. In pre-clinical experiments PC-SOD has a 4.5 times greater oxygen-radical scavenging effect, which leads to a 100-fold increase in protective effect against O_2^- induced vascular endothelial cell damage compared with unmodified SOD.(8) In addition, a stronger binding to human vascular endothelial cells was demonstrated.(9) Furthermore, studies in rats showed that PC-SOD had a prolonged residence time, compared to unmodified SOD and was effective in various animal models.(2;3;10-19) These characteristics make PC-SOD a potentially protective agent in various pathological conditions that involve free radical overproduction.

Previous phase I trials in Caucasians demonstrated that PC-SOD was well tolerated in doses up to 80mg, but the pharmacokinetics in other ethnic groups has not been reported yet. This may be of particular importance for the clearance of PC-SOD as apparently most differences caused by ethnic factors occur during drug metabolism.(20)

Therefore, a pharmacokinetic study with single iv doses (up to 160mg) and repeated iv doses (80mg/day for 7days) of PC-SOD in healthy Japanese volunteers was performed. As a previously performed PK study in Caucasians used the same methodology, the PK of the single iv 80 mg dose were compared.

SUBJECTS AND METHODS

Subjects

For the study performed in Japan, eligible for study participation were male Japanese volunteers, within 20% of the normal body weight range relative to height and frame size. All subjects were screened prior to study participation and considered healthy based on history, physical examination and laboratory assessment. This study protocol was approved by IRB of The Kitasato Institute, Research Center for Clinical Pharmacology (formerly known as The Kitasato Institute Bio-Iatric Center). The study in Caucasian subjects was performed as previously described.(21) This protocol was approved by the Medical Ethics Committee of Leiden University Medical Center. From both Japanese and Caucausian subject's written informed consent was obtained before screening.

Study design

The study in Japanese subjects was done in three cohorts of six male volunteers who received escalating single doses of PC-SOD (40, 80 and 160 mg) in an open-label fashion and a single cohort of eight male volunteers who received seven daily doses of PC-SOD (80 mg) in a placebo-controlled design (6 active treatment, 2 placebo). Dose escalation occurred when no clinically significant safety issues were observed in the previous dose-level. The multiple dose part of the study started after completion of the highest dose of the single dose study.

The study in Caucasian subjects consisted of eight healthy subjects (4 female and 4 male) who received single doses of PC-SOD (20, 40 and 80 mg) in a double blind, placebo-controlled, 4-way cross-over study.

Trial medication

Recombinant human SOD (rhSOD) was produced in *Escherichia coli*, the exact procedure is described elsewhere.(8) One of the cysteine residues of rhSOD was converted to S-(2-hydroxyethyl-thio-) cysteine and phosphatidylcholine derivatives were then covalently bound to this modified rhSOD to produce PC-SOD. The specific activity of PC-SOD was about 3,000 U/mg of protein when assayed with the cytochrome C method using a xanthine-xanthine oxidase-cytochrome C system. Vials for injection containing 30 mg of PC-SOD were produced by a freeze-drying process with purified sucrose as an additive. The test drug was dissolved in xylitol 5% (Japan) or mannitol 5% (Netherlands). Placebo consisted of either xylitol or mannitol.

Study days (Japanese)

The subjects were admitted to the research unit after an overnight fast. After preparation and baseline measurements, the study drug was administered intravenously over 60 min. For the participants of the multiple-dose cohort the study drug was administered 7 times with an interval of 24 hours in between. During the study days, frequent measurements of vital signs, 12-lead ECG recording and evaluation of adverse events, blood sampling and fractionated urine collection took place. The subjects remained in the unit for 48 hrs (multiple dose: 72 hrs) and returned for follow-up assessments and blood sampling one and two weeks after (last) dosing. During the study days subjects had standard meals and abstained from using xanthine-containing drinks or food.

Sampling (Japanese)

PC-SOD serum concentrations were assessed before administration and at 30, 60, 90 minutes and 2, 3, 5, 9, 13, 25, and 48 hours after dosing (single dose). For the multiple dose part serum PC-SOD concentrations were assessed 60 minutes prior to each administration and at 30, 60, 90 minutes and 2, 3, 5, 9 and 13 hours on day 1 and 4. In addition PC-SOD concentrations were determined 23, 48, 72 and 168 hours after the last administration.

Cumulative urinary PC-SOD concentrations were measured at -12-0, 0-6, 6-12, 12-24, and 24-48 hours (and 48-72 hours for

the multiple dose cohort) after the start of administration, for the single dose cohorts and the first day and the last day of the multiple dose cohort. In addition during day 2 to 6 cumulative urinary PC-SOD concentration was measured for each 24 hour period.

For all cohorts safety laboratory assessments were done before each administration, at 24 hours and 1 week after PC-SOD administration. For the multiple dose cohort additional safety assessments were done at 48 and 72 hours after the last dose.

The study outline for the Caucasian subjects was comparable to those of the Japanese volunteers.(21)

Serum and urinary PC-SOD concentrations were measured using an enzyme linked immunosorbent assay (ELISA), consisting of an antibody against human Cu, Zn-SOD, and a second antibody against human Cu, Zn-SOD conjugated with horseradish peroxidase. The assay has a lower limit of quantification 0.626 µg/mL. The intra-assay variability and inter-assay was investigated at PC-SOD concentrations of 0.626, 2.50 and 10.0 µg/ml for serum and 0.626, 5.0 and 20.0 µg/ml for urine (each concentration in triplicate). The coefficients of variation for the intra-assay variability for the respective concentrations were 5.6, 3.2 and 1.0% in serum, and 7.3%, 2.3% and 2.3% in urine. The coefficients of variation for the inter-assay variability in serum and urine were 7.9, 2.7 and 1.3% and 4.9%, 8.2% and 1.2% respectively. Repeated freezing and thawing had no appreciable effects (CV < 10% after 3 freeze/thaw cycles).

Non-compartmental pharmacokinetic analyses

The data were analysed using non-compartmental analysis with estimation of the elimination half-life (ln2/Đz) using log-linear regression of the terminal part of the curve, where the number of included points was determined by the software program WinNonlin V5.0 (Pharsight Corp, Mountain View, CA). Extrapolation of the $AUC_{0-\infty}$ was done using the calculated AUC_{0-last} to which $C_{last/\lambda z}$ was added. The pharmacokinetic parameters of PC-SOD after single doses (for both the Japanese and Caucasian subjects, only 80mg data) were analysed for C_{max}, (maximum observed plasma drug concentration), AUC_{0-24} (area under the plasma drug concentration curve (AUC) from time 0 to 24 hours), AUC_{0-last} (AUC from time 0 to last point measured), $AUC_{0-\infty}$ (AUC from time 0 to infinity), clearance (Cl), volume of distribution (Vd) and terminal elimination half-life (t1/2). The degree of accumulation of PC-SOD expected during the multiple-dose regimen was predicted based on the single-dose data. The predicted accumulation ratio (Rpred) was defined as the $AUC_{0-\infty}$ of the 80mg single-dose cohort divided by AUC_{0-24} of the 80mg single-dose cohort.
After multiple-dose administration, the following parameters were determined from the PC-SOD concentration versus time data: C_{max}, $AUC_{0-\infty}$, AUC_{0-24} and t1/2 after the first administration and C_{max}, AUC_{int} (AUC over the 24 dosing interval during steady state) and t1/2 after the last administration. The observed accumulation ratio (Robs) was defined as AUC_{int} (AUC over the 24 hour dosing interval) on day 7 of the multiple-dose cohort divided by AUC_{0-24} on day 1. The accumulation of PC-SOD in serum at steady-state (Rss, steady-state accumulation ratio) was defined as the AUC_{int} on day 7 of the multiple-dose cohort divided by $AUC_{0-\infty}$ on day 1.

Compartmental pharmacokinetic analyses

Compartmental analysis was performed using the software program WinNonlin V5.0 (Pharsight Corp, Mountain View, CA). A 2-compartment model with macro-constants was used. Observations were iteratively reweighted using the square of the predicted concentration corresponding to a constant coefficient of variation residual error model. Using this model C_{max}, Cl, initial half-life ($t_{½,\ initial}$), terminal half-life ($t_{½,\ initial}$) and Vd were determined for both single- and multiple dose data .

Statistical analysis

Pharmacokinetic parameters were summarized using mean, standard deviation (SD), median, minimum and maximum. Tolerability and safety variables were summarized using descriptive statistics (n, mean, SD, median, minimum and maximum for continuous variables).

Dose-normalized C_{max} and total clearance were used to assess dose-linearity using single factor factorial analysis of variance on log-transformed data (ANOVA; factor dose) to assess dose-linearity. Mean differences and 90%-CI intervals in C_{max} (µg/mL), $t_{½}$ (hr), clearance (mL/hr), volume of distribution and $AUC_{0-\infty}$ (µgαhr/mL) between Japanese and Caucasian were determined using two-sample student t-tests on log-transformed data assuming unequal variances.

RESULTS

General

Twenty-six male Japanese volunteers (age: 20-32, mean BMI: 21.4 kg/m²) were included. In the Caucasian study eight subjects (4 female/4 male, age: 18-27, mean BMI: 23.4 kg/m²) participated.

All Japanese subjects completed the study. No adverse events were observed in 40 and 80mg single dose groups. The most common adverse event was mild diarrhea (twice in the 160mg-group, once in the 80mg multiple dose group, in one subject receiving placebo). These events were considered possibly related to the study drug. Other adverse events were headache, muscle pain, fatigue, pain in the right hip and influenza. These events occurred once and were considered not to be related to the study medication. In one subject in the multiple doses group antibodies against PC-SOD were detected at the first follow up. Follow up at 6 months showed that these antibodies were no longer present.

Safety analysis in Caucasians did not indicate any safety issues, results of the safety analyses are reported elsewhere.(21)

Non-compartmental pharmacokinetic analyses

The mean serum concentrations of PC-SOD versus time curves for the single-dose and the multiple-dose regimens are shown in figure 1 and 2 respectively. A summary of the pharmacokinetic parameters is given in table 1 (single dose) and table 2 (multiple-dose).

Following single-dose intravenous PC-SOD administration in Japanese serum PC-SOD concentrations were elevated above baseline for 24 hours in all doses used. Mean (SD) terminal half-life (t1/2) of PC-SOD was 24.7 (4.3), 24.9 (3.5), 31.3 (14.6) hours for the 3 ascending doses respectively. After 80mg single dose in Caucasian a terminal half-life of 26.1 (11.2) hours was found.

Dose-normalized C_{max} and clearance (Japanese) were 259.4 (31.4), 254.9 (24.7) and 358.0 (74.1) ng/ml/mg and 167.4 (27.4), 143.9 (18.9) and 119.4 (30.0) ml/hr for 40, 80 and 160mg PC-SOD respectively. These data indicated that the pharmacokinetics of PC-SOD is dose-dependent. (p-values 0.002 and 0.022).

Urinary PC-SOD concentrations were below the limit of quantification for the 40mg, and 80mg, but after 160mg PC-SOD the cumulative urinary excretion 0-48hr was 2.28 (1.34) mg, which is 1.4 (0.8)% of the administered dose.

After multiple-dose administration of PC-SOD 80mg C_{max}, day7 was 38.1 (2.1) µg/mL. The AUC_{int} was 649.7 (98.3) hr*µg /mL. Based on the seven trough serum PC-SOD concentrations, steady state was reached after 5 days. The Robs 2.6 (0.4) was greater than the value calculated from the single dose data (Rpred: 2.0 (0.2), p=0.02).Urinary PC-SOD concentrations were below limit of quantification during the multiple-dose regimen.

Compartmental pharmacokinetic analyses

When data were modeled using a 2-compartmental model a good fit was obtained. In two subjects (in the 80 and 160 mg single dose cohort) no adequate estimation of half-life could be calculated. The results after compartmental analyses were comparable to those obtained with non-compartmental analyses (table 3).

Comparison Caucasians-Japanese

The non-compartmental pharmacokinetics of the 80mg single dose administrations were compared between Japanese and Caucasians using C_{max}, clearance, volume of distribution, half-life and $AUC_{0-\infty}$.(table 1)

DISCUSSION

In this study we evaluated the pharmacokinetic profile of PC-SOD following single doses of 40, 80 and 160mg and multiple doses (80mg/day for 7 days) in Japanese volunteers. Additionally, pharmacokinetics of 80mg single dose PC-SOD in Japanese and Caucasian subjects was compared.

The mean plasma concentration versus time curve for the 48 hours following a single dose of PC-SOD was characterized by bi-exponential decline from peak plasma concentration. Half-lives were more than 24 hours for all investigated doses, which is substantially longer than previous reports in trials with unlecithinized SOD.(22;23) The excretion of PC-SOD is predominantly extra-renal, as urinary excretion was less than 2% in the 160mg cohort. This is in line with findings from a previous study in healthy Caucasians, but in contradiction with results in earlier trials with unlecithinized recombinant SOD, where urinary excretions up to 57% were reported. These data suggest that the diminished urinary excretion, and possibly the prolonged half-life, can be attributed to the addition of lecithin to SOD.(22;23) In contradiction with earlier studies in Japanese and Caucasians dose-dependency of the pharmacokinetic parameters was shown, likely because in this study higher doses were studied. As also, C_{max} showed dose-dependency, this strongly suggests a saturable clearance for PC-SOD.

After multiple dosing steady state was reached after 5 days. Pharmacokinetics after the multiple dose regimen showed a similar pattern of distribution and elimination as observed during the single dose cohorts. But some differences were observed. First, terminal half-life was longer than during single dose regimen (56.8 vs 24.9 hours), second a slightly higher accumulation ratio than predicted on the single dose data (R_{pred} 2.0 vs R_{obs} 2.6) was found.

For the higher than expected accumulation and longer half-life of PC-SOD after multiple dosing, some possible explanations can be given. First, during the multiple-dose regimen the final part of $AUC_{0-\infty}$ is better characterized due to longer sampling (48 hours vs. 168 hours in the single- and multiple dose cohort respectively). It is therefore highly likely that the calculated $AUC_{0-\infty}$ during single-dose and following the first dose in the multiple dose regimen is underestimated because of incomplete characterization of the terminal elimination phase. Second, it may be that at higher exposures as in the multiple-dose part makes the observed non-linearity in the single-dose cohorts clearer. When the pharmacokinetic profiles were modeled using a 2-compartment model the estimated pharmacokinetic parameters were comparable to those determined with non-compartmental methods, indicating that we adequately described the pharmacokinetic properties of PC-SOD. Nevertheless, the finding that steady state is reached after approximately 5 days, which is more compatible with a half-life of 24 hrs, may suggest that there is a 'deep' compartment containing very little amounts of drug.(24) Thus for practical reasons it seems that the relevant elimination half-life of PC-SOD is in the order of 24 hours.

Based on our data there are no indications that after 80mg single dose of PC-SOD there are differences of clinical significance between Japanese and Caucasian subjects.

Generally, PC-SOD was well tolerated in doses up to 160 mg. The observation that one of the Japanese subjects developed antibodies against PC-SOD after multiple doses of PC-SOD requires further investigation and the development of antibodies should be monitored in future trials.

In conclusion, this study demonstrates that PC-SOD concentrations were elevated above baseline for at least 24 hours after single doses of PC-SOD greater or equal of 40mg. Dose non-linearity was demonstrated after single doses, indicating saturable clearance. During the multiple-dose regimen steady state was reached after 5 days. Accumulation was slightly higher than expected. It was shown that PK after a single iv dose of 80 mg PC-SOD is similar for healthy Japanese and Caucasian subjects. The pharmacokinetics of PC-SOD make it is worthwhile to further investigate PC-SOD in patients with diseases characterized by high free radical overload.

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Table 1 Non-compartmental pharmacokinetic parameters in Japanese and Caucasian volunteers.

		Japanese			Caucasians	Caucasians vs. Japanese
DoseParameter		40 mg (N=6)	80 (N=6)	160 (N=6)	80 mg (N=8)	Mean of difference†
C_{max} (µg/mL)	Mean (SD)	10.4 (1.3)	20.4 (2.0)	57.3 (11.9)	18.4 (2.6)	0.86 (0.73-1.02)
	Median (min-max)	10.0 (9.3-12.6)	21.1 (17.7-22.9)	54.9 (44.0-79.4)	18.2 (14.3-23.0)	
t ½ (hr)	Mean (SD)	24.7 (4.3)	24.9 (3.5)	31.3 (14.6)	26.1 (11.2)	1.02 (0.77-1.34)
	Median (min-max)	25.3 (18.9-29.4)	25.5 (21.1-30.5)	25.3 (22.2-60.1)	23.0 (14.7-48.1)	
Clearance (mL/hr)	Mean (SD)	167.4 (27.4)	143.9 (18.9)	119.4 (30.0)	167.9 (35.3)	0.86 (0.73-1.02)
	Median (min-max)	169.2 (134.7-208.7)	153.9 (116.9-160.5)	121.4 (66.4-157.4)	173.4 (123.3-219.0)	
Vd (L)	Mean (SD)	5.62 (1.31)	4.88 (0.55)	4.58 (0.58)	5.81 (2.38)	1.02 (0.77-1.34)
	Median (min-max)	5.7 (3.9-7.0)	4.7 (4.3-5.7)	4.7 (3.8-5.4)	5.03 (3.73-10.74)	
$AUC_{0-\infty}$ (µgαhr/mL)	Mean (SD)	244.4 (40.0)	564.6 (81.2)	1440.0 (493.7)	496.3 (108.3)	1.16 (0.98-1.37)
	Median (min-max)	236.4 (191.6-297.0)	519.7 (498.4-684.3)	1318.5 (1016.7-2411.2)	461.4 (365.2-648.8)	
Percent extrapolation	Mean (SD)	25.1(6.4)	24.9(3.5)	30.7(12.2)	24.9(11.6)	NA
	Median (min-max)	27.1 (15.7-31.4)	25.5 (21.1-30.5)	25.5 (22.2-54.4)	21.9 (8.1-44.6)	

C_{max}, maximum observed serum drug concentration; $t_{1/2}$, half-life; V_d, volume of distribution; $AUC_{0-\infty}$, AUC from time 0 to infinity; † 90%-confidence intervals between brackets.

Table 2 Comparison of the pharmacokinetic parameters of intravenous single-dose and multiple-dose PC-SOD administrations.

Parameter		Single dose PC-SOD 80mg	Multiple dose PC-SOD 80 mg dose (after first dose)	Multiple dose PC-SOD 80 mg dose (after last dose)
Cmax (µg/mL)		20.4 (2.0)	20.4 (1.4)	38.1 (2.1)
		21.1 (17.7-22.9)	21.0 (18.0-21.8)	39.2 (35.2-39.9)
AUC0-24 (hrαµg/mL)	Mean (SD)	281.5 (30.6)	253.9 (53.9)	NA
	Median (min-max)	283.6 (241.3-321.9)	255.8 (170.7-321.5)	NA
AUCint (hrαµg/mL)	Mean (SD)	NA	NA	649.7 (98.3)
	Median (min-max)	NA	NA	673.8 (514.4-742.2)
$AUC_{0-\infty}$ (hrαµg/mL)	Mean (SD)	564.6 (81.2)	411.8 (151.3)	NA
	Median (min-max)	236.4 (191.6-297.0)	397.3 (215.0-620.3)	NA
Percent extrapolation	Mean (SD)	26.1 (3.9)	37.0(12.6)	20.0 (4.4)
	Median (min-max)	26.6 (21.2-31.8)	36.8 (22.0-51.3)	19.9 (14.5-25.5)
t ½ (hours)	Mean (SD)	24.9 (3.5)	16.5 (5.1)	56.8 (20.8)
	Median (min-max)	25.5 (21.1-30.5)	16.2 (10.6-22.1)	58.3 (34.0-87.1)
Predicted accumulation ratio, Rpred	Mean (SD)	2.0 (0.2)	NA	NA
	Median (min-max)	2.0 (1.8-2.2)	NA	NA
Observed accumulation ratio, Robs	Mean (SD)	NA	NA	2.6 (0.4)
	Median (min-max)	NA	NA	2.5 (2.0-3.2)
Steady-state accumulation ratio, Rss	Mean (SD)	NA	NA	1.7 (0.6)
	Median (min-max)	NA	NA	1.7(1.0-2.4)

C_{max}, maximum observed serum drug concentration; $t_{0-\infty}$, half-life; AUC_{0-24}, area under the plasma drug concentration versus time curve (AUC) from time 0 to 24 hours; AUC_{int}, AUC over 1 dosing interval during steady state; $AUC_{0-\infty}$, AUC from time 0 to infinity.
R_{pred}, predicted accumulation ratio, defined as $AUC_{0-\infty}$ divided by AUC_{0-24}; R_{obs}, observed accumulation ratio, defined at AUC_{int} on day 7 divided by AUC_{0-24} on day 1; R_{ss}, steady-state accumulation ratio, defined as AUC_{int} on day 7 of the multiple-dose cohort divided by $AUC_{0-\infty}$ on day 1.

Table 3 Compartmental analyses of pharmacokinetic parameters in Japanese volunteers.

Parameter		Single dose PC-SOD 40 mg intravenous		Single dose PC-SOD 80 mg intravenous		Single dose PC-SOD 160 mg intravenous		Multiple dose PC-SOD 80 mg dose	
		Estimate	StandardError	Estimate	StandardError	Estimate	StandardError	Estimate	StandardError
C_{max} (µg/mL)	Mean (SD)	11.3 (2.4)	0.8 (0.4)	20.1 (2.7)	0.9 (0.5)	51.4 (5.8)	3.1 (1.6)	19.9 (1.1)	1.2 (0.3)
	Median (min-max)	11.0 (8.9-15.2)	0.7 (0.5-1.5)	20.2 (16.8-24.1)	0.8 (0.3-1.4)	49.8 (45.3-61.7)	2.7 (1.8-6.1)	20.3 (18.1-20.9)	1.2 (0.9-1.6)
	%-CV	7.2 (2.5)		4.2 (2.0)		5.9 (2.3)		5.9 (1.3)	
Vd (L)	Mean (SD)	6.0 (1.5)	0.6 (0.3)	5.4 (1.0)	1.3 (1.6)	6.5 (2.2)	3.4 (3.0)	7.5 (1.4)	0.5 (0.3)
	Median (min-max)	6.1 (4.0-8.1)	0.6 (0.3-1.0)	5.4 (4.2-6.5)	0.7 (0.3-4.1)	6.4 (4.2-10.1)	2.8 (0.8-8.1)	7.1 (5.7-9.4)	0.4 (0.3-0.9)
	%-CV	9.5 (2.3)		21.8 (23.7)		46.4 (27.6)		6.4 (2.1)	
Clearance (mL/hr)	Mean (SD)	163.0(27.1)	15.5(7.4)	134.6(20.0)	20.5(18.7)	93.4(30.4)	45.3(29.0)	110.7(22.3)	4.1(2.3)
	Median (min-max)	162.5 (132.0-208.8)	13.3 (8.6-25.6)	127.8 (109.7-158.6)	14.5 (7.7-53.4)	95.0 (47.2-126.8)	46.4 (14.4-84.7)	106.0 (84.7-149.5)	3.3 (2.5-8.4)
	%-CV	9.5 (4.3)		15.8 (15.0)		58.2 (42.3)		3.6 (1.2)	
t ½, initial (hours)	Mean (SD)	1.4 (0.8)	0.8 (0.6)	3.9 (3.0)	2.6 (1.8)	4.5 (1.1)	2.2 (0.7)	5.5 (3.2)	1.7 (0.8)
	Median (min-max)	1.7 (0.4-2.3)	0.7 (0.2-1.9)	3.2 (0.7-8.0)	2.5(0.6-5.3)	4.3 (3.3-5.9)	2.0 (1.5-3.3)	4.9 (2.3-5.5)	1.7 (0.7-1.7)
	%-CV	56.0 (29.8)		72.0 (14.7)		50.9 (19.8)		32.5 (8.8)	
t ½, terminal (hours)	Mean (SD)	27.2 (6.0)	5.0 (2.6)	31.0 (9.4)	16.8 (22.5)	63.7 (35.7)	81.7 (71.9)	54.7 (10.2)	6.0 (2.6)
	Median (min-max)	28.4 (20.2-35.2)	4.7 (2.3-8.6)	27.7 (22.1-43.5)	6.6 (2.4-55.9)	54.8 (29.1-103.8)	75.0 (9.7-166.6)	56.5 (38.4-54.7)	6.5 (2.4-6.0)
	%-CV	17.3 (5.8)		43.6 (49.1)		104.6 (60.8)		10.6 (3.7)	

C_{max}, estimated maximum drug concentration; $t_{½\ initial}$,initial half-life; $t_{½\ initial}$, terminal half-life; V_d, volume of distribution; %-cv, mean coefficient of variation in percentage with standard deviation between brackets.

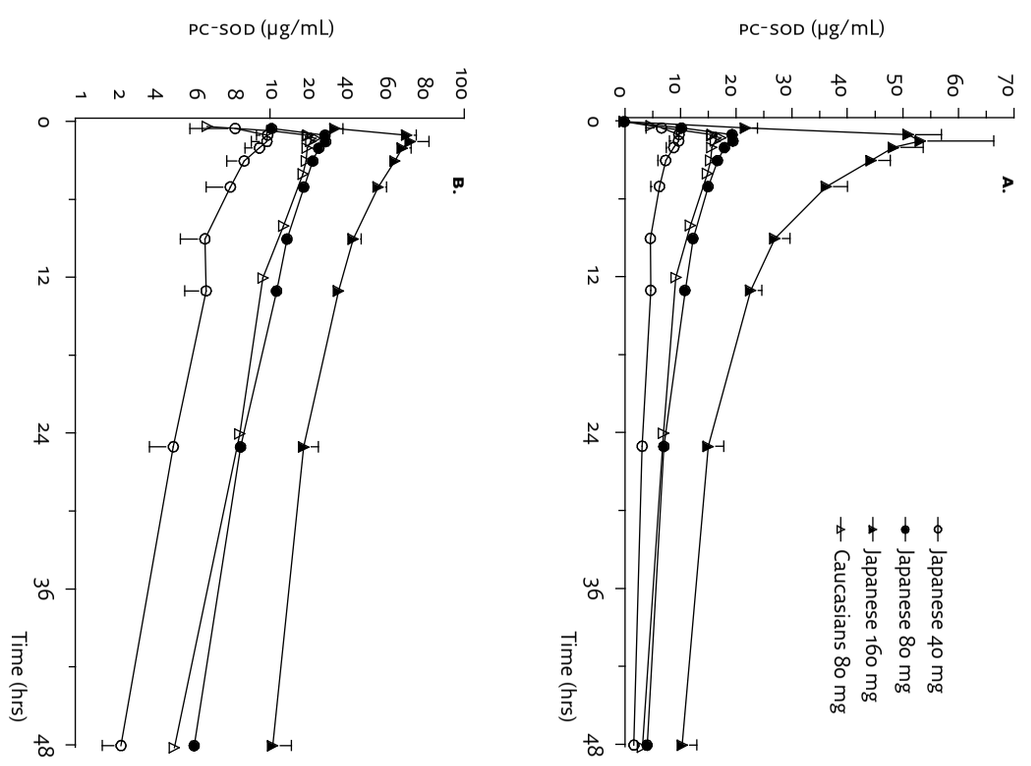
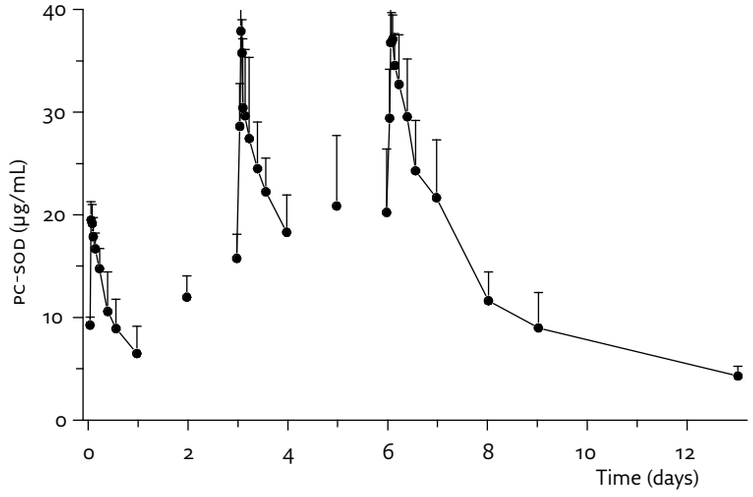


Figure 1A & B Serum PC-SOD concentration (mean ± SD) after single intravenous doses in Japanese (N=6, open circles 40 mg, closed circles 80 mg, closed triangles 160 mg) Caucasian (N=8, open triangles 80 mg) volunteers.

Figure 2 Serum PC-SOD concentration (mean) after repeated administration of 80 mg/day intravenously for seven days in Japanese volunteers.



CHAPTER 7

Evaluation of lecithinized human recombinant super oxide dismutase as cardioprotectant in anthracycline-treated breast cancer patients

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ABSTRACT

AIM Anthracycline-induced cardiotoxicity is (partly) mediated by free radicals overload. A randomized study was performed in breast cancer patients to investigate whether free-radical scavenger Super Oxide Dismutase (SOD) protects against anthracycline-induced cardiotoxicity as measured by changes in echo- and electrocardiography and an array of biomarkers.

METHODS AND RESULTS Eighty female, chemotherapy-naïve breast cancer patients (median age 49, range 24-67) scheduled for 4 or 5 courses of adjuvant three-weekly doxorubicin plus cyclophosphamide (AC) chemotherapy, were randomly assigned to receive 80 mg PC-SOD (human recombinant SOD bound to lecithin) or placebo, administered intravenously (IV) immediately prior to each AC course.

The primary end point was protection against cardiac damage evaluated using echocardiography, QT-assessments, and a set of biochemical markers for myocardial function, oxidative stress and inflammation. Assessments were performed before and during each course of chemotherapy, and at 1, 4 and 9 months after completion of chemotherapy regimen. In all patients cardiac effects such as increases in NT-proBNP concentration and prolongation of the QTc-interval were noticed. There were no differences between the PC-SOD and placebo-treated patients in systolic or diastolic cardiac function or for any other of the biomarkers used to assess cardiac effects of anthracyclines.

CONCLUSION PC-SOD at a dose of 80 mg IV is not cardioprotective in patients with breast carcinoma treated with anthracyclines.

CLINICAL TRIAL REGISTRATION INFORMATION The study is registered at www.controlled-trials.com, number ISRCTN56637853.

INTRODUCTION

Anthracyclines are widely used in treatment regimens of cancer, including breast cancer. Their use is hampered by occurrence of irreversible cardiotoxicity which typically manifests as congestive heart failure (CHF) months to years after anthracycline exposure. It is primarily related to cumulative anthracycline dose and it seems that females are affected more often than males.(1;2) The incidence increases from 5% in patients receiving doses up to 400 mg/m2 to 48% in patients receiving more then 700 mg/m2 of doxorubicin.(1) Although less toxic analogues such as epi-doxorubicin have been developed, anthracycline-induced cardiotoxicity remains a clinical problem.(3) As the decline in ejection fraction and clinically manifest CHF usually become apparent relatively late after anthracycline therapy, it is difficult to assess the cardiotoxic effects of anthracyclines early. However, anthracycline cardiac toxicity has also been reported to occur after only single dose administration.(1) This suggests that it may be possible to use (bio)markers of cardiac effects due to anthracyclines occurring early and that may be predictive of the late toxicity. Indeed, several markers such as QT-prolongation and changes in NT-proBNP and cardiac troponin levels have been suggested to be such early markers.(4-7) These markers can potentially also be used to assess the effects of putative protective strategies.(4)

The mechanism of anthracycline-induced cardiotoxicity has not been fully elucidated, but formation of reactive oxidative species (ROS), such as the superoxide (O_2-) radical seem to play a major role.(8) Superoxide dismutase (SOD) is an important scavenger of these ROS and its use to prevent organ damage mediated by free radical overload has been investigated.(8) However, the currently existing therapies using exogenous SOD as a protectant has been limited by for instance its short half-live and low affinity for the cell membrane.(9) Lecithinized SOD (PC-SOD) has a 100-200 fold higher affinity for the cell membrane and improved free radical scavenging properties.(10) Several animal-models, including a rodent doxorubicin-induced cardiotoxicity model, showed that PC-SOD protected against free-radical mediated injuries.(11-20)

Early clinical studies in healthy subjects showed that a single intravenous (IV) dose of 80 mg PC-SOD resulted in increased SOD-activity *in vivo* for 16-24 hours.(21;22)

The efficacy of PC-SOD as cardioprotective agent against anthracycline-induced cardiotoxicity was explored in an early phase II study in woman with breast cancer, using serial echocardiography measurements, electrocardiography and a set of (bio)markers, reflecting myocardial function, oxidative stress and inflammation.(4;7;23-28)

METHODS

Patient population

This multi-center, randomized, placebo-controlled trial was performed in female patients with early-stage breast cancer eligible for adjuvant doxorubicin and cyclophosphamide (AC) chemotherapy. Patients were scheduled to receive either 4 or 5 AC cycles according to national guidelines at that time. Prior or concomitant use of cardiotoxic medication was an exclusion criterion. Patients with distant metastases, a history of other malignant disease, a life expectancy of less than one year, pre-existing cardiovascular diseases, elevated transaminases above 3 times the upper limit of normal and patients of whom we were unable to obtain a good quality echocardiogram before study drug administration were excluded.

The institutional review board of Leiden University Medical Center (LUMC) approved the study protocol. All patients gave written informed consent before participation and the study was conducted in accordance with the declaration of Helsinki (South Africa 1996 amendment), Good Clinical Practice and all applicable local laws and regulations.

Study protocol

This study was coordinated and designed by the Centre of Human Drug Research (CHDR) and carried out in 5 oncology centers in The Netherlands. After randomization (1:1 to 80mg PC-SOD or placebo) of eligible patients baseline assessments were done and the patients started their scheduled chemotherapy (4 or 5 courses) consisting of a combination of doxorubicin (60 mg/m2 over approximately 30 min) and cyclophosphamide (600 mg/m2 over approximately 30 min) administered IV. Patients were admitted to the hospital in the morning of each chemotherapy course. After baseline assessments were completed the patients received PC-SOD or placebo as a 1-hr IV infusion. Immediately thereafter, anti-emetics followed by AC. After discharge in the afternoon, a 24 hour visit took place in the morning of the following day. A similar procedure was repeated during a maximum of four courses. Patients receiving 5 courses received the study drug at the third course but no measurements were done. Median volume loading during the courses was 300ml per hour (in total approximately 850 ml in 4 hours). After completion of chemotherapy, follow up visits took place at 1, 4 and 9 months.

Study medication

PC-SOD consists of an average of 4 molecules lecithin derivative covalently bound to the human derived CuZn-SOD, produced by genetic recombination using *E.coli* as a host cell.(10) The lecithinized product has 3x103 U SOD-activity per mg. A single batch of the lyophilized formulation was used. The PC-SOD formulation consisted of 80 mg PC-SOD and 133mg sucrose, the placebo formulation only consisted of sucrose. PC-SOD and placebo were prepared for use by dissolution in 5% mannitol diluted with distilled water; all study medication was prepared at the LUMC hospital pharmacy.

Outcome measures

EFFICACY Efficacy assessments included echocardiography (left ventricular ejection fraction [LVEF], E/A ratio), electrocardiography [ECG] (QT-assessments) and blood sampling for biomarkers of cardiac function or damage (NT-proBNP, CK-MB and troponin T),

inflammation (macrophage inhibiting protein 1 [MIP-1], high sensitivity C-reactive protein [hsCRP], tumor necrosis factor alfa [TNF-a] and soluble intercellular adhesion molecule-1 [sICAM]), and oxidative stress (oxLDL, urinary biopyrrin and non-protein bound iron [NPBI]).

Echocardiography, ECGs and blood sampling for determination of NT-proBNP, CK-MB (mass) and troponin T concentrations were done at baseline and 1 (including NPBI), 4 and 9 months after a full chemotherapy regimen.

ECG and blood sampling (for all biomarkers) was done before and at 24 hours after the start of each chemotherapy course. In addition ECG-recordings were made and blood was sampled for determination of CK-MB (mass), troponin T and TNF-α concentration at 4 hours after the start of each chemotherapy course.

SAFETY During the study period hematology and blood chemistry were frequently assessed. Glomerular filtration rate (GFR) was determined during the courses from 24 hours creatinine clearance, during the follow up visits the MDRD formula was used.(29) In addition, at the first follow-up visit antibodies against PC-SOD were determined.

All (serious) adverse events ((S)AE) were monitored from inclusion until last follow up and (S)AE's and concomitant medication were classified according to the World Health Organization Adverse Reaction Terminology and drug (WHOart and WHOdrug) classification system.

After completion of the last chemotherapy cycle for every 10th patient (until 60 patients were included), an interim safety report was reviewed by an independent Data Monitoring Committee (DMC). This report included all occurred (S)AE and laboratory safety data. After each report the DMC informed the principal investigator if in their opinion the data raised any safety concerns. The DMC was blinded during the whole study period, but could request emergency deblinding of (a part of) the data when deemed necessary.

QUALITY OF LIFE Quality of life (QoL) was assessed using two validated questionnaires developed by the European Organization for Research and Treatment of Cancer (EORTC): QoL (EORTC QLQ-C30, version 3) and QoL (EORTC QLQ-BR23).(30) Qol was assessed at baseline, during each course and 1, 4 and 9 months after completion of chemotherapy.

PHARMACOKINETICS Blood was sampled for the determination of PC-SOD serum concentrations during the first and last course at baseline and directly, 4 hours and 23 hours after the end of the infusion of PC-SOD or placebo.

ECHOCARDIOGRAPHY Echocardiography was performed at two locations in the Netherlands: the department of cardiology of LUMC, Leiden and the department of cardiology of Maasstad Ziekenhuis, Rotterdam. The examinations were performed by a single echographer in each center and all examinations were supervised by an experienced cardiologist. To exclude inter-observer variability all echo assessments for each individual patient were done at one center.

The investigations consisted of routine imaging, M-mode imaging for measurement of left ventricular end-diastolic and end-systolic wall thickness (septum, posterior wall), fractional shortening and left ventricular ejection fraction (LVEF, calculated according to Teichholz).(31) Measurements were made from the parasternal long-axis (or short-axis) view. The ratio of early rapid ventricular filling over atrial assisted filling (E/A ratio) was measured using pulsed-wave Doppler. Regional systolic function was evaluated with visual assessment of wall motion (and wall motion score index, WMSI) according to the 16-segment model. The examinations were performed using AGEvivid-7 echocardiograph equipped with pulsed-wave Doppler in the LUMC and using a Hewlett-Packard HP 5500 with a S3 probe in the Maasstad Ziekenhuis.

ECG RECORDINGS AND ANALYSIS For each patient 5-minute ECG recording were made using the CardioPerfect device (Welch Allyn, Delft, The Netherlands). ECG recordings were analyzed after fiducial segment averaging (FSA) to obtain heart rate, and QT-interval. This analysis was done using Intraval (Advanced Medical Systems, Maasdam, the Netherlands).(20)

For the analyses correction of the QT-interval for heart rate was done using Bazett's formula (QTcB= QT/√ (RR)), Fredericia's cubic root QTcF=QT * (1/RR)[13];and using the linear correction method according to Framingham Heart Study (QTcL= QT + 0.154 * (1 – RR)).

Assays

Samples were assayed for NT-proBNP, troponin T and CK-MB (mass) and NPBI at the Central Clinical Chemical laboratory (CKCL) of LUMC. Lower limits of detection (inter- and intra-assay variability) were 5 ng/L (< 5.8%), 0.1 ng/mL (< 2.5%), 0.01 µg/mL (< 5.6%) and 0.01 µmol/L (< 9.2) for NT-proBNP, cTnT, CK-MB (mass) and NPBI respectively. Assays for TNF-a, hsCRP, sICAM, MIP-1a, oxLDL and urinary bioppyrin were performed at the Netherlands Organization for Applied Scientific Research (TNO). The lower limits of detection of the assays (inter- and intra-assay variability) were 0.12 pg/mL (<12.5%), 0.1 µg/L (< 10%), 0.35 ng/mL (< 12.5%), 10 pg/mL (< 10%), 1 mU/L (< 7.5%) and 0.1 U/L (< 12.5%) for TNFa, hsCRP, sICAM, MIP-1a, oxLDL and urinary biopyrrin respectively.

Serum PC-SOD concentrations were measured using an enzyme linked immunosorbent assay (ELISA), consisting of an antibody against human Cu, Zn-SOD, and a second antibody against human Cu, Zn-SOD conjugated with horseradish peroxidase. The assay has a lower limit of quantification 626 ng/mL and the coefficients of variation did not exceed 7.9% which was observed for the lower concentrations. Antibody formation against PC-SOD was measured by quantification of specific IgE, IgG and IgM titres as described previously.(21)

Statistical analyses

POWER As both the incidence of sub-clinical cardiotoxicity and the treatment effects were unknown, the power calculation of the study has been performed using a number of assumptions: (1) The incidence of subclinical cardiomyopathy in patients is 33%; (2) Animal experiments suggest that PC-SOD treatment prevents cardiomyopathy in 100% of the cases. It has therefore been estimated that the protection by PC-SOD will reduce incidence of cardiomyopathy from 33% to 5.5% in the patients. In order to be able to demonstrate this treatment effect (power=80%; 2-sided test; $p<0.05$) a total of 72 patients is required. Second an exploratory power calculation on the biomarkers has been performed. These showed that this study has 80% power to detect (2 sided test; $p<0.05$) a difference in NT-proBNP levels between the groups of approximately 53%.

EFFICACY AND SAFETY POPULATION Eighty female breast cancer patients were randomized to PC-SOD or placebo. After randomization one patient was excluded because of an abnormal echography at baseline. During the trial 7 patients were replaced: 4 patients dropped out (2 patient's request, 1 because of discontinuation of chemotherapy due to extreme nausea), 4 patients had incomplete echocardiographic assessments (2 due to logistic problems, 2 due to equipment failure). Data of replaced patients are used in both safety and efficacy analyses. Two patients were excluded from the efficacy analyses because of anomalies in PK-results, hence the safety population and efficacy population consisted of 79 and 77 patients respectively.

TREATMENT EFFECTS First, for each course the difference between the 24 hour and the baseline measurement was calculated and this series of four differences was compared between placebo and PC-SOD treatment. This short term effect was analyzed using a mixed model analysis of variance (SAS proc mixed) with visit as repeated factor within patient, treatment (PC-SOD/placebo), group (4 or 5 courses) and treatment by group, treatment by time, group by time and treatment by group by time as fixed effects. For ECG parameters, TNFa and CK-MB (mass) the difference between the measurement at 4 hour and baseline at the occasion was analyzed the same way.

Second, the baseline measurement of each course except the first and the follow-up measurements were compared between Placebo and PC-SOD. The long-term treatment effect for the echocardiographic, ECG parameters and CK-MB and NT-proBNP was analyzed using a mixed model analysis of variance (SAS proc mixed) with visit (occasion) as repeated factor within patient, treatment,

group, treatment by time, treatment by group, time by group and treatment by time by group as fixed effects. The baseline value of the first course was included as covariate.

TIME EFFECTS To assess the 4 and 24 hour difference from baseline within a course, the estimated differences from baseline were compared to 0 (no difference from baseline) within the first treatment mixed model for the ECG and biomarker parameters. The estimated difference between the course 1 baseline and follow up measurements (long-term time-effects) for the echocardiographic, ECG parameters, CK-MB and NT-proBNP were compared to 0 (no difference from baseline) within the second treatment mixed model.

PHARMACOKINETICS Compartmental pharmacokinetic analysis was performed using NONMEM Version VI software (GloboMax LLC, Hanover, MD), maximum serum concentration and half-life were reported.

ADDITIONAL (SUB-GROUP ANALYSES) All data was also analyzed excluding patients who received trastuzumab or left-sided radiotherapy as concomitant therapy.

All statistical analyses were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA). The study is registered at www.controlled-trials.com, number ISRCTN56637853.

RESULTS

Baseline characteristics

The median age of the 79 patients who received at least one dose of PC-SOD and AC chemotherapy was 49 years (range 24-67 years). The median (min-max) number of courses was 4 (1 to 5) and 4 (2 to 5) for placebo and PC-SOD, combined with AC chemotherapy, respectively (table 1).

Safety

There were no clinically relevant findings related to PC-SOD treatment on clinical laboratory measurements, vital signs or ECG findings. GFR was stable during the study period. The AE pattern did not differ among treatment groups (table 2) and the majority of AE's could be attributed to the chemotherapeutics and were mild to moderate in intensity. Antibodies against PC-SOD were not detected in any of the patients

EFFICACY

Long-term effects

ECHOCARDIOGRAPHY LVEF (± SD) and E/A ratio (± SD) were 67% ± 6, 1.06 ± 0.28 and 64% ± 7, 1.1 ± 0.3 at baseline, in patients receiving placebo and PC-SOD respectively and the overall decline (95% confidence interval between brackets) was -1% (-2 to1%), 0.0 (0.0 to 0.0%) and -2% (-3 to -0%), 0.0 (0.0 to 0.0%) during the study (figure 1, table 3).

Differences (95%-confidence interval between brackets) between PC-SOD and placebo on LVEF and E/A ratio were -1% (-3 to 1%) and 0.0 (-0.1 to 0.0%) respectively.

WMSI did not change significantly during the trial and no differences between treatments were observed.

BIOMARKERS - MYOCARDIAL INJURY During courses and follow up the overall change (percentage change, 95%-confidence interval between brackets) of NT-proBNP and CK-MB was 32.0% (12.8 to 54.5), -5.7% (-12.4 to 1.4%) and 14.2% (-2.6 to 33.8%), -7.6% (-14.2 to -0.5%) in patients receiving placebo and PC-SOD respectively (figure 2, table 3).

The differences between PC-SOD and placebo were -13.5% (-30.9 to 8.2%) and -2.0% (-11.7 to 8.8%) for NT-proBNP and CK-MB respectively.

During the follow-up period in 10 patients (6 PC-SOD, 4 placebo) detectable (although not pathologically elevated) troponin levels were present.

ELECTROCARDIOGRAPHY Heart rate and QTc-interval (corrected using a linear method) increased during courses and follow up with 0.6 BPM (-1.5 to 2.8 BPM), 5 msec (3.8 to 11.3msec) and 4.0 BPM (1.9 to 6.2 BPM), 10.8 msec (7.0 to 14.7 msec) in patients with placebo or PC-SOD respectively (figure 3, table 3).

The differences between PC-SOD and placebo for heart rate, QT-interval, corrected QT-interval (Bazett) and corrected QT-interval (linear) were 3.4 BPM (0.4 to 6.5 BPM), -3.1 msec (-11.6 to 5.4 msec), 7.4 msec (1.9 to 12.9 msec) and 3.3 msec (-2.1 to 8.7 msec) respectively.

Effects within the courses

OXIDATIVE STRESS Urinary biopyrrin increased (percentage change, 95%-confidence interval between brackets) within the courses in the placebo group only, although this effect was not present for each individual course. Change at 24 hours was 13.0% (0.8 to 26.7%) and 3.4% (-7.9 to 16.0%) in patients receiving placebo and PC-SOD respectively. While OXLDL and NPBI levels did not change significantly between baseline and at 24 hours, the difference in percentage change (95%-confidence interval between brackets) between PC-SOD and placebo was 10.3% (-20.5 to 52.9%), 6.2% (0.2 to 12.5%) and -8.5% (-22.2 to 7.6%) for urinary biopyrrin, OXLDL and NPBI respectively (table 4).

MYOCARDIAL INJURY The overall increment at 24 hours post-dose for NT-PROBNP and CK-MB (mass) was 199.8% (154.6 to 253.0%), 8.2% (0.6 to 16.4%) and 263.8% (207.9 to 329.7%), 10.3% (2.5 to 18.8%) in patients receiving placebo and PC-SOD respectively.

The difference at 24 hours post-dose between PC-SOD and placebo was 21.4% (-3.9 to 53.3%) and 2.0% (-8.1 to 13.1%) for NT-PROBNP and CK-MB respectively (table 4).

INFLAMMATION Within the courses HSCRP and SLCAM did not change markedly, while TNF-α and MIP-1α declined with -23.2% (-30.5 to -15.1%), -46.9% (-64.5 to -20.4%) and -27.2% (-34.5 to -19.1%), -48.6% (-65.9 to -22.5%) in patients receiving placebo or PC-SOD respectively. For TNF-alpha this effect was already present at 4 hours post-dose. At 24 hours the difference between PC-SOD and placebo for HSCRP, SLCAM-1, TNF-α, MIP-1α was -2.9% (-19.0 to16.5%), -0.7% (-2.7 to 1.4%), -5.3% (-18.1 to 9.6%) and -3.3% (-45.7 to 72.1%) respectively (table 4).

ELECTROCARDIOGRAPHY Heart rate showed a small increment at 24 hour during the courses in the PC-SOD arm, changes were -1.2 BPM (-3.2 to 0.7 BPM) and 3.6 BPM (1.8 to 5.5 BPM) in patients receiving placebo and PC-SOD respectively. After each course the (corrected) QT-interval prolonged at 4 hours post-dose and increased further at 24 hours post-dose. Overall prolongation of the QTc interval (using a linear correction method) at 24 hours post-dose was 12.4 msec (8.8 to 15.9 msec) and 9.8 msec (6.4 to 13.2 msec) in patients receiving placebo and PC-SOD respectively.

The difference between PC-SOD and placebo at 24 hours after each chemotherapy cycle in heart rate, QT-interval, corrected QT-interval (Bazett), QT-interval (linear) was 4.9 BPM (2.2 to 7.6 BPM), -11.0 msec (-18.2 to -3.9 msec), 3.0 msec (-2.5 to 8.4 msec) and -2.7 msec (-7.4 to 1.9 msec) respectively (table 4).

Number of courses and other adjuvant therapy

It was also analyzed if other (potentially cardiotoxic) adjuvant therapy or the number courses influenced our results. As all analyses showed comparable results; only the full dataset was reported.

QUALITY OF LIFE In both treatment groups similar effects (decline) on QoL during the chemotherapy were observed (data not presented).

PHARMACOKINETICS Maximum serum concentrations were reached within 1 hour and amounted to 32.4 mg/L (SD 11.9) and 31.4 mg/L (SD 12.1) for the first and last visit respectively. The estimated half-life was approximately 20 hours.

PC-SOD did not show a protective effect on cardiotoxicity, as evidenced by differences in NT-proBNP concentration and prolongation of the QTc-interval, which occurred in all breast cancer patients undergoing AC chemotherapy. Also, echocardiographic systolic (LVEF) function or any of the array of the other biomarkers assessed did not show a clinical significant change during or following chemotherapy and were also not affected by PC-SOD.

Safety analyses did not show any unfavorable effects of PC-SOD at the administered dose, as laboratory assessments and AE patterns were similar between treatments.

The lack of a cardioprotective effect of PC-SOD at a dose of 80 mg IV on any of the markers of anthracycline-induced cardiotoxicity in chemo-naïve breast cancer patients may be explained by a lack of efficacy of PC-SOD at the dose used.

However, the negative findings in this study are in keeping with the results of several other studies, showing that exogenously administered free radical scavengers are not able to protect against anthracycline-induced cardiotoxicity and add to the increasing knowledge that free radical mediated injury is only partly involved in the pathogenesis of the cardiotoxicity of anthracyclines.(32-34) In addition, we were not able to demonstrate the occurrence of oxidative stress *in vivo*, as none of the biomarkers for oxidative stress changed after doxorubicin infusion.

Another reason for the lack of effects of PC-SOD (and maybe of free radical scavenging agents in general) could be that the therapeutic window of these agents seem to be narrow. This involves the observation that in animals a bell-shaped dose-response curve (higher doses of SOD showed less protection) is present after administration of (PC-) SOD.(11;35-39) If such a bell-shaped curve is also present in humans, this could implicate that in this study not the correct dosage was used. Although several mechanisms could be responsible for this bell-shaped effect curve, the most plausible explanation is (PC-) SOD causing excess ROS formation. In particular this concerns formation of H_2O_2 which has been shown to be capable to induce apoptosis in cardiomyocytes.(8;37;40-43)

Independent of the explanation of the failure of free radical scavenging agents as protective agents against anthracycline-induced cardiotoxicity, our study re-emphasizes the necessity to identify other strategies to reduce the risk of anthracycline-induced CHF.

We considered the possibility that the administered doses of anthracyclines did not induce sufficient myocardial damage to detect any prophylactic effect of PC-SOD, as LVEF and E/A ratio did not change markedly. However, the profound changes in NT-proBNP concentration and (corrected) QT-interval, indicate that in all patients indeed experienced some (subclinical) cardiotoxicity, as both markers are associated with the occurrence of anthracycline induced cardiac failure and an adverse outcome.(44)

A limitation of our study is that although the echo- and electrocardiographic and biochemical endpoints used in this study are well established markers of (anthracycline induced) cardiac damage and functional impairment, oxidative stress and inflammation, the study was not designed to detect differences in cardiac mortality or the occurrence of clinical CHF. Furthermore, some patients received additional potentially cardiotoxic treatments such as trastuzumab and/or radiotherapy. However, re-analysis of the data excluding these patients did not result in different findings. An additional yield of this trial is that we have demonstrated several robust biomarkers that are mechanistically associated with the Adriamycin-induced acute myocardial damage. These biomarkers could be used in future studies with other investigative agents that protect against this damaging effect.

In conclusion, we showed that IV administration of 80mg PC-SOD prior to each chemotherapy course was not efficacious as protective agent against anthracycline-induced cardiotoxicity, as evaluated by echocardiography, electrocardiography and a comprehensive array of biomarkers of myocardial damage, inflammation and oxidative stress, in female breast cancer patients treated with a combination of cyclophosphamide and doxorubicin for early stage breast cancer.

ROLE OF THE FUNDING SOURCE The funding source was involved in the design of the study. Data collection and analyses were performed by CHDR. All authors had access to all study data and had final responsibility for the decision to submit for publication.

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Table 1 Baseline Demographics and Clinical Characteristics

		Placebo (n = 40)	PC-SOD (n = 39)
Age, BMI and cumulative doxorubicin dose; median (range)			
Age (years)		47.0 (30 – 66)	50.0 (24 – 67)
BMI (kg/m2)		24.0 (19.4 – 37.7)	25.5 (19.6 – 38.5)
Cumulative doxorubicin dose (mg/m2)		240 (60 – 300)	240 (120 – 300)
Echocardiography; mean (SD)			
LVEF (%)		66 ± 6	64 ± 7
E/A ratio		1.06 ± 0.28	1.08 ± 0.28
Hemoglobin, renal function, and cardiac (bio)markers; mean (SD)			
Hemoglobin (mmol/L)		7.7 ± 0.7	8.0 ± 0.7
GFR (ml/min)		100.5 ± 23.9	106.3 ± 37.4
NT-proBNP (ng/L)		71 ± 46	84 ± 92
CK-MB mass (mg/L)		1.7 ± 0.9	1.7 ± 0.5
QTcL (msec)		426 ± 12.7	433 ± 24.0
Number of courses†; n (%)			
1		2 (3%)	0 (0%)
2		0 (0%)	1 (1%)
3		2 (3%)	0 (0%)
4		17 (22%)	20 (25%)
5		19 (24%)	18 (23%)
Adjuvant therapy; n (%)			
Hormonal therapy		24 (60%)	26 (67%)
Trastuzumab		0 (0%)	5 (13%)
Docetaxel		0 (0%)	4 (10%)
Gosereline		2 (5%)	3 (8%)
Radiotherapy; n (%)			
Right	Prior to chemotherapy	3 (8%)	3 (8%)
	After cessation of chemotherapy	3 (10%)	9 (23%)
Left	Prior to chemotherapy	4 (10%)	4 (10%)
	After cessation of chemotherapy	6 (15%)	9 (22%)

† Number of courses doxorubicin, cyclophosphamide (all patients received PC-SOD or placebo prior to their chemotherapy courses). No statistical comparison was done at baseline, as baseline values were included as covariates.

Table 2 Summary of Adverse Events

Adverse Event	Placebo (n = 40)		PC-SOD (n = 39)	
	No. of Patients	%	No. of Patients	%
General				
Fatigue	30	75	30	77
Malaise	6	15	8	21
Hot flushes	15	38	16	41
Surgery related AEs	7	18	8	21
Change of taste	11	28	8	21
Central nervous system disorders				
Headache	26	65	18	46
Dizziness	8	20	8	21
Gastro-intestinal system disorders				
Nausea	32	80	29	74
Constipation	11	28	11	28
Dyspepsia	7	18	13	33
Mucositis	5	13	9	23
Psychiatric disorders†	10	25	11	28
Respiratory system disorders				
Respiratory tract infections	13	33	17	44
Vision disorders				
(Kerato-) conjunctivitis	18	43	18	46

Note: Adverse events occurring during chemotherapy in more then 20 patients in one of the the 2 treatment groups.
† Nervousness, emotional lability, anxiety, agitation, insomnia, impaired concentration, abnormal thinking, depression and hallucinations

Table 3 Long-term effects

	Overall change over course baseline and follow up		Difference between treatments	95%-confidence interval	p-value
	Placebo	**PC-SOD**			
Echocardiographic parameters					
Left ventricular ejection fraction (%)	-1	-2	-1	-3 to 1	0.31
E/A ratio	-0.0	-0.0	-0.0	-0.1 to 0.0	0.48
Biomarkers of myocardial injury					
NT-proBNP (ng/L, % change)	32.0	14.2	-13.5	-30.9 to 8.2	0.20
CK-MB mass (mg/L, % change)	-5.7	-7.6	-2.0	-11.7 to 8.8	0.70
Electrocardiography					
Heart rate (BPM)	0.6	4.0	3.4	0.4 to 6.5	0.03
QT-interval (msec)	6.8	3.6	-3.1	-11.6 to 5.4	0.46
QTcB-interval (msec, corrected Bazett)	8.8	16.2	7.4	1.9 to 12.9	<0.01
QTcL-interval (msec, corrected Framingham)	7.5	10.8	3.3	-2.1 to 8.7	0.23

*** Significant change from baseline (p < 0.05)**

Table 4 Effects within the courses

	Overall change from course baseline (at 24 hours)		Difference between treatments	95%-confidence interval	p-value
	Placebo	PC-SOD			
Biomarkers of oxidative stress					
Urinary biopyrrin (µmol/g creatinine, % change)	13.0	3.4	10.3	-20.5 to 52.9	0.55
OxLDL (mU/L, % change)	-3.0	3.0	6.2	0.2 to 12.5	0.04
NPBI (µmol/L, % change)	15.9	5.1	-8.5	-22.2 to 7.6	0.28
Biomarkers of myocardial injury					
NT-proBNP (ng/L, % change)	199.8	263.8	21.4	-3.9 to 53.3	0.10
CK-MB mass (mg/L, % change)	8.2	10.3	2.0	-8.1 to 13.1	0.71
Biomarkers of inflammation					
hsCRP (µg/L, % change)	-2.0	-4.8	-2.9	-19.0 to16.5	0.75
sICAM-1 (ng/L, % change)	-1.1	-1.8	-0.7	-2.7 to 1.4	0.50
TNF-a (pg/L, % change)	-23.2	-27.2	-5.3	-18.1 to 9.6	0.46
MIP-1a (pg/mL, % change)	-46.9	-48.6	-3.3	-45.7 to 72.1	0.91
Electrocardiography					
Heart rate (BPM)	-1.2	3.7	4.9	2.2 to 7.6	<0.001
QT-interval (msec)	16.3	5.3	-11.0	-18.2 to -3.9	0.003
QTcB-interval (msec)	14.2	17.1	3.0	-2.5 to 8.4	0.23
QTcL-interval (msec)	14.2	11.5	-2.7	-7.4 to 1.9	0.24

* Significant change from baseline (p < 0.05)

Figure 1 Mean left ventricular ejection fraction (a) and E/A-ratio (b) for PC-SOD (open circles) and placebo (closed circles) and 95%-confidence intervals (PC-SOD down, placebo up) at baseline and 1, 4 and 9 months post-chemotherapy.

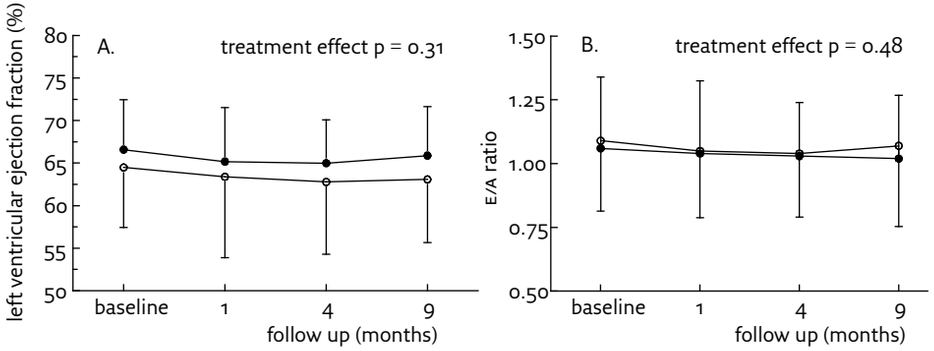
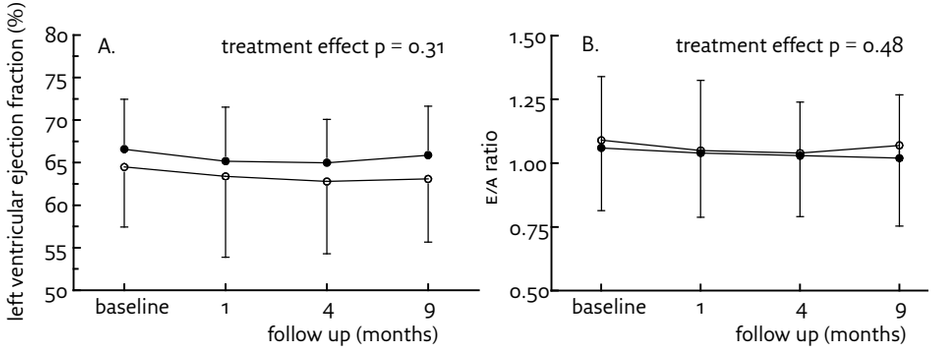


Figure 2 Mean NT-proBNP (a) concentrations, ng/L, and QTC (b), milliseconds, linear corrected for heart rate according to Framingham, during chemotherapy and follow up for PC-SOD (open circles) and placebo (closed circles) and 95%-confidence intervals (PC-SOD up, placebo down) during the course and 1, 4, 9 months post-chemotherapy.



CHAPTER 8

Summary and Discussion

SUMMARY AND DISCUSSION

Traditionally drug development starts with the evaluation of kinetics and tolerability, while in a later stage efficacy is evaluated. An alternative approach that includes biomarkers for clinical endpoints early in the clinical development has been advocated, with potential gains in time and information content of the development process.(1;2) This thesis described the such an approach for a drug to inhibit anthracycline-induced cardiotoxicity.

The thesis comprises two parts: in chapter 2, 3 and 4 we tried to further identify biomarkers suitable for detection of anthracycline-induced cardiotoxicity with an attempt to provide further insight in the pathophysiology of anthracycline-induced cardiotoxicity. In chapter 5, 6 and 7 the development of a novel compound shown to be effective against anthracycline-induced cardiotoxicity in animals, was described.

Biomarkers for clinical endpoints in anthracycline-induced cardiotoxicity

Although the underlying mechanisms are still not completely unravelled, reactive oxygen species (ROS), which are formed in the presence of non-protein bound iron (NPBI), are likely to play a pivotal role in anthracycline-induced cardiotoxicity. These ROS lead to apoptosis of cardiomyocytes eventually causing cardiomyopathy and clinical heart failure. Several stages in the development of anthracycline-induced cardiac failure may be evaluated using biomarkers. Therefore a comprehensive set of biomarkers, including markers of oxidative stress, myocardial injury and remodelling, and markers related to the inflammatory processes that accompany the injury were selected. Theoretically, a combination of these biomarkers can be used to assess the risk for the future development of cardiac failure. Secondly a model using a combination of these markers could be useful in the evaluation of new protective compounds against anthracycline-induced cardiotoxicity.

OXIDATIVE STRESS After anthracycline administration the damage to the myocardium begins with ROS. Therefore we included several parameters indicative of oxidative stress in our model. First oxidative damage was assessed by measuring the oxidation product of Low Density Lipoprotein (OXLDL). OXLDL is a predictor of mortality in congestive heart failure (CHF) and in a recent study it was shown that chronic exposure to OXLDL, as measured by antibodies against OXLDL, is associated with an increased morbidity and mortality in CHF.(3;4) Other markers of oxidative stress used in our studies was the measurement of oxidative metabolites of bilirubin in urine. Urinary biopyrrins are associated with (intracellular) oxidative stress and conditions associated with free radical overload, including congestive heart failure and acute coronary syndromes and could therefore also be useful in the detection of anthracycline-induced oxidative stress. (5;6)

In chapter 7 it was shown that OXLDL and urinary biopyrrins are not elevated after the administration of anthracyclines, suggesting that these markers are not suitable for the detection of anthracycline-induced cardiotoxicity in humans. This is in keeping with the knowledge that these markers were never directly linked to anthracycline-induced cardiotoxicity. The reason for this lack of response could be that these markers are not sensitive enough to detect oxidative stress caused by anthracyclines, or the dose used in our studies was too low to generate sufficient ROS (or a combination of both). Another possibility is that the effect of oxidative stress *in vivo* plays a less prominent role than previously thought.

In vitro and animal studies show that NPBI is an important factor in the generation of ROS.(7-9) NPBI is elevated after administration of the free radical generating chemotherapeutic bleomycin.(10) As discussed previously, NPBI has a pivotal role in the pathophysiology of anthracycline-induced cardiotoxicity.(11) In this thesis (chapter 3) we further explored NPBI as a marker for oxidative stress after the administration of anthracyclines. It was shown that NPBI was increased for a short time after the administration of anthracyclines. These results give further insight in the *in vivo* mechanism of anthracycline-induced cardiotoxicity and indicate that after administration of anthracyclines NPBI indeed is involved

in the mechanism of the cardiotoxicity of anthracyclines. The finding that NPBI is released after the administration could also explain the efficacy of the iron chelator dexrazoxane against anthracycline-induced cardiotoxicity. Therefore our results and those of others indicate that NPBI is an interesting marker to include in a pathophysiology-based model.

INFLAMMATION In the early stages of congestive heart failure pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), soluble ICAM and macrophage inhibiting protein (MIP) are elevated.(12) It can be hypothesized that when the injury by anthracyclines is supposed to occur (during and shortly after the anthracycline infusions) these biomarkers will (transiently) increase as a sign of the myocardial damage.

In our study no changes in these biomarkers were observed during or shortly after chemotherapy. This can be attributed to several causes. First, because anthracyclines (or the dose that was used) simply do not induce the production of pro-inflammatory cytokines. Another possibility is that anthracyclines suppress the production of pro-inflammatory cytokines by a yet unknown mechanism or that these cytokines exert a local reaction and that this goes unnoticed when using systemic venous blood samples. Finally, it cannot be excluded that the concomitant administration of corticosteroids suppressed the inflammatory reaction after anthracycline administration. Inflammatory markers therefore are of limited use in a model for early evaluation of anthracycline-induced cardiotoxicity.

CARDIAC INJURY All the earlier described processes eventually lead to cardiac injury and failure. It is necessary, therefore, to include markers of cardiac function. Unfortunately, traditional markers of cardiac function, such as left ventricular ejection fraction (LVEF), measured by echocardiography of nuclear imaging, are unsuitable for the early detection of anthracycline-induced cardiotoxicity.(11) This is merely because a decline in LVEF occurs in a stage of chronic pathology, when damage to the myocardium is irreversible. and compensatory mechanisms like remodeling are exhausted. Several biochemical markers, including natriuretic peptides and cardiac troponins, could be indicative of cardiac injury and have been suggested to detect anthracycline-induced cardiotoxicity in an early stage of pathology.(13-16) In chapter 2 of this thesis we further explored some of these markers (cardiac troponins, NT-proBNP and CK-MB) and showed that the administration of anthracyclines give an almost immediate two- to threefold increase of NT-proBNP, while no effects for the other markers was seen. As elevated levels of NT-proBNP are associated with an increased myocardial wall stretch, our findings suggest that the administration of anthracyclines causes an immediate increase in myocardial wall stress. It can only be speculated what the mechanism is for the increase in wall stress, as it is possible that the increase in NT-proBNP is either caused by direct damage to the myocardium or represents the neurohormonal response to the damage. Whatever the mechanism is, our findings suggest that NT-proBNP is suitable for the course-to-course evaluation of anthracycline-induced cardiotoxicity. Furthermore, it can be hypothesized that assuming this marker is an indication of myocardial wall stress, preventing the rise in this marker could be an indication of a cardioprotective. effect.. Indeed, there is evidence that the concurrent administration of ACE inhibitors (which reduce afterload) can protect against anthracycline-induced cardiotoxicity.(17;18)

In addition to biochemical markers, electrocardiographic parameters could also be indicative of cardiac failure; a marker that is of interest is cardiac repolarization. Repolarization is represented in the ECG by the length of QT-interval. The QT-interval may be prolonged in the failing heart and studies related QT-prolongation to the occurrence of heart failure.(19) Lengthening of the QT-interval has also been described after administration of anthracycline and has therefore been suggested as an early marker for anthracycline-induced cardiomyopathy.(16) The research described in this thesis showed that anthracyclines directly affect repolarization, as a prolongation of the QT-interval occurred after administration of anthracyclines. In addition, the results in chapter 4 suggest that repolarization reserve, which represents lability of repolarization, is affected by the administration of anthracyclines. It was shown that the repolarization reserve, as measured by a new method to assess the beat-to-beat variation in QT-interval, increases after the administration of anthracyclines.

As anthracyclines are not known to block cardiac ion channels, it is possible that both effects (prolongation of the QT-interval and the increased lability of repolarization), is an early sign of the cardiotoxic effects of anthracyclines. These results point to a potentially powerful non-invasive technique to evaluate anthracycline-induced cardiotoxicity at an early stage.

In summary, in this thesis an extensive array of (bio)markers was evaluated for the early detection of anthracycline-induced cardiotoxicity. It can be concluded that particularly the markers of cardiac failure (e.g. NT-proBNP and prolongation of the QT-interval) could be suitable for the early detection of anthracycline-induced cardiotoxicity.

The multi-marker approach described in this thesis could be a powerful tool in the development of cardioprotective compounds. This is in keeping with a research study that used a multi-marker approach to predict heart failure in the general community.(20)

Protective Strategies

The second part of this thesis consisted of the development of a new compound that could protect against anthracycline-induced cardiotoxicity.

The general belief was that the toxicity of anthracyclines could almost solely be explained by the effect of free radicals, which are formed intracellularly in the presence of iron. Therefore, numerous free radical scavengers have been evaluated in the past decade, both preclinically and clinically, to antagonize the toxic effects of anthracyclines – so far with limited effect, however.(21) As a possible explanation for this failure it has been suggested that the exogenously administered antioxidants did not reach the cytosol, or had too short a half-life. There is also evidence that the therapeutic range of antioxidants is narrow.(22;23) One of the most important antioxidants is superoxide dismutase (SOD), which is present in the cytosol, mitochondria and (in small amounts) extracellularly. SOD has an important function in scavenging free radicals formed in the presence of anthracyclines.

To overcome some of the disadvantages of SOD a lecithinized superoxide dismutase (PC-SOD) was developed which has a prolonged half-life and enhanced affinity for the cell membrane. (22;23) Indeed, studies in animals showed that PC-SOD could be efficacious against anthracycline-induced cardiotoxicity. In chapter 5 and 6 of the thesis the first-in-human studies of this compound are described. It was shown that in humans, too, PC-SOD had a prolonged half-life and an increased SOD activity until 24 hours after administration. It can be hypothesized that these characteristics make PC-SOD a possible candidate to be a protective agent in anthracycline-induced cardiotoxicity. Using the model described earlier, the efficacy of PC-SOD was evaluated in female breast-cancer patients, who received doxorubicin as adjuvant treatment. PC-SOD failed to show efficacy. Among the possible causes for this failure discussed in chapter 7, the most likely explanation is that free radical formation is not the sole explanation for the cardiotoxicity of anthracyclines. Evidence for this can be found in the fact that the anti-cardiotoxic effect of dexrazoxane is mediated by its ability to specifically inhibit doxorubicin-induced DNA damage in cardiomyocytes and probably not by counteracting the formation of free radicals.(24) Secondly, mitoxantrone, which is not known to cause free radical overload, also causes cardiotoxicity.(25-27) The findings in this thesis support the increasing knowledge that ROS are not solely responsible for anthracycline-induced cardiotoxicity and further preclinical research is necessary to elucidate the exact mechanism of anthracycline-induced cardiotoxicity.

Future directives

POTENTIALLY NEW BIOMARKERS In this thesis a comprehensive set of biomarkers is used, recently two new biomarkers have been discovered, ST2 and galectin-3.(12;28) Both markers are associated with cardiac fibrosis and remodeling and can be used to detect heart failure in an early stage.(12;28) There is a case to evaluate these markers in anthracycline-induced cardiotoxicity, as it can be hypothesized that administration of anthracyclines leads to cardiac remodeling and fibrosis. The latter is supported by the finding that late gadolinium enhancement, which is a marker of cardiac fibrosis, can be detected with cardiac MRI after

administration of anthracyclines.(29;30) In addition to these biochemical markers, some new echocardiographic parameters are worth exploring in anthracycline-induced cardiotoxicity. One promising technique is 2D speckle tracking which measures cardiac strain.(31). Recent studies demonstrated that patients treated with anthracyclines have impaired cardiac strain as measured by 2D speckle tracking.(32;33) These results are in keeping with the results in this thesis, which showed elevated levels of proBNP after the administration of anthracyclines. Moreover, late gadolinium enhancement, detected by cardiac MRI could in itself could be indicative of anthracycline-induced cardiotoxicity in an early stage, as incidental reports suggest that this technique could be suitable in the early identification of anthracycline-induced cardiotoxicity.(29;30) New techniques and markers may improve the biomarker model investigated in this thesis. However, the utility of any of these markers is dependent upon the availability of good cardioprotective agents.

NEW PROTECTIVE STRATEGIES The lack of efficacy of PC-SOD described in this thesis and the minimal effectivity of other agents indicate that it is unlikely that anthracycline induced cardiotoxicity is purely caused by free radical species.. The question remains how the distressing occurrence of late toxicity of anthracyclines can be prevented. So far, the only protective compound that has shown efficacy is dexrazoxane.(21) In the most recent ASCO guidelines it is advised to consider the administration of dexrazoxane in patients receiving 300/m2 or more of adriamycin. However, caution should be exercised when administering this agent in settings in which doxorubicin-based therapy has been shown to improve survival, as it has been suggested that the anti-tumor action of doxorubicin is impaired after the administration of dexrazoxane. So there is still need for other cardioprotectants or less cardiotoxic anti-tumor agents. A simple and relatively safe method that could be protective against anthracycline-induced cardiotoxicity is the concomitant administration of ACE inhibitors.(17;18) Additional studies should determine whether the concomitant administration of ACE inhibitors is indeed protective or just inhibits the neurohumoral response to damage. Other strategies than the development of protective agents should also be explored.

New chemotherapeutic agents like liposomal anthracyclines mat solve the problem entirely. A recent meta-analysis demonstrated that the occurrence of clinical and subclinical cardiotoxicity was considerably reduced with these agents when compared to doxorubicin.(34)

In addition, some other less ordinary strategies, such as modifying intracellular transcription factors, are worth exploring. The observation that concurrent treatment with the monoclonal antibody against the HER2/neu oncogene initially augments the occurrence of heart failure, leads to the hypothesis that upregulating HER2/neu could protect against anthracycline-induced cardiotoxicity. Indeed there is preclinical evidence that this protects cardiomyocytes from toxicity of anthracyclines.(35-37) Another interesting target is GATA4, a transcription factor that regulates myocyte differentiation and sarcomere synthesis, and influences survival and several cardiac genes that play a role in anti-apoptotic signaling.(38;39). Additionally it suppresses anthracycline-induced apoptosis.(40) As GATA4 overexpression by alfa-adrenergic agonists antagonizes anthracycline-induced cardiotoxicity it has been suggested that administration of alfa-adrenergic agonists could be cardioprotective in anthracycline induced heart failure. This seems paradoxical as increasing adrenergic drive has a deleterious influence on the outcome in heart failure. Nevertheless some authors suggest that the use of alfa-adrenergic agonists should be evaluated.(41) Finally, it has been suggested that exercise could prevent against anthracycline-induced cardiotoxicity by increasing neuregulin/erbB signaling.(41) It can be expected that when the exact mechanism of anthracycline-induced cardiotoxicity is further elucidated even more protective options will emerge.

Overall conclusion

In this thesis the development of a pathophysiology-based method for the early evaluation of anthracycline-induced cardiotoxicity was described. We evaluated a comprehensive array of biomarkers, representing several aspects of anthracycline-induced cardiotoxicity, including cardiac injury and remodeling, free

radical overload and the inflammation accompanying the injury. It was shown that predominantly the markers of cardiac injury may be suitable for the early detection of anthracycline-induced cardiotoxicity. In the second part of this thesis we evaluated a new, free-radical scavenging compound against anthracycline-induced cardiotoxicity using this approach. The failure of this compound to show efficacy against anthracycline-induced cardiotoxicity in our model suggests that a broader approach toward the mechanism of anthracycline-induced cardiotoxicity is necessary.

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Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Dit proefschrift bestaat uit 2 delen. In de eerste hoofdstukken wordt de ontwikkeling van een biomarkermodel voor anthracycline-geïnduceerd hartfalen beschreven. In het tweede deel wordt de ontwikkeling van een nieuw geneesmiddel tegen anthracycline-geïnduceerde cardiotoxiciteit beschreven.

HOOFDSTUK 1 – ALGEMENE INTRODUCTIE Anthracyclines worden veelvuldig gebruikt in de behandeling van kanker. Een nadeel van deze behandeling is het optreden van late hartschade, welke jaren na de laatste toediening nog kan optreden. Er zijn een aantal risicofactoren voor het optreden van deze hartschade, waarvan de cumulatieve dosering de belangrijkste is. Om die reden is men in de klinische praktijk beperkt in het behandelen met anthracyclines.

Het pathofysiologisch mechanisme van anthracycline-geïnduceerde cardiotoxiciteit is niet geheel bekend, maar een belangrijke rol wordt toegekend aan de formatie van vrije radicalen na toediening van anthracyclines, al dan niet in de aanwezigheid van vrij ijzer. Deze vrije radicalen veroorzaken via verschillende wegen apoptose van de hartspiercel.

Het blijkt lastig om in een vroeg stadium het optreden van anthracycline-geïnduceerd hartfalen te voorspellen, aangezien een daadwerkelijke daling van de systolische kamerfunctie vaak pas laat in het ziekteproces optreedt. Er zijn om die reden in de literatuur diverse (bio)markers beschreven die mogelijk voorspellend zijn voor het later optreden van anthracycline-geïnduceerd hartfalen, waaronder natriuretische peptiden, troponine en creatine kinase MB. Ook verlenging van de QT-tijd is gesuggereerd als mogelijke voorspeller.

Er zijn diverse manieren voorgesteld om deze late hartschade te voorkomen. Waaronder de ontwikkeling van minder schadelijke anthracyclines en andere doseringsschema's.

Gebaseerd op de gedachte dat vrije radicalen betrokken zijn bij het ontstaan van anthracycline-geïnduceerd hartfalen, zijn er verscheidene beschermende stoffen ontwikkeld, zoals vrije radicalen vangers en ijzerchelatoren. Helaas heeft dit tot op heden nog niet geleid tot een vermindering van het optreden van late hartschade. Integendeel zelfs, gezien het feit dat patiënten langer blijven leven (door verbeterde anti-tumor therapie, zal de incidentie van hartfalen na anthracycline bevattende chemotherapie alleen maar toenemen. Vandaar dat er een noodzaak blijft een oplossing te vinden voor dit grote klinische probleem.

HOOFDSTUK 2 – PILOT STUDIE BIOMARKERS In dit hoofdstuk worden de uitkomsten van een pilotstudie beschreven waarin diverse biomarkers, die mogelijk geschikt zijn voor de vroege detectie van anthracycline-geïnduceerde cardiotoxiciteit, werden onderzocht in een groep patiënten die voor diverse vormen van kanker anthracyclines toegediend kregen. De belangrijkste bevindingen in dit hoofdstuk zijn dat na toediening van anthracyclines verlenging van het QT-interval en een vrijwel directe stijging van het NT-proBNP optreedt. Dit suggereert dat deze markers mogelijk geschikt zijn voor de vroege detectie van anthracycline-geïnduceerde cardiotoxiciteit.

HOOFDSTUK 3 – VRIJ IJZER NA ANTHRACYCLINE BEVATTENDE CHEMOTHERAPIE IJzer (en dan met name in zijn niet eiwitgebonden vorm) lijkt belangrijk in het mediëren van de schadelijke effecten van anthracyclines. In dit hoofdstuk wordt de aanwezigheid van vrij ijzer na het toedienen van anthracycline bevattende chemotherapie bekeken. Het blijkt dat het toedienen van anthracycline-bevattende chemotherapie grote invloed heeft op het ijzermetabolisme. Vrijwel direct na het toedienen van anthracyclines ontstaat er een overschot aan ijzer. Dit blijkt uit het feit dat er een stijging zichtbaar is van de hoeveelheid (transferine-gebonden) ijzer en dat de ijzerverzadiging van transferrine vrijwel 100% is. Aangezien met name eiwit in zijn niet gebonden vorm theoretisch van belang lijkt voor het veroorzaken van vrije radicalen gemedieerde schade is er gekeken naar de aanwezigheid van dit vrije ijzer. Het blijkt dat er direct na toediening ook een toename is van vrij ijzer. Dit ijzer is mogelijk beschikbaar voor toxische reacties waarin vrije radicalen kunnen ontstaan en kan op die manier schade aan de hartspier geven.

HOOFDSTUK 4 – QT INTERVAL VARIABILITEIT Verlenging van het QT/QTC wordt gezien als een belangrijke risicofactor voor het ontstaan van arythmieën. Geneesmiddel geïnduceerde QT/QTC

tijd verlenging is een belangrijke reden om nieuwe geneesmiddelen niet toe laten tot de markt. Uit recent onderzoek blijkt dat de voorspellende waarde van verlenging van het QT/QTC interval voor het daadwerkelijk optreden van arythmieën beperkt is. Om die reden is er gezocht naar andere markers. Een belangrijke risicofactor voor het optreden van arythmieën blijkt de afname van de capaciteit van de cardiale celmembraan om te kunnen repolariseren: de zogenaamde repolarisatiereserve. Eén van de uitingen hiervan blijkt een toename van de slag-tot-slag variatie van het QT-interval. Uit onderzoek in diermodellen blijkt dat een toename van slag-tot-slag variatie in QT interval het optreden van arythmieën induceert.

Het is bekend dat anthracyclines na toediening arythmogeen kunnen zijn, het precieze mechanisme is echter niet bekend. In dit hoofdstuk wordt bekeken of er na het toedienen van anthracyclines een verandering is van de slag-tot-slag variatie van het QT-interval. Inderdaad blijkt de slag-tot-slag variatie in het QT interval toe te nemen na de toediening van anthracyclines, hetgeen aantoont dat anthracyclines kennelijk de repolarisatiereserve beïnvloeden. Mogelijk is dit een verklaring voor de bij anthracyclines geobserveerde arythmogeniciteit.

HOOFDSTUK 5 EN 6 – FARMACOKINETIEK LANGWERKEND SUPER-OXIDE DISMUTASE (PC-SOD) Theoretisch is één van de manieren om te beschermen tegen schade veroorzaakt door vrije radicalen het toedienen van exogene vrije radicalenvangers. Superoxide dismutase (SOD) is het enzym dat de reactie waarin het toxische superoxide radicaal wordt omgezet in waterstofperoxide catalyseert ($O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$). Een nadeel van exogeen SOD is dat de halfwaardetijd kort is en dat het een beperkte affiniteit heeft voor de celmembraan. Om die reden is PC-SOD, een recombinant superoxide dismutase, waaraan 4 lecithine staarten zijn gekoppeld, ontwikkeld. Preklinisch onderzoek liet zien dat PC-SOD een 4-voudige hogere affiniteit had voor de celmembraan en een 100-200 voudige hogere activiteit dan normaal SOD. Wij lieten zien dat PC-SOD circa 20 uur actief blijft in gezonde vrijwilligers, hetgeen PC-SOD mogelijk geschikt maakt als beschermend agens tegen ziekten veroorzaakt door vrije radicalen overproductie.
De kinetiek van geneesmiddelen kan verschillen tussen Kaukasiërs en Japanners, vandaar dat al vroeg in het ontwikkelingsproces de kinetiek moet worden vergeleken. In hoofdstuk 6 wordt beschreven dat de kinetiek van PC-SOD in Kaukasiërs en Japanners vergelijkbaar is.

HOOFDSTUK 7 – EFFECTIVITEIT PC-SOD IN VROUWELIJKE BORSTKANKER PATIËNTEN In dit hoofdstuk wordt de werkzaamheid van PC-SOD onderzocht in vrouwelijke borstkanker patienten, die een combinatie van adriamycine (een anthracycline) en cyclofosfamide kregen als adjuvante behandeling. Voor de beoordeling van de werkzaamheid werd gebruik gemaakt van een model met meerdere biomarkers, gebaseerd op de pathofysiologie van anthracycline-geïnduceerde hartschade. Hiervoor werden er (bio) markers die kenmerkend zijn voor hartschade, vrije radicalen overproductie en voor de ontstekingreactie (welke gepaard gaat met de anthracycline geïnduceerde hartschade) geselecteerd. Gebruikmakend van dit model blijkt PC-SOD niet werkzaam als beschermende stof tegen anthracycline-geinduceerde cardiotoxiciteit. Hiervoor zijn een aantal redenen te noemen: de belangrijkste zijn dat de pathofysiologie van anthracycline-geïnduceerde cardiotoxiciteit waarschijnlijk ingewikkelder is dan eerder werd aangenomen en dat het wegvangen van vrije radicalen alleen mogelijk onvoldoende bescherming biedt. Een andere reden zou kunnen zijn dat PC-SOD een nauwe therapeutische range heeft.

Samenvattend wordt in dit proefschrift een op de pathofysiologie van anthracycline-geïnduceerde cardiotoxiciteit gebaseerd evaluatiemodel beschreven. Een uitgebreide set biomarkers, die inzoomen op verschillende aspecten van anthracycline-geïnduceerde cardiotoxiciteit, waaronder cardiale schade en remodellering, vrije radicalen overproductie en de ontstekingsreactie die gepaard gaat met de schade. Met name de markers specifiek voor cardiale schade lijken geschikt voor vroege detectie van anthracycline-geïnduceerde hartschade. In het tweede deel van het proefschrift wordt de ontwikkeling van een nieuwe vrije radicalen vanger tegen anthracycline geïnduceerde hartschade gepresenteerd. Gebruikmakend van het eerder genoemde model blijkt dit middel niet effectief. Dit suggereert dat een bredere kijk op het mechanisme van anthracycline-geïnduceerde cardiotoxiciteit noodzakelijk is.

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CURRICULUM VITAE

Freerk Broeyer was born in Utrecht on April 9th 1979. He graduated from the Christelijk Gymnasium in Utrecht in 1997, and continued his education at Utrecht University medical school. As a medical student he was involved in research into the pathological assessment of Huntington's disease at the department of neurology at the Leiden University Medical Center (supervised by Dr. M. Maat-Schieman). He obtained his medical degree in 2004 after a senior elective associating lower adiponectin levels with metabolic syndrome in patients with coronary artery disease at the department of vascular medicine of Utrecht University Medical Center (supervised by Prof. dr. F.L.J. Visseren). In this rotation his interest for biomarkers in heart disease was aroused.

After a short period as a resident-not-training in internal medicine at the Sint Franciscus Gasthuis in Rotterdam (supervised by Dr. H.S.L.M. Tjen), He joined the Centre for Human Drug Research (CHDR) in 2005 and started this thesis project. In addition he was trained as a clinical pharmacologist, a training he finished successfully in 2009.

After his research at CHDR he was involved in the cardiovascular education of medical students at Utrecht University Medical Center. In 2010 he started his training in cardiology at the Utrecht University Medical Center (headed by Prof. Dr. P.A.F.M. Doevendans), the first part of which he commenced at the department of internal medicine of the Diakonessenhuis in Utrecht (supervised by Dr. A.F. Muller).

In his spare time Freerk enjoys listening to music and he sings in several choirs.

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