Cardiotoxicity of Doxorubicin

A Study of Methods and Protective Interventions in Rat Models

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Scientific environment

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Abbreviations

ACE-inhibitors	Angiotensin-converting enzyme inhibitors
ALL	Acute lymphoblastic leukemia
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocators
AoP	Aortic pressure
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BAX	Proapoptotic bcl-2-like protein
CHF	Congestive heart failure
CL	Clearance
CypD	Cyclophilin D
DIA	Diazoxide
DOX	Doxorubicin
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
EF	Ejection fraction
EPI	Epirubicin
HK	Hexokinase
HF	Heart failure
H_2O_2	Hydrogen peroxide
5-HD	5-hydroxydecanoate
HPLC	High performance liquid chromatography
HR	Heart rate
IDA	Idarubicin

IMM	Inner mitochondrial membrane
IUPAC	International Union of Pure and Applied Chemistry
KHBB	Krebs Henseleit bicarbonate buffer
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
LVEF	Left ventricular ejection fraction
LC-MS/MS	Liquid chromatography with mass spectrometry detection
LTM	Long-time model
M-CK	Myofibrillar creatine kinase
MDR	Multidrug resistance
MO	Morphine
mPTP	Mitochondrial permeability transition pore
Mt-CK	Mitochondrial creatine kinase
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NAL	Naloxone
$NO_{\bullet}$	Nitric oxide
OMM	Outer mitochondrial membrane
$O_{2\bullet}^{-}$	Superoxide radical
$OH_{\bullet}$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite
OR	Opioid receptor
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate buffered saline
PC	Pharmacological preconditioning

PKC	Protein kinase C
PKG	Protein kinase G
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RyR-2	Ryanodine receptor 2
SAL	Saline 0.9 % NaCl
SD	Standard deviation
SL	Sarcolemma
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
STM	Short-time model
TnT	Cardiac troponin T
VDAC	Voltage-dependent anion channel

Table of Contents

Abstract

Introduction: The clinical use of anthracyclines, like doxorubicin, is a double-edged sword. On one side, anthracyclines play an undisputed key role in the treatment of many neoplastic diseases. On the other side; administration of anthracyclines induces cardiomyopathy and congestive heart failure usually refractory to common medications. Therefore, interventions to reduce the cardiotoxicity of doxorubicin are important and clinically relevant, and research should be performed to achieve a better understanding of the toxic mechanisms of doxorubicin.

Generation of reactive oxygen species (ROS), cellular damage mediated by ROS, mitochondrial dysfunction, and impaired calcium handling have been proposed as toxic mechanisms to explain both acute and delayed cardiotoxicity of anthracyclines.

The mechanisms behind the cardiotoxicity of doxorubicin are numerous, and unfortunately not fully understood. However, the anticancer mechanisms and the cardiotoxic mechanisms seem to be quite distinct, leading us to hope that interventions targeted towards the cardiotoxic effects will not interfere or diminish the anticancer effect of this wildly used drug.

In order to intervene, and hopefully prevent cardiotoxicity and heart failure, we need tools and biomarkers for early detection of heart damage. A common clinical tool for evaluation of cardiac function is monitoring left ventricular ejection fraction (EF). A weakness in this method is that cardiac damage is usually detected only when an irreversible functional impairment has already occurred, which leaves little room for early, preventive strategies. Measurement of biomarkers, on the other hand, can be a useful diagnostic tool for early identification, assessment, and monitoring of cardiotoxicity. Release of biomarkers like cardiac troponin T (TnT) and indices of ROS generation like hydrogen peroxide (H_2O_2) are of relevance to study myocardial damage, and can be supplemented by measurement of

myocardial accumulation of doxorubicin and its metabolite doxorubicinol in experimental studies, in order to get a better understanding of the pharmacokinetics and pharmacodynamics of this drug. Thus, animal models where functional, biochemical and pharmacological indices can be studied within an acceptable time-frame, are of interest.

Aims: To establish a rat model to assess interventions to reduce cardiotoxicity of doxorubicin based on physiological, biochemical and pharmacological indices. To study and assess whether pharmacological pretreatment or pharmacological preconditioning with morphine and diazoxide, could reduce cardiotoxicity of doxorubicin.

Results: We developed a short-time model (STM) (Paper I) that reduced animal stress as well as time- and resource consumption in experimental protocols, compared to a long-time model (LTM). Furthermore, we found that morphine enhanced doxorubicin cardiotoxicity (Paper II), and we found diazoxide to be protective against doxorubicin cardiotoxicity (Paper III).

Conclusion: The principles of pharmacological preconditioning (mimicking ischemic preconditioning) represent promising protective interventions in doxorubicin cardiotoxicity, and can be studied in a STM. In our rat model, diazoxide, but not morphine, showed protective effects that could be related to preconditioning. However, the results could be related to other effects of the two drugs.

List of publications

This thesis is based on the following original publications, referred to in the text by Roman numerals.

Paper I **Lisa Drange Hole, Terje H. Larsen, Kjell O. Fossan, Fredrik Limé and Jan Schjøtt**

A short-time model to study relevant indices of cardiotoxicity of doxorubicin in the rat.

Toxicol Mech Methods, 2013; 23(6): 412-418

Paper II **Lisa Drange Hole, Terje H. Larsen, Kjell O. Fossan, Fredrik Limé and Jan Schjøtt**

Morphine enhances doxorubicin-induced cardiotoxicity in the rat.

Cardiovasc Toxicol, 2014; 14(3): 251-259.

Paper III **Lisa Drange Hole, Terje H. Larsen, Kjell O. Fossan, Fredrik Limé and Jan Schjøtt**

Diazoxide protects against doxorubicin-induced cardiotoxicity in the rat.

BMC Pharmacology and Toxicology, 2014, 15:28

1. Introduction

When we started working on this thesis we had two goals. Our first goal was to establish a rat model to assess interventions to reduce cardiotoxicity of doxorubicin based on physiological, biochemical and pharmacological indices. Our second goal was to study and assess whether pharmacological pretreatment or pharmacological preconditioning (mimicking the principles of ischemic preconditioning) could reduce cardiotoxicity of the anthracycline doxorubicin. We wanted the model to be able to induce significant myocardial damage rapidly (in less than 2 weeks), but at the same time allow assessment of protective interventions. Effects of cardiotoxicity on physiological function were assessed in a Langendorff *ex vivo* isolated rat heart model, and release of TnT and H_2O_2 was measured in addition to pharmacological indices. The establishment of a rat model for this purpose is presented in paper I.

The use of morphine as an analgesic is routine in human cancer treatment. Opioids have been reported to induce protection against ischemia and oxidative stress in animal models. Proposed mechanisms of cardiotoxicity of anthracyclines include elements of oxidative stress and impaired calcium homeostasis. Direct stimulation of myocardial δ_1-opioid receptors leads to opening of mitochondrial K_{ATP}-channels and a resultant increase in intracellular free radical signals *in vitro* (1). Thus, we hypothesized that morphine could represent an intervention to reduce cardiotoxicity of anthracyclines acceptable for patients.

We found however, that pretreatment with morphine *in vivo* was associated with a cardiodepressive effect in isolated hearts before doxorubicin exposure. After exposure to doxorubicin *ex vivo*, isolated hearts from rats pretreated with morphine were associated with increased release of H_2O_2, increased release of TnT, increased myocardial contracture and increased myocardial content of doxorubicin. The results from our study of pharmacological pretreatment with morphine are presented in paper II. These results, falsified our hypothesis that morphine could protect against doxorubicin cardiotoxicity. However, results from a

comparable study (2) found that morphine was protective against doxorubicin cardiotoxicity. Based on these opposing reports, we continued to search for a protective mechanism downstream of the δ_1-opioid receptor.

We now investigated whether direct opening of mitochondrial K_{ATP}-channels interacts with the cardiotoxic mechanisms of anthracyclines. We used diazoxide, a selective mitochondrial K_{ATP}-channel agonist, known to have protective properties against cardiac ischemia (3, 4). We also used 5-hydroxydecanoate (5-HD), a selective mitochondrial K_{ATP}-channel antagonist. 5-HD inhibits the increase in free radicals seen with δ_1-opioid receptor activation, and abolishes cardioprotection afforded by ischemic preconditioning (3). In this experiment we found that diazoxide protected against doxorubicin-induced cardiotoxicity in the rat. This protection was abolished by 5-HD. These results are presented in paper III.

1.1 Anthracyclines

Chemotherapy has been used in cancer treatment since World War I when mustard gas was used in chemical warfare and discovered to be a potent suppressor of hematopoiesis (5). Today more than 100 cytostatic drugs are in use, as mono therapy or in combination therapy.

A common target for chemotherapeutic drugs is mitosis or DNA synthesis in cells with short cell cycles. Anthracyclines are cytotoxic antibiotics and are among the most effective antitumor drugs ever developed. Since their discovery more than 50 years ago, anthracyclines have been used in the treatment of many cancers; solid tumors, soft tissue sarcomas and hematological malignancies.

Anthracyclines consists of a tetracyclic ring structure connected to a sugar group. Although similar in chemical structure, the physiological properties of the different anthracyclines, and their clinical application, are very diverse. As shown in Figure 1.1, doxorubicin and daunorubicin share aglyconic and sugar moieties. The aglycone consists of a

tetracyclic ring with adjacent quinone-hydroquinone groups in rings C-B, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13. The sugar, called daunosamine, is attached by a glycosidic bond to the C-7 of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety. The only difference between doxorubicin and daunorubicin is that the side chain of doxorubicin terminates with a primary alcohol, whereas that of daunorubicin terminates with a methyl-group. This minor difference has important consequences for the spectrum of activity of doxorubicin and daunorubicin. Doxorubicin is an essential in treatment of breast cancer, childhood solid tumors, soft tissue sarcomas, and aggressive lymphomas, while daunorubicin shows activity in acute lymphoblastic or myeloblastic leukemias. However, both doxorubicin and daunorubicin had adverse effects, such as development of resistance in tumor cells, toxicity in healthy tissues, and most notably chronic cardiomyopathy and congestive heart failure (CHF). To avoid the latter, the maximum recommended cumulative doses of daunorubicin and doxorubicin were tentatively set at 500 or 450 to 600 mg/m^2, respectively (6).

The search for a "better anthracycline" has resulted in about 2000 analogs, not surprising if one considers the number of chemical modifications or substitutions and/or conjugations that can be introduced in the tetracyclic ring, the side chain, or the aminosugar.

However, only few analogs have reached the stage of clinical development and approval, among them, epirubicin (EPI) and idarubicin (IDA) which both enjoy popularity as useful alternatives to doxorubicin or daunorubicin, respectively. Furthermore, pirarubicin, aclacinomycin A (aclarubicin), and mitoxantrone (a substituted aglyconic anthraquinone) have also attained clinical approval. Still, the activity and toxicity of the most commonly used anthracyclines indicate that a better anthracycline has yet to come. It is therefore not surprising that relatively old drugs like doxorubicin and daunorubicin remain the focus of clinical and preclinical research aimed at improving our understanding of their mechanisms of

activity and toxicity and at identifying new strategies for better use in cancer patients. Figure 1.1 shows the structures of the aforementioned anthracyclines.

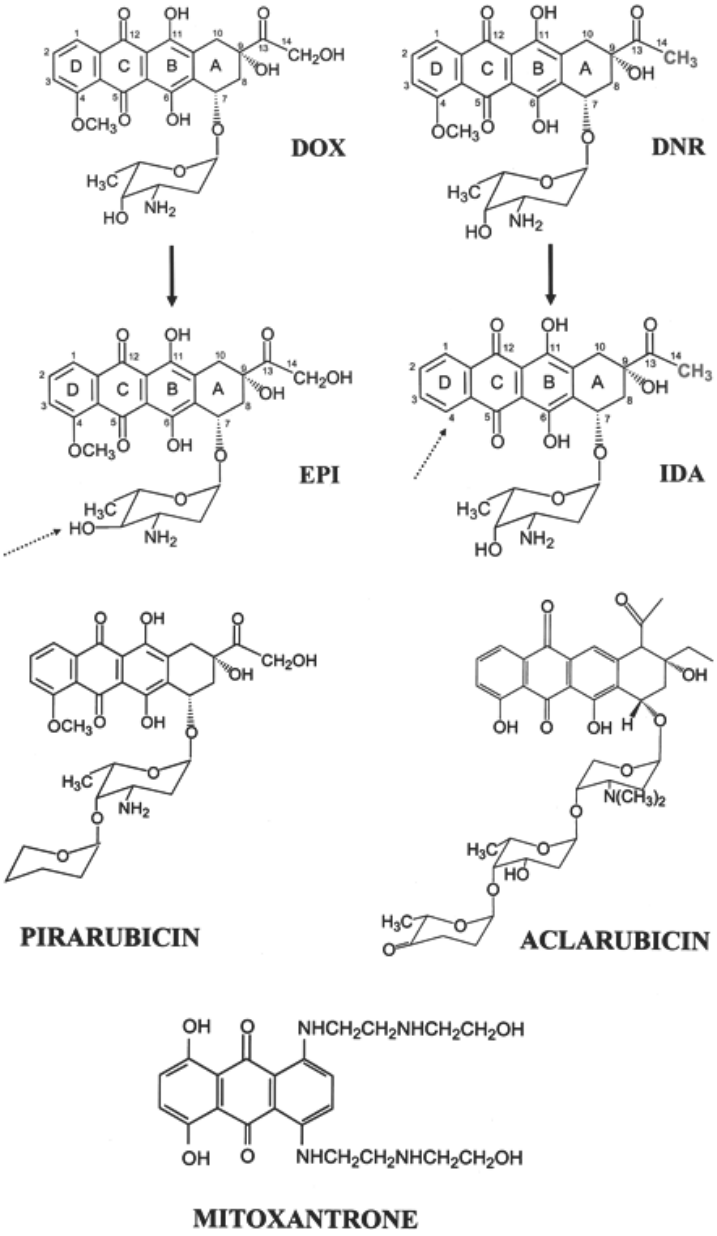

Figure 1.1 Structures of doxorubicin (DOX), daunorubicin (DNR), epirubicin (EPI), idarubicin (IDA), pirarubicin, aclarubicin and mitoxantrone. The side chain of DNR and IDA terminates with a methyl-group in place of a primary alcohol compared with doxorubicin or EPI. Dotted arrows indicate structural modifications in EPI compared with doxorubicin (axialto-equatorial epimerization of the hydroxyl group at C-4′ in daunosamine), or in IDA compared with DNR (lack of the methoxy group at C-4 in ring D). Figure borrowed from (6).

Despite intensive research, the underlying mechanisms responsible for the doxorubicin-induced cardiotoxicity, are not yet fully understood. Published literature so far has focused mostly on mitochondrial dysfunction with consequent oxidative stress, Ca^{2+} overload, and cardiomyocyte death, leading to heart dysfunction. The mechanisms of cardiotoxicity may be more complex than just mitochondrial dysfunction. Partnership of both basic and clinical research is needed to promote new strategies in diagnosis, prophylaxis and therapies, with concomitant cardioprotection, in order to achieve cancer treatment with acceptable cardiotoxicity along life span. See figure 1.2 for chemical structure of doxorubicin and its metabolites (7).

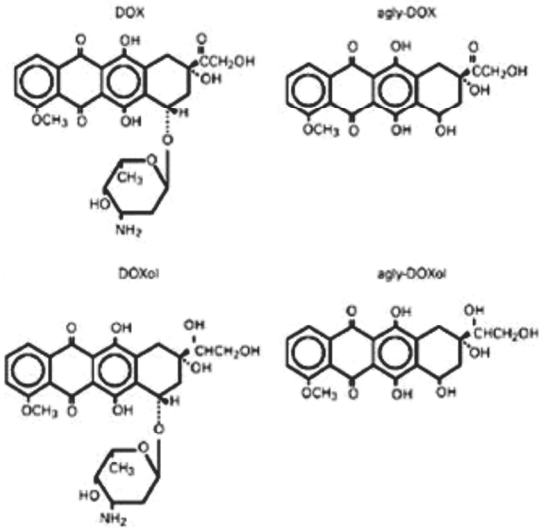

Figure 1.2 Chemical structures of doxorubicin (DOX), doxorubicinol (DOX_{ol}), aglycone-DOX (agly-DOX) and aglycone-DOX_{ol} (agly-DOX_{ol}) (Misiti, Giardina et al. 2003).

1.1.1 Anti-tumor mechanisms

The antitumor mechanisms of anthracyclines are numerous: Cross-linking of DNA, DNA alkylation, inhibition of topoisomerase II activity, inhibition of DNA replication and RNA transcription, generation of free radicals that cause damage to DNA and lipid peroxidation. In addition anthracyclines interfere with the uncoiling of DNA and strand separation and helicase activity and also cause direct membrane damage from lipid peroxidation (8, 9).

1.1.2 Cardiotoxicity

The cardiovascular system, one of the most metabolically active areas in the human body, is likely to be affected by anti-cancer therapies intended to impair cellular turnover and uncontrolled metabolism. Much effort has gone into finding ways to prevent anthracycline cardiotoxicity. However, heart failure remains a consequence of anthracycline exposure. Moreover, symptomatic heart failure often occurs years after cancer treatment, making it difficult to evaluate preventive strategies (10). After or during administration of an anthracycline, patients can experience acute cardiac toxicity which manifests as acute hypotension or transient rhythm disturbances. This is usually transient and resolves without intervention (11). Early chronic and late onset chronic cardiotoxicity manifests as a decrease in cardiac function which can lead to CHF. This is thought to be due to a decrease in left ventricular wall thickness, indicating a decrease in cardiac tissue (12-15).

The incidence of cardiac dysfunction after anthracycline therapy varies depending upon how cardiac dysfunction is defined and the length of time between the end of therapy and evaluation (16, 17). Female gender has also been associated with increased risk for cardiac disease in several studies (12, 18, 19). The reason female gender has been correlated with this increased risk is unknown. Lipshultz et al. hypothesized that it may be due to "differences in oxidative stress, differential expression of the multidrug-resistance gene, and

body composition" (12). However, one should also raise the question whether radiation may be contributing to the increased rate of cardiotoxicity in females in these studies. The specific mechanism of cardiomyocytes injury from anthracyclines remains unclear. Both enzymatic and mitochondrial respiratory chain generation of free radicals and other non-enzymatic formations of anthracycline-iron complexes are thought to be culprit mechanisms, both of which generate ROS (20).

1.1.3 Reactive oxygen species

Free radicals are characterized by the presence of one or more unpaired electrons. Due to this faculty they are extremely reactive compared to their electron paired counterparts. There are several different radicals, but those derived from oxygen are of most concern in biological systems, and are collectively known as ROS. ROS are produced as necessary intermediates in a variety of normal biochemical reactions where they act as intracellular signaling molecules (21). Under physiologic conditions, the level of ROS is kept low and in balance by biochemical antioxidant systems, when this critical balance is disrupted, oxidative stress occurs as a consequence of excess ROS. The mitochondrial respiratory chain is a major source of ROS, with 1-2 % of the consumed oxygen being converted to superoxide (22), and even more during hypoxia (23). A common type of ROS is the superoxide radical which is efficiently converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 may be converted into water by the enzymes catalase or glutathione peroxidase, or produce the highly reactive hydroxyl radical ($OH\cdot$) via the Fe^{2+}-catalyzed Fenton reaction (24). Unlike superoxide and H_2O_2, hydroxyl radicals are highly toxic compounds, reacting with any substance in its vicinity such as lipids, nucleic acids and proteins (25). Whenever free radicals are generated in living cells, the cellular response depends upon the cell type, intracellular localization, amplitude, life span and the type of reactive species (26).

Oxidative stress is the most widely accepted hypothesis of anthracycline-related cardiotoxicity (27-29). This hypothesis links the generation of ROS and lipid peroxidation of the cell membrane to injury of the cardiomyocytes. Anthracyclines enter the cells through passive diffusion (9). Cytosolic enzymes act on the quinone moiety of the anthracyclines, which then induces the formation of superoxide anions. These anions cause subcellular damage, either directly or through further conversion into hydrogen peroxide and the formation of highly reactive hydroxyl radicals, causing lipid peroxidation and DNA damage (9). Figure 1.3 shows how the chemical structure of doxorubicin is the basis for its ability to induce formation of ROS (30).

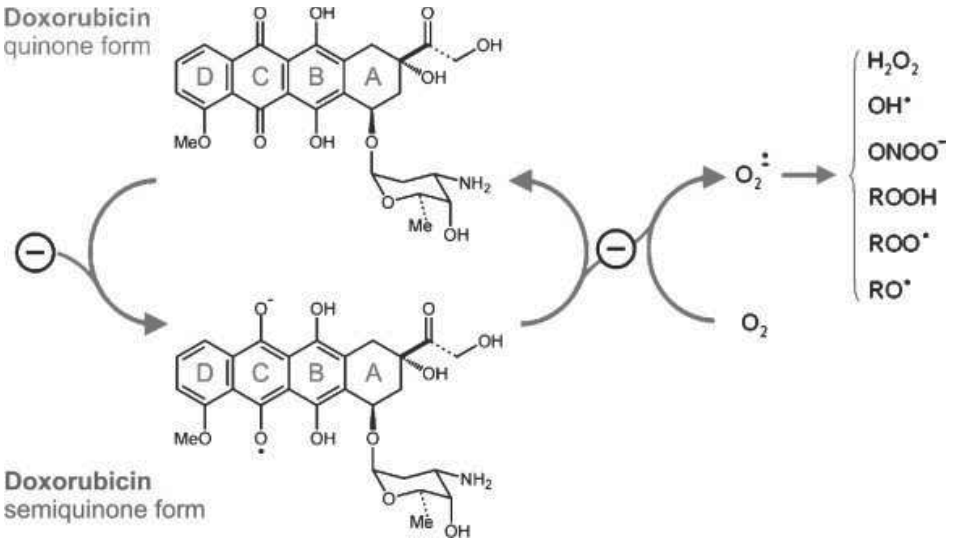

Figure 1.3 Chemical structure of doxorubicin and related redox cycling by enzymatic mechanism. Doxorubicin consists of a tetracyclic ring containing adjacent quinone-hydroquinone groups in rings C–B, coupled with the sugar daunosamine attached by a glycosidic linkage to the ring A. One-electron reduction of the quinone moiety mediated by the cellular oxidoreductases results in formation of a semiquinone radical that regenerates the parent quinone by reducing oxygen molecule (O_2) to superoxide radical ($O_2{\cdot}^-$). This initiates a reaction cascade leading to the formation of other ROS and RNS: superoxide dismutases can convert superoxide radical ($O_2{\cdot}^-$) into H_2O_2; $O_2{\cdot}^-$ and H_2O_2 can interact with iron or other transition metal ions to generate hydroxyl radical ($OH{\cdot}$); $O_2{\cdot}^-$can also initiate lipid peroxidation forming lipid peroxides and derived alkoxyl and peroxyl radicals (ROOH, $RO{\cdot}$, $ROO{\cdot}$) or react with nitric oxide ($NO{\cdot}$) to form peroxynitrite ($ONOO^-$). Besides the depicted enzymatic mechanism, doxorubicin can generate free radicals and other related reactive oxygen and nitrogen species through a nonenzymatic pathway involving complexation with iron (Fe^{3+}). Figure and text borrowed from (30).

Oxidative stress can also induce nitric oxide synthase, and thus nitric oxide ($NO{\cdot}$) and peroxynitrite ($ONOO^-$), which can inactivate key enzymes of the heart muscle, including myofibrillar creatine kinase (31). An important area of investigation addresses the sources and effects of ROS and reactive nitrogen species (RNS) in heart diseases and the factors responsible for their regulation. At low levels, ROS and RNS contribute to a basal endogenous redox buffering environment that reversibly interacts with specific cellular targets, thus creating conditions toward optimal performance (32). With respect to the

mechanism of doxorubicin-induced ROS production, it has previously been indicated that a significant source of the superoxide produced after treatment with doxorubicin is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In support of this observation it has been shown that NADPH oxidase inhibitor significantly attenuated doxorubicin-induced ROS production (33). The human heart is particularly susceptible to doxorubicin toxicity due to its low antioxidant enzyme activities. Therefore, the heart is more vulnerable to doxorubicin-generated ROS insults than other organs in the body. The level of doxorubicin-induced oxidative stress has been found to be up to 10 times greater in the heart than in the liver, kidney and spleen (34).

1.1.4 Mitochondria

The heart is an extremely hard working organ as it pumps on average 10 tons of blood with about 100,000 beats per day. Its mechanical and electrophysiological functions require efficient energy supply and high energy pools. β-Oxidation of fatty acids coupled with mitochondrial oxidative phosphorylation leads to a relatively efficient energy formation in the heart. These processes involve redox mechanisms where oxygen plays a major role, therefore ensuing the formation of significant amounts of ROS. Doxorubicin also enters mitochondria where it either directly inhibits the respiratory chain by binding to cardiolipin, or interacts with mitochondrial DNA (mtDNA) (35, 36). Somatically acquired and accumulating respiratory chain defects which perpetuate ROS formation and contributing quantitative and qualitative lesions in mtDNA (mtDNA depletion, deletions, and point mutations) represent a plausible mechanism for the delayed onset of the cardiomyopathy and the molecular basis of the dose memory for the cumulative doxorubicin exposure (29). Myocardial mitochondria also play an important role in the physiological regulation of the intracellular homeostasis of calcium ions by either sequestering or releasing Ca^{2+} (37). For this purpose, mitochondria and cardiomyocyte membranes are equipped with a complex array of Ca^{2+} transporters.

Doxorubicin interferes at various levels with the mitochondrial membrane and furthermore induces an influx of Ca^{2+} from the extracellular compartment into the cardiomyocytes (38).

1.1.5 Calcium

Ca^{2+} is a key regulator of muscle contraction and of important mitochondrial enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and ATP synthase (39, 40). Alterations in mitochondrial Ca^{2+} transport in the heart have been reported to range from inhibition of uptake by mitochondria upon exposure to doxorubicin *in vitro* to stimulation of uptake in rabbits after repeated doxorubicin exposure (41). It is not known whether the alterations in myocellular Ca^{2+} homeostasis are primary or secondary to the respiratory chain dysfunction in chronic doxorubicin cardiomyopathy. *In vitro* models suggest that the doxorubicin-induced Ca^{2+} uptake by cardiomyocytes and their mitochondria inhibits Complexes I and IV of the respiratory chain and enhances ROS generation, effects which may subsequently limit ATP production (42-44). These data are supported by the observation that decreased cellular ATP levels and thus mitochondrial dysfunction were preceded by increases in mitochondrial Ca^{2+} (45). These studies were carried out *in vitro* and therefore more closely reflect acute rather than chronic doxorubicin cardiotoxicity. A different study, investigating the effects of doxorubicin on the mitochondrial respiratory chain and calcium homeostasis, in a model mimicking the chronic course of the patients, suggest that the respiratory chain dysfunction in chronic doxorubicin cardiomyopathy precedes an intracellular and intramitochondrial accumulation of Ca^{2+} (46). Importantly, doxorubicin is a potent inducer of mitochondrial permeability transition pore (mPTP) associated with reperfusion injury described later in this thesis. Table 1.1 summarizes proposed mechanisms and myocardial cellular targets for doxorubicin cardiotoxicity.

Mechanism	Reference
Lipid peroxidation	(47, 48)
Inhibition of nucleic acids and protein synthesis; release of vasoactive amines	(20)
Changes in adrenergic function and adenylate cyclase	(35, 49, 50)
Inhibition of spontaneous or caffeine-induced sarcoplasmic reticulum Ca^{2+} release	(51)
Reduced expression of: α-Actin, myosin light chain 2 slow, troponin I SR Ca^{2+}-ATPase and Ca^{2+}-gated Ca^{2+} release channel (RyR-2) Phospholamban, calsequestrin Rieske iron-sulfur protein ADP/ATP translocase, phosphofructokinase, Mt-CK	(52-58)
Irreversible decrease in mitochondrial Ca^{2+} loading and ATP content	(59)
Impairment of membrane binding, assembly, and activity of MtCK	(60)
Peroxynitrite-dependent nitration/inactivation of Mt-CK or nitration/activation of metalloproteinases	(48, 61, 62)
Mitochondrial permeability transition pore (mPTP)	(63)

Table 1.1 The multiple mechanisms of cardiotoxicity of doxorubicin. M-CK, myofibrillar creatine kinase; Mt-CK, mitochondrial creatine kinase; RyR-2, ryanodine receptor 2; SR, sarcoplasmic reticulum. Table borrowed from (6).

1.2 Clinical interventions to reduce cardiotoxicity of anthracyclines

Over the past decades, progresses in cancer diagnosis and treatment have improved the life expectancy of countless patients worldwide. Radiotherapy techniques deliver higher dose intensities while narrowing the irradiation field in the interest of the patient's safety. Improved and more conservative surgical techniques inflict less physical and psychological strain on the patients. And finally, molecular biologists joined pharmacologists in the search for drugs that target cancer cells but not the healthy tissues. However, anticancer therapies still challenge patients' compliance, still cause hematologic and non-hematologic toxicities, in

particular cardiovascular toxicity. The incidence of cardiovascular toxicity from anticancer drugs is highly variable and depends on the type of drug being used, its regimen and schedule, the age of the patient at treatment, and the absence or presence of individual risk factors. Cardiotoxicity from anticancer drugs may occur any time after completing chemotherapy. New treatment regiments, combining old cytostatics with the new generation targeted drugs may induce new paradigms and clinical phenotypes of cardiovascular toxicity.

1.2.1 Dose-reduction

Anthracyclines are associated with a risk of heart failure, with the risk proportionate to the cumulative exposure; cardiac injury appears to occur with every dose, and cardiac-biopsy specimens obtained within hours after a single dose of an anthracycline (e.g. doxorubicin) show pathologic changes (64). The development of congestive heart failure can occur at any anthracycline dose, but the risk for development increases with increased cumulative dose of anthracycline, especially doses $\geq 300\ mg/m^2$ (14, 18, 19). Also, the longer it has been since a patient has received anthracycline treatment the higher their risk is for developing changes in cardiac function (15, 65, 66).

1.2.2 Iron-chelates

Dexrazoxane chelates iron, thus preventing the formation of iron-doxorubicin complexes, hence limiting their toxicity by diminishing ROS generation (67, 68). Dexrazoxane also prevents the formation of iron-doxorubicin complexes by interfering with topoisomerase 2β, inhibiting doxorubicin-induced DNA damage (69). Dexrazoxane has almost not been applied in clinical practice the last twenty years because clinicians believed it to reduce tumor response or stimulate second malignancies (70). Recent publications however, show that this is not the case, and that dexrazoxane indeed might provide long-term cardioprotection without

compromising oncological efficacy, frequency of second malignancies, or survival in doxorubicin-treated children with high-risk ALL or in adults treated for breast cancer (68, 71). In these studies the combination of dexrazoxane and doxorubicin resulted in less cardiac damage in children than caused by doxorubicin alone. These results were based on elevated cTnT levels, which were increased in 50% of children treated with doxorubicin alone but only in 21% treated with doxorubicin and dexrazoxane (67, 68). These two latter articles highlight the importance of administering dexrazoxane before each dose of doxorubicin during the first 80 days of therapy in children with high-risk ALL. However, an update committee of the American Society of Clinical Oncology (ASCO) concluded in 2009 that dexrazoxane was not recommended for routine use in breast cancer in adjuvant setting, or metastatic setting with initial doxorubicin-based chemotherapy. They suggested to consider use with metastatic breast cancer and other malignancies, for patients who have received >300 mg/m2 doxorubicin who may benefit from continued doxorubicin-containing therapy (72). The coming years will most certainly show whether dexrazoxane will become routine adjuvant treatment or not. As of today, the jury is still out.

1.2.3 Liposomal anthracyclines

Liposomal forms of anthracyclines (pegylated and non-pegylated) have a more favorable oncological profile and a safer cardiovascular profile than conventional anthracyclines (73). Pegylated anthracyclines circulate longer in the body because uptake by phagocytes is decreased (74). They also have a longer half-life, slower plasma clearance, and a smaller volume of distribution than do conventional forms. These characteristics ensure higher concentrations at the cancer site. In addition, liposomal anthracyclines cannot escape the tight junctions of the heart cells, which prevents them from concentrating in the heart (73). Endomyocardial biopsy specimens from patients receiving pegylated anthracyclines have

shown less cardiac damage than from patients receiving conventional forms (73). A meta-analysis of two studies of women with metastatic breast cancer found significantly lower rates of clinical and subclinical heart failure in patients treated with liposomal anthracyclines rather than conventional anthracyclines (75). Randomized trials testing the efficacy and safety of liposomal anthracyclines for first line therapy in children have yet to be conducted.

1.2.4 ACE-inhibitors

Angiotensin-converting enzyme inhibitors (ACE-inhibitors) are commonly used as cardioprotective agents. ACE-inhibitors are a class of drugs that are routinely administered in the clinic and have clearly shown positive therapeutic profiles for the treatment of heart failure caused by a number of cardiovascular diseases (76). ACE-inhibitors possess free radical scavenger and antioxidant properties (77), and have recently been shown in two clinical studies to be effective in the prevention of anthracycline-induced heart failure when administered early after chemotherapy regimens (78, 79). Administration of ACE-inhibitors in these trials and preclinical studies has not been linked with an increased rate of recurrent malignancy (80, 81). The mechanisms by which ACE-inhibitors can prevent anthracycline-induced cardiotoxicity is not yet fully understood, and only a few studies have been performed (77, 82). So far, the potential effects ACE-inhibition may have on anthracycline-induced mitochondrial dysfunction remain undescribed. One study, investigated potential *in vivo* protective effects of ACE-inhibition against doxorubicin-induced cardiotoxicity in a ten week long model in rats. This study found that concurrent administration of enalapril with doxorubicin ameliorated cytotoxic effects of doxorubicin but also prevented doxorubicin-induced free radical formation and preserved mitochondrial respiratory efficiency and cellular ATP content (83).

1.2.5 Pharmacogenetics

One of the goals of research in the field of pharmacogenetics is to identify genetic variants that predict the occurrence of adverse effects. Pharmacogenetics can help to identify patients at risk for developing a severe adverse effect of a drug. Anthracycline-induced cardiotoxicity after treatment for childhood cancer is a grave problem. Literally hundreds of studies have been performed to identify risk factors for this severe adverse effect. The cumulative dose of anthracyclines seems to be the most important risk factor. However, some children can tolerate a high dose of anthracyclines of 500 mg/m^2 (84). This observed individual variation in anthracycline cardiotoxicity might be explained by genetic susceptibility. Genetic variations in drug metabolizing enzymes and drug transport systems may lead to differences in drug exposure between individuals, resulting in severe toxicity in some of these patients (85). Limited data provide suggestive, but not conclusive, evidence for predisposing variants in genes involved in the metabolism and transport of anthracyclines, however, none of the suspected variants attained the stage of clinical applicability for screening the individual risk for developing CHF or other cardiac events induced by anthracyclines (86). Many enzymes are involved in the metabolism and transportation of anthracyclines. Variation in enzyme efficiency due to genetic factors can increase the concentration of anthracyclines and the risk of cardiotoxicity. Studies have identified associations between specific polymorphisms and doxorubicin-induced cardiotoxicity. However, most studies were performed on small study groups, included few candidate genes and have not been replicated (87). For future pharmacogenetic studies focusing on cardiotoxicity of anthracycline therapy, it will be essential to achieve sufficient statistical power. A large number of childhood cancer survivors is needed to identify a higher number of genetic risk factors, especially when there is not a very large effect of each genetic factor. Perhaps in the future, prediction rules that take both a patient's genetic susceptibility and his or hers other risk factors into account, can yield the

risk of anthracycline cardiotoxicity. In this manner, children with a high risk of anthracycline cardiotoxicity can get a personalized and safer treatment.

1.3 Experimental interventions to reduce cardiotoxicity

1.3.1 Ischemic preconditioning

Ischemic preconditioning (IPC) is a cardioprotective phenomenon in which one or more periods of myocardial ischemia prior to a subsequent prolonged ischemic insult result in an adaption which increases the myocardial tolerance against infarction. Both delayed cell injury and limited myocardial necrosis have been described (88). The phenomenon was first described by Murry et al. (89). They studied the effect of short term coronary occlusions on anaesthetized dogs and reported that ischemic preconditioning resulted in infarct sizes approximately 25% of those observed in untreated hearts (89). The phenomenon has an early phase in which cardioprotective effects last for 1-3 hour, and a delayed phase which appears approximately after 24 hours and may last up to 72 hours (90). The cardioprotection achieved by IPC is manifested in a reduction of infarct size, which is used as the most common measure (91). IPC reduces metabolic activity during the ischemic period which results in better protection of the cells energy storage of ATP (92). Furthermore damaging ionic alterations during ischemia and subsequent reperfusion is reduced. Pretreatment with both adenosine and adenosine receptor agonists induce protection against infarction in rabbit hearts similar to IPC via A_1-receptors (93). Furthermore, an adenosine antagonist administered before IPC abolishes the protective effect (93). Later, another endogenous substance, bradykinin, was demonstrated to induce protection associated with IPC (94). Within the research field of remote IPC, researchers are searching for a humoral factor that might convey cardioprotection to non-cardiac tissues and other organs. Importantly, this implies that

interventions other than ischemia can induce protection associated with IPC, but with a potential for acceptance in the clinical setting.

1.3.2 Pharmacological preconditioning

Pharmacological preconditioning, mimicking ischemic preconditioning, is suggested as an intervention to reduce doxorubicin cardiotoxicity (2). Several drugs and ligands have been proposed to induce pharmacological preconditioning, and it has been demonstrated with opioids including morphine through activation of delta and kappa opioid receptors (95, 96). Schultz et al. suggested that opioid receptor activation serves not only as a trigger of the response but also as a mediator of the memory phase of preconditioning in the rat myocardium (97). Thus, in early preconditioning, a memory phase of up to two hours after trigging in which protection is demonstrated. Morphine is a first-line analgesic in cancer treatment where anthracyclines are involved. Mitochondria are the major effectors of cardioprotection by mechanisms that open the mitochondrial K_{ATP}-channel, including ischemic and pharmacological preconditioning (98). Thus, intracellular signalling pathways as well as sarcolemmal receptors could be targets for pharmacological interventions to reduce cardiotoxicity.

1.3.3 Intracellular signalling pathways

In addition to their essential role in cell survival, mitochondria are regulators of cell death via apoptosis (programmed cell death) and necrosis (accidental cell death). Through feedback mechanisms between the mitochondria and the cytoplasm, cell survival, energy metabolism and homeostasis are maintained (99). Myocardial ischemia increases intracellular Ca^{2+} concentration and oxidative stress generation within the cytoplasm and the mitochondria. The cellular Ca^{2+} overload, oxidative stress and ATP reduction result in a permeability transition

associated with the formation of a non-specific mPTP in the inner mitochondrial membrane, with subsequent loss of ionic homeostasis. Low pH associated with ischemia prevents mPTP, but reperfusion and normalization of pH leads to mPTP (63). During a mild insult of stress, transient opening of the mPTP and/or irreversible pore opening can result in matrix swelling and outer membrane rupture, leading to the release of apoptotic proteins and induction of apoptosis (63). Apoptosis is only possible if a majority of the mitochondria are still able to synthesize ATP. During a severe insult of stress massive swelling and mitochondrial membrane depolarization result in enhanced production of ROS and ATP hydrolysis. Even though apoptotic proteins are released, cell death will occur via necrosis due to ATP depletion (99). Most likely there is a link between apoptosis and IPC. IPC prevents formation of mPTP, thus preventing major influx of electrolytes and water into the mitochondrial matrix, resulting in a decrease of apoptotic myocytes (100). Opioids, adenosine and bradykinin are assumed to give cardioprotection via stimulation of G-protein coupled receptors. As a result protein kinase C (PKC) enhances K_{ATP}-channel activity. It has been demonstrated that in isolated rabbit cardiomyocytes bradykinin triggered ROS generation, and this effect was abolished by inhibitors of both mitochondrial K_{ATP}-channel and protein kinase G (PKG) (94). Opioid receptor stimulation has also been shown to increase intracellular ROS which also activate PKC. Opening of mitochondrial K_{ATP}-channels contributes to an increased level of ROS, which further amplifies activation of K_{ATP}-channels (1). Opening of the K_{ATP}-channels are thought to result in a cascade of signals which in turn will lead to inhibition of mPTP formation in mitochondria (100).

1.3.4 Morphine

Morphine is a non-selective opioid receptor agonist. Opioid receptors are involved in regulation of cardiovascular tissue in both healthy and damaged myocardium. The G-protein

coupled receptors are localized in the central nervous system, peripherally on autonomic presynaptic nerve endings and on cardiac myocytes (101). Myocardial cells are a site for opioid peptide synthesis, storage and release, which are elevated during stress (102, 103). There are three primary subtypes of opioid receptors (OR); my (μ), delta (δ), and kappa (κ). Several studies have found that activation of specific ORs induce cardioprotection similar to IPC (97, 104, 105) and the δ- OR seem to be the primary OR to mediate the cardioprotective effect of IPC. Schultz et al suggested that the δ_1-subtype mediates IPC using BNTX, a selective δ_1-OR antagonist, and a TAN-67, a selective δ_1-OR agonist (106).

Remifentanil, a synthetic opioid analgesic drug, mediates cardioprotective effects through the κ- and δ-ORs, but not through the μ-ORs (107). Another interesting finding is that only κ-receptors are involved in mediating protection in infarct and in arrhythmia mediated by ischemia, while the δ-ORs only take part in the arrhythmia protection. Thus pretreatment with a κ-OR agonist might give a more beneficial protection compared with pretreatment with a δ-OR agonist. However, a higher concentration of the κ-OR agonist is required to generate cardioprotection (108).

Morphine is a commonly used analgesic that, besides having μ-OR mediated analgesic effects, also can stimulate the δ_1-OR. Pretreatment with morphine 24 hours before 25 minutes of regional ischemia followed by 2 hours of reperfusion reduced infarct size after 24 hours to 20%. The infarct size reduction with morphine was abolished by the μ-OR competitive antagonist naloxone (109). Direct stimulation of myocardial δ_1-opioid receptors leads to activation of mitochondrial K_{ATP}-channels and a resultant increase in intracellular ROS *in vitro*. This is an important component of the signalling pathways by which morphine mimics preconditioning in cardiomyocytes (1). Thus, in early preconditioning, a memory phase of up to two hours after trigging has been reported in which protection is demonstrated (90, 95).

Theoretically, this should allow for opioid receptor activation in the memory phase of preconditioning in the rat myocardium.

1.3.5 Diazoxide

Diazoxide has previously been described as an agent with a unique molecular target by opening of mitochondrial K_{ATP}-channels in cardioprotection. However, more recently a consensus seems to emerge that there are numerous effectors involved in the cardioprotective effects of diazoxide, and these effectors may synergistically contribute to its cardioprotective properties (3, 4). In the early 1960's, a study was designed to examine possible non-diuretic mechanisms by which benzothiadiazines lower blood pressure, and diazoxide was found to directly cause vasodilation of blood vessels independent of diuretic actions (110). Early reports, however, also demonstrated that some hypotensive drugs such as diazoxide led to elevated blood glucose levels (111). The following years saw a large rise in publications, mostly related to the hypotensive and hyperglycemic effects of diazoxide. Other actions, including effects on renal excretory function, also started to emerge (112, 113). Nevertheless, the compound became accepted for its oral use in the management of intractable hypoglycemia and intravenously in the management of hypertensive emergencies. The publication rate waned in the mid-1980s. Following the identification of diazoxide molecular effectors in pancreatic β-cells (114, 115) and vascular smooth muscle cells, a secondary rise in diazoxide-related publications occurred, which was further stimulated by the mid-1990's findings that diazoxide has powerful cardioprotective properties (4, 98, 116, 117).

1.3.6 Other drugs

Several other substances have been proposed to be candidates for pharmacological intervention involving targets above. Two examples with clinical relevance are nicorandil and

cyclosporine A. Nicorandil, an anti-angina agent with a mitochondrial ATP-dependent potassium channel opener and nitrate-like activity, reportedly improves prognosis in patients with angina pectoris via preconditioning effects and also exerted endothelial protective effects in both clinical settings and animal studies (118). Nicorandil has been shown to be cardioprotective through inhibition of cardiomyocyte apoptosis induced by oxidative stress and hypoxia (119). Ciclosporine A is fungal metabolite possessing potent immunosuppressive properties that is reported to inhibit reperfusion injury in rat through direct inhibition of mPTP (63, 120). Although these drugs are promising in an experimental setting as cardioprotective, their clinical relevance is hampered by nonselective effects.

2. Methods

2.1 Animals

Male Wistar rats, a strain of Rattus Norwegicus, were used in all three papers. Wistar rats are one of the most frequently applied experimental models in the world. The animals are more than 99% genetically identical, which allows for reproducible and reliable results. However, these rats are predominantly healthy and lack co-morbidities such as hypertension, coronary artery disease, diabetes and so forth, co-morbidities often seen in the average adult cancer patient. Also, using only male and not female animals, can be an important confounder, as a gender difference has been described in doxorubicin-induced cardiotoxicity (12, 121). Our study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Animal Care and User Committee in Norway.

2.2 Anesthesia and anticoagulation

We used intraperitoneal (i.p.) injections of sodium pentobarbithal for anesthesia. Sodium pentobarbithal is quickly absorbed and cheap. Although it is a cardiodepressant, this effect is reversible as the drug is washed out during the first minutes of stabilization in the isolated heart. Heparin i.p. was used to prevent blood clotting.

2.3 Langendorff perfusions

The Langendorff perfusion technique of the mammalian heart was first described by Oscar Langendorff more than a hundred years ago *Untersuchungen am überlebenden Säugetierherzen* "Investigation of the living mammalian heart", Pflügers Arch. 61: 291–332, 1895, is still one of the most widely used models for cardiac research. Langendorff's *ex vivo* model allows for isolated heart studies without influence from the nervous system or the endocrine system that may confound the physiology in question. The Langendorff set up allows for standardization and control over temperature, pH, ion concentration, energy substrates, perfusion pressure and administration of drugs in a controlled manner. A model of the real world will always be a compromise. An *ex vivo* model has many fundamental limitations, when compared to an *in vivo* model for example, similarities are, in many cases limited, hence, one should always be very cautious to extrapolate data generated from such an *ex vivo* model, to a clinical setting. In the Langendorff model, hearts are perfused retrogradely by way of a cannula inserted into the aorta, stopping right above the coronary ostia. When the perfusion medium or buffer flows down the cannula, it is forced into the coronary ostia, because the aortic valve is a one way valve and shuts automatically when pressure is exerted on it from above. The buffer flowing into the coronary ostia perfuses the myocard via the coronary arteries. The cardiac chambers however, are left empty, without any pump action. A constant hydrostatic pressure of 73 mmHg or 100 cm H_2O is maintained by a buffer reservoir

located above the heart. The buffer is a modified Krebs Henseleit buffer (KHBB). The heart is a "multi fuel" pump, able to utilize many different metabolic substrates, predominantly fatty acids in fasted resting state. We used glucose as the only metabolic substrate. This is very common for Langendorff preparations and makes for easy buffer preparation. Fatty acids or proteins would often froth the buffer, sometimes causing mechanical clogging of the coronaries. There are no red blood cells in the buffer. Oxygen is provided to the heart by oxygenation of the buffer with gas made up of 5% CO2 and 95% O2. Because of the buffer's low osmotic pressure, and the increased coronary flow, capillary leak is relatively high. Even so, the heart is stable for several hours with a steady decline of 5-10% in function per hour (122). Our model allows rapid switching between pressure-regulated and volume-regulated flow to allow experimental protocols involving drugs dissolved in KHBB or infused undiluted by a side arm of the aortic cannula. Of particular relevance to the present work is the ability in the Langendorff model to measure contractile indices and coronary variables without systemic effects. Doxorubicin cardiotoxicity is associated with cardiac dysfunction, and this can be studied in parallel with release of biochemical biomarkers of damage. Tissue concentrations of drugs or biomarkers can also be retrieved during or at the end of experiments.

2.4 Cardiac pacing

Cardiac pacing (300 beats per minute by electric stimulation of 5 V amplitude of 3 ms duration) was obtained by placing one electrode on the right auricle and one on the steel cannula of the Langendorff apparatus. We used cardiac pacing, which excludes the possibility of evaluating heart rate and electrocardiograms. This would have been valuable and interesting information considering the well-known association between arrhythmias and anthracyclines. However, assessment of cardiac function (recording of left ventricular developed pressure and secondarily derived contractility indices) was of particular importance

to us, thus, pacing was used to maintain a standard contractile response to the experimental drugs in the model not influenced by changes in heart rate and/or periods of arrhythmia.

2.5 H_2O_2 measurements

H_2O_2 in cardiac effluent was measured using an Apollo 4000 electrochemical detection system (World Precision Instruments, Sarasota, Florida, USA). The system is compatible with a range of specific electrodes with a replaceable tip containing a buffer. When H_2O_2 in the effluate interacts with the tip, an electrical signal is produced which are displayed on the system which allows continuous monitoring. The electrode was calibrated using 9 serial dilutions of H_2O_2 in phosphate buffered saline with added aniline. The current recorded from the effluent was then calculated as μM H_2O_2. Samples were kept at 37°C during measurement. The electrode was allowed 3 minutes of stabilisation and 1 minute of recording.

2.6 Troponin measurements

Cardiac troponins have been suggested as valuable biomarkers of anthracycline cardiotoxicity, both in animal and clinical studies (123, 124). The diagnosis of cardiotoxicity by the evidence of symptomatic heart failure or asymptomatic decrease in left ventricular ejection fraction precludes any chance of preventing its development. The time elapsed from the end of chemotherapy and the beginning of heart failure therapy is of crucial importance in determining complete, partial or non-recovery from anthracycline-induced cardiomyopathy, and this highlights the need for an early diagnosis of cardiac injury (78). By measuring troponins we have the opportunity to detect doxorubicin-induced cardiotoxicity at an early phase, well in time before any reduction in EF has occurred. Troponin is the gold standard biomarker for myocardial injury from any cause. Monitoring troponins during high-dose doxorubicin therapy permits early identification of patients at risk of developing cardiac

dysfunction (123), and stratification of cardiac risk after doxorubicin therapy (125). Thus allowing for preventive therapy in selected high-risk patients (79). We used a 3rd generation troponin T test, Elecsys 2010 immunoassay analyser. This test uses the same monoclonal antibodies (M11.7 and M7) as the 2nd generation ES 300 test, but is standardized with human recombinant cardiac TnT instead of bovine cardiac TnT, which has been used previously. However, previous studies (126) have concluded that there were no differences in coronary TnT concentrations in isolated rat heart effluent between 2nd and 3rd generation cardiac TnT assays, ES 300 and Elecsys 2010, respectively. The sensitivity and precision of the TnT assays have been improved in recent years, and clinically approved high-sensitivity assays, now detect cardiac troponins in nanogram per liter concentration.

2.7 Doxorubicin measurements

Doxorubicin and doxorubicinol were quantified by high performance liquid chromatography (HPLC-MS/MS) (1200 series RRLC, Agilent Technologies, USA) coupled to an Agilent 6410 triple quadrupole mass spectrometer using positive electrospray ionisation (Agilent Technologies, USA). Frozen left ventricular tissue was minced and weighted out in a glass tube with a screw cap and homogenized in physiological saline (2ml/100mg tissue) with a tissue homogenizer (Ultra Turrax, Sigma Aldrich, Germany). 1000µl of sample was added 100µl of daunorubicin as internal standard (IS), and 200µl of buffer (1M TRIZMA, pH 11.1) and mixed well before extraction with 4ml ethylacetate/heptane (80/20 vol/vol). The samples were mixed using a rotary blender for 15 minutes and then centrifuged at 3500rpm for 10 minutes at 10ºC. The organic phase was evaporated to dryness at 50ºC under nitrogen then dissolved in 100µl of methanol followed by 100µl of distilled water. The extract was mixed thoroughly and transferred to silanized vials before analysis. 25µl of extract was injected and separated on a Zorbax SB-Aq (2.1 x 50mm, 1.8µm particles, Agilent Technologies, USA)

column using gradient elution with acetonitrile and 0.1% formic acid in water. Quantification was performed using multiple reaction monitoring (MRM) mode at m/z 546.1 → 363.1 for doxorubicinol, m/z 544.1 → 361.1 for doxorubicin and m/z 528.1 → 321.1 for daunorubicin (IS). HPLC-MS/MS combines excellent sensitivity and specificity in analytical pharmacology. The latter is of importance to separate and quantify closely related analytes (drug and metabolites) through the principle of mass-to-charge ratio of charged particles.

2.8 Drugs

We only used drugs in daily clinical practice in the experiments and with formulations administrated to humans. Commercial saline injections of doxorubicin, morphine, diazoxide and naloxone were used to study interventions (see below) with concomitant vehicle controls. Drug administration in *ex vivo* experiments were obtained by infusion of undiluted saline formulation of doxorubicin at a constant volume rate which was mixed with KHHB in the aortic cannula. Any dead space in the tubes and cannula was avoided by visually observing doxorubicin (red colour) dripping from the tip of it. Reservoirs and tubes were protected from light by aluminium foil. All injections were performed by i.p. injections, and care was taken to follow stability information on packages and leaflets provided by the producers or providers. Drugs were refrigerated and protected by light when not in use when appropriate. Due to the importance of doxorubicin in all three papers in this thesis, a more elaborate description of this drug is included while the other drugs are mentioned briefly.

2.8.1 Doxorubicin

In the 1950s, an Italian research company, Farmitalia Research Laboratories, began an organized effort to find anticancer compounds from soil-based microbes in the area surrounding the 13th-century castle Castel del Monte. A new strain of Streptomyces

peucetius, which produced a red pigment, was isolated, and an antibiotic from this bacterium was effective against tumors in mice. Since a group of French researchers discovered the same compound at about the same time, the two teams named the compound daunorubicin, combining the name Dauni, a pre-Roman tribe that occupied the area of Italy where the compound was isolated, with the French word for ruby, rubis, describing the colour (127). Clinical trials began in the 1960s, and the drug was successful in treating acute leukemia and lymphoma. However, by 1967, it was recognized that daunorubicin could produce fatal cardiac toxicity (127). Researchers at Farmitalia soon discovered that changes in biological activity could be made by minor changes in the structure of the compound. A strain of *Streptomyces* was mutated, and this new compound was named Adriamycin, after the Adriatic Sea. The name was later changed to doxorubicin to conform to the established naming convention (128). Doxorubicin showed better activity than daunorubicin against mouse tumors, and especially solid tumors, its therapeutic index was higher, yet the cardiotoxicity remained. Doxorubicin is commonly used in the treatment of a wide range of cancers, including hematological malignancies such as leukemia and lymphoma, many types of solid tumours and soft tissue sarcomas. The drug is administered intravenously, as the hydrochloride salt. Doxorubicin is photosensitive. Brand names include Adriamycin PFS, Adriamycin RDF, or Rubex. Doxorubicin is also available in liposome-encapsulated/pegylated forms as Doxil, Caelyx and Myocet. Common adverse effects of doxorubicin include hair loss, myelosuppression, nausea and vomiting, oral mucositis, oesophagitis, diarrhoea, skin reactions including hand-foot syndrome and localized swelling and redness along the vein in which the drug is delivered. Less common, yet serious reactions include hypersensitivity reactions, anaphylaxis, liver dysfunction and most important for this thesis; cardiotoxicity (129). Figure 2.8.1 shows structure and systematic (IUPAC) name of doxorubicin.

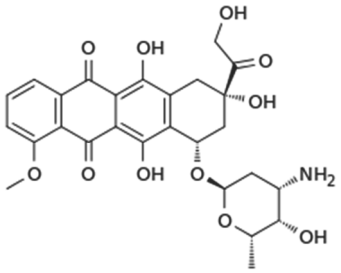

Figure 2.8.1 Structure of doxorubicin. Systematic (IUPAC) name: (7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione.

2.8.2 Morphine

Morphine is a non-selective opioid receptor agonist. Direct stimulation of myocardial δ_1-opioid receptors leads to activation of mitochondrial K_{ATP}-channels and a resultant increase in intracellular ROS *in vitro*. This is an important component of the signalling pathways by which morphine mimics preconditioning in cardiomyocytes (1, 130). Figure 2.9.1 shows structure and systematic (IUPAC) name of morphine.

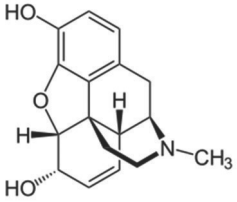

Figure 2.9.1 Structure of morphine. Systematic (IUPAC) name: (5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol.

2.8.3 Naloxone

Naloxone is a non-selective opioid receptor antagonist, used clinically to treat alcohol dependence. Naloxone works by stereospecific competition for receptors, that are mainly located in the central and peripheral nervous system. By way of competitive binding to these receptors, naloxone blocks the access for exogenous opiates. Figure 2.10.1 shows structure and systematic (IUPAC) name of naloxone.

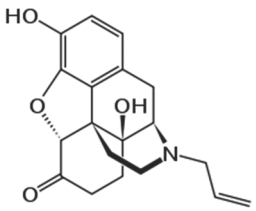

Figure 2.10.1 Structure of naloxone. Systematic (IUPAC) name: (1*S*,5*R*,13*R*,17*S*)- 10,17-dihydroxy- 4-(prop-2-en-1-yl)- 12-oxa- 4-azapentacyclo [9.6.1.$0^{1,13}$.$0^{5,17}$.$0^{7,18}$] octadeca- 7(18),8,10-trien- 14-one.

2.8.4 Diazoxide

Diazoxide has been in clinical use since the early 1960's to treat severe non-malignant and malignant hypertension in hospitalized adults and acute severe hypertension in hospitalized children. Diazoxide is also used to treat hypoglycaemia. The mechanisms of diazoxide's clinical action relate predominantly to the opening of pancreatic and smooth muscle K_{ATP}-channels (3). The protective properties against cardiac ischemia are thought to stem from diazoxide's selective mitochondrial K_{ATP}-channel agonistic properties (3, 4). Figure 2.11.1 shows structure and systematic (IUPAC) name of diazoxide.

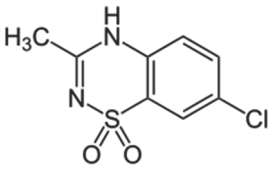

Figure 2.11.1 Structure of diazoxide. Systematic (IUPAC) name: 7-chloro-3-methyl-4*H*-1,2,4-benzothiadiazine 1,1-dioxide.

2.8.5 5-Hydroxydecanoate

5-HD (5-hydroxydecanoate) is a selective mitochondrial K_{ATP}-channel antagonist. 5-HD inhibits the increase in free radicals seen with δ_1-opioid receptor activation, and abolishes cardioprotection afforded by ischemic preconditioning (3). The existence of this channel is controversial as it has only been indirectly characterized by way of patch-clamp studies of the inner mitochondrial membrane (131) and subsequently isolated and purified (132). Figure 2.11.1 shows structure and systematic (IUPAC) name of 5-HD.

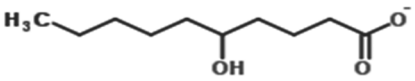

Figure 2.12.1 Structure of 5-hydroxydecanoate. Systematic (IUPAC) name: 5-hydroxydecanoate.

2.9 Statistics

All results were reported as absolute mean values ± standard deviation (SD) in Paper I-III. In experiments including a baseline measurement (Paper II), percent change from baseline was also reported. Groups were compared with regard to parameters with a Student t-test (Paper

I), or a one-way analysis of variance (ANOVA) in Paper II and III. Fisher's protected least significant difference (Fisher's PLSD) test was used as a post-hoc test. Fisher's PLSD is the most 'liberal' of the post-hoc tests in statistical software, and therefore the most likely to result in a Type 1 error (incorrect rejection of a true null hypothesis). Only experimental end-points after treatments (drugs, vehicles, blockers and combinations) were assessed and compared between groups. No repeated measurements descriptions or statistics were performed. SPSS for Windows version 17.0 (IBM, Kolbotn, Norway) was used, and $p < 0.05$ was considered statistically significant in all experiments.

3. Aims

The work presented in this thesis describes establishment of a rat model to assess interventions to reduce the cardiotoxicity of anthracyclines within an acceptable time-frame. Doxorubicin was chosen among anthracyclines. Interventions were based on the principles of pharmacological preconditioning, and included morphine and diazoxide. The primary objective was to assess if the model was cost- and time efficient, and suitable for evaluating protective interventions. We hypothesized that the experimental interventions could reduce cardiotoxicity of doxorubicin. Figure 3.1 summarizes aims, hypotheses and possible targets related to the experimental interventions in the thesis.

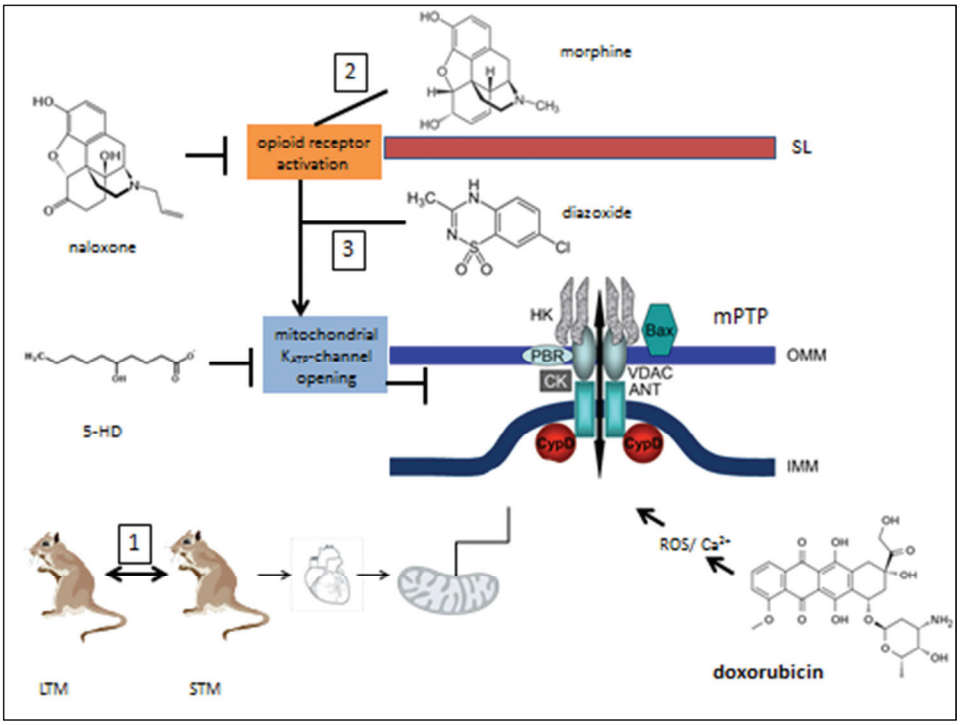

Figure 3.1 Summary of aims, hypotheses and potential pharmacological targets in the thesis. 1. Does a STM (short-time model) of 9 days yield the same information regarding cardiotoxicity of doxorubicin as a LTM (long-time model) of 9 weeks. 2. Does pharmacological pretreatment with morphine, mimicking ischemic preconditioning through opioid receptor activation, reduce the cardiotoxicity of doxorubicin? 3. Does opening of mitochondrial KATP-channels by use of diazoxide reduce the cardiotoxicity of doxorubicin? Also shown is the proposed structure of the mitochondrial permeability transition pore (mPTP) which could be caused by impaired Ca^{2+} and ROS homeostasis caused by doxorubicin. SL (sarcolemma), OMM (outer mitochondrial membrane), VDAC (voltage-dependent anion channel), IMM (inner mitochondrial membrane), ANT (adenine nucleotide translocators), CypD (cyclophilin D), HK (hexokinase), CK (creatine kinase), PBR (the peripheral benzodiazepine receptor), BAX (proapoptotic bcl-2-like protein 4), 5-HD (5-hydroxydecanoate). Modified figure based on (133).

3.1 Paper I

In paper I we investigated if a STM of 9 days yields the same information regarding cardiotoxicity as a LTM of 9 weeks. STMs to study the cardiotoxicity (acute or chronic) of doxorubicin in rats are of interest to assess protective interventions and pathways. A STM promotes more ethical animal treatment with less stress, and at a lower cost compared to established LTMs. We demonstrated that a STM is comparable to a LTM to study relevant indices of cardiotoxicity of doxorubicin in rat hearts. Biochemical parameters, such as H_2O_2 and TnT release, were more pronounced in the STM, while contractile effects were more pronounced in the LTM. Hence, we concluded that our STM could be a preferred model for preliminary studies of protective interventions.

3.2 Paper II

In paper II we investigated if pharmacological pretreatment with morphine, mimicking ischemic preconditioning, could reduce the cardiotoxicity of doxorubicin. The purpose of this study was to examine if pretreatment *in vivo* with morphine could reduce doxorubicin-induced cardiotoxicity *ex vivo* in a rat model, and to characterize the TnT and H_2O_2 responses in hearts treated with doxorubicin. We demonstrated that pretreatment with morphine is associated with a cardiodepressive effect, and enhances cardiotoxicity of doxorubicin, measured by increased myocardial accumulation of doxorubicin and physiological and biochemical indices. The negative effects observed in our rat model were abolished by naloxone, suggesting that stimulation of myocardial δ_1-opioid receptors prior to doxorubicin exposure could be a mechanism involved in cardiodepression and accumulation of doxorubicin in the myocardium.

3.3 Paper III

In paper III we wanted to investigate if opening of mitochondrial K_{ATP}-channels by use of diazoxide could reduce the cardiotoxicity of doxorubicin, by measuring TnT and H_2O_2 in effluate, and if this mechanism was protective against doxorubicin cardiotoxicity. Administration of diazoxide attenuated doxorubicin-induced cardiac dysfunction and reduced doxorubicin-associated H_2O_2-release. This effect was abolished by the K_{ATP}-channel-antagonist 5-HD. We concluded that diazoxide protects against doxorubicin-induced cardiotoxicity in the rat, possibly via opening of mitochondrial KATP-channels.

4. Summary of Results

Paper I

A short-time model to study relevant indices of cardiotoxicity of doxorubicin in the rat

A STM is comparable to a LTM to study relevant indices of cardiotoxicity of doxorubicin in rat hearts. Thus, STM could be a preferred model for further studies of protective interventions. STM is a better alternative than previously described LTMs, because it submits the animals to less stress and discomfort. In addition a STM is less time consuming, more cost effective and more in accordance with the idea of refinement for use of laboratory animals. This study shows that our STM is a promising alternative for future preclinical studies of cardiotoxic mechanisms of doxorubicin and doxorubicinol, and for studies of protective interventions. Furthermore, the model allows the possibility to elucidate important pathways associated with cardioprotective principles.

Paper II

Morphine enhances doxorubicin-induced cardiotoxicity in the rat

In conclusion, we found that pretreatment with morphine is associated with a cardio depressive effect in isolated rat hearts combined with increased release of H_2O_2 and TnT. After exposure to doxorubicin *ex vivo*, isolated hearts from rats pretreated with morphine is associated with increased release of H_2O_2 and TnT, increased myocardial contracture and accumulation of doxorubicin. Morphine increases intracellular free radical signals, and this is an important component of the signalling pathways by which morphine mimics preconditioning in cardiomyocytes. However, results in our pilot study (Aune M. A rat modell to assess interventions to reduce cardiotoxicity of anthracyclines. Does pre-treatment with morphine reduce the cardiotoxicity of doxorubicin? Master thesis in Pharmacy 2008, University of Bergen) and the present study suggest that these pathways, although promising interventions to reduce cardiotoxicity of anthracyclines, are also associated with risk of additional damage in the rat.

Paper III

Diazoxide protects against doxorubicin-induced cardiotoxicity in the rat

The main observation in the present results is that pretreatment with diazoxide attenuates doxorubicin-induced cardiac contractile dysfunction, and attenuates release of biomarkers of cardiotoxicity in effluate without decreasing the accumulation of doxorubicin. 5-HD completely abolished this effect of diazoxide in our rat model. A possible mechanism could be opening of mitochondrial K_{ATP}-channels. However, we cannot exclude other effects of the drug, including opening of vascular K_{ATP}-channels. The low specificity of 5-HD, calls for additional studies of mechanisms of a promising protective intervention.

5. Discussion

5.1 Choice of model

Our study in paper I shows that a 9 day STM is sufficient to demonstrate relevant indices of cardiotoxicity of doxorubicin in isolated Langendorff-perfused rat hearts, irrespective of flow mode. Our model allows parallel assessment of ROS and TnT release, myocardial content of anthracyclines and measurement of cardiac function. Notably, the reduction in contractile function is not so pronounced that any protective intervention would be masked. Biochemical differences between experimental groups are more pronounced in STM, while contractile differences are more pronounced in LTM. The latter reflects the accumulated myocardial contractile damage associated with doxorubicin in the tissue. However, a STM represents a preferred model for preliminary preclinical studies of protective interventions with less stress for the animals, and at a lower cost.

HPLC-MS/MS measurements of doxorubicin and its metabolite doxorubicinol showed significant accumulation of anthracyclines in the myocardial tissue. The results from the LTM, demonstrate the slow elimination of doxorubicin from myocardial tissue. Disposition of doxorubicinol in patients is formation rate limited, with the terminal half-life of the metabolite being similar to doxorubicin. The relative exposure of doxorubicinol, i.e., the ratio between area under the curve (AUC) for doxorubicinol compared to AUC for doxorubicin ranges between 0.3 and 0.6 (134, 135). Thus, doxorubicinol is present in a much lower concentration than doxorubicin, though it is proposed to be more toxic compared with doxorubicin, and it has been established that doxorubicinol is nearly 30 times more potent than doxorubicin at inhibiting calcium-handling proteins and at depressing contractility and systolic myocardial function (20, 136).

5.2 Flow mode

In our STM, a significant reduction in contractile parameters due to anthracyclines could be studied in Langendorff hearts irrespective of flow mode. Reduction of cardiac function can be directly related to measurements of myocardial release of relevant biomarkers and content of doxorubicin and doxorubicinol. With volume regulated flow, the effects on coronary vascular resistance of the anthracycline can be studied in parallel with effects on myocardial contractility. Thus, reduced contractility as a result of reduced coronary flow during pressure regulated perfusion could be assessed. Our results demonstrate that flow mode is of minor importance when studying myocardial effects of doxorubicin in this model.

In our STM, LVDP in doxorubicin treated rats were significantly reduced compared to controls both during pressure regulated and volume regulated perfusion. A similar short term model found comparable results (137), they studied cardiac performances of *ex vivo* perfused hearts from rats that had been treated with various anthracyclines within 12 days, administrated by repetitive injections. One group received 3mg/kg doxorubicin every other day for 12 days, up to a total cumulative dose of 18mg/kg. Even though they used a higher total cumulative dose compared to our study, they found that their model was able to predict correctly what was already known concerning the cardiotoxicity of anthracyclines. These findings support our STM.

5.3 Doxorubicin administration

Different laboratories use different doses of doxorubicin to induce cardiotoxicity. In general, i.p. injections of 2.0-4.0mg/kg are used, up to total cumulative doses of 10-20mg/kg, but higher doses have been reported. In paper I, we used 2mg/kg doxorubicin, up to a total cumulative dose of 10mg/kg, to induce cardiotoxicity. Selection of dose and concentration of doxorubicin was done to give the rats a total cumulative dose high enough to induce

myocardial damage, but at the same time low enough to keep them alive. Choice of dose is particularly important to reduce the possibility of masking beneficial effects of protective interventions. In our results, a reduction of 15% of LVDP compared to controls in STM provides a frame for evaluating such effects, and an increase in dose is possible. The dosage and concentration frame of doxorubicin used in this study has been applied in previous studies, and has yielded comparable results (137-139).

In paper II the concentration of doxorubicin in the perfusate was measured to 5.42 ± 1.98 μM. Again, a non-physiological concentration could mask a protective effect of morphine. However, C_{max} of plasma doxorubicin following a 30 mg/m^2 i.v. bolus dose in humans is reported to be 3 μmol/L, with cellular levels 30-100-fold higher than that of the plasma (140).

5.4 Evaluation of cardiac function

Reduction of the cardiotoxicity of doxorubicin and its main metabolite doxorubicinol, while retaining their anticancer effect, is desirable. Thus, interventions that reduce accumulation of anthracyclines and improve the drug's effect on cardiac function can be measured directly in our STM. Interestingly, diastolic dysfunction, reflecting poor ventricular compliance, is reported to be an early sign of anthracycline cardiotoxicity in the clinic (141, 142). Diastolic dysfunction and increased coronary vascular resistance was observed both in STM and LTM in the present results, and flow mode did not influence the results. Furthermore, an increase of LVEDP between 70-100% was apparent already in our 9 day model and was still present after 9 weeks. However, the increase in coronary vascular resistance was doubled in the same period from 13-30%. Thus, long time effects of doxorubicin in the heart, affect both function of the cardiomyocytes but also the coronary arteries.

Non-invasive transthoracic echocardiography is suitable for studying development and course of anthracycline-cardiomyopathy and development of heart failure in animal models (143). Our global isovolumetric STM cannot reflect *in vivo* heart function, and reduction of contractile indices represents only indirect measures of heart failure. However, our STM is ideal to study contractile function in parallel with release of relevant biochemical parameters in real time specific to the heart. Release of biomarkers in blood associated with *in vivo* models could be subject to several systemic effects and be less specific to the organ of interest.

5.5 The pilot study

In the pilot study, we established an *in vivo* model with 3 groups: 8 rats were pretreated with an i.p. injection of 3 mg/kg morphine and 8 rats were pretreated with an i.p. injection of 0.9 % saline, 60 minutes prior to a 3 mg/kg doxorubicin i.p. injection every other day for 11 days, up to a cumulative dose of 12 mg/kg doxorubicin. 5 rats were pretreated with an i.p. injection of 0.9 % saline 60 minutes prior to an i.p. injection of 0.9 % saline every other day for 11 days. On day 12, hearts from the three groups were planned to be excised and Langendorff perfused for comparison of physiological and biochemical indices of cardiotoxicity. However, 6 out of 8 rats in the group pretreated with morphine died before day 12, and did not complete the treatment protocol. Mortality was evident in the group by day 9 (n=1) and by days 10 and 11 (n=5). The remaining two rats were moribund and euthanasia was performed. Thus, physiological parameters from rats pretreated with morphine were not available for comparison to the other groups, and myocardial tissue was discarded due to risk of post-mortem redistribution of drugs. Based on these results, we hypothesized that the interaction between doxorubicin and morphine could result in increased cardiotoxicity. The results from the pilot study were unexpected because a comparable study (2) found morphine to be

protective against doxorubicin-induced cardiotoxicity in rat. This study pretreated rats with an i.p. dose of 10 mg/kg morphine 30 minutes prior to doxorubicin. Doxorubicin (1.25 mg/kg i.p.) was administrated 4 times per week for 4 weeks, with a total cumulative dose of 20 mg/kg. Cardioprotective efficacy of morphine was performed by analysing the electrocardiographic parameters (QRS complexes and ST segments) and contractility force of left ventricular papillary muscle, and these parameters were improved in rats pretreated with morphine. Morphine also reduced mortality in this study.

5.6 Morphine enhances cardiotoxicity

The main observation in paper II is that the increased mortality associated with morphine observed in the pilot study could be related to enhanced cardiotoxicity. We measured LVDP and derived indices. Enhanced mortality by a combination of morphine and doxorubicin has previously been demonstrated in mice where morphine pretreatment caused a dose-dependent increase in plasma doxorubicin (144). In our study, rats were pretreated *in vivo* with morphine before the hearts were isolated and exposed to doxorubicin *ex vivo*. Pretreatment with morphine *in vivo*, irrespective of dose, is associated with a cardio depressive effect in isolated hearts combined with increased release of H_2O_2 and TnT. After exposure to doxorubicin *ex vivo*, isolated hearts from rats pretreated with morphine, irrespective of dose, demonstrate increased release of H_2O_2 and TnT, increased myocardial contracture and increased myocardial accumulation of doxorubicin. These effects were abolished when naloxone was administrated before morphine in the pretreatment protocol. The enhanced cardiotoxicity of pretreatment with morphine was in particular evident by LVEDP. Myocardial contracture assessed by an increase in LVEDP suggests that pretreatment with morphine enhanced diastolic dysfunction during doxorubicin infusion. Diastolic dysfunction is proposed to reflect impaired calcium handling which together with generation of ROS are proposed as toxic

mechanisms of anthracyclines (145). Interestingly, diastolic dysfunction is proposed to precede systolic dysfunction in chronic anthracycline cardiotoxicity and suggested as an early marker of subsequent heart failure in the clinic (145).

5.7 Pharmacological preconditioning

The morphine dose is the same in our study as in the study that found morphine to be protective against doxorubicin cardiotoxicity (2). However, we delivered it 60 minutes prior to doxorubicin compared to 30 minutes in (2). By using i.p. administration of 4 mg/kg morphine, a study on rats found peak plasma concentrations of about 1 μM after 8 minutes with a half-life of about 46 minutes (146). Theoretically, all these protocols should allow for opioid receptor activation in the memory phase of preconditioning in the rat myocardium. In early preconditioning, a memory phase of up to two hours after trigging has been reported in which protection is demonstrated (90, 95). We used a dose of 3 mg/kg i.p, and added a dosage of morphine of 10 mg/kg as reported by others (2, 109, 147), to investigate whether the dose used in the pilot study was too low to illicit a protective effect. However, both 3 and 10 mg/kg morphine i.p. had a cardio depressive effect prior to exposure to doxorubicin. In this present study, cardio depression with a theoretical morphine peak plasma concentration of approximately 1 μM is in accordance with previous observations (148).

5.8 Free radical damage

In vitro studies have shown that opioids directly decrease the contractile response of isolated ventricular cardiomyocytes to electrical stimulation (149). One hypothesis explaining morphine-induced decreased contractility is that morphine induced the increase in generation of free radicals that we measured at baseline. A study of pretreatment of chick embryonic ventricular myocytes with1 μM morphine before one hour of ischemia and three hours of

reoxygenation found a two-fold increased free radical production before ischemia compared with controls (150). The increase in free radical signals with morphine was abolished by 5-hydroxydecanoate, a selective mitochondrial K_{ATP}-channel antagonist (1). We observed that the cardio depressive effect was associated with increased release of H_2O_2 in morphine treated hearts. However, after doxorubicin infusion, this was associated with further increased release of hydrogen peroxide and increased myocardial contracture evident by increased LVEDP. This raises the question whether an increase in generation of free radicals induced by morphine not only induced a cardio depressive effect prior to doxorubicin, but produced additive myocardial damage to that produced by doxorubicin. H_2O_2 is an important by product of oxidative metabolism and is a major contributor in oxidative stress-induced functional and metabolic dysfunction (151, 152). An experiment that exposed isolated perfused rat hearts to 200μM H_2O_2 for 30 minutes resulted in a time-dependent depression of myocardial contractility and a 1000% elevation in LVEDP (153). Similar results have been reported in isolated rat hearts (154, 155). We found a depression in LVDP of approximately 40% in the morphine groups and 30% in the saline pretreated group after 45 minutes of doxorubicin exposure. This effect was associated with approximately 400% elevation in LVEDP in the morphine groups, irrespective of dose, compared to 200% in the saline group with levels of H_2O_2 in the range of 35-45 μM. Thus, our results suggest that additive free radical damage generated by morphine and doxorubicin could result in contractile dysfunction. H_2O_2 mediates increased endothelial permeability and may increase the extravasation of doxorubicin and alter its distribution in the myocardium (156, 157). Thus, a potential change in endothelial permeability due to increased H_2O_2 can explain the increased content of doxorubicin in hearts from rats pretreated with morphine in our study. We observed that H_2O_2 levels are altered by morphine in combination with doxorubicin. However, there is insufficient assessment of why this might lead to enhanced damage, and of the possibility that

other reactive species may also be involved in the effects seen. Further studies are needed to assess this.

5.9 Diazoxide attenuates cardiac dysfunction

In paper III pretreatment with diazoxide attenuated doxorubicin-induced cardiac dysfunction in the rat. Doxorubicin treatment produced a significant loss in left ventricular developed pressure ($p<0.05$) both in volume- and pressure regulated perfusion similar to previous reports (158, 159). Diazoxide significantly attenuated this decrease in LVDP ($p<0.05$) in both perfusion protocols. Diazoxide also improved diastolic dysfunction in doxorubicin treated hearts, by attenuating LVEDP ($p<0.05$) in both perfusion protocols, compared to animals that received both diazoxide and 5-HD. The protective effect of diazoxide is equivalent to that of ischemic preconditioning, and diazoxide is often used as a pharmacological means to induce preconditioning (3). The drug has previously been described as an agent with a unique molecular target by opening of mitochondrial K_{ATP}-channels in cardioprotection. However, more recently a consensus seems to emerge that there are numerous effectors involved in the cardioprotective effects of diazoxide, and these effectors may synergistically contribute to its cardioprotective properties (3). During volume regulated perfusion, aortic pressure, an indirect measure of coronary resistance, was increased in hearts that received both diazoxide and 5-HD, or just 5-HD, before doxorubicin. However, diazoxide pretreatment attenuated this effect. The vasodilatory effects of diazoxide in vascular smooth muscle are due to opening of vascular K_{ATP}-channels. K_{ATP}-channels have a pronounced role in controlling coronary blood flow and the coronary reserve, particularly in the resistance arterioles (160). In some studies diazoxide has been noted to improve coronary flow, which is associated with cardioprotection, in perfused hearts (161), despite the fact that the coronary flow reserve is low in crystalloid-perfused hearts (162). Coronary flow was significantly higher ($p<0.05$)

during pressure regulated perfusion, in hearts form rats pretreated with diazoxide before doxorubicin, compared to hearts form rats pretreated with saline before doxorubicin. This effect was abolished with 5-HD. Based on these results, improved cardiac function associated with preserved coronary flow during pressure regulated perfusion, and attenuated increase in aortic pressure during volume regulated perfusion, could account for some of the cardioprotective effects of diazoxide.

5.10 Diazoxide reduced H_2O_2 in cardiac effluate

Diazoxide pretreated hearts had lower concentrations of H_2O_2 in cardiac effluate compared to hearts that were pretreated with 5-HD and diazoxide or 5-HD alone. Diazoxide is an inhibitor of the mitochondrial complex II protein, succinate dehydrogenase (SDH). This inhibition also occurs in the heart (163). The activity of SDH is a site of reactive oxygen species (ROS) generation, and the possibility has been raised that diazoxide and other SDH inhibitors mediate some of their cardioprotective effects via modulation of ROS production. Which ROS signals cardioprotection is mediated through is not fully understood (98). Opening of mitochondrial K_{ATP}-channels leads to increased ROS, leading to a persistent open state of the channel. The ROS responsible for this is not known (3). H_2O_2 has been proposed as one candidate for this effect, although one study concludes that H_2O_2 is not the mediator of mitochondrial K_{ATP}-channel-dependent ROS signalling (98). In our study we found higher levels of H_2O_2 in effluate from hearts that had not undergone diazoxide pretreatment before doxorubicin, or that had received both 5-HD and diazoxide before doxorubicin. The level of H_2O_2 associated with cellular signalling is much lower than the levels associated with cardiotoxicity (164). The present study was not designed to examine signalling effects, and our observations are thus associated with the reduced cardiotoxicity of doxorubicin. This is supported by our observation of diazoxide improving diastolic dysfunction in doxorubicin

treated hearts, by lowering the elevation of LVEDP. Diastolic dysfunction and contracture is proposed to be related to ROS generation (165, 166).

5.11 Diazoxide reduced TnT in cardiac effluate

Diazoxide pretreated hearts also had lower concentrations of TnT in cardiac effluate compared to hearts that were pretreated with 5-HD and diazoxide or 5-HD alone. Troponins are myocardial regulatory proteins, which regulate the calcium mediated actin and myosin interaction. Troponin-T is widely used as a specific biomarker to diagnose myocardial infarction. Doxorubicin is associated with increased TnT in serum and in heart effluate (158, 167, 168).

5.12 Langendorff bypasses pharmacokinetics

The Langendorff model bypasses the pharmacokinetic phase associated with drug metabolism and only measures the direct action of the drug in the myocardium. The interaction of morphine and doxorubicin *in vivo* could involve systemic effects that secondarily influenced the function of the heart. In paper II we wanted to investigate how doxorubicin affects a heart that has already been exposed to morphine *in vivo* based on our findings in the pilot study where morphine was administrated in a similar way. Ideally, heart functional parameters could be measured *in vivo* after morphine and subsequent doxorubicin administration, but direct cardiotoxic effects could be difficult to separate from indirect systemic effects. Furthermore, both morphine and doxorubicin could be administrated in sequence in Langendorff hearts to study their effects exclusively in the heart, but pharmacokinetic effects would lack as mentioned above. Furthermore, the effect of morphine-6-glucuronide (active metabolite) was not assessed in our experiments. Metabolism of morphine to morphine

glucuronides is fast, and mean plasma levels of morphine-6-glucuronide exceeds that of morphine 0,5 hour after administration of intravenous morphine (169).

5.13 Intraperitoneal administration of drugs

Repeated i.p. injections could give development of scar tissue and thickened skin with reduced and unpredictable absorption of drugs. Furthermore, doxorubicin, 5-HD and diazoxide could give local tissue damage and inflammation. We were careful to alternate injection sites, using correct injection techniques including a suitable needle to reduce this problem.

5.14 Distribution of doxorubicin and doxorubicinol

Precise cellular and subcellular distribution of the anthracycline is of relevance to understand mechanisms of cardiotoxicity. The compartmental distribution of doxorubicin in the myocardium or within cardiomyocytes is not known due to the fact that heart tissue was minced and homogenized. In paper II the main coronary vascular bed is unlikely due to our wash-out procedure. Furthermore, the reduced contractile function is not due to vasoconstrictive effects since we used a model with volume-regulated perfusion. However, doxorubicin could accumulate in the extracellular space as well as in the cardiomyocytes. The reduced contractile function suggests that at least a part of the accumulation affects the cardiomyocytes. In paper II pretreatment with diazoxide was not associated with decreased myocardial accumulation of doxorubicin or doxorubicinol which suggest that the protective effects did not involve a change of distribution of the anthracycline to the heart *in vivo*.

5.15 Histology and pathology

Assessment of development of cardiomyopathy is important in studies of anthracyclines. In our study, the condition of the heart at the two time points would be of interest. However, our model did not allow for cardiac pathology due to the tissue being used to detect doxorubicin and doxorubicinol.

5.16 Measuring TnT and H_2O_2 in cardiac effluate

Our STM does not allow measurement of intracellular production and compartmental distribution and release of TnT and H_2O_2. Thus, release in effluate represents a sum of venous drain (majority) and exudate from the surface of the heart (minority) from the organ. After nine weeks, pathological changes in the heart could allow for a different distribution and release of H_2O_2. However, release is specific to the organ compared to *in vivo* models where development of heart failure and subsequent hypotension would affect potential release from other organs.

5.17 Do Mitochondrial K_{ATP}-channels exist?

In this study we wanted to investigate if opening of mitochondrial K_{ATP}-channels by diazoxide is protective against doxorubicin cardiotoxicity. Mitochondrial K_{ATP}-channels have been implicated as important mediators of preconditioning (96, 117). However, the evidence for their role in preconditioning is primarily based on the effects of pharmacological agents, in particular, diazoxide and 5-HD. Diazoxide has been reported to be a specific opener of mitochondrial K_{ATP} channels, but studies have suggested that its mechanism of cardioprotection may be due to other actions, such as inhibition of succinate dehydrogenase (170, 171), activation of sarcolemmal K_{ATP} channels (172) or transient opening of the mitochondrial permeability transition pore (173). In addition, it might be argued that the

complex metabolic effects of 5-HD severely limit its usefulness as a 'selective' blocker of mitochondrial K_{ATP} channels (174). Furthermore, since their identification heavily relies on the use of diazoxide as a specific opener and 5-HD as a specific blocker, the very existence of mitochondrial K_{ATP} channels may be questioned. This skeptical view is supported by a study in which no changes in mitochondrial matrix volume induced by diazoxide or 5-HD could be detected (175). Hence, one view on 5-HD is that is should no longer be considered a useful tool for studying the role of mitochondrial K_{ATP} channels in preconditioning (174). Thus, although diazoxide attenuated doxorubicin-induced cardiac dysfunction in our results, our model was not designed to establish the precise protective mechanisms. Furthermore, we cannot exclude that the protective effects involved a change in distribution of doxorubicin to different compartments within the heart.

5.18 Not quite bench to bedside – yet.

The present STM is suitable for preclinical evaluation of new protective interventions to reduce anthracycline cardiotoxicity. However, protection in compressed doxorubicin regimens is not necessarily equiprotective when tested in a more clinically relevant chronic regimen. Such studies should be reserved for interventions proven effective and safe in STM. An experimental STM with a combination of *in vivo* doxorubicin administration and subsequent *ex vivo* evaluation of cardiotoxicity in the rat must make some compromises in comparison to the clinical administration of the drug. The development of cardiotoxicity in animal models with weekly injections is previously described in the literature (137). Repetitive low doses are preferred compared to a single high dose in the rat, and every-other-day administration has been proposed (176). In our STM we used daily i.p. injections to reduce duration of the experiments.

However, our study is of clinical relevance since we used drugs established in the clinic with a described safety profile. In principle, this allows for prospective clinical studies as well as examination of retrospective data. Morphine, for example, is used as an analgetic in oncology and is often combined with anthracyclines. For the time being, however, we are not aware of clinical studies of our hypotheses.

6. Concluding Remarks

A STM is comparable to a LTM to study relevant indices of cardiotoxicity of doxorubicin in rat hearts. STM is a better alternative than previously described LTMs, because it submits the animals to less stress and discomfort. In addition a STM is less time consuming, more cost effective and more in accordance with the idea of refinement for use of laboratory animals. This study shows that our STM is a promising alternative for future preclinical studies of cardiotoxic mechanisms of doxorubicin and doxorubicinol, and for studies of protective interventions. Furthermore, the model allows the possibility to elucidate important pathways associated with cardioprotective principles.

Pretreatment with morphine is associated with a cardio depressive effect in isolated rat hearts combined with increased release of H_2O_2 and TnT. After exposure to doxorubicin *ex vivo*, isolated hearts from rats pretreated with morphine is associated with increased release of H_2O_2 and TnT, increased myocardial contracture and accumulation of doxorubicin. Morphine increases intracellular free radical signals, which is an important component of the signalling pathways by which morphine mimics preconditioning in cardiomyocytes. These effects were blocked by naloxone, in our study (II) suggesting a receptor mediated mechanism. These pathways might be promising interventions to reduce cardiotoxicity of anthracyclines, however they are also associated with a risk of additional damage in the rat.

Pretreatment with diazoxide attenuates doxorubicin-induced cardiac contractile dysfunction, and attenuates release of biomarkers of cardiotoxicity in effluate without decreasing the accumulation of doxorubicin. 5-HD completely abolished this effect in our rat model. A possible mechanism could be opening of mitochondrial K_{ATP}-channels. However, we cannot exclude other effects of the drug, including opening of vascular K_{ATP}-channels. The low specificity of 5-HD, calls for additional studies of mechanisms of a promising protective intervention. Finally, we have no direct evidence of a protective mechanism involving mPTP to explain our results. However, this is an interesting hypothesis.

7. Future Perspectives

In order to prevent and treat doxorubicin-induced cardiotoxicity properly, we need to know more about the underlying mechanisms that cause toxicity by anthracyclines.

The present STM is suitable for preclinical evaluation of new protective interventions to reduce anthracycline cardiotoxicity. However, protection in compressed doxorubicin regimens is not necessarily equiprotective when tested in a more clinically relevant chronic regimen. Such studies should be reserved for interventions proven effective and safe in STM. Furthermore, the model allows the possibility to elucidate important pathways associated with cardioprotective principles.

We observed that H_2O_2 levels are altered by morphine in combination with doxorubicin. However, there is insufficient assessment of why this might lead to enhanced damage, and of the possibility that other reactive species may also be involved in the effects seen. Further studies are needed to assess this.

The Langendorff model bypasses the pharmacokinetic phase associated with drug metabolism and only measures the direct action of the drug in the myocardium. The interaction of morphine and doxorubicin *in vivo* could involve systemic effects that

secondarily influenced the function of the heart. In the present study we wanted to investigate how doxorubicin affects a heart that has already been exposed to morphine *in vivo* based on our findings in the pilot study where morphine was administrated in a similar way. Ideally, heart functional parameters could be measured *in vivo* after morphine and subsequent doxorubicin administration, but direct cardiotoxic effects could be difficult to separate from indirect systemic effects. Furthermore, both morphine and doxorubicin could be administrated in sequence in Langendorff hearts to study their effects exclusively in the heart, but pharmacokinetic effects would lack as mentioned above.

A possible mechanism for the protective effect of diazoxide that we observed, could be opening of mitochondrial K_{ATP}-channels. However, we cannot exclude other effects of the drug, including opening of vascular K_{ATP}-channels. The low specificity of 5-HD, calls for additional studies of mechanisms of a promising protective intervention.

Furthermore, we cannot exclude that the protective effects involved a change in distribution of doxorubicin to different compartments within the heart. The compartmental distribution of doxorubicin in the myocardium or within cardiomyocytes is not known in our experiments due to the fact that heart tissue was minced and homogenized. Precise cellular and subcellular distribution of the anthracycline is of relevance to understand mechanisms of cardiotoxicity.

Assessment of development of cardiomyopathy is important in studies of anthracyclines. In our study, the condition of the heart at the two time points would be of interest, and cardiac pathology ought to be performed.

Pharmacogenetics is a relatively new field, and mapping of potential genes involved in doxorubicin metabolism and genes that influence cardiotoxicity are highly relevant, especially in relation to personalized chemotherapy, in order to minimize adverse effects of cancer treatment.

Although KATP-channels and mPTP represent promising targets for pharmacological interventions, the lack of specificity and selectivity of intervening drugs for targets and tissue illustrates a general problem in experimental and clinical pharmacology. A beneficial effect in the heart could be cancelled by systemic adverse effects. This is of course exemplified by the cardiotoxicity of doxorubicin which limits its anti-cancer effect. Furthermore, the role of active metabolites like morphine-6-glucuronide and doxorubicinol should be examined. The rapid development in technology in molecular biology and drug development could perhaps overcome these problems in the near future.

Animal studies are primarily performed on young and healthy animals. Considering the Silver Tsunami, and the fact that «80 is the new 60», experimenting on young and healthy animals might be misleading. A large number of cancer patients have diabetes and/or cardiovascular disease, and new models should be designed to accommodate these comorbidities, because it is likely that they influence anthracycline cardiotoxicity in some way or other.

It is now 18 years since the contra-intuitive idea that pretreatment with ischemia could attenuate acute epirubicin-induced cardiotoxicity (177) was published, and this thesis represents an initiative to pursue that idea.

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