Clinical and pre-clinical aspects of monoHER
in combination with Doxorubicin

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Clinical and pre-clinical aspects of monoHER
in combination with Doxorubicin

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Chapter 1

General introduction
1. Doxorubicin

Anthracyclines antibiotics are considered to be some of the most effective anti-neoplastic agents for cancer therapy. Anthracyclines are fermentation products of Streptomyces fungi and the structures of the main anthracyclines are shown in Figure 1. Doxorubicin (DOX) has shown to be a potent anti-neoplastic agent used in a wide spectrum of malignancies including hematologic, solid and childhood cancers [Johnson& Richardson, 1998, Iarussi D et al, 2001, Minotti G et al, 2004]. Using DOX as part of treatment improved survival for e.g. in the adjuvant and metastatic breast cancer setting and in childhood cancer [Jensen BV, 2006, Lipshultz SE, 2006, Van Dalen EC, et al, 2006]. Since the development of colony stimulating factors, like G-CSF, and the development of better anti-emetic therapies, DOX-induced side effects such as bone marrow suppression and nausea and vomiting are reversible and better controlled. Unfortunately, the successful use of DOX is restricted by the occurrence of cardiotoxicity [Von Hoff DD, et al, 1979, Minotti G et al, 2004].

\[\text{Figure 1. Structures of doxorubicin (DOX), daunorubicin (DNR), epirubicin (EPI), and idarubicin (IDA). The side chain of DNR or IDA terminates with a methyl group instead of a hydroxymethyl group compared with DOX or EPI. Dotted arrows indicate structural modifications in EPI compared with DOX (axialto-equatorial epimerization of the hydroxyl group at C-4 in daunosamine), or in IDA compared with DNR (lack of the methoxy group at C-4 in ring D) (Figure obtained from Minotti et al.)}\]
1.1 Doxorubicin antitumor activity

The precise mechanism of cytotoxic action for DOX is not fully understood. The following mechanisms may contribute to the antitumor effect of DOX.

The DOX molecule consists of a daunosamine sugar attached to the aglycone part consisting of a tetracyclic ring, which represents the chromophore and includes the quinone structure (Figure 1). The tetracyclic ring is important for intercalation into DNA and it was thought that DOX exerted its activity by DNA intercalation and thereby inducing damage to the DNA and inhibiting synthesis of macromolecules [Pigram WJ, et al, 1972], but this explanation is not sufficient to explain the whole range of different actions of DOX. Topoisomerase II, an enzyme that promotes DNA strand breaks and is involved in resealing the breaks [Wang JC, 1996], is likely to be one of the primary target sites for the antitumor activity of DOX [Zunino & Capranico, 1990, Gerwirtz DA, 1999]. Probably the sugar moiety of DOX plays an important role in the formation and stabilization of the DNA-DOX-topoisomerase II complex [Capranico G, et al, 1994]. Finally, stimulation of the cleavage reaction and inhibition of the religation step of the topoisomerase-DNA cleavable complex by DOX, results in activation of the cascade of apoptosis [Godard T, et al, 2002]. It is suggested that the site of strand breaks induced through inhibition of topoisomerase II is a critical factor in the cytotoxic effect of DOX [Binaschi M, et al, 1997, Gerwirtz DA, 1999].

The association of DOX-induced cardiotoxicity and free radicals is well accepted, but involvement of DOX-induced free radicals in the antitumor effect of DOX is somewhat confusing. However, two reviews concluded that free radicals do not contribute significantly to DOX cytotoxicity in clinically relevant concentrations of DOX [Keizer HG, et al, 1990, Gerwirtz DA, 1999]. In addition, important evidence of dissociating DOX-induced cardiotoxicity from its antitumor effect is a clinical study showing protection against cardiac damage induced by DOX by the iron-chelating agent ICRF-187, without influencing its antitumor toxicity [Elbl L, et al, 2006] and in tumor bearing mice [Van Acker SABE, et al, 1997]

1.2 Doxorubicin-induced cardiotoxicity

DOX-induced cardiac injury is dose-dependent and because of the increased risk of developing cardiac damage at high cumulative doses of DOX, empirical dose limits are routinely used in clinical practice. Most treatment schedules for adult and childhood cancer limit the maximum cumulative dose of DOX to 450–550 mg/m² [Von Hoff DD, et al, 1979, Krischer JP, et al, 1997]. However, there is a considerable variation in the individual susceptibility to the cardiotoxic effects [Mason JW, et al, 1978] and ultrastructural changes in endomyocardial biopsies have been documented in patients treated with reportedly safe cumulative doses [Billingham ME, et al, 1978].
In a recent analysis 26% of patients treated with 550 mg/m^2 DOX developed congestive heart failure (CHF) and of these patients more than 50% experienced a reduction in left ventricular ejection fraction (LVEF) to ≤ 30% [Swain SM, et al, 2003]. The incidence of CHF is somewhat higher in this analysis than was described earlier by Lefrak et al. (> 550-600 mg/m^2 DOX, 18% CHF) [Lefrak EA, et al, 1973]. Beyond a cumulative dose of 550 mg/ m^2 DOX, the increase of CHF increases exponentially (> 36%) [Lefrak EA, et al, 1973, Jain D, 2000].


1.3 Cardiotoxicity in the clinic

DOX-induced cardiotoxicity can manifest itself in patients as subclinical and clinical heart failure. Subclinical heart failure is defined as cardiac abnormalities detected in asymptomatic persons by various methods. Both cannot only develop during therapy, but also years after finishing treatment with DOX [Shan K, et al, 1996]. This cardiotoxicity is usually classified by clinical presentation into three categories: acute, early-onset chronic progressive cardiotoxicity and late-onset chronic progressive cardiotoxicity [Giantris A, et al, 1998, Wouters KA, et al, 2005]. Acute complications of DOX therapy are observed in 0.4–41% of the patients and occur shortly after start of administration. Mostly, these disorders include sinus tachycardia, decreased QRS voltage and accessory ventricular and supraventricular beats [Wojtacki J, et al, 2005]. Discontinuation of DOX usually results in improvement. Less than 1% of these patients die of these acute cardiac complications [Wojtacki J, et al, 2005].

A much less frequently seen complication consists of myocarditis and pericardial effusion, which may appear some days or several weeks after the last dose of DOX [Zucchi & Danesi, 2003].

Early-onset chronic progressive cardiotoxicity occurs during treatment with DOX or within the first year after completion of therapy and is related to the earlier mentioned risk factors. This cardiotoxicity will persist or progress even after discontinuation of DOX treatment, and may evolve into a chronic dilated cardiomyopathy in adults and restrictive cardiomyopathy in children [Giantris A, et al, 1998]. Late-onset progressive cardiotoxicity manifests itself at least 1 year after completion of DOX treatment. After an asymptomatic period cardiac abnormalities appear to be progressive and manifest themselves as life-threatening
congestive heart failure (CHF), which is usually irreversible and has a high morbidity and mortality rate [Ferrans VJ, 1983]. Shortness of breath, tachycardia, edema of the ankles, hepatomegaly, paroxysmal nocturnal dyspnea, increasing fatigue and a dry cough are clinical symptoms that can be part of heart failure [Singal & Iliskovic, 1998]. Besides that CHF is associated with significant mortality, the quality of life is also significantly impaired in patients with CHF [Elliot P, 2006, Johnson SA, 2006]. Patients may experience a diminished ability to perform activities of daily living such as exercise intolerance [Dracup K, et al, 1992]. Without treatment the prognosis of patients with DOX-induced heart failure is grave, with a mortality rate of about 50% within two years of diagnosis [Jensen BV, et al, 2002]. When patients with DOX-induced CHF are treated with angiotensin-converting enzyme (ACE) inhibitors, cardiac function may improve for a certain period, but after several years patients return to pre-ACE inhibitor levels of myocardial function [Lipshultz SE, et al, 2002b]. It is thought that subclinical cardiac damage may be induced as early as the first dose of treatment and is progressive, leading to myocardial cell loss and long-term cardiac dysfunction [Elliot P, 2006, Johnson SA, 2006]. Indeed, several clinical studies showed that the incidence of abnormal cardiac function after treatment with DOX appears to increase with time [Lipshultz SE, et al, 1991, Steinherz LJ, et al, 2001, Kremer LCM, et al, 2002]. In survivors of childhood cancer the incidence of cardiac abnormalities also increased during the length of follow-up with lifelong consequences [Lipshultz SE, et al, 1991, Kremer LCM, et al, 2002].

1.4 Detection of cardiotoxicity
Regular monitoring of the heart function during treatment, at the end of the treatment and in the follow up of patients treated with DOX is important for several reasons. First, identification of (severe) cardiac damage can lead to discontinuation of DOX to prevent further deterioration of cardiac function. Second, detection of cardiac dysfunction can lead to early medical intervention to improve the prognosis. There are several different methods used at this moment either as a standard or as an investigational procedure in the detection of DOX-induced cardiotoxicity. No satisfactory method to regular monitoring DOX-induced cardiotoxicity has been recognized presently, because each technique has its own specific limitations [Meinardi M, et al, 1999]. In clinical studies of new anthracycline analogues or cardioprotective agents, early detection of anthracycline-induced cardiotoxicity is necessary. The most frequently used techniques are discussed below.
Endomyocardial biopsy

The golden standard for early detection of DOX-induced cardiotoxicity is the endomyocardial biopsy, because of its high sensitivity and high specificity [Singal & Iliskovic, 1998, Kilickap S, et al, 2005, Villani F, et al, 2006]. During percutaneous right or left heart catheterization three to four biopsy specimens from the septum or apex (depending on the method of approach) of the right or left ventricle are obtained and these specimens are prepared and assessed by light and electron microscopy [Mason JW, 1978, Mills A, et al, 1985]. Two main types of DOX-induced myocyte damage have been recognized: 1) myofibrillar loss, in which the cardiac cells are characterized by smaller and fewer mitochondria, and 2) vacuolar degeneration. The earliest manifestation is distension of the sarcoplasmic reticulum which eventually swells and coalesces to form large membrane-bound clear spaces in the cytoplasm [Billingham ME, et al, 1978] These two types of myocyte injury may occur in the same cell or in distinct cells and will eventually progress until death of the cardiomyocyte [Billingham ME, et al, 1978]. The morphologic grade determined from the specimens examined by electron microscopy was scored on a 6-point scale previously described by Billingham and Bristow [Billingham ME, et al, 1978, Bristow MR, et al, 1982]. The degree of morphologic cardiotoxicity assessed by the grading system proved to be related with the cumulative dose of DOX and to be predictive for the development of cardiac heart failure [Bristow MR, et al, 1978, Bristow MR, et al, 1981, Swain SM, et al, 2003]. Data of Billingham et al. [Billingham ME, et al, 1978] showed that anthracycline-associated myocardial degeneration occurred in nearly all patients treated with doses of 240 mg/m$^2$. Despite its invasiveness, it is the best manner to investigate early damage to the heart tissue at this moment [Berry G, et al, 1998, Meinardi M, et al, 1999].

Biochemical markers

For detection of cardiotoxicity at an earlier stage, the use of biochemical markers such as atrial and brain natriuretic peptides, endothelin-1 as well as cardiac troponin – T and – I have been investigated [Yamashita J, et al, 1995, Suzuki T, et al, 1998, Kilickap S, et al, 2005]. Cardiac troponin – T (cTnT) and – I are thin-filament contractile proteins present in high concentrations in the myocardium. The serum cTnT level increases after myocardial injury such as acute myocardial infarction [Katus HA, et al, 1991], but some studies also show an increased serum cTnT during DOX treatment [Lipshultz SE, et al, 1997, Auner HW, et al, 2003].

Brain natriuretic peptide (BNP) is mainly secreted from the heart, and mostly from the ventricles, whereas atrial natriuretic peptide (ANP) is mainly synthesized and released from the atria. It has been shown that levels of natriuretic peptides were increased in patients with severe CHF, but also in patients with asymptomatic left ventricular systolic dysfunction...
Francis GS, et al, 1990, Lerman A, et al, 1993]. Results from several studies indicate that BNP release is increased as left ventricular function deteriorates and that both increased wall stretch and increased tension are responsible for the elevated BNP level [Berry G, et al, 1998]. There are a few studies showing changes in natriuretic peptides during anthracycline therapy [Suzuki T, et al, 1998, Nousiainen T, et al, 1999].

Endothelin-1 is a peptide with a potent vasoconstrictive effect. It is synthesized in the vasculature and the myocardium by endothelial cells and ventricular myocytes. There is little information on the increased concentration of plasma endothelin-1 in patients after treatment with DOX who developed CHF. This data suggests that serial monitoring of plasma endothelin-1 levels may detect subclinical DOX cardiac damage [Yamashita J, et al, 1995]. However, large-scale studies are needed to address whether these biomarkers following DOX treatment are at increased risk of developing late onset overt heart failure.

**Left ventricular ejection fraction**

Currently, the most common method used to detect DOX-induced cardiac damage is the serial measurement of the left ventricular ejection fraction (LVEF) by radionuclide angiocardiography or echocardiography [Killickap S, et al, 2005, Lu P, 2005, Villani F, et al, 2006]. The latter, in comparison to radionuclide angiocardiography, is considered to possess a similar specificity and a lower sensitivity, but has the advantage of being easy to perform without radiation exposure. Serial studies of LVEF can detect a change in cardiac function over time and DOX treatment can be stopped when a certain decrease in LVEF is observed. Schwartz et al. [Schwartz RG, et al, 1987] proposed guidelines for monitoring DOX-induced cardiotoxicity, which resulted in a fourfold reduction in the incidence of CHF. Interpretation of the changes in LVEF values must be done with care, because the presence of factors such as fever or anaemia may affect these values [Jain D, 2000].

**Electrocardiography**

Electrocardiography used for monitoring patients treated with DOX shows transient ECG-abnormalities in 0 to 41% [Ganz WI, et al, 1996] of patients, but they do not reflect nor predict the development of CHF. Historically, a decrease of QRS-voltage was used to indicate chronic cardiotoxicity, but this seems to be a late phenomenon that coincides with the onset of cardiac failure. Besides, patients who develop pericardial effusion or pleural effusion because of tumor progression for instance can also have a decrease in QRS-voltage. Other ECG parameters that were proposed to be related with DOX-induced cardiotoxicity are for e.g. PQ interval, and QRS duration, but these changes appeared to be of low sensitivity and low specificity [Ganz WI, et al, 1996].
1.5 Mechanisms of DOX-induced cardiotoxicity

Over the years, different hypotheses have been proposed to explain DOX-induced cardiac damage. There is much evidence that the generation of free radicals plays a role in cardiomyocyte toxicity [Gianni L, et al, 1985, Olson & Mushlin, 1990, Wouters KA, et al, 2005], but besides this, several other mechanisms appear to be involved in the development of DOX-induced cardiac damage.

Free radicals and the selective cardiotoxicity

DOX can generate free radicals in various ways. The quinone moiety in the tetracyclic ring of DOX is converted into the semiquinone radical by oxoreductive enzymes like cytochrome P450 reductase and NADH dehydrogenase. Via oxidation of the DOX-semiquinone radical by molecular oxygen its original quinone form and superoxide anion radicals ($O_2^-$) are created. Superoxide ($O_2^-$) can be reduced to $O_2$ and hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD). Catalase (CAT) can convert $H_2O_2$ further into water and oxygen, whereas glutathione peroxidase uses glutathione to reduce and thereby inactivate $H_2O_2$ to water and oxidized glutathione [Myers C, 1998]. However, due to the relatively low concentration of the enzyme CAT in the heart tissue and in the presence of traces of iron salts, the Fenton or the Haber-Weiss reactions can occur (equation 1 and 2, respectively).

$$H_2O_2 + Fe^{2+} \rightarrow HO^+ + HO^- + Fe^{3+} \quad (1)$$
$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+}/Fe^{3+}} HO^+ + HO^- + O_2 \quad (2)$$

The hydroxyl radicals ($HO^+$) produced by these reactions are much more reactive and destructive oxidants than $O_2^-$ and $H_2O_2$.

In addition to the enzymatically generated radicals, they are also generated via the non-enzymatic reduction of DOX, which occurs after forming a complex of DOX with metal ions like $Fe^{3+}$, $Cu^{2+}$, $Mg^{2+}$ and $Zn^{2+}$. The free radicals cause cell damage to DNA, proteins and cells membranes [Myers C, 1998]. The role of iron in DOX-induced cardiac damage appears to be complex. Besides that anthracyclines were shown to induce dysregulation of iron homeostasis [Minotti G, et al, 2001, Kwok & Richardson, 2003], results of a recent study of Kaiserová et al. [Kaiserová H, et al, 2006] suggested that the Fenton derived oxidative stress is not crucial in the development of DOX-induced cardiac toxicity.

In comparison to cells of other organs, cardiac cells are more susceptible to free radical damage due to specific heart tissue properties. Heart tissue is 50% mitochondria of weight [Myers C, 1998] and contains cardiolipin, which is a phospholipid in their inner membrane. DOX has very high affinity for cardiolipin, which results in an accumulation of DOX inside cardiac cells [Goormaghtigh E, et al, 1999]. Because of the relatively low concentration of
CAT and SOD in cardiac cells, these enzymes cannot properly inactivate the large amounts of free radicals generated by DOX. In addition to this, DOX was found to rapidly destruct glutathione peroxidase, which is responsible for an important mechanism to detoxify hydrogen peroxide [Doroshow JH, et al, 1980]. Results of another in vitro study suggest that DOX-induced GSH depletion during apoptosis occurs mainly via an oxidative mechanism [Kotamraju S, et al, 2000]. To prevent or modify the cardiac damage caused by DOX-induced free radicals, several studies tried to achieve cardioprotection by for e.g.; increasing endogenous cell antioxidants, or by introducing exogenous antioxidants that would inactivate / neutralize the free radicals generated by DOX [Myers CE, et al, 1983, Yen H, et al, 1996, Matsui H, et al, 1999, Herman EH, et al, 2000].

**The metabolite doxorubicinol**

The secondary alcohol metabolite of DOX is doxorubicinol. This metabolite is found to be about 30-fold more potent than DOX in reducing the contractile force of isolated rabbit papillary muscle [Olson RD, et al, 1988]. On the other hand de Jong et al. found in the mouse left atrium model that doxol is two times more potent than DOX in reducing atrium contractility [de Jong J, et al, 1993]. It was demonstrated that animals with a cardiac-specific overexpression of human carbonyl reductase exhibited an increased conversion of DOX into doxorubicinol and an accelerated course of developing cardiac damage [Forrest GL, et al, 2000]. Recently it was shown that low concentration of both paclitaxel and docetaxel increased doxorubicinol formation. This may be an explanation for the induction of cardiotoxicity at lower than expected cumulative doses of DOX during concomitant administration of DOX and paclitaxel or docetaxel [Salvatorelli E, et al, 2006].

**Intracellular calcium overload**

Reports suggest that calcium accumulation within the cardiomyocytes is involved in the development of DOX-induced cardiotoxicity [Miwa N, et al, 1986, Earm YE, et al, 1994]. High levels of intracellular calcium lead e.g. to mitochondrial dysfunction, increased muscle stiffness and finally cell death [Olson & Mushlin, 1990]. Low concentrations of DOX were shown to enhance calcium influx via activation of a cAMP-dependent protein kinase that phosphorylates channel proteins [Olson & Mushlin, 1990]. Arai et al. demonstrated that treatment with DOX decreased the mRNA expression for sarcoplasmatic reticulum (SR) Ca^{2+} -ATPase 2, a major Ca^{2+} transport protein in SR that regulates intracellular Ca^{2+} concentrations and thereby increasing the cytoplasmic calcium concentration [Olson & Mushlin, 1990, Arai M, et al, 1998]. Later findings suggested that reactive oxygen intermediates induced by DOX partly inhibited the transcription of the (SR) Ca^{2+} -ATPase 2 gene [Arai M et al, 2000].
Recently, the mechanistic link between ROS generated from DOX metabolism in mitochondria and increased cytosolic calcium concentration was demonstrated in vitro, leading to the initiation of the apoptotic cascade [Kalivendi SV, et al, 2005].

**Role of histamine**

It is known that (DOX-induced) free radicals stimulate the release of histamine and besides this, histamine has been found to facilitate calcium influx via histamine H$_2$ receptors, suggesting that calcium may be involved [Klugmann BF, et al, 1986, Olson & Mushlin, 1990]. In this context, it has been shown that anthracyclines induce a dose-dependent histamine release from heart tissue in vitro, which is limited by the antiallergic mast-cell stabilizer sodium cromoglycate [Decorti G, et al, 1997]. This drug also protected against DOX-induced cardiotoxicity in animal models [Klugmann BF, et al, 1986].

**DOX-induced inflammation**

Several studies showed that inflammatory effects are directly and indirectly caused by treatment with DOX. Data of Fujihira and Hecker [Hecker JF, 1990, Fujihira S, et al, 1993] illustrated that DOX also induces inflammatory effects in the vasculature and in the myocardium. Besides this, the concentration of pro-inflammatory cytokines (TNF-α, IL-1β and IL-2) was increased after DOX treatment [Hou G, et al, 2005]. DOX also elevates NF-κB [Deepa & Varalakshmi, 2006] and the activation of several secondary messenger systems that increase the production of pro-inflammatory cytokines and adhesion molecules. In vitro it was shown that DOX in clinically achievable concentrations directly induced neutrophil adhesion of vascular endothelial cells via the overexpression of VCAM and E.selectin [Abou El Hassan MAI, et al, 2003b]. Results of another study suggested that treatment with DOX produced marked inflammatory changes in heart tissue, liver and kidneys in rats [Deepa & Varalakshmi, 2006]. Data of the earlier described studies support the indication that DOX-induced inflammatory effects may play a role in its cardiac damage. In addition to this, it is suggested that prostaglandins, thromboxanes and leukotrienes may also be involved in DOX-induced toxicity because anthracyclines alter arachidonic acid metabolism [Das UN, 1981].

Therefore, considering these data together with the histamine release, it is interesting to investigate the possible protective effect of anti-inflammatory drugs on DOX-induced cardiotoxicity.

**Apoptosis**

Several studies suggest that DOX-induced apoptosis in endothelial cells and cardiomyocytes contribute to the development of DOX-related cardiotoxicity [Childs AC, et al, 2002, Wu
S, et al, 2002, Kluza J, et al, 2004, Spallarossa P, et al, 2004]. DOX-induced cardiomyocyte apoptosis can be mediated by reactive oxygen species, which induce apoptosis via a caspase dependent pathway [Kotamraju S, et al, 2000, Youn HJ, et al, 2005], whereas data of another study suggested a caspase-independent, but p53-dependent pathway as mechanism for DOX induced apoptosis in cardiomyocytes [Childs AC, et al, 2002]. A recent investigation demonstrated that specific DOX-induced DNA lesions in the cardiac myocytes occur as an early event in DOX-induced cardiomyocyte injury and that cell death is mediated through activation of p53 and the mitochondria [L’Ecuyer T, et al, 2006]. Data of an in vitro study showed that \( \text{H}_2\text{O}_2 \) plays a crucial role in DOX-induced apoptosis in endothelial cells and cardiomyocytes, whereas it is largely independent of p53 activation. In contrast, not \( \text{H}_2\text{O}_2 \), but p53 plays an important role in inducing apoptosis in tumor cells [Wang SW, et al, 2004]. It appears that more than one mechanism can mediate programmed cell death in endothelial cells and cardiomyocytes and further investigation will be needed to explore the pathways of DOX-induced apoptosis in these cells. In addition to this, it is also important to compare these pathways with those of tumor cells and find out whether differences in apoptosis may result in a treatment option for cardiotoxicity without interfering with the antitumor effect of DOX.

**PARP activation**

Data of Pacher et al. demonstrated that activation of the enzyme poly (ADP-ribose) polymerase (PARP) may contribute to the cardiac damage induced by DOX. Pharmacological inhibition of PARP with the phenanthridinone PARP inhibitor PJ34 significantly improved cardiac dysfunction and increased the survival of mice treated with DOX [Pacher P, et al, 2002].

**Interaction with the actin-myosin contractile system**

DOX treatment is associated with changes in the cardiac actin-myosin interaction. In vitro it has been reported that DOX has a high affinity for cardiac actin [Lewis W, et al, 1982]. It has been found that repeated administration of DOX caused a decreased tension response, which is best explained by a direct interaction of the drug with the contractile system of heart muscle and most likely caused by changes in actin-myosin interaction [De Beer EL, et al, 2000]. De Beer et al. also investigated whether the cardioprotective effect of dexrazoxane prevented the impaired actin-myosin cross-bridge interaction, which was seen in rats after chronic DOX treatment. It has been concluded that pretreatment with dexrazoxane prevented the negative effects of DOX on the trabecular actin-myosin interaction [De Beer EL, et al, 2001].
Changes in cardiac energy metabolism

Large amounts of energy are necessary for cardiac tissue to sustain its contractile performance. Tokarska et al. focussed on the myocardial energetic network as a target of DOX induced cardiotoxicity [Tokarska-Schlattner M, et al, 2006]. For example, it is known that DOX has a high binding affinity for cardiolipin, a phospholipid that is located in the inner mitochondrial membrane. In addition to its mitochondrial structure function, cardiolipin is also very important for overall cardiac energy metabolism and cell survival [Schlame M, et al, 2000]. Impairment of cardiac energetics, like reducing both ATP and phosphocreatine and defects in the AMPK signaling pathway, has been primarily associated with compromised mitochondrial energy production. This mitochondrial damage, mostly cardiolipin-bound DOX, is mediated by oxidative stress [Minotti G, et al, 2004]. It was demonstrated that within the mitochondria, DOX accepts unpaired electrons specifically from complex I (NADH-reductase) of the electron transport chain initiating the redox cycle. This leads to ROS production in the mitochondrial compartment and subsequently to a decrease in respiratory control and inhibition of ATP synthesis, which leads to mitochondrial damage [Wallace KB, 2003].

1.6 Reduction of doxorubicin-induced cardiotoxicity

If the incidence of DOX-induced cardiotoxicity resulting in congestive heart failure dropped, the quality and extent of life for patients who survived cancer would improve. In addition, if the cardiac complications induced by DOX could be reduced or prevented, higher doses of DOX could potentially be used, thereby further increasing cancer cure rates. Different approaches have been investigated in an attempt to minimize or prevent DOX-induced cardiac damage.

Limiting the cumulative dose

The maximal cumulative dose of DOX is usually limited to 450–550 mg/m² for adults as well as for children, whereas the risk of cardiac abnormalities increases rapidly above this dose [Von Hoff DD, et al, 1979, Krischer JP, et al, 1997]. However, there is a considerable variation in the individual susceptibility to DOX-induced cardiac toxicity and thus there is no absolute safe dose of this agent [Mason JW, et al, 1978, Marty M, et al, 2006].

Altering administration schedule

DOX appeared to be less cardiotoxic when it was administered as a prolonged, continuous infusion [Legha SS, et al, 1982]. Besides this, another early report showed that treatment with DOX given weekly is less cardiotoxic and as effective as giving DOX in a three-weekly schedule [Torti FM, et al, 1983]. Later data showed no prevention in cardiac toxicity after DOX treatment by prolonged infusion in comparison to bolus infusion in pediatric patients.
General introduction


**Analogs of DOX**

Several analogs of DOX have been developed and used in order to obtain drugs with a similar antitumor activity but being less cardiotoxic. Epirubicin is one of these analogs, but although the risk of cardiotoxicity occurs at a higher cumulative dose than it does with DOX, the problem of cardiac toxicity is still present [Jensen BV, et al, 2002, Theodoulou & Clifford, 2004]. In addition, the other important anthracyclines daunorubicin and idarubicin also induce cardiotoxicity. It appeared that the use of the anthracyclonitide mitoxantrone also produced cardiac effects in several patients [Aviles A, et al, 1993].

**Liposomal anthracyclines**

Another way of reducing DOX-induced cardiotoxicity involves liposomal encapsulation, which alters the distribution in the tissue and the pharmacokinetics of DOX. Liposomes can penetrate into tissues and organs lined with cells that are not tightly joined (tumor), while in areas that have tight capillary junctions, such as the heart muscle, they cannot escape the circulation [Theodoulou & Clifford, 2004]. In this way, toxicity in for e.g. heart tissue should be limited. In metastatic breast cancer patients treatment with Caelyx (pegylated liposomal DOX) has shown to have significant efficacy with reduced cardiotoxicity [Overmoyer B, et al, 2005]. Another liposomal anthracycline, Myocet (non-pegylated liposomal DOX) has been examined in two large randomized phase III studies. Both studies in patients with metastatic breast cancer treated with Myocet showed a comparable antitumor effect to DOX with a reduced cardiac toxicity [Batist G, et al, 2001, Harris L, et al, 2002]. While Caelyx showed toxicity (hand-foot syndrome and myelotoxicity, each 30%), Myocet showed less toxicity and appears to be a promising cardiac safe formulation of DOX.

**Addition of cardioprotectors to DOX treatment**

The cardioprotective effect of several agents has been studied during co-administration with DOX. Some of these agents like carvedilol and amifostine demonstrated promising results in vivo studies in animals, but were not tested yet as a cardioprotectant in humans, whereas with a few agents like N-acetylcycteine, which were studied in humans, no protection was observed against DOX-induced cardiotoxicity [Myers CE, et al, 1983, Matsui H, et al, 1999, Herman EH, et al, 2000].

The only compound that has been shown to offer a considerable protection against the cardiotoxic effects of DOX in clinical studies in adults and children is dexrazoxane (ICRF-187) [Swain SM, et al, 1997, Lipshultz SE, et al, 2004, Marty M, et al, 2006]. In the cell,
the bisdioxopiperazine dexrazoxane undergoes hydrolysis to its presumably active rings-open metal ion binding form ADR-925, which has the ability to strongly chelate iron, or to quickly remove iron from the iron-doxorubicin complex and thereby preventing oxygen radical formation [Hasinoff BB, 1998]. Besides this, dexrazoxane is also a potent catalytic and non-cleavable complex-forming inhibitor of DNA topoisomerase II [Larsen AK, et al, 2003]. Because topoisomerase II is also one of the targets of DOX, it is possible that dexrazoxane may interfere with the antitumor activity of DOX. Thus, while it has been shown that dexrazoxane is effective in reducing DOX-induced cardiotoxicity in humans, long-term follow-up of these patients has to give a definite answer on the question whether dexrazoxane interferes with the antineoplastic activity of DOX. No complete cardioprotection against DOX induced damage was observed when dexrazoxane was co-administered. Therefore the search for more potent cardioprotecting agents, which furthermore lack an interference with the antitumor effect of DOX, is warranted.

2. Flavonoids

Flavonoids are a group of more than 5000 polyphenolic compounds that occur naturally in vegetables, fruit and plant-derived beverages such as wine and tea [Ren W, et al, 2003, Nichenametla SN, et al, 2006]. They all share a common three-ring structure, which is identified by two benzene rings A and B linked through a heterocyclic pyran (with a double bond) ring C in the middle (Figure 2). Flavonoids are classified according to their chemical structure. Major flavonoid classes are flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanidins and chalcones as major classes (Figure 2) [Van Acker FAA, et al, 2001]. In addition to naturally occurring flavonoids, semisynthetic flavonoids have been developed e.g. rutin has been hydroxyethylated to enhance its metabolic stability and water solubility yielding hydroxyethylrutosides (HERs).
2.1 **Intake of flavonoids and prevention of diseases**

The intake of flavonoids per day varies among the population [Middleton E Jr, et al, 2000]. Several years ago, the Dutch average intake of all flavonoids together has been estimated to be 23 mg per day. Quercetin was the mostly consumed flavonoid. The richest sources of flavonoids consumed in general were tea, onions and apples [Hertog MG, et al, 1993]. Recently, analysis of soy protein and isoflavone intake suggested that ≤ 10% of the people of four Asian countries consume as much as 25 g of soy protein or 100 mg of isoflavones per day [Messina M, et al, 2006].

In recent years, several epidemiological studies showed that flavonoids might have a potential protective role in various chronic diseases, including common cancers. When investigating the impact of dietary flavonoid intake on the risk of breast cancer in a population-based sample of US women, it was found that a decrease in breast cancer was associated with flavonoid intake [Fink BN, et al, 2006]. In another study conducted in Italy, also evidence was found for an inverse association of flavone and flavonols intake with breast cancer risk [Bosetti C, et al, 2005]. A Finnish study found a significant inverse association of flavonoid
intake with lung cancer, and also the incidence of asthma was lower at higher total flavonoid intake [Knekt P, et al, 2002]. Lagiou et al. studied the association of each major flavonoid class with coronary heart disease (CHD) risk and found evidence that intake of flavan-3-ols, mostly from wine and tea, is inversely associated with risk of CHD [Lagiou P, et al, 2004]. However, other studies did not show an association or even reported contradictory results [Rimm EB, et al, 1996, Knekt P, et al, 2000, Hirvonen T, et al, 2001, Knekt P, et al, 2002]. Further prospective studies are necessary to elucidate and confirm the role of flavonoids in the etiology of chronic disease.

2.2 Properties of flavonoids *in vitro* and *in vivo*


2.3 Flavonoids as cardioprotectors

Flavonoids have been recognized as new protective agents against DOX-induced cardiotoxicity, because of their radical scavenging and iron chelating properties [Van Acker SABE, et al, 1996, Bast A, et al, 2007]. This property is probably due to their structure, which has e.g. an easily oxidizable catechol moiety that interacts with free radicals and favours resonance of electrons after the radical-flavonoid interaction. After structure-activity relationship studies it was suggested that flavonols are the best antioxidants and in addition, our group has shown that the 3-OH group in combination with a C2-C3 double bound increases the scavenging activity of the flavonol [Van Acker SABE, et al, 1996, Lien EJ, et al, 1999]. Furthermore, flavonoids form complexes with transition metal ions such as Fe$^{2+}$, Fe$^{3+}$, Cu$^{2+}$ and Al$^{3+}$ at different sites in the molecule. Via chelation of the metal ions, the radicals are formed in the vicinity of the flavonoid, which thus can easily scavenge them. By this site-specific scavenging the flavonoid is able to effectively protect the cell against radical damage. The antioxidant activity in combination with the efficient metal ion chelation, renders the flavonoids potent protectors against DOX-induced cardiac damage.
Among the flavonoids, the semisynthetic hydroxyethylrutosides (HER) derived from the naturally occurring flavonol rutin are strong iron chelators and effective radical scavengers and thus promising candidates as cardioprotectors. Such a mixture is commercially available as the drug Venoruton® (a mixture of hydroxyethylrutosides) and registered for patients with chronic venous insufficiency. It was shown that Venoruton® and its individual constituents protect the heart against DOX-induced cardiac damage in an ex-vivo atrium model [Van Acker SABE, et al, 1993b]. Of these constituents, 7-mono-O- (β-hydroxyethyl)-rutoside (monoHER) (Figure 3) appeared to be the most powerful antioxidant [Van Acker SABE, et al, 1993a].

3. Monohydroxyethylrutoside (monoHER)

![Figure 3. Chemical structure of monoHER](image)

The scavenging activity of monoHER was measured by determining the inhibition of ferricytochrome c reduction and the inhibition of oxygen consumption, showing an inhibition of 91% and 70%, respectively [Van Acker SABE et al, 1993a]. Because of the favorable iron chelating and radical scavenging properties of monoHER, the semisynthetic flavonoid was tested as a protectant against DOX-induced cardiotoxicity. MonoHER protected for 92,7% against DOX-induced cardiotoxicity in the isolated atrium model [Van Acker SABE et al, 1993a]. In mice, the cardioprotective properties of monoHER were investigated and its protection was observed after the i.p. administration of 500 mg/kg of monoHER two or five times per week before DOX (4 mg/kg i.v.) for a period of six weeks [Van Acker SABE, et al, 1995]. The increase of the ST-interval of the ECG of every mouse was measured with a transmitter transplanted in the intraperitoneal cavity and used as measure for DOX-induced cardiotoxicity. A later performed study showed complete protection against DOX-induced cardiac damage in mice, when monoHER was given as a single i.p. injection (500 mg/kg) only once a week 1 hour before DOX (4 mg/kg, i.v.) [Van Acker FAA, et al, 2000b].
Considering the increasing incidence of DOX-induced cardiotoxicity in patients especially after a period of longer follow-up [Steinherz LJ, et al, 2001], it is important to investigate if the cardioprotective effect of monoHER will also be maintained after a longer period of time.

Pharmacokinetic (pk) studies of monoHER in mice after i.p. administration under protecting conditions (500 mg/kg) showed that the plasma concentration of monoHER reached a mean plateau level of about 130 µM between 5 and 15 min after administration in plasma as well as in the heart [Abou El Hassan MAI, et al, 2003a]. Thereafter, MonoHER disappeared from plasma and heart tissue with a half-life of about 30 min. Because it is known that the cardiotoxic effect of DOX is also related to its peak plasma drug concentration [Danesi R, et al, 2002], shortening the time interval between the administration of monoHER and DOX might be of influence on the cardioprotective effect of monoHER. On the other hand, active metabolites of monoHER may be formed, which possibly takes more than 1 h. When considering these aspects, the time interval between monoHER and DOX needs further investigation in order to optimize its dosing schedule.

Results of some studies support the suggestion that inflammation induced by DOX, plays a role in its cardiac damage. This aspect has been mentioned earlier in this introduction when describing the mechanisms of DOX-induced cardiotoxicity [Inchiosa MA Jr & Smith CM, 1990, Chen QM et al, 2005, Hou G et al, 2005]. In vitro, data showed that DOX affected both the viability and neutrophil adhesion of endothelial cells via the overexpression of VCAM and E.selectin with clinically achievable concentrations of DOX and monoHER protected against these DOX-induced inflammatory effects [Abou El Hassan MAI et al, 2003b]. To confirm the contribution of the inflammatory effects by DOX on its cardiotoxicity, the protective effect of anti-inflammatory drugs on DOX-induced cardiac damage needs to be investigated in an in vivo model. In parallel to these investigations, a biomarker for inflammatory stress as response to DOX treatment might be explored.

In addition to its protective properties against DOX-induced cardiac damage, a cardioprotector should not interfere with the antitumor activity of DOX. In vitro, it was found that monoHER did not influence the IC50 (which is the concentration of the drug giving a 50% inhibition of cell growth of treated cells when compared to the growth of control cells) of DOX in A2780, OVCAR-3 human ovarian cancer cells, and MCF-7 human breast cancer cells. Also in vivo, monoHER did not influence the antitumor effect of DOX on A2780 and OVCAR-3 xenografts in nude mice [Van Acker SABE, et al, 1997]. Both in vitro and in vivo, monoHER protected against the toxic effect of DOX on the cardiac cells without interfering with the cytostatic effect on cancer cells. It might be that besides its radical scavenging and chelating properties, monoHER plays a protective role on apoptosis induced by DOX in these cells, which needs further investigation.
Abou El Hassan et al. investigated the bioavailability of monoHER after different routes of administration. After oral administration, monoHER could not be detected in plasma, indicating that monoHER had a very poor oral bioavailability, thus monoHER cannot be administered orally [Abou El Hassan MAI, et al, 2003a]. The i.p. and s.c. bioavailabilities were about 30 and 40%, respectively. In this study, he also characterized the pk profile of monoHER under protecting conditions (500 mg/kg, i.p.). It was found in mice, that between 5 and 15 minutes after i.p. administration of 500 mg/kg monoHER, a $C_{\text{max}}$ of about 131 $\mu$M was obtained. The AUC$_{\infty}$ was 6.3 $\mu$M.min [Abou El Hassan MAI, et al, 2003a]. These values can be used as a possible endpoint for a phase I study with monoHER.

4. Aim and outline of the thesis

Up to the present, several preclinical studies were performed in which monoHER was found to protect against doxorubicin-induced cardiotoxicity in mice without influencing the antitumor activity of DOX. In addition to its radical scavenging and iron chelation properties, it is hypothesized that other properties may also play an important role in the cardioprotective effect of monoHER. Therefore the aim of this thesis is to perform a few more preclinical studies about the mechanism of action of monoHER and to examine the effect of monoHER in the clinic.

With regard to its mechanism of action in chapter 2 the anti-inflammatory effect of monoHER on dox-induced cardiac damage was studied, whereas in chapter 3 the anti-apoptotic role and the contribution to its selective protection was investigated.

In order to evaluate the influence of the time interval between monoHER and DOX administration on the protection against dox-induced cardiotoxicity, mice were treated with different time intervals between monoHER and DOX administration. Cardiac damage was evaluated by studying the morphological changes in the cardiac tissue of these mice (chapter 4).

In all previous studies, the cardioprotective effect of monoHER was evaluated during a 6-week treatment period and 2 weeks of observation thereafter. Because it is known that the incidence of ventricular dysfunction after DOX treatment continues to increase over time, it is important to know whether the protective effect of monoHER on the heart is still present after a long period of time. In chapter 5 the cardioprotective effect of monoHER is studied in DOX-treated mice during a period of 6 months.

The last part of this thesis concerns the clinical studies which were performed with monoHER. In chapter 6 the possible side effects and the pharmacokinetics of monoHER were evaluated in healthy volunteers in a Phase I study. With the dose recommended from the Phase I study,
the cardioprotective effect of monoHER was evaluated in a Phase II study in DOX-treated cancer patients. Cardiac damage was evaluated by histopathological scoring of the heart tissue obtained by endomyocardial biopsy (chapter 7). The outcome of these investigations and future perspectives are discussed in chapter 8.

References


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General introduction


Anti-inflammatory agents and monoHER protect against DOX-induced cardiotoxicity and accumulation of CML in mice

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Abstract

Cardiac damage is the major limiting factor for the clinical use of doxorubicin (DOX). Preclinical studies indicate that inflammatory effects may be involved in DOX-induced cardiotoxicity. Nε-(carboxymethyl) lysine (CML) is suggested to be generated subsequent to oxidative stress, including inflammation. Therefore, the aim of this study was to investigate whether CML increased in the heart after DOX and whether anti-inflammatory agents reduced this effect in addition to their possible protection on DOX-induced cardiotoxicity. These effects were compared with those of the potential cardioprotector 7-monohydroxyethylrutoside (monoHER).

BALB/c mice were treated with saline, DOX alone or DOX preceded by ketoprofen (KP), dexamethasone (DEX) or monoHER. Cardiac damage was evaluated according to Billingham. Nε-(carboxymethyl) lysine was quantified immunohistochemically. Compared to saline, a 21.6-fold increase of damaged cardiomyocytes was observed in mice treated with DOX (P < 0.001). Addition of KP, DEX or monoHER before DOX significantly reduced the mean ratio of abnormal cardiomyocytes in comparison to mice treated with DOX alone (P ≤ 0.02). In addition, DOX induced a significant increase in the number of CML stained intramyocardial vessels per mm² (P = 0.001) and also in the intensity of CML staining (P = 0.001) compared to the saline treated group. CML positivity was significantly reduced (P ≤ 0.01) by DOX-DEX, DOX-KP and DOX-monoHER. These results confirm that inflammation plays a role in DOX-induced cardiotoxicity, which is strengthened by the observed DOX-induced accumulation of CML, which can be reduced by anti-inflammatory agents and monoHER.
Introduction

Doxorubicin (DOX) is a successfully used anticancer drug. However, DOX-induced cumulative cardiotoxic effects, including cardiomyopathy and congestive heart failure, limit the use of this agent [Von Hoff DD et al, 1979, Signal PK & Iliskovic N, 1998, Gharib MI & Burnett AK, 2002]. Various molecular mechanisms have been suggested. DOX-induced free radicals are believed to play a central role in its cardiotoxicity [Yen HC et al, 1996, Horenstein MS et al, 2000, Xu MF et al, 2001].


Protein damage caused by oxidative stress, inflammation or hyperglycemia leads to carbohydrate-derived advanced glycation end products (AGEs), such as Nε–(carboxymethyl) lysine (CML) [Miyata T et al, 1997, Hudson BI et al, 2003]. Elevated levels of CML were demonstrated in patients with renal failure, in intramyocardial arteries of the heart of diabetic patients [Schalkwijk CG et al, 2004] and in patients with atherosclerosis having inflammatory / pro-oxidative environments [Degenhardt TP et al, 1997, Schleicher ED et al, 1997]. CML is produced under oxidative stress [Miyata T et al, 1997, Nagai R et al, 1997] and may therefore be regarded as a biomarker for local endogenous oxidative stress, next to local inflammatory stress [Baynes JW, 1991, Nerlich AG & Schleicher ED, 1999]. After binding to the receptor for AGE, CML activates endothelial cells as indicated by the induction of adhesion molecules such as VCAM-1 [Boulanger E et al, 2002]. Therefore, the first aim of our study was to investigate whether CML increases in intramyocardial arteries after treatment with DOX. Because inflammatory processes are involved the second aim of our study was to investigate whether anti-inflammatory agents would reduce DOX-induced CML increase.

In the past, we have shown the cardioprotective properties of the antioxidant 7-monohydroxyethylrutoside (monoHER) against DOX-induced cardiotoxicity in the mouse [Van Acker SA et al, 1997, Van Acker FA et al, 2000]. In vitro, we have also shown that monoHER protects against DOX-induced inflammatory effects [Abou El Hassan MAI et al, 2003]. Therefore, the effect of monoHER on DOX-induced CML increase was also investigated in the in vivo mouse model.
Furthermore, a possible protective effect of the anti-inflammatory drugs ketoprofen (KP) and dexamethasone (DEX) on DOX-induced cardiotoxicity in comparison to the protective effect of monoHER was investigated in this model.

**Materials and Methods**

**Chemicals**

7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). The drug was formulated and dissolved as described before, giving a final concentration of 33 mg/ml [Bruynzeel AME et al, 2006]. Formulated DEX (Dexamethasone 4 mg/ml) was obtained from the Pharmacy Department, VU medical center (Amsterdam, the Netherlands). Before injection, the content of the ampoule was diluted in sterile saline to obtain a concentration of 2 mg/ml. Formulated KP (1% ketoprofen) was obtained from Merial B.V. (Amstelveen, the Netherlands). A volume of 0.5 ml KP was added to 19.5ml PBS to obtain a concentration of 0.025% KP (0.25 mg/ml). Formulated DOX (Doxorubicin hydrochloride, 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, the Netherlands). Before injection, the content of the vial was diluted in a sterile 0.9% NaCl solution to a concentration of 1 mg/ml.

**Animals**

Thirty-six male BALB/c mice (20–25 g) obtained from Harlan Nederland (Horst, the Netherlands) were kept in a light and temperature-controlled room (21°C–22°C; humidity 60–65%). The animals were fed a standard diet (Harlan Teklad) and allowed to eat and drink tap water ad libitum. The animals were allowed to adapt to the laboratory housing conditions for 2 weeks before starting the experiment.

**Experimental design**

The protocol was approved by the ethics committee for animal experiments of the Vrije Universiteit (Amsterdam, the Netherlands) and the methodology was also in compliance with the UKCCCR guidelines on ethical use of animals.

Thirty mice were submitted to one of the following weekly dosing schedules for 6 weeks:

- **Group 1 (n = 6)**: 0.1 ml 0.9% NaCl solution i.v. + 0.3 ml 0.9% NaCl solution s.c. 60 min before i.v. injection, and 6, 24 and 48 hours after i.v. injection
- **Group 2 (n = 6)**: 4 mg/kg DOX i.v. + 0.3 ml 0.9% NaCl solution s.c. 60 min before DOX and 6, 24 and 48 hours after DOX
Group 3 (n = 6)  4 mg/kg DOX i.v. + 2 mg/kg KP s.c. 30 min before DOX, and 6, 24 and 48 hours after DOX
Group 4 (n = 6)  4 mg/kg DOX i.v. + 8 mg/kg DEX s.c. 60 min before DOX, and 6, 24 and 48 hours after DOX
Group 5 (n = 6)  4 mg/kg DOX i.v. + 500 mg/kg monoHER i.p. 60 min before DOX

DOX was administered via the tail vein. Six mice were sacrificed just before starting treatment (control group) and their heart tissue was used as a control at the beginning. During treatment and a 2-week observation period thereafter, body weight was determined twice a week as a measure of general toxicity. After the treatments and the observation period, the mice were killed.

**Tissue samples**
The hearts were excised and the central part of both ventricles was cut into 5-mm-thick pieces of 2–3 mm, which were fixed in 2% phosphate buffered glutaraldehyde solution or in 4% formalin.

**Histological analyses**
After fixation in 2% phosphate buffered glutaraldehyde solution the heart tissue was post-fixed in 1% osmium tetroxide. The tissue was then dehydrated through a graded series of ethanol solutions of 70–95% and embedded in JB-4 Plus resin. Thereafter 0.5–3.0-µm thick sections were cut with a glass knife. These semithin sections were examined by light microscopy and DOX-induced cardiac damage was evaluated according to Billingham et al (1978). For this purpose the percentage of cardiac cells, that had been damaged, was established. Cardiac myocytes with more than two vacuoles or loss of myofibrils were counted as deviant. The scoring area was measured, using a commercially available interactive video overlay-based measuring system (Q-Prodit, Leica, Cambridge, UK; Vermeulen EG et al, 2001). For each mouse the number of aberrant myocytes per mm² was scored.

**Immunohistochemical methods**
After fixation in 4% formalin the heart tissue was embedded in paraffin. Paraffin-embedded cardiac tissue sections (4 µm) were mounted on microscope slides and were deparaffinized for 10 minutes in xylene at room temperature and dehydrated by decreasing concentrations of ethanol. Sections were then stained with hematoxylin and eosin. Subsequent to deparaffinization and dehydration, sections were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Sections were not heated to prevent artificial induction of CML by this procedure [Dunn JA et al, 1989]. Sections were
preincubated with normal rabbit serum (1:50, Dako, Glostrup, Denmark) for 10 minutes and incubated for 60 minutes with anti-CML (1:500) both at room temperature. After washing in phosphate buffered saline (PBS), pH 7.4, sections were incubated for 30 minutes with rabbit anti-mouse biotin-labeled antibody (1:500, Dako) at room temperature and subsequently washed in PBS. After incubation with streptavidin horseradish peroxidase (1:200, Dako) for 60 minutes at room temperature, peroxidase was visualized with 3, 3-diamino-benzidine-tetrahydrochloride/H$_2$O$_2$ (DAB) (Sigma Chemical Company, St. Louis, MO, USA) for 3 to 5 min.

The CML staining intensity was scored in the intramyocardial arteries. For the intensity scoring each positive vessel was given a score of: 1 = weak positivity; 2 = moderate positivity or 3 = strong positivity, according to a previous study [Schalkwijk CG et al, 2004]. Subsequently, the scoring area was calculated as described before [Vermeulen EG et al, 2001]. For each mouse the total number of CML staining arteries per mm$^2$ was scored. Thereafter the difference in the CML staining intensity of the intramyocardial arteries per mm$^2$ was investigated between the experimental groups.

**Statistical Analysis**

For the analyses, the number of aberrant cardiac myocytes was log-transformed yielding an unskewed variable. Differences between experimental groups were assessed using Student’s two-sided $t$-test. The level of significance was set at 5%. Ninety-five percent confidence intervals (CI) on the original scale were obtained by exponentiating the upper and lower bounds of the 95% confidence intervals constructed on the log-scale. All calculations were performed with SPSS version 9.0 (SPSS, Chicago, IL). For the analyses, the difference between the experimental groups regarding the number of vessels positive for CML staining and the intensity scoring per mm$^2$ was assessed using Student’s two-sided $t$-test. The level of significance was chosen at 5%. These calculations were also performed with SPSS version 9.0. To examine whether the contribution of moderately and strongly stained CML vessel walls differed among treatment groups, Fisher’s exact test was applied and also Student’s two-sided $t$-test.

**Results**

Animals appeared lively throughout the study and no behavioral changes were observed between the treatment groups. There were no signs of decreased activity, indicating low general toxicity. No significant differences were observed in weight between the experimental groups. No signs of gastrointestinal toxicity were observed in the mice treated with KP.
**Histological examination of the cardiomyocytes**

Histology of the hearts from the control and saline group did not show damaged cardiac myocytes, indicating that environmental factors and treatment with saline did not influence cardiac health of the animals. Treatment with DOX alone induced a significant 21.6-fold (95%CI 6.2 – 74.5) increase of damaged cardiac myocytes in comparison to the saline-treated group ($P < 0.001$).

Heart tissue of all mice treated with DOX alone or in combination with KP, DEX or monoHER particularly showed vacuolar degeneration, whereas loss of myofibrils was rarely detected. Table 1 shows the ratio of the mean number of aberrant cardiac myocytes per mm$^2$ in all groups in comparison to the group treated with DOX. The addition of KP 30 minutes before and 6, 24 and 48 hours after DOX injection resulted in a significant protective effect by reducing the ratio of the mean number of abnormal cardiac cells per mm$^2$ with a factor 4.4 (95%CI 1.4 – 14.3, $P = 0.021$). When DEX was added 60 minutes before DOX injection and 6, 24 and 48 hours after DOX administration, a significant protective effect was also detected ($P = 0.006$). Co-treatment with DEX led to a 6.2-fold reduction of deviant cardiac cells (95%CI 1.9 – 20.0) compared to the mice treated with DOX alone. The protective effect by adding monoHER before DOX led to a significant 8.6-fold ($P = 0.002$, 95%CI 2.6 – 27.8) reduction of abnormal cardiomyocytes.

Table 1 also shows the ratio of the mean number of aberrant cardiac myocytes per mm$^2$ in treated versus saline treated animals. When KP or DEX was added before DOX administration, significantly more abnormal cardiac myocytes were observed in comparison to the saline group, indicating that the protection was not complete (for KP a 4.9-fold increase, 95%CI 1.4 – 17.0, $P = 0.014$; for DEX a 3.5-fold increase, 95%CI 1.0 – 12.0, $P = 0.049$). When monoHER was added before DOX treatment, no significant increase of aberrant cardiac myocytes was detected compared to the saline group ($P = 0.137$). No significant difference was found between the groups treated with the combinations DOX- monoHER, DOX-KP and DOX-DEX ($P > 0.05$).
Table 1. Ratios of the mean number of aberrant cardiac myocytes/mm$^2$

<table>
<thead>
<tr>
<th>Treatment group (n = 6 per group)</th>
<th>Fold increase (95%CI, $P$)</th>
<th>Fold reduction (95%CI, $P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>1 (Reference)</td>
<td>21.6 (6.2 – 74.5, &lt;0.001)</td>
</tr>
<tr>
<td>2. DOX</td>
<td>21.6 (6.2 – 74.5, &lt;0.001)</td>
<td>1 (Reference)</td>
</tr>
<tr>
<td>3. DOX + KP</td>
<td>4.9 (1.4 – 17.0, 0.014)</td>
<td>4.4 (1.4 – 14.3, 0.021)</td>
</tr>
<tr>
<td>4. DOX + DEX</td>
<td>3.5 (1.0 – 12.0, 0.049)</td>
<td>6.2 (1.9 – 20.0, 0.006)</td>
</tr>
<tr>
<td>5. DOX + MH</td>
<td>2.5 (0.73 - 8.7, 0.137)</td>
<td>8.6 (2.6 – 27.8, 0.002)</td>
</tr>
</tbody>
</table>

**Abbreviations**: DEX = dexamethasone; DOX = doxorubicin; KP = ketoprofen; MH = 7-monohydroxyethylrutoside.

Fold increase = geometric mean number of abnormal cells in treated animals / geometric mean number of abnormal cells in mice treated with saline, fold reduction = geometric mean number of abnormal cells in DOX treated animals / geometric mean number of abnormal cells in other treatment groups.

**Immunohistochemical staining of CML**

CML positivity was found in intramyocardial blood vessels, especially endothelium and partly smooth muscle cells in DOX-treated mice. DOX treatment induced a significant increase in the number of CML stained vessels per mm$^2$ compared to the group treated with saline ($P = 0.001$) irrespective of the intensity score. Figure 1A illustrates immunohistochemical detection of CML in heart tissue of a mouse after treatment with DOX alone, whereas figure 1B is a slide without addition of the primary antibody. Treatment of the animals with DOX in combination with DEX, KP or monoHER significantly reduced the amount of blood vessels positive for CML compared to the DOX-treated animals ($P = 0.004, 0.009$ and $0.006$, respectively). No difference was found in the number of vessels positive for CML between the groups treated with DOX combined with DEX, KP or monoHER and the animals treated with saline ($P = 0.633, 0.424$ and $0.514$, respectively). When comparing the amount of vessels positive for one of the three categories of intensity scores for CML (weak, moderate, strong) no difference was found between the five treatment groups for weakly stained positive CML vessels per mm$^2$ ($P = 0.887$), but when the mean number of moderately and strongly stained vessels per mm$^2$ were combined for each experimental group, a significantly enhanced staining for CML ($P = 0.001$) was found between the mice treated with DOX alone and the animals treated with saline (Figure 2). DEX, KP and monoHER reduced this enhancing effect of DOX significantly ($P = 0.003, 0.014$ and $0.007$, respectively). No significant difference in
staining was found between the animals treated with saline and those treated with the combination DOX-DEX, DOX-KP or DOX-monoHER ($P = 0.659, 0.275$ and $0.424$, respectively). These results indicate that all three combinations significantly reduce the enhancing effect of DOX on CML intensity.

Figure 1. Immunohistochemical detection of CML in the mouse (63x). Arrow: CML deposition on endothelial cells in intramyocardial blood vessel. (A) immunohistochemical detection of CML in the heart tissue of a mouse after DOX treatment alone, whereas (B) is an image without addition of the primary antibody.
Figure 2. The mean number of vessels per mm$^2$ weakly, moderately and strongly positive after staining for CML in intramyocardial vessels in the heart tissue of treated mice. A significant difference ($P = 0.001$) was found between the mice treated with DOX alone and the animals treated with saline when the mean number of moderately plus strongly stained vessels per mm$^2$ were considered (* no significant difference in the mean number of strongly plus moderately stained CML vessels per mm$^2$ in comparison to the saline-treated group and $P \leq 0.01$ when compared to the DOX-treated group).

Discussion

In this study, we showed that addition of ketoprofen and dexamethasone during treatment with DOX reduced its cardiac damage in vivo. In addition, it was demonstrated that treatment with DOX induces an increase of CML in intramyocardial arteries in mice, which is reduced by these anti-inflammatory agents and monoHER.

Although DOX-induced free radicals are believed to play a central role in its cardiotoxicity [Yen HC et al, 1996, Horenstein MS et al, 2000, 57, Xu MF et al, 2001], the precise mechanism of myocardial impairment remains unclear. Several studies showed that inflammatory effects are directly and indirectly caused by treatment with DOX. In vitro it was shown that DOX directly induced neutrophil adhesion of vascular endothelial cells via the overexpression of VCAM and E.selectin [Abou El Hassan MAI et al, 2003], whereas results of another study suggest that treatment with DOX produced marked inflammatory changes in heart tissue, liver and kidneys [Deepa PR & Varalakshmi P, 2005]. Results of our study confirm
the contribution of inflammation in DOX-induced cardiotoxicity, because anti-inflammatory agents can at least in part reduce DOX-induced cardiotoxicity.

It has been suggested that DOX also induces endothelial dysfunction [Kotamraju S et al, 2002, Wolf MB & Baynes JW, 2006], because it has been demonstrated in vivo that treatment with DOX caused oxidative stress and myeloperoxidase (MPO) activity [Fadillioglu E et al, 2004]. CML can be formed by oxidative stress [Baynes JW, 1991, Nerlich AG & Schleicher ED, 1999], and also by the enzyme MPO [Anderson MM et al, 1999]. In a recent study was found that CML positivity colocalized with E-selectin-positive endothelial cells in the heart [Baidoshvili A et al, 2006]. Earlier it was demonstrated that DOX induced neutrophil adhesion that was mediated via overexpression of E-selectin [Abou El Hassan MAI et al, 2003]. Therefore, it is tempting to speculate that CML is derived from these pathways and could play a role in DOX-induced vascular endothelial injury and subsequent cardiotoxicity.

It is known that CML interacts with cells through a specific receptor system for AGEs (RAGE) [Zill H et al, 2001]. Activation of RAGE by binding of CML is thought to lead to the nuclear translocation of NF-κB [Sousa MM et al, 2000] and the activation of several secondary messenger systems that increase the production of proinflammatory cytokines and adhesion molecules [Boulanger E et al, 2002]. These events lead to progressing inflammation and a further increase of formation and accumulation of CML. Several approaches have been used to block the formation of AGE or the interaction of AGEs with RAGE to reduce complications [Brownlee M et al, 1986, Panagiotopoulos S et al, 1998, Bucciarelli LG et al, 2002]. From these studies it appeared that reduction or even prevention of the formation of CML seems to be important to prevent endothelial dysfunction and besides, this also reduces inflammation. In line with this, we have demonstrated in another study increased accumulation of CML in intramyocardial arteries of diabetic patients and suggested that CML contributes to the increased risk of heart complications in diabetes mellitus (Schalkwijk and Niessen, unpublished observation).

In the present study we showed that monoHER significantly reduced CML positivity and intensity of intramyocardial arteries. As monoHER has been shown to have radical scavenging properties [Haenen GRMM et al, 1993, Van Acker SABE et al, 1993, Van Acker SABE et al, 1997, Van Acker FA et al, 2000], this again points to a role of free radicals in CML production by DOX. We also found that anti-inflammatory agents decreased CML positivity and intensity in intramyocardial arteries (Figure 1). It has indeed been suggested that inflammation is another source of CML formation [Daugherty A et al, 1994, Anderson MM & Heinecke JW, 2003]. It has, however, to be noticed, that glucocorticoids and NSAIDs also have antioxidant properties [Hamburger SA & Mc Cay PB, 1990, Kataoka M et al, 1997, Chen QM et al, 2005, Ozmen I, 2005, Yamada K et al, 2006] besides their anti-inflammatory properties [Koehler L et al, 1990, Auphan N et al, 1994, Masferrer JL & Seibert K, 1994, Scheinman RI et al, 1995, Morteau O, 2000].
As a representative of the NSAIDs, we used KP because it is a strong non-selective COX-inhibitor and it is available for s.c. injection. DEX was chosen as a representative of the glucocorticosteroids, because of its known strong anti-inflammatory properties. By using the earlier mentioned treatment schedules for KP and DEX, we intended to maintain the presence of the anti-inflammatory agents when DOX was administered and during the first 2 days thereafter, because the high peak levels of DOX during that period [Van der Vijgh WJF et al, 1990] are considered of major importance in the development of DOX-induced cardiotoxicity [Von Hoff DD et al, 1979].

Up to the present, two studies reported protective effects of cotreatment with ibuprofen and glucocorticoids on DOX-induced cardiac damage. The first study [Inchiosa MA Jr & Smith CM, 1990] only evaluated survival, whereas the effect of glucocorticoids on DOX toxicity was only evaluated in vitro [Chen QM et al, 2005]. At present, our study quantifies to what extent cardioprotection occurred in animals cotreated with DEX, KP and monoHER. It strongly confirms the role of inflammation in DOX-induced cardiotoxicity and indicates a possible way to protect (in part) against this toxicity.

High dose DEX or prednisone is part of the DOX containing therapeutic treatment regimens in patients with aggressive Non-Hodgkin’s lymphoma or multiple myeloma (VAD, CHOP). Considering the results of our study, we reviewed data of these clinical studies concerning the cardiac consequences of the combined use of DOX and glucocorticoids for these patients and found out that up to the present little is known about the long-term-effects on their cardiac tissue [Limat S et al, 2003, Elbl L et al, 2006]. These clinical aspects merit further attention.

As mentioned earlier, it was believed that the cardioprotective effect of monoHER was mainly owing to its radical scavenging and iron-chelating properties; however, the results of the present study in combination with the in vitro study of Abou El Hassan [Abou El Hassan MAI et al, 2003] indicate that monoHER also has anti-inflammatory properties. Recently, anti-inflammatory activity was also shown for the flavonoids quercetin [Comalada M et al, 2005], myricetin [Kang BY et al, 2005] and luteolin [Kim JS & Jobin C, 2005]. A quantitative comparison between the three compounds (KP, DEX and monoHER) regarding their intrinsic anti-inflammatory and / or radical scavenging activities is not possible yet, because none of the doses nor the dosing regimes of the investigated protectors are optimised.

In conclusion, two anti-inflammatory agents of different classes, ketoprofen (NSAID) and dexamethasone (synthetic glucocorticoid) clearly protected against DOX-induced cardiotoxicity in mice by decreasing the number of abnormal cardiac myocytes. These results establish the suggestion that inflammatory effects owing to treatment with DOX are involved in the development of DOX-induced cardiotoxicity. The role of DOX-induced
inflammation in the development of its cardiac damage is confirmed by the observation that DOX induced accumulation of CML in intramyocardial arteries, which is significantly reduced after treatment with DEX, KP and monoHER. Further investigations are warranted to develop anti-inflammatory agents as a protector against DOX-induced cardiotoxicity.

References


Chapter 3

Caspase-dependent and -independent suppression of apoptosis by monoHER in Doxorubicin treated cells

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Abstract

Doxorubicin (DOX) is an antitumor agent for different types of cancer, but the dose-related cardiotoxicity limits its clinical use. To prevent this side effect we have developed the flavonoid monohydroxyethylrutoside (monoHER), a promising protective agent, which did not interfere with the antitumor activity of DOX. To obtain more insight in the mechanism underlying the selective protective effects of monoHER, we investigated whether monoHER (1 mM) affects DOX-induced apoptosis in neonatal rat cardiac myocytes (NeRCaMs), human endothelial cells (HUVECs) and the ovarian cancer cell lines A2780 and OVCAR-3. DOX-induced cell death was effectively reduced by monoHER in heart, endothelial and A2780 cells. OVCAR-3 cells were highly resistant to DOX-induced apoptosis. Experiments with the caspase-inhibitor zVAD-fmk showed that DOX-induced apoptosis was caspase-dependent in HUVECs and A2780 cells, whereas caspase-independent mechanisms seem to be important in NeRCaMs. MonoHER suppressed DOX-dependent activation of the mitochondrial apoptotic pathway in normal and A2780 cells as illustrated by p53 accumulation and activation of caspase-9 and -3 cleavage. Thus, monoHER acts by suppressing the activation of molecular mechanisms that mediate either caspase-dependent or -independent cell death. In light of the current work and our previous studies, the use of clinically achievable concentrations of monoHER has no influence on the antitumor activity of DOX whereas higher concentrations as used in the present study could influence the antitumor activity of DOX.
Introduction

Doxorubicin (DOX) is highly effective against various types of cancers, including leukaemias, breast and ovarian cancer. DOX induces pleiotropic cytotoxic effects of which DNA intercalation and topoisomerase II inhibition have been proposed to play an important role in its mechanism of action, causing growth arrest and the subsequent activation of apoptosis [Capranico G et al, 1990, Gewirtz DA, 1991, Binaschi M et al, 1997]. The contribution of DOX-induced reactive oxygen species (ROS) to its antitumor activity is still a matter of debate [Keizer HG et al, 1991, Shacter E et al, 2000, Gouaze V et al, 2001, Suresh A et al, 2003].


MonoHER – a semisynthetic flavonoid that was developed in our laboratory [Willems AM et al, 2006] and presently in a Phase II clinical trial is both a radical scavenging and a metal ion chelating agent [Haenen GRMM et al, 1993, van Acker SABE et al, 1993]. In our studies the scavenging activity of monoHER was measured by determining the inhibition of ferricytochrome c reduction and the inhibition of oxygen consumption, showing an inhibition of 91% and 70%, respectively [van Acker SABE et al, 1993]. Because of these favourable properties, monoHER was tested as a protectant against DOX-induced cardiac damage in in vivo models and appeared a potent protector against DOX-induced cardiotoxicity without influencing its antitumor effects [van Acker SABE et al, 1997, van Acker FA et al, 2000].

The present study was initiated to determine possible differences in the effects of monoHER against DOX-induced apoptosis in cardiac myocytes and vascular endothelial cells in comparison to ovarian cancer cell lines. The protective effects of monoHER on cell cycle progression and molecular determinants of mitochondrial-dependent apoptosis were also studied. The clinical relevance of our results regarding the use of protectors such as monoHER in combination with chemotherapy is discussed.
Materials and Methods

Chemicals
7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). Doxorubicin HCl was purchased from Pharmacia Upjohn BV (Woerden, the Netherlands). The following reagents were used in this study: bovine serum albumin, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium vanadate (NaVO₃) (all from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands)); human serum (Central Laboratory for Blood Transfusion, Amsterdam, the Netherlands); M199 medium and Hanks Balanced salt solution (without calcium) (Invitrogen, Breda, the Netherlands); Dulbecco’s Modified Eagle’s Medium (DMEM) and HEPES (Cambrex Bio Science, New Jersey, US); Fetal calf serum (FCS) (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands); Penicillin-G (Yamanouchi Pharma B.V., the Netherlands); Streptomycin (Fisiopharma, Milano, Italy); l-glutamine (ICN Biochemicals, Cleveland, Ohio); horse serum (Life Technologies NV, Merelbeke, the Netherlands); Glycerol (J.T. Baker, Deventer, the Netherlands); protease inhibitor tablets (Roche, Almere, the Netherlands); Endothelial cell growth factor (ECGF) was extracted from bovine hypothalamus as previously described [Maciag T et al, 1979].

Cell culture
All cells were maintained at 37°C in humidified air containing 5% CO₂. A2780 and OVCAR-3 cells were grown in DMEM with 10% FCS and 20 mM HEPES. Human endothelial cells (HUVECs) were prepared as described previously by Verheul et al. (2000). In brief, the endothelial cells were cultured in gelatin-coated (1%) tissue culture plates with culture medium consisting of M199 supplemented with 10% human serum, 10% FCS, 5 units/ml heparin, 200 I.E./ml penicillin and 200 µg/ml streptomycin, 0.29 mg/ml l-glutamine and 50 µg/ml ECGF. Cells were grown to confluence at 37°C in 5% CO₂. Endothelial cells after 3 passages (P3) were used during the whole study. Neonatal rat cardiac myocytes (NeRCaMs) were isolated as previously described by Abou El Hassan et al. [Abou El Hassan MAI et al, 2003a]. The cardiac cells were divided over tissue culture plates with a cell density of 75x10³cells/cm². After plating, the cells were incubated with medium consisting of equal portions of DMEM and Ham’s F-10 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 5% Horse Serum. The cultures contained at least 90% of synchronously beating NeRCaMs. After 24 h the medium was refreshed and 48 h later the NeRCaM cultures were ready for use in the experiments.
Flow Cytometric Analysis of PI-stained cells
Cardiac myocytes, endothelial, A2780 and OVCAR-3 cells were treated with different concentrations of DOX (0.1-10 µM) alone or in the presence of 1mM monoHER for 48 h, respectively. Apoptotic cell fraction (subG1) in PI-stained cells was measured by flow cytometry as described previously [Swain SM et al, 1997, Suresh A et al, 2003]. In brief, after trypsinization the cells were resuspended in PI staining solution (50 µM/ml Pl, 0.1% sodium citrate, 0.1% TritonX-100, 0.1 mg/ml Rnase in PBS). The cells were incubated for at least 30 min at 4°C in the dark before analysis by flow cytometry using a FACScan (Becton, Dickinson and Company, NJ USA). When indicated cells were pre-treated for 1 hour with 50 µM of the broad caspase inhibitor z-VAD-fmk prior to DOX exposure or co-treated with monoHER (1mM).

Western Blot analysis
Cells were treated with DOX with or without 1 mM monoHER for 24 and 48h at 37°C. The cells were trypsinized and lysed for 20 min on ice in lysis buffer consisting of 20 mM HEPES/KOH (pH 7.4), 50 mM β-glycerophosphate, 50 mM KCl, 0.2 mM EDTA, 1% (w/v) Triton X-100, and 10% (w/v) glycerol, supplemented with protease inhibitors and 1 mM NaVO₃. Protein concentrations were determined using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard using a spectrophotometer. Equal amounts of protein (15 µg) were loaded and electrophoresed on 7–12% SDS-polyacrylamide gels and transferred into polyvinylidene difluoride membranes (Amersham, Braunschweig, Germany). Subsequently, membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated at 4°C overnight with the indicated primary antibodies followed by a 1 h incubation at room temperature with a secondary antibody, either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:2000). Protein loading equivalence was assessed by the expression of β-actin (1:10000). Proteins were visualized by enhanced chemiluminescence (ECL kit, Amersham, Braunschweig, Germany). Primary antibodies used were against: p53 (human specific, DAKO, Glostrup, Denmark), Poly (ADP-ribose) polymerase (PARP) (Roche Diagnostics Netherland BV, Almere, the Netherlands), Caspase-9 (human specific or mouse mAb), p53 and PARP (rat specific, all from Cell Signaling Technology Inc., Beverly, MA, USA), Bax and Caspase-3 (BD Transduction Laboratories NJ, USA) and β-actin (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands).
### Results

**Effect of monoHER on DOX-induced apoptosis**

Normal cells were more sensitive to DOX-induced apoptosis than the selected tumor cell lines. The IC$_{50}$ values were 0.75, 0.5 and 1.5 $\mu$M for HUVECS, NeRCaMs and A2780 cells, respectively. However, OVCAR-3 was highly resistant with a maximum of only 10 to 12% cells in subG1 phase at 10 $\mu$M (Figure 1 and Table 1). HUVECs were relatively resistant to low concentrations of DOX and reached a plateau of apoptotic cell fraction at a concentration of 1 $\mu$M. NeRCaMs displayed a gradual dose-dependent increase of apoptotic cells at low DOX concentrations reaching a plateau of 60% killing at 1$\mu$M DOX. A2780 cells also showed a dose-dependent increase in apoptotic cells, reaching a plateau of about 50% at DOX concentrations $\geq$ 1 $\mu$M. The ratios of the IC$_{50}$ values shown in Table 1 indicate that 1 mM monoHER protected 13 and 15 fold against DOX-induced apoptosis in HUVECs and NeRCaMs, respectively. A2780 cells were only protected with a factor 5.3, whereas OVCAR-3 did not show any protection by monoHER.

**DOX-induced caspase activation**

The differences found in sensitivity to the apoptotic effects of DOX in the examined cell panel prompted us to investigate the role of caspases in mediating cell death. To that purpose, HUVECs, NeRCaMs, A2780 and OVCAR-3 cells were treated with 1 $\mu$M DOX with or without the addition of the broad-spectrum caspase inhibitor z-VAD-fmk (Figure 1C). A strong suppression (leaving only 15% apoptotic cells) was observed in HUVECs whereas no significant protection was observed in NeRCaMs. In tumor cells, z-VAD-fmk almost completely protected against the moderate apoptotic effects of DOX in A2780 cells (only 10% apoptotic cells left). Z-VAD-fmk exhibited no significant protection against DOX-induced apoptotic effects in OVCAR-3, however the interpretation of this finding is difficult in light of the strong apoptosis-resistance found in these cells. Taken together, these results indicate that DOX-induced apoptosis in HUVECs and A2780 cells was principally mediated by caspases, whereas NeRCaMs and OVCAR-3 cells displayed less or hardly any contribution of caspases to DOX toxicity. In addition, the effect of a higher dose of DOX (10 $\mu$M) with or without the addition of z-VAD-fmk was examined inducing a more robust cell death than 1 $\mu$M DOX. Under these conditions z-VAD-fmk showed comparable results as obtained at 1 $\mu$M DOX (results not shown).
Caspase-dependent and independent suppression

A

B

% Apoptotic cells

Concentration DOX (µM)

% Apoptotic cells

Concentration DOX (µM)
Figure 1. The effect of monoHER (1 mM) on DOX-induced apoptosis in cardiomyocytes (NeRCaMs), endothelial cells (HUVECs) (A) and ovarian tumor cells A2780 and OVCAR-3 (B) after 48 h of incubation. The bars indicate the levels of the sub-G1 cell fractions of apoptotic cells after PI staining. The effect of 50 µM zVAD-fmk (a broad-spectrum caspase inhibitor) on DOX (1 µM, 48 h)-induced cell death was also investigated (C). Results are presented as the mean (± s.d.) of three independent experiments except for the NeRCaMs in Figure 1A, where results of two experiments are shown.

Table 1. IC\(_{50}\) values (µM) for endothelial (HUVECs), cardiac cells (NeRCaMs), OVCAR-3 and A2780 cells. ND = not determined.
Cell cycle effects of DOX treatment
The effect of the combined treatment of DOX (1 µM, 48 h) with monoHER or z-VAD-fmk on cell cycle progression in the panel of non-tumor and tumor cells was determined by FACS analysis of PI-stained cells (Figure 2). DOX alone induced apoptosis as illustrated by the accumulation of cells in subG1. HUVECs were most sensitive to DOX and monoHER-dependent suppression of DOX-induced sub-G1 accumulation was clearly accompanied by G2/M arrest. The cell cycle profile obtained after caspase inhibition displayed accumulation of HUVECs in all cell cycle phases with a distribution resembling that of untreated control cells. In NeRCaMs co-exposure with monoHER protected against apoptosis, and a small increase in G2/M accumulation was observed. A2780 tumor cells showed strong accumulation in G2/M upon apoptosis suppression by co-treatment with monoHER; z-VAD-fmk also led to G2/M accumulation and strong S-phase arrest. In apoptosis-resistant OVCAR-3 cells DOX mediated an S-phase arrest that was strongly prevented by monoHER, leading to a large accumulation in G2/M. Co-treatment with z-VAD-fmk resulted in accumulation of cells in the G2/M phase. Taken together, it appears that monoHER delays DOX-treated cells in the G2/M phase, particularly in the examined tumor cells. Caspase-inhibition by z-VAD-fmk was most notable in HUVECs where DOX apoptotic effects seem to be merely caspase-dependent, whereas other cells types showed partial inhibition or in case of OVCAR-3 almost no effect at all. Although both monoHER and z-VAD-fmk had overlapping effects on the cell cycle distribution of DOX-treated cells, mainly G2/M accumulation, z-VAD-fmk resulted also in the delay of the tumor cells in S-phase, and in G1-phase in case of HUVECs or NeRCaMs. Because monoHER can suppress DOX cytotoxicity in all these cells it apparently suppresses both caspase-dependent and -independent forms of cell death.

Effect of monoHER on DOX-dependent activation of p53, Bax and caspases
To obtain more insight in the molecular mechanism underlying the apoptosis-inducing effect of DOX and the apoptosis suppressing effects of monoHER, the expression of several regulators and executors of apoptosis was studied in the panel of cells by Western blotting. To that purpose, cells were exposed to the corresponding IC₅₀ concentrations of DOX in the presence or absence of monoHER for 24 and 48 h.
In HUVECs (Figure 3A), DOX treatment resulted in an accumulation of p53 at 24 h exposure which decreased at 48 post-treatment, most probably caused by the strong activation of apoptosis resulting in the degradation of cellular proteins. This finding was supported by a decrease in β-actin levels. Interestingly, co-exposure with monoHER suppressed p53 accumulation. A small increase in p53 levels may be observed after 24 h treatment with monoHER alone, which was not detected after 48 h. This increase was not seen in the other cells tested (see below). Although we have no clear explanation for this small increase it may
reflect a more ROS-sensitive regulation of p53 in untreated HUVECs than in the other cells. The pro-apoptotic Bcl2 family member Bax was constitutively expressed in untreated HUVECs and a shorter form of Bax of approximately 18 kDa appeared after treatment with DOX probably representing a cleaved or truncated form. The occurrence of this shorter variant was, however prevented by monoHER. As predicted by the z-VAD-fmk sensitivity of DOX-induced apoptosis in these cells, robust caspase-9 and -3 cleavage was seen accompanied by processing of the caspase substrate PARP. MonoHER suppresses caspase activation. As controls, DMSO and monoHER alone did not trigger caspase activation.

In NeRCaMs, p53 expression was only detectable after DOX treatment and monoHER appeared to influence the post-translational modification of p53 as indicated by changes in the mobility of the p53 specific bands (Figure 3B). DOX potently triggered caspase-9 activation as indicated by the strong decrease or even absence of the pro-form band at 24 and 48 h after treatment. Also strong PARP cleavage was observed. In light of the observed lack of effect of z-VAD-fmk treatment on DOX-induced cell death this may suggest that a caspase-independent form of cell death can be activated in parallel in these cells. Regardless of this, monoHER very effectively suppressed caspase-9 activation and PARP cleavage. We were not able to detect Bax or caspase-3 expression in NeRCaMs as the available antibodies did not cross-react with the rat counterparts.

Next, the ovarian cancer cell lines were investigated. A2780 cells (wild-type p53) showed clear DOX-induced p53 accumulation and PARP cleavage that could be suppressed by monoHER (Figure 3C). However, the mechanism of DOX-induced caspase activation in A2780 cells was different from that in HUVECs, with no or hardly any Bax, caspase-9 and -3 cleavage/activation, suggesting no primary role for the mitochondrial pathway in mediating DOX-dependent apoptosis in these cells. Nonetheless, A2780 cells displayed clear PARP cleavage after DOX exposure. In contrast, OVCAR-3 cells showed little or no evidence for DOX-induced p53, Bax or caspase activation or PARP cleavage (Figure 3D). This was not unexpected because these cells contain mutant p53 and show resistance against apoptosis.
Figure 2. Effect of 1 mM monoHER and 50 µM zVAD-fmk on cell cycle progression in cardiomyocytes, endothelial cells, and ovarian cancer cells treated with 1 µM DOX for 48 h. The percentage of cells in the different cell cycle fractions was determined by flow cytometry.
### A. HUVECs

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<tr>
<th>Protein</th>
<th>Control</th>
<th>DMSO</th>
<th>MH 24</th>
<th>DOX 24</th>
<th>D+M 24</th>
<th>MH 48</th>
<th>DOX 48</th>
<th>D+M48</th>
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<td>β-actin</td>
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kDa:
- p53: 53
- Bax: 21
- Caspase-9: 18
- Cleaved Caspase-9: 47
- Caspase-3: 32
- PARP: 116
- β-actin: 46

### B. NeRCAms

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<th>DOX 24</th>
<th>D+M 24</th>
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kDa:
- p53: 53
- Caspase-9: 47
- PARP: 37
- β-actin: 46
Figure 3. Effect of monoHER on DOX-induced p53, Bax and caspase activation. The expression of the indicated proteins was examined by Western blotting in HUVECs (A), NeRCaMs (B), and in A2780 (C) and OVCAR-3 tumor cells (D) after 24 and 48 h of DOX exposure at their IC 50. The molecular weight of the bands is indicated and β-actin was included as a control for protein loading.
Discussion

The antitumor effects of DOX result from preventing DNA replication and repair leading to cell cycle arrest and apoptosis [Capranico G et al, 1990, Gewirtz DA, 1991, Binaschi M et al, 1997]. DOX-induced cardiotoxicity was related to the activation of apoptosis in cardiomyocytes and endothelial cells [Wu S et al, 2002, Yamanaka S et al, 2003, Spallarossa P et al, 2004]. In these cells the main effects of DOX were mediated via the production of ROS, known for its potent cell damaging effects. Thus DOX-induced apoptosis, although via different routes, is a common mechanism in normal and cancer cells.

Interestingly, the potent ROS scavenger monoHER, which is presently in a clinical phase II study, showed a strong protection against the cardiotoxic effects of DOX without modulating its antitumor effects both in vivo and in vitro [van Acker SABE et al, 1997, van Acker FA et al, 2000, Abou El Hassan MAI et al, 2003a, Abou El Hassan MAI et al, 2003d]. The anti-apoptotic role played by monoHER and the contribution to its selective protection is not studied yet. The present study was therefore aimed to compare the protective effects of monoHER against DOX-induced apoptosis in normal and cancer cells in order to unravel the mechanism of its selective protection.

DOX induced concentration-dependent apoptosis both in HUVECs and NeRCaMs, which is in line with an earlier study [Wu S et al, 2002]. A2780 cells were also sensitive to induction of apoptosis by DOX, whereas OVCAR-3 cells were highly resistant. This resistance could be attributed to the presence of mutant p53, but may also be caused by other yet unknown apoptotic blockades. Employing the broad caspase inhibitor z-VAD-fmk revealed that DOX triggered caspase-dependent apoptosis in HUVECs and A2780 cells, and caspase-independent cell death in NeRCaMs. Combination treatment with monoHER was effective in suppressing both caspase-dependent (HUVECs and A2780) and -independent apoptosis (NeRCaMs). Assessment of the fold anti-apoptotic protection achieved showed that HUVECs and NeRCaMs were stronger protected by monoHER than A2780 cells. Examination of the molecular mechanisms that underlie the protective effects of monoHER indicated that it can strongly reduce the activation of DOX-induced p53 accumulation in cardiomyocytes, endothelial cells and A2780 cells, which contain wild-type p53 [Camarda G et al, 2002], whereas as expected, no p53 response was detected in OVCAR-3 cells that express mutant p53. It is conceivable that the radical scavenging properties of monoHER cause the reduction of p53 accumulation, which is a known sensor of ROS-dependent toxicity [Uberti D et al, 1999], leaving only topoisomerase 2-DNA damage as a trigger for p53 activation.

The suppressive effect of monoHER on the activation of caspase-9 and -3 and the substrate PARP can also be explained by the neutralization of ROS-dependent triggers of caspase activation. Evidence for mitochondria-dependent activation of caspases was only obtained
Caspase-dependent and independent suppression in HUVECs, in which DOX exposure resulted in the occurrence of a shorter form of BAX, which was suppressed by monoHER. This shorter form may be produced by cleavage of full-length Bax or may represent an alternatively spliced variant with mitochondria destabilising activity leading to the apoptosome-dependent activation of caspase-9 and the subsequent activation of apoptotic cell death [Youn HJ et al, 2005]. In A2780 cells the mitochondrial pathway seemed to be less involved in DOX-induced apoptosis because DOX did not show clear changes in BAX or pro-caspase-9 or -3 levels. However, PARP cleavage was clearly evident in A2780 cells and together with the fact that zVAD-fmk strongly suppressed DOX-induced apoptosis we postulate that other yet unconfirmed mechanisms of caspase-dependent apoptosis are activated, such as for example the extrinsic pathway and caspase-8. The opposite holds true for NeRCAms in which DOX activated p53 as shown by caspase-9 and PARP cleavage, whereas the addition of z-VAD-fmk only partially suppressed apoptosis indicative of caspase-independent cell death. In NeRCAms, the observed caspase-9 activation seems to be merely a co-phenomenon of DOX-induced apoptosis. In line with that Youn et al. (2005) reported that DOX-induced death of the rat cardiac muscle cell line H9c2 was associated with p53 upregulation and caspase-independency. Altogether, this indicates the involvement of caspase-independent apoptotic routes in mediating death in cardiac myocytes by DOX. This cannot be generalized because in other cell types (in our study and in previous reports) caspases seem to play a role in DOX-induced cell death [Nakamura T et al, 2000, Grassilli E et al, 2004, Kalivendi S et al, 2005]. Together this supports the idea that more than one mechanism can mediate programmed cell death, which depends on both the death stimulus provided and the cell type studied [Bröker LE et al, 2005]. MonoHER appears to protect against these diverse DOX-induced cell death signals possibly because of the ability of monoHER to neutralise ROS. OVCAR-3 cells were different in their response to DOX compared to the other cell types. They showed hardly any apoptosis within the concentration range of 0.5 to 10 \( \mu \)M, which is comparable to clinically achieved concentrations of DOX (0.3-5 \( \mu \)M) [Gerwitz DA, 1999]. MonoHER did cause cell cycle delay in G2/M in these cells but did not affect the small amount of DOX-induced cell death.

The finding that monoHER not only suppresses apoptosis in endothelial cells and cardiomyocytes at clinically relevant concentrations of DOX, but also in ovarian cancer cells raises the question whether monoHER may reduce the antitumor effects of DOX in the clinic. It should be noted that in the present study a concentration of 1 mM monoHER is used. This concentration is about threefold or sevenfold higher than the maximal peak plasma concentrations of monoHER found in the clinic (360 \( \mu \)M) during the phase I study [Willems AM et al, 2006] or in mice (131 \( \mu \)M) under protecting conditions [Abou El Hassan MAI et al, 2003c]. Thus, monoHER provides a potent protective effect against various routes of doxorubicin-induced cell death allowing an efficient protection of heart and endothelial cells.
previously reported as major targets in doxorubicin-induced cardiotoxicity. Importantly, the present study addresses the necessity not to raise the dose of monoHER above the presently used 1500 mg/m$^2$ to avoid apoptosis-protecting concentrations, which might adversely affect the antitumor activity of DOX.

Finally, we postulate that the tendency of monoHER to protect normal cells more than cancer cells may be attributed to the inherent proliferation capacity of cancer cells, even in confluent cultures as used in this study where the growth of tumor cells is less affected by contact inhibition. It can be envisioned that in slow- or non-proliferating cells, such as normal cardiomyocytes and endothelial cells, monoHER predominantly acts against the apoptotic effects of DOX and consequently offers more protection. This is supported by our results showing restricted monoHER protection of confluent but not proliferating endothelial cells after DOX treatment in vitro [Abou El Hassan MAI et al, 2003d]. On the other hand, the different effects of monoHER in terms of apoptosis suppression may reflect the activity of the intrinsic ROS defence systems present in cells, which may also be associated with different mechanisms of cell death activation. In this respect, multiple antioxidant defence systems / enzymes (for example glutathione, thioredoxin and SOD) are active to varying degrees in different cell types thereby forming complex ROS-neutralising networks that are related to apoptosis [Haddad JJ, 2004]. In addition, cardiomyocytes were reported to have intrinsically low levels of antioxidant enzymes that may explain the potent protective function of monoHER in these cells [Hrdina R et al, 2000]. Also high activity of the MRP1/GS-X pump in these cells was reported [Krause MS et al, 2007], which may export DOX as glutathione-S-conjugate out of the cells, thereby modifying its cytotoxic effects. However, previously we found that monoHER did not affect DOX levels in cardiac cells in mice or influence the biodistribution of DOX [Abou El Hassan et al, 2003b]. Thus, although the level of ROS defense is likely to at least partially determine the protective effect of monoHER in cells, the high complexity of these defense mechanisms makes it difficult to discriminate between their relative contribution to monoHER protection and the relevance for counteracting DOX-induced apoptosis, and will require a systematic, more detailed analysis.
Caspase-dependent and independent suppression

References


The influence of the time interval between monoHER and doxorubicin administration on the protection against doxorubicin-induced cardiotoxicity in mice

Anna M.E. Bruynzeel, Paula P.N. Mul, Johannes Berkhof, Aalt Bast, Hans W.M. Niessen, Wim J.F. van der Vijgh

Abstract

Despite its well-known cardiotoxicity, the anthracyclin doxorubicin (DOX) continues to be an effective and widely used chemotherapeutic agent. DOX-induced cardiac damage presumably results from the formation of free radicals by DOX. Reactive oxygen species particularly affect the cardiac myocytes because these cells seem to have a relatively poor antioxidant defense system. The semisynthetic flavonoid monohydroxyethylrutoside (monoHER) showed cardioprotection against DOX-induced cardiotoxicity through its radical scavenging and iron chelating properties. Because of the relatively short final half-life of monoHER (about 30 min), it is expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER. Therefore, the aim of the present study was to investigate this possible effect.

Six groups of 6 BALB/c mice were treated with saline, DOX alone or DOX (4 mg/kg i.v.) preceded by monoHER (500 mg/kg i.p.) with an interval of 10, 30, 60 or 120 min. After a 6-week treatment period and additional observation for 2 weeks, the mice were sacrificed. Their cardiac tissue was processed for light microscopy, after which cardiomyocyte damage was evaluated according to Billingham.

Microscopic evaluation revealed that treatment with DOX alone induced significant cardiac damage in comparison to the saline control group ($P < 0.001$). The number of damaged cardiomyocytes was 9.6-fold (95%CI 4.4 – 21.0) higher in mice treated with DOX alone than that in animals of the control group. The ratio of aberrant cardiomyocytes in mice treated with DOX preceded by monoHER and those in mice treated with saline ranged from 1.6 – 2.8 (mean 2.2, 95%CI 1.2 – 4.1, $P = 0.019$). The mean protective effect by adding monoHER before DOX led to a significant 4.4-fold reduction ($P < 0.001$, 95%CI 2.3 – 8.2) of abnormal cardiomyocytes. This protective effect did not depend on the time interval between monoHER and DOX administration ($P = 0.345$). The results indicate that in an outpatient clinical setting monoHER may be administered shortly before DOX.
Introduction

Doxorubicin (DOX) is a potent antitumor agent and is widely used to treat a range of cancers, including solid tumors and lymphomas. A major limitation for its use is the development of cardiomyopathy at high cumulative doses (> 550 mg/m2) [Lefrak EA et al, 1973, Singal PK et al, 1998, Hrdina R et al, 2000]. Although several hypotheses concerning the cause of DOX-induced cardiotoxicity have been suggested in the literature, the formation of free radicals by DOX semiquinones is supposed to be the most important mechanism of DOX-induced cardiotoxicity [Horenstein MS et al, 2000, Wojtacki J et al, 2000]. The cardiac myocyte is particularly susceptible to the free radicals because protective enzymes, like superoxide dismutase (SOD), catalase and GSH-peroxidase are present in a lower concentration in heart tissue than in to other tissues [Hrdina R et al, 2000, Iarussi D et al, 2000]. The generation of free radicals is facilitated by the formation of DOX-iron complexes [Myers C, 1998, Minotti G et al, 2004]. Scavengers of free radicals and iron chelators showed a protective effect against DOX-induced free radical production and cardiotoxicity in vitro and in vivo [Swain SM et al, 1997, Liu X et al, 2002, Oliveira PJ et al, 2004]. Up to now, the cardioprotectant dexrazoxane, a chelating agent that binds iron intracellularly, is the only drug that has been cautiously introduced into the clinic in patients treated with anthracyclines [Swain SM & Vici P, 2004]. The histopathological features of DOX-induced cardiac damage, characterized by endomyocardial biopsies of patients and laboratory animals, include the loss of myofibrils and vacuolar degeneration [Rosenoff SH et al, 1975, Billingham ME et al, 1978].

In previous studies we have shown the iron chelating and radical scavenging properties of monoHER [Haenen GRMM et al, 1993, Van Acker SABE et al, 1993]. Because of these properties monoHER was tested as a protectant against DOX-induced cardiotoxicity. It appeared that monoHER protected the heart tissue of mice against DOX-induced cardiotoxicity in vivo [Van Acker FAA et al, 2000] and ex vivo [Van Acker FAA et al, 2001]. In vitro and in vivo experiments also showed that monoHER did not interfere with the antitumor effect of DOX [Van Acker SABE et al, 1997]. The cardiotoxicity of DOX is not only related to its cumulative dose but also to its peak plasma drug concentration [Pai VB & Nahata MC, 2000, Xu MF et al, 2001]. Considering this, it seems plausible that maximal monoHER concentrations should be present during the highest circulating concentrations of DOX, assuming that a maximal plasma concentration of monoHER offers a maximal protection against DOX-induced cardiotoxicity. Because of the relatively short final half-life of monoHER (about 30 min) it is expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER.
Therefore, the aim of the present investigation was to evaluate the influence of the time interval between monoHER and DOX administration on the protection against DOX-induced cardiotoxicity by evaluating the morphological changes in the cardiac tissue of mice treated with different time intervals between monoHER and DOX administration.

Materials and Methods

Chemicals
7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). The drug was formulated under aseptic conditions by the Pharmacy Department, VU medical center, Amsterdam, the Netherlands. The required amount of monoHER was dissolved in 20 ml dextrose 5% for intraperitoneal (i.p.) use, adjusted to pH = 9.3 using sodium hydroxide 4M. After dissolution of the drug, the solution was readjusted to pH = 8.4 with hydrochloric acid 1M, giving a final concentration of 33 mg/ml. Formulated doxorubicin (Doxorubicin hydrochloride, 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, the Netherlands). Before injection, the content of the vial was dissolved in sterile 0.9% NaCl solution to a concentration of 1 mg/ml.

Animals
Thirty-six male BALB/c mice (20–25 g) obtained from Harlan Nederland (Horst, the Netherlands) were kept in a light- and temperature-controlled room (21°C–22°C; humidity 60–65%). The animals were fed a standard diet (Hope Farms, Woerden, the Netherlands) and allowed to eat and drink tap water ad libitum. The animals were allowed to adapt to the laboratory housing conditions for 2 weeks before starting the experiment.

Experimental design
The protocol was approved by the Ethics Committee for animal experiments of the Vrije Universiteit (Amsterdam, The Netherlands).

With an interval of 2 months, two groups of 18 mice (each subdivided into six groups of three mice) were treated according to one of the following schemes for 6 weeks:

Group 1 (n = 6) 0.5 ml 0.9% NaCl solution i.p, followed by 0.1 ml 0.9% NaCl solution i.v.
Group 2 (n = 6) 0.5 ml 0.9% NaCl solution i.p, followed by 4 mg/kg DOX i.v.
Group 3 (n = 6) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 10 min.
Group 4 (n = 6) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 30 min.
Group 5 (n = 6) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 60 min.
Group 6 (n = 6) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 120 min.
The influence of the time interval between monoHER and DOX

The i.v. injections were administered in the tail vein. During treatment and 2 weeks of observation thereafter, body weight was determined once a week as a measure of general toxicity. After the 8-week period, the animals were sacrificed. The hearts were excised and the central part of both ventricles were cut into 5-mm-thick pieces of 2–3 mm, which were fixed in 2% phosphate buffered glutaraldehyde solution.

**Histological analyses**

After fixation in 2% phosphate buffered glutaraldehyde solution, the heart tissues were postfixed in 1% osmium tetroxide. The tissues were then dehydrated through a graded series of ethanol solutions of 70–95% and embedded in JB-4 Plus resin. Thereafter, 0.5–3.0 µm-thick sections were cut with a glass knife. These semithin sections were examined by light microscopy and the myocardial damage was evaluated according to Billingham [Billingham ME et al, 1978]. For this purpose, the percentage of cardiac myocytes, which had been damaged, was established. Cardiac myocytes with more than two vacuoles and / or loss of myofibrils were considered to be damaged. The circumference of the scoring area was measured using a commercially available interactive video overlay based measuring system (Q-Prodit, Leica, Cambridge, UK) [Brinkhuis M et al, 1995]. For each mouse, the number of aberrant myocytes/mm² was scored.

**Statistical Analysis**

For the analyses, the number of aberrant cardiac myocytes was log-transformed yielding normally distributed variables. Differences between experimental groups were assessed using two-sided t-tests. The influence of time between monoHER and DOX administration on the number of aberrant myocytes was examined by linear regression. The level of significance chosen was 5% (P < .05). All calculations were done with SPSS version 9.0.

**Results**

Behavior of the animals appeared normal in all treatment groups. Animals appeared lively throughout the study and there were no signs of decreased activity, which would indicate low general toxicity. No significant differences in weight gain between the groups were observed.

Microscopic evaluation revealed that treatment with DOX alone induced significant myocardial damage in comparison to the saline-treated control group, which was demonstrated by the appearance of vacuoles (P < .001). Treatment with DOX alone showed a 9.6-fold (95%CI
4.4 – 21.0) increase of damaged cardiac myocytes compared to the control group. Loss of myofibrils was not found.

The mean ratio of aberrant cardiac myocytes/mm² in treated versus the saline control group is presented in Table 1. When monoHER was added before DOX treatment, the mean presence of aberrant cardiac myocytes was 2.2-fold (95%CI 1.2 – 4.1) higher than that in the saline control group ($P = 0.019$). Compared to the mice treated with DOX alone, the addition of monoHER before DOX showed a significant ($P < 0.001$) protective effect by reducing the mean ratio of aberrant myocytes with a factor 4.4 (95%CI 2.3 – 8.2). The protective effect did not depend on the time interval between monoHER and DOX administration ($P = 0.345$).

Table 1. Ratio of aberrant cardiac myocytes/mm² in treated versus control (= saline treated) mice (95% CI).

<table>
<thead>
<tr>
<th>Group (n = 6 per group)</th>
<th>Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>Reference</td>
</tr>
<tr>
<td>2. DOX</td>
<td>9.6 (4.4 – 21.0)</td>
</tr>
<tr>
<td>3. MH 10 min before DOX</td>
<td>1.9 (1.1 – 3.5)</td>
</tr>
<tr>
<td>4. MH 30 min before DOX</td>
<td>1.6 (0.8 – 3.1)</td>
</tr>
<tr>
<td>5. MH 60 min before DOX</td>
<td>2.8 (1.6 – 5.1)</td>
</tr>
<tr>
<td>6. MH 120 min before DOX</td>
<td>2.5 (1.3 – 4.8)</td>
</tr>
</tbody>
</table>

Discussion

In the past it was shown that the presently used DOX schedule, i.e. 4 mg/kg/DOX once every week for 6 weeks results in an appreciable cardiotoxicity which is well suited to investigate cardioprotectors and still acceptable for the animal [Van Acker SABE et al, 1996]. It had also been demonstrated that the flavonoid monoHER is a very effective protector against DOX-induced cardiotoxicity in vitro and in vivo without interfering with the antitumor effect of DOX [Van Acker SABE et al, 1997, Van Acker FAA et al, 2000, Abou El Hassan MAI et al, 2003]. It also appeared that monoHER injected in mice as an i.p. dose of 500 mg/kg did not exert any effect on their heart tissue [Van Acker SABE et al, 1995].
Van Acker et al. showed the protection of monoHER against DOX-induced cardiac damage in mice when given once as an i.p. dose of 500 mg/kg 1 hour before DOX [Van Acker FAA et al, 2000]. After an i.v. bolus injection of DOX, maximal plasma levels are immediately obtained and also various tissues, including the heart, immediately achieve high concentrations of DOX (followed by a further distribution and a slow elimination phase) [Van der Vijgh WJF et al, 1990]. High peak serum levels, which follow after rapid administration, seem to be correlated with cardiotoxicity. Legha et al. showed that decreasing peak plasma levels of DOX by using continuous infusion reduced cardiotoxicity [Legha SS et al, 1982]. Also DOX divided over a weekly schedule is associated with less anthracycline-induced cardiac damage than the same amount of DOX given in the conventional 3-week schedules [Torti FM et al, 1983]. Because of these considerations, high concentrations of monoHER should be present during the peak concentrations of DOX. Pharmacokinetic (pk) studies of i.p. administered monoHER in mice showed that $C_{\text{max}}$ was obtained at about 10 minutes after administration in plasma as well as in tissues. Thereafter, monoHER disappeared rapidly within a few hours [Abou El Hassan MAI et al, 2003]. Based on these findings and the mechanism of action of monoHER – i.e., scavenging of DOX-induced radicals – it was postulated that protection by monoHER could be optimized by giving DOX 10 min after i.p. monoHER. Our data, however, do not indicate a significantly better protection against DOX-induced cardiac damage when reducing the time interval between monoHER and DOX from 2 h to 10 min.

In this experiment, and also in earlier studies, the protective properties of monoHER were evaluated after an additional observation time of only 2 weeks after the 6-week treatment period. Several clinical studies showed that the incidence of ventricular dysfunction continues to increase with time [Lipshultz SE et al, 1991, Kremer LCM et al, 2001, Steinhertz LJ, 2001]. It is therefore important to know whether the protective effect of monoHER is still present after a longer period of time and it may be that the influence of the time interval between monoHER and DOX administration becomes of significant importance during such a longer observation period. The present data, however, suggest that in an (out-patient) clinical setting monoHER may be administered in a convenient short time before DOX.
References


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Chapter 5

Long-term effects of 7-monohydroxyethylrutoside (monoHER) on DOX-induced cardiotoxicity in mice

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Abstract

Doxorubicin (DOX) is a potent antitumor agent for different types of cancer, but the cumulative, dose-related cardiotoxicity limits its clinical use. The incidence of abnormal cardiac function after treatment with DOX appears to increase with time. Therefore, late cardiotoxicity is – especially in young surviving patients – a major concern. The aim of this study was to evaluate in mice whether the semisynthetic flavonoid 7-monohydroxyethylrutoside (monoHER) also protected against DOX-induced cardiotoxicity after a long period of follow-up.

Four groups of 6 Balb/c mice were treated weekly during 6 weeks with saline, DOX alone (4 mg/kg i.v.), DOX preceded by monoHER (500 mg/kg i.p.), or DOX preceded by monoHER followed by long-term weekly monoHER injections during the observation period of 6 months. Half of the mice treated with DOX only developed DOX-induced heart failure and died within 6 months of observation. Two mice co-treated with monoHER showed weight loss and shortness of breath, whereas one mouse was found dead in its cage known with weight loss. The group receiving DOX plus long-term repeated doses of monoHER started to lose weight. Five out of six mice in this group developed shortness of breath and died before the end of the study with symptoms of cardiac failure induced by DOX. Statistical comparison of the histological heart damage between the different experimental groups was not possible, because the animals died at different time-points in the observation period and DOX-induced cardiotoxicity progressed with time. Nevertheless, it was clear that the initial cardioprotective effect of monoHER was not prolonged during the half-year observation period. It was even suggested that addition of repeated doses of monoHER tended to aggravate DOX-induced cardiotoxicity. It cannot be excluded that the dose and frequency of monoHER administration is crucial in obtaining an optimal antioxidant activity without a pro-oxidant activity of monoHER.
Introduction

The clinical efficacy of the chemotherapeutic agent doxorubicin (DOX) is severely limited by its dose-limiting cardiotoxicity in patients with cancer [Lefrak EA et al, 1973, Singal PK & Iliskovic N, 1998, Hrdina R et al, 2000, Swain SM et al, 2003]. The most common hypothesis for the mechanism by which DOX induces cardiac damage includes the formation of free radicals [Horenstein MS et al, 2000, Xu MF et al, 2001]. Heart tissue is particularly susceptible to free radicals because the concentration of enzymes protecting against oxidative damage is lower than that in other tissues [Hrdina R et al, 2000, Iarussi D et al, 2000].

Several clinical studies showed that the incidence of abnormal cardiac function after treatment with DOX appears to increase with time [Lipshultz SE et al, 1991, Kremer LCM et al, 2001, Steinherz LJ et al, 2001]. In survivors of childhood cancer, the incidence of cardiac abnormalities also increased with the length of follow-up [Lipshultz SE et al, 1991, Kremer LCM et al, 2001]. More than 65% of children with cancer achieve long-term survival and because many of these survivors will have received DOX or another anthracycline, late anthracycline cardiotoxicity is a major concern [Grenier MA & Lipshultz SE, 1998, Sorensen K et al, 2003].

The importance to minimize or completely prevent this severe side effect of DOX treatment is clear. In the past we have identified the cardioprotective properties of 7-monohydroxyethylrutoside (monoHER) against DOX-induced cardiotoxicity in mice [Van Acker SABE et al, 1997, Van Acker FAA et al, 2000] without influencing the antitumor effect of DOX. Its cardioprotection is believed to result from protection against DOX-induced free radicals through radical scavenging and iron chelating properties of monoHER [Haenen GRMM et al, 1993, Van Acker SABE et al, 1993]. The cardioprotective effect of monoHER was dose-dependent and a dose of 500 mg/kg monoHER 1 h before DOX completely protected against DOX-induced cardiotoxicity [Van Acker SABE et al, 1997]. In all previous studies the protective properties of monoHER were evaluated during a 6-week treatment period and 2 weeks thereafter. Because the incidence of ventricular dysfunction continues to increase with time it is important to know whether the cardioprotective effect of monoHER is still present after a long period of follow-up.

Therefore, the aim of the present study was to evaluate the cardioprotective effect of monoHER in DOX treated mice during a period of 6 months. The morphological changes in the cardiac tissue were evaluated by microscopic examination.
Chapter 5

Materials and Methods

Chemicals
7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health (Nyon, Switzerland). The drug was formulated and dissolved as described before, giving a final concentration of 33 mg/ml [Bruynzeel AME et al, 2006]. Formulated doxorubicin (Doxorubicin hydrochloride, 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, the Netherlands). Before injection, the content of the vial was dissolved in a sterile 0.9% NaCl solution to obtain a concentration of 1 mg/ml.

Animals
Twenty-four male BALB/c mice (20–25 g) obtained from Harlan Nederland (Horst, the Netherlands) were kept in a light- and temperature-controlled room (21°C–22°C; humidity 60–65%). The animals were fed a standard diet (Harlan Teklad) and allowed to eat and drink tap water ad libitum. The animals were allowed to adapt to the laboratory housing conditions for 2 weeks before starting the experiment.

Experimental design
The protocol was approved by the Ethics Committee for animal experiments of the Vrije Universiteit (Amsterdam, The Netherlands).

The mice received one of the following weekly dose-schedules for 6 weeks:

Group I ($n = 6$) 0.5 ml 0.9% NaCl solution i.p, followed by 0.1 ml 0.9% NaCl solution i.v.
Group II ($n = 6$) 0.5 ml 0.9% NaCl solution i.p, followed by 4 mg/kg DOX i.v
Group III ($n = 6$) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 60 min.
Group IV ($n = 6$) 500 mg/kg monoHER i.p, followed by 4 mg/kg DOX i.v. after 60 min. In addition the i.p. injection with 500 mg/kg monoHER was continued once every week during the observation period of 26 weeks.

DOX was administered via the tail vein. During treatment and the 26 weeks of observation thereafter, body weight was determined once a week as a measure of general toxicity. After this long period of observation or earlier when necessitated by the bad condition of an animal, the animals were sacrificed. The hearts were excised and the central part of both ventricles was cut into 5-mm-thick pieces of 2–3 mm, which were fixed in 4% phosphate buffered formaldehyde solution.
Histological analyses
After fixation in 4% phosphate buffered formaldehyde solution, the heart tissue was post fixed in 1% osmium tetroxide. The tissue was then dehydrated through a graded series of ethanol solutions of 70–95% and embedded in JB-4 Plus resin. Thereafter 0.5–3.0 µm-thick sections were cut with a glass knife. These semithin sections were examined by light microscopy and the myocardial damage was evaluated according to Billingham [Billingham ME et al, 1978]. For this purpose, the percentage of cardiac myocytes that had been damaged was determined. Cardiac myocytes with more than 2 vacuoles and/or loss of myofibrils were considered to be damaged. The scoring area was measured using a commercially available interactive video overlay based measuring system (Q-Prodit, Leica, Cambridge, UK) [Vermeulen EG et al, 2001]. For each mouse the number of aberrant myocytes per mm² was scored.

Results
Effect on behavior and condition of the mice
The mice of the saline group appeared lively throughout the whole period of the study and no behavioral changes were observed. Their weight increased during these months. After the first 8 weeks of treatment, behavior of the animals appeared normal in all treatment groups. No signs of decreased activity and no significant differences in weight gain between the groups were observed, indicating low general toxicity. Table 1 illustrates characteristics of the mice per experimental group during the whole study. During the observation period of 26 weeks, nine mice from the groups treated with DOX were sacrificed. All of them had shortness of breath, a decreased activity, loss of appetite and finally as a consequence loss of weight with more than 10%. Their fur looked bad and the animals arched their back. These symptoms were indicated as clinical signs of congestive heart failure (CHF). Three mice were found dead in their cage during the observation period. Two of them already showed loss of weight, but the third mouse died unexpectedly. The remaining animals in group II and III survived without problems (no signs of decreased activity or significant differences in weight gain) until the end of the study. Five out of six animals of the fourth group treated with monoHER once every week during the observation period ended up in a bad condition, showing the earlier described features. Therefore, the mice were sacrificed, or died before the end of the study. Mouse IV-21 was not in a bad condition, but died unexpectedly.
Symptoms of CHF observed in sacrificed and death animals

Nine mice from group II – IV were sacrificed before the end of the study. Eight of them were evaluable. The data of animal IV -19 got lost. All nine mice showed loss of weight progressing rapidly during the last days before their sacrifice. During autopsy, it was clear that all animals were starved. The heart was enlarged in the eight mice and some of them had also some pleural fluid, which is indicative for CHF. Of the three animals found dead in their cage, one (IV -20) showed an enlarged heart. No signs of cardiac failure or another cause of death was found during autopsy in the other two mice, but one of them (III -13) showed signs of starvation.

Microscopic evaluation of the heart tissue of all animals

A number of animals died at different times in the follow-up period. Because DOX-induced cardiotoxicity is progressing with time, a statistical comparison of myocardial damage in the mice of the four experimental groups was not feasible anymore. Nevertheless, the percentage of damaged cardiac myocytes was evaluated. Cardiac myocytes with more than two vacuoles and / or loss of myofibrils were considered damaged. An overview of the aberrant cardiac myocytes / mm\(^2\) in the animals of the experimental groups is also presented in Table 1. Overall, heart tissue of animals treated with saline had a low score. The difference between the score of the saline-treated mice and the animals of the other experimental groups was remarkable, whereas between the three groups treated with DOX with or without monoHER hardly any difference was observed. Besides the (almost) absence of vacuolar degeneration in the cardiac myocytes of the mice treated with saline, their cardiac cells also showed a higher concentration of myofibrils in comparison to the other experimental groups.

Table 1. Characteristics of the mice per experimental group.

<table>
<thead>
<tr>
<th>Mouse (Group, #)</th>
<th>Time of death (weeks)*</th>
<th>Sacrificed (S) / Death (D)</th>
<th>Pleural fluid (Y/U)</th>
<th>Enlarged heart (Y/U)</th>
<th>Weight Loss</th>
<th>Score/ mm(^2)</th>
</tr>
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<tbody>
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</table>
### Discussion

Several studies showed that the long-term effect of DOX on cardiac tissue may progress in time to more severe myocardial injury resulting in cardiomyopathy or even CHF [Kremer LCM et al, 2001, Steinherz LJ et al, 2001]. Therefore, advances in long-term survival achieved over the last two decades for e.g; acute leukaemia in childhood necessitate obtaining favourable long-term effects of DOX-containing anticancer treatment [Langebrake C et al, 2002]. Thus, it is desirable to dispose of a compound giving long-term cardioprotection. MonoHER, a flavonoid with proven cardioprotecting properties [Van Acker SABE et al, 1997, Van Acker

<table>
<thead>
<tr>
<th>Mouse (Group, #)</th>
<th>Time of death (weeks)*</th>
<th>Sacrificed (S) / Death (D)</th>
<th>Pleural fluid (Y/U)</th>
<th>Enlarged heart (Y/U)</th>
<th>Weight Loss</th>
<th>Score/mm²</th>
</tr>
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<td>18</td>
<td>S</td>
<td></td>
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</table>

**Groups:** I = saline, II = DOX alone, III = DOX-monoHER, IV = DOX-monoHER + monoHER once every week; *weeks from the start of the observation period; Y = yes, U = unknown, information was lost; Score / mm²: abnormal cardiac myocytes / mm² scored according to Billingham; N = no material available, NE = tissue not suitable for evaluation.
FAA et al., 2000, Bruynzeel AME et al., 2006), and at present in a clinical phase II study, is expected to be such a compound.

The results of this study corroborate that monoHER given 1 hour before DOX is protecting in vivo against DOX-induced cardiotoxicity within the first 8 weeks of treatment. Its cardioprotective effect is present for a longer period of time thereafter, but towards the end of 26 weeks of observation the cardioprotection by monoHER is not present anymore and toxicity becomes comparable to that in DOX-treated animals. Continuation of weekly injections of monoHER (after DOX administration was ended) during the observation period even seems to aggravate the development of DOX-induced cardiotoxicity.

At the end of the study no difference in survival was observed between the mice treated with DOX alone and the animals treated with DOX-monoHER (group III), while the mice treated with monoHER once every week during the observation period even showed a decreased survival rate in comparison to the DOX-treated mice.

It is known however that flavonoids which can act as antioxidants, may display pro-oxidant action at higher doses [Breinholt VM et al., 2003, Niering P et al., 2005, Thomson AR et al., 2005] and/or when administered for an extended period of time [Breinholt VM et al., 2003]. One of the reasons for this change may be that compounds such as flavonoids and carotenoids which act as effective scavengers of reactive oxygen species, may themselves become reactive oxidation products [Breinholt VM et al., 2003, Boots AW et al., 2005]. These secondary formed toxic products may interact with lipids, proteins and DNA giving rise to (pro-oxidant) cytotoxic as well as genotoxic effects.

Also, Boots AW et al. (2005) showed that during its antioxidant activity quercetin becomes oxidized. This oxidized form is toxic since it reacts with for example the thiol glutathione (GSH), which results in a decreased amount of this cellular antioxidant.

Because of these properties, it may be that monoHER – being a structural analog of quercetin – was administered at a too high dose in the animals receiving monoHER just before DOX (group III) and especially in the animals receiving monoHER also once every week during the observation period (group IV). Therefore, it could be that the created overdose of the antioxidant monoHER started to behave as a pro-oxidant as shown for other flavonoids before [Sugihara N et al., 1999, Hibatallah J et al., 1999, Niering P et al., 2005, Thomson AR et al., 2005]. Another possible explanation may be that monoHER becomes a reactive oxidation product and thus depletes the already small amount of cardiac antioxidants.

The physical problems in the mice treated with monoHER every week (group IV) developed earlier and were more severe in comparison to the mice treated with DOX-monoHER (group III). This seems to underline the concentration-dependent pro-oxidative properties of monoHER. The right balance between the dose of monoHER and its anti- and pro-oxidant properties has not been established yet. This will play a key role in the further development of monoHER as a cardioprotector.
During microscopic evaluation of the cardiac damage we observed that the cardiac cells of mice treated with saline showed a higher density of myofibrils than the surviving animals treated with DOX either with or without monoHER. This observation – besides the morphological changes in the cardiac tissue which are included in the Billingham score (vacuolar degeneration, loss of myofibrils) – seems characteristic for long-term DOX-induced cardiac damage, because it was not seen in heart tissue of DOX treated mice after a short observation time.

The present study indicates that the dose of monoHER may be crucial in providing an optimal anti-oxidant effect without a pro-oxidant effect, thus obtaining the desired long-term cardioprotective effect. These aspects have to be elucidated in more detail.

References


Breinholt VM, Mølck AM, Svendsen GW, Daneshvar B, Vinggaard AM, Poulsen M, Dragsted LO (2003) Effects of dietary antioxidants and 2-amino-3-methylimidazo[4,5-f]- quinoline (IQ) on preneoplastic lesions and on oxidative damage, hormonal status, and detoxification capacity in the rat. Food Chem Toxicol 41: 1315-1323


Haenen GRMM, Jansen FP, Bast A (1993) The antioxidant properties of five O- (beta-hydroxyethyl) rutosides of the flavonoid mixture Venoruton. Phlebology Suppl.1: 10-17


Chapter 6

A phase I study of monohydroxyethylrutoside in healthy volunteers

Anja M. Willems, Anne M. Bruynzeel, Marc A. Kedde, Cees J. van Groeningen, Aalt Bast, Wim J. van der Vijgh

Abstract

The flavonol monohydroxyethylrutoside (monoHER) has demonstrated protection against doxorubicin-induced cardiotoxicity in *in vitro* and *in vivo* studies without affecting the antitumor effect. In the present phase I study, the possible side effects and the pharmacokinetics of monoHER were evaluated in healthy volunteers with the aim to develop a safe and feasible dose to be evaluated in cancer patients treated with doxorubicin. The study was performed as a single blind, randomized trial in healthy volunteers (age between 19 and 56 years). At each dose level, six subjects received monoHER and three placebo. MonoHER was solubilized in 100 ml dextrose 5% and administered as an i.v. infusion in 10 minutes. The placebo consisted of 100 ml dextrose 5%. The starting dose of monoHER was 100 mg/m². Dose escalation by 100% of the preceding dose took place after finishing each dose level until the protecting pharmacokinetic values for $C_{\text{max}}$ and $AUC^\infty$ (as observed in mice after 500 mg/kg monoHER i.p.) were reached and/or serious side effects were observed. The dose was escalated up to 1,500 mg/m². The mean values of $C_{\text{max}}$ and $AUC^\infty$ were $360 \pm 69.3 \mu M$ and $6.8 \pm 2.1 \mu mol.min/ml$, respectively. These values were comparable to the $C_{\text{max}}$ and $AUC^\infty$ observed under the protecting conditions in mice. No serious side effects occurred during the entire study. Thus, 1,500 mg/m² is a feasible and safe dose to be evaluated in a phase II study to investigate the protective properties of monoHER against doxorubicin-induced cardiotoxicity in cancer patients.
Introduction

Since the 1960s anthracyclines are used in a wide variety of malignancies. Unfortunately, these extensively used drugs have serious dose-limiting side effects. The cumulative dose-related cardiotoxicity is an important problem [Blum RH & Carter SK, 1974, Young RC et al, 1981, Hortobágyi GN, 1997], particularly in survivors of childhood cancers, who might experience ventricular dysfunction, heart failure, arrhythmias and sudden death, especially in the presence of stressors, such as pregnancy and sports [Hrdina R et al, 2000, Pai VB & Nahata MC, 2000].

Anthracycline-induced cardiotoxicity seems to be, at least partly, caused by oxidative stress [Hrdina R et al, 2000]. Anthracyclines can initiate hydroxyl radical formation, especially after complexation with iron. Owing to a lack of the oxidative defense system in the heart [Horenstein MS et al, 2000, Iarussi D et al, 2000] protection can be obtained by protecting compounds, which either scavenge radicals and/or prevent radical formation by iron chelation. ICRF-187 is the only registered and clinically successful cardiotoxicity modulator. By chelating iron, it can inhibit the formation of the oxygen free radicals. However, in a clinical trial in women with advanced breast cancer, the response rate to doxorubicin-based treatment was significantly lower in the group receiving ICRF-187 (48%) than in the placebo group (63%) [Anonymous, 1995]. In patients who received ICRF-187, bone marrow suppression was also more severe [Van Acker SABE et al, 1995, Weijl NI et al, 1997].

Monohydroxyethylrutoside (monoHER) is a potential new protective agent against doxorubicin-induced cardiotoxicity [Van Acker SABE et al, 1993a, Van Acker FAA et al, 2000]. Inhibition of lipid peroxidation through radical scavenging and iron chelation is supposed to be the mechanism of action [Van Acker SABE et al, 1993a,b]. The protection of monoHER against doxorubicin-induced cardiotoxicity was found to be dose-dependent [Van Acker SABE et al, 1997]. In vitro and in vivo experiments showed no influence of monoHER on the antitumor activity of doxorubicin. No side effects were observed in mice treated with monoHER [Van Acker SABE et al, 1997].

The present study describes the possible side effects of monoHER and the pharmacokinetics of monoHER in healthy volunteers during a phase I study using escalating dose levels with the aim to develop a safe and feasible dose to be evaluated in cancer patients treated with doxorubicin.
Materials and Methods

Study population
Volunteers were accrued by advertisement in weekly papers of the Vrije Universiteit and the Vrije Universiteit Medical Center. Volunteers (m/f) had to be healthy and in the age between 18 and 65 years. No medication was allowed since the week preceding the administration of monoHER.

Nine volunteers were entered at each dose level. Characteristics of the volunteers are shown in Table 1. Six subjects received monoHER whereas three subjects received placebo. This study was performed as a randomized, single blind study. Randomization was carried out by drawing a sealed envelope with a volunteer number. This number determined the medication of subsequent dose levels. Replacement of a volunteer meant continuation of the medication indicated in the lane of the respective volunteer number by the replacing volunteer. Because the same volunteers could participate in more than one dose level the randomization of medication was carried out in a way that every volunteer did get at least one placebo (Figure 1). Hematology was studied separately at the highest dose in the same volunteers (see Figure 1).

<table>
<thead>
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<th>Study</th>
<th>Dose mg/m²</th>
<th>Volunteer numbers</th>
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<tr>
<td>Hematology</td>
<td>1500</td>
<td>1 15 – – 6 16 8 9</td>
</tr>
</tbody>
</table>

Figure 1. Randomization schedule used in the phase I study and the hematology study (black square placebo; gray square monoHER). Number identifies the volunteer.

Ethical consideration
This study was approved by the Medical Ethical Review Committee of the Vrije Universiteit Medical Center. The outline of the study was explained to the volunteers before obtaining their written informed consent.
Table 1. Characteristics of the volunteers participating in the study

<table>
<thead>
<tr>
<th>Volunteer no</th>
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<th>Age (years)</th>
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<th>Body Surface Area (m²)</th>
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Data analysis

Concentration-time curves of monoHER were obtained from three volunteers at each dose level. At the highest dose level (1,500 mg/m²) six curves were analyzed. The area under the concentration-time curve (AUC∞), mean residence time (MRT), clearance (Cl), volume of distribution and half-lives were determined by using a NONLIN fit with two or three exponential terms using the pharmacokinetic computer program WinNonlin, version 1.5 (Pharsight Corporation, Mountain View, USA).
Treatment

MonoHER

Unformulated drug was provided by Novartis Consumer Health, Nyon, Switzerland. *Formulation of the drug* The drug was formulated under aseptic conditions by the Department of Pharmacy, Vrije Universiteit medical Center, Amsterdam, The Netherlands. The required amount of monoHER was dissolved in 100 ml dextrose 5% for intravenous use, adjusted to pH 9.3 using sodium hydroxide 4M. After dissolution of the drug, the solution was readjusted to pH = 8.4 with hydrochloric acid 1M. The final solution was filtered through a sterile 0.2 μm filter and transferred into a sterile 100 ml IVAC infusion system (Alaris Medical BV, Amersfoort, The Netherlands). The solution was chemically stable for at least 24 hours at room temperature [Abou El Hassan M et al, 2000]. MonoHER or placebo were administered to the volunteer within 24 hours after preparation.

*Administration of the drug* MonoHER was administered as an intravenous infusion during exactly 10 minutes. Because 10% of the registered drug Venoruton® is monoHER and 1,500 mg of Venoruton® could be administered intravenously to patients without any side effect [Neumann HAM et al, 1992], the starting dose of monoHER was 100 mg/m². After finishing a dose level the dose was escalated by 100% (of the preceding dose) until values of $C_{max} \geq 131 \mu M$ and $AUC^\infty \geq 6.3 \mu mol.min/ml$ were obtained under the protecting conditions in mice after administration of 500 mg/kg i.p. [Abou El Hassan M et al, 2003] and / or serious side effects were observed. Although monoHER is yellow-colored the placebo was not colored, because of possible side-effects of the additive. Instead, the IVAC infusion system was wrapped in aluminum foil and the infusion links were covered with a yellow extension tube for blinding. Hundred milliliters of placebo was administered in the same way as monoHER.

Safety parameters

Before administration of monoHER blood pressure, pulse frequency, liver function (serum bilirubin, SGOT, SGPT, $\gamma$-GT) and renal function (serum creatinine) were measured and a routine 12-lead ECG was performed. Blood pressure and pulse frequency were repeated directly after the infusion and 1, 2 and 3 h thereafter. An ECG was also repeated 3 h after administration. Measurements of renal and liver function were repeated 24 h and 3 days after the end of the infusion.

After the phase I study, hematology was checked in six healthy volunteers receiving 1,500 mg/m² of monoHER as a 10 min i.v. infusion. Hb, Ht, platelets, WBC and its differential count were determined before the infusion and 24 h and 7 days after the end of the infusion.
Pharmacokinetics
The pharmacokinetics of monoHER was measured at each dose level with the aim to obtain at least the same peak plasma concentration ($C_{\text{max}}$ of 131 µM) and a comparable concentration-time profile (AUC$^\infty$ of 6.3 µM.min) in healthy volunteers as obtained in mice [Abou El Hassan M et al, 2003]. At the same time linearity of the pharmacokinetics was checked.

Venous blood samples (2 ml) were collected from all volunteers (including those receiving placebo) just before infusion, just before the end of the infusion, 15, 30, 60 and 90 min and 2, 4, 6, 8, 21 and 24 h after the end of the infusion. Blood was collected in a cooled sodium heparin-containing glass tube (3 ml), which was kept on ice until processed shortly thereafter. Blood cells were spun down in a cooled centrifuge (4°C) at 4,000 rpm for 3 min. Aliquots of the plasma (supernatant) were transferred into polypropylene micro-test tubes (> 150 µl/tube). Each sample was frozen immediately at −80°C until analysis.

Urine was collected in three portions of 2 hours since the start of the infusion. Before infusion a blank urine sample was collected. Urine samples were collected in wide-mouthed polyethylene bottles. Each 2-h urine portion was gently shaken and the total volume was measured. Four aliquots of 2 ml were transferred into polypropylene micro-test tubes and frozen at −80°C until analysis.

HPLC analysis
Concentrations of monoHER in plasma and urine were analyzed by HPLC with electrochemical detection as previously described by Abou El Hassan et al [Abou El Hassan MA et al, 2001]. In short, monoHER was extracted from plasma and urine with methanol. After centrifugation (9,000 rpm, 3 min, 1°C), two portions of supernatant were transferred into a polypropylene micro-test tube (1.5 ml) and diluted with 25 mM phosphate buffer (pH 3.33). After centrifugation (9,000 rpm, 3 min, 1°C), the supernatant was transferred into a new polypropylene micro test tube (1.5 ml) and placed in the autosampler (Basic Marathon with cooled tray, 4°C, Spark Holland, The Netherlands). The level of monoHER was measured in duplicate. Calibration standards and quality control samples were freshly prepared on each day of analysis.
Results

Side effects
The side effects were expressed using the NCI’s Common Toxicity Criteria, Version 2. No serious side effects were observed at the different dose levels. After 100 mg/m² monoHER one volunteer complained about a burning sensation at the injection site during less than 1 min. She also complained about light-headedness during the whole day, but it did not interfere with her daily pursuits. The day after the infusion of 200 mg/m² monoHER one volunteer complained about paresthesia in all her fingertips at the site of the infusion. This was reversible and disappeared the day thereafter. One volunteer in the placebo group at the 400 mg/m² dose level experienced a vasovagal episode without loss of consciousness during the infusion. One volunteer reported a slight tension headache two hours after monoHER infusion at a dose of 400 mg/m². This headache disappeared five hours later. He was still able to work. One volunteer mentioned a slight feeling of nausea during and after infusion of monoHER at a dose of 400 mg/m² and 1,500 mg/m² without mentioning this during or after receiving placebo at the 800 mg/m² dose level.

Pulse frequency and blood pressure showed intra-subject variation within the normal range. No relation could be found with the administration of monoHER or placebo. Liver function (bilirubin, γ-GT, ASAT, ALAT) and kidney function (creatinine) also showed intra-subject variability, but could not be related to administration of monoHER or placebo.

Two volunteers were discovered with abnormalities on their initial ECG. One volunteer, a 19-year old female, was diagnosed with a Wolff-Parkinson-White syndrome. This volunteer received placebo without any change in the ECG. She was excluded from further participation in the study. In another volunteer, a 42-year old female, the initial ECG showed ischemic changes. This volunteer did not receive any treatment and she was excluded from the study. Administration of monoHER or placebo did not show any change in the ECG’s of the participating volunteers.

Hematological parameters (Hb, Ht, platelets, WBC and differential count) were analyzed at the earlier described time points. The administration of monoHER had no influence on these hematological parameters.

Pharmacokinetics in plasma
The mean plasma concentration-time curve of monoHER after 1,500 mg/m² in 6 healthy volunteers is shown in Figure 2. At this dose the mean peak plasma concentration was 360 ± 69.3 µM; the mean AUC∞ was 6.8 ± 2.1 µmol.min/ml. Because these values were comparable or higher than the targeted values, no further dose escalation was applied.
At the highest dose (1,500 mg/m²) monoHER disappeared triphasically from the plasma compartment with a $t_{1/2a}$ of 5.0 ± 1.6 min, a $t_{1/2b}$ of 27.0 ± 11.2 min and a $t_{1/2c}$ of 168 ± 148 min, respectively. The mean residence time (MRT), body clearance (Cl) and volume of distribution at steady state ($V_{d,ss}$) were 33.4 ± 8.8 min, 0.70 ± 0.22 l/min and 22.9 ± 8.7 l, respectively. The pharmacokinetics of monoHER seemed to be non-linear as shown by the plots of $C_{max}$ and $AUC_{\infty}$ versus the dose (Figures 3 and 4, respectively).

**Pharmacokinetics in urine**

Mean amounts of monoHER excreted in the urine (expressed as percentage of the dose) after administrations of monoHER at the different dose levels are shown in Figure 5. Urinary excretion of monoHER principally took place during the first two hours since the start of the infusion. Thereafter only small amounts of monoHER were excreted in the urine. At the highest dose of 1,500 mg/m² the mean (± SD) amount excreted within the first two hours since the start of the infusion was 11.5 ± 8.4% of the total dose (D) of monoHER administered. The urinary excretion from 2–4 h and 4–6 h were 0.7 ± 0.6% and 0.1 ± 0.0% D, respectively. A large inter-individual variability was observed. In total, about 12% of monoHER was eliminated by the kidneys within 6 h after the start of the infusion. No significant difference was measured between the mean amounts excreted at the various dose levels.

![Figure 2. Mean concentration (± SD) versus time curve at a dose of 1,500 mg/m² monoHER in six volunteers](image-url)
Figure 3. Peak plasma concentration ($C_{\text{max}}$) versus the dose of monoHER (circle individual value; thick line mean)

Figure 4. Area under the curve extrapolated to infinity ($\text{AUC}^\infty$) versus the dose monoHER(circle individual value; thick line mean)
A phase I study of monoHER

Discussion

In this study the pharmacokinetics and possible side effects of an i.v. infusion of monoHER, a very promising new agent in protecting the heart against anthracycline-induced toxicity [Van Acker SABE, 1995, Van Acker FAA et al, 2000] were investigated in human. Up to the highest dose of 1,500 mg/m², monoHER was well tolerated and no serious side effects were observed. The maximal tolerated dose (MTD) of monoHER was not investigated, because the pharmacokinetic end-points ($C_{\text{max}} \geq 131$ $\mu$M and $\text{AUC}_{\infty} \geq 6.3$ $\mu$mol.min/ml) were obtained and the solubility of monoHER at pH 8.4 ($\approx 3000$ mg/100 ml) limited the administered dose to about 1,500 mg/m² when given in 100 ml as a 10 min i.v. infusion.

An important pharmacokinetic characteristic of monoHER was the rapid distribution and elimination of monoHER from the body plasma as indicated by the low values found for its half-lives and mean residence time (MRT). The rapid distribution and elimination of monoHER from the plasma compartment was also observed in various animal species [Hackett AM & Griffiths L, 1977, Hackett AM & Griffiths LA, 1979, Abou El Hassan M et al, 2003]. The short distribution half-life of monoHER corresponded with the rapid uptake of monoHER in heart tissue as found in mice ($C_{\text{max}}$ in heart tissue within 5 min after i.p. administration of monoHER) [Abou El Hassan M, 2003]. The volume of distribution at steady state ($V_{dss}$ = 22.9 ± 8.7 l) as found in the present phase I study indicates that monoHER is distributed at

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**Figure 5.** The amount of monoHER excreted in the urine at each dose level expressed as percentage of the total dose. $n = 6$; light gray area 0–2 h; medium gray area 2–4 h; black area 4–6 h)
least in the extracellular fluid, and might be distributed in the intracellular fluid as well. This finding supports the idea that monoHER can intracellularly exert its effect against doxorubicin-induced cardiac damage. Our observations correspond with the previous findings of monoHER in mice studies [Griffiths LA & Hackett MA, 1978, Abou El Hassan M et al, 2003]. The $V_{dss}$ of monoHER lies in the range of those observed in phase I studies of other flavonoids, e.g. 24–38 l/m², 161 l and 6.2 – 92.6 l for flavone acetic acid [Havlin KA et al, 1991, De Forni M et al, 1995], genistein [Setchell KDR et al, 2001] and quercetin [Graefe E et al, 1999], respectively; thus indicating that these investigated flavonoids also seem to penetrate into the intra- and extracellular fluid.

The distribution and elimination half-lives of monoHER in plasma (5.0, 27.0 and 168 min) of our volunteers were comparable to those found in mice [Hackett AM & Griffiths L et al, 1977] and dogs [Hackett AM & Griffiths LA et al, 1979]. It also corresponded with the final half-lives found for other flavonoids during phase I studies, e.g. 3.1 h, 0.7 – 2.4 h and 3.2 – 7.1 h for flavone acetic acid [De Forni M et al, 1995], quercetin [Gugler R et al, 1975, Ferry DR et al, 1996] and genistein [Setchell KDR et al, 2001, Busby MG et al, 2002], respectively. Thus, the investigated flavonoids, including monoHER, stay for a relatively short time in the plasma compartment. This is not a drawback, because doxorubicin-induced cardiotoxicity seems to be related to the peak serum level of doxorubicin, as the incidence of doxorubicin-induced heart failure decreased after a low-dose weekly schedule or a prolonged infusion period [Bielack SS et al, 1989]. Because of the very short initial half-life of doxorubicin [Mross K et al, 1988], high plasma levels of doxorubicin are only present during the first 2 hours after the start of the infusion. At least during this time, high levels of monoHER are present to prevent radical formation and to scavenge radicals from doxorubicin, which seems to be enough to cause the observed cardioprotective effect.

In the present phase I study, a high dose (1,500 mg/m²) of the semi-synthetic flavonoid monoHER administered intravenously did not cause serious side effects. Only one volunteer complained about a slight feeling of nausea after 400 mg/m² and after 1,500 mg/m² monoHER, but not after the placebo, indicating that nausea could be a side effect related to monoHER infusion. The administration of monoHER did not influence the liver and kidney function. These favorable observations are in agreement with the toxicity studies in animals. At the highest single dose given orally to mice (16 g/kg), rats (16 g/kg) and dogs (32 g/kg) no side effects were observed related to the drug (Internal Report Zyma SA, 1973). Also, no teratogenic effects were observed in pregnant mice, rats and rabbits after an oral dose of 2,700 mg/kg given daily for 10 days during pregnancy (Internal Report Zyma SA, 1974). In contrast with ICRF-187, administration of monoHER did not influence the hematological parameters [Koeller JM et al, 1981, Hensley ML et al, 1999]. Thus, monoHER at a dose of 1,500 mg/m² i.v. can be administered safely.
A phase I study of monoHER

A few flavonoids have been investigated as a drug in a phase I study. For instance, quercetin, the main flavonol in our diet, caused a dose-limiting nephrotoxicity at 2,000 mg/m² intravenously [Graefe E et al, 1999]. Flavone acetic acid, a synthetic flavonoid, caused a dose-limiting hypotension at a dose of 10,000 mg/m² i.v.. At doses less than or equal to 5,000 mg/m² side effects were minimal [Havlin KA et al, 1991, De Forni M et al, 1995]. It cannot be excluded that an MTD of monoHER would have been found at dose levels not too far from 1,500 mg/m². This may be promoted – and also warns for care if further dose escalation would be considered – by the possible non-linear pharmacokinetic behaviour of monoHER. Nonlinear pharmacokinetics was also observed for tri- and tetra-HER after administration of Venoruton to healthy volunteers as a 6-h i.v. infusion [Balant LP et al, 1984]. In general, the liver is the main organ involved in the metabolism of polyphenols of which the flavonoids are a subgroup [Bravo L, 1998, Scalbert A & Williamson G, 2000]. In particular, monoHER was mainly excreted in bile as was found in several mice and rat experiments [Barrow A & Griffiths LA, 1974, Hackett AM & Griffiths LA, 1979]. This corresponds with the low excretion of monoHER by the kidneys (about 12% of the total dose) as found in the present study, which is in agreement with previous data from studies in mice, rats and dog [Barrow A & Griffiths LA, 1974, Hackett AM & Griffiths L, 1977, Hackett AM & Griffiths LA, 1979]. This suggests that the nonlinear pharmacokinetics of monoHER might be caused by a rate-limiting step in the metabolism and/or excretion by the liver.

Side effects might also appear after cumulative dosages of monoHER, but this does not seem very likely. Seven volunteers received two or more dosages of monoHER, but no cumulative dose-related side effects were observed in those volunteers. These findings are in agreement with a chronic toxicity study on Beagle dogs receiving monoHER at a dose of 150 mg/kg/day for 160 days without showing any side effects of the drug (Internal Report Zyma SA, 1974). Also, flavone acetic acid when administered intravenously at dosages of 6.4 g/m² with an interval of 48 h did not result in severe toxicity nor showed evidence of drug accumulation [De Forni M et al, 1995]. Thus, monoHER at a dose of 1,500 mg/m² can be administered safely. This dose will be used in a phase II study in which the cardioprotection of monoHER will be investigated in cancer patients receiving doxorubicin.
References


A phase I study of monoHER


Chapter 7

The effect of monohydroxyethylrutoside on doxorubicin-induced cardiotoxicity in patients treated for metastatic cancer in a phase II study

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Submitted
Abstract

Aim of the study was to investigate the cardioprotective effect of the semisynthetic flavonoid monoHER on DOX-induced cardiotoxicity in a phase II study in patients with metastatic cancer.

Eight patients with metastatic cancer were treated with DOX preceded by a 10 min i.v. infusion of 1500 mg/m$^2$ monoHER. Five patients were examined by endomyocardial biopsy after reaching a cumulative dose of 300 mg/m$^2$. Histopathological changes in the cardiomyocytes (Billingham score) were compared with those described in literature for patients treated with DOX only.

The mean biopsy score of the patients was higher (2.7) than the mean score (1.4) of historical data of patients who received similar cumulative doses of DOX. Although there is a considerable variability in the few investigated patients, it was indicative that monoHER enhanced DOX-induced cardiotoxicity. However, the antitumor activity of DOX seemed better than expected: three of the four patients with metastatic soft tissue sarcoma had a partial remission, the fourth patient stable disease.

It is likely that the relatively high dose of monoHER is responsible for the lack of cardioprotection and for the high response rate in patients with soft tissue sarcoma possibly by depleting the glutathione defense system in both heart and tumor.
Introduction

The anthracycline doxorubicin (DOX) is widely used in the treatment of several malignancies in adult and pediatric patients. Treatment with DOX is limited by a dose-dependent cardiotoxicity, which may lead to late side effects resulting in severe morbidity and mortality [Steinherz LJ et al, 2001, Lipshultz SE et al, 2005]. Although the 5-year survival of childhood cancer has improved from 30% to 70% in the last 40 years, the risk of death from cardiac events in these survivors is eight times higher than that in the normal population [Wouters KA et al, 2005]. Besides this, combining DOX with other anticancer drugs, e.g. taxanes and trastuzumab, increases efficacy, but unfortunately also augments cardiotoxicity [Seidman A et al, 2002, Minotti G et al, 2004].

Although the mechanism of DOX-induced cardiotoxicity is still not fully understood, a major role has been ascribed to the induction of free radicals [Horenstein MS et al, 2000, Hrdina R et al, 2000, Xu MF et al, 2001]. The cardiomyocyte is particularly vulnerable to free radical injury because of properties such as a low antioxidant status [Doroshow JH et al, 1980, Iarussi D et al, 2000].

Presently, the cardioprotectant dexrazoxane is the only drug with proven efficacy [Cvetkovic RS & Scott L Jr, 2005]. A recent review recommended the use of dexrazoxane if the risk of cardiotoxicity is high. However, clinicians should weigh its cardioprotective effect against the risk of a possible decrease of antitumor activity [Van Dalen EC et al, 2005].

Preclinical experiments showed that the flavonoid 7-monohydroxyethylrutoside (monoHER) is a potential protective agent against DOX-induced cardiotoxicity without interfering with its antitumor activity [Van Acker SA et al 1997, Van Acker FA et al, 2000]. Radical scavenging and iron chelating properties are the supposed mechanisms of action of monoHER [Haenen GRMM et al 1993, Van Acker SABE et al, 1993]. No serious side effects were observed in a clinical phase I study up to a dose of 1500 mg/m². At this dose the pharmacokinetic end-points were reached, i.e. $C_{max}$ and $AUC_{\infty}$ were comparable to those obtained in mice under protecting conditions. Therefore this dose was evaluated in a phase II study [Willems AM et al, 2006].

In the present study, we evaluated the cardioprotective properties of 1500 mg/m² monoHER given as a 10 minute i.v infusion before DOX in patients with metastatic cancer. For the early sensitive and specific detection of DOX-induced cardiotoxicity endomyocardial biopies were taken [Meinardi MT et al, 1999].
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Patients and Methods

Patient selection
Patients with metastatic solid tumors were entered when they received a DOX-based chemotherapy regimen with a dosage of DOX ≥ 50 mg/m$^2$/cycle and an infusion duration ≤ 1 hour. Patients had a WHO performance status of ≤ 2 and a life expectancy of ≥ 3 months. They also had adequate organ functions. Their left ventricular ejection fraction (LVEF) was > 50%. Patients were excluded if they had received prior anthracyclines, had prior or actual cardiovascular disease or had prior radiotherapy to the mediastinum.

All patients gave written informed consent and the protocol was approved by the medical ethical review committee of the VU University Medical Center (VUMC). Patients were enrolled between September 2003 and March 2006.

Treatment
7-Monohydroxyethylrutoside (monoHER) was provided by Novartis Consumer Health (Nyon, Switzerland). The drug was formulated by the Department of Pharmacy, VUMC, Amsterdam as described before [Willems AM et al, 2006]. Formulated doxorubicin (Doxorubicin hydrochloride, 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, the Netherlands). MonoHER was administered i.v. in 10 min at a dose of 1500 mg/m$^2$ 60 min before every DOX administration. If cardiotoxicity would be observed in the first three evaluable patients, administration according to this dosing scheme would be changed and the following patients would receive DOX infusion either immediately after monoHER (because in plasma and heart $C_{max}$ of monoHER is obtained immediately after the end of infusion [Abou El Hassan MAI et al, 2003, Willems AM et al, 2006]) or with an interval of 2 hours (to give monoHER the opportunity to convert into an active metabolite, if any).

Patient evaluation
Before starting, patients were evaluated by a full blood count, serum biochemistry including liver function tests, lactate dehydrogenase and cholesterol. Risk factors for cardiovascular disease were also evaluated. An LVEF and an ECG were performed before entry into the study.

Every subsequent administration of monoHER and DOX was preceded by a full blood count, liver enzymes, serum creatinine and a routine 12-lead ECG. A complete blood count was also done 10 days after chemotherapy.

After a cumulative dose of 300 mg/m$^2$ DOX an endomyocardial biopsy was performed and the LVEF was measured. The latter was repeated at least 3 weeks after the last dose of DOX with a biopsy if possible. For logistic reasons, the biopsy of patient #8 was done after a cumulative dose of 375 mg/m$^2$ DOX.
Endomyocardial biopsy
During a left heart catheterization, a 104 cm, 7 french biopsy forceps was used to obtain tissue from the left ventricle. Three to four specimens 0.5 to 1 mm in diameter were obtained. The specimens were fixed in 4% buffered formaldehyde and prepared for electron microscopy.

Histological analysis and biopsy scores
After fixation in 4% buffered formaldehyde, the heart tissue was post fixed in 1% osmium tetroxide. The tissue was then dehydrated through a graded series of ethanol solutions of 70–95% and embedded in JB-4 Plus resin. Thereafter, 0.5–3.0 \( \mu \)m thick sections were cut with a glass knife. These semithin sections were processed for electron microscopy. Cardiomyocytes with > 2 microvacuoles, macrovacuoles and/or loss of myofibrils were counted as deviant. The morphological grade determined from the specimens examined by electron microscopy was scored on a 6-point scale previously described by Billingham [Billingham ME et al, 1978, Bristow MR et al, 1982]: in grade 0 cells are normal; in grade 1, 1.5, 2 and 2.5 deviant cells are < 5%, 5–15%, 16–25% and 26–35%, respectively; in grade 3 cell damage is > 35%.

Off-study criteria
Patients went off study in case of progressive disease, a serious cardiac event, or other events that precluded further treatment. Criteria described by Shapiro et al. [Shapiro C et al, 1999] were used for diagnosis of cardiac events. Episodes of cardiac dysfunction were characterized according to the NYHA functional classification [Seidman A et al, 2002].

Assessment of tumor response
Assessment of tumor response was done every 2–3 cycles by CT-scan, using standard ECOG criteria [Oken MM et al, 1982].

Statistical analysis
This trial was an open-labelled, controlled study. We compared the data of our patients with those of 14 patients from the study of Torti et al. [Torti FM et al, 1986] treated with a cumulative dose of DOX between 200–300 mg/m\(^2\) alone using the Chi-Square test. Our hypothesis was that adding monoHER to DOX would eliminate its cardiotoxicity up to a cumulative dose of at least 300 mg/m\(^2\), which was the upper limit of the dose interval of DOX from Torti’s patients. In order to achieve a power of 80%, 11 patients would be required. This sample size was obtained when applying the Chi-Square test with significance level 0.05 and assuming a response rate (i.e. no cardiac damage) in the experimental arm of 80%. If at least 5 patients show DOX-induced damage, statistical significance cannot be achieved anymore and the study should be stopped.
Results

Eight patients meeting the inclusion criteria were enrolled (Table 1). Analysis of risk factors indicated that patient #6 was treated for hyperhomocysteinemia. Two other patients had an elevated body mass index of 29.1 (#1) and 30.1(#7), indicating overweight and obesity, respectively. Patient #2 had hyperlipidemia (LDL of 6.3 mmol/l; normal values ≤ 5.0). None of the other patients had risk factors for cardiovascular disease. All patients were classified as NYHA class I and had a performance status (WHO) ≤ 2.

Five patients received a cumulative dose ≥ 300 mg/m$^2$ DOX and underwent a biopsy. In the other three patients DOX was discontinued before this dose, due to progressive disease.

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Total dose of dox (mg/m$^2$)</th>
<th>Response on dox</th>
<th>Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>62/F</td>
<td>Breast cancer</td>
<td>100</td>
<td>PD</td>
<td>N</td>
</tr>
<tr>
<td>02</td>
<td>54/F</td>
<td>Adrenal cortical cancer</td>
<td>150</td>
<td>PD</td>
<td>N</td>
</tr>
<tr>
<td>03</td>
<td>25/M</td>
<td>Malignant peripheral nerve sheet tumor</td>
<td>480</td>
<td>PR</td>
<td>Y</td>
</tr>
<tr>
<td>04</td>
<td>64/M</td>
<td>Malignant fibrous histiocytoma</td>
<td>450</td>
<td>PR</td>
<td>Y</td>
</tr>
<tr>
<td>05</td>
<td>55/F</td>
<td>Breast cancer</td>
<td>100</td>
<td>PD</td>
<td>N</td>
</tr>
<tr>
<td>06</td>
<td>48/F</td>
<td>Breast cancer</td>
<td>300</td>
<td>SD</td>
<td>Y</td>
</tr>
<tr>
<td>07</td>
<td>45/F</td>
<td>Malignant fibrous histiocytoma</td>
<td>300</td>
<td>PR</td>
<td>Y</td>
</tr>
<tr>
<td>08</td>
<td>56/F</td>
<td>Malignant fibrous histiocytoma</td>
<td>375</td>
<td>SD</td>
<td>Y</td>
</tr>
</tbody>
</table>

Age in years; sex F female, M male; Assessment of tumor response was done by using standard ECOG criteria (PD = progressive disease; PR = partial remission; SD = stable disease); Y yes, N no.

Treatment with DOX preceded by monoHER

During monoHER infusion two patients reported adverse events. One patient described a sensation of fullness in his stomach which developed during infusion of monoHER, which disappeared soon after the end of the infusion. The other patient experienced itching in the skin of the neck during monoHER infusion and disappearing rapidly after the infusion. Both patients experienced the events during each cycle. A causal relationship cannot be excluded. The other 6 patients did not experience adverse events.
None of the patients had a delay in receiving subsequent cycles of chemotherapy. As expected, all patients developed chemotherapy-related leucopenia, which recovered before the start of the next cycle. No disturbances of liver enzymes and serum creatinine were noted.

**Biopsy scores**

The endomyocardial biopsy scores are shown in Table 2. After 300 mg/m² the first three evaluable patients showed abnormalities consistent with DOX-induced cardiotoxicity i.e. the presence of microvacuoles dominated (Figure 1). In patient #3 and #4 microvacuoles were present in 52 and 20% of the cardiomyocytes, respectively. In patient #6 33% of the cardiomyocytes had > 2 small vacuoles per cardiomyocyte. Because each of the first 3 evaluable patients showed cardiotoxicity, it was decided to stop this dosing scheme. The time-interval between monoHER and DOX administration was changed and patient #7 received DOX immediately after monoHER. In the biopsy of this patient microvacuolization was observed in 60% of the cardiomyocytes. After this result the interval was changed again and patient #8 received DOX 2 h after monoHER administration. In this patient microvacuolization was detected in 49% of the cardiomyocytes. After these results, the study had to be considered negative, because no cardioprotective effect of monoHER was observed in the five patients.

**Table 2.** Patients who received at least a cumulative dose (cum. dose) of 300 mg/m² DOX and underwent an endomyocardial biopsy.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Biopsy score cum.dose / Δt / grade</th>
<th>LVEF before dox</th>
<th>LVEF after ≥ 300 mg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>03</td>
<td>300 / 60 / Grade 3</td>
<td>71%</td>
<td>67%</td>
</tr>
<tr>
<td>04</td>
<td>300 / 60 / Grade 2</td>
<td>72%</td>
<td>60%</td>
</tr>
<tr>
<td>06</td>
<td>300 / 60 / Grade 2.5</td>
<td>52%</td>
<td>ND</td>
</tr>
<tr>
<td>07</td>
<td>300 / 10 / Grade 3</td>
<td>75%</td>
<td>63%</td>
</tr>
<tr>
<td>08</td>
<td>375 / 120 / Grade 3</td>
<td>65%</td>
<td>63%</td>
</tr>
</tbody>
</table>

Δt = time between end of monoHER infusion and start of DOX infusion in min.

The morphological grade was scored on a 6-point scale previously described by Billingham and Bristow; LVEF = left ventricular ejection fraction.
Chapter 7

Figure 1. Heart tissue from patient #3 after 300 mg/m² DOX. Evaluation by electron microscopy demonstrated an abundant presence of microvacuoles in the cardiomyocytes.

Second biopsies were also taken after 450–480 mg/m² of DOX in two patients (Table 3). The score of patient #4 had increased from 2.0 to 2.5, whereas the score of patient #3 remained 3. Striking was the increase in loss of myofibrils in addition to the microvacuoles observed in the cardiomyocytes after 300 mg/m² (Figure 2).

Ten months after his last cycle of DOX, patient #4 underwent a third biopsy. This time the cardiomyocytes showed recuperation of myofibrils, whereas the number of abnormal cardiac cells (microvacuolization) was less than that in the first two biopsies (score 2).

No complications occurred during the 7 biopsy procedures in the first four patients. However, the last patient (#8) developed a small pericardial effusion after the biopsy. This was preceded by chest pain and transient supraventricular rhythm disturbances. These sequelae disappeared within a few days and after a week she recovered and received the sixth cycle of DOX.
Table 3. Two patients with more than one endomyocardial biopsy.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Cum.dose / biopsy score / LVEF</th>
<th>Biopsy score / LVEF 10 months after last cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>03</td>
<td>480 / Grade 3 / 53%</td>
<td>ND / 51%</td>
</tr>
<tr>
<td>04</td>
<td>450 / Grade 2.5 / 66%</td>
<td>Grade 2 / 62%</td>
</tr>
</tbody>
</table>

The morphological grade was scored on a 6-point scale previously described by Billingham and Bristow; LVEF = left ventricular ejection fraction; ND = not done.

Figure 2. Heart tissue from patient #3 after 480 mg/m$^2$ DOX. In addition to the vacuolization, a loss of myofibrils is observed with electron microscopy.

Monitoring and evaluation of cardiac function

In all patients the ECG remained unchanged during therapy and no cardiac dysfunction occurred.

The 5 patients who received at least 300 mg/m$^2$ DOX started with a LVEF > 50% (Table 2). After 300–375 mg/m$^2$ DOX, the LVEF decreased in 4/5 patients. This decrease was not related with the biopsy score or the time interval between monoHER and DOX infusions. Unfortunately, no information on the LVEF was available for patient #6.
After 480 mg/m\(^2\) DOX, the LVEF in patient \#3 decreased to 53%, which is a decline of 25% compared to the LVEF before starting the study (71%). The patient had no symptoms of cardiac failure. Ten months after his last cycle of chemotherapy the LVEF remained stable and he remained without cardiac symptoms. The LVEF of patient \#4 showed an initial drop from 72% before starting chemotherapy to 60% after 300 mg/m\(^2\) DOX (decline of > 15%). This value remained stable up to 450 mg/m\(^2\) of DOX, and at 10-month follow-up the patient still had an excellent physical condition.

**Evaluation of tumor response**

Response to the chemotherapy was remarkable in the 4 patients with metastatic soft tissue sarcoma (STS). Three of them developed a partial remission (PR) as observed on the CT scan (Table 1). In two of these patients (\#4 and \#7) PR was maintained up to the present, i.e., 30 and 16 months after the start of chemotherapy, respectively. The other patient (\#3) had progressive disease after a PR of nine months duration. The fourth patient (\#8) achieved stable disease for at least 7 months, while continuing therapy with DOX up to a cumulative dose of 495 mg/m\(^2\).

**Discussion**

Based on the promising results with monoHER observed in preclinical experiments, we performed the present phase II study in patients with metastatic cancer. The cardioprotective effect of monoHER on DOX-induced cardiotoxicity was evaluated by endomyocardial biopsy. However, the results indicated that the preclinical observations were not translated into protection against DOX-induced heart damage in humans.

The golden standard for early detection of DOX-induced cardiotoxicity is the endomyocardial biopsy, because of its high sensitivity and high specificity [Torti FM et al, 1986]. Currently, the most common method used to detect DOX-induced cardiac damage is the evaluation of the LVEF, but usually at later stages [Kilickap S et al, 2005, Villani F et al, 2006]. For detection of cardiotoxicity at an earlier stage, the use of biochemical markers such as atrial and brain natriuretic peptides, endothelin-1 as well as cardiac troponin-T and –I have been investigated [Yamahita J et al, 1995, Suzuki T et al, 1998, Kilickap S et al, 2005]. However, large-scale studies addressing whether these biomarkers following DOX treatment will be predictive for the development of late onset heart failure, are lacking. Therefore, despite its invasiveness, we chose the endomyocardial biopsy for this evaluation.

Data of Billingham et al. [Billingham ME et al, 1978] showed that anthracycline-induced myocardial damage occurred in nearly all patients treated with cumulative DOX doses of
A Phase II study with monoHER and DOX

240 mg/m\(^2\). Therefore we could expect detectable heart damage after a cumulative dose of 300 mg/m\(^2\) which would allow the registration of protection by monoHER. However, all 5 patients undergoing the biopsy procedure after a median cumulative dose of 300 mg/m\(^2\) showed DOX-induced cardiotoxicity with a mean biopsy score of 2.7 according to Billingham [Billingham ME et al, 1978]. This score was independent of the time interval between monoHER and DOX infusion and much higher than the biopsy score of 1.4, which is expected after a cumulative dose of 300 mg/m\(^2\) DOX according to the linear regression analysis of Torti et al. [Torti FM et al, 1986]. In our patients mainly one of the three morphological changes, i.e. small vacuoles of varying size were observed after 300 mg/ m\(^2\) DOX, whereas in Torti’s study also partial or total myofibrillar loss was observed [Torti FM et al, 1986]. However, in Torti’s study it is not clear after which dose this occurred. In addition to microvacuolization, loss of myofibrils was demonstrated in two patients after higher cumulative doses of DOX. This suggests that the occurrence of microvacuoles as such is a marker of cytotoxicity of DOX treatment. Billingham et al. [Billingham ME et al, 1977] described that these microvacuoles, appear early as a swelling of the sarcoplasmic reticulum, which eventually coalesce to form large spaces in the cytoplasm (macrovacuoles). When, or if microvacuoles always become macrovacuoles is not clear from literature.

Thus, instead of protection, our patients had higher biopsy scores than the historical controls. However, it should be noted that the data of Torti et al. were obtained after a cumulative dose of 200–300 mg/m\(^2\) with biopsies from the right ventricle [Torti FM et al, 1986], whereas biopsies from our patients were obtained after a cumulative dose of 300 mg/m\(^2\) from the left ventricle. Previously, it was shown that ultrastructural abnormalities of cardiomyocytes were more pronounced in biopsy specimens from the left ventricle than those from the right ventricle [Mortensen SA et al, 1986]. In addition to this, there is a considerable variability in patient sensitivity to the cardiotoxic effects of DOX [Mason JW et al, 1978, Ferrans VJ, 1978]. This data may explain a possible overestimation of the toxic effect of DOX on the heart tissue in our patients. Although heart failure is directly related to the degree of myocyte damage [Bristow MR et al, 1978], none of our patients developed heart failure, although the LVEF dropped by 16% in two patients, whereas it decreased in two other patients by only 4–6% (in comparison to the initial LVEF).

In the third biopsy procedure of patient #4, the score of the cardiac tissue had improved. A recuperation of myofibrils in the cardiomyocytes was observed and the microvacuolization was less. These findings are in agreement with a previous study reporting that some improvement of the histological damage may occur [Mackay B et al, 1994]. This is in contrast with other results, which indicated that DOX-induced cardiotoxicity is an ongoing progressive process [Billingham ME et al, 1978].
The contrasting effects of monoHER found in animal and human studies may be attributed to differences in metabolism between the species. Therefore, patient #8 was treated with a 2 hour interval. However, this interval change did not reduce DOX-induced cardiotoxicity. In addition, no metabolites of monoHER have been detected until now. On the other hand, it cannot be excluded that during scavenging of the reactive oxygen species, the antioxidant monoHER is converted into a reactive oxidation product, which like the oxidation product of quercetin maybe prone to form adducts with thiol groups from glutathione and proteins [Boots AW et al, 2005a]. Depletion of glutathione may in addition to the low antioxidant status of the cardiomyocyte [Nowak D & Drzwoski J, 1996] reduce cardioprotection, while monoHER-protein adducts may cause additional toxicity [Boots AW et al, 2005b].

Another unexpected observation in our study was that 3 of the four patients with STS had objective remissions, while the fourth patient had stable disease. Normally, objective responses on DOX in STS patients without prior chemotherapy are approximately 25% [Santoro A et al, 1995]. Although our observation was done in a very limited number of patients, our result is much better than expected, because the chance of observing an objective response in 4 consecutive STS patients treated with DOX is 0.4%. Thus, it is suggestive that monoHER enhances the antitumor activity of DOX in STS.

This observation is in agreement with potentiating antitumor effects of a few flavonoids observed in vitro [Elangovan V et al, 1994, Sliutz G et al, 1996, Debes A et al, 2003]. The background for this effect may be that the concentration of GSH may play a role in STS chemoresistance [Hochwald SN et al, 1997], and that GSH depletion may increase the antitumor efficacy [Siemann DW & Beyers KL, 1997]. Thus, the same mechanism may play a role as hypothesized for the cardiomyocytes.

As a consequence of the above-mentioned aspects, there may be a dose-depending transition in the effect of monoHER i.e. a high dose (≥ 1500 mg/m²) for obtaining a potentiating effect of the antitumor effect for at least STS and a low dose (somewhere below 1500 mg/m²) for obtaining cardioprotection. These aspects have to be elucidated further in the near future.

It may be concluded that monoHER at a dose of 1500 mg/m² did not protect against DOX-induced cardiotoxicity in patients with metastatic disease, but may have an enhancing effect on the antitumor activity of DOX in patients with metastatic STS. Further preclinical and clinical investigations seem to be warranted to investigate the postulated dose-depending transitional effect of monoHER.
References


Chapter 8

Summary and general discussion
Anticancer treatment with the anthracycline doxorubicin (DOX) is associated with a dose-dependent cardiotoxicity, which increases rapidly above a cumulative dose of 550 mg/m². As a consequence, most treatment schedules for adult and childhood cancer limit the maximum cumulative dose of DOX to 450–550 mg/m². However, because of the considerable variability in the individual susceptibility to the cardiotoxicity, there is no absolute safe dose. In addition, DOX-induced cardiac damage increases during the length of follow-up. Different hypotheses have been proposed to explain this cardiac damage and there is a lot of evidence that the generation of free radicals plays an important role. However, several other mechanisms appear to be involved in the development of DOX-induced cardiac damage. If the incidence of DOX-induced cardiotoxicity resulting in congestive heart failure would drop, the quality and extent of life for patients surviving cancer would improve. Different approaches have been investigated in an attempt to minimize or prevent DOX-induced cardiotoxicity.

In preclinical studies, the protective effect of monoHER against DOX-induced cardiotoxicity has been demonstrated in vivo, when monoHER was given as a single i.p. injection (500 mg/kg) once a week 1 hour before DOX (4 mg/kg, i.v.) for a period of six weeks. MonoHER did not affect the antitumor activity of DOX, which was demonstrated in vitro and in vivo. In addition to its radical scavenging and iron chelating properties, it was found in vitro that part of its protective effects may also be due to the anti-inflammatory effect of monoHER. The pharmacokinetic profile of monoHER under protecting conditions (500 mg/kg, i.p.) was characterized and it was found that 5–15 minutes after i.p. administration of monoHER in mice, a mean peak plasma level of about 131 µM was obtained whereas the mean AUC∞s was 6.3 µM.min. This thesis focuses on more insight in the mechanisms of action of monoHER, optimizing the administration schedule between monoHER and DOX and finally its evaluation and potential cardioprotective effect in a clinical Phase II and I study.

In chapter 2 it was shown that addition of anti-inflammatory agents during treatment with DOX reduced its cardiac damage in mice. In addition, it was demonstrated that treatment with DOX induces an increase of Nε – (carboxymethyl) lysine (CML) in intramyocardial arteries in mice. The induced increase in CML, which can be regarded as a biomarker for local endogenous stress, is reduced by these anti-inflammatory agents and monoHER. These results suggested that DOX-induced inflammatory effects are involved in the development of DOX-induced cardiotoxicity and also indicated that monoHER has anti-inflammatory properties besides its radical scavenging and iron chelating properties.

In earlier studies from our group, monoHER showed a strong protection against the cardiotoxic effects of DOX without decreasing its antitumor effects, both in vivo and in vitro. Because it is known that DOX induces apoptosis, the effect of monoHER hereon was investigated in neonatal rat cardiac myocytes (NeRCaMs), human endothelial cells (HUVECs) and ovarian
cancer cell lines in chapter 3. Assessment of the fold anti-apoptotic protection achieved showed that HUVECs and NeRCaMs were stronger protected by monoHER than A2780 tumor cells. Employing the broad caspase inhibitor z-VAD-fmk revealed that DOX triggered caspase-dependent apoptosis in HUVECs and A2780 cells, and caspase-independent cell death in NeRCaMs. Thus, combination treatment with monoHER was effective in suppressing both caspase-dependent and -independent apoptosis. When examining molecular mechanisms that underlie the protective effects of monoHER, it was demonstrated that monoHER strongly reduced the activation of DOX-induced p53 accumulation in these cells. Probably, the radical scavenging properties of monoHER caused the reduction of p53 accumulation, which is a known sensor of ROS-dependent toxicity. The suppressive effect of monoHER on the activation of caspase-9 and -3 and the substrate PARP can also be explained by the neutralization of ROS-dependent triggers of caspase activation. However, the suppressive effect of monoHER on DOX-induced apoptosis in cancer cells raised the question whether monoHER may reduce the antitumor effects of DOX in the clinic. The concentration of monoHER used in this study was much higher than that found in mice under protecting conditions. Thus, it was therefore concluded that besides its potent protective effect against various routes of doxorubicin-induced cell death, it is important not to raise the dose of monoHER above the concentrations that demonstrated protection in vivo, because this might adversely affect the antitumor activity of DOX. The tendency of monoHER to protect normal cells more than cancer cells may be attributed to the inherent proliferation capacity of cancer cells. This is supported by earlier investigations showing restricted protection of confluent but not proliferating endothelial cells after treatment with DOX. The different effects of monoHER in terms of apoptosis suppression may reflect the activity of the intrinsic ROS defence systems present in cells, which may also be associated with different mechanisms of cell death activation.

Earlier, the protection of monoHER against DOX-induced cardiac damage was shown in mice when monoHER was given once as an i.p. dose of 500 mg/kg 1 hour before DOX. Because of the relatively short final half-life of monoHER (about 30 min), it was expected that the time-interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER i.e. becomes better with a shorter time interval and worse when lengthening the time interval. Our data described in chapter 4, did not indicate a significant change in protection against DOX-induced cardiac damage over the time interval between monoHER and DOX from 2 h to 10 min.

It is known that the long-term effect of DOX on cardiac tissue may progress in time to more severe myocardial injury resulting in cardiomyopathy or even chronic heart failure. Previous studies showed the protective effect of monoHER against DOX-induced cardiotoxicity within the first 8 weeks of treatment. Our data in chapter 5 showed that the cardioprotective effect
of monoHER is lasting for a longer period of time thereafter, however towards the end of 26 weeks of observation the cardioprotection by monoHER is not present anymore and toxicity becomes comparable to that in DOX-treated animals. Continuation of weekly injections of monoHER (after 6 weeks of DOX administration) for another 26 weeks even seems to aggravate the development of DOX-induced cardiotoxicity. It has been demonstrated before that flavonoids, which can act as antioxidants, may display pro-oxidant action at higher doses and/or when administered for an extended period of time. Because of these properties, it is possible that monoHER was administered at a too high dose in the animals receiving monoHER just before DOX and especially in the animals receiving monoHER also once every week during the observation period. Therefore, it could be that the assumed overdose of the antioxidant monoHER started to behave as a pro-oxidant as shown for other flavonoids before. A possible explanation for the pro-oxidant activity may be that monoHER is converted into a reactive oxidation product which subsequently depletes the already small amount of cardiac antioxidants. Thus, the right balance between the dose of monoHER and its anti- or pro-oxidant properties has not been established yet.

In chapter 6 the possible side effects and the pharmacokinetics of monoHER were evaluated in a clinical phase I study with healthy volunteers. Up to the highest dose of 1,500 mg/m², monoHER was well tolerated and no serious side effects were observed. It was not attempted to establish the maximal tolerated dose (MTD) of monoHER, because the pharmacokinetic end-points were obtained i.e. a mean peak plasma concentration of $360 \pm 69.3 \mu M$ and a mean $AUC_{\infty}$ of $6.3 \pm 2.1 \mu mol.min/ml$ at a dose of 1,500 mg/m². At this dose level the solubility of monoHER in the i.v. infusion fluid was also reached. MonoHER was rapidly distributed and eliminated from the plasma compartment, which corresponds with the rapid uptake in and elimination from heart tissue as found before in mice. Our conclusion was that 1,500 mg/m² of monoHER would be a potential effective dose which could be administered safely. This dose was used in a clinical phase II study in which the cardioprotection of monoHER was investigated in cancer patients receiving DOX as described in chapter 7. Eight patients with metastatic cancer were treated with DOX preceded by a 10 min i.v. infusion of 1,500 mg/m² monoHER. Of them, five patients received a cumulative dose of $\geq 300$ mg/m² and underwent an endomyocardial biopsy. Three patients were treated with a time-interval ($\Delta T$) of 1 hour between monoHER and DOX, one patient with $\Delta T = 10$ min and one patient with $\Delta T = 2$ hours. No difference in biopsy score was found between the patients. The mean biopsy score of the five patients was higher (2.7) than the mean score (1.4) from historical data of patients who received a similar cumulative dose of DOX. Although there is a considerable variability in the few investigated patients, it was indicative that monoHER enhanced DOX-induced cardiotoxicity.
An interesting finding was that all four patients with metastatic soft tissue sarcoma (STS) responded (3PR, 1SD) to the combination therapy, which is much higher than expected. Although these effects of monoHER are in contrast to the earlier animal studies, they show a certain correspondence with the results of chapter 5. The differences may be explained by a possible difference in monoHER metabolism between mice and patients. In both patients (this chapter) and mice (chapter 4) it was found that the time interval between monoHER and DOX was not relevant. On the other hand, the dose of monoHER may be crucial as found in mice (chapter 5) and in patients (chapter 7). A possible explanation may be that during scavenging of reactive oxygen species, the antioxidant monoHER is converted into an oxidation product, which is reactive with thiols. Such a reaction may lead to toxicity in two ways. First, to a reduction of the antioxidant status of the cardiomyocyte and thus to a decrease in the protection against cardiotoxicity. Secondly, the oxidation product of monoHER may react with other thiols, such as protein thiols. These adducts may accumulate in the cell and cause additional toxicities. Previous studies have demonstrated that the concentration of the antioxidant glutathione may play a role in the antitumor effect in soft tissue sarcoma cells, and thus the same mechanism may play a role as that hypothesized for the cardiomyocytes. It was concluded that the dose of monoHER may play a crucial role: at a dose of 1500 mg/m² there was no protection against DOX-induced cardiotoxicity in patients with metastatic disease, but it may have an enhancing effect on the antitumor activity of DOX in patients with metastatic STS.

Conclusion

The semisynthetic flavonoid 7-monohydroxyethylrutoside (monoHER) has besides its radical scavenging and iron chelation properties, anti-inflammatory capacity which is indicated by inhibiting DOX-induced neutrophil adhesion of HUVECs and DOX-induced VCAM and E-selectin overexpression in vitro and its reducing effect on DOX-induced CML increase in vivo. In addition, anti-apoptotic properties of monoHER were demonstrated. This effect is probably also due to the ability of monoHER to neutralize ROS. Although, considering the relatively short half-life of monoHER, it was expected that the time interval between monoHER and DOX might be of influence on the cardioprotective effect of monoHER, no influence was observed. In contrast, data in this thesis indicate that the dose of monoHER may be important. High concentrations of monoHER (> 7 times of the maximal plasma concentration found in mice under protecting conditions) demonstrated protection of ovarian cancer cells in vitro. This observation indicates that the dose of monoHER may not lead to a concentration above that showing protection in vivo (131 µM). In addition,
repeated dosing of monoHER after the treatment period with DOX, tended to aggravate the development of DOX-induced cardiac damage in mice. It is supposed that the created overdose of the antioxidant monoHER starts to behave as a pro-oxidant as shown before for other flavonoids. Therefore, it seems that fine-tuning of dose and frequency of monoHER administration is crucial in obtaining an optimal and desired effect (anti-oxidant activity or pro-oxidant activity) of monoHER. These conclusions are in agreement with the findings in patients during the Phase II study.

Implications and further research

The present research indicates that the dose and frequency of monoHER administration are crucial in the protection against DOX-induced cardiotoxicity. There may be a dose-dependent transition in the effect of monoHER i.e. a high dose (≥ 1500 mg/m²) for obtaining a potentiating effect of the antitumor effect for at least soft tissue sarcomas and a low dose (somewhere below 1500 mg/m²) for obtaining cardioprotection. Several interesting research lines are open for the future. The first is to investigate the potentiating effect of monoHER on the effect of DOX on human soft tissue sarcoma cell lines. If these effects are positive, its mechanism of action has to be evaluated. If possible the findings have to be extrapolated to other tumor types. In the mean time a clinical Phase II study can be started to investigate the antitumor effect of monoHER in combination with DOX in patients with STS.

A second question to be answered is whether differences in metabolism of monoHER in mice and patients are responsible for (part) of the differences in cardiac response between the species.

A third area of special attention is the critical dose of monoHER necessary for obtaining long-term cardioprotection. After obtaining more insight in the changes of the intracellular metabolism due to monoHER, new in vivo experiments can be started.
Samenvatting en algemene discussie

De behandeling tegen kanker met het anthracycline doxorubicine (DOX) is geassocieerd met een dosis afhankelijke cardioxiciteit, die snel toeneemt boven een cumulatieve dosis van 550 mg/m². Als gevolg hiervan is de maximale cumulatieve dosis DOX in de meeste behandelingsschema’s voor volwassenen en kinderen beperkt tot 450–550 mg/m². Desondanks is er door een aanzienlijke variabiliteit in de individuele gevoeligheid voor de hartschade, geen absolute veilige dosis DOX. Hierbij komt dat DOX geïnduceerde hartschade toeneemt met de tijd na behandeling. Verschillende hypotheses zijn voorgesteld om deze hartschade te verklaren en er is veel bewijs dat de vorming van vrije radicalen een belangrijke rol speelt. Hiernaast lijken vele andere mechanismen een rol te spelen in de ontwikkeling van DOX geïnduceerde schade aan het hart.

Als de incidentie van DOX geïnduceerde hartschade die kan leiden tot hartfalen zou afnemen, zou de kwaliteit en de duur van het leven van patiënten die kanker overleven, verbeteren. In de muis is het beschermende effect van monoHER tegen DOX geïnduceerde hartschade in vivo aangetoond wanneer monoHER werd gegeven als een intraperitoneaal toegediende injectie (500 mg/kg) 1 uur voor DOX (4 mg/kg, i.v.), die éénmaal per week gedurende een periode van zes weken werd toegediend. Zowel in vitro als in vivo werd aangetoond dat monoHER geen invloed had op de antitumor activiteit van DOX. Behalve de radicaalvangende en ijzerchelerende eigenschappen, werd in vivo geobserveerd dat een gedeelte van de beschermende effecten van monoHER veroorzaakt zou kunnen worden door het anti-inflammatoire effect van monoHER. Tijdens het karakteriseren van het farmacokinetische profiel van monoHER onder beschermende omstandigheden (500 mg/kg, i.p.) werd 5–15 minuten na de i.p. toediening in muizen een gemiddelde piek plasma concentratie verkregen van ongeveer 131 µM, terwijl de gemiddelde AUC∞ 6.3 µM.min was. In dit proefschrift zullen de werkingsmechanismen van monoHER verder worden bestudeerd, zal de invloed van het tijdsinterval tussen monoHER en DOX op het beschermend effect van monoHER worden bestudeerd en zal monoHER en het mogelijke cardioprotectieve effect van monoHER worden geëvalueerd in een klinische Fase I en II studie.

In hoofdstuk 2 wordt aangetoond dat het toevoegen van anti-inflammatoire middelen tijdens de behandeling met DOX de hartschade in muizen verminderd. Ook wordt aangetoond dat behandeling met DOX een toename van Nε-(carboxymethyl) lysine (CML) in de intramyocardiale arteriën van de muis induceert. De geïnduceerde toename in CML, dat wordt gezien als een marker voor locale endogene stress, wordt verminderd door anti-inflammatoire middelen en monoHER. Deze resultaten suggereren dat DOX geïnduceerde anti-inflammatoire effecten betrokken zijn bij de ontwikkeling van DOX geïnduceerde
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hartschade. Tevens indiceren de resultaten dat monoHER naast zijn radicaalvangende en ijzerchelerende, ook anti-inflammatoire eigenschappen heeft.

In eerdere studies van onze groep liet monoHER zowel in vitro als in vivo, een sterke bescherming zien tegen de cardiotoxische effecten van DOX zonder hierbij de antitumor werking te verminderen. Omdat DOX apoptose induceert, is het effect van monoHER hierop bestudeerd in neonatale hartcellen van ratten (NeRCaMs), humane endotheelcellen (HUVECs) en ovarium kankercellinen in hoofdstuk 3. Aangetoond wordt dat monoHER de NeRCaMs en de HUVECs beter beschermt tegen DOX geïnduceerde apoptose dan A2780 tumorcellen. Het gebruik van de brede caspase remmer z-VAD-fmk laat zien dat DOX caspase-afhankelijke apoptose veroorzaakt in HUVECs en A2780 cellen en een caspase-onafhankelijke cel dood in NeRCaMs. Het blijkt dat als monoHER wordt toegevoegd aan DOX, monoHER effectief is in het onderdrukken van zowel caspase-afhankelijke als caspase-onafhankelijke apoptose. Tijdens het bestuderen van de moleculaire mechanismen die ten grondslag liggen aan het beschermende effect van monoHER, blijkt dat monoHER de door DOX-geïnduceerde toename van p53 in deze cellen vermindert. Waarschijnlijk zorgen de radicaalvangende eigenschappen van monoHER voor een afname van p53, waarvan bekend is dat het een sensor is van reactive oxygen species (ROS)-afhankelijke schade. Ook het onderdrukkende effect van monoHER op de activatie van caspase-9 and –3 en het substraat PARP kan worden verklaard door het neutraliseren van de ROS, die caspase activiteit stimuleren. Omdat monoHER de door DOX geïnduceerde apoptose in kankercellen onderdrukt, rijst de vraag of monoHER de antitumor werking van DOX in de kliniek zou kunnen verminderen. De concentratie monoHER die in deze studie gebruikt wordt is echter veel hoger dan die is bereikt in de muizen onder beschermende omstandigheden. Vandaar dat wordt geconcludeerd dat naast het beschermende effect dat wordt gezien bij verschillende routes van door DOX geïnduceerde celdood, het belangrijk is dat de dosis van monoHER niet hoger wordt gegeven dan de concentraties die bescherming lieten zien in vivo, omdat anders dit de antitumor activiteit van DOX negatief zou kunnen worden beïnvloed. De neiging van monoHER om de normale cellen meer te beschermen dan kankercellen, zou kunnen worden toegeschreven aan de proliferatie capaciteit van kankercellen. Dit wordt ondersteund door eerdere onderzoeken die alleen bescherming lieten zien van confluente cellen en niet van delende cellen die behandeld waren met DOX. De verschillende effecten die monoHER laat zien tijdens het onderdrukken van apoptose, zou de activiteit van het aanwezige intrinsieke verdedigingssysteem van de cellen tegen ROS kunnen weergeven. Ook zou het geassocieerd kunnen zijn met de verschillende mechanismen die celdood kunnen veroorzaken.

Eerder is de bescherming van monoHER tegen DOX-geïnduceerde hartschade in muizen aangetoond als monoHER werd gegeven als een i.p. dosis van 500 mg/kg 1 uur voor DOX. Vanwege de relatief korte halfwaarde tijd van monoHER (ongeveer 30 min), was de
verwachting dat het tijdsinterval tussen monoHER en DOX van invloed zou kunnen zijn op het cardioprotectieve effect van monoHER, namelijk dat het beter wordt bij een korter tijdsinterval en slechter als het tijdsinterval toeneemt. Onze data beschreven in hoofdstuk 4 laten zien dat er geen significante verandering is in de bescherming tegen door DOX-geïnduceerde hartschade als het tijdsinterval tussen monoHER en DOX varieert tussen 2 uur en 10 minuten. Het is bekend dat de toxiciteit van DOX op het hartweefsel kan toenemen met de tijd en een ernstige schade aan het hart kan veroorzaken, die uiteindelijk resulteert in cardiomyopathie of zelfs chronisch hartfalen. Eerdere studies toonden het beschermende effect aan van monoHER op DOX-geïnduceerde hartschade gedurende de eerste 8 weken van behandeling. Onze resultaten in hoofdstuk 5 laten zien dat het cardioprotectieve effect van monoHER nog langere tijd hierna aanwezig is, maar dat tegen het einde van de observatie periode van 26 weken de bescherming van monoHER niet meer aanwezig is. Hierna wordt de toxiciteit vergelijkbaar met die van de met DOX behandelde dieren. Het continueren van de wekelijkse monoHER injecties (na de 6 weken van DOX toediening) gedurende 26 weken lijkt zelfs de ontwikkeling van DOX-geïnduceerde hartschade te verergeren. Deze waarneming sluit aan bij eerdere bevindingen, nl. dat flavonoiden, die bekend staan als antioxidant, een pro-oxidante werking kunnen uitoefenen bij hogere doseringen en/of wanneer toegediend voor een langere periode. Door deze eigenschappen is het mogelijk dat monoHER in onze studie in een te hoge dosering is toegediend aan de dieren die monoHER vóór DOX hebben ontvangen en met name aan de dieren die monoHER ook nog elke week gedurende de observatie periode ontvingen. Het kan zijn dat de veronderstelde overdosis van de antioxidant monoHER zich als een pro-oxidant is gaan gedragen zoals eerder is gezien bij andere flavonoiden. Een mogelijke verklaring voor de pro-oxidant activiteit zou kunnen zijn dat monoHER wordt omgezet in een reactief oxidatie product dat vervolgens in het hartweefsel de al kleine hoeveelheid endogene antioxidant uitput. De juiste balans tussen de dosis monoHER en zijn anti- of pro-oxidante eigenschappen is nog niet vastgesteld. In hoofdstuk 6 worden de mogelijke bijwerkingen en de farmacokinetiek van monoHER geëvalueerd in een klinische fase I studie met gezonde vrijwilligers. Totaan de hoogste dosis monoHER van 1500 mg/m², wordt monoHER goed verdragen en worden er geen ernstige bijwerkingen waargenomen. Omdat de van tevoren vastgestelde farmacokinetische eindpunten werden bereikt, namelijk een gemiddelde piek plasma concentratie van 360 ± 69.3 µM en een gemiddelde AUC∞ van 6,3 ± 2,1 µmol.min/ml bij een dosis van 1500 mg/m², werd geen poging meer gedaan de maximaal toeleneerde dosis (MTD) van monoHER te bereiken. Bij dit dosis niveau is tevens de oplosbaarheid van monoHER voor een intraveneuze oplossing bereikt. De gebonden snelle verdeling en eliminatie van monoHER uit het plasma compartiment correspondeert met een snelle opname van monoHER in het muizenhart.
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vanwaar het ook weer snel wordt geëlimineerd. Onze conclusie is dat 1500 mg/m² monoHER een potentieel effectieve dosis is die veilig toegediend kan worden.

In hoofdstuk 7 wordt beschreven dat deze dosis wordt gebruikt in een klinische fase II studie waarin de cardioprotectie van monoHER wordt onderzocht in kankerpatiënten die DOX ontvangen. Acht patiënten met gemetastaseerde ziekte zijn behandeld met DOX voorafgegaan door een 10 minuten durend intraveneus infuus met een dosis van 1500 mg/m² monoHER. Van hen bereiken 5 patiënten een cumulatieve dosis van 300 mg/m² DOX en ondergaan dan een endomyocardbiopt. Van deze patiënten worden 3 behandeld met een tijdsinterval (Δ T) van 1 uur tussen monoHER en DOX, één met een Δ T = 10 min en één met een Δ T = 2 uur. Tussen de patiënten wordt geen verschil in biopsie score gevonden. De gemiddelde biopsie score van de vijf patiënten (2,7) is hoger dan de gemiddelde score (1,4) van patiënten die beschreven zijn in de literatuur die een vergelijkbare cumulatieve dosis DOX hebben ontvangen. Ondanks de grote variabiliteit binnen het kleine aantal onderzochte patiënten, lijkt monoHER de door DOX-geïnduceerde hartschade te verergeren.

Een interessante bevinding is dat alle vier de patiënten die een gemetastaseerd weke delen sarcoom hadden gunstig reageren (3 PR, 1SD) op de combinatie behandeling. Dit is veel meer dan verwacht wordt bij deze groep. Ondanks het feit dat deze effecten van monoHER tegengesteld zijn aan wat eerder is gezien in dierenstudies, laten deze resultaten een zekere overeenkomst zien met de resultaten van hoofdstuk 5. De verschillen zouden verklaard kunnen worden door een mogelijk verschil in het monoHER metabolisme tussen muizen en patiënten. In zowel patiënten (dit hoofdstuk) als in muizen (hoofdstuk 4) is aangetoond dat het tijdsinterval tussen monoHER en DOX niet relevant is. Aan de andere kant zou de dosis monoHER cruciaal kunnen zijn zoals gevonden is in muizen (hoofdstuk 5) en in patiënten (hoofdstuk 7). Een mogelijke verklaring kan zijn dat de antioxidant monoHER bij de reactie met de reactieve oxygen species wordt omgezet in een oxidatie product dat reactief is met thiolen. Een dergelijke reactie kan op twee manieren tot toxiciteit leiden. Ten eerste door een reductie van de antioxidant status van de cardiomyocyt waardoor de bescherming tegen cardiotoxiciteit afneemt. Ten tweede doordat het oxidatie product van monoHER zou kunnen reageren met andere thiolen, zoals eiwit thiolen. Deze adducten kunnen accumuleren in de cel en zo additionele toxiciteit veroorzaken. Eerdere studies hebben laten zien dat de concentratie van de antioxidant glutathione een rol kan spelen bij het antitumor effect in de weke delen sarcoom cellen. Hierdoor zou hetzelfde mechanisme een rol kunnen spelen zoals is voorgesteld voor de hartcellen. Geconcludeerd is dat de dosis van monoHER een cruciale rol speelt: bij een dosis van 1500 mg/m² wordt geen bescherming waargenomen tegen DOX-geïnduceerde hartschade in patiënten met gemetastaseerde ziekte. Daarentegen zou een versterkend effect aanwezig zijn op de antitumor werking van DOX in de patiënten die een gemetastaseerd weke delen sarcoom hebben.
Conclusie

Het semisynthetische flavonoid 7-monohydroxyethylrutoside (monoHER) heeft naast zijn radicaalvangende en ijzerchelerende eigenschappen, anti-inflammatoire activiteit. Dit is aangetoond door remming van door DOX-geïnduceerde neutrofiel adhesie aan endotheelcellen die plaatsvindt via de door DOX geïnduceerde VCAM en E-selectin overexpressie in vitro en het reducerende effect op de door DOX-geïnduceerde toename van CML in vivo. Hiernaast zijn anti-apoptotische eigenschappen van monoHER gevonden. Dit effect wordt waarschijnlijk veroorzaakt door de eigenschap van monoHER ROS te kunnen neutraliseren.

Door de relatief korte halfwaardetijd van monoHER werd verwacht dat het tijdsinterval tussen monoHER en DOX van invloed zou zijn op het cardioprotectieve effect van monoHER. Er werd echter geen invloed waargenomen. De beschreven onderzoeksresultaten in dit proefschrift geven wel aan dat de dosis van monoHER belangrijk zou zijn. Hoge concentraties monoHER (> 7 keer de maximale plasma concentratie die onder beschermende omstandigheden gevonden is bij de muis) in combinatie met DOX gaven bescherming van ovarium kankercellen in vitro. Deze observatie suggereert dat de dosis monoHER niet mag leiden tot een concentratie die hoger is dan de concentratie die bescherming laat zien in vivo (131 µM). Hiernaast neigt herhaalde dosering van monoHER na de behandelperiode met DOX tot verergering van de ontwikkeling van DOX-geïnduceerde hartschade in muizen.

Verondersteld wordt dat de gecreëerde overdosis van de antioxidant monoHER zich gaat gedragen als een pro-oxidant zoals eerder is gezien bij andere flavonoiden. Daarom is het waarschijnlijk cruciaal de dosis en de frequentie van monoHER toediening te verfijnen om het optimale en verlangde effect (antioxidant activiteit of pro-oxidant activiteit) van monoHER te verkrijgen. Deze conclusies zijn in overeenstemming met de bevindingen in de patiënten tijdens de fase II studie.

Implicaties en onderzoek in de toekomst

Het huidige onderzoek suggereert dat de dosis en de frequentie van monoHER toediening cruciaal zou zijn in de bescherming tegen DOX-geïnduceerde hartschade. Er zou een dosis-afhankelijke overgang kunnen zijn in het effect van monoHER, namelijk een hoge dosis (≥ 1500 mg/m²) om het versterkende effect op de antitumor werking voor weke delen sarcomen te verkrijgen en een lage dosis (ergens lager onder 1500 mg/m²) om cardioprotectie te krijgen.
Verschillende interessante onderzoekslijnen zijn open voor de toekomst. De eerste is het bestuderen van het versterkende effect van monoHER op het effect van DOX in humane weke delen sarcoma cellijnen. Als deze effecten positief zijn, zal het onderliggende mechanisme geëvalueerd worden. Als het mogelijk is zullen de bevindingen geëxtrapoleerd worden naar andere tumortypes. In de tussentijd wordt een klinische fase II studie gestart waarin het antitumor effect van monoHER in combinatie met DOX in patiënten met WDS zal worden bestudeerd.

Een tweede vraag die beantwoord moet worden is of mogelijke verschillen in het metabolisme van monoHER tussen muis en patiënten (deels) verantwoordelijk zijn voor de waargenomen verschillen in de hart toxiciteit tussen de species.

Een derde gebied van speciale aandacht is de kritische dosis van monoHER die noodzakelijk is voor cardioprotectie op de lange termijn. Nadat meer inzicht is verkregen in de veranderingen van het intracellulaire metabolisme veroorzaakt door monoHER, zullen nieuwe in vivo experimenten worden gestart.