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PREVENTING ANTHRACYCLINE CARDIOTOXICITY: FROM IRON CHELATION TO CARBONYL REDUCTASE INHIBITION

Doctoral dissertation

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The investigations described in this thesis were carried out at the Department of Biochemical Sciences of the Faculty of Pharmacy of the Charles University in Prague and at the Department of Pharmacology and Toxicology of the Faculty of Medicine of the University of Maastricht. The author's research was supported by the grants GAUK No. 97/2005, GAČR No. 305/05/P156, GAČR No. 305/03/1511 and the Research Center LN00B125 of the Czech Ministry of Education.

Acknowledgements

My sincere thanks belong to both my supervisors, Prof. RNDr. Eva Kvasničková, CSc. and Prof. Dr. Aalt Bast for all their help, professional guidance, sharing their precious experience with me and shaping my scientific career.
I would also like to thank to my close colleague and friend Dr. Tomáš Šimůnek, for offering a helping hand whenever necessary. I treasure the time we spent discussing research together as well as his help with experimental work, careful revision of the manucripts, presentations and, finally, this thesis.
I am indebted to Dr. Gertjan J.M. den Hartog for his perfect organization of my stay in the Netherlands.
Special thanks go to all the PhD students from the Department of Biochemical Sciences – Romča, Ivča, Viktor, Yogee, Bohunka, Verča, Lucka, Michal and Marek – who created a cheerful and collegial working environment and who will always remain my dear friends.
I would like to express my gratitude to Mrs. Alenka Pakostová not only for her excellent technical assistance but also for being a true friend.
My research work of course would not be possible without a stable and supportive background. My greatest thanks therefore go to my parents as well as to my boyfriend Jakub for their endless love, understanding and overall support.

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1. Introduction

Anthracycline antibiotics rank among the most efficient and widely used anticancer drugs ever developed (Weiss, 1992) and remain an important part of many chemotherapy protocols in oncological practice both in children and adults (Greiner and Lipshultz, 1998; Jensen, 2006, Johnson, 2006). The original anthracycline, daunorubicin, was isolated from a sample of Italian soil and was identified as a fermentation product of the bacterial strain *Streptomyces* (*S. peuceticus, S. ceruleorubidus*) more than 40 years ago. Although not very active against solid tumors, the drug is still widely used in the treatment of acute leukemias. Numerous derivatives, either isolated from bacterias or semi-synthetic analogs, have been studied since, nevertheless, only a few found their use in clinical settings (Table 1).

Table 1. The anthracycline analogs in clinical use and their main indications

Anthracycline	**Trade names**	**Indications**
Doxorubicin	Adriblastina, Rubex, Doxil*, Caelyx*, Myocet*	breast, bladder, stomach, ovary, lung and thyroid cancer, lymphomas, bone tumors, soft tissue sarcomas, neuroblastoma, Wilms´ tumor, multiple myeloma, uterine, urothelial, carcinoid, cervical, endometrial, esophageal, islet cell and hepatocellular cancers
Daunorubicin	Cerubidine, DaunoXome*	acute myelogenous and lymphoblastic leukemia
Epirubicin	Pharmorubicin, Ellence	gastric and breast cancer
Idarubicin	Idamycin, Zavedos	acute myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphomas, breast cancer
Valrubicin	Valstar	bladder cancer (intravesical use)
Mitoxantrone**	Novantrone	breast cancer, acute promyelocytic or myelogenous leukemias, androgen-independent prostate cancer

* liposomal formulations
** anthracene derivate resembling the anthracyclines by both its chemical structure and mechanism of action; it is frequently classified as an anthracycline.

Doxorubicin, the 14-hydroxy derivative of daunorubicin, possesses a much broader spectrum of anticancer activity. It is not only effective against hematological malignancies but also against a wide range of solid tumors such as breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas e.g. the Hodgkin's disease (Minotti et al., 2004).

The molecule of the anthracycline basically consists of two parts – a conjugated tetracyclic skeleton (aglycone), which is bound to an aminosugar daunosamine thus forming a glycoside. Interestingly, the structure of all clinically used anthracycline analogs is similar (Figure 1),

but their biological activity and indications differ. The quinone moiety in ring B allows the anthracyclines to redox-cycle as will be discussed later.

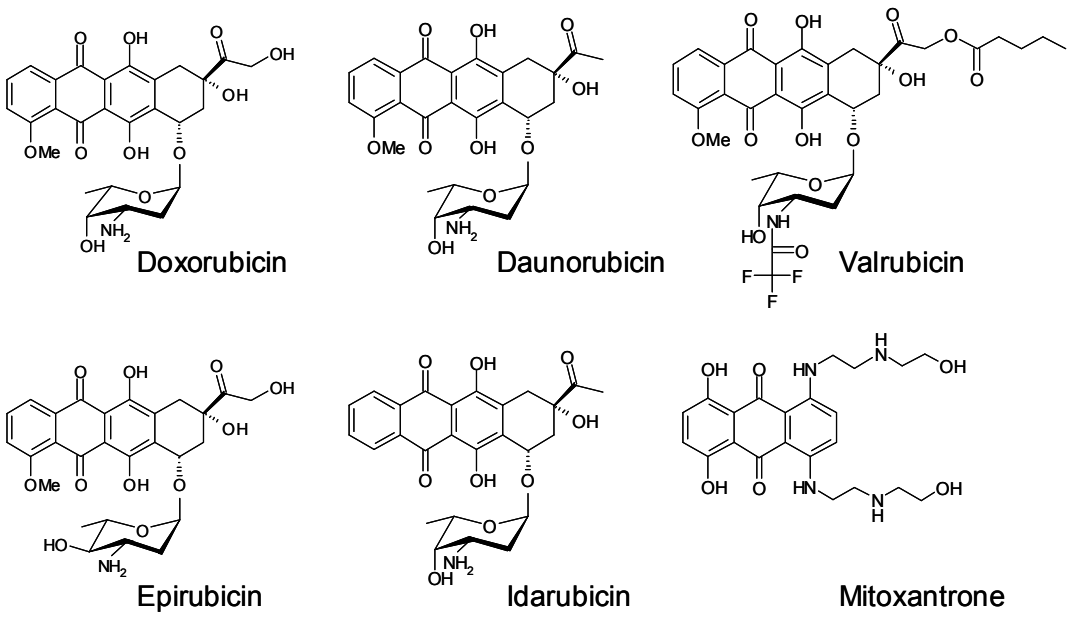

Figure 1. Chemical structures of the anthracycline derivatives

In anticancer chemotherapy, the administered dose largely determines the severity of the side-effects. Common severe toxic effects associated with most anticancer drugs are bone marrow toxicity and gastro-intestinal toxicity (nausea, vomiting). Apart from these effects, anthracycline treatment is frequently accompanied by the development of cardiac toxicity after repeated administration and typically manifested as congestive heart failure (von Hoff et al., 1979). Whereas toxicity to bone marrow and gastro-intestinal tract are nowadays manageable by means of concomitant administration of hematopoietic growth factors and modern antiemetics (5-HT_3 antagonists), it is the cardiac toxicity of the anthracyclines that determines the cumulative maximal tolerable doses of these drugs. The severity of the anthracycline-associated cardiotoxicity stimulates a search for less toxic (and equally active) analogs. Among plenty of analogs synthesized, only epirubicin (a stereoisomer of doxorubicin) partially fulfilled these expectations as it was reported to allow an increase of the cumulative dose of the anthracycline without an increase in cardiotoxicity and myelotoxicity (Ganzina, 1983). The anticancer spectrum of epirubicin is equivalent to that of doxorubicin. The main advantage of idarubicin, an anthracycline derived from daunorubicin lies in its increased lipophilicity with possibility of oral administration (Toffoli et al., 1997)

and a broader spectrum of activity (Borchman et al., 1997). Nevertheless, doxorubicin still remains the basic anthracycline for the treatment of various types of malignant diseases and its cardiotoxicity remains a serious clinical problem.

2. Metabolism of the anthracyclines

A large number of studies on anthracycline metabolism and distribution have been carried out in various animal species as well as in humans. All anthracyclines are metabolized extensively. The major metabolic routes are (1) reduction of the C13-keto group forming C13-dihydroanthracyclines (also called 13-hydroxyanthracyclines) and (2) aglycone formation after the removal of daunosamine sugar. Semiquinone radical is involved in the latter step as a reactive intermediate with the consequences that will be discussed separately (see 4.1.1.). Phase II metabolism only takes place with epirubicin and results in the formation of epirubicin(ol)-glucuronide conjugates.

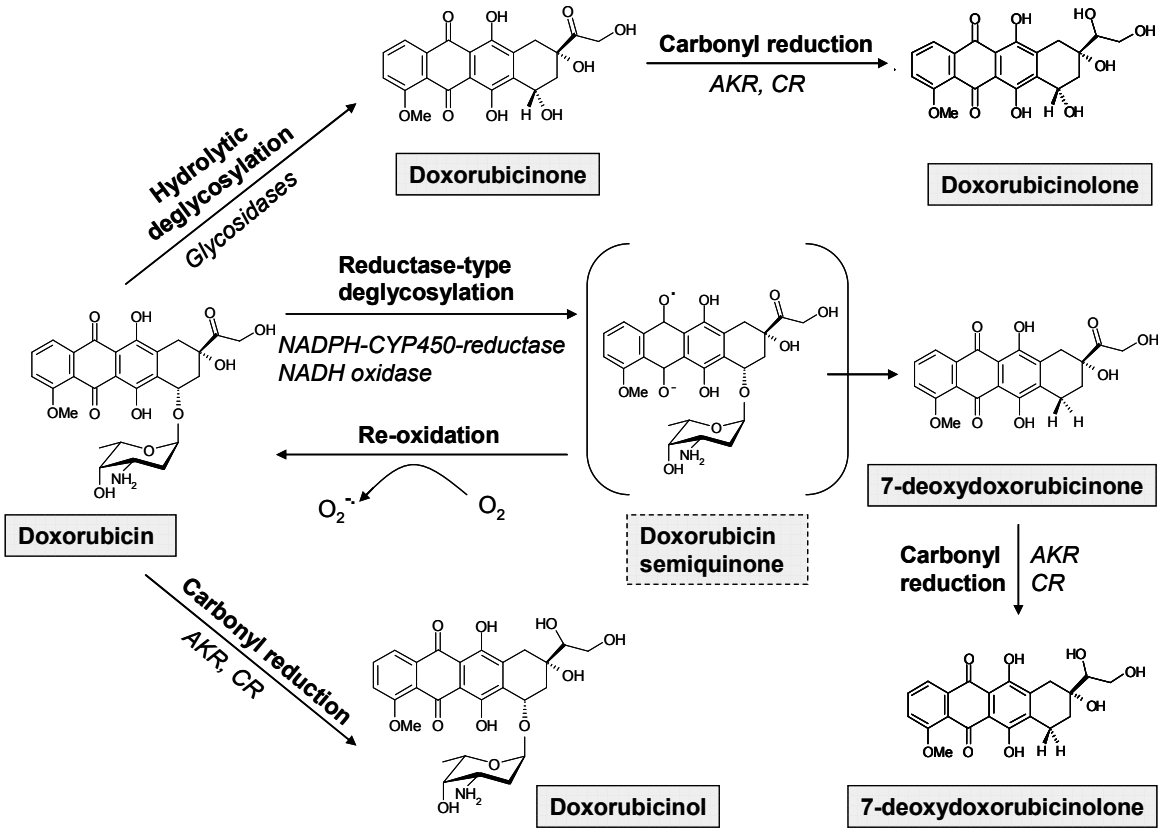

Figure 2. The major pathways of anthracycline metabolism (demonstrated on example of doxorubicin).

2.1. C13-carbonyl reduction

Formation of C13-dihydrometabolites of the anthracyclines represents the most prominent metabolic pathway of these drugs (with exception of epirubicin, see below). Several ubiquitous cytosolic enzymes, such as carbonyl reductases (CR) and aldo-keto reductases participate in the formation of C13-dihydroanthracyclines (Slupe et al., 2005; Mordente et al., 2001). These metabolites have been shown to be more cardiotoxic than the parent drugs (Cusack et al., 1993; Olson et al., 1988). CR, in particular, seems to play an important role in anthracycline-induced cardiotoxicity as can be deduced from the studies with CR-overexpressing or knock-outed animals (Forrest et al., 2000; Olson et al., 2003). On the other hand, it has been shown that an inhibition of aldo-keto reductases also led to a decreased doxorubicinol formation *in vitro* and *in vivo* in rats, although the cardioprotective effects were only deduced from a decrease in a non-specific creatine kinase serum concentration (Behnia and Boroujerdi, 1999). The contradictory findings on the participation of various reductases in anthracycline C13-metabolite formation demand further investigations.

It has been shown that the rate of daunorubicin reduction is about 300-fold lower in the heart than in the liver (Pröpper and Maser, 1997) suggesting that most of the C13-dihydroanthracyclines present in the circulation originate from liver metabolism. However, a possible role of intracardiac metabolism has also been suggested to explain the observed intracardiac accumulation of these relatively polar metabolites, which might encounter difficulties to cross the membranes (Licata et al., 2000). The mechanistic studies of anthracycline C13-carbonyl reduction are complicated by the findings of Mordente et al. (2003), who found out that the reduction of doxorubicin and daunorubicin to their respective C13-dihydrometabolites in human myocardium might be conveyed by virtue of different reductases. Moreover, differences have also been described in the rate of metabolite formation between the rabbits and humans (Mordente et al., 2003).

Secondary alcohol metabolites are significantly less effective than their parent drugs at producing oxygen radicals, presumably because they exhibit reduced affinity for one-electron quinone reductases (Licata et al., 2000). This could eventually explain their lower cytotoxicity. Other factors, e.g. decreased uptake of the reduced anthracyclines by tumor cells, however, should be considered as well.

2.2. Aglycone formation

Anthracycline and C13-dihydro anthracycline aglycones result from a loss of aminosugar moiety from their molecule. This might occur via hydrolytic or redox processes leading to hydroxyaglycones or 7-deoxyaglycones, respectively (Licata et al., 2000). The formation of hydroxyaglycones was previously attributed to instability and spontaneous hydrolysis of the anthracyclines. The results of Licata, however, suggest an involvement of NADPH-dependent hydrolase-type glycosidases that are present in the cytosol and tightly coupled to other enzymes of anthracycline biotransformation. Deoxyaglycone formation, on the contrary, seems to be initiated by microsomal or mitochondrial oxidoreductases rather than by cytosolic enzymes. It has been shown that one-electron reduction of the quinone moiety of the anthracycline tetracyclic ring by the microsomal NADPH-cytochrome P450 reductase (Powis, 1989), the mitochondrial exogenous NADH oxidase (Gille and Nohl, 1997) or cytochrome P450 (Goeptar et al., 1993) resulted in formation of a semiquinone radical, which regenerated the parent quinone by reducing and cleaving the glycosidic bond to give deoxyaglycones. Under aerobic conditions, however, molecular oxygen tends to compete with a glycosidic bond as an electron acceptor for a semiquinone making the reductive pathway of deoxyaglycone formation less likely. Aglycone metabolites have also been proposed to be linked with anthracycline cardiotoxicity. Being much less polar that the parent drugs, they are able to penetrate cell membranes very easily, reach mitochondria and generate hydroxyl radicals more effectively than would be expected if the parent anthracycline remained in the cytosol-membrane interface (Licata et al., 2000).

2.3. Glucuronidation

Unlike other anthracyclines, epirubicin has a unique metabolic pathway, glucuronidation, which may result in faster clearance and eventually lower cardiotoxicity compared to doxorubicin (Weenen et al., 1984). The majority of epirubicin is metabolized to glucuronides: 78% to epirubicin glucuronide, 19.3% to epirubicinol glucuronide and only 0.2% of epirubicin is metabolized to epirubicinol. The formation of epirubicin glucuronide is catalyzed by liver UDP-glucuronosyl transferase (UGT). Innocenti et al. (2001) showed that epirubicin was converted exclusively by the UGT2B7 isoform in human liver microsomes. A study by Hu et al. (1989) showed that epirubicin metabolism correlated with response to the treatment. They found that the patients with nasopharyngeal cancer treated with epirubicin were more likely to relapse if they rapidly metabolized epirubicin. A single nucleotide

polymorphism in the enhancer region of UGT2B7 has been identified and some researchers suspect this polymorphism to be responsible for the interindividual variability in epirubicin metabolism (Innocenti et al., 2001).

3. Cytotoxic action of the anthracyclines

The mechanism by which the anthracyclines induce cell death remains unclear and paradoxical findings can be found in the literature. Most of the theories presume the presence of the anthracyclines in the nucleus (e.g. DNA intercalation, stabilization of the DNA-topoisomerase II complex), however, it was also shown that anthracyclines might retain their cytotoxicity under conditions when the nucleus cannot be reached e.g. when anthracyclines are bound to the polymers (Hovorka et al., 2002). This indicates possible involvement of direct membrane effects independent on other mechanisms.

Interference with DNA and RNA biosynthesis

The first mechanism proposed to explain the cytotoxicity of the anthracyclines was an intercalation of the planar moiety of the anthracyclines between base pair of DNA-strand with subsequent inhibition of DNA and RNA biosynthesis (Pigram et al., 1972). This effect might be enhanced by the site-specific radical formation in the presence of transition metals (Berlin and Haseltine, 1981). Direct inhibition of DNA polymerases has also been suggested to contribute to the inhibition of DNA biosynthesis (Momparler et al., 1976). A number of studies, however, failed to detect direct effects on DNA synthesis at lower concentrations of the drugs. It has been suggested that the DNA synthesis inhibition may perhaps relate only to a cytostatic component of the anthracycline action whereas other effects are responsible for their lethal effects on the cells (Gewirtz, 1999).

Interaction of the anthracyclines with topoisomerase II

Topoisomerases act by catalyzing the breakdown and rejoining reactions in the phosphodiester backbone of DNA. Topoisomerase I reversibly cleaves a single strand in duplex DNA molecule, whereas topoisomerase II breaks and rejoins both DNA strands. Topoisomerase II appears to be one of the primary targets for the antitumor activity of the anthracyclines (Tewey et al., 1984). It has been shown that the anthracycline-resistant tumor cells have reduced levels or altered activity of the enzyme with concomitant reduced levels of anthracycline-induced DNA-strand breaks (Deffie et al., 1989). Because anthracyclines act as

topoisomerase II poisons rather than as classical competitive inhibitors, their action is dependent upon the level of the enzyme in the cells. Rapidly proliferating cells, which contain relatively higher levels of topoisomerase II, appear to be more sensitive to these agents.

Role of free radicals

The contribution of free radicals generation to the cytotoxic effects of the anthracyclines remains controversial. Anthracyclines are undoubtedly capable to induce free radical formation under certain conditions. The question is whether these effects occur at clinically relevant concentrations and under hypoxic conditions of tumor cells. Most studies have shown that GSH depletion sensitized tumor cells to anthracycline cytotoxicity (Dusre et al., 1989; Hamilton et al., 1984). Protection of various cancer cell lines against cytotoxicity of the anthracyclines using catalase and/or superoxide dismutase has also been demonstrated (Kang et al., 2002; Doroshow et al., 1991). A question arises whether and how these large proteins reach intracellular compartment when added exogenously.

On the other hand, 5-iminodaunorubicin, a drug that cannot redox-cycle seems to retain a great deal of its cytotoxicity (Glazer et al., 1982). The fact, that a cardioprotector dexrazoxane selectively prevents the injury to cardiac cells while not altering the cytotoxic effect of the anthracyclines also weakens the putative role of free radicals in the cytotoxic effects exerted by the anthracyclines.

Effects on membranes

Triton et al. performed a series of experiments that indicated that the anthracyclines must be present in the extracellular environment to exert their cytotoxic activity (reviewed in Triton, 1991). Their findings are, however, somewhat weakened by the fact that intracellular concentration of the drug was not measured and the contribution of the interaction of the anthracyclines with intracellular targets could have been initiated before their washing out from the medium. Moreover, the fact that the resistance to anthracycline treatment is associated with drug efflux pumps renders the “membrane hypothesis“ unlikely.

Induction of apoptosis

There is a clear evidence that anthracycline-treatment induces apoptosis. However, most studies consistently indicated that the apoptosis is only initiated with low levels of the drug (up to 1 μM) whereas no apoptosis is evident at higher doses (Ling et al., 1993; Zaleskis et al., 1994). Interestingly, the extent of apoptosis does not seem to influence the ultimate extent of

cell death (Han et al., 1997) and the apoptosis may therefore not be a critical factor in cell sensitivity to cell death.

4. Anthracycline cardiotoxicity

Cardiotoxic side-effects of the anthracycline treatment represent a major limitation factor of their clinical use. The clinicians distinguish between four basic types of anthracycline-induced cardiotoxicity; acute, subacute, chronic and delayed. Acute toxicity is directly associated with the administration of the anthracyclines and is manifested as cardiac dysrhythmias with typical changes in ECG and usually resolves spontaneously within few hours (Lefrak et al., 1973). Subacute toxicity, whose incidence is very low, is characterized with pericarditis-myocarditis syndrome (Harrison and Sanders, 1976). Very rarely, myocardium infarction or sudden death occurs within the first hours of anthracycline infusion. Whereas acute and subacute toxicity do not represent a serious clinical problem, chronic and delayed toxicity do. The manifestations include dilated cardiomyopathy with progressive impairment of left ventricular function and the development of chronic heart failure. The onset of chronic anthracycline cardiotoxicity is typically observed within weeks to months since the chemotherapy has been finished whereas in delayed cardiotoxicity there is a lag-period that lasts for 4-20 years before the symptoms finally occur. Delayed cardiotoxicity became a particularly serious complication in anthracycline-treated childhood cancer survivors (Steinherz et al., 1991).

4.1. Pathophysiology of anthracycline cardiotoxicity

Since the anthracycline-induced chronic heart failure is largely refractory to medicamental treatment due to its progressive character, it is crucial to characterize the pathophysiological events that lead to its development in order to design the most convenient strategies for cardioprotection. Understanding the pathophysiology of anthracycline cardiotoxicity is further necessary for choosing the most appropriate techniques (markers) of cardiotoxicity monitoring. It should be noted that the mechanisms by which the anthracyclines induce their cardiac side-effects have not been completely elucidated yet and the cardiotoxicity development is likely to be a multifactorial process. Some of the proposed mechanisms are discussed below.

4.1.1. Free radical generation

The redox cycling of the anthracycline quinone ring has been described shortly after the discovery of the anthracyclines and the production of free radicals as a by-product of anthracycline metabolism is considered to be the main mechanism of anthracycline-induced cardiotoxicity (Keizer et al., 1991; Rajagopalan et al., 1988). The one-electron reduction of the quinone to the semiquinone can occur either enzymatically (see paragraph 2.2.) or non-enzymatically. The semiquinone moiety of the anthracycline is oxidized under aerobic conditions yielding parent anthracycline in its quinone form and superoxide radical ($O_2^{\cdot-}$). Two molecules of $O_2^{\cdot-}$ are dismutated with superoxide dismutase (SOD) or, at much lower rate, spontaneously into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (1). Removal of H_2O_2 is catalyzed by the catalase (CAT), which converts H_2O_2 into water and O_2 (2). In catalase-poor tissues (e.g. heart) abundant H_2O_2 might react with $O_2^{\cdot-}$ or ferrous ions according to Fenton or Haber-Weiss reactions, respectively (3 and 4). In this way hydroxyl radicals ($HO^{\cdot}$) are formed. Hydroxyl radicals are extremely reactive due to their short half-life (10^{-9} s) and instaneously react with the macromolecules in their vicinity causing lipid peroxidation, DNA or protein damage (Griffin-Green et al., 1988; L'Ecuyer et al., 2006; Chen et al., 2006)

$$2\ O_2^{\cdot-} + 2\ H^+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad (1)$$

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2 \qquad (2)$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\cdot} + HO^- + Fe^{3+} \qquad (3)$$

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad (4)$$

The non-enzymatic reduction of the anthracycline quinone ring can also occur spontaneously in aqueous solutions, albeit much more slowly compared to the enzymatic reduction. The non-enzymatic reduction occurs after the complexation of the anthracyclines with transition metal ions (Fe^{3+}, Cu^{2+}, Mg^{2+}, Zn^{2+}). The complexation with Fe^{3+} (Figure 3) is followed by a transfer of one electron to the iron, which subsequently delivers the electron to one molecule of molecular oxygen yielding $O_2^{\cdot-}$.

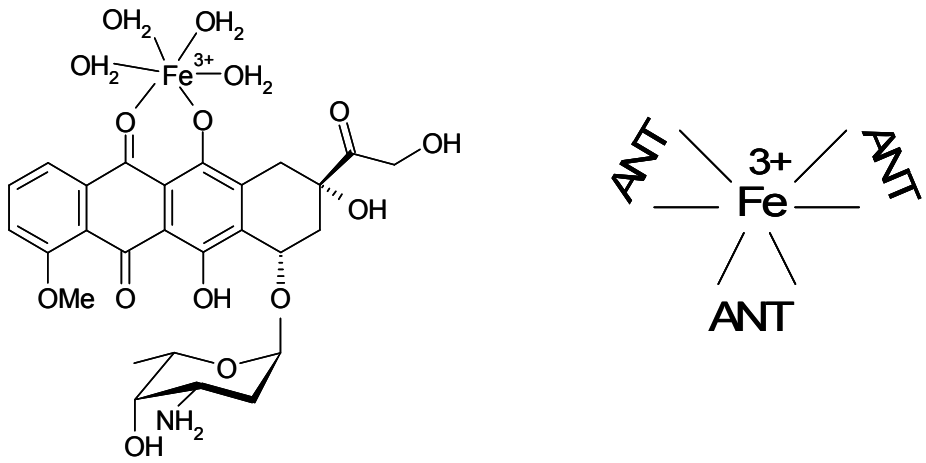

Figure 3. Anthracycline-iron complexes.

The specific sensitivity of the heart to free radical-induced damage can be attributed to the nature of the heart tissue. Firstly, the myocardium is rich in mitochondria (Keizer et al., 1991). Anthracyclines have high affinity to cardiolipin, a mitochondrial membrane phospholipid, which renders the heart (mitochondria) a pool of anthracycline accumulation (Goormaghtigh et al., 1990). Secondly, the natural antioxidant defenses (CAT and SOD activities) of the heart are poor (Doroshow et al., 1980), moreover the anthracyclines were found to reduce the levels of cardiac glutathione (GSH), which is already low in the heart (Mohamed et al., 2000).

4.1.2. C13-dihydro metabolites

In addition to the free radical hypothesis, the attention is paid to the role of C13-dihydrometabolites in the induction of the anthracycline cardiotoxicity. It was demonstrated that doxorubicinol was ten-fold more potent than doxorubicin in reducing inotropy of rabbit papillary muscle, and even a hundred-fold when comparison took place on a tissue concentration basis instead of the concentration in the incubation bath (Olson et al., 1988). The authors attributed these distinct effects of doxorubicin and doxorubicinol to the inhibition of the membrane-associated pumps e.g. ATPase, whose activity was much more inhibited by doxorubicinol compared to doxorubicin in both cardiac mitochondrial and sarcosomal membrane fractions. Obviously, for C13-dihydrometabolites to be a significant factor *in vivo*, they have to be available in sufficient quantities in the heart tissue after administration of the parent anthracycline. Doxorubicinol has been shown to accumulate in rat heart after repeated dosing of doxorubicin (Peters et al., 1981) and there is some evidence that the same occurs in

man (Stewart et al., 1993). Other studies showed that although doxorubicin and doxorubicinol were equally active in inducing $O_2^{\cdot-}$ production as measured by cytochrome *c* reduction, doxorubicinol had much greater activity in potentiating the respiratory burst (Nielson et al., 1986). On the other hand, there are also studies showing that C13-dihydrometabolites were less cardiotoxic than the parent anthracyclines in isolated perfused rat heart (Platel et al., 2001) or in rats *in vivo* (Danesi et al., 1986). The latter study, however, does not take into account the differences in tissue uptake of both drugs and no firm conclusions about the relative cardiotoxicity of the parent anthracyclines vs. their C13-dihydrometabolites can therefore be drawn.

4.1.3. Impairment of iron homeostasis

Iron is essential for all known organisms. At cellular level, iron homeostasis is maintained by a well-controlled system of iron regulatory proteins (IRPs) in order to prevent both iron overload and iron deficiency. Based on the actual cytosolic 'iron pool', IRPs post-transcriptionally regulate the expression of the iron storage protein ferritin and the receptor for iron transporter transferrin (Mladěnka et al., 2006). It has been reported that, *in vitro*, anthracyclines decreased the binding of IRPs to the so called iron-responsive elements (IRE) of mRNA, thus modifying expression of the proteins that are critical for maintaining optimal intracellular iron levels (Minotti et al., 2001). Others have shown that doxorubicin induced an accumulation of iron in ferritin and prevented its mobilization, which might possibly result in relative iron depletion (Kwok and Richardson, 2003). It is however not known whether and to which extent these effects contribute to anthracycline cardiotoxicity in a clinical setting. Using an *in vivo* rabbit model of chronic anthracycline-induced heart failure, no changes of myocardial iron content were observed either in animals treated for ten weeks with daunorubicin or in the group where dexrazoxane was co-administered (Šimůnek et al. 2005a).

4.1.4. Calcium overload

Anthracyclines were found to cause changes in calcium metabolism, including dysfunction of the sarcoplasmic reticulum and decreased expression of calcium-handling proteins such as the ryanodine receptor (Gambliel et al., 2002). An increased intracellular calcium concentration leads to muscle stiffness and impaired contractile function followed by cell death. However, it has been suggested that an increase in intracellular calcium concentration is rather a result than a cause of anthracycline-induced cardiotoxicity. The increase in intracellular calcium could be a result of membrane damage due to the anthracycline-produced free radicals, with a

subsequent loss of control of the flow of proteins and ions to/from the cell. This hypothesis is supported by the occurrence of ATP level depletion in anthracycline-treated cells (Jensen et al., 1986)

4.1.5. Anthracycline mitochondriopathy (role of mtDNA lesions)

Recent data suggest that somatically acquired alterations of mitochondrial DNA (mtDNA) and concomitant mitochondrial dysfunction might play an important role in the onset of chronic anthracycline cardiotoxicity (Zhou et al., 2001; Lebrecht et al., 2003). The mitochondrial toxicity of the anthracyclines is evidenced by the fact that the anthracyclines can enter mitochondria, inhibit the respiratory chain by binding to cardiolipin and interact with mitochondrial DNA (mtDNA) either directly or by generating reactive oxygen species (ROS). The recorded interferences with oxidative phosphorylation, calcium-loading, mtDNA adducts and histopathology are irreversible and persistent for up to 5 weeks (25 drug half-lives) following the last injection of the drug (Serrano et al., 1999). It has been suggested that the pathogenesis of chronic cardiomyopathy hinges on the fact that respiratory chain dysfunction is associated with mtDNA damage and can subsequently generate free radicals, which then in turn may either attack the respiratory chain itself or damage mtDNA. The important thing is that these vicious circles can continue to operate after doxorubicin treatment has been finished and account for the delayed manifestation of the cardiomyopathy and at least contribute to the molecular mechanism of "dose memory". To support the hypothesis that mtDNA is an important target of doxorubicin toxic effects, it has been recently shown that the artificial mtDNA-free A549 subline displayed decreased sensitivity to doxorubicin compared to the parent cell line (Lo et al., 2005)

4.1.6. Myofilament degradation

Titin, the largest myofilament protein, is an essential component of the sarcomere, making important contributions to sarcomere organization and myocyte compliance. Previous studies have reported disorganization and loss of titin in failing human hearts (de Jonge et al., 2002). It has been recently shown that titin proteolysis is an early event of doxorubicin-induced cardiomyocyte injury and that titin degradation is mediated by calcium-dependent proteases - calpains (Lim et al., 2004). The authors showed that calpain inhibitors preserved titin and reduced myofibrilar disarray while inhibitors of caspases did not. These results suggest that

titin degradation may predispose cardiomyocytes to myofilament instability and cell death by necrosis.

4.1.7. Alterations of the adrenergic and cholinergic responses of the myocardium

Anthracyclines appear to have a direct toxic effect on the adrenergic function of the myocardium as documented by down-regulation of β_1-adrenergic receptors or decreased norepinephrine contents in the ventricles (Bocherens-Gadient et al., 1992; Nagami et al., 1997). Another study showed that doxorubicin inhibited the negative chrono- and inotropic responses to parasympathetic nerve stimulation due to the inhibition of acetylcholine release from nerve varicosities in the heart (Hoyano et al., 1996).

4.1.8. Inflammatory effects of the anthracyclines

Anthracyclines also elicit inflammatory effects. Doxorubicin was found to stimulate histamine release from mast cells of the heart and histamine on its own produced lesions in the hearts of treated subjects, which were similar to those induced by doxorubicin (Bristow et al., 1983; Klugmann et al., 1986). Several immune regulatory factors such as prostaglandins, leukotrienes, thromboxane and platelet activating factor were also observed to increase after anthracycline treatment. Doxorubicin was found to inhibit the production of vascular dilator nitric oxide (NO) leading to imbalance between NO and $O_2^{\cdot-}$ (Luo and Vincent, 1994). The dominance of $O_2^{\cdot-}$ results in vascular injuries exhibited as inflammatory reactions and skin injuries in anthracycline-treated patients. Anthracyclines were also shown to induce neutrophil adhesion via increased levels of endothelin-1 or xanthine oxidase (Lopez Farre et al., 1993; Terada et al., 1997) and doxorubicin was found to activate transcription factor NF-κB, which is known to mediate activation of endothelial cell adhesion molecules (Gerritsen and Bloor, 1993). Interestingly, the flavonoid monoHER, which has been found to protect against doxorubicin cardiotoxicity also prevented doxorubicin-induced inflammatory effects in human umbilical cord vascular endothelial cells (HUVECs) suggesting a possible link between the cardiotoxic and inflammatory effects of the anthracyclines (Abou El Hassan et al., 2003).

4.2. Synergic cardiotoxicity of the anthracyclines and other anticancer drugs

Paclitaxel

Anthracycline cardiotoxicity might be aggravated by a concomitant use of paclitaxel (Saad et al., 2004; Perroti et al., 2003). This combination is very active in breast cancer. Studies with isolated human heart cytosol showed that paclitaxel was able to stimulate an NADPH-dependent reduction of doxorubicin to its toxic secondary alcoholic metabolite doxorubicinol. Both paclitaxel and docetaxel cause nonlinear disposition of doxorubicin and its metabolites, leading to persistence of the elevated plasma concentrations of the anthracyclines (Salvatorelli et al., 2006).

Trastuzumab

Patients treated with trastuzumab developed cardiac dysfunction and chronic heart failure that were similar to those induced by anthracyclines. The patients treated concomitantly with anthracyclines and trastuzumab as well as patients with prior anthracycline treatment, were at higher risk of trastuzumab-associated cardiac dysfunction and heart failure (Slamon et al., 2001). These observations suggest a 'cardiac memory' for prior anthracycline treatment and early detection of chemotherapy-induced cardiac damage, even if subclinical, is warranted.

4.3. Detection of anthracycline cardiotoxicity

The main goal of cardiotoxicity detection techniques is to predict cardiac failure as early as possible i.e. before the symptomatic disease occurs. This is, however, an extremely challenging task.

Electrocardiography (ECG)

ECG is routinely performed in patients treated with anthracyclines, although ECG changes are non-specific (Lefrak et al., 1973). Exercise testing in combination with LVEF assessment probably does not improve the diagnostic sensitivity of early cardiomyography, although some authors believe it is helpful (Ewer, 2004).

Radionuclide angiography (ventriculography)

Multi-gated radionuclide angiography (MUGA) utilizes intravenously injected ^{99}Tc, that binds to erythrocytes and, therefore, a cardiac pool can be visualized with γ-camera. Serial assessment of cardiac function with radionuclide ventriculography after anthracycline therapy

is elaborate, expensive and not widely available. Furthermore, radionuclide ventriculography only detects systolic dysfunction and by that time myocardial damage might have already significantly progressed (Suter and Meier, 2002).

Echocardiography

Echocardiographically-assessed left ventricular ejection fraction (LVEF), or, in children, left ventricular fractional shortening (LVFS) are the most frequently used methods for anthracycline cardiotoxicity detection in clinical practice. However, the latter parameters may not be very useful for early cardiotoxicity disclosure (Benvenuto et al. 2003). This can be better achieved with exercise LVEF and Echo-Doppler assessment of left ventricular diastolic function (Bountioukos et al. 2003).

Endomyocardial biopsy

Endomyocardial biopsy with electron microscopic evaluation of myocardial tissue is the most invasive technique. Despite its sensitivity (Ewer et al., 1984), its invasive nature and the need for the specialist input for performing the procedure and interpreting the findings represent its main limiting factors.

Troponins

Troponin is a contractile regulatory protein of the thin filament of striated muscle and the troponin complex consists of three subunits; troponin C, T and I. Elevations of troponin T (TnT) levels were recorded following initial therapy with doxorubicin, with the magnitude of elevation predicting left ventricular (LV) thickness and wall thinning nine months later (Lipshultz et al., 1997). Elevated troponin I (TnI) levels have been documented in anthracycline-treated patients compared to healthy controls and anthracycline-naive patients (Missov et al., 1997). Kilickap et al. (2005) showed an increase in TnT in 41 patients treated with anthracycline-based chemotherapy and an association with diastolic function was observed.

Natriuretic peptides

A new tool for the diagnosis and assessment of heart failure is the 32-amino acid polypeptide - B-type natriuretic peptide (BNP). The synthesis of BNP occurs in the ventricles of the heart and the serum level correlates with the severity of heart failure and with left (as well as right) ventricular pressure. Since the negative predictive value of BNP is as high as 98%, low BNP plasma levels make ventricular dysfunction highly unlikely and BNP might therefore be the

long awaited tool to screen for anthracycline-induced cardiotoxicity (Suter and Meier, 2002). So far, two small studies have evaluated the usefulness of BNP as a predictor of anthracycline-induced cardiotoxicity (Okumura et al., 2000; Nousiainen et al., 2002). Both have shown promising results and the findings suggest that BNP might not only be able to detect overt anthracycline-induced cardiotoxicity but also subclinical left ventricular diastolic dysfunction (Nousiainen et al., 2002).

Atrial natriuretic peptide (ANP) has also been identified as a potentially useful marker of anthracycline cardiotoxicity. However, ANP is mainly synthesized in the atria in response to atrial overload. The mechanism of its release is therefore secondary to any change in ventricular dysfunction. BNP therefore appear to be superior to ANP in its predicting value (Koh et al. 2004).

5. Cardioprotection strategies

A number of approaches have been used for minimizing anthracycline cardiotoxicity. The major principles of these attempts are to modulate the pharmacokinetics or formulation of the anthracyclines or to use less cardiotoxic analogs of the anthracyclines or cardioprotectors.

5.1. Tailoring the cumulative dose of anthracyclines

The basic strategy is to administer anthracyclines up to a cumulative dose of 550 mg/m^2 of doxorubicin or daunorubicin. With this dose the incidence of severe cardiotoxicity is limited. The retrospective analysis of 399 patients treated with doxorubicin showed that chronic heart failure developed in 4% of those treated with a maximum cumulative dose of 500-550 mg/m^2, but the incidence increased to 18% at cumulative doses of 550-600 mg/m^2 and 36% at doses > 600 mg/m^2 (Lefrak et al., 1973). Cumulative doses for other anthracyclines derivates might differ from those of doxorubicin and daunorubicin. For example the threshold for epirubicin lies between 900 and 1,000 mg/m^2 (Ryberg et al., 1998).

5.2. Modification of anthracycline formulation

Liposomal anthracyclines

Encapsulating the anthracyclines in a liposomal delivery system engineered to target these drugs away from the heart has become a successful strategy to modify the pharmacokinetic and pharmacodynamic profile of the anthracyclines. Not all the liposomes are the same, as

they differ in lipid character, ratio and size. These characteristics result in altered biophysical properties including stability, drug release rate and pharmacokinetics (Swenson et al., 2003). One of the most favorable liposomal formulations of doxorubicin has polyethylene glycol embedded in the lipid layers ("pegylated liposomal doxorubicin", Doxil/Caelyx). This formulation results in delivery of greater quantities of doxorubicin to tumors while being less cardiotoxic (Overmoyer et al., 2005). The principle toxicities of pegylated liposomal doxorubicin are hand-foot syndrome (palmar-plantar erythrodysesthesia) and mild myelosuppression (Chia et al., 2006). The hand-foot syndrome appears to be dose limiting and the dosage of pegylated liposomal doxorubicin is therefore different from that of free doxorubicin, even if the efficacy is equivalent. Non-pegylated liposomal doxorubicin (Myocet) was shown to be equipotent to free doxorubicin (mg to mg) while Myocet exerted dramatically reduced cardiotoxicity (Harris et al., 2002; Batist et al., 2001).

Anthracycline-antibody immunoconjugates

Monoclonal antibodies directed at tumor-associated antigens provide another attractive means to deliver cytotoxic agents to the tumor while potentially sparing normal tissue (Trail and Bianchi, 1999). An immunoconjugate of doxorubicin and a tumor-specific monoclonal antibody, BR96-DOX (SGN-15), targets chemotherapy to the cells that express LewisY antigen (lung, breast, ovary, colon) (Hellstrom et al., 2001). *In vitro* and in animal models, binding of BR96-Dox to the Le^Y antigen results in rapid internalization of the complex and intracellular release of doxorubicin by acid hydrolysis in the acidic environment of endosomes and lysosomes (Braslawsky et al., 1991). In animal studies, BR96-Dox produced cure in 70% of mice bearing a large tumor burden and overall prolongation of survival when compared with untreated mice or mice treated with maximum doses of doxorubicin (Trail et al., 1993). Unfortunately, clinical studies in humans remain rather contradictory. The phase II study of Ajani et al. (2000) showed insufficient efficacy of BR96-DOX in patients with Le^Y-positive gastric carcinoma. In another study, a combination of BR96-DOX with paclitaxel was well tolerated and effective in non-small cell lung cancer patients (Ross et al., 2006). It should be noted, that these studies focused on a decrease in overall toxicity of doxorubicin rather than the cardiotoxicity in particular. The potential of the immunoconjugates to relieve anthracycline cardiotoxicity therefore needs to be established.

5.3. Anthracycline analogs

Various anthracycline analogs have been synthesized during the past four decades, however, only a few seem to have potential advantage to doxorubicin. Epirubicin appears to be less cardiotoxic at comparable doses (van Dalen, 2006), however, the risk of chronic-heart failure is not completely abated. No single analog has been found to be consistently free of cardiotoxicity at equivalent antitumor doses.

5.4. Modulation of the administration schedule

It has been suggested that anthracycline cardiotoxicity is related to the peak plasma drug concentration whereas the antineoplastic activity depends on the total systemic exposure or the tissue concentration over time (Legha et al., 1982a). Indeed, continuous infusion schedules and/or distribution of the total anthracycline dose in more boluses seemed to produce less cardiotoxicity in several studies (Legha et al., 1982a; Chlebowski et al., 1980). In contrast to the above, more recent studies have not found a significant difference in cardiotoxicity between different administration schedules (Lipshultz et al., 2002).

5.5. Cardioprotective agents

The use of cardioprotective agents received wide attention to overcome anthracycline cardiotoxicity. An extensive array of compounds has been evaluated and few of them showed certain degree of protection. These compounds can act against one or more of the damaging effects of the anthracyclines, namely compensate for ATP depletion, reduce histamine release, inhibit reductive biotransformation of the anthracyclines to the cardiotoxic C13-dihydrometabolites, scavenge free radicals or chelate metal ions.

5.5.1. Antioxidants

The use of antioxidants as protectors against anthracycline cardiotoxicity is based on a free radical theory of its development and aims to mitigate the oxidative stress-induced damage of the myocardium. A large amount of either natural or (semi)-synthetic antioxidants has been investigated including vitamin E, probucol, N-acetylcysteine, curcumin, glutamine, selenium, melatonin or various flavonoids. Contrary to the expectations, many excellent antioxidants (vitamin E, acetylcysteine) gave contradictory or negative results with respect to cardioprotection against anthracyclines (van Vleet et al., 1980; Legha et al., 1982b). Probucol protected the heart tissue against toxicity of doxorubicin in rats *in vivo* in the study of Singal

(1995), however, the pharmacological properties of this drug are rather complex and therefore its protective effect cannot be clearly attributed to its antioxidant activity. Alterations of pharmacokinetics of doxorubicin with probucol have also been suggested (El-Demerdash, 2003). The same holds for the flavonoids, which are among the most interesting antioxidants with potential to relieve anthracycline cardiotoxicity (see paragraph 5.5.3).

5.5.2. Iron chelators

Whereas common antioxidants scavenge free radicals only after they have been formed, iron chelators are able to prevent the formation of the reactive species (e.g. $HO^{\cdot}$) or at least remove the redox-active iron from the vicinity of the macromolecules.

Iron has six coordination sites and, to make it redox-inactive, it is necessary to occupy all these sites. We can distinguish between the chelators according to the number of free electron pairs that can be used to form a coordination bond – thus we recognize the bidentate, tridentate and hexadentate chelators. In the case of hexadentate chelators (e.g. deferoxamine, DFO), a single molecule of a chelator occupies all six coordination sites of iron. When bidentate or tridentate ligands are employed, the ligand-iron ratio should be 3:1 or 2:1, respectively, to prevent the formation of redox-active partial complexes. It is complicated to design new chelators that would be both hexadentate and possess a good bioavailability (Hider and Zhou, 2005). Therefore, most of the newly synthesized compounds belong to the group of bidentate or tridentate chelators.

Iron chelators are typically not designed directly for use in prevention of anthracycline cardiotoxicity but rather for the treatment of chronically iron-overloaded patients such as those with β-thalassemia. The chelators that have been tested as protectors against anthracycline cardiotoxicity are depicted in Table 2. Apparently, the findings coming from different studies depend on many factors: chelator dose, model used or parameters followed. A major limitation of many of the models is the fact that they reflect rather an acute toxicity whereas it is the chronic cardiotoxicity that makes anthracyclines dangerous. Chronic models of anthracycline-induced cardiomyopathy therefore form an important part of preclinical evaluation of new chelators of iron (Šimůnek et al., 2004).

Dexrazoxane

The concept of iron chelation as a way how to prevent anthracycline cardiotoxicity was born with the discovery of the cardioprotective effects of dexrazoxane (ICRF-187) (Herman and Ferrans, 1981). This bisdioxopiperazine compound is metabolized into a metal-chelating

EDTA-like product (ADR 925), and its cardioprotective action was therefore assumed to be due to the chelation of redox active iron and thus reducing doxorubicin-induced oxidative stress (Schroeder and Hasinoff, 2002). Recently, other mechanisms by which dexrazoxane might eventually prevent anthracycline cardiotoxicity have been proposed. Burke et al. (2000) showed that dexrazoxane ameliorated the anthracycline-induced decrease in expression of the gene for ryanodine receptor (a calcium-handling protein), thus suggesting a potential iron-independent pathway of its cardioprotective activity. Today, dexrazoxane serves as the basic and reference agent to prevent cardiotoxicity of doxorubicin, being the only clinically approved cardioprotector. Despite its high effectiveness, its use in clinical practice suffers from several disadvantages: bone-marrow toxicity (which is cumulative with that of anthracyclines), low bioavailability (i.v. application), high costs and possible antagonism with the antitumor effect of the anthracyclines (Swain, 1998). Therefore, alternative chelators of iron with lower toxicity, higher specificity for iron, oral availability and no interference with the antiproliferative effects of the anthracyclines are being studied.

Table 2. Iron chelators as protectors against anthracycline cardiotoxicity.

Chelator	Study	Model	Protection (+/-)	Methodology
Deferoxamine (DFO)	Hershko (1993)	rat cardiomyocytes	+ (iron loaded cells) - (normal cells)	LDH, contractility
	Herman (1994)	spont. hypertensive rats	-	histology
	Voest (1994)	isolated mouse left atria	+ (200 μM DFO) - (500 μM DFO)	contractility
	Saad (2001)	rats *in vivo* (acute toxicity)	+	CK-MB, LDH, AST, GSH, MDA
Deferiprone (L1)	Barnabe (2002)	rat cardiomyocytes	+	LDH
	Link (1996)	rats *in vivo*	+	LDH, contractility, mitochondrial function
Deferasirox (ICL670)	Hasinoff (2003)	rat cardiomyocytes	-	LDH
Aroyl hydrazones (PIH analogs)	Šimůnek (2005b)	rabbits *in vivo*	+/- (PIH) *	mortality, histology, biochemistry, functional parameters
	Štěrba (2006)	rabbits *in vivo*	+ (o-108)	mortality, histology, biochemistry, functional parameters

*significantly increased survival of the animals, cardioprotection did not reach statistical significance

Deferoxamine

The excellent iron-chelating properties of a hexadentate hydroxamate deferoxamine (DFO) make it a good candidate for the protection against anthracycline cardiotoxicity. A number of studies have been performed with DFO yielding mixed results. It was found that iron-loading aggravated the doxorubicin cardiotoxicity both *in vitro* and *in vivo* and this effect could be prevented with DFO, however, in models with normal iron stores, the protection was lost (Link et al., 1996; al-Harbi et al., 1992). In the study of Voest in isolated mouse atrium (1994), the protective effect of deferoxamine was dose-dependent and disappeared with higher dose of the chelator (500 μM vs. 200 μM). This is in contrast with dexrazoxane, for which a greater inhibition of doxorubicin-induced decrease in atrial contractile function was observed at a higher concentration in the same study. *In vivo* studies in rats performed by the same authors have shown that DFO was able to prevent the histopathological abnormalities and the decrease in heart function. Unfortunately, DFO proved to be quite toxic and the mortality in DFO+DOX-treated group of animals was actually higher than in those receiving DOX only (Voest, 1993). No cardioprotective effects of DFO were also found in spontaneously hypertensive rats by the group of Herman (1994). On the other hand, studies using an acute model of doxorubicin cardiotoxicity (single injection of DOX in a large dose) gave positive results as demonstrated by an improvement of various biochemical markers of cardiac damage as well as myocardial histopathology (Saad et al., 2001). It is, however, questionable, how these result relate to a repeated exposure to the anthracyclines as seen in oncologic patients.

Deferiprone (L1)

Deferiprone represents a bidentate, orally active iron chelator that slowly seems to replace deferoxamine in the treatment of iron-overloaded patients. Deferiprone has been previously shown to protect isolated cardiomyocytes against doxorubicin-induced LDH leakage (Barnabe et al., 2003). Recent study of Xu et al. (2006) in spontaneously beating isolated rat atria suggested an attenuation of doxorubicin-induced negative inotropic effects and mitochondrial ultrastructure and function with deferiprone. Partial prevention of doxorubicin-induced drop of mitochondrial function was also observed *in vivo* in rats (Link et al., 1996). Although deferiprone seems to be one of the most promising cardioprotector at the moment, further *in vivo* studies are clearly needed to confirm its beneficial effects.

Deferasirox (ICL670)

Deferasirox is a tridentate, orally absorbed chelator of iron, which is intended as a once-daily monotherapy for transfusional iron overload. Contrary to deferiprone, it lacked any protective effect against doxorubicin toxicity in isolated rat cardiomyocytes in a study of Hasinoff et al. (2003), despite its high lipophilicity (membrane permeability) and strong iron-chelating potency (Hider and Zhou, 2005). So far, no further studies concerning a prevention of anthracycline toxicity are available.

Aroylhydrazone analogs

The mother compound of these tridentate ligands, pyridoxal isonicotinoyl hydrazone (PIH), was discovered in the late 1970s by Ponka and colleagues (1984). Many analogs have been derived by now and the compounds with enhanced antiproliferative properties are also being developed as new anticancer drugs (Richardson et al. 1995). PIH and its analogs with mild to moderate toxicity, however, could be used in prevention of anthracycline cardiotoxicity. The major drawback of these compounds is their short plasma half-life, possibly due to the hydrolysis of unstable hydrazone bond (Kovaříková et al. 2006). This disadvantage can be overcome for example by means of creating stable pro-chelators that are only activated at the site where oxidative stress occurs (Charkoudian et al., 2006, Yiakouvaki et al., 2006).
To date the cardioprotective effects of the three aroylhydrazone analogs, PIH, SIH and o-108 (Fig. 4) have been evaluated.

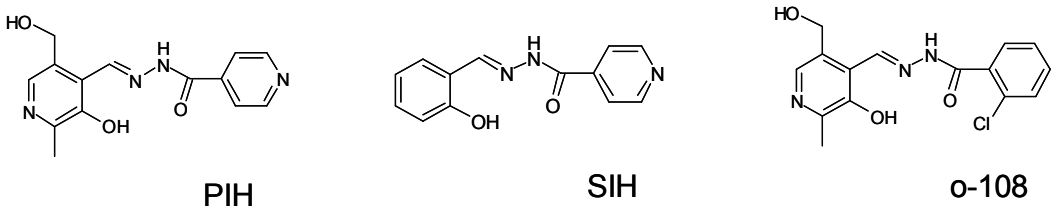

Figure 4. Structures of the three aroylhydrazone analogs investigated for their potential cardioprotective properties. PIH, pyridoxal isonicotinoyl hydrazone; SIH, salicylaldehyde isonicotinoyl hydrazone; o-108, 2-chlorobenzoyl hydrazone.

SIH was previously found to prevent oxidative stress-induced mitochondrial injury in H9c2 cardiomyoblast cells *in vitro* (Šimůnek et al., 2005c) thus giving a rationale for its use in prevention of anthracycline cardiotoxicity. Using a rabbit model of chronic daunorubicin cardiomyopathy, PIH, SIH as well as o-108 were found to prevent premature deaths and

improve daunorubicin induced impairment of cardiac function (LVEF, dp/dtmax), biochemical markers (TnT) as well as morphological parameters. Interestingly, chelator dose escalation not only did not provide additional protection, but on the contrary it hampered virtually all the beneficial effects of the chelators regarding both cardioprotection and overall mortality (Šimůnek et al., 2005b, Štěrba et al., 2006).

5.5.3. Flavonoids

Although flavonoids could easily be ranked among both antioxidants and iron chelators, they will be discussed separately due to their rather unique physico-chemical, biochemical as well as pharmacological properties.

Flavonoids are a group of naturally occurring benzo-γ-pyron derivatives (found in fruits, vegetables, nuts and flowers), which are estimated to be over 4000 identified structures so far. Their biological importance as vitamin-like compounds was first proposed by Szent-György at the beginning of the 20th century. The pharmacological properties of the flavonoids can be collectively summarized as being antithrombotic, anti-inflammatory, antibacterial, antiviral, vasodilatatory and protective against some chronic diseases (Gryglewski et al., 1987; Ferrandiz and Alcaraz, 1991; Middleton et al., 2000; Duarte et al., 1993).

Besides naturally occurring flavonoids, semi-synthetic structures were developed. Rutin was hydroxyethylated to enhance its metabolic stability and water solubility yielding hydroxyethylrutosides (HERs), whose mixture forms a basis of Venoruton, the preparation used in the treatment of chronic venous insufficiency (Cesarone et al., 2006).

Chemistry and classification

Despite the huge number of flavonoids they can be classified in a few groups. They all share a typical flavan structure as a core. Its respective rings are identified by A, B and C. Since the structure of the flavonoids strongly influences their biological activities (Cody, 1988), they are classified according to their structure and the relative orientation of the various moieties in the molecule. The major classes are flavonols, flavones, flavanones, flavanols (catechins), anthocyanidins, dihydroflavonols and chalcones (Figure 4). Ring B attached to C3 instead of C2 forms a subgroup called the isoflavonoids.

Pharmacokinetics

Absorption of the flavonoids from the diet was long considered to be negligible, as most of the flavonoids present in plants are bound to sugars. It has been shown that the glycosidic bonds are splitted by the colonic microflora, which facilitate the absorption of the aglycones

(Griffiths et al., 1972). On the other hand, bacteria might also contribute to degradation of the aglycones decreasing the bioavailability of the flavonoids. Later, it has been found that the flavonoid glycosides can also be absorbed without prior hydrolysis (Hollman and Katan, 1998).

As the natural flavonoids already possess several polar hydroxyl groups, phase I metabolism has not been reported but for the synthetic structure lacking hydroxyl groups (Hackett, 1986). Conjugation with glucuronic acid, sulfate or glycine represents the phase II metabolism of the flavonoids. Metabolism by the colonic flora (hydrolysis of the glycosides and the conjugates, ring cleavage) also contributes to the flavonoid biotransformation. The metabolic degradation products are excreted into the bile or urine (Manach et al., 2004).

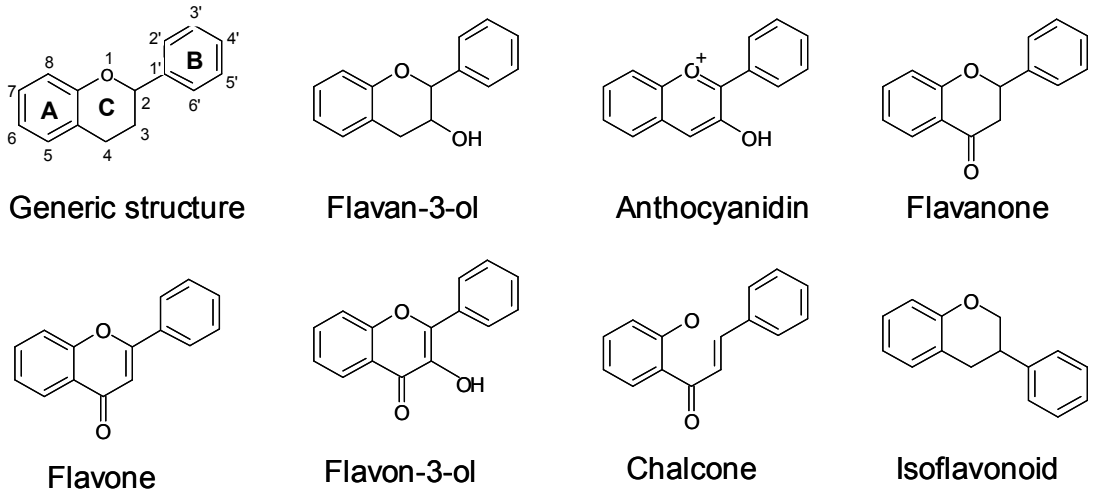

Figure 4. Molecular structures of the flavonoid subclasses

Flavonoids as cardioprotectors

In section 4.1.1. the role of ROS in anthracycline cardiotoxicity was discussed. Flavonoids have long been recognized as excellent radical scavengers and potent inhibitors of lipid peroxidation. This property can be derived form their structure, which has an easily oxidizable catechol moiety that interacts with free radicals and favors resonance of electrons after the radical-flavonoid interaction. Several studies have established structure-activity relationships (SAR) with respect to the antioxidant behavior of the flavonoids (van Acker et al., 1996; Catapano et al., 1997). It has been suggested that flavonols are the best antioxidants among flavonoids. This can be explained by the possession of a catechol moiety, a carbonyl group in

ring C, a 2,3-double bond conjugated with 4-oxo function and 3-(5-) hydroxyl groups, which allow extensive resonance between the three-ring system of the flavonols (Lien et al., 1999). Furthermore, flavonoids form complexes with all kinds of transition metal ions, such as Fe^{2+}, Fe^{3+}, Cu^{2+} and Al^{3+} (Hider et al., 2001). These transition metal ions can form complexes with flavonoids at various sites. If a catechol moiety is present, each hydroxyl group can occupy one coordination site of the ion. This also hold true for flavonoids containing 4-oxo function combined with a 5-OH or a 3-OH.

The combination of antioxidant and iron-chelating properties renders flavonoids very interesting as potential protectors against anthracycline cardiotoxicity. In addition, there is one more characteristics of the flavonoids that is worth investigating, i.e. their inhibitory action towards carbonyl reductases (CRs). CRs have been implicated to be involved in the metabolization of the anthracyclines to the corresponding C13-alcohols (see paragraph 2.1.). Quercetin, quercitrin and rutin are known inhibitors of CRs (Forrest and Gonzales, 2000). Detailed SAR on the flavonoids with respect to their inhibitory effects are, however, scarce (Silvestrini et al., 2006; Imamura et al., 2000) and to date no attempts have been made to determine the correlation between the inhibitory effects of the flavonoids and their cardioprotective potential.

Hydroxyethylrutosides (HERs) are semi-synthetic flavonoids derived from rutin and the mixture of structurally related HERs forms the active ingredient of Venoruton. 7-monohydroxyethylrutoside (monoHER) has been previously identified as the most powerful antioxidant of this mixture and it was selected for cardioprotective studies (Haenen et al., 1993). MonoHER was found to completely prevent doxorubicin-induced cardiotoxicity in mice (van Acker et al., 2000). Importantly, monoHER did not influence the antitumor effect of doxorubicin in various cancer cell lines *in vitro*, nor did it influence the effects of doxorubicin on the growth of A2780 and OVCAR-3 xenografts in nude mice (van Acker et al., 1997). A phase I clinical study in healthy volunteers concluded that 1500 mg/m2 of monoHER i.v. is a feasible and safe dose to be evaluated in a phase II study to investigate its potency against doxorubicin cardiotoxicity in cancer patients (Bruynzeel et al., 2006).

Grape seed proanthocyanidin extract (GSPE) has been recently shown to inhibit doxorubicin-induced cardiotoxicity as demonstrated by reduced serum creatine kinase (CK) activity, DNA damage and histopathological changes in the cardiac tissue of mice (Bagchi et al., 2003). The authors of this study suggested that the cardioprotective properties of GSPE

may be at least partially attributed to its ability to block anti-death signaling mediated through the proapoptotic transcription factors and genes.

Pycnogenol, a mixture of water soluble bioflavonoids extracted from the bark of pine trees also prevented the elevation of serum creatine-phosphokinase activity and the decrease in heart rate in doxorubicin-treated mice (Feng et al., 2002).

Catechin was cardioprotective in a single study of Kozluca et al. (1996) in rats receiving doxorubicin +/- catechin. The prevention of doxorubicin-induced damage was documented by electrocardiography, myocardial contractility and electron microscopy.

6. Outline and scope of the thesis

Iron chelator dexrazoxane is a long-known protector against anthracycline cardiotoxicity, however, its side effects limit its use. Previously, a series of new lipophilic chelators from aroylhydrazone class has been synthesized and three of these chelators – PIH, SIH and o-108 have shown promising protection against daunorubicin-induced chronic cardiomyopathy in the rabbit model. Flavonoids represent another group of compounds with potential to relieve anthracycline cardiotoxicity. This thesis deals with the search for mechanisms by which iron chelators and flavonoids prevent anthracycline-induced cardiac injury with focus on biotransformation aspects of the anthracyclines and the cardioprotectors under investigation.

It has been suggested that the protective effects of iron chelators are due to prevention of anthracycline-induced hydroxyl radical formation via Fenton and Haber-Weiss chemistry. This hypothesis was tested in A549 cells by comparing the effects of various iron chelators (PIH, SIH, deferoxamine, dexrazoxane, monoHER) against doxorubicin- and hydrogen peroxide/iron-induced oxidative stress. Moreover, we investigated whether these chelators do not hamper antiproliferative and pro-apoptotic effects of doxorubicin (***Chapter II***).

The anthracyclines are metabolized by microsomal cytochromes P450 and cytosolic reductases. We have therefore evaluated the effect of the anthracyclines on the activity of these enzymes in isolated hepatocytes as well as an influence of the co-administration of iron chelators PIH and SIH on the anthracycline-induced biotransformation effects (***Chapter III***).

Carbonyl reduction of the anthracyclines is particularly interesting to investigate since the C13-dihydroanthracyclines have been shown to be more cardiotoxic than the parent drugs.

This metabolic conversion can be catalyzed either by carbonyl reductases or aldo-keto reductases. To elucidate the proportion of their contribution to the formation of C13-dihydroanthracyclines (i.e. to indicate which enzyme is the most important anthracycline C13-reductase) we conducted an inhibition study of daunorubicin reduction with specific enzyme inhibitors (***Chapter IV***).

Since the flavonoids quercetin, quercitrin or rutin are known inhibitors of carbonyl reductases they represent an attractive tool to mitigate the anthracycline cardiotoxicity. Moreover, the flavonoids possess iron-chelating and antioxidant properties, which have been extensively studied. In the last chapter we have aimed to find out, which of those properties of the flavonoids are crucial for their cardioprotective effects (***Chapter V***)

The conclusions and future perspectives are discussed in ***Chapter VI***.

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II

Iron is not involved in oxidative stress-mediated cytotoxicity of doxorubicin and bleomycin.

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Br J Pharmacol 2006; **149**:920-930

Summary

Background and purpose. The anticancer drugs doxorubicin (DOX) and bleomycin (BLM) are notorious because of their oxidative stress-mediated side effects in heart and lung, respectively. It is frequently suggested that iron is involved in DOX and BLM toxicity. We therefore aimed to elucidate whether iron chelation prevents the oxidative stress-mediated toxicity of DOX and BLM and whether it affects the antiproliferative/proapoptotic effects of DOX and BLM.

Experimental approach. We investigated the interactions between five iron chelators and the oxidative stress-inducing agents (DOX, BLM and H_2O_2) in the A549 cells. Oxidative stress and cellular damage were quantified by TBARS formation, GSH consumption and LDH leakage. Cell proliferation and caspase-3 activity were determined to evaluate the antitumour/proapoptotic effects of the compounds.

Key results. All the tested chelators, except for monohydroxyethylrutoside (monoHER), strongly prevented hydroxyl radical formation induced by H_2O_2/Fe^{2+} in EPR studies. However, only salicylaldehyde isonicotinoyl hydrazone (SIH) and deferoxamine (DFO) protected intact A549 cells against H_2O_2/Fe^{2+}. Inversely, in DOX and BLM-induced oxidative stress only dexrazoxane (DXZ) and monoHER were found to relieve cellular damage, while the other chelators did not.

Conclusions and implications. We have shown that the ability to chelate iron as such is not the sole determinant of a compound protecting against DOX or BLM-induced cytoxicity. Our data challenge the putative role of iron in the oxidative stress-mediated toxicity of DOX and BLM and have implications for the direction in which new protectors should be developed.

Abbreviations

BLM, bleomycin; DFO, deferoxamine; DOX, doxorubicin; DXZ, dexrazoxane; EPR, electron paramagnetic resonance $HO^{\bullet}$, hydroxyl radical; LDH, lactate dehydrogenase; monoHER, monohydroxyethylrutoside; PIH, pyridoxal isonicotinoyl hydrazone; SIH, salicylaldehyde isonicotinoyl hydrazone; TBARS, thiobarbituric acid reactive substances

Introduction

Modern chemotherapy employs a wide range of efficient cytostatic agents. However, perilous side effects sometimes hamper the therapy and may lead to serious or even fatal organ dysfunctions. Among the anticancer drugs, doxorubicin (DOX) is notorious for its cardiotoxicity (Minotti et al., 2004; Keizer et al., 1990) while bleomycin (BLM) is known to elicit severe interstitial pulmonary fibrosis (Mir et al., 1996; Azambuja et al., 2005). These two drugs belong to different classes: DOX is an anthracycline antibiotic whereas BLM represents a glycosylated peptide antibiotic. They however share some similarities viz. (1) chronic organ toxicity frequently develops upon administration of cumulative doses of both drugs (Azambuja et al., 2005; Singal & Iliskovic, 1998), (2) reactive oxygen species (ROS) were shown to be involved in the toxicity of both DOX and BLM (Zhou et al., 2001; Manoury et al., 2005) and (3) interactions of both drugs with iron are considered to be of importance in exerting their deleterious effects to the healthy tissues and to the antineoplastic activity of the drugs. The exact mechanisms leading to DOX-induced cardiotoxicity and BLM-induced lung toxicity, however, remain unclear and it is generally accepted that more mechanisms are involved.

The interactions of DOX with iron are complex (for review, see Xu et al., 2005). Some of these include involvement of ROS whereas others are oxidative stress-independent. Iron can either promote hydroxyl radical generation via the Fenton or Haber-Weiss reaction or form DOX-Fe(III) redox active complexes. ROS-independent effects include for example disturbance of either iron regulatory proteins (Minotti et al., 1999) or iron mobilization from ferritin (Kwok & Richardson, 2003). The role of iron in DOX cardiotoxicity became particularly evident after successful application of the iron chelator dexrazoxane (DXZ, ICRF-187) as a cardioprotector (Cvetkovic & Scott, 2005; Hasinoff et al., 2003). DXZ, a prodrug of an EDTA analogue, likely acts via displacing iron from DOX-Fe(III) complexes and removing free iron from the vicinity of biomolecules. We have previously shown that another iron chelating compound, monohydroxyethylrutoside (monoHER), is protective against DOX-induced cardiotoxicity (Van Acker et al., 2000; Abou El-Hassan et al., 2003a). Besides iron chelation, also the antioxidant or anti-inflammatory effects might contribute to the cardioprotective effect of the flavonoid monoHER. (Van Acker et al., 1998; Abou El Hassan et al., 2003b).

We also found that monoHER did not affect the antitumour effects of DOX in MCF-7, OVCAR-3 and A2780 cell lines and in graft hosted nude mice (Van Acker et al., 1997). The antiproliferative activity of DOX is complex. The interaction with the DNA-topoisomerase II complex is considered a primary trigger for cell growth arrest (Gewirtz, 1999). Formation of free radicals, directly affecting the cell membrane or the effects via DNA cross-linking, DNA intercalation or alkylation have also been reported. The precise contribution of free radicals to cancerous cell death is still under debate. It has been shown that doxorubicin retains toxicity under hypoxic conditions when ROS cannot be formed (Tannock et al., 1981). Co-treatment of DOX with the iron chelator dexrazoxane also did not affect the antitumour effects of DOX (Wu & Hasinoff, 2005). On the other hand, some other studies demonstrated that DXZ compromised the antitumour properties of DOX (e.g. Zhang et al., 1996). The mechanisms by which DOX can interact with cardiac and cancer cells are depicted in Figure 1.

In the case of BLM, there is convincing evidence that its antitumour action is linked with free radical formation. BLM binds iron and oxygen thus forming an activated complex capable of releasing damaging oxidants in close proximity to DNA (El-Medany et al., 2005). However, some investigators observed that bleomycin was equally effective in normal and iron-deprived mice (Lyman et al., 1989). In another study, O-phenantroline, a metal ion chelator, fully inhibited BLM-induced DNA cleavage (Larramendy et al., 1989). BLM does not only form ROS but it can also act as an intercalating agent thanks to its bithiazole structural moiety. ROS are clearly involved in the development of lung fibrosis. Studies with antioxidants such as N-acetylcysteine or bilirubin showed effective protection of rats against BLM lung fibrosis. There is also growing evidence that imbalances in various metalloproteinases and their inhibitors are crucial elements in BLM fibrogenic process (Manoury et al., 2005).

In the present study, we have employed five iron chelators with clinical applicability: DXZ, monoHER, deferoxamine (DFO) and two aroyl hydrazones PIH and SIH (Figure 2). These compounds differ in their physico-chemical properties but share a well-documented iron chelating capacity. We investigated the interactions between these iron chelators and the oxidative stress-inducing agents (DOX, BLM and for comparison H_2O_2) in order to find out (1) whether the iron chelators are able to prevent DOX and BLM oxidative stress-mediated cytotoxicity and (2) whether the antiproliferative effects of DOX and BLM in A549 human lung adenocarcinoma cells are affected by iron chelation.

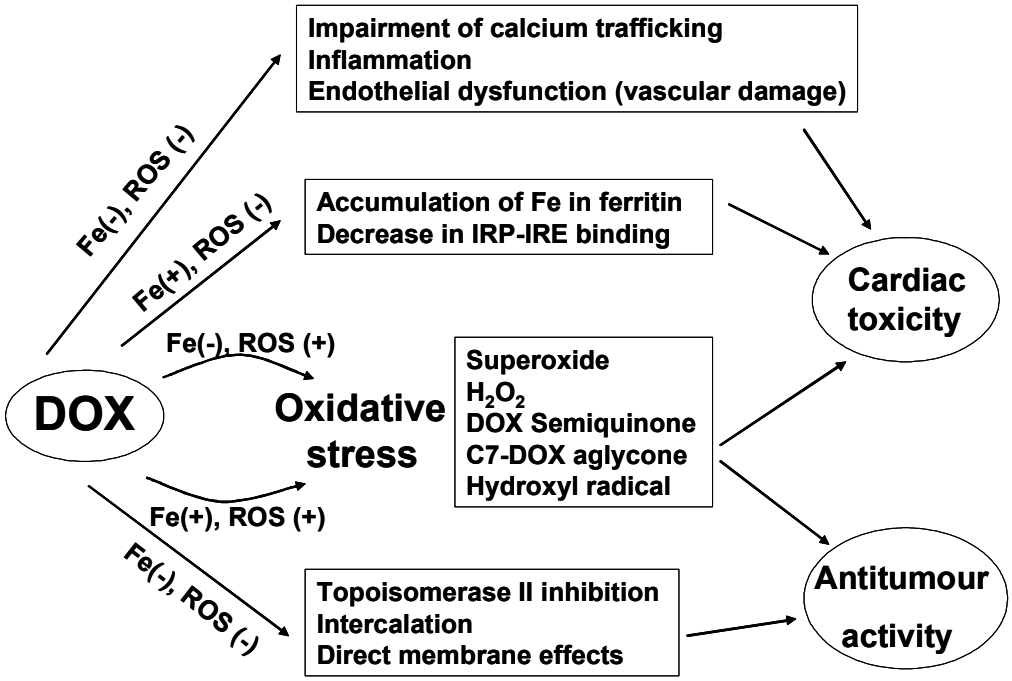

Figure 1 A simplified scheme of the proposed interactions of DOX with tumour and cardiac cells illustrates their complexity. With regard to iron, the effects of DOX can be divided to Fe-dependent and Fe-independent. The most prominent characteristic of DOX is its ability to induce oxidative stress. ROS can be formed in the absence of Fe (semiquinone radical, C7-aglycone radical, superoxide, H_2O_2) but their generation can also be Fe-catalyzed (Fenton reaction, Haber-Weiss reaction) bringing to life highly damaging $HO^{\bullet}$ radical. DOX can also form DOX-Fe(III) complexes that also lead to ROS. ROS may induce damage to both cardiac and neoplastic cells. The higher susceptibility of heart tissue to oxidative stress is often explained by its poor antioxidant defences and/or abundance of mitochondria, which are both important source, and target of ROS. The major mechanisms that lead to cancerous cell death are, most probably, inhibition of topoisomerase II and DNA intercalation - the effects independent on oxidative stress and iron. Other effects that do not depend on ROS are thought to contribute predominantly to their cardiotoxicity: dysregulation of iron homeostasis via interaction with iron regulatory protein and inhibition of Fe mobilization from ferritin are examples of Fe-mediated effects whereas inflammation, endothelial dysfunction and calcium homeostasis impairment are both iron and ROS independent.

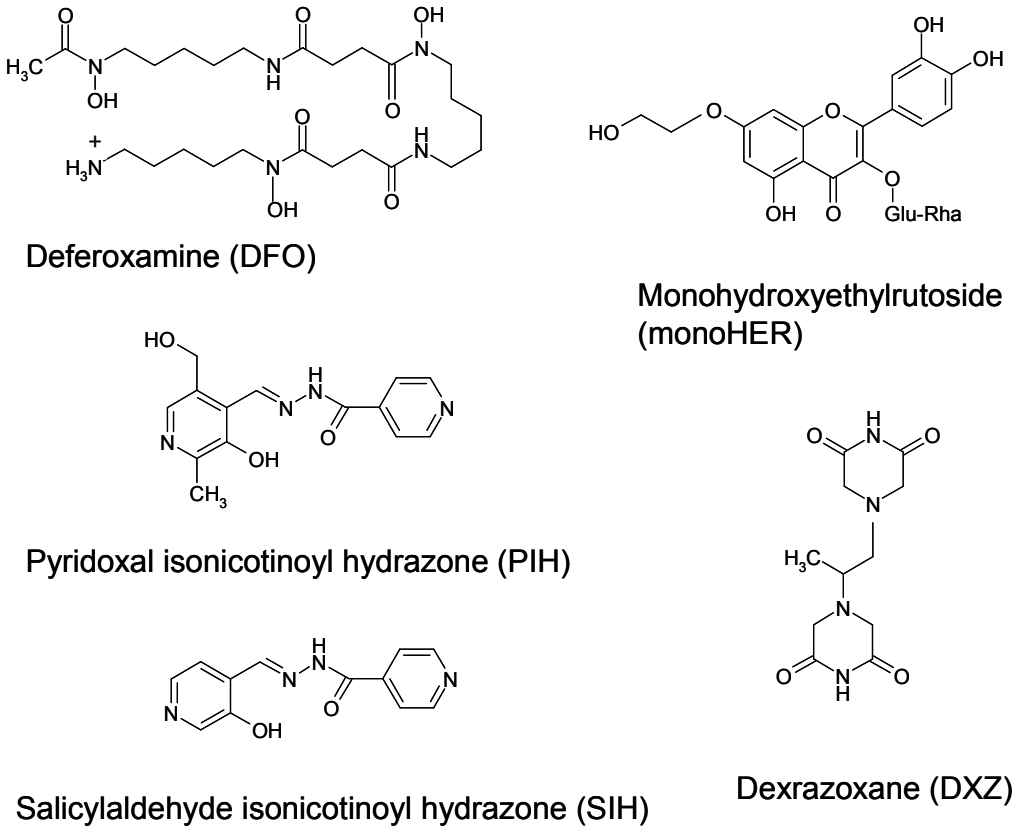

Figure 2 Chemical structures of the iron chelators used in this study: deferoxamine (DFO), pyridoxal isonicotinoyl hydrazone (PIH), salicylaldehyde isonicotinoyl hydrazone (SIH), monohydroxyethylrutoside (monoHER) and dexrazoxane (DXZ, ICRF-187).

Methods

Cell culture

Human lung adenocarcinoma cell line (A549) was maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 2 mM of L-glutamine under humidified atmosphere containing 5% CO_2 at 37°C. Cell passages between 25 and 40 were used for experiments described in this study.

For lipid peroxidation assay, GSH determination and LDH leakage, subconfluent cultures were trypsinized and seeded in 6 cm Petri dishes at a density of 1.9 x 10^5 /ml (in a total volume of 4.5 ml). Cells were left to attach for 24 h after which the culture medium was renewed for the

medium containing test compounds and cells were cultivated for further 48 h. Cells were 40-50% confluent when treated and confluent after 48 h incubation. The concentration of DMSO used to dissolve PIH, SIH and monoHER was adjusted to 0.2% in all the solutions including controls. After the end of incubation period 200 µl of medium was aspirated from each dish, mixed with 50 µl of 15% BSA and stored at –80°C for later LDH determination. The rest of the medium was removed and the dishes were kept on ice. Cell monolayers were washed with ice cold PBS and scrapped from the dishes with 600 µl of fresh PBS per dish. Cells were sonicated on ice briefly (15 s) and 300 µl of the cell lysate were removed to another microtube containing 33 µl 15% SSA and stored at –20°C for GSH determination. The rest of the lysate was also stored at –20°C and used for determination of thiobarbituric acid reactive substances.

Cell proliferation assay (Trypan Blue exclusion)

For cell proliferation assay, subconfluent cultures were trypsinized and seeded in six well plates at a density of 1.9 x 10^5/ml (in a total volume of 2 ml). They were left to adapt for 24 h after which the medium was changed for the medium containing test compounds using the same solutions as for the assays described above. Cells had been exposed to substances for 48 h and after that they were trypsinized with 300 µl of trypsin/well, 700 µl of serum containing medium was added, and cell suspension was mixed thoroughly in the well and transferred to a microtube. Samples were then vortexed and an aliquot was mixed with a Trypan Blue solution (5 mg/ml) 1:1. Living cells were counted using a Bürker's chamber and cell viability was expressed as a percentage of control.

Caspase-3 activity determination

Cells were seeded on 10-cm Petri dishes at the density of 1.9x10^5 cells/ml (in 9 ml total volume). After the 24 h adaptation period cell were exposed to the compounds. The chelators were added 30 min prior to DOX or BLM. At 12, 24 and 48 h cells were harvested by centrifugation (600 x g, 5 min) and lysed on ice for 20 min in a lysis buffer containing 50 mM HEPES, 5 mM CHAPS and 5 mM DTT. The lysates were centrifuged at 14,000 x g, 10 min, 4°C, the supernatants were collected and stored at -80°C. The enzyme activity was measured in a 96-well microplate using a kinetic fluorometric method based on the hydrolysis of the peptide substrate Ac-DEVD-AMC by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. Ac-DEVD-CHO, a specific inhibitor of caspase-3, was used to confirm the specificity of the

cleavage for caspase-3. Fluorescence was recorded at λ_{ex} 360 nm and λ_{em} 465 nm. The concentration of the AMC released was calculated from a standard curve performed with defined AMC solutions. Caspase-3 activity was expressed as nmol AMC/min/ml and correlated to mg of protein.

TBARS assay for lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured by means of HPLC according to Lepage et al. (1991). Briefly, 100 µl of cell lysate or malondialdehyde (MDA) standard were mixed with 900 µl reagent composed of 10 parts of reagent A (0.012 M 2-thiobarbituric acid (TBA), 0.32 M H_3PO_4 and 0.01% EDTA) and 1 part of reagent B (butylated hydroxytoluene (BHT) in ethanol 1.5 mg/ml). Standards were prepared using 0-10 µM MDA solutions in PBS and derivatized during the same analytical measurement as the samples. The tubes were covered with marbles and heated for 1 h at 99°C. After cooling down the product was extracted in 500 µl of butanol by vigorous shaking and the tubes were centrifuged for 5 min at 5000 r.p.m. 30 µl of the extract were injected to a Nucleosil C18 column, 150 mm x 3.2 mm (Supelco Inc.) and eluted with 65% water and 35% methanol + 0.05% TFA. Fluorescence was recorded at λ_{ex} 532 nm and λ_{em} 553 nm. The peak of TBA-MDA product was integrated and concentration of MDA was calculated by means of linear regression. The amount of MDA was correlated to the protein contents and the results were expressed as a percentage of TBARS formation taking control sample as 100%.

GSH determination

Samples preserved in SSA were thawed, sonicated for 5 min on ice, centrifuged for 5 min at 5000 r.p.m. and the supernatants were used for the assay. Total glutathione was determined according to Tietze (1969) using the enzymatic recycling method with DTNB and glutathione reductase in a microplate format. The rate of TNB formation was recorded at 405 nm for 2 min and the slope was compared to that of the standard curve of GSSG. The concentration of total GSH was calculated using the method of linear regression, the results were protein correlated and expressed in relative values taking total GSH in control sample as 100%.

LDH leakage assay

For LDH activity determination, 50 µl of sample were mixed with 950 µl of reagent consisting of 1.2 mM of sodium pyruvate and 0.1 mM NADH in 50 mM Na^+/K^+ phosphate buffer pH 7.4. The rate of NADH oxidation was followed at 340 nm for 1 min. Enzyme activity was calculated using extinction coefficient of NADH 6.32 $l.mmol^{-1}cm^{-1}$ and relative LDH leakage was expressed as percentage of control taking LDH leakage in control sample (medium from non treated cells) as 100% release.

Protein determination

Protein was determined spectrophotometrically using bicinchoninic acid (Brown et al., 1989), with bovine serum albumin as a standard.

Electron paramagnetic resonance (EPR)

H_2O_2/Fe^{2+} induced hydroxyl radical formation and its modulation by iron chelators was measured by EPR spectroscopy. Measurements were performed under the following conditions: microwave power, 2 mW; modulation amplitude, 1 G; scan width, 50 G; modulation frequency, 100 kHz; and temperature, 30 °C using spectrometer Bruker EMX (Bruker GmbH, Freiburg, Germany). A typical reaction mixture contained 66 µl of 750 mM DMPO, 300 µl of 6.6 mM H_2O_2, 2 µl of 50 mM PIH or SIH in DMSO (or DMSO alone in case of control sample) and 582 µl of deaerated MilliQ water. The reaction was started with 50 µl of 200 µM ferrous sulphate and the spectrum was recorded after 2-3 min. DFO and DXZ were dissolved in MilliQ instead of DMSO. DMSO was adjusted to 0.2% (v/v) in all the measurements. The final concentrations of H_2O_2, Fe^{2+}, chelators and DMSO for the EPR experiments were the same as used for cell culture experiments.

Drugs and chemicals

Pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde hydrazone (SIH) were a kind gift from P. Ponka (Mc Gill University, Montréal, Canada). MonoHER was kindly provided by Novartis Consumer Health (Nyon, Switzerland). Dexrazoxane (Cardioxane®) was from Chiron B.V. (Amsterdam, The Netherlands), and deferoxamine (Desferal®) was from Novartis (Switzerland). Doxorubicin (Adriblastina R.T.U.®) was from Pfizer (USA) and bleomycin (Bleomycine 15U PCH®) was purchased from Pharmachemie B.V. (Haarlem, the Netherlands).

Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), oxidized glutathione (GSSG) and reduced glutathione (GSH), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase, β-NADPH, trypan blue, pyruvate, hydrogen peroxide, ferrous sulphate, Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), sulfosalicylic acid (SSA), dimethylsulfoxide (DMSO), 7-amino methyl coumarin (AMC), HEPES and malondialdehyde (MDA) were purchased from Sigma (St. Louis, MO, USA). Hanks' balanced salt solution (HBSS), trypsin, fetal bovine serum (FBS), penicillin and streptomycin (P/S) were from Life Technologies (Breda, The Netherlands). NADH was obtained from ICN Biochemicals (Ohio, USA) and bicinchoninic acid (BCA) was obtained from Pierce. N-acetyl Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) and N-acetyl-Asp-Glu-Val-Asp-CHO (aldehyde) (Ac-DEVD-CHO) were from Alexis Biochemicals. CHAPS was from Fluka.

Data analysis

Results are given as mean ± SD. Statistical analysis was performed by SigmaStat for Windows 3.0.1 (SPSS). Comparisons between groups were made by One-way ANOVA with Tukey´s post-hoc test. The differences were considered significant when $p < 0.05$.

Results

The effects of iron chelation on H_2O_2/Fe^{2+}- induced oxidative stress

The iron chelators DFO, DXZ, PIH and SIH markedly decreased the H_2O_2/Fe^{2+}-induced $HO^{\bullet}$ formation, measured as DMPO-OH adduct, to 43%, 23%, 13% and 15% respectively, compared to control (Fig.1). MonoHER was only a weak $HO^{\bullet}$ scavenger (84% of control). Besides the characteristic spectra of DMPO-OH adducts, additional peaks were observed by EPR after adding DXZ and to a smaller extent in spectra after PIH. This suggests that besides $HO^{\bullet}$, other radicals are being formed. In contrast, simple spectra, similar to that of H_2O_2/Fe^{2+} alone resulted when DFO, SIH or monoHER were added. These results indicate that there are differences in the mechanism of action between the chelators.

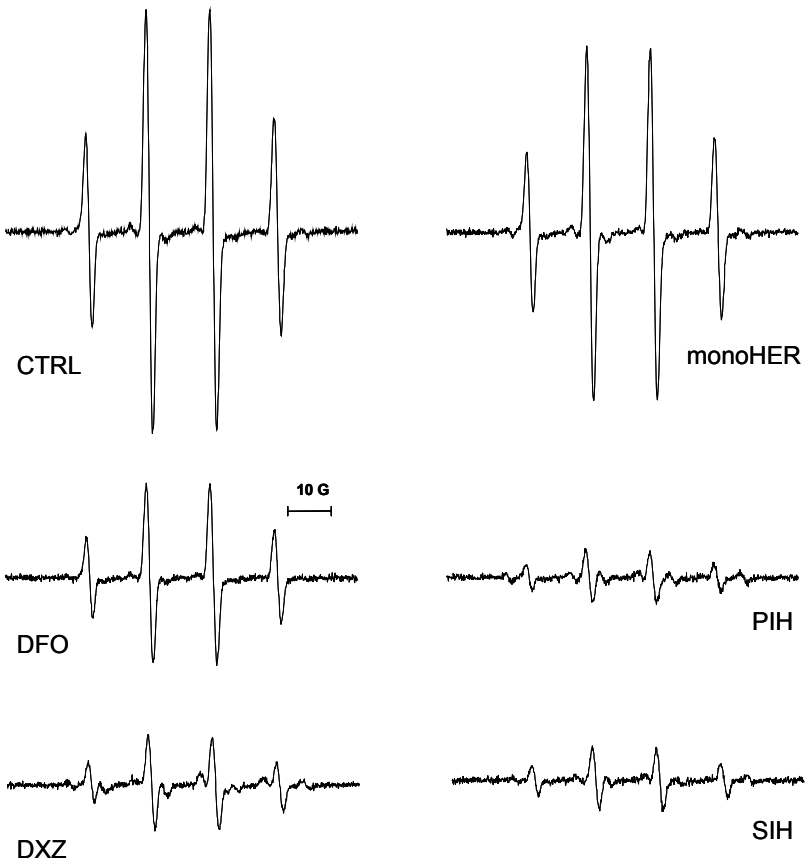

Figure 3 Representative EPR spectra of DMPO radicals produced by Fenton reagents (2 mM H_2O_2, 10 μM Fe^{2+}) (Control) and its modulation by 100 μM of DFO, DXZ, monoHER, PIH and SIH. The concentration of the reagents used in this experiment correspond to those used in cell culture. For details, see Materials and Methods and Results.

Incubation of the intact A549 cells with the chelators and subsequent exposure to H_2O_2/Fe^{2+} showed that SIH effectively reduced H_2O_2/Fe^{2+}-induced oxidative damage as demonstrated by diminished TBARS formation and conserved GSH levels (Tables II-III, the last column). Cell viability was increased and LDH leakage was prevented by SIH (Table I, the last column) suggesting its high efficacy in prevention of Fenton-derived toxicity. On the other hand, DFO offered only partial protection (marked preservation of GSH levels while the other parameters did not change significantly). The other chelators PIH, DXZ and monoHER, were not effective. To

exclude a confounding effect of DMSO, which by itself can act as a $HO^{\bullet}$ scavenger, the DMSO concentration was adjusted to 0.2% in both EPR experiments and the cell culture experiments.

The effects of iron chelation on DOX and BLM-induced cytotoxicity and oxidative stress

Leakage of the cytosolic enzyme LDH from A549 cells increased upon 48 h exposure to 1 μM DOX while a comparable concentration of BLM did not induce LDH leakage (Table I). No LDH release was observed with PIH- or monoHER-treated cells. DXZ caused a slight increase in LDH leakage while the effect of SIH and DFO was a significant increase. Combining the chelators with either DOX or BLM resulted in a significant cellular protection against DOX induced LDH leakage by DXZ and monoHER whereas other chelators were not protective.

Table 1. Effects of various iron chelators on DOX, BLM and H_2O_2/Fe^{2+}-induced LDH leakage in the A549 cells[a]

Chelator	Control[c] (%)	DOX (%)	BLM (%)	H_2O_2/Fe^{2+} (%)
Control[b]	**100**	232 ± 19 *	86 ± 5	259 ± 11 *
DXZ	137 ± 11	121 ± 14 †	135 ± 7 †	279 ± 26 *
DFO	260 ± 37 *	260 ± 37 *	221 ± 49 * †	234 ± 19 *
monoHER	86 ± 3	150 ± 20 * †	93 ± 8	305 ± 22 * †
PIH	91 ± 11	241 ± 24 *	97 ± 4	262 ± 9 *
SIH	214 ± 32 *	262 ± 37 *	223 ± 26 * †	131 ± 6 * †

[a]The A549 cells were pretreated with 100 μM of the chelator for 30 min at 37 °C before DOX (1μM), BLM (1 μg/mg) or H_2O_2/Fe^{2+} (2 mM /10 μM) were added. The exposure period was 48 h. Values represent the mean ± SD (n ≥ 3). Data are expressed as a percentage of LDH leakage relative to untreated controls. Control values were 18.3 ± 0.7 U/l.min.

[b]Cells not pretreated with any chelator

[c]Cells not treated with any oxidative stress-inducing agent

*$p < 0.05$ vs. untreated control; †$p < 0.05$ vs. DOX, BLM and H_2O_2/Fe^{2+}, respectively

Thiobarbituric acid-reactive substances, a marker of lipid peroxidation, increased in the cells exposed to DOX and to a lesser extent also upon exposure to BLM. An explanation is that BLM does not easily cross membranes (Tounekti et al., 1993) whereas DOX enters the cell rather quickly. Except for monoHER and PIH, all the chelators were able to induce TBARS formation directly. None of the chelators showed any protection against DOX or BLM-induced lipid peroxidation (Table II).

Table 2. Effects of various iron chelators on DOX, BLM and H_2O_2/Fe^{2+}-induced TBARS formation in the A549 cells[a]

Chelator	Control[c] (%)	DOX (%)	BLM (%)	H_2O_2/Fe^{2+} (%)
Control[b]	**100**	389 ± 132 *	176 ± 51	8998 ± 940 *
DXZ	435 ± 16 *	513 ± 55 *	435 ± 91 *†	11936 ± 1744 *
DFO	386 ± 17 *	469 ± 72 *	441 ± 83 *†	9457 ± 2158 *
monoHER	100 ± 9	458 ± 88 *	207 ± 58	11110 ± 2258 *
PIH	133 ± 34	409 ± 82 *	144 ± 43	8856 ± 2702 *
SIH	282 ± 149 *	659 ± 263 *†	470 ± 262 *†	3224 ± 76 †

[a]The A549 cells were pretreated with 100 μM of the chelator for 30 min at 37 °C before DOX (1μM), BLM (1 μg/mg) or H_2O_2/Fe^{2+} (2 mM /10 μM) were added. The exposure period was 48 h. Values represent the mean ± SD ($n \geq 3$). Data are expressed as a percentage of TBARS formation relative to untreated controls. Control values were 0.29 ± 0.06 nmol MDA/mg.

[b]Cells not pretreated with any chelator

[c]Cells not treated with any oxidative stress-inducing agent

*$p < 0.05$ vs. untreated control , †$p < 0.05$ vs. DOX, BLM and H_2O_2/Fe^{2+}, respectively

The intracellular antioxidant GSH decreased after DOX and to a lesser extent also after the BLM treatment (Table III). Notably, the chelators that were effective in reducing the H_2O_2/Fe^{2+}-induced decrease in GSH (i.e. SIH and DFO) failed to protect the A549 cells against the fall in GSH induced by DOX and BLM. On the contrary, the chelators DXZ and monoHER, which did not prevent the loss in GSH induced by H_2O_2/Fe^{2+} significantly protected against the DOX induced GSH decrease. Only monoHER prevented BLM-induced GSH depletion.

Table 3. Effects of various iron chelators on DOX, BLM and H_2O_2/Fe^{2+}-induced GSH decrease in the A549 cells[a]

Chelator	Control[c] (%)	DOX (%)	BLM (%)	H_2O_2/Fe^{2+} (%)
Control[b]	**100**	50 ± 9 *	82 ± 5 *	8 ± 1 *
DXZ	99 ± 7	81 ± 13 *†	82 ± 4 *	11 ± 5 *
DFO	56 ± 5 *	26 ± 13 *†	51 ± 6 *†	39 ± 5 *†
monoHER	103 ± 10	83 ± 15 †	98 ± 5	10 ± 4 *
PIH	102 ± 16	50 ± 10 *	76 ± 7 *	14 ± 6 *
SIH	54 ± 4 *	44 ± 6 *	81 ± 18 *	109 ± 11 †

[a]The A549 cells were pretreated with 100 μM of the chelator for 30 min at 37 °C before DOX (1μM), BLM (1 μg/mg) or H_2O_2/Fe^{2+} (2 mM /10 μM) were added. The exposure period was 48 h. Values represent the mean ± SD ($n \geq 3$). Data are expressed as a percentage of GSH decrease relative to untreated controls. Control values were 51 ± 5 nmol GSH/mg.

[b]Cells not pretreated with any chelator

[c]Cells not treated with any oxidative stress-inducing agent

*$p < 0.05$ vs. untreated control; †$p < 0.05$ vs. DOX, BLM and H_2O_2/Fe^{2+}, respectively

The effects of iron chelation on the antiproliferative and proapoptotic activity of DOX and BLM

The influence of the two anticancer drugs and the iron chelators on cell proliferation was followed in time (12, 24 and 48 h; Figure 4). In comparable concentrations (1 μM of DOX, 1 μg/ml of BLM), DOX was a much more efficient antiproliferative agent than BLM with IC_{50} values of 0.03 μM and 5 μg/ml, respectively after the 48 h exposure (data not shown). Among the chelators, DFO, DXZ and SIH (all at 100 μM) exerted their own remarkable antiproliferative effect. In contrast, PIH was only moderately effective and monoHER did not have any direct influence on cell proliferation. When combined with DOX, the chelators DFO, PIH, SIH did not hamper the cytotoxic properties of DOX. A minor attenuation of the antiproliferative effect of DOX by DXZ and monoHER after 48 h incubation was observed. The antiproliferative effect of BLM, which could not be observed earlier than after 48 h of treatment, was not diminished by any of the chelators.

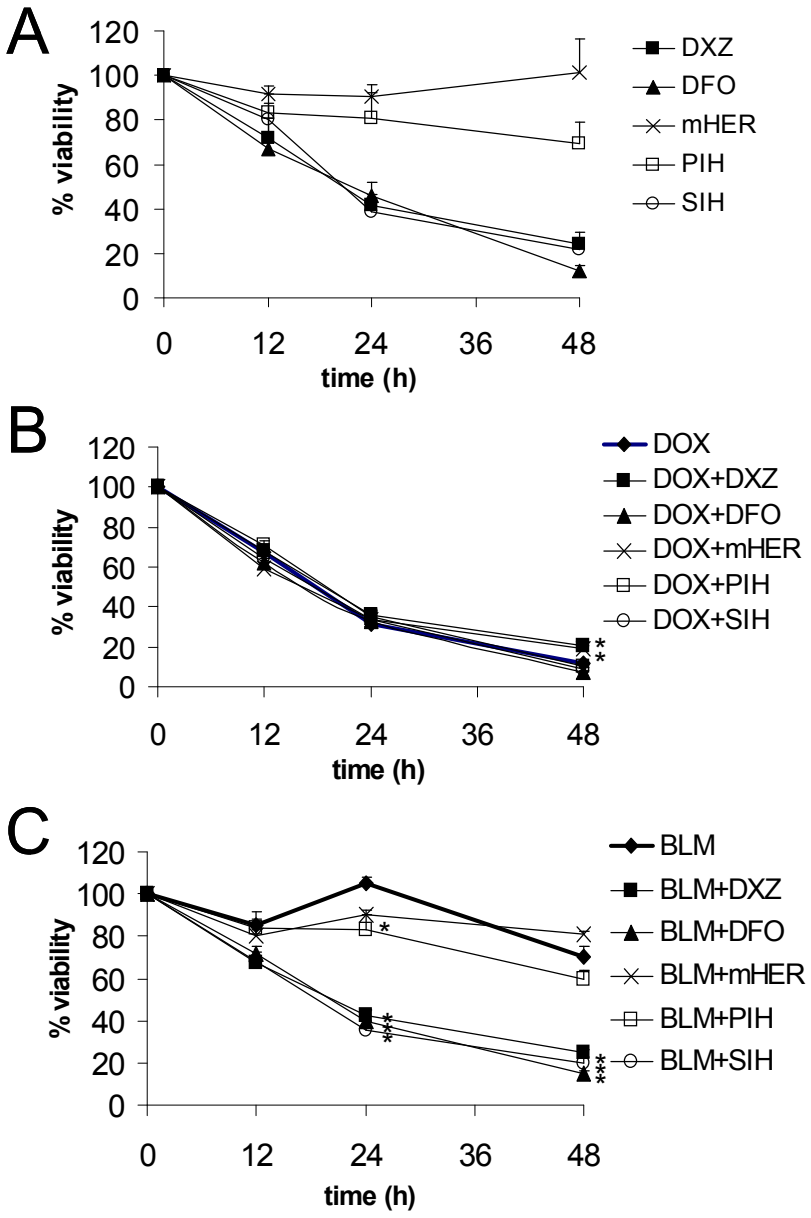

Figure 4 Inhibition of tumour cell proliferation by various iron chelators (A) and the effects of these chelators on the antiproliferative activity of DOX (B) and BLM (C). The A549 cells were pretreated with the chelators (100 μM) for 30 min at 37 °C before 1 μM of DOX or 1 μg/mg of BLM were added. Cells were incubated for further 12, 24 or 48 h, harvested by trypsinization and viable cells were counted using Trypan Blue staining. Cell viability (mean ± SD) is expressed as percentage of viable control (untreated) cells at the three respective time points. $^{*}p < 0.05$ vs. DOX and BLM, respectively. For sake of clarity, significance vs. control is not indicated in the graphs.

To investigate the nature of cell death induced by DOX, BLM and the chelators we employed the caspase-3 activity assay to quantify apoptosis in time (Figure 5).

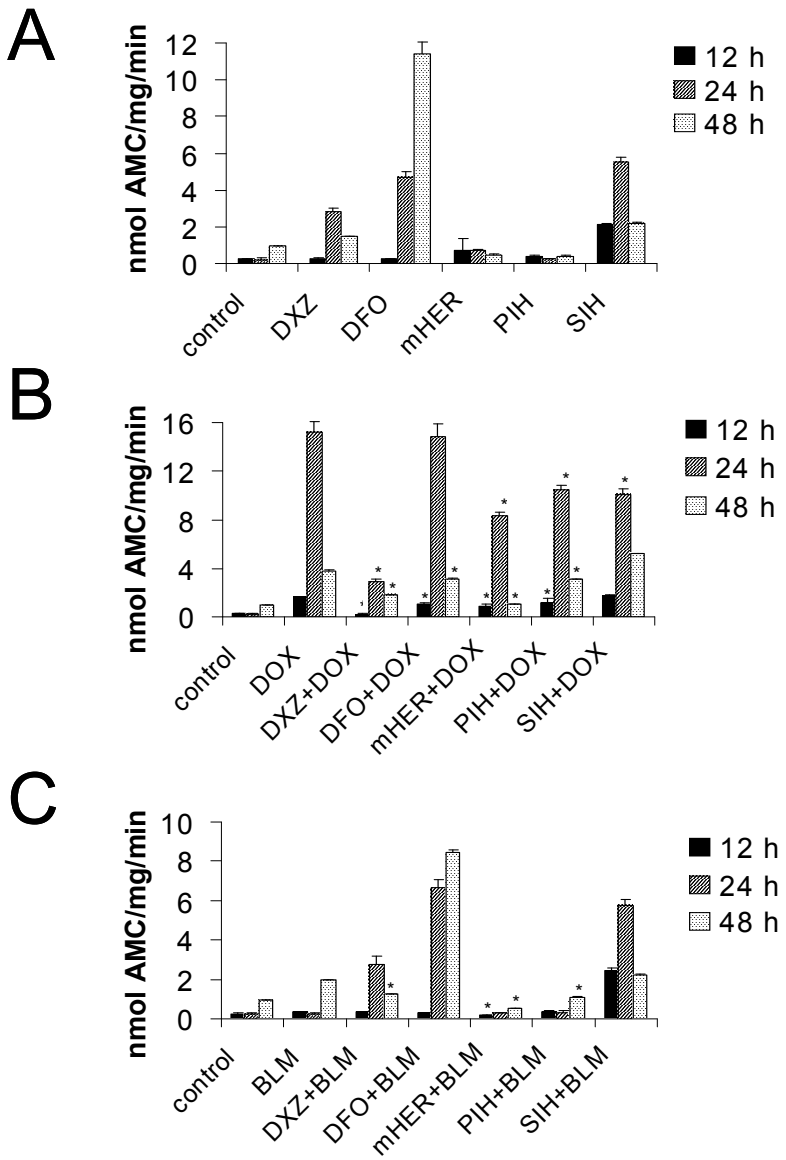

Figure 5. Induction of apoptosis in the A549 cells treated by iron chelators (A) and the effects of these chelators on the DOX and BLM-induced apoptosis (B and C, resp.) at 12, 24 and 48 h. Caspase-3 activity was measured as described in Materials and Methods. The results are expressed as nmol of the fluorescent product (AMC) formed by a caspase-3 cleavage of its specific substrate. Data are given as means ± SD. $^{*}p < 0.05$ vs. DOX and BLM, respectively. For sake of clarity, significance vs. control is not indicated in the graphs

The onset of apoptosis in A549 cells was much more rapid with DOX than with BLM. With DOX, an increase in caspase-3 activity was detected as early as after 12 h of incubation with maximum activity after 24 h, whereas BLM only induced apoptosis after 48 hours of incubation. Moreover, BLM-induced caspase-3 activation was relatively weak compared to the effect of DOX. Among the chelators, PIH and monoHER did not induce apoptosis whereas DXZ, SIH and DFO acted as pro-apoptotic agents, SIH being able to induce the earliest cellular response (at 12 h). All chelators, except for DFO, reduced the DOX-induced apoptosis after 24 h, where the differences between the groups were the most obvious. DXZ was the most efficient chelator in this respect. At 12 and 48 h, SIH was the only chelator, which did not cause a significant decrease in apoptosis induced by DOX. Among the chelators that prevented DOX-induced apoptosis, DXZ and monoHER were the most effective compounds throughout all time points.
Interestingly, we observed that the treatment of the cells with DFO+DOX caused less caspase-3 activation than DFO alone. Evaluation of the effects of the chelators on BLM-induced apoptosis was only meaningful after 48 h incubation. MonoHER, PIH and DXZ effectively reduced BLM-induced caspase-3 activation while SIH and DFO did not.

Discussion and conclusions

We investigated (1) the role of iron in the oxidative stress-mediated toxicity of DOX and BLM and (2) the consequences of iron chelation on the antiproliferative effects of DOX and BLM. To this end, we used the A549 human lung adenocarcinoma cells and five different chelators of iron whose iron-chelating capacity has been well documented in a variety of *in vitro* and *in vivo* models. DXZ (ICRF-187) is a cell permeable pro-drug of an EDTA-like chelator (known as ADR-925) and is approved for clinical use in the prevention of DOX cardiotoxicity. DXZ was also shown to act against hypoxia-reoxygenation damage (Hasinoff, 2002) and more recently, it has successfully been used to prevent the extravasation tissue injury by anthracyclines (Langer et al., 2000; Hasinoff, 2006). DFO is a strong hydrophilic iron chelator that is currently used for the treatment of iron-overload diseases. MonoHER is a flavonoid, which was shown to protect against DOX cardiotoxicity (Abou-El-Hassan et al., 2003a; Van Acker et al., 2000) and recently entered phase II clinical trials. It has both antioxidant properties as well as iron-chelating capacity

(Haenen et al., 1993; Van Acker et al., 1998). PIH and SIH are two low-molecular, lipophilic iron chelators of the aroyl hydrazone class. (Ponka et al., 1979; Baker et al., 1992; Hoy et al., 1979; Cabantchik et al., 1996).

We show that the chelators presented here induce different cellular responses in many respects. The most striking difference in the activity of the compounds is the one between PIH and SIH, the most structurally related compounds of this study. A mere replacement of the pyridoxal moiety for the salicylaldehyde moiety completely changed the behaviour of the molecule in the cell, PIH being relatively intact whereas SIH acted as a pro-oxidant and apoptosis inducer. On the other hand, SIH was as an excellent protector against H_2O_2/Fe^{2+}-induced injury. In EPR experiments, both chelators were equally effective in prevention of $HO^{\bullet}$ formation induced by H_2O_2/Fe^{2+}. Their effect was comparable to that of DXZ and larger than that of DFO and monoHER. However, of all the chelators tested only SIH and partially DFO protected the A549 cells against H_2O_2/Fe^{2+}-induced oxidative damage (as documented by TBARS formation, LDH leakage and GSH consumption). The different efficacy of these compounds is probably due to different lipophilicity, i.e. membrane permeability. Sufficient lipophilicity is important to allow the compounds to reach the cellular compartments where ROS are produced. This would certainly explain the effect of SIH, which was previously also found to be protective against H_2O_2-induced injury in isolated cardiomyocytes (Horackova et al., 2000) and in the H9c2 cardiomyoblast cells (Simunek et al., 2005a). However, it remains somewhat puzzling with regard to DFO, a relatively large, water-soluble molecule with a low partition coefficient and molecule. Although the cellular protection by DFO was weaker than that of SIH, DFO was still more effective than other more lipophilic chelators - e.g. PIH. Membrane permeability is therefore not the only prerequisite of the action of an iron chelator in the cell. For example, its affinity to iron and stability of the iron-chelator complexes might be of importance. Although DFO is not freely diffusible through the biological barriers, it is known that it is taken up by the cells by endocytosis (Persson et al., 2003). The effective antioxidant behaviour of DFO is also because it is a hexadentate chelator, which binds to all six coordination sites of iron making it unreactive. On the other hand, we have found that in the A549 cells, DFO and SIH were able to induce considerable oxidative stress on their own (as demonstrated by GSH and lipid peroxidation assays). However, when the cells were preincubated with DFO or SIH and subsequently exposed to H_2O_2, these chelators efficiently prevented the oxidative damage. DXZ

was shown to have relatively weaker pro-oxidant properties while PIH and monoHER had no such effects at all. In conclusion, SIH and partially also DFO protected against H_2O_2/Fe^{2+}-induced oxidative stress, whereas DXZ, monoHER and PIH did not.

Next, we investigated the effect of iron chelation on DOX and BLM-induced toxicity. Based on preliminary experiments, the concentrations of 1 μM and 1 μg/ml* were found to be optimal for DOX and BLM-induced toxicity, respectively. At these concentrations both DOX and BLM were capable of inducing cellular oxidative damage after a 48 h incubation. A shorter incubation period did not result in significant differences in oxidative stress markers between the groups (data not shown). However, for cell proliferation purposes 12 and 24 h time points were found to be useful and illustrative.

Although DFO and SIH were effective in prevention of oxidative stress induced by H_2O_2/Fe^{2+}, these chelators failed to protect against DOX and BLM-induced oxidative injury. On the contrary DXZ and monoHER reduced DOX cellular toxicity and partially also the toxicity of BLM but had no effect on H_2O_2/Fe^{2+}- induced oxidative stress. These data imply that the Fenton-type reaction is not involved in the toxicity of DOX. We suggest that the lack of protection against DOX and BLM-induced oxidative stress can be explained by involvement of other than hydroxyl radicals in DOX and BLM toxicity while iron chelators protect via prevention of $HO^{\bullet}$ formation. It was previously demonstrated in mice *in vivo* that DOX cardiotoxicity was relieved by administration of lecithinized copper-zinc superoxide dismutase (Den Hartog et al., 2004) showing that it is rather the superoxide radical, which is crucial in pathophysiology of DOX toxicity. As for bleomycin, protection against lung fibrosis in mice overexpressing extracellular SOD was also described (Bowler et al., 2002).

However, there is evidence that iron chelators can protect against DOX cardiotoxicity and dexrazoxane (ICRF-187) became a clinically approved drug in this indication. The same compound was successfully used to prevent BLM lung toxicity in mice *in vivo* (Herman et al., 1995). PIH was shown to attenuate daunorubicin-induced histological and biochemical changes in the rabbit heart *in vivo* and increased the survival of the animals (Simunek et al., 2005b). In isolated hepatocytes, SIH also prevented daunorubicin-induced loss in CYP450 activities

* 1 μg/ml of BLM approximately corresponds to 0.7 μM. Bleomycin is a mixture of several molecules (Bleomycin A, Bleomycin B, …) in variable ratios and thus the molecular weight is not specified in most commercially available products.

(Schroterova et al., 2004). It seems safe to conclude that Fe is somehow involved in the cellular toxicity of DOX but differently than via the previously accepted Fenton-derived production of $HO^{\bullet}$ radicals. Minotti et al. (1999) for example suggested that the anthracyclines (or possibly their C13-alcoholic metabolites) disrupt a delicate iron homeostasis through their interaction with iron regulatory proteins (IRPs), which regulate the expression of transferin receptor and ferritin according to the cellular needs. It was also shown that DOX as well as other redox-cycling agents induce an accumulation of iron in ferritin in both myocardial and neoplastic cells and this effect can be prevented by some iron chelators (DFO but not DXZ) (Kwok & Richardson, 2003). It is, however, not clear, whether and in which way this effect contributes to the anthracycline toxicity.
The cardioprotective potential of another compound, the flavonoid monoHER, although originally selected because of its iron chelating and antioxidant properties, has recently been linked with other features like anti-inflammatory effects, which could be of importance (Abou El-Hassan et al., 2003a). PIH analogues also seem to possess antioxidant properties in addition to their iron chelating capacity (Hermes-Lima et al., 2000). Interestingly, it was shown that systolic heart failure induced by the anthracyclines is accompanied by chronic calcium overload and DXZ was able to restore normal myocardial calcium content (Simunek et al., 2005c). Interference of daunorubicin with calcium-handling proteins such as ryanodine receptor and its normalization by DXZ was also demonstrated (Burke et al., 2000). It is therefore possible that the protective effects by many chelators - including DXZ - are not exclusively due to the iron chelation.
Before introducing new iron chelators in chemotherapy protocols, it is essential to establish whether the compounds do not interfere with the antitumour effect of the cytostatics. Our results have shown that the pretreatment of the A549 cells with 100 μM PIH, SIH and DFO did not affect the antiproliferative effects of either DOX or BLM. On the other hand, we have observed a weak reduction of DOX-induced apoptosis and cell death by DXZ and monoHER. Although clinical trials showed that DXZ did not hamper the efficiency of DOX chemotherapy in patients (Marty et al., 2006), studies in hypertensive rats and in CHO cells, however, confirmed an antagonism (Zhang et al. 1996; Hasinoff et al., 1996). It was suggested that the mechanism, by which DXZ diminishes the antiproliferative effects of DOX is that the active chelatable form of DXZ (ADR-925)-Fe(III) complexes oxidatively degrade α-ketol side chain of DOX possibly changing its antitumour efficiency (Malisza & Hasinoff, 1995). This interaction promotes formation of $HO^{\bullet}$, which would also explain certain pro-oxidant activity of DXZ. On the other

hand, addition of DXZ to the DOX-containing chemotherapy protocols allows increasing the cumulative dose of the anthracycline without concurrent increased cardiac risk. Moreover, it was demonstrated that in vitro DXZ significantly delayed the development of multidrug resistance (MDR), a frequent complication of DOX chemotherapy (Sargent et al., 2001). All the chelators, except for monoHER, were shown to possess antiproliferative properties by themselves (DFO ≈ SIH ≈ DXZ > PIH). It was likely that an additive effect would be observed when these chelators were combined with DOX or BLM due to apparently different mechanisms, by which these compounds inhibit cell growth. Nevertheless, this was not the case. It might be that a possible interference of the chelators with the antitumour activity of the cytostatic drugs is sufficiently counteracted by the antiproliferative action of the chelators. On the other hand, Wu et al. (2004) previously showed that the antiproliferative effect of BLM was not compromised by dexrazoxane in a specially developed dexrazoxane-insensitive CHO cell line in which the confounding effect of the intrinsic antiproliferative effect of the chelator was eliminated.

Three of the five chelators tested (DFO, SIH, DXZ) induced apoptosis in the A549 cells. SIH derivatives (aroylhydrazones) were previously shown to induce apoptosis in the Jurkat T lymphocytes and the K562 cells (Buss et al., 2003) and the chelators with enhanced antiproliferative and pro-apoptotic properties are continuously being developed for further evaluation as new anticancer drugs (Lovejoy & Richardson, 2002). It was suggested that the apoptosis caused by these compounds is triggered by redox-cycling of their iron complexes. (Buss et al., 2004). Other studies demonstrated that DXZ also induced apoptosis in tumour cells, which is likely due its inhibitory action on topoisomerase II (Hasinoff, 2001). Interestingly, DFO was recently shown to act as a cytostatic agent in the MCF-7 breast cancer cells but it caused neither apoptosis nor cell cycle arrest (Hoke et al., 2005). The concentration of DFO used in this study was, however, 4-30 μM compared to 100 μM used in this study. We therefore propose that the mechanism of the antitumour effect of DFO is concentration-dependent. It is well-known that iron is involved in some crucial metabolic pathways in the cell such as DNA synthesis or oxygen transport (Mladenka et al., 2006). We suggest that in high concentration of the chelator, not only free iron, but also the iron bound to the macromolecules might be depleted and cellular apoptosis initiated. Possible differences between various cell types also have to be considered when extrapolating the data collected from the A549 cells to non-cancerous cells, for example the cardiomyocytes. Nevertheless, we suggest that the mechanisms by which the compounds (DOX

and BLM vs. H_2O_2) induce oxidative stress, and the way in which this can be prevented, may not be cell-type dependent. Moreover, our data offer a reasonable argument to support this statement, as the two iron chelators that had been previously shown to protect cardiac cells against DOX toxicity (DXZ, monoHER) also prevented the DOX-induced oxidative damage and increased the cell survival of the A549 cells in the present study.

In conclusion, using the A549 cells we established that (1) the mechanism by which DOX and BLM induce oxidative stress to the cells is not iron-mediated (i.e. Fenton reaction-dependent). We have clearly shown that the ability to chelate iron as such is not the sole determinant of an effective protective compound. (2) The effect of iron chelation on the antiproliferative activity of DOX and BLM is only minor. These findings have impact on the direction in which new protective agents should be developed.

Acknowledgements

This work was supported by Grant Agency of Charles University in Prague (grant No.97/2005) and Ministry of Education of Czech Republic (grant MSM 0021620820). We thank Marc A.J.G. Fischer for his excellent technical assistance.

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III

The effect of new lipophilic chelators on activities of cytosolic reductases and P450 cytochromes involved in the metabolism of anthracycline antibiotics. Studies in vitro.

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Physiol Res 2004; **53**:683-91

Summary

A major obstacle to the therapeutic use of anthracyclines, highly effective anticancer agents, is the fact that their administration results in dose-dependent cardiomyopathy. According to the currently accepted hypothesis, anthracyclines injure the heart by generating oxygen free radicals. The ability of new iron chelators, pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) to protect against lipid peroxidation and their suitable biological, physical and chemical properties make the compounds promising candidates for prevention of anthracycline cardiotoxicity. The effect of PIH and SIH on activities of carbonyl reductase CR (1.1.1.184), dihydrodiol dehydrogenase DD2 (1.3.1.20), aldehyde reductase ALR1 (1.1.1.2) and P450 isoenzymes (CYP1A1, CYP1A2, CYP2B, CYP3A) were studied since these enzymes appear to be involved in the anthracycline metabolism. Possible interferences with the biotransformation effects of the anthracyclines (doxorubicin and daunorubicin) were also investigated. Various chelator, daunorubicin and doxorubicin concentrations were used for *in vitro* study in isolated hepatocytes. A significant decrease of activity of the enzymes under investigation was not observed but at PIH and SIH concentrations that are higher than those presumed to be used in therapy. The results show that *in vitro,* PIH and SIH have no effect on the activities of the enzymes studied and allow us to believe that they will not negatively interfere with the metabolism of co-administered drugs. Most importantly, daunorubicin (Da) and doxorubicin (Dx) significantly reduced cytochrome P450 activity in the hepatocytes while the pretreatment of the cells with both SIH and PIH (50 μM) was able to prevent this decrease and restored the activity to 70 – 90 % of relevant controls.

Introduction

Pyridoxal isonicotinoyl hydrazone (Fig.1, PIH) was studied back in the 1950s as a coenzyme of pyridoxal-dependend enzymes (Davison 1956, Duhault et al. 1967). Later, several authors found that it can chelate Fe ions (Ponka et al.1979a, 1979b, 1994; Hoy et al. 1979; Brittenham 1990) and can be therapeutically used in the treatment of secondary iron overload pathologies such as

β-thalassemias, porphyria cutanea tarda and alcoholic cirrhosis (Pippard 1994). Apart from being used to treat Fe overload, PIH may also be useful against free radical injury (Schulman et al. 1995). At present, the only Fe chelators for widespread clinical use are desferrioxamine (DFO) and dexrazoxane (Lombardo et al. 1996, Wexler 1998). However, their therapeutical administration suffers from several drawbacks – e.g. poor intestinal absorbtion, high cost, potential to develop allergy due to the mast-cell activation effect (Magro and Brai 1983, Lombardo et al. 1996). Last but not least, they require repeated long-term infusions leading to patient´s discomfort. PIH and its analogue SIH (Fig. 1) have been tested *in vivo* in rats after parenteral or *per os* administration (Bláha et al. 1998, Richardson and Ponka 1998). The results indicated that pyridoxal isonicotinoyl hydrazone is not affected by hydrolytic enzymes and low pH in the gastrointestinal tract, is well absorbed by the intestine, has a high affinity to Fe bound in hemosiderin, ferritin and transferin and, at the same time, low affinity to Fe bound in hemoglobin, cytochromes, myoglobin and other cations of physiological significance. Clinical studies in patients with iron overload demonstrated good PIH tolerance and iron excretion, while no distinct adverse effects were observed (Brittenham 1990).

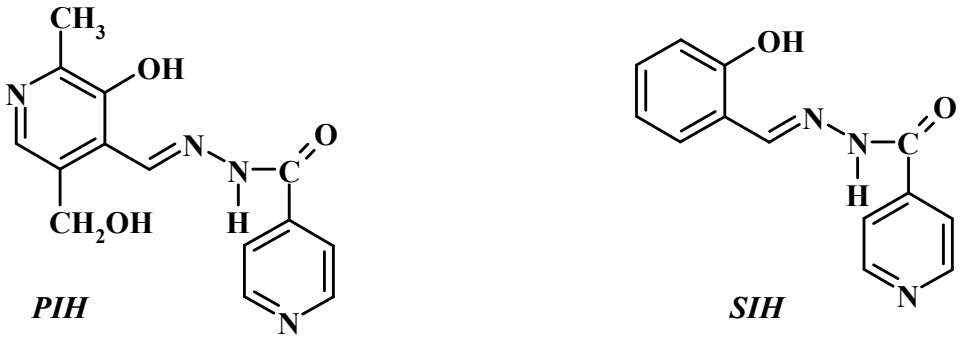

Figure 1. Structure of pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH)

One of the theories explaining the cardiotoxic effects of antracyclines is the ″iron and free radical hypothesis″. According to the hypothesis, anthracyclines impair cardiac function by oxygen free radicals generated in reactions catalysed by cytochromes P450 (CYP) (Hoy et al. 1979, Buzdar et al. 1985, Brittenham 1990, Olson and Mushlin 1990, Rhoden et al. 1993, Goeptar et al. 1994). Cytochromes P450 represent a large super family of heme-containing proteins that play a central role in the metabolism of drugs, other xenobiotics and some lipophilic endogenous substrates. In mammals, the main drug-metabolising families of CYP (CYP1, CYP2, CYP3) are primarily expressed in liver (Testa 1995). CYP3A, CYP2B and NADPH-cytochrome P450 reductase (Goeptar et al. 1994) catalyse reductive transformation of daunorubicin and doxorubicin to semiquinone radicals, which are reoxidized by molecular oxygen. In a single-electron transfer reaction, the oxygen molecule forms superoxide radical that either initiates lipid peroxidation or is converted by dismutase to hydrogen peroxide. The presence of Fe enables subsequent radical chain reactions. Although the free radical hypothesis has received a lot of criticism, it is still the principal hypothesis to explain myocardial injury by anthracyclines.

Another theory links the toxicity of anthracyclines to their toxic metabolite C13-ol, generated by cytosolic carbonyl reductases. Subcellular fractions from liver and heart cells were used to examine the kinetics of the enzymes and organ distribution (Cusack et al. 1993, Propper and Maser 1997). In comparison with its substrate, C13ol was found to be a 30-fold more potent inhibitor of cardiac contractility in rabbit papillary muscles (Olson and Mushlin 1990, Maser and Bannenberg 1994, Minotti et al. 1999). High levels of C13-ol persisting for several days were also found in rabbit heart tissue upon a single-dose administration of the substrate (Cusack et al. 1993, Pouna et al. 1996).

We have investigated the activities of oxidative and reducing enzymes in rabbit liver cells upon the administration of various doses of anthracyclines Da and Dx, PIH and SIH as well as their combinations to examine whether the prospective chelators affect the activities of the enzymes which are involved in the metabolism of the anthracyclines and/or other drugs that might be co-administered to the patients. Such changes of enzyme activity might result in modification of metabolic transformation rates, pharmacological effects or the duration of therapeutic effects of the drugs.

Methods

Animals

Rabbits (*Oryctolagus cuniculus var. Chinchilla*, about 3 kg, from Velaz, Prague, Czech Republic) were fed with standard diet and fasted 12 h prior to the experiment. The animals were sacrified, their liver removed and immediately used for the isolation of the hepatocytes. All the procedures were performed in accordance with the "Guide for the care and use of laboratory animals" (1996) and supervised by the Ethical Committee of the Faculty of Pharmacy, Charles University in Prague, Hradec Kralove, Czech Republic.

Chemicals and reagents

Coenzyme NADPH was obtained from ICN Biochemicals, Inc. California, 7-benzyloxyresorufin, 7-methoxyresorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, Williams´medium E, nutrient mixture F-12 HAM, penicillin G, streptomycin sulphate, albumin were purchased from Sigma-Aldrich. Collagenase was obtained from Sevapharma. Pyridoxal isonicotinoyl hydrazone and salicylaldehyde isonicotinoyl hydrazone – gift of Dr. Ponka, McGill University, Montreal, Canada. Daunorubicin (Cerubidine) was obtained from Rhône-Poulenc – France. Doxorubicin (Adriablastina) was obtained from Pharmacia & Upjohn – Italy. Other chemicals and solvents were of analytical grade and were obtained from common commercial sources.

Cell preparation and culture

Hepatocytes were isolated from male rabbits (without any treatment), by a two-step collagenase perfusion (Seglen 1976) with some modifications. First, the liver lobe was perfused with a salt solution containing a calcium-binding component (0.4 mM EGTA). Subsequently, the liver was perfused (for 5 - 7 min) with a buffer containing collagenase (50 mg /100 ml) and 2 mM $CaCl_2$. The whole perfusion was performed at 37 °C and pH 7.4. Hepatocytes were centrifuged three-times (40x g for 5 min at 4 °C) in cold buffer containing 2 mM $CaCl_2$ and the last pelet was resuspended in plating medium. The cells were plated on 6-cm tissue culture dishes at a density of 3x 10^6cells/culture dish in 3 ml of medium and were left to attach under a humidified atmosphere of air (95 %) and CO_2 (5 %) at 37 ºC. Culture medium (ISOM) was supplemented with 3 % (v/v) fetal bovine serum and antibiotics (60 μg/ml penicillin, 100 μg/ml streptomycin).

After 3-4 hours, the medium was replaced by fresh medium without serum, with 5 μM insulin and antibiotics (3 ml of medium per dish) and the tested compounds were added. Incubation periods were 24 and 48 hours.

Incubations

Chelators PIH and SIH were incubated with the hepatocytes in a monolayer at concentrations of 50, 100, 150, 250 and 750 μM. Anthracyclines (daunorubicin and doxorubicin) were incubated with the cells at a concentration of 10 μM. When combinations of the chelators and the anthracyclines were used, incubations were started with chelators only (50 and 150 μM) and the anthracyclines (10 μM) were added after 60 minutes. Control medium for PIH, SIH and combinations of chelators with doxorubicin contained 1 % (v/v) DMSO, control medium for daunorubicin contained D-mannitol (1.5 μM), control medium for combinations of chelators and daunorubicin contained 1 % (v/v) DMSO and D-mannitol (1.5 μM), control medium for doxorubicin was additive-free.

Isolation of subcellular fractions

At the end of incubation, hepatocytes were homogenized in the ratio of 1:3 in the 0.1 M sodium-phosphate buffer pH 7.4 in the Potter-Elvehjem homogeniser. Microsomal fraction was obtained by fractional ultracentrifugation of the liver homogenate (Gillette 1972). Microsomes were resuspended in the same buffer containing 20 % glycerol and stored in –80 °C.

Protein and enzyme assay

Protein was determined by a modification of the Lowry method (Peterson 1979).

7-Ethoxyresorufin O-dealkylase, 7-methoxyresorufin O-dealkylase, 7-pentoxyresorufin O-dealkylase activity and 7-benzyloxyresorufin O-dearylase activities were determined by a modification of the method described by Prough et al. (1978).

Activities of selected cytosolic reductases were measured spectrophotometrically monitoring the oxidation rate of NADPH at 340 nm (Ohara et al. 1995). Carbonyl reductase, CR (E.C.1.1.1.184) and dihydrodiol dehydrogenase, DD2 (E.C.1.3.1.20) were assayed in kalium-phosphate buffer (0.1 M, pH=6.0). TRIS-HCl buffer (0.2 M, pH=8.5) was used for determination of aldehyde reductase ALR1 (E.C. 1.1.1.2). The reaction mixture contained daunorubicin (0.01 mM) as a

substrate.Enzyme activities were calculated in pmol/min per mg of protein, and expressed as % values of relevant controls.

Cytotoxicity assay

The MTT (tetrazolium dye) assay was performed according to (Martin and Clynes 1993) with some modifications. Cell suspension (1x 10^6cells/ml) was plated into a 96-well plate (50 μl/well) and incubated for 24 and 48 hours with tested compounds. At the end of incubation, 2.5 mg/ml of MTT was added and incubated for 1 hour (37 °C, 5 % CO_2). Formazan crystals were dissolved in HCl-isopropanol. Optical density was measured at 595 nm.

Lactate dehydrogenase (LDH) activity released into the culture medium was measured spectrometrically monitoring the rate of NAD^+ reduction at 340 nm (Clynes 1998).

Cytotoxicity of tested chemicals was expressed as % of control.

Instrumentation

Hepatocytes were isolated and incubated in flowbox AURA2000 M.A.C. BIOAIR Instruments and CO_2-incubator HERAcell Heraeus Instruments. Microsomal fractions were prepared using Avanti J-30I Beckman Coulter centrifuge and Biofuge Stratos Heraeus Instruments. Cytochrome P450 isozymes and reductases activities were measured using luminiscence spectrofluorimeter Perkin Elmer LS 50B and UV-VIS spectrometer Helios β Spectronic Unicam. MTT assay was performed using Micro Plate Reader 550 BioRad.

Results

Figure 2 shows the activities of 3A, 1A1, 1A2 and 2B of P450 isoenzymes upon the addition of PIH and SIH chelators in 50, 100, 150, 250 and 750 μM concentrations after 24-hr incubation. The highest PIH concentration employed significantly reduced the activity of the isoenzymes. In the same-type experiment, SIH in 250 and 750 μM concentrations reduced the activity of the isoenzymes in comparison with controls. The highest concentrations of the chelators used in the experiments are, however, higher than those used in pre-clinical *in vivo* tests and presumed therapeutical doses in humans.

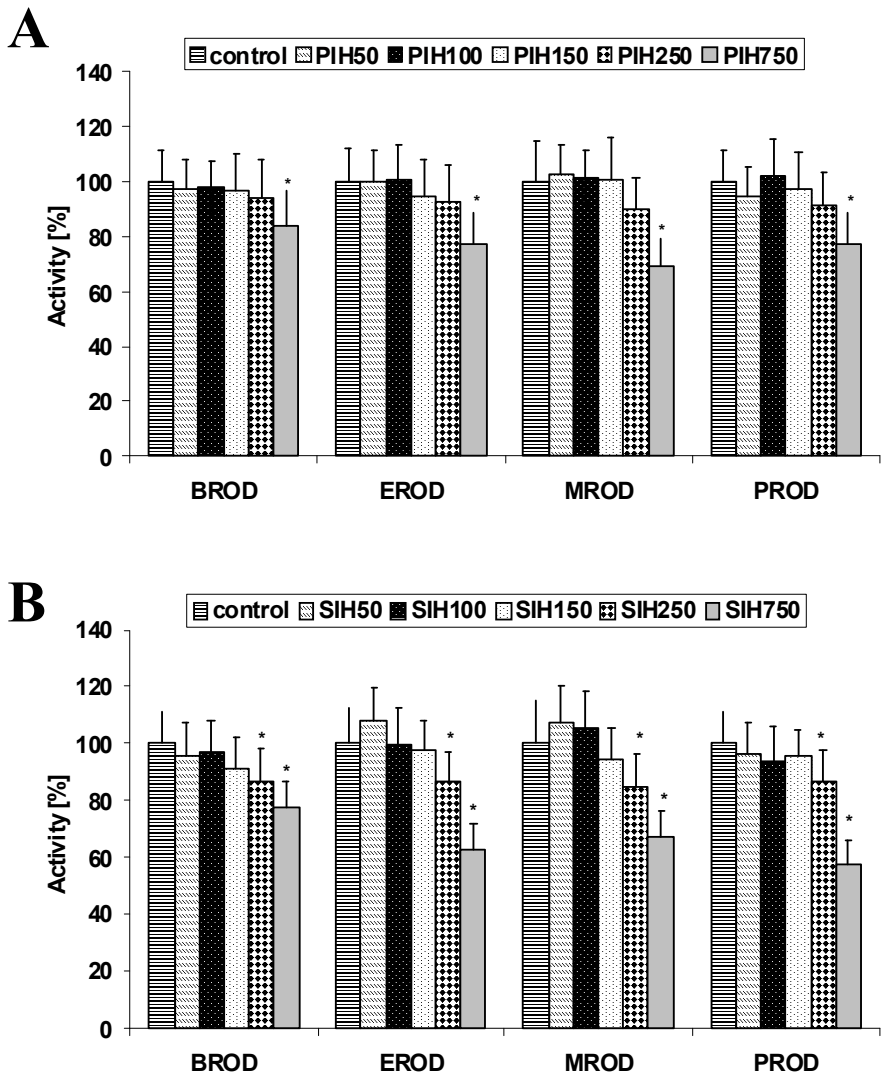

Figure 2. Effect of pyridoxal isonicotinoyl hydrazone (PIH) (A) and salicylaldehyde isonicotinoyl hydrazone (SIH) (B) on P450-dependent monooxygenases. Incubation time of primary culture of hepatocytes from eight rabbits was 24 hours. Hepatocytes were incubated with five concentrations (50, 100, 150, 250 and 750 μM) of PIH or SIH. Values are expressed as % of control (1 % DMSO in hepatocytes medium). Enzymatic activity of control: BROD (benzyloxyresorufin O-dearylase activity) – 41.5 pmol/min/mg, EROD (ethoxyresorufin O-dealkylase activity) – 35.2 pmol/min/mg, MROD (methoxyresorufin O-dealkylase activity) - 27.6 pmol/min/mg, PROD (pentoxyresorufin O-dealkylase activity) – 9.3 pmol/min/mg.
*$P<0.05$ (Student's t-test); significantly different from control

Figure 3 shows that the chelators at the same concentrations (50 - 250 μM) and incubation conditions had no effect on the activities of cytosolic reductases (carbonyl reductase E.C. 1.1.1.184, aldehyde reductase E.C. 1.1.1.2 and dihydrodiol dehydrogenase E.C. 1.3.1.20) in comparison with controls.

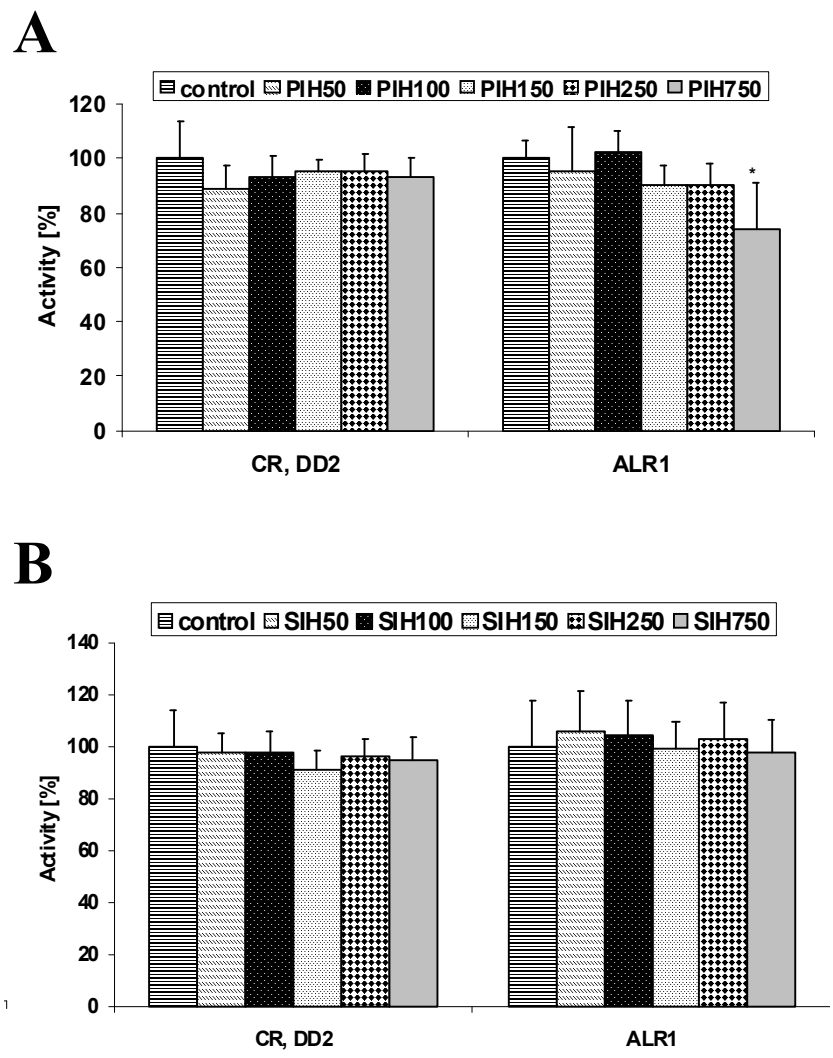

Figure 3. Effect of pyridoxal isonicotinoyl hydrazone (PIH) (A) and salicylaldehyde isonicotinoyl hydrazone (SIH) (A) on reductases in rabbit liver hepatocytes. Incubation time was 24 hours. Hepatocytes were incubated with five concentrations (50, 100, 150, 250 and 750 μM) of PIH or SIH. Values are expressed as % of control (1 % DMSO in hepatocytes medium). CR – carbonyl reductase, DD2 – dihydrodiol dehydrogenase, ALR1 – aldo-keto reductase
*$P<0.05$ (Student's t-test); significantly different from control

The addition of anthracyclines (10 μM) into the incubation mixture significantly reduced the activities of all P450 isoenzymes in comparison with controls (full horizontal line – 100 %). However, the activities were significantly increased when daunorubicin or doxorubicin was combined with chelators (50 a 150 μM) in comparison with the samples incubated with the anthracyclines only. (Figure 4)

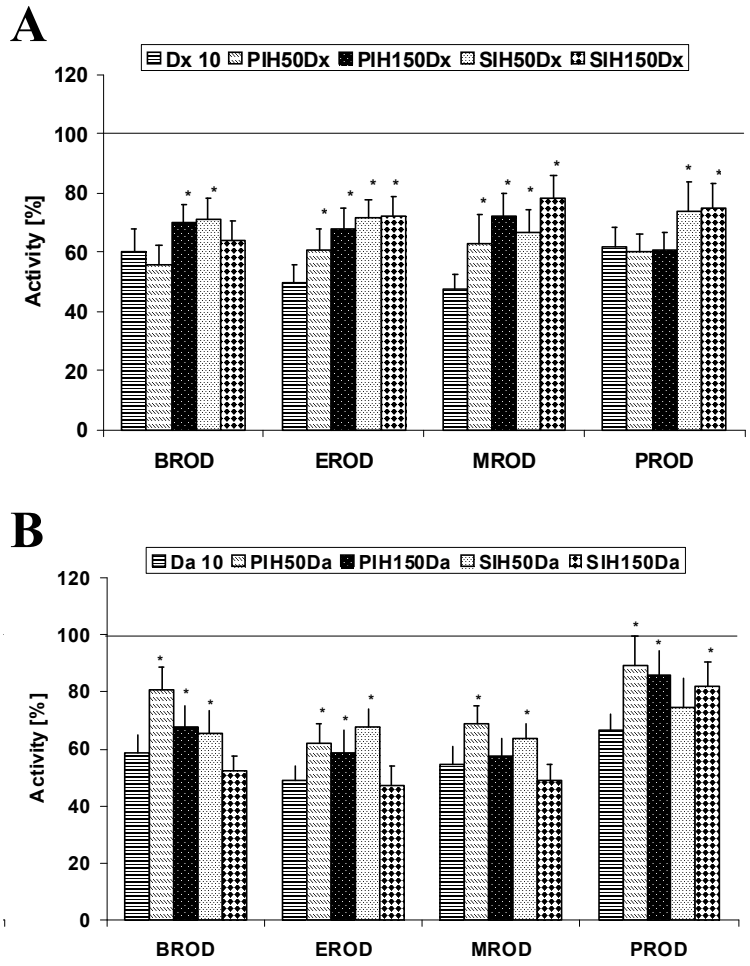

Figure 4. Effect of doxorubicin (Dx) (A), daunorubicin (Da) (B) +/- PIH/SIH on P450-dependent monooxygenases in rabbit liver hepatocytes (24 h incubation). Cells were incubated with Dx, Da (10 μM) +/- PIH or SIH (50 and 150 μM). Anthracycline were added after 60 minutes of incubation with the chelators. DMSO concentration in the medium did not exceed 1% (v/v). Values are expressed as % of control. BROD – benzyloxyresorufin O-dearylase activity, EROD – ethoxyresorufin O-dealkylase activity, MROD –methoxyresorufin O-dealkylase activity, PROD – penthoxyresorufin O-dealkylase activity
*$P<0.05$ (Student's t-test); significantly different from Dx, Da.

No results after 48-hr incubations are presented here since such a long incubation period cannot guarantee reproducible results due to toxic effect/s of the antibiotics on cellular biomolecules. This was confirmed by tests of cytotoxicity (MTT test, LDH activity).

A comparison of the activities of rat hepatocyte cytosolic reductases incubated for 24 hrs with daunorubicin with controls (Figure 5) shows significantly higher CR and DD2 activities in the incubations with the antibiotic. No effect of daunorubicin on ALR1 was observed. The addition of PIH and SIH (50 and 150 μM) chelators had no effect on the activities of the cytosolic reductases in comparisons with the samples incubated with the antibiotics only.

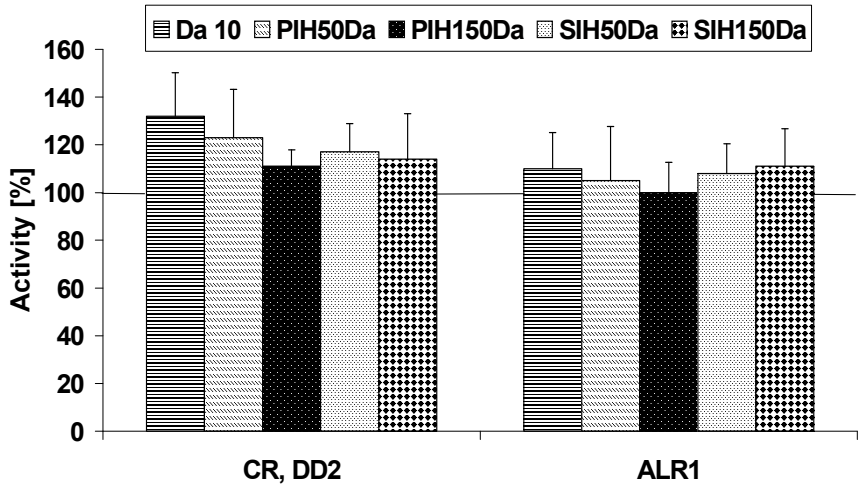

Figure 5. Effect of daunorubicin (Da) and combinations (Da and PIH/SIH) on reductases in rabbit liver hepatocytes. Incubation time was 24 hours. Hepatocytes from six animals were incubated with Da (10 μM) +/- PIH or SIH (50 and 150 μM). Anthracycline was added after 60 minutes of incubation with the chelators. Values are expressed as % of control. DMSO concentration in the medium did not exceed 1 % (v/v). CR – carbonyl reductase, DD2 dihydrodiol dehydrogenase, ALR1 – aldo-keto reductase
*P<0.05 (Student's t-test); significantly different from Da

Discussion

Properties of an ideal chelator defined by Chaberek and Martell (1969) involve sufficient biospecifity, high affinity for iron, low affinity for other cations of biological importance, simple oral administration, good intestinal absorption and bloodstream transport, minimal adverse effects and accumulation in tissues, particularly in fat tissue. The low cost is also a favorable factor.

Desferrioxamine (DFO) is the only clinically available chelator for therapy of iron-overload diseases and the chelator dexrazoxane is an effective cardioprotective drug. (Lombardo et al. 1996, Wexler 1998). Although DFO and dexrazoxane are highly effective drugs, they suffer from a number of adverse effects. A considerable effort has been therefore exerted to develop new, biologically active chelators with improved properties.

Chemical properties of pyridoxal isonicotinoyl hydrazone and its analogue salicylaldehyde isonicotinoyl hydrazone most probably could be administered *per os*. At physiological pH, these compounds are mostly (80 %) neutral molecules; the addition of calcium carbonate effectively prevent their hydrolysis at pH 0.8 – 1.5 or, alternatively they could be administered *per os* before meal. They easily cross the cellular membranes to reach the intracellular iron pools and are well-absorbed by the intestine. Last but not least, their synthesis is inexpensive (Richardson and Ponka 1998).

Since both the chelators are metabolised by hydrolysis (Brittenham 1990), we did not expect they would interact with the binding sites of P450 or cytosolic reductases, but it was necessary to eliminate potential interactions between the very lipophilic xenobiotic/s with other enzymes and/or biomolecules that would eventually affect the activities of the enzymes of interest (i.e. P450 and the anthracycline cytosolic reductases). We therefore studied the effect of PIH and SIH on the activities of these enzymes using primary rabbit hepatocyte cultures. Chelator concentrations (50 μM - 750 μM) selected for those experiments were within the range of expected therapeutic levels except for the highest concentration (750 μM), which only served for experimental purposes.

In the concentration range of 50 – 250 μM, PIH had no effect on the activities of P450 1A1, 1A2, 2B, and 3A, while SIH at the two highest concentrations tested significantly reduced the activities of all the enzymes.

Figure 3 shows the results of the same type of experiment, in which the effect of PIH and SIH on the activities of carbonyl reductase (CR), dihydrodiol reductase (DD2) and aldehyde reductase (ALR1) was examined. None of the followed enzymes was affected by the chelators at concentrations up to 250 μM. Enzyme activities were assayed at their optimal pH; CR and DD (with the same pH optimum 6.0) were assayed as a single enzyme activity. These studies were carried out with respect to the fact that at intracellular pH 7.4, it is carbonyl reductase (EC 1. 1. 1. 184) rather than DD2, that catalyzes the reduction of Da and Dx to C13-ol (Propper a Maser 1997).

Based on the results presented on Figures 2 and 3, it can be concluded that pyridoxal isonicotinoyl hydrazone and its analog salicylaldehyde isonicotinoyl hydrazone do not affect the activities of the oxidases and reductases investigated in this study. The results of cytotoxicity tests (MTT test and LDH leakage test) further support the safety of these drugs – none of the PIH and SIH concentrations tested proved to be cytotoxic.

In subsequent *in vitro* experiments in rabbit hepatocytes, the effect of anthracycline antibiotics on the activities of P450 and cytosolic reductases was studied after 24- and 48-hr incubation, and the results were compared with incubations in which Da or Dx (10 μM) were combined with PIH and SIH chelators (50 and 150 μM). Those concentrations were chosen to mimic plasma levels of the anthracyclines and the chelators used in our *in vivo* experiments (Gersl et al. 1999, Adamcova et al. 2002).

Figure 4 depicts the effect of Da and Dx (10 μM) incubated with hepatocytes in primary culture for 24 hrs. Both anthracyclines significantly depressed the activity of all P450 isoenzymes studied when compared to the controls (100 %). Interestingly, the co-administered chelators (at concentrations of 50 and 150 μM) partly reversed the negative effect of Da and Dx.

The "protective" effect of the chelators, i.e. their ability to chelate iron ions and suppress the generation of hydrogen peroxide and free radicals along the anthracycline biotransformation pathway seems to have another benefit – it reduces cytotoxic effect of the antibiotics. In our

experiments, the addition of PIH or SIH (50 and 150 μM) significantly increased the viability of the hepatocytes incubated with Da or Dx (10 μM), as determined by MTT test and LDH leak test. In comparison with controls, the activity of cytosolic reductases CR and DD2 was significantly increased after the hepatocytes were incubated with Da or Dx (10 μM). A similar rise in reductase activity after the anthracycline treatment was previously observed *in vitro* in tumor cell lines (Ax et al. 2000). The authors suggested that the observed induction of carbonyl reductase activity might be (partially) responsible for the development of acquired resistance to anthracyclines. We are currently working on elucidating the mechanism/s through which healthy liver cell increases the activities or the two cytosolic reductases.

No increase of the activity of any cytosolic reductase studied was observed when the chelators were co-administered with the anthracyclines.

Based on these *in vitro* studies, one can conclude that the PIH and SIH chelators affect neither the activities of P450 1A1, 1A2, 2B, and 3A, nor those of cytosolic reductases CR, DD2 and ALR1, i.e. the enzymes involved in oxidative or reductive transformation of a vast majority of drugs, hormones and other xenobiotics. The role of these enzymes in the toxic effects of the anthracyclines has been well established (Propper and Maser 1997, Ax et al. 2000, Forrest et al. 2000, Lee et al. 2001, Mordente et al. 2001). It is thus evident that the new lipophilic chelators, PIH and SIH do not affect the activity of the enzymes and may hopefully be used in clinical settings. This, however, must be verified in subsequent pre-clinical (animal) and primary clinical studies.

Acknowledgments

This study was supported by Research Project LN00B125 of the Czech Ministry of Education.

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IV

Inhibition study of rabbit liver cytosolic reductases involved in daunorubicin toxication.

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J Enz Inh Med Chem 2005; **20**: 477-483

Abstract

Anthracycline cardiotoxicity represents the most unfavorable side effect of these highly efficient anticancer drugs. Several biotransformation enzymes have been described to contribute to their cardiotoxicity. Besides the activities of CYP450 isoforms which lead to the generation of reactive oxygen species (ROS), the cytosolic reductases have attracted attention nowadays. The reductases known to metabolize anthracyclines to C13-hydroxyanthracyclines are carbonyl reductase CR (1.1.1.184) and the aldo-keto reductases AKR1C2 (1.3.1.20) and AKR1A1 (1.1.1.2). Their participation on the formation of the toxic C13-hydroxymetabolite has been investigated in rabbit using the diagnostic inhibitors of CR and AKR1C2. The kinetics and the type of reductase inhibition exerted by the two inhibitors have been described and it was found that CR was the main daunorubicin reductase at both optimal and physiological pH with the kinetic parameters of daunorubicin reduction of K_m =17.01 ± 1.98 μM; V_{max} = 139.60 ± 5.64 pcat/mg. The IC_{50} values for quercitrin and flufenamic acid were 5.45 ± 1.37 μM and 3.68 ± 1.58 μM, respectively. The inhibiton was uncompetitive for both inhibitors and irreversible in the case of flufenamic acid.

Abbreviations

ROS – reactive oxygen species, DAU-daunorubicin, QUER-quercitrin, FA-flufenamic acid, CR – carbonyl reductase, AKR – aldo-keto reductase, C13-OL – C13-hydroxyanthracycline

Introduction

Anthracyclines are among the most efficient and widely used antineoplastic agents (Hortobagyi, 1997). It was about 50 years ago that daunorubicin (DAU) and doxorubicin were first isolated from the strain of bacteria *Streptomyces* and their antibiotic and antimitotic effects described (Arcamone et al., 1969). However, the anthracyclines were found to be too toxic for the therapy of human infectious diseases and they finally found their place in the chemotherapy of cancer. Many derivatives were synthesized (Giannini, 2004; Monerret, 2001; Cottin et al., 1998) but even now none of them has actually replaced the first two parent compounds of the class – daunorubicin and doxorubicin.

Like many other cancer chemotherapeutics, the anthracyclines possess a series of side effects which complicate therapy such as hair loss, nausea, vomiting etc. but the most inconvenient and potentially life-threatening effect of anthracyclines is their cardiotoxicity (Shadle et al., 2000). The cardiotoxicity can be observed at different levels from acute to chronic and delayed toxicity (Ferrans et al., 1997) from which the latter is especially serious for it can occur suddenly many years after the chemotherapy has finished. It is well known that anthracycline-induced cardiomyopathy is most likely to develop upon administration of cumulative doses of anthracyclines of about 550 mg/m^2 (the exact value depends on the particular anthracycline) (Singal and Illiskovic, 1998). The cause of the cardiotoxicity has not yet been fully explained. There are many hypotheses about its development and several biotransformation enzymes seem to be involved. According to the popular freeradical theory, the cardiac injury results from the redox cycling of anthracyclines which leads to the generation of reactive oxygen species (ROS) and oxidative damage of the cell (Hrdina et al., 2000; Keizer et al., 1990). One-electron redox cycling of a quinone moiety of the molecule is catalyzed by CYP450 isoforms (Goeptar et al., 1993) but some other enzymes have also been proposed to contribute to this proccess (e.g. nitric oxide synthase, NADH dehydrogenase) (Mordente et al.. 2003). However, this freeradical mechanism can hardly explain the development of the chronic toxicity. Moreover, it has been shown (*in vitro*) that the generation of ROS by anthracyclines can paradoxically protect the cardiac cell against iron-mediated toxicity (Corna et al., 2004). The role of iron in a freeradical-independent mechanism of cardiotoxicity has been studied intensively and it was suggested that anthracyclines disturb the homeostasis of iron by interferring with key iron regulatory proteins (Minotti et al., 2001; Cairo et al., 2002).

Some investigators have shown that the cardiotoxicity correlates with the reduction of anthracyclines to their C13-alcoholic metabolites (Cusack et al., 1993; Olson et al., 1990; Boucek et al., 1987) (Fig. 1). This metabolic conversion is catalyzed by cytosolic reductases of the aldo-keto reductase class (AKR) and short chain dehydrogenase class (SDR) (Mordente et al., 2003). The reducing capacity of the heart was shown to be much lower than that of the liver (Propper and Maser, 1997), however, the cumulation of the metabolite in the heart has been described (Cusack et al., 1993) suggesting that the alcoholic metabolites might be transported and selectively trapped by cardiac cells. Three enzymes have actually been described to metabolize

anthracyclines to C13-hydroxymetabolites (Ax et al., 2000): carbonyl reductase CR (1.1.1.184) (Forrest and Gonzales, 2000; Imamura et al., 1993), AKR1C2 (1.3.1.20) (Miyabe et al., 1995; Hara et al., 1996) and AKR1A1 (1.1.1.2.) (Flynn and Green, 1993). The latter two were previously referred as dihydrodiol dehydrogenase DD2 and aldehyde reductase ALR1, respectively. Unfortunately not much is known about the participation of these particular reductases on C13-alcoholic metabolites formation and it seems useful to know which enzyme is the most active regarding anthracycline reduction since this would allow the design of appropriate cardioprotective drugs on the basis of reductase inhibition. In this study we characterized the activities of anthracycline reductases in rabbit liver cytosol, the kinetics of daunorubicin reduction and the parameters of the reductase inhibition with quercetin and flufenamic acid.

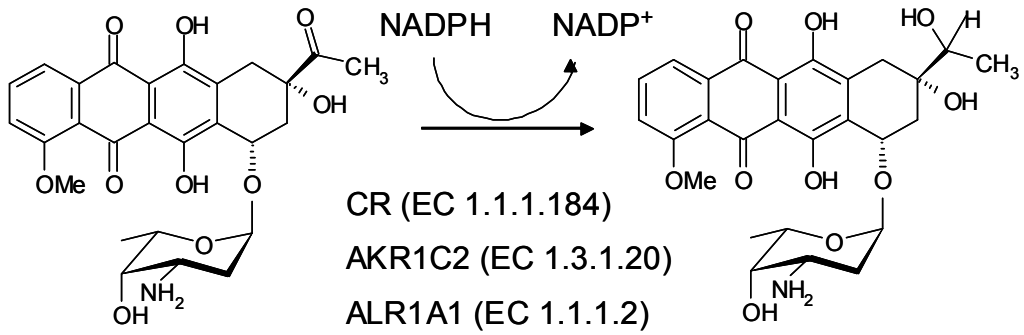

Figure 1. Scheme of metabolic conversion of daunorubicin to its C13-hydroxymetabolite daunorubicinol. The cytosolic reductases involved in this process are indicated.

Matherials and Methods

Animals

Male rabbits (*Oryctolagus cuniculus var.Chinchilla*) about 3 kg weight were obtained from Velaz, Prague, Czech Republic. They were kept on standard laboratory diet *ad libitum*. The animals were sacrificed by bleeding to death under anaesthesia, their liver removed and stored at

–80 °C. All the procedures were permitted and supervised by the Ethics Committee of the Faculty of Pharmacy, Charles University in Prague.

Chemicals and Reagents

Daunorubicin (Cerubidine® inj.) was obtained from Rhône-Poulenc, France. NADPH was obtained from ICN Biochemicals Inc., California, USA. Quercitrin, flufenamic acid and protein assay reagents (BCA kit) were obtained from Sigma-Aldrich. All other chemicals and solvents used were of analytical grade and were obtained from local commercial sources.

Isolation of Liver Cytosolic Fractions

Rabbit livers stored at –80 °C were thawn at room temperature (all the next procedures took place at 4 °C). 20 g of liver tissue per rabbit were cut into small pieces and homogenised with 100 ml of sodium-phosphate buffer pH 7.4 using Potter-Elvehjem´s homogeniser. The homogenate was distributed in 8 cuvettes, the buffer was added to the total volume of 50 mL per cuvette and the homogenate was centrifuged at 5,000 x g. The supernatant was centrifuged at 20,000 x g in new cuvettes. After this, the supernatant was ultracentrifuged at 100,000 x g for 1 hour. The supernatant represented cytosolic fraction and was stored at –80 °C for later use.

Reductase Activity Assay

Activities of selected cytosolic reductases were measured spectrophotometrically monitoring the oxidation rate of NADPH at 340 nm (Felsted and Bachur, 1980; Penning et al., 1984). Kalium-phosphate buffer 0.1 M, pH 6.0 was used for CR and AKR1C2 assay and TRIS-HCl buffer 0.2 M, pH 8.5 was used for AKR1A1 assay. The incubation mixture contained 930 μl of appropriate buffer, 50 μL of cytosolic fraction, 10 μL of NADPH (final concentration 0.1 mM) and 10 μL of daunorubicin (final concentration 0.01 mM).

Inhibition Assays

Quercitrin, the specific inhibitor of CR (Forrest and Gonzales, 2000; Atalla and Maser, 2001) and flufenamic acid (NSAID), the specific inhibitor of AKR1C2 (Miyabe et al., 1995; Hara et al., 1996) were used to separate the activities of the two enzymes at pH 6.0. Inhibitors were dissolved in DMSO so that a series of stock solutions (1000-fold concentrated) was prepared to give final concentrations within the range of 0.5-50 μM. The concentration of DMSO inreaction mixture

never exceeded 0.1%, which did not affect enzyme activity. Dicoumarol was dissolved in 15 mM NaOH to give final concentration between 1-50 μM. Typically, 50 μL of cytosolic fraction and 10 μL of 10 mM NADPH were put in 930 μL of buffer, 1 μL of inhibitor stock solution was added and the reaction mixture was preincubated 5 min. The reaction was then started by adding 10 μL of 1 mM DAU and the residual reductase activity was determined as described above. Sodium-phosphate buffer 0.1 M was used for measuring activity at pH 7.4. The fraction of total reductase activity that was not inhibitable by quercetin was considered to be due to AKR1C2 activity, whereas the fraction of total activity that was not sensitive to flufenamic acid was considered as due to CR activity; these values allowed calculation of the CR/AKR1C2 ratios at pH 6.0 and 7.4.

Reversibility assay (dialysis)

Reversibility of inhibition was tested by comparing the activities of inhibited reactions before and after dialysis. Briefly, after the reductase activity of inhibited sample was determined, the reaction mixture was placed in dialysis tubing (Visking®, MWCO 14,000, Carl Roth GmbH) and dialysed against PBS for 12 h at 4°C with the buffer being changed every 2 h. After 12 h, the contents of each dialysis tube was carefully moved to the cuvette and the reductase activity was measured again after adding 10 μL of 10 mM NADPH and 10 μL of 1 mM DAU. The non-inhibited reaction was dialysed and measured under the same conditions and served as a control to eliminate the influence of spontaneous loss of activity and/or minor changes in the enzyme concentration due to the sample manipulation during the experiment.

Protein Determination

Protein was determined spectrophotometrically using bicinchoninic acid (Brown et al., 1989), with bovine serum albumin as a strandard.

Data analysis

The data were analysed using Graph Pad Prism v. 3.00. All values are given as means of at least 3 measurements ± SD.

Results

The reductase activities of CR, AKR1C2 and AKR1A1 were determined spectrophotometrically at their respective optimal pH (Fig. 2). However, the pH optimum for CR and AKR1C2 is the same (6.0) so that we were initially not able to distinguish between the activities of the two enzymes and for this purpose we used quercitrin and flufenamic acid, the diagnostic inhibitors of CR and AKR1C2, respectively.

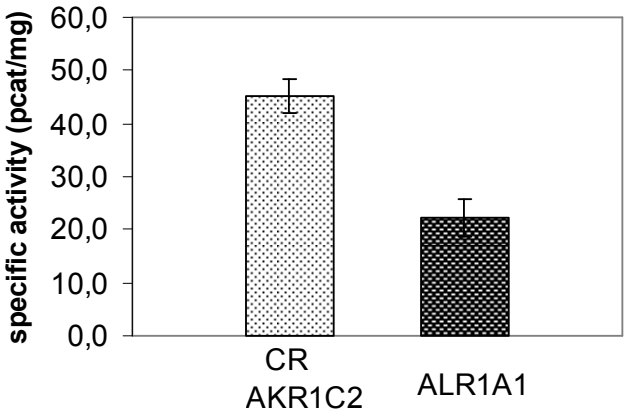

Figure 2. The activities of the three cytosolic reductases measured at their optimal pH. The first column represents the sum of activities of CR and AKR1C2 with the pH optimum at 6.0. The activity of AKR1A1 could be measured separately at the pH 8.5.

We measured the dependence of residual reductase activity on increasing inhibitor concentration and from the resulting inhibition curves (Fig. 3) estimated the IC_{50} values as 5.45 ± 1.37 μM for quercitrin and 3.68 ± 1.58 μM for flufenamic acid. We also evaluated the contribution of CR and AKR1C2 to the total activity at pH 6.0 which was 3:1. The test of inhibitor sensitivity was repeated at pH 7.4 as well, to get closer to physiological conditions and we realized that the difference between CR and AKR1C2 activity is then even more pronounced – in the ratio of 5:1. As the method for reductase activity determination is based on measuring the rate of NADPH oxidation instead of direct product determination (C13-OL) it could be regarded as relatively non-specific. We therefore investigated the possible interference of another cytosolic NADPH-dependent reductase which is known to metabolize daunorubicin, DT-diaphorase (EC 1.6.99.2).

DT-diaphorase reduces the quinone moiety of the tetracyclic ring of daunorubicin and is not involved in C13-OL formation but theoretically it could interfere with our kinetic study. However, it was found that that dicoumarol, an inhibitor of DT-diaphorase, had no effect on NADPH-dependent reduction of daunorubicin in our system indicating that the reduction of quinone moiety of daunorubicin did not interfere with the determination of C13-carbonyl reduction (data not shown).

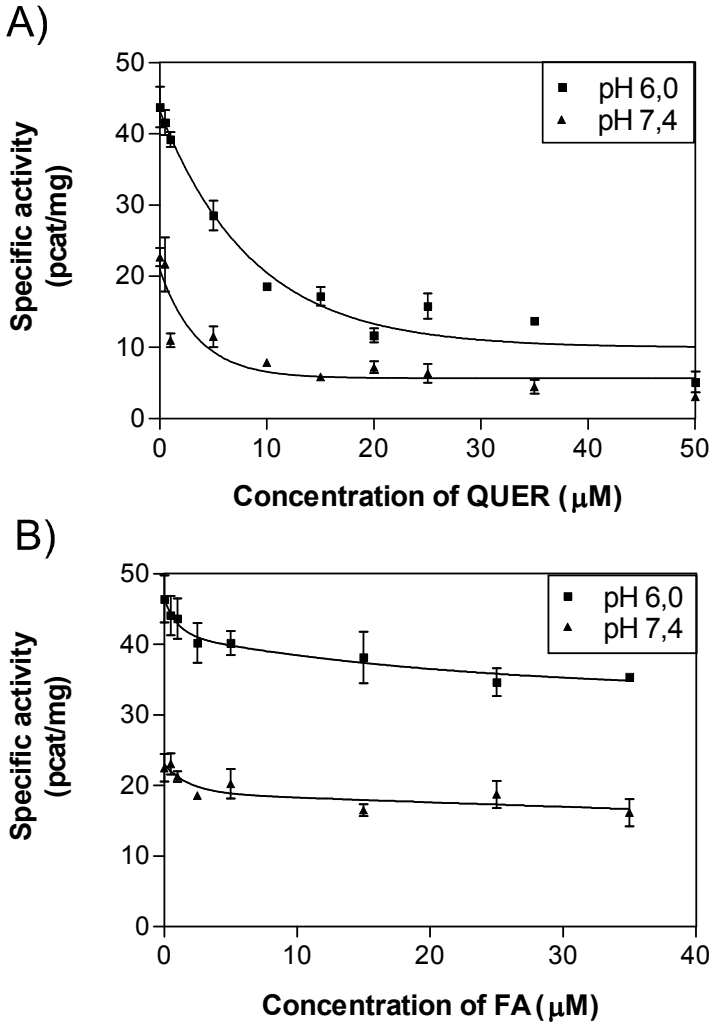

Figure 3. The inhibition curves of quercitrin (A) and flufenamic acid (B) – pH 6.0 and pH 7.4 comparison. The participation of CR and AKR1C2 to the total reductase activity was calculated to be 3:1 at pH 6.0 (optimal) and 5:1 at pH 7.4 (physiological). The IC50 values at pH 6,0 were estimated to be 5.45±1.37 μM for quercitrin and 3.68±1.58 μM for flufenamic acid

As the next step we were interested discovering the mechanism of reductase inhibition exerted by quercitrin and flufenamic acid (this was done at optimal pH 6.0). Initially, the kitetics of non-inhibited daunorubicin reduction were studied (Fig. 4) and gave K_m =17.01 ± 1.98 μM and V_{max} = 139.60 ± 5.64 pcat/mg.

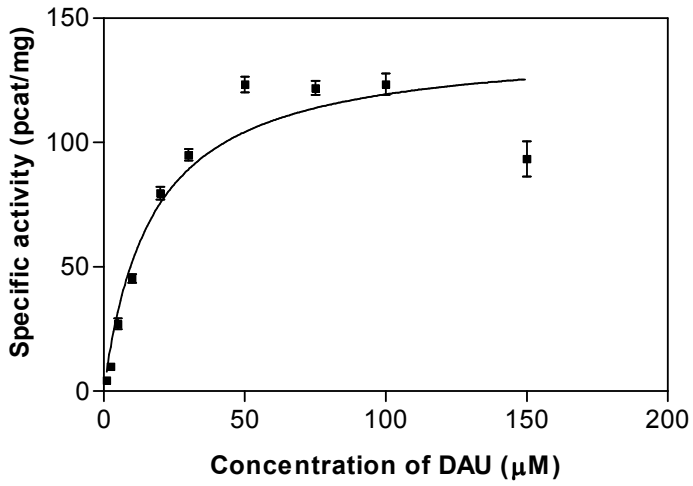

Figure 4. Michaelis-Menten kinetics of daunorubicin reduction at pH 6.0. The kinetic parameters calculated from the curve were: K_m = 17.01±1.98, V_{max} = 139.60±5.64.

Four different concentrations of substrate (5-30 μM) and two concentrations of inhibitors (the lower concentration immitated its IC_{50} value and the other one was 3-fold higher) were used. The kinetics of the inhibited reactions were investigated (Fig. 5), the data were transformed according to Lineweaver and Burk and the K_i values were calculated for each concentration of inhibitor (Table 1). An uncompetitive mechanism of inhibition was found in both inhibitors (Fig. 6). However, we ar aware that attempting to estimate the kinetics of the minore component (AKR1C2) brought certain difficulties. The differences between the inhibitor concentrations were small and the error became relatively large so that the calculated Ki value for flufenamic acid presented here did not come out as accurate as desired.

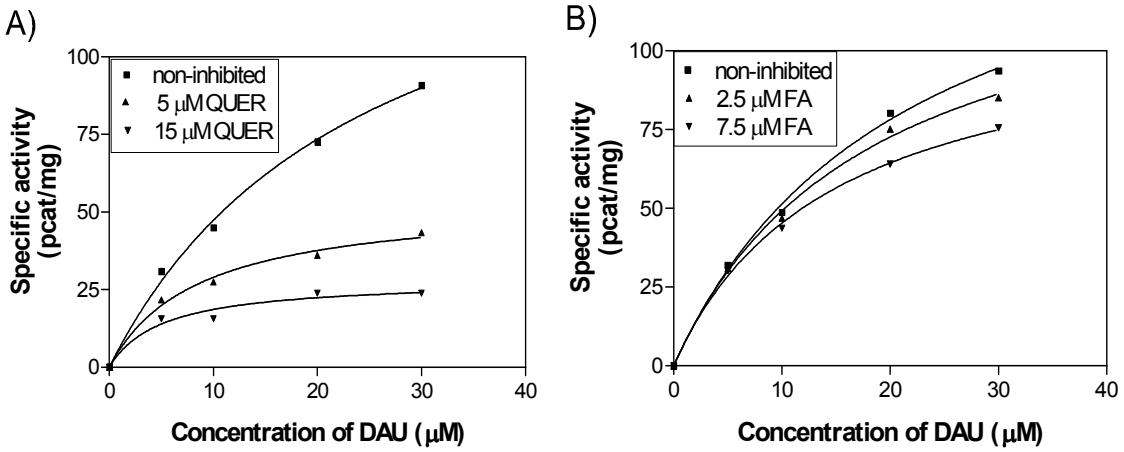

Figure 5. Michaelis-Menten kinetics of daunorubicin reduction at pH 6.0 inhibited by quercitrin (A) and flufenamic acid (B) in two different concentrations. Comparison with non-inhibited reduction.

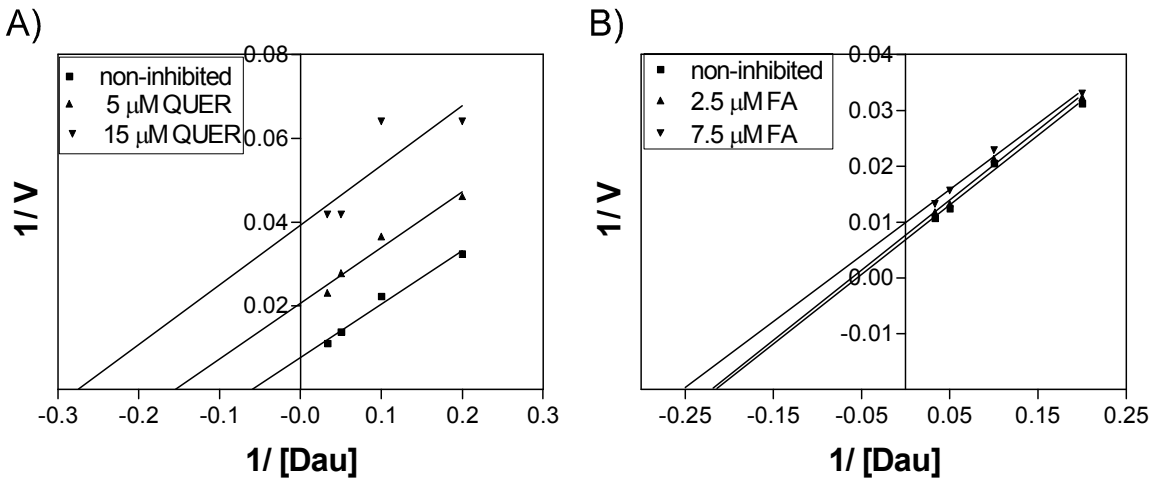

Figure 6. Linearization of the data according to Lineweaver and Burk. Inhibition of daunorubicin reduction by quercitrin (A) and flufenamic acid (B) (at pH 6.0).

Table 1. IC_{50} values, K_i constants and inhibition types of quercitrin and flufenamic acid for rabbit liver carbonyl reductase CR and aldo-keto reductase AKR1C2, respectively

Inhibitor	IC_{50} value (μM)[†]	[i] (μM)	K_i constants (μM)	Inhibition type
Quercitrin	5.45 ± 1.37	5	3.21	Uncompetitive/ Reversible
		15	7.23	
Flufenamic acid	3.68 ± 1.58	2.5	20.13*	Uncompetitive/ Irreversible
		7.5	14.06*	

*see text, [†]DAU concentration = 10 μM

The reversibility of inhibition was tested by measuring the rate of inhibition before and after the dialysis and comparing these values with those of control (i.e. non-inhibited) sample to avoid misinterpretation of irreversibility of inhibition due to possible loss of activity during dialysis. The inhibition of CR by quercitrin was found to be reversible while flufenamic acid acted as an irreversible inhibitor of AKR1C2 (Fig. 7).

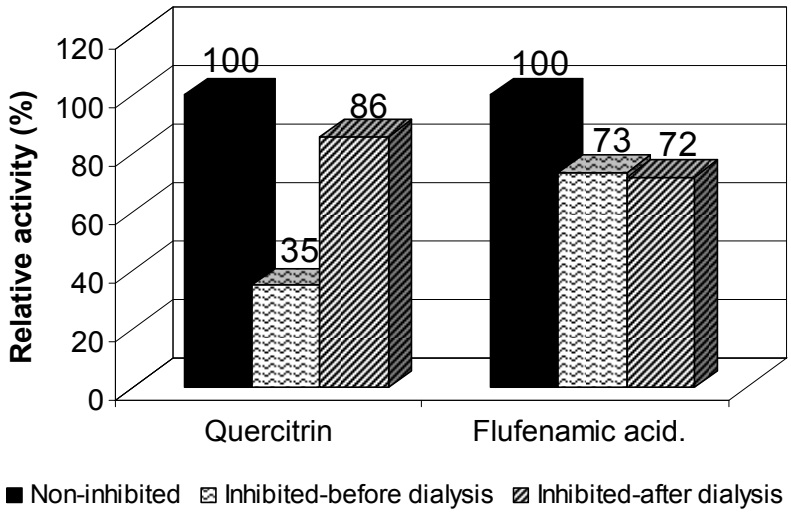

Figure 7. Inhibition reversibility assay. The reduction was inhibited by 10 μM quercitrin and 5 μM flufenamic acid to 35% and 73% of control, respectively. After 12 h of dialysis in PBS the inhibition by quercitrin was reversed and the activity reached 86% of control. In case of flufenamic acid no significant changes in activity were observed due to the dialysis suggesting reversible inhibition of CR by quercitrin and irreversible inhibition of AKR1C2 by flufenamic acid.

Discussion

The cardiac toxicity of anthracyclines is the main obstacle of their clinical use (Shadle et al., 2000). This is quite unfortunate because anthracyclines are otherwise very efficient for the chemotherapy of a number of solid tumors as well as haematological malignancies (Hortobagyi, 1997). The mechanism of the cardiotoxicity development is not clear but it is believed that it is different from those toxic effects that anthracyclines exert against cancer cells (Kluza et al., 2004). The generation of ROS during the redox-cycling of anthracyclines is well described (Hrdina et al., 2000; Keizer et al., 1990) and generally accepted as an important factor that can contribute to the cardiotoxicity but it is certainly not the only one. The role of cytosolic reductases have also been implied (Mordente et al., 2003; Propper and Maser, 1997; Ax et al., 2000) and the cardiodepressive effects of the secondary alcoholic metabolites were described (Cusack et al., 1993; Olson et al., 1990). It has also been proved that the metabolites are cumulated in the heart and moreover that they are less potent cytostatic drugs than the parent compounds (Ax et al., 2000). Three cytosolic reductases (CR, 1.1.1.184; AKR1C2, 1.3.1.20; AKR1A1, 1.1.1.2) have been described as potentially participating on C13-carbonyl reduction of anthracyclines (Ax et al. 2000), however, not much is known about the contribution of the particular enzymes to the formation of C13-OL. It was previously indicated that AKR1A1 which has the pH optimum at 8.5 was not sensitive to its specific inhibitors (barbiturates) at pH 7.4 suggesting it was not involved in the process of C13-OL formation from daunorubicin under physiological conditions (Propper and Maser, 1997). In that study the authors also showed that carbonyl reductase was inhibited by its inhibitor at physiological pH and suggested that this single enzyme was responsible for the C13-OL formation. However, the third enzyme – AKR1C2 was not investigated. The authors formulated their conclusions on the basis of a kinetic study at pH 7.4 which they indicated to be a single enzyme kinetics. The slight deviations from this model were explained by non-enzymatic daunorubicinol formation. Therefore it was our interest to find out whether it could be due to contribution of enzymatic reduction by AKR1C2.

This study was designed to estimate the contribution of CR and AKR1C2 to the total reductase activity at pH 6.0 (optimal) as well as at pH 7.4 (physiological). Using diagnostic inhibitors of the two enzymes it was found out that the CR/AKR1C2 ratio is 3:1 at pH 6.0 and 5:1 at pH 7.4. These results indicate that CR is the major enzyme of daunorubicin reduction but there is still

some residual activity due to AKR1C2 even at physiological pH and this should not be neglected. On the other hand the Propper´s group observation was confirmed that AKR1A1 was not not active at pH 7.4 (Propper and Maser, 1997). This is due to the fact that the residual reductase activities in the presence of quercitrin or flufenamic acid altogether accounted for 100% of activity so that it is very unlikely that any enzyme other than CR and AKR1C2 participates in daunorubicinol formation at pH 7.4.

Uncompetitive inhibition was found for both inhibitors of daunorubicin reduction in this study, unfortunately with low accuracy in the case of flufenamic acid which was unavoidable while working with the whole cytosolic fraction and not using purified enzymes. As for AKR1C2, it is evident that the members of AKR1C class have been much more studied in relation to the oxidation of the *trans*-dihydrodiols of aromatic hydrocarbons and their role in carcinogenesis (Palackal et al., 2002; Burczynski et al., 1999). The uncompetitive inhibition of AKR1C2 by flufenamic acid was previously described with respect to $NADP^+$ (i.e. in the direction of oxidation) (Hara et al., 1996). Enzymes, however, do not necessarily have to exert the same kinetic properties for the reactions of oxidation and reduction, which is why we performed our own experiments using daunorubicin as the substrate of interest and examined its NADPH-dependent reduction. No information about the mechanism of inhibition of CR by quercitrin are available in the literature, where quercitrin was only described as a noncompetitive inhibitor of lens aldose reductase (EC 1.1.1.21) (Varma et al., 1975).

In conclusion, we described here how CR and AKR1C2 participated on C13-OL formation and that CR was the more important enzyme in this process and, moreover, we characterised the mechanism of inhibition of these enzymes by their specific inhibitors. This information can be especially valuable for designing the new effective cardioprotective agents since it is clear that the C13-OL metabolites are at least partially responsible for the anthracycline-induced cardiotoxicity. It is of interest that quercitrin which belongs to a class of flavonoids is a potent inhibitor of C13-OL formation from daunorubicin. Flavonoids have been extensively studied as potential cardioprotectors since they act as antioxidants and iron-chelating agents (Korkina and Afanas'ev, 1997). With regard to the freeradical theory and the involvement of iron in the development of cardiotoxicity it seems that the flavonoids could offer good multi-factorial protection. However, the group of flavonoids (either natural or semi-synthetic) is huge and while the relations between their structure and their antioxidant, and iron-chelating properties have been

at least partially described in several studies (van Acker et al., 1996, 1998), very little is known about the correlation with their inhibitory/induction effects which could perhaps be crucial for their clinical efficacy in protecting the heart from anthracycline-induced injury.

Acknowledgements

This work was supported by the Research Centre LN00B125 of Czech Ministry of Education and GA ČR No. 305/03/1511

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V

Flavonoids as protectants against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductases

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(submitted)

Abstract

Anthracycline antibiotics (e.g. doxorubicin and daunorubicin) are among the most effective and widely used anticancer drugs. Unfortunately, their clinical use is limited by the dose-dependent cardiotoxicity. Flavonoids represent a potentially attractive class of compounds to mitigate the anthracycline cardiotoxicity due to their iron-chelating, antioxidant and carbonyl reductase-inhibitory effects. The relative contribution of various characteristics of the flavonoids to their cardioprotective activity is, however, not known. A series of ten flavonoids including quercetin, quercitrin, 7-monohydroxyethylrutoside (monoHER) and seven original synthetic compounds were employed to examine the relationships between their inhibitory effects on carbonyl reduction, iron-chelation and antioxidant properties with respect to their protective potential against doxorubicin-induced cardiotoxicity. Cardioprotection was investigated in the neonatal rat ventricular cardiomyocytes whereas the H9c2 cardiomyoblast cells were used for cytotoxicity testing. Iron chelation was examined via the calcein assay and antioxidant effects and site-specific scavenging were quantified by means of inhibition of lipid peroxidation and hydroxyl radical scavenging activity, respectively. Inhibition of carbonyl reductases was assessed in cytosol from human liver. None of the flavonoids tested had better cardioprotective action than the reference cardioprotector, monoHER. However, a newly synthesized quaternary ammonium analog with comparable cardioprotective effects has been identified. No direct correlation between the iron-chelating and/or antioxidant effect and cardioprotective potential has been found. A major role of carbonyl reductase inhibition seems unlikely, as the best two cardioprotectors of the series are only weak reductase inhibitors.

Abbreviations

MonoHER – 7-monohydroxyethylrutoside; CR – carbonyl reductase(s); LDH – lactate dehydrogenase; NRU – neutral red uptake; SIH – salicylaldehyde isonicotinoyl hydrazone; DR – 2-deoxyribose; TBA – thiobarbituric acid; BHT – butylated hydroxytoluene; LPO – lipid peroxidation; k_s – second-order rate constant; ANOVA – analysis of variance

Introduction

Anthracyclines, of which doxorubicin is the leading compound, are among the most potent anticancer drugs, however, their use is limited by the risk of severe cardiotoxicity (Jones et al., 2006). Various plausible hypotheses have been proposed to explain the doxorubicin-induced cardiotoxicity (for reviews see Minotti et al., 2004; Hrdina et al., 2000) and today there is general agreement that more mechanisms are involved. Therefore, pharmacological agents that could interfere with multiple cellular targets might be particularly useful in the prevention of doxorubicin cardiotoxicity compared to the compounds that act via a single mechanism.

Flavonoids are a group of benzo-γ-pyron derivatives, naturally found in the diet, which exhibit numerous pharmacological properties that are beneficial for human health (Havsteen, 1989). With respect to doxorubicin cardiotoxicity, their antioxidant activity, iron-chelating properties and inhibitory effects on carbonyl reductases are of interest. Evidence has been given that the flavonoids indeed have a strong potential to relieve doxorubicin cardiac side-effects (van Acker et al., 1995; van Acker et al., 2001, Psotová et al., 2004). However, it is not fully elucidated yet, which of their pharmacological properties are essential for their cardioprotective action.

In vivo, anthracyclines undergo a two-electron NADPH-dependent reduction to C13-dihydrometabolites (doxorubicinol, daunorubicinol, etc.), which have been shown to be more cardiotoxic than the parent drugs (Boucek et al., 1987; Olson and Mushlin, 1990). Several ubiquitous cytosolic enzymes, such as carbonyl reductases (CR) and aldo-keto reductases participate in the formation of C13-dihydroanthracyclines. CR, in particular, seem to play an important role in anthracycline-induced cardiotoxicity (Forrest et al, 2000; Olson et al., 2003; Kaiserová & Kvasničková, 2005). Flavonoids like quercetin or rutin are known inhibitors of CR (Forrest & Gonzales, 2000) and might therefore also act as pharmacological inhibitors of doxorubicinol formation.

Doxorubicin generates reactive oxygen species (ROS), which have been suggested to play an important role in its cardiotoxicity (Hrdina et al., 2000). Free radical scavengers have therefore been proposed to protect cardiac tissue from doxorubicin-induced oxidative stress and thus to relieve its cardiotoxicity. Most of the flavonoids possess excellent antioxidant properties and the relationships between their structure and antioxidant activity have been well described (van Acker et al., 1996).

Whereas common antioxidants inactivate ROS only after they have been formed, iron chelators are able to prevent their formation. Iron can redox-cycle between its two redox states - Fe^{2+} and Fe^{3+} - and acts as a catalyst of hydroxyl radical formation (Fenton and Haber-Weiss reactions). Iron chelation is considered to be an important tool to decrease anthracycline cardiotoxicity as documented by the beneficial effect of dexrazoxane (Schroeder & Hasinoff, 2002) as well as other chelators of iron (Štěrba et al., 2006; Šimůnek et al., 2005). In flavonoids, the antioxidant and iron chelating properties are closely related and their activity may include two steps - iron is first chelated by the flavonoid and the ROS, which are formed in its vicinity, are subsequently scavenged by the flavonoid. In this way, the radicals are quenched at the same place where they are formed. This concept has been called site-specific scavenging (Haenen et al., 1993).

For this comparative study, we have used a series of ten differently substituted flavonoids (Fig.1), of which seven were newly synthesized (van Acker et al., 2000) and three were commercially available. Some of these compounds have been well-characterized by now, for example 7-monohydroxyethylrutoside has already proved to be highly efficient against the cardiotoxicity of doxorubicin (van Acker et al., 1995; Bruynzeel et al., 2006) and has recently entered phase II clinical trials. Quercetin and its *O*-glucoside, quercitrin, also represent extensively studied flavonoids being among the most common dietary polyphenols and the components of various food nutrients. The flavonoids were synthesized with the objective to discern the structural requirements that are essential for a good cardioprotective activity of the flavonoids.

The aim of this work was to describe and compare the inhibitory effects of the flavonoids on doxorubicin carbonyl reduction, their iron-chelating and antioxidant properties as well as their general toxicity: all in relation to their molecular structure. Most importantly, we aimed to assess, which of those features are especially important for the cardioprotective effect of the flavonoids.

Materials and Methods

Chemicals

7-monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health, Nyon, Switzerland, quercetin and quercitrin were obtained from Sigma-Aldrich, Prague, Czech

Republic and the substituted flavonoids were synthesized as described elsewhere (van Acker et al., 2000). Formulated doxorubicin (doxorubicin hydrochloride 2 $mg.mL^{-1}$) was obtained from TEVA (Pharmachemie B.V., Haarlem, The Netherlands). Doxorubicinol was a kind gift from Assoc. Prof. Bruce G. Charles (University of Queensland, Brisbane, Australia). SIH was obtained from Prof. P. Ponka (Mc Gill University, Montréal, Canada). All other chemicals were of the highest grade available.

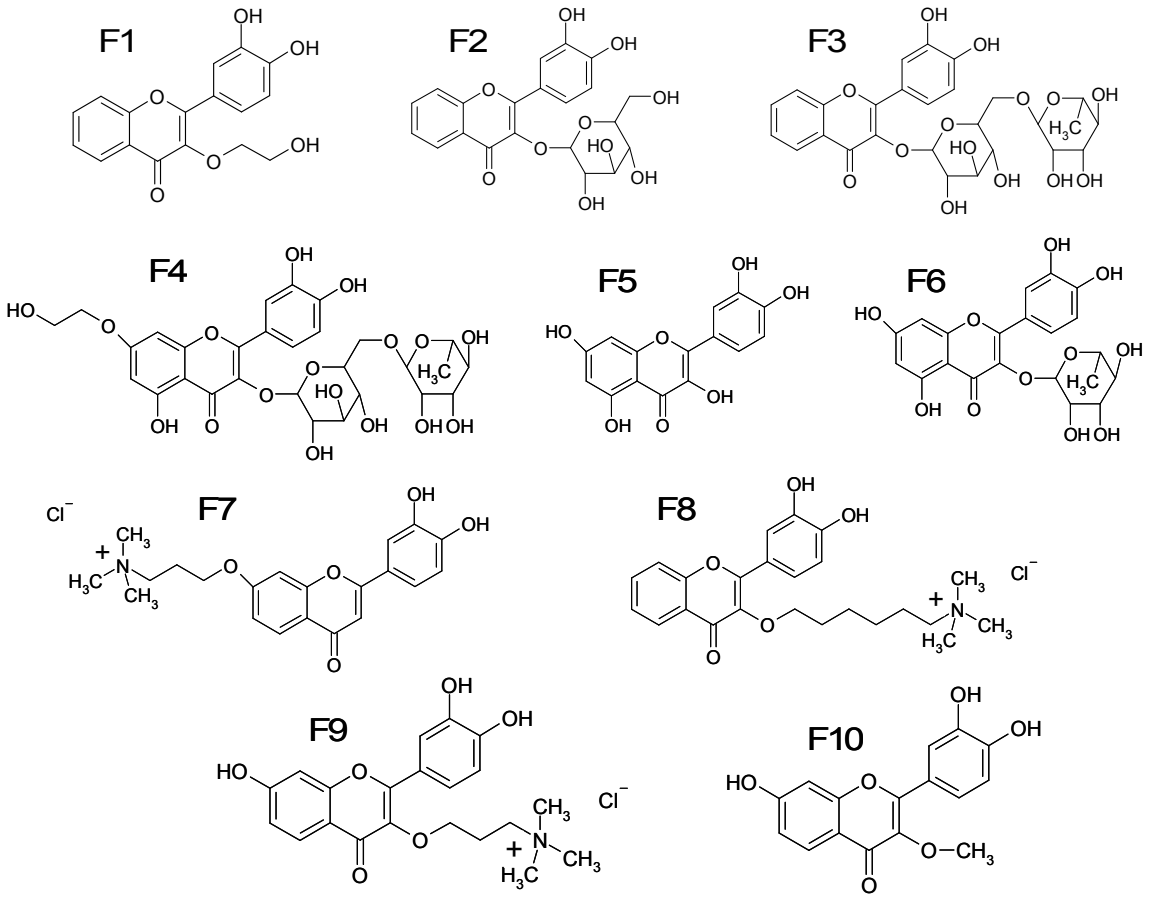

Figure 1. Chemical structures of the flavonoids under investigation. F4 = 7-monohydroxyethylrutoside (monoHER), F5 = quercetin, F6 = quercitrin.

Cell culture

H9c2 rat cardiomyoblast cell line (ATCC, Manassas,VA, USA) was maintained in DMEM supplemented with 10% heat-inactivated FBS (Cambrex Inc., Walkersville, USA), 100 $U.mL^{-1}$ of penicillin and 100 $\mu g.mL^{-1}$ of streptomycin (PAA, Pasching, Austria) and grown under humidified atmosphere containing 5% CO_2 at 37°C. Cell passages between 25 and 40 were used for cytotoxicity assessments.

Primary cardiomyocyte cultures were prepared from 2-day-old neonatal Wistar rats (BioTest, Konárovice, Czech Republic) according to Vlasblom et al. (2004). All the procedures have been conducted in accordance with the Declaration of Helsinki and approved and supervised by the Ethical Committee of the Faculty of Pharmacy in Hradec Králové, Charles University in Prague. The animals were anaesthetized with CO_2 and decapitated. The chests were opened and the hearts were collected in an ice-cold ADS buffer. The ventricles were thoroughly minced and serially digested with a mixture of collagenase II (0.25 $mg.mL^{-1}$; Gibco) and pancreatin (0.4 $mg.mL^{-1}$; Sigma) solution at 37°C The obtained cell suspension was placed on a 15-cm covered Petri dish and left for 2 hours at 37°C in order to separate the myocytes (floating in the medium) from fibroblasts (attached to the dish). The myocyte suspension was collected and viable cells counted using trypan blue exclusion. Cells were plated on the gelatine-coated 12-well plates at a density of 800,000 cells per well in the DMEM/F12 (1:1) growth medium containing 10% horse serum, 5% FCS, 1% Pen/Strep and 4% sodium pyruvate. After 40 h, the medium was renewed and the serum concentration was lowered to 5% (FCS). The medium was changed once more after another 24 h and the experiments were performed at the 4^{th} day after the isolation using both serum and pyruvate-free medium.

LDH leakage assay

The cardiomyocytes were preincubated with the test compounds dissolved in DMSO (0.2% final concentration) for 30 min. After that, 1 μM of doxorubicin was added and the medium was sampled after 24, 48 and 72 hours. Activity of released LDH was assayed in Tris-HCl buffer pH 8.9 containing 35 mM of lactic acid (Sigma Aldrich, Prague, Czech Republic) and 5 mM of NAD^+ (MP Biomedicals, Irvine, CA, USA). The rate of NAD^+ reduction was monitored spectrometrically at 340 nm (Helios β, Unicam). LDH activity was calculated using molar absorption coefficient $\varepsilon = 6.22 .10^3\ M^{-1}.cm^{-1}$.

Neutral red uptake (NRU) cytotoxicity assay

In order to assess the cytotoxicity of the tested flavonoids to the target cells, the viability of the H9c2 rat cardiomyoblast cell line was assayed by the NRU method. The cells were plated in flat-bottom microtiter plates at a cell density of 10,000 cells per well. After a 24-hour preincubation period, cells were incubated with the flavonoids for another 48 or 96 hours and tested for viability. Briefly, 40 $\mu g.mL^{-1}$ of neutral red (Sigma-Aldrich, Prague, Czech Republic) was added to each well. After incubation for 3 h at 37° C, the supernatant was discarded, cells were fixated with 1% $CaCl_2$ in 0.5% formaldehyde for 15 min, washed twice with PBS and solubilized with 1% acetic acid in 50% ethanol. The optical density of soluble neutral red was measured at 540 nm using a BioRad microplate reader. Cytotoxicity was expressed as the IC_{50} values, which were determined using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA).

Preparation of cytosolic fractions

The human liver samples from five male (18, 24, 52, 56 and 60 years old) and one female (55 year old) donors were obtained from the Cadaver Donor Programme of the Transplant Centre of the Faculty of Medicine, Charles University, Hradec Králové. Cut part of liver (*lobus hepatis sinister*) in ice-cooled Eurocollins solution was transported from the hospital to the laboratory (less than 30 min) and stored in the freezer (−80°C). Frozen liver samples were thawed at room temperature (up to 15 min) and homogenized at 1:6 (w/v) ratio in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandeline, Germany). The cytosolic fractions were isolated by fractional ultracentrifugation of the resulting homogenate (the first 105,000 x g supernatant was considered the cytosolic fraction). Protein concentration was assayed using the bicinchoninic acid method (Brown et al., 1989) and the cytosolic fractions were stored at −80°C.

Doxorubicin carbonyl reduction assay

Cytosolic carbonyl reductases (CR) catalyze the formation of the most characteristic metabolite of doxorubicin – C13-dihydrodoxorubicin (doxorubicinol). We have therefore followed the rate of doxorubicinol formation in human liver cytosol to describe the potency of various flavonoids to inhibit CR. Unless otherwise indicated, C13 carbonyl reduction of doxorubicin was assayed by incubating 1 $mg.mL^{-1}$ of cytosolic fraction with 1 mM of doxorubicin in 50 mM sodium

phosphate buffer (total volume of 150 μL) and the reaction was started with 0.5 mM NADPH (Serva, Heidelberg, Germany). To assess an inhibitory action of the flavonoids on carbonyl reduction, the test compounds were preincubated with the cytosolic fractions for 5 min prior to the addition of doxorubicin and NADPH. Control experiments were performed without biological material. The reactions were carried out at 37°C and stopped after 60 min by adding an equal volume (150 μL) of 0.2 M Na_2HPO_4 pH 8.4 while cooling the reaction mixture on ice. The anthracyclines were extracted with 1.2 mL of a 9:1 (v/v) chloroform/1-heptanol mixture. After 15 min of vigorous shaking, samples were centrifuged at 5,000 x g for 10 min to separate the layers. The lower organic phase was carefully removed to another microtube and re-extracted with 150 μL of 0.1 M *o*-phosphoric acid. After 1 min of vigorous shaking the upper aqueous layer was removed to a vial and subjected to the HPLC analysis (Fogli et al., 1999). The activity of the flavonoids was expressed as IC_{50} being the concentration of the flavonoid causing 50% reduction in doxorubicinol formation.

Determination of doxorubicinol

Following the extraction, doxorubicin and doxorubicinol were separated and detected using the Agilent 1100 series HPLC system (Agilent Technologies, Inc.). Reverse-phase chromatography was performed with a Supelco Discovery C18 analytical column (15 cm x 4 mm, 5 μm) protected with a guard column. The analytes (25 μL) were isocratically eluted with a freshly prepared mobile phase consisting of 50 mM sodium phosphate buffer pH 4.0 and acetonitrile in a 75:25 (v/v) ratio. The flow rate was 1.5 $mL.min^{-1}$. Under these conditions the substances eluted at 2.2 min (doxorubicinol) and 3.8 min (doxorubicin) as monitored spectrofluorimetrically with excitation wavelength of 480 nm and emission wavelength of 560 nm. Metabolite quantification was performed with the aid of a calibration curve constructed by using known concentrations of authentic doxorubicinol. All data are mean from at least 3 separate experiments performed in duplicates. Standard deviations were less than 10%.

Calcein assay for iron chelation

Chelator efficiency was determined using a calcein assay according to Cabantchik et al. (1996). Fluorescence of free calcein (Molecular Probes, Eugene, OR, USA) was measured with a LS50B Perkin Elmer spectrofluorimeter equipped with a magnetic stirrer. The measurements (λ_{ex} = 486 nm, λ_{em} = 517 nm) were done at room temperature and recorded as a function of time. Briefly,

calcein (20 nM in HEPES-buffered saline, pH 7.2) was incubated for 1 h with ferrous ammonium sulphate (200 nM), after which at least 95% of fluorescence was quenched. The calcein-iron complexes were then exposed to test compounds (5 μM) for 500 sec. After that, in order to obtain maximal dequenching, 5 μM of SIH was added and the reaction was followed for another 500 sec. Background fluorescence did not exceed 3% throughout the experiment. Data were analyzed using FL WinLab Software (Perkin Elmer) and normalized to the maximal fluorescence attained by the treatment with SIH, a reference iron chelator, which was previously shown to efficiently shield the cellular labile iron pool and completely block the Fenton reaction-mediated oxidative damage (Šimůnek et al., 2005).

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity was assayed by the 2-deoxyribose method according to Halliwell et al. (1987). It is based on the competition between the flavonoids and 2-deoxyribose for hydroxyl radicals. Upon the reaction with hydroxyl radicals, 2-deoxyribose is degraded into a mixture of thiobarbituric acid (TBA)-reactive products, which can be quantified spectrophotometrically. The incubation mixture contained: H_2O_2 (2.8 mM), EDTA (when indicated, 100 μM), 2-deoxyribose (2.8 mM), ascorbate (100 μM) and ferric chloride (20 μM). The concentrations of the flavonoids ranged from 100-500 μM. The reaction mixtures were incubated at 37°C for 1 h. The reaction was stopped by the addition of TBA reagent, freshly prepared by mixing 9 parts of reagent A (2.8 mM TBA, 0.125 M HCl, 16.8% (w/v) trichloracetic acid) and 1 part of reagent B (1.5 $mg.mL^{-1}$ solution of butylated hydroxytoluene in ethanol). Subsequently, the reaction mixture was heated for 15 min at 99°C. After centrifugation (3000 x g, 7 min), the absorbance (532 nm) was measured. The second-order rate constant of the scavenger (k_s) with the hydroxyl radical can be calculated using a rate constant of 3.1 x 10^9 $M^{-1}s^{-1}$ for deoxyribose (k_{DR}). The absorbance at 532 nm (A) depends on the concentration of the scavenger ([S]) and the absorbance found without scavenger (A_0). The k_s value of the scavenger is obtained from the slope of the linear plot of the reciprocal value of A versus [S] and calculated as follows: k_s = slope x k_{DR} x [DR] x A_0 (Halliwell et al., 1987).

Lipid peroxidation assay

LPO was assayed as described elsewhere (van Acker et al., 2001). Briefly, heat-inactivated microsomes from rat liver were incubated with ascorbate (200 μM) and ferrous sulphate (10 μM)

at 37°C for up to 60 min. At t = 0, 5, 10, 15, 30, 45 and 60 min, an aliquot of 0.3 ml was mixed with 2 mL of TBA-trichloroacetic acid-HCl-butylated hydroxytoluene solution to stop the reaction. The reagent was prepared as described in the hydroxyl scavenging assay. After heating (15 min, 80°C) and centrifugation (15 min), the absorbance at 535 vs. 600 nm was determined. The IC_{50} was determined by measuring the percentage of LPO inhibition at several concentrations and calculating the concentration at which 50% inhibition was obtained.

Data evaluation

Unless otherwise indicated, the data are given as a mean of at least three separate experiments ± SD. One-way ANOVA with Tukey's post test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Assessment of cardiac protection

Using a model of neonatal rat ventricular cardiomyocytes we investigated the protective activity of the flavonoids against doxorubicin toxicity. Cellular damage was quantified via measurement of the time-dependent LDH release from the cells (Table 1). We found that after 24 h incubation of the cells with 1 μM of doxorubicin and 100 μM of the flavonoids, four compounds (F4, F5, F7, F10) offered more than 40% protection, four compounds (F2, F3, F6, F9) reached 20-30% protection whereas the two remaining flavonoids (F1, F8) were not protective. After 48 and 72 h incubation, only F4 and F7 maintained their high protective effect (> 40%). An initial promising effect of F5 and F10 decreased dramatically, most probably due to their high intrinsic toxicity (see the cytotoxicity section, Table 2). Although the protective action of quercetin (F5) was still significant at 48 h, it disappeared completely after 72 h. On the other hand, the effect of moderate protectors – F2, F3, F6 and F9 was more stable during the incubation period, albeit weaker. In summary, none of the flavonoids was more effective than F4 (monoHER), F7 being the only one which had a comparable effect throughout the whole experiment (up to 72 h). The flavonoids F1 and F8, which lacked any protective properties from the beginning, and also F5 (quercetin) and F10, which showed a clear short-term protection that however quickly disappeared, are clearly the least interesting agents for further investigation as potential cardioprotectors.

Table 1. Protection of the flavonoids against doxorubicin-induced toxicity in rat neonatal ventricular cardiomyocytes (NVCM). Data express LDH activity in the extracellular media and the percentual ability of the flavonoids (100 μM) to reverse DOX (1 μM)-induced LDH leakage (100% = complete protection).

	24 h		48 h		72 h	
	LDH activity ($mU.L^{-1}.min^{-1}$)	Protection vs.DOX (%)	LDH activity ($mU.L^{-1}.min^{-1}$)	Protection vs.DOX (%)	LDH activity ($mU.L^{-1}.min^{-1}$)	Protection vs.DOX (%)
Control	18 ± 4		26 ± 8		43 ± 1	
DOX	72 ± 8		130 ± 7		156 ± 3	
F1 + DOX	92 ± 2	none	138 ± 4	none	173 ± 10	none
F2 + DOX	53 ± 10	37	101 ± 14 *	29	146 ± 4	8
F3 + DOX	54 ± 10	35	105 ± 9	25	142 ± 5	12
F4 + DOX	39 ± 10 **	58	68 ± 7 **	55	90 ± 10 **	58
F5 + DOX	23 ± 3 **	91	96 ± 15 *	34	154 ± 10	1
F6 + DOX	57 ± 18	24	94 ± 10 *	30	135 ± 0	18
F7 + DOX	47 ± 6 *	46	86 ± 13 **	42	109 ± 3 **	41
F8 + DOX	69 ± 12	8	119 ± 8	12	151 ± 1	5
F9 + DOX	57 ± 9	29	108 ± 11	22	140 ± 4	14
F10 + DOX	36 ± 10 **	69	114 ± 16	17	150 ± 1	5

*p < 0.05; **p < 0.001 (One-Way ANOVA with Tukey´s post-hoc test)

Carbonyl reductase inhibition and cytotoxicity evaluation

First of all, we have optimized the method for doxorubicinol determination in our cytosolic samples using various substrate (doxorubicin) and coenzyme (NADPH) concentrations as well as incubation time points (Fig. 2). As a result we standardly used 1 mM of doxorubicin, 0.5 mM of NADPH and the incubations were stopped after 60 min, during which the reaction rate was linear. We have found that all of the tested flavonoids were capable of inhibiting doxorubicinol formation, although their potency strongly differed. The IC_{50} values for the inhibition of doxorubicinol formation are shown in Table 2. Quercetin and quercitrin (F5, F6) were the most effective inhibitors in the series of the present study. The only synthetic flavonoid with comparable potency was F10, indicating that neither the methylation of C3-OH nor the presence of C4-OH has influence on the CR inhibitory effect. However, the substitution of C3-OH with an

aliphatic chain containing a quaternary ammonium moiety decreased the inhibitory effect (F9 vs. F10).

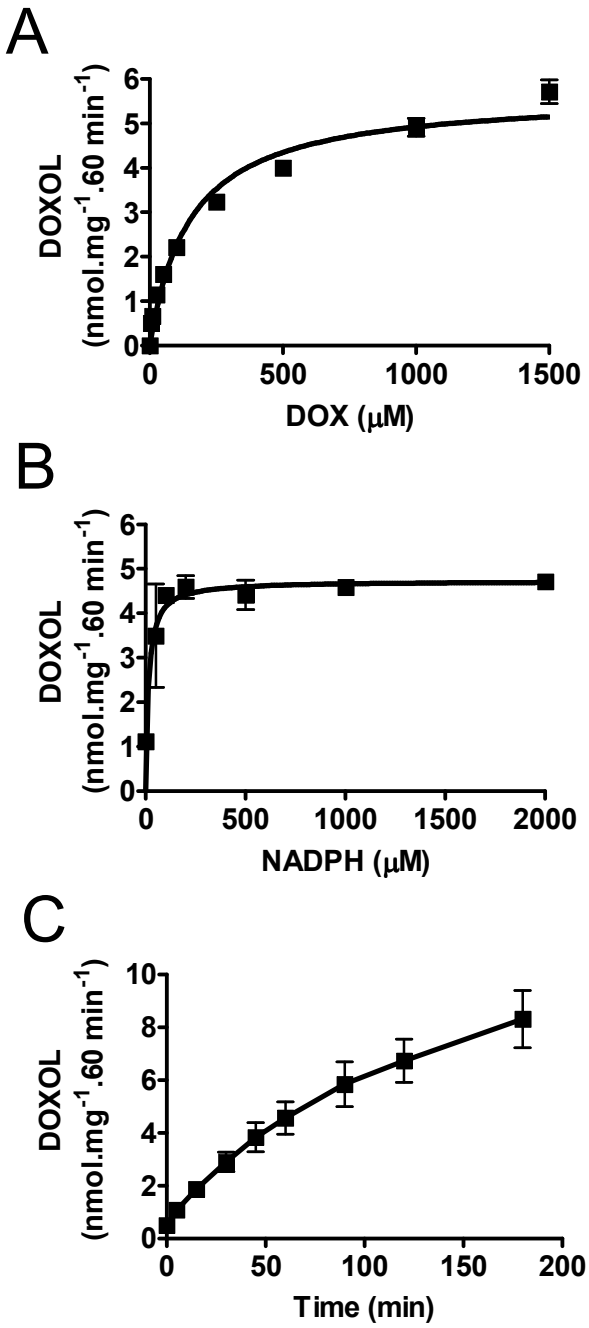

Figure 2. Optimization of the reaction conditions for the carbonyl reduction inhibition studies. (A) Michaelis-Menten plot of doxorubicinol formation as a function of the doxorubicin concentration, (B) the effect of NADPH concentration using a fixed (1 mM) concentration of doxorubicin and (C) doxorubicinol formation in time (DOX 1 mM, NADPH 0.5 mM).

Table 2. Carbonyl reductase inhibition by the flavonoids in human liver cytosol and their cytotoxicity in the rat cardiomyoblast cell line (H9c2). IC_{50} = the concentration of a flavonoid which caused 50% decrease in doxorubicinol formation or 50% decrease in cell viability, respectively.

	CR inhibition	Cytotoxicity to H9c2	
	IC_{50} (μM)	IC_{50} 48h (μM)	IC_{50} 96h (μM)
F1	35 ± 6	96 ± 23	44 ± 8
F2	50 ± 7	> 500	271 ± 29
F3	71 ± 13	> 500	417 ± 108
F4	84 ± 27	340 ± 123	463 ± 182
F5	18 ± 2	133 ± 33	44 ± 6
F6	12 ± 4	> 500	> 500
F7	170 ± 40	> 500	> 500
F8	67 ± 13	341 ± 144	146 ± 36
F9	46 ± 4	> 500	466 ± 133
F10	20 ± 2	243 ± 38	37 ± 10

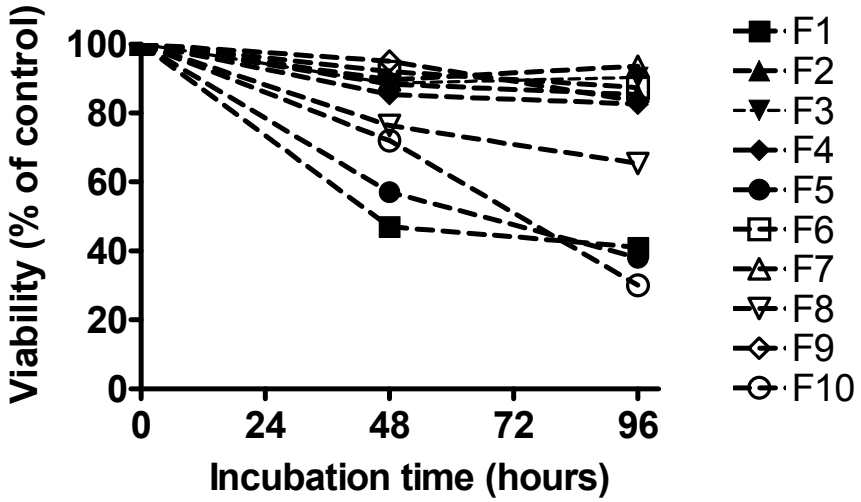

Figure 3. Time-dependent plot of the cytotoxic effects of the flavonoids (100 μM) in H9c2 cells. Error bars not indicated for sake of clarity.

The viability of the H9c2 cells is shown as a function of either concentration of the flavonoids (Table 2) or time (Fig. 3). It is clear that the toxicity decreases with the degree of substitution of the hydroxyl groups, because the simplest structures, e.g. F1, F5 or F10, are the most toxic ones. Glycosylation (F2, F3, F4 and F6) or introduction of the charged and/or bulky moieties (F7, F8, and F9) lead to a decrease in the toxicity.

Iron chelation and antioxidant effects

All the tested flavonoids were found to chelate iron as demonstrated by their ability to displace iron from the iron-calcein complex. Nevertheless, their efficacy was considerably lower than the reference iron chelator SIH, which is able to displace iron from the iron-calcein complex both quickly and completely. The flavonoids did not vary strongly in their ability to chelate iron and after 500 sec. of incubation, the activity of all the compounds reached from 12 to 39% of the maximal effect (Table 3, first column). The most efficient chelators of iron were F5 and F6. This can be explained by the presence of an additional iron-chelating moiety in their molecule (between 5-OH and 4-oxo groups) whereas other structures (with an exception of F4) appear to chelate iron only when the *o*-catechol group in ring B is present.

In addition, the deoxyribose assay for hydroxyl radical scavenging was performed in the absence or presence of the strong metal chelator, EDTA and the second-order rate constants of the flavonoids with hydroxyl radicals (k_s) are presented in the second and third column of Table 3. Our results show that the k_s values of all tested flavonoids increased when EDTA was not present. This effect was particularly pronounced with F4, F5 and F6 (Table 3, fourth column) and seems to be the result of an additional chelating site in those molecules, represented by 4-oxo group combined with 5-OH.

Lipid peroxidation (LPO) inhibition assays confirm that most of the compounds studied are excellent antioxidants with F2, F5, F8, F9, F10 being particularly effective (Table 3, last column). Interestingly, the ability of the flavonoids to inhibit LPO does not seem to correlate with hydroxyl radical scavenging potency where F3, F8, F9 acted as the best scavengers. On the contrary, F5 (quercetin) promoted hydroxyl radical formation although it was very effective against LPO. The high efficiency of F8 and F9 could be attributed to the quaternary ammonium group; nevertheless, this would not explain the relatively lower efficiency of F7 and high efficiency of F3 at the same time.

Table 3. Site-specific scavenging of hydroxyl radicals expressed as second-order rate constants of the flavonoids with hydroxyl radicals (k_s), relative iron (III) chelation expressed as percentage of the effect of the reference chelator SIH and prevention of lipid peroxidation by the flavonoids. IC_{50} = the concentration of the flavonoid at which 50% inhibition of LPO was obtained.

	Fe^{3+} chelation*	k_s ** $[M^{-1}s^{-1}] \times 10^9$		w/o EDTA : with EDTA ratio	LPO***
	(% of SIH)	w/o EDTA	with EDTA		IC_{50} (μM)
F1	15 ± 2	57 ± 18	10 ± 2	6	22 ± 6
F2	13.7 ± 0.6	53 ± 12	9 ± 2	6	2.8 ± 0.9
F3	14.8 ± 0.5	69 ± 5	28 ± 2	2	16 ± 5
F4	11.7 ± 0.2	106 ± 15	8 ± 2	13	13 ± 4
F5	39.4 ± 0.1	20 ± 4	-1 ± 0	15	5.1 ± 0.1
F6	29.1 ± 0.9	62 ± 11	6 ± 2	10	n.d.
F7	22.8 ± 0.1	53 ± 13	10 ± 2	5	37 ± 2
F8	18.3 ± 0.4	52 ± 8	19 ± 2	3	1.4 ± 0.3
F9	16.4 ± 0.8	107 ± 16	24 ± 2	4	3.8 ± 0.1
F10	18.5 ± 0.3	48 ± 11	11 ± 1	4	1.6 ± 0.7

* Measured as displacement of Fe^{3+} from its complexes with calcein after 500 seconds of incubation (complete displacement by SIH).

**Hydroxyl radicals were generated in presence of H_20_2 (2.8 mM), Fe^{3+}(20 μM), ascorbate (100 μM), EDTA (100 μM, where indicated) and 2-deoxyribose (2.8 mM), which was used as a detector molecule.

***Lipid peroxidation was induced with Fe^{2+} (10 μM) and ascorbate (200 μM) in heat-inactivated microsomes in presence/absence of various concentrations of the flavonoids.

n.d. – not determined

Discussion

For many years, flavonoids attract the attention or researchers because they possess multiple pharmacological properties (Scalbert et al., 2005). Some of their characteristics, namely the antioxidant, iron-chelating and carbonyl reductase-inhibitory effects, render them particularly interesting to investigate them as new protective compounds against doxorubicin cardiotoxicity. Evidence has been given that the semi-synthetic flavonoid monoHER is cardioprotective in animal models (van Acker et al., 1995). This compound was also shown not to interfere with the anticancer effects of doxorubicin (van Acker et al., 1997). Because monoHER has to be administered at a high dose and because of its low oral bioavailability, a series of new synthetic derivatives with potentially enhanced antioxidant properties and/or better intestinal absorption

have been synthesized (van Acker et al., 2000) in order to identify a compound with increased cardioprotective properties. To achieve this goal, various types of substitutions have been tested. An attempt was also performed to increase the cardioselectivity of the flavonoids via introduction of the quaternary ammonium moiety in various positions of the flavonoid structure (Grisar et al., 1991).

Antioxidant properties were long considered to be the major or even sole determinants for efficient protectors against doxorubicin cardiotoxicity because reactive oxygen species and oxidative stress are considered to be involved in the pathophysiology of its development (Kaiserová et al., 2006; Gille et al., 2002; Gille and Nohl, 1997). However, it has been shown that there are pronounced differences in the cardioprotective effects also among the flavonoids with comparable antioxidant properties (van Acker et al., 2001).

Apart from their antioxidant effects, other factors are obviously involved in the protective properties of the flavonoids. We conducted this study in order to explore these other features that are likely to be involved in their cardioprotection. Iron chelation was chosen because of an apparent involvement of iron in doxorubicin-induced cardiotoxicity (Xu et al., 2005) and the effects on carbonyl reduction of doxorubicin were examined because its inhibition will prevent formation of the cardiotoxic C13-dihydrometabolites of the anthracyclines (Olson et al., 2003; Wang et al., 2001). The antioxidant properties and general cytotoxicity of the compounds have also been evaluated. We attempted to determine structure-activity relationships and most importantly, the collected data were related to their cardioprotective potential in order to define the characteristics that are essential for their cardioprotective potency. This knowledge is vital for designing new efficient and safe cardioprotectors.

The cardioprotective effects of the selected flavonoids were assessed in neonatal rat cardiomyocytes. These experiments were designed with concentrations of doxorubicin (1 μM), which corresponded with plasma concentration in human patients and allowed us to prolong the incubation period (up to 72 h). In a previously used model of an electrically paced isolated mouse left atrium (van Acker et al., 2001) the acute doxorubicin toxicity (1 h incubation) was measured using a higher dose of doxorubicin (35 μM), which corresponded with heart concentrations in mice (van der Vijgh et al., 1990). Both models gave similar results and demonstrated that the compounds F4 (monoHER) and F7 are the best protectors of the series. For F5 and F10, a remarkable decrease in protection against the doxorubicin-induced cardiomyocyte toxicity was

found during incubation (24 vs. 48 and 72 h). This might be due to their own toxicity. It is known that the metabolite of quercetin (F5), quercetin-quinone methide, is thiol-reactive and rapidly forms adducts with glutathione. This reaction may lead to toxic effects such as increased membrane permeability or altered function of the SH-containing enzymes (Boots et al., 2005). The structures of F10 and F1 resemble that of quercetin the most and indeed, their biochemical behaviour is similar in many aspects.

Low intrinsic toxicity of the compounds is of course an important requirement to allow eventual clinical application. We have tested the cytotoxicity of the flavonoids in the H9c2 cells. With exception of F1, F5 and F10, all the compounds had acceptable toxicity. F7 was the least toxic compound of the series. Apparently high cytotoxicity has consequences in cardioprotection: the beneficial effects of the compounds (if any) do not last for long as they are overwhelmed by another type of toxicity. In this way our study points out the limitations of short-term (acute) models of doxorubicin cardiotoxicity. For example, quercetin was previously designated as an excellent cardioprotector in neonatal cardiomyocyte model (Psotová et al., 2004), however, its effects were only followed for 8 hours, which may lead to an overestimation of the beneficial effects of these types of compounds (i.e. compounds with reactive intermediates). We have found that the protection by quercetin was still high at 24 h but it decreased during the next 48 h, whereas the effect of F4 (monoHER) or F7 lasted during the whole experiment.

All the tested flavonoids were able to chelate iron and it was found that the number of chelating sites present in the molecule determines the degree of iron chelation. However, no link was found between the cardioprotective effects of the flavonoids and their iron-chelating ability. The discrepancy between the effect of monoHER (F4) in the calcein assay (low) and site-specific scavenging assay (high) may lie in a different affinity of the flavonoids for Fe^{2+} and Fe^{3+}. Although iron is introduced in its ferrous form in the calcein assay, it is rapidly oxidized to its ferric form under the conditions of the experiment. It is not possible to keep it in the ferrous form using reducing agents (e.g. ascorbate) because this leads to a degradation of the probe (Hasinoff et al., 2003). Therefore, calcein assay gives information about the Fe^{3+}-chelating ability whereas in the 2-deoxyribose assay, iron is present in its Fe^{2+} form due to the presence of ascorbate. It has been suggested that the flavonoids chelate iron as Fe^{2+} and their ability to chelate Fe^{3+} is related to their capacity to reduce Fe^{3+} to Fe^{2+} before association (Mira et al., 2002), which is apparently larger in F5 and F6 than in F4.

The deoxyribose assay for hydroxyl radical scavenging, that has been employed to investigate iron chelation, also gives information about antioxidant behaviour of the compounds. In the presence of EDTA, information on the direct antioxidant behaviour of the flavonoids (i.e. without the participation of iron chelation) is provided. An increase of scavenging activity in the absence of EDTA is indicative for iron chelation by the flavonoids and the influence of iron chelation on total antioxidant capacity by the flavonoids. Interestingly, we have found that under the conditions of the assay, the compound F5 (quercetin) did not scavenge hydroxyl radicals in presence of EDTA (which does not allow site-specific scavenging). On the contrary, it acted as mild pro-oxidant. On the other hand, quercetin acted as a powerful protector against lipid peroxidation (LPO). This discrepancy is not very surprising, the fine balance between the anti- and pro-oxidant properties of the flavonoids is well known and it obviously depends on the exact conditions of the assays. Similar results were previously achieved by Laughton et al. (1989). As can be seen from the k_s values and the IC_{50} values for LPO, all the compounds possess good antioxidant properties. Indeed, it was previously found that the catechol moiety in combination with C2-C3 double bond and 4-oxo function are the essential structural elements for potent antioxidant activity and that 3-substituted compounds are superior to the 7-substituted compounds in LPO assay (Van Acker et al., 2000). Based on our findings, we conclude that neither hydroxyl radical scavenging nor inhibition of lipid peroxidation seem to predict the cardioprotective potential of the flavonoids.

The inhibition of doxorubicin carbonyl reduction was assessed in order to evaluate the involvement of cardiotoxic metabolite formation in prevention of the doxorubicin-induced cardiotoxicity. There is one additional reason for blocking doxorubicin reduction to doxorubicinol. It has been shown that an increased carbonyl reduction, which is inducible by anthracyclines, might also lead to the development of resistance to chemotherapy (Ax et al., 2000). To date, very few studies have been conducted on the inhibitory effects of the flavonoids on carbonyl reductases (Imamura et al., 2000; Silvestrini et al., 2006), although it is known for a long time that the flavonoids quercetin, quercitrin or rutin are excellent CR inhibitors. To our knowledge, this study is the first to combine the biochemical inhibition of doxorubicinol formation with cardioprotection evaluation. Based on the IC_{50} values we have identified the compounds F5, F6 and F10 as the best inhibitors of carbonyl reductases of the series. On the contrary, F7 was the worst inhibitor. The quaternary ammonium moiety is not only bulky but it is

also positively charged and thus it can affect the appropriate electrostatic interaction between enzyme and inhibitor. From the three flavonoids with a quaternary ammonium group, F9 was the best inhibitor (preserved 7-OH, substituted 3-OH) and F7 was the worst (substituted 7-OH). This means that an absence (F1, F2, F3, F8) or even a substitution (F4, F7) of C7-OH clearly decrease the degree of inhibition. The fact that F7 was a weak inhibitor further confirms the role of C7-OH in the enzyme-inhibitor interaction and the negative influence of the quaternary ammonium moiety on the inhibitory properties of the flavonoids. If we take into account that F7 is one of the best cardioprotectors of the present study, the importance of carbonyl reductase inhibition seems unlikely. Moreover, F4 (monoHER) is also a poor inhibitor while its cardioprotective effects are marked.

In summary, it can be stated that the efficient cardioprotective compounds are not cytotoxic by themselves. High efficacy in carbonyl reductase inhibition is not critical for the cardioprotective action of the flavonoids. Moreover, no correlation was found between the inhibition of LPO or hydroxyl radical scavenging and the cardioprotective effects. It was found that iron chelation increased the scavenging capacity of the flavonoids (through site-specific scavenging). The present study shows that besides monoHER also compound F7 has excellent cardioprotective properties. Cardioprotection is however not clearly associated with a single physico-chemical or biochemical property of the flavonoid.

Acknowledgement

This work was financially supported by the grants GAUK 97/2005 (HK) and GAČR 305/05/P156 (TŠ). The authors are indebted to Assoc. Prof. Bruce G. Charles for providing us with pure doxorubicinol. We would also like to thank Mrs. Alenka Pakostová for her skilful technical assistance.

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VI

Conclusions
&
Future perspectives

Conclusions and future perspectives

Iron chelation appears to be a very successful strategy how to diminish the anthracycline-induced cardiotoxicity. The clinical efficacy of dexrazoxane (a prodrug, yielding a metal-chelating product) boosted the extensive search for new molecules that would possess the same cardioprotective effect while reducing the side-effects of dexrazoxane (e.g. bone-marrow toxicity, low bioavailability). The chelators of aroylhydrazone class appear to be promising cardioprotectors in animal models, however their protective effects disappear upon the escalation of chelator dose. These worriesome findings warrant more preclinical studies. In this thesis, we investigated the effects of two aroylhydrazones – PIH and SIH on selected biotransformation enzymes, which are involved in the anthracycline metabolism i.e. cytochrome P450 (redox cycling of the quinone moiety) and cytosolic reductases (formation of C13-dihydroanthracyclines), in search for possible interactions. In our *in vitro* study in isolated hepatocytes, we have not found significant interference between the chelators and the enzymatic systems under investigations except for very high concentrations (> 250 μM), which exceed expected plasma levels of these drugs *in vivo*. On the contrary the anthracyclines (both doxorubicin and daunorubicin) decreased the activity of all four CYP450 isoforms studies (1A1, 1A2, 3A, 2B) and both PIH and SIH were able to prevent this negative effect and restore the activities up to 90% of control levels. This finding, together with the fact that both chelators showed little cytotoxicity to the hepatocytes, has to be regarded as cytoprotective and beneficial.

As the cardioprotective effects of iron chelators are generally assumed to be due to the prevention of hydroxyl radical formation, we compared the effects of five iron chelators (PIH, SIH, deferoxamine, dexrazoxane and 7-monohydroxyethylrutoside = monoHER) on oxidative stress mediated by either doxorubicin or hydrogen peroxide/ferrous iron (Fenton system) in A549 cell line. We have shown that the chelators that were protective against doxorubicin-induced oxidative stress i.e. dexrazoxane and monoHER were inefficient against hydrogen peroxide/iron toxicity while SIH and DFO were the only agents which were able to prevent hydrogen peroxide/iron-induced oxidative damage and cell death. These data suggest that the oxidative toxicity of the anthracyclines might not be due to hydroxyl radicals but rather some other reactive oxygen species (e.g. superoxide radicals) and thus, chelation of redox-active iron is not the sole determinant of an effective protector. Iron, however, might still play role in some oxidative

stress-independent mechanisms of anthracycline toxicity. Despite the fact that these studies were performed in cancer cell line, it is possible that these observations can be extrapolated for cardiomyocytes as well and this remains to be confirmed.

An important question that has to be answered when developing new protectors against anthracycline cardiotoxicity is whether these compounds do not affect the anticancer potency of the anthracyclines. We have found that the effect of all five iron chelators under investigation on the antiproliferative activity of doxorubicin was none (PIH, SIH, deferoxamine) or only minor (dexrazoxane, monoHER) in A549 cells. The investigations of the nature of cell death, however, showed that both monoHER and dexrazoxane significantly reduced the initiation of apoptosis in these cells (measured as caspase-3 activity), suggesting kind of antagonism between these compounds and doxorubicin. Although most clinical studies have not found decreased antitumour response to doxorubicin in dexrazoxane-treated patients, the risk/benefit ratio of dexrazoxane should be carefully considered before its administration to each individual patient. On the other hand, the aroylhydrazone chelators, PIH and SIH, which possess the antiproliferative properties on their own, can only be expected to further increase the antitumour effect of the anthracyclines.

The C13-dihydroanthracyclines have been shown to be more cardiotoxic compared to the parent drugs and their reductase-catalyzed formation could be regarded as a risk factor for anthracycline cardiotoxicity development. In our experiments with cytosolic fractions from rabbit liver, we identified the carbonyl reductase (EC 1.1.1.184) as the most important daunorubicin-C13-reductase i.e. superior to the members of AKR class, whose contribution to C13-dihydrometabolite formation is only minor. These findings have implications for the design of new cardioprotectors as pharmacological inhibitors of carbonyl reductases. Since these ubiquitous cytosolic reductases are particularly sensitive to flavonoid inhibitors such as quercetin or rutin, we have used a series of synthetic original flavonoids as well as three flavonoids from commercial sources in order to describe the relationships between the carbonyl reductase-inhibitory effects and molecular structure of these compounds. We have found that all the tested flavonoids inhibited doxorubicinol formation in human liver cytosol. Quercetin and quercitrin were the best inhibitors, while most types of substitutions (except for those on C3-OH) decreased the inhibitory potency. More importantly, we aimed for identifying the characteristics of the flavonoids that are crucial for their protective effects. Flavonoids are not only inhibitors of carbonyl reductases, but also well-known antioxidants and iron-chelating agents. All these

characteristics fit in some part of proposed pathogenetic pathway of the anthracyclines. Only two flavonoids significantly protected the isolated neonatal rat cardiomyocytes against doxorubicin toxicity throughout the whole 72-hour experiment – monoHER (whose cardioprotective efficiency has already been known) and the 7-trimethylammoniumpropyloxy-derivative of quercetin. However, cardioprotection by flavonoids has not been found to be associated with a single physico-chemical or biochemical property of the flavonoid. It can be stated that high efficacy in carbonyl reductase inhibition is not critical for the cardioprotective action of the flavonoids, since both monoHER and quaternary ammonium group-containing analog were weak inhibitors.

In conclusion, the aroylhydrazone iron chelators and (some of) the flavonoids deserve further investigations as they have proven to possess biochemical features that are favorable for their use as protectors against anthracycline cardiotoxicity. The exclusion of some mechanisms that have been thought to mediate the anthracycline toxicity (hydroxyl radical formation, carbonyl reductase activity) will facilitate the design of new protectors. It is also challenging for more mechanistic investigations.

VII

Summary / Souhrn

Summary

Anthracycline antibiotics rank among the most efficient and widely used anticancer drugs ever developed and remain an important component of many chemotherapy protocols in oncological practice. Unfortunately, their use is limited by the risk of development of severe cardiac toxicity, whose incidence greatly increases upon the administration of a cumulative dose exceeding 550 mg/m^2 (for doxorubicin and daunorubicin). This toxicity is particularly unpleasant in young patients, who in principle have high life expectancy. These survivors of childhood cancers with history of anthracycline treatment are endangered by chronic cardiac failure even after 15-20 years of follow-up.

It has been assumed that the mechanisms of anthracycline cardiotoxicity can be separated from their antitumor effect albeit not completely. Anthracyclines interfere with DNA replication via interactions with topoisomerase II and intercalation of its molecule into the DNA double strand. Inhibition of DNA replication is detrimental for rapidly proliferating tumors. On the other hand, cardiotoxicity is thought to be largely mediated by reactive oxygen species generated during redox-cycling and/or formation of C13-dihydroanthracycline metabolites. A number of other hypotheses have been proposed, it is however difficult to distinguish between the causes and mere consequences of anthracycline-induced cardiac injury.

Among the various strategies of limiting anthracycline cardiotoxicity, liposomal encapsulation and the use of cardioprotective agents appear to be the most successful ones. An extensive array of compounds has been evaluated whereas only few of them showed certain degree of protection. The most interesting cardioprotectors arise from the group of iron chelators (with dexrazoxane as the leading compound) and the flavonoids (e.g. 7-monohydroxyethylrutoside, monoHER).

The investigations described in this thesis aimed to shed light on the mechanisms by which cardioprotectors prevent the anthracycline toxicity. We have focused on the relationships between anthracycline metabolism and biochemical behavior of iron chelators and the flavonoids. The data presented here result from *in vitro* experiments performed either with the whole cells (primary cultures of hepatocytes and cardiomyocytes, A549 and H9c2 cell lines) or subcellular fractions from rabbit and human liver.

Our group has previously described the cardioprotective potential of aroylhydrazone iron chelators (PIH, SIH, o-108) in a rabbit *in vivo* model. Before new drugs are introduced in clinical trials it is necessary to search for possible interactions with key biotransformation enzymes.

Therefore, we investigated the effects of the two aroylhydrazones – PIH and SIH on the enzymes involved in the anthracycline metabolism - cytochrome P450 and cytosolic reductases. Using isolated hepatocytes, we have not found significant interference between the chelators and the enzymatic systems under investigations except for very high concentrations (> 250 μM), which exceed expected plasma levels of these drugs *in vivo*. On the contrary, the anthracyclines decreased the activity of all four CYP450 isoforms studies (1A1, 1A2, 3A, 2B) and both PIH and SIH were able to prevent this negative effect and restored the activities up to 90% of control levels. Low cytotoxicity of both compounds to the hepatocytes was also found.

The cardioprotective effects of iron chelators are generally assumed to be due to the prevention of hydroxyl radical formation. However, we have shown that the chelators that were protective against doxorubicin-induced oxidative stress i.e. dexrazoxane and monoHER were inefficient against hydrogen peroxide/ferrous iron (Fenton reagent)-toxicity while SIH and deferoxamine were only able prevent hydrogen peroxide/iron-induced oxidative damage and cell death in A549 cell line. PIH was ineffective in either of the two systems. These data suggest that the oxidative toxicity of the anthracyclines might not be due to hydroxyl radicals but rather some other reactive oxygen species (e.g. superoxide radicals) and thus, chelation of redox-active iron is not the sole determinant of an effective protector.

An important question that has to be answered when developing new protectors against anthracycline cardiotoxicity is whether these compounds do not affect the anticancer potency of the anthracyclines. We have found that the effect of the five iron chelators under investigation on the antiproliferative activity of doxorubicin was none (PIH, SIH, deferoxamine) or only minor (dexrazoxane, monoHER) in A549 cells. As the aroylhydrazones possess antiproliferative properties on their own, it is plausible that they can further enhance the response to the anticancer chemotherapy of the anthracyclines. The C13-dihydroanthracyclines have been shown to be more cardiotoxic compared to the parent drugs and their reductase-catalyzed formation might be regarded as a risk factor for anthracycline cardiotoxicity development. In our experiments with cytosolic fractions from rabbit liver, we identified the carbonyl reductase (EC 1.1.1.184) as the most important daunorubicin-C13-reductase.

Carbonyl reductases are particularly sensitive to flavonoid inhibitors such as quercetin or rutin. We have studied a series of synthetic original flavonoids as well as three flavonoids from commercial sources and investigated their reductase-inhibitory effects. All the tested flavonoids

inhibited doxorubicinol formation in human liver cytosol. Quercetin and quercitrin were the best inhibitors, while most types of substitutions (except for those on C3-OH) decreased the inhibitory potency. More importantly, we aimed to identify the biochemical determinants that are crucial for the protective effects of the flavonoids, focusing on inhibition of carbonyl reductase, antioxidant and iron-chelating properties. Of ten agents studied, only two flavonoids significantly protected the cardiomyocytes against doxorubicin toxicity throughout the whole 72-hour experiment – monoHER (whose cardioprotective efficiency has already been known) and the 7-trimethylammoniumpropyloxy-derivative of quercetin. However, cardioprotection by flavonoids has not been found to be associated with a single physico-chemical or biochemical property of the flavonoid. The role of carbonyl reductase inhibition was found irrelevant, since both monoHER and quaternary ammonium group-containing analogue were weak inhibitors.

In conclusion, we have not found any adverse effects of the aroylhydrazone iron chelators *in vitro* and further research of these compounds is encouraged. More attention should be paid to alternative mechanisms of their cardioprotective effects than a mere prevention of hydroxyl radical formation. Some flavonoids were also found to be protective against anthracycline-induced damage in cellular models. New flavonoid cardioprotectors do not have to exert pronounced inhibitory effects on carbonyl reductases.

Souhrn

Antracyklinová antibiotika patří mezi nejefektivnější a široce využívaná protinádorová léčiva a do dnešní doby zůstávají významnou složkou mnoha chemoterapeutických protokolů. Jejich podání je bohužel limitováno rizikem rozvoje závažné kardiální toxicity, jejíž incidence výrazně stoupá při překročení kumulativních dávek antracyklinů (pro daunorubicin a doxorubicin – 550 mg/m^2). Tato toxicita nabývá na významu zvláště u pacientů, kteří přežili nádorové onemocnění v dětském věku a byli léčeni antracykliny. Tito mladí lidé zůstávají v ohrožení vývoje chronického srdečního selhání dokonce i v případech kdy od ukončení chemoterapie uběhlo 15 i více let.

Všeobecně se má za to, že mechanismus antracyklinové kardiotoxicity je odlišný od mechanismu jejich protinádorového působení. Antracykliny zasahují do buněčné replikace DNA, pravděpodobně prostřednictvím interakce s topoisomerasou II a interkalací planární části jejich molekuly mezi baze dvoušroubovice. Proces replikace DNA je naprosto zásadní zejména pro růst rychle proliferujících nádorových buněk. Naproti tomu, vznik antracyklinové kardiotoxicity je spojován zejména s tvorbou reaktivních forem kyslíku, které se tvoří v průběhu tzv. redoxního cyklu, a dále s tvorbou C13-dihydrometabolitů antracyklinů. Byla navržena celá řada dalších hypotéz, otázkou ovšem zůstává, jak rozlišit příčiny a následky působení antracyklinů na srdeční buňku.

Byly studovány různé přístupy ke snížení antracyklinové kardiotoxicity, jako nejefektivnější možnosti se zdají být terapie liposomálními antracykliny a zejména pak použití kardioprotektivních látek. Pouze zlomek z velkého množství testovaných látek vykázal alespoň částečnou protekci. V současnosti lze za nejdůležitější skupiny potenciálních kardioprotektiv považovat chelátory železa (osvědčený dexrazoxan) a flavonoidy (v současnosti je v klinickém testování monoHER – 7-monohydroxyethylrutosid).

Cílem předkládané práce bylo přispět k objasnění mechanismů, kterými kardioprotektiva snižují antracyklinovou toxicitu. Důraz byl kladen na vztahy mezi metabolismem antracyklinů a biochemickými vlastnostmi chelátorů železa a flavonoidů. Data byla získána z *in vitro* experimentů provedených jak na buněčné úrovni (primární kultury hepatocytů, kardiomyocytů, buněčné linie A549 a H9c2), tak na úrovni subcelulárních frakcí z králičích i lidských jater.

V dřívějších experimentech byl zjištěn kardioprotektivní potenciál chelátorů železa ze skupiny aroylhydrazonů (PIH, SIH, o-108) na modelu daunorubicinem navozené chronické

kardiomyopatie u králíka. Nezbytnou součástí preklinického hodnocení nových léčiv před jejich uvedením do klinických studií je zjištění jejich případných interakcí s klíčovými biotransformačními enzymy. Zabývali jsme se proto účinky aroylhydrazonů PIH a SIH na vybrané enzymy, které se účastní metabolismu antracyklinů - cytochromy P450 a cytosolické reduktasy. V izolovaných hepatocytech nebyla nalezena významná interference mezi chelátory a sledovanými enzymovými systémy s výjimkou velmi vysokých koncentrací chelátorů (> 250 μM), které již převyšují očekávané plazmatické koncentrace *in vivo*. Samotné antracykliny snížily aktivitu všech sledovaných isoforem cytochromu P450 (1A1, 1A2, 3A, 2B), zatímco oba chelátory PIH a SIH tomuto poklesu efektivně bránily (samotný doxorubicin nebo daunorubicin – 50% aktivity kontroly vs. koinkubace s chelátory – 70-90% aktivity kontroly). Oba chelátory zároveň vykazovaly na hepatocytech nízkou cytotoxicitu.

Kardioprotektivní účinky chelátorů železa jsou tradičně přisuzovány prevenci tvorby hydroxylových radikálů. V našich pokusech jsme ovšem pozorovali výrazné rozdíly v působení různých chelátorů proti oxidačnímu stresu navozenému doxorubicinem a peroxidem vodíku s železnatými ionty (Fentonovo činidlo). Dexrazoxan a monoHER byly na buněčné linii A549 účinné proti oxidačnímu poškození buněk doxorubicinem a neúčinné proti peroxidové toxicitě, zatímco u SIH a deferoxaminu tomu bylo právě naopak. PIH se ukázal být neúčinným v obou případech. Tyto výsledky naznačují, že oxidační toxicita antracyklinů je pravděpodobně primárně vyvolána jiným typem radikálů než hydroxylovými (např. superoxidovými) a chelatace redox-aktivního železa není jediným předpokladem účinné kardioprotektivní látky.

Při vývoji nových kardioprotektiv musí být zodpovězena otázka, zda tyto látky nesnižují protinádorový účinek antracyklinů. V buněčné linii A549 jsme nezaznamenali snížení antiproliferačních účinků doxorubicinu vlivem PIH, SIH ani deferoxaminu. Mírné snížení bylo pozorováno u dexrazoxanu a monoHER. Protože aroylhydrazony samy o sobě vykazují antiproliferační účinky, je pravděpodobné, že mohou naopak protinádorovou účinnost antracyklinů zvyšovat.

Bylo zjištěno, že C13-dihydroantracykliny působí více kardiotoxicky než parentní antracykliny. Jejich tvorbu (katalyzovanou cytosolickými reduktasami) je nutné považovat za potenciální rizikový faktor rozvoje kardiotoxicity. V našich experimentech s cytosolickou frakcí z králičích jater byla jako hlavní C13-reduktáza daunorubicinu identifikována karbonylreduktasa (EC. 1.1.1.184), příspěvek aldo-keto reduktas je poměrně menší (1:5 při fyziologickém pH).

Karbonylreduktasy jsou citlivé k flavonoidním inhibitorům typu quercetinu či rutinu. Studovali jsme inhibiční vlastnosti série původních syntetických a tří komerčně dostupných flavonoidů. Všechny látky byly schopny inhibovat vznik doxorubicinolu v cytosolu z lidských jater, i když v různém rozsahu. Quercetin a quercitrin byli nejlepšími inhibitory ($IC_{50} < 20$ μM), zatímco většina typů substitucí (s výjimkou hydroxylace na C3) snižovala inhibiční efekt. Dále jsme se pokusili popsat biochemické vlastnosti, které jsou zásadní pro protektivní účinek flavonoidů (se zaměřením na inhibici karbonylreduktasy, antioxidační a chelatační vlastnosti). Pouze dva z deseti sledovaných flavonoidů ochránily izolované srdeční buňky proti doxorubicinové toxicitě po celou dobu experimentu (72 h) – monoHER (jehož kardioprotektivní účinky jsou již dobře známy) a 7-trimetylamoniumpropyloxyderivát quercetinu. Bohužel, nebyl nalezen jednoznačný vztah mezi kardioprotektivním působením a jedinou fyzikálně-chemickou nebo biochemickou vlastností flavonoidů. Role inhibice karbonylreduktasy (tj. tvorby doxorubicinolu) se nicméně jeví jako nevýznamná, jelikož obě výrazně protektivní látky patří mezi slabé inhibitory.

Souhrnně: Na buněčné a podbuněčné úrovni nebyly nalezeny žádné nežádoucí účinky aroylhydrazonových chelátorů PIH a SIH a lze tedy doporučit další studie s těmito nadějnými kardioprotektivy. Pozornost by se měla upírat k mechanismům odlišným od prevence tvorby hydroxylových radikálů. Naše výsledky rovněž podporují použití flavonoidů jako potenciálních kardioprotektiv. Nová flavonoidní protektiva ze skupiny flavonoidů ale nemusí být dobrými inhibitory karbonylreduktas.

VIII

List of publications

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Kaiserová H, Šimůnek T, Štěrba M, den Hartog GJM, Schröterová L, Popelová O, Geršl V, Kvasničková E, Bast A. New iron chelators in anthracycline-induced cardiotoxicity. *(submitted)*

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Curriculum vitae

Helena Kaiserová was born on the 27th September 1979 in Bohumín, where she also attended a high school (from 1991 to 1998). After her graduation, she studied pharmacy at the Faculty of Pharmacy in Hradec Králové, Charles University in Prague. She obtained her Master's degree in 2003. She continued with her doctoral education in Biochemistry (Pathobiochemistry and xenobiochemistry) at the same institution under the supervision of Prof. RNDr. Eva Kvasničková, CSc., who previously guided her diploma thesis. In 2005, she spent 6 months at the Department of Pharmacology and Toxicology, University of Maastricht, The Netherlands, where she was supervised by Prof. Dr. Aalt Bast and worked on the oxidative stress-related part of her project. She was active in the field of anthracycline cardiotoxicity for 6 years. She gave several lectures for both domestic and international audience, once as an invited speaker (Como, Italy 2006). During her postgraduate studies, she participated in the practical education of the undergraduate students in General Biochemistry.

Starting January 2007, she became a junior researcher at the Institute of Organic Chemistry and Biochemistry in Prague, where she joined the group of Prof. RNDr. Antonín Holý, DrSc. (Department of Nucleic Acid Chemistry). She will work on the biochemical evaluation of new nucleotide and nucleoside analogs as anticancer and/or antiviral drugs.