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The Effect of New Lipophilic Chelators on the Activities of Cytosolic Reductases and P450 Cytochromes Involved in the Metabolism of Anthracycline Antibiotics: Studies in Vitro

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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 pyridoxal isonicotinoyl hydrazone (PIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) – new iron chelators – to protect against peroxidation as well as their suitable biological, physical and chemical properties make the compounds promising candidates for pre-clinical and clinical studies. 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) involved in the metabolism of daunorubicin, doxorubicin and other drugs or xenobiotics were studied. Various concentrations of the chelators were used either alone or together with daunorubicin or doxorubicin for *in vitro* studies in isolated hepatocytes. A significant decrease of activity was observed for all enzymes only at PIH and SIH concentrations higher than those presumed to be used for therapy. The results show that PIH and SIH have no effect on the activities of the enzymes studied *in vitro* and allow us to believe that they will not interfere with the metabolism of co-administered drugs and other xenobiotics. Daunorubicin (Da) and doxorubicin (Dx) significantly reduce cytochrome P450 activity, but the addition of SIH and PIH chelators (50 μM) reverses the reduction and restores the activity to 70-90 % of the activity of relevant controls.

Key words
Oxidative stress • P450 • Cytosolic reductases • Iron chelators • Pyridoxal isonicotinoyl hydrazone

Introduction

Pyridoxal isonicotinoyl hydrazone (Fig. 1, PIH) was studied back in the 1950s as a coenzyme of pyridoxal-dependent enzymes (Davison 1956, Duhault *et al.* 1967). Later, several authors.(Hoy *et al.* 1979, Poňka *et al.* 1979, 1979, 1994, Brittenham 1990) found that it can chelate Fe ions and can be used as a chelator in the

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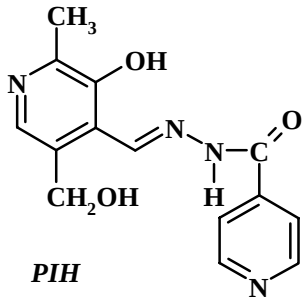
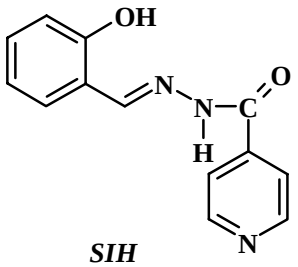
treatment of secondary iron overload pathologies that occur in iron-loading anemias such as β-thalassemias, porphyria cutanea tarda and alcoholic cirrhosis (Pippard 1994). Apart from being used to treat iron overload, PIH may also be a useful therapeutic agent against free radical injury (Schulman *et 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 has some drawbacks, e.g. poor intestinal absorption, high cost, potential to develop allergy due to mast cell activation (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* (Bláha *et al.* 1998, Richardson and Poňka 1998) on rats after parenteral or peroral administration. 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 for Fe hemosiderin, ferritin and transferrin and, at the same time, low affinity for Fe 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).



PIH



SIH

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

One of the hypotheses explaining the toxic effects of anthracyclines is the "iron and free radical hypothesis". According to this hypothesis, anthracyclines impair cardiac function by oxygen free radicals generated in reactions catalyzed by CYP enzymes (Hoy *et al.* 1979, Buzdar *et al.* 1985, Brittenham 1990, Olson and Mushlin 1990, Rhoden *et al.* 1993, Goeptar *et al.* 1994). CYP enzymes are a large superfamily 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-metabolizing families of CYP (CYP1, CYP2, CYP3) are primarily expressed in liver (Testa 1995). CYP3A, CYP2B and NADPH cytochrome reductase (Goeptar *et al.* 1994) catalyze reductive transformation of daunorubicin (Da) and doxorubicin (Dx) and semiquinone radicals generated in those reactions are reoxidized by molecular oxygen. In a single-electron transfer reaction, the oxygen molecule forms superoxide radical that either initiates lipid peroxidation reactions or is converted by dismutase to hydrogen peroxide. The presence of Fe enables subsequent reactions of free radicals. Although the free radical hypothesis has a number of critics, it is still the principal hypothesis that explains myocardial injury by anthracyclines.

Another hypothesis links the toxicity of anthracyclines to their toxic metabolite C13-ol generated by cytosolic carbonyl reductases. Subcellular fractions of 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, C13-ol was found to be a 30 times more potent inhibitor of cardiac contractility in rabbit papillary muscles (Olson and Mushlin 1990, Maser and Bannenberg 1994, Minotti *et al.* 1999), and high level of C13-ol persisting for several days were found in the rabbit heart tissue upon a single-dose administration of the substrate (Cusack *et al.* 1993, Pouna *et al.* 1996).

We studied the *in vitro* activities of oxidative and reductive enzymes in rabbit liver cells upon the administration of various doses of anthracyclines daunorubicin and doxorubicin, PIH and SIH as well as their combinations in order to examine whether the prospective chelators affect the activities of enzymes 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 modifications of metabolic transformation rate, pharmacological effects and the durations of therapeutic effects of the drugs.

Methods

Animals

Rabbits (*Oryctolagus cuniculus var*. Chinchilla, about 3 kg, Velaz, Prague, Czech Republic) were fed a standard diet and fasted 12 h prior to the experiment. The animals were euthanized; their liver excised and used fresh for the isolation of hepatocytes. All procedures were done in compliance with the "*Guide for the care and use of laboratory animals*" (1996) and were supervised by the Ethics Committee of the Faculty of Pharmacy, Charles University in Prague, Hradec Králové, 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 and albumin were purchased from Sigma-Aldrich. Collagenase was obtained from Sevapharma. Pyridoxal isonicotinoyl hydrazone and salicylaldehyde isonicotinoyl hydrazone were a gift of Dr. Poňka (McGill University, Montreal, Canada). Daunorubicin (Cerubidine) was obtained from Rhône-Poulenc, France and doxorubicin (Adriablastina) was from Pharmacia and Upjohn, Italy. Other chemicals and solvents were of analytical grade and were obtained from general commercial sources.

Cell preparation and culture

Hepatocytes were isolated from male rabbits (without treatment), by 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) by a buffer solution with 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 a cold buffer containing 2 mM $CaCl_2$ and finally with a plating medium. Hepatocytes were cultured using ISOM medium supplemented with 3 % (v/v) fetal calf serum and antibiotics (60 μg/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere of air (95 %) and CO_2 (5 %) at 37 ºC. The cells were plated on 6-cm tissue culture dishes at a density of $3x10^6$ cells/culture dish in 3 ml of medium and were left to attach. After 3-4 h, the medium was replaced by a fresh medium without serum, with 5 μM insulin and antibiotics (3 ml of medium per dish) and containing the tested compounds. Incubation times were 24 and 48 h.

Incubations

Chelators PIH and SIH were incubated with hepatocytes in monolayers in concentrations 50, 100, 150, 250 and 750 μM. Anthracyclines daunorubicin and doxorubicin were incubated with hepatocyte monolayers in concentrations 10 μM. When combinations of chelators and anthracyclines were used, incubation was started only with chelators (50 and 150 μM). Anthracyclines (10 μM) were added after 60 min of incubation with chelators. A control medium for PIH, SIH and combinations of chelators with doxorubicin contained 1 % (v/v) DMSO, control medium for daunorubicin contained D-mannitol (150 μM), control medium for combinations of chelators and daunorubicin contained 1 % (v/v) DMSO and D-mannitol (150 μM). No additional substances were added to the control medium for doxorubicin.

Isolation of subcellular fractions

Hepatocytes after cultivation were homogenized in the ratio of 1:3 in a 0.1 M Na-phosphate buffer at pH 7.4 in the Potter-Elvehjem homogenizer. The microsomal fraction was obtained by fractional ultracentrifugation the liver homogenates (Gillette 1972). Microsomes were resuspended in the same buffer with 20 % glycerol and stored at –80 °C.

Protein and enzymatic assay

Proteins were determined by a modification of the Lowry method (Peterson 1979). 7-ethoxyresorufin O-dealkylase, 7-methoxyresorufin O-dealkylase, 7-pento-xyresorufin O-dealkylase activity and 7-benzyloxy-resorufin O-dearylase activity 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 a potassium-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.

Enzymatic activities were calculated in

pmol/min per mg of protein, and expressed as % values of relevant controls.

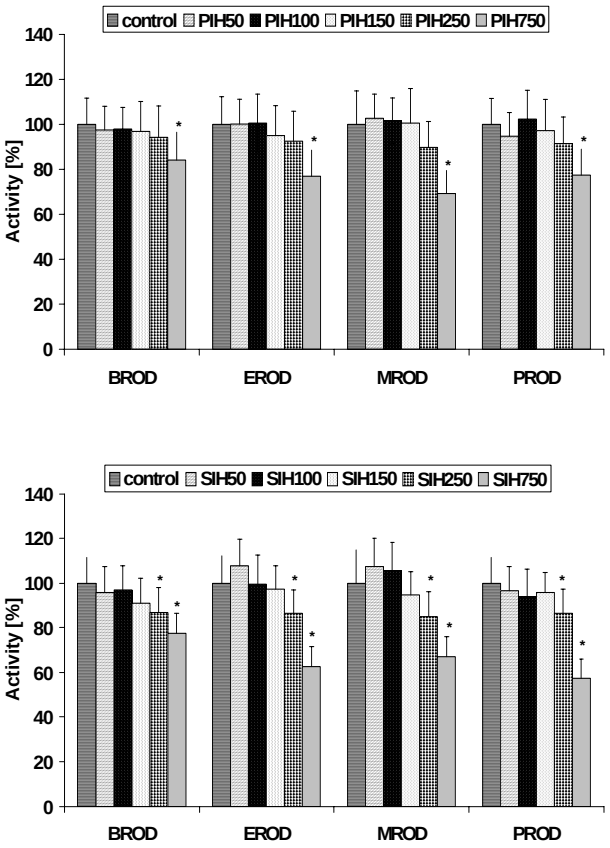


Fig. 2. Effect of pyridoxal isonicotinoyl hydrazone (PIH), salicylaldehyde isonicotinoyl hydrazone (SIH) on P450-dependent monooxygenases. Incubation time of primary culture of hepatocytes from eight rabbits was 24 h. Hepatocytes were incubated with five concentrations (50, 100, 150, 250 and 750 μM)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

Cytotoxicity assay

The MTT (tetrazolium dye) assay was performed according to Martin and Clynes (1993) with some modifications. Cell suspension ($1x10^6$ cells/ml) was plated into the 96-well plate (50 μl/well) and incubated for 24 and 48 h with tested solutions. At the end 2.5 mg/ml MTT was added and incubated for one hour (37 °C, 5 % CO_2). Formed formazan crystals were dissolved in HCl-isopropanol. Optical density was measured at 595 nm.

Lactate dehydrogenase (LDH) activity was measured in a medium spectrophotometrically monitoring the optical density of NADH at 340 nm (Clynes 1998).

Cytotoxicity of tested chemicals was calculated as % of the control.

Instrumentation

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

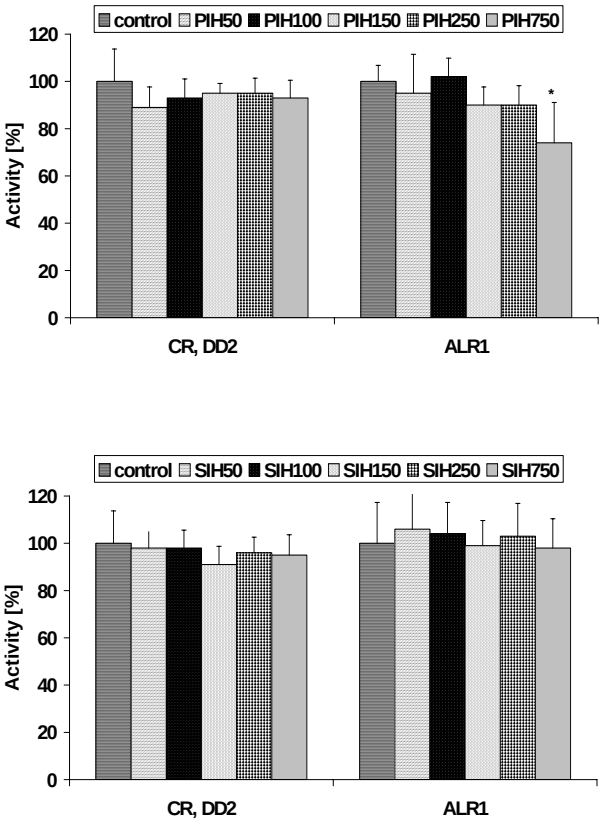


Fig. 3. Effect of pyridoxal isonicotinoyl hydrazone (PIH), salicylaldehyde isonicotinoyl hydrazone (SIH) on reductases. Incubation time of primary culture of hepatocytes from eight rabbits was 24 h. Hepatocytes were incubated with five concentrations (50, 100, 150, 250 and 750 μM) of PIH or SIH.Values are expressed as % of control (1 % DMSO in hepatocytes medium). Enzymatic activity of control: CR + DD2 3.20 nmol/min/mg, ALR1 1.21 nmol/min/mg. CR – carbonyl reductase, DD2 - dihydrodiol dehydrogenase, ALR1 – aldo-keto reductase. * P<0.05 (Student's t-test); significantly different from control.

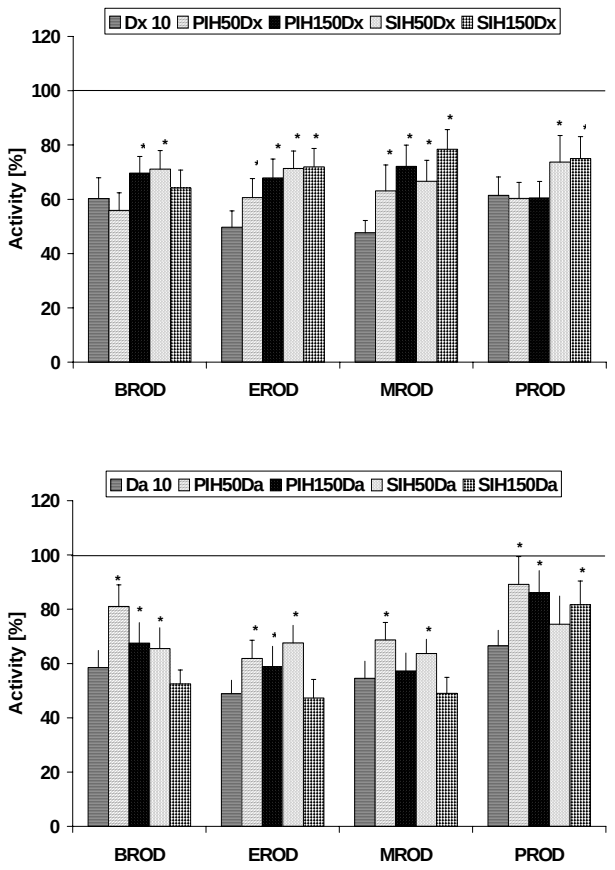


Fig. 4. Effect of doxorubicin (Dx), daunorubicin (Da) and combinations (Dx and PIH/SIH; Da and PIH/SIH) on P450-dependent monooxygenases. Incubation time of rabbit liver hepatocytes from six animals was 24 h. Hepatocytes were incubated with Dx, Da (10 μM), for combinations were used doxorubicin and daunorubicin plus two concentrations of PIH or SIH (50 and 150 μM). Anthracyclines were added after 60 min of incubation with chelators. Values are expressed as % of control. Control medium for Dx+SIH/PIH contained 1 % (v/v) DMSO. Enzymatic activity of control: BROD 50.5 pmol/min/mg, EROD 44.3 pmol/min/mg, MROD 32.7 pmol/min/mg, PROD 12.0 pmol/min/mg. Control medium for Dx did not contain any added compound. Enzymatic activity of control: BROD 56.4 pmol/min/mg, EROD 48.3 pmol/min/mg, MROD 37.4 pmol/min/mg, PROD 15.1 pmol/min/mg. Control medium for Da+SIH/PIH contained 1 % (v/v) DMSO + D-mannitol (150 μM). Enzymatic activity of control: BROD 55.1 pmol/min/mg, EROD 47.3 pmol/min/mg, MROD 41.1 pmol/min/mg, PROD 11.1 pmol/min/mg. Control medium for Da contained D-mannitol (150 μM). Enzymatic activity of control: BROD 55.4 pmol/min/mg, EROD 47.1 pmol/min/mg, MROD 37.9 pmol/min/mg, PROD 14.7 pmol/min/mg. BROD – benzyloxyresorufin O-dearylase activity, EROD – ethoxyresorufin O-dealkylase activity, MROD – methoxyresorufin O-dealkylase activity, PROD – pentoxyresorufin O-dealkylase activity. * $P<0.05$ (Student's t-test); significantly different from Dx, Da.

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-h incubation. The highest PIH concentration employed significantly reduced the activity of the isoenzymes. In the same type of 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 were higher than those used in preclinical *in vivo* tests and than presumed therapeutical doses.

Figure 3 shows that the chelators at the same concentrations (50-250 μM) and under the same 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 the controls.

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 (Fig. 4).

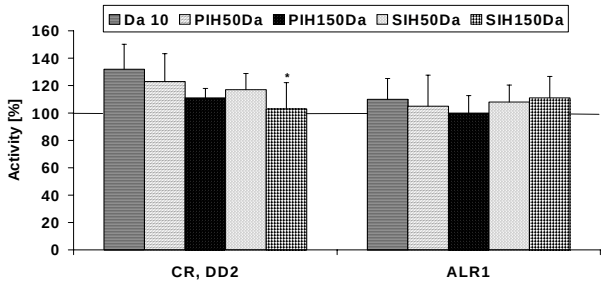


Fig. 5. Effect of daunorubicin (Da) and combinations (Da and PIH/SIH) on reductases. Incubation time of rabbit liver hepatocytes from six animals was 24 h. Hepatocytes were incubated with Da (10 μM), for combinations were used daunorubicin (10 μM) plus two concentrations of PIH or SIH (50 and 150 μM). Anthracycline was added after 60 min of incubation with chelators. Values are expressed as % of control. Control medium for Da+SIH/PIH contained 1 % (v/v) DMSO + D-mannitol (150 μM). Enzymatic activity of control: CR + DD2 3.51 nmol/min/mg, ALR1 1.51 nmol/min/mg. Control medium for Da contained D-mannitol (150 μM). Enzymatic activity of control: CR + DD2 3.36 nmol/min/mg, ALR1 1.13 nmol/min/mg. CR – carbonyl reductase, DD2 - dihydrodiol dehydrogenase, ALR1 – aldo-keto reductase. * $P<0.05$ (Student's t-test); significantly different from Da.

As for cytosolic reductases, the activities of CR and DD2 in rabbit hepatocytes incubated for 24 h with daunorubicin were significantly higher than in the

controls (Fig. 5). 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 comparison with the samples incubated with antibiotics only.

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

Discussion

Properties of a suitable 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 bloodstream transport and intestinal absorption, minimal adverse effects and accumulation in tissues, particularly in the fat tissue. The low price 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 with a few adverse effects, they have a number of serious problems. A considerable effort has therefore been exerted to develop new, biologically active chelators with improved properties.

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

Since both the chelators are metabolized 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 likely interactions between very lipophilic xenobiotics with other enzymes and/or biomolecules that might affect the activities of enzymes with important roles in the metabolism of xenobiotics.

We therefore studied their effect on the activities of enzymes involved in biotransformation using primary rabbit hepatocyte cultures. The concentrations of the chelators used in our experiments were within the range of expected therapeutic levels except for the highest concentration (750 μM) which served for experimental purposes only.

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

Figure 3 shows the results of the same type of experiments in which the effect of PIH and SIH chelators was examined on the activities of carbonyl reductase (CR), dihydrodiol reductase (DD2) and aldehyde reductase (ALR1). No effect of the chelators was observed in concentrations 50-250 μM on any of the enzymes was observed. Enzyme activities were assayed at optimum pH for each enzyme; CR and DD (with the same pH optimum 6.0) were assayed as a single enzyme. These studies were carried out with respect to the fact that at intracellular pH 7.4 it is carbonyl reductase (EC1.1.1.184) rather than DD2 that catalyses the reduction of daunorubicin and doxorubicin to C13-ol (Propper and Maser 1997).

Based on the results shown in Figures 2 and 3, it can be concluded that pyridoxal isonicotinoyl hydrazone and its analog salicylaldehyde isonicotinoyl hydrazone do not affect the activities of oxidases and reductases studied in our experiments and that they do not interfere with the metabolism of anthracycline cytostatics, additional co-administered drugs and/or other xenobiotics. The results of toxicity tests (MTT test and LDH leak test) support this conclusion – 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-h incubation, and the results were compared with incubations in which daunorubicin or doxorubicin (10 μM) were combined with the PIH and SIH chelators (50 and 150 μM). These concentrations were chosen to mimic plasma levels of the substrates and chelators used in our *in vivo* experiments (Geršl *et al.* 1999, Adamcová *et al.* 2002).

Figure 4 depicts the effect of daunorubicin and doxorubicin (10 μM) incubated with hepatocytes in primary culture for 24 h. Both antibiotics significantly

depressed the activity of all P450 isoenzymes in comparison with the controls (100 %). However, chelators co-administered (in concentrations of 50 and 150 μM) with the antibiotics partly reversed the negative effect of daunorubicin and doxorubicin.

The "protective" effect of these chelators, i.e. their ability to chelate iron ions and suppress the generation of hydrogen peroxide and free radicals along daunorubicin and doxorubicin biotransformation pathway, seems to have another beneficial effect – it reduces the cytotoxic influence of the antibiotics. In our experiments, the addition of PIH or SIH (50 and 150 μM) significantly increased the viability of cells incubated with daunorubicin or doxorubicin (10 μM), as determined by the MTT test and LDH leak test.

In comparison with the controls, the activity of cytosolic reductases CR and DD2 was significantly higher when hepatocytes were incubated with daunorubicin or doxorubicin (10 μM). A similar increase was observed in *in vitro* experiments with tumor cell lines (Ax *et al.* 2000); the authors explain this effect by tumor cell resistance to chemotherapeutics. We are currently working on elucidating the mechanisms through which healthy liver cells increase the activities or the two cytosolic reductases.

No increase of the activity of any of the cytosolic reductases studied was observed in incubations in which the chelators were co-administered with the antibiotics.

Based on the *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. of the enzymes involved in oxidative or reductive transformation of a vast majority of drugs, hormones and other xenobiotics. The role that the studied enzymes play in the mechanism of the toxic effects of 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 studied in our experiments do not affect the activity of the enzymes and may hopefully be used in clinical medicine. This, however, must be verified in subsequent preclinical and primary clinical studies.

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