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RESEARCH PAPER

# Anthracycline toxicity to cardiomyocytes or cancer cells is differently affected by iron chelation with salicylaldehyde isonicotinoyl hydrazone

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**Background and purpose:** The clinical utility of anthracycline antineoplastic drugs is limited by the risk of cardiotoxicity, which has been traditionally attributed to iron-mediated production of reactive oxygen species (ROS).
**Experimental approach:** The aims of this study were to examine the strongly lipophilic iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH), for its ability to protect rat isolated cardiomyocytes against the toxicity of daunorubicin (DAU) and to investigate the effects of SIH on DAU-induced inhibition of proliferation in a leukaemic cell line. Cell toxicity was measured by release of lactate dehydrogenase and staining with Hoechst 33342 or propidium iodide and lipid peroxidation by malonaldehyde formation.
**Key results:** SIH fully protected cardiomyocytes against model oxidative injury induced by hydrogen peroxide exposure. SIH also significantly but only partially and with no apparent dose-dependency, reduced DAU-induced cardiomyocyte death. However, the observed protection was not accompanied by decreased lipid peroxidation. In the HL-60 acute promyelocytic leukaemia cell line, SIH did not blunt the antiproliferative efficacy of DAU. Instead, at concentrations that reduced DAU toxicity to cardiomyocytes, SIH enhanced the tumoricidal action of DAU.
**Conclusions and implications:** This study demonstrates that iron is most likely involved in anthracycline cardiotoxicity and that iron chelation has protective potential, but apparently through mechanism(s) other than by inhibition of ROS-induced injury. In addition to cardioprotection, iron chelation may have considerable potential to improve the therapeutic action of anthracyclines by enhancing their anticancer efficiency and this potential warrants further investigation.
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**Keywords:** anthracycline cardiotoxicity; leukaemia; daunorubicin; iron chelation; salicylaldehyde isonicotinoyl hydrazone (SIH)

**Abbreviations:** 2-MPG, 2-mercaptopropionyl glycine; DAU, daunorubicin; MDA, malondialdehyde; NVCMs, neonatal ventricular cardiomyocytes; PI, propidium iodide; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute medium; SIH, salicylaldehyde isonicotinoyl hydrazone

## Introduction

Anthracycline antibiotics, such as daunorubicin (DAU), doxorubicin and epirubicin, are among the most effective antineoplastic agents ever developed. They remain important components of many current chemotherapy protocols of both haematological malignancies and solid tumours. However, repeated administration of these drugs is accompanied by the risk of serious and irreversible cardiomyopathy and the development of heart failure (Adams and Lipshultz, 2005; Jones *et al*., 2006; Barry *et al*., 2007). Despite the four decades of intensive research, the precise pathophysiology of anthracycline-induced cardiotoxicity remains highly controversial (Minotti *et al*., 2004a; Chen *et al*., 2007). Nevertheless, the iron (Fe)-catalysed production of reactive oxygen species (ROS), proposed originally by Myers *et al*. (1982) and confirmed by numerous subsequent studies (Sarvazyan, 1996; Zhou *et al*., 2001; Lou *et al*., 2006; Berthiaume and Wallace, 2007), has been presented as the primary mechanism even in the most current textbooks and review articles (Adams and Lipshultz, 2005; Brunton and Parker, 2005; Ewer and Yeh, 2006; Barry *et al*., 2007).

The importance of ROS and Fe in the aetiopathogenesis of anthracycline cardiotoxicity has been confirmed by the high

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protective efficiency of dexrazoxane (ICRF-187)—the only clinically approved cardioprotectant so far (Swain and Vici, 2004). It is generally accepted that dexrazoxane protects cardiomyocytes against the anthracycline-induced damage through its potent metal-chelating hydrolysis product, ADR-925 (an EDTA analogue), which reportedly acts by displacing Fe from the complexes with the anthracycline or by chelating free or loosely bound intracellular Fe, and thus preventing Fe-based free radical injury (Hasinoff *et al.*, 1998). Furthermore, it has been well demonstrated that, in iron-overload conditions, anthracycline-induced cardiotoxicity is markedly exacerbated (Hershko *et al.*, 1993; Link *et al.*, 1996; Panjrath *et al.*, 2007). Taken together, this provides a strong rationale for the investigation of intracellular iron chelators as cardioprotectants against anthracycline cardiotoxicity.

Multiple studies examining various available chelators (both registered drugs already being used in clinical practice and novel experimental agents) have been published since the early 1990s with very heterogeneous results: from positive and encouraging (Barnabe *et al.*, 2002), through ambiguous and inconclusive (Voest *et al.*, 1994) to purely negative (Hasinoff *et al.*, 2003; for review see Kaiserova *et al.*, 2007).

Our group has focused on the aroylhydrazone group of iron chelators—the analogues of pyridoxal isonicotinoyl hydrazone. These ligands are small lipophilic molecules, which possess neutral charge at physiological pH. These properties allow their easy passage through cell membranes and access to intracellular labile Fe pools (Buss *et al.*, 2002). Aroylhydrazones and salicylaldehyde isonicotinoyl hydrazone (SIH) in particular were demonstrated to have a very strong antioxidant activity due to their Fe-chelating properties (Horackova *et al.*, 2000; Simunek *et al.*, 2005a; Kurz *et al.*, 2006), which has rendered them ideal candidates for use in anthracycline cardioprotection studies.

Using an *in vivo* model of DAU-induced chronic cardiotoxicity in rabbits, we have recently demonstrated that the repeated administration of three chelators—pyridoxal isonicotinoyl hydrazone, SIH and *o*-108 (pyridoxal *o*-chlorbenzoyl hydrazone)—was able to fully prevent the DAU-induced mortality (Simunek *et al.*, 2005b; Sterba *et al.*, 2006, 2007). SIH and *o*-108 were particularly effective in reducing chronic DAU cardiotoxicity: they significantly improved the impairment of left ventricular contractility, as well as markedly reducing the severity of DAU-induced histopathological lesions (Sterba *et al.*, 2006, 2007). However, even a moderate (2- to 2.5-fold) increase of the optimal chelator dose resulted in the disappearance of their beneficial effects in terms of both mortality and cardioprotection (Sterba *et al.*, 2006, 2007). As such doses of the chelators were well tolerated when administered to animals alone (Klimtova *et al.*, 2003), we could only speculate that it was the combination of pronounced intracellular Fe chelation with the effects of the anthracycline, rather than the toxicity of the chelators, that was responsible for the loss of protection. This unexpected and puzzling bell-shaped dose–response observed in the animal experiments seemed to contradict the putatively straightforward role of Fe-mediated ROS production as a pivotal pathophysiological mechanism responsible for anthracycline cardiotoxicity. Altogether, questions arising from the above-described animal studies encouraged us to embark on further investigations of the interactions of iron and iron chelation with anthracycline cardiotoxicity at the cellular level. For our *in vitro* cardiotoxicity/cardioprotection experiments, we have used primary cultures of rat neonatal ventricular cardiomyocytes (NVCMs), a model that has been well established and repeatedly used for this purpose (Hershko *et al.*, 1993; Link *et al.*, 1996; Barnabe *et al.*, 2002; Hasinoff *et al.*, 2003; Kwok and Richardson, 2003).

The objectives of the present study were: (i) to assess the ability of SIH to remove iron from a complex with DAU; (ii) to examine and compare the protective action of SIH against the cardiomyocyte injury induced by either DAU or hydrogen peroxide (model oxidative injury); (iii) to determine the inherent toxicity of SIH to cardiomyocytes; (iv) to establish possible mechanisms responsible for SIH-induced cardioprotection and/or toxicity (role of iron, oxidative stress and/or signal-transduction pathways) and (v) to investigate the effects of SIH on DAU-induced inhibition of leukaemic cell growth as well as the potential for antiproliferative activity of SIH itself.

## Methods

*Assessment of displacement of iron from DAU–$Fe^{3+}$ complexes with SIH*

The spectrophotometric assay was performed according to Hasinoff *et al.* (2003). The DAU–$Fe^{3+}$ (3:1) complex was prepared by adding $FeCl_3$ in 15 mM HCl to DAU solution. The resulting complex, which revealed a typical absorption band at 600 nm (a 20-fold increase as compared with uncomplexed DAU), was added to the reaction buffer (50 mM Tris/150 mM KCl, pH 7.4, room temperature) in the glass cuvette to yield a final concentration of 45 μM DAU/15 μM $Fe^{3+}$. After a 4-min equilibration period, SIH or other reference chelators (deferoxamine and EDTA) were added so as to yield a final concentration of 100 μM. The absorbance at 600 nm was then followed for another 4 min using a spectrophotometer (Helios Beta; Unicam, Cambridge, UK). In addition, spectral scans (400–650 nm) of solutions with DAU, DAU + Fe and DAU + Fe + SIH (at identical concentrations as given above) were taken after 8 min incubations. The absorbance of SIH (which absorbed light at $\lambda < 460$ nm) was subtracted from the DAU + Fe + SIH spectral scans. At 600 nm, SIH displayed zero absorbance.

*Isolation of NVCMs*

All animal procedures and the preparation of NVCM have been approved and supervised by the Ethical Committee of the Faculty of Pharmacy, Charles University in Prague. Primary cultures of NVCM were prepared from 2-day-old Wistar rats. The animals were anaesthetized with $CO_2$, and decapitated. The chests were opened and the hearts were collected in an ice-cold $Ca^{2+}$-free buffer, containing 116 mM NaCl, 5.3 mM KCl, 1.2 mM $MgSO_4$, 1.13 mM $NaH_2PO_4$, 5 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (pH 7.40). The ventricles were thoroughly minced and serially digested with a mixture of collagenase (0.25 mg $mL^{-1}$; Gibco, Paisley, UK) and

pancreatin (0.4 mg mL$^{-1}$; Sigma-Aldrich, Schnelldorf, Germany) solution at 37 °C. The cell suspension was placed on a large (15 cm) Petri dish and left for 2 h at 37 °C to separate the myocytes (floating in the medium) from fibroblasts (attached to the dish). The myocyte-rich suspension was collected and viable cells were counted using Trypan blue exclusion. Cells were plated on the gelatin-coated 12-well plates (TPP, Trasadingen, Switzerland) at a density of 800 000 cells per well ($2.2 \times 10^5$ cells cm$^{-2}$) in the Dulbecco's modified Eagle's minimal essential growth medium/F12 (1:1; Sigma) containing 10% horse serum, 5% foetal calf serum, 4% sodium pyruvate (all Sigma) and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria). After 40 h, the medium was renewed and the serum concentration was lowered to 5% (foetal calf serum). The medium was replaced once more after another 24 h. The isolation procedure resulted in a confluent cellular monolayer with ~90% of synchronically beating cardiomyocytes.

In vitro *cardiotoxicity studies*

All experiments were started on the fourth day after the isolation. Using both serum and pyruvate-free medium, NVCMs were incubated at 37 °C with the tested agents either alone or in combination. Unless stated otherwise, all compounds were added simultaneously and experiments lasted for 48 h. To dissolve SIH, dimethyl sulphoxide (0.2% v/v) was present in the culture medium of all groups. At this concentration, dimethyl sulphoxide had no effect on cellular viability.

The activity of lactate dehydrogenase (LDH) released from cardiomyocytes was determined in cell culture media as a standard marker of cytotoxicity and cellular breakdown. LDH activity was assayed in Tris-HCl buffer (pH 8.9) containing 35 mM of lactic acid (Sigma) and 5 mM of $NAD^+$ (MP Biomedicals, Illkirch, France). The rate of $NAD^+$ reduction was monitored spectrophotometrically at 340 nm. LDH activity was calculated using molar absorption coefficient $\varepsilon = 6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$. The cytotoxicity results were expressed as a percentage of total cardiomyocyte content of LDH, determined by cell lysis with 0.1% Triton-X100 (Fluka, Seelze, Germany). It was established that neither SIH (100 μM) nor DAU (10 μM) has any direct effect on LDH activity in cell culture medium for up to 72 h at 37 °C.

For additional viability assessments, NVCMs were incubated for 48 h under control or given experimental conditions using both serum and pyruvate-free Dulbecco's modified Eagle's minimal essential/F12 medium at 37 °C. Cells were then washed and loaded with 3 μg mL$^{-1}$ of Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and 3 μg mL$^{-1}$ of propidium iodide (PI; Molecular Probes), respectively, for 20 min at room temperature. Hoechst 33342 is a blue-fluorescent probe ($\lambda_{ex} = 360$ nm; $\lambda_{em} = 460$ nm) that stains cell nuclei. In apoptotic cells, chromatin condensation occurs, and apoptotic cells can thus be identified as those with condensed and more intensely stained chromatin. PI is a red ($\lambda_{ex} = 560$ nm; $\lambda_{em} = 630$ nm) DNA-binding dye, which is unable to cross the plasma membrane of living cells, but readily enters necrotic (or late-stage apoptotic) cells and stains their nuclei with red fluorescence. Sample fields with approximately 250 cells were randomly selected and evaluated using an inverted epifluorescence microscope Nikon Eclipse TS100, ×40 Nikon air objective, digital cooled camera (1300Q; VDS Vosskühler, Osnabrück, Germany) and software NIS-Elements AR 2.20 (Laboratory Imaging, Prague, Czech Republic). The cells were scored as 'intact' (normal appearance of dark-blue Hoechst 33342-stained nucleus as well as the absence of red PI staining); 'apoptotic' (condensed and/or fragmented nuclei with diameter <3 μm; presumably apoptotic) and/or 'PI+' (red PI staining; necrotic or late-stage apoptotic). The number of intact, apoptotic and PI-positive cells was expressed as a percentage of the total number of nuclei counted.

*Malondialdehyde assay for lipid peroxidation determination*

Oxidative injury to NVCM was quantified by measuring malondialdehyde (MDA) formation. Isolated cardiomyocytes ($2 \times 10^6$ cells per sample per well) were incubated for 48 h under control conditions, with 10 μM DAU—alone or with 3 or 100 μM SIH. Cells were then washed twice with ice-cold phosphate-buffered saline and scraped from the dishes with 300 μL of fresh phosphate-buffered saline per dish. Cells were briefly (15 s) sonicated on ice and the cell lysates were stored at −80 °C. After thawing the samples they were sonicated again, centrifuged (700 *g*, 10 min, 4 °C) and 250 μL samples of supernatant were taken and analysed as described by Pilz *et al*. (2000) with minor modifications. Briefly, 50 μL of 6 M NaOH was added to each sample and after vortexing the solution was incubated for 30 min at 60 °C. The solution was then cooled on ice and 125 μL of 35% perchloric acid was added. After centrifugation (13 200 *g*, 10 min, 4 °C) 250 μL of supernatant was taken and derivatization was performed using 25 μL of 5 mM 2,4-dinitrophenylhydrazine. After 10 min in the dark, the solution (30 μL) was analysed using HPLC system (Shimadzu) with ultraviolet detection (310 nm): column—EC Nucleosil 100-5 C18, 4.6 mm × 125 mm heated on 30 °C, mobile phase—acetonitrile–water–acetic acid 380:620:2 (v/v/v), flow rate −1.0 mL min$^{-1}$. MDA concentrations were related to the protein content in each sample, determined by the bicinchoninic acid assay (Sigma) according to the manufacturer's instructions.

*Proliferation studies with HL-60 cells*

The HL-60 human acute promyelocytic leukaemia cell line was obtained from American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 (Roswell Park Memorial Institute) medium (Sigma) supplemented with 10% heat-inactivated foetal calf serum (Sigma), 1% penicillin–streptomycin (PAA Laboratories) and grown in a humidified atmosphere at 37 °C in 5% $CO_2$. Medium was renewed every 2–3 days. For the experiments, cells between passages 8 and 20 were used. For proliferation studies, cells in the log phase of growth were diluted to a density of $10^5$ cells mL$^{-1}$. Tested substances or their combinations had been added and cells were allowed to proliferate for 72 h under standard conditions. For combination assays, 12 nM of DAU was used, which was previously shown to induce 50%

growth inhibition. To quantify the number of viable cells after each treatment, suspension samples were taken, mixed 1:1 with 0.4% Trypan blue solution (Sigma), and the living (unstained) cells were counted using a Bürker's haemocytometer under a light microscope.

*Data analysis*

Results are expressed as mean ± s.e.mean of a given number of experiments. Statistical software SigmaStat for Windows 3.0 (SPSS, Chicago, IL, USA) was used. Significances of the differences were determined using one-way ANOVA with a Bonferroni *post hoc* test (comparisons of multiple groups with corresponding control). Data without a normal distribution were evaluated using the non-parametric Kruskal–Wallis ANOVA on ranks with Dunn's test. $P<0.05$ was used as the level of statistical significance. The $IC_{50}$ values were calculated with CalcuSyn 2.0 software (Biosoft, Cambridge, UK).

*Chemicals*

Salicylaldehyde isonicotinoyl hydrazone was synthesized in-house as described previously (Edward *et al*., 1988). The structure and purity of the compound were confirmed by $^{1}H$ and $^{13}C$ NMR, IR spectroscopy and HPLC with ultraviolet detection (Kovarikova *et al*., 2004). Stock solutions of SIH (0.1 M) were prepared in dimethyl sulphoxide and stored at −20 °C for less than 2 months. DAU hydrochloride was obtained from Pharmacia (Nerviano, Italy), 3% hydrogen peroxide water solution was from Fluka. Constituents for various buffers as well as other chemicals (for example, various iron salts) were from Sigma-Aldrich (Schnelldorf, Germany), Fluka, Merck (Darmstadt, Germany) or Penta (Prague, Czech Republic) and were of the highest available pharmaceutical or analytical grade.

## Results

*Assessment of displacement of iron from DAU–$Fe^{3+}$ complexes with SIH*

Addition of 100 μM SIH to DAU–Fe complex solution quickly reduced the absorbance at 600 nm (by 82% after 4 min—Figure 1b) and produced a spectrum close to that of uncomplexed DAU (Figure 1a). This indicates efficient removal of iron from its complex with DAU. Two other chelators, deferoxamine and EDTA, showed similar or even slightly higher efficiency in $Fe^{3+}$ removal, with the observed absorbance changes at 600 nm being 83 and 88%, respectively. The rate of Fe displacement among these three chelators declined in the order SIH > EDTA > deferoxamine (Figure 1b).

In vitro *cardiotoxicity studies*

Using our model system of NVCMs, we investigated the toxic and/or protective activity of various agents and their combinations. First, the effects of SIH (0.3–100 μM) against the oxidative injury induced by 500 μM $H_2O_2$ were examined. As seen in Figure 2, exposure of NVCM to $H_2O_2$ resulted in 6.5-fold increase in LDH release as compared with untreated cardiomyocytes. Co-incubation with SIH efficiently and dose-dependently protected the cardiomyocytes with an $IC_{50}$ of about 2 μM. Complete protection was achieved at SIH concentrations ⩾10 μM.

The 48-h incubation of cardiomyocytes with 10 μM of DAU resulted in dramatic changes of cellular morphology (Figure 3). During the first 24 h, cardiomyocytes increased their cellular volume and their nuclear structure became obvious. Cytoplasmic vacuolization and granulation were observed, which later proceeded into a disruption of the cellular monolayer and cellular as well as nuclear shrinkage. Eventually, formation of cell debris was conspicuous.

The results of determinations of cell viability with Hoechst 33342 and PI stainings are shown in Table 1. Exposure of myocytes to DAU for 48 h dramatically increased the proportion of cells exhibiting features of both apoptosis (cells with condensed chromatin) and necrosis (PI-stained cells). Notably, most of the cells exhibited both nuclear shrinkage and PI positivity (Figure 3), suggesting that either both modes of cell death took place or, more likely, that the observed necrosis was secondary to late-stage apoptosis. SIH, assayed at 3 and 100 μM concentrations, significantly increased the number of viable cells lacking both nuclear

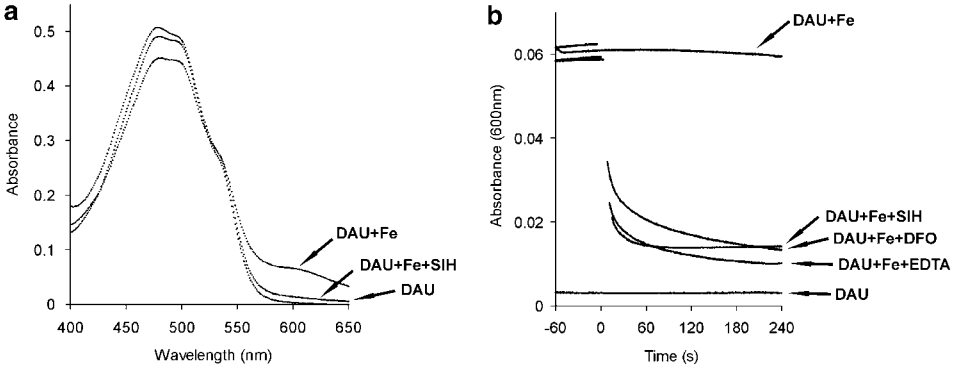


**Figure 1** Displacement of iron from its complex with daunorubicin (DAU). (**a**) Spectral scans of 45 μM DAU, complex of 45 μM DAU and 15 μM $FeCl_3$ (DAU + Fe) and DAU–Fe(III) complex 4 min after the addition of 100 μM salicylaldehyde isonicotinoyl hydrazone (SIH) (DAU + Fe + SIH). (**b**) Absorbance–time plots at 600 nm (absorbance peak of the DAU–Fe complex) before and after the addition of 100 μM SIH (or other two reference chelators—EDTA and deferoxamine (DFO)) to the complex of 45 μM DAU + 15 μM $FeCl_3$. Although the results shown are from a single experiment, they are each representative recordings of at least three repetitions.

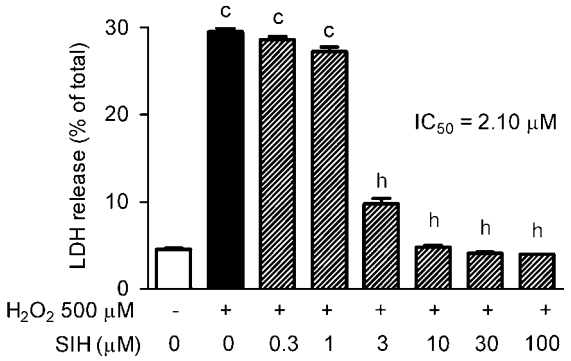


**Figure 2** Effects of salicylaldehyde isonicotinoyl hydrazone (SIH) (0.3–100 μM) on lactate dehydrogenase (LDH) release from neonatal ventricular cardiomyocytes 6 h after the exposure to 500 μM $H_2O_2$. $N=4$ experiments in each group; statistical significance (ANOVA; $P<0.05$): c—against the control (untreated) cells; h—against the $H_2O_2$-treated group.

**Table 1** Effects of SIH (3 and 100 μM) on cardiomyocyte (NVCM) viability after 48 h incubation with 10 μM daunorubicin (DAU)

| | *Intact cells (%)* | *Apoptotic cells (%)* | *PI + cells (%)* |
|---|---|---|---|
| Control | 90.3 ± 0.6 | 9.7 ± 0.6 | 1.1 ± 0.3 |
| DAU 10 μM | 9.7 ± 1.4[a] | 86.8 ± 2.6[a] | 74.6 ± 2.8[a] |
| DAU 10 μM + SIH 3 μM | 29.2 ± 1.3[a,b] | 68.6 ± 1.0[a,b] | 60.7 ± 4.0[a,b] |
| DAU 10 μM + SIH 100 μM | 22.4 ± 4.0[a,b] | 71.6 ± 4.3[a,b] | 75.3 ± 3.9[a] |

Abbreviations: NVCM, neonatal ventricular cardiomyocyte; SIH, salicylaldehyde isonicotinoyl hydrazone.
[a]Significantly different from control (untreated) group.
[b]Significantly different from DAU 10 μM group.
'Intact'—cells with normal appearance of dark blue Hoechst 33342-stained nucleus as well as the absence of a red propidium iodide (PI) staining; 'Apoptotic'—cells with distinctively condensed and intensively blue-stained nuclei; 'PI + '—cells with PI staining (necrotic/late-state apoptotic).
$P<0.05$: ANOVA; $N=4$ experiments.

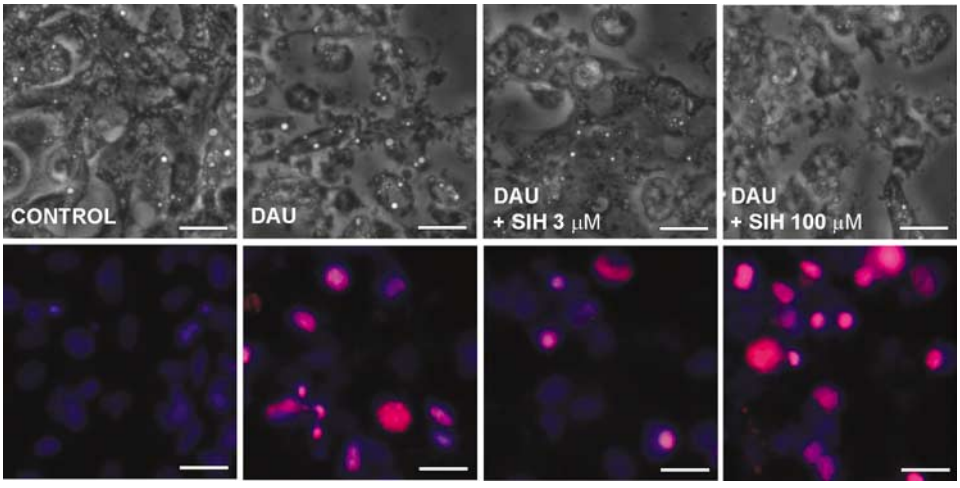


**Figure 3** Effects of daunorubicin (DAU), salicylaldehyde isonicotinoyl hydrazone (SIH) or their combination on neonatal ventricular cardiomyocytes (NVCMs). NVCMs were incubated for 48 h under control conditions, with DAU (10 μM) or DAU and SIH (3 or 100 μM) as indicated. Upper panels: brightfield microphotographs showing cellular morphology; lower panels: overlay of blue ($\lambda_{ex}=360$ nm; $\lambda_{em}=460$ nm) and red ($\lambda_{ex}=560$ nm; $\lambda_{em}=630$ nm) epifluorescence images of the same cells as above, double-stained with Hoechst 33342 and propidium iodide. Images were acquired with inverted epifluorescence microscope Nikon TS100, × 40 Nikon air objective, cooled digital camera VDS Vosskühler 1300Q and software NIS-Elements AR 2.20. Size bars: 10 μm.

condensation and PI positivity. The protection was, however, more pronounced with the lower, 3 μM SIH concentration (24%) than with 100 μM SIH (16%). Whereas 3 μM SIH significantly lowered the number of both apoptotic and necrotic cells, with the 100 μM SIH concentration, only a decrease in the number of apoptotic cells was observed (Table 1).

Treatment of NVCM with 10 μM DAU induced sixfold increase in LDH concentration in the cell culture medium as compared with the control (Figure 4b), which corresponded to 68% of the maximum LDH release following cell lysis with 0.1% Triton X-100. The effects of co-incubation of 10 μM DAU with SIH are shown in Figure 4b. SIH exerted significant cytoprotection at concentrations as low as 3 μM; however, this protection was only partial and was not dose-dependent.

Apart from this 48 h co-incubation with SIH and DAU, the protective potential of SIH was assayed in several other settings in search for more pronounced cytoprotective action: after 24 h incubation, toxicity of 10 μM DAU increased threefold over the control (as measured by LDH release) and whereas 3 μM SIH induced significant protection (38%; $P<0.05$), at higher concentrations of SIH, more variable protective effects were observed (from 8% protection at 10 μM to 22% protection at 100 μM SIH). Then, a 2 h exposure of cells to 10 μM DAU ± SIH with subsequent wash out and incubation for 72 h was tested. This resulted in a fivefold increase of LDH release by DAU and only very slight and nonsignificant protection (maximum 6%), which was observed only with 30 μM SIH. Lowering DAU concentrations from 10 to 1 or 3 μM did not result in better protective action of SIH either. Hence, we concluded that the limited protective potential of SIH against DAU-induced cardiotoxicity *in vitro* was not due to the particular experimental protocol. We therefore used a 48 h continuous exposure of 10 μM DAU and/or other studied substances, in subsequent experiments.

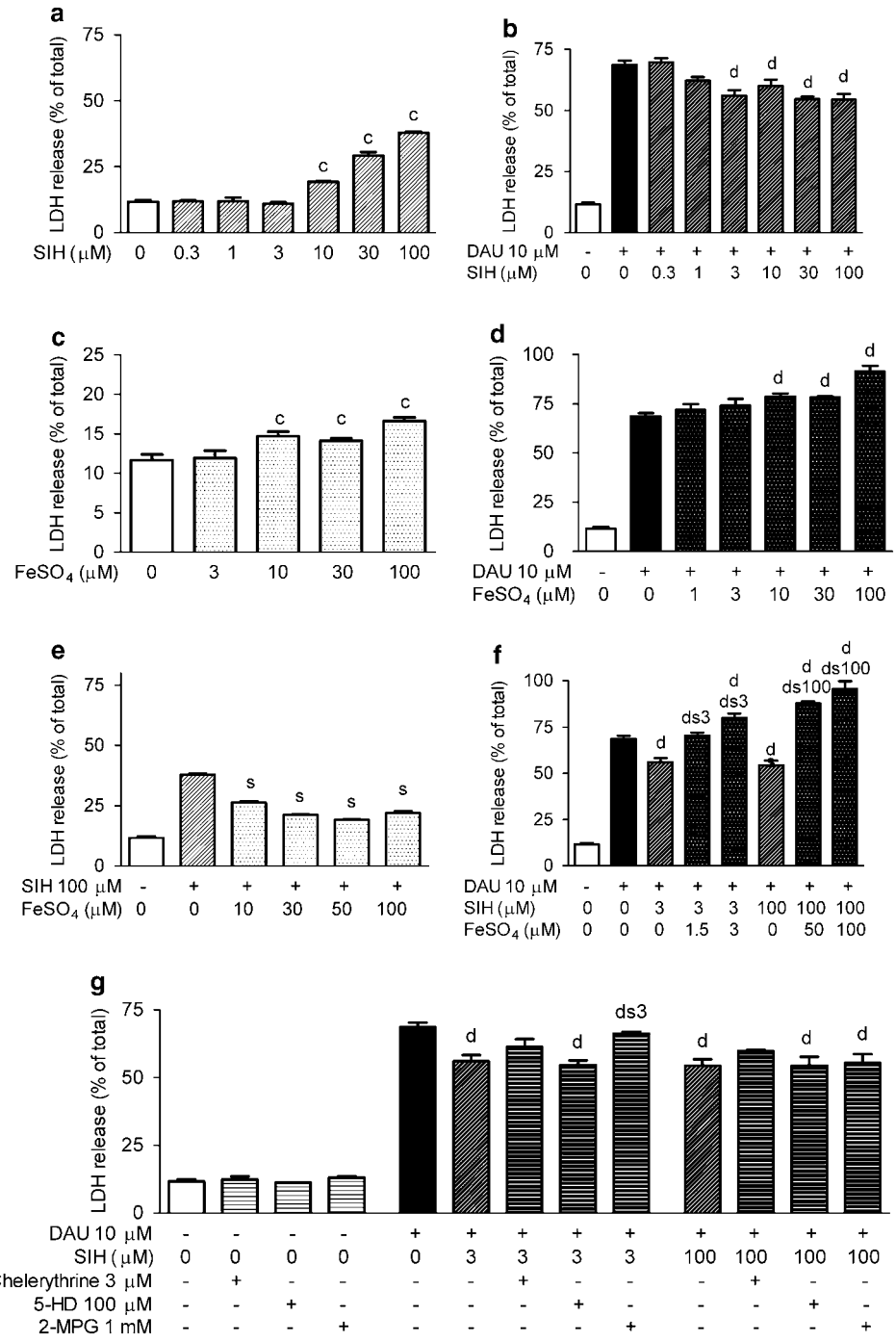


**Figure 4** Effects of 48 h incubation with various drugs or their combinations on lactate dehydrogenase (LDH) release from neonatal ventricular cardiomyocytes, expressed as a percentage of total cellular LDH content. (**a**) LDH release from cardiomyocytes exposed to SIH alone; (**b**) LDH release from cardiomyocytes exposed to daunorubicin (DAU, 10 μM)—alone or in combination with SIH; (**c**) LDH release from cardiomyocytes exposed to ferrous sulphate ($FeSO_4$); (**d**) effects of $FeSO_4$ on LDH release from cardiomyocytes induced by DAU; (**e**) effects of $FeSO_4$ on LDH release from cardiomyocytes induced by SIH; (**f**) effects of $FeSO_4$ on protection of cardiomyocytes from DAU-induced LDH release by 3 and 100 μM SIH; (**g**) effects of chelerythrine (3 μM; inhibitor of protein kinase C), 5-hydroxydecanoic acid (5-HD; 100 μM; blocker of mitochondrial $K_{ATP}$ channels) and 2-mercaptopropionyl glycine (2-MPG; 1 mM; reactive oxygen species (ROS) scavenger) on protection of cardiomyocytes from DAU-induced LDH release by 3 and 100 μM SIH. $N = 4$ experiments in each group; statistical significance (ANOVA; $P < 0.05$): c—against the control (untreated) cells; d—against the DAU (10 μM)-treated cells; s—against the cells incubated with 100 μM SIH; ds3—against the combination group of DAU (10 μM) with 3 μM SIH; ds100—against the combination group of DAU (10 μM) with 100 μM SIH. For the purpose of clarity, significant differences from control are not shown in (**b**, **d**–**g**).

Together with cytoprotection assays against DAU, the inherent toxicity of SIH was determined. As shown in Figures 4a and 7, although SIH was non-toxic at concentrations $\leqslant 3$ μM, at higher concentrations, it dose-dependently increased the LDH release, reaching 38% of maximum LDH release at 100 μM concentration.

Both SIH-induced protection and its own toxicity were shown to be iron dependent as these could be blunted by increasing iron ($FeSO_4$) content in the cell culture medium. Although the protection against DAU toxicity achieved with both 3 and 100 μM SIH could be fully abolished by iron supplementation (Figure 4f), the toxicity of 100 μM SIH was reduced significantly, but only incompletely (by 72%) with 50 μM $FeSO_4$ (Figure 4e). At iron concentrations exceeding a 2:1 SIH/Fe ratio (that is, the ratio when all iron coordination bonds are shielded), SIH toxicity was augmented (Figures 4e and f). $FeSO_4$ *per se* induced only limited (though statistically significant) toxicity to cardiomyocytes; it reached 17% of the maximal LDH release at 100 μM $FeSO_4$ (Figure 4c), whereas it markedly and dose-dependently potentiated the cardiotoxicity induced by DAU (Figure 4d). Apart from $FeSO_4$, ferric ammonium citrate was also tested with similar findings: dose-dependent loss of both SIH-induced protection against DAU toxicity and the low toxicity of the salt (data not shown).

In addition to iron salts, three inhibitors of various known cardioprotective pathways were examined for their ability to blunt the SIH-induced cytoprotection. As seen in Figure 4g, 3 μM chelerythrine (catalytic inhibitor of protein kinase C) showed a trend towards partial blockade of protection induced with both 3 and 100 μM SIH, which did not reach statistical significance. 5-Hydroxydecanoic acid (5-HD; a blocker of mitochondrial $K_{ATP}$ channels; 100 μM) displayed no effect. 2-Mercaptopropionylglycine (2-MPG; reducing agent and ROS scavenger; 1 mM) significantly (by 81%) reduced the protection induced by 3 μM SIH, but had no significant effect on protection by 100 μM SIH. At the concentrations used, none of these inhibitors caused measurable toxicity to cardiomyocytes, when used alone (Figure 4g).

*MDA assay for lipid peroxidation determination*

Exposure of NVCM to 10 μM DAU for 48 h resulted in significantly increased cellular MDA content, to 279% of control values. Co-incubation with either 3 or 100 μM SIH did not affect these raised MDA concentrations (Figure 5).

*Proliferation studies with HL-60 cells*

Under control conditions, suspension culture of HL-60 cells was propagated from the initial seeding density of $1 \times 10^5$ to $1.84 \pm 0.06 \times 10^6$ cells $mL^{-1}$ over the 72-h experimental period. During the pilot experiments, an $IC_{50}$ value of 12 nM was established for the antiproliferative effects of DAU. As seen in Figure 6b, low concentrations of SIH (<3 μM) had only little or no effect on the antiproliferative effects of DAU (12 nM), whereas 3–100 μM of SIH significantly and dose-dependently augmented its antiproliferative action. Furthermore, SIH itself displayed significant and dose-dependent inhibition of tumour cell growth at concentrations ⩾3 μM (Figure 6a). The $IC_{50}$ values for the antiproliferative activity of SIH were found to be 4.70 and 4.66 μM for SIH alone and SIH in the combination assay with DAU, respectively, suggesting that these actions of SIH and DAU were additive.

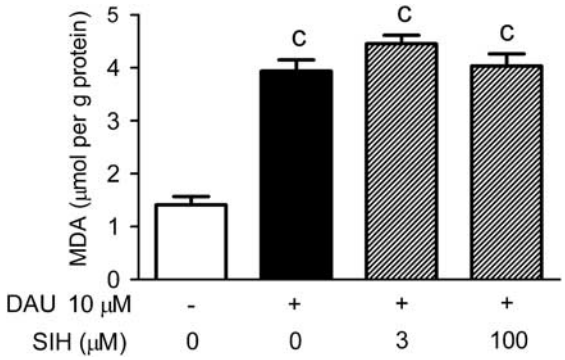


**Figure 5** Malondialdehyde (MDA) concentrations in neonatal ventricular cardiomyocytes incubated for 48 h under control conditions, with 10 μM daunorubicin (DAU)—alone or with 3 or 100 μM SIH. $N = 4$ experiments in each group; statistical significance (ANOVA; $P < 0.05$): c—against the control (untreated) group.

Experiments examining the effects of iron supplementation on proliferation of HL-60 cells and possible interferences with the actions of DAU and/or SIH are shown in Figures 6c–f. Under control conditions, $FeSO_4$ did not affect the rate of cell growth up to a concentration of 100 μM (Figure 6c). Similarly, when $FeSO_4$ was added to DAU (12 nM), no significant effect on DAU-induced inhibition of cell proliferation was observed (Figure 6d).

In contrast, $FeSO_4$ dose-dependently inhibited the antiproliferative action induced with 10 μM SIH, both alone and in its combination with DAU. In either of these two experimental settings, 5 μM $FeSO_4$ concentration (that is, a 2:1 SIH/Fe ratio) was just able to fully reverse effect of SIH (Figures 6e and f).

In Figure 7, the effects of exposing proliferating leukaemic HL-60 cells or non-proliferating NVCM to SIH are compared. The leukaemic cells were clearly more sensitive to SIH-induced iron deprivation. Significant inhibition of cellular proliferation was observed already at the very low micromolar range, and it further exponentially increased upon dose escalation. In contrast, with the same concentrations, the non-proliferating cardiac cells were relatively resistant to either SIH-induced iron deprivation or its eventual direct toxicity.

## Discussion

Anthracycline-induced cardiotoxicity remains a formidable and serious clinical problem (Adams and Lipshultz, 2005; Wouters *et al*., 2005; Jones *et al*., 2006; Barry *et al*., 2007). Production of ROS mediated by iron remains a prevailing explanation of anthracycline cardiotoxicity and intracellular iron chelation by the dexrazoxane hydrolysis product (ADR-925) is said to be responsible for its cardioprotection (Hasinoff *et al*., 1998; Adams and Lipshultz, 2005; Jones *et al*., 2006; Barry *et al*., 2007). If so, chelating agents with the same or even higher affinity and selectivity for iron should have similar protective effects. Therefore, for the present study, we have used SIH, an agent with very strong iron-chelating activity (Vitolo *et al*., 1990) and high antioxidant

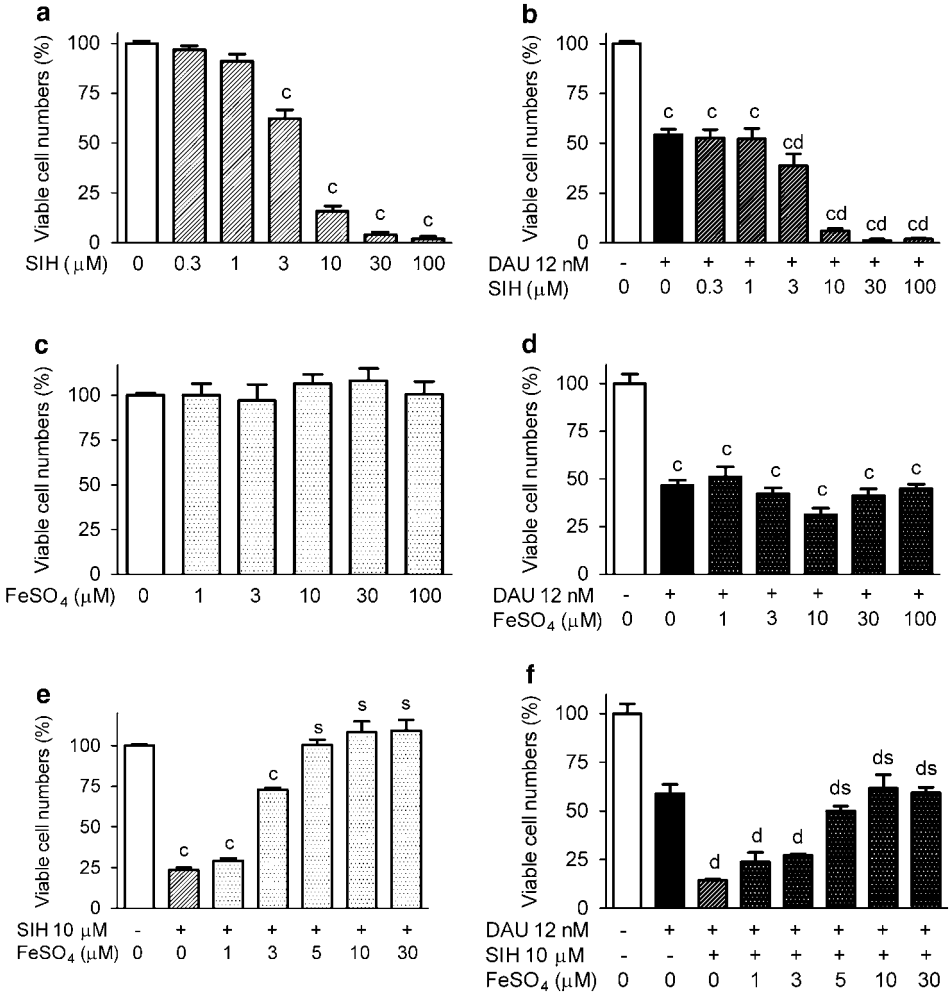


**Figure 6** Effects of 72 h incubation with various drugs or their combinations on the proliferation of HL-60 acute promyelocytic leukaemia cell line (percentage of viable cells relative to untreated control). (**a**) Proliferation of HL-60 cells exposed to SIH alone; (**b**) proliferation of cells exposed to daunorubicin (DAU, 12 nM)—alone or in combination with SIH; (**c**) proliferation of cells exposed to ferrous sulphate ($FeSO_4$); (**d**) effects of $FeSO_4$ on inhibition of HL-60 cells induced by DAU; (**e**) effects of $FeSO_4$ on inhibition induced by SIH; (**f**) effects of $FeSO_4$ on inhibition induced by combination of 12 nM DAU and 10 μM SIH. $N=4$ experiments in each group; statistical significance (ANOVA; $P<0.05$): c—against the control (untreated) cells; d—against the DAU (12 nM)-treated cells; s—against the cells incubated with 10 μM SIH; ds—against the combination group of DAU (12 nM) with SIH (10 μM). For the purpose of clarity, significances against control are not shown in (**f**).

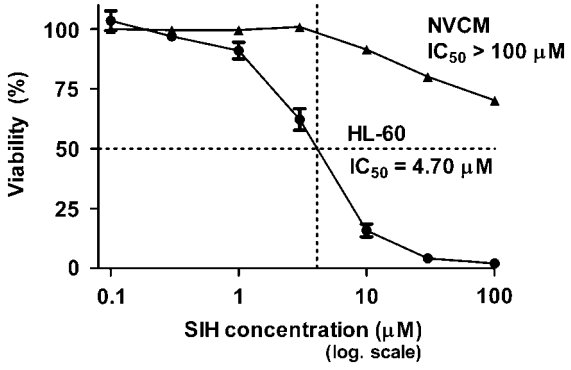


**Figure 7** Comparison of salicylaldehyde isonicotinoyl hydrazone (SIH) toxicity on non-proliferating neonatal ventricular cardiomyocytes (NVCMs) and the antiproliferative action of SIH in HL-60 cells. Viability of NVCM was determined as a percentage of total cellular lactate dehydrogenase (LDH) content not released to cell culture media following 48 h exposure to SIH. Proliferation of HL-60 cells—number of viable cells following 72 h exposure to SIH, relative to untreated control. $N=4$ experiments.

cytoprotective efficiency (Horackova *et al*., 2000; Simunek *et al*., 2005a; Kurz *et al*., 2006).

Two basic mechanisms of ROS production by anthracyclines have been described. The first mechanism is based on redox cycling of anthracycline aglycone, which produces superoxide and hydrogen peroxide, two substrates for the iron-catalysed Haber–Weiss reaction. The second mechanism proposes the formation of anthracycline–Fe(III) complexes that are allegedly responsible for further generation of free oxygen radicals (Myers *et al*., 1982). In this study as well as in previous experiments, SIH demonstrated the potential to interfere with both pathways. It has been repeatedly shown that SIH is able to rapidly enter many cell types (including cardiomyocytes) and bind free intracellular iron (Kakhlon and Cabantchik, 2002). Moreover, SIH provided complete protection of cardiomyocytes even against the toxicity of very high concentrations (500 μM) of $H_2O_2$, clearly demonstrating that shielding of intracellular free iron with this agent was able to efficiently prevent the Fenton and

Haber–Weiss-type reactions and associated cellular injury. Furthermore, SIH was demonstrated to quickly and efficiently remove iron from its complex with DAU.

Anthracycline cardiotoxicity was induced *in vitro* by a 48 h incubation of NVCM with 10 μM DAU. Treatment of cardiomyocytes with DAU resulted in considerable LDH release, which could be further dose-dependently increased by adding iron salts to cell culture media. This observation is consistent with previous reports showing exacerbation of anthracycline cardiotoxicity under conditions of iron overload in cultured cardiac cells (Hershko *et al*., 1993; Link *et al*., 1996) and in experimental animal models (Miranda *et al*., 2003; Panjrath *et al*., 2007).

SIH, assayed in a wide concentration range from 0.3 to 100 μM, significantly protected the myocytes, but only partially and without apparent dose dependency. The cytoprotective effect achieved with the 100 μM SIH concentration was comparable to that with the lowest effective SIH concentration—3 μM. The latter SIH dosage was, however, the only protective concentration that did not induce LDH release when added to cardiomyocytes alone. All the higher SIH concentrations dose-dependently increased LDH levels in culture media, indicating adverse effects of iron depletion after the prolonged incubation. It is possible that besides $Fe^{3+}$, SIH may scavenge low concentrations of $Fe^{2+}$, which is essential for the activity of many critically important enzymes.

The epifluorescent vital staining with Hoechst 33342 or PI confirmed the ability of SIH to reduce the toxicity of DAU to NVCM, as well as its rather limited efficiency and lack of further enhancement of the protective effect upon increase in concentration.

Indeed, the absence of a clear dose dependency in SIH-induced cardioprotection was also seen in the rabbit model of chronic DAU-induced cardiomyopathy, where the protective effects of SIH disappeared after moderate dose escalation (Sterba *et al*., 2007). Interestingly though, SIH did not induce any signs of cardiotoxicity after repeated 10-week administration to rabbits (50 mg $kg^{-1}$ i.p. weekly) as assessed by histopathological examination, functional cardiovascular measurements and cardiac troponin T plasma concentration determinations (Klimtova *et al*., 2003), showing that in the open *in vivo* system, SIH is able to protect the heart, but does not induce toxic iron depletion. Both the SIH-mediated protection of NVCM against DAU and its own toxicity were shown to be dependent on its iron chelation properties, as they could be dose-dependently antagonized by the addition of exogenous iron.

MDA is a product of polyunsaturated fatty acid peroxidation and it is a widely used biomarker of cellular oxidative stress (Halliwell and Gutteridge, 2007). MDA content significantly increased following 48 h exposure of NVCM to 10 μM DAU, but surprisingly neither 3 μM nor 100 μM SIH (both concentrations inducing significant cytoprotection) showed any decrease in MDA concentration; with 3 μM SIH even a slight increase could be seen. Previously, we have assayed and compared the effects of SIH on oxidative stress markers in the A549 human lung adenocarcinoma cell line (Kaiserova *et al*., 2006). Similarly as observed here with NVCM, SIH failed to mitigate the increased MDA formation and decreased glutathione (GSH) cellular content induced by doxorubicin. In sharp contrast, SIH efficiently prevented hydroxyl radical formation induced by the Fenton reagent $H_2O_2/Fe^{2+}$ (as shown by EPR spectroscopy experiments), and not only protected the A549 cells against the $H_2O_2/Fe^{2+}$ cytotoxicity but also efficiently prevented MDA production and GSH depletion (Kaiserova *et al*., 2006). This was also observed in H9c2 cardiomyoblasts where 10 μM SIH fully prevented the MDA formation induced by 100 μM $H_2O_2$ or tertbutyl-hydroperoxide (data not shown).

The observed discrepancies between the SIH-afforded protection against peroxide- and DAU-induced cardiomyocyte injury (with respect to efficiency, dose dependency, as well as markers of oxidative stress) strongly suggest that the protection against DAU—although iron dependent—is unlikely to be mediated by reduction of Fenton-type oxidative damage. It is plausible that the oxidative stress is not the primary cause of the anthracycline-induced cardiac injury, but rather its secondary by-product. A general failure of numerous antioxidants to efficiently protect against the chronic type of anthracycline cardiotoxicity (Myers *et al*., 1983; Berthiaume *et al*., 2005) further supports this hypothesis. Interestingly, some recent studies suggest that the clinically approved cardioprotectant, dexrazoxane, may act by mechanisms other than being a direct antioxidant. A very effective reduction of cardiac mitochondrial DNA lesions by dexrazoxane has been reported (Lebrecht and Walker, 2007) and this preservation of function of mitochondrially encoded respiratory chain enzymes can result in secondary oxidative stress reduction. In addition, Lyu *et al*. (2007) as well as Hasinoff and Herman (2007) have suggested that the dexrazoxane-induced cardioprotection may be linked to inhibition of topoisomerase II.

During the last decade, it has been shown that anthracyclines are able to interfere with cellular iron in a very complex manner and, without any doubt, this is not limited to the mere production of the toxic free oxygen radicals. Anthracyclines and/or their metabolites have been shown to perturb cellular iron metabolism by interacting with multiple molecular targets, including the iron regulatory proteins 1 and 2 (IRP1 and IRP2). The RNA-binding activity of these molecules regulates expression of the transferrin receptor 1 and ferritin, which are crucial proteins involved in iron uptake and storage, respectively (Minotti *et al*., 2001, 2004a, b; Kotamraju *et al*., 2002). Moreover, it has been shown that doxorubicin can induce accumulation of iron in ferritin and prevent its mobilization (Kwok and Richardson, 2003), which might possibly result in relative deficiency of iron for metabolic use.

Interestingly, iron chelators have also been shown to interact with cardiomyocytes in a more complex way than just serving as antioxidants. In a recent study, cardioprotection with deferoxamine has been linked to signal-transduction pathways involved in cardiac pre-conditioning (Dendorfer *et al*., 2005). Therefore, to examine the possibility that SIH activated cardioprotective pathways, which are generally dependent on protein kinase C, mitochondrial $K_{ATP}$ channels and ROS signalling, we have tested whether chelerythrine, 5-HD or 2-mercaptopropionyl glycine (2-MPG), the inhibitors of these three respective pathways, have any influence on protection exerted by SIH. Neither

chelerythrine nor 5-HD had any significant effects. 2-MPG abolished the protection, but only that mediated by SIH added in 3 μM concentration. Perhaps, low concentrations of SIH may incompletely chelate and/or redistribute free cellular iron and promote redox cycling and ROS formation. The slight increase in MDA content seen with the 3 μM SIH concentration also indicates that this could have taken place. Indeed, Buss *et al*. (2004) have shown that the SIH–$Fe^{3+}$ complex may be redox-active and toxic within the intracellular environment. In any case, the observed abolition of the 3 μM SIH-induced protection with antioxidant 2-MPG is another finding arguing strongly against the antioxidant mechanism of SIH action. On the other hand, other mechanisms might have taken place at the higher SIH concentration, as 2-MPG did not have any effect on SIH-afforded cardioprotection in this setting. Therefore, additional studies investigating the mechanisms of how iron chelation actually inhibits anthracycline-mediated cardiotoxicity are required.

Lack of interference with anticancer efficacy of anthracyclines is obviously a crucial prerequisite for any potential cardioprotective agent. In the present study, SIH concentrations that reduced the DAU toxicity to cardiomyocytes (that is, 3–100 μM) did not diminish the effectiveness of the antiproliferative action of DAU to the HL-60 promyelocytic leukaemia cell line. In contrast, SIH dose-dependently augmented its tumoricidal action. Indeed, aroylhydrazones are known for their ability to exhibit considerable antiproliferative action towards a great variety of cancer cell lines (Richardson *et al*., 1995) and, in the present study, SIH efficiently and dose-dependently inhibited the HL-60 cell growth. The antiproliferative action of SIH was shown to be mediated by iron chelation as it could be dose-dependently blocked by the addition of exogenous Fe, reaching complete inhibition at a 2:1 SIH/Fe ratio. Notably, this study points out marked differences in sensitivity to iron deprivation between cardiac and neoplastic cells (Figure 7). Proliferating tumour cells are known to be highly dependent on iron supply and are therefore sensitive to iron deprivation. The main target for this activity is probably an iron-dependent enzyme ribonucleotide reductase that is crucial for DNA synthesis (Cooper *et al*., 1996; Kolberg *et al*., 2004), although recently a multitude of other cell cycle control molecules have also been shown to be regulated by iron (Yu *et al*., 2007). Considering this, iron chelators can be very effective antiproliferative agents, and many of them have already shown great potential to be clinically applied in the treatment of cancer (Kalinowski and Richardson, 2005). Importantly, the present study suggests that they might also increase the curative effect of anthracyclines.

Results of this study encourage further *in vivo* experiments examining both cardioprotection and tumour response in a single experimental animal model. The original assumption of iron chelation-based cardioprotection proposed that chelators should protect the heart while allowing safer escalation of anthracycline dose. However, it would be of great interest to assess whether iron chelation with SIH or other suitable ligands may rather permit decrease of anthracycline dose with preserved anticancer efficiency and added cardioprotection.

In conclusion, iron chelation with SIH has been shown to possess a potential to reduce the toxicity of DAU to isolated cardiac cells, but only partially and most likely through mechanism(s) other than that originally proposed, inhibition of iron-catalysed ROS formation. In addition, iron chelators could further favourably influence the therapeutic action of anthracyclines by promoting their anticancer efficiency.

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## Conflict of interest

The authors state no conflict of interest.

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