Chemical Research in Toxicology®

Article

Structure-Activity Relationships of Nitro-Substituted Aroylhydrazone Iron Chelators with Antioxidant and Antiproliferative Activities

Katerina Hruskova, Eliska Potuckova, Lukas Opalka, Tereza Hergeselova, Pavlina Haskova, Petra Kovarikova, Tomas Sim#nek, and Katerina Vavrova

Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.7b00324 • Publication Date (Web): 16 May 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

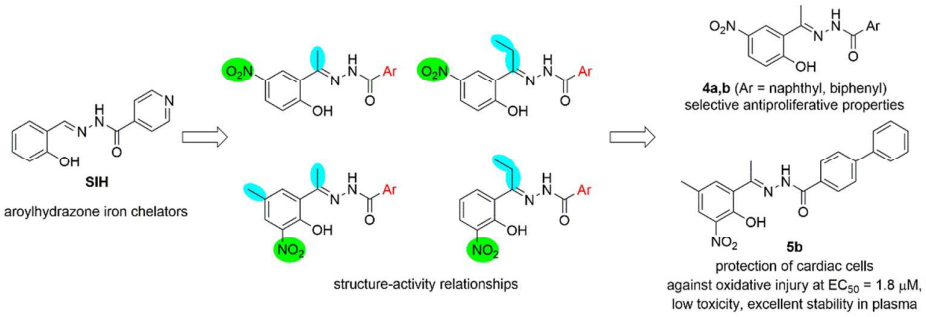
Structure-Activity Relationships of Nitro-Substituted Aroylhydrazone Iron Chelators with Antioxidant and Antiproliferative Activities

Kateřina Hrušková, Eliška Potůčková, Lukáš Opálka, Tereza Hergeselová, Pavlína Hašková, Petra Kovaříková, Tomáš Šimůnek, Kateřina Vávrová**

Charles University, Faculty of Pharmacy in Hradec Králové, Akademika Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

KEYWORDS. iron chelator; hydrazone; oxidative injury; anticancer; structure-activity relationships

TOC graphic


SIH
aroylhydrazone iron chelators
structure-activity relationships
4a,b (Ar = naphthyl, biphenyl)
selective antiproliferative properties
5b
protection of cardiac cells
against oxidative injury at EC50 = 1.8 μM,
low toxicity, excellent stability in plasma

ABSTRACT. Aroylhydrazone iron chelators such as salicylaldehyde isonicotinoyl hydrazone (SIH) protect various cells against oxidative injury and/or display antineoplastic activities. Previous studies have shown that a nitro-substituted hydrazone, namely, NHAPI, displayed markedly improved plasma stability, selective antitumor activity, and moderate antioxidant properties. In this study, we prepared four series of novel NHAPI derivatives and explored their iron chelation activities, anti- or pro-oxidant effects, protection against model oxidative injury in the H9c2 cell line derived from rat embryonic cardiac myoblasts, cytotoxicities to the corresponding noncancerous H9c2 cells, and antiproliferative activities against the MCF-7 human breast adenocarcinoma and HL-60 human promyelocytic leukemia cell lines. Nitro substitution had both negative and positive effects on the examined properties, and we identified new structure-activity relationships. Naphthyl and biphenyl derivatives showed selective antiproliferative action, particularly in the breast adenocarcinoma MCF-7 cell line, where they exceeded the selectivity of the parent compound NHAPI. Of particular interest is a compound prepared from 2-hydroxy-5-methyl-3-nitroacetophenone and biphenyl-4-carbohydrazide, which protected cardiomyoblasts against oxidative injury at 1.8 ± 1.2 μM with 24-fold higher selectivity than SIH. These compounds will serve as leads for further structural optimization and mechanistic studies.

Introduction

Iron is an essential element for virtually all organisms. However, this transition metal may catalyze the Fenton reaction, which yields hydroxyl radicals that are highly reactive and toxic to various biological macromolecules.[1] Therefore, the human body has a finely tuned mechanism for how to absorb, conserve, recycle and store iron, although its excretion is not regulated and is restricted to intestinal and skin cell desquamation, minor (e.g., menstrual) bleeding and urinary excretion.[2] Accumulation of iron and subsequent organ damage caused by iron-mediated oxidative stress is connected with numerous illnesses characterized by either local or generalized iron overload, such as hereditary hemochromatosis, some neurodegenerative and cardiovascular diseases, and thalassemia and other anemias requiring repeated blood transfusions.[3] Of note, several recent studies have demonstrated the complex roles of iron in cancer growth. This includes free radical formation with potentially promutagenic effects and, most importantly, the impaired regulation of iron metabolism, which can escalate cancer risk and stimulate tumor growth. Tumorigenesis appears to be associated with regulation of cellular iron intake through an iron efflux pump ferroportin and its regulator hepcidin . Cancer cells have impaired control of gene expression of proteins that regulate iron, including differences in miRNA, methylation, and acetylation.[4, 5]

Iron chelating agents have been successfully used in diseases with systemic iron overload in clinical practice.[6] In addition to systemic iron overload conditions, a substantial evidence suggests that iron chelation is also beneficial to prevent iron-mediated worsening of oxidative stress, which can take place locally even with normal body iron levels.[7] Furthermore, cancer cells require increased iron supplementation due to their fast and sustained growth, and the iron deprivation of cancer cells may represent another promising therapeutic effect of iron chelators.[8]

Salicylaldehyde isonicotinoyl hydrazone (SIH, Figure 1A) is a tridentate chelator that selectively forms 2:1 complexes with Fe^{3+}. SIH has low molecular weight and optimal lipophilicity, and can be administered orally.[9] It readily penetrates the cell membrane, and strongly chelates the intracellular labile iron pool; hence, it efficiently prevents the formation of hydroxyl radicals.[9] SIH has previously been shown to effectively protect guinea pig and rat isolated cardiomyocytes, as well as other cell types, against various types of oxidative injury.[10-13] In addition, SIH displayed the best ratio of cytoprotection to its inherent toxicity among seven iron chelating agents of several distinct chemical structures, including three clinically used drugs: desferrioxamine (DFO), deferiprone (L1, CP20) and deferasirox (ICL670A).[14] Aroylhydrazone iron chelators also showed antiproliferative activity, especially those of the 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311) or 2-pyridylketone isonicotinoyl hydrazone (PKIH) class.[8]

However, SIH (as well as other aldehyde-based aroylhydrazone chelators) undergoes fast hydrolysis in plasma and has a short half-life *in vivo*.[15] Recently, the plasma stability of aroylhydrazones has been substantially increased by alkylation of the hydrazone carbon, *i.e.*, by replacing salicylaldehyde with 2-hydroxyacetophenone (compound HAPI, Figure 1A) or 2-hydroxypropiophenone (HPPI).[16] Both HAPI and HPPI retained the cytoprotective activity of SIH despite having antiproliferative activities that were even higher than that of SIH.[16, 17] Further studies on HAPI and HPPI derivatives with modified hydrazide scaffolds showed that these compounds can be tuned to promote antioxidant (pyridazine derivative) or antiproliferative properties (naphthyl and biphenyl derivatives).[18] In contrast, the reduction of the C=N bond or the introduction of branching close to this bond decreased both the antioxidant and antiproliferative properties.[19]

Further substitution of the phenol ring in HAPI resulted in the identification of several promising substances. In particular, the nitro group-containing (*E*)-*N′*-[1-(2-hydroxy-5-nitrophenyl)ethylidene]isonicotinoylhydrazide (NHAPI; Figure 1A) showed exceptional plasma stability and selective antitumor activity against the MCF-7 human breast adenocarcinoma and HL-60 human promyelocytic leukemia cell lines. Although the nitro group has long been considered to be a source of toxicity and is generally avoided in drug design, compound NHAPI was one of the least toxic in H9c2 cardiomyoblasts among the aroylhydrazones studied.[16, 17] Of note, several nitro group-containing drugs have recently been introduced into clinical practice.[20, 21]

In this work, we further explored the structure-activity relationships in nitro-substituted aroylhydrazone iron chelators. We synthesized four series of compounds that were designed as hybrids of NHAPI[16] and compounds described in our most recent work[18] by combining the nitro group-containing ketones with hydrazides bearing naphthyl, biphenyl, pyridine-3-yl, pyridine-4-yl, pyrazine-2-yl and pyridazin-4-yl groups (Figure 1B, compounds a, b, c, d, e and f, respectively). The first series of compounds, **4a-f**, are based on the same ketone as NHAPI, *i.e.,* 2-hydroxy-5-nitroacetophenone (Figure 1B – series **4**). Compounds of series **9** are homologs of series **4**, with an ethyl group instead of a methyl group at the hydrazone carbon, while series **10** has the nitro group shifted to position 3 relative to the hydrazone (Figure 1B). Finally, aroylhydrazones of series **5** are isomers of series **10**, with the ethyl group replaced by two methyl groups, one at the hydrazone carbon and the second in position 5 of the phenyl ring (Figure 1B).

The prepared compounds were examined for their iron-chelating ability, capability to mobilize iron and prevent its uptake from transferrin, anti- and pro-oxidative properties, ability to protect H9c2 cardiomyoblasts against hydrogen peroxide-induced model oxidative

injury, inherent toxicity in H9c2 cardiomyoblasts, and antiproliferative activity in the MCF-7 human breast adenocarcinoma and HL-60 human promyelocytic leukemia cell lines. The most promising compounds were further examined for their stability in plasma *in vitro*.

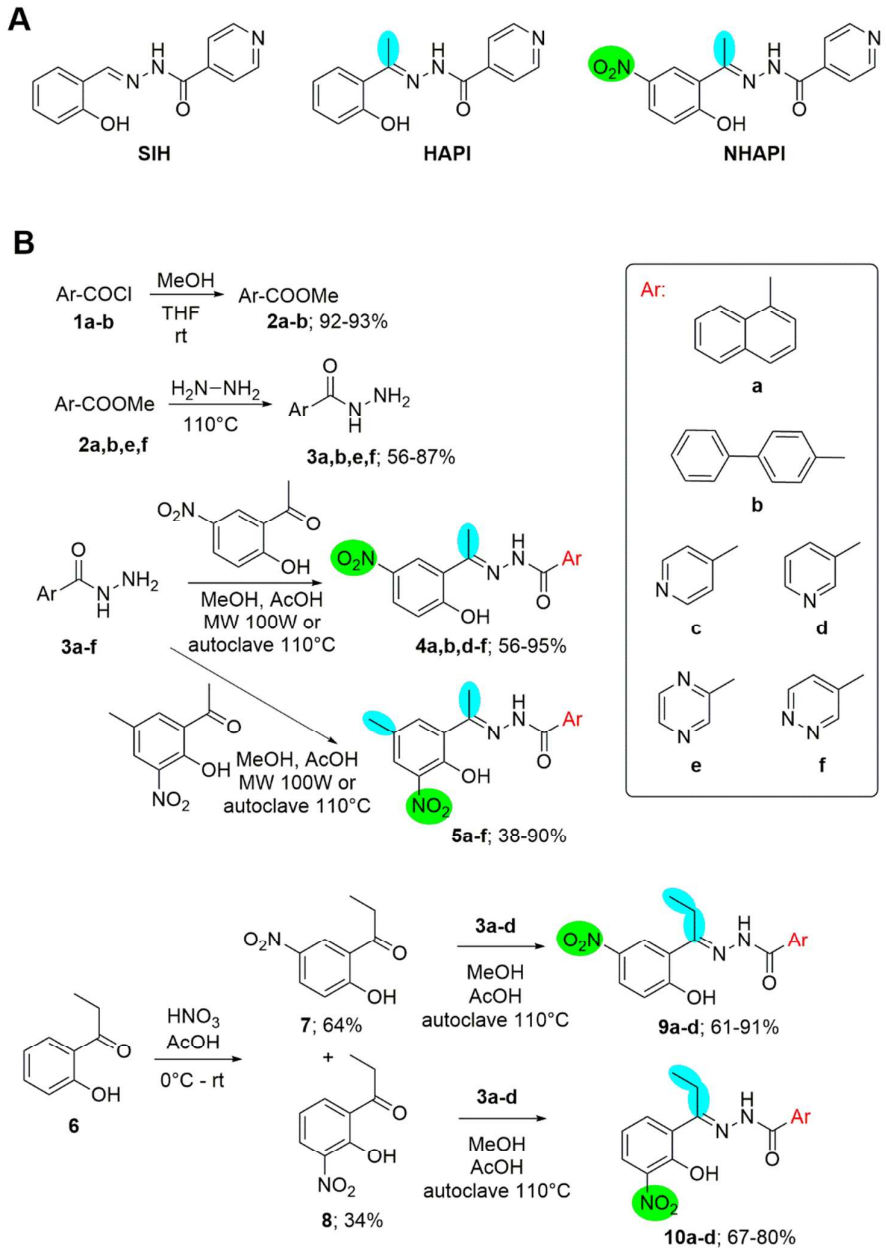


Figure 1. Structures of known aroylhydrazone iron chelators (panel A) and syntheses of the examined compounds (panel B).

Experimental procedures

Chemicals and instrumentation

Syntheses of the ligands are described in the Supporting Information. Methyl 1-naphthoate (**2a**), 1-naphthohydrazide (**3a**), methyl biphenyl-4-carboxylate (**2b**), biphenyl-4-carbohydrazide (**3b**), pyrazine-2-carbohydrazide (**3e**), and pyridazine-4-carbohydrazide (**3f**) were prepared as described previously.[18] All other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany), Merck (Darmstadt, Germany) or Penta (Prague, Czech Republic) and were of the highest available pharmaceutical or analytical grade. Thin layer chromatography was performed on TLC sheets (silica gel 60 F_{254}) from Merck (Darmstadt, Germany). Microwave reactions were conducted in a Milestone Micro-SYNTH Ethos 1600 URM apparatus (Milestone Inc., Shelton, CT, USA). Melting points were measured on a Kofler apparatus and are uncorrected. IR spectra were measured on a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA). All products were characterized by NMR (Varian Mercury Vx BB 300 or VNMR S500 NMR spectrometers, Varian, Palo Alto, CA, USA). Chemical shifts were reported as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal. Elemental analysis was measured on a CHNS-OCE FISONS EA 1110 apparatus (Fisons, Ipswich, UK). MS experiments (ESI+) were performed with an Agilent 500 Ion Trap LC/MS (Agilent Technologies, Santa Clara, California, USA). HPLC-HRMS experiments (ESI+) were performed using an HRMS system Acquity UPLC I-class and a Synapt G2Si Q-TOF mass spectrometer (Waters, Milford, MA, USA).

Cell cultures

The H9c2 and HL-60 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and the MCF-7 cell line was from the European Collection of Cell Cultures (ECACC; Salisbury, UK). H9c2 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with and without phenol red, respectively, (DMEM; Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lonza), 1% penicillin/streptomycin solution (Lonza) and 10 mM HEPES buffer (Sigma-Aldrich). HL-60 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution (Lonza). Each cell line was cultured in 75 cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) at 37°C in a humidified atmosphere of 5% CO_2. Subconfluent cells (H9c2 and MCF-7) or cell suspensions (HL-60) were subcultured every 3-4 days.

The cells were seeded in 96-well plates (TPP) at a density of 10,000 cells per well (H9c2 and HL-60) or 5,000 cells per well (MCF-7). H9c2 cells were seeded in the plates 48 h prior to the addition of a studied compound, and 24 h prior to the experiment, the medium was exchanged for serum- and pyruvate-free DMEM (Sigma-Aldrich). MCF-7 cells were seeded in the plates 24 h prior to the addition of the studied compound.

Proliferation and cytotoxicity studies, protection against oxidative injury

The cytotoxic/antiproliferative activities of the examined hydrazones were studied at different concentrations after a 72-h incubation. 0.1% DMSO (v/v) was added to increase the hydrazone solubility in the medium. At this concentration, DMSO had no effect on cellular proliferation. Model oxidative injury in H9c2 cells was induced by the addition of 200 μM H_2O_2 to the culture medium at the same time as the chelator. The protective effect was

evaluated at various concentrations of the tested substances after 24-h of incubation. Again, 0.1% DMSO (v/v) was used in the culture medium.

The cell viabilities of H9c2 and MCF-7 cells were evaluated by a neutral red assay, which measures the incorporation of the dye into the viable lysosomes.[22] The optical density of the neutral red dye was measured using a Tecan Infinite 200M plate reader (Tecan Group, Männedorf, Switzerland) at λ = 540 nm. Viabilities of HL-60 cells were evaluated by the ability of active mitochondria to reduce yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) to purple formazan according to the manufacturers' instruction. The optical density of MTT was measured at λ = 570 nm with the subtraction of the λ = 690 nm background using a Tecan Infinite 200M plate reader. The viabilities of hydrazone-treated cells were expressed as a percentage of untreated controls (100%).

Determinations of iron chelating efficiencies in solution

To assess iron chelation potential of the new hydrazones in solution, calcein fluorescence[23] was measured. Calcein is a fluorescent probe that forms complexes with iron ions, causing fluorescence quenching. The addition of the iron-chelating agent to the Fe-calcein removes iron from its calcein complex with a new complex forming. This is manifested by an increased fluorescence. A complex of calcein (free acid, 20 nM) with ferrous ammonium sulfate (FAS; 200 nM) was prepared in HEPES-buffered saline (150 mM NaCl, 40 mM HEPES; pH 7.2). Calcein and FAS were stirred continuously in the dark for 45 min, after which > 90% of fluorescence was quenched. Next, 995 µl of the complex was transferred into a stirred cuvette, and a baseline was measured. After 100 s, 5 µl of studied compound solution

was added yielding a final concentration of 5 μM. Fluorescence intensity change was measured as a function of time at 25°C using a Perkin Elmer LS50B fluorimeter (Perkin Elmer, Waltham, MA, USA) at λ_{ex} = 486 nm and λ_{em} = 517 nm for 350 s. The iron chelation efficiency in solution was expressed as the percentage of efficiency of the reference chelator SIH (100%).

Ascorbate oxidation assay for analysis of redox activity of iron complexes

The ascorbate oxidation assay was employed to evaluate the redox activities of the iron complexes of chelators in buffered solution using an established protocol.[24, 25] In brief, 100 μM ascorbic acid was prepared immediately prior to the experiment and incubated either alone or in the presence of 10 μM Fe^{3+} (added as ferric chloride in a 50-fold molar excess in 500 μM citrate) and chelators. Chelators were evaluated at iron binding equivalents (IBE) of 0.1 (excess of Fe), 1 (fully charged Fe-chelator complexes) and 3 (excess of free chelator). The chelators EDTA and DFO were used as positive and negative controls, respectively, as their redox activity has been well characterized.[26] The decrease in absorbance at 265 nm, which is proportional to ascorbate oxidation, was measured after 10 and 40 min incubation at room temperature using the Tecan Infinite 200M plate reader (Tecan, Austria). The decrease in absorbance between the two time points was calculated and expressed as a percentage of the control group without chelator (100%).

The effects of hydrazones on mobilizing cellular ^{59}Fe

Human transferrin (Tf) (Sigma-Aldrich) was labeled with ^{56}Fe or ^{59}Fe (PerkinElmer, Massachusetts, USA) to produce $^{56}Fe_2$-Tf or $^{59}Fe_2$-Tf, respectively, as previously described.[27, 28] Unbound ^{59}Fe was removed by exhaustive vacuum dialysis against a large excess of 0.15 M NaCl buffered at pH 7.4 with 1.4% $NaHCO_3$ by standard methods.[27, 28]

To study the potencies of the prepared hydrazones to mobilize ^{59}Fe from MCF-7 cells, Fe efflux experiments were carried out using established techniques.[25, 29] In brief, after pre-labeling the cells with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C, the cell cultures were washed four times with ice-cold phosphate buffered saline (PBS) and were then incubated with each chelator (25 μM) for 3 h at 37°C. The overlying medium containing released ^{59}Fe was then separated from the cells. Radioactivity was measured in both the cell pellet and supernatant using a γ-scintillation counter (Wallac Wizard 3, Turku, Finland).

Effect of the hydrazones on preventing cellular ^{59}Fe uptake from ^{59}Fe-Tf

The ability of the compounds to prevent cellular ^{59}Fe uptake from ^{59}Fe-Tf was examined using standard techniques.[25, 29] In brief, MCF-7 cells were incubated with ^{59}Fe-Tf (0.75 μM) for 3 h at 37°C in the presence of the chelators (25 μM). The cells were then washed four times with ice-cold PBS, and internalized ^{59}Fe was determined via established methods by incubating the cell monolayer for 30 min at 4°C with the general protease Pronase (1 mg/ml; Sigma-Aldrich). The cells were then harvested from the monolayer with a plastic spatula and centrifuged for 1 min/12,000 g. The supernatant represents membrane-bound, Pronase-sensitive ^{59}Fe that was released by the protease, while the Pronase-insensitive fraction represents internalized ^{59}Fe.[25, 28, 29] The amount of internalized Fe was expressed as a percentage of Fe internalized by the control (untreated) cells (100%).

Stability in plasma

Heparinized drug-free rabbit plasma was spiked with a standard solution of each chelator in DMSO (1 mg/ml) to obtain a concentration of 100 µM. The plasma was incubated at 37°C in a Thermomixer comfort (Eppendorf, Germany) for 10 h and gently shaken (500 rpm) during the experiment. Fifty microliters of plasma was taken at 0, 1, 2, 4, 6, 8 and 10 h, spiked with an internal standard and precipitated with methanol (200 µl). The clear supernatant was then analyzed.

HPLC assays were performed on an LC 20A Prominence (Shimadzu, Duisburg, Germany) chromatographic system with a PDA detector. Separation was achieved on an Ascentis® C18 column (100 × 3 mm, 3 µm) protected with a guard column (2 × 4 mm, 3 µm, both from Sigma-Aldrich, Schnelldorf, Germany). The mobile phase was composed of 10 mM NaH_2PO_4 with 2 mM EDTA and methanol in either a 20:80 (**4a** and **4b**) or 25:75 (**5b**) ratio. Quantification was performed at 279 nm. A flow rate of 0.3 ml/min, a column temperature of 25°C and an injection volume of 20 µL were used. Either (*E*)-*N′*-[1-(2-hydroxyphenyl)propylidene]biphenyl-4-carbohydrazide or (*E*)-*N′*-[1-(2-hydroxyphenyl)propylidene]-1-naphthoylhydrazide were used as internal standards for the analysis of **4a-b** and **5b**, respectively. All methods fulfilled the general validation criteria for bioanalytical methods.

Data analysis

The concentrations of chelators inducing a 50% proliferation decrease (IC_{50}) or 50% cellular protection (EC_{50}) were calculated using CalcuSyn 2.0 software (Biosoft, Cambridge, UK). SigmaStat for Windows 3.5 (Systat Software, San Jose, CA, USA) was used for

statistical analysis. Data are presented as a mean ± SD of a given number of experiments. Statistical significance was determined using ANOVA with a Bonferroni *post hoc* test (comparisons of multiple groups against a corresponding control). The results were considered to be statistically significant at $p < 0.05$.

Results

Synthesis

The target aroylhydrazones were synthesized via acetic acid-catalyzed condensation of a ketone with the respective hydrazide in a microwave reactor at 100 W or in an autoclave at 110°C (Figure 1). Ketones **7** and **8** were prepared by nitration of 2-hydroxypropiophenone in glacial acetic acid; the two positional isomers (**7** and **8**) were separated by column chromatography. Hydrazides **3a**, **3b**, **3e** and **3f** were synthesized from the corresponding methyl esters as described previously.[18] The calculated values of molecular weight (MW), lipophilicity (logP) and acidity of the phenol group (pKa) are given in Table 1.

Table 1. The chemical properties of the studied compounds, their protective effects against model oxidative injury of H9c2 cardiomyoblasts, their inherent toxicities to H9c2 cells, and their antiproliferative potential in MCF-7 and HL-60 cell lines compared to the lead compounds SIH and NHAPI.

Comp.	MW	logP	pKa	EC_{50} H9c2 (μM)	TC_{50} H9c2 (μM)	IC_{50} MCF-7 (μM)	IC_{50} HL-60 (μM)	Ratio TC_{50} H9c2/ IC_{50} MCF-7	Ratio TC_{50} H9c2/ IC_{50} HL-60
SIH [a]	241.3	1.64	9.2	18.2	28.9 ± 6.7	12.4 ± 0.6	5.7 ± 0.5	2.3	5.1
NHAPI (**4c**)	300.3	0.97	7.8	68.5	241.5 ± 52.9	19.4 ± 6.0	2.3 ± 0.5	12.6	104
4a	349.4	3.20	7.8	NA	153.9 ± 3.6	4.7 ± 0.4	5.5 ± 1.3	**33.1**	28.2
4b	375.4	3.52	7.8	22.8 ± 2.2	45.0 ± 1.6	3.0 ± 0.7	1.6 ± 0.2	**15.2**	27.6
4d	300.3	0.97	7.8	NA	37.1 ± 9.7	18.1 ± 2.5	5.1 ± 2.0	2.1	7.2
4e	301.3	0.09	7.8	NA	> 200*	28.3 ± 7.6	24.6 ± 4.0	> 7	> 8
4f	301.3	3.41	7.8	NA	> 200*	> 200*	> 200*	NA	NA
5a	363.4	3.62	5.4	12.4 ± 12.1	16.2 ± 4.5	7.2 ± 1.5	2.8 ± 0.3	2.3	5.9
5b	389.4	3.93	5.4	1.8 ± 1.2	69.7 ± 3.9	6.8 ± 1.8	6.0 ± 1.9	10.5	11.7
5c	314.3	1.39	5.4	NA	69.7 ± 0.8	53.0 ± 7.2	10.8 ± 1.9	1.3	6.5
5d	314.3	1.39	5.4	61.2 ± 3.4	44.9 ± 7.4	22.3 ± 1.8	9.1 ± 3.1	2.0	4.9
5e	315.3	0.51	5.3	NA	> 200*	28.4 ± 6.5	21.0 ± 5.6	> 7	> 10

5f	315.3	3.82	5.4	NA	> 200*	> 200*	> 200*	NA	NA
9a	363.4	3.66	7.7	10.3 ± 1.7	35.9 ± 2.7	5.1 ± 1.3	5.1 ± 0.9	7.0	7.0
9b	389.4	3.97	7.8	1.4 ± 1.0	1.0 ± 0.4	3.5 ± 0.4	2.3 ± 1.5	0.3	0.4
9c	314.3	1.43	7.8	NA	28.2 ± 4.2	21.3 ± 3.8	4.7 ± 2.1	1.3	6.0
9d	314.3	1.43	7.8	NA	44.7 ± 7.8	14.2 ± 2.3	4.6 ± 2.0	3.2	9.8
10a	363.4	3.66	5.3	8.5 ± 2.2	23.1 ± 4.5	6.4 ± 1.1	4.1 ± 0.7	3.6	5.7
10b	389.4	3.97	5.4	2.7 ± 0.1	7.0 ± 4.0	2.8 ± 0.2	1.6 ± 0.4	2.5	4.4
10c	314.3	1.43	5.4	NA	> 100*	56.5 ± 2.6	23.9 ± 1.8	> 1.8	> 3
10d	314.3	1.43	5.4	93.4 ± 6.2	> 100*	27.8 ± 4.1	15.6 ± 1.6	3.6	6.4

[a] Data from Refs.[16, 17]; * limited solubility beyond this concentration; NA = not active

MW, molecular weight; logP, the logarithm of the octanol/water partition coefficient; and pKa, the negative logarithm of the acid dissociation constant calculated using ChemBioOffice Ultra 13.0. The EC_{50} values (concentration that reduced the toxicity induced by 200 μM H_2O_2 to 50% of the untreated control) were calculated after a 24-h incubation with H9c2 cells. The TC_{50} values (concentration that reduced cellular viability to 50% of the untreated control) were calculated after 72-h of incubation with H9c2 cells. The IC_{50} values (concentration that reduced cellular proliferation to 50% of the untreated control) were calculated after 72-h of incubation with MCF-7 or HL-60 cells. The ratios of the concentrations (TC_{50} in H9c2 cells/IC_{50} in cancer cells) indicate the selectivity of the antiproliferative action. Mean ± SD; $n \geq 4$ experiments.

The ability to chelate iron ions in solution

The iron binding dynamics of the synthesized aroylhydrazones compared to those of the model and well-established iron chelator SIH were assayed using a weak chelator calcein, because iron complexation quenches its fluorescence (Figure 2A). The studied compounds compete with calcein for the iron ions; thus, the fluorescence of free (dequenched) calcein is proportional to the chelating efficiency of the studied substances. The chelation activities of the new compounds were expressed as a percentage of the efficiency of the SIH (100%). Compounds **4a**, **4e, 4f, 9a**, **9b** and **9d** showed an ability to chelate iron comparable to that of SIH (92-97%). In contrast, biphenyl-containing compounds **4b** and **5b** were apparently much weaker iron chelators with only 41% and 29%, respectively, of the SIH activity. In general, the compounds of series **5** and **10**, *i.e.,* those with a nitro group in position 3, showed 10-19% lower iron binding than the corresponding compounds of series **4** and **9** with 5-nitro substitution.

The ability to mobilize cellular ^{59}Fe and prevent cellular ^{59}Fe uptake from ^{59}Fe-transferrin

The ability of the aroylhydrazones to mobilize the cellular ^{59}Fe was expressed as the percentage of total ^{59}Fe loaded into MCF-7 cells (Figure 2B). In agreement with the calcein assay, biphenyl compound **4b** had a weak ability to mobilize cellular iron. In contrast, compound **5b**, which had a low iron binding ability in solution, released 40% of cellular iron, whereas compounds **4f**, **5f** and **9b**, which showed good iron chelation in solution, did not significantly mobilize cellular iron. All naphthyl derivatives (**4a**, **5a**, **9a**, and **10a**), as well as compounds **4d-e**, **5e** and **9c-d**, mobilized more than 50% of the cellular iron.

For the ability of the novel compounds to influence iron metabolism in the cell, their capability to prevent the cellular uptake of ^{59}Fe from ^{59}Fe-labeled transferrin[27, 30] is also important. The uptake was expressed as a percentage of ^{59}Fe internalized by MCF-7 cells from a control medium without a chelator (100%; Figure 2C). The uptake correlated with the ability of the ligand to mobilize iron, and the best results were found using compounds **4a, 4d-e, 5a, 5c-e, 9a, 9c-d** and **10a**. The low ability of compounds **4b** and **5b** to prevent the uptake of iron from ^{59}Fe-Tf can be explained by their low chelation capacity. Nevertheless, compounds **9b**, **10c**, **4f** and **5f** were not able to mobilize the ^{59}Fe from MCF-7 cells or to prevent the uptake of ^{59}Fe from ^{59}Fe-Tf despite displaying chelation activities of 69-94% compared to SIH in the calcein assay. A plausible explanation of this effect may be their poor ability to cross cellular membranes.

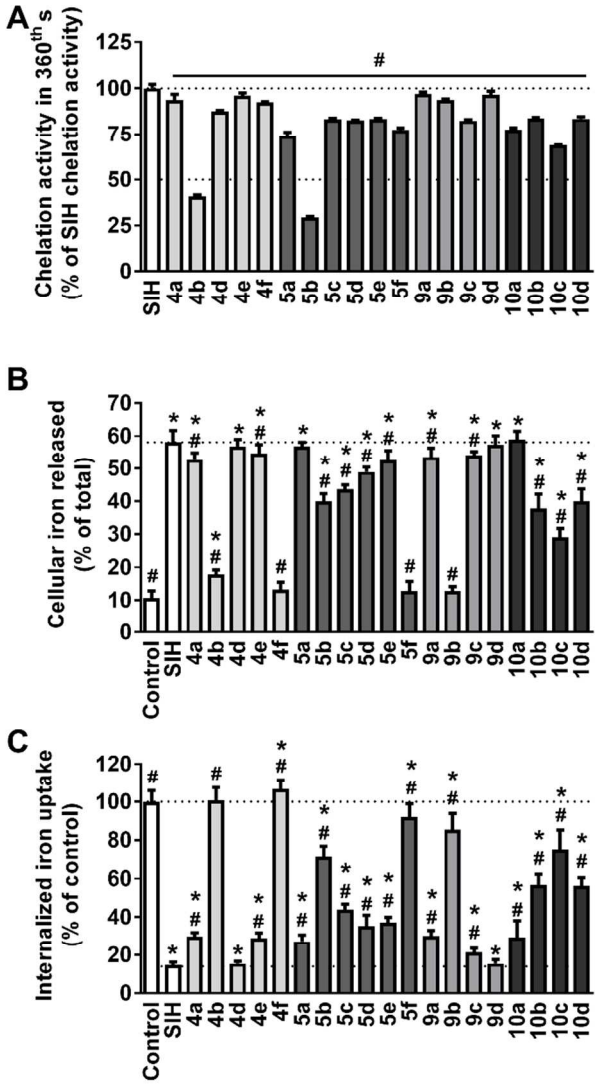


Figure 2. Iron chelation and mobilization by the studied compounds. (A) The chelation dynamics of the compounds in solution using the calcein assay for 360 s expressed as the percentage of the chelation activity of the reference chelator SIH (100%). Mean ± SD; $n \geq 3$ experiments. # significant difference compared to SIH at $p < 0.05$ (ANOVA). (B) The abilities of the hydrazones to promote ^{59}Fe mobilization from ^{59}Fe prelabeled MCF-7 cells and (C) to inhibit the uptake of ^{59}Fe from ^{59}Fe-Tf by MCF-7 cells after a 3-h incubation. Mean ± SD; $n \geq 3$

experiments. Statistical significance (ANOVA, $p < 0.05$): # compared to the reference chelator SIH. * compared to the control (untreated) group.

Pro-oxidant/antioxidant activities of iron-chelator complexes

Pro-oxidant properties of iron chelators have been previously identified as one of the plausible mechanisms of their antiproliferative effect.[25] The ability to form redox-active complexes was therefore assessed by an ascorbate oxidation method.[24, 25] The rate of ascorbate oxidation was calculated as the percentage of the control group (ascorbate with free Fe^{3+}, Figure 3). Three well-established chelators were used as antioxidant (DFO, SIH) or pro-oxidant (EDTA) controls.[26] The iron complexes of DFO and SIH demonstrated a typical antioxidant profile: the ascorbate oxidation was lower at an iron-binding equivalent (IBE) of 3 (*i.e.*, excess of chelator) than at an IBE of 0.1 (*i.e.*, excess iron). In contrast, the iron complex of EDTA showed a pro-oxidant profile, as it led to higher ascorbate oxidation at an IBE of 3 (429% of control) compared to that at an IBE of 0.1 (155% of control).

Our model compound SIH showed antioxidant properties comparable to DFO (11% of control at an IBE of 3). Similar activities were found for compounds **4d-f, 5c-5f** and **10c-d** with the level of oxidized ascorbate at an IBE of 3 reaching 22-57% of control. In contrast, biphenyl derivatives **4b**, **5b** and **10b** were apparently pro-oxidant with the levels of oxidized ascorbate at an IBE of 3 reaching 133-159% of control. All other compounds were neither antioxidant nor pro-oxidant, as the levels of oxidized ascorbate were similar to those of the control at all IBEs.

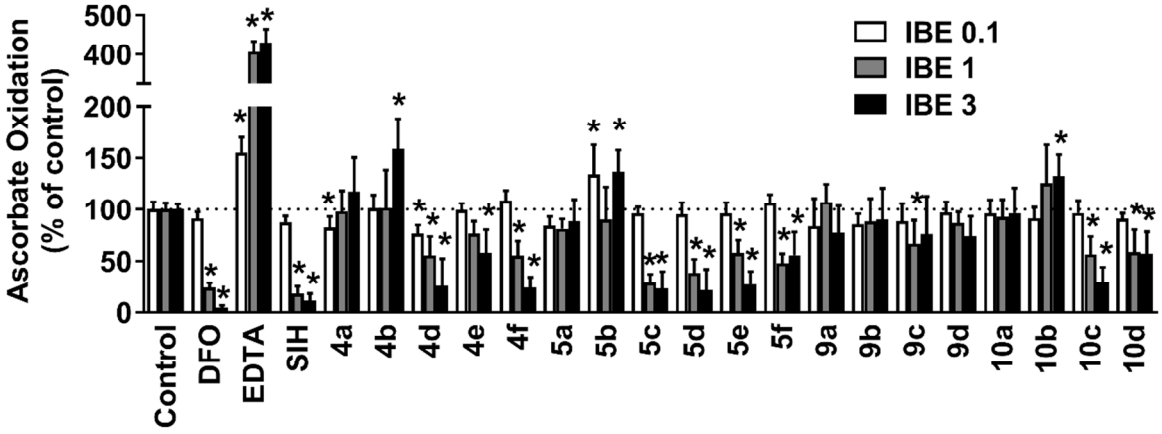


Figure 3. Pro-oxidant or antioxidant properties of the studied hydrazones assessed by their effects on Fe-induced oxidation of ascorbic acid at pH 7.4. Compounds were examined at iron binding equivalents (IBEs) of 0.1 (excess of Fe), 1 (iron-chelator complexes with fully filled coordination spheres) and 3 (excess of free chelator). DFO and SIH were used as negative (antioxidant) controls, and EDTA was used as a positive (pro-oxidative) control. The results are expressed as the percentage of the control group in the absence of chelator (100%). Mean ± SD; $n \geq 3$ experiments. * significant differences compared to the control group (Fe^{3+} with ascorbate) at $p < 0.05$ (ANOVA).

Prevention of H_2O_2-induced oxidative injury to cardiac-derived H9c2 cells

The ability of the studied substances to protect H9c2 cells from hydrogen peroxide-induced model oxidative stress was evaluated after 24-h co-incubation of the cells with 200 μM H_2O_2 and various concentrations of the tested compounds (Figure 4, Supplementary Figure S1 and Table 1). The level of protection was expressed as half-maximal effective concentration (EC_{50}), which

is the concentration that reduced the cytotoxicity induced by H_2O_2 to 50% compared to the control.

Heterocyclic aroylhydrazones did not show any protective activities (except for nicotinic acid derivatives **5d** and **10d**, which increased cell viability to 50% at concentrations over 60 μM). Naphthyl derivatives showed protection at concentrations similar to SIH, except for **4a**, which was inactive. The most promising results were found for the biphenyl compounds, particularly **5b**, **9b** and **10b**, which showed protective activities in the μM range (EC_{50} values of 1.4-2.7 μM), that is, at approximately 10-fold lower concentrations than SIH.[16] At higher concentrations of the ligands, the cell viabilities decreased, which was probably caused by the toxicity of the ligands (Figure 4). Compound **5b** increased cell viability by over 75% at concentrations of 3-30 μM, which was the best activity/toxicity ratio among the studied compounds (Figure 4C).

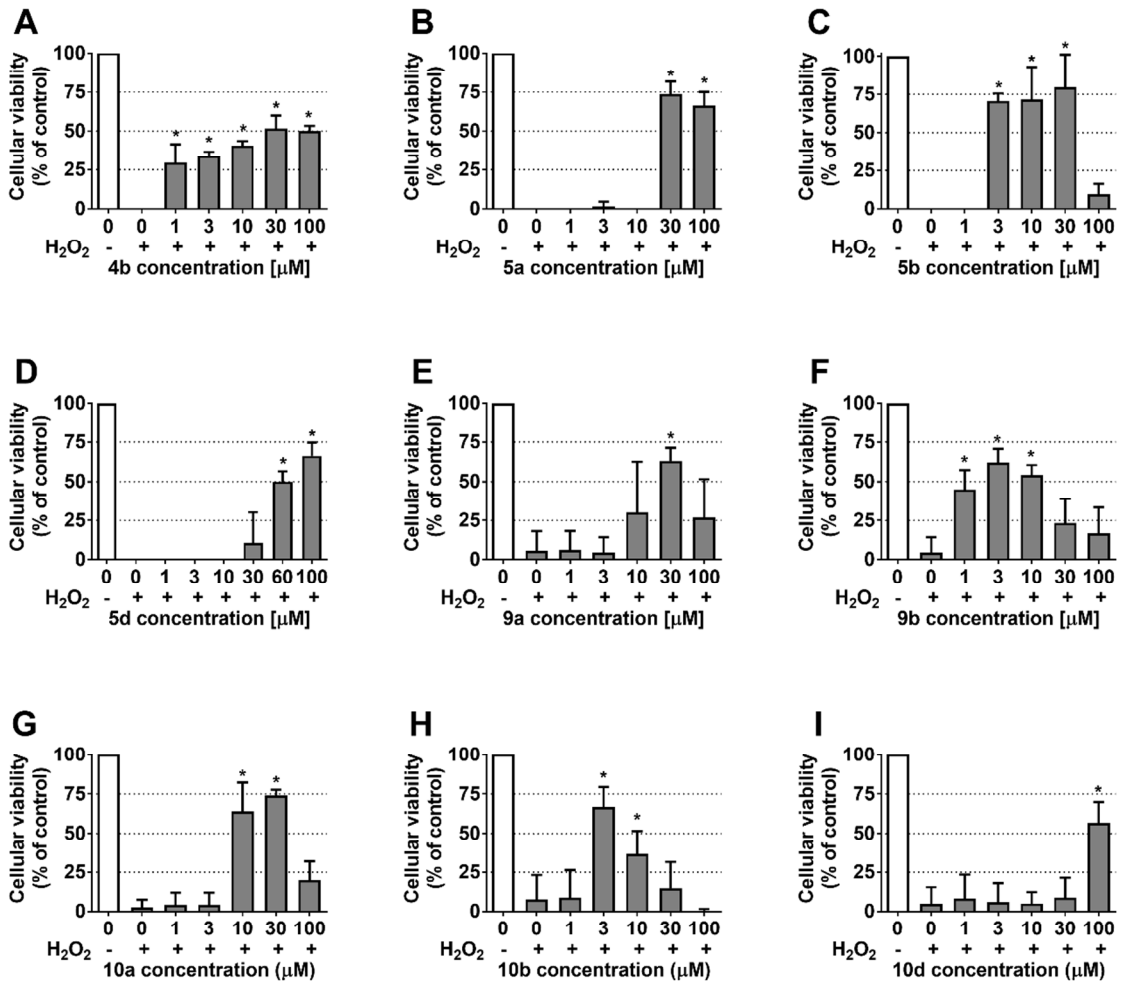


Figure 4. Protection of H9c2 cells against model oxidative damage induced by H_2O_2 (200 μM) exposure for 24 h. Only the compounds that significantly protected H9c2 cells (**4b, 5a, 5b, 5d, 9a, 9b, 10a, 10b** and **10d**) are shown. The results for the other compounds (**4a, 4d-f, 5c-f** and **10c**) are included in the Supplementary content (Supplementary Figure S1). Mean ± SD; $n \geq 4$ experiments. * significant differences compared to the H_2O_2 group without hydrazone co-treatment at $p < 0.05$ (ANOVA).

Inherent toxicities in cardiac-derived H9c2 cells

The inherent toxicities of the studied hydrazones were determined after their 72-h incubation with the (non-cancerous) H9c2 cells and are expressed as half-maximal toxic concentration (TC_{50}) values (Figures 5-6 and Table 1). Toxicity after a 24-h incubation was not determined because the short-term toxicities of aroylhydrazones are usually low and the TC_{50} values could not be calculated due to their limited solubility.[16] All pyrazine and pyridazine-based hydrazones (**4e-f** and **5e-f**) and compounds **10c-d** revealed very low toxicities even after 72 h; they did not reach TC_{50} values within their solubility limits. Additionally, compounds **4a, 5b** and **5c** displayed toxicities above 60 μM. Other compounds showed comparable toxicities to SIH, except for the biphenyl-containing propiophenones **9b** and **10b**, which were toxic to cardiac-derived H9c2 cells at concentrations of 1-7 μM.

Although the protection and toxicity values are not directly comparable because they were determined at different time points (and hence overestimate toxicity), the TC_{50}/EC_{50} ratio can give an estimate of the selectivity of the protective action. Compound **5b** showed the most advantageous combination of protection (1.8 ± 1.2 μM) and toxicity (69.7 ± 3.9 μM) to H9c2 cells, which gives a TC_{50}/EC_{50} ratio of 39. This ratio is much higher than 1.6 for SIH or 3.5 for NHAPI.[16]

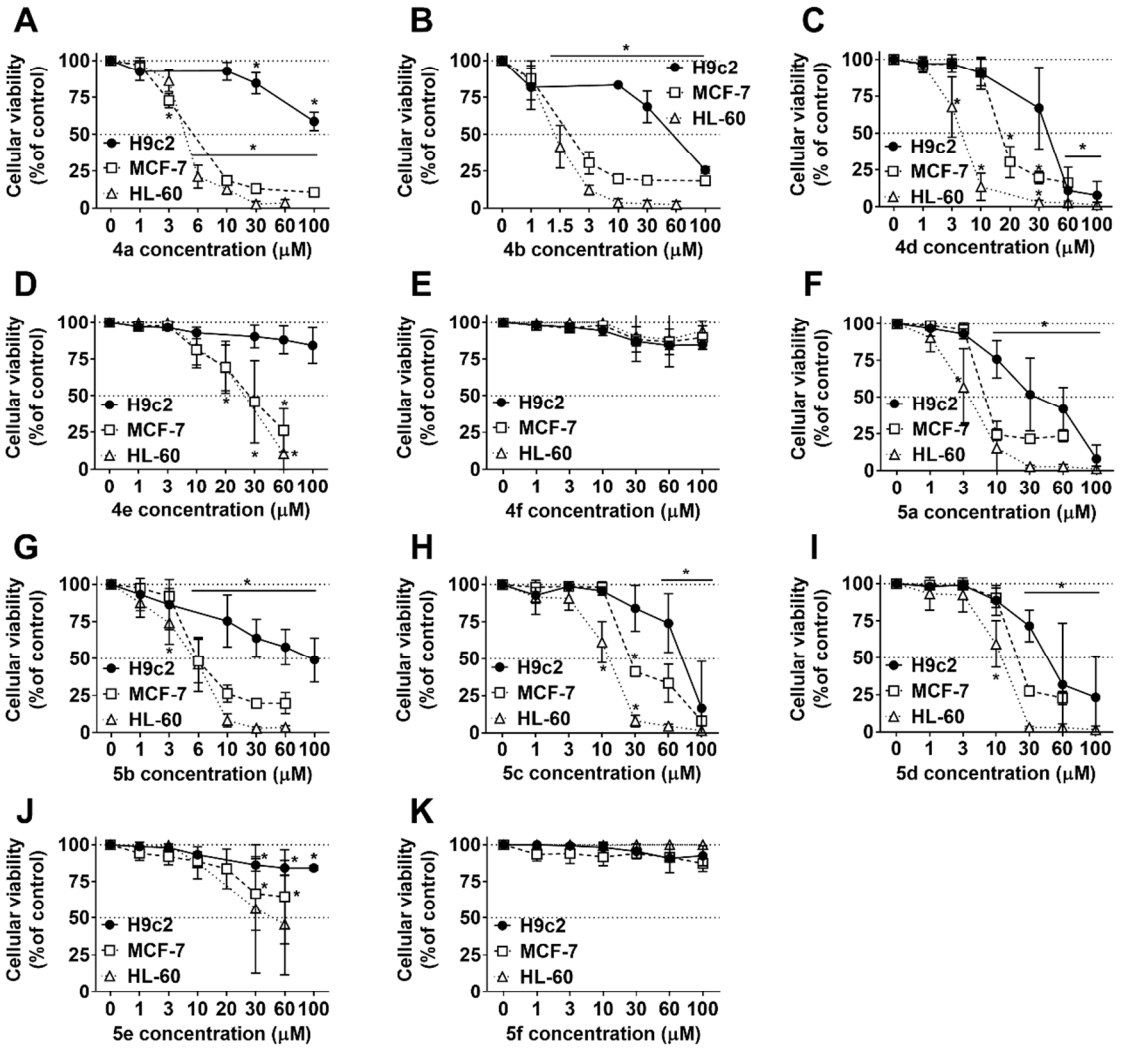


Figure 5. Cytotoxicities of series **4** and **5** of the hydrazones towards non-cancerous and cancerous cell lines. The viabilities of H9c2 cardiomyoblasts were assessed after a 72-h incubation with the tested compounds. The antiproliferative activity was determined after incubation of MCF-7 breast adenocarcinoma cells and HL-60 promyelocytic leukemia cells with the compounds for 72 h. Mean ± SD; $n \geq 4$ experiments. * significant differences compared to the control (untreated) group at $p < 0.05$ (ANOVA).

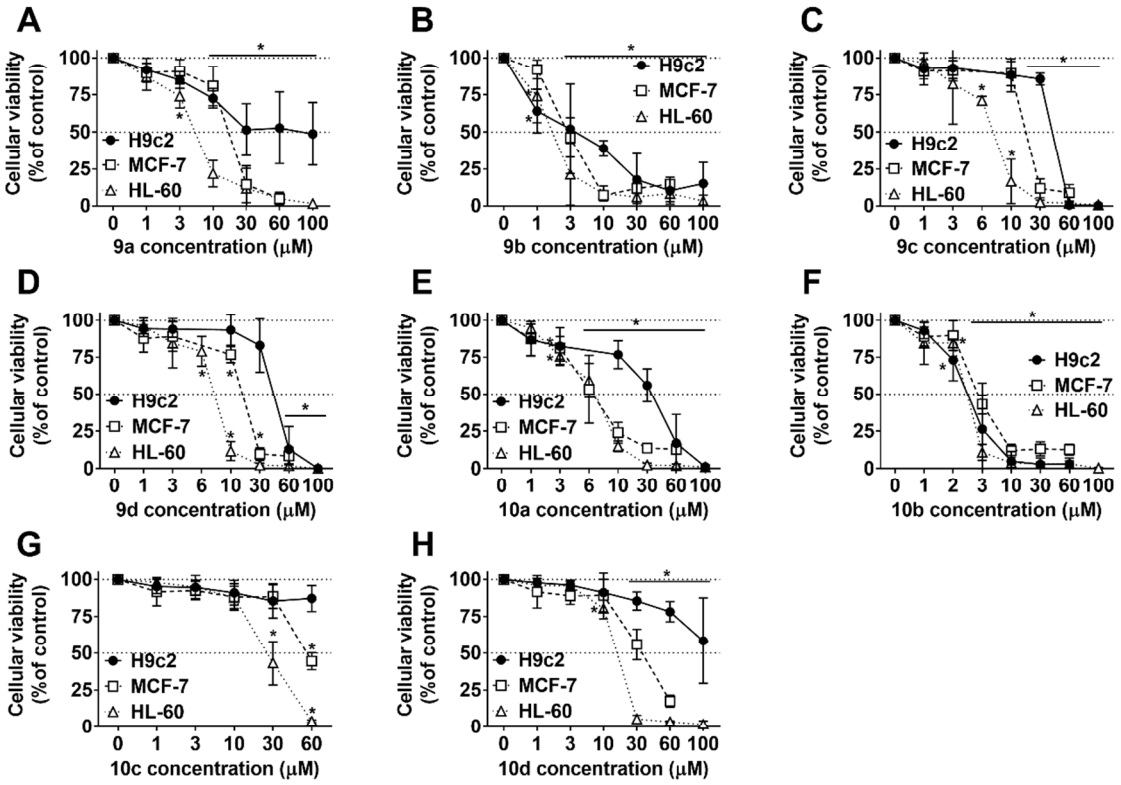


Figure 6. Cytotoxicities of series **9** and **10** of the hydrazones towards non-cancerous and cancerous cell lines. The viabilities of H9c2 cardiomyoblasts were assessed after a 72-h incubation with the tested compounds. The antiproliferative activity was determined after incubation of MCF-7 breast adenocarcinoma cells and HL-60 promyelocytic leukemia cells with the compounds for 72 h. Mean ± SD; $n \geq 4$ experiments. * significant differences compared to the control (untreated) group at $p < 0.05$ (ANOVA).

Antiproliferative activity towards MCF-7 and HL-60 cancer cell lines

The antiproliferative activities of the examined hydrazones against two cancerous cell lines, human breast adenocarcinoma MCF-7 and human promyelocytic leukemia HL-60, were assessed

after a 72-h incubation with the tested compounds (Figures 5 and 6 and Table 1). The MCF-7 and HL-60 cancer cell lines used in the present study have been widely used in various experimental panels of malignant cells and represent model solid and hematological cancer cell types, respectably. They have also been used in our previous studies[17, 18] and therefore allow direct comparisons of the present results with our previous data. Compounds with naphthyl (**4a**, **5a**, **9a** and **10a**) and biphenyl moieties (**4b**, **5b**, **9b** and **10b**) showed antiproliferative potential at μM concentrations (1.6-7.2 μM) in both MCF-7 and HL-60 cells. Similar IC_{50} values were also found for pyridine derivatives **4d**, **5c**, **5d**, **9c** and **9d** in the HL-60 cell line. Pyrazines **4e** and **5e** had IC_{50} values >20 μM, whereas pyridazines **4f** and **5f** did not show any toxicity towards the studied tumor cell lines.

Considering the inherent toxicities of the studied hydrazones in H9c2 cardiomyoblasts, many of them showed higher selectivities for the malignant cell lines than SIH. No compound showed better selectivity than NHAPI towards HL-60 cells. However, naphthyl and biphenyl derivatives of NHAPI, *i.e.*, **4a** and **4b,** with TC_{50}/IC_{50} ratios of 33 and 15, respectively, exceeded the selectivity of NHAPI for MCF-7 cells.

For most of the novel compounds, their antiproliferative activity was higher against the HL-60 cell line than against the MCF-7 cell line. This effect could also be observed for the model compound NHAPI, whose IC_{50} values were 19.1 ± 6.0 μM for MCF-7 cells and 2.3 ± 0.5 μM for HL-60 cells (*i.e.*, 8 times lower than that for the MCF-7 cells). Among the newly synthesized aroylhydrazones, a striking contrast between the toxicities of the two cell lines was found for compounds **4d**, **5c**, **9c** and **9d**. In our previous work investigating compounds containing the same hydrazides with ketones without a nitro group, no marked differences in the toxicities to both the MCF-7 and HL-60 cell lines were observed.[16]

Stability in plasma

Finally, the stabilities of hydrazones with promising protective (**5d**) or antiproliferative activities (**4a-b**) in plasma were estimated. The results are given as the percentage of a compound remaining intact in the plasma during a 10-h incubation at 37°C (Figure 7). All compounds showed good plasma stability, particularly compounds **4b** and **5d**, the concentration of which did not significantly decrease after 10 h.

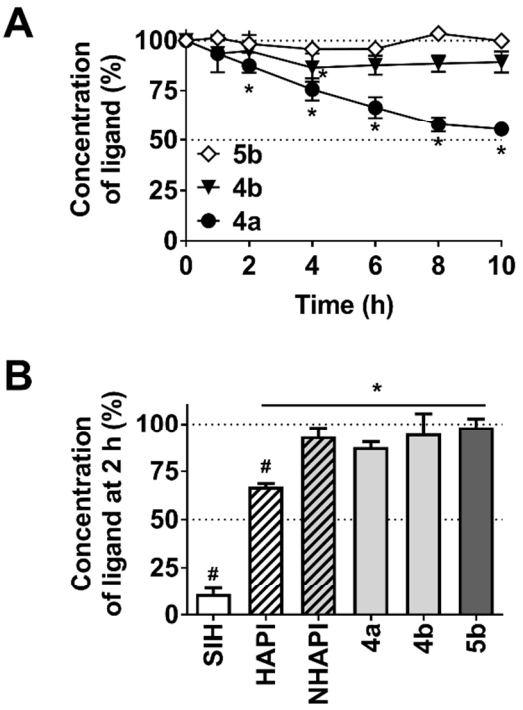


Figure 7. Stabilities of selected hydrazones **4a-b** and **5b** in rabbit plasma at 37°C shown as the percentage of the remaining compound over time (panel A) and comparison to the reference chelators SIH, HAPI and NHAPI at 2 h (panel B). Mean ± SD; $n \geq 3$ experiments. Statistical significance (ANOVA; panel B): * $p < 0.05$ compared to SIH and $^{\#}$ $p < 0.05$ compared to NHAPI.

Discussion

*Series **4** derived from 2-hydroxy-5-nitroacetophenone*

The first series of the prepared aroylhydrazones, compounds **4a-4f**, was derived from NHAPI (which would be **4c** according to the abbreviations in this work); *i.e.*, they had a nitro group in position 5 of the phenolic ring and a methyl at the hydrazone carbon. Naphthyl and biphenyl derivatives **4a** and **4b** respectively, with almost 3 orders-of-magnitude higher lipophilicities than NHAPI, showed the most promising antiproliferative activities among the compounds examined in this study. This is in agreement with the previously suggested positive role of lipophilicity in the cytotoxic activity of aroylhydrazones, as seen in compound 311 derived from 2-hydroxyacetonaphtone.[28, 31-33] These positive effects of lipophilicity can be explained by the better ability of more lipophilic compounds to enter the cells and interact with molecular targets. In particular, the selectivity for the MCF-7 cells of hydrazone **4a** was 2.6-fold higher than that of NHAPI, and the selectivity of **4b** was comparable to that of NHAPI. The selectivities of hydrazones **4a** and **4b** for the examined cell lines are comparable to our previous results on biphenyl- and naphthyl-containing hydrazones without a nitro group[18] and to compound 311 (although it was evaluated using different cancer cell lines).[34] Thus, the nitro group had neither a negative nor a positive effect on the antitumor properties of this class of aroylhydrazones. This is rather surprising given the positive effect of 5-nitro substitution of HAPI leading to NHAPI, which increased the selectivities for MCF-7 and HL-60 cells by approximately 2- and 20-fold, respectively.[17]

Compounds **4a** and **4b** displayed stark differences in the iron chelation assays: naphthyl derivative **4a** chelated iron ions in solution, mobilized cellular iron ions and prevented their

uptake from transferrin in a manner comparable to SIH, whereas biphenyl derivative **4b** was inactive in all these experiments. These differences indicate that the mechanisms of their antiproliferative action are more complex than merely chelating the labile iron pool. Similar behavior was previously observed for antiproliferative biphenyl and naphthyl hydrazones without nitro group substitution.[18] A possible reason for the different outcomes of the calcein assay for compounds **4b** and **9b**, which differ only by one carbon (methyl vs. ethyl), may be their different geometries (*E/Z* isomers) at the double bond, which change their ability (or dynamics) to compete with calcein. The *E/Z* ratio differed between the similar methyl and ethyl compounds HAPI and HPPI.[15] Another important difference in the behavior of compounds **4a** and **4b** is that **4b** but not **4a** formed a redox-active complex with Fe, although the pro-oxidant potential of this complex was lower than that of the pro-oxidant control EDTA. Thus, the antiproliferative abilities of compounds **4a** and **4b** may involve divergent mechanisms despite their similar IC_{50} values. The redox-active **4b-**Fe chelate could also be responsible for the higher toxicity of biphenyl **4b** to the H9c2 cardiomyoblasts than naphthyl derivative **4a**.

The substantial differences between compounds **4a** and **4b** are not easy to explain, as the lipophilicities of these two compounds, as well as the acidities of their phenolic hydroxyls, which are involved in iron chelation, are similar. The biphenyl moiety in **4b** is the least electron-withdrawing group among the studied substituents, which may influence the electron density of the hydrazide carbonyl and its ability to chelate iron (and other metals). Weak redox-activity of iron-chelator complexes was also found in biphenyls **5b** and **10b** but not in biphenyl-containing compound **9b** based on 5-nitropropiophenone and previously studied biphenyls without a nitro group on the phenolic ring.[18] The pro-oxidative activity of the abovementioned biphenyl compounds was abolished by the exchange of biphenyl for naphthyl, with the introduction of

nitrogen-containing heterocycles (**c-f**) returning their antioxidative properties. In fact, compounds **4b**, **5b** and **10b** (combining electron-withdrawing nitro groups on one side and poorly electron-withdrawing biphenyls on the other side of the metal ligating central scaffold) and API[19] (with a softer pyridine base instead of a hard phenolic oxygen base) are the only pro-oxidative aroylhydrazones identified in our studies thus far. Interestingly, the pro-oxidant properties of biphenyl compounds **4b**, **5b** and **10b** did not increase their antiproliferative activities compared to redox-inactive compounds **4a**, **5a** and **10a**. Compound **4b** (as the only compound from series **4**) also protected cardiomyoblasts from oxidative injury at 3-fold lower concentrations than NHAPI. However, the inherent toxicity of **4b** was higher than that of NHAPI.

Given the antiproliferative potential of compounds **4a** and **4b**, their stability in plasma was verified because rapid hydrolysis is a serious drawback of SIH.[15] Both of these novel aroylhydrazones were more stable in plasma than SIH or HAPI; the stability of biphenyl derivative **4b** was even comparable to that of NHAPI.

Compound **4d** is an isomer of NHAPI derived from nicotinic instead of isonicotinic acid. This change in the nitrogen position from 4 to 3 (relative to the hydrazide carbonyl) was highly disadvantageous. Specifically, it resulted in a loss of protective ability, gave an approximately 6-fold higher inherent toxicity, and provided similar or decreased antiproliferative activity despite the excellent ability of **4d** to chelate iron in solution and in cells and its comparable antioxidant activity to SIH. Similar differences between 3- and 4-pyridyl iron chelators were observed previously.[18] A possible explanation for the different properties of these isomeric compounds is that they may differ in their dissociation constants[35] or in their selectivity towards other metal ions, which can influence their toxicity. For example, zinc(II)-thiosemicarbazone complexes

entered the lysosomal compartment where they transmetallated with copper ions that led to their toxicity.[36]

Pyrazine derivative **4e** is the least lipophilic compound among the examined aroylhydrazones. Although the toxicities of this compound to H9c2 and MCF-7 cells were comparable to those of NHAPI, its activity on HL-60 cells was approximately 10-fold lower than that of NHAPI. Very similar IC_{50} values were found for compound **5e**, another pyrazine derivative with a nitro group in position 3 and a methyl in position 5 of the phenol ring. Pyridazine-based compounds **4f** and **5f** are isomers of **4e** and **5e**, respectively, with markedly higher lipophilicities, comparable to those of naphthyl and biphenyl-containing derivatives **4a-b**. However, both pyridazines **4f** and **5f** were completely inactive (within their solubility limits); thus, an increase in lipophilicity in structurally related compounds does not ensure an increase in activity. Both compounds also lost any protective ability against model oxidative injury in cardiomyoblasts, which was disappointing because the structurally similar compound without a nitro group ((*E*)-*N′*-[1-(2-hydroxyphenyl)propylidene]pyridazine-4-carbohydrazide) showed an activity/toxicity ratio five times higher than that of SIH.[18] A possible explanation is the lack of the ability of pyridazines **4f** and **5f** to mobilize iron ions in the cells and prevent ^{59}Fe uptake from transferrin. Thus, in this case, the nitro substitution was disadvantageous, and pyrazine and pyridazine derivatives were not further prepared in series **9** and **10**.

*Enlarged alkyl group at the C=N bond: series **9** derived from 2-hydroxy-5-nitropropiophenone*

Previous studies have identified that when the aldimine hydrogen is replaced with an unbranched alkyl increases the stability and antiproliferative activity of aroylhydrazones.[16, 19]

Thus, we prepared series **9** based on 2-hydroxy-5-nitropropiophenone. Here, all compounds (**9a-d**) had similar antiproliferative activity to their lower homologs **4a-d** and NHAPI but exhibited an order-of-magnitude higher toxicity to cardiac-derived H9c2 cells (except for **9d**, the toxicity of which was similar to that of **4d**), leading to unfavorable selectivities. Compound **9b** was the most toxic substance to H9c2 cells among the examined aroylhydrazones, followed by another propiophenone-derived biphenyl, compound **10b**. Both of these compounds were also significantly toxic to the tumor cell lines with micromolar IC_{50} values. These two compounds, **9b** and **10b**, were the most lipophilic hydrazones in this study; hence, they might more easily penetrate cellular membranes and induce rather non-selective toxicity. Despite their toxicity in the 72-h experiments, naphthyl and biphenyl derivatives **9a-b** protected cardiomyoblasts against H_2O_2-induced injury in the 24-h assay at concentrations lower than SIH. Interestingly, none of the compounds in series **9** showed pro- or antioxidant properties.

*Effects of nitro group position: series **10** derived from 2-hydroxy-3-nitropropiophenone*

To explore the protective potential of compounds **9a-b** further, the position of the nitro group was changed from 5 to 3. Although this is the same position relative to the hydrazone, it changed from *para* to *ortho* relative to the phenolic hydroxyl. This led to a markedly increased acidity of this hydroxyl, making it fully ionized at physiological pH. Interestingly, this structural change re-established the antioxidant properties in both pyridine-containing compounds (**10c-d**) and the pro-oxidant potential in biphenyl-containing hydrazone **10b**. Nevertheless, no marked improvement in activity over series **9** was observed.

*Two methyl groups instead of ethyl: series **5** derived from 2-hydroxy-5-methyl-3-nitroacetophenone*

In an effort to combine the abilities of the compounds in series **9** and **10** to protect cardiomyoblasts from oxidative injury at micromolar concentrations and to lower the toxicities of series **4**, the additional methyl group was "moved" to position 5 of the phenolic ring; *i.e.*, 2-hydroxy-5-methyl-3-nitroacetophenone was condensed with various hydrazides, leading to compounds **5a-d**. Indeed, this structural change resulted in the identification of compound **5b** with an excellent ability to protect cardiomyoblasts from oxidative injury while simultaneous demonstrating low inherent toxicity to these cells. The "selectivity" of compound **5b** was 24-fold higher than that of reference compound SIH. Hydrazone **5b** also displayed excellent plasma stability, which makes it a promising candidate for further study. Interestingly, this compound showed a lower ability to mobilize cellular iron and to prevent its uptake from transferrin than SIH. The ascorbate assay indicated a pro-oxidant potential of **5b** (which might have interfered with the calcein assay, because the free calcein fluorescence decreases in an oxidative milieu,[37] leading to apparently low chelation in solution). A similar finding (*i.e.*, a pro-oxidative compound displaying significant protective effects against peroxide-induced cytotoxicity) was previously shown for a thiosemicarbazone, Dp44mT.[14] This effect was strongly concentration dependent: at 1 μM, Dp44mT maintained viability of H9c2 cardiomyoblasts, (likely due to a strong Fe-chelation), while higher concentrations led to a decreased viability (because of the inherent toxicity of the ligand). A similar inherent toxicity of compound **5b** was also found but only at a concentration of 100 μM. Although it is hard (if not impossible) to define universally valid structural features that make an aroylhydrazone more protective, antiproliferative or toxic, the four structurally similar series of compounds described in this study suggested the following

principles: (i) heterocycles in the hydrazide scaffold usually lead to antioxidant properties, whereas the pro-oxidant capacity was observed only in the biphenyl-4-carbohydrazide-derived hydrazones (when considering hydrazones with an *O,N,O* ligating pattern), (ii) naphthyl and biphenyl derivatives display better antiproliferative selectivities than the heterocycles in the hydrazide scaffold studied, (iii) a nitro group does not increase the inherent toxicity of an aroylhydrazone but instead decreases it, and (iv) even structurally very close compounds (isomers or homologs) may have significantly different biological activities or act at different concentrations. Hence, the mechanisms involved in the biological actions of aroylhydrazone iron chelators are more complex than mere iron chelation or manipulation of the redox activity of Fe.

Conclusions

In this study, we prepared four series of nitro group-substituted aroylhydrazones and examined their iron chelation and antioxidant/pro-oxidant abilities, potential to prevent hydrogen peroxide-induced model oxidative injury to cardiomyoblast cells , inherent toxicity, and antiproliferative activities in two cancer cell lines. The presence of a nitro group had both negative and positive effects on the examined properties, and we identified interesting structure-activity relationships, especially between isomeric compounds. Thus, the biological activities of aroylhydrazones are particularly prone to small structural changes, and the outcome of those changes is hardly predictable. Considering antiproliferative activities, naphthyl and biphenyl derivatives **4a** and **4b** displayed good selectivity for cancerous cells, particularly for the human breast adenocarcinoma MCF-7 cell line, where they exceeded the selectivity of the parent compound NHAPI. However, the most interesting result from this work is the identification of compound **5b**, which protected cardiomyoblasts against oxidative injury at 1.8 ± 1.2 μM with a 24-fold higher selectivity than SIH. Hence, this compound presents a lead for further structural optimization and mechanistic studies.

.

ASSOCIATED CONTENT

Supporting Information.

[Syntheses of the ligands, supplementary Figure S1, copies of NMR spectra (PDF)] This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

* TŠ: Department of Biochemical Sciences, Charles University, Faculty of Pharmacy in Hradec Králové, Akademika Heyrovského 1203, 500 05 Hradec Králové, Czech Republic. Tel.: +420-495-067422. Fax: +420-495-067168. E-mail: Tomas.Simunek@faf.cuni.cz.

* KV: Department of Organic and Bioorganic Chemistry, Charles University, Faculty of Pharmacy in Hradec Králové, Akademika Heyrovského 1203, 500 05 Hradec Králové, Czech Republic. Tel.: +420-495-067497. Fax: +420-495-067166. E-mail: Katerina.Vavrova@faf.cuni.cz.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

We thank Assoc. Prof. Jiří Kuneš for NMR data and Assoc. Prof. Lucie Nováková for HRMS data.

Funding Sources

This study was supported by the Czech Science Foundation (project 13-15008S) and the Charles University in Prague (project SVV 260401).

ABBREVIATIONS

DFO, desferrioxamine; EC_{50}, half-maximal effective concentration; H9c2, rat cardiomyoblasts; HL-60, human promyelocytic leukemia cell line; IC_{50}, half-maximal inhibitory concentration; MCF-7, human breast adenocarcinoma cell line; NHAPI, (*E*)-*N′*-[1-(2-hydroxy-5-nitrophenyl)ethylidene]isonicotinoylhydrazide; SIH, salicylaldehyde isonicotinoyl hydrazone; $TC_{50,}$ half-maximal toxic concentration.

REFERENCES

(1) Halliwell, B., and Gutteridge, J. M. C. (2007) *Free radicals in biology and medicine*. 4th ed., Oxford University Press, Oxford ; New York.

(2) Ponka, P. (1999) Cellular iron metabolism. *Kidney Int Suppl 69*, S2-11.

(3) Fleming, R. E., and Ponka, P. (2012) Iron overload in human disease. *N Engl J Med 366*, 348-359.

(4) Manz, D. H., Blanchette, N. L., Paul, B. T., Torti, F. M., and Torti, S. V. (2016) Iron and cancer: recent insights. *Ann N Y Acad Sci 1368*, 149-161.

(5) Torti, S. V., and Torti, F. M. (2011) Ironing out cancer. *Cancer Res 71*, 1511-1514.

(6) Kwiatkowski, J. L., Kim, H. Y., Thompson, A. A., Quinn, C. T., Mueller, B. U., Odame, I., Giardina, P. J., Vichinsky, E. P., Boudreaux, J. M., Cohen, A. R., Porter, J. B., Coates, T., Olivieri, N. F., Neufeld, E. J., and Thalassemia Clinical Research, N. (2012) Chelation use and iron burden in North American and British thalassemia patients: a report from the Thalassemia Longitudinal Cohort. *Blood 119*, 2746-2753.

(7) Galey, J. B. (2001) Recent advances in the design of iron chelators against oxidative damage. *Mini Rev Med Chem 1*, 233-242.

(8) Kalinowski, D. S., and Richardson, D. R. (2005) The evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol Rev 57*, 547-583.

(9) Buss, J. L., Hermes-Lima, M., and Ponka, P. (2002) Pyridoxal isonicotinoyl hydrazone and its analogues. *Adv Exp Med Biol 509*, 205-229.

(10) Horackova, M., Ponka, P., and Byczko, Z. (2000) The antioxidant effects of a novel iron chelator salicylaldehyde isonicotinoyl hydrazone in the prevention of H(2)O(2) injury in adult cardiomyocytes. *Cardiovasc Res 47*, 529-536.

(11) Lukinova, N., Iacovelli, J., Dentchev, T., Wolkow, N., Hunter, A., Amado, D., Ying, G. S., Sparrow, J. R., and Dunaief, J. L. (2009) Iron chelation protects the retinal pigment epithelial cell line ARPE-19 against cell death triggered by diverse stimuli. *Invest Ophthalmol Vis Sci 50*, 1440-1447.

(12) Simunek, T., Boer, C., Bouwman, R. A., Vlasblom, R., Versteilen, A. M., Sterba, M., Gersl, V., Hrdina, R., Ponka, P., de Lange, J. J., Paulus, W. J., and Musters, R. J. (2005) SIH--a novel lipophilic iron chelator--protects H9c2 cardiomyoblasts from oxidative stress-induced mitochondrial injury and cell death. *J Mol Cell Cardiol 39*, 345-354.

(13) Sterba, M., Popelova, O., Simunek, T., Mazurova, Y., Potacova, A., Adamcova, M., Guncova, I., Kaiserova, H., Palicka, V., Ponka, P., and Gersl, V. (2007) Iron chelation-afforded cardioprotection against chronic anthracycline cardiotoxicity: a study of salicylaldehyde isonicotinoyl hydrazone (SIH). *Toxicology 235*, 150-166.

(14) Bendova, P., Mackova, E., Haskova, P., Vavrova, A., Jirkovsky, E., Sterba, M., Popelova, O., Kalinowski, D. S., Kovarikova, P., Vavrova, K., Richardson, D. R., and Simunek, T. (2010) Comparison of clinically used and experimental iron chelators for protection against oxidative stress-induced cellular injury. *Chem Res Toxicol 23*, 1105-1114.

(15) Kovarikova, P., Mrkvickova, Z., and Klimes, J. (2008) Investigation of the stability of aromatic hydrazones in plasma and related biological material. *J Pharm Biomed Anal 47*, 360-370.

(16) Hruskova, K., Kovarikova, P., Bendova, P., Haskova, P., Mackova, E., Stariat, J., Vavrova, A., Vavrova, K., and Simunek, T. (2011) Synthesis and initial in vitro

evaluations of novel antioxidant aroylhydrazone iron chelators with increased stability against plasma hydrolysis. *Chem Res Toxicol 24*, 290-302.

(17) Mackova, E., Hruskova, K., Bendova, P., Vavrova, A., Jansova, H., Haskova, P., Kovarikova, P., Vavrova, K., and Simunek, T. (2012) Methyl and ethyl ketone analogs of salicylaldehyde isonicotinoyl hydrazone: novel iron chelators with selective antiproliferative action. *Chem Biol Interact 197*, 69-79.

(18) Hruskova, K., Potuckova, E., Hergeselova, T., Liptakova, L., Haskova, P., Mingas, P., Kovarikova, P., Simunek, T., and Vavrova, K. (2016) Aroylhydrazone iron chelators: Tuning antioxidant and antiproliferative properties by hydrazide modifications. *Eur J Med Chem 120*, 97-110.

(19) Potuckova, E., Hruskova, K., Bures, J., Kovarikova, P., Spirkova, I. A., Pravdikova, K., Kolbabova, L., Hergeselova, T., Haskova, P., Jansova, H., Machacek, M., Jirkovska, A., Richardson, V., Lane, D. J., Kalinowski, D. S., Richardson, D. R., Vavrova, K., and Simunek, T. (2014) Structure-activity relationships of novel salicylaldehyde isonicotinoyl hydrazone (SIH) analogs: iron chelation, anti-oxidant and cytotoxic properties. *PLoS One 9*, e112059.

(20) Makarov, V., Manina, G., Mikusova, K., Mollmann, U., Ryabova, O., Saint-Joanis, B., Dhar, N., Pasca, M. R., Buroni, S., Lucarelli, A. P., Milano, A., De Rossi, E., Belanova, M., Bobovska, A., Dianiskova, P., Kordulakova, J., Sala, C., Fullam, E., Schneider, P., McKinney, J. D., Brodin, P., Christophe, T., Waddell, S., Butcher, P., Albrethsen, J., Rosenkrands, I., Brosch, R., Nandi, V., Bharath, S., Gaonkar, S., Shandil, R. K., Balasubramanian, V., Balganesh, T., Tyagi, S., Grosset, J., Riccardi, G., and Cole, S. T. (2009) Benzothiazinones Kill Mycobacterium tuberculosis by Blocking Arabinan Synthesis. *Science 324*, 801-804.

(21) Singh, R., Manjunatha, U., Boshoff, H. I. M., Ha, Y. H., Niyomrattanakit, P., Ledwidge, R., Dowd, C. S., Lee, I. Y., Kim, P., Zhang, L., Kang, S., Keller, T. H., Jiricek, J., and Barry, C. E., III. (2008) PA-824 Kills Nonreplicating Mycobacterium tuberculosis by Intracellular NO Release. *Science 322*, 1392-1395.

(22) Repetto, G., del Peso, A., and Zurita, J. L. (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc 3*, 1125-1131.

(23) Esposito, B. P., Epsztejn, S., Breuer, W., and Cabantchik, Z. I. (2002) A review of fluorescence methods for assessing labile iron in cells and biological fluids. *Anal Biochem 304*, 1-18.

(24) Mladenka, P., Kalinowski, D. S., Haskova, P., Bobrovova, Z., Hrdina, R., Simunek, T., Nachtigal, P., Semecky, V., Vavrova, J., Holeckova, M., Palicka, V., Mazurova, Y., Jansson, P. J., and Richardson, D. R. (2009) The novel iron chelator, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone, reduces catecholamine-mediated myocardial toxicity. *Chem Res Toxicol 22*, 208-217.

(25) Richardson, D. R., Sharpe, P. C., Lovejoy, D. B., Senaratne, D., Kalinowski, D. S., Islam, M., and Bernhardt, P. V. (2006) Dipyridyl thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity. *J Med Chem 49*, 6510-6521.

(26) Chaston, T. B., Watts, R. N., Yuan, J., and Richardson, D. R. (2004) Potent antitumor activity of novel iron chelators derived from di-2-pyridylketone isonicotinoyl hydrazone involves fenton-derived free radical generation. *Clin Cancer Res 10*, 7365-7374.

(27) Richardson, D. R., and Milnes, K. (1997) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents II: the mechanism of action of ligands derived from salicylaldehyde benzoyl hydrazone and 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone. *Blood 89*, 3025-3038.

(28) Richardson, D. R., Tran, E. H., and Ponka, P. (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood 86*, 4295-4306.

(29) Becker, E., and Richardson, D. R. (1999) Development of novel aroylhydrazone ligands for iron chelation therapy: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone analogs. *J Lab Clin Med 134*, 510-521.

(30) Baker, E., Richardson, D., Gross, S., and Ponka, P. (1992) Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture. *Hepatology 15*, 492-501.

(31) Richardson, D. R. (2005) Molecular mechanisms of iron uptake by cells and the use of iron chelators for the treatment of cancer. *Curr Med Chem 12*, 2711-2729.

(32) Kalinowski, D. S., Yu, Y., Sharpe, P. C., Islam, M., Liao, Y.-T., Lovejoy, D. B., Kumar, N., Bernhardt, P. V., and Richardson, D. R. (2007) Design, synthesis, and characterization of novel iron chelators: structure-activity relationships of the 2-benzoylpyridine thiosemicarbazone series and their 3-nitrobenzoyl analogues as potent antitumor agents. *J. Med. Chem. 50*, 3716-3729.

(33) Merlot, A. M., Pantarat, N., Lovejoy, D. B., Kalinowski, D. S., and Richardson, D. R. (2010) Membrane transport and intracellular sequestration of novel thiosemicarbazone chelators for the treatment of cancer. *Mol. Pharmacol. 78*, 675-684.

(34) Chaston, T. B., Lovejoy, D. B., Watts, R. N., and Richardson, D. R. (2003) Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311. *Clin Cancer Res 9*, 402-414.

(35) Merlot, A. M., Pantarat, N., Menezes, S. V., Sahni, S., Richardson, D. R., and Kalinowski, D. S. (2013) Cellular Uptake of the Anti-Tumor and Anti-Metastatic Agent, Dp44mT, Occurs via a Saturable, Temperature-Dependent Mechanism Consistent with Carrier/Receptor-Mediated Transport. *Mol. Pharmacol.*, mol. 113.088393.

(36) Stacy, A. E., Palanimuthu, D., Bernhardt, P. V., Kalinowski, D. S., Jansson, P. J., and Richardson, D. R. (2016) Zinc (II)-thiosemicarbazone complexes are localized to the lysosomal compartment where they transmetallate with copper ions to induce cytotoxicity. *Journal of Medicinal Chemistry*.

(37) Zhang, X., Li, M., Cui, Y., Zhao, J., Cui, Z., Li, Q., and Qu, K. (2012) Electrochemical behavior of calcein and the interaction between calcein and DNA. *Electroanalysis 24*, 1878-1886.