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Characterization of cytoprotective and toxic properties of iron chelator SIH, prochelator BSIH and their degradation products

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Abstract

Free cellular iron catalyzes the formation of toxic hydroxyl radicals and therefore chelation of iron could be a promising therapeutic approach in pathological states associated with oxidative stress. Salicylaldehyde isonicotinoyl hydrazone (SIH) is a strong intracellular iron chelator with well documented potential to protect against oxidative damage both *in vitro* and *in vivo*. Due to the short biological half-life of SIH and risk of toxicity due to iron depletion, boronate prochelator BSIH has been designed. BSIH cannot bind iron until it is activated by certain reactive oxygen species to active chelator SIH. The aim of this study was to examine the toxicity and cytoprotective potential of BSIH, SIH, and their decomposition products against hydrogen peroxide-induced injury of H9c2 cardiomyoblast cells. Using HPLC, we observed that salicylaldehyde was the main decomposition products of SIH and BSIH, although a small amount of salicylic acid was also detected. In the case of BSIH, the concentration of formed salicylaldehyde consistently exceeded that of SIH. Isoniazid and salicylic acid were not toxic nor did they provide any antioxidant protective effect in H9c2 cells. In contrast, salicylaldehyde was able to chelate intracellular iron and significantly preserve cellular viability and mitochondrial inner membrane potential induced by hydrogen peroxide. However it was consistently less effective than SIH. The inherent toxicities of salicylaldehyde and SIH were similar. Hence, although SIH - the active chelating agent formed following the BSIH activation - undergoes rapid hydrolysis, its principal decomposition product salicylaldehyde accounts markedly for both cytoprotective and toxic properties.

Keywords

Salicylaldehyde isonicotinoyl hydrazone (SIH); iron chelation; boronate salicylaldehyde isonicotinoyl hydrazone (BSIH); prochelator; salicylaldehyde

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1. Introduction

Iron (Fe) is a biometal that plays indispensable roles in various essential life processes including energy metabolism, DNA synthesis and cellular respiration (Wang and Pantopoulos 2011). However, unless appropriately shielded, this transition metal is hazardous for the cells as it can readily cycle between its ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states and thus catalyze the Haber-Weiss reaction of superoxide radical and hydrogen peroxide (H_2O_2) yielding hydroxyl radicals – the most reactive and toxic form of reactive oxygen species (ROS) (Halliwell and Gutteridge 2007; Jomova and Valko 2011).

Oxidative stress is a common denominator of a wide range of cardiovascular disorders. These include ischemia/reperfusion injury, cardiac arrhythmias, congestive heart failure, myocarditis, atherosclerosis, hypertension and the cardiotoxicity of various redox-cycling drugs (Griendling and FitzGerald 2003). There are several approaches of prevention and/or treatment of cardiovascular diseases, including the prevention of ROS production. This can be achieved with Fe chelators. Depending on their chemical structure, they may form redox-inactive complexes with Fe^{2+} and/or Fe^{3+} and block the Fe-dependent and site-specific production of hydroxyl radicals (Galey 2001; Kalinowski and Richardson 2005; Liu and Hider 2002). Fe chelators that contain “soft” donor atoms, such as nitrogen-containing ligands, prefer Fe^{2+} but there is a risk of toxicity caused by interaction with biologically important bivalent metals (Zn^{2+} or Cu^{2+}). Therefore, Fe chelators that prefer Fe^{3+} are preferable in clinical practice (Liu and Hider 2002).

Salicylaldehyde isonicotinoyl hydrazone (SIH, Fig. 1) is a tridentate biocompatible metal chelator that easily penetrates cell membranes and firmly binds predominantly Fe^{3+} of the intracellular labile Fe pool (Simunek et al. 2005). Previous results from our laboratory as well as those of other groups have shown its promising cardioprotective potential both *in vitro* and *in vivo* (Bendova et al. 2010; Hašková et al. 2011; Horackova et al. 2000; Sterba et al. 2007). It has been shown as highly effective in the protection of guinea pig or rat isolated cardiomyocytes, H9c2 cells and other cell types against damage induced by H_2O_2 (Horackova et al. 2000; Jansová et al. 2014; Kurz et al. 2006; Lukinova et al. 2009), *tert*-butylhydroperoxide (Bendova et al. 2010) as well as catecholamines (Hašková et al. 2011). Interestingly, SIH has shown the most favorable ratio of own toxicity and cytoprotective efficiency against cellular oxidative injury from a group of various Fe chelators, including the clinically used agents desferrioxamine (DFO), deferiprone (L1) and deferasirox (ICL-670A) (Bendova et al. 2010). SIH has also been shown to protect cells against ionizing radiation (Berndt et al. 2010) as well as against the cardiotoxicity of anthracycline daunorubicin – both *in vitro*, using rat neonatal ventricular cardiomyocytes (Simunek et al. 2008), and *in vivo* using the chronic rabbit model of anthracycline-induced heart failure in rabbits (Sterba et al. 2007). Of note, apart from the direct antioxidative action, SIH can be also protective by activation of the Nrf2 transcription factor that regulates the expression of antioxidant proteins (Caro et al. 2015). Despite these promising outcomes, previous studies have revealed a short biological half-life following administration of SIH to rabbits, apparently due to the fast hydrolysis of its hydrazone bond in plasma (Buss and Ponka 2003; Kovářková et al. 2005; Kovářková et al. 2008).

The concept of prochelation may be a viable strategy to overcome these limitations. Boronyl salicylaldehyde isonicotinoyl hydrazone (BSIH, Fig. 1) has been prepared (Charkoudian et al. 2006) as a prochelator of SIH, which contains a boronic ester in place of a phenolic hydroxyl that is a key metal-binding site for SIH. BSIH does not bind Fe ions until the protective boronyl mask is removed by reaction with H_2O_2 under conditions that are unique for diseases associated with oxidative stress (Charkoudian et al. 2007). Hence, unlike SIH, BSIH does not alter physiological Fe distribution nor compete with Fe-binding proteins, which are the significant drawbacks of using classical Fe chelators in states without systemic Fe overload (Galey 2001). In our recent study, BSIH was shown to be considerably more stable in plasma than SIH and importantly also showed significant improvement in biological half-life (Bureš et al. 2015).

The stability assessment of any promising drug candidate together with the characterization of potential degradation products plays a vital role in the process of novel drug development. It is of particular importance for aroylhydrazones and their prochelators, as the stability of hydrazone-containing compounds has been previously identified. Hence, the aim of this study was to detect the possible decomposition products of BSIH and SIH and determine their biological properties with respect to their iron chelation, cytoprotective and toxic effects. Apart from BSIH and SIH, we focused on isoniazid (INH, Fig. 1), salicylaldehyde (SAL, Fig. 1) and its possible oxidation products, namely salicylic acid (SA, Fig. 1) and pyrocatechol (PRC, Fig. 1).

2. Methods

2.1. Chemicals

The tested compounds (Fig. 1, Fig. S2), SIH, BSIH, BASIH and PKIH (di-2-pyridyl ketone isonicotinoyl hydrazone – an internal analytical standard – IS) were synthesized as described previously (Buss et al. 2002; Charkoudian et al. 2006; Charkoudian et al. 2007). INH, SAL, SA, PRC and constituents for various buffers as well as other chemicals (including these used for HPLC analysis) were purchased from Sigma (Germany), Fluka (Germany), Merck (Germany), or Penta (Czech Republic) and were of the highest available pharmaceutical or analytical grade.

2.2. Cell culture

The H9c2 cardiomyoblast cell line derived from embryonic rat heart tissue (Kimes and Brandt 1976) was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Lonza, Belgium), 1% penicillin/streptomycin (Lonza, Belgium) and 10 mM HEPES buffer (Sigma, Germany) in 75 cm² tissue culture flasks (Techno Plastic Products (TPP), Switzerland) at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells were subcultured twice weekly, once they reached approximately 80–90% confluence. 24 h before experiments, the medium was changed to serum- and pyruvate-free DMEM (Sigma, Germany). Serum deprivation was used to stop cellular proliferation to mimic the situation in post-mitotic cardiomyocytes, whereas pyruvate was omitted because it is an antioxidant and may interfere with ROS-

related toxicity. The lipophilic compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma, Germany), which was used at final concentration of 0.2 % in all experimental groups. At this concentration DMSO had no effect on cellular viability.

2.3. Degradation study

H9c2 cells seeded in a 60 cm² Petri dish at a density 0.45 million cells per dish in DMEM were incubated with the tested compounds (100 µM) alone or in combination with H₂O₂ (200 µM). These concentrations were used based on previous studies, particularly the pilot *in vivo* pharmacokinetics studies of SIH and BSIH. Here, following the intravenous administrations of 10 mg/kg of (pro)chelator to rabbits or rats the maximal plasmatic concentration ($c_{\max}$) values were determined around 100 µM (SIH - 108 µM; BSIH - 63 µM) (Bureš et al. 2015; Kovariková et al. 2005). Furthermore, these compounds were also incubated under the same conditions in DMEM without cells. The samples were collected at time points of 0, 1, 3, 6 and 24 hours after addition of tested compounds and analyzed using HPLC.

HPLC analyses were performed on a chromatographic system Prominence LC 20A (Shimadzu, Germany) equipped with SPD-20AC detector. The data were processed by LC solution software, version 1.21 SP1 (Shimadzu, Germany).

All analyses were performed using a chromatographic column Zorbax Bonus-RP (150 × 3 mm, 3.5 µm), protected by the same type of a guard column (Zorbax Bonus-RP, 20 × 3 mm, 3.5 µm) both purchased from Agilent Technologies (USA). Mobile phase was composed of a part A (2 mM EDTA in 7 mM phosphate buffer, pH 7.0) and a part B (a mixture of methanol and acetonitrile in a ratio of 30:70, v/v) in the following gradient: 0–7 min (10–60% B), 7–10 min (60% B), 10.0–10.1 (60–10% B), 10.1–15.0 min (10% B). To detect pyrocatechol, the gradient was slightly modified: 0–12 min (10–60% B), 12–15 min (60% B), 15.0–15.1 min (60–10% B), 15.1–20.0 min (10% B). A column oven was set at 25 °C and an autosampler at 5 °C. A flow rate of 0.4 mL/min and an injection volume of 40 µl were used. PKIH was selected as an IS (Fig. S2C). Wavelength of 254 nm was used for detection of SAL and 297 nm for the rest of the analytes.

The linearity, precision and accuracy of the method were tested in concentrations from either 2.5 µM (SIH, SAL) or 5 µM (BSIH, SA) to 100 µM (all analytes). Blank DMEM spiked with known amount of the analytes were used for validation proposes. Selectivity was confirmed by an analysis of blank DMEM. All evaluated parameters reached acceptable values (FDA 2001).

2.4. Neutral red uptake assay for assessment of cellular viability

The cellular viability of H9c2 cells was determined using an assay based on the ability of viable cells to incorporate neutral red (NR; Sigma, Germany). This weak cationic dye readily penetrates cell membranes and accumulates in the intact lysosomes of viable cells (Repetto et al. 2008). H9c2 cells seeded in a 96-well plate at a density 10,000 cells per well were incubated with the tested compounds or H₂O₂ (alone or in combinations) for 24 h. At the end of incubation, half of the medium volume had been removed from each well and then the same volume of medium with NR was added yielding final NR concentration of 40

µg/mL. After 3 h at 37 °C, the supernatant was discarded and the cells were fixed in 0.5% formaldehyde supplemented with 1% CaCl₂ for 15 min. The cells were then twice washed with phosphate buffered saline (PBS; Sigma, Germany) and solubilized with 1% acetic acid in 50% ethanol during 30-min continuous agitation. The absorption of soluble NR was measured using the Tecan Infinite 200 M (Tecan, Austria) plate reader at $\lambda = 540$ nm. The viability of experimental groups was expressed as a percentage of the untreated control (100 %).

2.5. Determination of mitochondrial inner membrane potential

Photomicrographs were obtained using the inverted epifluorescence microscope Nikon Eclipse Ti (Nikon, Japan) equipped with digital cooled sCMOS camera (Andor Zyla, Germany) and software NIS-Elements AR 4.20 (Laboratory Imaging, Czech Republic). Mitochondrial activity was assessed using the JC1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; Molecular Probes/Invitrogen, Czech Republic). JC1 non-specifically accumulates in cellular cytosol as a green-fluorescent monomer ($\lambda_{\text{ex}} = 480$ nm; $\lambda_{\text{em}} = 535$ nm). In metabolically-active mitochondria with polarized inner membrane, JC1 monomers flock into red fluorescent J-aggregates ($\lambda_{\text{ex}} = 560$ nm; $\lambda_{\text{em}} = 630$ nm). Cells were seeded on 3.5 cm Petri dishes suitable for microscopy and incubated for 24 h with tested compounds and then loaded with 2 µM JC1 for 30 min at 37 °C after which the medium was replaced with PBS. The mitochondrial inner membrane potential was also measured using the plate reader Tecan Infinite 200 M (Tecan, Austria). Cells were seeded in 12-well plates and incubated for 24 h with tested compounds and then loaded with 2 µM JC1 for 15 min at 37 °C after which the medium was replaced with PBS.

2.6. Calcein-AM assay for measurement of Fe-chelation efficiency in cells

The experiments were performed according to Glickstein *et al.* (Glickstein et al. 2006) with slight modifications. H9c2 cells were seeded in 96-well plates (10,000 cells per well). Prior to the experiment, cells were loaded with Fe by incubating with 100 µM ferric ammonium citrate (FAC) in serum-free medium for 24 h and then washed. To prevent potential interference (especially with regard to various trace elements), the medium was replaced with ADS buffer (116 mM NaCl, 5.3 mM KCl, 1.2 mM MgSO₄·7H₂O, 1.13 mM NaH₂PO₄·H₂O, 20 mM HEPES, pH 7.4) supplemented with 1 mM CaCl₂, and 5 mM glucose. Cells were then loaded with 1 µM of calcein green acetoxymethyl ester (Calcein-AM, Molecular Probes/Invitrogen, Czech Republic) for 30 min at 37 °C and washed. Calcein-AM is cell-permeable, and is converted into cell membrane-impermeable calcein by intracellular esterases that cleave the acetoxymethyl groups. Fluorescence of intracellular calcein is quenched by FAC. Intracellular fluorescence ($\lambda_{\text{ex}} = 488$ nm; $\lambda_{\text{em}} = 530$ nm) was then followed as a function of time (1 min before and 10 min after the addition of tested compounds with or without H₂O₂) using a Tecan Infinite 200 M micro-plate spectrophotometer (Tecan, Austria).

2.7. Data analysis

In this study, the statistical software SigmaStat for Windows 3.5 (SPSS, USA) was used. Data are presented as mean $\pm$ SD (standard deviation). Statistical significance was determined using ANOVA with a Bonferroni post hoc test (comparisons of multiple groups

against a corresponding control). Results were considered to be statistically significant when $p < 0.05$. The concentrations of tested compounds inducing 50% toxicity/viability decrease (median toxic concentration; TC_{50}) and the concentration leading to 50% protection from toxicity induced by H_2O_2 (median effective concentration; EC_{50}) were calculated using CalcuSyn 2.0 software (Biosoft, UK). The graphs were created using GraphPad Prism 6 for Windows (GraphPad Software, USA).

3. Results

3.1. Degradation study on BSIH and SIH

We tested the decomposition of BSIH and SIH in DMEM medium with H9c2 cells at 37°C using a validated HPLC assay. To examine whether the decomposition is spontaneous or whether H9c2 cells metabolically contribute, the same experiments were done also in DMEM medium without cells. Apart from exact quantification of BSIH and SIH we focused on determination of SAL and SA the most important degradation products of SIH. PRC another possible decomposition product of SIH was not detected in any samples in this degradation study. For representative chromatogram demonstrating applicability of the HPLC assay see Fig. S2.

3.1.1. Decomposition in DMEM medium—BSIH was relatively stable in DMEM medium (Fig. 2A), which is in agreement with our previous results (Bureš et al. 2015). After 24 hours, the concentration of this prochelator decreased only to 85% of its initial amount (100 μ M). As a consequence, the degradation products were found only in trace amounts, deeply below LLOQ of the HPLC method (2.5 and 5 μ M for SIH, SAL and SA, respectively). The only exception was SAL at the time of 24 hours, where 2.6 μ M were assayed. In contrast, BSIH exposed to 200 μ M of H_2O_2 showed rapid activation to SIH (Fig. 2B). However, we observed also marked formation of SAL, concentrations of which were double compared to SIH. The highest concentration of SAL was determined 6 hours after the start of the experiment (almost 40 μ M), which was followed by gradual decrease probably due to its volatility. No SA was detected at any time of the experiments of BSIH incubation, both with and without H_2O_2 .

SIH showed rapid decomposition in DMEM medium, with 33% and 13% of its initial concentration remaining at 6 and 24 hours of the experiment, respectively, which is well in line with our previous study (Bureš et al. 2015). Formation of SAL was observed with maximum concentration of 48 μ M after 6 hours (a time interval at which its concentration exceeded that of SIH). Identical concentration profiles were obtained exposing SIH to H_2O_2 , indicating that H_2O_2 had no effect on SIH decomposition (Fig. 2C, D).

SAL was very unstable when incubated in DMEM medium with or without H_2O_2 (Fig. 2E, F). Only approximately 25% of its initial concentration was assayed at the end of the 24-h experiment. However, no SA could be detected at any of the time points.

Interestingly, BSIH was the only compound where the results differed in the presence of H_2O_2 .

3.1.2. Decomposition in DMEM medium with H9c2 cells—The presence of H9c2 cells effected the amount of BSIH in the experiments without H₂O₂, as the concentration of detected BSIH decreased further than in the cell-free medium (63% of BSIH initial concentration remained unchanged after 24 hours). However, none of the assayed potential decomposition products could be detected. In contrast to the cell-free experiments, the H₂O₂-induced conversion of BSIH to SIH was less efficient in the presence of H9c2 cells. The concentrations of SIH can be assayed till 6 hours of the experiments while later fell below LLOQ of the assay (2.5 µM). Also here, SAL was consistently present at higher concentrations than SIH, reaching a maximum in the first 3 hours of the experiment (13 µM), which was followed by a decrease in concentration to 7 µM after 6 hours. As for SIH, SAL concentration was below LLOQ of the assay at the end of the experiment (24 hours). The less efficient conversion of BSIH to SIH may be caused by the decomposition of H₂O₂ by antioxidant enzymatic systems of the cells. Whereas the decrease in detectable BSIH over the course of the first 6 hours was more significant in the presence of H₂O₂ than without H₂O₂, the final amount of detectable BSIH after incubation with cells for 24 hours was consistently ~55% for both conditions (Fig. 3A, B).

Slightly faster decomposition of SIH was observed in the presence of H9c2 cells compared to the medium without cells. On the other hand, we detected lower overall concentrations of SAL, mainly in the last two time points, where this decrease was statistically significant. The concentrations of SA were below LLOQ of the assay till 6 hours of incubation. Interestingly, about 5 µM concentration of SA was detected at the end of the experiment with cells, whereas it was undetected in cell-free conditions. Addition of H₂O₂ had no apparent effect on this process (Fig. 3C, D).

Also SAL showed considerably faster decrease in the medium with the cells. It was completely undetectable after 24 hours of the experiment. However, SA a possible decomposition product was not detected. Again, H₂O₂ did not influence this process (Fig. 3E, F).

3.2. Inherent toxicity of tested compounds

The inherent toxicity of tested compounds to H9c2 cells was assessed by the neutral red uptake assay (Fig. 4). BSIH, INH and SA induced virtually no viability reduction after 24-h incubations with cells in the concentration range (3 – 600 µM). SIH and SAL displayed similar dose-dependent toxicity profiles. They significantly reduced H9c2 viability by 10–25% at concentrations greater than 30 and 60 µM, respectively.

3.3. Protective effect of tested compounds against oxidative injury

The neutral red uptake assay was used for assessment of the ability of examined agents to protect H9c2 cells against model oxidative injury induced by 24-hour exposure to 200 µM H₂O₂ (Fig. 5). SIH, BSIH and SAL dose-dependently protected cells against the complete viability loss caused by H₂O₂. SIH significantly protected H9c2 cells even at the 10 µM concentration with EC₅₀ value (concentration reducing the H₂O₂ toxicity to 50 % of the viability of control cells) being calculated as 8.0 ± 0.4 µM. BSIH significantly protected cells at concentrations 60 µM (EC₅₀ = 84 ± 6 µM) and at higher concentrations its

protective efficiency was comparable or even slightly better than the parent chelator SIH. SAL was also able to preserve viability at concentrations 60 μ M with $EC_{50} = 333.4 \pm 15.8$ μ M. Neither SA nor INH displayed any increase in cellular viability at any of the tested concentrations.

3.4. Inherent toxicities and protective properties of tested compounds based on assessment of mitochondrial inner membrane potential

The JC1 probe was used to assess the inner mitochondrial membrane potential (Ψ_m) and these results were observed by epifluorescence microscopy and quantified fluorometrically. H9c2 cells were incubated for 24 h with tested compounds (all 100 μ M) alone or in combination with H_2O_2 (200 μ M). As seen in Figs. 6 and 7, SIH and SAL induced slight but significant reduction of red fluorescence intensity and also slight increase in green fluorescence, indicating slow and transient loss of Ψ_m . BSIH, SA and INH did not induce any signs of mitochondrial depolarization. The H_2O_2 -treated cells displayed complete transition to green fluorescence indicating complete Ψ_m dissipation. Whereas SA and INH were unable to effect H_2O_2 -induced mitochondrial injury, SIH, BSIH and SAL significantly protected H9c2 cells from the H_2O_2 -induced Ψ_m loss, with SIH and BSIH providing the most protection.

3.5. Determination of intracellular Fe-chelation efficiency

The abilities of the tested compounds to pass through the H9c2 plasma membrane and chelate the intracellular labile Fe pool were assessed using the calcein-AM assay. Changes in intracellular fluorescence intensity were monitored during a 10 min incubation of cells with examined agents in concentration of 100 μ M or in combination with H_2O_2 (200 μ M). The increase in intracellular fluorescence intensity is caused by the removal of Fe from the intracellularly trapped complex with calcein. The results are presented as percentages of chelating effectiveness of the reference chelator SIH at $t = 10$ min (Fig. 8).

Prochelator BSIH did not show any significant increase in calcein fluorescence, but in the presence of H_2O_2 it reached ≈ 70 % of the chelating activity of SIH. SAL was able to significantly chelate intracellular Fe and its efficiency reached almost 50 % of the fluorescence increase induced by SIH. A slight, yet significant, increase in calcein fluorescence was observed also with SA compared to untreated cells (Fig. 8B). INH did not display any significant chelating activity.

4. Discussion

Chelators of Fe (e.g. DFO, L1 or ICL-670A) have been used in clinical practice for management of β -thalassemia major and other pathological states associated with Fe overload. Their beneficial effect largely depends on prevention of cardiac mortality due to Fe-catalyzed and ROS-mediated cardiomyopathy (Berdoukas et al. 2013; Hershko et al. 2005; Nienhuis and Nathan 2012). The protective potential of Fe chelation has been also described in pathological states without Fe overload, such as ischemia/reperfusion injury, myocardium remodeling after myocardial infarction and heart failure (Han et al. 2009; Luo et al. 2009; Mishra et al. 2010). Because Fe is an essential cellular nutrient that is critical for

DNA synthesis, Fe chelation has also shown promise in the treatment of cancer (Richardson et al. 2009).

In this study, we examined the experimental lipophilic and orally active chelator SIH that belongs to the group of pyridoxal isonicotinoyl hydrazone analogs (Kalinowski and Richardson 2005). Using the calcein-AM assay, we confirmed its high Fe-chelating efficiency in H9c2 cells and ability to effectively protect the H9c2 cardiomyoblast cells against toxicity induced by H_2O_2 . However, the major drawbacks of SIH are its toxicity caused by Fe depletion and labile hydrazone bond resulting in fast hydrolysis. A key finding of the current study, however, is that the two key decomposition products of SIH are either innocuous (INH) or exhibit bioactivity on their own (SAL). In this study, INH displayed no toxicity to H9c2 cells, but neither did it provide any protective effect against H_2O_2 toxicity or intracellular iron chelating ability. On the other hand, SAL itself reduced cellular viability by ~25% after 24 hours exposure to 60 μM or higher concentrations. While the maximum 24-hour toxicity of SAL and SIH were similar, the appearance of toxicity at a lower concentration of SIH compared to SAL (30 μM vs 60 μM , respectively), indicates that intact SIH is inherently more toxic than its SAL decomposition product.

In spite of some inherent toxicity, we found that SAL significantly protected H9c2 cells against H_2O_2 -induced damage. Again, the observation that bioactivity requires lower concentrations of SIH compared to SAL (10 μM vs 60 μM , respectively for cytoprotection) suggests that intact SIH is a more potent compound. Consistent with this supposition is that 600 μM concentrations of SAL preserved about 50 % of the cellular viability lost to H_2O_2 , whereas 30 μM SIH preserved more than 75 % viability. These results closely correlated with the ability to bind intracellular Fe, where 100 μM SAL reached almost 50 % of the chelation efficiency of SIH in the cellular calcein assay.

Apart from SIH, HPLC analysis was able to assay SAL as a product of hydrolytic cleavage of the hydrazone bond, while INH could not be detected due to its coelution with the solvent front. We found that SAL appears as SIH disappears, but not in the expected equimolar amount. A possible explanation can be poor inherent stability of SAL during incubation. Hence SA was also monitored as a suspected oxidative degradation product. While we observed that it was formed during the decomposition of SIH in DMEM medium incubated with cells (Fig. 3C, D), only a rather small amount $\approx 5 \mu M$ was detected. SA did not display any significant toxicity on H9c2 cells. Although we observed slight chelating activity, we have not observed any protective effect of SA against H_2O_2 -induced damage. Furthermore, we supposed that one of the possible additional decomposition products could be PRC, as it can be prepared by reacting SAL with H_2O_2 in alkaline conditions (Dakin 1923). However, none of our analyses confirmed this hypothesis as no PRC has been detected.

A second hypothesis for the smaller than expected quantification of SAL from SIH decomposition was its volatility. To examine this, we repeated this experiment in tightly closed Eppendorf tubes instead of Petri dishes. Compared to experiments in Petri dishes, where only $\approx 25 \%$ of SAL was present at the end of the experiment (24 h; Fig. 2E), in closed Eppendorf tubes, almost 75 % of SAL from its initial amount was detected (Fig. S3). This result indicates strongly that SIH decomposes in DMEM cell culture medium into its

component hydrolysis products: SAL and INH, the former of which is volatile and therefore detected at variable concentrations depending on handling conditions, and the latter of which elutes at the solvent front and therefore not assayed by our current HPLC method. Importantly, SAL contributes to both the toxic and cytoprotective bioactivities of SIH, although it is not as potent.

The boronate-masked prochelator BSIH was designed to minimize the disadvantages of SIH while maximizing its protective benefits. Former studies verified that BSIH had protective properties against toxicity caused by H_2O_2 as well as the redox-cycling herbicide paraquat (Charkoudian et al. 2008, Jansová et al. 2014). It has been shown that once BSIH dissolves in water solution, its pinacol group (2,3-dimethyl-2,3-butanediol) of the aryl boronic ester dissociates to generate its boronic acid form (BASIH, Fig. 1) (Bureš et al. 2015) and NMR data suggest that in aqueous media BASIH may exist in equilibrium with its components INH and the boronic acid/ester version of SAL (BSAL), where BASIH strongly prevails. A comparison of biological properties of BSIH and BASIH showed that they both are nontoxic compounds after 24 h exposure to H9c2 cells and have practically identical protective properties against H_2O_2 -induced damage (Fig. S1). While both starting compounds provided similar bioactivities in these cases, we chose to use BSIH in our experiments as it is a well-established prochelator used in previous studies.

In agreement with previous studies (Bureš et al. 2015, Charkoudian et al. 2008, Charkoudian et al. 2006), BSIH was in this study considerably more stable in cell culture medium compared to SIH. Although neither INH nor BSAL could be assayed in our HPLC method, these degradation products at relevant concentrations were shown not to interfere with assay of BSIH. The integrity of the BSIH signal over time (Figs. 2A and 3A) confirms the greater stability of BSIH compared to SIH under cell culture conditions.

Using the calcein-AM assay, we confirmed that BSIH has little affinity for Fe ions until the protective mask is removed by reaction with H_2O_2 . Similar to previous results, however, neither the cytoprotection against peroxide nor the efficiency of Fe chelation by BSIH + H_2O_2 matched that of SIH itself. These discrepancies suggest that conversion of BSIH to SIH under cell culture conditions is incomplete. Indeed, HPLC analysis of the reaction products revealed less than 25% SIH formation in culture medium, and less in the presence of cells (Fig 2B; Fig 3B). Whereas SAL was not detected from BSIH alone, it was observed following activation of BSIH by H_2O_2 in DMEM medium as well as in DMEM medium incubated with H9c2 cells. The amount of SAL detected from BSIH and H_2O_2 was consistently higher than SIH (Fig 2B; Fig 3B). Given the volatility of SAL, the concentrations of this species formed in the BSIH + H_2O_2 experiments are likely even higher.

The formation of significant levels of SAL from BSIH exposure to H_2O_2 could arise from at least two pathways. BSIH/BASIH itself could equilibrate into INH and BSAL, which could then react directly with H_2O_2 to form SAL. Alternatively, intact BSIH/BASIH can react with H_2O_2 to form SIH, which subsequently hydrolyzes to SAL and INH. Both pathways could occur simultaneously, with preference depending on concentration, pH, and other factors. The recognition that SAL itself contributes to the bioactivity of SIH is therefore also relevant

for BSIH. Another key finding of the current study is that BSIH prevents any negative effects of both SIH and SAL, as BSIH is consistently found to be less toxic than either of these species.

In conclusion, in the present study we confirmed advantageous properties of nontoxic and stable prochelator BSIH that does not interfere with physiological iron homeostasis but is converted in conditions associated with oxidative stress to active chelating agents. We observed that the highly effective iron chelator SIH was formed, however SAL was consistently detected in higher concentrations. SAL is also able to bind iron and thereby inhibit Fenton reaction and therefore it had significant cytoprotective properties. Thanks to the unique properties of both the parent compound and its decomposition products, BSIH is a promising candidate for further investigations using more complex models of ROS-related cardiovascular diseases as well as a thorough study of its pharmacokinetics and metabolism *in vivo*. These studies further suggest that BSIH derivatives or drug delivery strategies that maximize the concentration of SIH formed following BSIH administration to oxidatively stressed cells are desirable. Efforts in this direction are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BASIH	boronyl salicylaldehyde isonicotinoyl hydrazone, free acid form
BSAL	boronyl salicylaldehyde
BSIH	boronyl salicylaldehyde isonicotinoyl hydrazone – pinacol ester form
DFO	desferrioxamine
DMSO	dimethyl sulfoxide
FAC	ferric ammonium citrate
ICL670A	deferasirox
INH	isoniazid
L1	deferiprone
PKIH	di, 2-pyridyl ketone isonicotinoyl hydrazine
PRC	pyrocatechol

ROS	reactive oxygen species
SA	salicylic acid
SAL	salicylaldehyde
SIH	salicylaldehyde isonicotinoyl hydrazone

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Highlights

- Fe prochelator BSIH is nontoxic, stable and does not interfere with Fe homeostasis.
- BSIH is activated by ROS to Fe chelator SIH that is prone to hydrolysis.
- Salicylaldehyde is the main decomposition product of SIH and BSIH.
- Salicylaldehyde retains chelating activity and significant cytoprotective properties.
- BSIH is promising compound with multiple active degradation products.

Fig. 1.

Chemical structures of the compounds examined in this study (SIH - salicylaldehyde isonicotinoyl hydrazone; BSIH – boronyl salicylaldehyde isonicotinoyl hydrazone – pinacol ester form; BASIH - boronyl salicylaldehyde isonicotinoyl hydrazone - free acid form; INH – isoniazid; SA – salicylic acid; SAL – salicylaldehyde; PRC - pyrocatechol).

Fig. 2.

Decomposition of BSIH (A), SIH (C) and SAL (E) alone or in combination with 200 μM H₂O₂ (B, D, F). The tested compounds were incubated in DMEM medium at 100 μM initial concentration for 24 h. The decomposition was evaluated using HPLC analysis. The concentrations below LLOQ of the method (2.5 μM for SIH and SAL or 5 μM for BSIH or SA) are shown as zero. Data are presented as means ± SD.

Fig. 3.

Decomposition of BSIH (A), SIH (C) and SAL (E) alone or in combination with 200 μM H₂O₂ (B, D, F). The tested compounds were incubated in DMEM medium with H9c2 cells (1.2 million cells per 60 cm² Petri dish) at initial 100 μM concentration for 24 h. The decomposition was evaluated using HPLC analysis. The concentrations below LLOQ of the method (2.5 μM for SIH and SAL or 5 μM for BSIH and SA) are shown as zero. Data are presented as means ± SD.

Fig. 4.

Inherent toxicities of tested compounds (BSIH, SIH, SAL, SA, INH). H9c2 cells were incubated with increasing concentrations of tested compounds for 24h. The cellular viabilities were assessed using neutral red uptake assay and expressed as a percentage of the untreated control group (100 %). Data are presented as means $\pm$ SD; n = 4; statistical significance (ANOVA), p < 0.05, * compared to the control.

Fig. 5. Protective effects of BSIH, SIH, SAL, SA, INH on cellular toxicity induced by 24-h exposure of H9c2 cells to 200 μM H₂O₂. The cellular viabilities were determined using neutral red uptake assay and expressed as a percentage of the untreated control group (100 %). Data are presented as means ± SD; n = 3–4; statistical significance (ANOVA), p 0.05, # compared to the H₂O₂-treated group.

Fig. 6. Mitochondrial inner membrane potential (Ψ_m) was assessed by epifluorescence microscopy. H9c2 cells were incubated for 24 h with tested compounds (BSIH, SIH, SAL, SA and INH) or H_2O_2 (alone or in combinations) and then stained with JC1 probe. Punctate or rod-shaped red emission reflects mitochondrial inner membrane potential-dependent accumulation of probe aggregates in actively respiring mitochondria, diffuse cytosolic green fluorescence indicates monomers of the probe released into cytoplasm after mitochondrial depolarization. Lack of fluorescence is indicative of probe release from necrotic or secondary apoptotic cells. Inherent toxicities of tested compounds (all 100 μM) are shown on the upper photographs and protective effects of these compounds against the dissipation of Ψ_m induced by 200 μM H_2O_2 are shown on the lower photographs. Scale bars represent 200 μm .

Fig. 7.

The mitochondrial inner membrane potential (Ψ_m), quantified as the ratio of intensities of red and green fluorescence of the JC1 probe. H9c2 cells were incubated for 24 h with tested compounds (BSIH, SIH, SAL, SA and INH; all 100 μ M) or H₂O₂ (200 μ M; alone or in combinations) and then stained with JC1 probe. Data are presented as percentage of control and as means $\pm$ SD; n = 3–4; statistical significance (ANOVA), p < 0.05, * - compared to the control group, # - compared to the H₂O₂-treated group.

Fig. 8.

Intracellular iron-chelating efficiencies of the tested compounds (BSIH, SIH, SAL, SA and INH) were assessed using the calcein-AM assay in H9c2 cells. The increase in fluorescence after displacement of intracellular labile iron from its complex with intracellularly-trapped calcein following the extracellular addition of tested compounds (all 100 μ M). (A) The time-course of fluorescence change following the addition of compounds. The prochelator BSIH was activated by 200 μ M H₂O₂. (B) Mean changes in fluorescence intensity following a 10 min incubation with studied compounds. The effectiveness of chelation is expressed as a percentage of the efficiency of SIH at $t = 10$ min (100 %). Data are presented as means $\pm$ SD; $n = 4$; statistical significance (ANOVA, $p < 0.05$): *, as compared to control; #, compared to SIH.