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INVOLVEMENT OF HEME OXYGENASE IN FERROPTOTIC CELL DEATH OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS AND OSTEOBLAST-LIKE CELLS

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ABSTRACT

Heme oxygenase (HO) is an integral enzyme of the cell's anti-oxidant stress-response and iron homeostasis. HO releases biliverdin, carbon monoxide and iron by degrading heme, and is thus involved in ferroptosis, a form of cell death induced by iron overload and lipid peroxidation. However, HO's involvement in ferroptosis is controversial, as both anti-oxidant and ferroptotic effects have been shown in different studies. Thus, the aim of this study was to test, whether HO contributes to ferroptosis of human bone marrow mesenchymal stromal cells (hBMSCs, undifferentiated cells) and thereof derived osteoblast-like cells (differentiated cells). In order to find out whether the differentiation state has an influence, undifferentiated and differentiated were analyzed in parallel. Cells were treated with ferroptosis inducing compounds, erastin and RSL3, and subjected to HO modulation using Zn^{2+}-protoporphyrin IX (ZnPP) and hemin. To confirm that cell death was executed via ferroptosis, ferroptosis inhibitor ferrostatin-1 (FS) was added as control. Cell death was determined using decrease of protein content via Bradford assay. HO activity was determined with a coupled photospectroscopic biochemical assay measuring the end product bilirubin. Our study showed that undifferentiated cells displayed only a moderate, differentiated cells no sensitivity at all against erastin. However, RSL3 exerted ferroptosis in cells of both differentiation states, with undifferentiated cells being more sensitive. ZnPP prevented ferroptosis induction at least partially in RSL3 treated undifferentiated cells, and fully rescued differentiated cells. Co-treatment with FS resulted in complete protection of undifferentiated cells treated with ZnPP and RSL3, indicating at least partial contribution of HO products to ferroptotic death of undifferentiated cells. Contrary to our expectation, HO upregulation by hemin did not increase ferroptotis rate upon RSL3 challenge, which indicates activation of multiple cytoprotective pathways, possibly involving BACH. This all suggests that HO is supporting ferroptosis in hBMSCs and osteoblast-like cells due to release of iron, and that differentiated cells possess a higher resistance against ferroptosis. Hemin treatment, however, is not suitable for enhancing ferroptosis in these cells.

ABSTRACT

Häm-Oxygenase (HO) ist ein integrales Enzym der anti-oxidativen Stressreaktion und der Eisenhomöostase. HO setzt Biliverdin, Kohlenmonoxid und Eisen durch den Abbau von Häm frei. Dadurch könnte HO an der Ferroptose beteiligt sein, einer Form des Zelltods, die durch Lipidperoxidation ausgelöst wird. Eine Beteiligung von HO an der Ferroptose von Osteosarkoma-Zellen ist jedoch umstritten, da unterschiedliche Studien sowohl zytoprotektive als auch ferroptotische Wirkungen von HO beschreiben. Ziel dieser Studie war es daher, zu prüfen, ob HO zur Ferroptose von human bone marrow mesenchymal stromal cells (hBMSCs, undifferenzierte Zellen) und den davon abgeleiteten osteoblast-like cells (differenzierte Zellen) beiträgt. Um herauszufinden, ob der Differenzierungszustand einen Einfluss hat, wurden beide Zelltypen parallel analysiert. Die Zellen wurden mit Ferroptose-induzierenden Subtanzen, Erastin und RSL3, behandelt und gleichzeitig die HO-Aktivität mit Zn^{2+}-Protoporphyrin IX (ZnPP) und Hemin moduliert. Um zu bestätigen, dass der Zelltod durch Ferroptose ausgelöst wurde, wurde der Ferroptose-Inhibitor Ferrostatin-1 (FS) als Kontrolle hinzugefügt. Zelltod wurde anhand der Abnahme des Proteingehalts mittels Bradford-Assay bestimmt. Die HO-Aktivität wurde mit einem gekoppelten photospektroskopischen biochemischen Assay bestimmt, bei dem das Endprodukt Bilirubin gemessen wurde. Unsere Studie zeigte, dass undifferenzierte Zellen nur eine mäßige, und differenzierte Zellen überhaupt keine Empfindlichkeit gegenüber Erastin zeigen. RSL3 löste jedoch in Zellen beider Differenzierungsstadien Ferroptose aus, wobei undifferenzierte Zellen empfindlicher waren. ZnPP verhinderte die Ferroptose zumindest teilweise in den mit RSL3 behandelten undifferenzierten Zellen und rettete differenzierte Zellen vollständig. Die gleichzeitige Behandlung mit FS führte zu einem vollständigen Schutz undifferenzierter Zellen, die mit ZnPP und RSL3 behandelt wurden, was auf einen zumindest teilweisen Beitrag von HO-Produkten zum ferroptotischen Tod hinweist. Entgegen unserer Erwartung führte die Hemin-Behandlung nicht zu einer Erhöhung der Ferroptose-rate. Dies könnte durch eine Aktivierung zytoprotektiver Pathways durch Hemin erklärt werden, möglicherweise unter Beteiligung von BACH1. Dies alles deutet darauf hin, dass HO die Ferroptose in diesen Zellen durch die Freisetzung von Eisen unterstützt wird und dass differenzierte Zellen eine höhere Resistenz gegen Ferroptose besitzen. Die Behandlung mit Hemin ist jedoch nicht geeignet, die Ferroptose in den untersuchten Zellen zu fördern.

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1. INTRODUCTION

1.1 Ferroptosis

Ferroptosis is an iron-dependent form of non-apoptotic regulated cell death. This type of cell death is induced by an overload of redox-reactive iron and a lethal accumulation of reactive oxygen species (ROS) and lipid peroxides [Fang et al., 2022]. It is mainly driven by the peroxidation of phospholipids impairing the cellular integrity of cellular membranes. Ferroptosis requires three hallmarks to be present: the presence of ferrous iron either being free or in the form of heme, enzymes like lipoxygenases and cytochrome P450 executing peroxidation at polyunsaturated fatty acid residues, and a failure of the anti-oxidant response, which is associated with a reduced availability of the lipid peroxide repair network, like glutathione peroxidase 4 (GPX4). The result is the accumulation of peroxidized phospholipids, which causes the ferroptotic cell death [Stockwell et al., 2021].

1.1.1 Iron and reactive oxygen species

Reduction and oxidation (red-ox) reactions are chemical processes, involving the acquisition and loss of electrons by atoms, resulting in a changed oxidation state. A loss of electrons is termed as oxidation, and reduction is the gain of electrons. Redox reactions are an important part of every living cell's metabolism. However, they can generate very reactive and short-lived intermediates, radicals, upon transfer of single-electrons, capable of attacking all cellular components. These species are important signaling compounds. However, they are also a major contributor to oxidative stress and can ultimately result in cell death [Kohen et al., 2002].

The most significant pro-oxidants produced by single electron transfer red-ox reactions are reactive oxygen species (ROS). They are byproducts generated in the cellular oxidative metabolism, for example during mitochondrial electron transport or by oxidoreductase enzymes. ROS plays a major role in the development of many diseases like cancer, degenerative diseases, inflammation, and aging, as well as cell death. To counteract increased levels of ROS, the cell possesses several anti-oxidant systems [Dickinson et al., 2011; Florean et al., 2019]. Iron in its low molecular weight form is an important catalyst of ROS formation. Therefore, iron levels need to be carefully balanced, involving transferrin and ferritin mainly, to avoid an oversaturation in iron, which can contribute to cell death [Zhao et al., 2020]. Iron is contributing

to ROS production through the Fenton reaction, which is a non-enzymatic chemical reaction. Ferrous iron (Fe^{2+}) is oxidized by hydrogen peroxide (H_2O_2), leading to the formation of hydroxyl radicals ($HO^{\bullet}$), which are highly reactive and able to inflict significant oxidative damage to cells [Fenton, 1894; Braughler et al., 1986]. Iron is also a cofactor of phospholipid peroxide producing enzymes cytochrome P450 and lipoxygenases. Thus, iron is involved in the production of ROS due to its ability to easily participate in electron transfer reactions by transitioning between the ferrous and ferric iron state [Koppenol et al., 2019; Stockwell et al., 2021].

1.1.2 Lipid peroxidation

Lipid peroxidation is a process during which oxidants like ROS attack and oxidatively oxidize polyunsaturated fatty acyl tails and polyunsaturated phospholipids, which turns them into lipid peroxyl radicals and hydroperoxides. Glycolipids, phospholipids, and cholesterol are also often targets of lipid peroxidation [Ayala et al., 2014]. Lipid peroxidation can occur via enzymatic and non-enzymatic mechanisms. Enzymes involved in lipid peroxidation are lipoxygenases (LOXs) and cytochrome P450 oxidoreductases, which both require iron as a prosthetic group, as well as cyclooxygenases. LOXs are iron-containing enzymes, which catalyze dioxygenation of polyunsaturated fatty acids to produce phospholipid peroxides. LOXs seem to be involved in ferroptosis, especially LOX-15 has been proven to be a key regulator in ferroptotic cell death, as inhibition of LOX-15 is cytoprotective [Yang et al., 2016; Stockwell et al., 2021; Shah et al., 2018].

Non-enzymatic lipid peroxidation is conducted in three steps, initiation, propagation, and termination. During initiation, pro-oxidants like superoxide anions ($O_2^{\bullet-}$) or hydroxyl radicals (HO•), which can be formed in the Fenton reaction influenced by iron, attack lipids and form a carbon-centered lipid radical (L•) by abstracting the allylic hydrogen. Those lipid radicals continue to react with oxygen, which is added to the radical L• to form a peroxyl radical (LOO•) during propagation. The chain oxidation during propagation is mediated by the transfer of a hydrogen atom to LOO• from another phospholipid, generating a new lipid radical L• and a lipid hydroperoxide (L-OOH). This newly formed lipid radical can also induce initiation again, leading to a chain reaction of lipid peroxidation. The reaction is terminated by anti-oxidants donating a hydrogen atom to the lipid peroxyl radical (LOO•), forming a nonradical product.

Once lipid peroxidation is initiated, the chain propagation will continue until the process is terminated by the reaction of anti-oxidants and the creation of termination products [Ayala et al., 2014; Yin et al., 2011]

Lipid peroxidation can be inhibited by glutathione peroxidase 4 (GPX4) by reducing the lipid hydroperoxides (L-OOH) and neutralizing them to water or their corresponding lipid-alcohols. GPX4 possesses a highly nucleophilic selenocysteine in their catalytic center, enabling fast reduction of the hydroperoxides, using glutathione (GSH) as a reductant, a tripeptide composed of glutamate, cysteine and glycine [Ayala et al., 2014]. GPX4 oxidizes GSH to glutathione disulfide (GSSG) during this reaction, buffering the oxidative stress, and can be recycled back to GSH using NADPH/H+ [Conrad et al., 2015]. GPX4 is dependent on the activity of system X_{c-}, a cystine-glutamate antiporter of the cell membrane, as the imported cystine is reduced and needed for GSH synthesis. System X_{c-} is a heterodimer composed of two subunits, and transfers glutamate out of the cell and cystine into the cell. Without GSH as a cofactor GPX4's activity is impaired, resulting in the accumulation of peroxidized phospholipids and ferroptotic cell death [Stockwell et al., 2021; Zhao et al., 2020].

1.1.3 Ferroptosis and cancer cells

Cancer is a disease caused by the uncontrolled division of cells, which have mutated due to genetic predispositions or damaging environmental factors and show different behaviors than normal body cells. Those developments are mostly detrimental to the host's body, as the uncontrollable division can extrude the normal tissue, metastasize, and compromise the function of organs and of the whole organism, often leading to death. During tumor development cancer cells can execute different forms of regulated cell deaths, which can change during their mutation process. Due to the fact that apoptotic cell death mechanisms may have been suppressed due to genetic mutations in some cancers, many therapeutic approaches aim at activating alternative regulated cell death mechanisms. Most current chemotherapeutic treatments aim at inducing apoptosis, however cancer cells can further mutate and undergo apoptotic escape, becoming resistant to the treatment and leading to a worse prognosis [Zhao et al., 2021]. Therefore, ferroptotic cell death may have a great potential as a new cancer therapeutic, as it can overcome the development of resistance against chemotherapeutics. Research has shown that ferroptosis can inhibit the proliferation of malignant cells in several

kinds of cancer like liver, pancreatic, prostate, breast, and other cancers. Especially some highly malignant cancers showed to be inherently vulnerable to ferroptosis [Zhao et al., 2020]. Several cancers like renal cell carcinoma are highly susceptible to ferroptosis induced by GPX4 inhibition [Zhang et al., 2022]. Erastin, which inhibits system X_c-, was shown to induce irreversible cell death, without affecting normal cells of the same phenotype [Zhao et al., 2020]. Activation of the p53 tumor suppression pathway has also been shown to be to be essential for ferroptosis in some cancers. It inhibits cystine uptake through the cystine/glutamate antiporter, limiting GSH production. [Zhang et al., 2018; Zhang et al., 2022]. Some cancers even adapted to avoid ferroptosis, like lung adenocarcinoma due to the high oxygen environment, which would otherwise lead easier to oxidative stress. Also avoiding exposure to high iron levels are a strategy to evade ferroptosis. Therefore, melanoma cells preferably metastasize via the lymphatic path. [Alvarez et al., 2017; Ubellacker et al., 2020]. All this indicates that there is high potential ferroptosis inducing compounds in cancer treatment [Codenotti et al., 2018; Zhao et al., 2020].

1.1.4 Small molecules for inducing ferroptosis

Ferroptosis inducers are small molecules, capable to induce ferroptotic cell death. They are divided into two classes; class I which targets system X_c-, and class II that inhibit GPX4 activity. Class I ferroptosis inducers include erastin, a quinazolinone derivative and related compounds, as well as sulfasalazine (SAS) and sorafenib (SRF). They all inhibit the cystine importer system X_c-, subsequently reducing GSH synthesis. Erastin also induces ferroptosis by interacting with p53 and voltage-dependent anion channels, which are mitochondrial membrane exchange channels and whose change of permeability induces mitochondrial disorders which produces ROS [Zhao et al., 2020; Zuo et al., 2020] (*See section 1.1.2* for further information).

Class II of ferroptosis inducers directly inhibit GPX4 activity or promote its degradation, causing increased lipid peroxidation and thus ferroptosis by blocking the key repair systems for phospholipid peroxides in cells. Cancer cells capable to synthesize cysteine through amino acid metabolic pathways, system X_c- inhibitors have no effect on them; however, class II molecules can still affect them. Such compounds are the synthetic RAS selective lethal small molecules (RSLs), ferroptosis-inducing agents and diphenylene iodonium chlorides. RSL3 inactivates GPX4 by alkylation of the selenocysteine [Xie et al., 2016; Zuo et al., 2020].

1.1.4.1 Role of ferrostatin

Radical trapping agents such as ferrostatin-1 (FS) inhibit ferroptosis induction by interrupting the lipid peroxidation process, with its iron complex trapping the radicals. FS is an arylalkylamine and anti-oxidant, which scavenges initiating alkoxyl radicals produced by ferrous iron, preventing the start of the lipid peroxidation chain reaction. FS is regenerated by being reduced back by ferrous iron, which also decreases the levels of free ferrous iron, limiting its pro-oxidative activity [Skouta et al., 2014; Miotto et al., 2020]. As FS inhibits ferroptosis, it can be used to determine whether the cause of a cell's death is indeed ferroptosis, and not another form of cell death.

1.2 Heme oxygenase

Heme oxygenase (HO) is an enzyme important for iron homeostasis, anti-oxidant defense, adaption to cell stress and signaling pathways with carbon monoxide (CO) as a messenger. HO catalyzes the NADPH-dependent oxidation of heme to biliverdin, with heme being both, substrate and prosthetic group. Heme itself originates from hemoglobin and myoglobin, as well as other hemoproteins. Heme can be released due to tissue injury, and this heme release causes further damage due to oxidative stress. During the HO reaction equimolar amounts of CO, free ferrous iron (Fe^{2+}) and biliverdin (BV) are formed. During the oxidation of heme three molecules of O_2 and seven electrons are consumed. BV is then converted into the end product bilirubin (BR) by the cytosolic enzyme biliverdin reductase (BVR). HO has cytoprotective effects by degrading heme and is also involved in a wide variety of anti-inflammatory, anti-oxidant, and anti-apoptotic pathways [Duvigneau et al., 2019; Montellano, 2000]. The HO pathway is depicted in *Figure 1*.

HO activity can be stimulated by its substrate hemin/heme and is inhibited by porphyrin analogues, such as metalloporphyrins with different metal ion complexes within the tetrapyrrole ring. These are structural analogs of heme, binding to the heme binding pocket of the HO enzyme, without being degraded and thereby inhibiting it. An example is zinc protoporphyrin IX (ZnPP), which inhibits HO activity and can be used as a cancer therapeutic [Schulz et al., 2012].

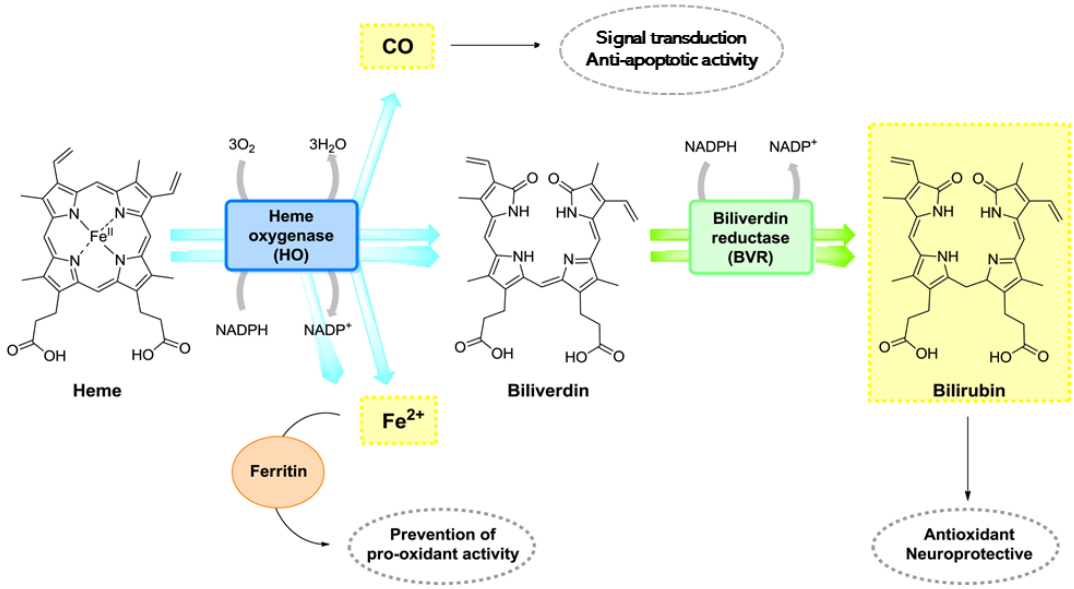


Figure 1: Heme oxygenase pathway and function of its end products. *Heme oxygenase catalyzes the NADPH-dependent degradation of heme into biliverdin (BV), ferrous iron (Fe^{2+}) and CO. BV gets further degraded by biliverdin reductase into bilirubin. The products of heme oxygenase mediate different functions in the cell, as depicted above [Modified from Intagliata et al., 2019].*

1.2.1 Heme oxygenase-1 and heme oxygenase-2

There are two main catalytically active isoforms of HO in mammalian cells, heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2). Both isoforms contribute to the cell's overall capacity to convert heme [Muñoz-Sánchez & Chánez-Cárdenas, 2014]. Heme oxygenase-1 is a member of the heat shock protein family, induced by stressful stimuli, also called heat shock protein 32 (HSP32). HO-1 is a microsomal/mitochondrial enzyme only expressed in low levels in most tissues under physiological conditions, and expression is increased under stressful conditions like hypoxia, heavy metal exposure, toxins, inflammation, ultraviolet irradiation, and ischemia. HO-1 transcription is suppressed by Broad-complex, Tramtrack, and Bric-à-brac (BTB) and Cap'n'collar (CNC) homolog 1 (BACH1), a transcriptional repressor which binds to the stress-responsive elements (StREs). BACH 1 antagonizes nuclear factor erythroid 2-related factor-2 (Nrf2), which is a master regulator of cytoprotective cell responses. Upon oxidative stress and exposure to electrophiles Nrf2 is released from its receptor Keap1 and translocated to the nucleus, where it induces the transcription of Nrf-2 multiple regulated genes, including HO-1.

Some of the cytoprotective genes influenced by Nrf-2 are ferritin light and heavy chain, which sequesters free iron, the iron exporter ferroportin, GPX4, and system Xc-. Heme can bind to BACH1 and induces conformational modifications, which inhibits binding to StREs and induces nuclear export, polyubiquitination and subsequent degradation of BACH1. Thus, the HO substrate heme by interacting with BACH1 results in cytoprotective and anti-oxidant effects via an enhanced expression of multiple targets.

Application of heme or hemin leads to an increase of HO activity [Müllebner et al., 2015]. The cytoprotective effects of HO-1 are most likely conferred by the removal of heme, and by the generation of heme degradation products, which play important roles as cytoprotective compounds. BR/BV and CO possess anti-inflammatory and anti-oxidant properties, reducing inflammation and apoptosis, and inducing cell proliferation and angiogenesis [Duvigneau et al., 2019; Kwon et al., 2015; Belcher et al., 2010]. BR has been shown to have anti-inflammatory effects decreasing P- and E-selectin expression, preventing leucocyte rolling and inhibiting the complement cascade [Nitti et al., 2017]. Relevantly, cancer cells frequently exploit the cytoprotective capacity of HO, and consequently HO-1 overexpression has been found in hepatoma, melanoma, thyroid and lung carcinomas. Additionally, some cancerous cells use the HO mediated anti-apoptotic effects for evading cytotoxicity of many chemotherapeutics [Banerjee et al., 2012].

In contrast to HO-1, which is mainly expressed in selective tissues under physiological conditions, HO-2 is the constitutive isoform and is expressed in all tissues at different levels. Under physiologic conditions HO-2 contributes in major parts to the cellular HO activity. It has been suggested that HO activity via the release of free iron contributes to ferroptosis [Chiang et al., 2018].

HO, due to the generation of cytoprotective products, may confer protection against ferroptosis. On the other hand, the release of ferrous iron may contribute to ferroptosis. In the latter case, tumor cells, particularly when overexpressing HO, may be selectively targeted by ferroptosis inducing compounds. For osteosarcoma cells, which originate from bone marrow mesenchymal cells, the role of HO in ferroptosis induction is controversially discussed. [Banerjee et al., 2012; Jozkowicz et al., 2007].

1.3 Bone marrow derived mesenchymal stromal cells, osteoblast-like cells and osteosarcoma cells

Human bone marrow mesenchymal stromal cells (hBMSCs) are multipotent stromal cells found in the bone marrow. Due to their secretory properties and their capacity to differentiate into osteoblasts, chondrocytes and adipocytes, hBMSCs constitute promising therapeutic cells for tissue remodeling and differentiation-induced metabolic adaptation during osteogenic differentiation [Zavadskis et al., 2020]. HO-1 promotes differentiation towards the osteoblast pathway and inhibits adipogenesis. [Barbagallo et al., 2010]. Osteogenic pathways can be triggered by external stimuli like increased mechanical stimulation of bones or hypoxia, while adipogenesis can be increased due to high caloric intake. The cells are also involved in immunological processes [Gao et al., 2021]. Osteoblasts are bone-forming cells, actively secreting extracellular matrix and building up bone matrix. The counterpart are osteoclasts, which resorb the bone matrix, and remodel the bone continuously together with osteoblasts. Their balance is important for physiological bone function [Swolin-Eide, 2020]. Osteoblast-like cells are also often found in epithelial cancers, where they cause calcification [Scimeca et al., 2017].

Osteosarcoma most likely originates from disruption of differentiation of osteoblast precursor cells and mesenchymal stem cells, with broad genetic and molecular alterations. HO-1 expression also plays a significant role in osteoblast differentiation, positively regulating it via reduction of ROS, protecting cells against oxidative stress [Vanella et al., 2010]. Intracellular iron levels are higher in osteosarcoma cells in comparison to normal body cells, supporting ROS creation and promotion of cancer cell proliferation. These ROS levels have been suggested to help cancer cells to proliferate [Bai et al., 2015]. Therefore, ferroptosis inducing compounds may be suitable to treat osteosarcoma cells [Tang et al., 2008; Bai et al., 2015; Abarrategi et al., 2016; Mutsaers et al., 2014]. However, for osteosarcoma cells, which originate from bone marrow mesenchymal cells, the role of HO in ferroptosis induction is controversially discussed. [Banerjee et al., 2012; Jozkowicz et al., 2007]. In order to understand the impact of the differentiation state on the sensitivity towards ferroptosis, both, differentiated osteoblast-like cells and their progenitors, the undifferentiated bone-marrow derived mesenchymal stem cells, will be compared in the presented thesis.

2 AIM OF THE STUDY

2.1 Aim of the study

Data regarding the role of heme oxygenase are conflicting. While one study has shown that HO supports ferroptotic cell death of cancer cells [Banerjee et al., 2012], another study reported protection against ferroptotic cell death would require active HO [Jozkowicz et al., 2007]. Theoretically, HO could exert both, a supportive role in ferroptosis, due to the release of redox active iron during heme degradation. On the other hand, the generation of BR and CO are potentially active in preventing cell death due to their anti-oxidant and anti-apoptotic effects. We therefore aimed at clarifying the impact of HO activity on killing of human bone marrow mesenchymal stromal cells and osteoblast-like cells provoked by ferroptosis inducer. We further questioned whether the differentiation state of these cells has an influence on the cell killing executed by ferroptosis inducers.

2.2 Approach

Undifferentiated human bone marrow mesenchymal stromal cells and thereof derived differentiated osteoblast-like cells should be treated with two ferroptosis-inducing compounds, erastin and RSL3. These compounds differ in their mechanism to induce ferroptotic cell death, and may involve free ferrous iron released by HO or heme, which is degraded by HO. In order to understand the role of the differentiation state, both, undifferentiated and differentiated osteoblast-like cells should be tested for their sensitivity towards ferroptosis induction. Using ZnPP, a competitive HO inhibitor and hemin, an inducer of HO-1, the contribution of HO to ferroptotic cell death provoked by erastin and RSL3 should be analyzed. Reduction in cell mass should be verified by quantifying the protein content present in the cell culture wells after the incubation period. In order to discriminate between an inhibition of proliferation and cell killing, LDH-activity within the cell culture medium was taken into account. These data were provided to me. To determine, whether cell killing was indeed provoked by ferroptosis, and not by other types of cell death, ferrostatin-1 was used as a control substance, as it inhibits ferroptotic cell death. Modulation of HO activity should be verified using a biochemical assay determining formation of the heme degradation end product BR by means of photospectroscopy.

2.3 Hypothesis

We hypothezised that 1) in case that HO supports ferroptosis, its inhibition rescues the cells against ferroptotic cell death, while HO-upregulation aggravates cell killing; 2) in case that HO prevents ferroptosis, HO-upregulation by hemin rescues the cells and HO inhibition aggravates ferroptotic cell killing.

3 MATERIAL AND METHODS

3.1 Cell types and cell culture

Human bone marrow mesenchymal stromal cells (hBMSCs) and osteoblast-like cells (Lonza, Basel, Switzerland), which were generated by inducing differentiation as was described elsewhere [Zavadski et al., 2020] were used. Throughout this thesis, hBMSCs will be referred to as undifferentiated cells or undifferentiated osteo stem cells, while osteoblast-like cells will be referred to as differentiated cells or osteoblast-like cells. The cells were provided to me in a frozen state at the end of the incubation period with the different stimulants. The experiments with the cells and the determination of LDH activity in the supernatant were conducted under the supervision of Andrey V. Kozlov at the Ludwig Boltzmann Institute of Experimental and Clinical Traumatology (LBI).

Briefly, cells were seeded on T75 tissue culture flasks and expanded in hBMSC culture medium, which consists of Dulbecco´s Modified Eagle´s Medium high glucose (DMEM- HG) 90 ml supplemented with 10 % fetal bovine serum (FBS) 10 ml, 100 U/ml penicillin and 100 µg/ml streptomycin 1 ml (Pen/Strep), 2 mM L-glutamine 1 ml, and 1 ng/ml basic fibroblast growth factor (bFGF) 50 ml (Peprotech, Rocky Hill, CT, USA). Differentiation to osteoblast-like cells was induced through osteogenic induction for 14 days in DMEM-HG supplemented with 10 % FCS, 1 % Pen/Strep, 2 mM L-glutamine, 10 nM dexamethasone, 50 µM ascorbic acid-2-phosphate, and 10 mM ß-glycerophosphate [Zavadskis et al., 2020].

The cells were used for an endpoint measurement after a 24-hour treatment following modulation of the HO activity in combination with the induction of ferroptosis as is described in *section 3.1.1.* The treatment conditions are given in *section 3.1.2.* Measurements of lactate dehydrogenase (LDH) activity in the cell culture supernatant at the end of the experiment, were conducted by Yuliya Dvoretskaya and Giulia Bottau at the LBI. In order to discriminate between cell death and inhibition of proliferation, without killing cells, these data were considered. However, since I was not involved in the generation of these data, they will not be presented within the results section, but considered for correctly interpreting the protein data.

3.1.1 Experimental setup

One experimental series generally consisted of two 12-well plates with 266.000 cells/wells that were subjected to different ferroptosis inducers, HO modulators and controls. This results in 24 wells with a different combination of stimulants per experiment. In each series, one 12-well plate was treated with erastin at three different concentrations (0.75 µM, 1 µM, 2 µM), while the other 12-well plate was treated with RSL3 at three concentrations (1 µM, 2.5 µM, 5 µM). Each plate was treated with modulators of the HO activity, either the inhibitor zinc protoporphyrin IX (ZnPP) (20 µM) or the inducer hemin (10 µM), as well as the vehicle controls. Additionally, ferrostatin-1 (FS) (10 µM) was added as ferroptosis inhibitor (ferroptosis control). The setup and the concentrations used are described in *section 3.1.2.* and is shown for each series in *Fig. 1.*

Ten experimental series have been used in total. Four series were carried out using undifferentiated cells.

In three experimental series ZnPP was used, resulting in six 12-well plates. Only one 12-well plate was treated with hemin combination with RSL3, while the corresponding plate with erastin has been omitted. As the cells were difficult to obtain, culture and maintain, not enough cells were available to test all conditions. Thus, no tests have been performed including undifferentiated cells treated with hemin and erastin, and only one condition with a combination of hemin and RSL3.Therefore, a total of seven 12-well plates have been prepared for the undifferentiated cells. For the differentiated osteoblast-like cells six experimental series have been prepared, resulting thus in a total of twelve 12-well plates. In three series, the cells were treated with ZnPP, while three other series were treated with hemin.

The table below shows the different series provided for this thesis.

Table 1. Experimental series provided as cell culture plates.

HO activity modulators:	**hemin**		**ZnPP**	
Ferroptosis inducing agents:	**erastin**	**RSL 3**	**erastin**	**RSL 3**
Undifferentiated cells	No experiment	1 x 12-wells	3 x 12-wells	3 x 12-wells
Differentiated cells	3 x 12-wells	3 x 12-wells	3 x 12-wells	3 x 12-wells

3.1.2 Treatment of cells

The cells were seeded in 12-well plates at a density of 266.000 cells/wells and treated as follows: as ferroptosis inducing agents erastin (stock solution 10 mM) or RSL 3 (stock solution 5 mM) were used, both solved in dimethyl sulfoxide (DMSO). Since treatment with both compounds was expected to kill the cells, different concentrations were applied, to allow data elaboration in at least some of the samples. The following final concentrations were applied to the cell culture wells: erastin (2 µM, 1 µM, 0.75 µM) and RSL 3 (5 µM, 2.5 µM, 1 µM). To modulate heme oxygenase activity, either ZnPP (stock solution 20 mM) or hemin (stock solution 10 mM in DMSO) were added to the respective 12-well plate to reach a final concentration of 20 µM ZnPP or 10 µM hemin per well. As ferroptosis inhibitor ferrostatin-1 (stock solution 5 mM in DMSO) was used and added at a final concentration of 10 µM per well. Since all substances were solved in DMSO as a vehicle, which is why different volumes of DMSO were included as a corresponding vehicle control. Cells were incubated at 30 °C, 99 % humidity and 5 % CO_2 for 24 hours (Labotec C42, Labotect Labor-Technik-Göttingen GmbH, Germany). The plate setup is shown in Fig. 2.

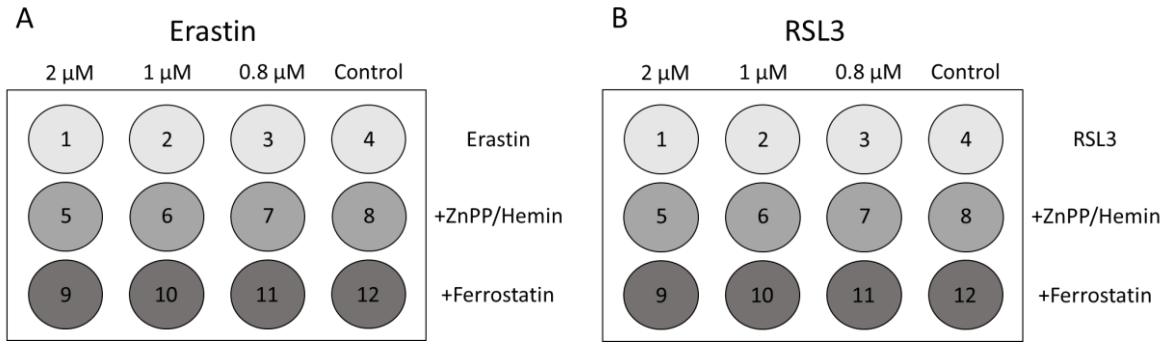


Figure 2: Setup scheme of treatment of cells in 12-well plates. *Cells were seeded with a density of 266.000 cells/well and incubated for 24 hours with the ferroptosis-inducing compounds erastin (A) or RSL3 (B) at indicated concentrations. In the second row the HO modulators, ZnPP (20 µM) or hemin (10 µM) were added in parallel to the ferroptosis inducing agents. In the third row the ferroptosis inhibitor ferrostatin (10 µM) was added to the ferroptosis inducer.*

After the incubation time the medium was collected, snap frozen, and stored until being used for further analyses, such as determination of lactate dehydrogenase activity. The cell layer was carefully washed with either phosphate buffered saline (PBS) or sodium chloride (NaCl), and then 300 µl of heme oxygenase assay buffer (HO-assay buffer) (100 mM potassium phosphate

buffer; 1 mM EDTA; pH 7.4) was added to the cells. Thereafter the plates were frozen, transported to the Veterinary University Vienna in a frozen state and stored at -80 °C until being processed. These frozen plates were kindly provided to me and analyzed in the frame of this study.

3.1.3 Preparation of cell homogenates

The frozen plates (*see section 3.1.1 and 3.1.2*) containing the cells, which were treated with the different additives, and loaded with approximately 300 µL HO-assay buffer per well, were slowly thawed on ice. The cells were scraped of the bottom of the well with a cell scraper for detaching. The cell scraper was cleaned with distilled water and dried with a paper towel prior to be used for a different well in order to avoid cross contamination. To homogenize the cells, the solution was pipetted up and down several times, while the well was tilted to gather all of the solution containing detached cells. The homogenates were transferred in labeled 1.5 ml reaction tubes, snap frozen in liquid nitrogen, and then stored at -80 °C until being used.

3.2 Determination of heme oxygenase activity

Activity of HO was determined using a coupled spectrophotometric assay by quantifying the formation of the end product bilirubin (BR), as was recently described [Duvigneau et al., 2020]. The processing of the samples is schematically shown below (*Fig. 3*).

3.2.1 Assembly of the heme oxygenase assay

The previously prepared and frozen cell homogenates were unfrozen at 37 °C in a water bath (Water Bath GFL 1092, Burgwedel, Germany) for 30 seconds, vortexed and then stored on ice. The assay was assembled in 2 ml reaction tubes (Greiner Bio-One, Austria), allowing to provide enough oxygen for the reaction. The tubes and all required compounds were set on ice except for hemin, which was kept at room temperature in the darkness, in order to prevent degradation by light. All further steps were performed in a light-dimmed room. The assay consisted of the following components: 120 µl cell homogenate of each sample, 4 µl hemin (Fluka, 1 mM, solved in DMSO) as substrate, 5 µl deferoxamine mesylate (DFO) (Sigma, 100 mM in water) as iron chelator and 25 µl NADPH (Sigma, 10 mM in HO-assay buffer) as co-substrate (*see table 8 supplements and solutions for pipetting scheme*). The residual cell homogenate was used for generating a sample pool to be used as negative control, and for the determination of protein

concentration with the Bradford method (see *section 3.4.*). Two negative controls were included per experiment, consisting of the pooled sample of equal amounts of remaining homogenates of cells treated with the same HO modulator (ZnPP or hemin) or without modulator. One negative control only contained the samples in which an HO modulator had been added, the other negative control consisted of samples without an HO modulator, as blank control to correct the measurements. In the negative controls, 120 µl of pooled homogenates were used by replacing 25 µl NADPH by 25 µl HO-Assay-Buffer, and hemin was substituted by 4 µl of DMSO as vehicle control.

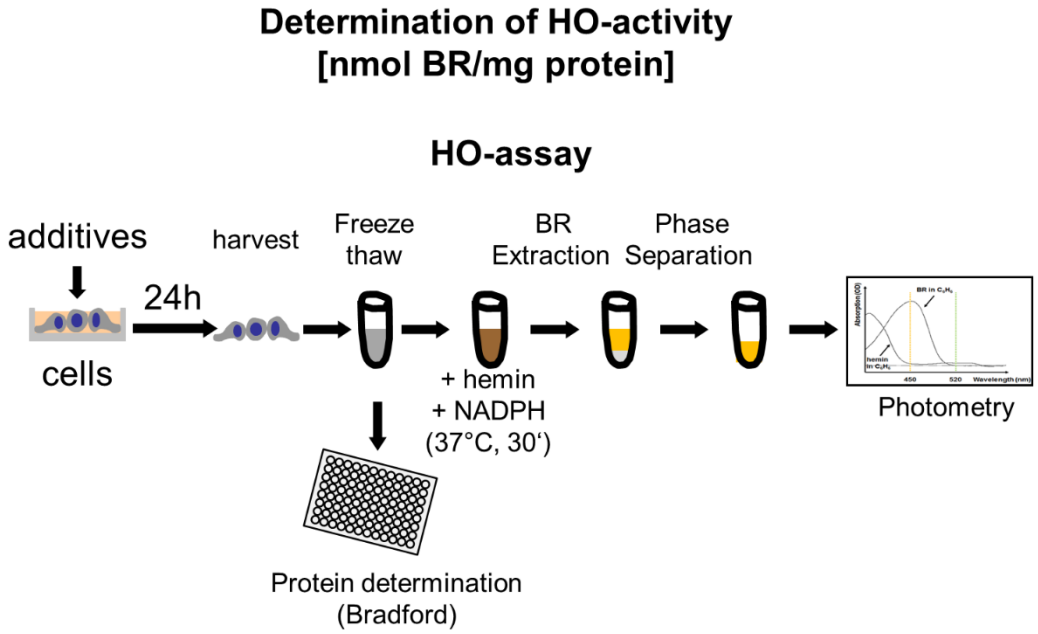


Figure 3: Scheme of the determination of HO activity using a coupled enzymatic assay. *Cells were treated as described in 3.1.1 and 3.1.2. Cells homogenized in HO assay buffer were incubated with the HO substrate hemin and NADPH for 30' at 37 °C. Thereafter BR, the end product of the heme degradation, was extracted into benzene and after phase separation BR concentration was determined by photospectroscopy in the extracts. Extracts were scanned (600-400 nm) using a double beam photometer. BR specific extinction was used to calculate amount of BR in nmol using the extinction maximum of 450 nm corrected for the reference extinction at 520 nm. In order to correct for the differences in the underlying protein concentration present in the assay, amount of protein was determined in the residual homogenates using Bradford (3.4.)*

The compounds were then vortexed with a Vortex mixer (Classic Advanced Vortex Mixer, VELP scientifica, Italy) to assure thorough mixing of the reagents and incubated in at 37 °C for exactly 30 minutes under constant agitation (Water Bath GFL 1092, Burgwedel, Germany), to

enable the enzymatic reaction. Thereafter the samples were transferred on ice to stop the reaction.

3.3 Determination of bilirubin quantity

For the determination of the BR content of the samples, the BR was extracted using a liquid-liquid extraction technique with benzene, and BR content was analyzed by spectrophotometry in the organic extracts.

3.3.1 Extraction of bilirubin using benzene

In order to allow an efficient extraction, 50 µl caffeine solution (*see table 9 in supplements and solutions*) was added to facilitate dislocation of BR from proteins in the sample. This mixture was thoroughly vortexed and incubated for 5 minutes at room temperature. To disable formation of an emulsion in the subsequent extraction step, 50 µl of saturated potassium chloride (KCl) (*see table 9 in supplements and solutions*) was added. After vortexing the samples, 1 ml benzene (C_6H_6) was added under the fume hood at room temperature. The mixture was vigorously mixed following a standardized vortex protocol, assuring a thorough extraction of the bilirubin into the benzene phase. The vortexing scheme consisted of the following three steps:1x long for 4 seconds continuously on the vortex set to medium speed, 3x short for 2 seconds in quick succession up and down on the vortex set to highest speed, 1x long for 4 seconds continuously set to medium speed. The resulting two phases, aqueous and organic phases, were separated by centrifugation at 10.000 x g for 5 minutes at room temperature in a small table centrifuge (Centrifuge MiniSpin®, Eppendorf AG, Hamburg, Germany). The upper organic benzene phase was carefully separated from the aqueous phase using a pipette set to 800 µl and transferred into a 1.5 ml reaction tube. The tubes were frozen at -20 °C, to allow separation from possible aqueous contamination, which would thus collect at the bottom of the tube. Until the measurement the extracts were stored in the -20 °C freezer, usually until the next day.

3.3.2 Quantification of bilirubin by spectrophotometry

The quantity of the bilirubin in the benzene extract was determined by spectrophotometry, using a double beam spectrophotometer (U-3000 Spectrophotometer, Hitachi, Japan) in a dimmed room, to avoid decomposition of the bilirubin in the samples due to light. Prior to the

measurement the benzene extracts were allowed to unfreeze in the dark at room temperature for 30 minutes. The spectrophotometer was allowed to warm up for at least 30 minutes, to ensure optimal working conditions and temperature of the wolfram light bulb. The inbuilt deuterium lamp was switched off during the measurements. The following settings defined in the method "HO-act" using the inbuilt software of the photometer (UV Solutions 2.2, Hitachi, Japan) were applied: slit: 5 nm; scan rate: 120 nm/min; Photomultiplier: autogain, high resolution on; replicates: 2; sampling interval 2 nm. Measurements were performed as scans from 600 nm to 400 nm, light source wolfram lamp only. Prior to the measurement of the samples, benzene was used to record a baseline. For the measurements approximately 700 µl of the extracts were transferred carefully into quartz cuvettes (precision cells made of special optical glass 10 mm, Hellma™, Germany) using a pipette, leaving a few µl in the tube, to assure that aqueous contaminants would not be transferred into the cuvette and impair the measurements. The extinction was determined against a reference cuvette equipped with benzene. The surface of the cuvettes was cleaned with ethanol using microfiber paper towels (precision wipes, KIMTECH® science), to remove any dust particles or benzene residues for avoiding diffraction. The extinction was recorded twice as wavelength scans from 600 nm to 400 nm with benzene as the reference and baseline. From scans without impairment extinctions at the wavelengths 520 nm and 450 nm were recorded and the difference of 450-520 nm (DiffOD), which is the biliburin specific absorption, was calculated. For further processing the means of the two DiffOD were used. DiffOD from negative controls were used to correct the respective mean DiffOD resulting in a negative-control-corrected DiffOD. To determine the amount of nmol of bilirubin formed during the assay, the negative-control-corrected DiffOD was divided by a proportionality factor (0.039/nmol), which had been determined in a previous experiment [Duvigneau et al., 2020]. The resulting BR amount was then corrected for the protein amount, which was determined using the Bradford method described in *section 3.4*. The HO activity was given as nmol BR formed per mg of protein present in the homogenates during 30 minutes of reaction time.

3.4 Determination of protein concentration in the cell homogenates with the Bradford method

The protein concentration of the residual cell homogenates and negative controls was determined using the Bradford method. The cell homogenates were diluted 1:5 using 30 µl of the homogenate added to 120 µl of 0.9 % NaCl. Samples with a concentration below the detection limit were diluted once again, using a 1:2.5 dilution. In order to determine the protein concentration, a standard dilution series of BSA (bovine serum albumin) was prepared using 1 mg/ml aliquots stored at -20 °C. This solution was diluted with 0.9 % NaCl as is indicated in the scheme below (*table 2).*

Table 2. Preparation of BSA standards S1-S5 (100-5 µg/ml) for protein determination

STANDARDS	CONCENTRATION (µg/ml)	Preparation
STANDARD 1 (S1)	100	450µl NaCl + 50µl BSA (1mg/ml)
STANDARD 2 (S2)	75	50µl NaCl + 150µl S1
STANDARD 3 (S3)	50	100µl NaCl + 100µl S1
STANDARD 4 (S4)	25	150µl NaCl + 50µl S1
STANDARD 5 (S5)	5	190µl NaCl + 10µl S1

Of each sample 50 µl were pipetted into the predefined wells of a 96-well microtiter plate (MTP) (96-well microplate, Greiner Bio One GmbH, Austria). The following samples were pipetted in duplicates: blanks (0.9 % NaCl), standards S1-S5, diluted samples, and negative controls. To each sample 200 µl of Coomassie Brilliant Blue solution (*see table 10 in supplements and solutions*) were added for staining the proteins in the wells. The 96-well microtiter plate was analyzed using a Microplate reader (Spark® multimode microplate reader, Tecan Trading AG, Switzerland) with the inbuilt software Sparkcontrol Magellan V2.2 (Version 2.2, Tecan Trading AG, Switzerland). The absorbance of the samples at 590 nm was determined using Spark multimode microplate reader with the settings wavelength 590 nm, number of flashes 10 ms and 50 ms settle time. The protein concentration was calculated using the inbuilt software (Sparkcontrol Magellan V2.2), using the regression of the standard-calibration curve. The measured protein concentrations of the duplicates were calculated to build the mean concentration (µg/ml), which was used for the further calculations taking into account dilution and volume of the sample as is described in *Section 3.3.3*. Loading of the microtiter plate is shown below (*table 3*):

Table 3. Pipetting scheme of the 96-well microtiter plate for the Bradford assay.

wells	**1**	**2**	**3**	**4**	**5**	**6**
A	Blank (NaCl 0.9 %)	Blank (NaCl 0.9 %)	Sample 2	Sample 2		
B	S5	S5	Sample 3	Sample 3		
C	S4	S4	Sample 4	Sample 4		
D	S3	S3	Etc.	Etc.		
E	S2	S2				
F	S1	S1				
G	(Empty)	(Empty)				
H	Sample 1	Sample 1				

3.5 Statistics and data analysis

The data were examined and analyzed using Microsoft Excel (Microsoft 365 MSO, Version 2203, 32 Bit on Windows 10). To enable comparison, the values were calculated relative to the vehicle controls, which were included in each plate, when effect of only one component was to be visualized. In case that a combination of substances was analyzed, the values were additionally calculated relative to one of the components alone (then referred to as 'relative to the respective control') for better visualizing the effects elicited by the added substances. The group means, standard deviation (SD) and standard error of mean (SEM) were calculated from these values for each group. Statistical significances were determined using T-test (two-sided, paired test), with the probability value (p-value) of $p<0.05$ considered as statistically significant. Visualization was performed in Excel, as well as Microsoft PowerPoint (Microsoft 365 MSO, Version 2203, 32 Bit on Windows 10).

4 RESULTS

4.1 Suitability of HO activity modulators

4.1.1 ZnPP and hemin are suitable for modulating HO activity in undifferentiated and differentiated cells

Bone marrow-derived mesenchymal stem cells (undifferentiated cells) treated with ZnPP at a concentration of 20 µM showed a significant decrease of approximately 50% in their HO activity. But it must be taken in consideration that ZnPP treatment also decreased the protein concentration of the cells, potentially indicating cell killing (*Figure 4*).

Hemin treatment of the undifferentiated cells resulted in a more than two-fold increase of HO activity (*Figure 4*). Although there had been only one sample of hemin treated undifferentiated cells. The hemin treated cells did not show a decrease in cell protein, suggesting the HO activity upregulation does not negatively affect cell viability (*Figure 6*).

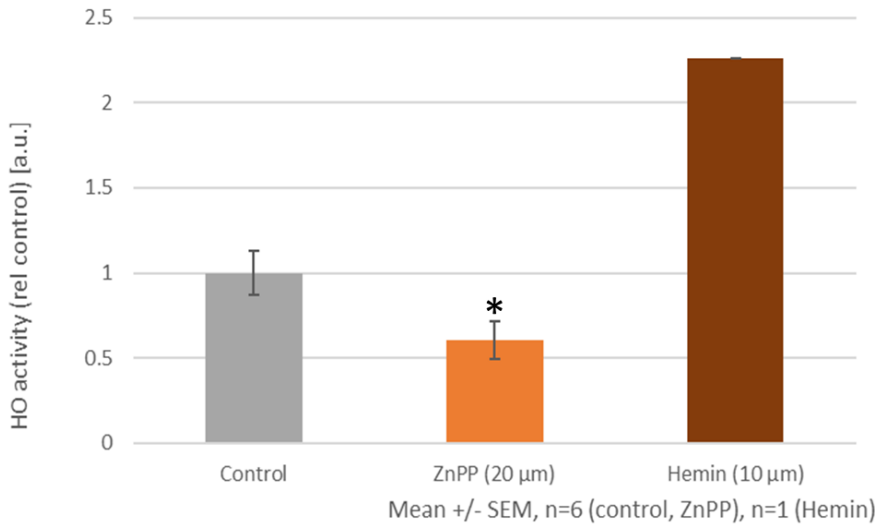


Figure 4: Effect of HO modulators, ZnPP and hemin on the HO activity of undifferentiated bone marrow derived mesenchymal stem cells. *Cells were grown in 12-well plates (266.000 cells/well) and incubated for 24h with vehicle (DMSO, control, grey bar), ZnPP (20 µM, orange bar) or hemin (10 µM, brown bar). HO activity was determined as described in Material and Methods and is given in nmol BR formed within 30 minutes per mg protein present in the cell culture dish [nmolBR/mgProt]. Each group consisted of 6 biological replicates, n=6, except of hemin, which was analyzed only once. Data are displayed as mean values relative to the control (rel control) indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

In differentiated osteoblast-like cells the HO activity appears to be modulated to a lesser extent (*Figure 5*). Opposite to the undifferentiated cells, ZnPP did not decrease HO activity in differentiated. However, since ZnPP decreased the cell number the residual cell amount was just sufficient of producing a BR amount that was slightly over detection limit. Therefore, this might have resulted in the higher variation and makes results less reliable (*Figure 5*). Hemin stimulation upregulated HO activity less than two-fold, which is less than the upregulation in undifferentiated cells, which had been modified to more than two-fold. This could indicate that differentiated cells may be less sensitive towards HO modulation, in comparison to their undifferentiated counterparts. Protein amount was not affected in differentiated cells by hemin (*Figure 7*).

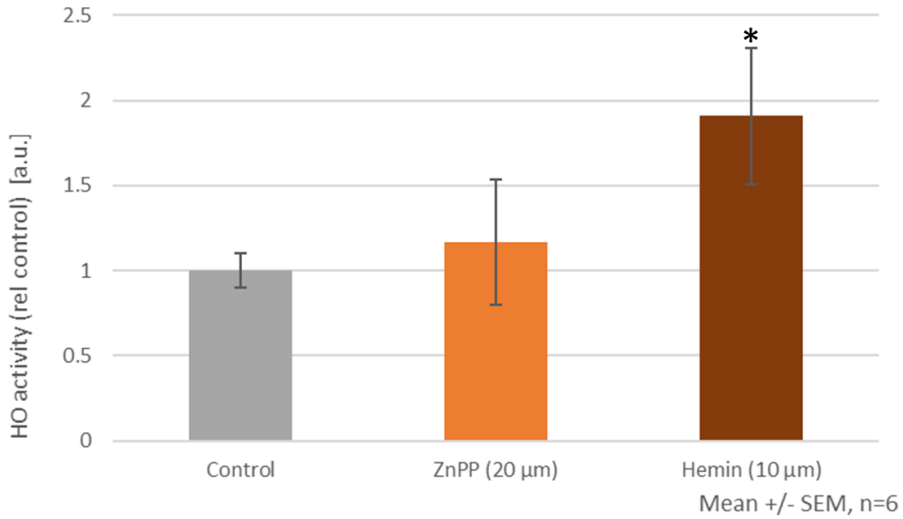


Figure 5: Effect of HO modulators, ZnPP and hemin on the HO activity of differentiated osteoblast-like cells. *Cells were grown in 12-well plates (266.000 cells/well) and incubated for 24h with vehicle (DMSO, control, grey bar), ZnPP (20 µM, orange bar) or hemin (10 µM, brown bar). HO activity was determined as described in Material and Methods and is given in nmol BR formed within 30 minutes per mg protein present in the cell culture dish [nmolBR/mgProt]. Each group consisted of 6 biological replicates, n=6. Data are displayed as mean values relative to the control (rel control) indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

Since both compounds were capable to modulate HO activity in a sufficient degree, we considered the concentrations suitable for our experiments. HO activity was both verifiable modulated with ZnPP and hemin, in both undifferentiated and differentiated bone marrow derived cells, they are suitable HO modulators for the following experiments.

4.1.2 Inhibition of HO activity reduces cell protein by decreasing cell proliferation

In undifferentiated bone marrow derived mesenchymal stem cells shown in *Figure 4* the ZnPP treatment significantly decreased HO activity. This effect was accompanied by a significant decrease of protein concentration (about 20%) (*Figure 6*). Protein content further decreased with additionally added ferrostatin-1 to the ZnPP treated cells. To conclude that cells were killed diminished protein content should go in parallel with increased LDH. In the following part, without showing LDH data, since I was not involved in the measurements, a decreased protein content, which goes together with an increased LDH activity, will be referred to as 'cell death'. A decrease in protein content without simultaneously increased LDH activity will be taken as sign for a decrease in cell proliferation.

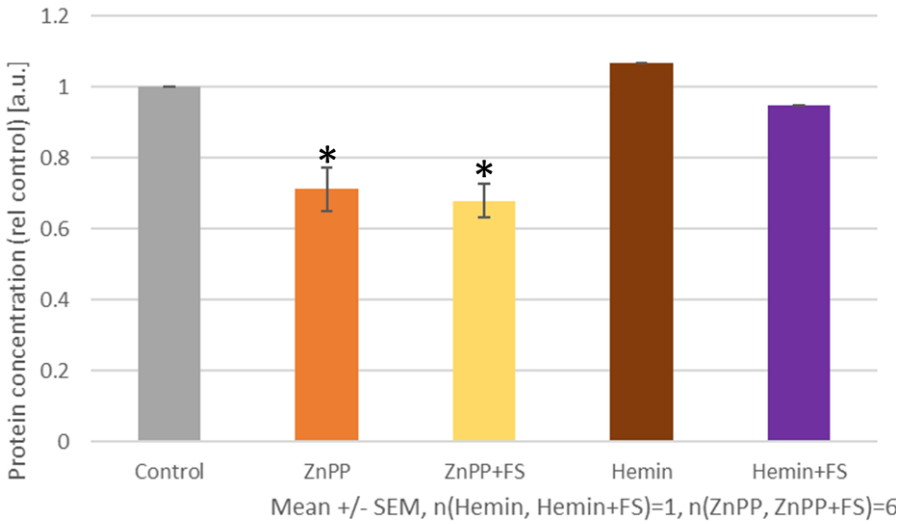


Figure 6: Effect of HO modulators in combination with ferroptosis inhibitor ferrostatin on protein concentration of undifferentiated bone marrow derived mesenchymal stem cells. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with vehicle (DMSO, control, grey bar), ZnPP (20 µM, orange bar), with ZnPP plus ferrostatin-1 (20 µM ZnPP+ 10 µM FS, yellow bar), or with hemin (10 µM, brown bar), or with hemin plus ferrostatin-1 (10 µM Hemin+10 µM FS, violet bar). Protein concentration [mg/ml] was determined as described in Material and Methods and is given in mg protein present per ml of the homogenated cell mass present in cell culture dish after 24h and are shown relative to the values of the untreated control wells. Each group consisted of 6 biological replicates, except for hemin and hemin+FS, which was analyzed only once. Data are displayed as means indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

LDH levels of undifferentiated cells treated with ZnPP were not increased (data not shown), indicating that protein decrease was caused by diminished cell proliferation. An increased HO activity caused by treatment with hemin did not decrease the protein concentration of

undifferentiated cells, in contrast, there might even be a slight increase of protein content be visible. However, adding FS slightly decreased protein amount.

ZnPP affects differentiated osteoblast-like cells and undifferentiated cells in a similar extent, as the protein concentration was also significantly lower (about 20%). A decrease of protein content was also measured in the ZnPP plus FS treated cells, which suggests that FS could not prevent ZnPP mediated diminished cell proliferation. LDH did not increase with ZnPP treatment, but it slightly increased with the added FS, which could indicate a slight cell killing. Hemin treatment did not decrease protein concentration, remaining constant at control levels. FS slightly decreased protein amount again (*Figure 7*).

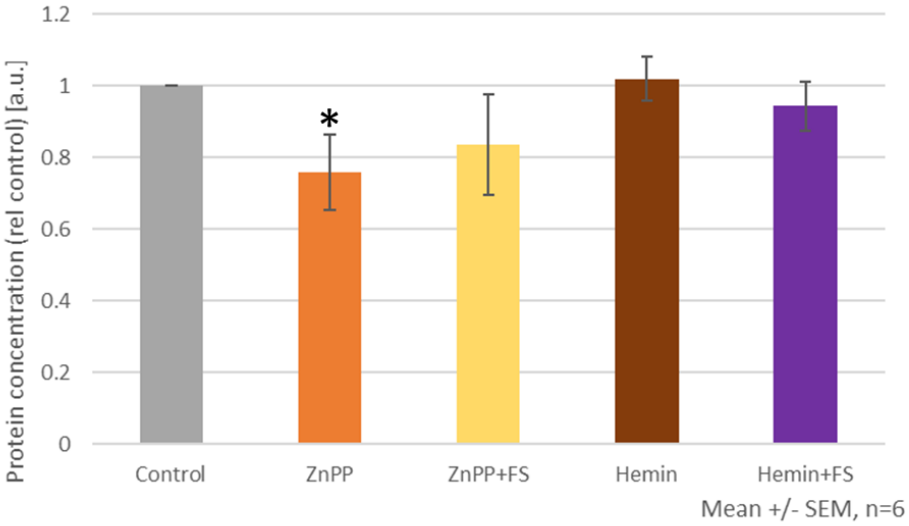


Figure 7: Effect of HO modulators in combination with ferroptosis inhibitor ferrostatin on protein concentration of differentiated osteoblast-like cells. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with vehicle (DMSO, control, grey bar), ZnPP (20 µM, orange bar), with ZnPP plus ferrostatin-1 (20µM ZnPP+10 µM FS, yellow bar), or with hemin (10 µM, brown bar), or with hemin plus ferrostatin-1 (10 µM Hemin+10 µM FS, violet bar). Protein concentration [mg/ml] was determined as described in Material and Methods and is given in mg protein present per ml of the homogenated cell mass present in cell culture dish after 24h and are shown relative to the values of the untreated control wells. Each group consisted of 6 biological replicates. Data are displayed as means indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

We can conclude that neither ZnPP nor hemin kill undifferentiated or differentiated cells. The decreased protein concentration caused by ZnPP treatment can be attributed to a decreased cell proliferation, but cell viability was not negatively influenced (data not shown). To correctly attribute effects of the single substances, when applied in combination, the data must be

calculated to the respective control. Therefore, in the following sections, all data will be displayed in this manner, relative to the respective control, which is set to 1 (*Figure 7*).

4.2 Suitability of erastin and RSL3

4.2.1 Cells are sensitive to both, erastin and RSL3, but sensitivity depends on differentiation state

To determine a suitable concentration of the ferroptosis-inducing agents, erastin and RSL3, we tested three different concentrations. For our experiments we defined a suitable concentration as a dose which does not completely kill all cells, sufficient amount of cells should remain allowing determination of HO activity. Erastin treatment was performed with concentrations of 0.8 µM, 1 µM and 2 µM, which have previously been established as suitable in the osteosarcoma cell line MG63 [Bottau, 2021]. Proportional to the increasing erastin amount, the protein concentration decreased. The undifferentiated cells showed a significant decrease of protein content at the highest erastin concentration (about 20%). Differentiated cells seem to be less sensitive to erastin (*Figure 8*).

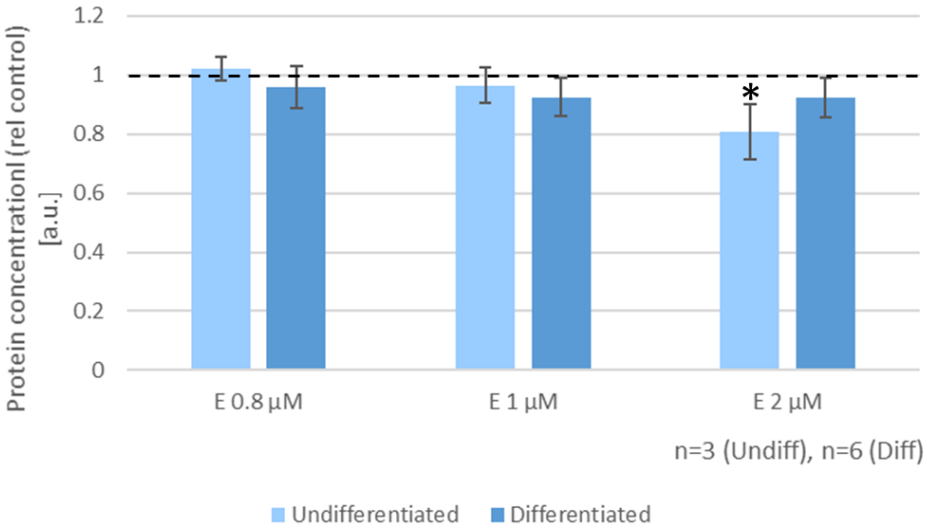


Figure 8: Comparison of the susceptibility of undifferentiated and differentiated cells towards ferroptosis induction by erastin using protein concentration as indicator for survival. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with erastin at three different concentrations (E 0.8 µM; E 1 µM; E 2 µM), with undifferentiated cells as light blue, and differentiated cells as darker blue. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the values of the untreated control (rel control), visualized as a dashed line. Undifferentiated cells consisted of 3 replicates, while differentiated cells of 6 replicates. Data are displayed as means indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

RSL3 concentrations used were 1 µM, 2.5 µM and 5 µM, and have also been found suitable in MG63 cells [Bottau, 2021]. RSL3 treatment decreased protein concentration in both cell types. Undifferentiated cells were far more sensitive to RSL3, all concentrations resulted in a significant decrease (about 80%). In differentiated cells the RSL3 treatment decreased the protein dose dependently with the highest concentration resulting in a significant decrease in comparison to the control (approximately 40%). (*Figure 9*). Thus, we can conclude that erastin and RSL3 exert cell killing effects both in undifferentiated and differentiated cells, although the different cell types show different sensitivities.

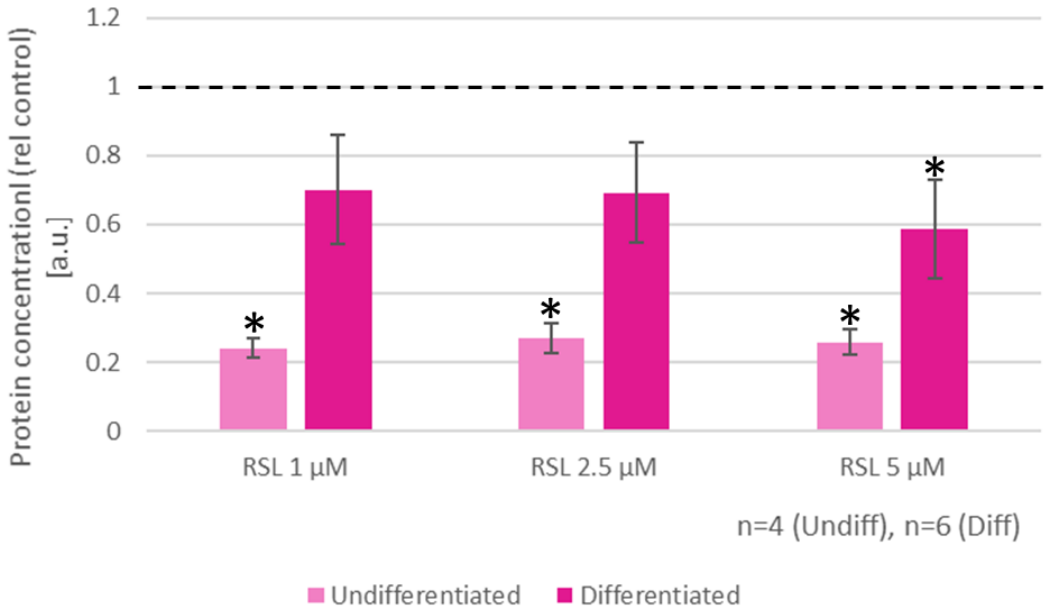
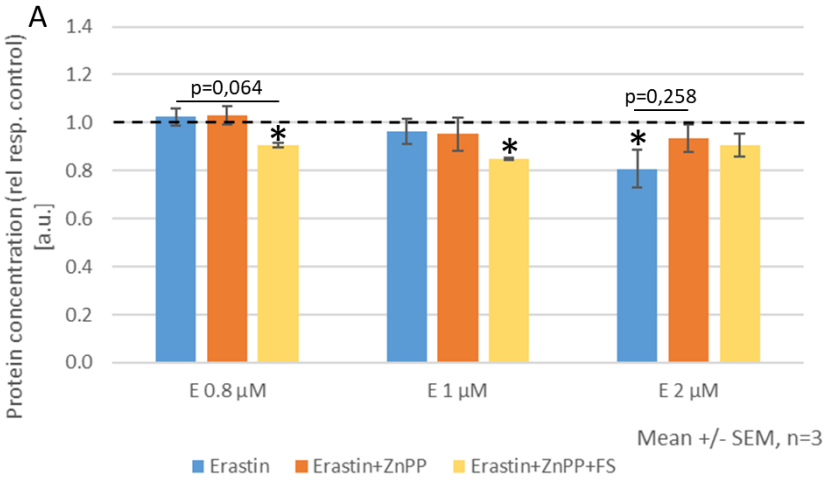


Figure 9: Comparison of the susceptibility of undifferentiated and differentiated cells towards ferroptosis induction by RLS3 using protein concentration as indicator for survival. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with RSL3 at three different concentrations (RSL 1 µM; RSL 2.5 µM; RSL 5 µM), light pink bars are undifferentiated, and dark pink differentiated cells. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the values of the untreated control (rel control), visualized as a dashed line. Undifferentiated cells consisted of 4 replicates, while differentiated cells of 6 replicates. Data are displayed as means indicating +/- SEM. Significant differences (Student's t-test, paired, two-sided) to the untreated control group are indicated (*, $p<0.05$).*

4.3 Effect of HO modulation on ferroptosis induced by ZnPP-treatment

4.3.1 HO-inhibition by ZnPP rescues against loss of cell protein provoked by erastin

Adding ZnPP rescues cell protein indicating cell protection, against killing mediated by erastin in undifferentiated cells. This effect is visible at the highest concentration of erastin, which induced a significant cell killing when applied alone. Additional supplementation with FS exerted unfavorable effects and decreased cell protein further. A significant decrease in protein concentration with added FS occurred at erastin concentration 0.8 µM and 1 µM (*Figure 10 A*). In differentiated osteoblast-like cells, although they were less sensitive to the effects of erastin, a slight improvement of increased cell protein can be noted by adding ZnPP. Also here, this effect is abrogated, when FS is given in combination with ZnPP, lowering cell protein again, especially at the highest erastin concentration (*Figure 10 B*).



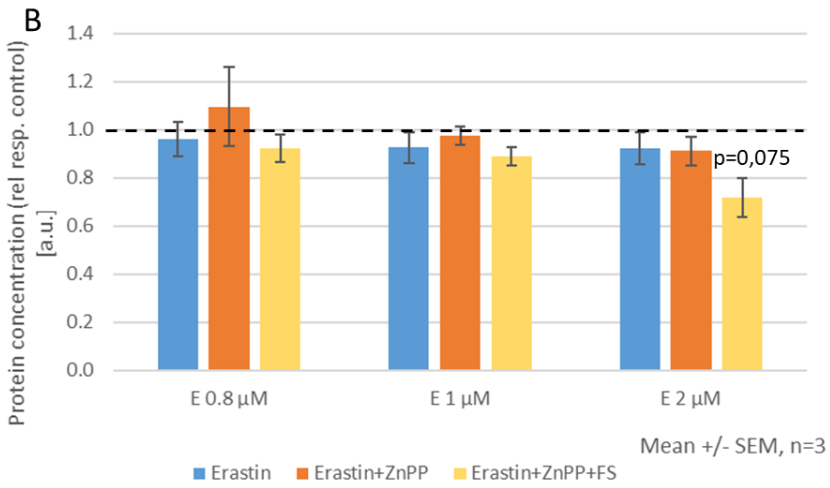


Figure 10: Co-treatment with ZnPP favors survival of bone marrow derived cells against erastin treatment but exerts unfavorable effects in combination with ferrostatin-1. *Undifferentiated cells (A) and differentiated cells (B) were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with erastin at three different concentrations (E 0.8 µM; E 1 µM; E 2 µM, blue bars) or in presence of ZnPP (erastin+20 µM ZnPP, orange bars), or with ZnPP in combination with ferrostatin-1 (erastin+20 µM ZnPP+10 µM FS, yellow bars), as ferroptosis inhibitor. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the respective control (without erastin). The respective control group (rel resp. control) is visualised by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/- SEM. Differences were analyzed using paired, two-sided Student's t-test. Differences are indicated above the bars when significant to the respective control group (*, p<0.05). P-values are indicated when differences were close to the setted significance level.*

This shows that HO inhibition, caused by ZnPP, rescued cells against erastin mediated killing. We can thus conclude that the killing of undifferentiated cells by erastin was mediated through the activity of HO.

4.3.2 ZnPP partially rescues cell protein, indicating protection against RSL3 mediated cell killing

Adding ZnPP to undifferentiated cells led to a higher protein content. However, when comparing to the untreated control (dashed line in *Figure 11*), the protein content remained below the levels determined in the control. This indicates that cells cannot be fully rescued by HO inhibition. However additional supplementation with FS to cells challenged with RSL3 restored protein levels to more than those of the control. This indicates that FS exerts a protective effect (*Figure 11 A*).

Differentiated osteoblast-like cells were less sensitive to the RSL3 mediated cell killing, showed a full restoration of protein upon HO inhibition by ZnPP. Here adding FS lowers the protein concentration however without reaching statistical significance. As we have already seen before (*Figure 6 from 4.1.2*) the added FS exerts negative effects on protein amount (*Figure 11 B*). This indicates that HO inhibition at least partially rescued cells against RSL3 induced cell death, in both cell types. However, while HO inhibition could only partially rescue undifferentiated cells against RSL3 mediated killing, the differentiated cells were fully protectable against RSL3 by HO inhibition. But HO only contributes to RSL3 mediated cell killing, as there are also other mechanisms responsible for inducing cell killing.

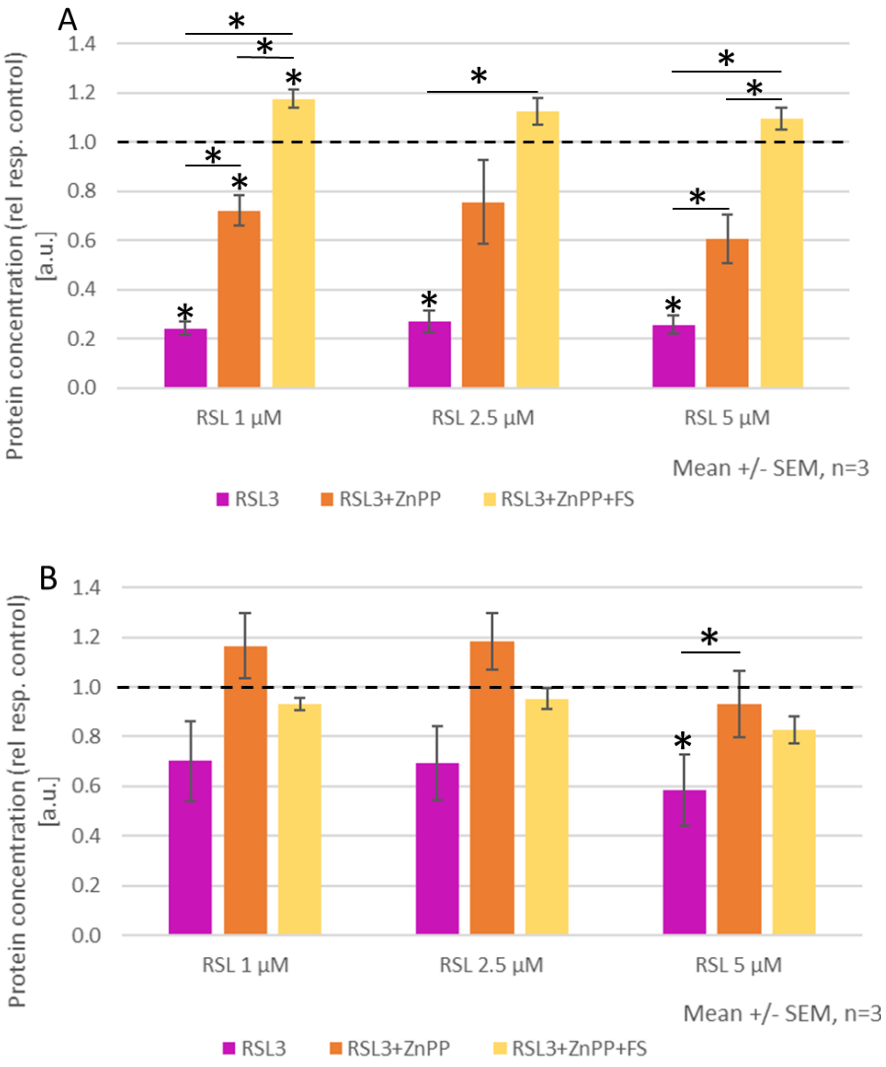


Figure 11: Co-treatment with ZnPP favors survival of bone marrow derived cells against RSL3 treatment, ferrostatin-1 shows additional protective effects. *Undifferentiated cells (A) and differentiated cells (B) were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with RSL3 at three different concentrations (RSL 1 µM; RSL 2.5 µM; RSL 5 µM, pink bars) or in presence of ZnPP (RSL3+20 µM ZnPP, orange bars), or with ZnPP in combination with ferrostatin-1 (RSL3+20 µM ZnPP+10 µM FS, yellow bars), as ferroptosis inhibitor. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the respective control (without RSL3). The respective control group (rel resp. control) is visualized by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/-SEM. Differences were analyzed using paired, two-sided Student's t-test. Significant differences are indicated above the bars (*, $p<0.05$).*

4.4 Effect of HO stimulation on ferroptosis induced by hemin-treatment

4.4.1 Although HO inhibition exerted cell protection, hemin stimulation did not increase cell death mediated by erastin

Since we found that HO activity is necessary for supporting cell death mediated by erastin, we expected more cell killing when HO activity is increased using hemin. Only data of differentiated osteoblast-like cells is available, since no experiments with erastin and heme have been conducted with undifferentiated cells, due to little amount of available cells. Contrary to our expectation, adding hemin to erastin-challenged cells did not increase cell killing, since no decrease in cell protein occurred. In contrast, adding hemin resulted in protein levels that were as high as those found in the untreated control (*Figure 12)*.

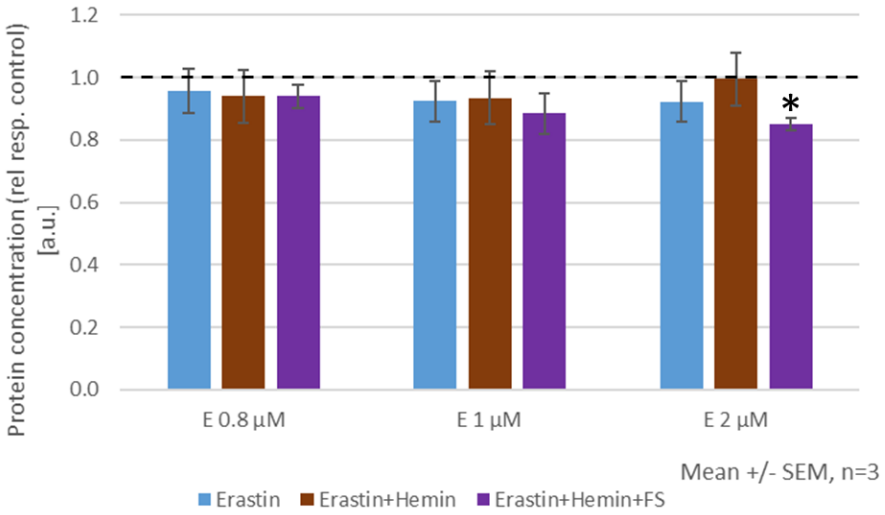


Figure 12: Effect of erastin-treatment on protein concentration of differentiated osteoblast-like cells and co-treatment with hemin and ferrostatin-1. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with erastin at three different concentrations (E 0.8 µM; E 1 µM; E 2 µM, blue bars) or in presence hemin (erastin+10 µM hemin, brown bars), or with hemin plus ferrostatin-1 (erastin+10 µM hemin+10 µM FS, violet bars), as ferroptosis inhibitor. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the respective control (without erastin). The respective control group (rel resp. control) is visualized by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/- SEM. Differences were analyzed using paired, two-sided Student's t-test. Significant differences are indicated above the respective bars or above the respective bar, when difference to the respective control group was significant (*, $p<0.05$).*

However, upon additional FS treatment, a significant loss of cell protein occurred, suggesting enhanced cell killing, which may indicate loss of hemin-mediated protection (*Figure 12*). In

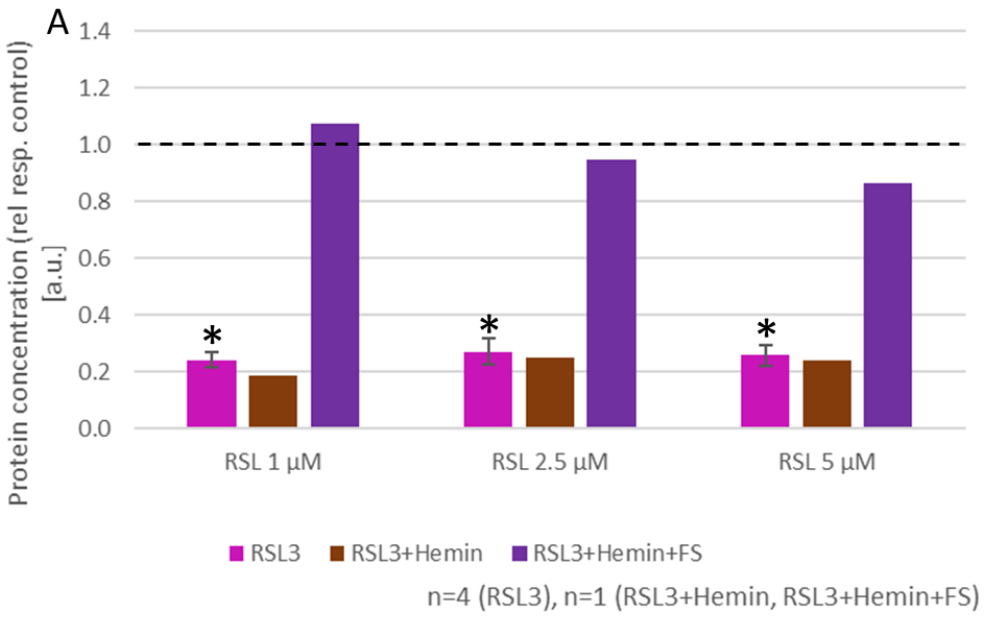
conclusion heme treatment did not aggravate cell killing mediated by erastin. However, FS supplementation might have increased cell killing.

4.4.2 Although HO inhibition exerted cell protection, HO stimulation did not increase cell death mediated by RSL3, while ferrostatin-1 restored cell protein

As mentioned before, it was expected that increased HO activity would increase RSL3 mediated cell death. The protein concentration did in fact decrease with added hemin, but just slightly and not significantly. Instead, treatment with FS fully restores cell protein and thus protects against RSL3 mediated cell killing.

As undifferentiated bone marrow derived mesenchymal stem cells were more sensitive to RSL3 mediated cell killing. However, added FS did not completely succeed in restoring protein content in contrast to other cells. But there were only one sample for undifferentiated cells with hemin, as well as hemin and FS (*Figure 13 A*). But in differentiated cells a complete recovery of protein concentration was possible with the added FS (*Figure 13 B*).

Thus, we can conclude that HO stimulation does not enhance cell killing mediated by ferroptosis-inducers, since the added hemin did not decrease the cell protein. The upregulated HO activity appears to not contribute to ferroptotic cell killing. However, treatment with FS together with hemin induced a complete recovery against RSL3 mediated cell killing.



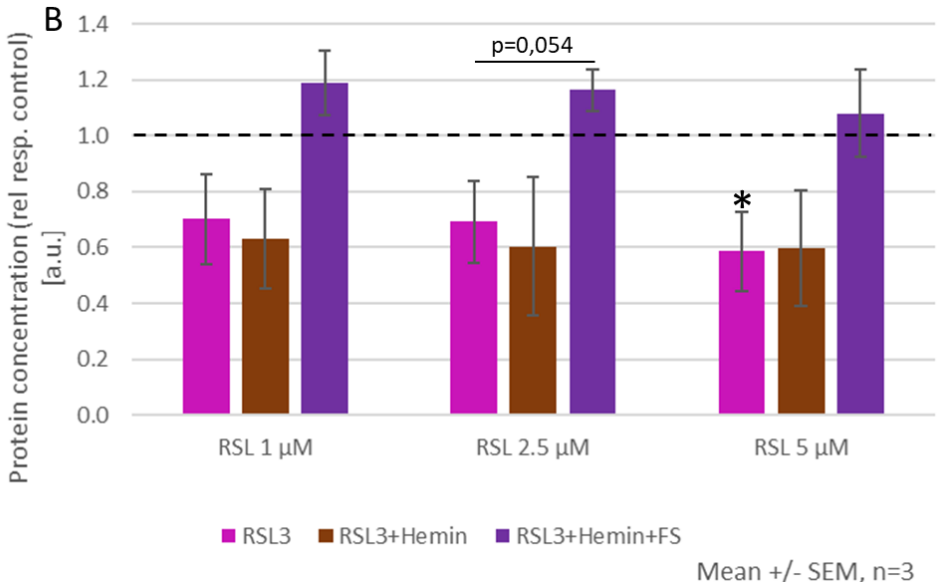


Figure 13: Effect of RSL3-treatment on protein concentration of bone marrow derived cells and co-treatment with hemin and ferrostatin-1. *Undifferentiated cells (A) and differentiated cells (B) were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with RSL3 at three different concentrations (RSL 1 µM; RSL 2.5 µM; RSL 5 µM, pink bars) or in presence hemin (RSL3+10 µM hemin, brown bars), or with hemin plus ferrostatin-1 (10 µM, RSL3+10 µM hemin+10 µM FS, violet bars), as ferroptosis inhibitor. Protein concentration [mg/ml] was used as marker for cell killing and was determined as described in Material and Methods and is shown relative to the respective control (without RSL3). The respective control group (rel resp. control) is visualized by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/- SEM. Differences were analyzed using paired, two-sided Student's t-test. Significant differences are indicated above the respective bars or above the respective bar, when difference to the respective control group was significant (*, $p<0.05$). Values close to significance are added as p-values.*

4.5 Effect on HO activity of ferroptosis inducers and HO modulators

4.5.1 HO activity, indicating cell stress, is significantly upregulated by erastin. Co-application with hemin adds on cell stress level. Ferrostatin-1 abrogates heme effect

Since we did not determine a decrease in cell killing upon adding hemin to the cells treated with ferroptosis inducer we checked if HO activity in these conditions has been modulated (*Figure 12 from 4.4.1*). HO activity is an indicator for cell stress, a high HO activity indicates that the cell is fighting against a stressor. A relief of cell stress would thus go along with lower HO activity. The data is only available for differentiated cells because no experiments were conducted with undifferentiated cells with erastin and hemin. The HO activity increased due to

the added stress of exposure to erastin. Hemin further increased HO activity significantly. FS significantly decreased the HO activity again, indicating less cell stress, abrogating the effect of hemin (*Figure 14*). It might be possible that erastin and hemin have a synergistic effect, with erastin potentiating the hemin-mediated HO induction.

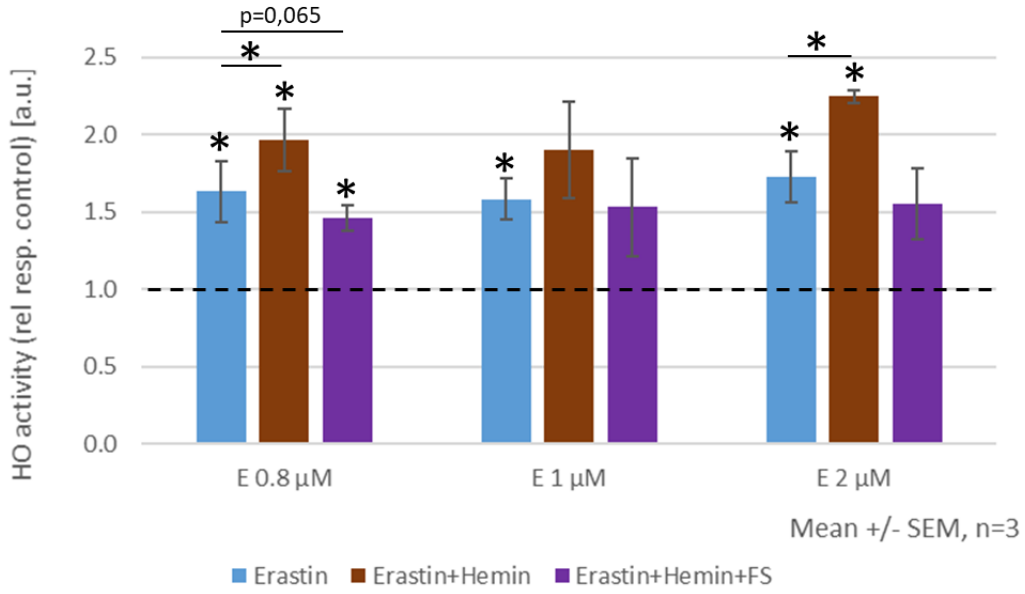


Figure 14: Effect of erastin-treatment on HO activity of differentiated osteoblast-like cells and co-treatment with hemin and ferrostatin-1. *Cells were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with erastin at three different concentrations (E 0.8 µM; E 1 µM; E 2 µM, blue bars) or in presence hemin (erastin+10 µM hemin, brown bars), or with hemin plus ferrostatin-1 (erastin+10 µM hemin+10 µM FS, violet bars), as ferroptosis inhibitor. HO activity was determined as described in Material and Methods and is given in nmol BR formed within 30 minutes per mg protein present in the cell culture dish [nmolBR/mgProt] and is shown relative to the respective control (without erastin). The respective control group (rel resp. control) is visualized by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/- SEM. Differences were analyzed using paired, two-sided Student's t-test. Significant differences are indicated above the respective bars or above the respective bar, when difference to the respective control group was significant (*, p<0.05). Values close to significance are added as p-values.*

4.5.2 HO activity, indicating cell stress, is significantly upregulated by RSL. Co-application with hemin abrogates RSL3 mediated HO upregulation

HO activity was significantly increased by RSL3, in both cell types. In undifferentiated cells the HO activity was lower than in differentiation. They were more sensitive to RSL3 cell

stressing effects, which more than doubles HO activity at low concentrations of RSL3. The HO activity slightly decreased with the higher concentrations. Adding hemin did barely increase HO activity, and FS also has no significant effects on HO activity (*Figure 15 A*). In differentiated cells the HO activity was much higher increased, up to 3.5 times the HO activity in the highest concentrations of RSL3. Hemin did not significantly influence HO activity, but FS did slightly increase the HO activity in the highest concentration of RSL3 (*Figure 15 B*). RSL3 did not potentiate the effect of hemin's HO activity induction like erastin did. The lower HO activity might also be indicative of lesser cell stress.

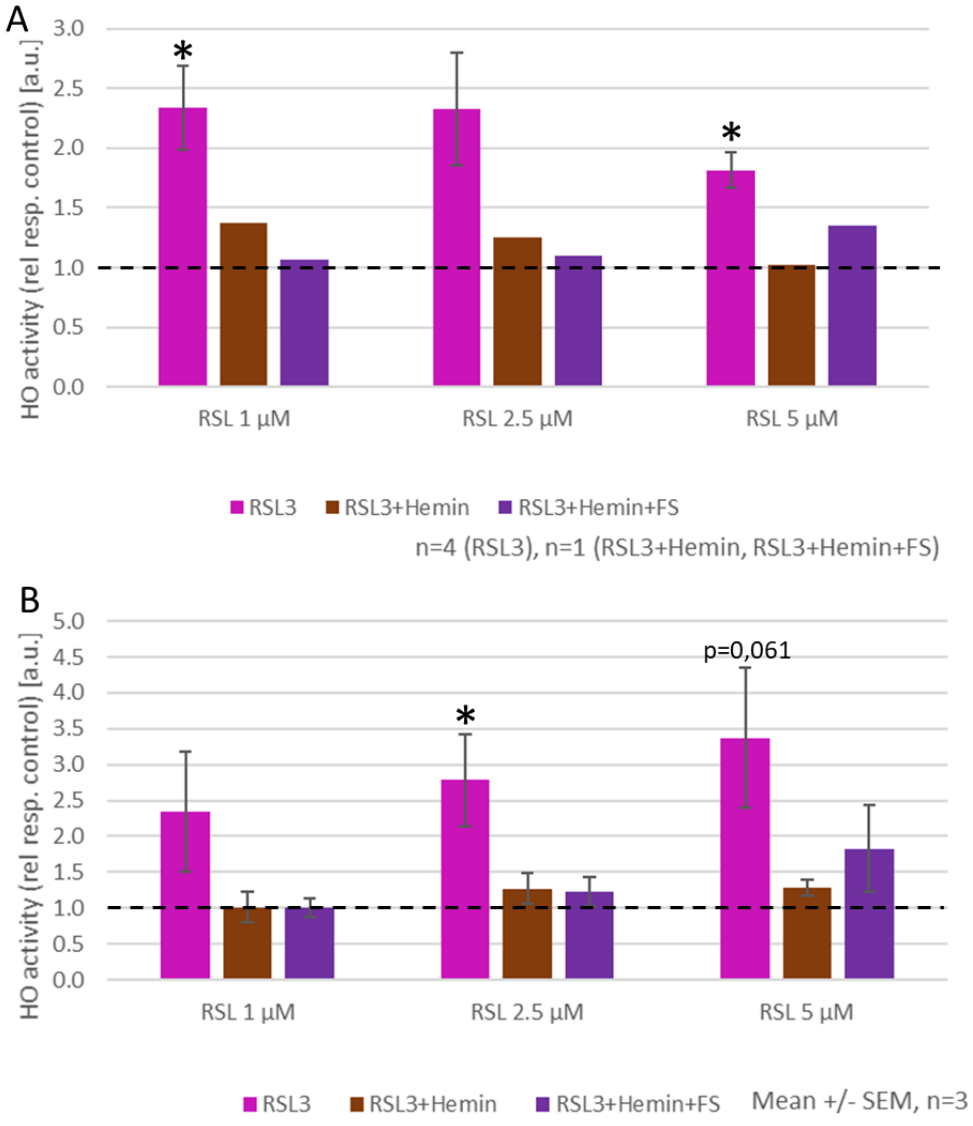


Figure 15: Heme-treatment abrogates the increase of the capacity to convert heme in response to RSL3 in differentiated osteoblast-like cells, irrespectively of co-application with ferrostatin.

Undifferentiated cells (A) and differentiated cells (B) were grown in 12 well-plates (266.000 cells/well) and incubated for 24h with RSL3 at three different concentrations (RSL 1 µM; RSL 2.5 µM; RSL 5 µM, pink bars) or in presence of RSL3 plus hemin (RSL3+10 µM hemin, brown bars), or additionally supplemented with ferrostatin-1 (RSL3+10 µM hemin+10 µM FS, violet bars), as ferroptosis inhibitor. HO activity was determined as described in Material and Methods, is given in nmol BR formed within 30 minutes per mg protein present in the cell culture dish [nmolBR/mgProt] and is shown relative to the respective control (rel resp. control; substances without RSL3). The respective control group is visualized by the dashed line. Each group consisted of 3 independent biological replicates. Data are displayed as means indicating +/- SEM. Differences were analyzed using paired, two-sided Student's t-test. Significant differences are indicated above the respective bars or above the respective bar, when difference to the respective control group was significant (, $p<0.05$). P-Values are indicated, when close to set significance level.*

5 DISCUSSION

The aim of this study was to understand the role of HO activity in ferroptotic cell death induced by RSL3 or erastin in human bone marrow mesenchymal stromal cells (hBMSCs) and osteoblast-like cells. To inspect the influence of the differentiation state in terms of sensitivity towards ferroptosis, both, undifferentiated and differentiated cells, which were generated by inducing osteoblast-like differentiation [Zavadskis et al., 2020], were used. Bone resident cells are especially iron-dependent and are a suitable test subjects to explore, if ferroptosis induction could be used in the elimination of cancerous bone cells, i.e. osteosarcoma cells. Erastin and RSL3 have been proven to trigger ferroptosis in previous experiments [Shintoku et all., 2017; Stockwell et al.,2020]. In order to understand the role of HO in ferroptosis, HO activity was modulated using ZnPP to inhibit and hemin to upregulate its activity. In case that HO products, especially ferrous iron, support ferroptosis, we expected to see a rescuing effect against cell killing when HO activity is inhibited. In case that HO protects against ferroptosis, we expected to see an enhanced cell killing, when HO activity is inhibited, or when heme is supplemented exogenously.

5.1 Setting up the experimental approach

Three different concentrations of ferroptosis inducers have been used, to make sure that however strong the cell killing effects was, there were still cells remaining for being used to determine HO activity and to verify a detrimental or protective effect exerted by HO modulators. But at the same time the dose had to be high enough to kill a sufficient number of cells for confirming induction of ferroptosis. To accurately determine whether a diminished cell number was indeed due to cell killing, two parameters were considered: protein content of the cell layer present within cell culture wells at the end of the incubation period, representing number of living cells, and LDH activity in the cell culture medium, as a determinant for cell death, as LDH is released by stressed or dying cells. Thus, a lower protein content, which went in parallel with an increased LDH activity was taken as an indicator of cell killing. However, a lower protein content without simultaneous increase in LDH activity was taken as an indicator for a decreased cell proliferation.

5.2 Suitability of HO modulators

Adding 10 µM hemin to undifferentiated and differentiated cells doubles the capacity of the cell homogenate to produce bilirubin, which stands for the *in-vitro* HO activity [nmolBR/mg protein] in both cell types. The increase in HO-activity was associated without simultaneously decreasing cell protein, thus not having any cytotoxic effects. As ferrous iron (Fe^{2+}) is formed in equal amounts as BR, the iron levels must have also risen to a similar extent. To determine the suitability of ZnPP, it was necessary to find out whether ZnPP was indeed able to decrease BR formation and thus inhibit HO activity in the used cell types. In a previous study using osteosarcoma cells of the line MG63, it was found that a concentration of 20 µM sufficiently blocked formation of BR and its appearance in the cell culture medium. Using the undifferentiated cells, we found that upon treatment with ZnPP at a concentration of 20 µM, the capacity to degrade heme was decreased by half, but there was no decrease in HO activity in response to ZnPP in the differentiated cells. But since ZnPP also decreased the cell number, the BR-amounts generated by the homogenate were only slightly above the detection limit (of 2 pmol BR), which was therefore difficult to quantify. Thus, both, ZnPP and hemin appeared to be suitable for modulating HO activity in both, undifferentiated hBMSCs and differentiated osteoblast-like cells.

We further found that ZnPP, which induced a decrease of cell protein of cells in both differentiation states, reduced cell proliferation, but did not exert toxic effects, since LDH levels were not increased. A similar effect was also observed in osteosarcoma cells of the MG63 line [Bottau, 2021]. As ZnPP inhibits HO activity, these findings suggest that HO activity is required for supporting cell viability and proliferation. This hypothesis is also supported by findings of another study, which showed that silencing of HO-1 in MG63 cells prevented induction of HO-1, increased cell death, and increased intracellular ROS levels in MG63 cells in response to an electrophile stress [Zhong et al., 2014], indicating a role for HO in the development of this cancer type. Further, induction of HO-1 restores proliferation of osteosarcoma cells, whose proliferation has been stopped due to co-cultivation with a parental prostate cancer cell line [Ferrando et al., 2013].

The slight increase in cell protein seen in the hemin treated cells, and the significant decrease in cell protein found in cells treated with the HO-inhibitor ZnPP indicates that hBMSCs and osteoblast-like cells require functional HO for supporting proliferation.

Further, it can be assumed that, since functional HO is necessary in these cells, they are not only capable to remove heme, but also to produce heme degradation products. Additionally, treatment with hemin resulted in an approximately two-fold increased BR production of the cell homogenates, indicating upregulated HO-activity (see below). It is therefore expected that ongoing HO activity results in steady state levels of free ferrous iron, one of the HO degradation products.

5.3 Suitability of ferroptosis inducers

The concentrations of ferroptosis inducers were taken from our previous experiments using MG63 osteosarcoma cells, as was described recently [Bottau, 2021]. Using these cells the concentration range of both, erastin and RSL3, was adjusted to kill only a part of the cells [Bottau, 2021]. Using these concentrations, we found that hBMSCs and osteoblast-like cells showed a different sensitivity compared to MG63 cells (see below). Additionally, we found that the percentage of cells that were killed, varied from experiment to experiment, indicated by the high variation. For this heterogeneity several reasons could account for. One explanation is, that the conditions were not exactly reproduced throughout the experiments. Possibly, the routine of the experimentators might have played a role. However, despite this variation, the measured effects were reproducible in terms of the direction. To combat the variability, values were calculated relative to the controls, and for determining statistical significance analyses were executed with a paired t test.

5.4 Cell killing exerted by erastin

Erastin decreased the cell mass of undifferentiated cells by about 20%, which was evident only at the highest concentration of 2 µM. This decrease in protein was associated with an increase of LDH levels, confirming that erastin-treatment resulted in cell killing. No reduction of cell mass was observed in differentiated cells, suggesting that their differentiation state protected them. Compared to the previous experiments using MG63 cells, in which a concentration of

2 µM was found to kill about 95% of the cells [Bottau, 2021], osteoblast-like cells, thus, revealed a much higher resistance against erastin.

Erastin induces ferroptosis by inhibiting system X_c-, a cysteine-glutamate antiporter, which leads to the inhibition of glutathione synthesis due to the decreased availability of cysteine [Zhao et al., 2020]. This causes a depletion of glutathione in the cell, limiting the activity of glutathione peroxidases (GPX), amongst others GPX4, which is required to counteract lipid peroxidation [Stockwell et al.,2020; Dixon et al., 2012]. However, erastin does not affect only one pathway, but acts via multiple targets. It is therefore not exclusively operating via increased lipid peroxidation and GPX4 [Sato et al., 2018]. It has been shown that increased levels of Nrf2 are associated with resistance against erastin [Kwon et al., 2020]. Also cells capable to enhance synthesis of cysteine in sufficient amounts are known to be protected against ferroptosis [Hayano, 2016]. These cell types may generate metabolites of the transsulfuration pathway, which have been shown to not affect iron levels [Hayano, 2016], thus explaining, why these cells were far less sensitive towards erastin. Additionally, it is possible that the differentiation state is associated with an increased capacity to counteract stress related to electrophiles and ROS, which might originate during metabolic adaptation [Allen-RG, 1991]. Either way, increased ROS and deprivation of the cellular thiol pool has been shown to result in the activation redox sensitive transcription factors, such as NfkB and KEAP/Nrf2 [Antelmann & Helmann, 2011]. These transcription factors increase the expression of anti-oxidant enzymes, such as HO-1 [Nishizawa, 2022]. We found that HO activity was clearly upregulated in response to erastin, similarly to the treatment with hemin. This indicates that in differentiated osteoblast-like cells an upregulation of multiple anti-oxidant targets may have occurred, in the attempt to counteract erastin-mediated deprivation of glutathione. Thus, erastin might be useful for treating osteosarcoma, since undifferentiated and differentiated bone marrow-derived cells are expected to be less vulnerable.

5.5 Cell killing exerted by RSL3

While erastin treatment decreases the intracellular thiol pool, and thereby decreases the anti-oxidant capacity more generally, RSL3 acts specifically on one pathway, by covalently inhibiting GPX4. This leads to an irreversible inhibition of the repair system for peroxides of phospholipids and leads to increased lipid-peroxidation resulting in ferroptotic cell death

[Stockwell et al., 2020]. Also, the concentrations of RSL3 were previously determined using MG63 cells [Bottau, 2021]. In these cells RSL3 killed about 100% at the highest concentration of 10 µM. Osteoblast-like cells and hBMSCs appeared to be much more sensitive towards RSL3. The undifferentiated cells displayed a killing rate of about 80% at the highest concentration of 5 µM RSL3. RSL3 affected the differentiated cells to a lesser extent. It resulted in a cell killing of about 40% at the highest concentration. Thus, also RSL3-treatment revealed undifferentiated cells being more vulnerable to the cell killing, which corresponds to the effect of erastin. Compared to the MG63 cells, however, RLS3 mediated inhibition of GPX4 was far more effective in hBMSCs and osteoblast-like cells.

GPXs, particularly GPX4, play an important role for the regulation of cell proliferation and malignancy [for review see: Brigelius-Flohé & Kipp, 2009]. Several cancer cell types, such as pancreatic and breast cancer cells [Cejas et al., 2007; Liu et al., 2006] display a reduced expression of GPX4 compared to the non-transformed cell type. It was shown that overexpression of GPX4 in pancreatic tumor cells was associated with a lowering of the proliferation rate and a decreased malignancy [Liu et al., 2006]. Thus, it is possible that osteoblast-like cells and hBMSCs depend in a higher extend on functional GPX4 and cannot easily compensate its inactivation. This may be associated with their dependence on ferrous free iron. Differentiation and metabolism are regulated by iron in a particular balanced fashion [for review see: Balogh et al., 2018].

5.6 Role of HO inhibition in erastin and RSL3 mediated cell killing

Although erastin treatment did not succeed to exert a substantial cell killing effect at the used concentrations, adding ZnPP clearly increased the protein amount. This was found for undifferentiated cells treated with the highest concentration of erastin. Here, adding ZnPP restored cell mass and yielded values similarly to those seen with ZnPP alone. This indicates that ZnPP fully inhibited cell death caused by erastin and protected the cells against thiol deprivation. This indicates that HO supports cell killing via its product, ferrous iron, which is released during degradation of endogenous heme. Consequently, cell death is suppressed when HO activity is inhibited by ZnPP treatment. These findings are in line with previous findings of ZnPP preventing erastin-triggered ferroptotic cell death of fibrosarcoma cells [Kwon, et al., 2015]. Thus, not endogenous heme, but an HO product supports erastin-mediated cell killing.

Whether or not erastin killed the cells via ferroptosis, cannot be clarified. However, instead of preventing cell death, adding FS slightly supported cell death, and counteracted the protection exerted by ZnPP: This indicates that lipid radical formation was not the predominant mechanism of erastin-mediated cell killing in hBMSCs and osteoblast-like cells.

Contrary to erastin treatment, RSL3 induced ferroptotic cell death in all cell types at all concentrations, with undifferentiated cells being more sensitive. ZnPP was able to rescue cells against RSL3 mediated cell killing to a substantial extent in both differentiation states. The cell rescuing effect was less pronounced in the undifferentiated cells, and ZnPP could not fully restore cell mass. This indicates that HO only partially contributed to the induction of cell death in this cell type. In differentiated cells the effect of RSL3 was less severe compared to the undifferentiated cells, and ZnPP fully recovered cell mass. RSL3 resulted in ferroptosis of both cell types at all concentrations, which was completely prevented by supplementing with FS. Since FS mediated rescue effect is considered as a sign to protect specifically against ferroptosis, we conclude that RSL3 induced killing of osteoblast-like cells and hBMSCs was exerted exclusively via ferroptosis. FS cytoprotective actions are caused by the block of lipoxyl-radicals production and detoxification of ferrous iron [Miotto et al., 2020]. As HO generates free iron, our data indicate that HO activity contributes at least partially to iron mediated ferroptosis induced by RSL3.

5.7 Role of heme mediated HO upregulation in erastin and RSL3 mediated cell killing

We expected that the adding of exogenous heme would result in an increased ferroptotic cell killing in response to ferroptosis inducer, due to the higher availability of ferrous iron. Since adding 10 µM hemin to undifferentiated and differentiated cells doubled the *in-vitro* HO activity in both cell types (see above), we expected to also increase the release of ferrous iron (Fe^{2+}). As ferrous iron can induce lipid peroxidation and thus ferroptosis, it would be expected that the added hemin should cause increased cell death via ferroptosis. However, contrary to our expectation, adding hemin to cells treated with ferroptosis inducer did not increase cell death in terms of protein amount and LDH activity in the supernatant. These findings are in line with those determined in osteosarcoma cells of the line MG63 [Bottau, 2021].

Thus, heme exhibited no enhanced cell killing effect in cells treated with erastin or RSL3, which poses the question how this was prevented. One possibility could be that the byproducts of

heme degradation, CO and BR, with their established anti-oxidant and antiapoptotic effects, balanced out the pro-oxidant effects of the released iron [Belcher et al., 2010]. However, heme also has other effects than just inducing HO-1. It functions as an intracellular regulator of gene expression by its ability to interact with the heme-sensitive repressor BACH 1 and 2 [for review see: Igarashi & Sun, 2006]. This transcription factor cooperates with Nrf2, which is a master regulator of cytoprotective cell responses and stimulates the synthesis of several anti-oxidant enzymes, amongst others proteins required for safe iron storage. It has been shown that heme/BACH interaction not only induces HO-1, but additionally upregulated ferritin light and heavy chains simultaneously [Gozzelino & Soares, 2014]. This upregulation has been suggested to mediate the protective effect of HO-1 against oxidative stress. [Cheng et al., 2015]. Thus, hemin-treatment might have induced an improved iron handling capacity, accounting for the possible protective effects of hemin application, as already mentioned [Otterbein et al., 2003; Mafra et al., 2022]. Further, heme/BACH interaction derepresses also the iron exporter ferroportin. An increased ferroportin expression has been shown to prevent ferroptotic cell death [Namgaladze et al., 2022]. Thus, interaction of hemin with BACH may counteract ferroptosis. Our findings suggest that despite its implication in ferroptosis, hemin-treatment is not a suitable approach to enhance ferroptotic cell killing.

5.8 Role of HO activity in hBMSCs and osteoblast-like cells

This study showed that HO supports ferroptosis, since inhibition of its activity by ZnPP was cytoprotective. ZnPP mediated cytoprotection was found for both, undifferentiated and differentiated cells. Thus, it can be concluded that iron, released during heme degradation, is a potential trigger of lipid peroxidation. Interestingly, HO activity was shown to be relevant for cell proliferation in both differentiation states. An inhibition of HO decreases cell proliferation, which was also shown in several other studies that HO activity is important for cell proliferation [Belcher et al., 2010]. Therefore, induction of ferroptosis will benefit from the dependence of the cellular metabolism on functional HO activity.

Erastin significantly upregulated HO activity in differentiated cells at all concentrations, while no experimental data is available for undifferentiated cells. Hemin supplementation further increased HO activity. Upon adding FS to erastin and hemin treated cells, levels of HO activity decreased to the levels seen without added heme, indicating that FS could abrogate hemin's

effects. This could indicate that cell stress, which was exerted by erastin, and which upregulated HO activity, was neutralized by FS.

Erastin not only affects system X_c- to induce ferroptosis, but also affects other cellular pathways, which could have caused HO activity to be upregulated. The other two products of HO, CO and BR, also showed cytoprotective effects in cancer cells, providing the selective advantage for tumor cells to overcome the increased oxidative stress [Nitti et al., 2017]. This could also explain how the HO activity is increased, but no cell killing occurred, due to HO products possibly exerting cytoprotective effects. Further, HO and hemin also activate other cytoprotective pathways involving BACH/Nrf2, which can protect the cell against exacerbation of stress and enhancing iron handling capacities. [Loboda et al., 2015; Nishizawa et al., 2022].

RSL3 treatment increased HO activity in both differentiation states, higher than erastin did, which only increased about 1,5-fold, while RSL3 more than doubled HO activity. Differentiated cells showed a higher increase in HO-activity than undifferentiated cells, which also appeared to exhibit higher stress levels, although less cells were killed. Differentiated cells were less sensitive to the effects of RSL3, showing lower rates of cell killing, but an increase of HO activity. Contrary to the situation with erastin, adding hemin did not further increase HO activity in bone marrow derived cells, irrespectively of their differentiation state. In contrast, HO activity decreased to baseline levels, while protein amount remained the same. This may indicate a reduced stress level or may reflect downregulation of HO activity in order to lower the release of ferrous iron. This is in line with the findings, that HO inhibition is protective for RSL3 treated cells. Additional supplementation with FS did not increase HO activity, staying close to baseline, indicating that FS-treatment decreased cell stress.

5.9 Undifferentiated and differentiated cells

One of the aims of this study was to check whether the differentiation state has influence on the sensitivity towards ferroptotic inducing treatments. Undifferentiated stem cells seemed more sensitive towards both erastin and RSL3 treatment, indicating that during the differentiation process cells become less sensitive and develop better strategies to deal with oxidative and ferroptotic cell stress. Undifferentiated cells were very affected by inhibition of GPX4 by RSL3, inducing ferroptotic cell death. HO-1 activity is shown to play a role in osteoblast proliferation, and inhibits osteoblastic apoptosis by CO synthesis, and HO activity is increased during

osteoblast stem cell development. HO activity supports osteoblast differentiation by reducing ROS [Vanella et al., 2010]. This could be the reason, why differentiated osteoblast-like cells are less sensitive towards ferroptosis, as the cells have adapted their iron handling capacities. Interestingly, basal HO activity was higher in undifferentiated than in differentiated cells, which also could have made them more vulnerable to the treatment with ferroptosis inducer. Thus, our data show, that due to their dependence on HO, hBMSCs and osteoblast-like cells are more vulnerable to ferroptosis induction by GPX4-inhibition exerted by RSL3, while better protected against glutathione deprivation, which is induced by erastin treatment. Upon differentiation cells acquire strategies for being better protected.

6 CONCLUSION

Our data suggests that heme oxygenase activity is supporting ferroptosis in hBMSCs and osteoblast-like cells with its product, ferrous iron. Our experiments show that bone marrow derived cells are susceptible to ferroptotic cell death by RSL3 but are much less affected by erastin at the used concentrations. Although it was expected that hemin treatment, which upregulates HO activity, enhances ferroptosis, it did in fact not further increase ferroptotic cell death. In contrast, our data suggest that heme, besides upregulating HO-1, induced protective pathways, capable to counteract iron mediated stress. This cytoprotective effect is possibly involving Nrf-2 and BACH pathways. Our findings further suggest that undifferentiated cells are more sensitive towards RSL3 and erastin effects than differentiated cells, and thus more sensitive towards ferroptosis and HO modulation, suggesting that changes in HO activity are involved in the differentiation process. As hBMSCs and osteoblast-like cells are less sensitive towards erastin-induced cell killing, it could be used for targeting osteosarcoma, without significantly affecting mesenchymal stem cells or osteoblast-like cells.

Thus, our data suggests that, similarly to osteosarcoma cells, functional HO is a prerequisite for mediating ferroptotic cell death in osteoblast-like cells. However, hemin treatment is not a suitable strategy for critically increasing intracellular iron levels.

7 SUMMARY

Heme oxygenase (HO) is an integral enzyme of the stress response of the cell, iron homeostasis and anti-oxidant defense. HO oxidatively cleaves heme, degrading it into biliverdin (BV), ferrous iron and carbon monoxide (CO), which promote the anti-inflammatory, anti-oxidant, and antiapoptotic effects of HO. However, due to HO's release of iron, it's involvement in ferroptosis is being questioned and may limit its pro-survival function. Ferroptosis is form of non-apoptotic regulated cell death dependent on iron. It can be induced by an overload of iron in the cell, which leads to lipid peroxidation and an accumulation of lethal reactive oxygen species (ROS). ROS fulfil important signaling function, however, when in excess they cause oxidative stress, oxidatively damaging the cells organelles and membranes, and induce programmed cell death. Ferroptosis can be induced by small molecules, such as erastin and RSL3, which can interfere with the cell's capacity to counteract lipid peroxidation. Erastin induces ferroptosis by inhibiting cystine importing system X_{c-}, and thereby GSH synthesis, which reduces the cellular ability to handle oxidative stress. GSH is required for GPX4 activity, an anti-oxidant enzyme, which repairs lipid peroxides. RSL3 directly inhibits GPX4, and thus increases lipid peroxidation in the cell, resulting in ferroptotic cell death. It was the aim of this study to investigate the effect of HO function on ferroptosis in human bone marrow mesenchymal stromal cells (hBMSCs) (undifferentiated cells) and osteoblast-like cells (differentiated cells), which particularly depend on iron. Since differentiation state of osteoblast-like cells may also play a role in ferroptosis, both, adherently growing undifferentiated and differentiated cells were used. Cells were treated with erastin and RSL3, and in parallel with hemin and zinc protoporphyrin IX (ZnPP) for inhibiting and increasing HO activity respectively. To test whether the cell death was resulting from ferroptosis, ferrostatin-1 (FS) was added, together with ferroptosis inducers and HO modulators. As indicator of cell death, protein content of the cell mass present at the end of the experiment was determined using the Bradford method. HO activity of homogenated cells was measured using a coupled photospectroscopic biochemical assay. Undifferentiated cells displayed only a weak, differentiated cells no sensitivity at all against erastin. However, RSL3 exerted ferroptosis in cells of both differentiation state, with undifferentiated cells being more sensitive. ZnPP prevented ferroptosis induction at least partially in RSL3 treated undifferentiated cells, and fully rescued differentiated cells. Co-treatment with FS resulted in complete protection of

undifferentiated cells treated with ZnPP and RSL3, indicating at least partial contribution of HO products to ferroptotic death of undifferentiated cells. Contrary to our expectation, heme did not increase ferroptosis rate upon RSL3 challenge, which indicates upregulation of multiple cytoprotective pathways, possibly involving BACH. This all suggests that HO activity is supporting ferroptosis in hBMSCs and osteoblast-like cells due to release of iron, and that undifferentiated cells are more sensitive towards those effects. Hemin treatment, however, appears to be no suitable approach for enhancing ferroptosis.

8 ZUSAMMENFASSUNG

Häm-Oxygenase (HO) ist ein integrales Zellenzym, beteiligt an der Stressreaktion der Zelle, der Eisenhomöostase und der anti-oxidativen Abwehr. HO spaltet Häm oxidativ in Biliverdin (BV), Eisen und Kohlenmonoxid (CO), welche die entzündungshemmende, anti-oxidative und anti-apoptotische Wirkung von HO vermitteln. Da HO Eisen freisetzt könnte seine anti-apoptotische Funktion eingeschränkt werden, weshalb seine Beteiligung an der Ferroptose in Frage gestellt. Ferroptose ist eine eisenabhängige Form des nicht-apoptotischen, regulierten Zelltods. Sie kann durch eine Überladung der Zelle mit Eisen ausgelöst werden, was zu Lipidperoxidation und einer Anhäufung von letalen reaktiven Sauerstoffspezies (ROS) führt. ROS haben eine wichtige Signalfunktion, verursachen jedoch im Übermaß oxidativen Stress, der die Zellorganellen und -membranen oxidativ schädigt und den programmierten Zelltod auslöst. Die Ferroptose kann durch kleine Moleküle wie Erastin und RSL3 ausgelöst werden, die die Fähigkeit der Zelle, der Lipidperoxidation entgegenzuwirken, beeinträchtigen können. Erastin löst die Ferroptose aus, indem es das Cystin-Import-System X_{c-} und damit die GSH-Synthese hemmt, wodurch die Fähigkeit der Zelle, oxidativen Stress zu bewältigen, verringert wird. GSH ist für die Aktivität von GPX4 erforderlich, einem anti-oxidativen Enzym, das Lipidperoxide repariert. RSL3 hemmt GPX4 direkt und erhöht so die Lipidperoxidation in der Zelle, was zum ferroptotischen Zelltod führt. Ziel dieser Studie war es, die Auswirkungen der HO-Funktion auf die Ferroptose in Knochenmarkszellen wie human bone marrow mesenchymal stromal cells (hBMSCs) (undifferenzierte Zellen) und osteoblast-like cells (differenzierte Zellen), die besonders eisenabhängig sind, zu untersuchen. Da der Differenzierungsgrad der Knochenmarkszellen eine Rolle bei der Ferroptose spielen könnte, wurden von adhärent wachsenden Zellen sowohl undifferenzierte als auch differenzierte Zellen verwendet. Die Zellen wurden mit Erastin oder RSL3 behandelt, mit Zusatz von Hemin oder Zinkprotoporphyrin IX (ZnPP), um die HO-Aktivität zu hemmen bzw. zu erhöhen. Um zu prüfen, ob der ausgelöste Zelltod auf Ferroptose zurückzuführen ist, wurde Ferrostatin-1 (FS) zu den Ferroptose-Induktoren und HO-Modulatoren hinzugefügt. Als Indikator für den Zelltod wurde der Proteingehalt, der am Ende des Experiments vorhandenen Zellmasse mit der Bradford-Methode bestimmt. Die HO-Aktivität der homogenisierten Zellen wurde mit einem gekoppelten photospektroskopischen biochemischen Test gemessen. Undifferenzierte Zellen zeigten nur eine geringe, differenzierte Zelle überhaupt keine Empfindlichkeit gegenüber

Erastin. RSL3 jedoch bewirkte Ferroptose in Zellen beider Differenzierungsstadien, wobei undifferenzierte Zellen empfindlicher waren. ZnPP verhinderte die Ferroptose-Induktion zumindest teilweise in mit RSL3 behandelten undifferenzierten Zellen und rettete differenzierte Zellen vollständig. Die gleichzeitige Behandlung mit FS führte zu einem vollständigen Schutz undifferenzierter Zellen, die mit ZnPP und RSL3 behandelt wurden, was auf einen zumindest teilweisen Beitrag von HO-Produkten zum ferroptotischen Zelltod undifferenzierter Zellen hinweist. Entgegen unserer Erwartung erhöhte Hemin die Ferroptose-rate nach einer RSL3-Behandlung nicht, was auf eine Hochregulierung mehrerer zytoprotektiver Signalwege hinweist, möglicherweise unter Beteiligung von BACH. Das alles deutet darauf hin, dass die HO-Aktivität Ferroptose in hBMSCs und osteoblast-like cells durch die Freisetzung von Eisen unterstützt und dass undifferenzierte Zellen empfindlicher auf diese Effekte reagieren. Die Behandlung mit Hemin scheint jedoch kein geeigneter Ansatz zur Förderung der Ferroptose zu sein.

9 ABBREVIATIONS

BACH1 BTB and CNC homolog 1

bFGF basic fibroblast growth factor

BMSC bone marrow stem cell

BR bilirubin

BR bilirubin

BSA bovine serum albumine

BTB Broad-complex, Tramtrack, and Bric-à-brac

BV biliverdin

BVR biliverdin reductase

C_6H_6 benzene

CNC Cap'n'collar

CO carbon monoxide

DFO deferoxamine mesylate

DiffOD differential optical density

DMEM- HG Dulbecco´s Modified Eagle´s Medium high glucose

DMSO dimethyl sulfoxide

DPIs diphenylene iodonium chlorides

Fe^{2+} ferrous iron

Fe^{3+} ferric iron

FBS fetal bovine serum

FS Ferrostatin-1

GPX4 glutathione peroxidase 4

GSH glutathione

GSSG glutathione disulfide

H_2O_2 hydrogen peroxide

hBMSCs human bone marrow mesenchymal stromal cells

HO heme oxygenase

HO-assay buffer heme oxygenase assay buffer

HSP32 heat shock protein 32

KCl potassium chloride

Keap1 Kelch-like ECH-associated protein 1

L• lipid radical

LDH Lactate dehydrogenase

LOO• lipid peroxyl radical

L-OOH lipid hydroperoxide

LOXs lipoxygenases

NADPH nicotinamide adenine dinucleotide phosphate

Nrf2 nuclear factor erythroid 2-related factor-2

$O_2^{\bullet-}$ superoxide anions

$HO^{\bullet}$ hydroxyl radicals

OD optical density

OS osteosarcoma

P- value probability value

PBS phosphate buffered saline

Pen/Strep Penicillin and Streptomycin

Red-ox reduction-oxidation

ROS reactive oxygen species

RSL3 Ras-selective lethal 3

SAS sulfasalazine

SD standard deviation

SEM standard error of mean

SRF sorafenib

StREs stress-responsive elements

ZnPP zinc protoporphyrin IX

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11 SUPPLEMENTS AND SOLUTIONS

Table 4. Reagent's preparations and conditions for cell cultures.

Medium compounds	**Amount**	**Final concentration**	**Details**	
FCS	2 ml	10%	Sigma	Stored at -20°C
L-glutamine	200µl	1%	Sigma	Stored at -20°C
DMEM	17.8 ml	/	D6546-500ML	Stored at 4°C. Also used for washing cells.

Table 5. Stimulant's preparation and details.

Reagents	**MW**	**Amount reagents**	**Amount DMSO [µl]**	**Stock concentration [mM]**	**Details**	
Erastin	440.9	5mg	457.0	20	S7242-5mg	Stored at -20°C.
RSL3	262.4	5mg	1134.1	10	S8155-5mg	
Hemin	651.94	1g	153388.3	10	CAS-16009-13-5	
Ferrostatin-1	547.0	5mg	3811.7	5	S7243-5mg	
ZnPP Zinc (II) Protoporphyrin IX	626.0	25mg	1996.7	20	CAS 15442-64-5	

Table 6. HO-Assay Buffer preparation for cell-culture.

Reagent formula	Final concentration [mM]	Amount (g)	Details	
KH_2PO_4	25mM	0.3402 g	pH = 7.4 Add KOH if too acidic	
K_2HPO_4	75mM	1.306 g		
Aqua dest.		100 ml		

Table 7. Color reagent components.

Color reagent components	**Amount**	**Final concentration [µM]**	**Details**
β-Nicotinamide adenine dinucleotide sodium salt	0.898 g	6.55	Sigma, cat. No. 20111-18-6
INT	0.35 g	1.465	Sigma, cat. No. 146-68-9
Aqua dest.	200 mL	/	/
PMS	0.09 g	3.46	Sigma, cat. No. 299-11-6
			Stored at -20°C aliquoted in 2ml and protected from the light.

Table 8. Pipetting scheme of the assay for the determination of heme oxygenase activity.

	Well Sample	NegContr
ID	Test HO	Negative Control

HO-assay-buffer [µl]	0	25
Cell homogenase [µl]	120	120
Hemin [µl]	4	0
DMSO [µl]	0	4
DFO [µl]	5	5
NADPH [µl]	25	0
Caffeine [µl]	50	50
KCl [µl]	50	50
C_6H_6 [µl]	1000	1000
Total Volume [µl]	1254	1254

Table 9. Reagent's preparations and conditions for determination of heme oxygenase activity.

Reagents	Final concentration	Description
HO Assay Buffer	0.1 M	KH_2PO_4 (25 mM): 3.402 g K_2HPO_4 (75 mM): 17.12 g Dist. Aq.: 1000 ml pH: 7.4 (Add KOH if too acidic). Store at -20°C. Thawing at RT
Hemin (Fluka 51280)	1 mM in DMSO	Weight in mg and dissolve in enough DMSO to produce a 10mM solution.

		Make a 1:10 dilution with DMSO to produce a 1mM solution. Store the solution at -80°C. Thawing away from the light.
DMSO	100 uM	Store at -80°C. Thawing away from the light at 37°C.
DFO		Store at -80°C. Thawing at 37°C
NADPH (Sigma N-6505)	10 mM in HO Assay Buffer	Weight aliquotes that are approximately 8mg/tube. The aliquotes are stored as a powder at -20°C. Dissolve in enough HO Assay Buffer to produce a 10mM solution (8.334 mg/mL = 10 mM). Store the solution at -80°C. Thawing at 37°C.
Caffeine solution	0,25 M	Caffeine: 50 g (F.c[2].: 0.25 M) Sodium Benzoate: 75 g (F.c.: 0.52 M) Sodium Acetate: 125 g (0.9 M) Adest: 1000 ml Store at -20 °C. Thawing at RT.
KCl		Put as much KCl in Adest until sediment does not solve nomore. Store at RT.

[2] *F.c.: = final concentration.*

Table 10. Recipe for Coomassie Brilliant Blue.

Reagents	**Amount**	**Molecular Weight**	**Details**

Coomassie Brilliant Blue® G250	0.075mg	854.0	Order number: 35050 Serva Feinbiochemica Heidelberg, Germany	Filled up with distilled water until 1000ml.
Ethanol 96%	50ml			In the dark at 4 °C
Phosphoric acid conc.	100ml			

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