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Redox Modulation and Induction of Ferroptosis as a New Therapeutic Strategy in Hepatocellular Carcinoma

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ABSTRACT

Ferroptosis, a newly discovered form of cell death mediated by reactive oxygen species (ROS) and lipid peroxidation, has recently been shown to have an impact on various cancer types; however, so far there are only few studies about its role in hepatocellular carcinoma (HCC). The delicate equilibrium of ROS in cancer cells has found to be crucial for cell survival, thus increased levels may trigger ferroptosis in HCC.
In our study, we investigated the effect of different ROS modulators and ferroptosis inducers on a human HCC cell line and a human hepatoblastoma cell line. We identified a novel synergistic cell death induction by the combination of Auranofin and buthionine sulfoxime (BSO) or by Erastin and BSO at subtoxic concentrations. We found a caspase-independent, redox-regulated cell death, which could be rescued by different inhibitors of ferroptosis. Both cotreatments stimulated lipid peroxidation. All these findings indicated ferroptotic cell death. Both cotreatments affected the canonical ferroptosis pathway through GPX4 downregulation. We also found an accumulation of Nrf2 and HO-1, indicating an additional effect on the non-canonical pathway. Our results implicate that targeting these two main ferroptotic pathways simultaneously can overcome chemotherapy resistance in HCC.

Introduction

The evasion of programmed cell death and the imbalance of redox homeostasis contribute to tumor formation and lead to failure of anticancer therapies [1–3]. The identification of novel drugs, which reinduce cell death in tumor cells by addressing the redox system through modulation of ROS could be a promising new therapeutic strategy. Ferroptosis has recently been discovered as a new form of programmed non-apoptotic, oxidative cell death, which is characterized, inter alia, by fenton reaction caused by redox-active iron pools, increased ROS production and accumulation of lipid peroxidation [4]. Cells undergoing ferroptosis show morphological changes such as mitochondrial shrinkage, rupture and condensation of the mitochondrial membrane and vanishing of the mitochondrial crista [4–6]. Induction of ferroptosis was found in various kinds of cancer cells including renal cell carcinoma, diffuse large B-cell carcinoma, breast cancer, lung cancer, pancreatic cancer and others [5,7–9].

Two main pathways for inducing ferroptotic cell death have been described: first, the canonical pathway, which is characterized by degradation or blocking of glutathione (GSH) peroxidase 4 (GPX4), a protein which protects cell membranes against lipid peroxidation [10,11], and second, the non-canonical pathway which is mediated by activation of heme oxygenase-1 (HO-1), resulting in an increase of the labile Fe-(II) pool thereby inducing ferroptosis. The non-canonical pathway is mainly regulated by decreased levels of Kelch-like ECH-associated protein 1 (KEAP1) resulting in accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2), which subsequently translocates into the nucleus [10,11]. Target genes of Nrf2, e.g. thioredoxin reductase (TrxR), are involved in GSH synthesis and elimination of ROS [12].

Ferroptosis-inducing compounds can be further differentiated based on their mode of inhibition of GPX4 [5]. Class 1 inducers lead to GSH depletion, for example by blocking its synthesis with substances like BSO or by inhibiting the Xc^--system, which delivers cystine for GSH regeneration. A known Xc^--inhibitor and ferroptosis inducer is Erastin [4,5,13]. A second

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class, e.g. Ras selective lethal 3 compound (RSL3), directly inhibits GPX4 without GSH depletion [5].

The delicate ROS homeostasis plays an important role in protecting cells from lipid peroxidation and is therefore another interesting target for inducing ferroptosis in cancer, especially since cancer cells appear to be more easily damaged by ROS imbalance due to their already elevated basal ROS levels [1,12,14]. Essential for redox homeostasis is the thioredoxin (Trx) system, which protects DNA from oxidative stress-associated damage and lipid peroxidation [15,16]. Auranofin, a gold complex used in antirheumatic therapy, which can inhibit TrxR-1, an enzyme that maintains the supply of antioxidant Trx, could be another promising anticancer agent [16,17].

Addressing ROS homeostasis and ferroptosis might be a new promising strategy for anticancer therapies, especially for human HCC, which is known for its resistance to most chemotherapeutical regimens. Because of the late onset of symptoms, HCC is often too advanced to be treatable via surgery, ablation or radioembolization at the time of diagnosis, and there are only limited therapeutic alternatives. The effect of approved molecular targeted agents, so far consisting solely of Sorafenib or Regorafenib, is still unsatisfactory, showing a median overall survival benefit of only 3 months compared to placebo [18,19]. Being the second leading cause of cancer death worldwide with increasing incidence in Europe and North America, it is crucial to find new therapeutic approaches to treat HCC [20]. Recently we showed that ROS is a mediator to induce apoptotic cell death in HCC [21–23]. The fact that Sorafenib, which is by now known to induce ferroptosis, induces HCC cells to undergo cell death, might suggest that other ferroptosis-inducing regiments could be effective as well [24]. And indeed, several studies have shown first promising results concerning the induction of ferroptosis in HCC [16,25–27].

Therefore, in the present study we investigated the role of different ROS modulators and ferroptosis inducers in the induction of cell death in human HCC cells.

Materials and Methods

Cell Culture and Reagents

The human HCC cell line Huh7 and human hepatoblastoma cell line HepG2 were purchased from Japan Collection of Research Biosources (JCRB) Cell Bank (Osaka, Japan) and cultured in DMEM medium (high glucose, GlutaMAX™; Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1 mM sodium pyruvate (Invitrogen). All cell lines were maintained in a humidified atmosphere at 37 °C with 5% CO_2.

Auranofin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Necrostatin-1 (Nec-1) from Calbiochem (Darmstadt, Germany) and the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) from Bachem (Heidelberg, Germany). RSL3 was kindly provided by B. Stockwell (Columbia University, New York, NY, USA). All other chemicals were purchased from Sigma-Aldrich or Carl Roth (Karlsruhe, Germany) unless indicated otherwise.

Determination of Cell Viability, Cell Death, ROS Production and Lipid Peroxidation

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Cell death was determined by analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei or forward/side scatter (FSC/SSC) analysis of PI-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany) as described previously [28].

To analyze ROS production cells were incubated with 5 μM CM-H_2DCFDA (Molecular Probes, Inc., Eugene, OR, USA) or 5 μM of MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Inc.) according to the manufacturer's protocol. ROS production was measured by flow cytometry before cells succumbed to cell death. For measuring lipid peroxidation, cells were incubated with 5 μM BODIPY™ 581/591 C11 (Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at 37 °C according to the manufacturer's protocol and immediately analyzed by flow cytometry before cells underwent to cell death.

Determination of TrxR Activity

TrxR activity was measured with the Thioredoxin Reductase Assay Kit Colorimetric (Abcam, Cambridge, UK) following the instructor's manual. Protein content of the lysates was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 80 μg of protein were used for each analysis.

Western Blot Analysis

Western blot analysis was performed as described previously [28] using the following antibodies: mouse anti-β-Actin (Sigma-Aldrich), rabbit anti-Nrf2 (Abcam, Cambridge, United Kingdom), rabbit anti-KEAP1 (Proteintech Group, Rosemont, IL, USA), rabbit anti-HO-1 (Enzo Life Science, Lörrach, Germany) and rabbit anti-GPX4 (R&D Systems, Minneapolis, MN, USA). Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amershan Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown.

Apoptosis Protein Array

For detection of different apoptotic proteins, we used an apoptosis array purchased by R&D Systems (Minneapolis, MN, USA). Shortly, HCC cells were treated for 24 hours with Auranofin and BSO or Erastin and BSO. Approximately 200 μg of protein lysates were used, and the assay was performed in accordance with the manufacturer's protocol. The apoptotic proteins are visualized using chemiluminescent detection reagents.

Statistical Analysis

Statistical significance was assessed by Student's t-Test (two-tailed distribution, two- sample, unequal variance).

Results

Auranofin/BSO and Erastin/BSO Cotreatment Synergistically Induced Cell Death in HCC Cells

To investigate whether HCC cells are susceptible to oxidative stress-mediated cell death we tested the effects of different ROS modulators and ferroptosis inducers alone and in combination, including Auranofin, Erastin, BSO and RSL3, in the human liver cancer cell lines Huh7 and HepG2. Interestingly, Auranofin combined with BSO as well as Erastin combined with BSO acted in concert to reduce cell viability compared to treatment with either agent alone in both cell lines (Figure 1*A*). Other combinations exerted no or minimal effects on cell viability loss or exhibited moderate effects in only one of the two cell lines (Suppl. Figure 1). For confirming the cooperative cell death induction and performing kinetic analysis we used another cell death assay. Similarly, Auranofin and BSO as well as Erastin and BSO acted together to induce cell death in both cell lines (Figure 1*B*). Kinetic analysis showed that Auranofin/BSO- and Erastin/BSO-cotreatment induced a time-dependent increase in cell death starting after 24 to 48 hours (Figure 1*B*). Together, these experiments demonstrate that cotreatment of Auranofin/BSO and Erastin/BSO synergistically induced cell death in both HCC cell lines.

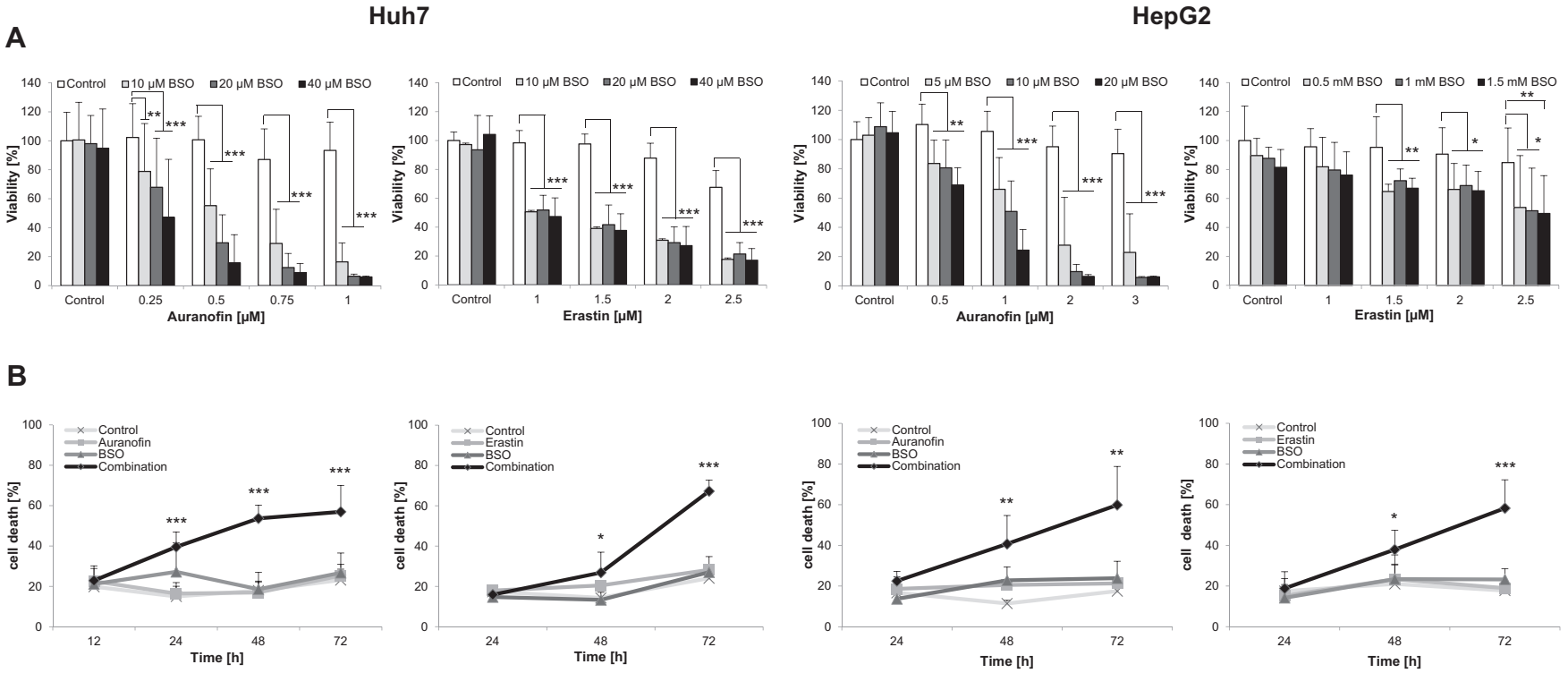

Figure 1. The effect of different ROS modulators and ferroptosis inducers on the human liver cancer cell lines Huh7 and HepG2. (A) Huh7 and HepG2 were treated for 72 hours with indicated concentrations of Auranofin, BSO and Erastin. (B) Huh7 cells were treated with 0.5 µM Auranofin and/or 10 µM BSO or 1 µM Erastin and/or 10 µM BSO for indicated times. HepG2 cells were treated with 1 µM Auranofin and/or 10 µM BSO or 2 µM Erastin and/or 1.5 mM BSO for indicated times. Cell viability was determined by MTT assay (A) and cell death by analysis of PI-stained nuclei using flow cytometry (B). Mean and SD of three different experiments performed in triplicate are shown. * $P <$ .05; ** $P <$ .01; *** $P <$ .001.

Ferrostatin-1 (Fer-1) Inhibited Auranofin/BSO- and Erastin/BSO-Induced Cell Death

Next, we asked which type of cell death was activated upon cotreatment with Auranofin/BSO or Erastin/BSO. To address this question we determined cell death in the absence or presence of Fer-1, a pharmacological inhibitor of ferroptosis [4]. Importantly, addition of Fer-1 significantly decreased Auranofin/BSO- or Erastin/BSO-induced cell death in both cell lines (Figure 2, *A* and *B*). Since ferroptosis is known to be caspase-independent [4,10], we hypothesized that Auranofin/BSO- and Erastin/BSO-cotreatment triggered a caspase-independent cell death in HCC cells. To clarify whether caspases are required for the induction of cell death we used the pan-caspase inhibitor zVAD.fmk. zVAD.fmk was unable to provide protection against Auranofin/BSO- or Erastin/BSO-mediated cell death (Figure 3, *A* and *B*), whereas it significantly decreased cell death upon treatment with Sorafenib (Sora) and oleanolic acid (OA) that was used as positive control for caspase-dependent cell death induction [21,23]. Furthermore, analysis of caspase-3 activation by protein array showed no caspase-3 activation upon Auranofin/BSO or Erastin/BSO cotreatment, underlined by the missing expression of active cleaved form of caspase-3 (Figure 3*C*). As positive control, both cell lines were treated with Sorafenib and oleanolic acid [21,23], which triggered the activation of pro-caspase-3 and its processing into the active cleaved caspase-3 (Figure 3*C*).

Together, these findings are consistent with caspase-independent, ferroptotic cell death upon Auranofin/BSO or Erastin/BSO cotreatment.

Lipoxygenase (LOX) Inhibitors and Ferroptosis Inhibitors Blocked Auranofin/BSO and Erastin/BSO Cotreatment-Induced Cell Death and Lipid Peroxidation

To confirm the hypothesis that Auranofin/BSO or Erastin/BSO cotreatment triggers ferroptosis we tested Liproxstatin-1 (Lip-1) as another pharmacological inhibitor of ferroptosis that acts via inhibition of accumulation of lipid hydroperoxides [29]. Importantly, Lip-1 potently rescued both cell lines from Auranofin/BSO- or Erastin/BSO-induced cell death (Figure 4, *A* and *B*). Since LOX has recently been implicated in the regulation of ferroptosis [10], we also determined the effect of LOX inhibitors. Of note, the addition of nordihydroguaiaretic acid (NDGA), a pan-LOX inhibitor [30] and Baicalein, a selective 12/15-LOX inhibitor [31], significantly reduced Auranofin/BSO- or Erastin/BSO-induced cell death in both cancer cell lines (Figure 4, *A* and *B*).

In the next step, we assessed lipid peroxidation by using BODIPY-C11, a fluorescent dye that detects lipid peroxidation [32,33]. Auranofin/BSO and in particular Erastin/BSO cotreatment caused a significant increase in lipid peroxidation in both cell lines (Figure 5, *A* and *B*). This Auranofin/BSO- or Erastin/BSO-stimulated increase in lipid peroxidation was significantly reduced in the presence of the ferroptosis inhibitors Lip-1 and Fer-1 (Figure 5, *A* and *B*). These findings confirm that the Auranofin/BSO and Erastin/BSO cotreatment trigger ferroptosis in HCC cells.

Auranofin/BSO and Erastin/BSO Cotreatment Led to ROS Production and ROS-Dependent Cell Death

Since ferroptosis is characterized by the accumulation of lipid-based ROS [4], we next used the fluorescent dye CM-H_2DCFDA, which has been

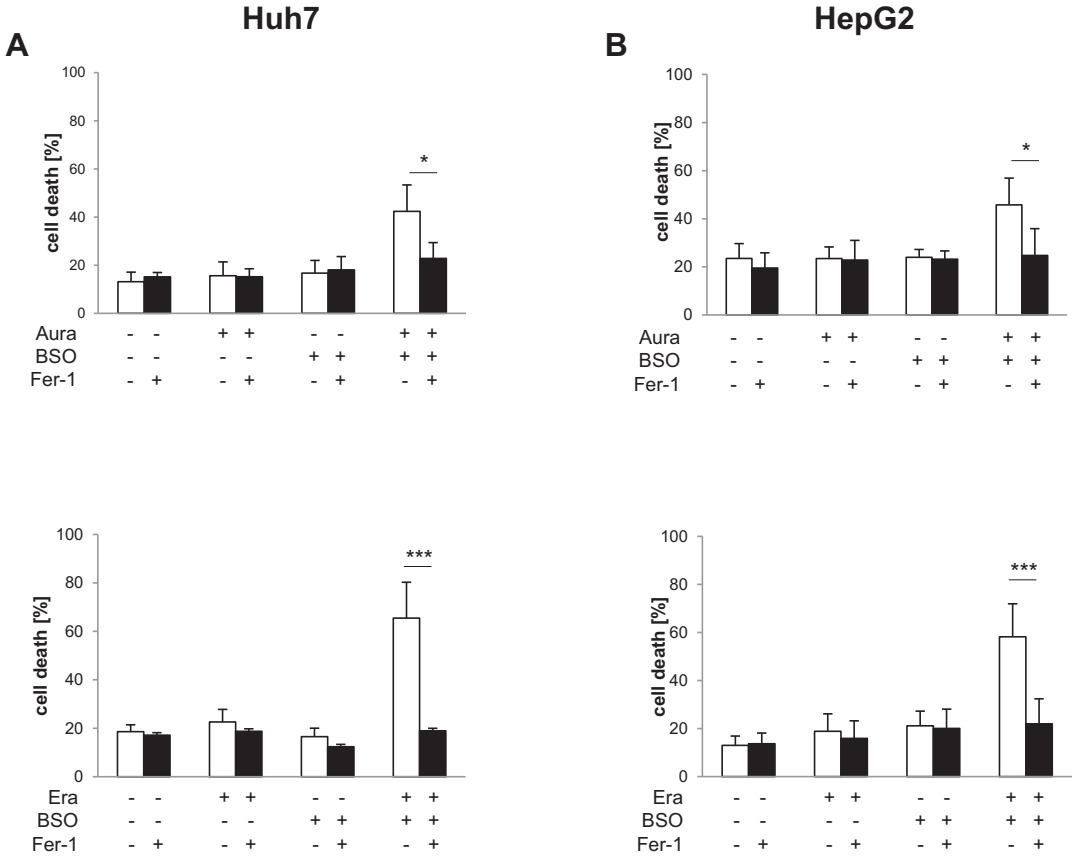

Figure 2. Fer-1 inhibited Auranofin/BSO- and Erastin/BSO-induced cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and/or 10 μM BSO for 48 hours or 1 μM Erastin and/or 10 μM BSO for 72 hours in the presence or absence of 10 μM Fer-1. (B) HepG2 cells were treated with 1 μM Auranofin and/or 10 μM BSO or 2 μM Erastin and/or 1.5 mM BSO for 72 hours in the presence or absence of 10 μM Fer-1. Cell death was determined by the analysis of PI-stained nuclei using flow cytometry. Mean and SD of three different experiments performed in triplicate are shown. * $P < .05$; *** $P < .001$.

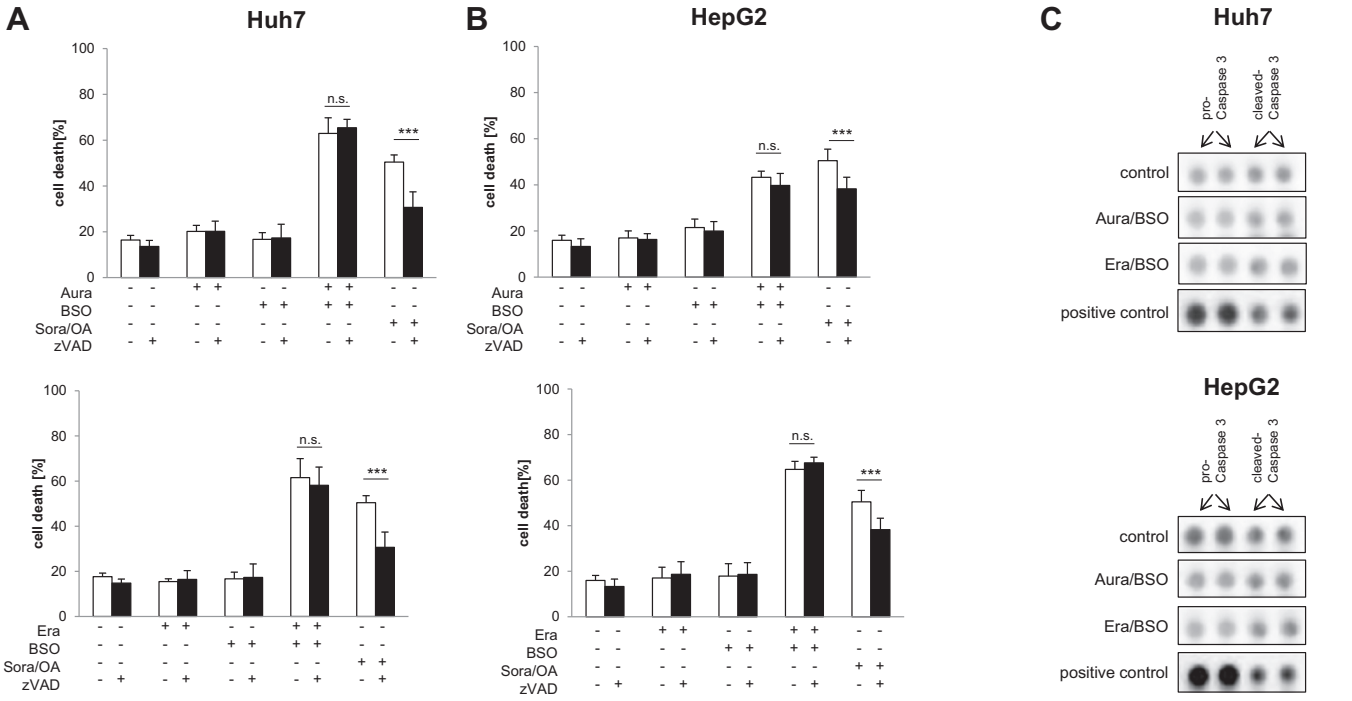

Figure 3. Auranofin/BSO- and Erastin/BSO-cotreatment triggered caspase-independent cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and/or 10 μM BSO for 48 hours or 1 μM Erastin and/or 10 μM BSO for 72 hours in the presence or absence of 50 μM zVAD.fmk. (B) HepG2 cells were treated with 1 μM Auranofin and/or 10 μM BSO or 2 μM Erastin and/or 1.5 mM BSO in the presence or absence of 50 μM zVAD.fmk for 72 hours. Cell death was determined by the analysis of PI-stained nuclei using flow cytometry. As positive control for caspase-dependent cell death, we treated both cell lines with 5 μM Sorafenib (Sora) and 60 μM oleanolic acid (OA). Mean and SD of three different experiments performed in triplicate are shown. n.s. = not significant; *** $P < .001$. (C) Caspase-3 activation was measured with a Proteome Profiler® Human Apoptosis Array Kit as described in the Material & Methods section. Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO or 1 μM Erastin and 10 μM BSO for 24 hours. HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO or 2 μM Erastin and 1.5 mM BSO for 24 hours. As positive control, both cell lines were treated with 5 μM Sorafenib and 60 μM oleanolic acid for 24 hours. Representative blots are shown.

reported to detect ROS such as hydrogen peroxides, hydroxyl radicals or peroxyl radicals. Remarkably, Auranofin/BSO as well as Erastin/BSO cotreatment led to a significant increase in ROS production (Figure 6*A*). This increase in ROS production was confirmed by another ROS dye, i.e. MitoSOX™ Red (Suppl. Figure 2), which is known to be a selective dye for detection of mitochondrial ROS [34,35]. Addition of the lipophilic ROS scavengers α-Tocopherol (α-Toc), a Vitamin-E derivate [36], significantly reduced Auranofin/BSO- or Erastin/BSO-stimulated ROS production in both cell lines (Figure 6*A*). By comparison, N-acetyl-cysteine (NAC), an antioxidant and GSH precursor [37,38], suppressed ROS production by Auranofin/BSO or Erastin/BSO in Huh7 cells, while NAC failed to prevent the increase in ROS levels in HepG2 cells (Figure 6*A*).

Next, we explored whether these changes in redox signaling contribute to cell death upon Auranofin/BSO or Erastin/BSO cotreatment. Importantly, the addition of α-Toc or NAC almost completely rescued HCC cells from Auranofin/BSO- or Erastin/BSO-induced cell death (Figure 6*B*). This indicates that Auranofin/BSO and Erastin/BSO cotreatment trigger a redox-dependent form of cell death.

Auranofin/BSO and Erastin/BSO Cotreatment Increased Levels of Nrf2 and HO-1 and Decreased GPX4 Levels

To further explore changes in redox regulation upon Auranofin/BSO or Erastin/BSO cotreatment we monitored TrxR activity by ELISA. Auranofin/BSO cotreatment caused a significant reduction of TrxR activity in both cell lines (Figure 7*A*), consistent with the reported inhibition of TrxR by Auranofin, leading to a reduction of the antioxidant Trx [17,39]. By comparison, Erastin/BSO cotreatment did not suppress TrxR activity (Figure 7*A*).

Furthermore, monitoring of regulators of redox homeostasis and ferroptosis by Western blotting revealed decreased protein levels of KEAP1 along with increased levels of Nrf2 (Figure 7*B*), consistent with oxidative stress. Interestingly, both Auranofin/BSO and Erastin/BSO decreased protein expression of GPX4 (Figure 7*B*), the only GPX that is able to reduce hydroperoxides within membranes [40]. In addition, Auranofin/BSO or Erastin/BSO cotreatment caused a strong increase in expression levels of HO-1(Figure 7*C*), which has been reported to promote lipid peroxidation by increasing the Fe-(II) pool [11].

In summary, Auranofin/BSO and Erastin/BSO cotreatment alters redox homeostasis by increasing levels of Nrf2 and HO-1 and decreasing GPX4 levels.

Discussion

Addressing redox homeostasis in cancer cells could be a promising novel therapeutic approach, since cancer cells often harbor increased ROS levels [1]. On the one hand, ROS has been shown to be implied in tumorigenesis, because it activates survival pathways, induces DNA damage, leads to mutations and helps tumor cells to escape senescence [1]. On the other hand, excessive ROS accumulation in cancer cells can limit tumor formation and progression by promoting cell death [1,16,41]. Lipid ROS plays a key role in a recently identified form of cell death, i.e. ferroptosis [4]. Since different animal studies have shown that inhibition of ferroptosis could be a new treatment possibility in pathological cell death conditions (e.g. ischemia/reperfusion injury, Huntington's disease), many publications are currently presenting the induction of ferroptosis as a new option for cancer therapy as well [7,9,11,42–44].

In the present study, we therefore investigated the question whether the inhibition of antioxidant pathways that protect HCC cancer cells from oxidative stress and the induction of ferroptosis provides a new therapeutic approach in HCC cells. Here, we identified a novel synergistic interaction of Auranofin/BSO and Erastin/BSO in HCC cells. Mechanistic studies showed

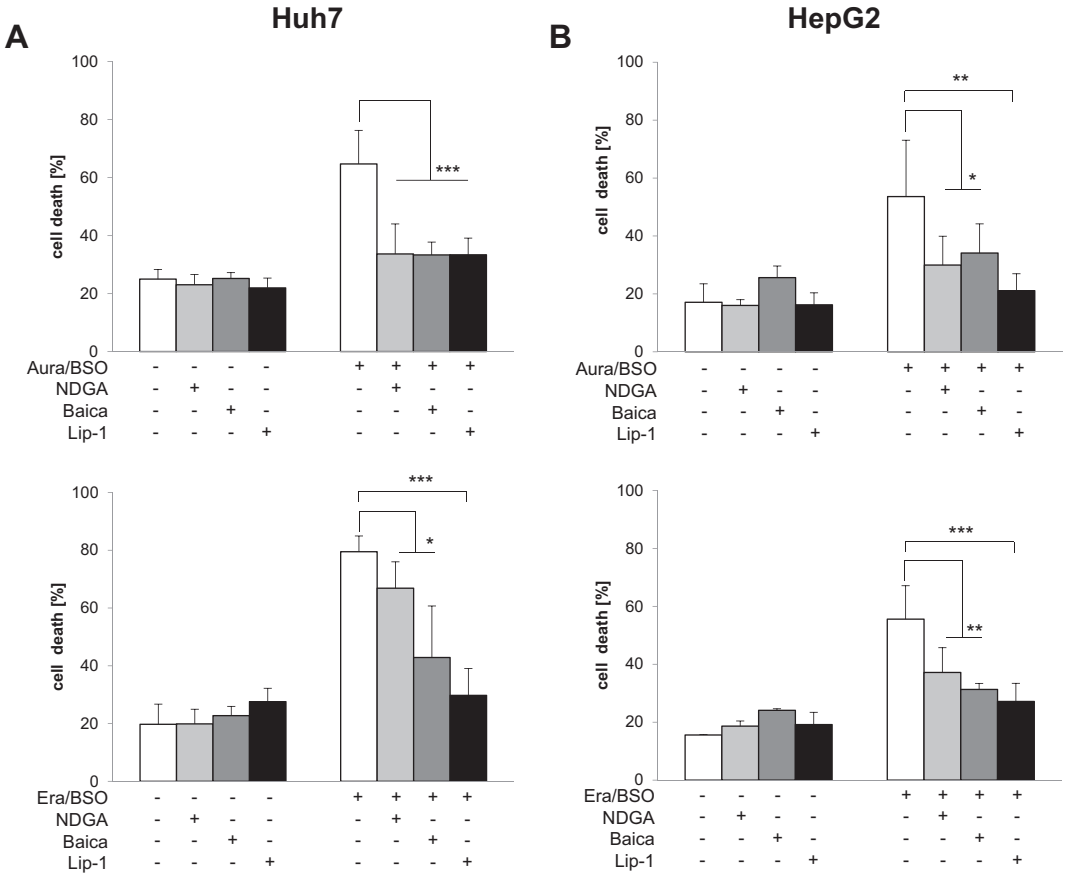

Figure 4. Auranofin/BSO- and Erastin/BSO-cotreatment induced ferroptotic cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO for 48 hours or 1 μM Erastin and 10 μM BSO for 72 hours in the presence or absence of 1 μM NDGA, 0.5 μM Baicalein or 25 nM Lip-1. (B) HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO in the presence or absence of 3 μM NDGA, 0.5 μM Baicalein or 25 nM Lip-1 for 72 hours or were treated with 2 μM Erastin and 1.5 mM BSO in the presence or absence of 2 μM NDGA, 2 μM Baicalein or 25 nM Lip-1 for 72 hours. Cell death was determined by the analysis of PI-stained nuclei using flow cytometry. Mean and SD of three different experiments performed in triplicate are shown. * $P < .05$; ** $P < .01$; *** $P < .001$.

that Auranofin/BSO and Erastin/BSO cotreatment triggered a redox-dependent ferroptotic cell death in HCC cells. This conclusion is supported by data showing that a) pharmacological inhibitors of ferroptosis rescued cell death in both cancer cell lines and b) Auranofin/BSO- or Erastin/BSO-stimulated cell death is associated with lipid peroxidation, a typical feature of ferroptosis, which was reversed by inhibitors of ferroptosis. Consistently, we demonstrated that Auranofin/BSO and Erastin/BSO cotreatment led to a ROS-dependent form of cell death.

Our findings are in line with different studies postulating that the imbalance of ROS production and its removal are involved in liver fibrosis and hepatocarcinogenesis [45–47]. Interestingly, the level of mitochondrial ROS in HCC is associated with tumor progression and with the prognosis of patients with HCC [48]. Another study showed that ROS levels in sera from patients with HCC treated with Sorafenib can predict the response to Sorafenib [49]. Cellular redox homeostasis and protection of cells from oxidative stress are mainly regulated by the Trx system and the GSH-dependent system [15,50,51]. It seems that in certain cancer cells addressing only one antioxidant pathway by stimulating ROS accumulation could be compensated by increased ROS-scavenging enzymes caused by redox adaptation through another existing antioxidant pathway [52].

In our study, we showed that simultaneous pharmacological inhibition of the two main antioxidant pathways, i.e. the Trx system and the GSH synthesis pathway, through Auranofin/BSO cotreatment could be a promising new anticancer strategy in HCC, especially given the fact that neither Auranofin nor BSO monotherapy prompted cell death in HCC cells. The addressing of the Trx system is shown by the significant reduction of TrxR activity by the Auranofin/BSO cotreatment. Another work by Lee et al. demonstrated that pharmacological inhibition of TrxR-1 by Auranofin suppressed tumor growth and sensitizes HCC cells to Sorafenib [16]. Further promising results of Auranofin have been presented in different studies and in first human clinical trials for the treatment of leukemia, lymphoma, non-small lung cancer and ovarian cancer [53,54]. Also, the combination of BSO and Auranofin could be an anticancer strategy, for example in head and neck cancer and rhabdomyosarcoma cells, and could sensitize breast cancer stem cells to radiation therapy [55–58]. The anticancer activity of BSO in combination with melphalan in high-risk neuroblastoma in pediatric patients has been described in first clinical trials [59,60].

In addition to the combination of Auranofin/BSO, we identified Erastin/BSO cotreatment as another new approach to induce ferroptotic cell death in HCC. Erastin is an inhibitor of the x_c^- cystine/glutamate system which mediates cystine uptake into the cell, thereby maintaining the thiol-containing pool of ROS scavengers, particularly of GSH [4]. Consequently, Erastin/BSO cotreatment leads to a significant depletion of intracellular GSH [42] resulting in a reduction of GPX4, a GSH-dependent enzyme, as shown in our work. Furthermore, the impairment of cystine uptake by Erastin leads to the production of lethal lipid ROS [4]. The fact that only Erastin/BSO cotreatment induces cell death in HCC goes in line with the above described hypothesis that cancer cells have the capacity to adapt their antioxidant systems. The Erastin analogue PRLX 93936 has been tested in two clinical phase I/II trials in patients with multiple myeloma (NCT01695590) and in various advanced cancer forms (NCT00528047).

We showed, that Auranofin/BSO as well as Erastin/BSO cotreatment have prompted the activation of the canonical pathway of ferroptosis via

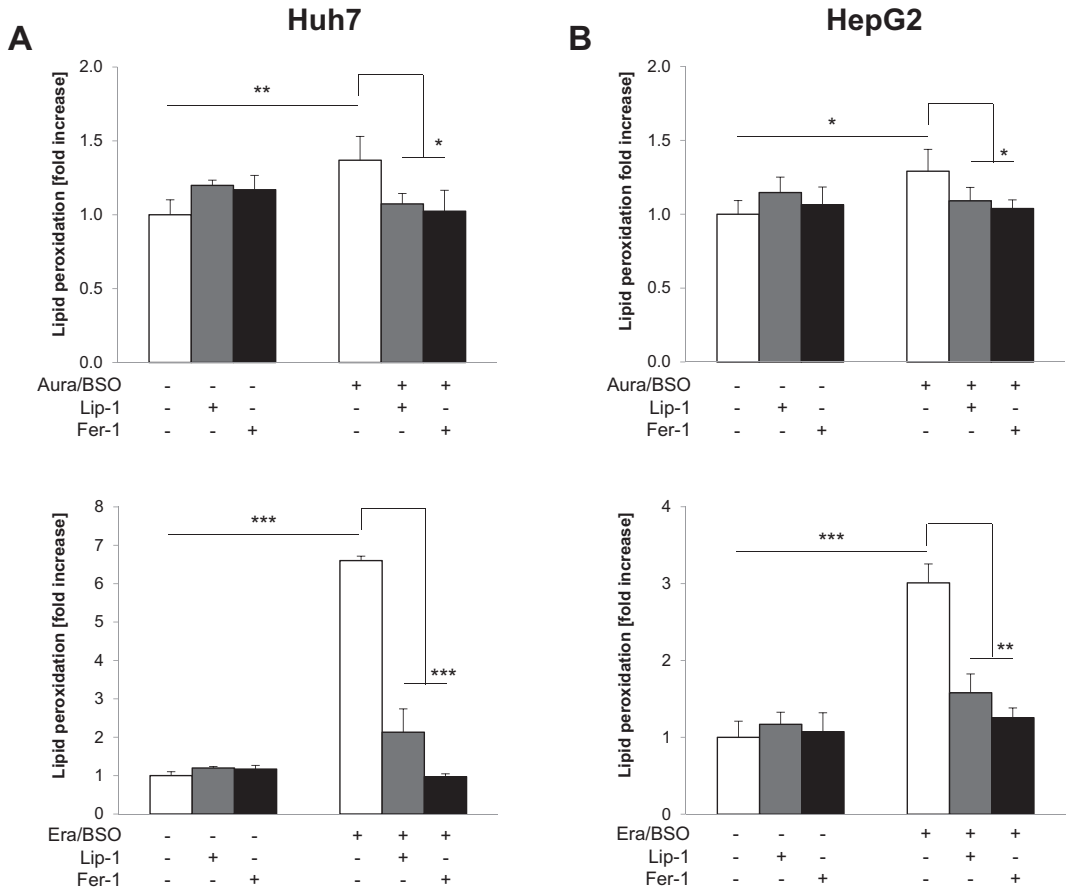

Figure 5. Lipid peroxidation contributed to Auranofin/BSO- and Erastin/BSO-induced cell death and could be rescued by different inhibitors of ferroptosis and lipid peroxidation. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO or 1 μM Erastin and 10 μM BSO in the presence or absence 25 nM Lip-1 or 10 μM Fer-1 for 24 hours. (B) HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO in the presence or absence of 25 nM Lip-1 or 10 μM Fer-1 for 24 hours or were treated with 2 μM Erastin and 1.5 mM BSO in the presence or absence of 25 nM Lip-1 or 10 μM Fer-1 for 24 hours. Lipid peroxidation was assessed by flow cytometry in PI-negative cells using the fluorescent dye BODIPY-C11 and is shown as fold increase compared to untreated cells. Mean and SD of three different experiments performed in triplicate are shown. * $P < .05$; ** $P < .01$; *** $P < .001$.

GPX4 inhibition. Additionally, we also observed an involvement of the non-canonical ferroptotic pathway via the Nrf2/KEAP1 system, seen in both cotreatments. Remarkably, the Nrf2/KEAP1 pathway has been shown to be one of the most frequently mutated pathways in HCC [61,62]. The non-canonical pathway has been described to involve activation of HO-1, a detoxification enzyme which plays a dual role in cancer cells: On the one hand, elevated HO-1 levels contribute to cancer progression and chemotherapy resistance by protecting cells from oxidative stress and, on the other hand, very high cellular ROS levels can enforce HO-1 to become a mediator for ferroptosis by promoting uncontrolled iron, Fe-(II) accumulation through heme degradation, finally leading to lipid peroxidation [63].

There are only few studies exploring ferroptosis as promising anticancer therapy in HCC. Mainly, these studies focus on investigating the role of the first-line therapy drug Sorafenib in ferroptosis and/or improving its therapeutic efficacy by combination therapies. Louandre et al. showed that the cytotoxic effects of Sorafenib were mediated by oxidative stress and led to ferroptosis in HCC cells [24]. But Sorafenib is also able to induce apoptosis [21,23,64,65]. Louandre et al. suspected that the induced pathway (apoptosis vs. ferroptosis) depends on the state that the cells are in while adding Sorafenib [24]. In pro-apoptotic states (e.g. by adding other pro-apoptotic agents or sensitizer) Sorafenib tends to induce apoptosis. Furthermore, they assumed that Sorafenib used as a single compound is a probably a better inducer of ferroptosis than apoptosis in HCC cell lines [24]. They further detected the retinoblastoma protein, which is induced by Sorafenib, as a regulator of ferroptosis in HCC [66]. The loss of function of this retinoblastoma protein is a common event during hepatocarcinogenesis [66].

Another study described that Haloperidol, a psychotropic drug, augmented the effect of Erastin- or Sorafenib-induced ferroptosis in HCC by inducing the expression of the sigma receptor 1, which seems to be involved in oxidative stress metabolism [26,67]. Beyond that, Sauzay et al. investigated the effect of Sorafenib on the regulation of protein biosynthesis and discovered that Sorafenib can both prompt ferroptosis as a single agent and protect HCC cells from Erastin-induced ferroptosis by inhibition of protein biosynthesis with increasing the availability of amino acids for GSH synthesis [24,26,66,68].

Despite promising data on the induction of ferroptosis as an anticancer strategy, the role of ferroptosis in the development in liver fibrosis and cirrhosis remains poorly understood and the data are partially contradictory.

The work by Tsurusaki et al. see ferroptosis as an important trigger for chronic inflammation of the liver and the development of steatohepatitis, which could lead to liver fibrosis, cirrhosis and finally to the development of HCC [69]. In contrast, another work showed that the induction of ferroptosis by Sorafenib and Erastin in hepatic stellate cells remarkably improved liver fibrosis [70]. Due to the contradictory data, further work and studies are required to finally evaluate the importance of ferroptosis in patients with liver cirrhosis or fibrosis.

In our study, we achieved important results for the future development of ROS-modulating therapies in HCC. First and foremost, Auranofin/BSO and Erastin/BSO cotreatment could be promising approaches for new

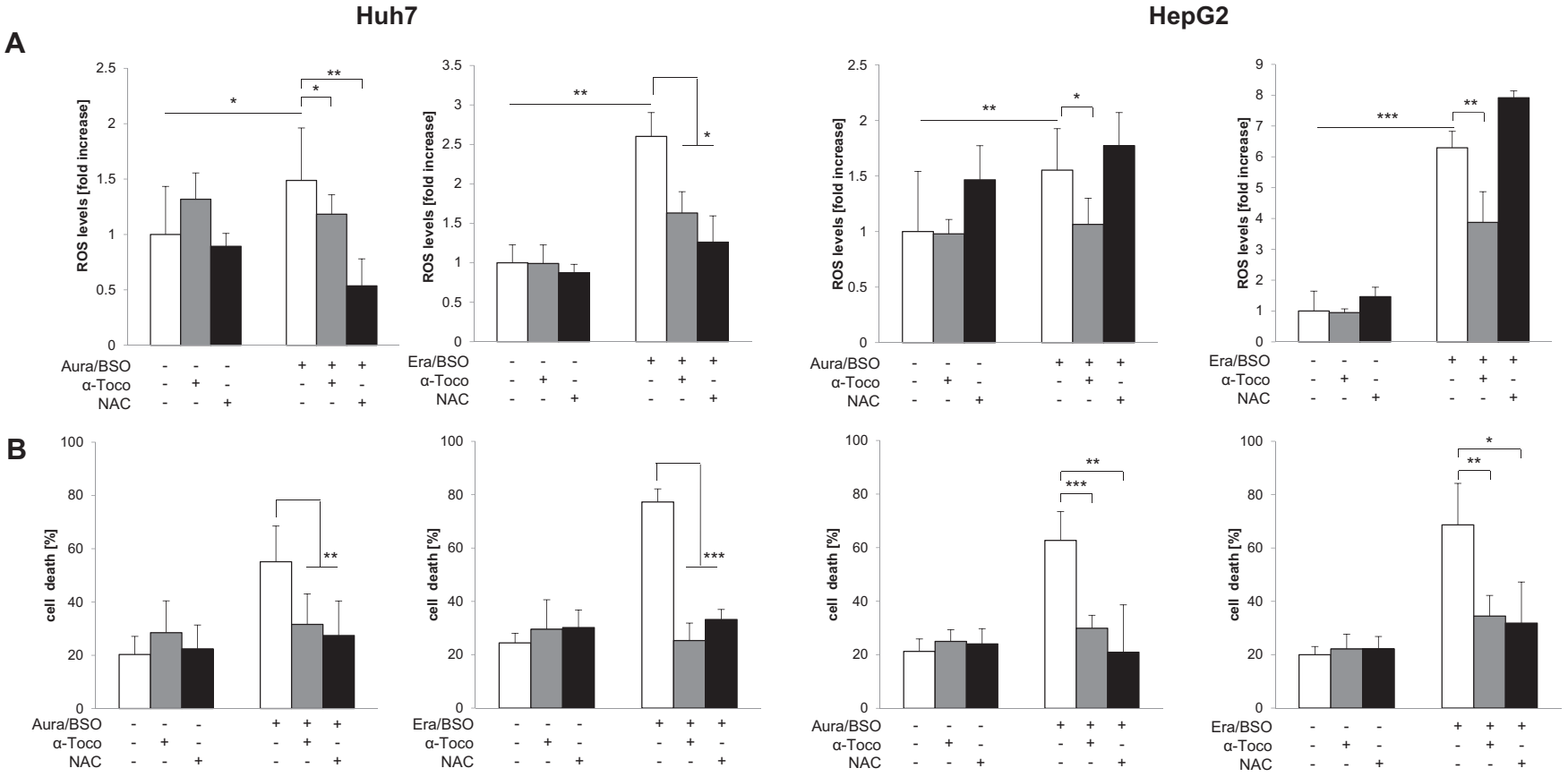

Figure 6. Auranofin/BSO and Erastin/BSO cotreatment led to ROS production and ROS-dependent cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO for 6 hours or 1 μM Erastin and 10 μM BSO for 24 hours in the presence or absence of 50 μM α-Toc or 1.25 mM NAC. HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO or 2 μM Erastin and 1.5 mM BSO for 24 hours in the presence or absence of 50 μM α-Toc or 2.5 mM NAC. ROS levels were determined by flow cytometry in PI-negative cells using the fluorescent dye CM-H_2DCFDA and is shown as fold increase compared to untreated cells. (B) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO for 48 hours or 1 μM Erastin and 10 μM BSO for 72 hours in the presence or absence of 50 μM α-Toc or 1.25 mM NAC. HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO or 2 μM Erastin and 1.5 mM BSO for 72 hours in the presence or absence of 50 μM α-Toc or 2.5 mM NAC. Cell death was determined by the analysis of PI-stained nuclei using flow cytometry. Mean and SD of three different experiments performed in triplicate are shown. * $P < .05$; ** $P < .01$; *** $P < .001$.

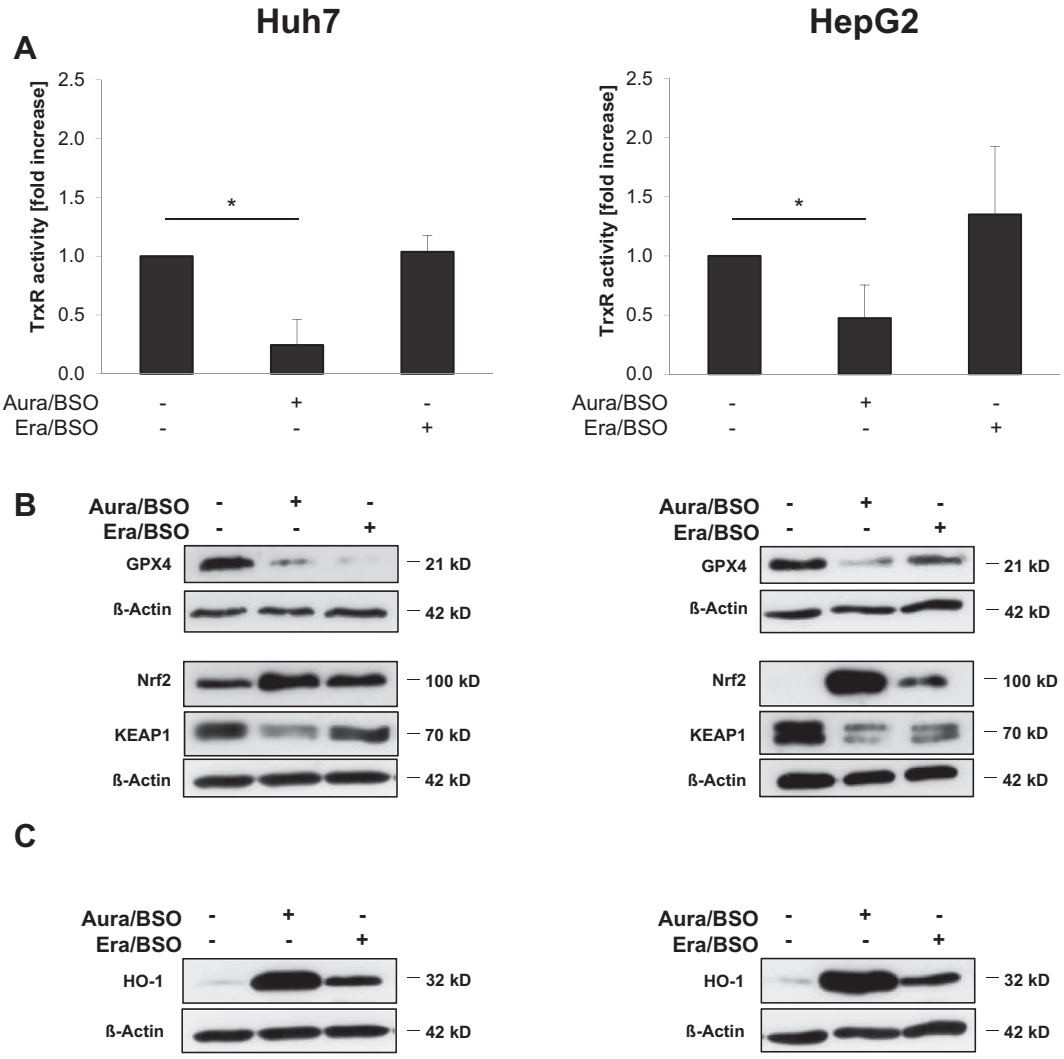

Figure 7. Auranofin/BSO and Erastin/BSO cotreatment decreased TrxR activity, led to Nrf2 accumulation, promoted activation of HO-1 and prompted GPX4 decrease. Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO or 1 μM Erastin and 10 μM BSO for 24 hours. HepG2 cells were treated with 1 μM Auranofin and 10 μM BSO or 2 μM Erastin and 1.5 mM BSO for 24 hours. TrxR activity was measured as described in the Material & Methods section and is shown as fold increase compared to untreated cells (A). Mean and SD of three different experiments performed in triplicate are shown. * $P < .05$. (B) Protein expression of Nrf2, KEAP1 and GPX4 were determined by Western blotting. Representative blots are shown. (C) HO-1 protein expression was determined by Western blotting. Representative blots are shown.

therapies for the treatment of HCC. Second, the combination of the compounds described above at subtoxic concentrations resulted in synergistic ferroptotic cell death induction. Third, Auranofin/BSO- and Erastin/BSO-induced cell death is redox-regulated. Fourth, simultaneous targeting of different antioxidant systems, as shown by the coactivation of the canonical and the non-canonical pathway, can overcome chemotherapy resistance in cancer cells.

In conclusion, addressing ROS homeostasis and ferroptosis by Auranofin/BSO or Erastin/BSO cotreatment could be an interesting anticancer strategy in HCC, which warrants further investigations.

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Declaration of competing interest

None to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100785.

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