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Redoxmodulation und Ferroptose als neue Therapiestrategie im hepatozellulären Karzinom

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1. Inhaltsverzeichnis

2. Zusammenfassung

Für die Funktion und das Überleben von Zellen ist die Aufrechterhaltung und Regulation der Redoxhomöostase durch Produktion und Elimination von radikalen Sauerstoffspezies (ROS) von entscheidender Bedeutung. In Tumorzellen finden sich höhere basale ROS-Level als in gesunden Zellen, was jedoch trotz des vermehrten oxidativen Stresses nicht zur Zelltodinduktion führt, da kompensatorisch antioxidative Mechanismen ebenfalls gesteigert sind. Vor allem zwei antioxidative Systeme sind hauptsächlich bei der Elimination von ROS involviert: das Glutathion (GSH)-System und das Thioredoxin (TRX)-System. Hierbei spielt eine beständige Regeneration von GSH, als auch von TRX, eine wichtige Rolle, da diese bei der Reduktion von Sauerstoffradikalen verbraucht werden.
Im hepatozellulären Karzinom (HCC) ist die gestörte Redoxhomöostase mit gesteigerten ROS Leveln ein wichtiger Faktor in der Karzinogenese. Das HCC wird oft erst im fortgeschrittenen, nicht mehr kurativen Stadium diagnostiziert und ist resistent gegenüber nahezu allen Formen von Chemotherapien. Dies verdeutlich die immense Bedeutung der Erforschung der teilweise unverstandenen Tumorgenese, aber auch die Notwendigkeit für die Entwicklung von neuen Therapien.

In der hier dargestellten experimentellen Arbeit gingen wir deshalb der Frage nach, ob die alleinige Inhibierung der antioxidativen Schutzmechanismen durch sog. ROS Modulatoren ausreicht, um eine Zelltodinduktion in humanen HCC-Zelllinien herbeizuführen und damit eine potenzielle neue Therapiestrategie aufzuzeigen.
Wir konnten zeigen, dass die simultane Inhibierung dieser zwei antioxidativen Hauptmechanismen im HCC durch die Kombination von Auranofin (TXR-Inhibitor) mit Buthionine-Sulfoximin (BSO, Glutathion-Inhibitor) und Erastin (indirekter Glutathion-Inhibitor) mit BSO zur ROS-abhängigen Zelltodinduktion im HCC *in vitro* führt. Interessant ist, dass die gesteigerten ROS-Level jedoch nicht den Zelltod im HCC induzierten, wenn nur eines der beiden antioxidativen Systeme inhibiert wurde. Offenbar ist die Tumorzelle in der Lage durch Hochregulierung anderer antioxidativer Systeme das induzierte ROS zu neutralisieren. Unsere Untersuchungen zum Wirkmechanismus der Zelltodinduktion durch die Kombinationsbehandlungen Auranofin + BSO bzw. Erastin + BSO identifizierten

unerwarteterweise eine Caspasen-unabhängige, nicht-apoptotische Zelltodform, die sog Ferroptose, welche durch ROS-Produktion und Lipidperoxidierung charakterisiert ist. Weitere Experimente konnten untermauern, dass mit der Induktion der Ferroptose durch die selektive ROS-Modulation die Apoptoseresistenz der HCC Zellen umgangen werden kann.
Mechanistisch kann diese erstmals 2012 beschriebene eisenabhängige Zelltodform, Ferroptose, durch zwei verschiedene Signalwege induziert werden: Erstens durch den kanonischen Pfad, bei welchem die Inhibierung der Glutathionperoxidase 4 (GPX4), einem Protein, welches die Zellmembran vor Lipidperoxidation schützt, eine zentrale Rolle spielt, und zweitens durch den nicht-kanonischen Pfad, welcher durch eine Anhäufung von zweiwertigem Eisen u. a. durch Aktivierung von Hämoxygenasen (HO), vermittelt durch den Transkriptionsfaktor Nuclear factor erythroid 2-related factor 2 (Nrf2), gekennzeichnet ist. Wir konnten feststellen, dass in unseren Kombinationsbehandlungen beide Pfade involviert sind und eine Herunterregulierung von GPX4 als auch eine Akkumulation von Nrf2 und Hämoxygenase-1 (HO-1) besteht.

Zusammenfassend konnte unsere Arbeit zeigen, dass mittels pharmakologischer Adressierung zweier antioxidativer Systeme die Therapieresistenz der HCC-Zellen umgangen werden kann, und dass die Induktion der Ferroptose zukünftig eine vielversprechende Therapieoption im HCC darstellen könnte.

3. Abstract

The maintenance and regulation of redox homeostasis through the production and elimination of reactive oxygen species (ROS) is of crucial importance for the function and survival of cells.

In tumor cells there are higher basal ROS levels than in healthy cells. However, this does not lead to cell death induction despite the increased oxidative stress, as compensatory antioxidant mechanisms are increased as well. Particularly, two antioxidant systems are mainly involved in the elimination of ROS, the glutathione (GSH) system and the thioredoxin (TRX) system. A constant regeneration of both GSH and TRX plays an important role here, as these are being depleted for the reduction of ROS.

In hepatocellular carcinoma (HCC) the disturbed redox homeostasis with increased ROS levels is an important factor for carcinogenesis. HCC is often diagnosed in an advanced, no longer curative stage, and is resistant to almost all forms of chemotherapy. This illustrates not only the immense importance of research into the still not sufficiently understood tumorigenesis, but also the need for developing new therapies.

In the experimental work presented here, we therefore investigated whether the inhibition of the antioxidative pathways, by so-called ROS modulators, is sufficient to induce cell death in human HCC cell lines, and if this could be a new therapeutic strategy.

We were able to show that simultaneously inhibiting the above-mentioned two main antioxidant mechanisms in HCC through combinations of auranofin (TXR-inhibitor) with BSO (glutathione inhibitor) and Erastin (indirect glutathione inhibitor) with BSO led to ROS-dependent cell death induction in HCC *in vitro*. Interestingly, the increased ROS levels did not induce cell death in HCC if only one of the two antioxidant systems was inhibited. Apparently, the tumor cells are able to neutralize the induced ROS by upregulating other antioxidant systems. Investigating the underlying mechanism of cell death induction by our combination treatments auranofin + BSO or Erastin + BSO unexpectedly showed a caspase-independent, non-apoptotic form of cell death, the so-called ferroptosis, which is characterized by ROS production and lipid peroxidation. Further experiments were able to confirm that ferroptosis induction through selective ROS modulation can circumvent apoptosis resistance of HCC cells.

Mechanistically this iron-dependent form of cell death, ferroptosis, first described in 2012, can be induced by two different signaling pathways, firstly by the canonical pathway, in which the inhibition of glutathione peroxidase 4 (GPX4), a protein that protects the cell membrane from lipid peroxidation, plays a central role, and secondly through the non-canonical pathway, characterized, by an accumulation of divalent iron. The latter is caused, among other factors, by activation of hemoxygenases (HO) mediated by the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2). We were able to demonstrate that both pathways were involved in our combination treatments and that a down-regulation of GPX4 occurred as well as an accumulation of Nrf2 and heme oxygenase-1 (HO-1).

In summary, we were able to show that pharmacologically addressing two antioxidant systems can circumvent the resistance of HCC cells to therapy and thus, the induction of ferroptosis could be a promising therapeutic option in HCC in the future.

4. Abkürzungsverzeichnis

BSO	–	L-Buthionin-S,R-sulfoximin
DNA	–	Desoxyribonukleinsäure
Fer-1	–	Ferrostatin-1
GPX4	–	Glutathionperoxidase 4
GSH	–	Glutathion
HCC	–	Hepatozelluläres Karzinom
HO	–	Hämoxygenase
HO-1	–	Hämoxygenase-1
KEAP1	–	Kelch-like ECH-associated protein 1
Lip-1	–	Liproxstatin-1
LOX	–	Lipoxygenase
MTT	–	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid
NAC	–	N-Acetylcystein
NDGA	–	Nordihydroguaiarsäure
Nrf2	–	Nuclear factor erythroid 2-related factor 2
PD-L1	–	Programmed death-ligand 1
PI	–	Propidiumiodid
ROS	–	Radikale Sauerstoffspezies
RSL3	–	Ras selective lethal 3 compound
TRX	–	Thioredoxin
TrxR-1	–	Thioredoxinreduktase 1
ZVAD.fmk	–	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketon

5. Übergreifende Zusammenfassung

5.1 Einleitung

Das hepatozelluläre Karzinom (HCC) steht weltweit an zweiter Stelle der durch Krebs verursachten Todesfälle [1]. Es entsteht meist auf dem Boden einer Leberzirrhose, oft verursacht durch virale Hepatitiden, chronischen Alkoholabusus aber auch Steatohepatitis [2,3]. Die Diagnose erfolgt häufig erst im fortgeschrittenen Tumorstadium, in welchem kurative Therapien in Form von Leberresektion oder -transplantation bzw. radiologisch-interventionellen Verfahren wie Ablation oder Embolisation aufgrund der Tumorausdehnung oft nicht mehr möglich sind. Das HCC ist auch dadurch gekennzeichnet, dass es gegenüber nahezu allen klassischen Chemotherapien resistent ist. Zwar stehen mit den Multikinase-Inhibitoren Sorafenib und Regorafenib und mit dem Programmed death-ligand 1 (PD-L1) - Inhibitor Atezolizumab in Kombination mit dem Angiogenesehemmer Bevacizumab medikamentöse Therapien zur Verfügung, sie finden jedoch nur in palliativen Situationen Anwendung [4-6]. Die schlechte Prognose des HCCs und das Fehlen von effektiven systemischen Therapien verdeutlichen die Notwendigkeit der Entwicklung neuer medikamentöser Therapieansätze.

Möglich ist dies jedoch nur durch Einblicke in die Pathogenese des HCC, wobei die genauen zellulären Mechanismen hierbei bisher nur unzureichend erforscht sind.

In Tumorzellen ist die Redoxhomöostase oft gestört: Da es durch eine gesteigerte aerobe Glykolyse zu vermehrtem Sauerstoffverbrauch kommt, entsteht ein Übermaß an ROS, insbesondere in den Mitochondrien [7]. Dieser oxidative Stress kann einerseits zur Tumorentstehung und zum Überleben von Tumorzellen beitragen, vor allem durch DNA-Schädigung und Initiierung von Zellüberleben-begünstigenden Mutationen, andererseits führt die Akkumulation von radikalen Sauerstoffspezies zur Zellschädigung bis hin zum Zelltod, sobald die antioxidativen Mechanismen der Tumorzelle überschritten werden [8,9].

Die Redoxhomöostase wird in Zellen hauptsächlich über das Thioredoxin (TXR)- und über das Glutathion (GSH)-abhängige System reguliert [9-11]. Hemmung eines der beiden Systeme kann zu einer Kompensation mittels des anderen Systems führen und vice versa, was zur Therapieresistenz von Tumorzellen beitragen kann [12].

In der Entstehung des HCC scheint die gestörte Redoxhomöostase eine besondere Rolle zu spielen: Mehrere Studien konnten zeigen, dass eine Erhöhung der ROS-Produktion in der Leber zur Karzinogenese beiträgt [13,14]. Andererseits konnte gezeigt werden, dass eine weitere Steigerung der ROS-Level zum Zelltod führen kann, sobald die Kapazitäten zur Kompensation durch antioxidative Mechanismen überschritten werden [15-17].
All die Punkte legen nahe, dass es sich bei der Adressierung des Redoxsystems um eine vielversprechende Strategie zur Überwindung der Chemotherapieresistenz des HCC handeln könnte.

5.2 Darstellung der Publikation

In der hier im Folgenden dargestellten Publikation gingen wir deshalb der Frage nach, ob die alleinige Modulation des Redoxsystems in der Tumorzelle ausreicht, um mit der Induktion eines ROS-abhängigen Zelltodes im HCC einen neuen und innovativen experimenteller Therapieansatz im HCC aufzuzeigen.
Folgende ROS-Modulatoren sollten in ersten *in vitro* Versuchen in den humanen HCC-Zelllinien Huh7 und HepG2 zum Einsatz kommen: 1. Auranofin, ein Goldkomplex, welcher seit Jahrzehnten als Antirheumatikum klinisch eingesetzt wird, dem aber durch die Hemmung der Thioredoxinreduktase 1 (TrxR-1) auch eine mögliche antikanzerogene Wirkung zugeschrieben wird [18]; 2. Erastin, ein Hemmer des natrium-abhängigen Cystin-Glutamat-Antiporters, welcher Cystin im Austausch gegen Glutamat nach intrazellulär transportiert und somit, nach Reduktion von Cystin zu Cystein, ein wichtiges Substrat zur GSH-Regeneration liefert [19-21]; 3. Ras selective lethal 3 compound (RSL3), ein direkter Glutathionperoxidase 4 (GPX4)-Inhibitor [20]; und 4. BSO, welches die GSH-Synthese hemmt und damit zum ROS-Anstieg in der Zelle beiträgt [20].

ROS Modulatoren führen zur Zelltodinduktion im HCC

In dem ersten Versuchsteil untersuchten wir, ob die Mono- oder erst die Kombinationsbehandlung mit den ROS-Modulatoren Auranofin, BSO, Erastin und RSL3 zur Zelltodinduktion in den beiden humanen Tumorzelllinien (Huh7, HepG2) führt.

Zu Beginn führten wir den 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid (MTT) Assay zur Messung des Einflusses der ROS-Modulatoren auf die Tumorzellproliferation und -viabilität durch. Interessanterweise führten lediglich die Kombinationsbehandlungen von Auranofin mit BSO und Erastin mit BSO, jeweils in subtoxischen Konzentrationen der Einzelsubstanzen, zur synergistischen Zelltodinduktion in den Tumorzellen. Die Bestätigung und Quantifizierung der Zelltodinduktion sowie kinetische Untersuchungen wurden mit einer zweiten Methode, durchflusszytometrisch mittels Propidiumiodid (PI)-Färbung, durchgeführt. Hier zeigte sich ebenfalls eine signifikante Zelltodinduktion in den Kombinationsbehandlungen Auranofin+BSO (nach 24 Stunden in Huh7; nach 48 Stunden in HepG2) und Erastin+BSO (nach 48 Stunden in beiden Zelllinien).

Die Zelltodinduktion ist ROS-abhängig

Mittels zweier verschiedener ROS-Färbungen (MitoSOX®; CM-H_2DCFA) konnten wir in der Durchflusszytometrie eine signifikant gesteigerte Freisetzung von ROS nachweisen. In den folgenden Experimenten gingen wir deshalb der Frage nach, ob die Zelltodinduktion durch die Auranofin+BSO- bzw. Erastin+BSO-Behandlung ROS-abhängig ist. Hierfür verwenden wir die bekannten ROS-Fänger α-Tocopherol, ein Vitamin-E Derivat, sowie N-Acetylcystein (NAC), einem Vorläufer von GSH [22,23]. Deren Einsatz zeigte nicht nur eine signifikante, zelllinienabhängige Verminderung der ROS-Produktion, sondern auch eine Verhinderung der Zelltodinduktion durch beide Kombinationsbehandlungen. Dieses Versuchsset beweist die ROS-Abhängigkeit der Zelltodinduktion durch unsere Kombinationsbehandlungen Auranofin+BSO und Erastin+BSO.

Die Kombinationsbehandlungen führen zur Ferroptose-Induktion

In den folgenden mechanistischen Experimenten untersuchten wir die zugrundeliegende Zelltodform. Bei der klassischen Apoptose kommt es u. a. zur Aktivierung von verschiedenen Caspasen, welche einen essenziellen Bestandteil in der Signalkaskade der Apoptose-Auslösung bilden [24,25]. Die Zugabe des sog. irreversiblen Pan-Caspasen-Inhibitors, Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketon (ZVAD.fmk)

konnte die Zelltodinduktion durch Auranofin+BSO bzw. Erastin+BSO nicht verhindern. Auch mittels Proteome Profiler® Human Apoptosis Array Kit konnte keine Aktivierung von Caspase-3 gezeigt werden. Als Positivkontrolle verwendeten wir eine Kombinationsbehandlung aus Sorafenib mit Oleanolsäure, die in anderen Arbeiten zur apoptotischen und ebenso ROS-abhängigen Zelltodinduktion führte [17,26]. Aus den Ergebnissen dieser beiden Versuche schlussfolgerten wir, dass Apoptose als zugrunde liegende Zelltodform unwahrscheinlich ist.

Es ist bekannt, dass Erastin zu einer Eisen- und ROS-abhängigen sowie Caspasen-unabhängigen Zelltodform, der sog. Ferroptose führen kann [19,20]. Interessanterweise zeigte der Einsatz von Ferrostatin-1 (Fer-1), einem Ferroptose-Inhibitor [19], einen signifikanten Rückgang der Zellsterblichkeit durch oben genannte Kombinationen.

Ferroptose als zugrundeliegenden Zelltodform unserer Kombinationsbehandlung schien unter diesen Aspekten immer wahrscheinlicher, deswegen untersuchten wir anschließend, ob es neben einem ROS-Anstieg auch zu vermehrter Lipidperoxidation kommt, welche ein charakteristisches Merkmal der Ferroptose darstellt [19]. Mittels fluoreszierender BODIPY-C11-Färbung konnten wir schließlich eine gesteigerte Lipidperoxidation in beiden Zelllinien detektieren. Sowohl durch Fer-1 als auch durch den Lipidperoxidationsshemmer Liproxstatin-1 (Lip-1) konnte dieser Effekt verringert und die Induktion des Zelltods durch Auranofin+BSO sowie Erastin+BSO verhindert werden. Auch durch direkte Inhibierung von Ferroptose-auslösenden Lipoxygenasen (LOX) mittels Nordihydroguaiarsäure (NDGA) und Baicalein zeigte sich eine signifikante Zelltod-Reduktion. All diese Ergebnisse sprechen dafür, dass Auranofin+BSO und Erastin+BSO Ferroptose in den HCC-Zellen induzieren.

Die Kombinationsbehandlungen aktivieren beide Ferroptose-Signalwege

Ferroptose kann durch zwei Signalwege ausgelöst werden: 1. der kanonische Weg, basierend auf einer Blockade oder Herunterregulierung von GPX4, ein Enzym, welches radikale Sauerstoffspezies unter Verbrauch von GSH reduzieren kann und somit die Lipidperoxidation verhindert; sowie 2. der nicht-kanonische Weg, welcher durch eine Herunterregulierung von Kelch-like ECH-associated protein 1 (KEAP1) konsekutiv zur Akkumulation von Nrf2 führt [20,27]. Letzterer spielt als Transkriptionsfaktor eine wichtige

Rolle bei der Regulierung von antiinflammatorischen Zielstrukturen, wie beispielsweise HO-1 [28]. Beide dieser Wege basieren auf einem Ungleichgewicht von radikalen Sauerstoffspezies als zugrundeliegende Antriebskraft. Im nächsten Schritt unserer Arbeit erfolgten deshalb Western-Blots, in welchen sowohl eine Herunterregulation von GPX4 sowie KEAP1, als auch eine Hochregulation von Nrf2 in beiden Zelllinien dargestellt werden konnte. Zusätzlich konnte durch die Kombination von Auranofin mit BSO eine signifikante Reduktion der TrxR-Aktivität mittels ELISA in beiden Zelllinien gemessen werden. Dieses Versuchsset spricht für die Involvierung beider Ferroptose-induzierender Signalwege.

Unsere Publikation zeigt nicht nur einen vielversprechenden und innovativen Ansatz für die Entwicklung von neuen Therapiestrategien im HCC, sondern untermauert einmal mehr die Bedeutung der ROS Modulation und der Ferroptose für die Überwindung der Chemotherapieresistenz im HCC.

5.3 Diskussion

Ferroptose ist eine erstmals durch Dixon im Jahr 2012 beschriebene Zelltodform. Sie ist gekennzeichnet durch morphologische Veränderungen der Mitochondrien, wie beispielsweise Volumenreduktion und Verdichtung der Membran, durch Akkumulation von zweiwertigem Eisen sowie Lipidperoxidation und ROS-Abhängigkeit [19,20,29,30].

Die Bedeutung der Ferroptose bei der Entstehung von inflammatorischen Lebererkrankungen, Leberzirrhose und Steatose ist in Studien widersprüchlich. Einige Arbeiten sehen die Ferroptose als Verstärker oder gar als Auslöser von chronischer Entzündung der Hepatozyten, was wiederum Fibrose und Zirrhose zur Folge haben kann [31]. Andererseits konnten Zhang et al. zeigen, dass eine Ferroptoseinduktion mittels Erastin und Sorafenib in den Kupffer‘schen Sternzellen zu einer Verringerung der Fibrose führt [32]. Unsere Arbeit reiht sich in anderen Arbeiten ein, die in der Induktion der Ferroptose eine neue Therapieoption für das HCC sehen [28,33,34].

Zu den von uns verwendeten Substanzen gibt es verschiedene Studien, welche sich mit der Ferroptoseinduktion in anderen Tumorentitäten beschäftigen: So wurde eine

Wirksamkeit von Auranofin zur Behandlung von Leukämie und Lymphomen, sowie beim Ovarialkarzinom und nichtkleinzelligem Lungenkarzinom beschrieben [18,35]. Weiterhin scheint die Kombination von Auranofin und BSO zum einen sowohl in Kopf-Hals-Tumoren, als auch im Rhabdomyosarkom Wirksamkeit zu zeigen, zum anderen kann es als Radiosensitizer in Brustkrebs-Stammzellen wirken [36-38].

Bisher veröffentlichte Ergebnisse zur Ferroptoseinduktion als Therapiestrategie im HCC fokussierten sich oft auf Sorafenib. [34]. Interessanterweise scheint Haloperidol, bekannt als antipsychotisch wirkende Substanz, den Effekt von sowohl Sorafenib- als auch Erastin-induzierter Ferroptose zu verstärken [39]. Die Arbeit von Werth et al. zeigt, dass Sorafenib in HCC-Zellen wichtige Regulatorproteine des Eisenstoffwechsels, wie beispielsweise HO-1, phosphoryliert. Sie vermuten deshalb, dass die Auslösung von Ferroptose mittels Phosphorylierung reguliert wird [40].

In der Regulierung der Ferroptose im HCC scheint aber auch das Glykoprotein Ceruloplasmin, welches vor allem als Transportprotein von Kupfer bekannt ist, eine Rolle zu spielen, wobei hier eine vermehrte Expression mit einer Inhibierung der Ferroptose einhergeht. Sowohl Erastin als auch RSL3 konnten in dieser Arbeit die Expression von Ceruloplasmin verringern und damit zur Ferroptoseinduktion im HCC führen [41].

Interessant ist zudem, dass mehrere retrospektive Studien zeigen konnten, dass die Expression Ferroptose-regulierenden Gene mit der Prognose des HCCs korreliert [42-44].

Abschließend ist hervorzuheben, dass die von uns verwendeten ROS-regulierenden Substanzen bereits teilweise klinische Anwendung finden. Die zu Erastin analoge Substanz PRLX 93936 wurde in klinischen Phase I und II – Studien zur Therapie des Multiplen Myeloms und bei fortgeschrittenen soliden Tumoren getestet (NCT01695590, NCT00528047). Weiterhin gibt es bereits erste klinische Studien zur Effektivität von BSO in Kombination mit Melphalan beim kindlichen Neuroblastom, mit ersten hoffnungsvollen Ergebnissen [45,46].

Limitationen unserer Arbeit ergeben sich zum einen aus der Tatsache, dass sämtliche Versuche nur *in vitro* durchgeführt wurden und erste *in vivo* Versuche erst anstehen. Weiterhin sind die möglichen Wechselwirkungen der ROS-Modulatoren und deren Effekte

auf nicht-tumoröse Zellen, aber auch auf die vorbeschädigte, zirrhotische Leber unbekannt und müssen in weiteren Studien untersucht werden.

Zusammenfassend konnten wir in unserer Arbeit einen vielversprechende und innovative synergistische Zelltodinduktion durch Auranofin mit BSO, respektive Erastin mit BSO, in HCC-Zellen identifizieren. Unsere Ergebnisse zeigen weiterhin, dass erst die simultane Aktivierung von zwei antioxidativen Signalwegen, des TRX- und des GSH-Systems, zum ROS-abhängigen Zelltod der Tumorzellen führt. Die Induktion der Ferroptose durch die selektive ROS-Modulation in unserer Arbeit kann die Apoptoseresistenz der Tumorzellen umgehen.
Damit stellt die Modulation der Redoxhomöostase und die Induktion der Ferroptose in den Tumorzellen eine geeignete neue Perspektive zur Überwindung der Chemotherapieresistenz des HCCs dar und sollte Schwerpunkt zukünftiger Forschungsprojekte sein.

6. Veröffentlichte Publikation

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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.

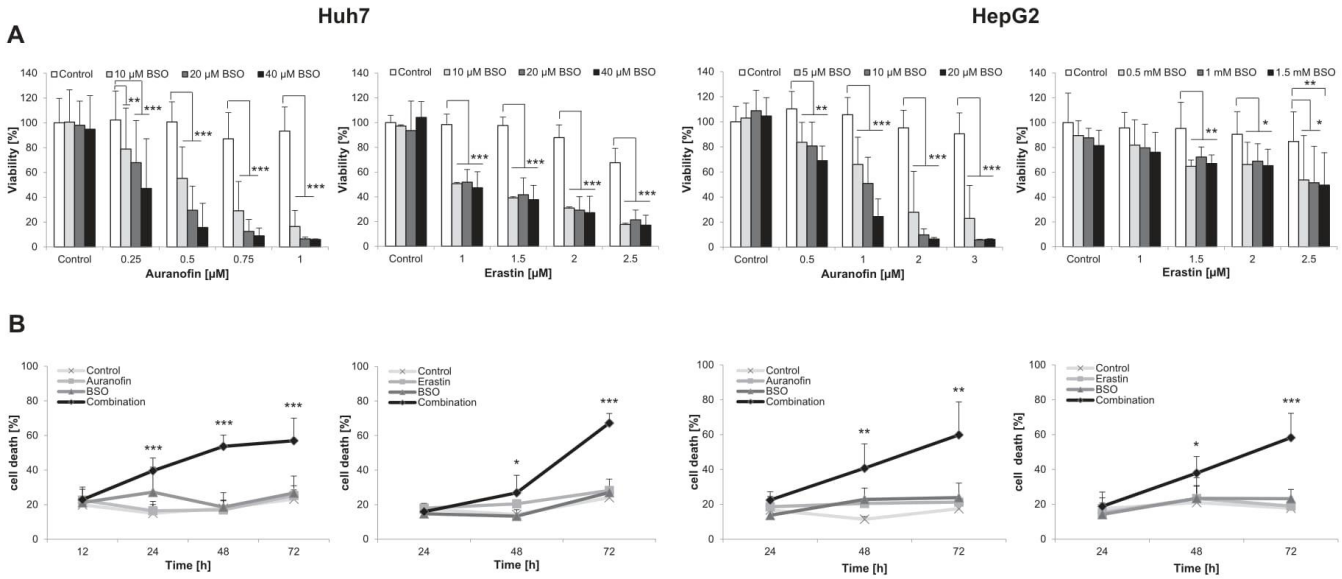

Figure 1. The effect of different ROS modulators and ferroptosis inducers on the human liver cancer cell lines Huh7 and HepG2. (A) Huh7 and HepG2 were treated for 72 hours with indicated concentrations of Auranofin, BSO and Erastin. (B) Huh7 cells were treated with 0.5 µM Auranofin and/or 10 µM BSO or 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

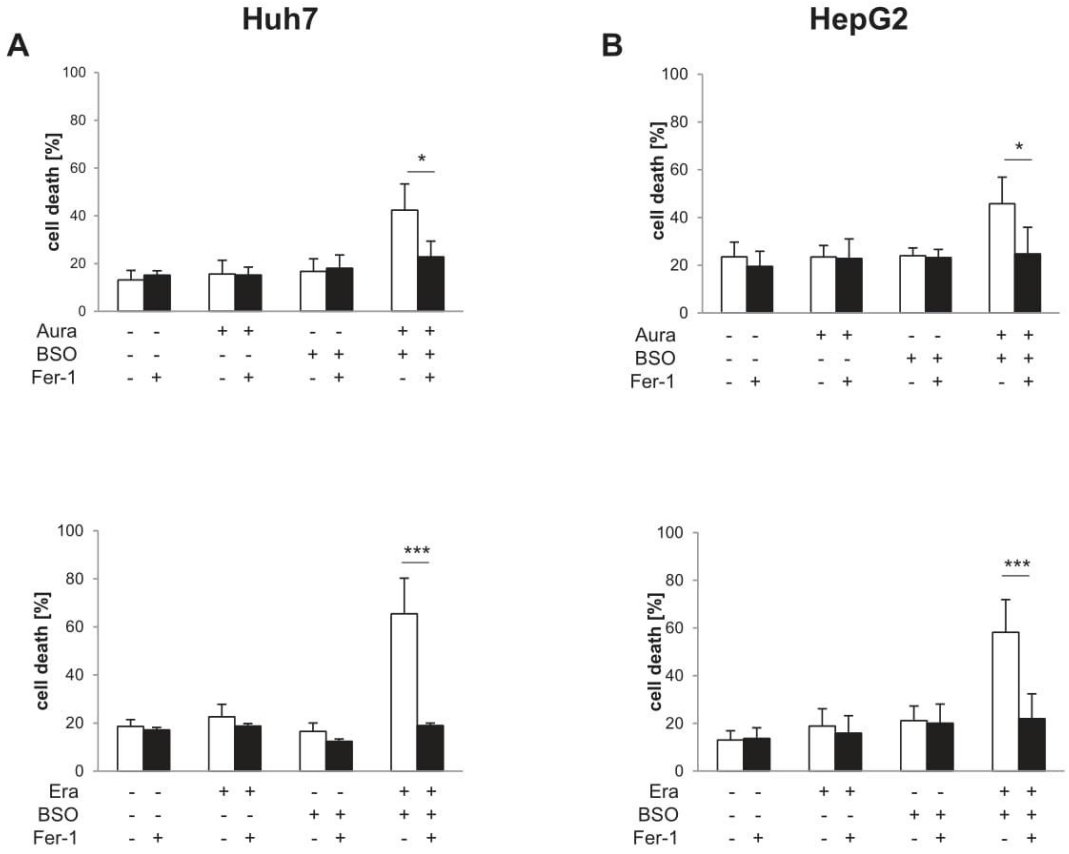

Figure 2. Fer-1 inhibited Auranofin/BSO- and Erastin/BSO-induced cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and/or 10 μM BSO for 48 hours or 1 μM Erastin and/or 10 μM BSO for 72 hours in the presence or absence of 10 μM Fer-1. (B) HepG2 cells were treated with 1 μM Auranofin and/or 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. * $P < .05$; *** $P < .001$.

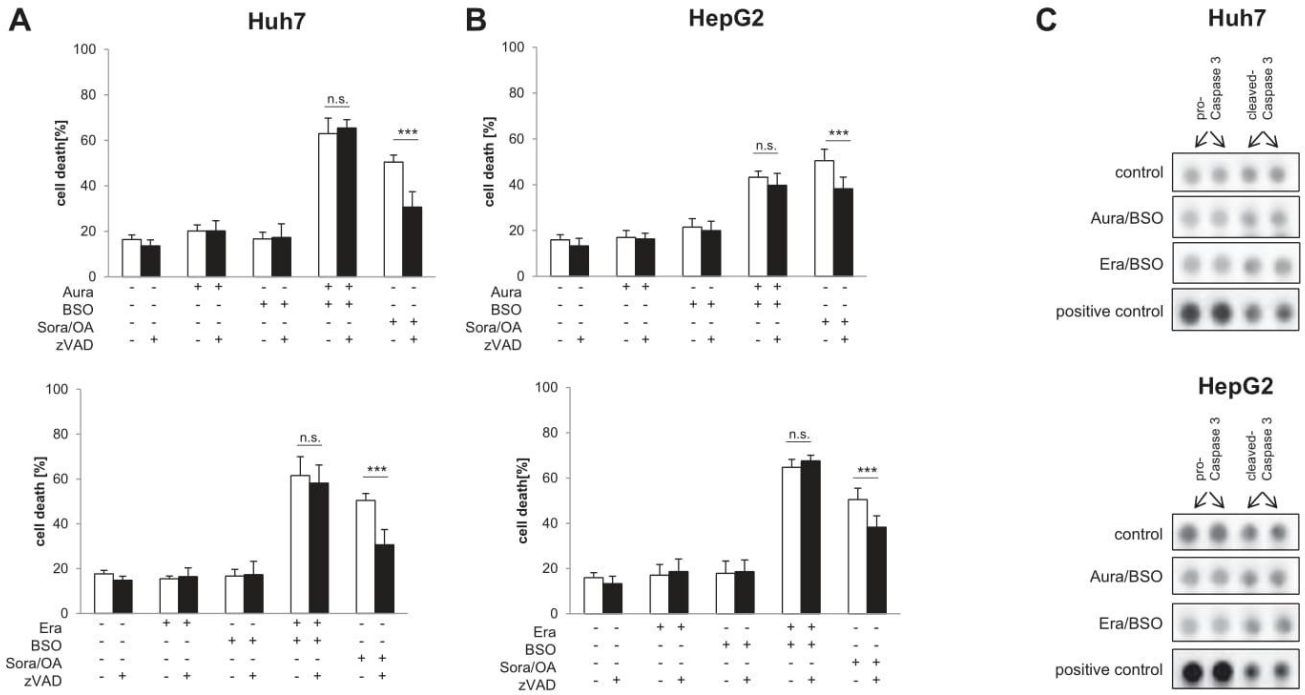

Figure 3. Auranofin/BSO- and Erastin/BSO-cotreatment triggered caspase-independent cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and/or 10 μM BSO for 48 hours or 1 μM Erastin and/or 10 μM BSO for 72 hours in the presence or absence of 50 μM zVAD.fmk. (B) HepG2 cells were treated with 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

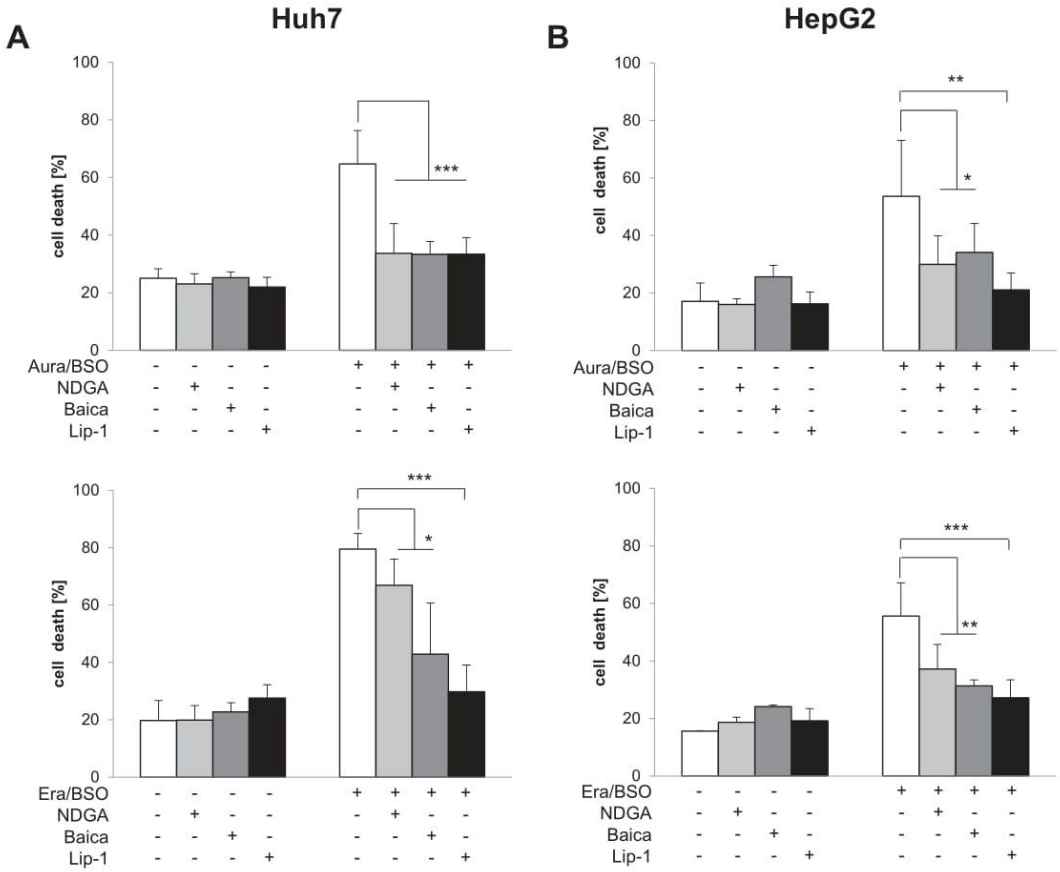

Figure 4. Auranofin/BSO- and Erastin/BSO-cotreatment induced ferroptotic cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO for 48 hours or 1 μM Erastin and 10 μM BSO for 72 hours in the presence or absence of 1 μM NDGA, 0.5 μM Baicalein or 25 nM Lip-1. (B) HepG2 cells were treated 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

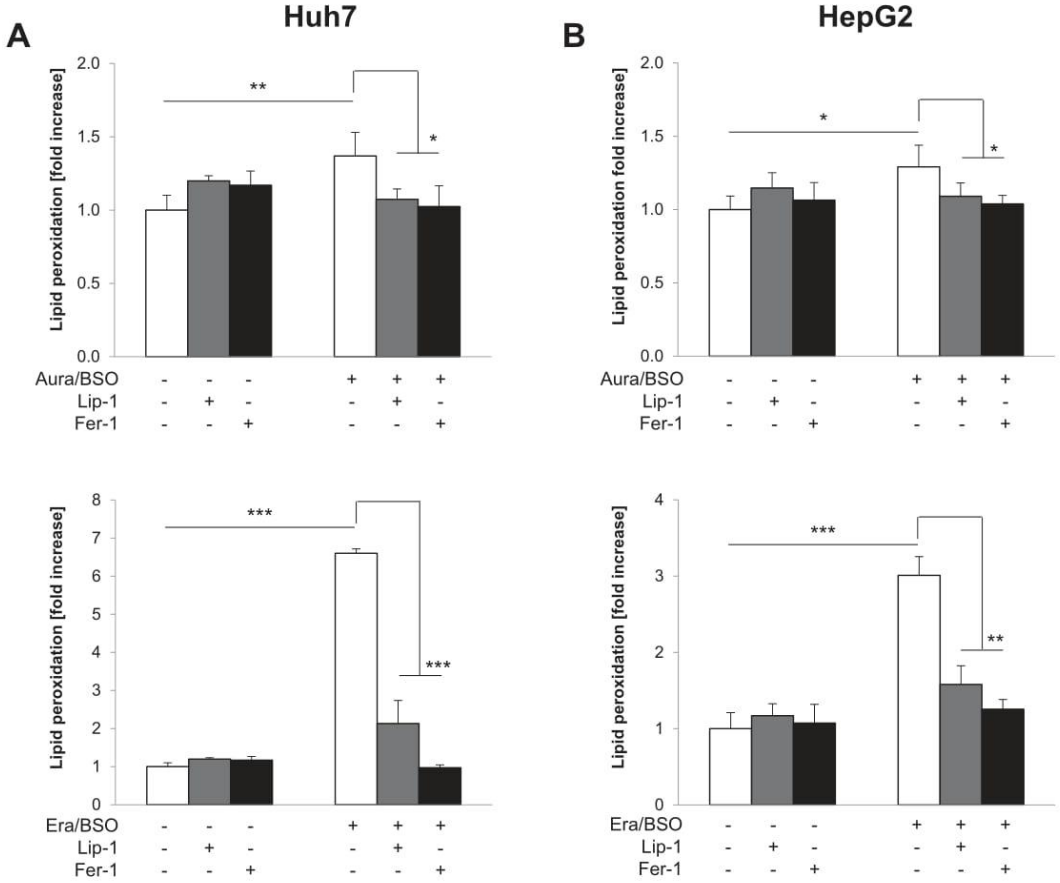

Figure 5. Lipid peroxidation contributed to Auranofin/BSO- and Erastin/BSO-induced cell death and could be rescued by different inhibitors of ferroptosis and lipid peroxidation. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO or 1 μM Erastin and 10 μM BSO in the presence or absence 25 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

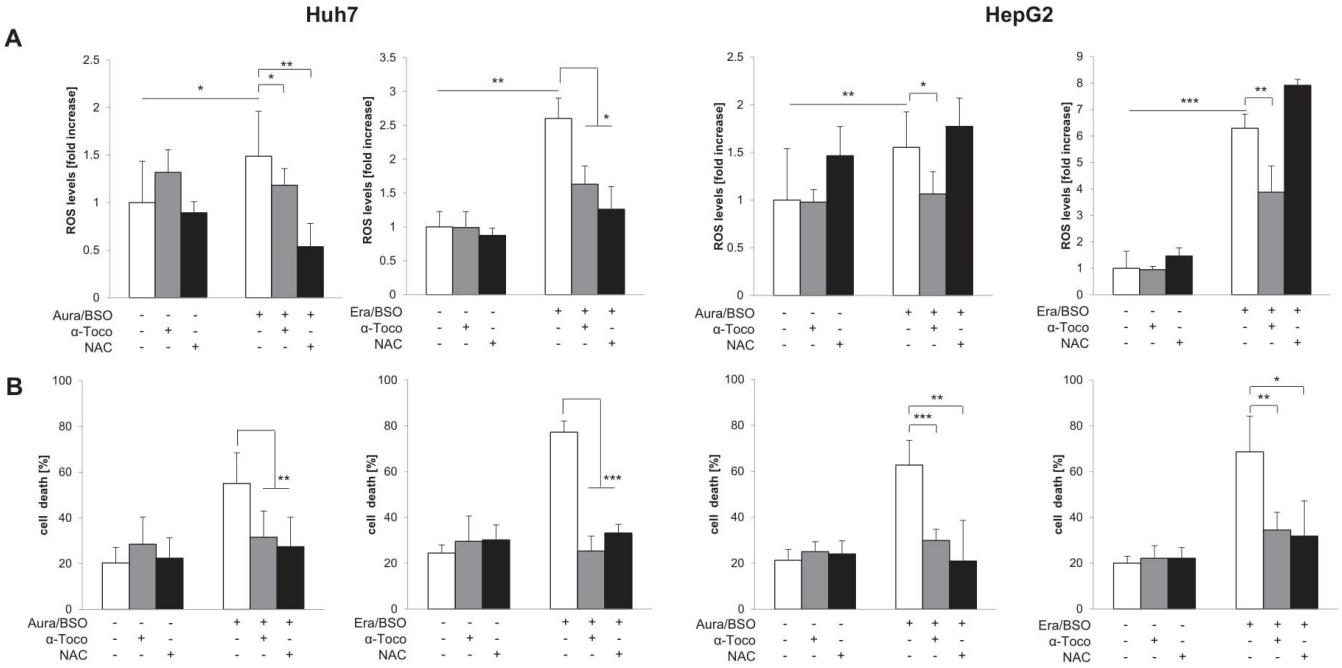

Figure 6. Auranofin/BSO and Erastin/BSO cotreatment led to ROS production and ROS-dependent cell death. (A) Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO for 6 hours or 1 μM Erastin and 10 μM BSO for 24 hours in the presence or absence of 50 μM α-Toc or 1.25 mM NAC. HepG2 cells were treated 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. * $P < .05$; ** $P < .01$; *** $P < .001$.

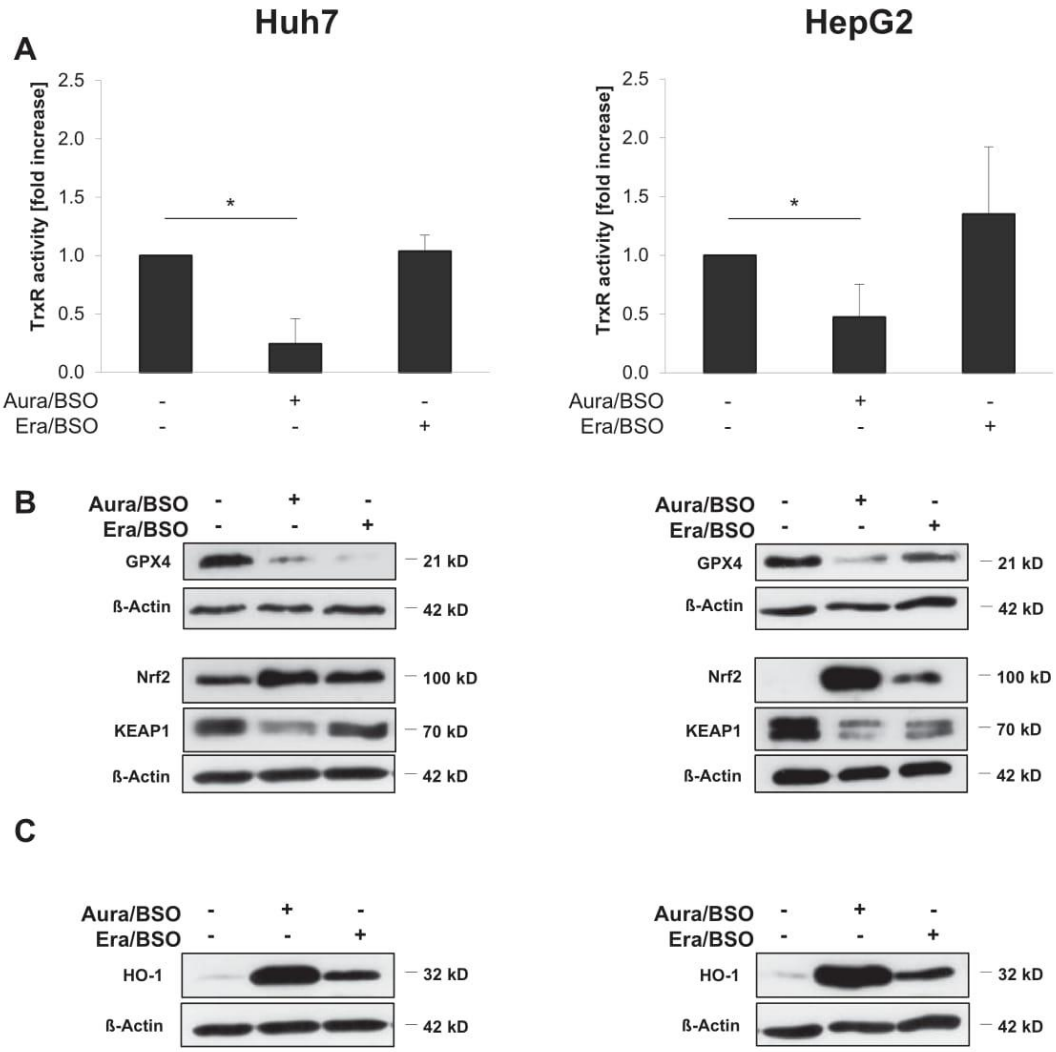

Figure 7. Auranofin/BSO and Erastin/BSO cotreatment decreased TrxR activity, led to Nrf2 accumulation, promoted activation of HO-1 and prompted GPX4 decrease. Huh7 cells were treated with 0.5 μM Auranofin and 10 μM BSO or 1 μM Erastin and 10 μM BSO for 24 hours. HepG2 cells were treated with 1 μM Auranofin 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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7. Darstellung des eigenen Anteils am publizierten Manuskript

Ich habe mich über die universitäre Frankfurter Promotionsbörse auf die Ausschreibung einer Promotionsstelle zum Thema „Redoxmodulation im HCC als neue Therapiestrategie“ unter Betreuung von Frau PD Dr. Liese und Frau Prof. Fulda beworben. Nach Vorgesprächen erstellte ich gemeinsam mit Frau PD Dr. Liese einen genauen Arbeitsplan zur Literaturrecherche, Einarbeitung in die Laborarbeit und zur Durchführung der ersten erforderlichen experimentellen Arbeiten. Im Verlauf der Promotion wurde der Arbeitsplan und die Abläufe der Versuche engmaschig besprochen und den aktuellen Ergebnissen angepasst.

Bereits während der Einarbeitung eignete ich mir ein umfangreiches Wissen über den aktuellen Stand der HCC-Forschung an und diskutierte die Publikationen und deren Relevanz für meine Promotion mit Frau PD Dr. Liese.

Die experimentelle Laborarbeit führte ich, nach ausführlicher Einarbeitung und Erklärung durch Frau PD Dr. Liese, selbstständig durch. Lediglich einzelne Western Blots stellten die Ausnahme dar (ca. 95% Eigenanteil). Sowohl die Datenauswertung als auch deren Interpretation erfolgte, unter Supervision von Frau PD Dr. Liese, selbstständig durch mich. Hierfür zog ich relevante Publikationen auf diesem Gebiet heran. Meine Schlussfolgerungen konnte ich ausführlich mit meiner Betreuerin diskutieren.

Nach erfolgreichem Abschluss der experimentellen Arbeiten und deren Auswertung verfasste ich auch große Teile des publizierten Manuskriptes. Hierbei erhielt ich Hilfestellung sowohl durch PD Dr. Liese als auch durch Prof. Fulda.

Die hier vorliegende Dissertation wurde vollständig von mir verfasst.

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Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Redoxmodulation und Ferroptose als neue Therapiestrategie beim hepatozellulären Karzinom

Im Zentrum für Kinder- und Jugendmedizin, Institut für experimentelle Tumorforschung in der Pädiatrie am Universitätsklinikum Frankfurt unter Betreuung und Anleitung von PD Dr. Juliane Liese ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden (oder werden) in folgendem Publikationsorgan veröffentlicht:
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