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Identification of a novel oxidative stress induced cell death by Sorafenib and oleanolic acid in human hepatocellular carcinoma cells

Matthias Lange [a,b], Behnaz Ahangarian Abhari [a], Tobias M. Hinrichs [a,b], Simone Fulda [a,c,d,*], Juliane Liese [a,b,c,d]

[a] Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Frankfurt, Germany
[b] General and Visceral Surgery, Goethe-University, Frankfurt, Germany
[c] German Cancer Consortium (DKTK), Heidelberg, Germany
[d] German Cancer Research Center (DKFZ), Heidelberg, Germany

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ABSTRACT

The lack of effective chemotherapies in hepatocellular carcinoma (HCC) is still an unsolved problem and underlines the need for new strategies in liver cancer treatment. In this study, we present a novel approach to improve the efficacy of Sorafenib, today's only routinely used chemotherapeutic drug for HCC, in combination with triterpenoid oleanolic acid (OA). Our data show that cotreatment with subtoxic concentrations of Sorafenib and OA leads to highly synergistic induction of cell death. Importantly, Sorafenib/OA cotreatment triggers cell damage in a sustained manner and suppresses long-term clonogenic survival. Sorafenib/OA cotreatment induces DNA fragmentation and caspase-3/7 cleavage and the addition of the pan-caspase inhibitor zVAD.fmk shows the requirement of caspase activation for Sorafenib/OA-triggered cell death. Furthermore, Sorafenib/OA co-treatment stimulates a significant increase in reactive oxygen species (ROS) levels. Most importantly, the accumulation of intracellular ROS is required for cell death induction, since the addition of ROS scavengers (i.e. α-tocopherol, MnTBAP) that prevent the increase of intracellular ROS levels completely rescues cells from Sorafenib/OA-triggered cell death. In conclusion, OA represents a novel approach to increase the sensitivity of HCC cells to Sorafenib via oxidative stress.

1. Introduction

HCC is the second most common cause of death from cancer worldwide and estimated to be responsible for nearly 745,000 deaths in 2012 [1]. Only 30–40% of patients are eligible for curative treatment, including liver resection, transplantation and percutaneous ablation [2,3]. At present, there is no effective systemic chemotherapy available for patients with advanced HCC. Sorafenib, an oral multikinase inhibitor, is currently the only routinely used chemotherapeutic drug, which improves the median survival and the time to radiologic progression up to 2.8 months compared to patients who received a placebo treatment [4]. Unfortunately, due to its toxicity, the administration of Sorafenib is reserved only to a limited group of patients. The underlying liver dysfunction in HCC patients, which leads to an even lower tolerance of treatment toxicity, presents a significant problem in the standard therapy with Sorafenib [5].

This highlights the need to develop novel strategies for the induction of cell death in HCC cells. Due to the lack of more effective treatment strategies in HCC, one strategy is to improve the efficacy of Sorafenib in HCC cells. Sorafenib has been reported to induce the generation of ROS in human HCC cell lines *in vivo* and *in vitro* in a dose-dependent manner [6,7]. The levels of ROS in Sorafenib-treated HCC patients correlate with the clinical efficacy of Sorafenib [6]. Another approach to induce ROS and cell death in HCC cells is the use of OA, a natural triterpenoid [8–10]. In Chinese medicine, OA has been used for many decades in the

Abbreviations: CI, combination index; DR5, death receptor-5; ER, endoplasmatic reticulum; FCS, fetal calf serum; Fer-1, ferrostatin-1; FSC/SSC, forward/side scatter; HCC, hepatocellular carcinoma; IAP, Inhibitor of Apoptosis; MTT, inhibitor N-benz yloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Nec-1, necrostatin-1; OA, oleanolic acid; PI, propidium iodide; RIP, receptor-interacting protein; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; zVAD.fmk, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

* Corresponding author at: Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Komturstr. 3a, 60528 Frankfurt, Germany.
E-mail address: simone.fulda@kgu.de (S. Fulda).

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treatment of liver disorders such as viral hepatitis [8]. Recently, an antitumor effect of OA *in vitro* and *in vivo* has been shown in HCC [8]. We previously identified a novel synergistic induction of ROS production and cell death by combining the Smac mimetic BV6, which antagonizes Inhibitor of Apoptosis (IAP) proteins [11,12], and OA in human HCC cells [10]. Searching for new strategies to overcome Sorafenib resistance in HCC, in the present study we investigated the effects of the combination of Sorafenib and OA on human HCC cells.

2. Materials and methods

2.1. Cell culture and reagents

The human HCC cell lines Huh7 and HepG2 were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM medium (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. The pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany), and necrostatin (Nec)-1s from Biomol (Hamburg, Germany). All other chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany) unless indicated otherwise.

2.2. Determination of cell death and cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) or by crystal violet staining (0.75% crystal violet, 50% ethanol, 0.25% NaCl and 1.57% formaldehyde). Cell death was determined by analysis of DNA fragmentation of propidium iodide

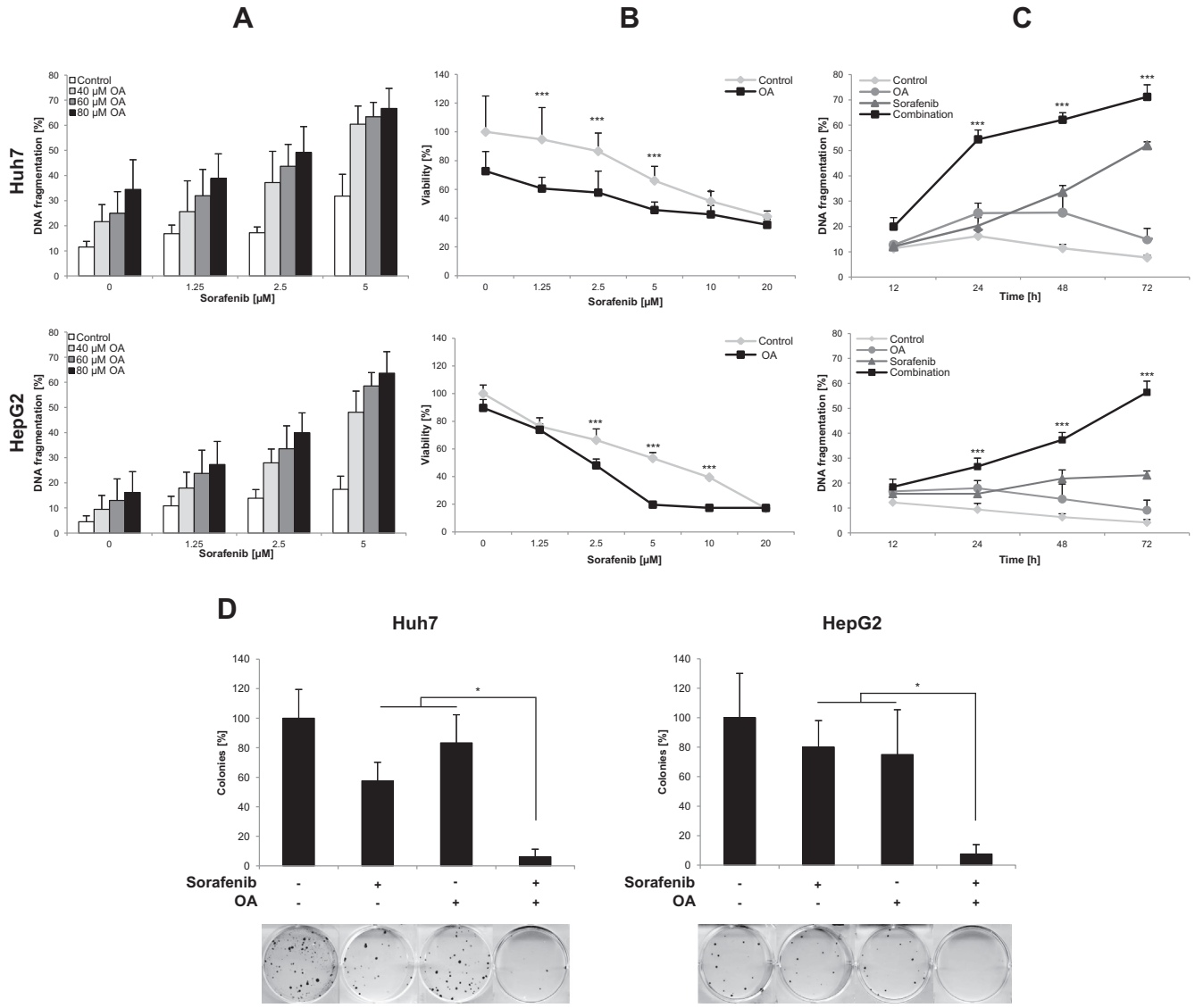

Fig. 1. Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival. (A)–(C) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with indicated concentrations of Sorafenib and OA (A, B) or for indicated times with 5 µM Sorafenib and/or 60 µM OA (C). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (A, C), cell viability was determined by MTT assay (B). (D) HCC cells were treated with 5 µM Sorafenib and/or 60 µM OA for 72 h and colony formation was assessed as described in Section 2. The percentage of colonies relative to untreated control (upper panels) and representative results (lower panels) is shown. Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{***}P < 0.001$. In (B) and (C), Sorafenib/OA-cotreated samples were compared to Sorafenib-treated samples.

(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 [13].

2.3. Colony formation assay

To determine colony formation, 0.2×10^6 cells were seeded in a 6-well tissue culture plate, and allowed to settle for 24 h. Cells were then treated with Sorafenib and OA for 48 h, trypsinized and re-seeded with a total count of 200 cells (Huh7) or 400 cells (HepG2) per well in a second 6-well tissue culture plate. After 12 days of cultivation, cells were stained with crystal violet solution, colonies were counted and the percentage of surviving colonies relative to the untreated controls was calculated.

2.4. Caspase-3/7 activity assay

The caspase-3/7 activity assay was performed using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Cells were seeded in 96-well plates and treated with Sorafenib and OA for 12 and 24 h. 100 µl of Apo-ONE® Caspase-3/7 reagent were added to each well. Contents were gently mixed on a plate shaker for 30 s. After one hour of incubation time the fluorescence emission at 530 nm of each well was measured using Tecan® reader Infinite® 200 PRO (Tecan Group, Ltd. Männedorf, Switzerland).

2.5. ROS staining and lipid peroxidation

ROS production was measured by flow cytometry using 5 µM of MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol. For measuring lipid peroxidation cells were stained with 5 µM of BODIPY-C11 (Invitrogen) and analyzed by flow cytometry according to the manufacturer's protocol.

2.6. Statistical analysis

Statistical significance was assessed by Student's *t*-test (two-tailed distribution, two-sample, unequal variance). Drug interactions were analyzed by the combination index (CI) method based on the publication by Chou [14] using CalcuSyn software (Biosoft, Cambridge, UK). A calculation of CI value of <0.9 indicates synergism, 0.9–1.1 additivity, >1.1 antagonism.

3. Results

3.1. Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival

To investigate whether OA can prime HCC cells to Sorafenib, we used two different human HCC cell lines (Huh7, HepG2). Interestingly, Sorafenib and OA acted in concert to trigger DNA fragmentation, a typical marker of apoptotic cell death, compared to treatment with either agent alone (Fig. 1A). The calculation of CI revealed a synergistic interaction of Sorafenib and OA in both HCC cell lines (Table 1). The cooperative interaction of Sorafenib and OA was confirmed by another assay in which Sorafenib and OA acted together to reduce cell viability of HCC cells in a dose-dependent manner (Fig. 1B). Kinetic analysis showed that Sorafenib/OA cotreatment induced a significant time-dependent increase in DNA fragmentation in Huh7 and HepG2 cells starting around 24 h compared to single treatment with Sorafenib (Fig. 1C). To explore the impact of cotreatment with Sorafenib and OA on long-term clonogenic survival, colony formation assay was performed. Of note, Sorafenib/OA cotreatment significantly reduced colony formation compared to either agent alone in Huh7 and HepG2 cells (Fig. 1D), demonstrating that the cotreatment also affects long-term clonogenic survival of HCC cells. Together, these experiments demonstrate that Sorafenib and OA synergistically induce cell death in HCC cells and suppress long-term clonogenic survival.

Table 1
Synergistic induction of cell death by Sorafenib and OA.

	OA (µM)	Sorafenib (µM)	CI
Huh7	40	1.25	1.068
	40	2.5	0.664
	40	5	0.261
	60	1.25	1.002
	60	2.5	0.631
	60	**5**	**0.283**
	80	1.25	0.881
	80	2.5	0.585
	80	5	0.294
HepG2	40	1.25	0.662
	40	2.5	0.336
	40	5	0.109
	60	1.25	0.53
	60	2.5	0.307
	60	**5**	**0.088**
	80	1.25	0.527
	80	2.5	0.274
	80	5	0.087

Combination index (CI) was calculated as described in Section 2. Values of CI <0.9 indicates synergism, 0.9–1.1 additivity, >1.1 antagonism. Drug concentrations used in that study are indicated in bold.

3.2. Sorafenib/OA cotreatment cooperates to trigger caspase activation and caspase-dependent apoptosis

To identify the underlying mechanisms of the synergistic induction of cell death by Sorafenib and OA, we monitored caspase activity by using an enzymatic caspase-3/7 activity assay. Sorafenib/OA cotreatment significantly increased caspase-3/7 activity at 12 and 24 h in both Huh7 and HepG2 cells (Fig. 2A). To further investigate the question whether caspases are required for the induction of cell death, we used the pan-caspase inhibitor zVAD.fmk. Addition of zVAD.fmk significantly decreased Sorafenib/OA-induced DNA fragmentation in both cell lines (Fig. 2B). As positive control for caspase-dependent cell death, both cell lines were treated with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the presence or absence of zVAD.fmk (Fig. 2B). This set of experiments shows that Sorafenib and OA cooperate to trigger caspase-3/7 activation, which is required for Sorafenib/OA-induced cell death.

3.3. Receptor-interacting protein (RIP)1 kinase activity is not required for Sorafenib/OA-induced cell death

Necroptosis has recently been discovered as another form of programmed cell death depending on the kinases RIP1 and RIP3 [15]. However, the role of necroptosis in Sorafenib/OA-induced cell death is still unknown. To address this question, we determined the cell death induction in the presence and absence of the RIP1 kinase inhibitor Necrostatin-1s (Nec-1s). To analyze apoptotic or necroptotic cell death, we used DNA fragmentation as a marker of apoptotic cell death and PI-staining to determine the loss of plasma membrane integrity as a parameter of necroptotic cell death. Inhibition of RIP1 kinase activity by Nec-1s failed to rescue Sorafenib/OA-induced cell death, as determined by analysis of DNA

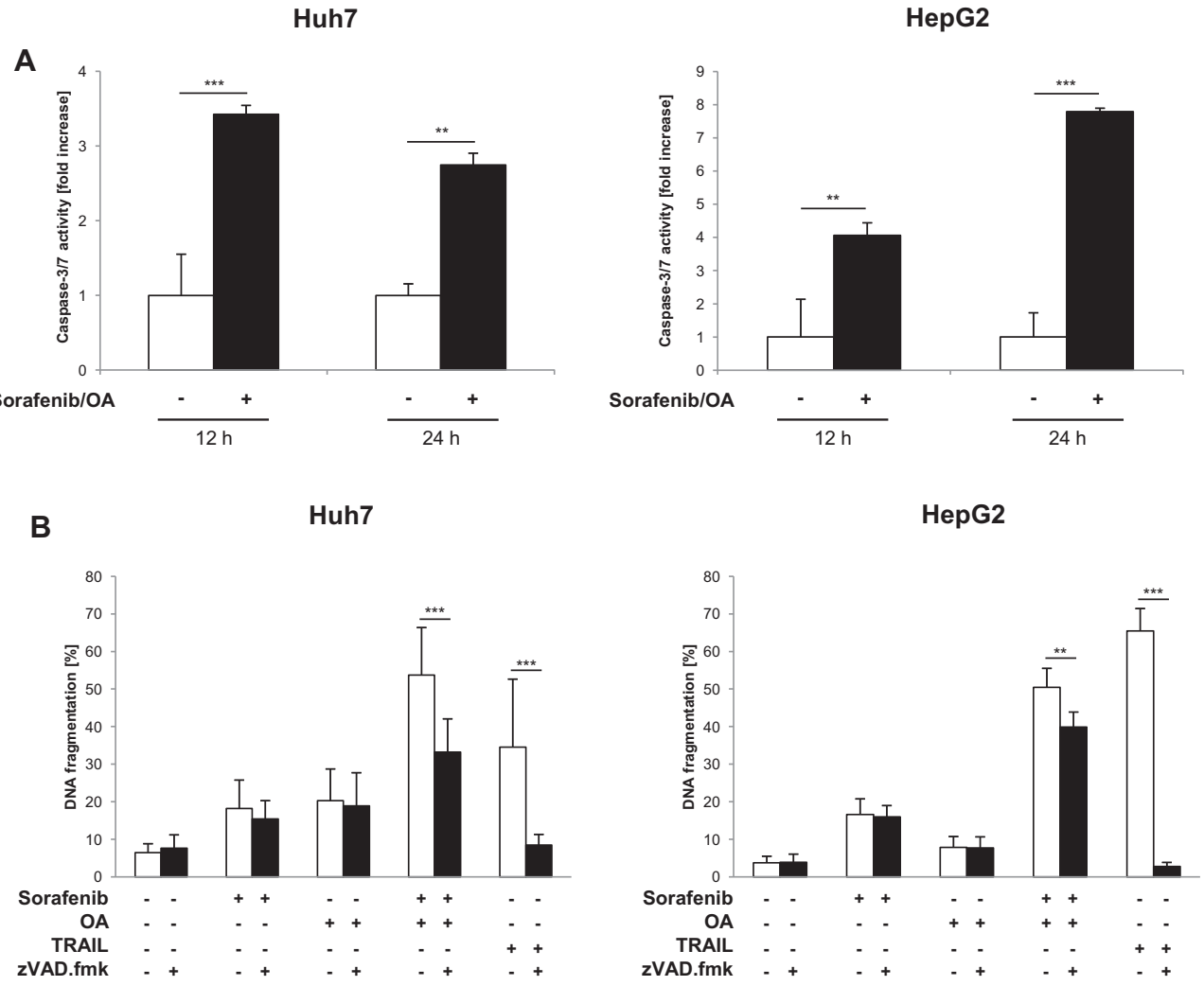

Fig. 2. Sorafenib and OA trigger caspase activation and caspase-dependent apoptosis. (A) HCC cells were cotreated for 12 h and 24 h with 5 μM Sorafenib and 60 μM OA. Caspase-3/7 activity was determined as described in Section 2 (white bar = control; black bar = Sorafenib and OA). (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM zVAD.fmk (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. As positive control, cells were treated for 48 h (Huh7) or 72 h (HepG2) with 40 ng/ml TRAIL in the presence or absence of 50 μM zVAD.fmk. Mean and SD of three independent experiments performed in triplicate are shown; $^{**}P < 0.01$; $^{***}P < 0.001$.

fragmentation or by PI-staining (Fig. 3A and B). This indicates that Sorafenib/OA cotreatment induces cell death independently of RIP1 kinase activity.

3.4. Lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner

Recently, it has been reported that Sorafenib can trigger ferroptosis [16–18]. Ferroptosis is a form of regulated cell death that is characterized by iron-dependent generation of lipid-based ROS and lipid peroxidation [17,19,20]. To investigate the role of lipid peroxidation in Sorafenib/OA co-treatment, we analyzed cell death in the presence and absence of Ferrostatin-1 (Fer-1), described as a small-molecule inhibitor of lipid peroxidation [21]. Addition of Fer-1 failed to rescue HCC cells from Sorafenib/OA-induced cell death as determined by DNA fragmentation in both cell lines and by PI-staining in the cell line Huh7, whereas Fer-1 partially rescued Sorafenib/OA-induced cell death as determined by PI-staining in HepG2 cells (Fig. 4A and B). To investigate whether Sorafenib/OA co-treatment stimulates lipid peroxidation, we used the fluorescent dye BODIPY-C11. Sorafenib/OA cotreatment significantly increased lipid peroxidation in HepG2, but not in Huh7 cells, which was completely blocked by the addition of Fer-1 (Fig. 4C). We also noted that treatment with OA alone induced lipid peroxidation in both cell lines, which could, however, not be blocked in the presence of Fer-1 (Fig. 4C). As positive control for lipid peroxidation-dependent cell death, we used Erastin that has been reported to trigger ferroptosis by blocking the cysteine/glutamate antiporter (system X_c^-) at the plasma membrane [19]. Fer-1 significantly decreased Erastin-stimulated lipid peroxidation and cell death in both cell lines (Fig. 4A–C). These findings indicate that lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner.

3.5. Sorafenib/OA cotreatment triggers ROS-dependent cell death

Different studies showed that ROS are involved in Sorafenib- or OA-induced cell death when they were used as single agents [6,8,10,22]. To investigate whether Sorafenib/OA cotreatment stimulates ROS production, we assessed ROS levels by using the ROS-sensitive fluorescent dye MitoSOX™ Red. Of note, Sorafenib alone and in combination with OA significantly increased ROS production in both Huh7 and HepG2 cells (Fig. 5A). To explore whether this increase in ROS production is critically required for

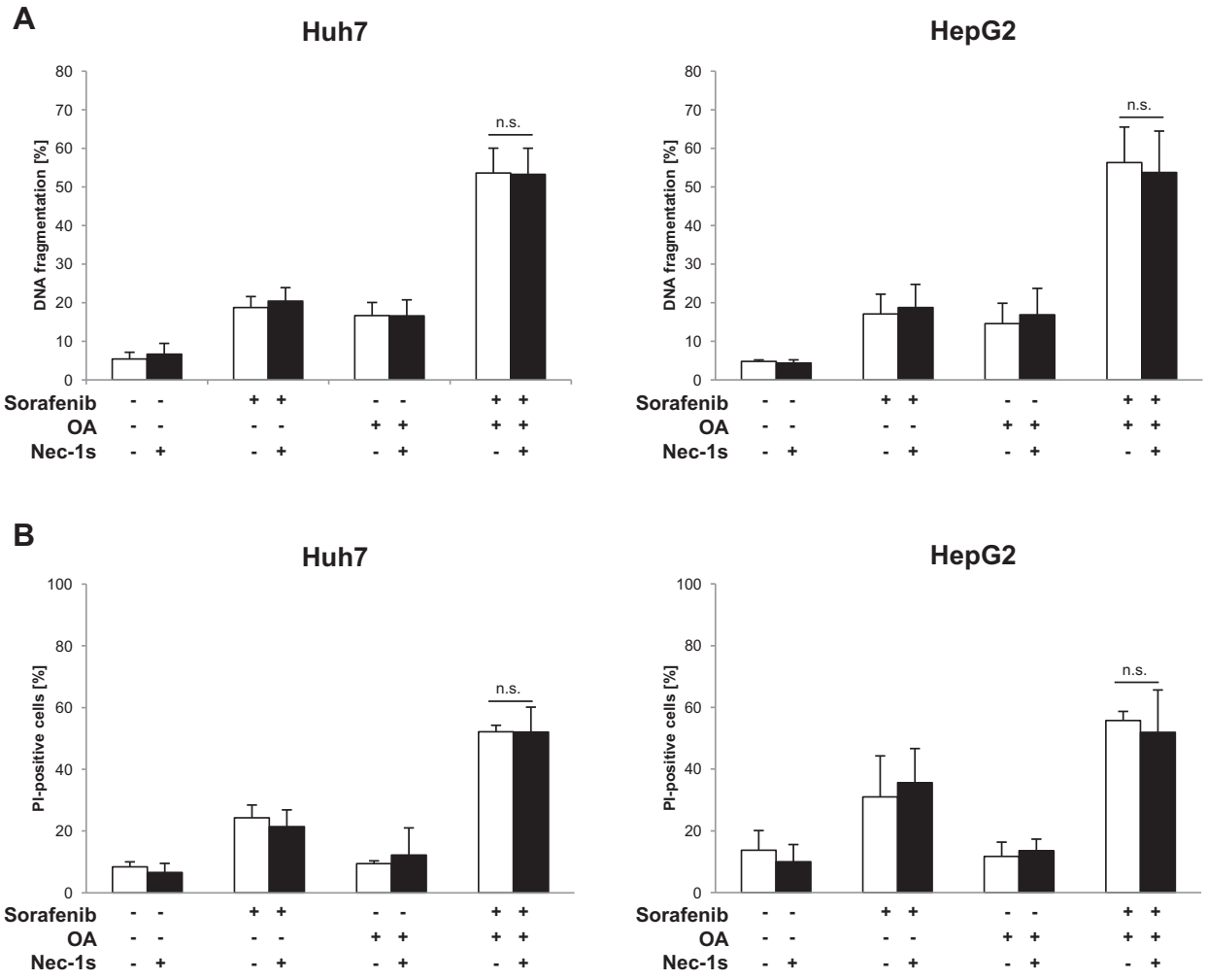

Fig. 3. RIP1 kinase activity is not required for Sorafenib/OA-induced cell death. (A) and (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 30 μM Nec-1s (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei (A) or FSC/SSC scatter analysis of PI-stained nuclei (B) using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; n.s. = not significant.

the induction of cell death, we blocked ROS production by using the ROS scavenger α-Tocopherol, a vitamin E derivative [23], and MnTBAP, a cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger [24]. Importantly, the addition of either α-Tocopherol or MnTBAP significantly reduced Sorafenib/OA-triggered DNA fragmentation in both cell lines (Fig. 5B). In addition, the combined use of both α-Tocopherol and MnTBAP almost completely rescued HCC cells from Sorafenib/OA-induced DNA fragmentation (Fig. 5C), consistent with a potent blockage of ROS production (Fig. 5A). This demonstrates that Sorafenib/OA cotreatment triggers ROS-dependent cell death.

4. Discussion

HCC is the most frequent primary liver cancer and one of the most aggressive tumors worldwide with an increasing incidence [3,25]. Dysregulation in the apoptotic program caused by different underlying liver diseases could explain chemotherapy resistance of HCC [26]. Due to its aggressive tumor growth, the majority of patients are in need of a palliative treatment. At present, Sorafenib is the treatment of choice for advanced HCC, but only shows a modest ability to extend the median survival [4]. Sorafenib resistance of HCC cells highlights the need for new strategies in HCC treatment.

In this study we identify a novel synergistic combination of Sorafenib and OA, which improves the efficacy of Sorafenib in HCC cells. In addition to increasing cell death in short-term assays, Sorafenib and OA also inhibit long-term clonogenic survival of HCC cells. Mechanistic studies showed that the combination of Sorafenib and OA triggers caspase-dependent cell death. This conclusion is supported by data showing that Sorafenib/OA cotreatment stimulates caspase activation and that the pan-caspase inhibitor zVAD.fmk partially prevents cell death. Furthermore, we demonstrate that Sorafenib/OA cotreatment leads to ROS production, which is required for cell death induction. In rescue experiments, ROS scavengers protect HCC cells from Sorafenib/OA-mediated cell death. Our findings are in line with recent publications reporting that ROS production is involved in OA-induced [8,22] or Sorafenib-induced cell death [6]. Different studies showed that Sorafenib inhibits the MEK/ERK pathway that controls ROS production in HCC [6,27,28]. Coriat et al. reported that Sorafenib dose-dependently stimulates ROS production in the human HCC cell line HepG2 and that the ROS scavenger MnTBAP significantly reduces the Sorafenib-mediated effect on tumor growth of HCC in mice experiments, emphasizing the relevance of ROS for the antitumor activity of Sorafenib [6]. This conclusion is underlined by an *in vivo* ROS analysis of sera from patients treated with Sorafenib, as the best response to Sorafenib has been reported for patients with high ROS levels during the treatment with Sorafenib [6].

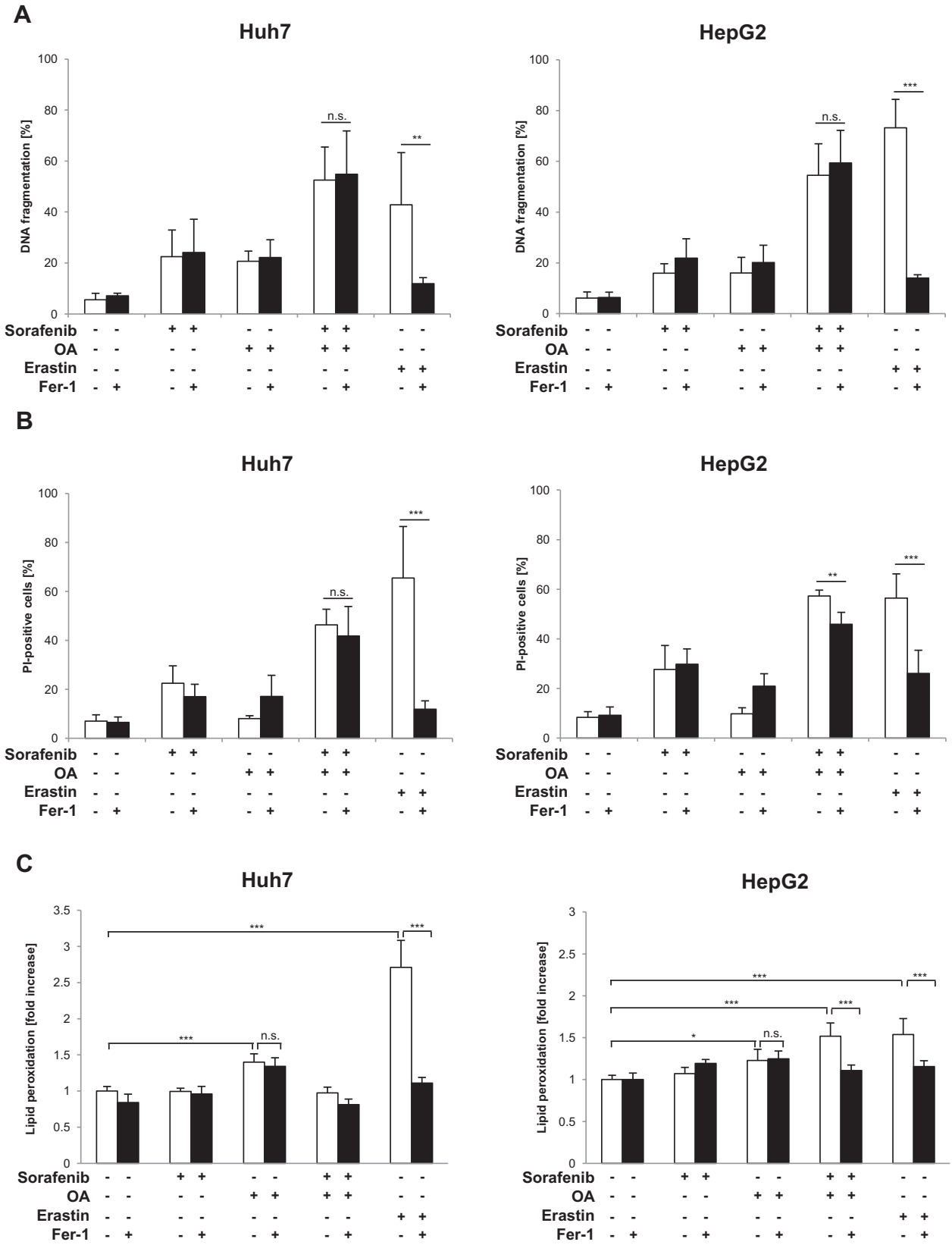

Fig. 4. Lipid peroxidation contributes to Sorafenib/OA-induced cell death in a cell line-dependent manner. (A) and (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 10 μM Fer-1 (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei (A) or FSC/SSC scatter analysis of PI-stained nuclei (B) using flow cytometry. As positive control, cells were treated for 48 h (Huh7) or 72 h (HepG2) hours with 50 μM Erastin in the presence or absence of 10 μM Fer-1. C, HCC cells were treated for 24 h with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 10 μM Fer-1 (black bars). Lipid peroxidation was determined by BODIPY-C11 using flow cytometry. As positive control, cells were treated for 24 h with 50 μM Erastin in the presence or absence of 10 μM Fer-1. Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = not significant.

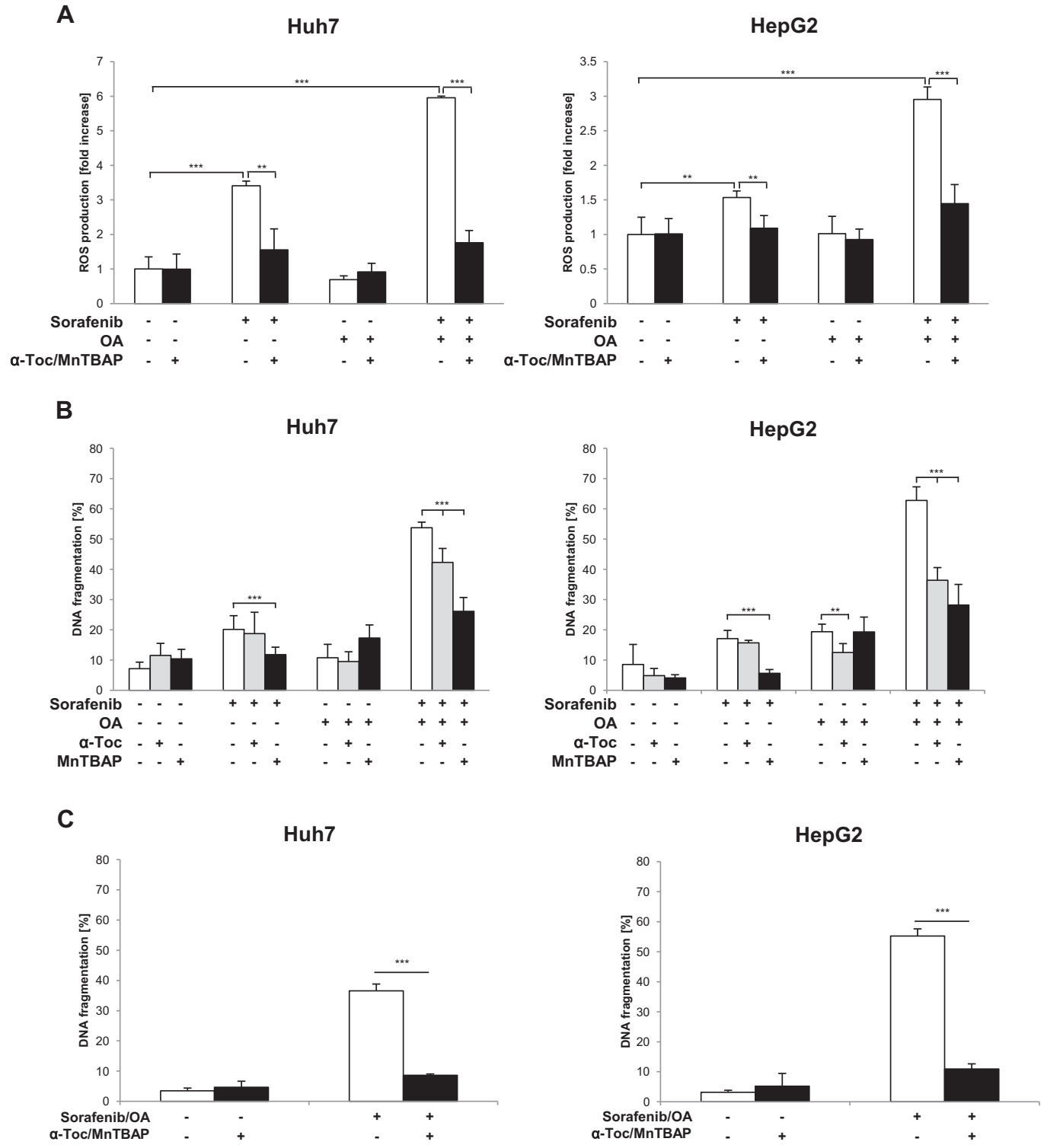

Fig. 5. ROS scavengers rescue Sorafenib/OA-induced cell death. (A) HCC cells were treated for 12 h with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of a combination of 50 μM α-Tocopherol and 300 μM MnTBAP (black bars). ROS production was determined by MitoSOX™ Red using flow cytometry. (B) HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM α-Tocopherol (gray bars) or 300 μM MnTBAP (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. C, HCC cells were treated for 48 h (Huh7) or 72 h (HepG2) with 5 μM Sorafenib and/or 60 μM OA (white bars) in the presence or absence of 50 μM α-Tocopherol and 300 μM MnTBAP (black bars). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. Mean and SD of three independent experiments performed in triplicate are shown; $^{**}P < 0.01$; $^{***}P < 0.001$.

Although the underlying mechanisms of the antitumor effects of OA remain largely unknown, several studies with various tumor types have stated that OA and its derivatives exert an inhibitory effect on tumor growth *in vitro* and *in vivo* and induce apoptosis [8,9,29–31]. Also, OA was reported to cause ROS generation in different cancer types [22,32,33]. In lung cancer cell lines, the synthetic OA derivate CDDO-Me has been described to induce endoplasmatic reticulum (ER) stress as well as upregulation of

death receptor-5 (DR5) and caspase activation [34]. In addition, OA and its synthetic derivatives were shown to activate the ERK pathway [8,32,35] and inhibition of ERK was shown to enhance the antitumor activity of OA in lung and pancreatic cancer cells, which was associated with increased ROS production [32]. In line with these findings, we show that combining subtoxic concentrations of OA, which do not yet stimulate ROS production, together with Sorafenib, which inhibits ERK [6,27], results in a significant increase of ROS production.

Several pathways of programmed cell death, including apoptosis and necroptosis, have been implicated during inflammation-associated tumorigenesis of HCC [36]. In prostate cancer Sorafenib has been reported to promote the interaction of p62 with RIP1 kinase leading to cell death by necroptosis [37]. Therefore, we investigated the role of necroptosis in Sorafenib/OA-induced cell death in HCC. However, so far we have no indication that Sorafenib/OA-induced cell death involves necroptosis, since inhibition of RIP1 kinase activity by Nec-1 failed to rescue HCC cells from Sorafenib/OA-induced cell death. In addition, RIP3 protein, another key component of necroptosis [38] and an inhibitor of inflammatory hepatocarcinogenesis [36], was not detectable by Western blotting in the HCC cell lines Huh7 and HepG2 [10]. Our findings do therefore not point to an involvement of necroptosis in Sorafenib/OA-induced cell death.

Another form of regulated cell death, which has been described in HCC, is ferroptosis occurs, for example, upon treatment with Sorafenib [16,17,39]. Ferroptosis involves iron-dependent accumulation of ROS and lipid peroxidation [19]. However, our data do not suggest that Sorafenib/OA-induced cell death is mediated by the increase of ROS from lipid peroxidation, as addition of Fer-1, which inhibits accumulation of ROS from lipid peroxidation [21], failed to consistently protect HCC cells from Sorafenib/OA-mediated cell death.

Sorafenib is since years in clinics [4] and its effects and toxicities are well known [5]. Also for OA, first clinical trials showed in lymphoma patients, a minimal toxicity (maximum orally tolerated dose 900 mg/day) and a good tumor response [9,40–42]. Furthermore, the antitumor activity and *in vivo* bioactivity of OA and its derivates were tested in different animal tumor models [9] and pharmacokinetic studies in humans were successfully performed [43,44]. Besides the antitumor effects of OA a hepatoprotective effect for acute hepatic damage and chronic liver disorders, e.g. viral hepatitis, is described [8,45]. We previously reported that the used concentration of OA exerted no detectable toxicity on a human hepatocyte cell line or primary human hepatocytes [10].

Target proteins of the Sorafenib/OA combination for induction of oxidative stress and its cascade for caspase activation and cell death have yet to be identified and investigated in further studies.

In the present study, we demonstrated for the first time that OA sensitizes HCC cells for Sorafenib. Since OA and its synthetic derivatives showed promising results in early clinical trials for the treatment of lymphoma [9,40,41], the Sorafenib/OA combination could be a new approach in the therapy of HCC.

Conflict of interest

None to declare.

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