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Combined 3-*O*-acetylbetulin treatment and carbonic anhydrase IX inhibition results in additive effects on human breast cancer cells

Marina Petrenko [a,*], Antje Güttler [a], Anne Funtan [b], Jacqueline Keßler [a], Daniel Emmerich [b], Reinhard Paschke [b], Dirk Vordermark [a], Matthias Bache [a]

[a] *Department of Radiotherapy, Martin Luther University Halle-Wittenberg, Ernst Grube Straße 40, D-06120, Halle, Germany*
[b] *Biozentrum, Martin Luther University Halle-Wittenberg, Weinbergweg 22, D-06120, Halle, Germany*

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ABSTRACT

Hypoxia plays a key role in tumor progression and resistance to radiotherapy. Expression of the transmembrane-tethered enzyme carbonic anhydrase IX (CA IX) is strongly induced by hypoxia. High CA IX expression levels correlate with poor prognosis in cancer patients. Previously, we showed that the downregulation of CA IX expression by siRNA interference and the inhibition of CA IX activity results in increased cytotoxicity, inhibition of migration and radiosensitization of hypoxic cancer cells. Betulinic acid (BA) is a natural compound derived from birch bark. It has shown promising anti-tumor effects due to its cancer cell specific cytotoxic properties. We have shown that BA inhibits the HIF-1α pathway, resulting in apoptosis, inhibition of migration and enhanced cytotoxicity of breast cancer cells. In this study, we investigate the effects of the novel betulin derivative 3-*O*-acetylbetulin (3-AC) and carbonic anhydrase inhibitors (CAI) octyl disulfamate (OCT) or 4-(3-[4-fluorophenyl] ureido)benzenesulfonamide (SLC-0111), on cellular and radiobiological parameters in MDA-MB-231 and MCF-7 cells. Treatment with 3-AC or OCT alone only caused moderate cytotoxicity, reduction in cell migration, ROS production and DNA damage. However, the combined treatment with 3-AC and CAI strongly enhanced radiosensitivity, increased cytotoxicity, inhibited cell motility and enhanced DNA damage. Our findings suggest that the combination of two bioactive drugs 3-AC and a CAI, such as OCT or SLC-0111, could be a promising therapeutic approach for targeting hypoxic tumor cells.

1. Introduction

Breast cancer is the most common malignancy in women worldwide [1]. Treatment consists of surgery either alone or in combination with radiation and/or chemotherapy and/or hormonal therapy [2]. Additionally, targeted therapy and multimodal therapy concepts are promising strategies in anticancer therapy [3]. However, the survival of metastatic disease is still limited [4]. It is generally accepted that tumor hypoxia is a negative prognostic factor in cancer therapy. Hypoxia contributes to cancer cell invasiveness and migration [5,6], chemoresistance and radioresistance, angiogenesis and metastasis [7]. Consequently, there is a need to further identify novel agents that sensitize hypoxic tumor cells to chemotherapy and/or radiotherapy.

Endogenous hypoxia markers, such as hypoxia-inducible factor 1 (HIF-1), are often overexpressed in breast cancer. An extensive meta-analysis of approximately 3000 breast cancer patients showed that increased HIF-1α protein levels are correlated with shortened overall survival [8]. Another endogenous hypoxia marker is carbonic anhydrase IX (CA IX), which is strongly regulated by HIF-1 [9]. A recent study suggested that CA IX is an excellent marker for the detection of tumor hypoxia in breast cancer cells *in vitro* and *in vivo* [10]. At the cell surface of solid tumors, carbonic anhydrases (CAs) catalyze the reversible hydration of carbon dioxide (CO_2) into a proton (H^+) and bicarbonate (HCO_3^-) and therefore maintain extracellular and intracellular pH balance [11,12]. As the hypoxia-induced enzyme CA IX is known to contribute to extracellular acidification, overexpression of CA IX is correlated with poor prognosis in patients, cell aggressiveness and cell migration [13,14]. Recently, evidence has arisen that cells cultured in acidic media display radiation resistance [15]. Several studies have proven an enhanced therapeutic effect of tumor irradiation after

* Corresponding author.
E-mail addresses: marina.petrenko@uk-halle.de (M. Petrenko), antje.guettler@uk-halle.de (A. Güttler), anne.funtan@biozentrum.uni-halle.de (A. Funtan), jacqueline.kessler@uk-halle.de (J. Keßler), daniel.emmerich@biozentrum.uni-halle.de (D. Emmerich), reinhard.paschke@biozentrum.uni-halle.de (R. Paschke), dirk.vordermark@uk-halle.de (D. Vordermark), matthias.bache@uk-halle.de (M. Bache).

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inhibition of CA IX in colorectal carcinoma, kidney carcinoma and breast cancer cells [16–20]. Hence, inhibition of CA IX is a possible anticancer strategy aimed at enhancing radiosensitivity.

Sulfonamide- or sulfamate-based drugs have been described as effective carbonic anhydrase inhibitors (CAIs) and have been used in different investigations [21]. We recently evaluated the effects of betulin-based CAIs with sulfamate groups on cell and radiobiological parameters in breast cancer cells. Our findings indicated that the drugs induced apoptosis, inhibited cell migration, arrested the cell cycle and increased radiosensitivity [22,23].

Betulinic acid (BA), a pentacyclic triterpene (Fig. 1 A), is a promising antineoplastic agent that induces apoptosis in melanoma cells and in different tumor entities [24,25]. Moreover, treatment with BA results in reduced migration and has a growth-inhibitory effect. It should be emphasized that BA has been found to have increased cytotoxicity in cancer cells compared to normal cells and therefore provides tumor selectivity [26]. Combined treatment with irradiation and BA has also been illustrated to have additive effects on radiosensitivity in tumor cells [27–29]. This observation is supported by our results revealing enhanced cytotoxicity and radiosensitivity in glioma cells under hypoxia [30]. Further studies have confirmed the effectiveness of BA treatment in human breast cancer cells *in vitro* and in animal models [23,31–34]. Therefore, BA delivery targeted to hypoxic regions might be a useful adjunct in combination with CA IX inhibition as a breast cancer treatment. However, application of BA is limited due to its poor solubility. Therefore development of derivatives with better solubility but equal cytotoxicity is on focus of research. Addition of functional groups (for example sulfamate) increases polarizability and therefore solubility of the derivatives [35].

3-*O*-acetylbetulin (3-AC, Fig. 1 B) is a precursor for the synthesis of betulin derivatives, for example for 3-acetoxybetulin 28-sulfamate, which is a cytotoxic compound with anti-tumor characteristics [22,23]. Therefore, in the present study, we observed the cellular and radiobiological effects of the betulin derivative 3-AC and the CA IX inhibitors octyl disulfamate (OCT, Fig. 1 C) or 4-(3-[4-fluorophenyl]ureido)benzenesulfonamide (SLC-0111, Fig. 1 D) in breast cancer cells under normoxic and hypoxic conditions.

In addition, OCT (K_i (hCA IX) = 4.0 nM [36]) has two sulfamate groups at the end of the chain and provides effective CA IX inhibition [36]. SLC-0111 is a well-characterized CAI (K_i (hCA IX) = 45.1 nM [37]) and is currently tested in Phase Ib/II clinical trials [38]. The characteristics of hypoxic tumor cells are high aggressiveness with a low rate of apoptosis and functions resulting in the acidification of the microenvironment. Thus, the aim of the combination of 3-AC and CAI was to target different tumor-specific characteristics under hypoxic conditions: first, activating cytotoxicity and cell death with 3-AC and second, inhibiting CA IX with CAI. Furthermore, we wanted to investigate the impact of the combination of 3-AC, CAI and radiation on hypoxic tumor cells.

In this light, our study demonstrates that targeting the hypoxic cell populations and CA IX simultaneously increases the radiosensitivity of breast cancer cells while reducing cancer cell survival. We show that the combination of 3-AC and OCT or SLC-0111 causes cytotoxicity by increasing the intracellular ROS levels, increasing the DNA damage and cell migration and improving the radiosensitivity of the breast cancer cell lines MCF-7 and MDA-MB-231. Consequently, our study supports the therapeutic rationale of targeting both tumor hypoxia and CA IX activity in order to improve the outcome of radiotherapy and thus patient survival.

2. Material and methods

2.1. Cell lines, treatment with 3-AC and/or OCT or SLC-0111, irradiation

The human breast cancer cell lines MDA-MB-231 and MCF-7 (obtained from Prof. Dr. Jürgen Dittmer, Department of Gynecology, Martin Luther University Halle-Wittenberg, Halle, Germany) were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (Lonza, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Karlsruhe, Germany), 1% sodium pyruvate (Invitrogen, Karlsruhe, Germany), 185 U/mL penicillin (Invitrogen, Karlsruhe, Germany) and 185 g/mL streptomycin (Invitrogen, Karlsruhe, Germany). Hypoxia (<0.1% O_2) was achieved using a gas generator system (BD, Franklin Lakes, USA). The chemical drugs 3-*O*-acetylbetulin (3-AC) and octyl disulfamate (OCT) were kindly provided by BioSolutions (Halle, Germany). 4-(3-[4-Fluorophenyl]ureido)benzenesulfonamide (SLC-0111) was obtained from R & D Systems (Minneapolis, MN, USA). The structures are shown in Fig. 1. All of the compounds were dissolved in dimethyl sulfoxide (DMSO) to achieve a 20 mM stock solution. Cells were seeded in cell culture flasks 24 h before treatment; the cells were treated with 3-AC and/or OCT for 24 h at 37 °C under normoxic or hypoxic conditions and were subsequently irradiated. Irradiation was performed using 6 MV photons and adequate bolus material on a linear accelerator (Siemens ONCOR, Erlangen, Germany)

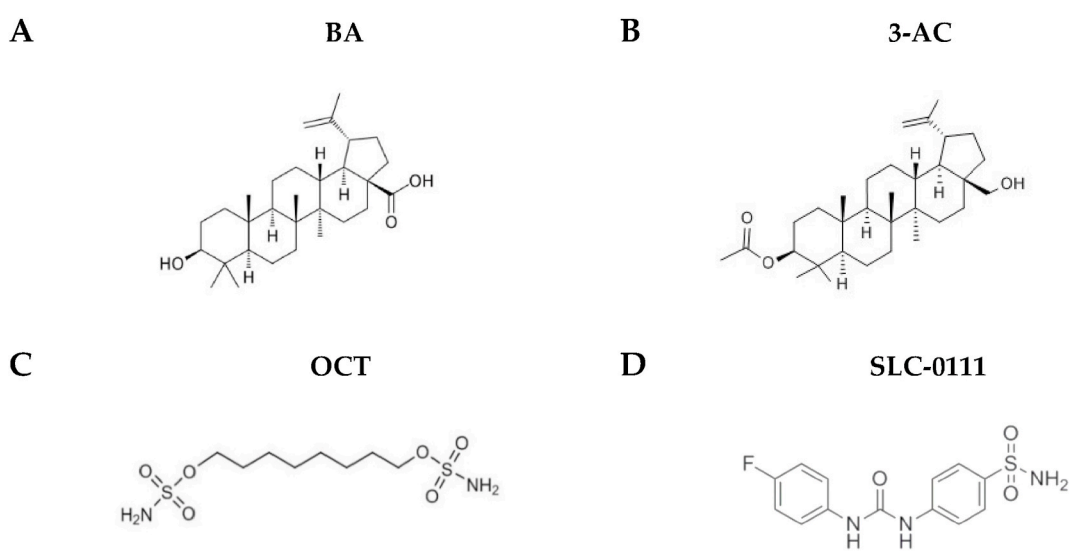

Fig. 1. Structures of (**A**) betulinic acid (BA), (**B**) 3-*O*-acetylbetulin (3-AC), (**C**) octyl disulfamate (OCT), and (**D**) 4-(3-[4-fluorophenyl]ureido)benzenesulfonamide (SLC-0111).

at a dose rate of 2 Gy/min.

2.2. SRB assay

The cytotoxicity of the drugs was determined by sulforhodamine B (SRB) assay as previously described [23]. Exponentially growing cells were seeded into 96-well plates. After 24 h, the cells were treated with a dilution series of 3-AC and/or OCT (0–40 μM) for 72 h. Further, cells were fixed with 10% trichloroacetic acid at 4 °C for 1 h and washed with H_2O. After the addition of SRB, the plates were washed with 1% acetic acid and dried. A 20 mM Tris base solution was added, and the absorbance was measured at 540 nm using a plate reader (TECAN GENios, Männedorf, Switzerland). Relative cell survival was determined based on 100% survival of DMSO-treated cells.

2.3. Wound-healing assay

Cells were grown in 24-well plates until confluence and were treated 2 h with 10 μg/mL mitomycin c prior to wounding. After wounding the cells with a 200 μl-tip and washing them twice with PBS, medium containing 20 μM 3-AC and/or OCT was added. To evaluate the migration rate, the initial wound area and the final wound area after a 16 h incubation time were measured from photographs using Adobe Photoshop CS6.

2.4. Clonogenic survival assay and radiosensitivity assay

Cells were treated with 50 μM 3-AC and/or 100 μM SLC-0111 under hypoxia for 24 h, irradiated (0 Gy, 2 Gy, 6 Gy, 10 Gy), and trypsinized. Then, 150–5000 cells were plated based on the optimal plating density.

Cells were cultured in RPMI supplemented with 10% FBS in a humidified atmosphere of 3% CO_2 at 37 °C. The cells were allowed to form colonies over 12–14 days, which were then fixed with paraformaldehyde (Sigma, Deisenhofen, Germany) and stained with 10% Giemsa solution (Sigma, Deisenhofen, Germany). Only colonies of >50 cells were scored to determine the surviving fraction (SF), or the ratio of the number of colonies formed after irradiation with 2 Gy, 6 Gy or 10 Gy to the number of colonies formed in unirradiated controls. The dose modification factor (DMF) was defined as the ratio of the surviving fraction of DMSO-treated control cells to that of 3-AC and/or SLC-0111-treated cells and was dependent on the dose of irradiation. The data represent the results of at least three independent experiments.

2.5. ROS

For measurement of intracellular ROS levels, cells were plated in 24-well plates for 24 h. After treatment with 20 μM 3-AC and/or OCT under normoxia or hypoxia for 24 h, the cells were incubated with the indicator CM-H2DCFDA (Thermo Fisher, Schwerte, Germany) at a concentration of 0.5 μM in PBS (complemented with $CaCl_2$ and $MgCl_2$) for 30 min at 37 °C. The cells were irradiated with 0 Gy or 4 Gy, and after washing with PBS, the cells were incubated with trypsin-EDTA (0.5%) (Life Technologies, Carlsbad, USA) for 3 min at 37 °C. Trypsin was decanted, and the cells were detached with PBS. Fluorescent ROS signals were measured with an LSRFortessa™ flow cytometer (BD Biosciences, Heidelberg, Germany).

2.6. γH2AX assay

For analysis of DNA damage, cells were plated in 8-well slides (Ibidi, Martinsried, Germany) for 24 h. The cells in the chambers were treated with 20 μM 3-AC and/or OCT under normoxia or hypoxia for 24 h and irradiated with 0 Gy or 4 Gy. The cells were again incubated for 24 h before analysis of residual γH2AX foci. After fixation with 4% formaldehyde (Sigma, Steinheim, Germany) for 20 min, the cells were washed with PBS. Permeabilization was performed with 0.5% Triton X-100/PBS for 15 min, and nonspecific binding was blocked with 1% BSA in PBS for 1.5 h. After incubation with a primary antibody against p-histone H2AX (S139) overnight at 4 °C and an Alexa Fluor 488-conjugated secondary antibody (both from Cell Signaling Technology, Danvers, USA) for 1 h, cell nuclei were stained with DAPI. After fluorescence microscopy, evaluation of the number of γH2AX foci per cell nucleus was performed with ImageJ (version 1.4.3.67).

2.7. Statistical analysis

Statistical analysis was performed by two-way ANOVA (Dunnet's test). Classification of significant results was based on a p-value of <0.05 in comparison with DMSO-treated cells.

3. Results

3.1. Effects of 3-AC and/or OCT on cytotoxicity and cell migration

On the basis of immunoblotting, CA IX expression in the breast cancer cell lines MDA-MB-231 and MCF-7 was examined under normoxic and hypoxic conditions (Fig. 2). Both cell lines showed hypoxia-dependent CA IX expression. To investigate drug-induced cytotoxicity, SRB screenings were performed, and cell survival was determined (Table 1). Treatment with 3-AC or OCT caused moderate effects. After incubation for 72 h with 40 μM 3-AC, cell survival values of 50.95% in MDA-MB-231 cells and 63.51% in MCF-7 cells (67.21% and 73.73%, respectively, for the cell lines after incubation with OCT) were obtained. The combination of 40 μM 3-AC and OCT exerted a profound effect, with significant cell survival rates of 44.88% ($p = 0.008$) in MDA-MB-231 cells and 51.64% ($p = 0.03$) in MCF-7 cells.

The influence of 3-AC and/or OCT on migration was assessed using a wound-healing assay under normoxic conditions (Fig. 3). A substantial reduction in migration activity was observed in both cell lines. Compared with DMSO-treated control cells, cells of both cell lines incubated with single drugs showed slight decreases in cell migration. The strongest inhibition of cell motility was triggered by treatment with both drugs. The combination of 3-AC and OCT caused a decrease in migration activity to 67.1% of control levels ($p = 0.002$) in MDA-MB-231 cells and to 55.4% of control levels in MCF-7 cells, therefore showing additional effects beyond those of single-drug treatment (Fig. 3).

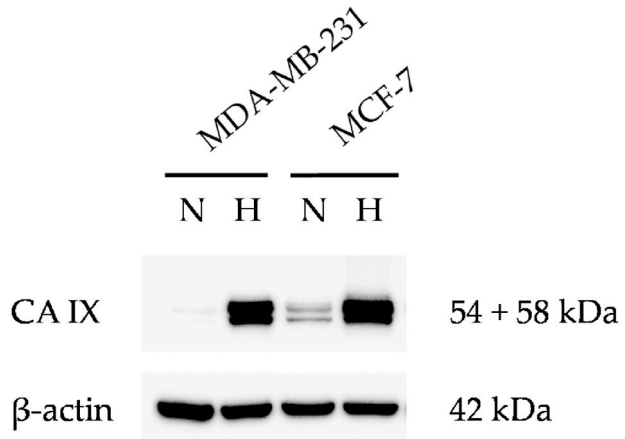

Fig. 2. CA IX expression in cell lines MDA-MB-231 and MCF-7 was confirmed under normoxia and hypoxia using immunoblotting. β-Actin was used as a loading control.

Table 1
The relative cell survival values (%) after 20 μM and 40 μM 3-AC, OCT or combination of 3-AC and OCT treatment were measured by using an SRB assay after an incubation period of 72 h in MDA-MB-231 cells and MCF-7 cells under normoxia (21% O_2). The data represent the average values (+SD) of four independent experiments. The significance level is based on *p*-values (*$p \leq 0.05$; **$p \leq 0.001$) compared to DMSO-treated cells.

	MDA-MB-231		MCF-7	
	20 μM	40 μM	20 μM	40 μM
3-AC	86.52 ± 12.6	50.95 ± 7.3	83.02 ± 5.5	63.51 ± 12.3
OCT	85.24 ± 31.3	67.21 ± 20.6	76.63 ± 19.3	73.73 ± 25.5
3-AC + OCT	**61.28*** ±3.3	**44.88**** ± 4.2	71.72 ± 7.8	**51.64*** ± 3.6

3.2. Effects of 3-AC and/or OCT on reactive oxygen species (ROS) generation and DNA damage

We further investigated ROS formation after treating cells with 3-AC and/or OCT by measuring the conversion of CM-H_2DCFDA to 5.6-chloromethyl-2.7-dichlorofluorescein. Interestingly, both cell lines showed 2- to 3-fold higher ROS levels when treated under hypoxia than when treated under normoxic conditions (data not shown). However, MDA-MB-231 cells achieved higher ROS levels than MCF-7 cells, especially under hypoxic conditions (data not shown). Under hypoxia, a significant 2.5-fold ($p \leq 0.05$) increase in ROS induction was observed after treatment with 3-AC compared to DMSO treatment in MDA-MB-231 cells, and 3-AC-treated MCF-7 cells showed 1.8-fold ($p = 0.06$) higher ROS generation than DMSO-treated control cells (Fig. 4). A lower effect was shown after incubation with OCT (1.7-fold elevation in MDA-MB-231 cells and 1.3-fold elevation in MCF-7 cells). Compared to control treatment, treatment with a combination of 3-AC and OCT under hypoxia caused a 2.3-fold ($p = 0.14$) increase in ROS levels in MDA-MB-231 cells and a 1.7-fold ($p = 0.13$) increase in ROS levels in MCF-7 cells (Fig. 4). Therefore, incubation with the combination of drugs caused no additional increases in ROS formation.

To assess whether 3-AC and/or OCT could induce cellular DNA damage, we performed a γH2AX assay (Fig. 5). Essentially, there were more γH2AX foci/cell after treatment under normoxic conditions than under hypoxic conditions (Fig. 5). In MDA-MB-231 cells, incubation with 3-AC or OCT induced DNA damage when compared to DMSO treatment. This was independent from oxygenation status (Fig. 5 A and B). The combination of 3-AC and OCT induced the highest numbers of γH2AX foci/cell in MDA-MB-231 cells (0 Gy: 4.75, normoxia). Similar outcomes of treatment with 3-AC, OCT or the combination of both drugs were observed in MCF-7 cells (Fig. 5 C and D). For example, OCT-treated MCF-7 cells showed higher numbers of γH2AX foci/cell (0 Gy: 6.41, normoxia) than 3-AC-treated MCF-7 cells (0 Gy: 2.80, normoxia). With 9.77 foci/cell, cells treated with the combination of both drugs displayed enhanced formation of γH2AX foci (0 Gy, normoxia).

In both cell lines, additional irradiation with 4 Gy resulted in increases in the number of γH2AX foci/cell, independent of treatment or

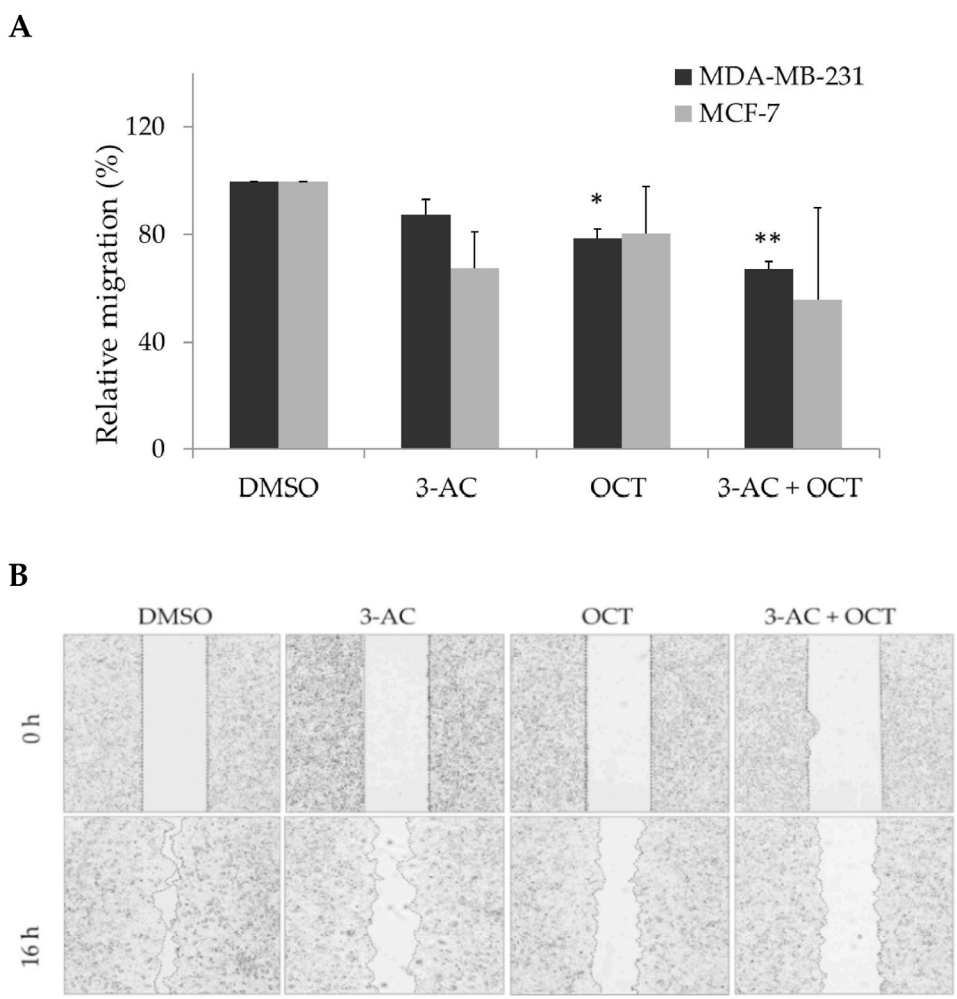

Fig. 3. The influence of 3-AC, OCT or combination 3-AC and OCT treatment on wound closure was measured in (**A**) MDA-MB-231 and MCF-7 cells using a wound-healing assay. (**B**) Microscopy analysis of MDA-MB-231 cells was conducted. The data represent the average values (+SD) of three independent experiments. *$p < 0.05$ and **$p < 0.01$.

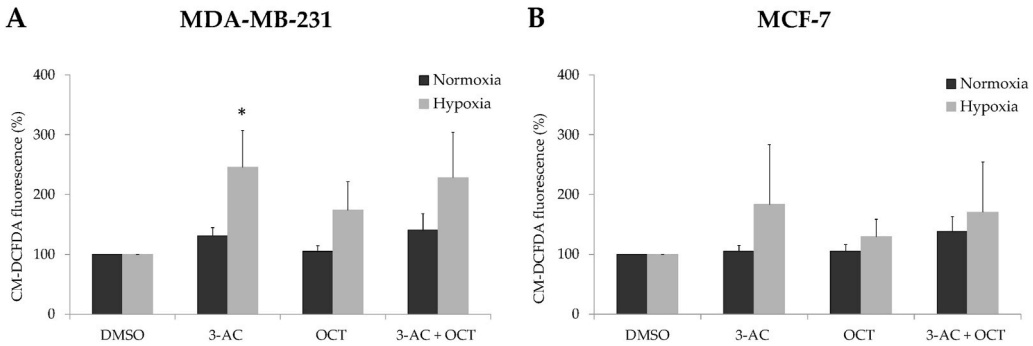

Fig. 4. The level of intracellular ROS was measured by CM-DCFDA fluorescence after treatment with 3-AC, OCT or a combination of 3-AC and OCT in (**A**) MDA-MB-231 cells and (**B**) MCF-7 cells under normoxia (21% O_2) and hypoxia (0.1% O_2). The data represent the average values (+SD) of three to four independent experiments. *$p < 0.05$.

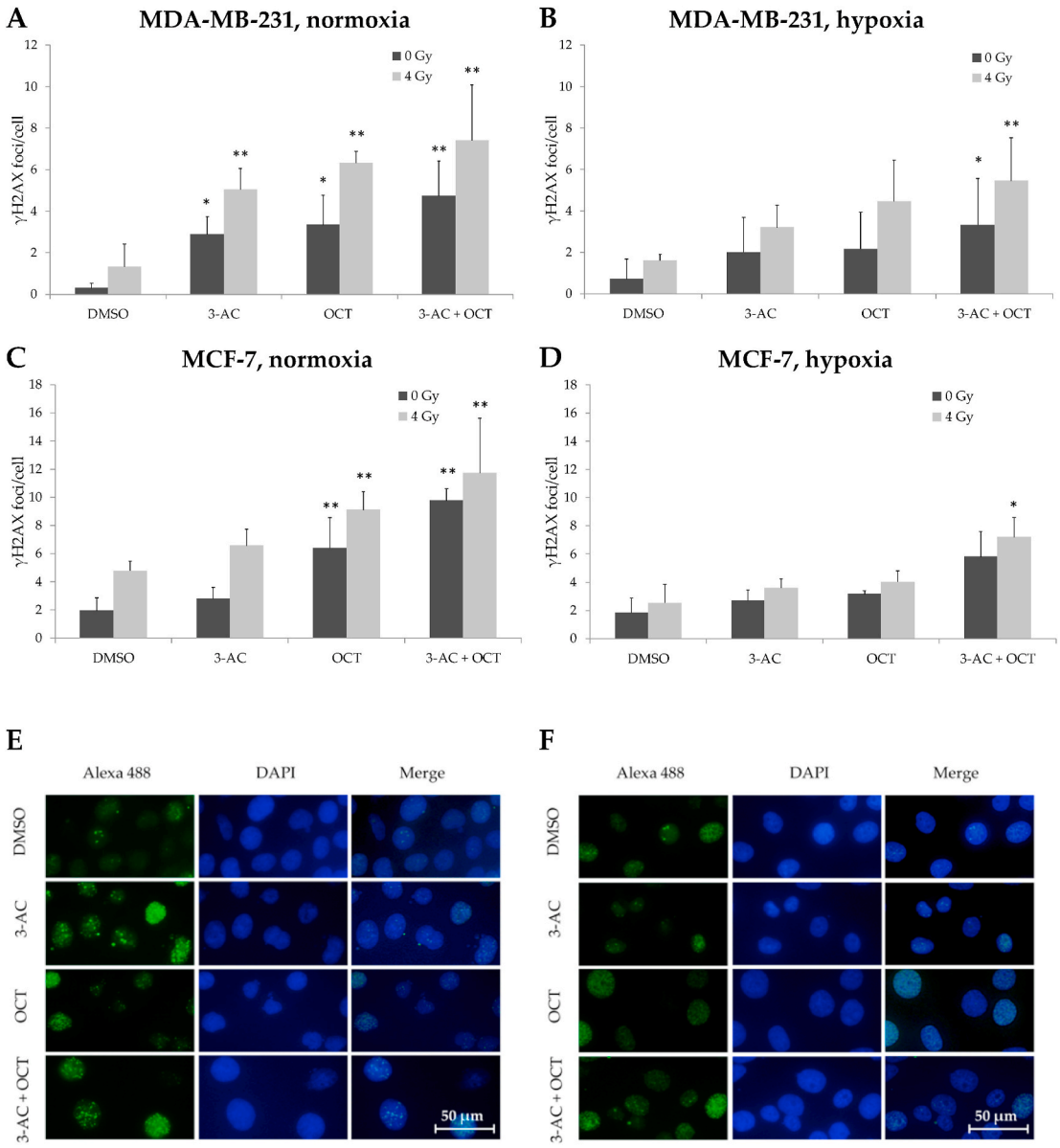

Fig. 5. Effects of 3-AC, OCT or the combination of 3-AC and OCT on the accumulation of residual γH2AX foci after irradiation with 0 Gy or 4 Gy were measured in (**A** and **B**) MDA-MB-231 cells and (**C** and **D**) MCF-7 cells under normoxia (21% O_2) and hypoxia (0.1% O_2). The data represent the average values (+SD) of three to four independent experiments. *$p < 0.05$ and **$p < 0.01$. In (**E**) and (**F**), representative staining of γH2AX in MDA-MB-231 cells after treatment with 3-AC, OCT or the combination of 3-AC and OCT under normoxia (21% O_2) and hypoxia (0.1% O_2) is shown. Green: γH2AX foci; blue: cell nuclei (DAPI).

oxygenation status (Fig. 5). Fig. 5 E & F shows representative staining of γH2AX foci and cell nuclei in the MDA-MB-231 cell line under normoxia and hypoxia.

3.3. Effects of 3-AC and/or the CAI SLC-0111 on radiosensitivity

CAI SLC-0111 was further used to investigate the effects of combined treatment with 3-AC, CAI and irradiation on the radiosensitivity of breast cancer cells under hypoxic conditions.

Treatment with only 3-AC or SLC-0111 had moderate effects on clonogenic survival in both cell lines (Fig. 6 C & E: 0 Gy). An enhanced reduction in clonogenic survival was detected after incubation with the combination of 3-AC and SLC-0111 to 44.3% of control levels ($p \leq 0.05$) in MDA-MB-231 cells and to 38.0% of control levels ($p = 0.03$) in MCF-7 cells. In addition to treatment with 3-AC and/or SLC-0111, cells were irradiated with 2 Gy, 6 Gy or 10 Gy (Fig. 6 C and E). Incubation with only 3-AC or SLC-0111 had no significant impact on radiosensitivity in MDA-MB-231 cells (3-AC: $DMF_{10} = 1.07$, $p = 0.54$; SLC-0111: $DMF_{10} = 1.0$, $p = 0.97$) or MCF-7 cells (3-AC: $DMF_{10} = 1.21$, $p = 0.30$; SLC-0111: $DMF_{10} = 1.18$, $p = 0.03$). However, the combination of 3-AC and SLC-0111 significantly enhanced the radiosensitivity of MDA-MB-231 cells ($DMF_{10} = 1.46$, $p = 0.09$) and MCF-7 cells ($DMF_{10} = 1.63$, $p = 0.02$) (Fig. 6 D & F).

4. Discussion

Recently, advances have been made regarding the use of natural compounds, including BA, to improve tumor treatment [39]. Our

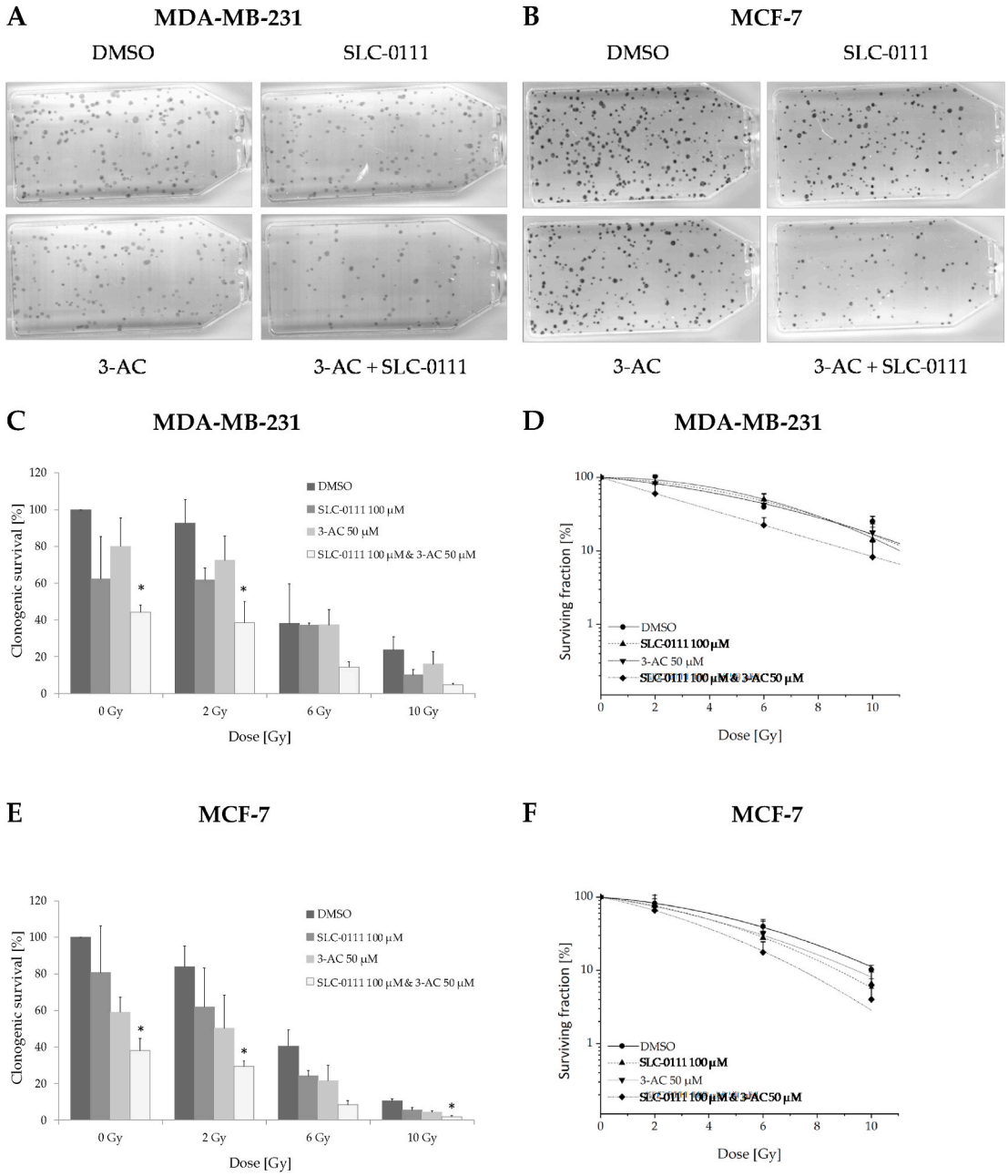

Fig. 6. The images of MDA-MB-231 cells (**A**) and MCF-7 cells (**B**) display colony formation in 25 cm^2 flasks after treatment with DMSO, 3-AC and/or SLC-0111 (0 Gy). The effect of 3-AC, SLC-0111 or the combination of 3-AC and SLC-0111 on clonogenic survival and radiosensitivity in (**C** and **D**) MDA-MB-231 cells and (**E** and **F**) MCF-7 cells after irradiation with 2 Gy, 6 Gy or 10 Gy under hypoxia (0.1% O_2) was tested. The data represent the average values (+SD) of three to four independent experiments. *$p < 0.05$.

previous studies revealed increased cytotoxicity and radiosensitivity in glioma cells after incubation with BA, particularly under hypoxia [30]. Investigations with betulin derivatives have shown that these agents enhance cytotoxicity, decrease cell migration and induce apoptosis without additional effects under hypoxic conditions [40]. Sulfamates and sulfonamides are considered effective CAIs and can also inhibit hypoxia-induced CA IX [21]. Inhibition of the CA IX pathway reduces hypoxia-induced extracellular acidosis, invasion and metastasis in various tumors. However, moderate cytotoxicity has also been observed in different cell lines after treatment with CAIs in multiple studies [16,41,42]. Recently, betulinyl-based CAIs have been found to enhance cytotoxicity and apoptosis, inhibit migration and increase radiosensitivity [22,23]. This is the first study to validate a combined therapy of a betulin derivative and a CAI. We investigated the effects of the betulin derivative 3-AC; a CAI, namely, OCT or SLC-0111; and the combination of 3-AC and CAI on cellular and radiobiological parameters in two breast cancer cell lines.

We have shown particularly that incubation with 3-AC or OCT alone induced moderate cytotoxic effects in both breast cancer cell lines (Table 1 & Fig. 6 C–F). In accordance with our findings, moderate cytotoxicity and cell line specificity have also been observed in other cell lines after treatment with sulfonamides in independent studies [41,42]. In agreement with this, Ivanova and colleagues reported reductions in the self-renewal capacity of breast cancer cells and synergistic effects after treatment with doxorubicin and the CAI acetazolamide [43]. Moreover, there have been investigations indicating that CA IX knockdown enhances the effect of bevacizumab treatment, reducing tumor growth rates *in vivo* [44].

Upon evaluating additional anticancer properties of 3-AC and/or OCT, we observed a slight reduction in cell migration after single-drug treatment. By contrast, incubation with a combination of 3-AC and OCT had stronger inhibitory effects on cell migration in both cell lines than incubation with any single agent (Fig. 3). BA-induced inhibition of cell migration has been proven in previous studies in glioblastoma cells and vascular smooth muscle cells [30,45]. Additionally, the BA derivatives B10 and NVX-207 have an inhibitory effect on cell migration in glioblastoma cells [40]. Moreover, betulinyl-based CAIs similarly effectively inhibit cell motility in breast cancer cells [23]. Our results appear consistent with those regarding the effects of other CAIs in MDA-MB-231 cells under normoxic and hypoxic conditions [41]. The CAI S4 also shows a significant inhibitory effect on cell migration in MDA-MB-231 cells *in vivo* [46]. Moreover, migration and metastasis can be reduced after benzenesulfonamide treatment in 4T1 cells *in vivo* [37]. Due to the additive effects of the drugs, the combination of 3-AC and OCT is suitable to strengthen the inhibition of migration in breast cancer cells.

Sedlakova and colleagues noted that a high expression level of CA IX causes tumor acidification and radiotherapy resistance [47]. Other studies have reported radiosensitization resulting from CA IX inhibition. There is inhibition of tumor growth in LS174Tr cells in mice after *CA 9* and *CA* 12 shRNA-mediated knockdown and irradiation [18]. However, few studies have investigated the influence of CA IX inhibition on radiosensitivity. In this study, we found enhanced radiosensitivity in MDA-MB-231 and MCF-7 cells after incubation with 3-AC and the CAI SLC-0111 (Fig. 6B and D). While treatment with 3-AC or SLC-0111 resulted in no or only slight radiosensitization, the results after treatment with a combination of 3-AC and SLC-0111 suggested enhanced radiosensitivity in both cell lines. We have previously verified that combined treatment with BA and irradiation results in additive effects in glioblastoma cells, particularly under hypoxic conditions [30]. In previous studies, we used the CAI SLC-0111 as an effective radiosensitizer in breast cancer cells under hypoxic conditions [48]. We have also tested betulinyl sulfamates, which act as radiosensitizers in combination with irradiation in breast cancer cells under hypoxic conditions [23]. Based on other findings, incubation with BA increases the radiosensitization and suppresses the growth and colony-forming ability of melanoma cells [29]. Furthermore, it has been shown that BA and irradiation additively inhibit cell survival in head and neck squamous cell carcinoma [28]. In comparison, the use of the betulin derivatives B10 and NXV-207 also causes slight radiosensitization in glioblastoma cells [40]. Further treatment with other CAIs (11c and AZA) and therefore inhibition of the CA IX pathway also causes enhanced radiosensitivity [20]. However, combination of the nitroimidazole based sulfonamide DH348 and irradiation did not change radiosensitivity *in vitro* but *in vivo* in HT29 colon carcinoma cells [19]. Moreover, the CAI FC9403A in combination with irradiation has a radiosensitizing effect on breast cancer spheroids [16]. Taken together, these data suggest that the combination of a betulin derivative and a CAI with irradiation constitutes a promising approach for improving radiosensitization in breast cancer cells.

Considering the effects of 3-AC and CAI on cytotoxicity (alone or in combination), migration and radiation, we analyzed ROS formation and DNA damage with or without the addition of radiation. Our data indicate that 3-AC- or OCT-treated cells have slightly increased intracellular ROS production under hypoxia compared to control cells. However, combined treatment with 3-AC and OCT had no additional effect. ROS formation has been detected in several cancer cell lines treated with BA [49,50]. Additionally, Yu and colleagues determined that BA induces ROS generation, which inhibits EBV replication through SOD2 suppression, in a lymphoblastoid cell line [51]. Further studies have illustrated increased ROS levels after treatment with the BA derivative 2c in HT-29 cells [52]. In accordance with these findings, another BA derivative (B5G9) has recently been found to increase ROS levels in HepG2 cells in a time-dependent manner [53]. There have been no studies concerning CAI-induced ROS generation thus far. We observed a slight increase in ROS levels after OCT treatment (Fig. 4).

Interestingly, we determined that there were higher ROS levels under hypoxia than under normoxia in both breast cancer cell lines (Fig. 4). It is clear that ROS levels are dependent on oxygen content. Previous studies have shown decreased ROS formation under normoxia [54], while some reports indicate that ROS generation is induced under hypoxia [55–58].

Additionally, we recorded increases in residual γH2AX foci that occurred in a concentration- and dose-dependent manner after treatment with 3-AC and/or OCT and irradiation in both cell lines. Although incubation with the combination of 3-AC and OCT induced the highest amount of DNA damage among the treatments, it has to be emphasized that 3-AC and OCT affected the formation of γH2AX foci even without irradiation. Different analyses have suggested that there is increased formation of γH2AX foci after treatment with the triterpenoid ganoderic acid [59]. Notably, triterpenoids have many biological and pharmacological properties, including anti-inflammatory and anti-HIV properties [60]. A previous study claimed that pentacyclic triterpenoids, such as oleanolic acid and pomolic acid, may have inhibitory effects on DNA polymerase β. This enzyme is responsible for DNA repair after exposure to damage-causing agents, such as cisplatin [61,62]. One area of interest for further study may be examination of whether BA plays a role in DNA polymerase β inhibition.

ROS and DNA damage do not necessarily show the same effects. Bell and colleagues submitted a report indicating that there is no relationship between ROS generation and DNA damage [63]. However, we are one of the few research groups who have observed DNA damage with the formation of γH2AX foci after treatment with a CAI. Recently, the accumulation of γH2AX in MCF-7 cells was detected with flow cytometry after incubation with a naphthalenesulfonamide agent [64]. Thus, 3-AC, OCT, or, in particular, the combination of these two drugs can be an effective treatment for DNA repair. In summary, betulin derivatives cause increased ROS formation, whereas CAIs influence DNA damage: combined treatment with these two agents results in increased cytotoxicity and radiosensitivity under hypoxia.

5. Conclusion

In conclusion, the betulin derivative 3-AC and the CAI OCT moderately affect cytotoxicity, cell migration, ROS production and DNA damage in human breast cancer cells when used separately. Intriguingly, compared to single-drug treatment, combined treatment with the betulin derivative and a CAI had stronger cytotoxic effects, caused greater inhibition of cell migration, and, in particular, mediated greater radiosensitivity. Given these findings, combined treatment with a betulin derivative and a CAI is a promising therapeutic approach for hypoxic tumors. Currently, there are no *in vivo* studies or clinical trials regarding the two used drugs. Consequently, the combination of 3-AC and OCT should be tested in animal tumor models.

Future studies have great potential to elucidate the applicability of combination therapy involving a betulin derivative and a CAI alongside radiation treatment.

Author contributions

D.V., M.B., and R.P. designed the study; A.G. and M.P. performed the experimental procedures; M.B., A.G., and M.P. analyzed the data and reviewed the manuscript; R.P., A.M., and D.E. synthesized the chemical drugs; J.K. contributed to the methods; and M.P. wrote the paper. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Sample CRediT author statement

Dirk Vordermark, Matthias Bache and Reinhard Paschke: study design; Antje Güttler and Marina Petrenko: experimental procedures; Matthias Bache, Antje Güttler and Marina Petrenko: data analysis and manuscript revision; Reinhard Paschke, Anne Funtan and Daniel Emmerich: drug synthesis; Jacqueline Keßler: method contribution; Marina Petrenko: writing, reviewing and editing. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marina Petrenko, Antje Güttler, Anne Funtan, Jacqueline Keβler, Daniel Emmerich, Reinhard Paschke, Dirk Vordermark, Matthias Bache.

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