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MSBA-S – A pentacyclic sulfamate as a new option for radiotherapy of human breast cancer cells

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ABSTRACT

Many pentacyclic triterpenoids show anti-cancer and anti-inflammatory properties. Recently, we detected a pronounced cytotoxicity and radiosensitivity of two betulinyl sulfamates in human breast cancer cells. Besides betulinic acid scaffold (BSBA-S), we synthesized several new sulfamate-coupled scaffolds from oleanolic acid (OSBA-S), ursolic acid (USBA-S), platanic acid (PSBA-S) and maslinic acid (MSBA-S). Highest cytotoxicity was monitored in breast cancer cell lines after MSBA-S treatment showing in SRB assays IC_{50} values between 3.7 μM and 5.8 μM. Other sulfamate/triterpene conjugates, however, were less cytotoxic holding IC_{50} values between 6.6 μM and >50 μM, respectively. MSBA-*S*-treated breast cancer cells displayed significantly reduced clonogenic survival and an increased rate of apoptosis as compared to the other conjugates. In addition, MSBA-S in combination with irradiation resulted in effects on radiosensitivity in MDA-MB-231 cells ($DMF_{10} = 1.14$). In particular, ROS formation was strongly assessed in MSBA-*S*-treated breast cancer cells. Our findings suggest that the sulfamate derivative of maslinic acid MSBA-S might be a new option for the radiation therapy in breast cancer cells.

1. Introduction

Breast cancer is one of the commonly diagnosed cancer types and provides the second highest cancer-related mortality rate in women [1]. The four classified distinct subtypes, such as luminal A or B, Her 2-positive (Her2+), and basal-like of breast cancer cell lines provide different response to treatment and outcome [2]. Especially the triple-negative breast cancers (TNBC) that lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 are associated with an adverse prognosis [3,4]. The diagnosis of TNBC ensures important implications for the choice of systemic therapies. Thus, standard breast cancer treatment consists of surgery, radiotherapy, chemotherapy, hormonal therapy and/or immunotherapy [5,6]. Radiotherapy is a conventional treatment method and is systematically performed as it decreases the rate of local recurrence and mortality. Since therapy is often limited and burdened with side-effects, it is essential that multi-targeted and effective therapies are developed to enhance chances for poor prognosis especially in TNBC. To reduce side-effects and improve the outcome of radiotherapy, combination with pharmacologically relevant therapeutic agents is of utmost importance.

For a long time, natural drugs have comprised a wide spectrum of medicinal and pharmacological activities. Due to its multi-targeting advantage in drug development and noteworthy anti-cancer properties, pentacyclic triterpenes came in the focus of scientific interest.

Naturally occurring pentacyclic triterpenoids such as ursolic acid (UA), betulin, betulinic acid (BA), oleanolic acid (OA), platanic acid (PA), or maslinic acid (MA) and derivatives thereof exhibit pharmacological benefits (Fig. 1). Notably, modification on positions C-2 and C-28 of the pentacyclic triterpenoidic skeleton resulted in profound efficiency and enhanced cytotoxicity, whereas the introduction of ester substituents at C-3 gave compounds of higher bioavailability, and C-28-amides held improved the solubility and higher cytotoxicity [7]. Anti-cancer properties of several pentacyclic triterpenes and their derivatives have been described

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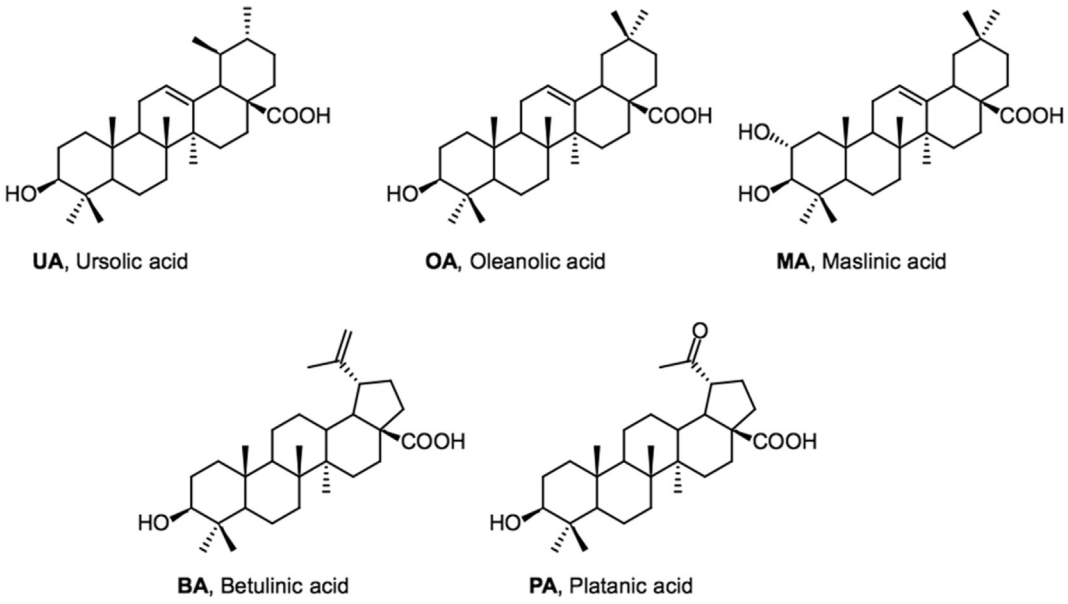


Fig. 1. Structures of used pentacyclic triterpenoic acids.

in vitro as well as *in vivo*: ursolic [8–10], betulinic [11–13], oleanoic [14,15], platanic [16,17] or maslinic acid [18,19]. Besides induction of apoptosis, triterpenes also modulate the tumor environment causing anti-angiogenic, anti-inflammatory, anti-oxidative, cardioprotective as well as anti-viral, anti-bacterial and anti-fugal effects [20,21]. In addition, terpenoids and derivatives provide inhibition of cell proliferation, induction of tumor cell death by targeting different genes and multiple pathways, such as Bcl-2, NF-κB, STAT3, TNF, angiogenesis, PI3K/Akt/mTOR, and TLR [22,23].

Previously, a few pentacyclic sulfamate triterpenes have been examined as carbonic anhydrase inhibitors (CAIs) [24–26]. Based on their structure, these compounds were capable to inhibit tumor-associated carbonic anhydrases (CA), such as hCA II, hCA IX and hCA XII. Thereby, sulfamates and sulfonamides bind directly to the zinc ion in the centre in the enzymes' active site [27]. Initial own studies showed anti-cancer benefits after incubation of pentacyclic sulfamate triterpenes, resulting in enhanced cytotoxicity and induction of apoptosis in different tumor entities as compared to their parent compounds [26,28,29]. Furthermore, betulinyl sulfamates revealed inhibition of migration and enhanced radiosensitivity [29]. Further, our very recent study suggested that combination of a betulin-derived triterpene and a CAI resulted in additive cytotoxicity and radiosensitization on breast cancer cells [30]. As a consequence, to identify effective pentacyclic sulfamate triterpenes with a balance of impairing tumor-selective drugs together with enhanced radiosensitization was called for.

In this light, we synthesized and investigated cell- and radiobiological effects of new pentacyclic sulfamate triterpenes in breast cancer cell lines.

2. Results and discussion

2.1. Synthesis of the triterpenoid sulfamates

We aimed to investigate the anti-cancer effect and radiosensitivity of the a small series of newly synthesized pentacyclic sulfamate triterpenes. As the presence of a benzylamide moiety at position C-28 was described to provide higher cytotoxic effects as well as increased tumor-selectivity compared to esters, benzylamides were chosen as cytotoxic parent compounds for further syntheses to access even more effective anti-tumor active triterpene derivatives [31]. Previous investigations already showed the cytotoxicity of BA to be greatly enhanced (concerning the proliferation of MCF-7 cells) by using a conjugate at position C-28 thus providing access to compounds holding low IC_{50} values [32]. Moreover, C-2/C-3-sulfamates of triterpenes have been reviewed as effective and competetive inhibitors of CA II [25].

To access the target structures **16**–**20**, parent triterpenoidic oleanolic acid, ursolic acid, betulinic acid, platantic acid and maslinic acid (Fig. 1) were acetylated to yield acetates **1**–**5** (Sch. 1). These acetates were activated with oxalyl chloride followed by the addition of benzyl chloride to give amides **6**–**10**, whose deacetylation furnished **11**–**15**. Reaction of these compounds with sulfamoyl chloride finally gave target compounds **16**–**20**, respectively (Sch. 2).

2.2. Effects of the sulfamates *16*–*20* on cytotoxicity

Betulinyl sulfamates have been found to enhance cytotoxicity in comparison to BA in cancer cells [26,29]. To determine the drug-induced cytotoxicity, SRB screenings were performed with breast cancer cell lines (Table 1). Treatment with OSBA-S (**16**) and PSBA-S (**19**) caused lowest cytotoxic effects with IC_{50} values varying from 9.3 μM to ≥ 50 μM. Incubation of BSBA-S (**18**) and USBA-S (**17**) resulted in moderate cytotoxicity of the IC_{50} values, varying from 6.6 μM to 26.9 μM in the breast cancer cell lines.

However, IC_{50} values of BA varied from 13 μM in MCF-7 breast cancer cell line and 18 μM in MDA-MB-231 cells in our recently

Table 1
Cytotoxicity of the compounds shown by IC_{50} values (μM) from SRB assays after an incubation period of 96 h in one basal-like breast cancer cell line (MCF-7) and three TNBC cells (HS578T, MDA-MB-231, BT-20). Cut-off was set to 50 μM. The data represent the average values (±SD) of at least three independent experiments.

compound	HS578T	MDA-MB-231	MCF-7	BT-20
BSBA-S	15.6 ± 2.5	23.3 ± 2.8	6.6 ± 1.6	26.9 ± 6.9
OSBA-S	15.8 ± 2.2	≥50	≥50	16.2 ± 6.4
USBA-S	18.8 ± 6.4	15.2 ± 7.1	7.5 ± 3.2	16.2 ± 4.0
PSBA-S	16.9 ± 4.5	≥50	9.3 ± 2.5	32.0 ± 4.5
MSBA-S	3.8 ± 2.4	3.7 ± 0.8	4.9 ± 1.9	5.8 ± 1.6

submitted work (under review). Derivatization at position C-28 for drug targets BSBA-S (**18**), USBA-S (**17**) and PSBA-S (**19**) brought about enhanced cytotoxicity in MCF-7 cells, whereas these modifications did not result in additive cytotoxic effects on the TNBC cell lines. Notably, the MA derivative was found to be toxic for tumor cell lines holding the lowest IC_{50} values as compared to other triterpenoids [25,28]. In particular, MSBA-S (**20**) caused the strongest effects on all cell lines in our study. Only MSBA-S (**20**) assessed strong cytotoxic effect on all breast cancer cell lines, with IC_{50} values from 3.7 μM to 5.8 μM.

This results parallel previous findings for triterpenoids structurally modified at positions C-2/C-3/C-28 [28]. Moreover, this study revealed strong cytotoxicity of sulfamate derived from maslinic acid with IC_{50} values from 2.2 μM to 4.0 μM for different cancer cells.

In conclusion, BSBA-S (**18**), USBA-S (**17**) and PSBA-S (**19**) exerted enhanced cytotoxicity, especially in MCF-7 breast cancer cells. Incubation of all four cell lines with MSBA-S (**20**) resulted in strong cytotoxicity, with rather low IC_{50} values.

2.3. Effects of the sulfamates **16**–**20** on clonogenic cell survival

In agreement with the SRB assay, highly significant impact on the reduction of the clonogenic survival was monitored after incubation of MSBA-S (**20**) in TNBC HS578T cells up to 16.5% ($p < 0.0001$) and in MDA-MB-231 cells up to 42.1% (Fig. 2). For the other sulfamates decreased effects were observed showing reduction levels of the clonogenic survival levels of 73.1%–81.3% in HS578T cells and of 80.6%–84.5% in MDA-MB-231 cells, respectively. For comparison, incubation of the betulinyl CAIs on breast cancer cells resulted in similar inhibition of the clonogenic survival and cytotoxic results within the SRB-Assay in our previous work [29].

We also observed an inhibition of cell migration of 21% after incubation with BSBA-S (**18**) with the wound healing assay in MDA-MB-231 cells (data not shown). In agreement with these findings, BA and betulinyl sulfamates caused strong inhibition of migration in MDA-MB-231 and MCF-7 cells as well [29]. Interestingly, OA has been found to increase MDA-MB-231 human breast cancer cell migration and hence to show promising properties for wound healing [32]. The inhibitory effect on cell migration might be induced in a cell-specific pattern.

2.4. Effects of the sulfamates **16**–**20** on cell death

On account of the strong effect of MSBA-S (**20**) on the clonogenic survival in TNBC cells, further investigations were focused on cell death analyses. Amides derived from BA, OA, UA, MA as well as from PA have been shown in several studies to exhibit anti-cancer activities by evoking apoptosis in human tumor cells [12,17,32,33]. Significant increase of secondary necrosis inducted by triterpenes was perceived in human tumor cells as well [31].

DAPI staining (Fig. 3 A and B) showed that apoptosis of the breast cancer cells was elevated after treatment with the cells with the sulfamates showing the typical morphologic shrinkage hallmark of the cell nuclei (Fig. 3C). Incubation with OSBA-S (**16**), PSBA-S (**19**), USBA-S (**17**) and BSBA-S (**18**) had moderate effects on cell death in the HS578T cell line with 3.5%–7.1% apoptotic cells. MSBA-S (**20**) assessed strong significant effect with 22.1% ($p = 0.03$) apoptotic cells. MDA-MB-231 cells showed lower effects after application of PSBA-S (**19**), USBA-S (**17**) and BSBA-S (**18**) with 2.1%–6.7% apoptotic cells. Moderate effects were gained after incubation with OSBA-S (**16**) and MSBA-S (**20**) with 10.5% and 8.9% apoptotic cells.

Quantification of vital (Q3), necrotic (Q1), late apoptotic (Q2) and apoptotic (Q4) cells was gained by flow-cytometry using Annexin V and propidium iodide (PI) staining (Fig. 4 A). Annexin V analysis (Fig. 4 B and C) was performed. BSBA-S (**18**) triggered moderate effects in HS57T cells, with 0.8% necrotic cells, 2.8% apoptotic and 12.1% late apoptotic cells. MSBA-S (**20**) caused strong cell death with 2.6% necrotic cells, 8.7% apoptotic and 44.4% late apoptotic cells, respectively. However, in MDA-MB-231 cells, the compounds had moderate effects (BSBA-S: 0.5% necrotic cells, 1.1% apoptotic and 4.8% late apoptotic cells; MSBA-S: 1.6% necrosis, 7.4% apoptosis and 18.4% late apoptosis).

Besides slight effects after incubation with the other pentacyclic sulfamate triterpenes, BSBA-S (**18**) and especially MSBA-S (**20**) treatment resulted in strongest impact on the TNBC cells. Previously, it could be shown that betulinyl sulfamates caused apoptosis, mediated by activation of caspases [26,29]. This parallels our results, showing induced cell death (Fig. 3) after incubation of the pentacyclic triterpene derivatives and late apoptosis after application of MSBA-S (**20**) in the Annexin V-PI assay (Fig. 4). In accordance with our results, betulinyl triterpenes caused necrosis and apoptosis, respectively [26,34].

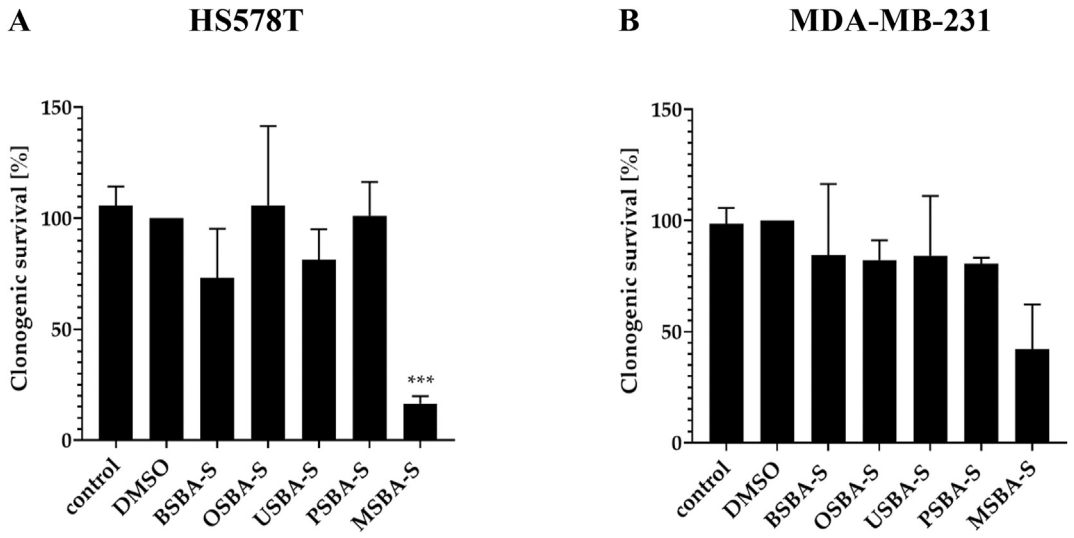


Fig. 2. Clonogenic survival after 24 h treatment with the sulfamates BSBA-S OSBA-S, USBA-S, PSBA-S and MSBA-S in TNBC cells (**A**) MDA-MB-231 and (**B**) HS578T. The data represent the average values (+SD) of three independent experiments; ***$p < 0.0001$.

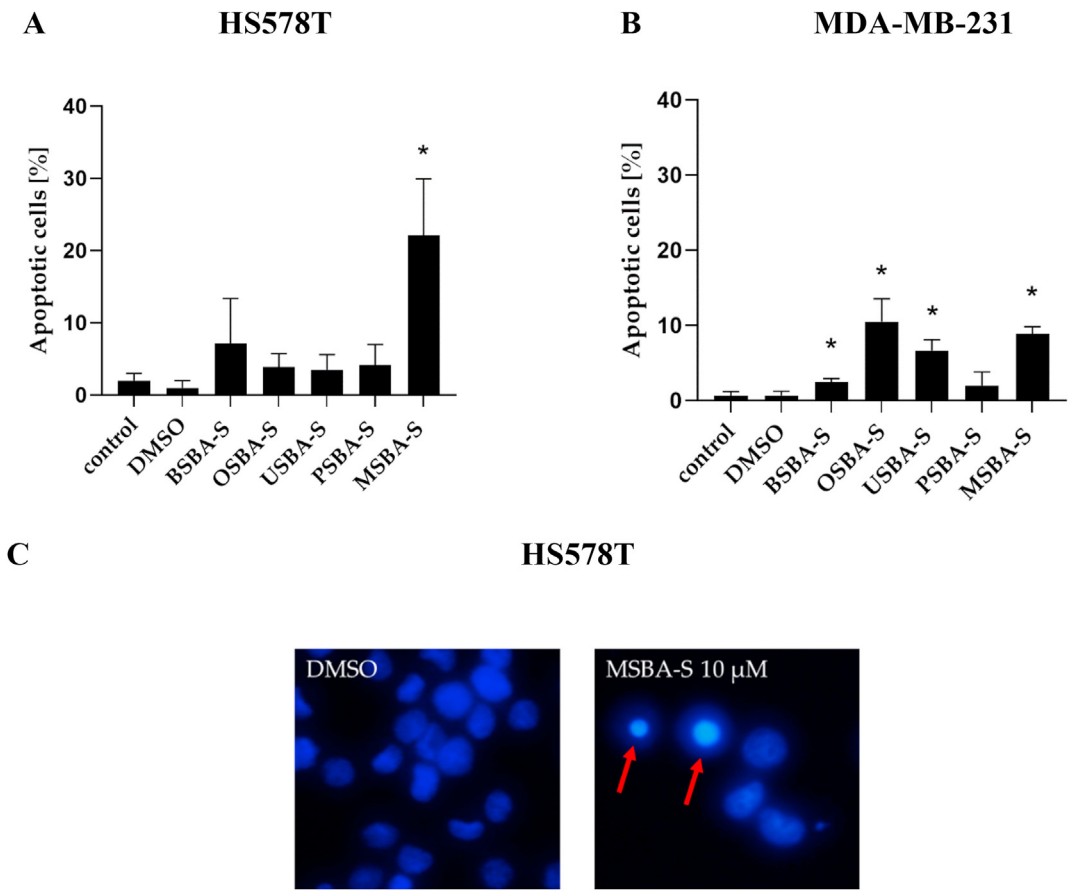


Fig. 3. Apoptotic cell death analysis (DAPI) in TNBC cells (**A**) HS578T and (**B**) MDA-MB-231 after 72 h treatment with BSBA-S OSBA-S, USBA-S, PSBA-S and MSBA-S. **C** displays typical apoptotic cell nuclei in HS578T cells after 72 h treatment with MSBA-S. The data represent the average values (+SD) of three independent experiments; *p < 0.05.

*2.5. Effects of the sulfamates **16**–**20** on reactive oxygen species (ROS) generation*

Another triterpene derivative was found to significantly suppress cell proliferation, invasion and migration in human tumor cells. Highlighting, it induced ROS-mediated ER-stress dependent apoptosis in human tumor cells [32].

ROS formation was detected after treatment of cells with BSBA-S (**18**) and MSBA-S (**20**). This effect was measured by the conversion of CM-H_2DCFDA to 5,6-chloromethyl-2,7-dichlorofluorescein. The drugs caused increased ROS formation in the two TNBC cell lines HS578T and MDA-MB-231 (Fig. 5). Compared to DMSO treatment, treatment with BSBA-S (**18**) resulted in a 2.7-fold ($p = 0.005$) significant increase in ROS levels in MDA-MB-231 cells and a 2.8-fold increase in ROS levels in HS578T cells. Significant 3.1-fold ($p = 0.01$) increase in ROS induction was detected after treatment with MSBA-S (**20**) in HS578T cells and a 5.7-fold increase in MDA-MB-231 cells was observed in comparison to DMSO-treated cells.

Highest ROS formation was achieved after treatment with MSBA-S (**20**). Studies revealed ROS induction after incubation of BA on tumor cells [35,36]. In addition, betulinyl triterpene derivatives contributed to the ROS formation in tumor cells as well [30,37,38]. However, few studies have been examined ROS formation after treatment with triterpenes. Our own study indicate enhancement of radiosensitivity in association with ROS formation [30].

*2.6. Effects of the sulfamates **16**–**20** on radiosensitivity*

Sensitivity of radiotherapy to carcinoma cells was previously found to be increased by a several dammarane-type triterpenes [32]. Radiosensitization was also observed in a rat model after combined application of OA [15]. There have been studies with BA and betulinyl triterpene derivatives, displaying enhanced radiosensitivity after incubation of the drugs [34,39,40]. Furthermore, radiosensitization was detected after incubation with betulinyl sulfamates and irradiation in human breast cancer cells under hypoxic conditions [29].

However, there is a lack of MA or MA derivatives studies of radiosensitivity so far. In our analysis, in addition to treatment with MSBA-S (**20**), cells were irradiated (Fig. 6). Incubation with MSBA-S (**20**) had impact on radiosensitivity in MDA-MB-231 cells with the $DMF_{10} = 1.14$ ($p = 0.13$). The association of ROS formation and radiosensitivity was observed.

Eventually, our findings suggest that application of MSBA-S (**20**) resulted in increased apoptosis and ROS formation and slight radiosensitization under hypoxic conditions in particular in TNBC cells. There is a need for further investigations of the effect of MSBA-S (**20**) on tumor behaviour *in vivo*.

3. Conclusion

In conclusion, our findings suggest that application of a substantial lower concentration of MSBA-S (**20**) in comparison to the other pentacyclic sulfamate triterpenes results in an increased cytotoxicity, significant reduction of clonogenic survival, apoptosis induction and increased ROS formation in TNBC cell lines. Additionally, enhanced radiosensitivity was shown in MDA-MB-231 cells. The other potential natural compounds OSBA-S (**16**), USBA-S (**17**), PSBA-S (**19**) and BSBA-S (**18**) have been simultaneously well been characterized but showed only marginal effects

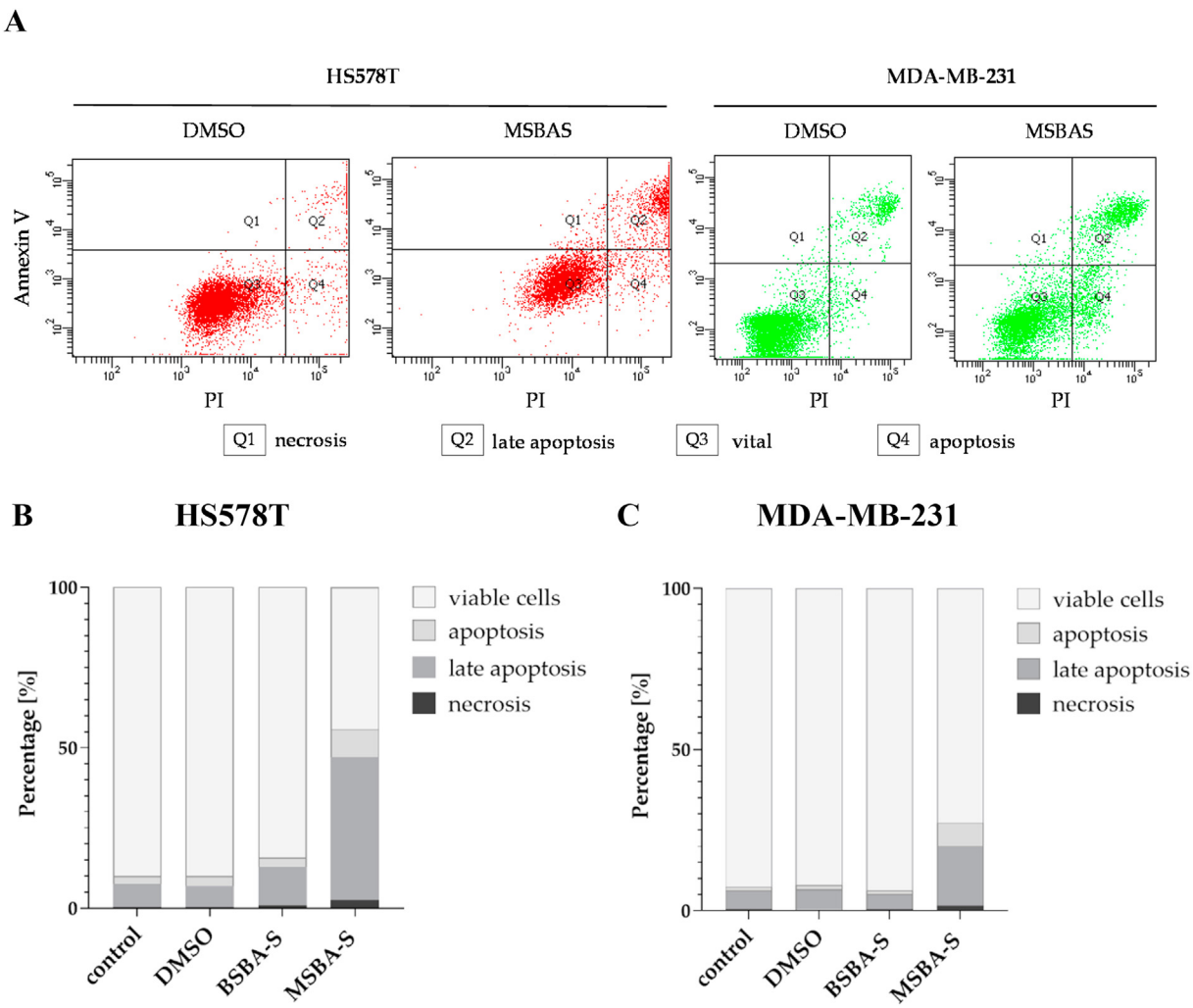


Fig. 4. Analysis of Annexin V-PI staining in TNBC cells (**B**) HS578T and (**C**) MDA-MB-231 after 24 h incubation of BSBA-S or MSBA-S. **A** displays flow cytometry analysis of Annexin V-PI staining and the classification of vital (Q3), necrotic (Q1), late apoptotic (Q2) and apoptotic (Q4) cells.

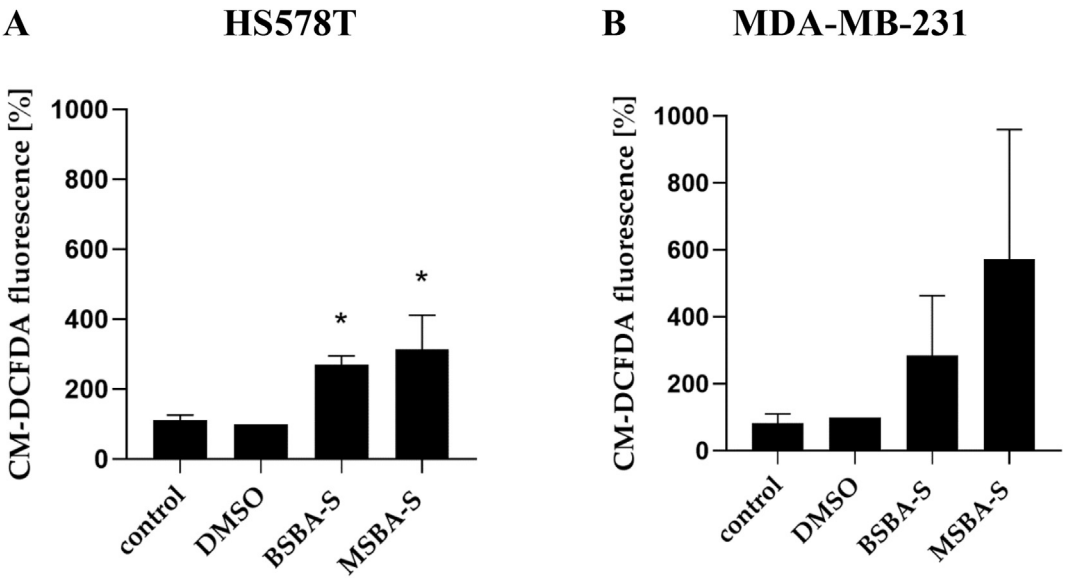


Fig. 5. ROS formation in TNBC cells (**A**) HS578T and (**B**) MDA-MB-231 after 24 h treatment with BSBA-S and MSBA-S. The data represent the average values (+SD) of three independent experiments; *p < 0.05.

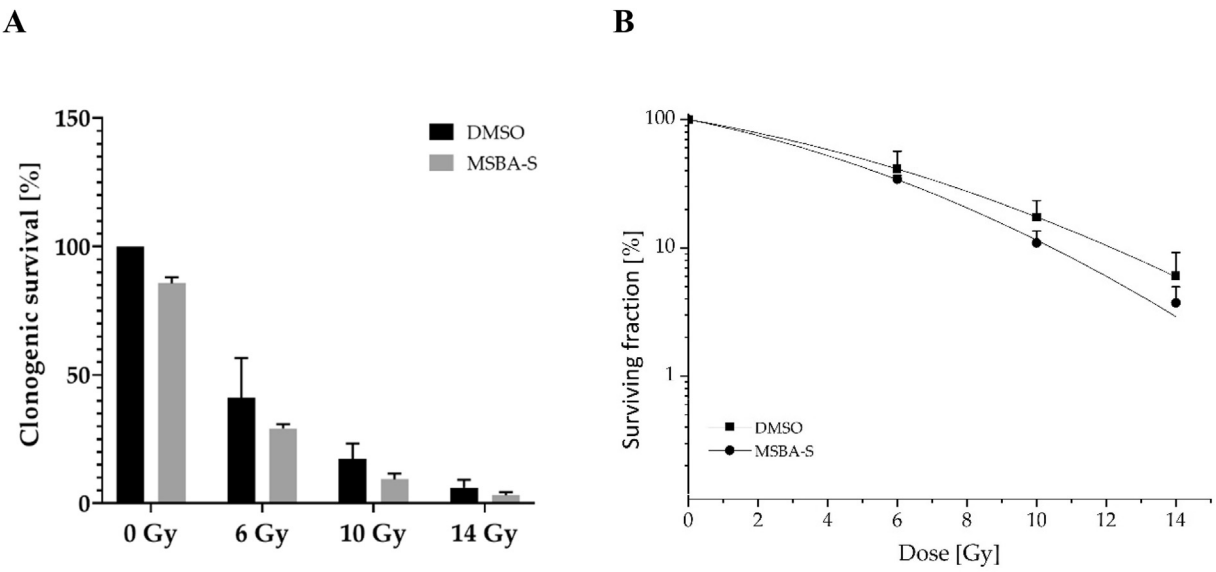


Fig. 6. The effects of MSBA-S on radiosensitivity in TNBC cells MDA-MB-231 after irradiation with 6 Gy, 10 Gy and 14 Gy under hypoxia (0.1% O_2) (**A** and **B**). The data represent the average values (+SD) of three independent experiments.

on cellular parameters. MSBA-S (**20**) might be considered as a potential anti-cancer drug in breast cancer cell lines due to its parameters being analyzed in this study.

4. Experimental

4.1. Syntheses

4.1.1. General

Melting points are uncorrected (Leica hot stage microscope or Büchi M – 565), NMR spectra were recorded using the Agilent spectrometers VNMRS or DD2 (δ given in ppm, J in Hz, internal Me_4Si or residual solvent peaks; typical experiments: H–H–COSY, HMBC, HSQC, NOESY, DQF-COSY), MS spectra were taken on a Finnigan MAT LCQ 7000 (electrospray, voltage 4.1 kV, sheath gas nitrogen) instrument; ASAP-MS spectra were taken on an Advion expression^L CMS-L mass spectrometer (source voltage: 77 V, APCI corona discharge: 4.2 μA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N_2). Optical rotations were measured on a JASCO P-2000 instrument. TLC was performed on silica gel (Merck 5554, detection with cerium molybdate reagent); IR spectra were recorded on a PerkinElmer Spectrum Two (UATR Two Unit). The solvents were dried according to usual procedures. Triterpenoic acids were obtained from "Betulinines" (Stříbrná Skalice, Czech Republic) and used as received.

4.1.2. General procedure of acetylation (GPA)

To a solution of the triterpenoic acid (OA, UA, BA, PA, MA, 1 equiv.) in dry DCM, acetic anhydride (3 equiv.), triethylamine (3 equiv.) and DMAP (C-at.) were added, and the mixture was stirred at 20 °C for 1 day. Usual aqueous work-up followed by recrystallization from ethanol furnished products **1**–**5**.

4.1.3. 3β-Acetyloxy-olean-12-en-oic acid (*1*)

Following GPA, compound **1** (2.45 g, 89 %) was obtained from OA as a colorless solid; $R_f = 0.70$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m. p. 287–290 °C (lit:[4, 5] 281–283 °C); $[\alpha]_D = +68.7°$ (*c* 0.25, $CHCl_3$) [(lit:[4, 5] $[\alpha]_D = +74.1°$ (*c* 0.4, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 499.1 ($[M+H]^+$), 516.2 (34 %, $[M + NH_4]^+$, 521.4 [30 %, $[M+Na]^+$.

4.1.4. 3β-Acetyloxy-urs-12-en-oic acid (*2*)

Following GPA, compound **2** (2.41 g, 89 %) was obtained from UA as a colorless solid; $R_f = 0.55$ (*n*-hexane/ethyl acetate, 3:1); m. p. 280–283 °C (lit:[6] 280–285 °C); $[\alpha]_D = +66.1°$ (*c* 0.35, $CHCl_3$) [lit:[6] [α]D = +65.59° (*c* 0.33, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 499.2 (11 %, $[M+H]^+$), 521.3 (35 %, $[M+H]^+$), 1019.2 (100 %, $[2\ M + Na]^+$).

4.1.5. 3β-Acetyloxy-lup-20(29)-en-28-oic acid (*3*)

Following GPA, compound **3** (4.90 g, 90 %) was obtained from BA as a colorless solid; $R_f = 0.59$ (*n*-hexane/ethyl acetate, 4:1); m. p. 276–278 °C (lit:[6] 282–285 °C); $[\alpha]_D = +20.4°$ (*c* 0.30, $CHCl_3$) [(lit:[6] $[\alpha]_D = +20.98°$ (*c* 0.33, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 487.0 (30 %, $[M - H]^-$, 995.1 (100 %, $[2M - H]^-$; 1018.4 (31 % $[2M-2H + Na]^-$).

4.1.6. 3β-Acetyloxy-20-oxo-30-norlupan-28-oic acid (*4*)

Following GPA, compound **4** (6.9 g, 84 %) was obtained from PA as a colorless solid; $R_f = 0.51$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10.5); m. p. 267–269 °C (lit:[7] 252–255 °C); $[\alpha]_D = -9.3°$ (*c* 0.30, $CHCl_3$) [(lit: $[\alpha]_D = -9.5°$ (*c* 0.5, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 999.4 (100 %, $[2M - H]^-$).

4.1.7. (2α, 3β) 2,3-Bis(acetyloxy)-olean-12-en-28-oic acid (*5*)

Following GPA, compound **5** (1.1 g, 92 %) was obtained from MA as a colorless solid; $R_f = 0.32$ (*n*-hexane/ethyl acetate, 3:1); m. p. 223–226 °C (lit: 221–223 °C); $[\alpha]_D = +30.5°$ (*c* 0.5, $CHCl_3$) [(lit: $[\alpha]_D = +29.3°$ (*c* 0.3, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 557.4 (52 %, $[M+H]^+$), 1135.1 (100 %, $[2\ M + Na]^+$).

4.1.8. General procedure for the synthesis of acetylated benzyl amides (GPB)

To a solution of the acetylated triterpenoic acid 1–5 (1 equiv.) in dry DCM, DMF (cat.) and oxalyl chloride (4 equiv.) were added followed by the addition of benzyl amine (1.5 equiv.) After stirring for 1 h at 20 °C followed by usual aqueous work-up and column chromatography, products 6–10 were obtained.

4.1.9. 3β-Acetyloxy-N-benzyl-olean-12-en-28-oic amide (*6*)

Following GPC, compound **6** (1.1 g, 95 %) was obtained from **1** as a colorless solid; $R_f = 0.35$ (*n*-hexane/ethyl acetate, 7:1); m. p.

243–244 °C (lit:[6] 247–249 °C); $[\alpha]_D = +29.8°$ (*c* 0.33, $CHCl_3$), [(lit:[6] $[\alpha]_D = +30.66°$ (*c* 0.35, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 588.2 (90 %, $[M+H]^+$), 610.1 (22 %, $[M+Na]^+$), 1175.0 (46 %, $[2\ M + H]^+$), 1197.3 (100 %, $[2\ M + Na]^+$).

*4.1.10. 3β-Acetyloxy-N-benzyl-urs-12-en-28-oic amide (**7**)*

Following GPC, compound **7** (1.4 g, 94 %) was obtained from **2** as a colorless solid; $R_f = 0.69$ (*n*-hexane/ethyl acetate, 7:1); m. p. 143–145 °C (lit:[6] 145 °C), $[\alpha]_D = +18.4°$ (*c* 0.4, CHCl3), [(lit: $[\alpha]_D = +17.89°$ (*c* 0.35, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 588.0(43 %, $[M+H]^+$), 610.3 (20 %, $[M+Na]^+$), 1175.2 (66 %, $[2\ M + H]^+$), 1197.1 (74 %, $[2\ M + Na]^+$).

*4.1.11. 3β-Acetyloxy-N-benzyl-lup-20(29)-en-28-oic amide (**8**)*

Following GPC, compound **8** (2.1 g, 84 %) was obtained from **3** as a colorless solid; $R_f = 0.24$ (*n*-hexane/ethyl acetate, 7:1); m. p. 143–146 °C (lit:[6] 138–144 °C), $[\alpha]_D = +22.5°$ (*c* 0.37, $CHCl_3$), [(lit:[6] $[\alpha]_D = +22.19°$ (*c* 0.36, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 588.1 (40 %, $[M+H]^+$), 610.1 (23 %, $[M+Na]^+$), 1197.2 (100 %, $[2\ M + Na]^+$).

*4.1.12. 3β-Acetyloxy-N-benzyl-20-oxo-30-norlupan-28-oic amide (**9**)*

Following GPC, compound **9** (0.95 g, 88 %) was obtained from **4** as a colorless solid; $R_f = 0.38$ (*n*-hexane/ethyl acetate, 7:1); m. p. 291–293 °C (lit:[8] 290 °C), $[\alpha]_D = +2.2°$ (*c* 0.32, $CHCl_3$), [(lit:[8] $[\alpha]_D = +0.5°$ (c 0.159, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 590.4 (100 %, $[M+H]^+$), 612.4 (28 %, $[M+Na]^+$).

*4.1.13. (2α, 3β) Bisacetyloxy-N-benzyl-olean-12-en-28-oic amide (**10**)*

Following GPB compound **10** (0.8 g, 84 %) was obtained from **5** as a colorless solid; $R_f = 0.70$ (*n*-hexane/ethyl acetate, 7:3); m. p. 143–145 °C (lit:[9] 143–145 °C); $[\alpha]_D = -7.3°$ (*c* 0.25, CHCl3) [(lit:[9] $[\alpha]_D = -7.0°$ (*c* 0.32, CHCl3)]; MS (ESI, MeOH): *m/z* 646.5 (100 %, $[M+H]^+$), 668.4 (50 %, $[M+Na]^+$).

4.1.14. General procedure for deacetylation (GPC)

To a solution of compounds **6**–**10** (1.0 equiv.) in methanol, methanolic KOH (1.2 equiv, freshly prepared) was added; the mixture was stirred at 20 °C until completion of the reaction (as indicated by TLC). The mixture was poured into ice cold aq. HCl (3.7 %), the precipitate was collected, dried, and subjected to column chromatography.

*4.1.15. 3β-Hydroxy-N-benzyl-olean-12-en-28-oic amide (**11**)*

Following GPC, compound **11** (0.62 g, 87 %) was obtained from **6** as a colorless solid; $R_f = 0.82$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); $R_f = 0.28$ (toluene/EtOAc/n-heptane/HCOOH, 80:26:10:5); m. p. 248–250 °C (lit: 249–251 °C), $[\alpha]_D = +30.3°$ (*c* 0.32, $CHCl_3$), [(lit: $[\alpha]_D = +30.42°$ (*c* 0.33, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 546.1 (54 %, $[M+H]^+$), 568.3 (15 %, $[M+Na]^+$), 1091.0 (45 %, $[2\ M + H]^+$), 1113.5 (100 %, $[2\ M + Na]^+$).

*4.1.16. 3β-Hydroxy-N-benzyl-urs-12-en-28-oic amide (**12**)*

Following GPC, compound **12** (0.72 g, 87 %) was obtained from **7** as a colorless solid; $R_f = 0.70$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m. p. 266–269 °C (lit:[10] 267–268 °C), $[\alpha]_D = +32.0°$ (*c* 0.33, $CHCl_3$), [(lit:[6] $[\alpha]_D = +32.33°$ (*c* 0.34, $CHCl_3$)]; MS (ESI, MeOH): *m/z* = 546.1 (100 %, $[M+H]^+$), 568.5 (12 %, $[M+Na]^+$), 1091.2 (72 %, $[2\ M + H]^+$), 1114.4 (94 %, $[2\ M + Na]^+$).

*4.1.17. 3β-Hydroxy-N-benzyl-lup-20(29)-en-28-oic amide (**13**)*

Following GPC, compound **13** (0.82 g, 71 %) was obtained from **8** as a colorless solid; $R_f = 0.57$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m. p. 254–256 °C (lit:[11] 254–256 °C), $[\alpha]_D = +4.1°$ (*c* 0.40, $CHCl_3$), [(lit:[11] $[\alpha]_D = +2.0°$ (*c* 0.3, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 546.2 (30 %, $[M+H]^+$), 568.4 (10 %, $[M+Na]^+$), 1091.5 (30 %, $[2\ M + H]^+$), 1113.1 (100 %, $[2\ M + Na]^+$).

*4.1.18. 3β-Hydroxy-N-benzyl-20-oxo-30-norlupan-28-oic amide (**14**)*

Following GPC, compound **14** (0.82 g, 92 %) was obtained from **9** as a colorless solid; $R_f = 0.40$ (toluene/ethyl acetate/heptane/formic acid, 80:26:10:5); m. p. 266–268 °C (lit:[8] 266–267 °C), $[\alpha]_D = -6.1°$ (*c* 0.3, $CHCl_3$), [(lit:[8] $[\alpha]_D = -5.9°$ (*c* 0.33, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 548.3 (100 %, $[M+H]^+$), 1095.1 (50 %, $[2\ M + H]^+$), 1117.2 (70 %, $[2\ M + Na]^+$).

*4.1.19. (2α, 3β) Dihydroxy-N-benzyl-olean-12-en-28-oic amide (**15**)*

Following GPC, compound **15** (0.34 g, 89 %) was obtained from **10** as a colorless solid; $R_f = 0.15$ (toluene/ethyl acetate/heptane/formic acid, 80:25:30:4); m. p. 147–149 °C (lit:[9] 148–151 °C); $[\alpha]_D = +28.2°$ (*c* 0.20, $CHCl_3$), [(lit:[9] $[\alpha]_D = +29.0°$ (*c* 0.29, $CHCl_3$)]; MS (ESI, MeOH): *m/z* 562.3 (40 %, $[M+H]^+$, 1145.3 (100 %, $[2\ M + Na]^+$).

4.1.20. General procedure for the synthesis of the sulfamates (GPD)

4.1.20.1. Sulfamoyl chloride. At 0 °C to vigorously stirred chlorosulfonyl isocyanate (24.6 mmol, 3.48 g, 2.14 mL), formic acid (24.6 mmol, 0.93 mL) was added, and stirring was continued until the evolution of gases had ceased. The precipitate was filtered off and dissolved in benzene and filtered again. The filtrate was evaporated to dryness, and the product was obtained as a colorless solid (2.45 g, 86 %) being used for all subsequent reactions without any further purification. It can be kept under argon in the dark at 20 °C for at least 2 months without deterioration.

To an ice-cold solution of the triterpenoic amides **11**–**15** (0.64 mmol) in THF (30 mL), NaH (0.90 mmol) was added, and the suspension was stirred at 25 °C for 30 min. A freshly prepared solution of sulfamoyl chloride (1.02 mmol) in THF (2 mL) was added, and the mixture was stirred for 5 days at 25 °C. The solvent was removed under diminished pressure, and the residue portioned between ether (100 mL) and H_2O (100 mL). The organic layer was separated, washed with brine (100 mL), dried ($MgSO_4$), concentrated under diminished pressure, and the residue was purified by column chromatography (silica gel).

*4.1.21. 3β-Aminosulfonyloxy-N-benzyl-olean-12-en-28-oic amide (**16**)*

Following GPD, compound **16** (0.43 g, 83 %) was obtained from **11** as a colorless amorphous solid; $R_f = 0.17$ (0.17, *n*-hexane/ethyl acetate, 7:3); $[\alpha]_D = +28.1°$ (*c* 0.385, CHCl3); IR (ATR): ν = 3406w, 2946 m, 2879w, 1639 m, 1635 m, 1520 m, 1497 m, 1464 m, 1455 m, 1434w, 1364s, 1266w, 1210w, 1203w, 1178s, 1010w, 961 m, 910vs, 871 m, 836 m, 698 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.36–7.22$ (m, 5H, 33-H + 33'-H + 34-H + 34'-H + H-35), 6.18 (dd, $J = 6.4, 4.4$ Hz, 1H, H-amide), 5.30 (dd, $J = 3.6$ Hz, 1H, H-12), 4.87 (s, 2H, NH_2-sulfamate), 4.62 (dd, $J = 14.7, 6.3$ Hz, 1H, 31-H_a), 4.22 (dd, $J = 12.0, 4.6$ Hz, 1H, 3-H), 4.14 (dd, $J = 14.7, 4.4$ Hz, 1H, 31-H_b), 2.54 (dd, $J = 13.0, 4.4$ Hz, 1H, 18-H), 2.09–1.93 (m, 2H, 2-H_a + 22-H_a), 1.92–1.71 (m, 5H, 2-H_b + 11-H_a + 11-H_b + 15-H_a + 19-H_a), 1.71–1.49 (m, 6H, 22-H_b + 15-H_b + 1-H_a + 16-H_a + 6-H_a + 9-H), 1.49–1.31 (m, 3H, 7-H_a + 6-H_b + 21-H_a), 1.31–1.13 (m, 3H, 7-H_b + 21-H_b + 19-H_b), 1.14 (s, 3H, 27-H), 1.03 (s, 3H, 24-H), 1.07–0.98 (m, 2H, 16-H_b + 1-H_b), 0.90 (s, 3H, 30-H), 0.90 (s, 3H, 29-H), 0.90 (s, 3H, 26-H), 0.86 (s, 3H, 23-H), 0.84–0.79 (m, 1H, 5-H), 0.67 (s, 3H, 25-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 178.0$ (C-28), 144.9 (C-13), 138.4 (C-32), 128.7 (C-33 + C-33'), 127.7 (C-34 + C-34'), 127.4 (C-35), 122.7 (C-12), 91.5 (C-3), 55.4 (C-5), 47.4 (C-9), 46.7 (C-19), 46.3 (C-17), 43.6 (C-31), 42.3 (C-18), 42.0 (C-14), 39.3 (C-8),

38.6 (C-4), 38.3 (C-1), 36.7 (C-10), 34.1 (C-21), 33.0 (C-30), 32.6 (C-15), 32.3 (C-7), 30.7 (C-20), 28.1 (C-24), 27.3 (C-16), 25.7 (C-27), 24.2 (C-2), 23.8 (C-22), 23.6 (C-29), 23.4 (C-11), 18.3 (C-6), 16.9 (C-25), 16.4 (C-23), 15.3 (C-26) ppm; MS (ESI, MeOH): *m/z* 528.4 (5 %, $[M + H-H_2NSO_3H]^+$), 625.3 (100 %, $[M+H]^+$), 647.3 (90 %, $[M+Na]^+$), 1271.3 (48 %, $[2\ M + Na]^+$); analysis calcd for $C_{37}H_{56}N_2SO_4$ (624.93): C 71.11, H 9.03, N 4.48; S 5.13; found: C 70.86, H 9.21, N 4.20, S 4.98.

4.1.22. 3β-Aminosulfonyloxy-N-benzyl-urs-12-en-28-amide (**17**)

Following GPD, compound **17** (0.62 g, 79 %) was obtained from **12** as a colorless amorphous solid; $R_f = 0.20$ (*n*-hexane/ethyl acetate, 7:3); $[\alpha]_D = +14.5°$ (*c* 0.355, $CHCl_3$); IR (ATR): ν = 3398w, 2973 m, 2921 m, 2872 m, 1646 m, 1628 m, 1524s, 1506 m, 1489 m, 1469 m, 1453 m, 1367s, 1180s, 1142 m, 1080 m, 908s, 871s, 837 m, 748 m, 724 m, 696 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.35–7.21 (m, 5H, 35-H + 34-H + 34'-H + 33-H + 33'-H), 6.16–6.12 (m, 1H, NH-amide), 5.21 (dd, *J* = 3.5 Hz, 3.5 Hz, 1H, 12-H), 4.87 (s, 2H, NH_2-sulfamate), 4.54 (dd, *J* = 14.5, 6.0 Hz, 1H, 31-H_a), 4.22 (dd, *J* = 12.0, 4.6 Hz, 1H, 3-H), 4.16 (dd, *J* = 14.6, 4.5 Hz, 1H, 31-H_b), 2.08–2.00 (m, 1H, 2-H_a), 2.00–1.95 (m, 1H, 15-H_a), 1.94–1.84 (m, 4H, 11-H_a + 2-H_b + 7-H_a + 18-H), 1.84–1.73 (m, 2H, 11-H_b + 15-H_b), 1.73–1.62 (m, 2H, 16-H_a + 1-H_a), 1.61–1.28 (m, 9H, 6-H_a + 21-H_a + 9-H + 22-H_a + 7-H_b +19-H +6-H_b + 21-H_b + 22-H_b), 1.08 (s, 3H, H-27), 1.09–1.04 (m, 2H, 16-H_b + 1-H_b), 1.04 (s, 3H, H-24), 0.97–0.91 (m, 1H, H-20),0.95 (s, 3H, H-30), 0.91 (s, 3H, H-26), 0.86 (s, 3H, H-24), 0.85 (d, *J* = 6.4 Hz, 3H, H-29), 0.83–0.78 (m, 1H, H-5), 0.70 (s, 3H, H-25) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 177.9 (C-28), 139.9 (C-13), 138.3 (C-32), 128.7 (C-34 + C-34'), 127.9 (C-33 + C-33′), 127.4 (C-35), 125.5 (C-12), 91.5 (C-3), 55.5 (C-5), 54.0 (C-18), 47.8 (C-17), 47.4 (C-9), 43.7 (C-31), 42.5 (C-14), 39.7 (C-19), 39.5 (C-8), 39.1 (C-20), 38.6 (C-4), 38.4 (C-1), 37.2 (C-7), 36.7 (C-10), 32.7 (C-22), 30.9 (C-21), 28.1 (C-24), 27.9 (C-16), 24.8 (C-15), 24.3 (C-2), 23.3 (C-11), 23.2 (C-27), 21.2 (C-30), 18.3 (C-6), 17.2 (C-29), 17.0 (C-25), 16.4 (C-23), 15.5 (C-26) ppm; MS (ESI, MeOH): *m/z* 528.5 (4 %, $[M + H-H_2NSO_3H]^+$), 625.3 (100 %, $[M+H]^+$), 647.3 (62 %, $[M+Na]^+$), 1249.2 (18 %, $[2\ M + H]^+$), 1271.4 (24 %, $[2\ M + Na]^+$); analysis calcd for $C_{37}H_{56}N_2SO_4$ (624.93): C 71.11, H 9.03, N 4.48; S 5.13; found: C 71.01, H 9.28, N 4.29, S 4.96.

4.1.23. 3β-Aminosulfonyloxy-N-benzyl-lup-20(29)-en-28-oic amide (**18**)

Following GPD, compound **18** (0.51 g, 80 %) was obtained from **13** as a colorless amorphous solid; $R_f = 0.20$ (*n*-hexane/ethyl acetate, 7:3); $[\alpha]_D = +17.2°$ (*c* 0.345, $CHCl_3$); IR (ATR): ν = 3347w, 2944 m, 2868 m, 1639 m, 1514 m, 1497 m, 1467 m, 1454 m, 1374s, 1362s, 1318w, 1246w, 1211w, 1178vs, 956 m, 930s, 911vs, 881s, 837 m, 698 m cm^{-1}; 1H NMR (400 MHz, $CDCl_3$): δ = 7.36–7.23 (m, 5H, 33-H + 33'-H + 34-H + 34'-H + 35-H), 5.89 (t, *J* = 5.8 Hz, 1H, NH-amide), 4.79 (s, 2H, NH2-sulfamate), 4.74 (d, *J* = 2.4 Hz, 1H, 29-H_a), 4.60 (dd, *J* = 2.4, 1.4 Hz, 1H, 29-H_b), 4.48 (dd, *J* = 14.7, 5.7 Hz, 1H, 31-H_a), 4.36 (dd, *J* = 14.7, 5.6 Hz, 1H, 31-H_b), 4.21 (dd, *J* = 12.0, 4.7 Hz, 1H, 3-H), 3.16 (td, *J* = 11.1, 4.1 Hz, 1H, 19-H), 2.50 (ddd, *J* = 12.9, 11.3, 3.6 Hz, 1H, 13-H), 2.07–1.96 (m, 2H, 2-H_a + 21-H_a), 1.97–1.82 (m, 2H, 15-H_a + 2-H_b), 1.80–1.67 (m, 3H, 22-H_a + 22-H_b + 12-H_a), 1.69 (s, 3H, 30-H), 1.62–1.21 (m, 12H, 18-H + 15-H_b + 1-H_a + 7-H_a + 7-H_b + 21-H_b + 11-H_a + 11-H_b + 16-H_a + 6-H_a + 6-H_b + 9-H), 1.15–1.08 (m, 1H, 16-H_b), 1.01 (s, 3H, 24-H), 0.99–0.93 (m, 1H, 1-H_b), 0.95 (s, 3H, 27-H), 0.91 (s, 3H, 26-H), 0.85 (s, 3H, 23-H), 0.84 (s, 3H, 25-H), 0.79–0.74 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 175.9 (C-28), 150.9 (C-20), 139.1 (C-32), 128.7 (C-34 + C-34'), 127.8 (C-33 + C-33′), 127.3 (C-35), 109.4 (C-29), 91.9 (C-3), 55.7 (C-5), 55.6 (C-17), 50.6 (C-9), 50.2 (C-18), 46.7 (C-19), 43.3 (C-31), 42.5 (C-14), 40.8 (C-8), 38.7 (C-4), 38.6 (C-1), 38.4 (C-22), 37.7 (C-13), 37.0 (C-10), 34.3 (C-7), 33.7 (C-15), 30.9 (C-21), 29.4 (C-16), 28.0 (C-24), 25.6 (C-12), 24.4 (C-2), 21.0 (C-11), 19.5 (C-30), 18.3 (C-6), 16.2 (C-25), 16.2 (C-23), 16.1 (C-26), 14.6 (C-27) ppm; MS (ESI, MeOH): *m/z* 528.3 (10 %, $[M + H-H_2NSO_3H]^+$), 625.3 (88 %, $[M+H]^+$), 647.2 (100 %, $[M+Na]^+$), 1271.3 (28 %, $[2\ M + Na]^+$); analysis calcd for $C_{37}H_{56}N_2SO_4$ (624.93): C 71.11, H 9.03, N 4.48; S 5.13; found: C 70.85, H 9.24, N 4.32, S 4.95.

4.1.24. 3β-AminosulfonyloxyN-benzyl-20-oxo-30-norlupan-28-amide (**19**)

Following GPD, compound **19** (0.40 g, 75 %) was obtained from **14** as a colorless amorphous solid; $R_f = 0.28$ (*n*-hexane/ethyl acetate, 7:3); $[\alpha]_D = -2.9°$ (*c* 0.340, $CHCl_3$); IR (ATR): ν = 3365w, 2946 m, 2866w, 1694 m, 1658 m, 1651 m, 1635 m, 1525 m, 1520 m, 1496w, 1469w, 1454 m, 1362s, 1248w, 1177s, 940s, 920vs, 909vs, 872 m, 837 m, 700 s cm^{-1}; 1H NMR (400 MHz, $CDCl_3$): δ = 7.38–7.24 (m, 5H, 32-H + 32′-H + 33-H + 33′-H + 34-H), 5.98 (t, *J* = 5.8 Hz, 1H, NH-amide), 4.48 (dd, *J* = 14.7, 5.7 Hz, 1H, 30-H_a), 4.33 (dd, *J* = 14.7, 5.4 Hz, 1H, 30-H_b), 4.19 (dd, *J* = 12.0, 4.7 Hz, 1H, 3-H), 3.47 (td, *J* = 11.2, 4.2 Hz, 1H, 19-H), 2.26 (td, *J* = 12.0, 4.1 Hz, 1H, 13-H), 2.17 (s, 3H, 29-H), 2.12–1.99 (m, 3H, 2-H_a + 2-H_b + 18-H), 1.94–1.56 (m, 5H, 15-H_a + 22-H_a + 1-H_a + 15-H_b + 22-H_b), 1.56–1.20 (m, 10H, 21-H_a + 7-H_a + 6-H_a + 7-H_b + 16-H_a + 11-H_a + 11-H_b + 9-H + 6-H_b + 16-H_b), 1.17–1.02 (m, 3H, 21-H_b + 12-H_a + 12-H_b), 1.01 (s, 3H, 24-H), 0.98 (s, 3H, 27-H), 0.95–0.89 (m, 1H, 1-H_b), 0.87 (s, 3H, 26-H), 0.84 (s, 3H, 23-H), 0.83 (s, 3H, 25-H), 0.80–0.73 (m, 1H, 5-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 213.0 (C-20), 175.7 (C-28), 138.9 (C-31), 128.7 (C-33 + C-33′), 127.7 (C-32 + C-32′), 127.4 (C-34), 91.7 (C-3), 55.6 (C-5), 55.5 (C-17), 51.0 (C-19), 50.4 (C-9), 49.9 (C-18), 43.3 (C-30), 42.3 (C-14), 40.7 (C-8), 38.7 (C-4), 38.5 (C-1), 38.0 (C-22), 37.0 (C-10), 36.7 (C-13), 34.2 (C-7), 33.0 (C-15), 30.3 (C-29), 29.4 (C-21), 28.6 (C-16), 28.0 (C-24), 27.2 (C-12), 24.4 (C-2), 21.0 (C-11), 18.3 (C-6), 16.2 (C-25), 16.2 (C-23); 16.1 (C-26), 14.7 (C-27) ppm; MS (ESI, MeOH): *m/z* 530.3 (18 %, $[M + H-H_2NSO_3H]^+$), 627.4 (100 %, $[M+H]^+$), 649.2 (90 %, $[M+Na]^+$), 1275.5 (28 %, $[2\ M + Na]^+$); analysis calcd for $C_{36}H_{54}N_2SO_5$ (628.90): C 68.97, H 8.68, N 4.47, S 5.11; found: C 68.70, H 8.83, N 4.26, S 4.96.

4.1.25. (2α, 3β) Bis(aminosulfionyloxy)-N-benzyl-olean-12-en-28-amide (**20**)

Following GPD, compound **20** (0.52 g, 78 %) was obtained from **15** as a colorless amorphous solid; $R_f = 0.15$ (*n*-hexane/ethyl acetate, 7:3); $[\alpha]_D = +7.3°$ (*c* 0.30, $CHCl_3$); IR (ATR): ν = 3338 m, 2949 m, 1626 m, 1524w, 1454 m, 1434 m, 1389s, 1367s, 1343vs, 1172s, 987 m, 950 m, 937 m, 830 m, 819s, 697 m cm^{-1}; 1H NMR (400 MHz, CD_3OD): δ = 7.29–7.26 (m, 5H, 35-H + 34-H + 34′-H + 33-H + 33′-H), 5.32 (dd, *J* = 3.6 Hz, 1H, 12-H), 4.72 (ddd, *J* = 11.6, 9.9, 4.7 Hz, 1H, 2-H), 4.37 (d, *J* = 14.7 Hz, 1H, 31-H_a), 4.26 (d, *J* = 14.4 Hz, 1H, 31-H_b), 4.22 (d, *J* = 10.0 Hz, 1H, 3-H), 2.83 (dd, *J* = 13.3, 4.3 Hz, 1H, 18-H), 2.36 (dd, *J* = 12.6, 4.7 Hz, 1H, 1-H_a), 2.13–2.02 (m, 1H, 22-H_a), 1.95–1.86 (m, 2H, 11-H_a + 11-H_b), 1.82–1.73 (m, 1H, 19-H_a), 1.72–1.30 (m, 9H, 9-H + 7-H_a + 7-H_b + 16-H_a + 22-H_b + 6-H_a + 6-H_b + 15-H_a + 21-H_a), 1.30–1.13 (m, 4H, 15-H_b + 21-H_b + 1-H_b + 19-H_b), 1.17 (s, 3H, H-27), 1.10 (s, 3H, H-23), 1.04–1.00 (m, 1H, 16-H_b), 1.02 (s, 3H, 25-H), 1.01–0.95 (m, 1H, 5-H), 0.95 (s, 3H, 30-H), 0.93 (s, 3H, 24-H), 0.91 (s, 3H, 29-H), 0.58 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, CD_3OD): δ = 178.6 (C-28), 144.0 (C-13), 139.0 (C-32), 128.0 (C-34 + C-34′), 127.4 (C-33 + C-33′), 126.7 (C-35), 122.1 (C-12), 90.2 (C-3), 77.3 (C-2), 54.7 (C-5), 47.3 (C-9), 46.2 (C-19), 46.1 (C-17), 44.6 (C-1), 42.9 (C-31), 41.6 (C-14), 41.2 (C-18), 40.1 (C-4), 39.2 (C-8), 37.9 (C-10), 33.7 (C-21), 32.9 (C-15), 32.1 (C-29), 32.1 (C-7), 30.2 (C-20), 27.8 (C-23), 27.1 (C-16), 25.0 (C-27); 23.2 (C-11), 22.6 (C-30), 22.6 (C-22), 18.0 (C-6), 16.5 (C-24), 16.3 (C-26), 15.3 (C-25) ppm; MS (ESI, MeOH): *m/z* 720.3 (100 %, $[M+H]^+$), 742.2 (84 %, $[M+Na]^+$), 1102.3 (10 %, $[3\ M+2Na]^{2+}$, 1461.3 (20 %,

[2 M + Na]$^+$); analysis calcd for $C_{37}H_{57}N_3S_2O_7$ (720.00): C 61.72, H 7.98, N 5.84; S 8.91; found: C 61.50, H 8.13, N 5.62, S 8.67.

4.2. Cell lines, treatment with pentacyclic triterpenoic acid derivatives

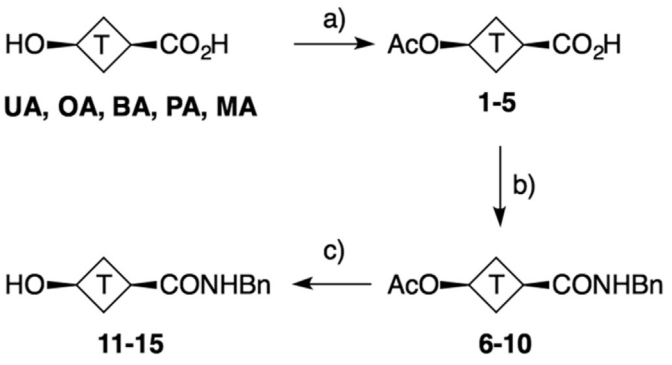
The human breast cancer cell lines MDA-MB-231, HS578T, MCF-7 and BT-20 were grown under standard conditions under 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (Lonza, Walkersville, MD, USA), enriched with L-Glutamine and 25 mM HEPES with additives of 10 % fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany), 1% pyruvate (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 2 % penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The pentacyclic sulfamate triterpene structures are shown in Fig. 1 and Schemes 1 and 2. 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 treatment was performed for 24 h.

4.3. SRB assay

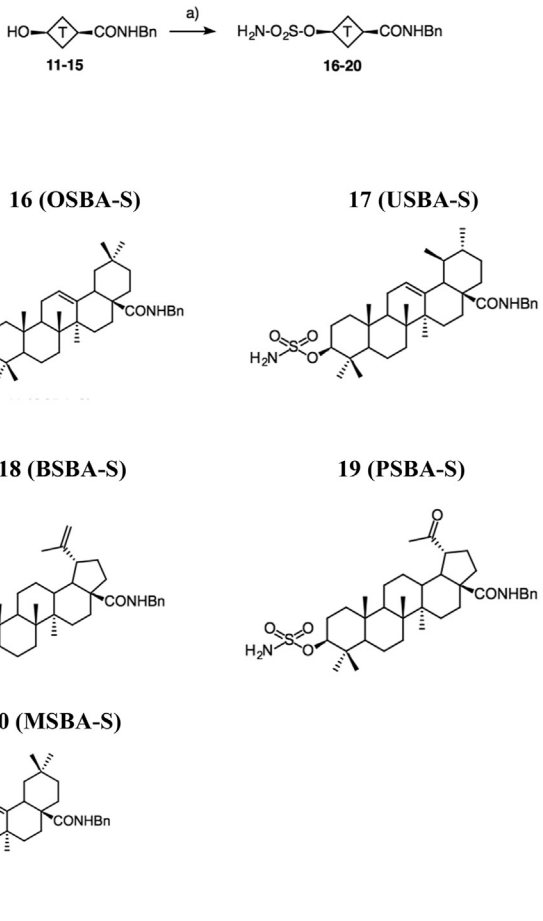
The cytotoxicity of the drugs was determined by Sulforhodamine B (SRB) assay. Treatment started 24 h after seeding the cells in 96-well plates. BSBA-S (**18**), OSBA-S (**16**), USBA-S (**17**), PSBA-S (**19**), and MSBA-S (**20**) in concentrations between 1 mM and 100 mM were applied for treatment lasting 96 h under standard conditions. Cells were fixed after incubation by use of 10 % trichloroacetic acid (Carl Roth GmbH, Karlsruhe, Germany). Following a wash step using iced water, a 0.4 % SRB solution (Sigma-Aldrich) was applied for staining. Another washing step with 1 % acetic acid (Carl Roth GmbH) was performed before drying. For extinction measurement at λ = 540 nm on TECAN GENios FL TWT (Tecan Treading Ag, Männedorf, Switzerland), fixed cells were dissolved in 300 μl 20 mM Trizma base (Sigma-Aldrich). The IC_{50} values were defined as the half-maximal inhibitory concentrations of the compounds.

4.4. Clonogenic survival assay and radiosensitivity

Incubation started 24 h after seeding cells in 6-well plates with concentrations of 40 μM BSBA-S (**18**), 40 μM OSBA-S (**16**), 40 μM USBA-S (**17**), 40 μM PSBA-S (**19**), and 10 μM MSBA-S (**20**). Following 24 h of incubation, trypsinization and counting of cells was performed for seeding of cells in small quantities (300 or 600 cells) in 75 cm^2 cell culture flasks. Allowing the cells to form colonies for about 12–14 days, they were fixed with paraformaldehyde (Sigma-Aldrich) and stained with 10% Giemsa solution (Sigma-Aldrich). Exclusively colonies counting more than around 50 cells were scored to determine the surviving fraction. The ratio of the number of colonies formed after irradiation with 6 Gy, 10 Gy or 14 Gy and treatment with 5 μM MSBA-S (**20**) was compared 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 pentacyclic triterpenoic acid derivatives-treated cells and was dependent on the dose of irradiation.



Scheme 1. Synthesis of compounds 1–15; reactions and conditions: a) Ac_2O, NEt_3, DCM, DMAP (cat.), 20 °C, 1 d: 1 (89 %), 2 (89 %), 3 (90 %), 4 (84 %), 5 (92 %); b) $(COCl)_2$, DCM, DMF (cat.), 20 °C, 1 h: 6 (95 %), 7 (84 %), 8 (84 %), 9 (88 %), 10 (84 %); c) KOH, MeOH, 20 °C: 11 (87 %), 12 (87 %), 13 (71 %), 14 (92 %), 15 (89 %).



Scheme 2. Synthesis of sulfamates **16**–**20**; reactions and conditions: a) NaH, THF 25 °C, 30 min, then $ClSO_2NH_2$, 25 °C, 5 d: **16** (83 %), **17** (79 %), **18** (80 %), **19** (75 %), **20** (78 %).

4.5. ROS

For measurement of intracellular ROS levels, cells were plated in 6-well plates for 24 h. After treatment with 5 μM MSBA-S (**20**) or 20 μM BSBA-S (**18**) 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. Following a washing step with PBS, cells were incubated with trypsin-EDTA (0.5%) (Life Technologies, Carlsbad, USA) for 3 min at 37 °C. After decanting Trypsin, cells were detached with PBS. Fluorescent ROS signals were measured with an LSRFortessa™ flow cytometer (BD Biosciences, Heidelberg, Germany).

4.6. DAPI analysis

After treatment with 20 μM OSBA-S (**16**), 40 μM BSBA-S (**18**), 40 μM USBA-S (**17**), 40 μM PSBA-S (**19**) and 10 μM MSBA-S (**20**) for 72 h under standard conditions, cells were trypsinized and centrifuged at 800 rpm, 4 min with the cell culture supernatant. The suspension was re-suspended in PBS and cells were fixed with 70% ethanol. Fixed cells were then centrifuged at 1500 rpm, 5 min with use of a cytospin chamber and fixed on a slide. For analysis of cell nuclei the fluorescent dye 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) (Serva, Heidelberg, Germany) was incubated for 5 min. Further the slide was washed three times with PBS, cells were covered with ProLong® Gold Antifade Reagent (Life technologies, Darmstadt, Deutschland) and a cover slip. The microscopy was performed with BZ-8000 (Keyence, Osaka, Japan), the apoptotic cell nuclei were counted.

4.7. Annexin V-PI assay

Cells were seeded in 6-wells and after growth time of 24 h, cells were treated with 40 μM BSBA-S (**18**) or 10 μM MSBA-S (**20**) for 24 h. Cell culture supernatant was collected and 1.0×10^6 cells were detached with accutase. After medium was detached, cells were re-suspended in 1 mL 1x binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 5 mM $CaCl_2$ x H_2O, pH = 7.4), 125 μl of the suspension was incubated for 15 min with the staining solution propidium iodide (PI) (50 μg/mL (Invitrogen, California, US) and/or Annexin V (Invitrogen, California, US). Before measurement with flow cytometry (FACS), 400 μl binding buffer was added.

4.8. Statistical analysis

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

Author contributions

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113721.

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