

BSC9 a betulin–acetazolamide conjugate enhances radiosensitivity in 2D and 3D HNSCC models

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Abstract

Bifunctional compounds, which combine carbonic anhydrase inhibitors (CAIs) with cytotoxic substances represent a potential strategy to advance cancer treatment. Therapeutic targeting of hypoxia-associated carbonic anhydrase IX (CA IX) has been a promising strategy to selectively treat radioresistant hypoxic tumor subpopulations. In this study, we evaluated BSC9, a bifunctional conjugate comprising the CA IX inhibitor acetazolamide (AZA) and 3-O-acetylbetulin (3-AC), a pentacyclic triterpene derivative with tumor-selective cytotoxic properties, in two head and neck squamous cell carcinoma (HNSCC) cell lines under both normoxic and hypoxic conditions. BSC9 exerted cytotoxic effects in the low micromolar range and increased sensitivity to radiation, particularly under hypoxic conditions in 2D cultures and in a two-colored 3D HNSCC model. In addition, this study revealed that BSC9 induced significant accumulation of reactive oxygen species (ROS), increased DNA damage and mitochondrial membrane depolarization. BSC9 triggered a complex transcriptional response characterized particularly under hypoxic conditions, by signaling pathways related to the cell cycle, cell adhesion, migration, metabolism, and proteostasis. These findings demonstrate that combining a CAI with a cytotoxic betulin derivative represents a promising strategy to counteract hypoxia-associated radioresistance and demonstrate the applicability of the two-colored 3D HNSCC model for evaluating treatment responses under distinct oxygenation conditions.

Highlights

- Cytotoxicity of BSC9 in 2D and 3D HNSCC models
- HNSCC shows Enhanced radiosensitivity by BSC9 HNSCC in 2D und 3D
- BSC9 preferentially radiosensitizes hypoxic HNSCC cell populations
- BSC9 promotes ROS formation, DNA damage and mitochondrial dysfunction
- Transcriptomic profiling supports a bifunctional CA IX- and BA-mediated mechanism

Introduction

Hypoxia induces transcriptional and posttranscriptional alterations that contribute to radio- and chemoresistance in head and neck squamous cell carcinoma (HNSCC). Hypoxia induces several adaptive mechanisms that promote treatment resistance, including the upregulation of carbonic anhydrases which regulates intra- and extracellular pH, CO₂ transport, ion and fluid homeostasis [1–3]. Several studies have demonstrated the overexpression of carbonic anhydrase IX (CA IX) in various human malignant tumor entities, including head and neck cancer [4, 5]. CA IX is a hypoxia-induced transmembrane glycoprotein that is strongly upregulated in perinecrotic regions of HNSCC while remaining largely absent from normoxic tissues [6, 7]. Through the regulation of extracellular acidification, CA IX further contributes to the modulation of chemotherapeutic efficacy [8, 9]. Our own studies show that CA9 mRNA and protein expression are significantly associated with poor prognosis in

oral squamous cell carcinoma, a major subgroup of HNSCC, identifying CA IX as an independent prognostic marker and potential therapeutic target [10].

Therapeutic strategies targeting hypoxia-associated pathways, including bifunctional approaches, are a major focus of ongoing research [11]. Targeted drug delivery approaches aim to exploit tumor-specific molecular features. Due to its selective overexpression in therapy-resistant hypoxic tumor cells, CA IX represents an attractive anchor for tumor-directed therapeutic strategies [12]. CA IX-binding drugs enable selective localization of therapeutic agents to hypoxic tumor regions, thereby enhancing antitumor efficacy [13, 14]. Accordingly, CA IX-directed inhibitors have demonstrated significant antitumor activity in preclinical models, impairing tumor cell viability, migration, invasion, and metastatic growth both in vitro and in vivo [15–18]. In addition to their targeting function, CA IX-drugs can also serve as anchoring moieties for cytotoxic effectors with intrinsic antitumor activity to generate bifunctional targeting drugs [19].

Within this concept of bifunctional approaches, several molecules have been developed that integrate CA IX-directed properties. A recent study has demonstrated high cytotoxicity of quinolone-sulfonamide hybrids in leukemia cell lines [20]. Our previous studies have shown that combining a CA IX inhibitor with a betulin derivative could be a promising therapeutic approach for combating hypoxic tumor cells [21]. Betulin and betulinic acid (BA), naturally occurring pentacyclic triterpenes derived from birch bark, induce apoptosis through the mitochondrial pathway while exhibiting low toxicity toward normal cells [22–25]. Notably, our former study demonstrated that BA increased cytotoxicity and radiosensitivity under hypoxic conditions, suggesting a particular suitability for targeting hypoxic tumor cells [26]. Chemical modification, especially at the C3 and C28 positions, has enabled the development of numerous derivatives with improved pharmacological properties and enhanced cytotoxicity [27]. Among these derivatives, betulin sulfamates and sulfonamides are of particular interest, as they combine intrinsic antitumor activity with carbonic anhydrase inhibition, thereby modulating tumor pH, enhancing cytotoxicity and radiosensitivity [28–32]. In this context, acetazolamide (AZA) provides a clinically established CA inhibitor that targets hypoxic tumor regions. In addition, AZA is currently being evaluated in early-phase clinical studies in combination with radio(chemo)therapy or radioimmunotherapy in various cancer types [33–35]. A preclinical study has shown that AZA improves survival rates and increases radiosensitivity in medulloblastomas [36]. We recently developed the bifunctional conjugate BSC9 (previously published as compound 9) consisting of AZA and a betulin derivative, which combines CA IX-inhibition with the tumor-selective cytotoxicity of pentacyclic triterpenes (structure in **Suppl. Fig. 1**) [37].

However, the preclinical evaluation of hypoxia-directed compounds remains challenging, as conventional 2D cultures insufficiently reproduce the spatial oxygen gradients and heterogeneous treatment responses characteristic of solid tumors [38–40]. More complex 3D tumor models may therefore provide a more relevant platform for assessing therapeutic efficacy under hypoxic conditions [41]. To evaluate the therapeutic potential of BSC9 in HNSCC models that reflects distinct oxygenation states, we proved their effects in a actually developed two-color HNSCC spheroid model [41]. The aim of this study was to

evaluate the applicability of BSC9 as a potential hypoxia-directed radiosensitizer in 2D and two-colored 3D HNSCC models and to identify potential mechanisms underlying its cytotoxic and radiosensitizing effects. In this study, BSC9 demonstrated cytotoxic and radiosensitizing effects in 2D and 3D HNSCC models, particularly under hypoxic conditions. These effects were further investigated by analyzing ROS production, DNA damage, mitochondrial membrane potential, and transcriptomic changes.

Materials and Methods

Cell culture conditions and treatment of cells

The HNSCC cell lines SAS and FaDu were kindly provided by Prof. Zips, Department of Radiation Oncology and Radiotherapy, University Hospital Charité Berlin, Germany. The cells were cultured in RPMI complete medium as previously described [41]. The chemical compound (3 β)-3-(acetyloxy)lup-20(29)en-28-yl-4-[[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl]amino]-4-oxobutanoate (BSC9; kindly provided from Prof. Dr. Reinhard Paschke, BioCenter, Martin-Luther-University Halle-Wittenberg, 06120 Halle (Saale), Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma, Steinheim, Germany) to prepare a 20 mM stock solution. The cells were treated with BSC9 for up to 72 h at 37°C, depending on the assay performed. To generate hypoxic conditions (approximately 0.1 % O), we used the GasPak EZ Anaerobic Pouch System (BD Biosciences, Heidelberg, Germany). Irradiation was performed with a Synergy linear accelerator (Elekta, Stockholm, Sweden). For determination of radiosensitivity, the cells were irradiated with 6 MV photon radiation between 2–16 Gy. For spheroid formation SAS and FaDu cells were lentivirally transduced with GFP- (pLV-eGFP, Addgene plasmid 36083) or mCherry (pLV-mCherry, Addgene plasmid 36084, both kindly provided from Pantelis Tsoulfas)-coding vectors as previously described [42]. Two-layered 3D spheroids consisting of inner GFP- and outer mCherry-labeled cells were generated to distinguish oxygen-dependent regions, as previously described [41].

Cell cytotoxicity

The effect of BSC9 in HNSCC cell lines was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) as previously described [41]. Briefly, SAS and FaDu cells were seeded at defined numbers and treated with BSC9 (0.5 to 80 μ M) under normoxic and hypoxic conditions. Cell viability was measured 24 h (2D) or 72 h (3D) after treatment. Half-maximal inhibitory concentration (IC₅₀) values were determined using Origin 2019 (OriginLab Corp., Northampton, MA, USA).

Clonogenic survival assay and radiosensitivity

Clonogenic survival assays were performed as previously described [41]. For determination of radiosensitivity, cells or spheroids were treated with BSC9 (2D: 10 μ M; 3D: SAS 20 μ M, FaDu 15 μ M) for 24 h and irradiated under hypoxic conditions at different doses (0 Gy–16 Gy) depending on cell line and cultivation conditions. In 2D model colonies were stained with Giemsa (Sigma–Aldrich) and quantified using a GelCount reader (Oxford Optronix, Milton, United Kingdom). In 3D HNSCC model colonies of

inner GFP- and outer mCherry-labeled cells were visualized via fluorescence imaging (ChemiDoc Imaging System (Bio-Rad, California, U.S.)) and counted using ImageJ (version 1.53). Survival curves were fitted using a linear-quadratic model ($-\ln S = \alpha D + \beta D^2$) via Origin 2019 and Dose Modifying Factor at 10 % survival (DMF_{10}) was calculated as previously described [29].

Reactive oxygen species (ROS)

The amount of intracellular ROS was measured by the incubation of cells with specific CM-H2DCFDA dye (Thermo Fisher Scientific) as previously described [21]. Following the treatment with BSC9 (10 μ M), the SAS and FaDu cells were stained with 0.5 μ M CM-H2DCFDA (in PBS supplemented with $CaCl_2$ and $MgCl_2$, Sigma-Aldrich). Fluorescence signals of single cells were analyzed using a LSRFortessa™ flow cytometer (BD Biosciences).

Quantification of γ H2AX foci formation

For the determination of γ H2AX Foci 12 mm coverslips (Marienfeld GmbH, Lauda Königshofen, Germany) were pretreated with 40% HCl/60% ethanol (both Carl Roth, Karlsruhe, Germany), washed, and stored in 70% ethanol. HNSCC cells (25.000 SAS or 37.500 FaDu per well) were plated into 24-well plates (TPP, Trasadingen, Switzerland) containing the prepared cover slips. After 24 h of treatment with BSC9 (10 μ M), cells were irradiated with 0 Gy or 4 Gy followed by further incubation under hypoxia for 24 h. Residual γ H2AX foci were analyzed by immunofluorescence as previously described [21].

Mitochondrial membrane potential (MMP)

To assess MMP, HNSCC cells were seeded in 6-well plates ($\geq 5 \times 10^5$ cells/well) overnight. Cells were treated with BSC9 (10 μ M) for 15 min at 37°C and a CCCP treated negative control (50 μ M (MedChemExpress, Monmouth Junction, USA) was prepared. After treatment, the medium was removed and cells were incubated with JC-1 (MedChemExpress) working solution for additional 15 min at 37°C in the dark. Following staining, cells were washed with PBS and the samples were analyzed immediately using a LSRFortessa™ flow cytometer (BD Biosciences). MMP was assessed by measuring the red (aggregates (ex.=488 nm, em.=595 nm)) to green (ex.=488 nm, em.=530 nm) fluorescence intensity ratio.

Western blot

Western blotting was performed as previously described [29, 41]. Briefly, cells were seeded in 6-well plates and treated with BSC9 (2.5, 5 and 10 μ M) under normoxic and hypoxic conditions before lysis and homogenization. Total protein concentrations were determined using the Bradford assay (Bio Rad). Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore). Membranes were blocked and incubated with primary antibodies against HIF1 α (1:1,000; BD

Biosciences), PARP (1:1,000; Cell Signaling, Leiden, Netherlands), CA IX (1:2,000; clone M75, Absolute Antibodies, Oxford, UK), LC3B (1:1,000; Cell Signaling), and β -actin (1:5,000; Sigma Aldrich). HRP conjugated secondary antibodies (1:2,000; DAKO, Jena, Germany) were applied, and signals were detected using enhanced chemiluminescence (Bio-Rad) with a ChemiDoc imaging system (Bio-Rad). Band intensities were quantified using ImageJ and normalized to β actin.

Immunohistochemistry (IHC)

IHC staining and quantification was performed as previously described [41]. Briefly, spheroids were treated with BSC9 (SAS 20 μ M and FaDu 15 μ M) for 72 h. Subsequently they were fixed in 4 % paraformaldehyde, embedded in agarose blocks using a 3D-printed mold (Xometry Europe GmbH), and processed for paraffin embedding. Sections (4 μ m) were deparaffinized, antigen retrieval was performed in citrate buffer (Zytomed Systems (10x)), and nonspecific binding was blocked (Zytomed Antibody Diluent, Bargeheide, Germany). Slides were incubated with CA IX (1:50, clone no. M75, Absolute Antibodies), HIF1 α (1:100, BD Biosciences), KI67 (1:150, Cell Signaling) and cleaved caspase-3 (1:150, Cell Signaling) followed by HRP-conjugated secondary antibodies (DAKO anti-rabbit/mouse polymer HRP) and DAB+ chromogenic detection (DAKO). Counterstaining was done using Mayer's hemalum (1:5 dilution; Sigma–Aldrich), and slides were scanned using a slide scanner (Axio Scan.Z1, Zeiss, Ostfildern, Germany). Quantification of CA IX, HIF1 α , KI67 and cleaved caspase-3 staining was performed using ImageJ based on threshold DAB signals.

mRNA sequencing and gene expression analysis

For mRNA sequencing, cells were treated with BSC9 (10 μ M) under normoxic and hypoxic conditions. Subsequently 2.5 μ g of total RNA per sample was used. PolyA-based library preparation and sequencing were performed by Genewiz (Leipzig, Germany). Paired-end strand-specific sequencing was performed with approximately 2x20 million reads per sample via the Illumina NovaSeq platform. Adapter sequences as well as low-quality read ends were clipped off via Cutadapt (v 4.4) [43]. The processed sequencing reads were aligned to the human reference genome (UCSC hg38) via HISAT2 (v 2.2.1) [44]. SAMtools (v 1.19.2) was used to extract primary alignments and to index the resulting bam files. FeatureCounts (v 2.0.6) was used to summarize the gene-mapped reads [45, 46]. ENSEMBL (GRCh38 v110) was used as an annotation basis [47]. Differential gene expression was determined via the R package edgeR (v 4.2.1) via trimmed mean of M values (TMM) normalization and the exact test function [48, 49]. Genes were ranked by a signed product score (p-score) integrating effect size and statistical significance ($|\log_2FC| \times -\log_{10}(FDR)$).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed via the R-packages clusterProfiler (v 4.12.1) and MSigDB gene sets (v2024.1. Hs) via the fgsea algorithm and setting the exponent parameter to 0 for

unweighted analyses of log2-fold-change-sorted gene lists obtained from differential gene expression analyses [50, 51].

Results and Discussion

BSC9 promotes cytotoxicity and radiosensitization

CA IX-directed dual-targeting approaches, combining CA IX-binding moieties with cytotoxic betulin derivatives, have emerged as a promising strategy to overcome hypoxia-associated resistance. Based on this, we investigated the cytotoxic effects of the betulin–acetazolamide conjugate BSC9 in HNSCC 2D and two-colored 3D spheroid models.

First, BSC9 was evaluated in 2D HNSCC models, where SAS and FaDu cells showed comparable cytotoxicity under normoxic and hypoxic conditions, with IC_{50} values in the range of 8–10 μ M (Fig. 1A, B). To better reflect the tumor microenvironment, we next evaluated BSC9 in 3D spheroid models. SAS and FaDu displayed a similar response to that observed in 2D models (IC_{50} between 15 and 20 μ M). Our recent study has also shown that BSC9 exhibits high cytotoxicity in other 2D and 3D tumor cell models [37]. In agreement with this, other triterpene sulfonamides and sulfamates also exhibit high cytotoxicity and effectively reduce clonogenic survival in different 2D tumor cell lines under normoxia and hypoxia [21, 28–32]. Additionally, our results show BSC9 resulted in significantly impaired spheroid growth, density and viability (**Suppl. Figure 2**), and strongly reduced clonogenic survival in both models (**Suppl. Figure 3A, B**).

To distinguish between the cytotoxicity and radiosensitivity of normoxic and hypoxic HNSCC cells in 3D models, we recently developed a two-layered spheroid model consisting of a GFP-labeled inner cell layer representing hypoxic regions and an outer mCherry-labeled layer reflecting normoxic conditions [41]. These two-layered spheroids were treated with BSC9 and irradiated with 2–16 Gy. Subsequently, radiosensitivity was determined by measuring the clonogenic survival of the inner (green) and outer (red) fluorescent cells as a function of radiation dose and enables the differential analysis of cytotoxicity and radiosensitivity in distinct tumor compartments within the same spheroid (Fig. 1C). Here, we observed reduced clonogenic survival in both mCherry- and GFP-labeled cells, with a more pronounced effect in the GFP-labeled hypoxic cell population (**Suppl. Figure 3C**).

Additionally, we investigated the radio sensitizing properties of BSC9. In 2D HNSCC models, BSC9 decreased radiosensitivity under both oxygen conditions, with the strongest effects observed under hypoxia (Fig. 1D, E **and** Table 1). Comparable effects were observed in two-layered 3D models. Here, treatment with BSC9 significantly enhanced the radiation response in both layers, with a more pronounced effect observed in the hypoxic GFP-labeled cell layer (Fig. 1F, G **and** Table 1). In both 2D and 3D HNSCC models, normoxic and, in particular, hypoxic tumor cells exhibited an enhanced radiosensitivity after BSC9 treatment. Consistent with previous reports, betulin sulfamates and betulin sulfonamides increased radiosensitivity in 2D breast cancer cells under hypoxia [21, 30, 31]. This

demonstrates the importance of bifunctional BS-CAI conjugates for the radiosensitivity of tumors in vitro. However, the radiosensitizing effect of BSC9 still needs to be demonstrated in vivo models.

Table 1
DMF₁₀ values of BSC9 in 2D HNSCC cells and the two-colored 3D HNSCC model relative to that of the corresponding control. Significant p values are highlighted with asterisks (* p < 0.05; ** p < 0.01, *** p < 0.001).

2D	SAS		FaDu	
	normoxic	hypoxic	normoxic	hypoxic
BSC9	1.14 ± 0.08*	1.83 ± 0.04*	1.08 ± 0.06	1.62 ± 0.11**
3D	mCherry	GFP	mCherry	GFP
BSC9	1.33 ± 0.15**	2.48 ± 0.08***	1.38 ± 0.10**	1.87 ± 0.09**

BSC9 promotes oxidative stress, DNA damage and mitochondrial dysfunction

To investigate the cellular response to cytotoxicity and radiosensitivity, the effects of BSC9 on ROS formation, DNA damage and mitochondrial membrane potential (MMP) under normoxic and hypoxic conditions were analyzed. BSC9 treatment increased ROS levels under normoxia (30-70-fold), with an enhanced effect under hypoxia (50-100-fold) in both cell lines (Fig. 2A, B). Consistent with increased ROS, γH2AX analysis revealed enhanced DNA damage upon BSC9 treatment, which was further augmented under hypoxic conditions, particularly in combination with irradiation (Fig. 2C, D). Studies of the MMP demonstrated that BSC9 treatment caused a pronounced proton leak across the mitochondrial membrane, indicative of mitochondrial membrane damage (Fig. 2E, F). Incubation with BSC9 led to a decrease in MMP under normoxia (17–20%). This was more pronounced under hypoxia (4–14%).

The elevated ROS levels together with the reduction in MMP suggest that mitochondrial dysfunction is a central mechanism of BSC9 activity, consistent with previous reports on BA and BA derivatives targeting mitochondrial integrity and redox homeostasis [52–54]. The increase in γH2AX foci further supports a ROS-mediated contribution to radiation-induced DNA damage. The stronger response under hypoxia may reflect an increased vulnerability of hypoxic cells to oxidative stress due to their altered redox balance, as described for related triterpenes [55]. The reduction in MMP further supports the role of mitochondria as a central target of BSC9, consistent with previous reports on BA derivatives inducing mitochondrial dysfunction and apoptosis [53, 54, 56, 57]. Overall, these findings suggest that BSC9 exerts its cytotoxic and radiosensitizing effects via oxidative stress and mitochondrial impairment, with enhanced efficacy under hypoxic conditions, potentially enabling the targeting of radioresistant tumor subpopulations.

BSC9 modulates hypoxia- and apoptosis-related protein expression

To further characterize of BSC9, expression levels of hypoxia- (HIF1 α , CA IX), cell death- (PARP, LC3B, and cleaved caspase-3) and proliferation-associated (Ki67) proteins was investigated using western blotting and immunohistochemistry (Fig. 3 and **Suppl. Figure 4**). In 2D models BSC9 induced PARP cleavage and elevated LC3B level, indicating activation of apoptotic and deregulation of autophagic pathways with a more pronounced effect observed under hypoxia (Fig. 3A). In 3D HNSCC models, Ki67 was strongly expressed in the proliferative periphery of untreated spheroids but nearly disappeared after BSC9 treatment, whereas cleaved caspase-3 increased mainly in the hypoxic core (Fig. 3B). Taken together, these findings demonstrate a combined induction of growth arrest and cell death, particularly within hypoxic tumor regions, which are typically resistant to therapy. CA IX plays a central role in the adaptation of tumor cells to hypoxic stress by regulating intracellular pH homeostasis and supporting proliferation and survival, its targeting may contribute to the observed antiproliferative and cytotoxic effects [18, 37]. In addition, the BA moiety likely enhances these effects, consistent with previous studies on BA, which also promotes apoptosis, autophagy and inhibits proliferation of tumor cells [56–58]. Thus, the cellular response to BSC9 appears to reflect effects associated with both individual substances.

To investigate the hypoxia-driven therapy resistance mediated by HIF1 α and its downstream effectors (e.g. CA IX), we assessed the impact of BSC9 on hypoxia-associated regulatory proteins. Under both normoxic and hypoxic conditions, BSC9 increased HIF1 α levels in 2D and 3D models (Fig. 3A). This is in contrast to reports on BA, where HIF1 α levels are typically decreased, indicating suppression of hypoxia signaling [26, 59–61]. However, BSC9 incubation resulted in a decrease of CA IX expression in both 2D (especially under hypoxia) and 3D HNSCC models (Fig. 3). Thus, despite overlapping downstream pathway effects, BSC9 in contrast to BA does not seem to inhibit the central hypoxia regulator HIF1 α . The reduction of CA IX expression upon BSC9 treatment is consistent with findings for classical carbonic anhydrase inhibitors, including our own data and previous studies, suggesting that binding of inhibitors to CA IX can promote its degradation [18, 37]. The simultaneous increase in HIF1 α and decrease in CA IX may indicate a functional uncoupling of HIF1 α stabilization from its canonical transcriptional output. In agreement with this, studies on CAI binding to CA IX demonstrate that this binding is dependent on hypoxia but not on HIF1 α [62].

BSC9 modulates transcriptomic signatures

The transcriptomic profiles of SAS and FaDu cells following treatment with BSC9 revealed a pronounced, oxygen-dependent transcriptional reprogramming (Fig. 4A). Compared to normoxia, BSC9 significantly increased the transcriptional response under hypoxia in overrepresentation analysis. The number of differentially expressed genes increased four- to fivefold (Fig. 4A). To identify the gene-level responses to BSC9, differentially expressed genes were ranked using a product score (**Suppl. Table 1, 2**). Under normoxia, the top dysregulated genes, INHBE, G0S2 and SLC6A9 were consistently upregulated and ANXA10 was downregulated in SAS and FaDu cells (Fig. 4B). This indicates alterations in metabolic regulation, amino acid transport and cellular stress adaptation [63, 64]. Additionally, SAS cells exhibited regulation of S100A7, S100A9 and CHAC1, suggesting alterations in epithelial/inflammatory signaling and redox homeostasis (**Suppl. Table 1**). Conversely, FaDu cells exhibited deregulation of SLC1A4, MT1F

and NUPR1, which are linked to amino-acid metabolism, oxidative stress protection and stress-induced survival responses (**Suppl. Table 2**) [64, 65]. Under hypoxia, the common response of MFSD2A, G0S2, CSF2 and ATF3, indicating enhanced metabolic and cellular stress signaling, while SAS-specific regulation of TIMP3, CCDC80 and FGFR3 suggested further effects on extracellular matrix and growth factor signaling (**Suppl. Table 1, 2**) [66–68]. In contrast, the regulation of MT1E, SLC30A1 and DUSP8 in FaDu cells indicates changes in metal/redox homeostasis and stress-responsive signaling (**Suppl. Table 2**) [69]. Notably, G0S2, a gene that plays a central role in lipid metabolism and cell death regulation, was the only gene among the top-ranked genes in all four conditions, indicating a conserved reaction of the BSC9 response [63].

GSEAs analysis revealed a total of 124 significantly enriched KEGG pathways in SAS and FaDu cells upon BSC9 treatment. Among these, 73 pathways were enriched in both cell lines, which were grouped into eight functional biological categories (Fig. 4C, **Suppl. Table 3**). In both HNSCC cell lines, BSC9 enriched 22 KEGG pathways independent of oxygen levels, 29 pathways under normoxia, and 22 pathways under hypoxia (Fig. 4C, **Suppl. Table 3**). Under normoxic conditions, BSC9 mainly affected pathways involved in signal transduction as well as tumor- and organ-specific signaling. In contrast, under hypoxia, deregulation affected pathways related to metabolism and detoxification, apoptosis, proteostasis, and cellular stress responses (**Suppl. Table 3**).

A comparison with the transcriptomic profile of BA shows that BSC9 and BA provoke similarly cellular response. Overall, 77% (49/64) of the KEGG pathways regulated by BA are also enriched by BSC9 (Fig. 4D, **Suppl. Table 3**). In agreement with this, BA pathway enrichment analyses from previous studies revealed a substantial overlap in enriched signaling programs with KEGG pathways enriched upon BSC9 treatment, particularly in pathways related to signal transduction and cell survival, as well as cell cycle regulation and apoptosis [54, 70–72]. Additionally, previous studies indicate that BA modulates MAPK signaling, p53-associated pathways, and mitochondrial function, while also affecting metabolic and detoxification pathways, such as mitochondrial metabolism and solute carrier systems, as well as proteostasis-related processes including autophagy and lysosomal function, consistent with the pathway alterations observed here [58, 71–73]. In line with our findings of this study, our recent transcriptomic analyses in MDA-MB231 and MCF7 breast cancer cells further demonstrate that BA significantly alters gene expression patterns, especially under hypoxic conditions [71]. However, a large-scale analysis across 60 NCI cell lines suggests that BA exerts cytotoxic effects through different, context-dependent mechanisms that vary substantially between tumor entities [73].

CAIs are known to act on pathways related to metabolism and detoxification and signal transduction by targeting pH regulation and hypoxia-associated pathways, particularly through inhibition of CA IX [18, 37]. However, our recent analyses show that various AZA derivatives effectively inhibit CA IX activity and influence pH regulation without exhibiting cytotoxic effects in tumor cell lines [37]. Supporting this, transcriptomic profiling of the AZA derivative revealed almost no differences in the gene expression profile of MDA-MB231 cells (unpublished data). Taken together, BSC9 induces a complex transcriptional profile in HNSCC cells that resembles that of BA. Together with the previously reported CA-inhibitory

properties of acetazolamide, these findings propose the concept that both structural components may contribute to the biological activity of BSC9. This broader activity profile may contribute to the observed cytotoxic and radiosensitizing effects of BSC9, particularly under hypoxic conditions. This underscores the dual mode of action of BSC9. Initial studies on the combination of dual-acting nitroimidazole-based sulfonamides and radiation therapy demonstrate enhanced effects in colorectal carcinoma xenograft models without systemic toxicity [74]. Future in vivo studies will be required to determine whether BSC9 preferentially accumulates in hypoxic CA IX-overexpressing tumors and to what extent its antitumor activity is linked to CA IX targeting.

Taken together, the presented results are consistent with the concept that the betulin and acetazolamide moieties contribute distinct properties to BSC9. However, direct evidence for a CA IX-dependent dual mechanism is currently lacking and will require further functional validation.

Conclusion

In this study, we demonstrate that BSC9 promotes cytotoxic and radiosensitizing effects in 2D and 3D HNSCC models, with enhanced effects under hypoxic conditions. BSC9 treatment was associated with increased oxidative stress, loss of mitochondrial membrane potential, DNA-damage and apoptosis. Similar to BA, BSC9 triggers a complex transcriptional response characterized, particularly under hypoxic conditions, by signaling pathways related to the cell cycle, cell adhesion, migration, metabolism, and proteostasis. Importantly, the application of a two-layered 3D HNSCC spheroid model enabled the evaluation of BSC9-mediated treatment responses under distinct oxygenation conditions and supported the hypoxia-dependent radiosensitizing activity observed in conventional 2D cultures. Together, these findings demonstrate the potential of BSC9 to enhance radiosensitivity under hypoxic conditions and are consistent with the proposed contribution of its betulin and acetazolamide moieties to its biological activity. The present findings are consistent with the proposed dual-function concept of BSC9, although direct functional evidence linking the acetazolamide moiety to CA IX-dependent targeting remains to be established. Further preclinical studies are warranted to evaluate BSC9 in combination with radiotherapy for HNSCC.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Consent to publish

The approval of each author to publish the manuscript has been obtained. The manuscript is not currently under review by any other journal and has not been published before.

Generative AI and AI-assisted technologies in the manuscript preparation process.

During the preparation of this work the author used ChatGPT (OpenAI) in order to assist with the interpretation of RNA sequencing results, the functional annotation of differentially expressed genes and the exploration of potential biological pathways and gene interactions. After using this tool, the authors reviewed, verified, and edited the content as needed and take full responsibility for the content of the published article.

- **Data availability**

The RNA-seq datasets generated in this study are available in the NCBI Gene Expression Omnibus (GEO) under accession number GSE324120 (FaDu) and GSE324120 (SAS). Processed and raw sequencing data are publicly accessible to ensure reproducibility and transparency of the analyses.

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- **Authors' Contribution**

Conceptualization: D.K., and M.B.; methodology: D.K., M.K., M.P, M.B. and.; validation: D.K, M.G., A.G., M.P. and M.B.; formal analysis: D.K., M.G., A.G. and M.P.; investigation: D.K.; resources: M.K., M.B., D.V., J.P., T.G. and F.T.; data curation: D.K., A.G. and M.G. M.B.; writing – original draft preparation: D.K.; writing – review and editing: D.K., M.K., M.G., A.G., M.P., J.P., T.G., F.T., D.V. and M.B.; visualization: D.K., supervision: D.K., M.K., D.V. and M.B., project administration: M.K., D.V., F.T. and M.B.; funding acquisition: M.K., D.V., F.T. and M.B

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Figures

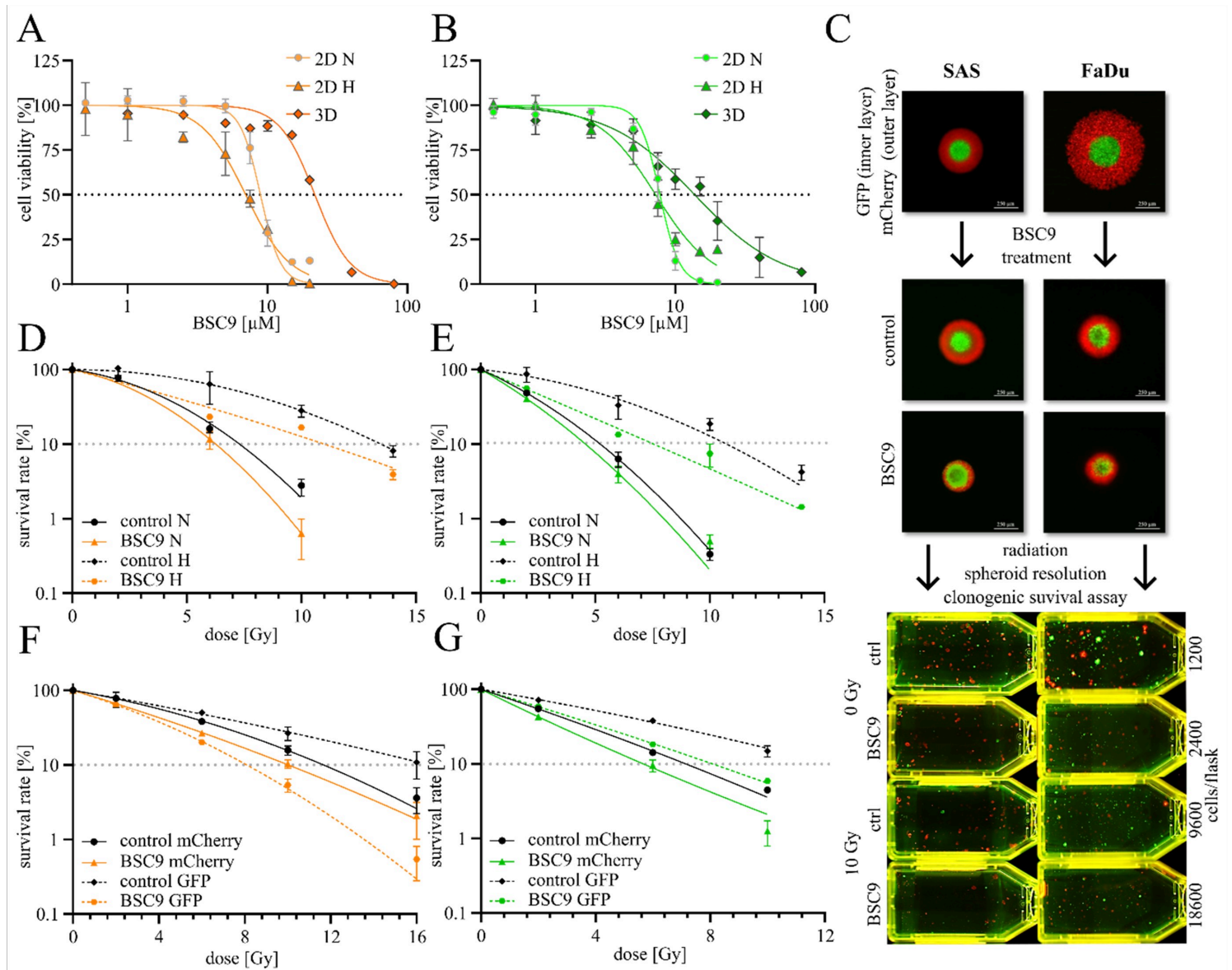


Figure 1

Cytotoxicity and radiosensitivity induced by BSC9 in 2D and 3D HNSCC cell models. Dose-dependent effects of BSC9 on cell viability were quantified by 2D and 3D CellTiter-Glo Luminescent Viability Assay for SAS (**A**) and FaDu (**B**) cells. BSC9 was tested at concentrations ranging from 0.5 to 80 μM under normoxia (N) and hypoxia (H). The dotted lines indicate a relative cell viability of 50 %. Representation of multilayered spheroids composed of a GFP-labeled inner cell layer and an outer mCherry-labeled cell layer after BSC9 treatment and radiation (**C, upper**). Representative clonogenic cell survival of inner and outer fluorescent cell layer is shown (**C, lower**). Radiosensitivity of 2D SAS (**D**) and FaDu (**E**) cells under normoxic and hypoxic conditions after treatment with BSC9 and radiation with 2–14 Gy; and of 3D HNSCC models of inner GFP- and outer mCherry-labeled SAS (**F**) and FaDu (**G**) cells after treatment with BSC9 and irradiation with 2–16 Gy. The dotted lines indicate a relative cell survival rate of 10 %. The data represent the mean values (± SDs) of at least three independent experiments.

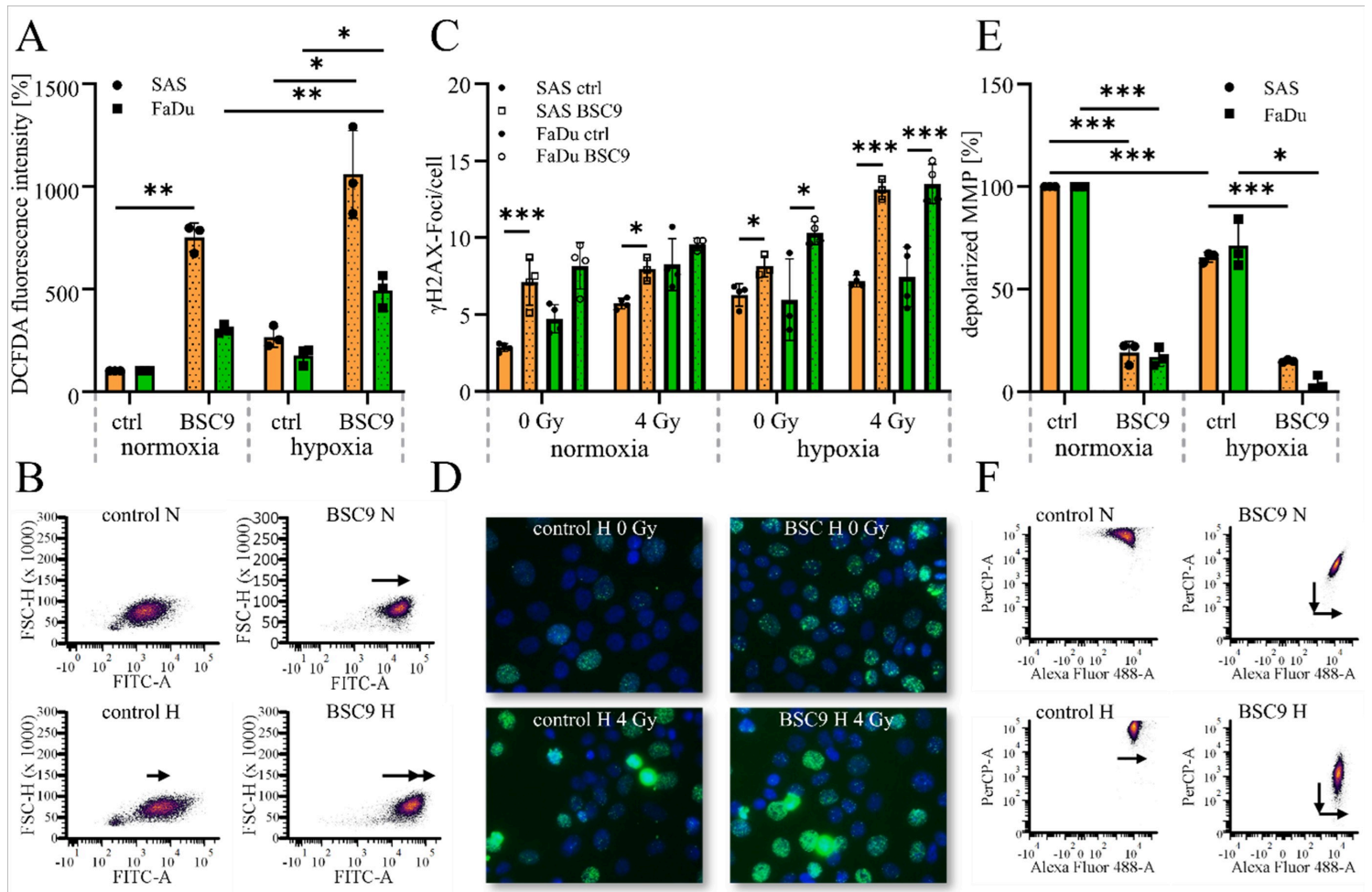


Figure 2

BSC9 induces reactive oxygen species, DNA damage, and mitochondrial dysfunction in HNSCC cells. SAS (orange) and FaDu (green) cells were treated with BSC9 under normoxic (N) and hypoxic (H) conditions and **(A)** ROS levels were quantified by flow cytometry. **(C)** DNA double-strand breaks were analyzed by γ H2AX staining after irradiation at either 0 or 4 Gy and **(E)** MMP was assessed and quantified by flow cytometry. Representative dot plots of ROS **(B)** and MMP **(F)**, as well as fluorescence images of γ H2AX staining **(D)** of SAS cells are shown. The data represent the mean values ($\pm$ SDs) of at least three independent experiments. Significant p values are highlighted with asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

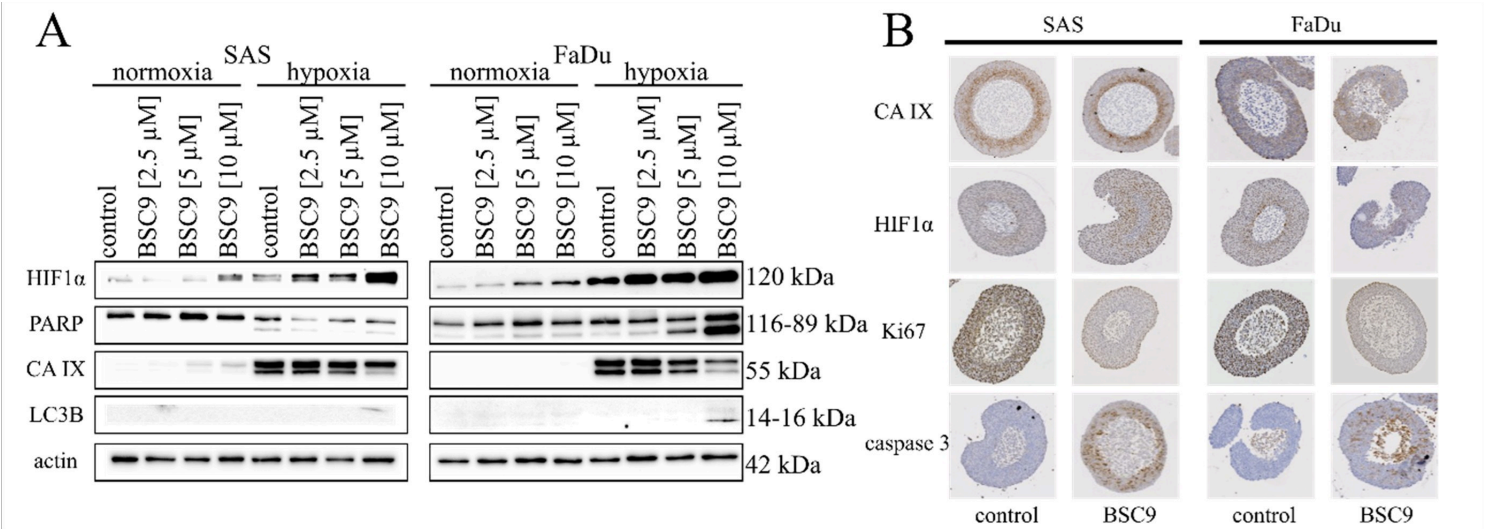


Figure 3

Protein expression of hypoxia- and cell death-induced proteins in 2D and 3D HNSCC cell models. (A) Western blot of HIF1α, PARP cleavage, CA IX, LC3B and actin of 2D SAS and FaDu models after BSC9 treatment (2.5 to 10 μM) under normoxia and hypoxia are shown in representative images. Actin was used as control. **(B)** Histological staining of CA IX, HIF1α, Ki67 and cleaved caspase-3 in 3D SAS- and FaDu models are shown in representative images. The spheroids were cultured under normoxic and hypoxic conditions in media with BSC9. Each image is representative of at least three independent experiments.

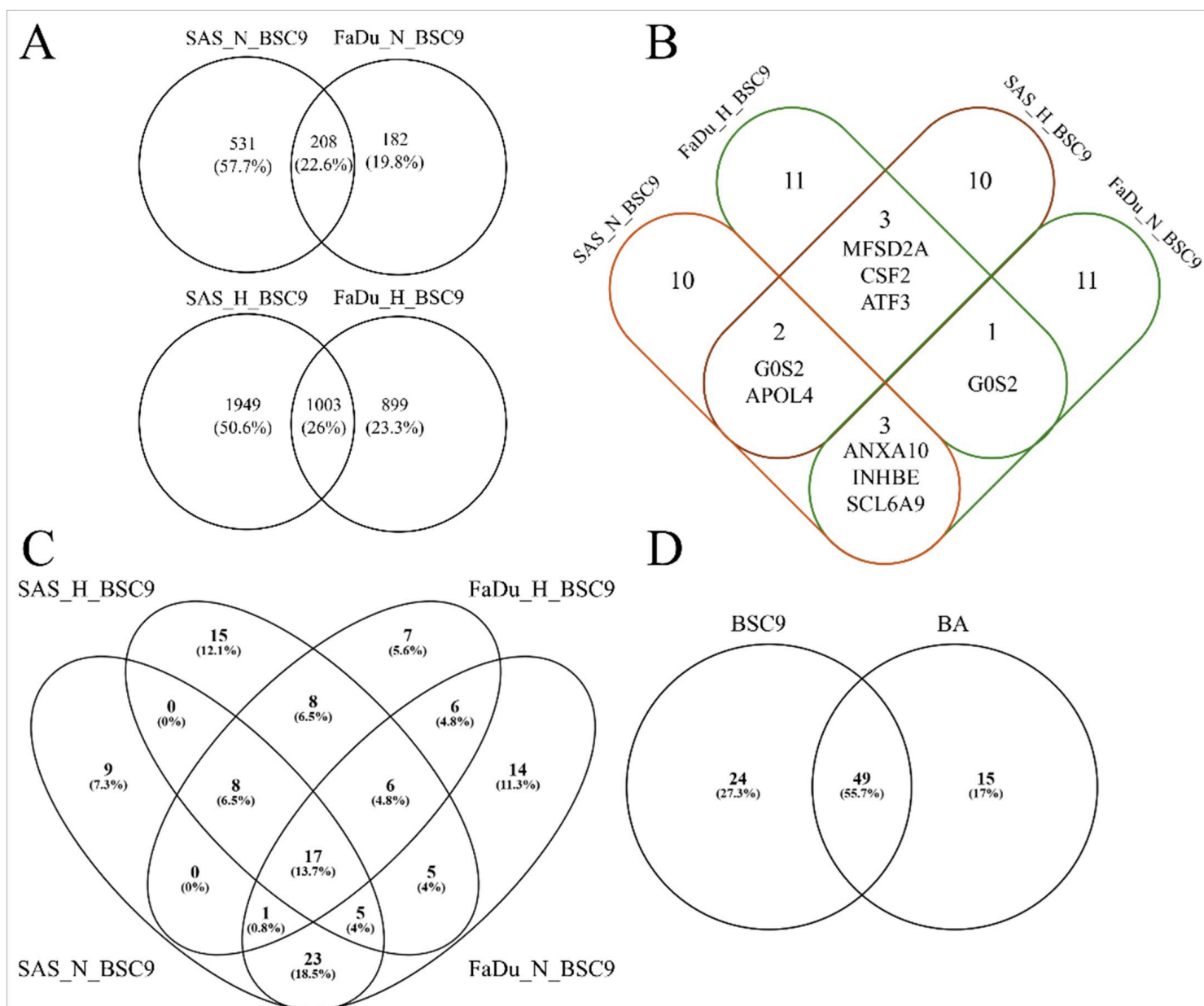


Figure 4

Transcriptomic profiling and GSEA analysis of BSC9 treated HNSCC cells under normoxia (N) and hypoxia (H). (A) Differential gene expression was calculated as $\log_2$ -fold-change > 1 or < -1 of DMSO control cells in at least four independent experiments ($p < 0.05$). (B) The top differentially expressed genes were ranked using a product score integrating effect size and statistical significance ($|\log_2FC| \times -\log_{10}(FDR)$). Shown are the top 15 genes. (C) KEGG pathway analysis of SAS and FaDu cells following BSC9 treatment under normoxia (N) and hypoxia (H) (D) Enriched KEGG-pathways in SAS and FaDu cells after treatment with BSC9 and BA under normoxia and hypoxia.

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