Combining TMZ and SB225002 induces changes of CXCR2 and VEGFR signalling in primary human endothelial cells *in vitro*

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Abstract. Standard of care therapy for glioblastoma (GBM) consisting of surgical removal, temozolomide (TMZ) and radiotherapy fails to cure the disease and median survival is limited to 15 months. Therapeutic approaches targeting vascular endothelial growth factor (VEGF)-mediated angiogenesis, one of the major drivers of tumour growth, have not prolonged patient survival as reported in clinical studies. Apart from VEGFR signalling, proangiogenic C-X-C motif chemokine receptor 2 (CXCR2) is of special interest as its ligands C-X-C motif chemokine ligand 2 (CXCL2) and interleukin-8 (IL8) are upregulated and associated with reduced survival in GBM patients. As CXCR2 is also expressed by endothelial cells, the aim of the present study was to elucidate the effect of combination therapy on gene and protein expression of primary human endothelial cells (HUVECs). To mimic the GBM specific CXCL2/IL8 oversupply environment [referred to as stimulation (STIM)], HUVECs were treated with a cocktail of CXCL2/IL8 and/or TMZ and/or CXCR2-antagonist SB225002 (SB). In brief, six treatment conditions were utilized: i) Control, ii) STIM (CXCL2/IL8), iii) TMZ + SB, iv) STIM + TMZ, v) STIM + SB, vi) STIM + TMZ + SB followed by either RNA-isolation and RT-qPCR for BAX, BCL2, vascular endothelial growth receptor (VEGFR)1/2, VEGF, CXCR1/2, CXCL2 and IL8 or immunofluorescence staining for VEGFR2 and CXCR2. SB and TMZ led to morphological changes of HUVECs and downregulated antiapoptotic BCL2 *in vitro*. In addition, gene expression of the alternative proangiogenic CXCL2/IL8/CXCR2 signalling pathway was significantly altered by the combination therapy, while the VEGF/VEGFR1/2 axis was only mildly affected. Furthermore, VEGFR2 and CXCR2 gene and protein expression regulation differed. VEGFR2 was not altered at the gene expression level, while combination therapy with TMZ and SB led to a 74% upregulation of VEGFR2 at the protein level. By contrast, CXCR2 was upregulated 5-fold by the combination therapy at the gene expression level and downregulated by 72.5% at the protein expression level. The present study provided first insights into the molecular changes of two major proangiogenic pathways in primary endothelial cells during treatment with TMZ and SB. Different gene and protein expression levels of the proangiogenic receptors CXCR2 and VEGFR2 *in vitro* must be taken into consideration in future studies.

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Introduction

To date, glioblastoma (GBM) is still the most common malignant brain tumour of glial descent in adults (1,2). Even with the combined standard of care therapy comprising of three major elements: Surgical removal of the tumour, followed by chemoradiation and subsequent adjuvant chemotherapy with temozolomide (TMZ), median survival is still limited to little over a year (2-5). To address this major issue, several new therapeutic approaches have been developed in recent years, showing promising results in preclinical and clinical studies (6-8). A number of these novel therapeutic approaches target the tumour microenvironment (6,9-11), that includes endothelial cells, pericytes, immune cells and particularly tumour-associated microglia/macrophages which make up to 30-50% of the tumour mass (12-16). They secrete soluble factors such as growth factors and chemokines and support tumour growth e.g., by initiating the formation of new blood vessels, the proliferation of tumour cells and by expressing immuno-suppressive molecules (12-15,17-20). For instance, VEGF is one of the most important proangiogenic growth factors in GBM (21,22). Nevertheless, targeting VEGF-mediated angiogenesis in GBM has not significantly improved patient survival (7,23). Apart from growth factors such as VEGF, chemokines and their respective receptors are crucial to numerous tumour-supporting processes and therefore are relevant targets for new therapeutic approaches in

GBM (6). Chemokine signalling axes, for instance the CXCR2 signalling pathway, have been investigated in preclinical *in vitro* and *in vivo* experimental models as well as in GBM patient *ex vivo* samples (11,18,19,24). As CXCR2 is expressed by glioma cells as well as endothelial cells and overexpression of its ligands CXCL2 and IL8 is associated with a reduced patient survival (11,18,25-28), this signalling pathway is a feasible target for GBM therapy. In this regard, SB225002 (SB), a commonly used small molecule CXCR2-antagonist, inhibits CXCR2 downstream signalling (11,18,24,29). In a previous study by the authors, it was demonstrated that anti-CXCR2 therapy with SB led to a reduced vessel density in an immunocompetent orthotopic mouse model *in vivo*. In addition, in that study, a diminished proliferation of glioma and murine endothelial cells was observed (11). Furthermore, SB also decreased angiogenesis in a 3D spheroid-based angiogenesis model utilizing primary human brain endothelial cells *in vitro* (18). Moreover, SB is known to inhibit CXCR2 signalling-mediated vascular mimicry *in vivo* (24).

Emerging evidence suggests that combined therapeutic approaches are superior to single therapies due to GBM heterogeneity and various resistance mechanisms (2-4). Based on the studies aforementioned, combining CXCR2-antagonisation with the standard-of-care TMZ therapy appears promising. In another previous study by the authors, it was demonstrated that a combination therapy *in vivo*, consisting of TMZ and SB, reduced tumour volume in immunocompetent mice (19). However, little is known about the molecular changes during treatment with the combination therapy. While proliferation was significantly reduced by the combination therapy, gene expression of proangiogenic pathways and pro- and antiapoptotic genes were not significantly altered within the tumour lysates (19). Nevertheless, certain tendencies were observed and the reduced tumour volume is likely based on changes in gene and protein expression (19). However, to take this promising approach from mouse experiments to a potential therapeutic approach in humans, more research is warranted. As tumour growth is dependent on the formation of new blood vessels and CXCR2 is widely expressed by endothelial cells, these cells are highly relevant for GBM therapy (25,27,30). Therefore, the aim of the present study was to elucidate the outcome of combined TMZ and SB treatment *in vitro*. The tumour microenvironment was mimicked in GBM patients with CXCL2 and IL8 oversupply (18) in comparison to normal culturing conditions and the effect of this treatment strategy on gene and protein expression of primary human endothelial cells was assessed.

Materials and methods

Culture of human endothelial cells. Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell GmbH and cultured in endothelial cell growth medium 2 (ECGM2; cat. no. C-22111) containing supplements (supplement mix for ECGM2; cat. no. C-39216; both PromoCell GmbH) and 0.1 mg/ml gentamicin in 75 mm^2 cell culture flasks (Falcon®, Corning, Inc.). The cells were incubated at 37˚C until they reached 90% confluency. For sub-culture of HUVECs the PromoCell Detach Kit was used following the instructions of the manufacturer. Cells from passages 3-4 were used for the experiments (Fig. 1A).

Treatment with TMZ and SB. HUVECs were seeded at $0.6x10^5$ and cultured on 6-well plates or 8-well-glass-bottom-μ-slides (both Sarstedt®; SARSTEDT AG & Co. KG) until they reached 80% confluency. Cells were starved for 4 h in 0.1% fetal calf serum (FCS) in endothelial cell basal medium 2 (ECBM2) (cat. no. C-22211; PromoCell GmbH). Subsequently, the cells were treated with a cocktail of 25 ng/ml CXCL2/IL8 (recombinant human CXCL2 (carrier-free), recombinant human CXCL8 (carrier-free); BioLegend, Inc.) and/or 10 μM TMZ (Temodal®; MSD; Merck & Co., Inc.) and/or 0.03 μM SB (SB225002; Tocris Bioscience) for 24 h as described below. A concentration of 25 ng/ml for CXCL2 and IL8 was selected as our previous study showed a significant effect on cells treated with that concentration (18). Furthermore, previous *in vitro* studies also revealed an effect of SB at 0.03 μM (11,18,31). To elucidate the efficacy of combined treatment with TMZ and SB, HUVECs were cultured in six different treatment conditions: i) Control, ii) STIM (stimulation by CXCL2 and IL8), iii) TMZ + SB (treatment with the combination therapy), iv) STIM + TMZ (treatment by CXCL2/IL8 and additional TMZ), v) STIM + SB (treatment by CXCL2/IL8 and additional SB), and vi) STIM + TMZ + SB (treatment by CXCL2/IL8 and additional TMZ + SB).

RNA isolation and reverse transcription-quantitative (RT-q)PCR. HUVECs were detached using cell scrapers (Corning®; Corning, Inc.) after application of 300 μl lysis buffer (PureLink RNA Mini Kit; Invitrogen; Thermo Fisher Scientific, Inc.) with 1% 2-mercaptoethanol per well. The PureLink RNA Mini Kit was used to isolate RNA according to the corresponding protocol. RNA concentration was measured with a plate photometer (Infinite M200; Tecan Group, Ltd.) and RNA quality was assessed using Agilent 2100 Bioanalyzer prior to eradication of DNA contamination. cDNA synthesis was carried out with 500 ng RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.) according to the manufacturer's instructions. The cDNA quantity was measured by photometry. RT-qPCR was performed for *BAX, BCL2, VEGF, VEGFR1*, *VEGFR2, IL8, CXCL2, CXCR1* and *CXCR2* using triplicates in a 10-μl reaction volume and the TB Green™ Premix Ex Taq™ Kit (Takara Bio, Inc.). *18S* was used as the reference gene. Primer sequences were designed with Primer BLAST by the National Center for Biotechnology Information, U.S. National Library of Medicine and purchased from TIB MOLBIOL (Table I). RT-qPCR was performed with the Quant Studio 6 Flex System (Thermo Scientific Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec; denaturation at 95˚C for 5 sec; annealing and elongation, each at 60˚C for 30 and 60 sec, respectively; and the hold stage at 4˚C. The number of performed cycles was 40. Target expression levels were normalized to *18S* mRNA. The relative quantification method $2^{-\Delta\Delta Cq}$ was used for analyses (32). Accordingly, fold change of target gene expression (relative expression level) was calculated in relation to the target gene expression of the control group (32) (Fig. 1B).

Immunofluorescence staining. HUVECs were cultured to 80% confluency on 8-well-glass-bottom-μ-slides (Sarstedt®;

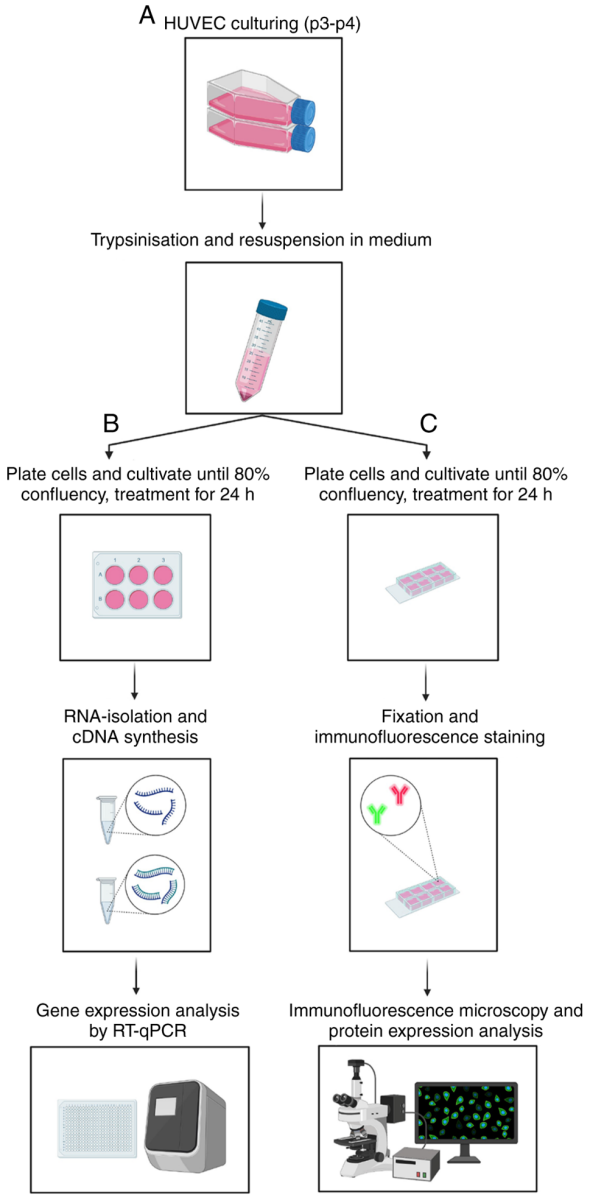

Figure 1. Methodological setup. (A) Cells were cultured and passaged. (B) HUVECs were seeded into 6-well plates and cultured until they reached 80% confluency. The cells were then starved for 4 h in 0.1% FCS in ECBM2, followed by treatment with 25 ng/ml CXCL2 and IL8 and/or 10 μM TMZ and/or 0.03 μM SB for 24 h. Subsequently, RNA-isolation was performed followed by quantitative PCR for *BAX*, *BCL2*, *VEGFR1*, *VEGFR2*, *CXCR1*, *CXCR2*, *VEGF*, *CXCL2* and *IL8*. (C) HUVECs were seeded into 8-well glass bottom plates and cultured until they reached 80% confluency. The cells were then starved for 4 h in 0.1% FCS in ECBM2, followed by treatment with 25 ng/ml CXCL2 and IL8 and/or 10 μM TMZ and/or 0.03 μM SB for 24 h. Immunofluorescence staining for the cell nucleus (DAPI), the cytoskeleton (Phalloidin), VEGFR2 and CXCR2 was carried out, followed by immunofluorescence microscopy. This figure was created using Biorender.org. HUVECs, human umbilical vein endothelial cells; FCS, fetal calf serum; ECBM2, endothelial cell basal medium 2; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8; TMZ, temozolomide; SB, SB225002; *VEGFR1*, vascular endothelial growth factor receptor 1; *VEGFR2*, vascular endothelial growth factor receptor 2; *CXCR1*, C-X-C motif chemokine receptor 1; *CXCR2*, C-X-C motif chemokine receptor 2; *VEGF*, vascular endothelial growth factor.

SARSTEDT AG & Co. KG) as aforementioned and then washed with PBS and fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min. The fixed cells were blocked in 1% Casein/PBS for 30 min. Primary antibodies rabbit anti-CXCR2 (1:200; product code ab14935; Abcam) or rabbit anti-VEGFR2 (1:200; product no. 2479S; Cell Signaling Technology, Inc.) combined with AlexaFluor™488-Phalloidin (1:200; cat. no. A12379; Thermo Fischer Scientific, Inc.) were applied in 0.5% Casein/PBS for 2 h at room temperature. Sections were then washed and treated with the secondary antibody (1:200; Cy™3 donkey anti-rabbit; code no. 711-165-152, lot no. 122296; Jackson ImmunoResearch Europe, Ltd.) for 1.5 h at room temperature. All sections were mounted with DAPI-containing medium (Dianova GmbH) and sealed with cover slips. Images were acquired using the same exposure time for every condition, with a 20X magnifying objective on a fluorescence microscope (Zeiss Axio Observer Z1; Zeiss MicroImaging GmbH). ImageJ 1.53c (available from: http://imagej.nih.gov/ij; National Insitutes of Health; accessed 28th June 2020) was used to analyse images (Fig. 1C).

Statistical analyses. Statistical analyses were performed using GraphPad Prism Software (v9.1.1; GraphPad Software, Inc.) expressed as the mean ± standard deviation (SD). If not indicated otherwise, all experiments were carried out at least three times. Groups were compared by one-way ANOVA with Bonferroni correction. A P-value ≤0.05 was considered to indicate a statistically significant difference.

Results

Treatment with TMZ and/or SB leads to morphological changes in HUVECs. To analyse the effect of the combination therapy consisting of TMZ and SB, HUVECs were treated as described in the previous section. In brief, six treatment conditions were utilized: i) Control, ii) STIM, iii) TMZ + SB, iv) STIM + TMZ, v) STIM + SB, and vi) STIM + TMZ + SB. The optimal concentration of the reagents for the experiments were based on previous experiments (11,18,31), as aforementioned. During culture, images by phase contrast microscopy were obtained regularly to investigate morphological changes. Alterations in cell morphology of HUVECs were observed in all groups treated with SB or TMZ (Fig. 2C-F). While cells in the control and STIM group (Fig. 2A and B) exhibited the typical long and thin phenotype, treatment with SB led to a rather rounded cell morphology (Fig. 2C, E and F) (18). Furthermore in the groups treated with either TMZ, SB or a combination of both, there were more detached cells and more cell debris (Fig. 2C-F). Therefore, these observations led us to speculate whether treatment with TMZ and/or SB triggered apoptosis.

Combination therapy induces downregulation of anti-apoptotic BCL2. It is known that SB inhibits proliferation and leads to apoptosis in leukaemia cells *in vitro* (29,31). Therefore, investigating the effect of SB in combination with TMZ on primary human endothelial cells was of special interest. Thus, HUVECs were cultured and RNA was extracted for qPCR to evaluate the expression level of two different genes involved in apoptosis: *BAX* (proapoptotic) and *BCL2* (anti-apoptotic). The expression of the proapoptotic molecule *BAX* was enhanced by the combination therapy (TMZ + SB) in the simulated CXCL2/IL8 oversupply environment compared to

Table I. Primer sequences.

Gene	Primer orientation	Sequence 5' → 3'	Tm (°C)	Fragment size (bp)
h18S[a]	Forward	GGCCCTGTAATTGGAATGAGTC	59	146
	Reverse	CCAAGATCCAACTACGAGCTT	58	
hVEGFR1	Forward	CAGGCCCAGTTTCTGCCATT	60	82
	Reverse	TTCCAGCTCAGCGTGGTCGTA	63	
hVEGFR2	Forward	CATGTACGGTCTATGCCATTCCTC	61	73
	Reverse	TTGGCGCACTCTTCCTCCAAC	63	
hVEGFA[a]	Forward	TGCAGATTATGCGGATCAAACC	59	81
	Reverse	TGCATTCACATTTGTTGTGCTGTAG	61	
hCXCR1	Forward	GCAGCTCCTACTGTTGGACA	60	84
	Reverse	GCCCTACCCCACAGAAAGTC	60	
hCXCR2	Forward	GGTGTCCTACAGGTGAAAAG	55	85
	Reverse	TGTCACTCTCCATGTTAAAA	52	
hCXCL2	Forward	TCCCTTGGACATTTTATGTCTTTC	57	89
	Reverse	TCTCTGCTCTAACACAGAGGGA	60	
hIL8[a]	Forward	CTGAGAGTGATTGAGAGTGG	55	113
	Reverse	ACAACCCTCTGCACCCAGTT	62	
hBAX	Forward	GCCCTTTTGCTTCAGGGTTT	59	121
	Reverse	TGAGACACTCGCTCAGCTTC	60	
hBCL2	Forward	TGCGGCCTCTGTTTGATTTC	59	120
	Reverse	GGCAGGCATGTTGACTTCAC	60	

[a]h18S (33), hVEGFA (34), hIL8 (35). VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; VEGFA, vascular endothelial growth factor A; CXCR1, C-X-C motif chemokine receptor 1; CXCR2, C-X-C motif chemokine receptor 2; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8.

the only simulated oversupply group (Fig. 2G). Interestingly, *BAX* expression was only minimally altered by the combination therapy in standard culturing conditions (Control vs. TMZ + SB) (Fig. 2G). Notably, antiapoptotic *BCL2* was significantly downregulated after combined treatment with TMZ and SB (Fig. 2H). Within the simulated oversupply group (STIM) gene expression of *BAX* and *BCL2* was not altered. Collectively, apoptosis was induced in all treatment groups receiving SB, however none reached the level of significance and *BCL2* was significantly downregulated by treatment with TMZ and SB.

CXCR2 gene and protein expression is altered differently by the combination therapy. As SB has been shown to i) affect the CXCR2 signalling pathway *in vitro* and *in vivo* (11,18,19) and ii) CXCR2 ligands, CXCL2 and IL8, are highly effective alternative proangiogenic molecules (18), the therapy-induced changes in gene and protein expression of proangiogenic pathways *in vitro* were investigated (Fig. 3). Apart from the standard proangiogenic receptors of *VEGF*, *VEGFR1* and *VEGFR2* (18,33-35), HUVECs express the receptors for *CXCL2* and *IL8*, *CXCR1* and *CXCR2* (18,25-28). Moreover, *CXCL2* and *IL8* are overexpressed in approximately one third of GBM patients, and overexpression is associated with a reduced overall survival (18). The analysis of the alternative proangiogenic receptors *CXCR1* and *CXCR2* revealed that the expression of the proangiogenic receptor *CXCR1* was not significantly changed in HUVECs. By contrast, *CXCR2* was upregulated by the combination therapy and by SB alone within and outside of the mimicked oversupply with CXCL2/IL8 (Fig. 3A and B). For instance, the combination therapy with TMZ and SB in the simulated oversupply environment significantly enhanced the expression of *CXCR2*. Under normal culturing conditions, combining TMZ and SB enhanced the expression of *CXCR2* (Fig. 3B). In summary, *CXCR2* was significantly highly expressed in all groups receiving treatment with SB alone or in combination with TMZ.

As *CXCR2* expression was affected by the combination therapy and thus appeared to be more relevant than *CXCR1*, the protein expression of CXCR2 was then evaluated using immunofluorescence staining. In the mimicked CXCL2/IL8 oversupply environment, CXCR2 protein expression was significantly increased (Fig. 3C and D). Furthermore, the combination therapy led to an enhanced CXCR2 protein expression under normal culturing conditions and a distinctively decreased CXCR2 expression under mimicked CXCL2/IL8 oversupply (Fig. 3C and D). In summary, any treatment group receiving SB (STIM + SB, TMZ + SB and STIM + TMZ + SB) exhibited an upregulation of CXCR2 at the gene expression level while at the protein expression level CXCR2 was differentially regulated. STIM and TMZ + SB exhibited an upregulation of CXCR2 whereas the combination therapy in the mimicked oversupply environment led to a downregulation of CXCR2 at the protein level.

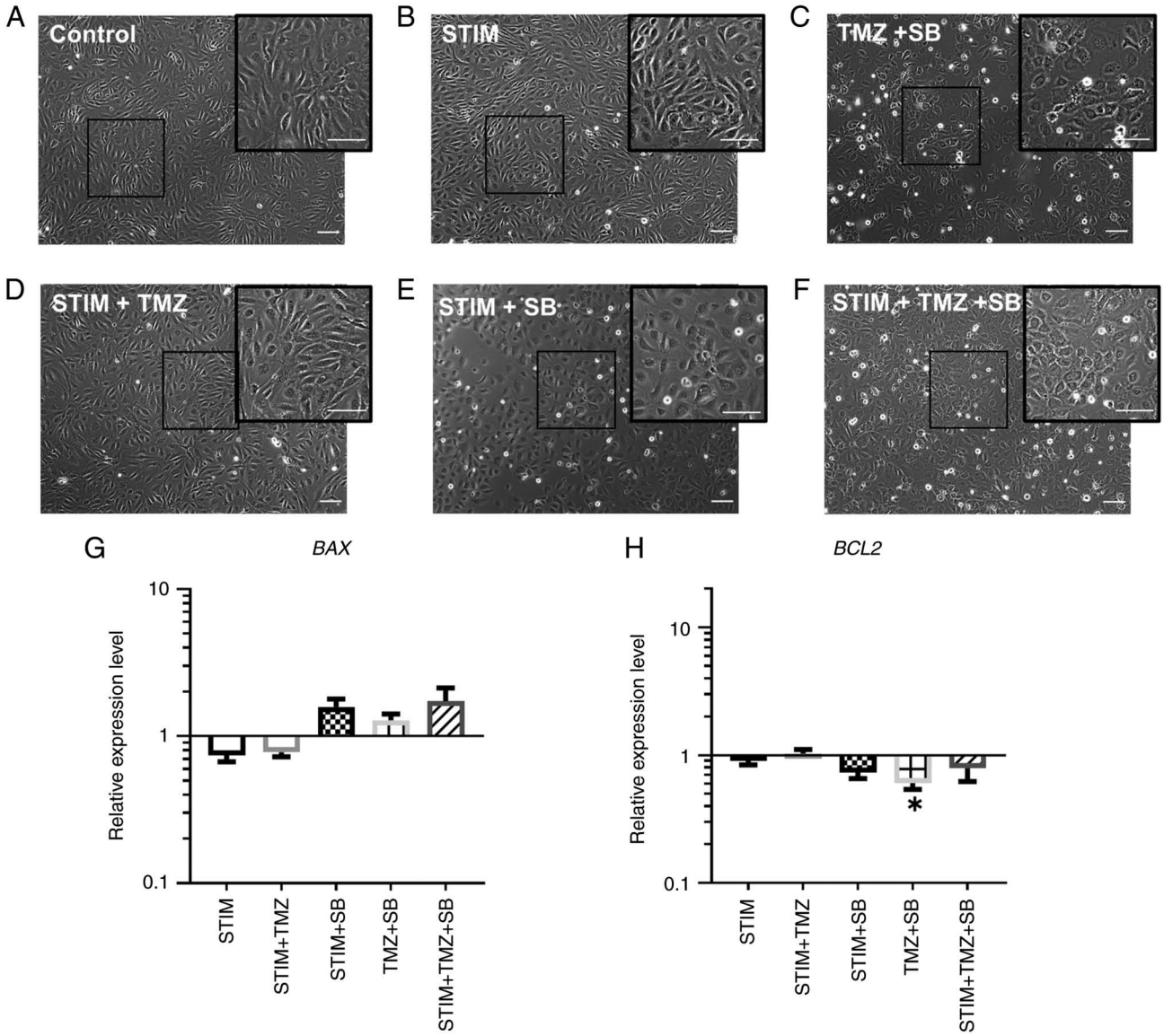

Figure 2. Combination therapy with TMZ and SB leads to morphological changes in HUVECs and downregulation of antiapoptotic *BCL2*. (A-H) HUVECs were stimulated with a cocktail of 25 ng/ml CXLC2 and 25 ng/ml IL8 combined with 10 μM TMZ, 0.03 μM SB or both for 24 h as indicated. Analysis of mRNA expression regarding *BAX* and *BCL2* expression was then performed. Gene expression was analysed using the relative quantification method ($\Delta\Delta$Cq) and compared to normal culturing conditions (control). Accordingly, expression of each target in the control group was set to 1. Medium containing 0.1% FCS/1% DMSO was used as the control. (A-F) Representative images by phase contrast microscopy are shown with more attached cells and debris in groups which included treatment with SB, TMZ or both; scale bar, 100 μm. (G and H) Changes of relative expression levels are shown for *BAX* and *BCL2* on a logarithmic scale. P-values indicated in the graph are in comparison to the control group. Other significant P-values for *BAX* expression: STIM vs. STIM + TMZ + SB, P=0.0253; STIM+ TMZ vs. STIM + TMZ + SB, P=0.0341. Other significant P-values for BCL2 expression: STIM + TMZ vs. STIM + SB, P=0.0259. Data represents multiple experiments with similar results (n=9/condition out of three independent experiments). *P<0.05; one-way ANOVA (Bonferroni correction); bar graphs represent the mean ± standard deviation. STIM, stimulation with 25 ng/ml CXCL2 and IL8; TMZ, temozolomide; SB, SB225002; HUVECs, human umbilical vein endothelial cells; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8; FCS, fetal calf serum; DMSO, dimethyl sulfoxide.

Expression of standard proangiogenic receptors, VEGFR1 and VEGFR2, is unaltered by combination therapy. VEGFR signalling is important for tumour angiogenesis and has been widely studied (21,36). It is known as the standard proangiogenic signalling in health and in disease (21,37). Furthermore, previous studies indicate a crosstalk between CXCL2/IL8 and VEGF signalling (38-40). Therefore, investigating changes in VEGFR signalling was of special interest. Treatment with TMZ, SB or a combination of both had no effect on the expression of *VEGFR2*, however *VEGFR1* expression was significantly reduced by SB in the mimicked oversupply environment with CXCL2 and IL8 and by the combination therapy under normal culturing conditions (Fig. 4A and B). The next aim was to evaluate the effect of the combination therapy on the protein expression of the most important proangiogenic VEGF receptor, VEGFR2 (7). Treatment of HUVECs with combined TMZ and SB *in vitro* was repeated, the cells were fixed and immunofluorescence staining for VEGFR2 was performed (Fig. 4C and D). At the protein level VEGFR2 was strongly upregulated by the combination therapy in the mimicked CXCL2/IL8 oversupply environment group and in the mimicked oversupply environment alone. However, VEGFR2 expression was unaltered by the combination

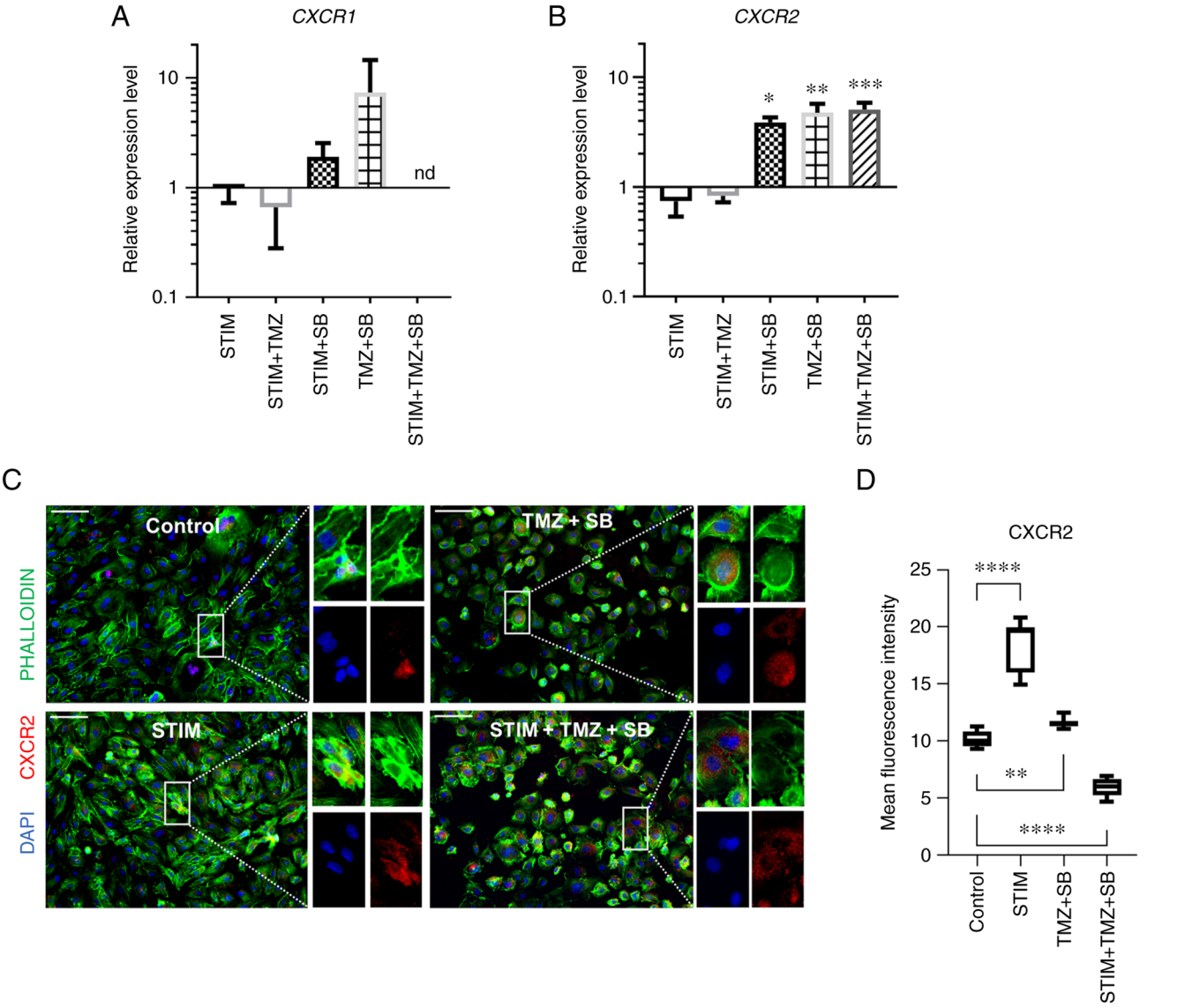

Figure 3. Combination therapy with TMZ and SB alters *CXCR2* gene and protein expression in HUVECs. (A-D) HUVECs were stimulated with a cocktail of 25 ng/ml CXLC2 and 25 ng/ml IL8 combined with 10 μM TMZ, 0.03 μM SB or both for 24 h as indicated. (A and B) Analysis of mRNA expression regarding *CXCR1* and *CXCR2*. Expression of the proangiogenic receptor *CXCR1* was not significantly changed. By contrast, *CXCR2* was upregulated by the combination therapy and by SB alone within and outside of the mimicked oversupply environment. Gene expression was analysed using the relative quantification method ($2^{-\Delta\Delta Cq}$) and compared to normal culturing conditions (control). Accordingly, expression of each target in the control group was set to 1. Medium containing 0.1% FCS/1%DMSO was used as the control. P-values indicated in the graph are in comparison to the control group. Other significant P-values for *CXCR2* expression: STIM vs. STIM + SB, P=0.0170; STIM vs. TMZ + SB, 0.0007; STIM vs. STIM + TMZ + SB, P=0.0002; STIM + TMZ vs. STIM + TMZ, P=0.0035; STIM + TMZ vs. STIM + TMZ + SB, P=0.0013. Data represents multiple experiments with similar results (n=9/condition out of three independent experiments). (C and D) Immunofluorescence staining of the cell nuclei (DAPI in blue), cytoskeleton (PHALLOIDIN in green) and CXCR2 (in red). Representative images of CXCR2 (C) in all four conditions, captured with the same exposure time; Scale bar, 100 μm. (D) Boxplots depicting mean intensity values under normal culturing conditions and in simulated oversupply with and without combination therapy with TMZ and SB. Mean intensity measurements revealed significant differences between the treatment groups. In contrast to gene expression CXCR2 protein was significantly downregulated by the combination therapy in the mimicked oversupply environment. (A-D) Medium containing 0.1% FCS/1% DMSO was used as the control. P-values indicated in the graph are in comparison to the control group. Other significant P-values for CXCR2 protein expression: STIM vs. TMZ + SB, P<0.0001; STIM vs. STIM + TMZ + SB, P<0.0001; TMZ + SB vs. STIM + TMZ + SB, P<0.0001; (n >10 images/condition). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. A, B and D, one-way ANOVA (Bonferroni correction); bar graphs represent the mean ± standard deviation. STIM, stimulation with 25 ng/ml CXCL2 and IL8; TMZ, temozolomide; SB, SB225002; CXCR2, C-X-C motif chemokine receptor 2; HUVECs, human umbilical vein endothelial cells; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8; CXCR1, C-X-C motif chemokine receptor 1; FCS, fetal calf serum; DMSO, dimethyl sulfoxide.

therapy under normal culturing conditions (Fig. 4D). Thus, VEGFR2 was differentially regulated at the gene and protein expression levels.

Expression of alternative proangiogenic molecules is altered by the combination therapy. As gene and protein expression of the important proangiogenic receptors VEGFR1/2 and CXCR1/2 were differently affected by the combination therapy, the changes in the expression of the associated ligands were evaluated. Expression of VEGF, CXCL2 and IL8 was not changed by the mimicked CXCL2/IL8 oversupply environment (Fig. 5A-C). Interestingly, CXCL2 and IL8 gene expression were altered by the treatment with SB (Fig. 5A and B). However, under normal culturing conditions only IL8 expression was

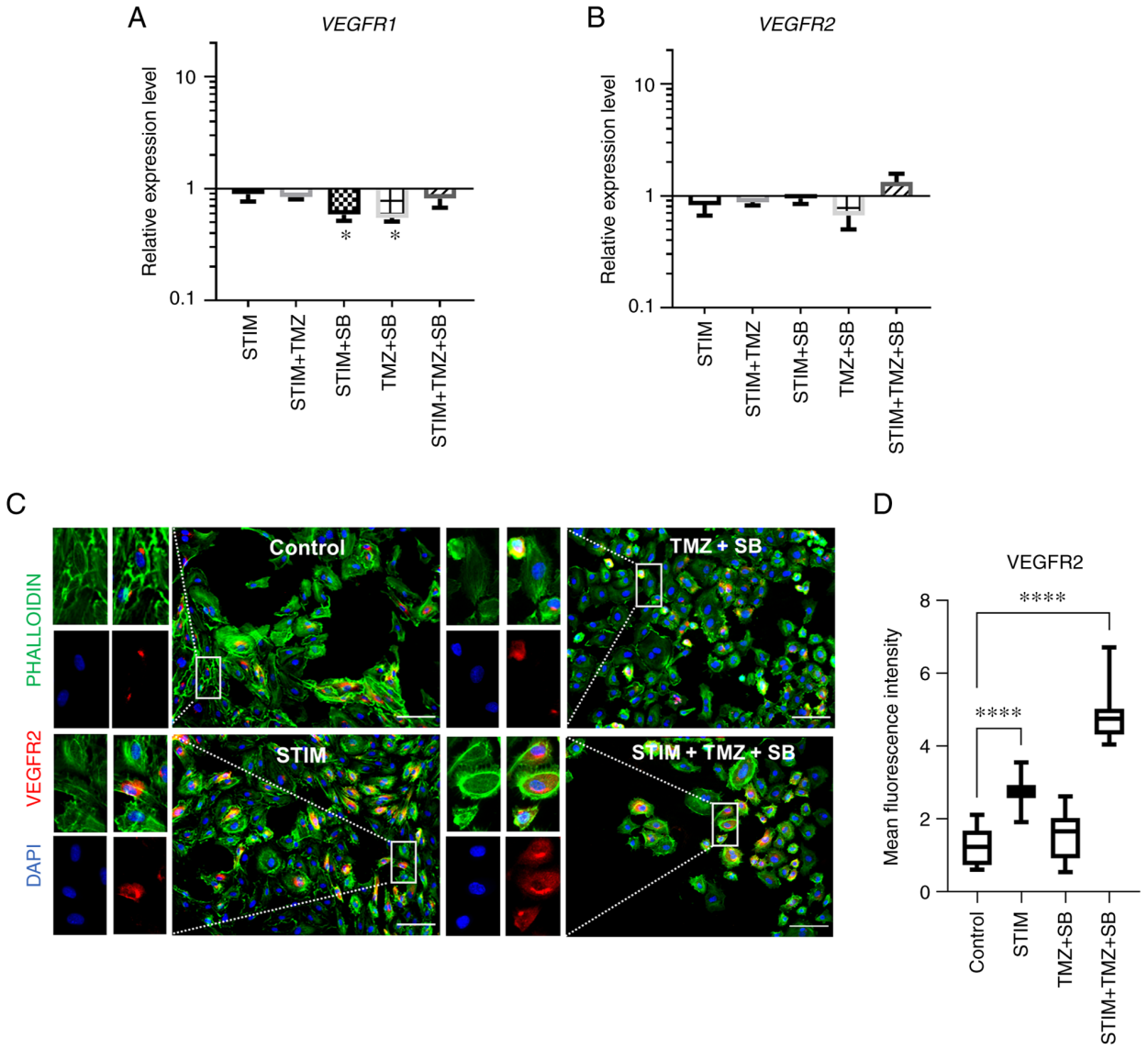

Figure 4. Combination therapy with TMZ and SB does not alter *VEGFR2* gene expression but changes protein expression of VEGFR2 in HUVECs. (A-D) HUVECs were stimulated with a cocktail of 25 ng/ml CXLC2 and 25 ng/ml IL8 combined with 10 μM TMZ, 0.03 μM SB or both for 24 h as indicated. (A and B) Analysis of mRNA expression regarding *VEGFR1* and *VEGFR2*. Expression of the proangiogenic receptor *VEGR1* and *VEGFR2* was not significantly changed in any group. Gene expression was analysed using the relative quantification method ($\Delta\Delta$Cq) and compared to normal culturing conditions (control). Accordingly, expression of each target in the control group was set to 1. Medium containing 0.1% FCS/1% DMSO was used as the control. Data represents multiple experiments with similar results (n=9/condition out of three independent experiments). P-values indicated in the graph are in comparison to the control group. (C and D) Immunofluorescence staining of the cell nuclei (DAPI in blue), cytoskeleton (PHALLOIDIN; green) and VEGFR2 (red). Representative images of VEGFR2 (C) in all four conditions, captured with the same exposure time; Scale bar, 100 μm. (D) Boxplots depicting mean intensity values under normal culturing conditions and in simulated oversupply with and without combination therapy with TMZ and SB. Mean intensity measurements revealed significant differences between the treatment groups. In contrast to gene expression, VEGFR2 was significantly upregulated by the combination therapy in a mimicked oversupply environment. (A-D) Medium containing 0.1% FCS/1% DMSO was used as the control. P-values indicated in the graph are in comparison to the control group. Other significant P-values for VEGFR2 protein expression: STIM vs. TMZ + SB, P<0.0001; STIM vs. STIM + TMZ + SB, P<0.0001; TMZ + SB vs. STIM + TMZ + SB, P<0.0001; (n >10 images/condition). *P<0.05, ****P<0.0001. A, B and D, one-way ANOVA (Bonferroni correction); bar graphs represent the mean ± standard deviation. STIM, stimulation with 25 ng/ml CXCL2 and IL8; TMZ, temozolomide; SB, SB225002; *VEGFR2*, vascular endothelial growth factor receptor 2; HUVECs, human umbilical vein endothelial cells; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8; *VEGFR1*, vascular endothelial growth factor receptor 1; FCS, fetal calf serum; DMSO, dimethyl sulfoxide.

induced by the combination therapy (TMZ + SB), although not reaching the level of significance (Fig. 5B). VEGF gene expression on the other hand was not altered by SB and TMZ alone or combined (Fig. 5C). Therefore, combination therapy with TMZ and SB under normal culturing conditions as well as in a mimicked oversupply environment mainly affected IL8 expression while the gene expression of CXCL2 and VEGF was unaltered in human primary endothelial cells. However, CXCL2 expression was upregulated by sole treatment with the CXCR2 antagonist SB.

Discussion

The data of the present study revealed that treatment with SB and TMZ led to morphological changes of primary human endothelial cells (HUVECs) and downregulated

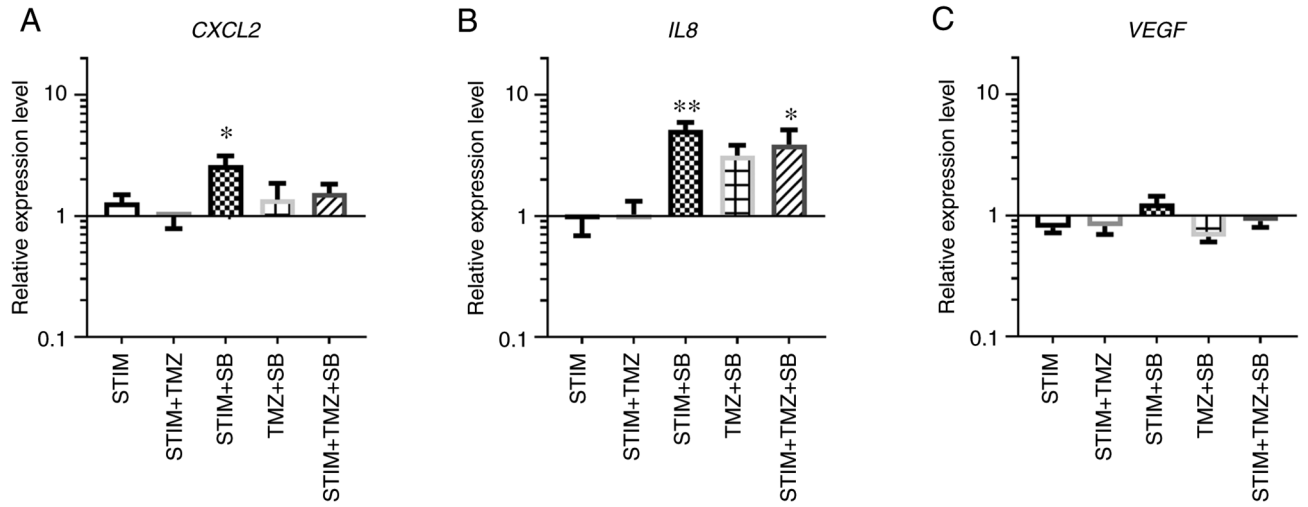

Figure 5. RNA expression of proangiogenic mediators is altered by treatment with TMZ and SB in human endothelial cells. (A-C) HUVECs were stimulated with a cocktail of 25 ng/ml CXLC2 and 25 ng/ml IL8 combined with 10 μM TMZ, 0.03 μM SB or both for 24 h. Analysis of mRNA expression regarding the indicated genes are depicted. Gene expression was analysed using the relative quantification method ($\Delta\Delta$Cq) and compared to normal culturing conditions (control). Accordingly, expression of each target in the control group was set to 1. Medium containing 0.1% FCS/1% DMSO was used as the control. Changes of relative expression levels are shown for *CXCL2*, *IL8* and *VEGF* on a logarithmic scale. P-values indicated in the graph are in comparison to the control group. Other significant P-values for *CXCL2* expression: STIM + TMZ vs. STIM + SB, P=0.0353. Other significant P-values for *IL8* expression: STIM vs. STIM + SB, P=0.0048; STIM + TMZ vs. STIM + SB, P=0.0036. Data represents multiple experiments with similar results (n=9/condition out of three independent experiments). *P<0.05, **P<0.01. A-C, one-way ANOVA (Bonferroni correction); bar graphs represent the mean ± standard deviation. TMZ, temozolomide; SB, SB225002; HUVECs, human umbilical vein endothelial cells; STIM, stimulation with 25 ng/ml CXCL2 and IL8; CXCL2, C-X-C motif chemokine ligand 2; IL8, interleukin 8; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; VEGF, vascular endothelial growth factor.

antiapoptotic *BCL2 in vitro*. In addition, gene expression of the alternative proangiogenic CXCL2/IL8/CXCR2 signalling pathway was altered by the combination therapy, while the VEGF/VEGFR1/2 pathway was only mildly affected. Furthermore, the data revealed that gene and protein expression of these two proangiogenic pathways were differentially regulated.

Resistance towards conventional GBM therapies requires new therapeutic approaches. Due to GBM heterogeneity, single treatments are destined to fail and combination therapies can target the tumour more effectively (6,41). In a previous study by the authors, it was demonstrated that single treatment with SB reduced the tumour burden, vessel density and infiltration of tumour-associated microglia/macrophages in an orthotopic immunocompetent mouse model, but failed to cure the mice (11). A follow-up study with a combination therapy consisting of TMZ and SB revealed promising first insights after just one cycle of combined treatment with TMZ and SB (19). The combination therapy was tolerated well *in vivo* and led to decreased tumour volumes in an orthotopic GBM mouse model (19). However, little is known about the molecular changes that the combination therapy may evoke in specified niches. In the present study, it was determined that protein and gene expression are differentially regulated *in vitro*. Notably, primary human endothelial cells exhibited a significant downregulation of antiapoptotic *BCL2* and a tendency to upregulate proapoptotic *BAX* after treatment with TMZ + SB compared with TMZ alone in a mimicked CXCL2 and IL8 oversupply environment, which would be beneficial if these findings could be translated to GBM tumours. This proapoptotic role of SB has also been reported in other studies (29,31). Furthermore, antitumoural effects have been shown in numerous tumour entities *in vitro* and *in vivo*, which can be explained by its proapoptotic functions (11,29,42-45). However, in the previous study performed by the authors (19), gene expression analyses of murine gliomas treated with the combination therapy did not exhibit an upregulation of *BAX* within the tumour in contrast to the findings in the present study. There, RNA was isolated from the tumour bulk whereas the present study specifically focused on endothelial cells (19). Furthermore, these differences could be explained by the abundant expression of CXCR2 in endothelial cells while glioma cells express CXCR2 to a lesser extent 11,18,25,46).

Several studies indicate that there is a crosstalk between CXCL2/IL8 and VEGF signalling (38-40). Through different mechanisms this crosstalk can lead to an upregulation of BCL2 and subsequent upregulation of IL8 in endothelial cells (39). As revealed by the data in the present study, SB alone and in combination with TMZ led to a significant downregulation of BCL2 and therefore may weaken the effect of this crosstalk between the two proangiogenic signalling pathways. Nevertheless, IL8 was upregulated in all treatment groups receiving the antagonist compared with the normal culturing conditions *in vitro*. This effect has been described before for SB in breast cancer and glioma cells *in vitro (*11,47). TMZ alone did not have any significant effect on all the targeted genes in the present study. Notably, IL8 and CXCL2 were regulated differently, even though they mediate their functions through the same receptor, CXCR2. This poses the question whether they have complementary functions. While IL8 has been studied extensively, less is known about CXCL2 (18,30,48). In a previous study by the authors, it was demonstrated that CXCL2 and IL8 were equally potent initiators of angiogenesis in primary brain endothelial cells while both molecules were less potent in HUVECs (18).

Furthermore, in a previous analysis of 38 patients with matched primary and recurrent GBM tumours, performed by the authors, it was revealed that CXCL2 was expressed by all patients in the primary tumour while IL8 was only expressed by 43% (19). However, IL8 was significantly upregulated to 67.5% in recurrent GBM tumours (19). This should be considered and further research with brain-derived endothelial cells is warranted to verify the results.

Furthermore, while gene expression of *VEGFR2* was not altered, protein expression was significantly upregulated in the STIM + TMZ + SB group. This raises the question of whether combination therapy could lead to an unexpected increase in angiogenesis. Nevertheless, in the previous study by the authors, *VEGFR2* expression was also unchanged by the combination therapy *in vivo* and vascular parameters e.g., vessel density and vessel size were not increased (19). However, VEGFR2 protein expression was not analysed *in vivo* (19). With regard to *VEGFR2* upregulation, it is important to highlight that VEGF is not the only signalling pathway relevant for angiogenesis in GBM. In the present study, CXCR2 expression was downregulated, supporting the theory of crosstalk between these two major proangiogenic signalling pathways, which may explain the differences in gene and protein expression underlining the importance of post-transcriptional processing. The impact of post-transcriptional processing as one reason for differences in mRNA and protein expression has been previously described in detail (49-51). Cheng *et al* suggest that these differences may be time-dependent and therefore, measuring gene and protein expression at the same time-point could lead to different results (49). Furthermore, the impact of other post-translational steps is controversially discussed in literature but could contribute to the differences in gene and protein expression (49,51).

Although the present study specifically focused on primary endothelial cells, it could function as a foundation for the development of a new GBM treatment protocol. The combination therapy was well tolerated in our previous *in vivo* study in an immunocompetent mouse model. Apart from inducing angiogenesis, proliferation and migration in endothelial cells as well as in glioma cells, CXCR2 signalling is known for its important role in vascular mimicry and the trans-differentiation of glioma cells into endothelial-like cells (18,24,46,52,53). Therefore, the molecular changes by the combination therapy may be similar in glioma cells. GBM is a very heterogeneous tumour and it is likely that this combination therapy would only target a subgroup of tumour cells (54-56). However, to date, a therapy that targets all tumour cells as well as the tumour microenvironment has yet to be discovered. In this regard combination therapies with SB appear promising. For instance, therapeutic approaches combining anti-CXCR4 therapy, CXCR4 is a different important chemokine receptor in GBM, with the well-established treatment options such as TMZ and/or radiotherapy are currently being investigated in clinical phase I and II studies and show encouraging results (57) (NCT03746080).

As aforementioned, the significance of the data may be limited, as the experiments were carried out with primary human endothelial cells. As demonstrated in a previous study performed by the authors, primary endothelial cells from the periphery may behave differently to primary brain endothelial cells (18). Furthermore, tumour cells, especially glioma cells may also behave differently. However, previous studies have shown that endothelial and glioma cell gene expression is similarly altered by SB *in vitro,* therefore, the combination therapy could have similar effects (11). However, it is unknown whether this can also be applied to an *in vivo* setting. Furthermore, additional research is warranted as protein expression was analysed solely by immunofluorescence staining. Other techniques may deliver more insight into the functional changes within the investigated pathways. A step forward could be to investigate the effect of the combination therapy in glioma organoids *in vitro*.

In conclusion, the data of the present study revealed that the combination therapy consisting of SB and TMZ altered the gene expression of antiapoptotic *BCL2* and the CXCR2 signalling pathway in primary endothelial cells. Furthermore, the combination therapy led to differential gene and protein expression of the proangiogenic receptors CXCR2 and VEGFR2 *in vitro*. The data provides first insights into the molecular changes of two major proangiogenic pathways during treatment with TMZ and SB on primary endothelial cells, which should be considered in future studies.

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Availability of data and materials

The datasets used and analysed during the present study are available from the corresponding author upon request.

Authors' contributions

RMU was involved in the conceptualisation of the project and acquisition of the funding. RMU carried out the experiments, curated, visualised and analysed the data and drafted the manuscript. CJ was involved in the interpretation of data, as well as reviewed and edited the manuscript. PV interpreted the data, was responsible for funding and supervision including reviewing and editing the manuscript. SB and GA were involved in the conceptualisation and supervision of the project, they curated the data and verified the data analysis as

well as reviewed and edited the manuscript. In addition, GA was involved in the acquisition of the funding. All authors confirm the authenticity of all the raw data and have read and approved the final manuscript.

Ethics approval and consent to participate

An ethical standards statement was provided by the supplier (PromoCell). Permission to publish the collected data was obtained from the Ethics Committee at Charité-Universitätsmedizin Berlin (EA1/090/22).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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