Research Article

Fermented Mistletoe Extract as a Multimodal Antitumoral Agent in Gliomas

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In Europe, commercially available extracts from the white-berry mistletoe (*Viscum album* L.) are widely used as a complementary cancer therapy. Mistletoe lectins have been identified as main active components and exhibit cytotoxic effects as well as immunomodulatory activity. Since it is still not elucidated in detail how mistle toe extracts such as ISCADOR communicate their effects, we analyzed the mechanisms that might be responsible for their antitumoral function on a molecular and functional level. ISCADOR-treated glioblastoma (GBM) cells down-regulate central genes involved in glioblastoma progression and malignancy such as the cytokine TGF-β and matrix-metalloproteinases. Using *in vitro* glioblastoma/immune cell co-cultivation assays as well as measurement of cell migration and invasion, we could demonstrate that in glioblastoma cells, lectin-rich ISCADOR M and ISCADOR Q significantly enforce NK-cell-mediated GBM cell lysis. Beside its immune stimulatory effect, ISCADOR reduces the migratory and invasive potential of glioblastoma cells. In a syngeneic as well as in a xenograft glioblastoma mouse model, both pretreatment of tumor cells and intratumoral therapy of subcutaneously growing glioblastoma cells with ISCADOR Q showed delayed tumor growth. In conclusion, ISCADOR Q, showing multiple positive effects in the treatment of glioblastoma, may be a candidate for concomitant treatment of this cancer.

1. Introduction

Glioblastoma (GBM) is the most common malignant brain tumor with an incidence of 3.5 cases per 100.000 people per year. GBM are among the most lethal neoplasms with a median survival of approximately one year after diagnosis even upon maximum current treatment strategies. Only few therapeutic regimens such as the chemotherapeutic drug temozolomide (TMZ) provide a short but significant increase in survival [1]. Additionally, tumor-intrinsic features including the methylation status of the O-(6)-methylguanine-DNA methyltransferase (MGMT) promoter are also predictive for the survival of GBM patients [2]. The failure of effective therapy regimens in malignant GBM is associated with its malignant characteristics which means that these tumors are highly resistant to cell death [3], possess immunosuppressive function [4] and show a highly invasive and destructive growth due to their migratory and invasive growth potential [5].

Extracts of the European mistletoe (*Viscum album* L.) have been widely used for decades as alternative, complementary treatment and adjuvant cancer therapy, especially in German-speaking countries. Cytotoxic glycoproteins, the mistletoe lectins (ML), are one active component of mistletoe extracts and can stimulate effector cells of the innate and adaptive immune system such as dendritic cells, macrophages, natural killer cells, and B and T lymphocytes, at least one mechanism that might be responsible for the

antitumoral properties of mistletoe extracts (ME) [6–13]. Beside their immune modulatory function, ME show direct growth inhibition and cell death induction in tumor cells such as induction of apoptosis or direct necrotic effects, dependent on the concentration used for treatment [14–21]. *In vivo,* preclinical activity of aqueous ME has been shown in a variety of transplantable rodent tumor models [22–26]. In clinical cancer therapy studies, adjuvant treatment with ME showed an impact on the patients' quality of life, reducing side effects of conventional therapies such as nausea, fatigue or reduced energy induced by chemotherapy or radiation, and is associated with prolonged survival [27–31], even in glioma [32].

One of the longest known mistletoe preparations is the fermented plant extract ISCADOR which is extracted from mistletoe plants growing on different host trees like apple (ISCADOR M), oak (ISCADOR Q), or pine (ISCADOR P). ISCADOR P contains only very small amounts of lectin, whilst lectin content in ISCADOR Q is high and medium in ISCADOR M. Especially natural killer (NK) cells as part of the innate immune system play an essential role in cell-mediated immune responses against tumor cells, also in glioma [33], and it has been shown before that ISCADOR treatment had a positive effect on NK cell function [9, 34]. There are first hints that the ISCADOR variants show positive effects using them as treatment agents for GBM, but the mechanisms responsible for these effects are not well elucidated until today [26, 32], In the present study, we especially aim to investigate the effects of lectin rich ISCADOR Q on GBM cell proliferation, cell death, cell motility and immune-cell mediated anti-GBM immune response to assess its antitumoral potential in gliomas.

2. Material and Methods

2.1. Cell Lines and Reagents. LNT-229 and LN-308 cell lines were provided by N. de Tribolet (Lausanne, Switzerland). These cells were chosen since both cell lines are well characterised, and differ in their growth capacity and genetic background [35]. Furthermore, LN-308 cells differ from LNT-229 cells in a reduced expression of the migration/invasion-relevant matrix metalloproteinases MMP-2 and -9 [36, 37]. LNT-229-Luc cells used for immune cell lysis assays were generated by stable transfection of the cells with pGL4.14-HSV-Luc expressing firefly luciferase. SMA560 cells were a friendly gift of G. J. Pilkington (Portsmouth, UK) [38, 39]. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO Life Technologies, Eggenstein, Germany) containing 10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 μg/mL), and the appropriate antibiotics in a humidified atmosphere containing 5% CO_2. Unless otherwise noted, other reagents were from Sigma (Munich, Germany).

2.2. Mistletoe Extracts. ISCADOR preparations were a friendly gift of Weleda, "Verein für Krebsforschung," Institute Hiscia (Arlesheim, Switzerland). Charge numbers of different ISCADOR preparations were as follow: ISCADOR Q_{200}:

1009/0325; ISCADOR P_{200}: 0809/811, ISCADOR M_{20}: 0711. As communicated by the manufacturer, total ML content in ISCADOR preparations were: 35 ng/mL (P), 899 ng/mL (M), and 15050 ng/mL (Q). Viscotoxin contents in the different ISCADOR preparations were: 160 μg/mL (P), 36 μg/mL (M) and 364 μg/mL (Q).

2.3. RNA Preparation and Quantitative RT-PCR. Total RNA was prepared using the RNA Isolation Kit (Roche, Mannheim, Germany) and was reverse transcribed using Superscript II (Invitrogen, Karlsruhe, Germany). Target gene expression was determined using SYBR green master mix (Thermo Fisher Scientific, MA, USA), on an ABI 7500 system. Relative mRNA expression was quantified ($[E^{\Delta CT}$ (specific primer)$/E^{\Delta CT}$ (GAPDH)]). The following primers were used: GAPDH-frwd TGCACCACCAACTGCTTAGC, GAPDH-rev GGCATGGACTGTGGTCATGAG; Ang1-frwd CGGTGAATATTGGCTGGGG; Ang1-rev GTCATACTG-TGAATAGGCTCGGTT, VEGFR2-frwd GTCCTAGAGCGT-GTGGCACC, VEGFR2-rev CATGATCTGTGGAGGGGG-ATTC. GAPDH primers were used for normalization.

2.4. PCR-Based Microarray Expression Analysis. Total RNA and cDNA were obtained as described. For expression analysis of 96 genes involved in tumor cell motility and angiogenesis, Human Angiogenesis 96-well StellARray qPCR array (Lonza, Basel, Switzerland) was prepared according to manufacturer's instruction using SYBR green master mix (Thermo Fisher Scientific, MA, USA) on an ABI 7500 system. Data were analyzed with Global Pattern Recognition Data Analysis Tool (Bar Harbor Biotechnology, Trenton, ME, USA) using the internal array control housekeeping gene expression for normalization.

2.5. Rembrandt Platform Analyses. The REMBRANDT database contains microarray data for probes from the Affymetrix U133 Plus 2.0 GeneChip (National Cancer Institute 2005; REMBRANDT home page: http://rembrandt.nci.nih.gov/ (accessed February 6, 2012)). At the time of accession, the database contained mRNA expression data of 228 glioblastomas and 28 normal control tissues. *P* values for median expression intensity changes between each glioma subgroup compared to normal CNS tissue controls were obtained.

2.6. ELISA. Cellular supernatants were collected from vehicle or ISCADOR treated LNT-229 cells (ISCADOR Q; 100 μg/mL) after incubation with serum-free medium. All ELISA kits were used following the manufacturer's protocol (TGF-β1-ELISA: eBioscience, Frankfurt, Germany; TGF-β2-ELISA: R&D Systems, Wiesbaden, Germany; Angiopoietin-1-ELISA: R&D Systems, Wiesbaden, Germany, VEGF-ELISA: Peprotech, Hamburg, Germany). Colorimetric quantification was performed on the ELISA-reader Multiskan Ex (Thermo Fischer Scientific, MA, USA) at 450 nm.

2.7. Growth and Viability Assays. Net cell culture growth and clonogenic survival was determined by crystal violet staining

as described [40]. Cell viability was determined by Trypan blue exclusion assay.

2.8. Analysis of Cell Death. For the caspase 3/7 activity assay cells were seeded in microtiter plates, allowed to attach, and treated with ISCADOR (100 μg/mL). 24 h later, the cells were lysed in 25 mM Tris-HCl (pH 8.0), 60 mM NaCl, 2.5 mM EDTA, and 0.25% NP40 for 10 min, and acetyl-DEVD-amc was added at 12.5 mM. Caspase activity was assessed by fluorescence using a Mithras LB940 fluorimeter (Berthold Technologies, Bad Wildbad, Germany) at 360 nm excitation and 480 nm emission wavelengths. To analyze total cell death, cells were trypsinized and stained with propidium iodide (PI). The content of PI-positive cells was determined by fluorescence activated cell sorting (FACS) analysis.

2.9. Immunoblot Analysis. The general procedure has been described [40]. Cellular supernatants were generated using serum-depleted cell culture medium and concentrated using Amicon concentrator columns (Millipore, Schwalbach, Germany). The following antibodies were used: anti-MMP-2 (Millipore, Schwalbach, Germany), anti-MMP-9 (R&D Systems, Wiesbaden, Germany), anti-GAPDH (Chemicon, Billerica, MA, USA), anti-MMP-1, and anti-MMP-3 (both from Biolegend, Fell, Germany), anti-MMP-10 (R&D Systems, Wiesbaden, Germany), anti-MMP-14 (Epitomics, Burlingame, CA, USA), and anti-TIMP-2 (R&D Systems, Wiesbaden, Germany). Protein contents were quantified using the Alpha-Innotec-LumiImager-Fluorchem-8900 (Cell Bioscience, Santa Clara, CA, USA).

2.10. Zymography. Cellular supernatants were collected, concentrated, and 20 μg protein was loaded on zymogram gels (Bio-Rad Laboratories GmbH, München, Germany). MMP activity was analyzed according to the manufacturer's protocol. Quantification was done using the Alpha-Erase-Software (Cell Bioscience, Santa Clara, CA, USA).

2.11. Measurement of Cell Migration and Invasion. The Scratch assay and the Boyden chamber migration assay have previously been described [41]. Matrigel-coated Boyden chambers (BD Biosciences, Heidelberg, Germany) were used for invasion assays.

2.12. FACS-Based Analysis. For expression analysis of NKG2D ligands, growing cells were detached from cell culture flasks using accutase (PAA, Cölbe, Germany). The cells were prepared according to the manufacturers instruction and surface protein expression was analyzed using purified ascites supernatants against MICA (clone AMO1), MICB (clone BMO1), ULBP1 (clone AUMO1), ULBP2 (clone BUMO1), or MHC I (clone W6.32) (kindly provided Alexander Steinle, Institute for Molecular Medicine, Frankfurt Main, Germany; [42]). For DNAX accessory molecule (DNAM)-1 ligands, anti-CD112-APC and anti CD155-PE (both from Ebioscience, Frankfurt, Germany) were used. Subsequently, the following secondary antibodies were used: Cy3-conjugated mouse IgG1 (Dianova, Hamburg, Germany)

or APC-conjugated mouse IgG1 or IgG2 (Immunotools, Friesoythe, Germany). The cells were subjected to flow cytometric analysis using a Cyan-ADP cytometer (DAKO GmbH, Hamburg, Germany).

2.13. Purification of PBL, Isolation, and Activation of Immune Effector Cells. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated peripheral venous blood of healthy donors by density gradient centrifugation (Biocoll, Biochrom KG, Berlin, Germany). Monocytes were removed from the PBMCs by allowing them to adhere to tissue culture plastic. The nonadherent fraction (PBLs) was then cocultured with irradiated RPMI 8866 feeder cells to obtain polyclonal NK cell populations [22]. CD3+ T cells were isolated from freshly isolated PBMCs using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD3+ T cells were then stimulated with 30 ng/mL of anti-CD3 antibody (clone OKT-3; eBioscience, Frankfurt, Germany) irradiated feeder cells and 50 U/mL human recombinant IL-2 (Immunotools, Friesoythe, Germany) as previously described [43]. On day 12 of the expansion cycle the activation status based on expression of CD25 (antihuman CD25-PE conjugated anitbody, Miltenyi Biotec) and HLA-DR (antihuman HLA-DR-FITC conjugated antibody, eBioscience) was examined by FACS analysis. These cells were used as effectors in the lysis assays at day 14 of their cycle.

2.14. Cellular Cytotoxicity Assay. Immune cell cytotoxicity against human GBM cells was analyzed using a nonradioactive assay measuring luciferase activity of stably transfected LNT-229-Luc cells. Briefly, target LNT-229-Luc cells were incubated with ISCADOR or control medium for 24 h. The cells were washed and human PBLs, isolated NK cells or activated CD3+ T cells of healthy donors were added to the target cells and incubated for 4 h at 37°C. Viable cells were determined by measuring luciferase activity using a Mithras LB940 fluorimeter (Berthold Technologies, Bad Wildbad, Germany). The experimental lysis was corrected by division by the spontaneous lysis of target cells at the corresponding ISCADOR concentration or control-treated cells. Percentage of lysis was calculated as followed: 100 − ((experimental lysis/spontaneous lysis) × 100). Experiments were done in triplets. For inactivation of NK cells, the cultures were preincubated with anti-NKG2D-antibodies (R&D Systems, Wiesbaden, Germany) for 0.5 h prior to coculture with GBM cells according to the manufacturer's instruction.

For analysis of NK cell attachment, purified NK cells were loaded with CSFE (10 μM) for 10 min and then cultivated on glioma cells for 1 h. Cocultivated cells were washed intensively with PBS to remove unattached NK cells and microscopically documented using a Zeiss Axiovert 200 M fluorescence microscope.

2.15. Mouse Experiments. Athymic CD1-deficient NMRI nude mice were purchased from Janvier (St. Berthevin, France). NMRI mice develop functional NK cells, but lack

T cells. VMDk mice [38, 39] were bred inhouse. Mice of 6–12 weeks of age were used in all experiments. The experiments were performed according to the German law, Guide for the Care and Use of Laboratory Animals (approval N3/09). LNT-229 or SMA560 cells were treated with ISCADOR Q (100 μg/mL) or left untreated. The cells were trypsinized, counted and viability was assessed by trypan-blue staining. Groups of 6 mice were injected s.c. with one million viable cells into the right flank. Mice were examined regularly for tumor growth using a metric caliper and sacrificed when tumors reached 200 mm^2. To avoid artifacts due to cytotoxicity or proliferation inhibition induced by ISCADOR, cell proliferation used for inoculation was analyzed. In brief, 1.000 cells were seeded in microtiter plates and allowed to attach. Cell density was monitored every 24 h by crystal violet staining. For treatment of tumors with ISCADOR, one million cells were implanted subcutaneously. Seven days later, 2 μg (20 μL of a 100 μg/mL stock) ISCADOR Q or vehicle (PBS) was injected intratumorally. In a further experiment, NMRI mice harboring subcutaneous tumors were periodically and subcutaneously injected on the contralateral body site with either PBS or with increasing concentrations (1 μg up to 100 μg in each 100 μL) of ISCADOR Q. Mice were examined regularly for tumor growth using a metric caliper, and killed when tumors reached 200 mm^2.

2.16. Statistical Analysis. Figures show data obtained in at least three up to ten independent experiments as indicated. Quantitative data were assessed for significant differences using t-test ($*P < 0.05$, $**P < 0.01$, $***P < 0.0001$). Statistical analysis of tumor growth in the animal experiments was done using the ANOVA test (SPSS18, SPSS Inc, Chicago, IL, USA). The results of the PCR-based microarray expression analysis were analyzed together with the results obtained for the corresponding genes in the REMBRANDT database by a contingency table analysis. Nominal scaled response variable (genes up- (1), non- (0), or down- (−1) regulated in glioblastomas versus normal brain tissue in the REMBRANDT database) and nominal explanatory variable (same gene up- or downregulated in PCR-based microarray expression analysis after ISCADOR treatment) were analysed and subsequently tested in a likelihood ratio test. A significance level of alpha = 0.05 was chosen for all tests. Statistical analysis to assess the association of the REMBRANDT and our PCR-based microarray was performed using JMP 8.0 software (SAS, Cary, NC, USA).

3. Results

3.1. ISCADOR Significantly Reduces the Expression of Genes Associated with Malignancy and Progression in Glioblastomas. To assess whether ISCADOR treatment might modulate gene expression in glioma cells, we first performed a PCR-based microarray expression analysis. This array contains a variety of genes involved in glioma-associated pathways such as proliferation, survival, migration, invasion, and angiogenesis as well as tumor-immunological processes. As shown in Figure 1(a), treatment of glioma cells with

ISCADOR Q reduced the expression of a variety of genes relevant for gliomagenesis: *epidermal growth factor receptor 2* (EGFR2/HER2/ERBB2) stimulates proliferation and blocks apoptosis [44], whereas BIRC5/Survivin is a potent inhibitor of apoptosis [45]. PKB/AKT1 protein kinase plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription, and cell migration [46, 47]. Constitutive signal transducer and activator of transcription 3 (STAT3) activation is associated with various human cancers, commonly suggests poor prognosis and provides antiapoptotic as well as proliferative effects [48]. Genes such as *matrix-metalloproteinases* (MMP), cell adhesion molecules such as *platelet/endothelial cell adhesion molecule* (PECAM)-1, or integrins are important players in cell motility whereas *vascular endothelial growth factor* (VEGF), VEGF receptor type II (VEGFR2), and *angiopoietin* (ANGPT)-1 and -related molecules as well as *transforming growth factor* (TGF)-β are genes involved in both cell motility and angiogenesis. The latter does not only enhance tumor cell motility and angiogenesis, but also is the central immunosuppressive molecule in GBM [5].

To verify reliability of microarray data, we exemplarily analyzed expression of differentially regulated genes by qPCR or ELISA. As shown in Figures 1(b)–1(e), there is a significant downregulation of TGF-β, ANGPT-1, VEGF, and VEGFR2 upon ISCADOR treatment. Reduced gene expression is not a result of enhanced cell death, since ISCADOR treatment conditions used for this analysis did not induce cell death (Figure 3). We further analyzed the data obtained from the microarray set for a potential association with genes regulated in glioblastomas versus normal CNS tissue in the REMBRANDT database (Figure 1(f)). ISCADOR treatment of LNT-229 glioma cells led to a relative increase of genes which are significantly higher in nonneoplastic CNS tissue specimens in the REMBRANDT database while the percentage of genes significantly upregulated in glioblastomas in the REMBRANDT cohort decreased ($P = 0.0047$).

3.2. ISCADOR Treatment Reduces Glioma Cell Growth in a Dose Dependent Manner. Since it has been shown that mistletoe extracts could induce cell death and hold antiproliferative activity in a variety of tumor cell lines derived from breast, lung, prostate, or renal cancer [14–19] and since we have shown that in GBM cells ISCADOR downregulated a variety of proliferation stimulating genes (Figure 1), we analyzed whether ISCADOR was able to reduce cell growth in human and mouse GBM cell lines, too. As shown in Figure 2, lectin rich ISCADOR M and Q reduced cell growth at doses of higher than 100 μg/mL both in human LNT-229 and in mouse SMA560 GBM cells, whereas ISCADOR P showed only weak effects on glioma cell growth. The cell death inducing effects of ISCADOR are dependent on their content of viscotoxins and mistletoe lectins [14, 15]. The cytotoxic and growth inhibitory effects seen for ISCADOR M and Q, but not for ISCADOR P in the GBM cell lines seemed therefore to be only a minor effect of viscotoxins present in these ME, since all variants of ISCADOR used so far contain viscotoxin, and even if the final concentration of viscotoxin is

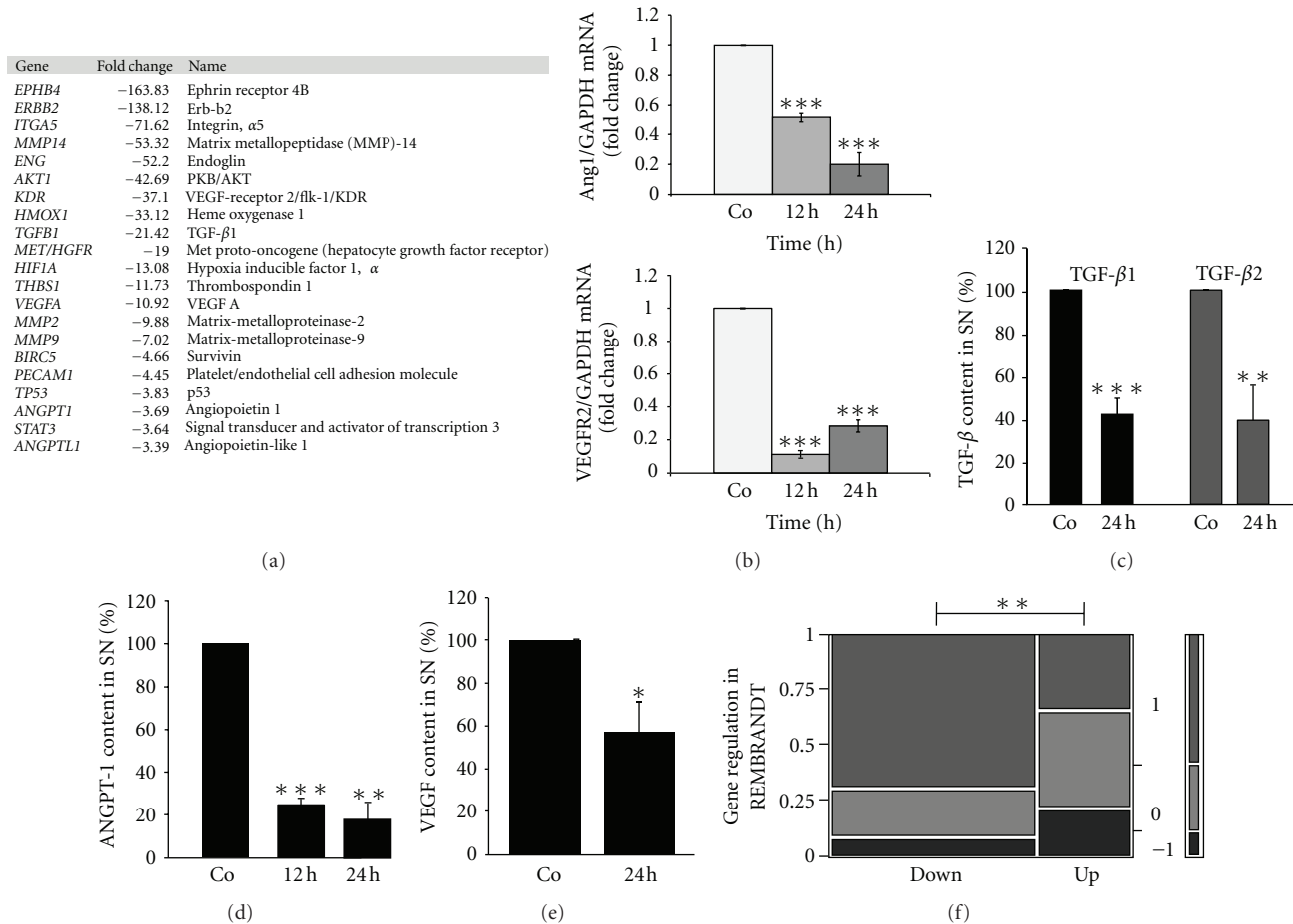| Gene	Fold change	Name
EPHB4	−163.83	Ephrin receptor 4B
ERBB2	−138.12	Erb-b2
ITGA5	−71.62	Integrin, α5
MMP14	−53.32	Matrix metallopeptidase (MMP)-14
ENG	−52.2	Endoglin
AKT1	−42.69	PKB/AKT
KDR	−37.1	VEGF-receptor 2/flk-1/KDR
HMOX1	−33.12	Heme oxygenase 1
TGFB1	−21.42	TGF-β1
MET/HGFR	−19	Met proto-oncogene (hepatocyte growth factor receptor)
HIF1A	−13.08	Hypoxia inducible factor 1, α
THBS1	−11.73	Thrombospondin 1
VEGFA	−10.92	VEGF A
MMP2	−9.88	Matrix-metalloproteinase-2
MMP9	−7.02	Matrix-metalloproteinase-9
BIRC5	−4.66	Survivin
PECAM1	−4.45	Platelet/endothelial cell adhesion molecule
TP53	−3.83	p53
ANGPT1	−3.69	Angiopoietin 1
STAT3	−3.64	Signal transducer and activator of transcription 3
ANGPTL1	−3.39	Angiopoietin-like 1

(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 1: In GBM cells, ISCADOR Q changes the expression of cancer associated genes. (a) PCR-based microarray expression analysis. LNT-229 cells were treated with ISCADOR Q (100 μg/mL) or were left untreated. 24 h later, the cells were harvested, mRNA was prepared, reverse transcribed into cDNA and used for microarray-based expression analysis on a panel of 96 migration-/invasion-angiogenesis-involved genes. The best downregulated genes with a threshold > 3 are shown. (b) RT-PCR-based validation of differentially regulated genes detected in (a) at 12 or 24 h of ISCADOR Q treatment ($n = 4$, SEM). (c) TGF-β ELISA of ISCADOR Q treated LNT-229 cells. The cells were treated with ISCADOR Q (100 μg/mL, 24 h) followed by incubation in serum-free medium for 48 h. Supernatants were harvested and TGF-β 1 or TGF-β 2 was quantified using ELISA ($n = 4$, SEM). (d) The cells were treated with ISCADOR Q (100 μg/mL, 12 or 24 h). Supernatants were harvested 48 h later. Angiopoietin-1 content was analyzed using ELISA ($n = 4$, SEM). (e) The cells were treated as in (c). Supernatants were harvested and VEGF content was analyzed using ELISA ($n = 4$, SEM). (f) Contingency table analysis of the PCR-based microarray expression data together with the corresponding genes of the REMBRANDT database. Nominal scaled response variable (genes significantly up- (1), non- (0), or down- (−1) regulated in glioblastomas versus normal brain tissue expression pattern in the REMBRANDT database) and nominal explanatory variable (same gene up- or downregulated in our PCR-based microarray expression analysis after ISCADOR treatment) were analysed and subsequently tested by the likelihood ratio test ($P = 0.0046$).

comparable when treating the cells with different ISCADOR variants, high concentrations of ISCADOR P presenting a higher amount of viscotoxin only marginally induced cell death. The main difference between the ISCADOR variants is their lectin concentration suggesting that ML are mainly responsive for the cytotoxic effects.

To analyze other effects of ISCADOR in the treatment of glioma cells beside the effects on cell growth, for further experiments an ISCADOR concentration of 100 μg/mL was chosen to avoid unwanted side effects induced by inhibition of proliferation or induction of cell death. The viscotoxin contents at the ISCADOR concentration of 100 μg/mL which we have used for further studies were 80 ng/mL (P),

180 ng/mL (M), and 182 ng/mL (Q), total ML concentrations were 0,017 ng/mL (P), 4,49 ng/mL (M), and 7,52 ng/mL (Q). At this concentration, no significant reduction in cell growth (Figure 3(a)) or induction of cell death (Figure 3(b)) or caspase activity (Figure 3(c)) were detectable. Even at higher concentrations of ISCADOR Q no caspase 3/7 activity was detectable, but cells became propidium iodide positive, suggesting that the cells died by necrosis (data not shown).

3.3. ISCADOR Enhances PBL-Mediated GBM Cell Lysis. Since we knew from the literature that ML can stimulate immune effector cells and since we have seen that the secretion of TGF-β, the most important immunosuppressive
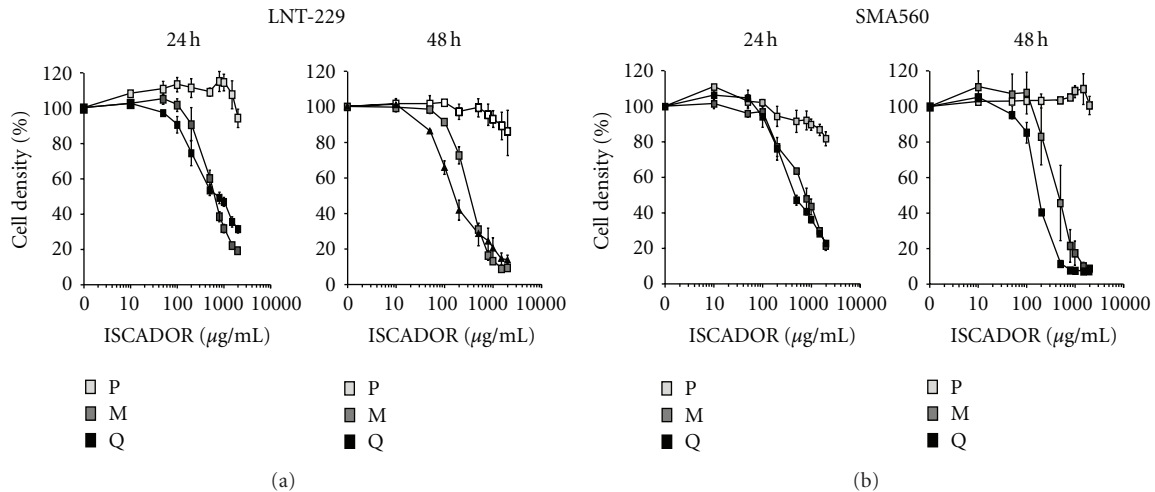
Figure 2: ISCADOR reduces glioma cell growth in a dose dependent manner. LNT-229 (a) or SMA560 cells (b) were treated of 24 or 48 h with increasing concentrations of ISCADOR or were left untreated. Cell density was assessed by crystal violet staining ($n = 3$, SEM).

cytokine in glioma, is decreased after treating the cells with ISCADOR Q (Figure 1), we asked whether ISCADOR might induce immune cell mediated GBM cell attack, too. Indeed, ISCADOR treatment of LNT-229 cells, enhanced PBL-mediated tumor cell lysis, dependent on the lectin content of different ISCADOR variants (Q > M > P; Figure 4(a) and data not shown). ISCADOR mediated GBM-cell lysis is dependent on the activity of NK cells, since preincubation of PBLs with an inactivating NKG2D antibody neutralized the lytic effect (Figure 4(b)), whereas coincubation of ISCADOR treated LNT-229 cells with purified NK cells also showed enhanced cell lysis (Figure 4(c)). NK cell mediated tumor cell lysis is dependent on the expression of so called danger/stranger signaling molecules on target cells such as the NKG2D ligands *major histocompatibility complex I class related* (MIC)-A, MIC-B, or *UL16 binding proteins* (ULBP) 1, 2, 3, or the *DNAX accessory molecule*-(DNAM)-1 ligands CD112 and CD115. To identify whether upregulation of these molecules was involved in the enhancing PBL mediated lytic effect of ISCADOR treated GBM cells, we performed FACS analysis to quantify surface protein expression. Neither NKG2D- nor DNAM-1-ligand expression was altered after treating the tumor cells with ISCADOR Q (data not shown). We therefore analyzed whether enhanced NK cell mediated glioma cell lysis might be an effect of increased attachment of NK cells to the target cells due to ML presented on the target cell surface. For this, we cocultivated CSFE labeled NK cells with ISCADOR Q pretreated glioma cells. As shown in Figures 4(d) and 4(e), more NK cells were attached to glioma cells if the target cells were pretreated with lectin rich ISCADOR Q, but not if the cells were treated with lectin poor ISCADOR P, suggesting the ML boost NK cell attachment to the GBM target cells.

In contrast to ISCADOR mediated enhancement of NK cell activity, cocultivation of LNT-229 glioma cells with purified and activated T cells did not show any difference in the lysis of untreated compared to ISCADOR treated glioma

cells, also no changes in the cell surface expression of MHC molecules were detectable on LNT-229 cells if these cells were treated with ISCADOR (data not shown).

3.4. ISCADOR Reduces GBM Cell Motility. To analyze whether the ISCADOR-mediated reduction in the expression of migration/invasion-relevant genes cells resulted in a reduction of glioma cell migration and invasion, we performed *in vitro* long lasting scratch assays, short time transwell boyden chamber migration as well as matrigel-invasion assays. As shown in Figure 5, treatment of human (LNT-229) and mouse (SMA-560) GBM cells with ISCADOR reduced cell migration (Figures 5(a) and 5(b)) and invasion (Figure 5(c)), revealing ISCADOR Q as the most potent antimigratory ISCADOR variant. Interestingly, in a second GBM cell line (LN-308) the antimigratory effect of ISCADOR Q was delayed compared to LNT-229 cells. LN-308 cells differ from LNT-229 cells in a reduced expression of migration-/invasion-relevant MMPs 2 and −9 ([36, 37] and data not shown), indicating that altered MMP expression or activity in ISCADOR-treated LNT-229 cells might be responsible for the reduction of migration and invasion. For this, we analyzed the expression and activity of a variety of MMPs known to be expressed in GBM in control and ISCADOR treated LNT-229 cells. As shown in Figures 6(a) and 6(b), ISCADOR Q reduced the amount of MMP-2 in cellular supernatants as well as the activity of all MMP-2 subforms (72 kD pro-MMP-2, 68 kD active MMP-2 and the putative 36 kD legumain-cleaved active MMP-2 variant [49]; Figures 6(c) and 6(d)), whereas no change of MMP-1, -3, and -10 protein expression was detectable. Even if we have demonstrated reduced MMP-9 mRNA expression upon ISCADOR treatment (Figure 1(a)), there was an increased secretion of MMP-9 in ISCADOR Q treated cells. However, MMP-9 activity was also impaired by ISCADOR Q treatment (Figures 6(c) and 6(d)). Since it has been described that the net MMP-2 activity correlates with
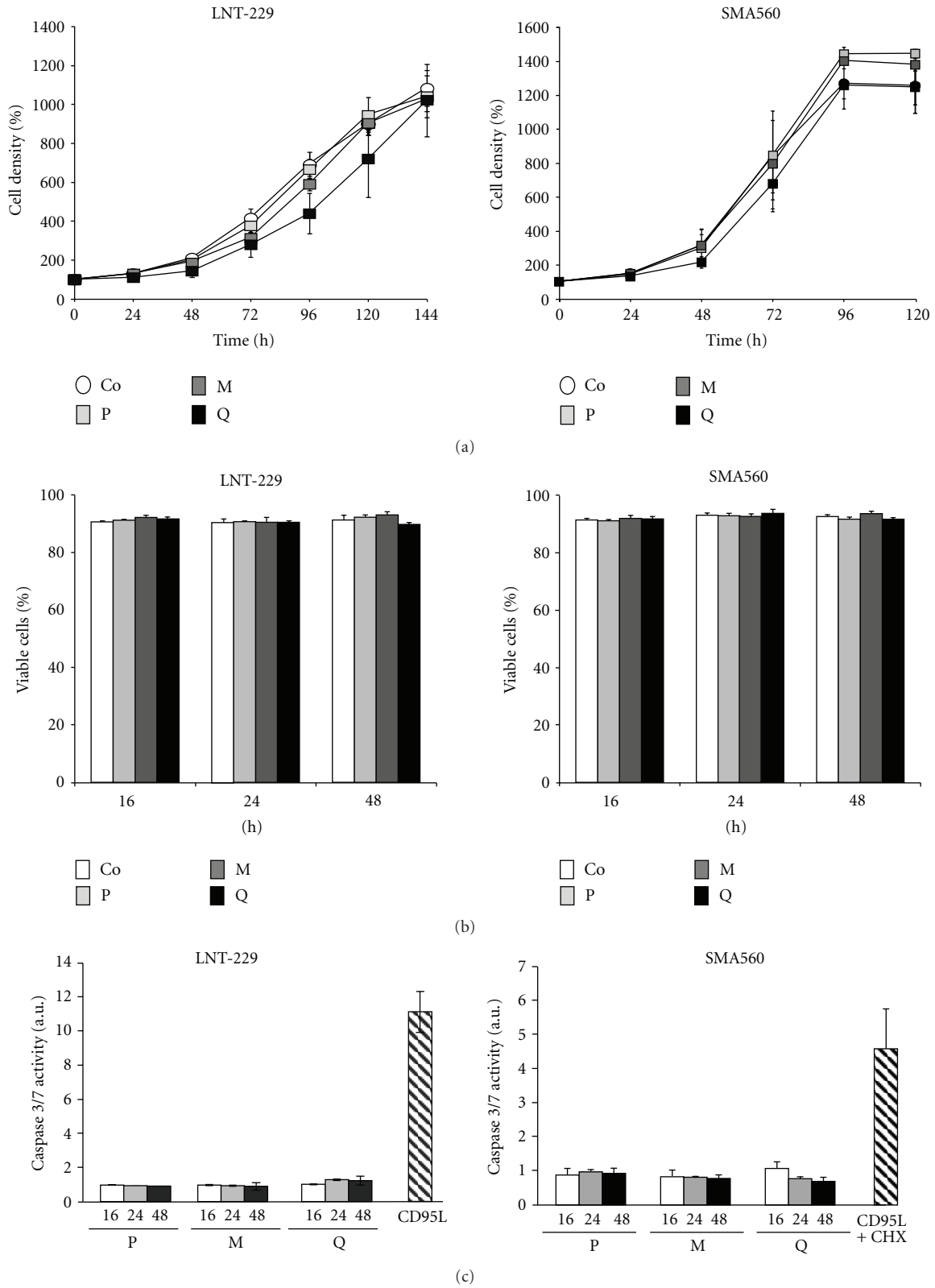
(a)

(b)

(c)

FIGURE 3: ISCADOR does not induce cell death at a concentration of 100 μg/mL. (a) LNT-229 cells were treated with ISCADOR (100 μg/mL, 24 h), or were left untreated, and then allowed to grow. Cell density was assessed by crystal violet staining every 24 h ($n = 9$ for LNT-229 cells, $n = 4$ for SMA560 cells, SEM). (b) The cells were treated as in (a). Viable cells were determined by trypan blue staining ($n = 3$, SEM). (c) Fluorescence based Caspase 3/7 activity assay. The cells were treated as indicated and caspase activity was analyzed 16, 24 and 48 h later using the fluorescent substrate DEVM-amc. As a positive control, the cells were treated with CD95L (100 U/mL + 10 μg/mL CHX) for 4 h ($n = 3$, SEM).
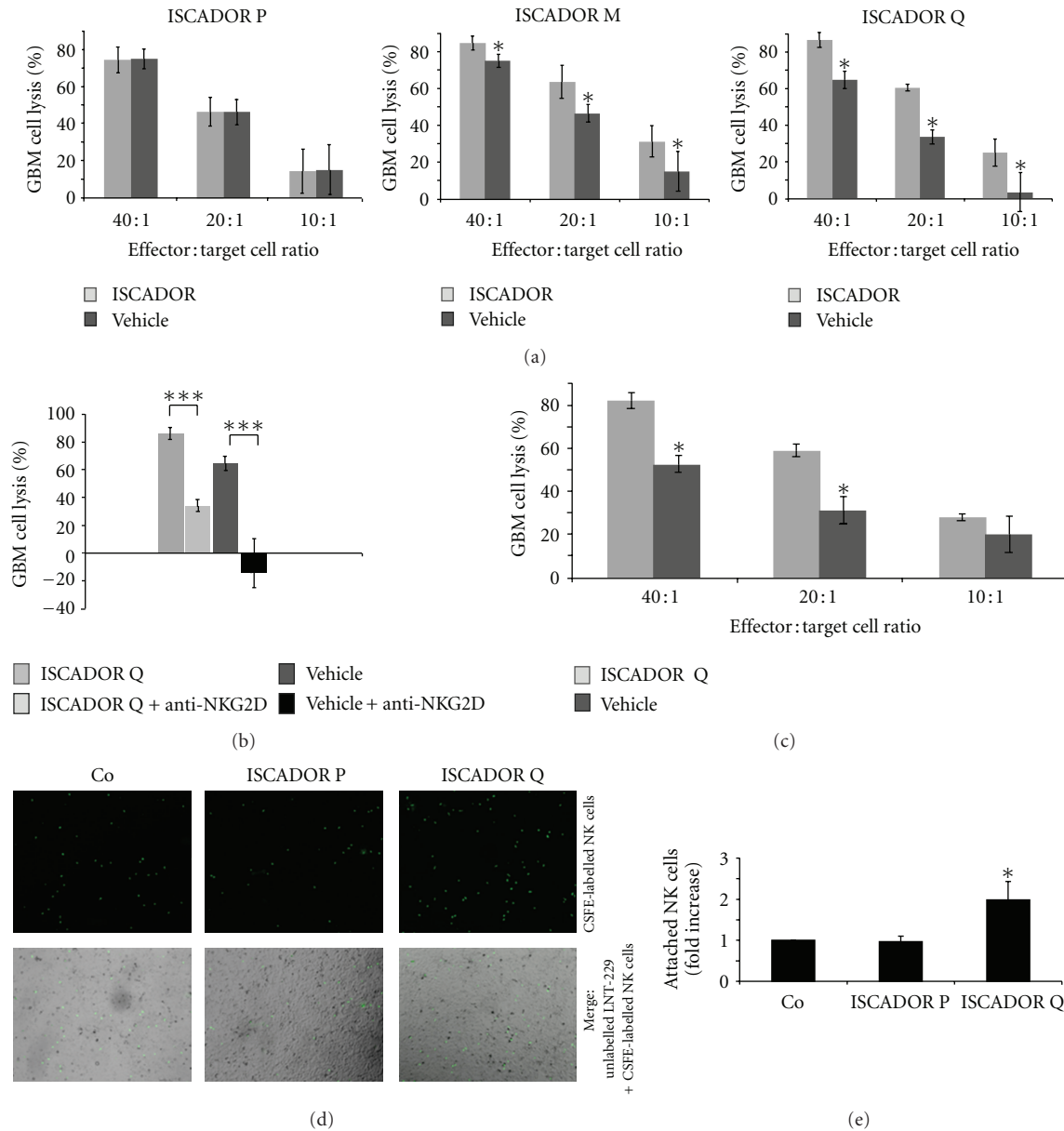
(a)

(b)

(c)

(d)

(e)

FIGURE 4: Treatment of GBM cells with ISCADOR enhances NK cell mediated tumor cell lysis. (a) LNT-229-Luc cells were treated with the appropriated ISCADOR variant ($100\,\mu$g/mL) for 24 h. The cells were washed and incubated with human PBLs at different E/T cell ratio for 4 h. Cell lysis was measured by quantifying luciferase activity. (b) LNT-229-Luc cells were treated as in (a). PBLs were incubated with neutralizing NKG2D antibody (R&D Systems, Wiesbaden, Germany, $10\,\mu$g/mL) or left untreated prior to coincubation with GBM cells at an E/T of 40 : 1. (c). LNT-229-Luc cells were treated as in (a) and coincubated with purified polyclonal NK cells ((a) to (c): one representative out of three independent experiments is shown, bars = SD). (d) CFSE labeled purified NK cells were cocultivated with LNT-229 glioma cells. After extensive washing, NK cells (upper panel) or coculture (lower panels) were microscopically documented ($n = 3$, one representative experiment is shown). (e) Quantification of NK cell attachment on LNT-229 cells ($n = 3$, SEM, $^{*}P = 0.035$).

the level of TIMP-2 expression [50], and knowing that under certain conditions TIMP-2 can activate MMP-2 [51], we analyzed TIMP-2 protein expression, demonstrating that TIMP-2 is also downregulated in ISCADOR treated GBM cells (Figure 6(a)).

3.5. ISCADOR Q Reduces Tumor Growth in Murine Glioma Models. To analyze the effects of ISCADOR Q on tumor growth *in vivo*, we used two different mouse models. We have chosen a xenograft model in which human LNT-229 GBM cells were subcutaneously implanted into the right flank of nude mice. For therapeutic intratumoral ISCADOR treatment, mouse SMA560 glioma cells, representing a spontaneously developed, poorly differentiated astrocytoma in a VMDk mouse, were implanted in immunocompetent VMDk mice [39]. As shown in Figures 7(a) and 7(b), both
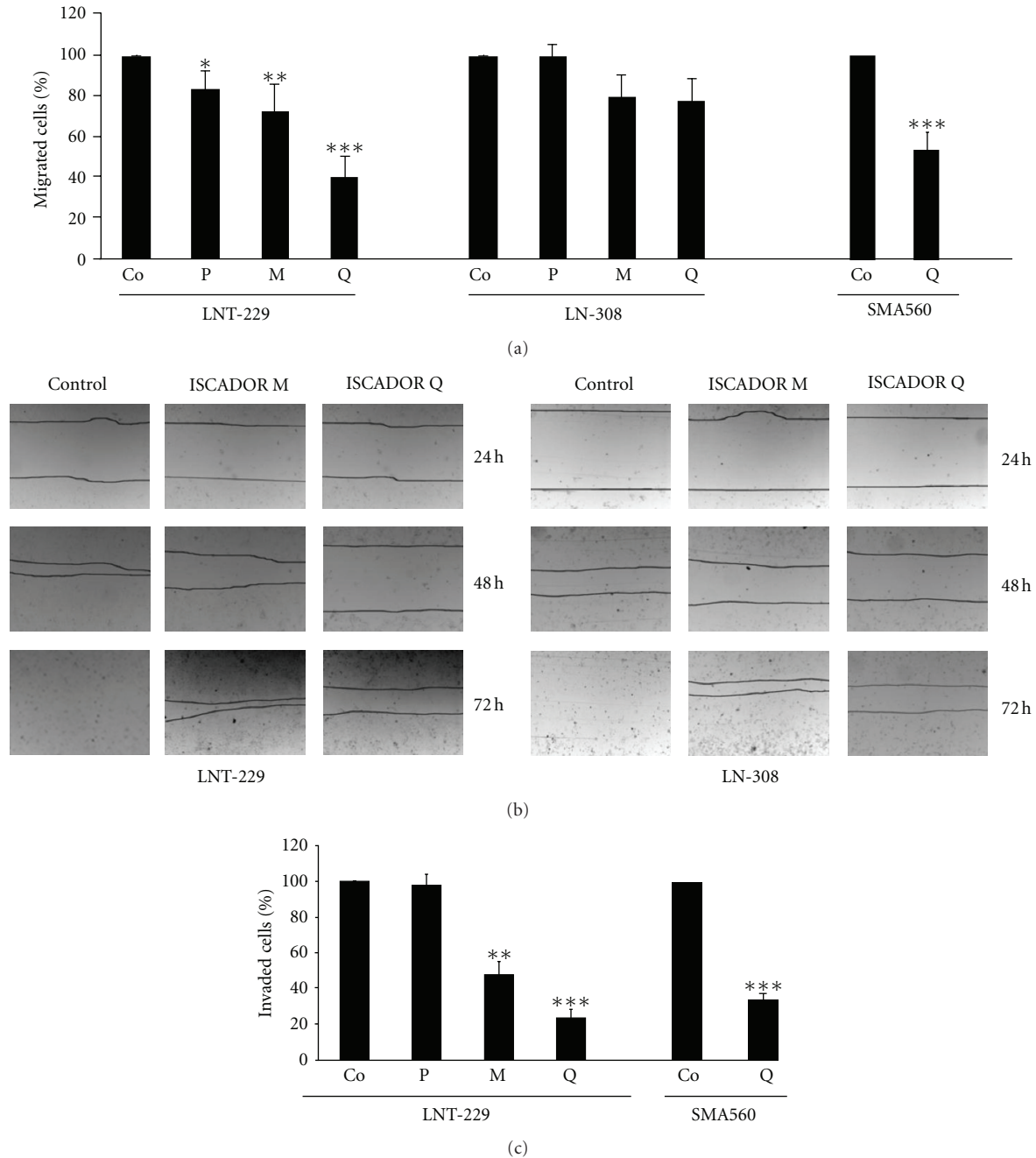
(a)

(b)

(c)

FIGURE 5: ISCADOR reduces cell motility and invasiveness in human and mouse GBM cell lines. (a) Boyden chamber migration assays. The cells were treated for 24 h with ISCADOR P, M, or Q (100 μg/mL). 20.000 treated cells were seeded in upper parts of Boyden chambers. Migrated cells were counted 21 h later ($n = 3$ for ISCADOR P, $n = 5$ for ISCADOR M, $n = 7$ for ISCADOR Q, SEM). (b) Scratch assay. The cells were seeded and treated as in (a) 24 h after treatment a scratch was set and migration of cells were documented photographically every 24 h. For better visualization, migration borders were marked with black lines ($n = 3$, one representative experiment is shown). (c) Boyden chamber matrigel invasion assay. The cells were treated as in (a) ($n = 3$, SEM).

in the xenograft and in the syngeneic mouse model, pretreatment of the implanted cells with ISCADOR Q (100 μg/mL) mitigated tumor growth indicating that the ISCADOR Q antitumor effects were also transferable to *in vivo* growing tumors. To exclude that the differences in tumor growth in ISCADOR Q and control treated tumors were a result

of altered cell proliferation or induction of cell death, we analyzed cell growth in parallel. There were no differences in proliferation in ISCADOR Q treated LNT-229 cells compared to vehicle treated cells. For SMA560 cells, ISCADOR Q delayed cell growth (insert in Figure 7(b)), but at 96 h after treatment, ISCADOR-treated cells reached the same
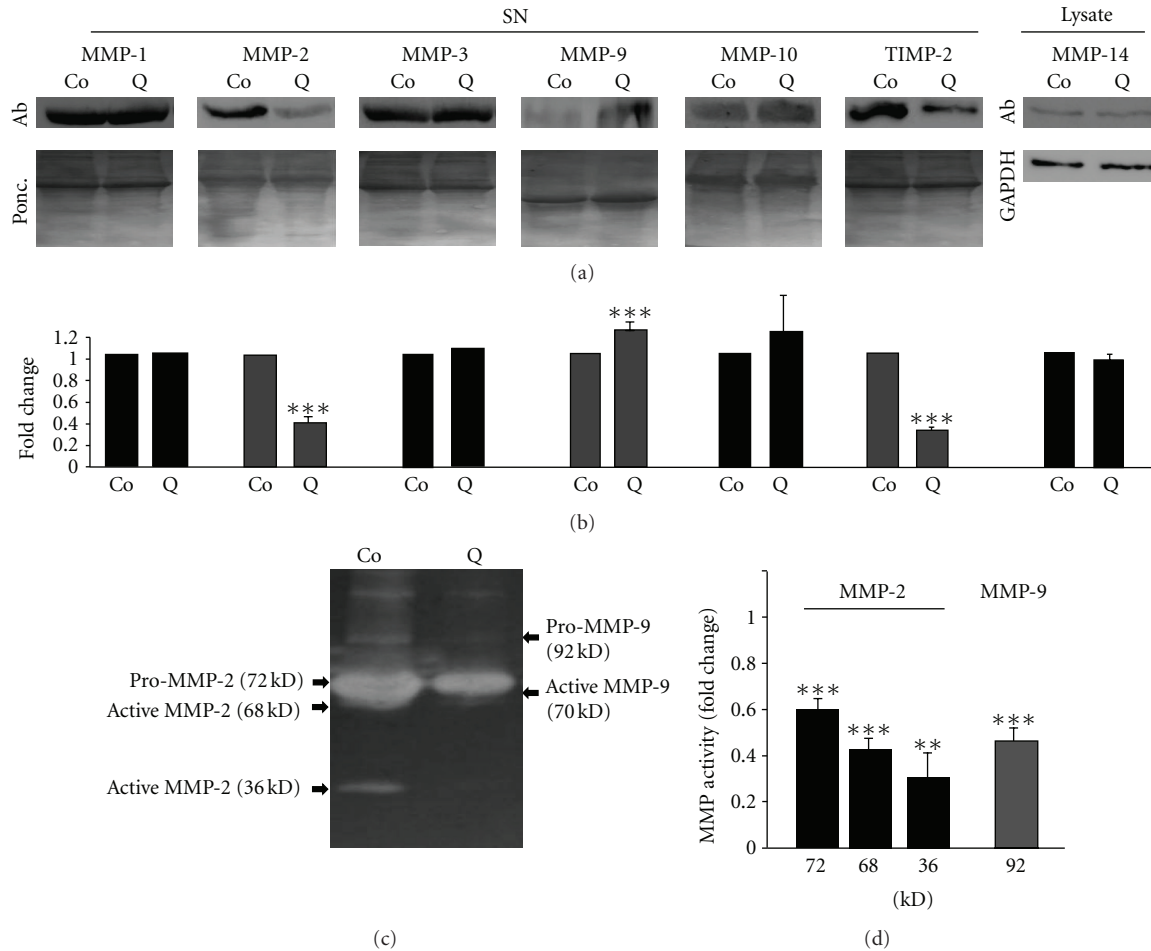
(a)

(b)

(c)

(d)

FIGURE 6: ISCADOR Q mediated reduction of cell motility is caused by reduced MMP-expression and activity. (a) LNT-229 cells were treated with ISCADOR Q or were left untreated. 24 h later, cellular supernatants (SN) or lysates were prepared. MMP or TIMP expression was analyzed by immunoblot (Ab; specific antibody, one representative experiment is shown). (b) Quantification of protein expression ($n = 3$ for MMP-1, $n = 9$ for MMP-2, $n = 3$ for MMP-3, $n = 8$ for MMP-9, $n = 2$ for MMP-10, $n = 4$ for MMP-14, $n = 5$ for TIMP-2). (c) Gelatinase zymogram analyzing MMP activity ($n = 10$, one representative experiment is shown). (d) Quantification of MMP gelatinase activity ($n = 8$ for MMP-9, $n = 10$ for MMP-2, $n = 4$ for 36 kD active MMP-2; SEM). Note that the activated form of MMP-9 could not be separated strictly from MMP-2. Therefore quantification of MMP-9 activity was only done for proMMP-9.

growth level than control cells suggesting that the differences in tumor growth were not due to altered proliferation or cell death. In a first therapy model mimicking conditions GBM patients use for ISCADOR treatment, the mice were treated with repeated subcutaneous injections of ISCADOR Q at increasing concentrations on the contralateral body site. This model will give information if ISCADOR Q is able to induce antitumoral activity by systemic application. As shown in Figure 7(c), there was a slight, but not significant reduction of tumor growth in ISCADOR Q treated mice. In a second therapy model we treated subcutaneously preimplanted tumors with a single intratumoral injection (20 μL, 100 μg/mL) of ISCADOR Q. This amount of ISCADOR was chosen since we wanted to gain information if an intratumoral application of ISCADOR might exhibit antitumor activity independent of its capacity to induce cell death or to inhibit proliferation (Figures 2(b) and 3(b)). Tumor growth was significantly reduced when ISCADOR Q was directly injected into the growing tumor mass. This suggests that ISCADOR Q might unfurl its antitumor effect best when in direct contact to the tumor cells.

4. Discussion

The failure of effective therapy regimens in malignant GBM is highly associated with its malignant characteristics which means that these tumors are highly resistant to cell death [3], possess immunosuppressive function [4] and show a highly invasive and destructive growth due to their migratory and invasive growth potential [5]. A therapeutic substance with multimodal function able to decrease GBM cell motility, to induce antitumoral immunity and to inhibit GBM cell proliferation might be of high value in tumor treatment. In this study, we showed for the first time that the mistletoe extract ISCADOR, and especially the lectin rich variant
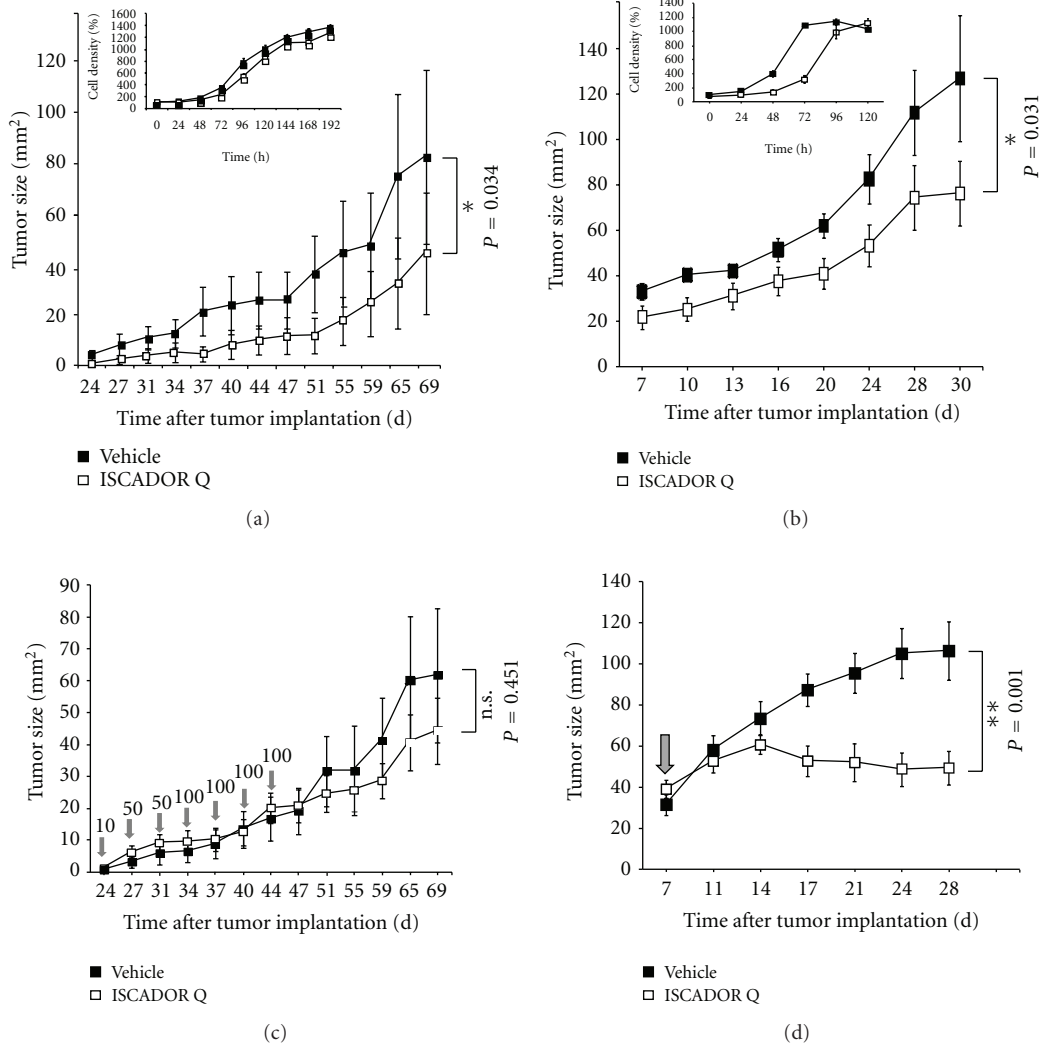
(a)

(b)

(c)

(d)

FIGURE 7: ISCADOR Q reduces the growth of subcutaneously growing GBM in mice. (a) and (b) The cells were pretreated with ISCADOR Q (100 μg/mL) or were left untreated. One million viable LNT-229 cells were implanted into the right flank of nude mice (a), or one million SMA560 cells into VMDk-mice (b) Tumor size was measured 3 times per week. Inlet: As a control and to avoid the measurement of artifacts induced by ISCADOR Q mediated inhibition of cell growth, proliferation of implanted cells was assessed by crystal violet staining in parallel. (c) One million LNT-229 cells were implanted into the right flank of nude mice and tumors were allowed to grow for six days. At day 7 subcutaneous injections twice a week with PBS or a weekly increasing dose of ISCADOR Q (1 up to 100 μg in total, arrows indicate ISCADOR injections) were given on the contralateral body site. After two weeks of treatment with the highest dosage, injections were stopped and tumor size was measured as in (a). (d) One million SMA560 cells were implanted into the right flank of VMDk mice and tumors were allowed to grow for six day. On day 7, a single intratumoral injection of ISCADOR Q (20 μL, 100 μg/mL, indicated by an arrow) was given into the palpable tumors. Tumor size was measured as in (a).

ISCADOR Q, not only reduced GBM cell growth and induced NK cell mediated GBM cell lysis, but also impaired the migration and invasion of GBM cells *in vitro* and delayed tumor growth *in vivo*. These effects seemed, at least in part, to be caused by an ISCADOR induced downregulation of glioma-associated genes involved in proliferation, survival and tumor cell motility (Figure 1(a)). In addition, ISCADOR treatment further led to a significant reexpression of genes which are usually downregulated in glioblastomas and a down-regulation of genes associated with malignant progression of gliomas (Figure 1(f)). These findings point to

a therapeutic effect of ISCADOR on gene clusters associated with gliomagenesis and glioma progression thereby exerting antitumor functions. The expression of several genes regulating proliferation (EGFR, PKB, STAT3) as well as survival (BIRC5/survivin) and especially the secretion of several factors relevant for cell motility was also mitigated by ISCADOR Q, the most important being TGF-β and MMP-2, knowing that TGF-β regulates MMP-2 [37]. Reduced secretion did not result from a general inhibition of protein secretion as there was no difference in the overall amount of secreted proteins (data not shown), and there were also

several other proteins we examined that did not show any difference in expression or secretion after ISCADOR Q treatment (Figures 4, and 6). Reduced secretion of MMP-2, being one of the most important proteins for ECM destruction and a well-known inducer of GBM cell motility [52, 53], was accompanied by the reduction of the activity of either pro- and mature MMP-2 variants as well as of MMP-9 (Figures 6(c) and 6(d)). Even if there was reduced expression of MMP-9 mRNA upon ISCADOR treatment (Figure 4(a)), there was an enhancement in extracellular pro-MMP-9 (Figures 6(a) and 6(b)) suggesting that ISCADOR might also alter biological processes differentially regulating protein secretion. But to unravel the mechanism of this observation needs further investigation. In this context, ISCADOR Q mediated downregulation of TIMP-2 (Figures 6(a) and 6(b)) should also be kept in mind. TIMP-2 was originally characterized as an inhibitor of MMP, and in this light, the downregulation of TIMP-2 and accompanied simultaneous reduction of MMP-2 activity seemed *prima facie* not fit. But meanwhile it has been shown that TIMP-2 can also act as an activator for MMP-2, thereby increased levels of TIMP-2 can lead to increased MMP-2 activity, too [50, 51]. For this reason ISCADOR Q mediated reduction of TIMP-2 fits well to decreased MMP-2 activity and impaired cell motility in ISCADOR Q treated GBM cells. The secretion of two additional factors related to tumor growth and motility as well as to angiogenesis, was also decreased in ISCADOR Q treated GBM cell: VEGF, whose expression is known in GBM to be correlated with MMP-2 [54] and Angiopoietin-1, a factor suggested to regulate, beside angiogenesis, GBM cell adhesion to the ECM via its receptor Tie-2 [55]. Reduced expression of VEGF and Ang-1 might therefore not only be suggested to contribute to the antimigratory/anti-invasive effect of ISCADOR, but also to contribute to reduced tumor neoangiogenesis.

In the literature ISCADOR has been described as an immune stimulating agent [6–10, 13, 34, 56, 57]. In the present study, ISCADOR treatment of GBM cells led to an increased NK cell mediated, but not T cell mediated tumor cell lysis (Figure 3). The mechanism by which ISCADOR enhanced NK cell dependent GBM cell lysis is still not completely unraveled, but it was clearly not an effect of directly striking NK cells nor of altered immune receptor/ligand surface expression on either GBM or immune cells. Neither regulation of NKG2DL, DNAM-1-ligand, or MHC class molecules on GBM cells, nor alterations of the appropriate receptors on NK cells, were detectable upon ISCADOR treatment. The immune stimulating effect of ISCADOR might be, on the one hand, an effect of reduced secretion of the prominent immunosuppressive cytokine TGF-β [4, 5, 58] in ISCADOR treated GBM cells, on the other hand might result from the strengthened attachment of NK cells to the surface of ISCADOR treated GBM cells, by this mechanism forcing the contact between immune effector and tumor target cells. Since the boosted attachment was restricted to the lectin rich ISCADOR variant Q, this effect is suggested to be a result of high ML concentration on the target tumor cell surface (Figure 4(d)). The immune stimulating effect of ISCADOR Q was best if tumor cells are directly exposed

to the agent, since we have shown that *in vitro* treatment of NK cells with ISCADOR provided no immune stimulation. *In vivo* using two different mouse models (syngeneic and xenograft) and two different therapeutic approaches (systemic or intratumoral ISCADOR Q application) we demonstrated that ISCADOR Q delayed tumor growth. Subcutaneous injection, of ISCADOR Q in mice harboring GBM tumors at the contralateral body flank showed only marginal and not significant tumor growth reduction. In contrast, local intratumoral treatment of tumors with ISCADOR Q significantly reduced tumor growth again suggesting that ISCADOR unfurls its antitumoral effect best when in direct contact to the target cells (Figures 7(c) and 7(d)).

5. Conclusions

In conclusion, we here present that ISCADOR treatment reduces the expression of genes associated with tumor progression, decreased GBM cell growth, mitigated GBM cell migration and invasion, and enhanced NK cell mediated GBM cells lysis. These transcriptional changes translated into a functionally relevant reduction of cell proliferation, a decrease of the migratory and invasory capacity as well as a decline in the immune evasory potential of glioma cells. *In vivo,* ISCADOR Q reduced tumor growth in both xenograft and syngeneic GBM mouse model. To display its best antitumoral function, ISCADOR Q and especially ML have to be in a close contact to the tumor cells. Thus, ISCADOR Q may hold promise as a multimodal antitumoral agent for concomitant treatment of human GBM.

List of Abbreviations

Ang-1:	Angiopoietin-1
APC:	Allophycocyanin
CFSE:	Carboxyfluorescein diacetate succinimidyl ester
CNS:	Central nervous system
DD:	Cluster of designation
DEVD-amc:	Acyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
DNAM-1:	DNAX accessory molecule 1
ECM:	Extracellular matrix
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
ELISA:	Enzyme linked immuno sorbant assay
E/T:	Effector: target cell ratio
FACS:	Fluorescence activated cell sorting
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanat
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GBM:	Glioblastoma multiforme
HLA-DR:	Human leukocyte antigen complex on chromosome 6
Luc:	Firefly luciferase
IgG:	Immunoglobulin G
i.t.:	Intratumoral
ME:	Mistletoe extracts

MGMT:	O-(6)-methylguanine-DNA methyltransferase
MHC:	Major histocompatibility complex
MICA/B:	MHC class I-related chain A or B
ML:	Mistletoe lectins
MMP:	Matrix-metalloproteinase
NK:	Natural killer
NKG2D:	Natural killer group 2D
PBS:	Phosphate buffered saline
PBL:	Peripheral blood lymphocyte
PECAM:	platelet/endothelial cell adhesion molecule
PCR:	Polymerase chain reaction
PE:	R-phycoerythrin
PI:	Propidium iodide
PKB:	Protein kinase B
REMBRANDT:	Repository of molecular brain neoplasia data
s.c.:	Subcutaneous
STAT3:	Signal transducer and activator of transcription 3
TGF-β:	Transforming growth factor beta
Tie-2:	Tyrosine kinase with Ig and EGF homology domains
TIMP-2:	Tissue inhibitor of metalloproteinase 2
TMZ:	Temozolomid
ULBP:	UL-16 binding protein
VEGF:	Vascular endothelial growth factor
VEGFR2:	Vascular endothelial growth factor receptor-2
a.u.:	Arbitrary unit

Conflict of Interests

All authors certify that there is no actual or potential conflict of interests in relation to this paper.

Acknowledgment

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