Silibinin inhibits invasive properties of human glioblastoma U87MG cells through suppression of cathepsin B and nuclear factor kappa B-mediated induction of matrix metalloproteinase 9

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Glioblastoma multiforme remains one of the most devastating human malignancies because of its high infiltrative capacity. This study aimed to investigate the effects of silibinin on human glioblastoma U87MG cells. The microculture tetrazolium test, bromodeoxyuridine cell proliferation assay, cell-based nuclear factor kappa B (NF-κB) activation assessment, cathepsin B activity assay, gelatin zymography, and quantitative real-time reverse transcription-PCR were performed to appraise the effects of silibinin on the metabolic activity, DNA synthesis, NF-κB phosphorylation, cathepsin B activity, and gelatinolytic activity of U87 cells. Silibinin inhibited metabolic activity, cell proliferation, NF-κB activation, cathepsin B enzymatic levels, and gelatinase B activity in U87 cells. In addition, an expressive decrease in mRNA levels of matrix metalloproteinase-9, cathepsin B, urokinase plasminogen activator receptor, urokinase plasminogen activator, and intercellular adhesion molecule 1 coupled with a significant induction in transcriptional levels of stefin A was observed. Altogether, these issues show for the first time that silibinin treatment could trammel invasive features of a highly invasive human glioma cell line, U87, through suppression of NF-κB-mediated stimulation of matrix metalloproteinase-9. Furthermore, silibinin might cripple the activation of gelatinase B by cramping transcriptional and enzymatic activities of cathepsin B in U87 cells.

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Introduction

Astrocytomas, the neoplasm of astrocytes, are the most frequent brain tumors of the central nervous system. According to the World Health Organization prognostic grading system, astrocytomas are classified from low- to high-grade gliomas (grades I–IV). Grade IV astrocytomas, also known as glioblastoma multiforme (GBM), are characterized with high invasive and proliferative capacities. Patients with GBM are thought to have a poor prognosis and short survival because of tumor cell invasion and spread to the neighboring normal brain tissue. The main hallmark of GBM is tumor cell detachment from the primary site and dissemination, which trammels the current therapeutic remedies including radiation, chemotherapy, and surgical resection. In this regard, there is a critical need to devise more efficacious curative regimens that target tumor cell spread and invasion in GBM [1].

An accumulating body of evidence underscores the pivotal roles of extracellular matrix (ECM)-degrading proteinases such as cysteine proteinases (cathepsin B), serine proteinases (urokinase-type plasminogen activator; uPA), and matrix metalloproteinases (MMPs) in glioma cell invasion [1,2]. In this regard, cathepsin B, a lysosomal proteinase, has been thought to play a cardinal role in tumor cell dissemination through ECM remodeling [3] and stimulation of pro-uPA, which in turn can activate MMPs [4]. Furthermore, cathepsin B has been suggested to be responsible for unbalanced expression of MMPs and their natural inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), through degradation of the latter [5]. In this regard, therapeutically disabling cathepsin B expression and enzymatic activity seems an excellent opportunity to disrupt cathepsin B/uPA/MMPs proteolytic cascade and, subsequently, tumor cell invasion and spread.

MMPs that are upregulated in human malignancies of varied origins are a family of zinc-dependent endopeptidades able to degrade almost all components of ECM and thereby play major roles in ECM remodeling and tumor invasion [6]. A comparative study of mRNA expression of MMP-2 (72 kDa gelatinase A), MMP-9

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(92 kDa gelatinase B), and membrane-type 1 MMP (MT1-MMP) in 47 brain tumor samples has shown that transcriptional levels of these MMPs are highly elevated in tumor samples compared with their normal counterparts [7]. Moreover, zymographic analysis showed that gelatinolytic activities of both MMP-2 and MMP-9 are enhanced in brain tumors in comparison with the control [7]. Furthermore, it has been suggested that although all three MMPs are involved in invasive behavior of gliomas, only the transcriptional and enzymatic levels of MMP-9 are correlated with tumor grade [7]. In this setting, pharmacologically quelling the signal transduction networks involved in transcriptional stimulation of MMPs seems an efficient therapeutic opportunity to reduce tumor cell dissemination.

A wealth of evidence highlights the principal roles of the nuclear factor kappa B (NF-κB) signaling module in multistage tumorigenesis. NF-κB has been manifested to govern all six hallmarks of cancer by controlling the genes involved in proliferation, evasion from apoptosis, inflammation, angiogenesis, and tumor invasion [8]. The determinant roles of NF-κB in triggering tumor invasion have been thought to be through the induction of MMP-2, MMP-9, and uPA [8]. Sustained activation of NF-κB in human glioblastoma has been reported [9]. Furthermore, a bulk of evidence suggests the fundamental roles of NF-κB in proliferation and invasion of glioblastoma [10,11]. In addition, it has been manifested that MMP-9 is induced in glioblastoma through the NF-κB signaling network [12]. In this regard, targeting the NF-κB module might suppress activation of MMP-9 and, subsequently, invasion in human glioblastoma.

Recently, silibinin, a natural flavonoid from milk thistle, has attracted a great deal of attention because of its strong pleiotropic anti-neoplastic effects against various tumor cells including glioblastoma [13], renal cell carcinoma [14], bladder [15], colon [16], lung [17], skin [18], prostate [19], and hepatocellular carcinoma [20]. In addition, an accumulating body of evidence indicates the anti-invasiveness potentials of silibinin against multiple human malignancies including oral squamous cell carcinoma [21], osteosarcoma [22], lung [17], breast [23], and hepatocellular carcinoma [20]. Moreover, there is substantial evidence that silibinin suppresses NF-κB [24,25].

Despite the variegated suppressive effects of silibinin on tumor cell invasion in various types of malignancies and the pivotal roles of NF-κB and cathepsin B in triggering tumor invasion in glioblastoma, to date no study has addressed the effects of silibinin on tumor cell invasion through the modulation of NF-κB activation, cathepsin B enzymatic activity, and mRNA expression in glioblastoma. This study aimed to evaluate the effects of silibinin on invasive features of the human glioblastoma cell line U87MG by moderating the NF-κB cascade as well as the transcriptional and enzymatic levels of cathepsin B.

Materials and methods

Cell line and silibinin treatment

The human glioblastoma cell line U87MG was grown as a monolayer in RPMI 1640 medium (Invitrogen, Auckland, New Zealand) supplemented with 10% fetal bovine serum (Invitrogen) in 5% CO_2 at 37°C. The cultures were then treated with 25, 50, 75, and 100 μmol/l of silibinin (Sigma, St. Louis, Missouri, USA) for 48 h.

Microculture tetrazolium test

A microculture tetrazolium (MTT) assay was performed to determine the inhibitory effect of silibinin on the metabolic activity of U87 cells. The cells were plated onto 96-well plates at a density of 5000 cells/100 μl/well. After incubation at 37°C for 24 h, the cells were exposed to silibinin at 25, 50, 75, and 100 μmol/l for 48 h. MTT solution (0.5 mg/ml) of 200 μl was added to each well and the cells were further incubated at 37°C for 2 h. After dissolving the precipitated formazan with 100 μl of dimethyl sulfoxide, the optical densitometry was measured at a wavelength of 578 nm. The inhibition rate of silibinin was evaluated using the following equation: inhibition rate $(\%) = (1 - \mathrm{OD}_{\mathrm{exp}}/\mathrm{OD}_{\mathrm{con}}) \times 100$, where $\mathrm{OD}_{\mathrm{exp}}$ and $\mathrm{OD}_{\mathrm{con}}$ are the optical densities of treated and untreated cells, respectively.

BrdU cell proliferation assay

The suppressive effect of silibinin on growth and proliferation of U87MG cells was measured using a colorimetric bromodeoxyuridine (BrdU)-based Cell Proliferation ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations. Briefly, the cells (5000 cells/100 μl/well) were treated with the desired concentrations of silibinin and then incubated with 10 μl of BrdU solution at 37°C for 48 h. The cells were then fixed and DNA was denatured using 200 μl of FixDenat solution provided with the kit. After incubation with peroxidase-conjugated anti-BrdU antibody at room temperature for 1 h, the cultures were exposed to 100 μl of substrate tetramethylbenzidine for 30 min at room temperature. To stop the peroxidase reaction, 25 μl of 1 M H_2SO_4 was applied and the samples were read at 450 nm in an ELISA reader.

Cell-based nuclear factor kappa B phosphorylation measurement by ELISA

The effect of silibinin on NF-κB activation was surveyed by Cellular Activation of Signaling ELISA (CASE Kit, SuperArray Bioscience, Frederick, Maryland, USA) according to the manufacture's protocol. Briefly, the cells were plated onto 96-well plates at a density of 5000 cells/well/100 μl. After 48 h of exposure with escalating concentrations of silibinin at 37°C, the cells were fixed in a 8% formaldehyde : phosphate-buffered saline solution and then stained with primary and secondary antibodies, followed by exposure to developing and stop solutions provided with the kit. After normalization with the

relative cell number obtained by a cell staining buffer at 578 nm, the ratio of phosphorylated NF-κB to total NF-κB was obtained at 450 nm.

Cathepsin B activity assay

To monitor the effect of silibinin on the activity of secreted cathepsin B in the medium of U87 cells, the cathepsin B activity assay kit (Biovision Research Products, Mountain View, California, USA) was applied following the manufacturer's instructions. Briefly, equal amounts of protein from conditioned media of each sample were incubated with 50 µl of reaction buffer and 2 µl of a specific synthetic substrate of cathepsin B on a 96-well plate at 37°C for 2 h. The substrate contains amino-4-trifluoromethyl coumarin, which is released because of cathepsin B activity. After reading the samples in a Varian Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Australia) with a 400 nm excitation filter and a 505 nm emission filter, the inhibition rate of cathepsin B activity was obtained by comparing the relative fluorescence units of samples with the control group.

Gelatin zymography

MMP-2 and MMP-9 gelatin zymographies were carried out as described earlier [20].

Analysis of gene expression by real-time quantitative PCR

The FastPure RNA kit (Takara Bio Inc., Otsu, Japan) was used to isolate total RNA from cultured cells. The quantity of RNA samples was assessed spectrophotometrically using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA). Changes in mRNA expression of the desired genes were surveyed by real-time PCR after reverse transcription (RT) of 1 µg of RNA from each sample with the PrimeScript RT reagent kit (Takara Bio) according to the manufacture's specifications. Quantitative real-time RT-PCR was performed on a light cycler instrument (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). SYBR Green master mix (10 µl) was added to 2 µl of cDNA samples, 0.5 µl of forward and reverse primers (10 pmol) in water and 7 µl of nuclease-free water (Qiagen, Hilden, Germany) to conduct PCR in 20 µl of reaction mixture. Thermal cycling conditions involved an initial activation step for 30 s at 95°C followed by 45 cycles including a denaturation step for 5 s at 95°C and a combined annealing/extension step for 20 s at 60°C. Melting curve analysis was applied to validate whether all primers yielded a single PCR product. The primers used are listed in Table 1. Hypoxanthine phosphoribosyltransferase1 was amplified as normalizer and the fold change in expression of each target mRNA relative to hypoxanthine phosphoribosyltransferase1 was calculated on the basis of $2^{-\Delta\Delta ct}$ relative expression formula [26].

Statistical analysis

Data are expressed as mean ± standard deviation. All experiments were performed in triplicate except for gelatin zymography, which was performed in duplicate. For statistical analysis, the Student's *t*-test and one-way analysis of variance were applied. To compare the control group with the experimental groups, Dunnett's multiple comparison tests was used. *P* values of less than 0.05 were considered significant.

Results

Silibinin inhibits metabolic activity of U87 cells

To determine whether silibinin treatment suppresses the metabolic potential of U87 cells, the MTT assay was carried out. Concentration-dependent experiments showed that silibinin inhibited the metabolic abilities of U87 cells. As shown in Fig. 1, treatment of cells with silibinin at 75 and 100 µmol/l after 48 h reduced cell metabolic capabilities by 19.66 and 31.85%, respectively.

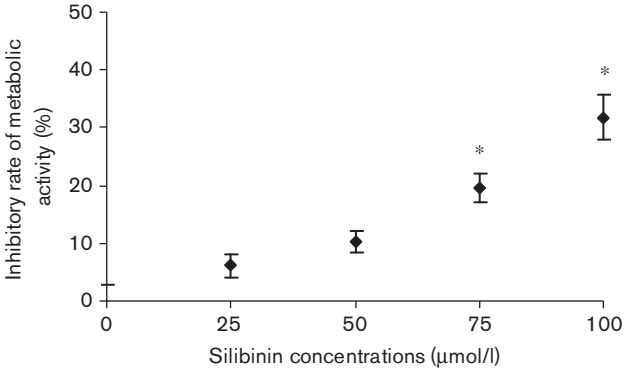
Silibinin inhibits proliferation of U87 cells

To explore whether silibinin treatment could suppress the growth and proliferation of U87 cells, a colorimetric BrdU proliferation assay was applied. A dose-dependent reduction in proliferative potential of U87 cells was observed after silibinin treatment. As shown in Fig. 2, a 20.21 and 27.74% decrease in cell proliferation was observed by silibinin at 75 and 100 µmol/l, respectively, after 48 h.

Table 1 Nucleotide sequences of the primers used for real-time RT-PCR

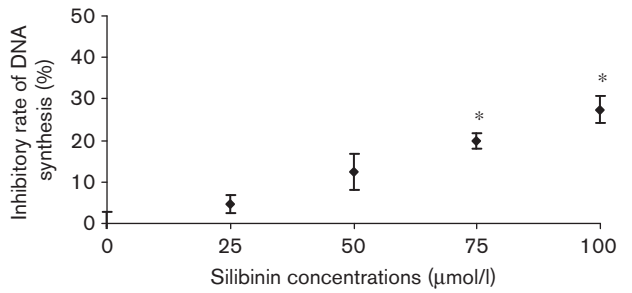
Gene	Accession number	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
MMP-2	NM_004530	CTTCCAAGTCTGGAGCGATGT	TACCGTCAAAGGGGTATCCAT	119
MMP-9	NM_004994	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC	139
Stefin A	NM_005213	TGATAAGGTTAAACCACAGCTTGAA	GTAGTAATTTGTTCCAGCAACAACTTG	103
TIMP-2	NM_003255	AAGCGGTCAGTGAGAAGGAAG	GGGGCCGTGTAGATAAACTCTAT	136
uPAR	NM_002659	TATTCCCGAAGCCGTTACCTC	TCGTTGCATTTGGTGGTGTTG	275
Cathepsin B	NM_000100	CTGTCGGATGAGCTGGTCAAC	TCGGTAAACATAACTCTCTGGGG	152
MT1-MMP	NM_004995	GAAGCCTGGCTACAGCAATATG	TGCAAGCCGTAAAACTTCTGC	119
uPA	NM_002658	TCAAAAACCTGCTATGAGGGGA	GGGCATGGTACGTTTGCTG	121
LRP1	NM_002332	GGCATCCTGATTGAGCACCT	CGGATCACACTCGTCTTCTGC	104
Cystatin C	NM_000099	TAGCTGGGGTGAACTACTTCTT	GATTTCGACAAGGTCATTGTGC	175
IKK1	NM_001278	AAGTTGAACCATGCCAATGTTGT	TCTCCTCCAGAACAGTATTCCAT	107
IKK2	NM_001556	CACCATCCACACCTACCCTG	CTTATCGGGGATCAACGCCAG	136
Nemo	NM_001099856	CTTCCAAGAATACGACAACCACA	CGGAACGGTCTCCATCACAAT	187

Fig. 1



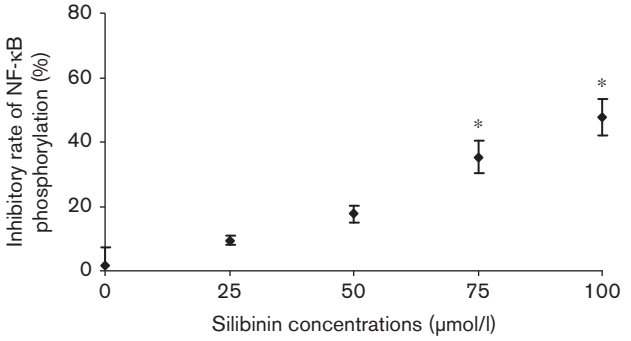
Effects of silibinin on metabolic activity of U87 cells. Using the microculture tetrazolium assay, the inhibitory effect of silibinin on metabolic characteristics of U87 cells was determined. The inhibitory rate of silibinin was measured as mentioned in materials and methods. Values are given as mean ± SD. Statistically different values of $*P<0.05$ were determined compared with the control.

Fig. 2



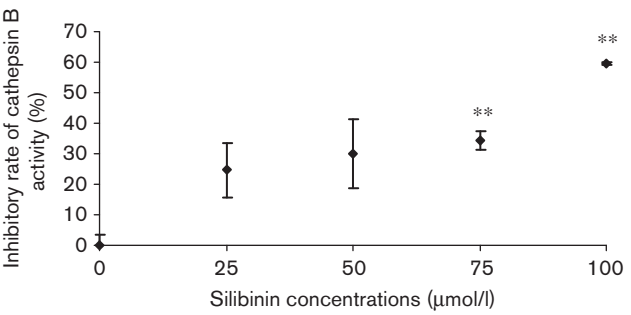
Effects of silibinin on proliferation of U87 cells. Bromodeoxyuridine incorporation was used to assess the proliferative capacity of silibinin-treated cells. After fixation of the cells and denaturation of DNA, the cells were stained with anti-bromodeoxyuridine. Tetramethyl-benzidine was applied as the substrate and the optical densitometries were measured at a wavelength of 450 nm. Values are given as mean ± SD. Statistically different values of $*P<0.05$ were determined compared with the control.

Fig. 3



Effects of silibinin on phosphorylation of NF-κB in U87 cells. Using a cell-based ELISA approach, relative phosphorylated levels of NF-κB were assessed. Fixed cells were stained with primary antibodies (against both phosphorylated and non-phosphorylated forms of NF-κB) and secondary antibody (horseradish peroxidase-conjugated). The ratio of activated NF-κB to total NF-κB was measured at 450 nm after normalizing with the relative cell number obtained at 578 nm. Values are given as mean ± S.D. Statistically different values of $*P<0.05$ were determined compared with the control. NF-κB, nuclear factor kappa B.

Fig. 4



Effects of silibinin on enzymatic activity of secreted cathepsin B in U87 cells. Conditioned media of treated cells were subjected to the synthetic substrate sequence RR labeled with amino-4-trifluoromethyl coumarin (AFC). The arginine–arginine–AFC substrate is cleaved by cathepsin B to release AFC, which is fluorometerically detected by 400 nm excitation and 505 nm emission filters. The inhibitory effect of silibinin on cathepsin B activity was quantified as $1-RFU_{exp}/RFU_{con}$ where RFU_{exp} and RFU_{con} are the relative fluorescence units of treated and untreated cells, respectively. Values are given as mean ± SD. Statistically different values of $**P<0.01$ were determined compared with the control. RFU, relative fluorescence units.

Silibinin inhibits nuclear factor kappa B activation

To assess whether silibinin exposure hinders NF-κB phosphorylation and activation in U87 cells, we next evaluated the influence of silibinin on the NF-κB signaling cascade using a cell-based ELISA assay for NF-κB protein phosphorylation. As shown by Fig. 3, silibinin exposure at 75 and 100 μmol/l reduced NF-κB phosphorylation by 35.2 and 47.78%, respectively, compared with the control.

Silibinin-inhibited enzymatic activity of cathepsin B in the medium of U87 cells

This activity was performed to investigate whether silibinin inhibits the activity of secreted cathepsin B in the medium of U87 cells using a fluorescence-based cathepsin B activity assay. As indicated in Fig. 4, silibinin at 75 and 100 μmol/l suppressed cathepsin B enzymatic activity by 34.4 and 59.91%, respectively, compared with the control.

Silibinin inhibits matrix metalloproteinase-9 enzymatic activity

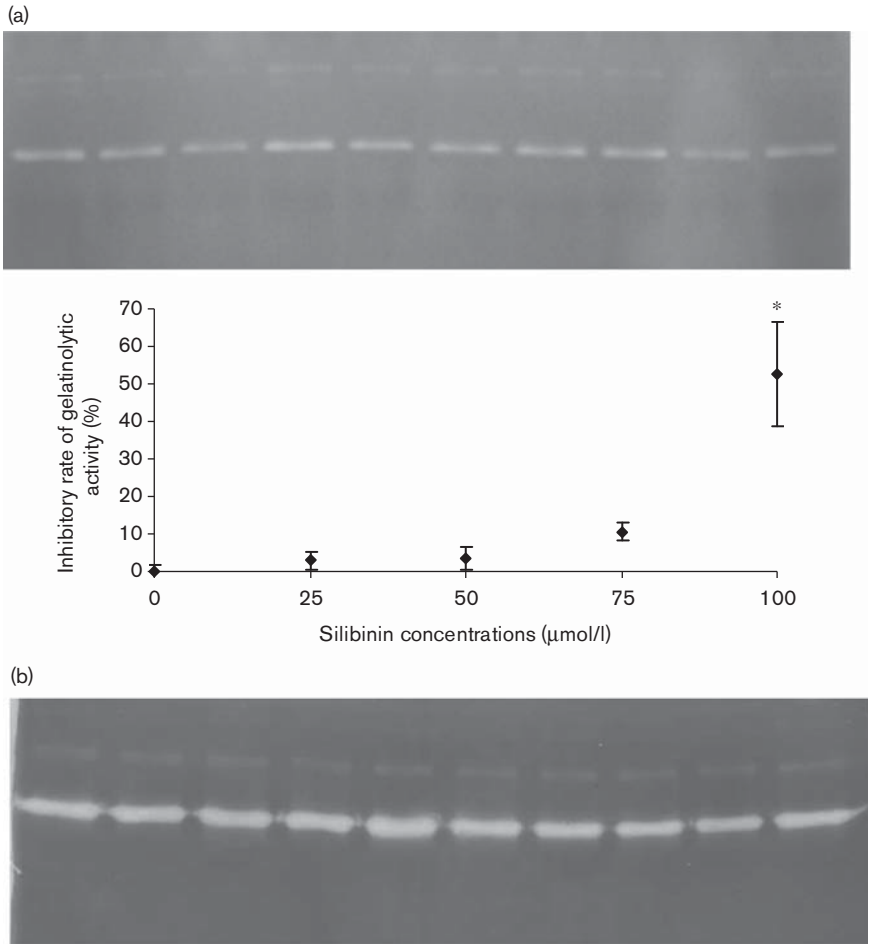
Zymoanalysis was carried out to monitor whether silibinin suppresses MMP-2 and MMP-9 activity in U87 cells. The gelatinolytic activities of MMP-2 and MMP-9 were

detected on gelatin A- and gelatin B-containing gels. Active MMPs were detectable as unstained bands against the blue background. The intensities of corresponding bands for MMP-9 diminished with the increase in silibinin concentration. As shown in Fig. 5a, MMP-9 activity was reduced in silibinin-treated cells after 48 h. Silibinin at 100 μmol/l inhibited MMP-9 enzymatic activity by 49.24% compared with the control. In comparison, as shown in Fig. 5b, silibinin had no inhibitory effect of gelatinolytic activity on MMP-2.

Silibinin downregulates mRNA levels of MMP-9, uPAR,uPA, cathepsin B, and ICAM1, and stimulates transcriptional levels of stefin A

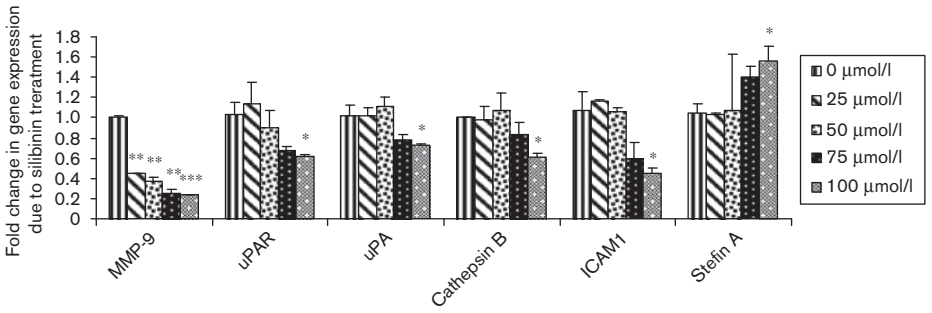
To explore whether silibinin trammels the invasive properties of U87 cells by attenuating the transcriptional activities of MMP-9, urokinase plasminogen activator receptor (uPAR), uPA, cathepsin B, intercellular adhesion molecule 1 (ICAM1), and the induction of mRNA levels of stefin A, we determined the effects of silibinin on mRNA levels of these genes. As shown in Fig. 6, silibinin significantly reduced the transcriptional levels of MMP-9 in a dose-dependent manner. Moreover, silibinin at 25 μmol/l caused an enhancement in mRNA levels of uPAR, but a dose-dependent reduction in the uPAR message was observed by silibinin at 50, 75, and 100 μmol/l. The mRNA levels of uPA were marginally enhanced by 25 and 50 μmol/l of silibinin, but displayed a dose-dependent decrease by silibinin at 75 and 100 μmol/l. Silibinin treatment at 50 μmol/l induced negligible increase in mRNA levels of cathepsin B, but exerted a concentration-dependent reduction in mRNA levels of cathepsin B at 75 and 100 μmol/l. Reduction in mRNA levels of ICAM1 was observed by silibinin at 75 and 100 μmol/l. Furthermore, silibinin exposure stimulated the mRNA levels of stefin A in a dose-dependent manner.

Fig. 5



Effect of silibinin on MMP-9 gelatinolytic activity. The conditioned media from U87 cells was collected and separated on a non-reducing polyacrylamide gel containing gelatin B. The intensities of clear bands representing the gelatinolytic activities of MMP-9 against the blue background of the stained gelatin were analyzed by Bio-Rad Multi-Analyst (Bio-Rad, Hercules, California, USA) (a). Values are given as mean ± SD. Statistically different value of $*P < 0.05$ was determined compared with the control. As shown in (b), silibinin had no suppressive effect of gelatinolytic activity on MMP-2. MMP, matrix metalloproteinase.

Fig. 6



Effect of silibinin on transcriptional levels of MMP-9, uPAR, uPA, cathepsin B, ICAM1, and stefin A. The relative mRNA expression of each gene was measured using real-time reverse transcription-PCR in silibinin-treated U87 cells after normalizing the cycle thresholds of each triplicate against their corresponding hypoxanthine phosphoribosyltransferase1. Values are given as mean ± SD. Statistically different values of $*P<0.05$, $**P<0.01$, and $***P<0.001$ were determined compared with the control. ICAM1, intercellular adhesion molecule 1; MMP, matrix metalloproteinase, uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

Discussion

Currently, there is no medication with proven efficacy for the treatment of GBM [27]. Surgical resection of the enhancing component of the GBM tumor mass might enhance the survival of these patients, but like chemotherapy and radiotherapy it is only palliative, and not curative for the non-enhancing invasive component [27]. Furthermore, the high infiltrative capacity of GBM, which is driven by the overexpressed proteases through degradation of ECM, has been thought of as being the major therapeutic obstacle in GBM patients [1]. In this regard, GBM seems a type of highly aggressive incurable malignancy for which it is of paramount importance to achieve more competent therapeutic interventions aimed at quelling both expression and enzymatic levels of ECM-degradative proteolytic pathways to weaken tumor invasion and infiltration.

A wealth of evidence indicates the cardinal roles of MMPs in glioblastoma invasion [28,29]. Elevated expression of mRNA and enzymatic levels of MMP-2 and MMP-9 has been shown to be strongly associated with the invasive and infiltrative capacities of glioblastoma *in vitro* [30] and *in vivo* [28]. Moreover, MMP-9 activity has been considered to correlate with glioma tumor grade in surgical samples [30]. Finally, transfection of human glioma SNB19 cells with MMP-9 antisense expressing vector significantly reduced MMP-9 mRNA transcription and gelatinolytic activity, and subsequently disabled the invasive features of these cells *in vitro* [29]. In addition, transfected cells were not able to form tumors, or formed very small tumors after intracranial implantation into null mice [29]. Similar results were obtained after transfection of SNB19 cells with an adenovirus expressing antisense-MMP-9 [31]. Transfected cells showed a lower transcriptional level and less enzymatic activity of MMP-9, and displayed lower matrigel invasion capacity [31]. Moreover, these cells developed undetectable tumors after intracerebral injection into nude mice [31]. More interestingly, infected cells caused regression of tumors established earlier by subcutaneous injection of U87MG cells into nude mice [31]. Taken together, these studies highlight the pivotal roles of MMP-9 in glioma invasion and infiltration and suggest MMP-9 as a promising therapeutic target to weaken tumor cell dissemination in gliomas. We then examined whether silibinin is able to repress transcriptional levels of MMP-9, MMP-2, and MT1-MMP. Our results indicate that silibinin exposure significantly preempted the MMP-9 message. In comparison, no inhibitory effect of silibinin on mRNA transcripts of MMP-2 and MT1-MMP was observed in this study (data not shown). Furthermore, the gelatin zymographic analysis of medium from silibinin-treated U87MG cells manifested that silibinin treatment resulted in reduction in gelatinolytic activity of MMP-9. In comparison, silibinin had no suppressive influence on enzymatic levels of MMP-2.

It is widely accepted that cancer metastasis is associated with imbalanced expression of MMPs and TIMPs, including overexpression of MMP-2 and MMP-9 accompanied with underexpression of TIMP-2 in human malignancies of varied origin, including gliomas [32]. In human glioblastomas, the expression of TIMP-2, which negatively regulates both MMP-2 and MMP-9, has been reported to be reduced [32]. In addition, experiments with cultured SF-188, highly invasive astrocytoma cells, showed that transfection of these cells with expression vectors bearing TIMP-1 and TIMP-2 cDNA significantly reduced *in vitro* invasive potential [33]. We next evaluated whether silibinin could stimulate the transcriptional level of TIMP-2. No inductionary impact of silibinin on mRNA expression of TIMP-2 was observed in our study (data not shown).

There is substantial evidence that glioma progression to an aggressive behavior is associated with enhanced expression and activity of uPA [34,35]. Moreover,

augmented levels of uPA have been shown to be strongly correlated with poor prognosis [36]. Concordantly, the increased levels of uPAR have been manifested to contribute to invasive features of glioblastoma cells [37]. In addition, the major molecular mechanism underlying the high activity of the uPA/uPAR system in high-grade gliomas has been considered to be the increased transcription [38]. In line with this, stable transfection of SNB19 cells with an antisense uPA vector hindered the transcriptional and enzymatic activities of uPA, and thereby the invasive ability of these cells in the Matrigel invasion assay [39]. Moreover, the transfected cells had much less tumorigenic potential when intracerebrally injected into nude mice [39]. In addition, Mohanam *et al.* [40] have shown that using an antisense strategy against uPAR in SNB19 cells culminates in transcriptional repression of uPAR, and weakened migratory, invasive and adhesive behaviors of transfected cells. Owing to the suppressive effects of silibinin on the mRNA and enzymatic levels of uPA on human lung cancer cell A549 [17] and the cardinal roles of the uPA/uPAR system in promoting tumor invasion in human gliomas, we then determined whether silibinin treatment could inhibit the mRNA levels of both uPA/uPAR in U87 cells. Our results indicate that silibinin is capable of negatively regulating the transcriptional levels of uPA and its receptor in a highly invasive glioblastoma cell line.

Recently, it has been documented that low-density lipoprotein receptor-related protein 1 (LRP1), a multiligand receptor that interacts with uPAR, triggers migration and invasion in U87 cells through induction of MMP-2 and MMP-9 by the activation of ERK 1/2 [41]. With regard to the inhibitory effect of silibinin on MMP-9, we next evaluated whether silibinin treatment could suppress the mRNA levels of LRP1. We observed no inhibitory effect of silibinin on the promoter activity of LRP1 in U87 cells (data not shown).

An accumulated body of evidence indicates that overexpression of cathepsin B coupled with underexpression of its inhibitors, stefin A and cystatin C, plays a determinant role in tumor cell invasion and infiltration in glioblastoma [42–44]. In line with this, there is ample evidence that treatment of malignant glioblastoma cells with cysteine–cathepsin inhibitor E64 results in suppression of cathepsin B activity, thereby reducing invasiveness in treated cells [42,45]. Concordantly, antisense cathepsin B and sense cystatin C stable clones have been shown to express lower levels of cathepsin B mRNA and protein and are less invasive in the Matrigel invasion assay, and formed undetectable tumors in nude mice [44,46]. In conclusion, a recent study indicates a pivotal role for cathepsin B in activation of MMP-2 and MMP-9 in a pH-dependent manner [47]. In this setting, it has been shown that treatment of CABA I ovarian carcinoma cells with E-64 results in development of gelatinase-negative tumor-shed vescicle. Moreover, using a specific short hairpin RNA against cathepsin B has shown that vesicles derived from depleted-cathepsin B cells have significantly reduced aggressiveness because of lack of gelatinase activity [47]. Owing to the prominent roles of cathepsin B in tumor invasion and metastasis, drugs that suppress its expression and activity may prove effective in metastasis interruption. Our study indicates that silibinin might weaken invasive features of U87 glioblastoma cells by inhibiting both enzymatic activity and transcriptional levels of cathepsin B, which bring about a significant reduction in gelatinase B activity. Furthermore, silibinin treatment resulted in transcriptional stimulation of stefin A. In comparison, no inductionary effect of silibinin on mRNA levels of cystatin C, an extracellular inhibitor of cathepsin B, was observed in this study (data not shown). Owing to the restrictive effects of silibinin on transcriptional activation of uPAR, uPA, and MMP-9, and its inhibitory impacts on both mRNA levels and enzymatic activity of cathepsin B observed in this study, it might be deduced that silibinin is capable of diminishing invasive characteristics of U87 cells by disabling the fatal cascade of cathepsin B/uPA/MMP-9.

It has been suggested that the NF-κB signaling module is constitutively activated in human glioblastoma [9]. In addition, Chintala *et al.* [12] have found a determinant role for the transcription factor NF-κB in promoting tumor invasion in glioblastoma through induction of MMP-9. In line with these findings, suppression of NF-κB through the overexpression of its inhibitor IκB by an adenovirus expressing IκB super repressor in U87 cells resulted in attenuated invasive properties of transfectants [12]. Furthermore, treatment of these cells with TNF-α, which activates the NF-κB, culminated in enhanced invasion in treated cells [12]. Collectively, these studies indicate that NF-κB plays a crucial role in tumor invasion and infiltration in glioblastoma through induction of MMP-9. As the results of this study indicate that silibinin inhibits the transcriptional and enzymatic levels of MMP-9, we next evaluated whether the suppressive effects of silibinin on gelatinase B might be achieved by preempting the NF-κB module using a cell-based NF-κB activation assay. Our results indicate that silibinin significantly suppressed the phosphorylation of the p65 subunit of NF-κB. Sustained stimulation of NF-κB has been reported to rely on phosphorylation and degradation of inhibitor proteins by an inhibitor of the NF-κB kinase (IKK) complex, which is composed of two catalytic subunits, NF-κB inhibitor kinase alpha (IKK1) and NF-κB inhibitor kinase beta (IKK2), and a regulatory subunit termed Nemo [48]. In this regard, we next determined whether silibinin could reduce the NF-κB cascade through transcriptional repression of IKK1, IKK2, and Nemo. In contrast to our expectations, no inhibitory effects of silibinin on the mRNA levels of these genes were observed in our study (data not shown). As cell adhesion plays a major role in triggering

tumor invasion in glioblastoma [49] and respecting the NF-κB-mediated induction of ICAM-1 and vascular cell adhesion molecule 1 [8], we next determined the effect of silibinin on the mRNA levels of these genes. The suppressive effects of silibinin on invasive capabilities of U87 cells were associated with transcriptional regression of ICAM-1. In comparison, no suppressive effect of silibinin on the vascular cell adhesion molecule message was observed in this study (data not shown).

With regard to the interactions among the proteolytic pathways involved in malignant progression, development of therapeutic strategies aimed at targeting more than one type of protease has been shown to be more effective than hitting only one proteolytic cascade [50,51]. In glioblastoma, the main challenging therapeutic hurdle is tumor cell invasion and infiltration into the adjacent normal tissues, and in this regard targeting the ECM-degradative enzymes is of vital importance. Our results show for the first time that silibinin may weaken the invasive potential in a GBM cell line, U87MG, through suppression of NF-κB-mediated stimulation of MMP-9. In addition, the suppressive effects of silibinin on the enzymatic activity of cathepsin B might reduce the activation of MMP-9. Anti-invasive activities of silibinin in this study were associated with transcriptional repression of uPAR and uPA, suggesting the efficacy of silibinin in disrupting the proteolytic cascade of cathepsin B/uPA/MMP9 cascade.

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