

Laboratory Investigation

Inhibition of matrix degrading enzymes and invasion in human glioblastoma (U87MG) Cells by isoflavones

Shilpa Puli, James C.K. Lai and Alok Bhushan

Department of Pharmaceutical Sciences, College of Pharmacy and Biomedical Research Institute, Idaho State University, Pocatello, ID, 83209, USA

Key words: glioblastoma, invasion, isoflavones, MMPs, uPAR

Summary

Glioblastoma multiforme is a primary brain tumor associated with extensive invasion into surrounding brain tissue. Matrix metalloproteinases (MMPs) and urokinase plasminogen activation (uPA) system are shown to be involved in tumor invasion as they help in degradation of extracellular matrix (ECM) proteins and thus assist in the movement of cells. MMP-2 and 9 were shown to be upregulated in gliomas, suggesting their involvement in invasion. Genistein and biochanin A are isoflavones commonly known as phytoestrogens and have some anticancer properties. We hypothesize that these two isoflavones can induce a lowering of tumor invasion by decreasing the activity of matrix degrading enzymes. In this study we investigated the effects of genistein and biochanin A on invasive activity of U87MG cells using the Calbiochem *in vitro* invasion assay system. Our results suggest that genistein and biochanin A induced a decrease in invasive activity of U87MG cells in a dose-related manner. Genistein also induced a decrease in EGF-stimulated invasion thereby implicating an involvement of EGF-mediated signaling in invasion. Our results also show that treatment of U87MG cells with the two isoflavones induced decreases in the enzymatic activity of MMP-9 and the protein levels of MT1-MMP and uPAR.

Introduction

Glioblastoma multiforme is a malignant primary brain tumor occurring in elderly patients. The patients with glioblastoma usually have a short survival time, which is less than a year [1]. In spite of current therapies that include surgery, radiation therapy, and chemotherapy, glioblastomas are associated with poor prognosis [2]. The major problem associated with treatment of glioblastomas is that total surgical removal of tumor is difficult as the tumor invades into the surrounding brain tissue [3,4].

Invasion is a process in which tumor cells migrate from the tumor mass and infiltrate into the surrounding normal brain tissue by degrading the extracellular matrix (ECM) [5]. Extracellular matrix of brain is composed of hyaluronic acid, collagen, and fibrous proteins [6]. These ECM proteins are degraded by various matrix degrading enzymes.

Matrix metalloproteases (MMPs) are zinc dependent endopeptidases that are secreted in the zymogen or in an inactive form [7,8]. Studies employing immunohistochemical analysis have shown that high-grade invasive glioblastomas express MMPs, whereas low-grade, non-invasive astrocytomas and normal brain do not express MMPs [9]. MMP-2 (72 kDa) and MMP-9 (92 kDa) are highly expressed in malignant brain tumors [10,11]. The latter two enzymes are also known as gelatinases as they digest gelatin in the ECM [12]. *In vitro* studies indicate that inhibiting MMP-2 activity leads to the blockade of invasion whereas increasing its activity results in increased glioma invasiveness [13]. Membrane-type

MMPs are metalloproteases expressed on the surface of tumor cells. Membrane-type 1 MMP (MT1-MMP) is upregulated in glioblastomas and is also involved in the activation of MMPs at the cell surface [14,15]. The overexpression of MMP-2 and MMP-9 correlates with invasive behavior of many cancers including breast, cervical, pancreatic, and prostate cancer [16–18].

Overexpression of epidermal growth factor receptor (EGFR) is associated with poor prognosis in many cancers including glioblastomas [11]. Studies have shown that EGF and amphiregulin upregulate the MMP-9 expression in human breast cancer cells [19]. In ovarian cancer cells, EGF stimulates the production of both urokinase plasminogen activation (uPA) and MMP-9 [20]. Furthermore, PI3K mediates this EGFR-coupled signaling to regulate MMP-9 [21]. In human squamous cell carcinomas, blockade of EGF induces the inhibition of angiogenesis and metastasis by inhibiting MMPs [22]. Non-small cell lung carcinoma (NSCLC) tumors have been shown to co-express MMP-9 and EGFR [23]. Various tyrosine kinase inhibitors inhibit migration and matrix metalloproteinases in malignant mesothelioma cells [24]. All these studies suggest that EGFR may play a role in invasive behavior by regulating MMPs.

Urokinase plasminogen Activator (uPA) is a serine protease that binds to urokinase Plasminogen Activator Receptor (uPAR), thereby converting the inactive plasminogen to the active plasmin. The active plasmin brings about the lysis of various ECM components resulting in cell migration [25]. uPAR is linked to plasma membrane by a glycoposphatidylinositol (GPI) linked

anchor and it lacks cytoplasmic as well as intracellular domain: thus, uPAR is thought to form multiprotein complex with several other proteins in order to mediate the signaling. Previous studies have shown that uPAR associates with integrin and signals to ERK which is disrupted by using integrin neutralizing antibodies. Previous studies have shown that cells expressing high levels of uPAR contain a complex that includes uPAR, EGFR, and $\alpha_5\beta_1$ [26]. uPA and uPAR are important in various physiological processes requiring cell migration, including inflammation, tissue repair, embryonic development, and cancer invasion and metastasis [27]. Elevated uPAR levels also correlate with tumor invasiveness in glioblastoma cells. Moreover, inhibition of uPAR expression by antisense oligonucleotides in glioblastoma cells results in inhibition of tumor formation in nude mice [28,29].

Epidemiological studies have shown that Asian population consuming diets rich in isoflavones have lower incidences of cancers of breast, prostate, and colon [30]. Genistein (4',5,7-trihydroxyisoflavone) and biochanin A (4'-methoxy, 5,7-dihydroxy isoflavone) are natural isoflavonoid phytoestrogens found in soy and subterranean clover, respectively [31]. Genistein exerts its anticancer properties via several mechanisms, including inhibition of tyrosine phosphorylation, weak estrogenic and antiestrogenic properties, as an antioxidant, inhibition of topoisomerase II, inhibition of angiogenesis, and induction of cell differentiation in breast cancer cells [32–34].

Most of the research using isoflavones has been carried out in hormone-dependent cancers; however, the effects of isoflavones on glioblastomas have not been fully elucidated. Thus, one goal of our studies was to investigate the utility of isoflavones to block glioblastoma invasion because genistein, an isoflavone present in soy, is a known protein tyrosine kinase inhibitor and competes with ATP for binding to the tyrosine kinase domain and thereby inhibits tyrosine kinase-mediated signaling [35]. Furthermore, genistein is known to inhibit EGF-stimulated phosphorylation in cultured A431 cells [36]. Studies have also shown that genistein inhibits glioblastoma invasion in an *in vitro* co-culture model by inhibiting EGFR tyrosine kinase activity [37,38]. However, it is not known whether biochanin A, another isoflavone, can block glioblastoma invasion. It has been reported that biochanin A has inhibitory potential on the development of lung tumors induced in mice by benzo(a)pyrene [39]. Both biochanin A and genistein have been shown to inhibit both serum and EGF stimulated growth of human prostate cancer cells [40,41]. Biochanin A has also been shown to inhibit the incidence and growth of LNCaP xenograft tumors in athymic mice [42]. We therefore hypothesize that biochanin A, an analog of genistein, can also inhibit glioblastoma invasion due to its structural similarity with genistein (Figure 1).

The studies described herein focused on elucidating the effects of biochanin A and genistein on the invasive properties of U87MG cells using a Calbiochem invasion assay system. We also determined the effects of both isoflavones on gelatinolytic activity of MMP-2 and

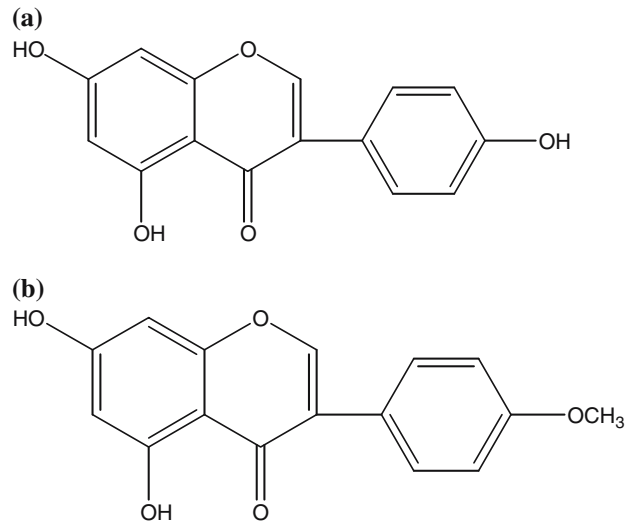


Figure 1. Chemical structures of the isoflavones, genistein (a) and biochanin A (b).

MMP-9 and the expression of MT1-MMP and uPAR proteins in U87MG cells.

Materials and methods

Materials

Genistein and biochanin A were obtained from LC laboratories (Woburn, MA). MT1-MMP and uPAR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Zymogram gels were obtained from BioRad (Hercules, CA). Invasion assay kits were obtained from Oncogene Research Products (San Diego, CA). The electrophoresis reagents were purchased from Boston Bioproducts (Ashland, MA), and Sigma Chemicals (St. Louis, MO).

Cell culture

Human glioblastoma cell line (U87MG) was purchased from American Type Culture Collection (Manassas, VA) and were maintained in Dulbeccos Modified Eagles Medium from Sigma chemicals (St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum from Atlanta Biologicals (Norcross, GA) at 37 °C in a humidified atmosphere containing 5% CO₂ in a Nuair tissue culture incubator (Plymouth, MN)

Western blot analysis

U87MG cells were treated with genistein and biochanin A at various concentrations for 72 h and cell lysates prepared using a lysis buffer (containing 1% (v/v) Triton X-100, 10 mM Tris base pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% (w/v) sodium azide, 0.5 μ M phenyl methyl sulfonyl fluoride, 0.2 μ g aprotinin, 0.4 μ g leupeptin, 100 μ g sodium orthovanadate, and distilled water at pH 7.6). Protein concentration was determined using BioRad reagents with photometric analysis. Twenty five

microgram of cell lysate proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred on to polyvinylidene fluoride membrane (PVDF) (Millipore, Bedford, MA). The membranes were incubated with primary antibody at 1:500 dilution in 5% (w/v) bovine serum albumin (BSA) and distilled water. The membrane was then treated with horseradish peroxidase-linked secondary antibody at 1:5000 dilution in Tris buffered saline with Tween (TBST) solution for 45 min at room temperature. The blots were then developed by using chemiluminescence reagents (Pierce biotechnology, Rockford, Illinois), according to the manufacturer's protocol and autoradiographed. The blots were scanned to determine the relative intensities of the bands using UNSCAN-IT software (Orem, UT). Western blot analysis using an antibody to β -Actin (SantaCruz biotechnology, CA) was carried out and its relative intensity was determined by UNSCAN-IT to normalize the intensities of the bands.

Zymography

U87MG cells were treated with various concentrations of genistein and biochanin A. The serum free conditioned media was collected after 72 h after treatment and centrifuged to remove the cell debris. The samples were electrophoresed on commercially available 10% (w/v) SDS polyacrylamide gels embedded with gelatin that were obtained from BioRad (Hercules, CA). After electrophoresis, the gels were washed with 2.5% (v/v) Triton X-100 solution for 1 h to remove SDS. Gels were then incubated over night at 37 °C in solution containing 50 M Tris base, 200 M NaCl, 5 M CaCl₂, and 0.02% (w/v) Brij-35 at pH 7.6. The gels were stained with Coomassie blue solution for 1 h and then destained using a solution containing 40% (v/v) methanol/10% (v/v) acetic acid/50% (v/v) water. Gelatinolytic activity was identified as a white band on blue background. The 72 kDa band corresponds to MMP-2 and 92 kDa band corresponds to MMP-9.

Invasion assay

Invasion studies were carried out using Calbiochem invasion assay as recommended by the commercial vendor (Calbiochem, San Diego, CA). A brief description is given below; 300 μ l of suspension of U87MG cells at a concentration of $0.5\text{--}1.0 \times 10^6$ cells/ml were suspended in serum free media along with the drug and this solution was added to the upper compartment of the cell invasion chamber (Oncogene research products, San Diego). 500 μ l of DMEM with 10% (v/v) fetal bovine serum was added to the lower compartment of the cell invasion chamber and incubated for 48 h in the tissue culture incubator. The culture insert was placed in wells containing cell detachment solution supplied by the manufacturer and incubated for 30 min. The culture insert was removed and then the cells that detached from the bottom of the insert were fluorescently labeled by incubating in calcein-AM solution provided by the manufacturer for about an hour and then fluorescence was measured at excitation wavelength 485 nm and emission wavelength of 520 nm. The amount of

fluorescence intensity is directly proportional to the number of cells that invade through the basement membrane. Equal volumes of the vehicle were loaded in all the treatment groups to ensure that vehicle to media ratio was around 1:1000.

Statistical analysis

SPSS software has been used for statistical analysis. One way ANOVA along with post-hoc Tukey test has been employed to analyze the difference between groups. The significance level has been set at ≤ 0.05 .

Results

Isoflavone treatment decreased invasion in U87MG cells

We used Calbiochem invasion assay to study the effect of isoflavones on the invasive activity of U87MG cells. Cells were treated with isoflavones, genistein and biochanin A, and allowed to invade through the filter coated with ECM for 48 h. Figure 2a shows the dose dependent effect on invasion on treatment with increasing concentration of genistein. Figure 2b shows a dose dependent effect on invasion on treatment with increasing concentrations of biochanin A. The results indicate that treatment with both isoflavones induced a dose dependent decrease in invasive activity of U87MG cells. Previous studies have shown that genistein blocks invasion in a co-culture model: results of this study confirmed that genistein blocks invasion of U87MG cells. Moreover, results of this study demonstrate that biochanin A, similar to genistein, also blocks invasion of U87MG cells.

Genistein treatment decreased EGF stimulated invasion in U87MG cells

The invasion assay was employed to examine the effect of genistein on EGF stimulated invasion of U87MG cells. Figure 2c shows that treatment of U87MG cells with EGF at 10 ng/ml stimulated invasion through the matrix and that this invasion is blocked on treatment with genistein at 10 μ M. Although not statistically significant these results are important as previous studies have shown that blocking EGFR with EGFR antibody blocked invasion of tumor cells [30] and addition of EGF ligand externally increased invasion of tumor cells, thereby implicating the role for EGFR in invasion [37].

Isoflavone treatment decreased MMP gelatinolytic activity in U87MG cells

MMP-2 and -9 are matrix degrading enzymes shown to be upregulated in high-grade invasive gliomas [9]. Gelatin zymography was performed to examine the effect of isoflavones on MMP-2 and MMP-9 gelatinolytic activity. Figure 3a shows that treatment of these U87MG cells with genistein induced a dose dependent decrease in the gelatinolytic activity of MMP-2 and MMP-9 in these cells. Figure 3b shows that treatment of these cells with biochanin A also induced a dose dependent decrease in

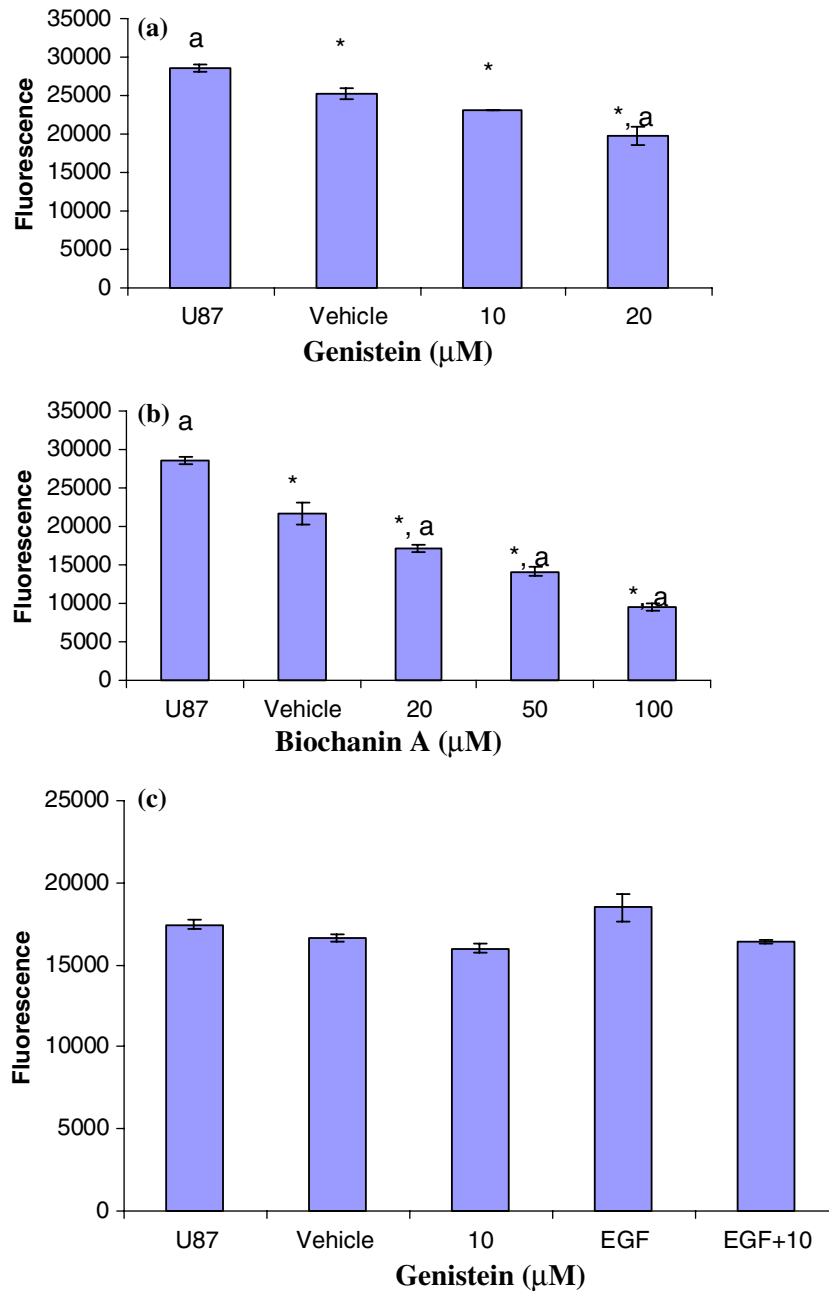


Figure 2. Graph showing the invasion of U87MG cells through the inserts using Calbiochem *in vitro* invasion assay. The U87MG cells were treated with genistein and biochanin A for 72 h and migration across the filter was assessed by fluorescent labeling of invaded cells. The relative fluorescence units are plotted in the graph. (a) Shows the effect of genistein on cell invasion. (b) Shows the effect of biochanin A on cell invasion. (c) Shows the effect of genistein (10 μM) on EGF (10 ng/ml) stimulated invasion. Values are means ± SEM and those marked with * represents statistically significant difference from U87MG cells with no treatment and those marked with 'a' represents statistically significant difference from cells treated with vehicle: $P \leq 0.05$.

the gelatinolytic activity of MMP-2 and MMP-9. We carried out these studies with highest concentration of genistein at 20 μM and biochanin A up to 50 μM in order to avoid a decrease in cell density due to cytotoxicity of these drugs at concentrations higher than that.

Isoflavones treatment decreased MT1-MMP protein levels in U87MG cells

MT1-MMP is a metalloproteinase expressed on cell surface is associated with activation of other metalloproteinases, and is shown to be up regulated in glioblastomas

[14]. Western blot analysis was therefore performed to examine the effect of isoflavones on MT1-MMP protein levels in U87MG cells. Figure 4a shows that treatment of U87MG cells with genistein induced a dose dependent decrease in MT1-MMP levels. Similarly, treatment of these cells with biochanin A also induced a dose related decrease in MT1-MMP levels in these cells (Figure 4b).

Isoflavone treatment decreased uPAR protein levels in U87MG cells

uPAR is a GPI linked receptor with a ligand binding domain for uPA and converts plasminogen to plasmin,

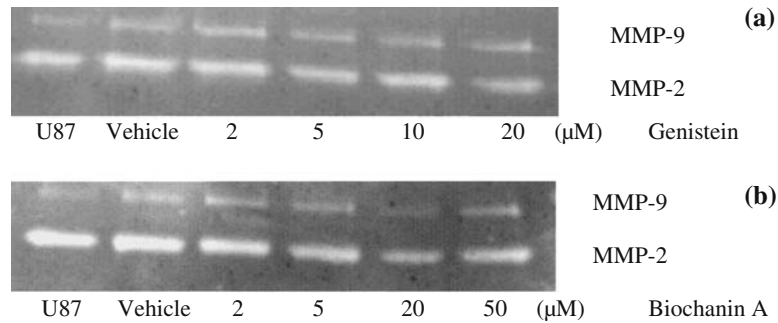


Figure 3. Gelatin zymographic analysis of MMP expression in U87MG cells after treatment with isoflavones. Conditioned medium was collected from U87MG cells after treatment with genistein and biochanin A and was run on gelatin zymography. (a) Shows the effect of increasing concentrations of genistein on MMP-2 and -9 activities. (b) Shows effect of increasing concentrations of biochanin A on MMP-2 and -9 activities.

which has ECM degrading properties. It has been shown that uPA binding to uPAR results in the expression of cathepsin B and MMP-9 in monocytic cells showing the role of uPAR in ECM degradation and migration of cells [43].

Western blot analysis was performed to examine the effect of isoflavones on uPAR protein levels in U87MG cells. Figure 5a shows the effect of genistein treatment on uPAR protein levels in U87MG cells and Figure 5b shows the effect of biochanin A treatment on uPAR protein levels. Our results indicate that treatment of U87MG cells with biochanin A induced the uPAR protein levels in U87MG cells but genistein treatment had no effect on uPAR protein levels in these cells.

Discussion

Glioblastoma multiforme is the most malignant, infiltrative tumor that invades into surrounding normal brain tissue. These tumors rarely metastasize out of the central nervous system, but their invasion away from the tumor mass makes it difficult for surgical resection of the tumor and for the treatment of these tumors [44]. Extensive research has been carried out in recent years to study the mechanisms involved in invasion and the

effect of agents thereon. Association of soy with decreased incidence of hormone dependent and independent cancers has led to the extensive research over two decades on isoflavones and their effect on cancer. These isoflavones have been implicated in cancer prevention based on some epidemiological studies as well as studies carried out in cell culture and in animal models [34,45–48]. Few studies have investigated the effect of isoflavones on gliomas, although Penar et al. have shown that genistein inhibits glioma invasion in an *in vitro* model [37,38]. Our study helps to elucidate the mechanism of inhibition of invasion by genistein as well as the effect of another isoflavone, biochanin A on glioma invasion. We hypothesize that biochanin A, similar to genistein, has the ability to inhibit invasion and that both genistein and biochanin A inhibit invasion by inhibiting matrix degrading enzymes.

Invasion of tumor cells into surrounding normal tissue involves interaction of tumor cells with ECM and surrounding cells, and their ability to secrete matrix degrading proteases. Several models have been used to study invasion, and most widely used ones being organotypic co-culture models and matrigel invasion assays [49–52]. We have used Calbiochem *in vitro* invasion assay that is similar to the matrigel invasion assay with wells containing filters coated with basement membrane

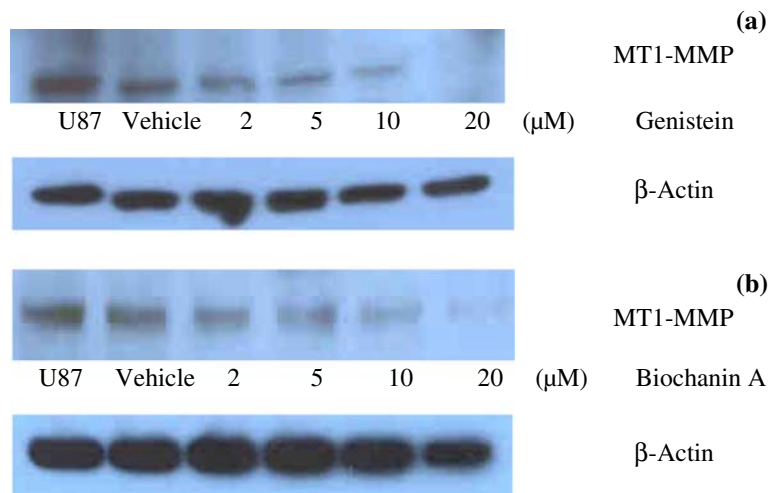


Figure 4. Western blot analysis of MT1-MMP expression after treatment with isoflavones. Western blot analysis was carried out on the cell lysates made from U87MG cells after treatment with isoflavones using a antibody to MT1-MMP. (a) Shows the effect of increasing concentrations of genistein on MT1-MMP protein levels. (b) Shows the effect of increasing concentrations of biochanin A on MT1-MMP protein levels.

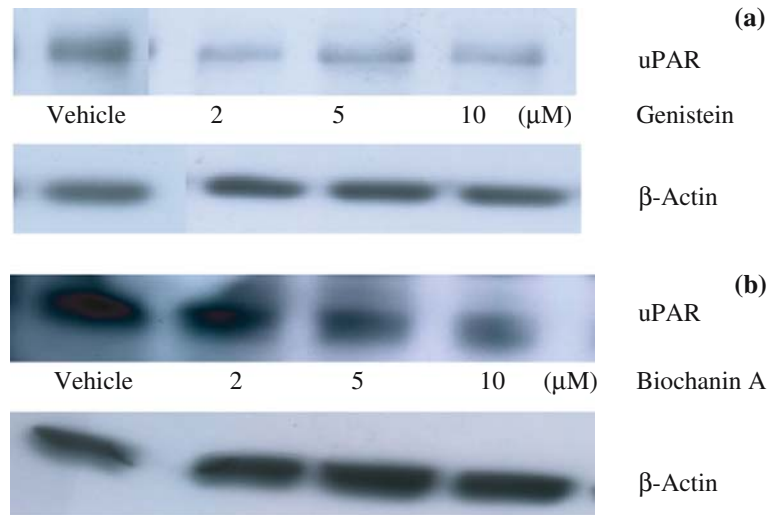


Figure 5. Western blots analysis of uPAR expression with isoflavones. Western blot analysis using a antibody to uPAR (Santa Cruz Biotechnology, CA) was carried out on the cell lysates prepared from U87MG cells, after treatment with isoflavones using a antibody to uPAR. (a) Shows the effect of increasing concentrations of genistein on uPAR protein levels. (b) Shows the effect of increasing concentrations of biochanin A on uPAR protein levels. The graphical representation of the blot is done by measuring the optical density of the MT1-MMP protein and β -actin protein (Santa Cruz Biotechnology, CA).

matrix. The advantage of this model over that of matrigel invasion assay is that this assay is quantitative and uses fluorescent labeling of cells for quantification.

Penar et al. have shown the effect of genistein at 10 μ M on glioma invasion in an *in vitro* co-culture model [37]. In addition, our results show a dose dependent decrease in glioma invasion with genistein confirming the results shown by Penar et al. using a different model (Figure 2a). In addition, our results show a dose dependent decrease in invasion when glioma cells were treated with biochanin A at or above 20 μ M (Figure 2b).

Previous studies have shown that addition of EGF stimulates the tumor invasiveness and this could be blocked by addition of antibody to EGF ligand binding domain [53,54]. Genistein is a known tyrosine kinase inhibitor and has been shown to inhibit invasion but till now none of the studies have shown the effect of genistein on EGF stimulated invasion [36]. Our goal was to examine the effect of genistein on EGF stimulated invasion. Our results show that addition of EGF at 10 ng/ml stimulates the invasion of U87MG cells slightly and this invasion is blocked on addition of genistein in combination with EGF showing that genistein blocks invasion that is mediated by EGF stimulated signaling (Figure 2c). It is known that genistein is a tyrosine kinase inhibitor and our results here indicate that genistein may block EGF stimulated invasion by inhibiting tyrosine phosphorylation.

The invasive phenomenon of gliomas depends on the combined actions of several proteolytic enzyme systems. Matrix metalloproteinases and plasminogen activation cascade are the two well known proteolytic systems under investigation [55]. Previous studies have shown that MMP-2 and MMP-9 are upregulated in gliomas and these enzymes have also been associated with malignancy of the gliomas [12,13]. Using gelatin zymography (Figure 3a and b), we demonstrated that

treatment of U87MG cells with increasing concentrations of genistein and biochanin A induced a concentration related decrease in active MMP-2 and MMP-9 levels. Our results suggest one of the mechanisms whereby isoflavones block the invasive capacity of U87MG cells may be through the inhibition of MMP activity.

MMP-2, the most abundant among all MMPs, is secreted in zymogen form that requires activation. This activation is an important event in the regulation of ECM degradation by MMPs. MMP-2 is activated by mechanisms involving membrane type-1 MMP (MT1-MMP) associated with cell surface [56]. Our results show that when U87MG cells were treated with increasing concentration of isoflavones, they expressed a dose related decrease in MT1-MMP levels (Figure 4a and b). These dose related decreases in MT1-MMP protein levels correlated with the isoflavone induced decreases in MMP-2 levels (Figure 3a and b). Increased expression and activation of laminin 5 γ 2, a basement membrane protein, has been associated with invasiveness of malignant cancers [57]. We also observed a decrease in laminin 5 γ 2 protein levels and its fragmentation into γ 2 and γ 2' proteins in U87MG cells treated with isoflavones (data not shown). Regulation of proteolysis of MMPs by MT1-MMP can act as an additional target for inhibition of tumor cell invasion. Thus, our results indicate that treatment with isoflavones may induce a decrease in the activity of MT1-MMP present on the membrane, thereby decreasing the matrix degrading activity at the leading edge of the cell [12].

The production of uPAR protein is high in high grade gliomas compared to that in low grade gliomas. uPA and uPAR expression are shown to be increased in human glioblastoma cells *in vivo* which show increased invasiveness into the surrounding brain tissue. Increased expression or upregulation of plasminogen activation system correlates with invasiveness of tumors [58–60].

We have examined the effects of isoflavones on uPAR protein levels in U87MG cells and our results show that treatment with biochanin A, but not with genistein, induced decreases in uPAR protein levels considerably in these cells (Figure 5a and b).

Understanding the mechanisms of tumor invasion are important in developing new therapeutic strategies. Elucidation of the effect of dietary agents such as soy isoflavones is important in identifying not only new therapeutic agents, but also new targets in cancer cells and in developing new cancer prevention techniques.

Acknowledgements

Our work was supported in part, by grants from Faculty Research Committee grant # 937, and University Research Committee grant # FY2002-09 at Idaho State University, and NIH/NCRR INBRE grant # P20RR16454.

References

- Central Brain Tumor Registry of the United States. Statistical report: primary brain tumors in the United States, 1995–1999. CBTRUS; 2002–2003
- Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, Frei E: Cancer Medicine 6. BC Decker, New York, 2003, pp 1195–1231
- Jeffrey Bruce: Glioblastoma Multiforme. eMedicine February, 2005
- Tremont-Lukats IW, Gilbert MR: Advances in molecular therapies in patients with brain tumors. *Cancer Control* 10(2): 125–37, 2003
- Bello L, Giussani C, Carrabba G, Pluderi M, Costa F, Bikfalvi A: Angiogenesis and invasion in gliomas. *Cancer Treat Res* 117: 263–284, 2004
- Binder DK, Berger MS: Proteases and the biology of glioma invasion. *J Neurooncol* 56(2): 149–158, 2002
- Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 92(8): 827–839, 2003
- Vihinen P, Kahari VM: Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *Int J Cancer* 99(2): 157–166, 2002
- Kachra Z, Beaulieu E, Delbecchi L, Mousseau N, Berthelet F, Moudjian R, Del Maestro R, Beliveau R: Expression of matrix metalloproteinases and their inhibitors in human brain tumors. *Clin Exp Metastasis* 17(7): 555–566, 1999
- Deryugina EI, Bourdon MA, Luo GX, Reisfeld RA, Strongin A: Matrix metalloproteinase-2 activation modulates glioma cell migration. *J Cell Sci* 110(19): 2473–2482, 1997
- Choe G, Park JK, Jouben-Steele L, Kremen TJ, Liao LM, Vinters HV, Cloughesy TF, Mischel PS: Active matrix metalloproteinase 9 expression is associated with primary glioblastoma subtype. *Clin Cancer Res* 8(9): 2894–2901, 2002
- Lampert K, Machein U, Machein MR, Conca W, Peter HH, Volk B: Expression of matrix metalloproteinases and their tissue inhibitors in human brain tumors. *Am J Pathol* 153(2): 429–437, 1998
- Abe T, Mori T, Kohno K, Seiki M, Hayakawa T, Welgus HG, Hori S, Kuwano M: Expression of 72 kDa type IV collagenase and invasion activity of human glioma cells. *Clin Exp Metastasis* 12(4): 296–304, 1994
- Fillmore HL, VanMeter TE, Broaddus WC: Membrane-type matrix metalloproteinases (MT-MMPs): expression and function during glioma invasion. *J Neurooncol* 53(2): 187–202, 2001
- Hess AR, Seftor EA, Seftor RE, Hendrix MJ: Phosphoinositide 3-kinase regulates membrane Type 1-matrix metalloproteinase (MMP) and MMP-2 activity during melanoma cell vasculogenic mimicry. *Cancer Res* 63(16): 4757–4762, 2003
- Ouyang YW, Peng ZL, Yao XY, Liu SL, He YD: The expression of matrix metalloproteinase-2 and -9 in cervical cancer and a study of their relationship. *Sichuan Da Xue Xue Bao Yi Xue Ban* 35(3): 330–333, 2004
- Gurevich LE: Role of matrix metalloproteinases 2 and 9 in determination of invasive potential of pancreatic tumors. *Bull Exp Biol Med* 136(5): 494–498, 2003
- Fan SQ, Wei QY, Li MR, Zhang LQ, Liang QC: Expression and clinical significance of MMP-2, MMP-9, TIMP-1, and TIMP-2 in breast carcinoma. *Ai Zheng* 22(9): 968–973, 2003
- Kondapaka SB, Fridman R, Reddy KB: Epidermal Growth Factor and Amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. *Int J Cancer* 17(6): 722–726, 1997
- Ellerbroek SM, Hudson LG, Stack MS: Proteinase requirements of epidermal growth factor induced ovarian cancer cell invasion. *Int J Cancer* 78(3): 331–337, 1998
- Ellerbroek SM, Halbleib JM, Benavidez M, Warmka JK, Wattenberg EV, Stack MS, Hudson LG: Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association. *Cancer Res* 61(1): 1855–1861, 2001
- Huang SM, Li J, Harari PM: Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. *Mol Cancer Ther* 1(7): 507–514, 2002
- Cox G, Jones JL, O'Byrne KJ: Matrix metalloproteinase 9 and the epidermal growth factor signal pathway in operable non-small cell lung cancer. *Clin Cancer Res* 6(6): 2349–2355, 2000
- Liu Z, Klominek J: Inhibition of proliferation, migration, and matrix metalloprotease production in malignant mesothelioma cells by tyrosine kinase inhibitors. *Neoplasia* 6(6): 705–712, 2004
- Andreasen PA, Kjoller L, Christensen L, Duffy MJ: The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 72(1): 1–22, 1997
- Liu D, Aguirre Ghiso J, Estrada Y, Ossowski L: EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell* 1(5): 445–457, 2002
- Aguirre Ghiso JA, Alonso DF, Farias EF, Gomez DE, de Kier Joffe EB: Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur J Biochem* 263(2): 295–304, 1999
- Mohanam S, Gladson CL, Rao CN, Rao JS: Biological significance of the expression of urokinase-type plasminogen activator receptors (uPARs) in brain tumors. *Front Biosci* 15(4): D178–D187, 1999
- Mohanam S, Chintala SK, Go Y, Bhattacharya A, Venkaiah B, Boyd D, Gokaslan ZL, Sawaya R, Rao JS: In vitro inhibition of human glioblastoma cell line invasiveness by antisense uPA receptor. *Oncogene* 14(11): 1351–1359, 1997
- Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE: Dietary effects on breast-cancer risk in Singapore. *Lancet* 337(8751): 1197–1200, 1991
- Persky V, Van Horn L: Epidemiology of soy and cancer: perspectives and directions. *J Nutr* 125(3): 709S–712S, 1995
- Barnes S, Peterson TG: Biochemical targets of the isoflavone genistein in tumor cell lines. *Proc Soc Exp Biol Med* 208(1): 103–108, 1995
- Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L: Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci USA* 90(7): 2690–2694, 1993
- Messina MJ, Persky V, Setchell KD, Barnes S: Soy intake and cancer risk: a review of the in vitro and in vivo data. *Nutr Cancer* 21(2): 113–131, 1994
- Chen WF, Huang MH, Tzang CH, Yang M, Wong MS: Inhibitory actions of genistein in human breast cancer (MCF-7) cells. *Biochim Biophys Acta* 1638(2): 187–196, 2003

36. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y: Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 262(12): 5592–5595, 1987
37. Penar PL, Khoshyomn S, Bhushan A, Tritton TR: Inhibition of epidermal growth factor receptor-associated tyrosine kinase blocks glioblastoma invasion of the brain. *Neurosurgery* 40(1): 141–151, 1997
38. Penar PL, Khoshyomn S, Bhushan A, Tritton TR: Inhibition of glioma invasion of fetal brain aggregates. *In Vivo* 12(1): 75–84, 1998
39. Lee YS, Seo JS, Chung HT, Jang JJ: Inhibitory effects of biochanin A on mouse lung tumor induced by benzo(a)pyrene. *J Korean Med Sci* 6(4): 325–328, 1991
40. Peterson G, Barnes S: Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* 22(4): 335–345, 1993
41. Hempstock J, Kavanagh JP, George NJ: Growth inhibition of prostate cell lines in vitro by phyto-oestrogens. *Br J Urol* 82(4): 560–563, 1998
42. Rice L, Samedí VG, Medrano TA, Sweeney CA, Baker HV, Stenstrom A, Furman J, Shiverick KT: Mechanisms of the growth inhibitory effects of the isoflavonoid biochanin A on LNCaP cells and xenografts. *Prostate* 52(3): 201–212, 2002
43. Rao NK, Shi GP, Chapman HA: Urokinase receptor is a multi-functional protein: influence of receptor occupancy on macrophage gene expression. *J Clin Invest* 96(1): 465–474, 1995
44. Laerum OD, Bjerkvig R, Steinsvag SK, de Ridder L: Invasiveness of primary brain tumors. *Cancer Metastasis Rev* 3(3): 223–236, 1984
45. Yamamoto S, Sobue T, Kobayashi M, Sasaki S, Tsugane S: Japan Public Health Center-Based Prospective Study on Cancer Cardiovascular Diseases Group. Soy, isoflavones, and breast cancer risk in Japan. *J Natl Cancer Inst* 95(12): 906–913, 2003
46. Wu AH, Ziegler RG, Nomura AM, West DW, Kolonel LN, Horn-Ross PL, Hoover RN, Pike MC: Soy intake and risk of breast cancer in Asians and Asian Americans. *Am J Clin Nutr* 68(6): 1437S–1443S, 1998
47. Wu AH, Ziegler RG, Horn-Ross PL, Nomura AM, West DW, Kolonel LN, Rosenthal JF, Hoover RN, Pike MC: Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol Biomarkers Prev* 5(11): 901–906, 1996
48. Zheng W, Dai Q, Custer LJ, Shu XO, Wen WQ, Jin F, Franke AA: Urinary excretion of isoflavonoids and the risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* 8(1): 35–40, 1999
49. Mikkelsen T, Bjerkvig R, Laerum OD, Rosenblum ML: *Brain Tumor Invasion: Biological, Clinical, and Therapeutic Considerations*. Wiley-Liss, 1998
50. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR: Basement membrane complexes with biological activity. *Biochemistry* 25(2): 312–318, 1986
51. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN: A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 47(12): 3239–3245, 1987
52. Bjerkvig R, Tonnesen A, Laerum OD, Backlund EO: Multicellular tumor spheroids from human gliomas maintained in organ culture. *J Neurosurg* 72(3): 463–475, 1990
53. Lund-Johansen M, Bjerkvig R, Humphrey PA, Bigner SH, Bigner DD, Laerum OD: Effect of epidermal growth factor on glioma cell growth, migration, and invasion in vitro. *Cancer Res* 50(18): 6039–6044, 1990
54. Lund-Johansen M, Forsberg K, Bjerkvig R, Laerum OD: Effects of growth factors on a human glioma cell line during invasion into rat brain aggregates in culture. *Acta Neuropathol* 84(2): 190–197, 1992
55. Yamamoto M, Ueno Y, Hayashi S, Fukushima T: The role of proteolysis in tumor invasiveness in glioblastoma and metastatic brain tumors. *Anticancer Res* 22(6C): 4265–4268, 2002
56. Nuttall RK, Pennington CJ, Taplin J, Wheal A, Yong VW, Forsyth PA, Edwards DR: Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells. *Mol Cancer Res* 1(5): 333–345, 2003
57. Guo P, Imanishi Y, Cackowski FC, Jarzynka MJ, Tao HQ, Nishikawa R, Hirose T, Hu B, Cheng SY: Up-regulation of angiopoietin-2, matrix metalloproteinase-2, membrane type 1 metalloproteinase, and laminin 5 gamma 2 correlates with the invasiveness of human glioma. *Am J Pathol* 166(3): 877–890, 2005
58. Lakka SS, Gondi CS, Yanamandra N, Dinh DH, Olivero WC, Gujrati M, Rao JS: Synergistic down-regulation of urokinase plasminogen activator receptor and matrix metalloproteinase-9 in SNB19 glioblastoma cells efficiently inhibits glioma cell invasion, angiogenesis, and tumor growth. *Cancer Res* 63(10): 2454–2461, 2003
59. Tsatas D, Kaye AH: The role of the plasminogen activation cascade in glioma cell invasion: a review. *J Clin Neurosci* 10(2): 139–145, 2003
60. Bhattacharya A, Lakka SS, Mohanam S, Boyd D, Rao JS: Regulation of the urokinase-type plasminogen activator receptor gene in different grades of human glioma cell lines. *Clin Cancer Res* 7(2): 267–276, 2001

Address for offprints: Alok Bhushan, College of Pharmacy, Idaho State University, Pocatello, ID, 83209, USA; Tel.: +1-208-282-4408; E-mail: abhushan@otc.isu.edu