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Original Research

Silibinin Inhibits Cell Invasion through the Inhibition of MMPs, *p*-p38, and IL-1β in Human Fibrosarcoma Cells

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Abstract

Background and Aims: Normal cells become tumorigenic owing to mutations in oncogenes and tumor suppressor genes modulating cell division. Cancer cells break down extracellular matrix to metastasize other tissues. Therefore, the development of natural and synthetic substances that suppress metastatic enzymes such as matrix metalloproteinase (MMP)-2 and MMP-9 is useful to inhibit metastasis. Silibinin is the main ingredient of silymarin extracted from the seeds of milk thistle plants having lung cancer-suppressing effects and liver protection. The purpose of this study was to investigate the inhibitory effect of silibinin on the invasion of human fibrosarcoma cells. **Methods**: The effect of silibinin on cell viability was measured in HT1080 cells using an MTT assay. The MMP-9 and MMP-2 activities were analyzed using a zymography assay. The expression of proteins in cytoplasm related to metastasis was examined by western blot analysis and immunofluorescence assay. **Results**: In this study, silibinin above 20 μM showed growth inhibitory effects. Silibinin above 20 μM remarkably inhibited the levels of MMP-2 and MMP-9 activation under phorbol myristate acetate (PMA) treatment conditions. Furthermore, silibinin at 25 μM reduced the levels of MMP-2, IL-1β, ERK-1/2, and *p*-p38 expression and silibinin above 10 μM inhibited cell invasion on HT1080 cells. **Conclusions**: These findings indicate that silibinin may have an inhibitory effect on the enzymes involved in invasion, hence it might influence the metastatic ability of tumor cells.

Keywords: silibinin; invasion; IL-1β; *p*-p38; MMPs

1. Introduction

Cancer cells that develop as a result of chronic inflammation move to peripheral tissues via blood vessels through angiogenesis. To metastasize to surrounding tissues, cancer cells need to degrade the extracellular matrix. In particular, the gelatinases such as matrix metalloproteinase (MMP)-2 and MMP-9 among MMPs degrade collagen 4, the main component of the basement membrane [1], involved in angiogenesis and metastasis. Therefore, it is crucial to regulate the expression of MMPs to limit the metastatic ability of cancer cells. Therefore, treatment of tumor cells with a substance that inhibits the expression of those inflammatory cytokines involved in MMPs regulation is also expected to limit metastasis formation.

While screening medicinal plants for anti-metastasis research, it was found that the inhibitory effect of milk thistle (*Cirsium japonicum*) extract was excellent. The silibinin employed in this study corresponds to 50–70% of the three isomers of silymarin which makes up roughly 2% of milk thistle's active component [2]. It has been known to have a role in the anti-tumor drug cisplatin's hepatoprotection, antioxidation, anti-angiogenesis, inhibition of inflammation, and nephrotoxicity [3]. The action mechanism of silibinin on metastasis remains unclear. The great efficacy of silibinin to target cancer cells' migratory and invasive features as well as their capacity to metastasize to distant organs has also been demonstrated in recent pre-clinical trials. According to thorough mechanistic investigations, silibinin targets signaling molecules that control the epithelial-to-mesenchymal transition (EMT), activation of proteases, adhesion, motility, and invasiveness as well as the components of the supporting tumor microenvironment, preventing metastasis [4]. Therefore, we tried to investigate whether silibinin could inhibit cell invasion and MMPs in the model of human fibrosarcoma cells (HT1080 cell line) widely used for the study of metastasis. Moreover, the expression of proteins such as MAPKs and IL-1β related to invasion and metastasis was examined to clarify the mechanism by which silibinin inhibits metastasis.

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA, and antibiotics such as penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL)/amphotericin (2500 μg/mL) reagents for cell culture were obtained from Life Technologies (Paisley, Scotland). MTT reagent, gelatin, agarose, RIPA lysis buffer and other reagents are obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Silibinin samples were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Cell Line and Culture

HT1080 cell line (ATCC No.CCL-12, Homo sapiens, fibroblast, lung) and IMR90 cell line (ATCC No.CCL-186, Homo sapiens, fibroblast) purchased from ATCC (American Type Culture Collection) were cultured using DMEM containing 10% of FBS and subcultured with trypsin-EDTA. Antibiotics such as penicillin/amphotericin/streptomycin were used to prevent cell culture from bacterial contamination. Mycoplasma testing was performed to authenticate the cell lines used in this study. This was accomplished using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Bend, OR, USA), which detects enzymatic activity associated with viable mycoplasma in cell cultures. Briefly, cells were harvested and lysed, and the resulting lysate was incubated with the MycoAlert™ substrate for 10 minutes at 37 °C. The fluorescence of each sample was then measured to check mycoplasma contamination. The silibinin was freshly dissolved in dimethyl sulfoxide (DMSO) as a solvent before use. Silibinin at 2.5, 5, 10, 15, 20, and 25 μM was used in all experiments. All doses, including blank and controls, were adjusted at 0.1% DMSO, which is the concentration contained in the highest silibinin dose used, and shown to have no cytotoxic effects.

2.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

Silibinin (500 μL) at 2.5, 5, 10, 15, 20, and 25 μM was reacted with DPPH (2,2-diphenyl-1-picrylhydrazyl) solution at 0.15 μM (500 μL), for 1 h at 25 °C. Vitamin C at 0.01% was used as a positive control. The optical density (OD) of the product was measured at 532 nm using a visible spectrophotometer (SpectraMax M3, Molecular Devices). The amount of DPPH-generated radicals was represented as % [(OD of silibinin treatment group/OD of the blank group) $\times$100]. Blank means the group containing DPPH spontaneously generated radicals and DMSO without silibinin.

2.4 Reducing Power Assay

Different doses of silibinin (6 μL) at 2.5, 5, 10, 15, 20, and 25 μM, 1% potassium ferricyanide (200 μL), distilled water (194 μL), and 200 mM phosphate buffer (200 μL) at pH6.6 were reacted in a microtube. After incubation for 20 min at 50 °C, trichloroacetic acid solution at 10% (200 μL) was added to the reaction product. After centrifugation at 2000 g for 10 min, the supernatant (250 μL) was mixed with distilled water (250 μL). Next, after ferric chloride (50 μL) was added, the optical density was measured at 700 nm using a UV spectrophotometer (SpectraMaxM3, Molecular Devices, San Jose, CA, USA). The blank group contained ferricyanide, $FeCl_3$, and DMSO without silibinin, and the positive control contained 0.001% of vitamin C. The level of antioxidant activity as a reducing power was displayed as % [(OD of silibinin treatment group/OD of the blank group) $\times$100].

2.5 MTT Assay

The growth inhibitory effect of silibinin on HT1080 cells was evaluated using 3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [5]. The cells at a density of 5 $\times$ 10^3 cells/well were inoculated into 96-well plates. After treatment with silibinin at 2.5, 5, 10, 15, 20, and 25 μM for 24 h, 20 μL of MTT (5 mg/mL) were added to each well for 4 h. DMSO (150 μL) was added to solubilize the formazan salts and measured the OD at 570 nm using a visible spectrophotometer (SpectraMax M3, Molecular Devices). Relative survival of cells was represented as a % compared to the blank group [(OD of the silibinin treatment group/OD of the blank group) $\times$ 100].

2.6 Gelatin Zymography

The activities of MMP-2 and MMP-9 were examined using gelatin zymography according to a previous study [6]. HT1080 cells were cultured in the presence of silibinin at 2.5, 5, 10, 15, 20, and 25 μM for 1 h. Then, phorbol myristate acetate (PMA) at 1 ng/mL was added for 3 days to induce the expression of MMP. The conditioned medium collected, cleared by centrifugation, and used for the analysis of gelatin zymography. The bands representing MMPs activity were detected as clear zones, and the degree of the bands' intensity was measured with Davinch-ChemiTM. The MMP enzyme activity was expressed as % in comparison to the blank group [(Silibinin treatment group / blank group $\times$ 100)].

2.7 Western Blot Analysis

Western blot analysis was carried out according to standard procedures. HT1080 cells were exposed to silibinin at 2.5, 5, 10, 15, 20, and 25 μM for 1 h. Then, they were stimulated using 1 ng/mL PMA for 24 h in the presence of silibinin. Next, cell lysis was performed with RIPA lysis buffer. The proteins from cell lysates were transferred from a 10% polyacrylamide gel to a nitrocellulose membrane. Thereafter, the membrane was treated with 5% of skim milk. Next, after the treatment of primary antibodies against the target protein such as MMP-2, TIMP-1, *p*-JNK, ERK-1/2, IL-1β and β-actin, secondary antibody treatment was performed. Target proteins were determined using a chemiluminescent ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The degree of band intensity was analyzed with a LAS3000 ® image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.8 Immunofluorescence Staining Assay

HT1080 cells were cultured in a slide chamber at 37 °C for 24 hours. After treatment with each concentration of silibinin, for 1 h, PMA at 1 ng/mL was added and incubated for 24 h. After fixing with 10% formalin for 15 min, the cells were permeabilized with phosphate-buffered saline (PBS) containing 0.5% of Tween 20 (PBS T-20) for 30 min

and washed 3 times with 0.1% PBS T-20. After blocking with 5% of donkey normal serum, primary antibodies (MMP-2) were added for 2 h. Then, after washing 3 times for 5 min each with 0.1% PBS T-20, secondary antibodies (donkey anti-goat conjugated CY3, donkey anti-mouse conjugated CY3, donkey anti-rabbit conjugated FITC) were added for 1 h. Then, after washing them, the slides were exposed to DAPI reagent for nuclei staining and observed with the iRiS Digital Cell Imaging System (Logos Biosystems, Gyeonggi-do, Korea). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.9 Cell Invasion Assay

The invasion of HT1080 cells was carried out in accordance with the Chemicon® methodology. The invasion chamber from the Cell Invasion Assay Kit (ECM550) consists of a 24-well tissue culture plate and 12 cell culture inserts containing polycarbonate membrane (8.0 μm pore size), over which a thin layer of ECMatrix solution is applied. HT1080 cells in 300 μL of serum-free media were added to each insert and 500 μL of media containing 10% fetal bovine serum (chemoattractant) was added to the lower chamber. After the cells adhered to insert, they were treated with each concentration of silibinin and stimulated with 1 ng/mL PMA. Chambers were then incubated in 5% CO_2 and at 37 °C for 72 hours. Non-invading cells as well as the ECM gel layer were removed using a cotton-tipped swab and washed with PBS. On the other side of the filter, invasive cells on the lower surface of the membrane were stained by dipping inserts in the staining solution for 20 min. After washing them with PBS, the stained cells were dissolved in 10% acetic acid. Then, the optical density was measured at 560 nm using a UV spectrophotometer (SpectraMaxM3, Molecular Devices). Relative invasion of cells was represented as a % compared to the blank group [(OD of the silibinin treatment group/OD of the blank group) $\times$ 100].

2.10 Statistical Analysis

Data were analyzed using ANOVA and post hoc (Duncan) test as means of values $\pm$ SD from three independent experiments (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$).

3. Results

3.1 Antioxidant Effect of Silibinin

The antioxidant activity of silibinin was examined using the DPPH radical scavenging assay and the reducing power assay. Vitamin C (Vit. C) at 100 μg/mL, used as a positive control, decreased by 65% of the radicals generated by DPPH, whereas silibinin showed no radical scavenging activity (Fig. 1A). The difference between blank group and silibinin treatment groups above 10 μM was significantly observed (Fig. 1B). When looking at the reducing power (Fig. 1B), vitamin C at 10 μg/mL (the highest dose of 100 μg/mL gave results out of scale) showed a 300% increase over the blank, and silibinin at doses of 10 μM and higher showed a modest but significant activity, which reached a 49% increase at 25 μM. These results indicate that silibinin has no radical scavenging activity, although being endowed with some reducing power. Therefore, its efficacy as an antioxidant is quite low.

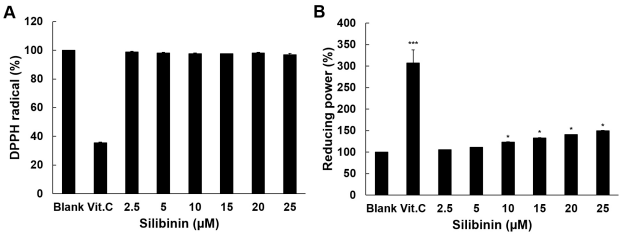

Fig. 1. Antioxidant activity of silibinin. (A) The scavenging effect of silibinin on DPPH radicals is shown in this experiment. Vitamin C was used as positive control at 100 μg/mL. (B) Reducing power of silibinin. Vitamin C was used as a positive control at 10 μg/mL. Data are shown as mean $\pm$ SD from three independent experiments, all run in triplicates. The level of significance between blank and silibinin treatment was evaluated statistically (*, $p < 0.05$; ***, $p < 0.001$) using ANOVA and post hoc (Duncan) test.

3.2 The Effect of Silibinin on Cell Viability in HT1080 Cells and IMR-90 Cells

The effect of silibinin on cell viability was examined in HT1080 and IMR-90 cells. In HT1080, silibinin at low dose (2.5 μM and 5 μM) resulted in a 37% and 38% increase in cell growth, respectively. On the contrary, at the higher doses (20 μM and 25 μM), silibinin induced a 26% and 29% reduction in cell viability, respectively (Fig. 2A). Silibinin effects on IMR-90 cells resulted in a milder growth inhibition (around 20%) at all doses, with no evident dose-effect. Moreover, the growth inhibitory effect of silibinin above 20 μM on HT1080 cells was significantly higher than that on IMR-90 cells, indicating some specificity of the effect, likely due to a different growth control in tumor versus normal cells.

3.3 The Effect of Silibinin on MMPs Activation in HT1080 Cells Stimulated with PMA

The effect of silibinin on MMPs activity was examined in HT1080 cells. Silibinin at low doses had no inhibitory effect, and only at the two higher doses of 20 μM and 25 μM reduced MMP-9 activity by 188% and 943% compared to the PMA group, respectively (Fig. 3). Silibinin effects on MMP2 were quite different, because there was a stimulation of its activity at the lower doses (5-10-15 μM), whilst at only the highest concentration of 25 μM it reduced MMP-2 activity by 147%, compared to the PMA group.

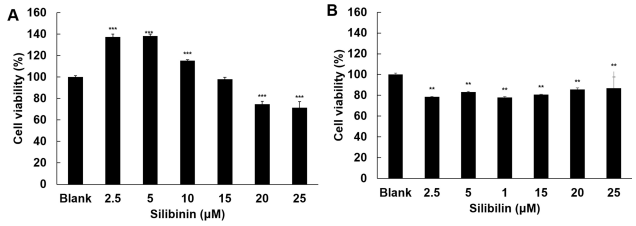

Fig. 2. Effect of silibinin on cell viability. The effects of silibinin on cell viability were examined in HT1080 cells (A) and IMR-90 cells (B), respectively. The cells were treated with silibinin at 2.5, 5, 10, 15, 20, and 25 μM. Cell viability was examined by MTT assay after 24 h treatment. Data show mean $\pm$ SD from three independent experiments, each one run in triplicate. The significance level between blank and silibinin treatment was evaluated statistically (**, $p < 0.01$; ***, $p < 0.001$) using ANOVA and post hoc (Duncan) test.

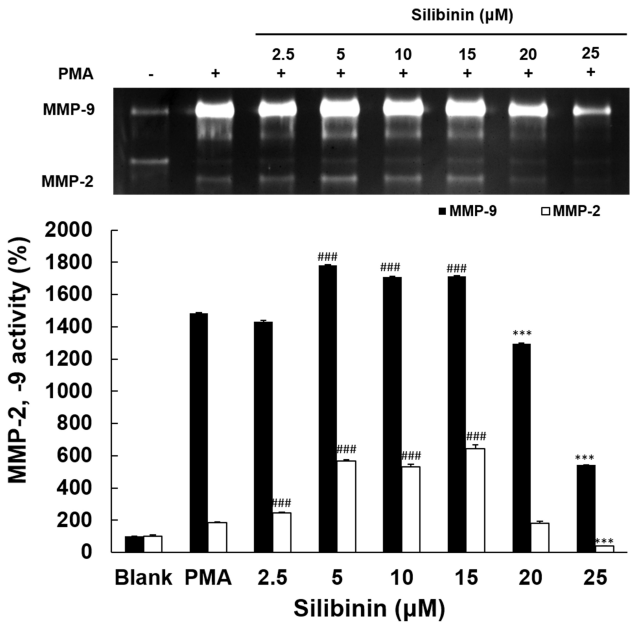

Fig. 3. Effects of silibinin on MMPs activation. The inhibitory effects of silibinin on the inhibitory activities of MMP-9 and MMP-2 was evaluated in PMA-stimulated HT1080 cells to induce MMPs expression. The silibinin at 2.5, 5, 10, 15, 20, and 25 μM was added under serum-free conditions for 72 h. MMP-9 and MMP-2 activities were analyzed using gelatin zymography assay. Data display means values $\pm$ SD from triplicate experiments. The significance level between PMA groups and silibinin treatment groups was determined statistically ($^{###}$, $p < 0.001$ for the activity increase of MMPs, ***, $p < 0.001$ for the activity decrease of MMPs) using ANOVA and post hoc (Duncan) test.

3.4 Effect of Silibinin on the Protein Expression Associated with Metastasis

The expressions of MAPK and other mediators regulating MMPs were examined to elucidate how silibinin influences the regulation of MMP-2 and MMP-9 expression and activity. The expression of ERK-1/2, *p*-p38, *p*-JNK (Fig. 4A), MMP-2, TIMP-1, and IL-1β (Fig. 4B) were analyzed in PMA-stimulated cells with or without silibinin presence, by using western blot. The expression of ERK-1/2 was diminished by 27% only in the presence of the highest dose (25 μM) of silibinin, whereas *p*-p38 expression was inhibited at all doses tested, with values between 23% and 50%. In particular, the expression level of *p*-JNK was significantly higher than in the PMA-treated control group in the presence of silibinin at 10 μM, with values of 28%. Silibinin at all doses progressively inhibited the expression of MMP2, TIMP1 and IL-1β and at the highest dose of 25 μM reduced the expression level of MMP-2 by 64%, TIMP-1 by 149% and IL-1β by 91% compared to the PMA-stimulated group.

3.5 Immunofluorescence Staining of p-p38, IL-1β and MMP-2 Associated with Metastasis

To investigate the effect of silibinin on the expression of metastasis-related proteins, immunofluorescence staining of MMP-2 was performed in PMA-stimulated HT1080 cells, with or without silibinin treatment. The cell's nuclei were observed in blue color after being stained with DAPI. MMP-2 were stained with CY3 and displayed in red color. Silibinin treatment at 25 μM decreased the degrees of MMP-2 compared to the PMA-stimulated group (Fig. 5), thus confirming that silibinin at 25 μM could reduce MMP2 expression.

3.6 Effect of Silibinin on Cell Invasion Related to Metastasis

Tumor cells degrade collagen in the extracellular matrix to obtain more nutrients and make space to move into other tissues through blood vessels. Therefore, in this study, an invasion assay was performed using HT1080 cells stimulated with PMA, in order to evaluate the efficacy of silibinin in the inhibition of cell invasion. Paradoxically, though in line with data on cell growth and effects on MMP2, silibinin treatment at low doses promoted invasion, while at concentrations above 10 μM progressively diminished cell invasion (Fig. 6). Silibinin at 25 μM inhibited cell invasion by 73% compared to PMA-stimulated cells, thus supporting the effect of silibinin as inhibitor of cancer metastasis.

4. Discussion

Chronic inflammation and the continuous release of inflammatory cytokines may contribute to cancer development and its evolution into a malignancy that demands oxygen and nutrition over time. As a result, matrix metalloproteinases (MMPs) can be induced in growing cancers, when the surrounding nutrients are low. MMP-2 and MMP-9 in particular digest collagen IV, a critical component of the extracellular matrix. Cancer cells then may reach and travel

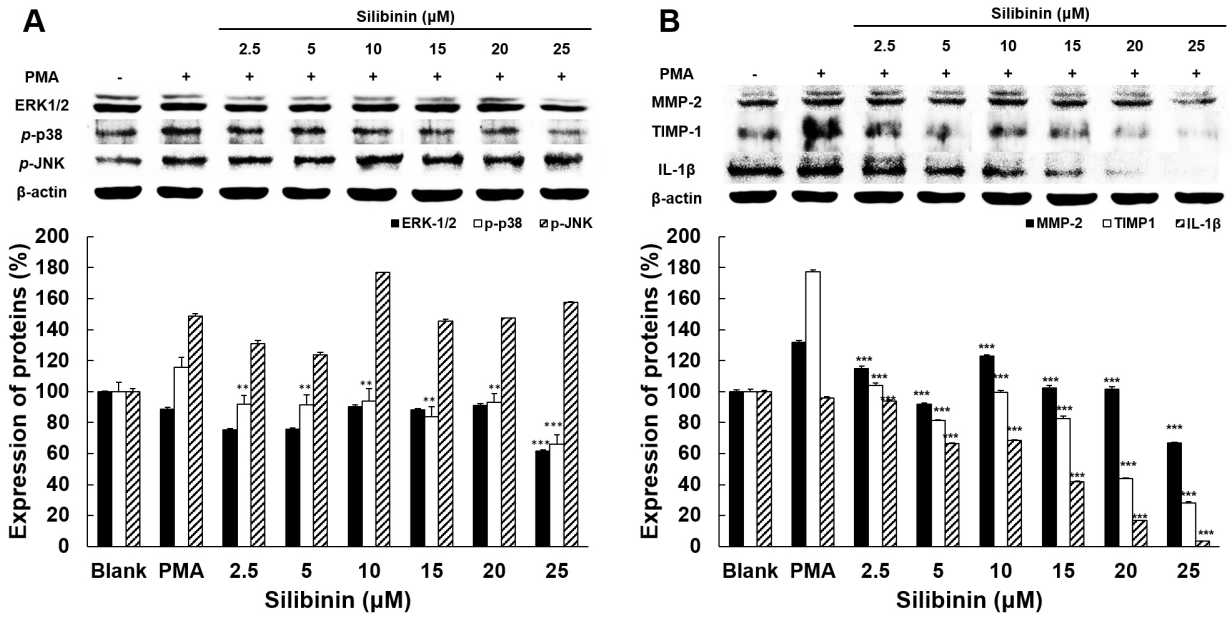

Fig. 4. The effect of silibinin on the expression of proteins associated with invasion and metastasis in HT1080 cells. (A) Effects of silibinin on the expressions of ERK-1/2, *p*-p38, and *p*-JNK. (B) Effects of silibinin on the expressions of MMP-2, TIMP-1, and IL-1β. The level of protein expression in cell lysates was determined by western blot analysis using the indicated antibodies. Target proteins were normalized using the expression of β-actin as housekeeping reference. Data represent means $\pm$ SD from triplicate experiments. The level of significance between PMA groups and silibinin treatment groups was identified statistically (**$p < 0.01$; ***, $p < 0.001$) using ANOVA and post hoc (Duncan) test.

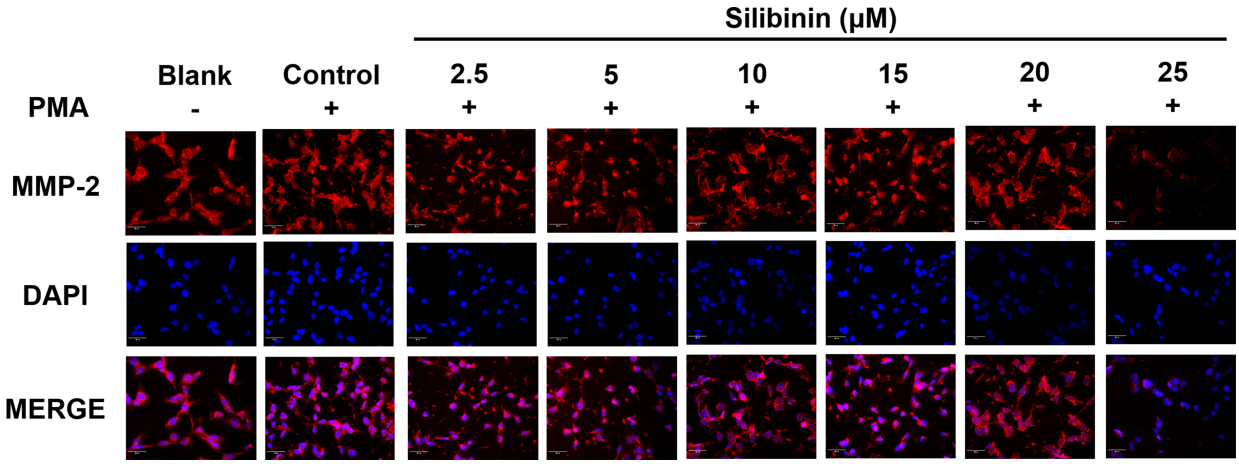

Fig. 5. The immunofluorescence staining analysis of MMP-2. HT1080 cells were treated with silibinin at 2.5, 5, 10, 15, 20, and 25 μM in the presence of PMA. Cells were detected with specific antibodies and counterstained with DAPI. The cells were observed at 200$\times$ of magnification using the iRiS™ Digital Cell Imaging System.

through the bloodstream, finally infiltrating metastatic cells into other permissive tissues. Therefore, blocking cell invasion may play a key role in cancer therapy and the prevention of cancer metastasis. In this study, we have shown that silibinin at the highest doses tested may suppress MMP-9 and MMP-2 activity and expression, as well as IL-1β and *p*-p38, with the final effect of preventing cell invasion by human fibrosarcoma cells (HT1080), previously stimulated by the tumor promoter PMA. This effect raises the intriguing possibility that silibinin could be used as a therapeutic candidate to prevent cancer cells invasion and metastasis. Previous study reported that silibinin possesses strong antioxidant activity and also modulates many molecular changes caused by xenobiotics and ultraviolet radiation to protect the skin [7]. Although we did not find an antioxidant activity of silibinin at the doses here tested, and only a modest reducing ability (Fig. 1), previous studies reported that silibinin was effective in blocking the cell cycle [8–12].

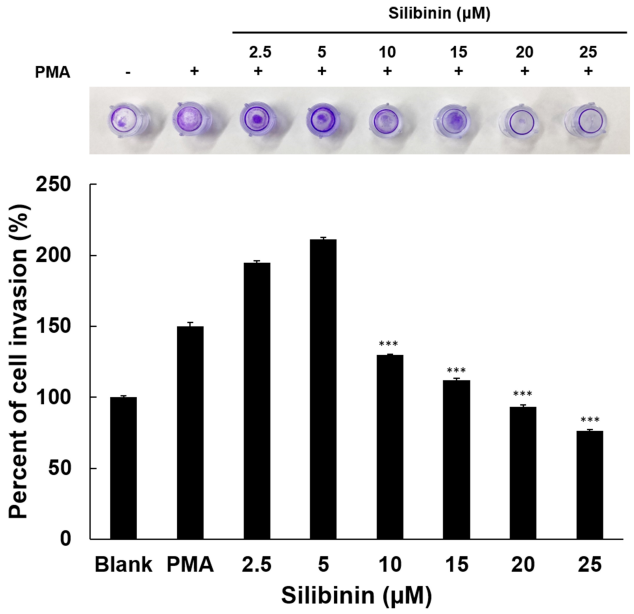

Fig. 6. Effects of silibinin on HT1080 cell invasion. Cell penetration into the ECM layer through the polycarbonate membrane was examined in the presence of silibinin. Using a 24-well tissue culture plate with an insert and a polycarbonate membrane with an 8 μm pore size, an invasion assay was carried out in the invasion chamber. To treat HT1080 cells, silibinin was used at concentrations of 2.5, 5, 10, 15, and 25 M. Data display means values $\pm$ SD from triplicate experiments. The level of significance between PMA groups and silibinin treatment groups was identified (***, $p < 0.001$) using ANOVA and post hoc (Duncan) test.

However, the doses of silibinin here used could inhibit the activity of gelatinases such as MMP-2 and MMP-9 in HT1080 cells previously stimulated with PMA. This effect is consistent with previously published results showing that silibinin inhibited the activity of MMP-2 and MMP-9 in osteoblasts [13].

During metastasis, AP-1 and NF-kB are the main mediators that promote the transcription of tumor necrosis factor (TNF) α, interleukin-1β, interleukin-6, growth factor, COX-2, and MMPs [14,15]. PMA is a cancer promoter, is an activator of protein kinase A and phosphorylates and activates ERK, JNK, and p38, all playing a crucial role in cell division and apoptosis. The activated MAPK migrates to the nucleus and regulates the production of MMPs by modulating the expression of transcription factors AP-1 and NF-kB, finally resulting in cell invasion and metastasis [16]. In this study, we have shown the inhibitory effects of silibinin the effects of silibinin on the protein expression levels of IL-1β, NF-kB and AP-1 as well as MMP-2, MMP-9 and MAPKs such as ERK-1/2, *p*-p38, and *p*-JNK were investigated in fibrosarcoma cells. It was shown that the expression of IL-1β, an inflammatory mediator, was also remarkably reduced by silibinin. Accordingly, silibinin was reported to inhibit the expression of the expression degree of IL-1β in HepG2 and RAW 264.7 cells [8,9], thereby suppressing inflammation and inhibiting metastasis [17,18]. Therefore, these findings are consistent with the previous result, suggesting that the inhibitory effect of silibinin on IL-1β could play a key role in the prevention of metastasis [19]. In addition, silibinin increased the activity of *p*-JNK, but decreased the expression of ERK-1/2 and inhibited the activation of p38. Along the same line, previous studies reported that oxymatrine and resveratrol inhibited the activity of *p*-p38 and ERK-1/2, thereby reducing MMP expression and inhibiting cancer metastasis [20,21]. Therefore, it is tempting to suggest that silibinin could suppress metastasis by inhibiting *p*-p38 and ERK-1/2, thereby inhibiting MMPs expression and activity, necessary for invasion and metastasis.

In addition, our results indicate that silibinin could modulate the MAPK signaling pathway of ERK and p38 via IL-1β, finally decreasing MMPs expression. This resulted in the inhibition of invasive abilities of fibrosarcoma cells, as shown by the invasion assay performed on HT1080 cells in the presence of PMA and silibinin (Fig. 6). Similarly, in a previous study it was also reported that metformin suppressed MMPs through AP-1 and NF-kB inhibition in MCF-7 cells finally reducing their metastatic ability [22] (Fig. 7).

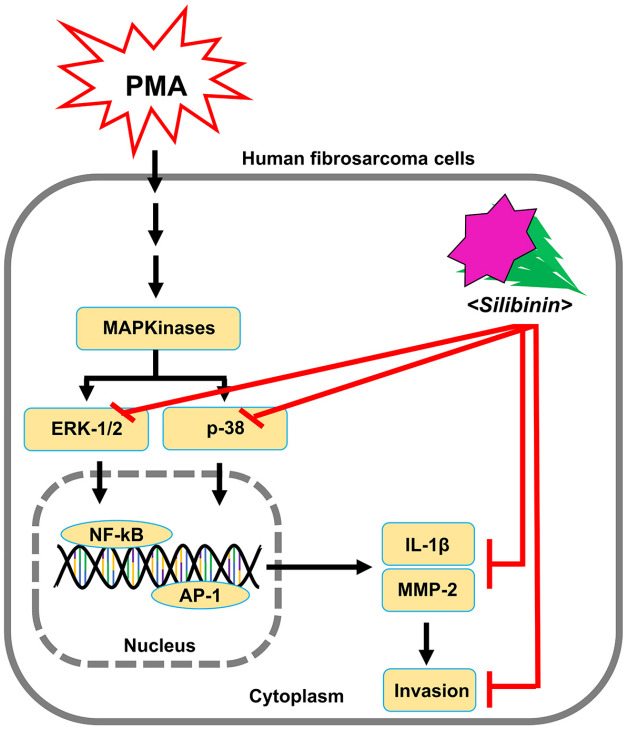

Fig. 7. Schematic diagram for the effect of silibinin on invasion and metastasis of HT1080 cells stimulated by PMA.

5. Conclusions

In the end, this study shows a paradoxical effect of silibinin on HT1080 cells. At low doses, it appears to promote growth and MMPs expression, thus favoring invasion. Only at the higher doses it shows inhibitory effects on these parameters, and therefore it might be supposed to inhibit the metastatic ability of cancer cells. *In vivo*, it is not predictable which dose will reach tumor cells within the tumor mass, and so it remains also unpredictable which effect is expected, whether promotion or inhibition of invasion and metastasis. More studies *in vivo*, at different doses, will be necessary to elucidate the pharmacokinetics and the pharmacodynamics of silibinin, in order to identify a dose which will exert the desired effect of preventing cell invasion and metastasis.

Abbreviations

ATCC, American Type Culture Collection; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ERK, extracellular signal-controlled kinases; FBS, fetal bovine serum; HT1080, human fibrosarcoma cells; IL-1β, Interleukin-1beta; MAPK, mitogen-activated protein kinase; MMP-2, Matrix metalloproteinase 2; MMPs, matrix metalloproteinase; MTT, 3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide; PMA, phorbol myristate acetate; *p*-JNK, phospho-c-Jun NH 2-terminal kinases; TCA, trichloroacetic acid; TIMP-1, TIMP metallopeptidase inhibitor 1; TNF, tumor necrosis factor; Vit. C, vitamin C.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

AIJ performed the experiments, analyzed the data, and assisted in writing the manuscript. Prof MMK proposed the concept, designed the experiment, analyzed the data, and revised the manuscript. Both authors read and approved the final manuscript and agree to be accountable for all aspects of the research.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2804064.

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