Silibinin Inhibits the Invasion of IL-6-Stimulated Colon Cancer Cells via Selective JNK/AP-1/MMP-2 Modulation in Vitro

Chiu-Mei Lin,[†,△,§] Yen-Hsu Chen,[○,⊗] Hon-Ping Ma,[□] Bao-Wei Wang,[⊥] Jen-Hwey Chiu,[#] Su-Kiat Chua,[‡] Jiann-Ruey Ong,[†,∥] and Kou-Gi Shyu*,[‡,▽,∥]

[†]Department of Emergency Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

[△]Institute of Injury Prevention and Control, College of Public Health, Taipei Medical University, Taipei, Taiwan

[§]Institute of Biotechnology, National Ilan University, Yilan County, Taiwan

[○]Division of Infectious Diseases, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung City, Taiwan

[⊗]Graduate Institute of Medicine, Tropical Medicine Research Center, College of Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan

[□]Faculty of Medicine, College of Medicine, Taipei Medical University, Taiwan

[⊥]Central Laboratory, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

[#]Institute of Traditional Medicine, School of Medicine, National Yang Ming University, Taiwan

[‡]Division of Cardiology, Department of Internal Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

[▽]Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

ABSTRACT: Silibinin is a flavonoid with antihepatotoxic properties and pleiotropic anticancer capabilities. This study investigated silibinin inhibition of cell invasion by down-regulating matrix metalloproteinase-2 (MMP-2) expression, via attenuation of activator protein-1 (AP-1) in IL-6-stimulated LoVo colon cancer cells. Western blot data showed that the expression of MMP-2 protein was reduced 1.6- or 1.7-fold over the control by treatment with silibinin or JNK inhibitor in the models. Similar results were revealed in zymography and confocal microscopy. Pretreatment with silibinin also abolished the binding activity of AP-1 and MMP-2 promoter activity via AP-1 binding, as observed by EMSA and luciferase assay. Finally, a [³H]-thymidine incorporation proliferation assay and cell migration assay demonstrated that silibinin inhibited IL-6-stimulated LoVo cell proliferation and invasion. Taken together, these data indicated that silibinin inhibits LoVo cell invasion with the reduction of MMP-2 presentation by attenuating AP-1 binding activity, suggesting a novel antimetastatic application for silibinin in colon cancer chemoprevention.

KEYWORDS: silibinin, IL-6, MMP-2, AP-1, colon cancer

■ INTRODUCTION

Colorectal cancer is one of the leading causes of cancer-related deaths in the world. Its mortality is usually attributed to metastasis of the cancer to distant organs.[1] Several molecular biomarkers of advanced colorectal cancers, such as interleukin 6 (IL-6), have been investigated as a way to determine the proper clinical prognosis and therapeutic response.[2,3] Increasing amounts of evidence suggest that IL-6 plays an important role in the progression and invasion of tumors by stimulating the production of matrix metalloproteinases (MMPs).[4,5] Among the various MMPs, matrix metalloproteinase-2 (MMP-2) is primarily responsible for the extracellular matrix (ECM) degradation seen in tumor cells and contributes to their metastasis and invasion.[6,7] The regulation of MMP-2 depends on mitogen-activated protein kinase (MAPK) family combinations of different signal transduction pathways in various cells.[8,9] Phosphatidylinositol 3-kinase (PI3K)−Akt signaling also plays a prominent role in several processes that are indicative of cancer.[10] However, the molecular regulation of MMP-2 signaling pathways in IL-6-stimulated colon cancer remains relatively unexplored. Therefore, the first aim of this study is to clarify the molecular regulation of MMP-2 in IL-6-stimulated colon cancer cells.

In response to potent pro-inflammatory cytokines, such as IL-6, MMP production is regulated by several signal transduction pathways, including c-Jun N-terminal kinase (JNK) and other MAPKs.[11] Consistent with the importance of these events in tumorigenesis, p38 MAPK and JNK are associated with various human cancers. MAPKs regulate a number of transcription factors, such as activator protein-1 (AP-1)[12] and nuclear factor-κB (NF-κB),[13] which act independently or cooperatively to regulate numerous genes involved in the modulation of MMP expression.

The discomfort, side effects, and toxicity that are associated with chemotherapy and radiotherapy can lead to the increased suffering of patients and diminished therapeutic benefits.

Treatment-related toxicity and side effects can also affect clinical cancer management plans.[1] Therefore, alternative medicine has been used to attenuate the burden of malignancy. In this context, dietary supplements are important phytotherapeutic agents. Recent studies on rodents suggest that silymarin and silibinin may be potentially useful for the chemoprevention of malignancies at a variety of sites, including the intestinal tract.[14] The fruit of the milk thistle plant has been used as a liver-protective remedy for 2000 years in China, and it has exhibited very low toxicity during acute or chronic administration in both animals and humans. The flavonoid silibinin (see Figure 1 for structure), is the major active component of
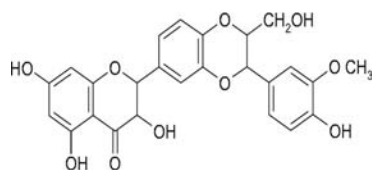

Figure 1. Structure of silibinin.

milk thistle and has been used as a safe dietary supplement for several decades.[12] Some studies have suggested that silibinin exhibits pleiotropic anticancer activities in various tumor cells, including prostate, skin, colon, and bladder cancers.[15] Additionally, Kaur et al. reported that silibinin suppresses cell growth and induces apoptotic death in LoVo colorectal cancer cells.[16] However, the molecular mechanism by which silibinin inhibits colon cancer remains unknown. Therapeutic approach to control colorectal cancer growth and metastasis, in comparison with conventional chemotherapy, is through its adjunctive intervention by natural phytochemicals. The second aim of this study is to elucidate the effects of potential phytochemicals on attenuation of tumor progression. Importantly, the novel molecular mechanisms of silibinin have been identified in LoVo cells, suggesting the strong implications of our findings in controlling human colorectal cancer growth.

■ MATERIALS AND METHODS

Chemicals. High-purity silibinin (>98%) was purchased from Wako Pure Chemical Industry (Osaka, Japan). DMEM was purchased from GIBCO (Invitrogen Co., Grand Island, NY, USA). Interleukin 6 (IL-6) was obtained from CytoLab Ltd. (Rehovot, Israel), and trysolol (aprotinin) was purchased from Bayer CropScience Co. Ltd. (Leverkusen, Germany). Dimethyl sulfoxide (DMSO), penicillin, and streptomycin were obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Matrigel basement membrane matrix was acquired from Becton Dickinson (Bedford, MA, USA). Antibodies were purchased from R&D Systems (Minneapolis, MN, USA), Cell Signaling Technology, Inc. (Beverly, MA, USA), or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). SP600125 (a potent, cell-permeant, selective, and reversible inhibitor of JNK, 25 μmol/L), SB203580 (a highly specific cell-permeant inhibitor of p38 MAPK, 3 μmol/L), PD98059 (extracellular-signal-regulated kinase, ERK, 50 μmol/L), wortmannin (a potent, selective, cell-permeable, and irreversible inhibitor of PI3K, 50 nmol/L), N-acetylcysteine (NAC, a free radical scavenger, 500 μmol/L), and MMP-2 inhibitor (working concentration, 12 nM) were purchased from Calbiochem (Merck, Darmstadt, Germany). Silibinin was dissolved with DMSO, and the DMSO concentration was below 1000.

Cell Lines and Cultures. LoVo colon cancer cells were obtained from the American Type Culture Collection (ATCC CCL-229) (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL of streptomycin and penicillin. The samples were maintained under the standard culture conditions of 37 °C, 95% humidified air, and 5% CO_2.

Proliferation Assay. The proliferation of LoVo cells was determined using the [^{3}H]-thymidine incorporation assay. LoVo cells were seeded on a ViewPlate (Packard Instrument) for 60 min at a density of 5×10^3 cells per well in serum-free medium and incubated overnight. Interleukin 6 (IL-6), silibinin, MMP-2 inhibitor, SP600125, and DMSO were then added to the plates. Additionally, LoVo cells were pretreated with the MMP-2 dominant vector and a control vector in serum-free medium and incubated overnight. Thymidine uptake was measured by the addition of 500 nCi/mL [^{3}H]-thymidine (Perkin-Elmer, Boston, MA, USA). Cells were washed twice with PBS. Nonspecific uptake was measured in the presence of 10 μM cytochalasin B and subtracted from the observed value. MicroScint-20 (50 μL) was added, and the plate was read with TopCount (Packard Instrument). The assay was done in triplicate, and each sample was normalized to the cells incubated without stimulation. All data in the experimental groups are expressed as the mean ± SD of three replicates from four separate experiments.

Cell Invasion Assay. A cell invasion assay was applied to examine the phenotypic alterations of LoVo colon cancer cells following the previously described modification of Liu's staining protocol.[17] All experiments were performed in triplicate, and each provided similar results.

Quantification of MMP-2 Proteins. MMP-2 antibodies were purchased from Oncogene (San Diego, CA, USA). LoVo cells were grown to confluence and subsequently stimulated with or without IL-6. Western blot analyses were performed as previously documented.[18] The assay was done in triplicate, and each sample was normalized to the cells incubated without stimulation that was used to determine the ratio of protein expression.

RNA Isolation and Reverse Transcription. RNA was isolated from cells using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. Total RNA (2 μg) was incubated with 200 U of Moloney murine leukemia virus reverse transcriptase in a buffer containing 50 mmol/L Tris-Cl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl$_2$, 20 U of RNase inhibitor, 1 μmol/L poly dT oligomer, and 0.5 mmol/L of each dNTP in a final volume of 20 μL. The reaction mixture was incubated at 42 °C for 1 h and then at 94 °C for 5 min to inactivate the enzyme. A total of 80 μL of water treated with diethyl pyrocarbonate was added to the reaction mixture before storage at −70 °C. The assay was done in triplicate, and each sample was normalized to the cells incubated without stimulation that was used to determine the ratio of protein expression.

Zymography. Degradation of the extracellular matrix (ECM) was detected by zymography. The protein was extracted from LoVo cells, and equal amounts of the protein samples were subjected to SDS-PAGE on gelatin-containing acrylamide gels [7.5% (w/v) polyacrylamide and 2 mg/mL gelatin] under nonreducing conditions. The zymography was performed as described previously, with minor modification.[18] The minor modification is using the collected cell culture media from 1×10^5 to 1×10^7 cells per Petri dish. The assay was done in triplicate.

MMP-2 Dominant Positive Plasmid. The MMP-2 dominant positive plasmid came from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan), and an MMP-2 functional assay was used in the zymography assay.

Electrophoretic Mobility Shift Assay (EMSA). An electrophoretic mobility shift assay was performed as described previously.[17] Oligonucleotide sequences included the AP-1 (activating factor 1) consensus binding region (5′-CGCTTGATGACTCAGCCGGAA-3′) and the AP-1 mutant oligonucleotide sequence (5′-CGCTTGATG-ACTTGGCCGGAA-3′). After annealing, these double-stranded oligonucleotides were labeled with (γ-^{32}P)-ATP probes by T4 polynucleotide kinase (Mission Biotech Co., Taipei, Taiwan). In each case, controls were performed in which mutant oligonucleotides competed with the labeled sequence. The binding reaction contained 20000 cpm of the DNA probe, 1 μg of poly(dIdC), 25 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, 10% glycerol, 5 mM MgCl$_2$, 0.45 M NaCl, and 5 μg of the nuclear proteins

collected from various conditions. Protein−DNA complex formation was conducted on ice for 20 min and resolved at 4 °C with a 5% nondenaturing polyacrylamide gel using 0.5 μg of a Tris borate–EDTA buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). The gels were dried, and bands were detected using autoradiographic film. Each assay was performed in triplicate.

Promoter Activity Assay. A −705 to +32 bp human MMP-2 promoter construct was generated as follows. Human genomic DNA was amplified with forward (CATTGTGGCTGATCATCTGTT) and reverse (GGCTCAGCAGGCAGCCCAGGA) primers. The amplified product was digested with *Mlu*I and *Bgl*II restriction enzymes and ligated into a pGL3-basic luciferase plasmid vector (Promega, Madison, WI, USA). The MMP-2 promoter contained AP-1 conserved sites (CA) at −365 to −366 bp. For the mutant, the AP-1 binding sites were mutated (TG) using the mutagenesis kit (Stratagene, La Jolla, CA, USA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into LoVo cells using a low-pressure accelerated gene gun (Biowave Technologies, Taipei, Taiwan) operated essentially according to the manufacturer's protocol. Two micrograms of the test plasmid and 0.02 μg of the control plasmid (pGL3-Renilla luciferases) were cotransfected in each well. After treatment, cell extracts were prepared using the Dual-Luciferase Reporter Assay System (Promega), and dual-luciferase activity was measured using a luminometer (Turner Designs). Each experiment was performed in triplicate.

Confocal Microscopy. LoVo cells were seeded onto laminin-coated coverslips and incubated at 37 °C for 2 h to allow for adherence. To identify the localization of MMP-2 in the LoVo cellular cytoplasm, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized in permeabilization buffer for 5 min, and blocked in DAKO diluent for 30 min at room temperature. The cells were then incubated with 1 μL/mL goat anti-MMP-2 antibodies (Oncogene, San Diego, CA, USA) at room temperature for 1 h and developed in the dark with 1:200 FITC-conjugated AffiniPure F(ab')$_2$ Fragment Donkey anti-goat antibodies (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 30 min. Rhodamine phalloidin (10 nM) (Cytoskeleton, Inc.) was added and allowed to incubate at room temperature in the dark for 30 min, followed by the addition of 2 μg/mL Hoechst (Molecular Probes, Inc.) and an additional 10 min of incubation at room temperature in the dark. Confocal microscopy at 400× magnification was performed using a Nikon confocal laser scanning microscope. Each experiment was performed in triplicate.

Statistical Analysis. The data were expressed as the mean ± SD of three replicates from four separate experiments. Statistical significance was determined by analysis of variance (GraphPad Software Inc., San Diego, CA, USA). The Dunnett's test was used to compare multiple groups to a single control group. The Tukey−Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. A value of $P < 0.05$ was considered to be statistically significant. ∗ indicates $p < 0.05$ versus IL-6-treated group (one-way ANOVA); # indicates $p < 0.05$ versus IL-6-treated group (Wilcoxon rank sum test).

■ RESULTS

Attenuated Proliferation Effects of Silibinin, MMP-2 Inhibitor, and SP600125 on LoVo Cells. To investigate the effects of silibinin on the IL-6-induced proliferation of LoVo cells, a [³H]-thymidine incorporation assay was performed. Figure 2 shows that treatment with IL-6 (10 ng/mL) or the MMP-2 dominant vector significantly increased the proliferation of LoVo cells. Pretreatment with silibinin, SP600125, or an MMP-2 inhibitor significantly attenuated the proliferation of LoVo cells stimulated by IL-6 (Figure 2).

Inhibitory Effects of Silibinin on the MMP-2 Protein. By Western blot analysis, MMP-2 protein expression was significantly increased for 24 h after IL-6 (10 ng/mL) stimulation in a time-dependent manner, reaching its peak
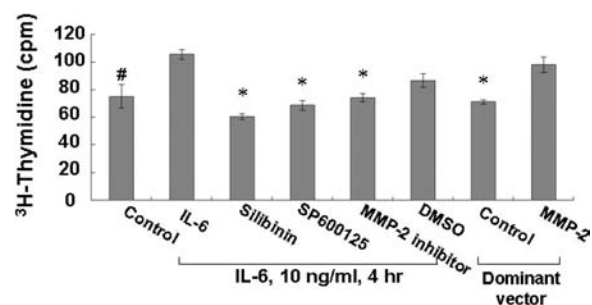
Figure 2. Attenuated proliferation of silibinin-treated LoVo colon cancer cells after stimulation by interleukin 6. A representative [³H]-thymidine incorporation assay shows the proliferation of LoVo cells after IL-6 (10 ng/mL) stimulation in the presence or absence of silibinin (10^{-6} M) or JNK inhibitor SP600125. ∗, $P < 0.05$ when compared to the IL-6-stimulated LoVo cell groups.

concentration after 4 h (Figure 3A). To test whether silibinin can inhibit the induction of MMP-2 expression by IL-6 in LoVo
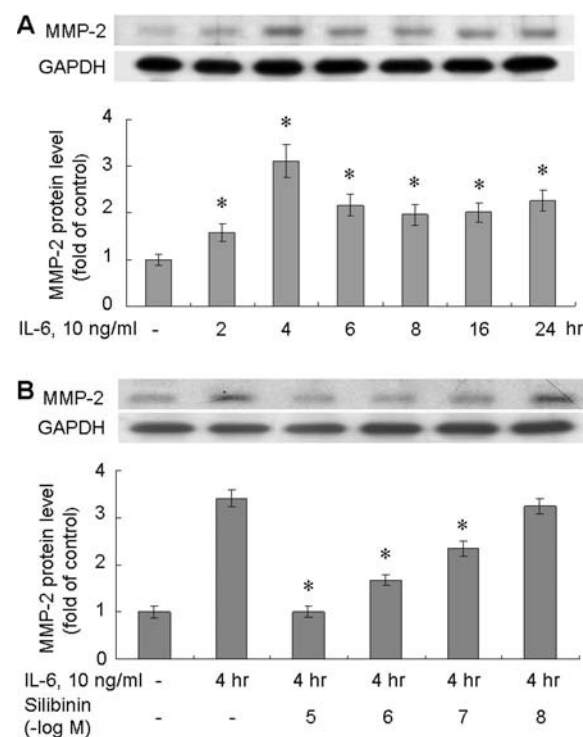
Figure 3. Interleukin 6 increased the expression of matrix metalloproteinase-2 (MMP-2), and silibinin decreased the expression of MMP-2 in cultured LoVo colon cancer cells: (A) representative Western blots and quantitative analysis for MMP-2 protein expression in LoVo cells stimulated by IL-6 (10 ng/mL) for various periods of time; (B) representative Western blots and quantitative analysis for MMP-2 protein expression in silibinin-treated (10^{-5}−10^{-8} M) LoVo cells stimulated by IL-6 (10 ng/mL) for 4 h. ∗, $P < 0.05$ when compared to the IL-6-stimulated group.

cells, various concentrations of silibinin were added before IL-6 stimulation. Silibinin inhibited the MMP-2 protein expression induced by IL-6 (10 ng/mL) in a concentration-dependent manner, but no inhibition was observed at 10^{-8} M (Figure 3B).

Silibinin Reduced MMP-2 in the Presence of IL-6. To investigate possible pathways for MMP-2 regulation, LoVo cells were treated with various inhibitors. SP600125, a JNK inhibitor, completely attenuated the MMP-2 protein expression stimu-

lated by IL-6 (10 ng/mL), and silibinin (10^{-6} M) revealed similar results (Figure 4A). PD98059 (an ERK inhibitor),
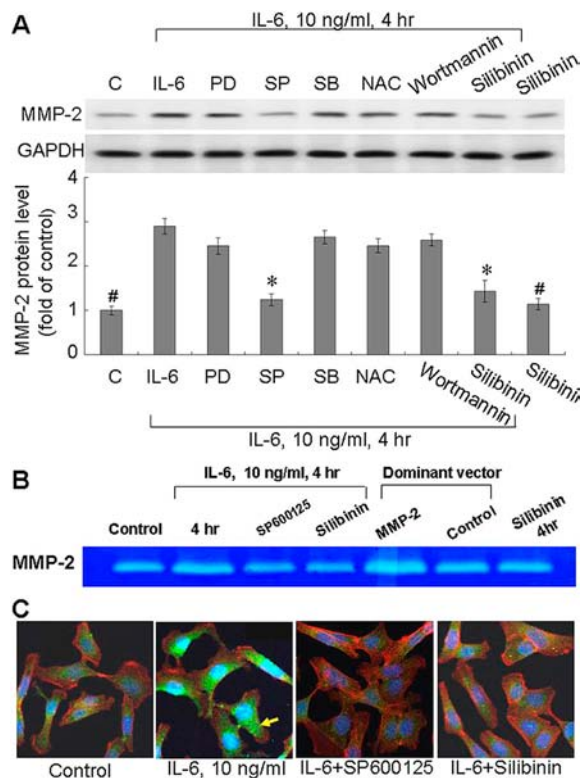

Figure 4. Effects of silibinin and other inhibitors on the expression of matrix metalloproteinase-2 (MMP-2) stimulated by interleukin 6 (IL-6). (A) The JNK pathway mediates IL-6-stimulated MMP-2 expression in LoVo colon cancer cells. The results are normalized to GAPDH levels. (B) Representative zymography for MMP-2 protein expression in LoVo cells stimulated by IL-6 (10 ng/mL) for various time periods. Dominant protein expression of MMP-2 showed augmented expression in LoVo cells. (C) The matrix metalloproteinase-2 (MMP-2) in serum-starved LoVo cells was stained with tetramethylrhodamine isothiocyanate−phalloidin (green dot) and observed with inverse fluorescence microscopy. Representative imaging shows that the amount of MMP-2 protein (green dot) is apparently decreased compared to the IL-6-stimulated plate upon treatment with a JNK inhibitor (SP600125) or silibinin (10^{-6} M). Original magnification was 200×. The assay was performed in triplicate, and the values from treated LoVo cells stimulated by IL-6 have been normalized to the values from the control cells ($n = 4$ per group). *, $P < 0.05$ when compared to the IL-6 stimulated group. #, $P < 0.05$ when comparing the control group to the IL-6-stimulated LoVo cells (Wilcoxon rank sum test).

SB203580 (a p38 MAPK inhibitor), NAC (an antioxidant scavenger), and wortmannin (a PI3K/Akt inhibitor) did not affect the MMP-2 protein expression induced by IL-6. These findings indicate that the JNK pathway is the main signaling pathway mediating the induction of MMP-2 protein expression by IL-6 stimulation. As shown in Figure 4B, the zymography demonstrated that the amount of activated MMP-2 significantly increased after stimulation by IL-6 (10 ng/mL) or the MMP-2 dominant vector for 4 h. Silibinin and a JNK inhibitor (SP600125) significantly attenuated the increased MMP-2 activity induced by IL-6. Furthermore, a confocal microscope study showed that the level of MMP-2 protein (green) in the cytoplasm of LoVo cells increased after 4 h of IL-6 stimulation.

Silibinin and SP600125 suppressed the induction of MMP-2 by IL-6 (Figure 4C).

Suppressive Effects of Silibinin on IL-6-Stimulated AP-1 Binding Activity and MMP-2 Promoter Activity. As seen in Figure 5A, the EMSA showed that IL-6 increased AP-1
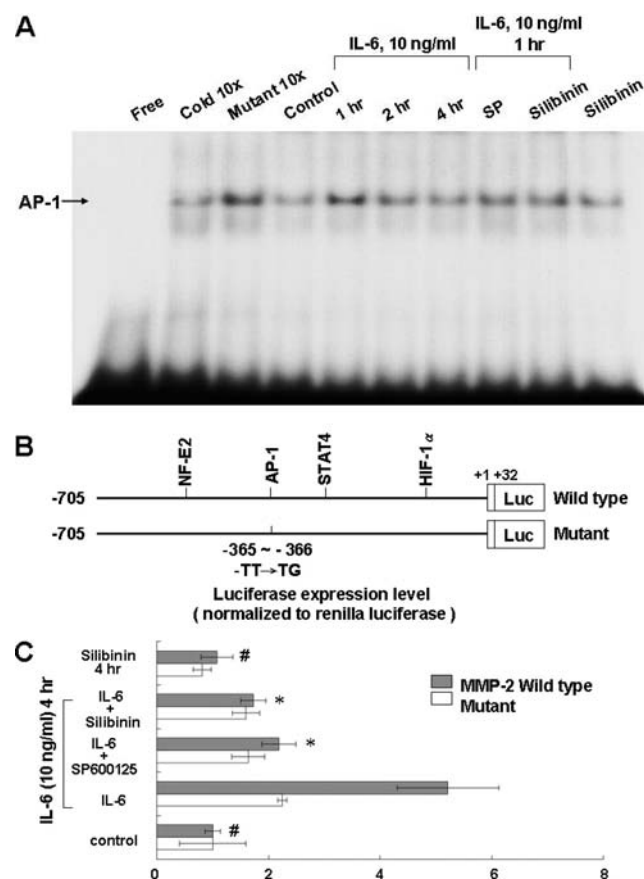

Figure 5. IL-6 enhanced the AP-1-binding activity, and silibinin inhibited the AP-1-binding activity. (A) Representative EMSA showing protein binding to the AP-1 oligonucleotide in nuclear extracts of LoVo cells after IL-6 (10 ng/mL) stimulation in the presence or absence of silibinin (10^{-6} M) or a JNK inhibitor (SP600125, 25 μmol/L). A significant supershifted complex (super) was observed after incubation with AP-1 antibodies. Cold oligo means unlabeled AP-1 oligonucleotides. (B) Constructs of AP-1 promoter gene. Positive +1 demonstrates the initiation site for the MMP-2 transcription. Mutant MMP-2 promoter indicates mutation of AP-1 binding sites in the MMP-2 promoter as indicated. (C) Quantitative analysis of MMP-2 promoter activity. Cultured LoVo cells were transiently transfected with pMMP-2 promoter-Luc by a low-pressure accelerated gene gun (Bioware Technologies, Taipei, Taiwan), essentially following the protocol from the manufacturer. The luciferase activity in the cell lysates was measured and normalized with renilla activity ($n = 3$ per group). *, $P < 0.001$ when compared to the control. +, $P < 0.01$ when compared to the 4 h time point.

DNA−protein binding activity, reaching its peak after 1 h. An excess of unlabeled probe with AP-1 binding site competing with the same labeled probe showed the binding activity decreased. An excess of labeled probe with a 2 bp substitution in the AP-1 binding site did not decrease AP-1 binding when competing with a nonmutated probe. Addition of SP600125 and silibinin 30 min before IL-6 (10 ng/mL) stimulation abolished the DNA−protein binding activity induced by IL-6. DNA-binding complexes induced by IL-6 could be supershifted

by a monoclonal AP-1 antibody, indicating the presence of this protein in these complexes. These results suggested that the AP-1 binding site in the MMP-2 promoter is critical for transcriptional regulation by IL-6 and that silibinin regulates MMP-2 promoter activity via the JNK/AP-1 pathway.

To study whether the MMP-2 expression stimulated by IL-6 is regulated at the transcriptional level, we cloned the promoter region of rat MMP-2 (−741 to +22) with or without an AP-1 binding site mutation (TT mutated to TG at −365 to −366) into the luciferase reporter vector (pGL3-Luc) (Figure 5B). Compared to the wild MMP-2 promoter, the IL-6-induced activity in LoVo cells containing the mutated MMP-2 promoter was significantly lower. This result indicates that MMP-2 expression is induced at the transcriptional level by IL-6, and the binding of AP-1 to this site plays a key role. Moreover, the addition of SP600125 or silibinin (10^{-6} M) suppressed the MMP-2 promoter activity induced by IL-6 (Figure 5C).

Inhibitory Effect of Silibinin on MMP-2 Phenotypic Alterations. To evaluate the effects of silibinin on phenotypic alterations of LoVo cells induced by IL-6, a cell migration assay was performed. Figure 6 shows that treatment with IL-6 (10
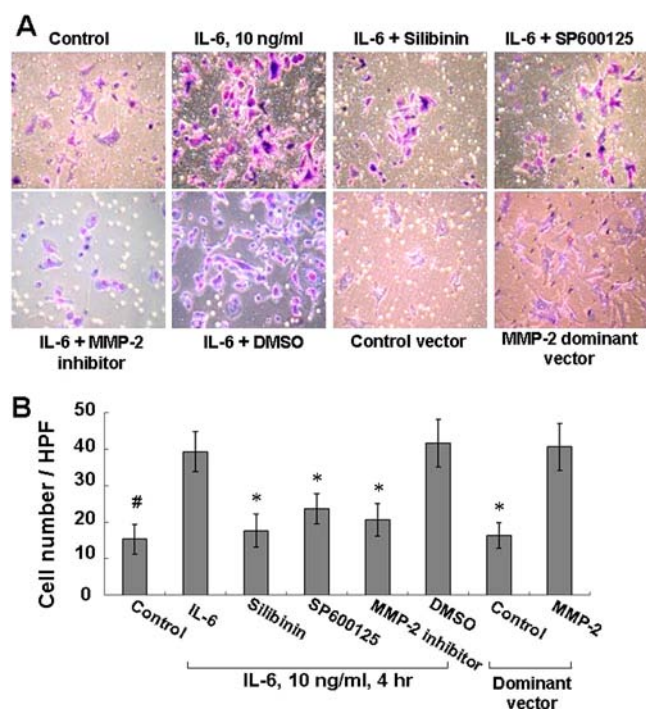
Figure 6. Effects of silibinin and a JNK inhibitor on tumor invasion in IL-6-induced LoVo cells: (A) effects of JNK inhibitor SP600125 and silibinin (10^{-6} M) on the invasion of LoVo cells through the matrigel-coated filter; (B) quantitative analysis of silibinin and the JNK inhibitor in IL-6-stimulated LoVo cells. The assay was performed in triplicate, and the values from the induced LoVo cells have been normalized to the values from the control cells ($n = 4$ per group). *, $P < 0.05$ when compared to the control. #, $P < 0.05$ when comparing the control cells to the IL-6-stimulated LoVo cells (Wilcoxon rank sum test).

ng/mL) or the MMP-2 dominant vector significantly increased the migratory activity of the LoVo cells. However, pretreatment with silibinin, SP600125, or an MMP-2 inhibitor abolished the migratory activity of LoVo cells stimulated by IL-6.

DISCUSSION

Silibinin was isolated from thistle, a Chinese plant used as a hepatoprotective agent in traditional Chinese medicine over 2000 years. Modern studies have suggested that silibinin produces nutraceutical and anticancer effects through the modulation of inflammation.[19] Some bioactivities have already been reported, including cytotoxicity and apoptotic effects in cancer cells. Moreover, silibinin has been proved to enrich the property of anticancers, including prostate cancer and skin cancer.[20,21] In these investigations, increasing evidence supported silibinin having the effect of anticancer through the modulation of inflammatory pathways. In comparison with the model of silibinin-treated cancers, our IL-6-induced model was used to mimic the pathophysiologic condition in colon cancers. Further investigation revealed that silibinin might inhibit colon cancer migration and invasion through inflammatory modulation. However, the influence of silibinin on the expression of MMPs and the invasiveness of colorectal cancer is still unclear. In our study, we examined the effect of silibinin on invasion/migration and its molecular mechanisms in human colorectal cancer cells for the first time. Previous study has reported that IL-6 and the IL-6R complex could induce clonogenic growth and invasiveness by mediating signals in the Ras/MAPK and PI3K/AKt pathways,[7] and the malignant phenotypes might be associated with the production of MMP-2 and MMP-9 after IL-6 stimulation in SW480 cancer cells or LoVo cells.[22] Our model is consistent with the existence of IL-6/MMP modulation in colon cancer cells. In addition, Sangeetha et al. reported that silibinin reduces the tumor incidence and preneoplastic lesions in a 32 week treatment period of colon cancer rat model;[23] meanwhile, our data explored the inhibitory property of invasion/metastasis in a short period (4 h) of silibinin-treated colon cancer cells. Our novel molecular investigation evaluated the attenuating effect of silibinin on IL-6-induced MMP-2 expression.

A large amount evidence has supported that silibinin attenuated MMP-2 regulation in prostate cancer and osteosarcoma MG-63 cells through different mechanisms.[24,25] Studies that included MAPK, PI3K, and ERK modulation suggested that silibinin inhibits A549 lung cancer cell invasion through inactivation of both the PI3K-Akt and MAPK signaling pathways.[26] Moreover, silibinin was able to abolish the invasion of oral squamous cell carcinomas by suppressing the ERK and MAPK pathways.[27] Kauntz et al. found that silibinin activated both the extrinsic and intrinsic apoptotic pathways and induced an autophage-mediated survival response in SW480 and SW620 cells.[28] In this study, we investigated the suppressive effect of silibinin in IL-6-stimulated LoVo cells and the molecular modulation of cell invasion and migration. The result revealed that the selective JNK pathway was inhibited in IL-6-stimulated LoVo cells by SP600125, but the inhibitors of MAPK, P38, and PI3K pathways were not. In the same experimental conditions treatment with silibinin revealed similar results (Figure 4A). In comparison with the previous studies, silibinin is able to attenuate cell invasion or augment the apoptotic effect through anti-inflammatory mechanism in various cancers.[26−28] Interestingly, results from Figures 4 and 5 revealed no inhibitory effect without IL-6 stimulation in LoVo cells. A similar finding was also noted in Chittezahath's study[29] that indicated silibinin would be exhibit the attenuated property after inflammatory cytokine stimulation.

MMPs are involved in remodeling of the extracellular matrix to modulate many normal and pathological processes, including morphogenesis, angiogenesis, tissue repair, and tumor invasion.[30] There are several line of evidence indicating that silibinin could be used to treat colon cancers through activation of antiproliferative, pro-apoptotic, and antiangiogenic pathways,[31] whereas attenuation of MMP-2 was not documented. In comparison with previous study, we presented gelatin zymography that silibinin inhibited the expression and activation of MMP-2 in LoVo cells (Figure 4B). Additionally, in the cell invasion/migration assay, treatment with an MMP-2 inhibitor and the MMP-2 dominant positive vector showed that MMP-2 plays a key role. This assay also revealed the inhibitory effect of silibinin on IL-6-stimulated LoVo cell migration. The above novel investigations indicated that silibinin might possess antimetastatic activity.

MAPKs regulate a number of transcription factors, such as AP-1 and NF-κB. Functional activation of the AP-1 transcription complex is implicated in tumor promotion and malignant transformations.[32] The transcription of MMP-encoding genes is regulated by their upstream sequences, including motifs corresponding to AP-1 or NF-κB binding sites.[33] In our study, treatment with silibinin could abolish MMP-2 expression through selective modulation of JNK but not MAPK or ERK pathways in IL-6-stimulated LoVo cells. Moreover, silibinin attenuated AP-1 binding activity by the EMSA (Figure 5A), and it also decreased luciferase expression levels upon AP-1 mutation (Figure 5B,C). Chu et al. have aimed to investigate the role of silibinin as a potential therapeutic target of A549 lung cancer, through MMP-2 and urokinase plasminogen activator (u-PA) pathway in antimetastasis.[34] In contrast, our data revealed that silibinin inhibited MMP-2 expression via the JNK/AP-1 pathway. Furthermore, Ravichandran and his colleagues have also reported that silibinin achieves its chemopreventive efficacy against azoxymethane-induced colon carcinogenesis in A/J mice by targeting the β-catenin and IGF-1R$_β$ pathways.[35] It was also indicated that silibinin protected against spontaneous intestinal polyps and tumors in APC (min/+) mice.[36] Meanwhile, silibinin has shown the ability to inhibit migration/invasion in our IL-6-stimulated LoVo cell model.

The data presented here demonstrate that silibinin suppresses LoVo colon cancer cell migration and invasion via the JNK/MMP-2 pathway by regulating the activation of transcription factor AP-1, thus leading to the suppression of tumor metastasis. Our study adds more evidence to the claim that silibinin has therapeutic potential for controlling colon cancer metastasis in future clinical applications, which may shed light on the clinical research of silibinin in cancer-related pathophysiology.

AUTHOR INFORMATION

Corresponding Author
*Postal address: Division of Cardiology, Shin Kong Wu Ho-Su Memorial Hospital, No. 95 Wen-Cheng Road, Shin Lin District, Taipei, Taiwan. E-mail: shyukg@ms12.hinet.net. Phone: 886-2-28332211-2094. Fax: 886-2-28365775.

Author Contributions
‖The corresponding author and J.-R.O. made equal contributions to this work.

Notes
The authors declare no competing financial interest.

ABBREVIATIONS USED

IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; MMP-2, matrix metalloproteinase-2; AP-1, activator protein-1

REFERENCES

(1) Mitry, E.; Guiu, B.; Cosconea, S.; Jooste, V.; Faivre, J.; Bouvier, A. M. Epidemiology, management and prognosis of colorectal cancer with lung metastases: a 30-year population-based study. *Gut* **2010**, *59*, 1383−1388.

(2) Li, Y. Y.; Hsieh, L. L.; Tang, R. P.; Liao, S. K.; Yeh, K. Y. Interleukin-6 (IL-6) released by macrophages induces IL-6 secretion in the human colon cancer HT-29 cell line. *Hum .Immuno.* **2009**, *70*, 151−158.

(3) Kemik, O.; Kemik, O.; Sumer, A.; Kemik, A. S.; Hasirci, I.; Purisa, S.; Dulger, A. C.; Demiriz, B.; Tuzun, S. The relationship among acute-phase response proteins, cytokines and hormones in cachectic patients with colon cancer. *World J. Surg. Onco.* **2010**, *8*, 85−90.

(4) Hong, D. Y.; Lee, B. J.; Lee, J. C.; Choi, J. S.; Wang, S. G.; Ro, J. H. Expression of VEGF, HGF, IL-6, IL-8, MMP-9, Telomerase in Peripheral Blood of Patients with Head and Neck Squamous Cell Carcinoma. *Clin. Exp. Otorhinolaryngol.* **2009**, *2*, 186−192.

(5) Loo, W. T.; Sasano, H.; Chow, L. W. Pro-inflammatory cytokine, matrix metalloproteinases and TIMP-1 are involved in wound healing after mastectomy in invasive breast cancer patients. *Biomed. Pharmacother.* **2007**, *61*, 548−552.

(6) Kossakowska, A. E.; Edwards, D. R.; Prusinkiewicz, C.; Zhang, M. C.; Guo, D.; Urbanski, S. J.; Grogan, T.; Marquez, L. A.; Janowska-Wieczorek, A. Interleukin-6 regulation of matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitor of metalloproteinase (TIMP-1) expression in malignant non-Hodgkin's lymphomas. *Blood* **1999**, *94*, 2080−2089.

(7) Hsu, C. P.; Chen, Y. L.; Huang, C. C.; Chou, C. C.; Liu, C. L.; Hung, C. H.; Kao, T. Y.; Chung, Y. C. Anti-interleukin-6 receptor antibody inhibits the progression in human colon carcinoma cells. *Eur. J. Clin. Invest.* **2011**, *41*, 277−284.

(8) Abe, Y.; Sakaguchi, M.; Furukado, S.; Okazaki, S.; Fujinaka, T.; Sakoda, S.; Yoshimine, T.; Kitagawa, K. Associations of local release of inflammatory biomarkers during carotid artery stenting with plaque echogenicity and calcification. *Cerebrovasc. Dis.* **2010**, *30*, 402−409.

(9) Mohamed, M. M.; Cavallo-Medved, D.; Rudy, D.; Anbalagan, A.; Moin, K.; Sloane, B. F. Interleukin-6 increases expression and secretion of cathepsin B by breast tumor-associated monocytes. *Cell Physiol. Biochem.* **2010**, *25*, 315−324.

(10) Lee, K. M.; Lee, D. E.; Seo, S. K.; Hwang, M. K.; Heo, Y. S.; Lee, K. W.; Lee, H,J. Phosphatidylinositol 3-kinase, a novel target molecule for the inhibitory effects of kaempferol on neoplastic cell transformation. *Carcinogenesis* **2010**, *31*, 1338−1343.

(11) Chakraborti, S.; Mandal, M.; Das, S.; Mandal, A.; Chakraborti, T. Regulation of matrix metalloproteinases: an overview. *Mol. Cell. Biochem.* **2003**, *253*, 269−285.

(12) Volate, S. R.; Davenport, D. M.; Muga, S. J.; Wargovich, M. J. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng, and rutin). *Carcinogenesis* **2005**, *26*, 140−146.

(13) Kidd, P. M. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Altern. Med. Rev.* **2009**, *14*, 226−246.

(14) Pierini, R.; Gee, J. M.; Belshaw, N. J.; Johnson, I. T. Flavonoids and intestinal cancers. *Br. J. Nutr.* **2008**, *99*, ES53−59.

(15) Cheung, C. W.; Gibbons, N.; Johnson, D. W.; Nicol, D. L. Silibinin–a promising new treatment for cancer. *Anticancer Agents Med. Chem.* **2010**, *10*, 186−195.

(16) Kaur, M.; Velmurugan, B.; Tyagi, A.; Deep, G.; Katiyar, S.; Agarwal, C.; Agarwal, R. Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. *Mol. Cancer Ther.* **2009**, *8*, 2366−2374.

(17) Lin, C. M.; Shyu, K. G.; Wang, B. W.; Chang, H.; Chen, Y. H.; Chiu, J. H. Chrysin suppresses IL-6-induced angiogenesis via downregulation of JAK1/STAT3 and VEGF: an in vitro and in ovo approach. *J. Agric. Food Chem.* **2010**, *58*, 7082−7087.

(18) Wang, B. W.; Chang, H.; Lin, S.; Kuan, P.; Shyu, K. G. Induction of matrix metaoolproteinase-14 and −2 by cyclical mechanical stretch is mediated by tumor necrosis factor-m in cultures human umbilical vein endothelial cells. *Cardiovasc. Res.* **2003**, *59*, 460−469.

(19) Gupta, S. C.; Kim, J. H.; Prasad, S.; Aggarwal, B. B. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Rev.* **2010**, *29*, 405−434.

(20) Singh, R. P.; Agarwal, R. Mechanisms and preclinical efficacy of silibinin in preventing skin cancer. *Eur. J. Cancer* **2005**, *41*, 1969−1979.

(21) Gu, M.; Singh, R. P.; Dhanalakshmi, S.; Agarwal, C.; Agarwal, R. Silibinin inhibits inflammatory and angiogenic attributes in photocarcinogenesis in SKH-1 hairless mice. *Cancer Res.* **2007**, *67*, 3483−3491.

(22) Knüpfer, H.; Preiss, R. Serum interleukin-6 levels in colorectal cancer patients–a summary of published results. *Int. J. Colorectal. Dis.* **2010**, *25*, 135−140.

(23) Sangeetha, N.; Aranganathan, S.; Panneerselvam, J.; Shanthi, P.; Rama, G.; Nalini, N. Oral supplementation of silibinin prevents colon carcinogenesis in a long term preclinical model. *Eur. J. Pharmacol.* **2010**, *643*, 93−100.

(24) Wu, K. J.; Zeng, J.; Li, L.; Fan, J.; Zhang, D.; Xue, Y.; Zhu, G.; Yang, L.; Wang, X.; He, D. L. Silibinin inhibits prostate cancer invasion, motility and migration by suppressing vimentin and MMP-2 expression. *Acta Pharmacol. Sin.* **2009**, *30*, 1162−1168.

(25) Hsieh, Y. S.; Chu, S. C.; Yang, S. F.; Chen, P. N.; Liu, Y. C.; Lu, K. H. Silibinin suppresses human osteosarcoma MG-63 cell invasion by inhibiting the ERK-dependent c-Jun/AP-1 induction of MMP-2. *Carcinogenesis* **2007**, *28*, 977−987.

(26) Chen, P. N.; Hsieh, Y. S.; Chiou, H. L.; Chu, S. C. Silibinin inhibits cell invasion through inactivation of both PI3K-Akt and MAPK signaling pathways. *Chem. Biol. Interact.* **2005**, *156*, 141−150.

(27) Chen, P. N.; Hsieh, Y. S.; Chiang, C. L.; Chiou, H. L.; Yang, S. F.; Chu, S. C. Silibinin inhibits invasion of oral cancer cells by suppressing the MAPK pathway. *J. Dent. Res.* **2006**, *85*, 220−225.

(28) Kauntz, H.; Bousserouel, S.; Gossé, F.; Raul, F. Silibinin triggers apoptotic signaling pathways and autophagic survival response in human colon adenocarcinoma cells and their derived metastatic cells. *Apoptosis* **2011**, *16*, 1042−1053.

(29) Chittezhath, M.; Deep, G.; Singh, R. P.; Agarwal, C.; Agarwal, R. Silibinin inhibits cytokine-induced signaling cascades and downregulates inducible nitric oxide synthase in human lung carcinoma A549 cells. *Mol. Cancer. Ther.* **2008**, *7*, 1817−1826.

(30) Nagase, H.; Woessner, J. F. Matrix metalloproteinases. *J. Bio. Chem.* **1999**, *274*, 21491−21494.

(31) Velmurugan, B.; Gangar, S. C.; Kaur, M.; Tyagi, A.; Deep, G.; Agarwal, R. Silibinin exerts sustained growth suppressive effect against human colon carcinoma SW480 xenograft by targeting multiple signaling molecules. *Pharm. Res.* **2010**, *27*, 2085−97.

(32) Huang, C.; Ma, W. Y.; Young, M. R.; Colburn, N.; Dong, Z. Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 156−161.

(33) Eferl, R.; Wagner, E. F. AP-1: A double edged sword in tumorigenesis. *Nat. Rev. Cancer* **2003**, *3*, 859−868.

(34) Chu, S. C.; Chiou, H. L.; Chen, P. N.; Yang, S. F.; Hsieh, Y. S. Silibinin inhibits the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. *Mol. Carcinog.* **2004**, *40*, 143−149.

(35) Ravichandran, K.; Velmurugan, B.; Gu, M.; Singh, R. P.; Agarwal, R. Inhibitory effect of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice. *Clin. Cancer Res.* **2010**, *16*, 4595−4606.

(36) Rajamanickam, S.; Velmurugan, B.; Kaur, M.; Singh, R. P.; Agarwal, R. Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin. *Cancer Res.* **2010**, *70*, 2368−2378.