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Butein Inhibits the Migration and Invasion of SK-HEP-1 Human Hepatocarcinoma Cells through Suppressing the ERK, JNK, p38, and uPA Signaling Multiple Pathways

Chia-Yu Ma,[†] Wei-Ting Ji,[‡] Fu-Shin Chueh,[§] Jai-Sing Yang,[∥] Po-Yuan Chen,[‡] Chien-Chih Yu,[⊥] and Jing-Gung Chung[*,‡,#]

[†]Department of Food and Beverage Management, Technology and Science Institute of Northern Taiwan, Taipei 112, Taiwan

[‡]Department of Biological Science and Technology, China Medical University, Taichung 404, Taiwan

[§]Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan

[∥]Department of Pharmacology, China Medical University, Taichung 404, Taiwan

[⊥]School of Pharmacy, China Medical University, Taichung 404, Taiwan

[#]Department of Biotechnology, Asia University, Taichung 413, Taiwan

ABSTRACT: Liver cancer is one of the most commonly diagnosed cancers and the leading cause of death in human populations. Butein, a tetrahydroxychalcone, has been shown to induce apoptosis in many human cancer cells, but the effects of butein on the migration and invasion of human liver cancer cells are not reported. Herein, we found that butein is effective in the suppression of migration and invasion in SK-HEP-1 human hepatocarcinoma cells by using the Matrigel cell migration assay and invasion system. The gelatin zymography assay indicated that butein inhibited the activity of matrix metalloproteinases 2 (MMP-2) and MMP-9. Western blotting analysis indicated that butein decreased the levels of MMP-2, -7, and -9, uPA, Ras, Rho A, ROCK1, ERK1/2, JNK1/2, p-p38, and p-c-Jun in SK-HEP-1 cells. Furthermore, butein inhibited the NF-κB binding activity in SK-HEP-1 cells by electrophoretic mobility shift assay. We also found that butein decreased the ERK, JNK, and p38 in SK-HEP-1 cells by in vitro kinase assay. In conclusion, this is the first study to demonstrate that butein might be a novel anticancer agent for the treatment of hepatocarcinoma through inhibiting migration and invasion.

KEYWORDS: Butein, migration, invasion, SK-HEP-1 human hepatocarcinoma cells, matrix metalloproteinase, MAPKs pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies and the third leading cause of cancer death worldwide.[1] In Taiwan, liver cancer is the first leading cause of cancer death. HCC accounts for about 6% of all human cancers annually.[2] It was suggested that DNA damage and reactive oxygen species (ROS) are involved in the process of hepatocarcinogenesis.[3] Currently, drug efficacy often presents undesirable side effects. For new therapies against liver cancer, HCC is especially needed to develop new agents.

Cancer invasion and metastasis were done through the breakdown of the extracellular matrix (ECM) by proteinases,[4,5] and matrix metalloproteinases (MMPs) are a family of ECM degrading proteinases. MMPs were originally implicated in cancer progression, invasion, and metastasis. On the basis of their structural and functional properties, MMPs can be divided into three distinct categories such as collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), and stromelysins (MMP-3, -10, and -11).[4−11]

Butein (3,4,20,40-tetrahydroxychalcone), a polyphenolic compound, was extracted from the stembark of cashews (*Semecarpus anacardium*) and *Rhus verniciflua* Stokes, which are used as a traditional herbal medicine in Chinese populations. Numerous studies have demonstrated that butein presents anticancer activities in a wide variety of human cancer cells including myelogenous leukemia,[12] breast carcinoma,[13,14] B16 melanoma,[15] lymphoma,[16,17] colon carcinoma,[18,19] osteosarcoma,[20] and hepatic stellate cells.[21] However, the anticancer mechanisms of butein are not fully understood. Recently, it was reported that butein can inhibit migration and invasion through the ERK1/2 and NF-κB signaling pathways in human bladder cancer cells, and this inhibitory effect may be associated with the reversal of epithelial-mesenchymal transition.[22] However, there is no available information to address how butein affected the migration and invasion of human hepatocarcinoma cells. Therefore, the present study was performed to evaluate the effects of butein on migration and invasion of SK-HEP-1 human hepatocarcinoma cells as well as to explore the underlying mechanisms. Our results indicated that butein is effective in down-regulating the migration and invasion of SK-HEP-1 human hepatocarcinoma cells through mitogen-activated protein kinases (MAPKs) and uPA signaling multiple pathways and then interacting with the levels of MMP-2, -7, and -9.

MATERIALS AND METHODS

Compounds and Reagents. Butein, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Butein was

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dissolved in DMSO and stored at −20 °C. The purity was >98%, as assessed by high-performance liquid chromatography (HPLC). Control cultures received the carrier solvent (0.5% DMSO). All of the antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Abcam (Science Park, Cambridge, United Kingdom).

Cell Culture, Morphology, and Cell Viability Assay. The human hepatocarcinoma cell line (SK-HEP-1) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 units/mL penicillin at 37 °C in an atmosphere of 5% CO_2. All culture medium and regents were obtained from Invitrogen Life Technologies (Carlsbad, CA). SK-HEP-1 cells (2×10^5 cells/well) maintained in 12-well plates with DMEM for 24 h and were treated with 0, 50, or 75 μM butein and incubated at 37 °C and 5% CO_2 and 95% air for 24 and 48 h. Cells then were photographed under a phase-contrast microscope and harvested from individual wells by centrifugation. For viability determination, isolated cells from each treatment onto 96-well plates were measured by using the MTT assay as previously described.[23,24]

Cell Migration Assay. Analysis of migration in SK-HEP-1 cells was assessed using an Oris Universal Cell Migration Assembly kit (Platypus Technologies, Madison, WI) following the manufacturer's instructions.[25,26] Briefly, SK-HEP-1 cells were first stained and labeled with 1 μM CellTracker Green CMFDA (Invitrogen Life Technologies) for long-term staining.[27] Cells were incubated for 45 min under growth conditions and then centrifuged and washed twice with cultured medium. Labeled SK-HEP-1 cells (1.5×10^4/100 μL) were seeded into each test well of the Oris plate with the well inserts (stoppers) and then incubated at 37 °C in a 5% CO_2 humidified chamber to permit cell attachment. After overnight incubation, the stoppers in each well were removed, and then, the wells were washed with PBS. SK-HEP-1 cells were incubated with 0.5% DMSO (as a control) and butein (50 or 75 μM), and migrated cells were determined and images of each well were taken for 0, 24, and 48 h periods of time by using a fluorescence microscope.[25,28] Migrated cells were assessed by area closure of the detection zone by using ImageJ software (Bethesda, MD) and calculated as follows: $[1 - (\text{control for 0 h})_{\text{area}}/(\text{treated groups})_{\text{area}}] \times 100$.

Boyden Chamber Assay for Cell Invasion and Migration. Cell invasion and migration assay were conducted with or without Matrigel (BD Biosciences, Franklin Lakes, NJ), respectively, by cell migration assay and invasion system as described previously.[29,30] Briefly, cell invasion was determined by using Matrigel-coated transwell cell culture chambers (8 μm pore size; Millipore Corp., Billerica, MA) as previously described.[29,30] SK-HEP-1 cells were kept for 24 h in serum-free medium and then were trypsinized and resuspended in serum-free DMEM and placed in the upper chamber of the transwell insert (5×10^4 cells/well) and incubated with 0.5% DMSO and butein (50 or 75 μM), and DMEM containing 10% FBS was added to the lower chamber. The plates were incubated in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C for 24 and 48 h. Noninvasive cells in the upper chamber were removed by wiping with a cotton swab, and invasive cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and stained with 2% crystal violet in ethanol. Cells in the lower surface of the filter that penetrated through the Matrigel were counted under a light microscope at 200×. Cell migration was determined as described for cell invasion assay except that the filter membrane was not coated with Matrigel. Cells located on the underside of the filter were counted under a light microscope at 200× magnification.[29,30]

Gelatin Zymography Assay. The secretions of MMP-2 and MMP-9 into culture medium were assayed by gelatin zymography as described previously.[31,32] SK-HEP-1 cells (5×10^5 cells/well) were cultured in 12-well plates and then treated with butein (50 or 75 μM) in each well with serum-free medium for 24 and 48 h. The conditioned medium was harvested and then centrifuged. The concentrated culture supernatant was resuspended in nonreducing loading buffer and incubated at 37 °C for 15 min. All samples were performed in 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) that had been cast with 0.2% gelatin. We then performed the electrophoresis, and at the end of electrophoresis, gels were incubated in renaturing buffer (2.5% Triton X-100) for 30 min, followed by incubation for 16 h at 37 °C in a developing buffer [50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$, and 150 mM NaCl]. Finally all gels were subsequently stained with Coomassie Brilliant Blue R-250 and destained by using 30% methanol and 10% acetic acid to detect gelatinase secretion.[31−33]

Western Blotting Analysis. The cell migration and invasion association protein expression in SK-HEP-1 cells were performed as previously.[29,34] SK-HEP-1 cells (1×10^6 cells/well) were cultured with DMEM in 6-well plates for 24 h. Then, butein was added to cells (each well) at a final concentration of 50 or 75 μM, while DMSO (solvent) alone was added to cells as control cells. All cells from experimental and control groups were incubated at 37 °C for 0, 24, and 48 h. Cells were harvested by centrifugation and then were resuspended in the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea). The homogenate was centrifuged at 13000*g* for 10 min at 4 °C to remove cell debris. The supernatant was collected, and total protein was determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin (BSA) as the standard. SDS gel electrophoresis and Western blotting were conducted to determine the effects of butein on protein levels as described previously.[29,35] After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes and blotted with the relevant antibodies, including anti-MMP-2, -9, and -7, uPA, and VEGF, which were purchased from Abcam as well as SOS1, GRB2, FAK, Rho A, ROCK1, Ras, MEKK3, ERK1/2, JNK1/2, p-p38, and p-c-jun, which were obtained from Santa Cruz Biotechnology, Inc. These blots then were detected by an enhanced chemiluminescence reagent (ECL, Amersham Biosciences, Piscataway, NJ) before we conjugated the secondary antibodies for 1 h at 37 °C. The bands were obtained and quantified using NIH ImageJ software.[34,36]

ERK, JNK, and p38 Activities Assay. The indicated substrate (ERK, MBP; JNK, ATF2; and p38, MBP) and Lipid Activator were prepared in the base reaction buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/mL BSA, 0.1 mM Na_3VO_4, 2 mM DTT, and 1% DMSO). Next, we delivered all required cofactors (1.5 mM $CaCl_2$, 16 μg/mL Calmodulin, and 2 mM $MnCl_2$) to the substrate solution mentioned previously. Afterward, the indicated kinase was sent into the substrate solution and mixed gently. Butein (7.81, 15.6, 31.3, 62.5, and 125 μM in DMSO) was then delivered into the kinase reaction mixture. ^{33}P-ATP (specific activity 0.01 μCi/μL final) was delivered into the reaction mixture to start the necessary reaction. Finally, we incubated the kinase reaction with ERK, JNK, and p38 proteins samples (20 μL) for 120 min at room temperature. Reactions are spotted onto P81 ion exchange paper (Whatman #3698-915, Maidstone, England, United Kingdom). We next extensively washed filters in 0.1% phosphoric acid and counted.[37−39]

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from butein-treated SK-HEP-1 cells by using the Nuclear Extraction Kit (Panomics, Inc., Redwood City, CA). The protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). The DNA-binding abilities were evaluated using the biotin-labeled oligonucleotide NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) probe.[40,41] Nuclear extract proteins were used with an EMSA kit (Panomics, Inc.) according to the protocol of the manufacturer. Five micrograms of nuclear extracts was subjected to denaturing 6% polyacrylamide native gel. The DNA was then rapidly transferred to a positive nylon membrane, UV cross-linked, probed with streptavidin-HRP

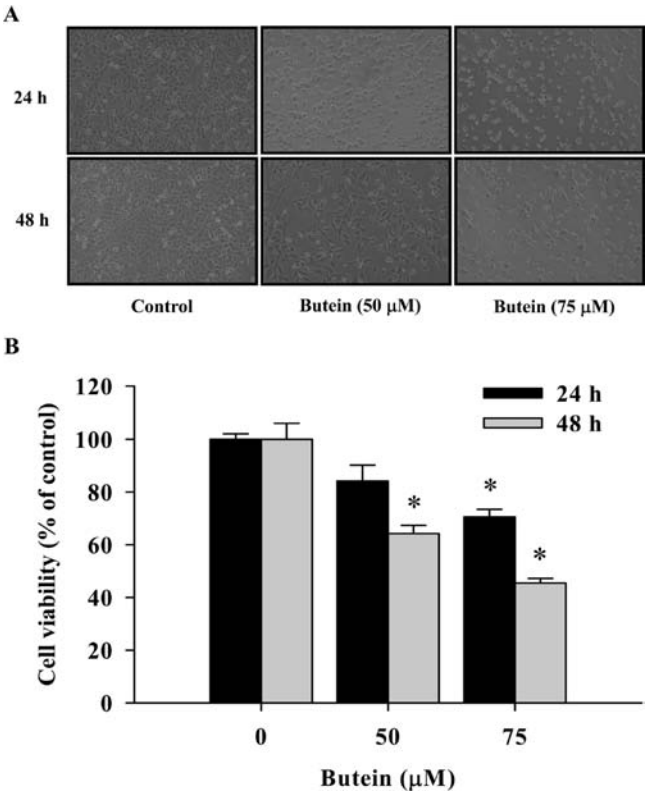

Figure 1. Butein affected the morphology and percentage of viable SK-HEP-1 human hepatocarcinoma cells. SK-HEP-1 cells were incubated with or without 50 or 75 μM butein for 24 and 48 h. Then, cells were examined and photographed by a phase-contrast microscope for morphological changes. Cells also were harvested for measuring the cell viability as described in the Materials and Methods. $^{*}p < 0.05$, significant difference between butein-treated groups and the control as analyzed by Student's t test.

conjugate, and incubated with the substrate of ECL kit (Millipore) as priviously described.[42,43]

Statistical Analysis. All results were expressed in triplicate as means ± SDs. Differences between the butein-treated and control groups were analyzed by Student's t test, with values of $^{*}p < 0.05$ and $^{***}p < 0.001$ considered significant.

RESULTS

Butein Affected the Morphology and Percentage of Viable SK-HEP-1 Human Hepatocarcinoma Cells. For examining the biological effects of butein, SK-HEP-1 cells were treated with 0, 50, or 75 μM butein for 24 and 48 h, and cell morphological changes and percentage of viable cells were determined. Results shown in Figure 1A,B indicated that butein induced cell morphological changes and the higher concentration of butein led to more cell morphological changes (Figure 1A). Butein decreased the percentage of viable cells (Figure 1B) through causing growth inhibition (Figure 1A,B) at the concentrations of 50 or 75 μM in SK-HEP-1 cells. Importantly, there were at least 50% viable cells as intervals of time increased in 50 or 75 μM butein treatment when compared to the control group ($p < 0.05$).

Butein Inhibited the Motility of SK-HEP-1 Cells in Vitro. To investigate the effect of butein on migration of SK-HEP-1 cells, cells were treated with different concentrations of butein (0, 50, or 75 μM) for 24, 48, and 72 h, and then, analysis of

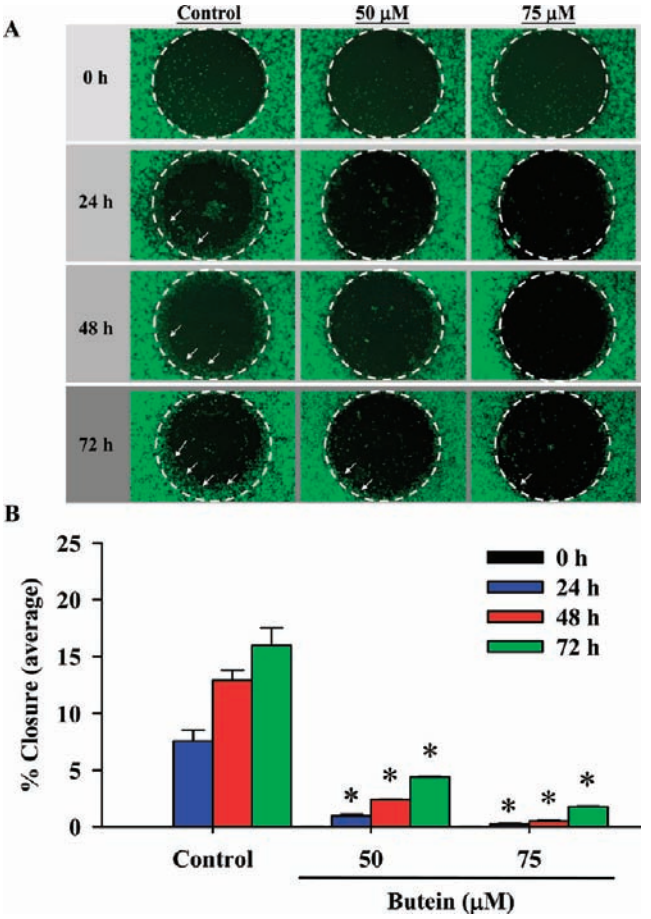

Figure 2. Butein suppressed the cell migration of SK-HEP-1 cells. Green-colored images of cells were prestained with CellTracker Green CMFDA, and then, cells were seeded on an Oris plate in the presence of vehicle only (control), 50 or 75 μM butein for 0, 24, 48, and 72 h (A). Quantification of area closure calculated from measured areas at the 0 h treatment in the control group and migration (B). Data represented the mean ± SD of at least three wells per condition.

migration in SK-HEP-1 cells was assessed using an Oris Universal Cell Migration Assembly kit. The results shown in Figure 2 indicated that butein at 50 or 75 μM significantly inhibited migration and motility of SK-HEP-1 cells (Figure 2A,B). These observations indicated that butein inhibited the migration of SK-HEP-1 cells in a dose- and time-dependent manner.

Butein Suppressed the Migration and Invasion of SK-HEP-1 Cells in Vitro. To investigate the effect of butein on migration and invasion of SK-HEP-1 cells, cells were treated with different concentrations of butein (0, 50, or 75 μM) for 24 and 48 h and seeded on transwell with uncoated (for migration) or Matrigel-coated (for invasion) filters. After 24 or 48 h of incubation, we examined migration activity and invasive potential of SK-HEP-1 cells, and the results are shown in Figure 3. Figure 3A,B indicates that butein at 50 or 75 μM significantly inhibited the migration of SK-HEP-1 cells. Figure 3C,D shows that both doses of butein significantly reduced the invasion of SK-HEP-1 cells. These observations indicated that butein suppressed the migration and invasion of SK-HEP-1 cells in dose- and time-dependent manners.

Quantitative Evaluations of MMPs Activities Were Shown in Butein-Treated SK-HEP-1 Cells by Gelatin Zymography. We investigated the inhibitory effects of butein on MMP-2 and -9

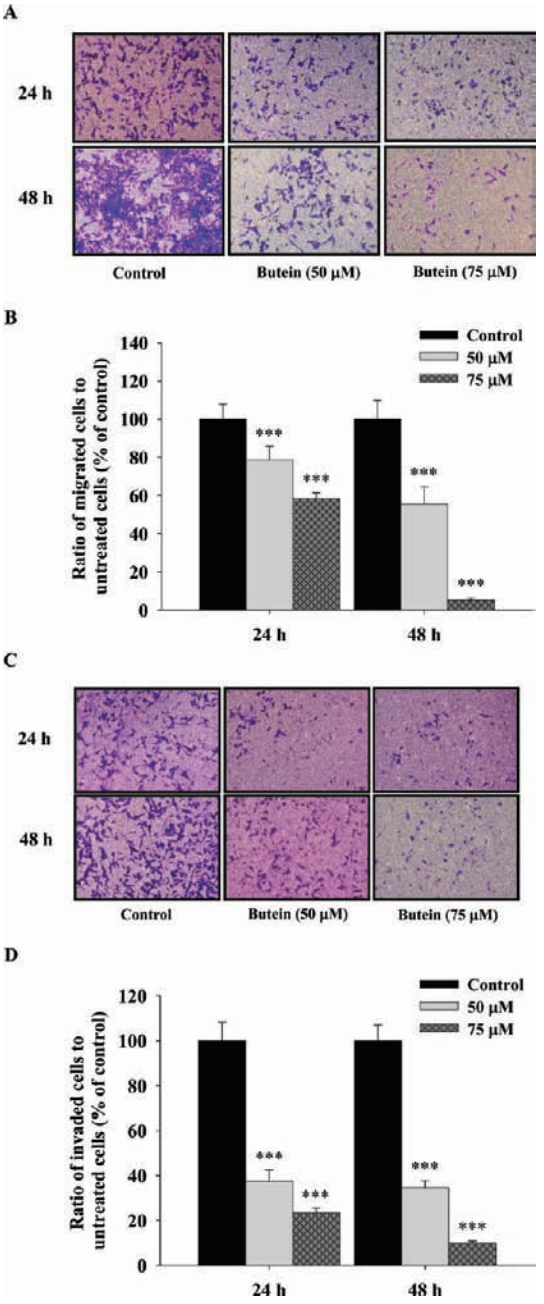

Figure 3. Butein suppressed the migration and invasion of SK-HEP-1 cells in vitro. SK-HEP-1 cells in 5×10^4 cells/well that penetrated through without or with the Matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at 200× (A and C). Quantification of cells in the lower chambers was performed by counting cells at 200× (B and D). Columns repeat the mean from three independent experiments. $^{***}p < 0.001$, significant difference between butein-treated groups and the control as analyzed by Student's *t* test.

productions by zymography in SK-HEP-1 cells. Cells were constitutively secreted highly of MMP-2 and low levels of MMP-9 (Figure 4A). Gelatin zymography assay indicated that the SK-HEP-1 cells secreted the 72 kDa protein with gelationlytic activity, corresponding to pro-MMP-2. As shown in Figure 4A, gelationlytic activity of MMP-2 and -9 in the zymogram was

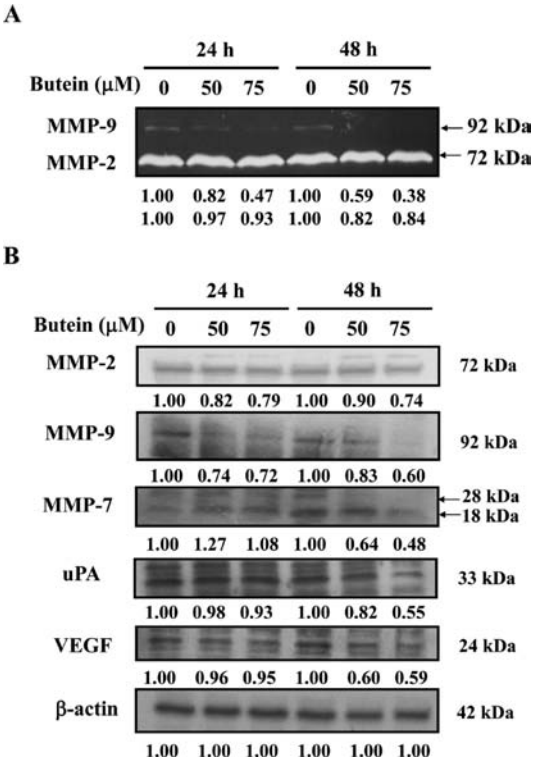

Figure 4. Quantitative evaluations of MMPs activities and protein levels in butein-treated SK-HEP-1 cells were determined by gelatin zymography and Western blotting analyses. Representative zymogram to detect the activity of secreted MMP-2 and MMP-9 using conditioned medium from SK-HEP-1 cells (A). The different activities of MMP-2 and -9 were determined by densitometric analysis. Cells from each treatment were also harvested for Western blotting to measure the changes of associated proteins of migration and invasion such as MMP-2, -9, and -7, uPA, and VEGF (B). β-Actin: a loading control.

inhibited by butein in a dose-dependent manner. Thus, both MMP-2 and -9 were down-regulated in butein-treated SK-HEP-1 cells.

Representative Western Blotting Analyses Demonstrated That Butein Affected the Changes in the Levels of Migration and Invasion-Associated Proteins in SK-HEP-1 Cells. Western blotting analysis was used to investigate the levels of invasion- and migration-associated proteins in SK-HEP-1 cells after treatment with butein for different time periods, and the results are shown in Figures 4B and 5. The data indicated that the levels of MMP-2, -9, and -7, uPA and VEGF (Figure 4B), SOS1, GRB2, FAK, Rho A, ROCK1, Ras, MEKK3 (Figure 5A) and ERK1/2, JNK1/2, p-p38, and p-c-Jun (Figure 5B) were lower in butein-treated cells than that of control cells.

Effects of Butein on NF-κB Activity in Vitro. SK-HEP-1 cells, after exposure to 50 μM butein, were checked for NF-κB activity by using EMSA. The results as can be seen in Figure 7 indicated that butein at 50 μM might inhibit the NF-κB activity, which is able to involve cell migration and invasion of SK-HEP-1 cells.

Effects of Butein on ERK, JNK, and p38 Activities in Vitro. Different concentrations of butein were determined in vitro kinases for ERK, JNK, and p38 activities that are shown in Figure 6, which indicated that butein at 15.6−125 μM inhibited the ERK and JNK activities and that at 62.5 and 125 μM suppressed the p38 activity. Also, these effects are a dose-dependent manner.

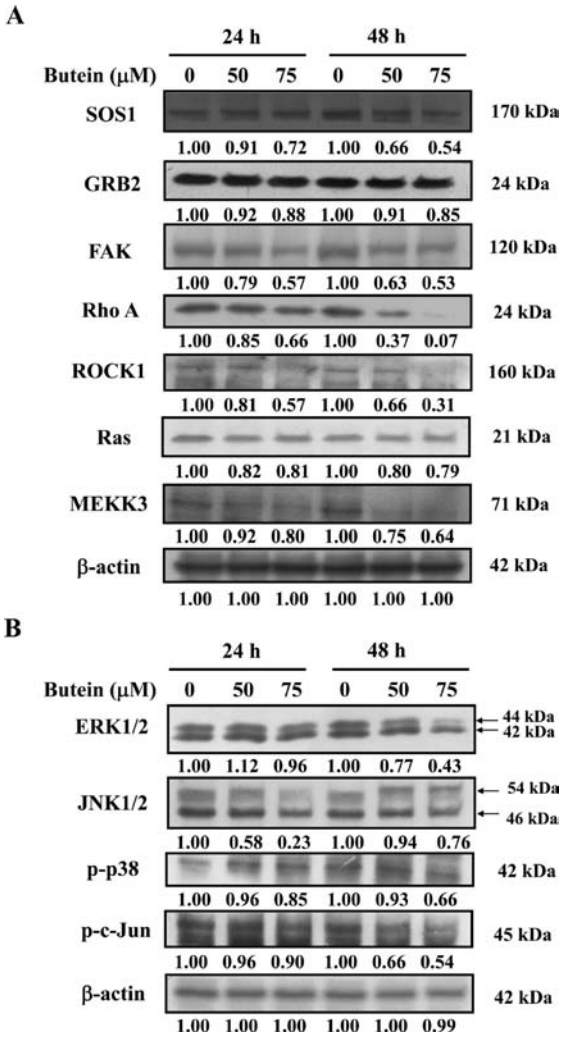

Figure 5. Representative Western blotting analysis demonstrated that butein affected the changes in the levels of cell migration and invasion-associated proteins in SK-HEP-1 cells. Cells (1×10^6 cells/well) were treated with butein at 0, 50, or 75 μM for different periods of time (0, 24, and 48 h). Cells were collected, and the total protein extracts were prepared and determined as described in the Materials and Methods. The levels of SOS1, GRB2, FAK, Rho A, ROCK1, Ras, and MEKK3 (A) as well as ERK1/2, JNK1/2, p-p38, and p-c-Jun (B) expressions were estimated by Western blotting as described in the Materials and Methods. β-Actin is used as a loading control.

DISCUSSION

HCC is the most common of all lethal malignancies and the third leading cause of cancer death worldwide. In the present study, we found that (1) butein effectively inhibits the migration and invasion of SK-HEP-1 human hepatocarcinoma cells; (2) butein inhibited the levels of GRB2, FAK, Rho A, and Ras in SK-HEP-1 cells; (3) butein inhibited the activities of ERK, JNK, and p38 in SK-HEP-1 cells; and (4) butein also inhibited the uPA with above effects led to the inhibitions of MMP-2/-9 via Ras, ERK, and uPA signaling pathways.

Herein, our results indicated that butein inhibited cell proliferation of liver cancer in dose-and time-dependent manners, which were in agreement with previous studies from other investigators who demonstrated that butein induced cytotoxic effects of hepatic stellate cells.[21] Butein inhibited the migration

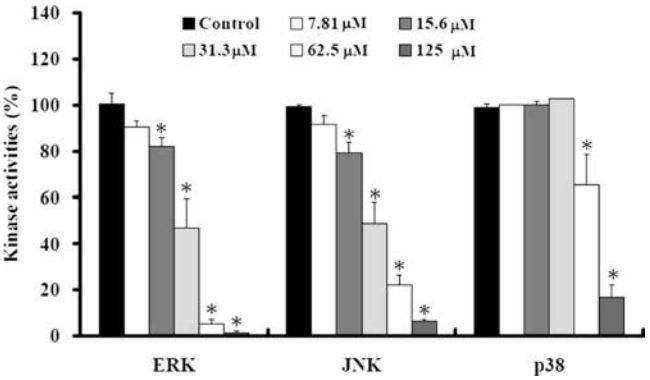

Figure 6. Butein decreased the ERK, JNK, and p38 activities by using in vitro kinase assay. Different concentrations (0, 7.81, 15.6, 31.3, 62.5, and 125 μM) of butein were measured for the activities of ERK, JNK, and p38 as described in the Materials and Methods. Data represent means ± SDs of three experiments. $^*p < 0.05$ was considered significant when compared to the control.

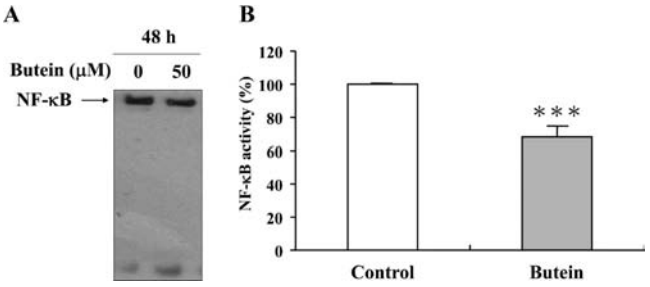

Figure 7. Butein reduced NF-κB activity in SK-HEP-1 cells. Cells were incubated with 50 μM butein for 48 h of exposure. Nuclear extracts were prepared from butein-treated SK-HEP-1 cells, and NF-κB activity was determined by EMSA (A). The NF-κB activity was calculated and quantified (B). $^{***}p < 0.001$ was considered a significant difference between the butein-treated group and the control.

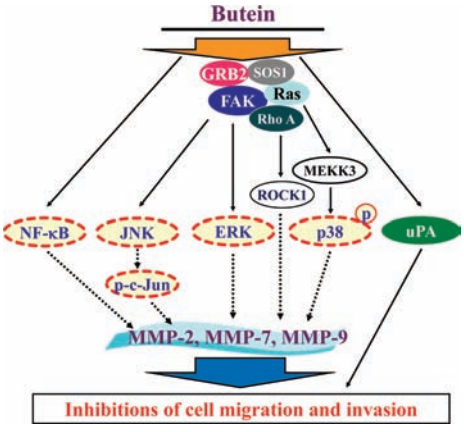

Figure 8. Possible signaling pathways for butein-inhibited cell invasion and migration in SK-HEP-1 human hepatocarcinoma cells.

and invasion of SK-HEP-1 cells, and then, we conducted further investigation for examining the effects whether or not it was through the inhibition of associated proteins. Thus, Western blotting and gelatin zymography assays were selected to investigate the protein levels in SK-HEP-1 cells. The results indicated

that butein inhibited the protein levels and activities of MMP-2, -7, and -9.

In our study, we found that butein decreased the levels of FAK and inhibited the downstream kinase activity such as ERK1/2 in SK-HEP-1 cells. These results suggest that the cooperation of FAK/Src with ERK1/2 plays a crucial role in butein-mediated cell migration in SK-HEP-1 cells. Furthermore, SK-HEP-1 cells were preincubated with U0126 (an ERK inhibitor) and then exposed to butein, resulting in enhancing the cell migration and invasion as well as MMP-2 and MMP-9 levels (data not shown). It was reported that FAK/Src signaling was implicated in ECM/integrin-mediated signaling pathways and plays an important role in tumor metastasis by increasing cell migration and invasiveness.[44,45] Other investigators also demonstrated that activated FAK (Tyr 397)/Src (Tyr 416) transducts signaling through multiple downstream targets, such as PI3K/AKT and Ras/ERK1/2 cascades in cancer cells.[46] It was reported that anticancer activity of butein may be involved in both ERK1/2 MAPK and NF-κB signaling pathway.[22,44] Our study investigated whether the MAPK pathway (ERK, JNK, and p38) is involved in butein-modulated migration and invasion of SK-HEP-1 cells and then regulated MMP-2, -7, and -9 levels in vitro.

Our results also showed that butein decreased the protein level of GRB2, and this is also in agreement with the other report that demonstrated that the formation of FAK/Src complex allows Src to phosphorylate Tyr 925 on FAK that will lead to mediate its interaction with growth factor receptor-bound protein 2 (GRB2), leading to the activation of Ras/ERK signaling pathway.[47] In the present study, we also found that butein decreased the protein levels of Ras (Figure 4). Our results also showed that butein decreased the levels of ERK1/2 and Rho A in SK-HEP-1 cells (Figure 4). Other reports also showed that the aberrant regulation of Rho proteins is associated with metastasis by promoting cancer angiogenesis and tumor cell motility.[48] Thus, our findings indicated that butein can be an effective inhibitor of ERK/Rho A signaling in SK-HEP-1 cells. In conclusion, we found that butein inhibited the migration and invasion of SK-HEP-1 cells through the inhibitions of FAK, GRB2, Rho A, and SOS1, leading to down-regulate ERK, JNK, and p38 or through uPA signal, resulting in the inhibitions of MMP-2, -7, and -9. Overall, in this study, utilizing the SK-HEP-1 liver cancer cell line, we have shown a novel anticancer effect of butein. This report also provided possible mechanisms responsible for their anti-invasive effects. Our further investigations will determine the tumor metastasis of liver cancer cells in vivo after butein exposure.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886 4 22053366 ext. 2161. Fax: +886 4 22053764. E-mail: jgchung@mail.cmu.edu.tw.

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