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RESEARCH ARTICLE

Gallic acid downregulates matrix metalloproteinase-2 (MMP-2) and MMP-9 in human leukemia cells with expressed Bcr/Abl

Ying-Jung Chen[1] *and Long-Sen Chang*[1,2]

[1] Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan
[2] Department of Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan

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Scope: The aim of the present study was to explore the signaling pathways associated with gallic acid induced matrix metalloproteinase-2 (MMP-2)/MMP-9 downregulation in human leukemia K562 cells.
Methods and results: Unlike the insignificant effect on human Bcr/Abl-negative leukemia U937 cells, gallic acid attenuated invasion of human Bcr/Abl-positive leukemia K562 cells with characteristic of decreased protein expression and mRNA levels of MMP-2 and MMP-9. Gallic acid induced β-TrCP upregulation evoked Bcr/Abl degradation in K562 cells, while overexpression of Bcr/Abl attenuated gallic acid induced MMP-2/MMP-9 downregulation. Overexpression of Bcr/Abl restored the levels of phospho-ERK and phospho-Akt but not JNK phosphorylation in gallic acid treated K562 cells. Gallic acid treatment repressed Akt/ERK-mediated c-Fos phosphorylation and JNK1-mediated ATF-2 phosphorylation. c-Jun inactivation was mediated through gallic acid induced Akt/ERK and JNK inactivation. Knockdown of c-Fos, c-Jun, and ATF-2 by siRNA and luciferase promoter assay reflected that c-Jun/ATF-2 and c-Jun/c-Fos were, respectively, responsible for MMP-2 and MMP-9 expression in K562 cells. Chromatin immunoprecipitating assay showed that gallic acid reduced the binding of c-Jun/ATF-2 and c-Jun/c-Fos with promoter region of MMP-2 and MMP-9 genes, respectively.
Conclusion: Our data indicate that MMP-2 and MMP-9 downregulation in gallic acid treated K562 cells are mediated through suppression of JNK1-mediated c-Jun/ATF-2 and Akt/ERK-mediated c-Jun/c-Fos pathways, respectively.

Keywords:
AP-1 / Bcr/Abl / Gallic acid / JNK1 / MMP-2/MMP-9

1 Introduction

Gallic acid (Fig. 1A) as a polyhydroxylphenolic compound is widely distributed in various plants, fruits, and foods [1]. Various biological activities of gallic acid have been reported, including antibacterial [2] and anti-inflammatory activities [3]. Moreover, anticancer activity of gallic acid has been reported in various cancer cells, such as leukemia [4, 5], prostate cancer [6–8], lung cancer [9–12], melanoma [13, 14], gastric, colon, breast, cervical, and esophageal cancer [15, 16]. Apoptosis induced by gallic acid is well demonstrated to be responsible for its anticancer activity [4, 17]. Gallic acid induced apoptosis is associated with mitochondria-mediated pathway activation in human lung cancer A549 cells, human leukemia HL-60 cells, and human melanoma A375.S2 cells [5, 12, 13], while gallic acid induced DNA damage elicits growth inhibition and cell cycle arrest in human prostate DU145 cells and PC-3 cells [6, 18]. You and Park [11] found that gallic acid induced lung cancer cell death is related to glutathione depletion and an increase in reactive oxygen species (ROS) generation. The gallic acid elicited death pathway is obviously cell-type dependent. Moreover, oral gallic acid feeding inhibits prostate cancer growth and progression to advanced-stage adenocarcinoma in mice model [19], suggesting the chemopreventive effect of gallic

Correspondence: Dr. Long-Sen Chang, Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan
E-mail: lschang@mail.nsysu.edu.tw
Fax: +886-7-5250197

Abbreviations: **ATF**, activating transcription factor; **ChIP**, chromatin immunoprecipitation; **CML**, chronic myeloid leukemia; **EGCG**, epigallocatechin-3-gallate; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **MMP-2**, matrix metalloproteinase-2; **VEGF**, vascular endothelia growth factor

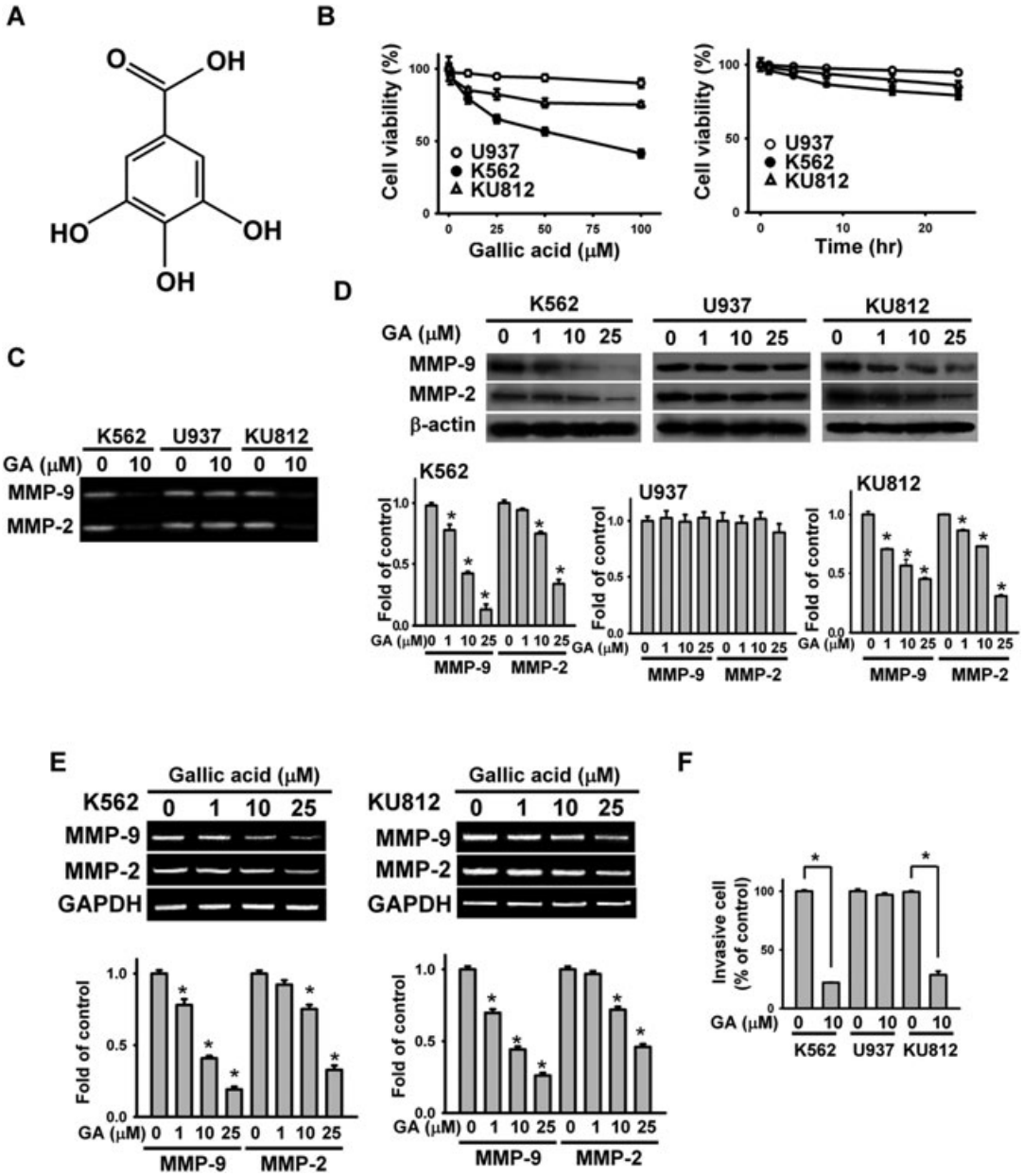


Figure 1. Effect of gallic acid on protein expression and mRNA levels of MMP-2 and MMP-9 in K562 cells, KU812 cells and U937 cells. (A) The structure of gallic acid. (B) Effect of gallic acid on viability of K562 cells, KU812 cells, and U937 cells. (Left panel) K562, KU812, and U937 cells were incubated with varying concentrations of gallic acid for 24 h. (Right panel) K562, KU812, and U937 cells were treated with 10 μM gallic acid for 24 h. Cell viability was determined by MTT assay. Results are expressed as the percentage of cell viability relative to the control. The values represent averages of three independent experiments with triplicated measurements (mean ± SD). (C) Gelatin zymography analyses of MMP-2 and MMP-9 on culture medium of gallic acid treated cells. K562, KU812, and U937 cells were treated with 10 μM gallic acid for 24 h. (D) Effect of gallic acid on MMP-2 and MMP-9 protein expression. K562, U937, and KU812 cells were treated with indicated concentrations of gallic acid for 24 h. Cell lysates were subjected to Western blot analyses. (Top panel) Western blot analyses. (Bottom panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$, gallic acid treated cells vs. control untreated cells). (E) Detecting the transcription of MMP-2 and MMP-9 mRNA using RT-PCR. K562 (left panel) and KU812 (right panel) cells were treated with indicated concentrations of gallic acid for 24 h. (Top panel) RT-PCR analyses. (Bottom panel) Quantification of MMP-2/MMP-9 expression from RT-PCR analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$, gallic acid treated cells vs. control untreated cells). (F) Effect of gallic acid on invasive ability of K562, KU812, and U937 cells. K562, KU812, and U937 cells were treated with 10 μM gallic acid, and invasion of leukemia cells were performed using a transwell assay as described in Section 2. Data represent mean ± SD of three independent experiments ($^{*}p < 0.05$, compared with control untreated cells).

acid on prostate cancer progression. On the other hand, gallic acid also exerts inhibitory effect on in vitro invasiveness in murine bladder cancer MB-49 cells, human glioblastoma U87 cells, human glioblastoma U25 cells, human gastric carcinoma AGS cells, human melanoma A375.S2 cells, human prostate cancer PC-3 cells, and human osteosarcoma U-2 OS cells [13, 14, 20–24]. These studies suggest that gallic acid induced matrix metalloproteinase (MMP) downregulation is involved in the gallic acid antimetastasis effect. Ho et al. [21] suggested that suppression of NFκB pathway and PI3K/Akt/small GTPase pathways may lead to MMP-2 and MMP-9 downregulation in gallic acid treated AGS cells. The results obtained by Liu et al. [23] revealed that blocking the p38 MAPK, JNK, protein kinase C, PI3K/Akt, and reducing the NFκB protein level are responsible for MMP-2/MMP-9 downregulation in gallic acid treated PC-3 cells. On the other hand, gallic acid treatment reduced the protein and mRNA level of MMP-2 in A375.S2 human melanoma cells via Ras pathway suppression [14], while suppression of protein kinase, mitogen-activated protein kinase, and PI3/Akt results in inhibiting MMP-2/MMP-9 expression in gallic acid treated U-2 OS cells [24]. However, the molecular mechanism of gallic acid in genetically regulating the expression of MMPs is not fully elucidated in these studies.

Increasing evidence suggests that MMPs promote tumor progression. The main role of MMPs in angiogenesis, tumor growth, and metastasis is degradation of extracellular matrix and release and/or activation of growth factors through their degradative activity [25]. MMP-2 and MMP-9 are presumed to be especially important for cell transmigration because these proteinases act on type IV collagen and other basement membrane components [26]. Our recent studies show that caffeine induces MMP-2/MMP-9 downregulation in human acute myeloid leukemia U937 cells [27], while caffeine treatment has insignificant effect on MMP-2/MMP-9 protein expression in human chronic myeloid leukemia (CML) K562 cells [28]. CML is characterized by the Philadelphia chromosome, which results from a reciprocal translocation between chromosome 9 and chromosome 22 [29]. This mutant gene encodes the constitutively active Bcr/Abl tyrosine kinase, which signals downstream to a variety of cytoprotective pathways including ERK, Akt, NF-κB, and Jak/STAT that collectively provide proliferative advantages and resistance to apoptosis [30, 31]. According to its role in malignant transformation, Bcr/Abl has served as a target for therapeutic intervention in CML [32, 33]. Noticeably, caffeine is unable to downregulate MMP-2 and MMP-9 protein expression in pcDNA3-Bcr/Abl (p210)-transfected U937 cells [28]. These results suggest that Bcr/Abl tyrosine kinase may play a role in MMP-2/MMP-9 genetic regulation of leukemia cells. This study was conducted to address (i) the Bcr/Abl tyrosine kinase effect on MMP-2/MMP-9 expression in gallic acid treated human leukemia cells and (ii) genetic regulation of gallic acid induced MMP-2/MMP-9 protein expression.

2 Materials and methods

Gallic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), U0126 (MEK1 and MEK2 inhibitor), SP600125 (JNK inhibitor), LY294002 (PI3K inhibitor), MG132, *p*-aminophenylmercuric acetate (APMA), quercetin, epigallocatechin-3-gallate (EGCG), syringic acid, methyl gallate, 3,4,5-trimethoxybenzoic acid, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO), and anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-ERK, anti-ERK, anti-JNK, anti-c-Fos, anti-c-Jun, anti-ATF-2, anti-c-Abl, and anti-β-Trcp antibodies were products of Cell Signaling Technology (Beverly, MA). Anti-MMP-2, anti-MMP-9, anti-phospho-JNK, anti-phospho-c-Fos (Ser374), anti-phospho-ATF-2 (Thr71), and anti-phospho-c-Jun (Ser73) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce (San Diego, CA). Cell culture supplies were purchased from GIBCO/Life Technologies (Carlsbad, CA). Unless otherwise specified, all other reagents were of analytical grade.

2.1 Cell culture and cell viability assay

Human CML K562 cells (Bcr/Abl-positive cells) and human acute myeloid leukemia U937 cells (Bcr/Abl-negative cells) were obtained from ATCC (Rockville, MD, USA). Human CML KU812 cells (Bcr/Abl-positive cells) were obtained from BCRC (Hsinchu, Taiwan). K562 cells, U937 cells, and KU812 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM glutamine and penicillin (100 units/mL)/streptomycin (100 μg/mL) in an incubator humidified with 95% air, and 5% CO_2. After specific treatment, cell viability was determined using MTT assay.

2.2 RNA preparation and RT-PCR

Total RNA was isolated from untreated control cells or gallic acid treated cells using the RNeasy minikit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. Reverse transcriptase reaction was performed with 2 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's recommendations. A reaction without reverse transcriptase was performed in parallel to ensure the absence of genomic DNA contamination. After initial denaturation at 95°C for 10 min, PCR amplification was performed using GoTaq Flexi DNA polymerase (Promega) followed by 30 cycles at 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s. After a final extension at 72°C for 5 min, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide transillumination under UV light. Primer sequences were as follows:

MMP-2 forward:
5′-GTGCTGAAGGACACACTAAAGAAGA-3′
MMP-2 reverse: 5′-TTGCCATCCTTCTCAAAGTTGTAG-3′
MMP-9 forward: 5′-A-3′GTGGCACCACCACAACAT-3′
MMP-9 reverse: 5′-TCCTGGGTGTAGAGTCTCTCG-3′
β-TrCP forward: 5′-TCTGCAACATAGGTTTAAGAT-3′
β-TrCP reverse: 5′-CACTTAGACAGACATACAACA-3′

The PCR reaction yielded PCR products of 323, 496, and 309 bp for MMP-2, MMP-9, and β-TrCP, respectively. Each reverse-transcribed mRNA product was internally controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR using primers 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TGTGGTCATGAGTCCTTCCA-3′ (reverse), yielding a 512 bp PCR product. The MMP-2 and MMP-9 reverse transcriptase-PCR products were subsequently confirmed by direct sequencing.

2.3 Gelatin zymography

Cells were incubated in serum-free medium overnight with or without gallic acid. Culture medium or cell lysates were mixed with SDS loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, and 0.02% bromophenol blue) and incubated for 30 min at 37°C. Samples were electrophoresed in a 10% polyacrylamide gel containing 0.1% gelatin. The gel was then washed in 2.5% Triton X-100 to remove SDS. The gel was incubated at 37°C for 48 h in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 5 mM $CaCl_2$. After staining with Coomassie Blue R-250, gelatinases were identified as clear bands.

2.4 In vitro cell invasion assays

Cell invasion was assessed using transwell (Millipore, Billerica, MA) insert using polycarbonate membranes of 8-μm pore size, and was performed using transwell precoated with Matrigel (Becton Dickinson Labware, Bedford, MA). In brief, 5×10^3 cells resuspended in a 100 μL serum-free medium containing indicated concentrations of gallic acid were placed in the upper chamber and allowed to invade for 24 h. The lower part of the transwell unit was filled with 10% fetal calf serum medium. The total number of invaded cells on the lower chamber (which were localized on the bottom surface of polycarbonate filters) were fixed in formaldehyde, stained with Giemsa solution, and counted under a microscopy. Each experiment was done in triplicate, and results from three independent experiments were expressed as mean ± SD.

2.5 Chromatin immunoprecipitation assays (ChIP)

K562 cells were grown on 10 cm dishes. Formaldehyde was directly added to the medium at a final concentration of 1% for 10 min at 37°C to cross-link nuclear proteins with genomic DNA. Cells were washed twice in ice-cold PBS before being scraped in PBS containing protease inhibitors (1 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 mM PMSF). Cells were centrifuged for 3 min at 2000 rpm and resuspended in 350 μL lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, and 10 mM EDTA) containing protease inhibitors on ice for 10 min. After brief sonication, the resulting supernatant contained DNA fragments ranging from ~200 to 1000 bp. To remove nonspecific protein binding, the supernatants were precleared with protein G plus/protein A agarose suspension (Calbiochem, San Diego, CA) for 1 h at 4°C with rotation. After tenfold dilution of the sonicated cell supernatants in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, and 1.2 mM EDTA, 167 mM NaCl) supplemented with protease inhibitors, supernatants were incubated with anti-c-Jun, anti-c-Fos, or anti-ATF-2 antibodies at 4°C with overnight rotation and then mixed with protein G plus/protein A agarose suspension for 1 h at 4°C with rotation. Monoclonal GFP antibody (Santa Cruz Biotechnology) was used as a control. The immunoprecipitates were then isolated by centrifugation and washed with ChIP buffer. Protein-DNA complexes were recovered from agarose beads with elution buffer containing 1% SDS and 0.1 M $NaHCO_3$. To reverse cross-links, chromatin complexes were incubated in 5 M NaCl for 6 h at 65°C. Then proteinase K was added for overnight at 37°C, and the DNA was recovered by phenol–chloroform extraction and ethanol precipitation. Pellets were dissolved in TE buffer and used as a template for PCR. Primers used for amplifying the −1359/−1108 region of the MMP-2 gene were 5′-CATCTCTGGGCCATTGTCAATGTTCC-3′ (forward) and 5′-CCTGTGACAACCGTCTCTGAGGAATG-3′ (reverse). Primers used for amplifying the -183/+37 of MMP-9 gene were 5′-GGTGTAAGCCCTTTCTCATGCTGGTG-3′ (forward) and 5′-CCAGGAGCACCAGGACCAGGGGC-3′ (reverse). PCR was performed using the cycling parameters: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for a total of 30 cycles. PCR products (252 bp for MMP-2, 221 bp for MMP-9) were separated on a 2% agarose gel stained with ethidium bromide and were visualized under UV light.

2.6 DNA transfection

The pCMV-MEK1 (expressed the constitutively active MEK1) and CA-Akt (constitutively activated myristoylated Akt) vectors were generous gifts from Dr. W. C. Hung (National Sun Yat-Sen University, Taiwan). The pcDNA3.1-Flag-β-TrCP-WT and -β-TrCPΔF plasmids were obtained from Dr. K. Y. Choi (Yonsei University, Korea). The pcDNA3-Bcr/Abl (p210) was obtained from Dr. B. J. Druker (Division of Hematology

and Medical Oncology, Oregon Health & Science University). The pSRα-3HA-JNKK2-JNK1 (JNKK2-JNK1 fusion protein acted as constitutively active c-Jun kinase) plasmid was kindly provided by Dr. A. Lin (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, China). The plasmids were transfected into K562 cells using Pipette-type Electroporator (MicroPorator-MP100, Digital Bio Tech. Co., Seoul, Korea).

2.7 RNA interference

c-Jun siRNA (catalog number sc-29223), c-Fos siRNA (catalog number sc-29221), ATF-2 siRNA (catalog number sc-29205), and negative control siRNA (catalog number sc-37007) were purchased from Santa Cruz Biotechnology. pKD-JNK1 shRNA and pKD-Negcon-V1 plasmids were purchased from Upstate Biotechnology. For the transfection procedure, cells were grown to 60% confluence, and c-Jun siRNA, c-Fos siRNA, ATF-2 siRNA, control siRNA, and pKD-JNK1 shRNA were transfected using Lipfectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.8 Luciferase activity assay

The luciferase construct, pGL3-MMP-9, containing the promoter region between −670 and +54 of human MMP-9 gene was provided by Dr. D. Boyd (Anderson Cancer Center, USA). Full-length human MMP-2 promoter-luciferase construct was in a eukaryotic expression vector pGL2 carrying a luciferase reporter gene downstream of the inserted MMP-2 gene was kindly provided by Dr. E. N. Benveniste (University of Alabama, USA). The plasmids and siRNA were cotransfected into K562 cells using Lipofetamine™ 2000 (Invitrogen). Luciferase assay was performed with the Luciferase Reporter Assay System (Promega).

2.9 Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was taken as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments with similar results. Results of Western blots and RT-PCR were quantified by a scanning densitometer. Fold changes in protein and mRNA expression were determined on the basis of β-actin and GAPDH loading control, respectively.

2.10 Other tests

MTT cell viability assay, Western blot analysis, measurement of intracellular ROS production and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) were performed in essentially the same manner as previously described [27, 34].

3 Results

As shown in Fig. 1B, treatment of cells with gallic acid ranging from 10 to 100 μM showed an approximately 3–5% decrease in viability of U937 cells. Compared with that on U937 cells, gallic acid exerted a notable cytotoxicity on K562 and KU812 cells. Treatment with 10 μM gallic acid for 24 h caused an approximate loss of 3, 10, and 20% viability of U937, KU812, and K562 cells, respectively (Fig. 1B). In order to reduce the effect of gallic acid induced cell death on MMP-2/MMP-9 expression, a single dose of 10 μM gallic acid concentration was employed to assess the effect of gallic acid on MMP-2/MMP-9 expression in leukemia cells. As shown in Fig. 1C, the results of gelatin zymography showed that treatment with 10 μM gallic acid notably attenuated the secretion of MMP-2 and MMP-9 of K562 and KU812 cells into the culture medium, while the secretion of MMP-2 and MMP-9 of U937 cells show no significant change after treatment with gallic acid. The molecular weights of MMP-2 and MMP-9 in zymography gel were approximately 64 and 92 kDa, respectively. After incubating the culture media of leukemia cells with 1 mM APMA for 1 h at 37°C, APMA-treated MMP2 showed the typically changed zymographic pattern: an increased band of the active form at 62 kDa and a decreased band of the proMMP-2 at 64 kDa (Supporting Information Fig. 1). A similar change in the MMP-9 pattern was also observed. Obviously, the secreted MMP-2 and MMP-9 in leukemia cells were latent but activatable.

Figure 1D shows that gallic acid treatment reduced protein expression of MMP-2 and MMP-9 in cell lysate of K562 and KU812 cells in a concentration-dependent manner, while MMP-2 and MMP-9 protein expression in U937 cells were not significantly reduced by gallic acid. Figure 1E reveals that transcriptional levels of MMP-2 mRNA and MMP-9 mRNA of gallic acid treated K562 and KU812 cells were lower than those of untreated control cells as evidenced by RT-PCR assay. Figure 1F shows that gallic acid inhibited invasion of K562 and KU812 cells but insignificantly affected invasion of U937 cells. These results suggested that gallic acid attenuated invasion of K562 and KU812 cells was mediated through downregulation of MMP-2 and MMP-9 at transcriptional level.

Given that K562 and KU812 cells are Bcr/Abl-positive cells, the role of Bcr/Abl in MMP-2/MMP-9 expression was examined. Figure2A shows that gallic acid downregulated Bcr/Abl protein expression in K562 and KU812 cells. Overexpression of Bcr/Abl (p210) suppressed the ability of gallic acid to downregulate MMP-2/MMP-9 protein expression and mRNA levels (Fig. 2B and C). Consistently, the results of gelatin zymography showed that gallic acid attenuated marginally the secretion of MMP-2 and MMP-9 in pcDNA3-Bcr/Abl (p210)-transfected K562 cells

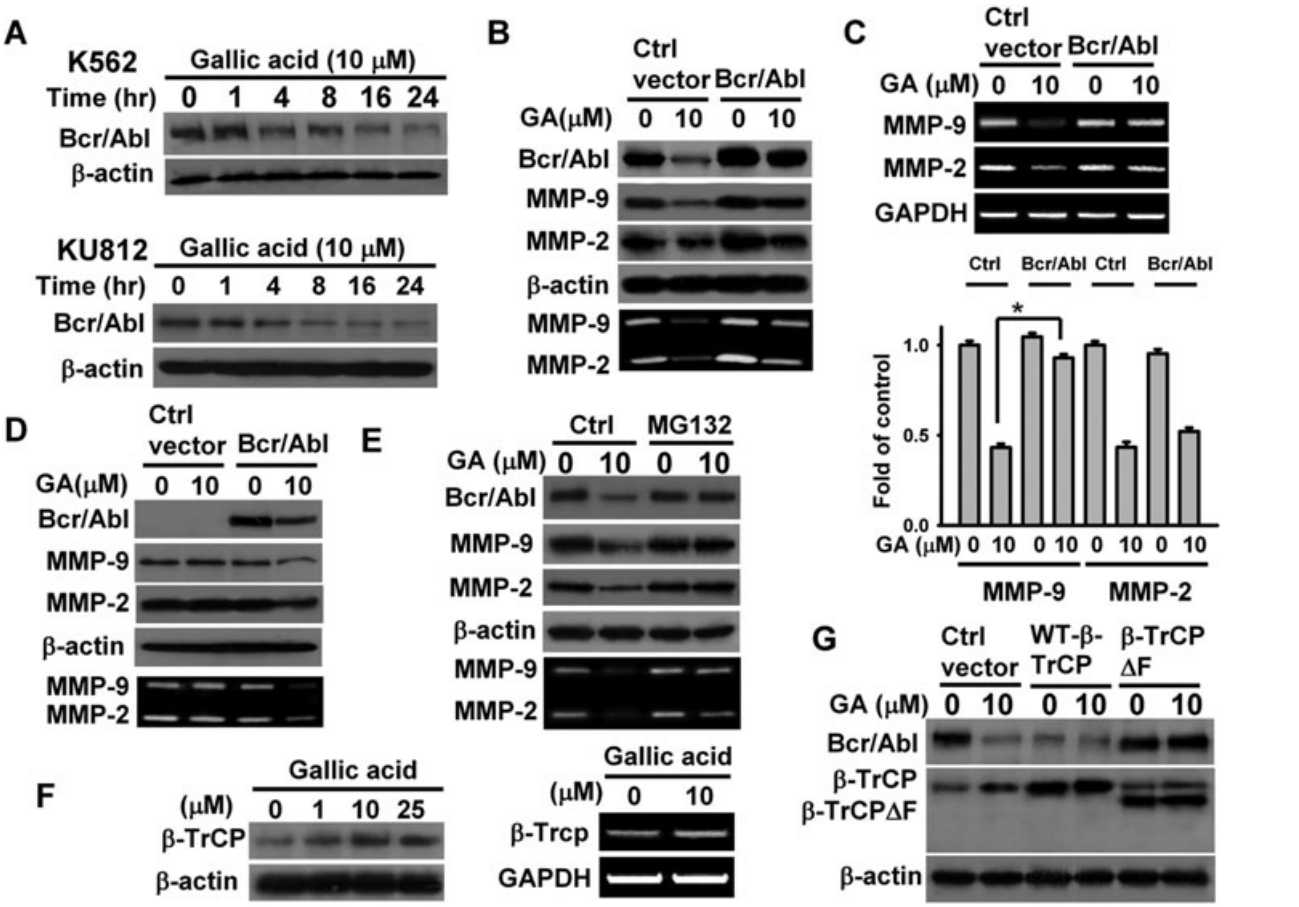


Figure 2. Effect of gallic acid induced Bcr/Abl downregulation on protein expression and mRNA levels of MMP-2 and MMP-9 in K562 cells. GA stands for gallic acid. (A) Effect of gallic acid on protein expression of Bcr/Abl. K562 and KU812 cells were treated with 10 μM gallic acid for indicated time periods. (B) Effect of gallic acid on MMP-2/MMP-9 protein expression in pcDNA3-Bcr/Abl (p210)-transfected K562 cells. K562 cells were transfected with an empty expression vector or pcDNA3-Bcr/Abl (p210), respectively. After 24 h post-transfection, the control vector-transfected cells and pcDNA3-Bcr/Abl (p210)-transfected cells were treated with 10 μM gallic acid for 24 h. (C) Effect of gallic acid on mRNA levels of MMP-2 and MMP-9 in pcDNA3-Bcr/Abl (p210)-transfected cells. K562 cells were transfected with an empty expression vector or pcDNA3-Bcr/Abl (p210), respectively. After 24 h post-transfection, the cells were treated with 10 μM gallic acid for 24 h. (Top panel) RT-PCR analyses. (Bottom panel) Quantification of MMP-2/MMP-9 expression from RT-PCR analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$). (D) Effect of gallic acid on MMP-2/MMP-9 protein expression in pcDNA3-Bcr/Abl (p210)-transfected U937 cells. U937 cells were transfected with an empty expression vector or pcDNA3-Bcr/Abl (p210), respectively. After 24 h post-transfection, the control vector-transfected cells and pcDNA3-Bcr/Abl (p210)-transfected cells were treated with 10-μM gallic acid for 24 h. (E) Effect of MG132 on gallic acid induced downregulation of Bcr/Abl, MMP-2/MMP-9 protein expression, and MMP-2/MMP-9 gelatinase activity. K562 cells were pretreated with 1 μM MG132 (proteasome inhibitor) for 1 h, and then incubated with 10 μM gallic acid for 24 h. (F) Effect of gallic acid on protein expression and mRNA level of β-TrCP. K562 cells were treated with indicated concentrations of gallic acid for 24 h. (Left panel) Western blot analyses. (Right panel) RT-PCR analyses. RT-PCR was conducted according to the procedure described in Section 2. (G) β-TrCP was involved in gallic acid induced downregulation of Bcr/Abl. K562 cells were transfected with plasmids encoding β-TrCP or β-TrCPΔF proteins. After 24 h post-transfection, cells were treated with 10 μM gallic acid for 24 h.

(Fig. 2B). Figure 2D shows that gallic acid induced downregulation of MMP-2/MMP-9 expression and the secretion of MMP-2/MMP-9 in pcDNA3-Bcr/Abl (p210)-transfected U937 cells. Moreover, gallic acid induced a reduction in Bcr/Abl expression in pcDNA3-Bcr/Abl (p210)-transfected U937 cells. These results suggested that overexpression of Bcr/Abl (p210) altered genetic regulation of MMP-2/MMP-9 in U937 cells. In fact, previous studies showed that cellular signaling pathways and phenotypic properties of U937 cells changed following Bcr/Abl expression [35, 36]. Cotransfection of pcDNA3-Bcr/Abl (p210) with p-EGFP into K562 cells showed that transfection efficiency exceeded by 80% as evidenced by examining GFP expression under fluorescent microscopy (data not shown), while Bcr/Abl protein expression in pcDNA3-Bcr/Abl (p210)-transfected K562 cells was approximately twofold that in control vector-transfected K562 cells. Compared with that of pcDNA3-Bcr/Abl (p210)-transfected U937 cells, gallic acid modestly reduced Bcr/Abl

expression in pcDNA3-Bcr/Abl (p210)-transfected K562 cells. This reflected that coexpression of endogenous and exogenous Bcr/Abl compensatorily maintained the protein expression in pcDNA3-Bcr/Abl (p210)-transfected K562 cells before and after gallic acid treatment.

Figure 2E shows that MG132 pretreatment suppressed gallic acid induced Bcr/Abl downregulation, reflecting that gallic acid induced increasingly Bcr/Abl degradation. Previous studies indicated that upregulating the β-TrCP expression, an F-box component of Skp1-Cul1-F-box protein E3 ubiquitin ligase, is linked with Bcr/Abl degradation in K562 cells [37]. Figure 2F shows that gallic acid induced upregulation of β-TrCP protein expression and mRNA level, suggesting that gallic acid transcriptionally regulated β-TrCP expression. Compared with that of the control vector, transfection of pcDNA3.1-Flag-β-TrCP-WT led to Bcr/Abl degradation regardless of gallic acid treatment (Fig. 2G). Overexpression of β-TrCPΔF, which acts as a dominant-negative mutant [38], increased the stability of Bcr/Abl in gallic acid treated K562 cells (Fig. 2G), thus resulting in accumulation of higher level of Bcr/Abl relative to the wild-type β-TrCP control. Consistently, gallic acid induced inappreciably Bcr/Abl downregulation in pcDNA3.1-Flag-β-TrCPΔF-transfected K562 cells. Meanwhile, overexpression of wild-type β-TrCP in K562 cells resulted in enhanced Bcr/Abl ubiquitination (Supporting Information Fig. 2). These observations indicated that β-TrCP-mediated Bcr/Abl degradation led to MMP-2/MMP-9 downregulation in gallic acid treated K562 cells.

Previous studies revealed that Bcr/Abl regulates phosphorylation of Akt and ERK [30, 39]. Thus, the effect of gallic acid on phosphorylation of MAPKs and Akt was examined. Figure 3A shows that phospho-JNK, phospho-ERK, and phosphor-Akt reduced notably in gallic acid treated K562 cells, while the level of phospho-p38 MAPK was not significantly altered by gallic acid treatment. Likewise, gallic acid reduced notably phosphorylation of Akt, ERK, and JNK in KU812 cells, while the level of phospho-p38 MAPK decreased marginally in gallic acid treated KU812 cells. Meanwhile, gallic acid marginally reduced the levels of phospho-p38 MAPK and phospho-Akt but not phospho-JNK and phospho-ERK in U937 cells. Transfection of Bcr/Abl (p210) abrogated the suppressed effect of gallic acid on Akt and ERK phosphorylation, but had no significant effect on the level of phospho-JNK (Fig. 3B). These findings suggested that gallic acid induced Bcr/Abl downregulation was involved in Akt and ERK inactivation, and that JNK inactivation was independent of Bcr/Abl-mediated pathway in K562 cells. On the other hand, gallic acid induced inactivation of Akt/ERK but insignificantly affected JNK and p38 MAPK phosphorylation in pcDNA3-Bcr/Abl (p210)-transfected U937 cells (Fig. 3C), again reflecting that gallic acid inactivated Akt/ERK in Bcr/Abl-expressing cells.

Treatment with LY294002 (PI3 kinase inhibitor), U0126 (MEK1 and MEK2 inhibitor) or SP600125 (JNK inhibitor) suppressed MMP-2/MMP-9 protein expression and the secretion of MMP-2/MMP-9 in the culture medium (Fig. 4A), emphasizing the notion that inactivation of ERK, Akt, and JNK was related to MMP-2/MMP-9 downregulation in K562 cells. Noticeably, treatment with LY294002 (PI3K inhibitor) or U0126 (MEK1 and MEK2 inhibitor) suppressed both Akt and ERK activation in K562 cells (Fig. 4A). Obviously, active Akt is crucial for ERK activation and vice versa. Consistent evidence of crosstalk between the Raf/MEK/ERK and Akt pathway has previously been described [40, 41]. Overexpression of either constitutively active Akt or constitutively active MEK1 attenuated gallic acid induced Akt/ERK inactivation, but had no significant effect on gallic acid induced JNK inactivation (Fig. 4B). Although transfection of constitutively active Akt or MEK1 suppressed gallic acid induced MMP-2/MMP-9 downregulation, the protein level of MMP-2 and MMP-9 could not restore to that of control untreated cells. This again suggested that Akt/ERK- and JNK-mediated pathway regulated MMP-2 and MMP-9 protein expression separately.

Given that c-Jun, c-Fos, and ATF-2 have been demonstrated to be involved in MMP-2/MMP-9 protein expression [42–47], the effect of gallic acid on phosphorylation of c-Jun, c-Fos, and ATF-2 was examined. Figure 5A shows that gallic acid treatment reduced c-Jun, c-Fos, and ATF-2 phosphorylation. Figure 5B shows that transfection of constitutively active Akt- and pCMV-MEK1 attenuated gallic acid induced c-Fos and c-Jun inactivation but restored insignificantly the level of phospho-ATF-2 in gallic acid treated cells. Moreover, phosphorylation of c-Fos and c-Jun was attenuated by LY294002 and U0126, while SP600125 reduced the levels of phospho-ATF-2 and phospho-c-Jun (Fig. 5C). These findings suggested that gallic acid induced c-Jun inactivation was mediated through inactivation of Akt/ERK and JNK, and that Akt/ERK and JNK were, respectively, responsible for c-Fos and ATF-2 phosphorylation in K562 cells. Transfection of JNK1 shRNA suppressed c-Jun and ATF-2 activation and downregulated MMP-2/MMP-9 protein expression in K562 cells (Fig. 5D), while transfection of pSRα-3HA-JNKK2-JNK1 (JNKK2-JNK1 fusion protein acted as constitutively active c-Jun kinase) restored the levels of phospho-ATF-2 and phospho-c-Jun in gallic acid treated cells (Fig. 5E). Moreover, overexpression of JNKK2-JNK1 fusion protein attenuated gallic acid induced MMP-2 downregulation and modestly rescued MMP-9 expression in gallic acid treated cells (Fig. 5E). As shown in Fig. 5F, knockdown of c-Jun expression by siRNA suppressed significantly protein expression of MMP-2 and MMP-9 in K562 cells. Transfection of c-Fos and ATF-2 siRNA suppressed selectively MMP-9 and MMP-2 protein expression, respectively. Taken together, these results suggested that MMP-2 and MMP-9 expression was regulated by c-Jun/ATF-2 and c-Jun/c-Fos, respectively. Consistently, the results of zymography analyses revealed that downregulation of c-Jun/ATF-2 and c-Jun/c-Fos by siRNA reduced, respectively, the secretion of MMP-2 and MMP-9 into the culture medium (Fig. 5F).

Figure 6A shows that gallic acid, LY294002, U0126, and SP600125 suppressed luciferase activity of MMP-2 and MMP-9 promoter constructs. Knockdown of c-Jun and ATF-2 by siRNA reduced luciferase activity of MMP-2 promoter

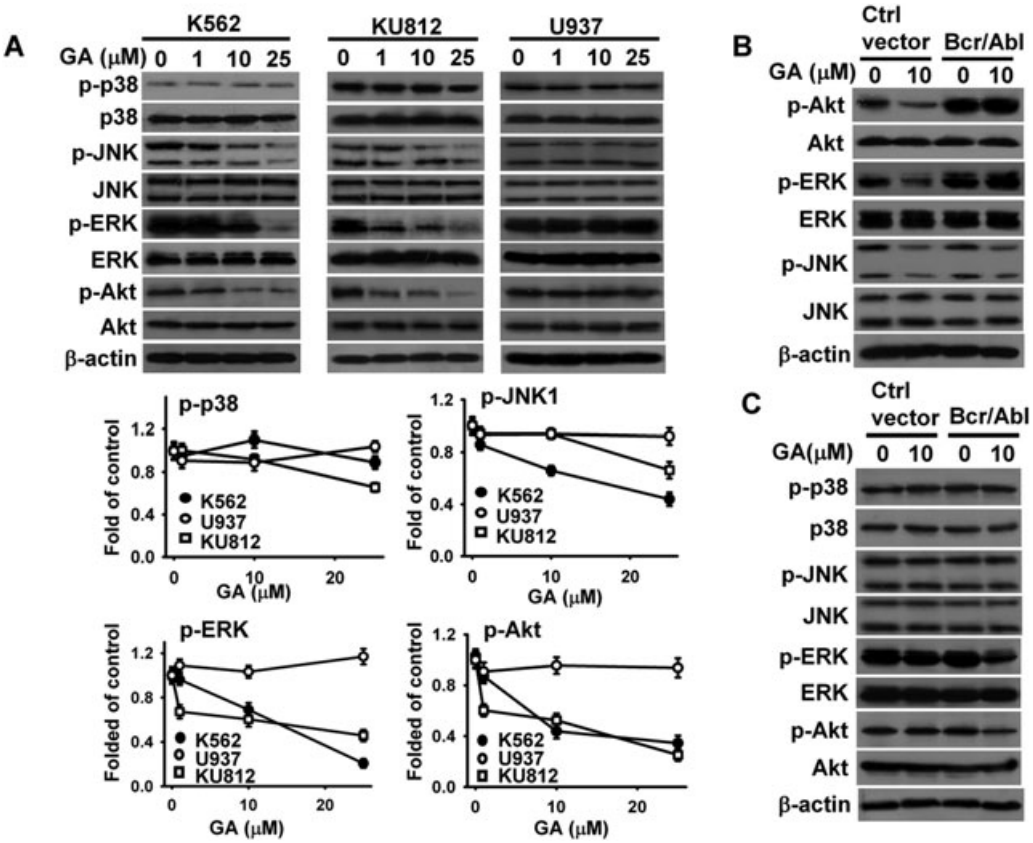


Figure 3. Effect of gallic acid on ERK, p38 MAPK, JNK, and Akt phosphorylation in K562, KU812, and U937 cells. GA stands for gallic acid. (A) Western blot analyses of phospho-p38 MAPK, phospho-ERK, phospho-JNK, and phospho-Akt in gallic acid treated cells. Cells were treated with indicated concentrations of gallic acid for 24 h. (Top panel) Western blot analyses. (Bottom panel) Quantification of phospho-p38 MAPK, phospho-ERK, phospho-JNK, and phospho-Akt from Western blot analyses. Three independent experimental results were analyzed by densitometry. (B) Overexpression of Bcr/Abl suppressed gallic acid induced inactivation of Akt and ERK. K562 cells were transfected with an empty expression vector or pcDNA3-Bcr/Abl (p210), respectively. After 24 h post-transfection, the cells were treated with 10 μM gallic acid for 24 h. (C) Gallic acid induced inactivation of Akt and ERK in pcDNA3-Bcr/Abl (p210)-transfected U937 cells. U937 cells were transfected with an empty expression vector or pcDNA3-Bcr/Abl (p210), respectively. After 24 h post-transfection, the cells were treated with 10 μM gallic acid for 24 h.

construct, while knockdown of c-Jun and c-Fos by siRNA reduced luciferase activity of MMP-9 promoter construct (Fig. 6B). Previous studies revealed that an activating protein-1 (AP-1) site at −1265 of MMP-2 promoter region was responsible for MMP-2 expression [47], while an AP-1 site at −79 of MMP-9 promoter region is crucial for MMP-9 expression [42]. The AP-1 transcription factor is a collective term referring to dimeric transcription factors composed of Jun, Fos, or activating transcription factor (ATF) subunits that bind to a common DNA site, the AP-1-binding site [48]. Thus, the binding of c-Jun, c-Fos, and ATF-2 on the −1359/−1108 region of MMP-2 gene and the −183/+37 region of MMP-9 gene was detected using chromatin immunoprecipitating assay. Chromatin immunoprecipitating assay showed that gallic acid reduced the binding of c-Jun and ATF-2 with MMP-2 promoter, and the binding of c-Jun and c-Fos with MMP-9 promoter (Fig. 6C and D). Meanwhile, no significant interaction between c-Fos and −1359/−1108 region of MMP-2 gene or between ATF-2 and −183/+37 region of MMP-9 gene was detected. These results suggested that suppression of c-Jun/ATF-2 and c-Jun/c-Fos was involved in gallic acid induced downregulation of MMP-2 and MMP-9, respectively.

4 Discussion

Our data show that gallic acid induced MMP-2/MMP-9 downregulation is mediated through Bcr/Abl degradation and JNK1 inactivation in K562 cells (Fig. 7). Bcr/Abl degradation further led to Akt/ERK inactivation in gallic acid treated K562 cells. Finally, suppression of Akt/ERK-mediated c-Jun/c-Fos phosphorylation and JNK1-mediated c-Jun/ATF-2 phosphorylation downregulates MMP-2/MMP-9 expression in gallic acid treated K562 cells. As shown in Supporting Information Fig. 3. gallic acid, syringic acid, and methyl gallate similarly suppressed MMP-2/MMP-9 expression in K562 cells.

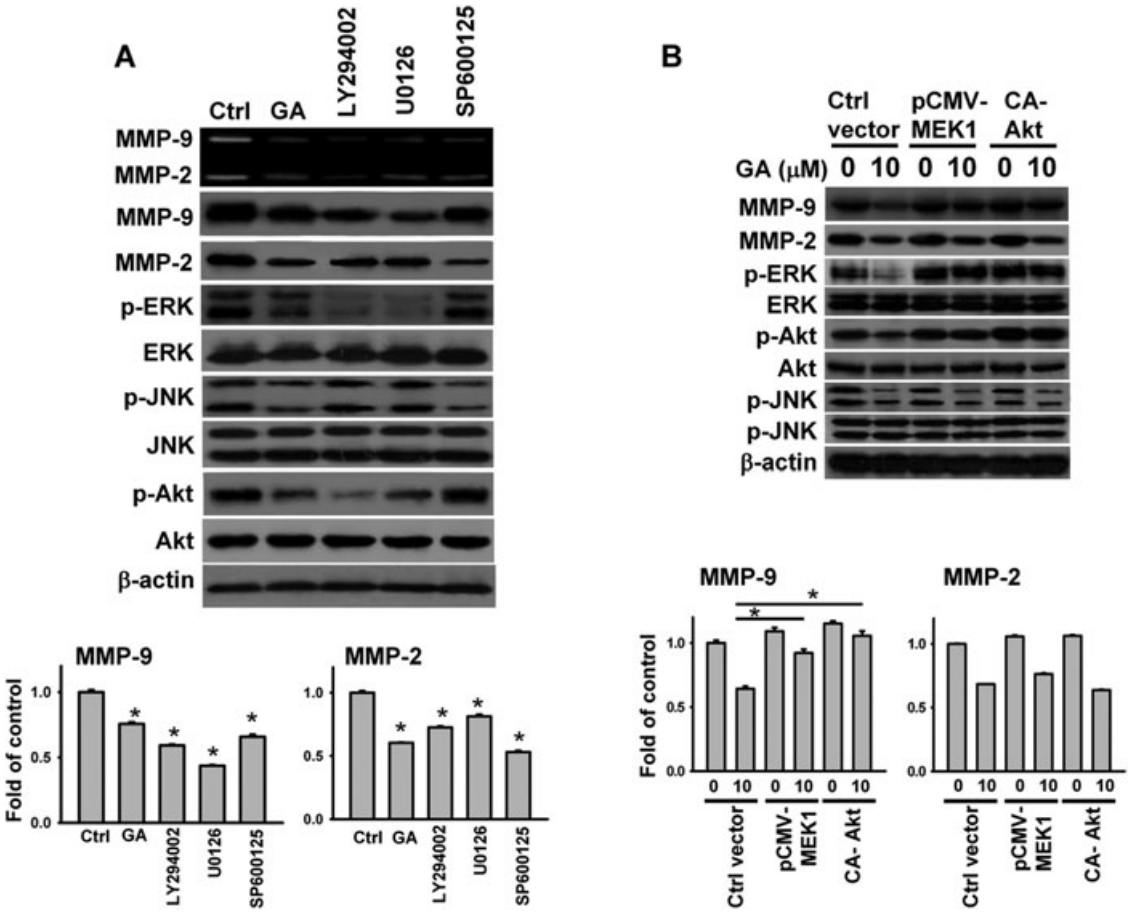
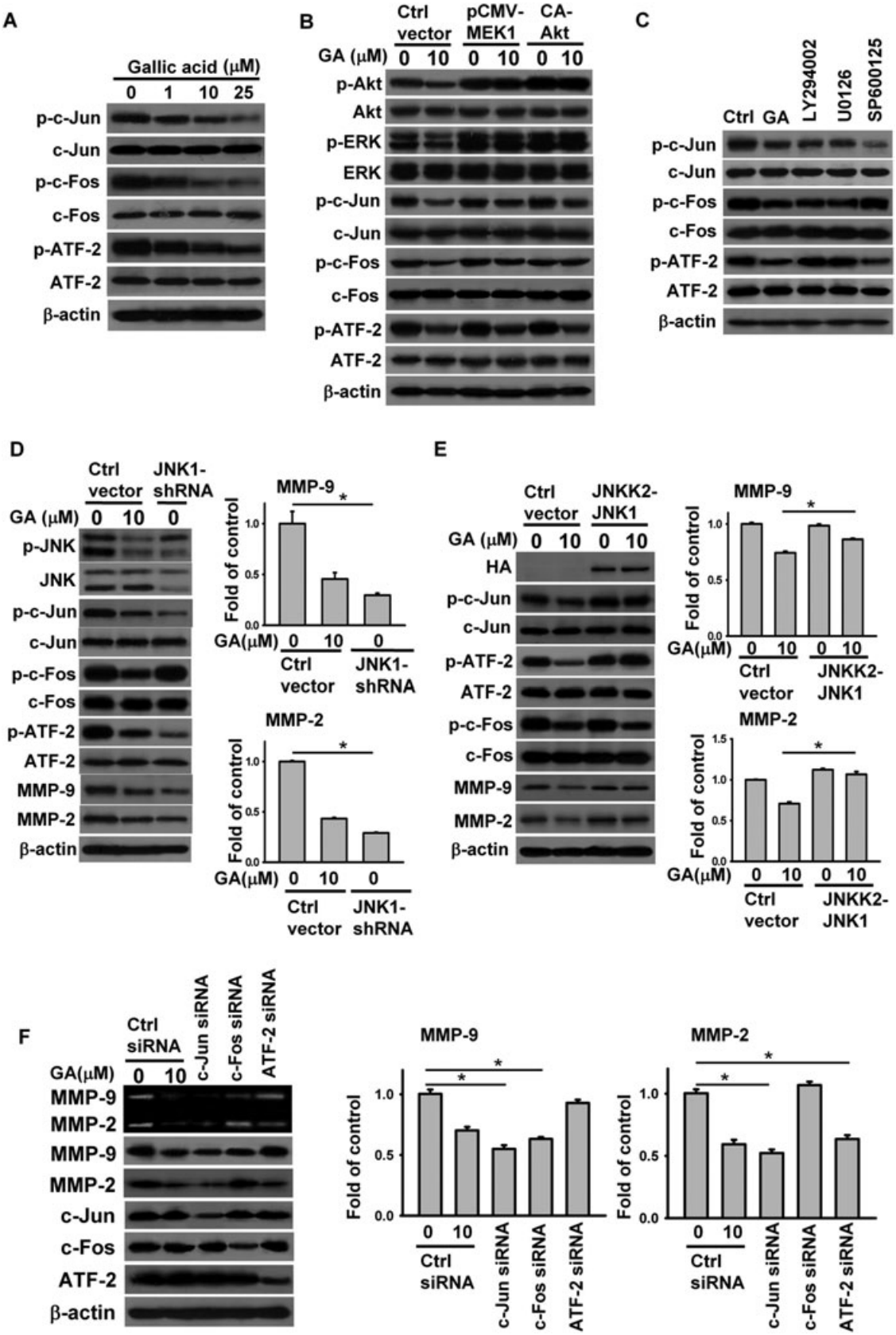


Figure 4. Gallic acid induced inactivation of Akt, ERK, and JNK was involved in MMP-2/MMP-9 downregulation in K562 cells. GA stands for gallic acid. (A) Effect of LY294002, U0126, and SP600125 on MMP-2/MMP-9 protein expression and gelatinase activity in K562 cells. K562 cells were pretreated with 10 μM gallic acid, 10 μM LY294002, 10 μM U0126, and 10 μM SP600125 for 24 h. (Top panel) Western blot analyses. (Bottom panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$, compared with control untreated cells). (B) Effect of constitutively active MEK1 and Akt on MMP-2/MMP-9 protein expression and gelatinase activity in gallic acid treated cells. K562 cells were transfected with an empty expression vector, pCMV-MEK1, or constitutively active Akt (CA-Akt), respectively. After 24 h post-transfection, the control vector-, pCMV-MEK1-, and CA-Akt-transfected cells were treated with 10 μM gallic acid for 24 h. (Top panel) Western blot analyses. (Bottom panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$).

Compared with gallic acid, syringic acid and methyl gallate, 3, 4, 5-trimethoxybenzoic acid marginally induced MMP-2/MMP-9 downregulation (Supporting Information Fig. 3B and C). These results suggested that the hydroxyl group at the *para*-position to the carboxylic group is important for the gallic acid effect on MMP-2/MMP-9 downregulation. Treatment with gallic acid induced an increase in $[Ca^{2+}]i$ but a reduction in ROS production in K562 cells (Supporting Information Fig. 4A and B). BAPTA-AM (Ca^{2+} chelator) pretreatment attenuated gallic acid induced downregulation of MMP-2/MMP-9 and JNK inactivation, but insignificantly altered gallic acid induced Bcr/Abl downregulation in K562 cells (Supporting Information Fig. 4C). These observations suggested that $[Ca^{2+}]i$ was responsible for gallic acid induced JNK inactivation, and indicated that gallic acid induced Bcr/Abl degradation and JNK inactivation were mediated through different pathway. The findings that gallic acid insignificantly affects the level of phospho-JNK in pcDNA3-Bcr/Abl (p210)-transfected U937 cells support this proposition. On the other hand, treatment with SP600125 (JNK inhibitor) modestly reduced MMP-2/MMP-9 expression in U937 cells (Supporting Information Fig. 5), indicating that the JNK pathway was related to MMP2/MMP-9 expression. However, gallic acid itself was unable to inactivate JNK in U937 cells, suggesting the absence of the signaling pathway responsible for gallic acid induced JNK inactivation in U937 cells.

Previous studies showed that the transepithelial rate of gallic acid is lower than other phenolic acid in either colon cancer Caco-2 cell monolayer or rats [49, 50]. Nevertheless, analyses on the bioavailability of polyphenols in human reveal



that gallic acid is better than the other polyphenols [51]. In humans, the plasma concentration for gallic acid was found to reach 1.8–2.1 μM at approximate 1.5 h with 50 mg gallic acid oral administration, and plasma gallic acid is undetectable after gallic acid intake for 12 h [52]. The studies of Roberts et al. [53] showed that approximately 8–10 μM concentration of gallic acid is detected in the serum of health volunteers after oral intake of a combination of dietary herbal supplement and 800 mg gallic acid. Obviously, the gallic acid concentration used in this study is physiologically attainable. Nevertheless, with respect to short elimination half-lives [51, 52], gallic acid should be combined with other chemotherapeutic drugs in suppressing the invasion of cancer cells. Alternatively, the finding that the gallic acid plasma concentration could be enhanced by repeated dosing in rats [54] suggests a manner for maintaining the gallic acid plasma concentration.

◀ **Figure 5.** Causal relationship of JNK, ERK, and Akt with phosphorylation of c-Fos, c-Jun, and ATF-2 in gallic acid treated cells. GA stands for gallic acid. (A) Effect of gallic acid treatment on levels of phospho-c-Jun, phospho-c-Fos, and phospho-ATF-2. K562 cells were treated with indicated concentrations of gallic acid for 24 h. (B) Effect of constitutively active MEK1 and Akt on phosphorylation of c-Jun, c-Fos, and ATF-2 in gallic acid treated cells. K562 cells were transfected with an empty expression vector, pCMV-MEK1, or constitutively active Akt (CA-Akt), respectively. After 24 h post-transfection, the control vector-, pCMV-MEK1-, and CA-Akt-transfected cells were treated with 10 μM gallic acid for 24 h. (C) Effect of LY294002, U0126, and SP600125 on levels of phospho-c-Jun, phospho-c-Fos, and phospho-ATF-2 in gallic acid treated K562 cells. K562 cells were treated with 10 μM gallic acid, 10 μM LY294002, 10 μM U0126, and 10 μM SP600125 for 24 h. (D) Effect of JNK1 shRNA on gallic acid induced MMP-2 and MMP-9 downregulation. K562 cells were transfected with control pKD-Negcon-V1 vector or pKD-JNK1 shRNA, respectively. After 24 h post-transfection, the control pKD-Negcon-V1 vector-transfected cells were treated with 10 μM gallic acid for 24 h. (Left panel) Western blot analyses. (Right panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$). (E) Effect of JNKK2-JNK1 on phosphorylation of c-Jun and ATF-2 and MMP-2/MMP-9 protein expression in gallic acid treated cells. K562 cells were transfected with an empty expression vector or pSRα-3HA-JNKK2-JNK1, respectively. After 24 h post-transfection, the control vector- and pSRα-3HA-JNKK2-JNK1-transfected cells were treated with 10 μM gallic acid for 24 h. (Left panel) Western blot analyses. (Right panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$). (F) Effect of c-Jun siRNA, c-Fos siRNA, and ATF-2 siRNA on gallic acid induced downregulation of MMP-2/MMP-9 protein expression and gelatinase activity. K562 cells were transfected with 100 nM control siRNA, c-Jun siRNA, c-Fos siRNA, and ATF-2 siRNA, respectively. After 24 h post-transfection, the control siRNA-transfected cells were treated with 10 μM gallic acid for 24 h. (Left panel) Western blot analyses. (Right panel) Quantification of MMP-2/MMP-9 protein expression from Western blot analyses. Three independent experimental results were analyzed by densitometry ($^{*}p < 0.05$).

In addition to gallic acid, other phytochemicals including flavonoids and catechins also show antimetastatic activity [55, 56]. Previous studies showed that EGCG (catechin) and quercetin (flavonoid) suppressed MMP-2/MMP-9 expression in cancer cells and inhibited MMP-2/MMP-9 activity [57–62]. To examine whether flavonoids and catechins downregulated MMP-2/MMP-9 expression in leukemia cells, K562 and U937 cells were treated with EGCG and quercetin. As shown in Supporting Information Fig. 6A, EGCG and quercetin reduced the viability of K562 and U937 cells in a concentration-dependent manner. In order to reduce the effect of EGCG- and quercetin-induced cell death on MMP-2/MMP-9 expression, 0–50 μM EGCG and 0–100 μM quercetin were employed to examine EGCG and quercetin effect on MMP-2/MMP-9 expression in K562 cells. On the other hand, 0–50 μM EGCG and 0–25 μM quercetin were employed to examine EGCG and quercetin effect on MMP-2/MMP-9 expression in U937 cells. As shown in Supporting Information Fig. 6B, EGEG insignificantly reduced MMP-2/MMP-9 expression in K562 cells compared with quercetin. Both EGCG and quercetin did not affect MMP-2/MMP-9 expression in U937 cells (Supporting Information Fig. 6C). Bcr/Abl protein expression and JNK phosphorylation remained unchanged in EGCG-treated K562 cells, while quercetin insignificantly affected Bcr/Abl protein expression but reduced JNK phosphorylation in K562 cells (Supporting Information Fig. 6B). These results indicated that, compared with EGCG and quercetin, gallic acid effectively suppressed MMP-2/MMP-9 expression in Bcr/Abl-positive leukemia cells via Bcr/Abl downregulation.

Previous studies show that the promoter regions of many MMPs, including MMP-1, -3, -7, -9, -10, -12, and -13 are highly conserved with respect to the AP-1 site that is located between −65 and −79 [63]. Consistently, activated c-Jun stimulated MMP-9 expression via the AP-1 site [64, 65]. Crowe and Brown [43] suggested that c-Fos binds to its response element at −79 on the MMP-9 promoter. These observations are in good agreement with our data showing that gallic acid reduces the binding of c-Fos and c-Jun with the AP-1 site at −79. In contrast to MMP-9 and other MMPs, the promoter region of the MMP-2 gene appears to lack the conserved AP-1 site at −79 [63]. Song et al. [44] and Singh et al. [47] found that a functional AP-1 site at position −1265 mediates MMP-2 transcription. Activation of ATF-2 and c-Jun that bind at the AP-1 site is suggested to be responsible for MMP-2 expression [44, 47]. Consistent with these observations, our data suggest that gallic acid induced c-Jun/ATF-2 inactivation suppresses MMP-2 expression. Taken together, it is conceivable that the AP-1 site at −79 of MMP-9 promoter and the AP-1 site at −1265 of MMP-2 promoter are responsive element for gallic acid treatment.

Previous studies showed that Bcr/Abl-transfected murine pro-B lymphocytic FL5.12 cells (an interleukin (IL)-3-dependent murine prolymphocytic cell line) show an increased secretion of MMP-2 and MMP-9 in the culture

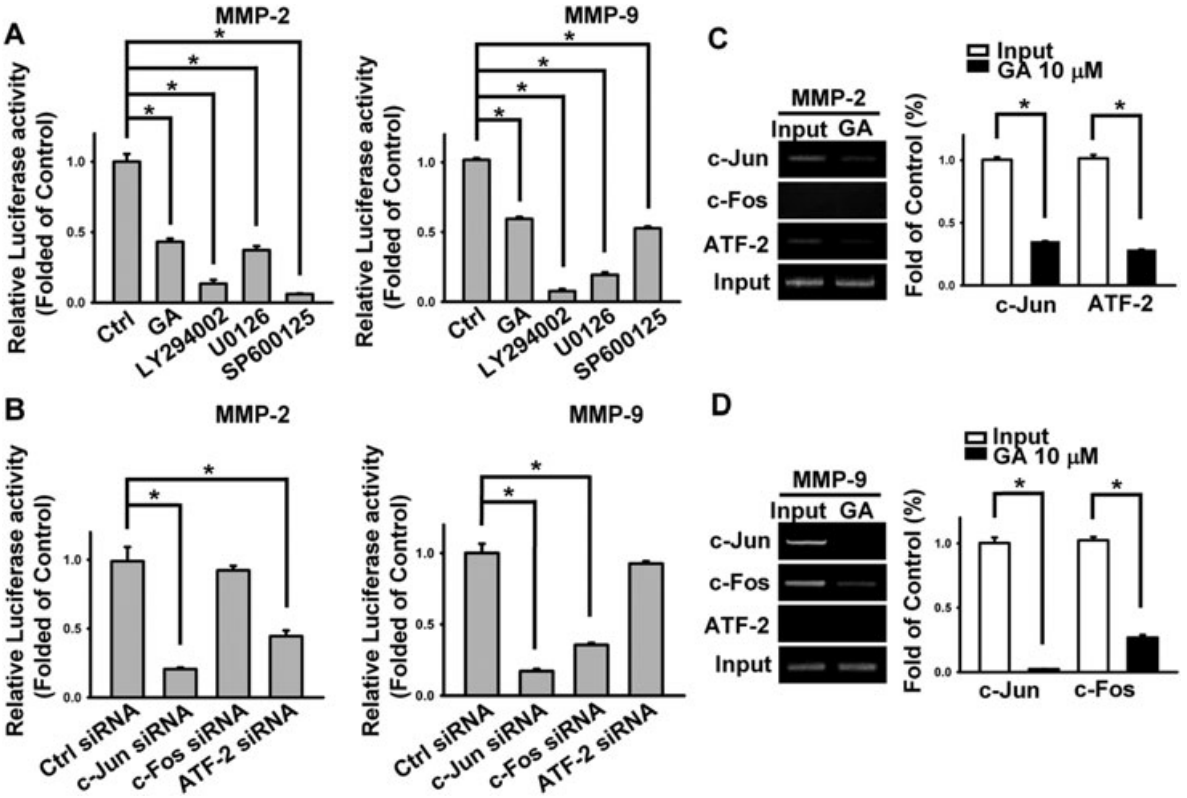


Figure 6. Analyses of gallic acid responsive elements in the promoter region of MMP-2 and MMP-9 gene. (A) Effect of gallic acid, LY294002, U0126, and SP600125 on luciferase activity of the MMP-2 and MMP-9 promoter constructs. After transfection with indicated plasmids for 24 h, MMP-2-transfected cells (left panel) and MMP-9-transfected cells (right panel) were treated with 10 μM gallic acid, 10 μM LY294002, 10 μM U0126, and 10 μM SP600125 for 24 h and then harvested for measuring luciferase activity (mean ± SD, $^{*}p < 0.05$). (B) Effect of c-Jun siRNA, c-Fos siRNA, and ATF-2 siRNA on luciferase activity of the MMP-2 and MMP-9 promoter constructs. K562 cells were cotransfected with MMP-2/MMP-9 promoter constructs and siRNA (100 nM control siRNA, c-Jun siRNA, c-Fos siRNA, or ATF-2 siRNA). After 24 h post-transfection, the transfected cells were harvested for measuring luciferase activity (mean ± SD, $^{*}p < 0.05$). (C) Gallic acid reduced the binding of c-Jun and ATF-2 with MMP-2 promoter. Cells were treated with 10 μM gallic acid for 24 h and then ChIP assay was performed according to the procedure described in Section 2. (Left panel) PCR amplification was conducted using the primers specific for detecting −1359/−1108 region, which contained AP-1 site (−1265) of the MMP-2 promoter. GA stands for gallic acid. Input, nonimmunoprecipitated cross-linked chromatin. (Right panel) Quantitative analyses of amplified PCR products related to the binding of c-Jun and ATF-2 with MMP-2 promoter. (D) Gallic acid reduced the binding of c-Jun and c-Fos with MMP-9 promoter. Cells were treated with 10 μM gallic acid for 24 h and then ChIP assay was performed according to the procedure described in Section 2. (Left panel) PCR amplification was conducted using the primers specific for detecting −183/+37 region, which contained AP-1 site (−79) of the MMP-9 promoter. GA stands for gallic acid. Input, nonimmunoprecipitated cross-linked chromatin. (Right panel) Quantitative analyses of amplified PCR products related to the binding of c-Jun and c-Fos with MMP-9 promoter.

medium and an increased expression of the vascular endothelial growth factor (VEGF) [66]. Compared with those of healthy individuals, peripheral blood and bone marrow mononuclear cells of CML patients show an increase in the expression of MMP-2/MMP-9 and VEGF [66–70]. Janowska-Wieczorek et al. [66] suggested that stimulation of angiogenesis by angiogenic factors including MMPs and VEGF plays an important role in the pathogenesis of CML. Thus, gallic acid induced MMP-2/MMP-9 downregulation may beneficially improve leukemia therapy by reducing metastasis. Noticeably, gallic acid is reported to be toxic to human microvascular endothelia cell culture when the gallic acid concentration used is higher than 10 μM [71]. Recent studies reveal that 100 μM tetradecyl gallate increases effectively the survival rate of preclinical melanoma mouse model through reducing lung metastasis in vivo, but show low systemic toxicity in the animals [72]. Thus, gallic acid derivatives should be a good candidate for additional evaluation as a potential therapeutic agent for reducing or treating hemotologic malignances.

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The authors have declared no conflict of interest.

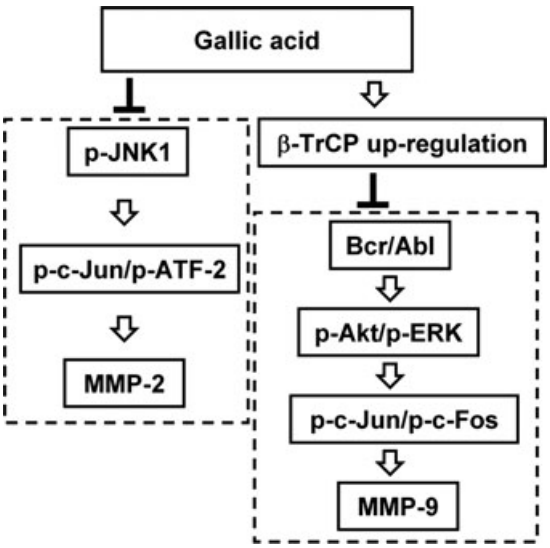

Figure 7. Signaling pathways elucidate the effect of gallic acid induced MMP-2/MMP-9 downregulation in K562 cells. Gallic acid induced β-TrCP upregulation led to Bcr/Abl degradation, which inactivated Akt/ERK-mediated c-Jun/c-Fos phosphorylation. In the meantime, gallic acid induced JNK1 inactivation suppressed c-Jun and ATF-2 activation. Inactivation of c-Jun and ATF-2 led to MMP-2 downregulation, while inactivation of c-Jun and c-Fos led to MMP-9 downregulation. JNK1 in concert with Akt/ERK was involved in c-Jun phosphorylation, and JNK1 and Akt/ERK were responsible for activation of ATF-2 and c-Fos, respectively.

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