Molecules and Cells

Microtubule Acetylation-Specific Inhibitors Induce Cell Death and Mitotic Arrest via JNK/AP-1 Activation in Triple-Negative Breast Cancer Cells

Suyeon Ahn[1,3], Ahreum Kwon[1,3], Youngsoo Oh[1], Sangmyung Rhee[2,*], and Woo Keun Song[1,*]

[1]Cell Logistics Research Center, School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 61005, Korea, [2]Department of Life Science, Chung-Ang University, Seoul 06974, Korea, [3]These authors contributed equally to this work.
*Correspondence: wksong@gist.ac.kr (WKS); sangmyung.rhee@cau.ac.kr (SR)
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Microtubule acetylation has been proposed as a marker of highly heterogeneous and aggressive triple-negative breast cancer (TNBC). The novel microtubule acetylation inhibitors GM-90257 and GM-90631 (GM compounds) cause TNBC cancer cell death but the underlying mechanisms are currently unknown. In this study, we demonstrated that GM compounds function as anti-TNBC agents through activation of the JNK/AP-1 pathway. RNA-seq and biochemical analyses of GM compound-treated cells revealed that c-Jun N-terminal kinase (JNK) and members of its downstream signaling pathway are potential targets for GM compounds. Mechanistically, JNK activation by GM compounds induced an increase in c-Jun phosphorylation and c-Fos protein levels, thereby activating the activator protein-1 (AP-1) transcription factor. Notably, direct suppression of JNK with a pharmacological inhibitor alleviated Bcl2 reduction and cell death caused by GM compounds. TNBC cell death and mitotic arrest were induced by GM compounds through AP-1 activation *in vitro*. These results were reproduced *in vivo*, validating the significance of microtubule acetylation/JNK/AP-1 axis activation in the anti-cancer activity of GM compounds. Moreover, GM compounds significantly attenuated tumor growth, metastasis, and cancer-related death in mice, demonstrating strong potential as therapeutic agents for TNBC.

Keywords: JNK/AP-1 signaling, microtubule acetylation, triple-negative breast cancer

INTRODUCTION

Triple-negative breast cancer (TNBC) is the most aggressive and metastatic subtype associated with extremely high mortality rates (Foulkes et al., 2010). This cancer type is resistant to endocrine and HER2-directed therapy owing to the lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression on the cell surface (Brenton et al., 2005). Several molecular targets, including poly(ADP-ribose) polymerase (PARP) and programmed cell death ligand-1 (PD-L1), with potential utility in alleviating the clinical consequences of TNBC have been reported. Inhibitors of PARP and PD-L1 specifically target tumor cells with mutated BRCA or overexpression of PD-L1, both commonly observed in TNBC (Li et al., 2021; Pardoll, 2012). However, these therapeutic agents do not show efficacy in all cases (Mittendorf et al., 2014; Rashid et al., 2016), necessitating additional mechanistic studies on biomarkers directly targeting TNBC.

Recent studies have shown that microtubules in TNBC cell lines and patient tissues are highly acetylated (Boggs et al., 2015). Microtubules are hollow tubular structures consisting

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of α- and β-tubulin heterodimers that are part of the cellular cytoskeleton with important roles in cell motility, division and intracellular transport (Magiera and Janke, 2014). These structures dynamically undergo a growth-shrinkage phase during cellular processes (Aher and Akhmanova, 2018) and their dynamics and arrangement are precisely regulated by post-translational modifications (PTMs) (Janke and Bulinski, 2011). Acetylation of microtubules occurs when α-tubulin N-acetyltransferase 1 (αTAT1) acetylates lysine 40 of the α-tubulin subunit in the luminal side (Soppina et al., 2012), leading to improvement of flexural rigidity and mechanical stress resistance (Eshun-Wilson et al., 2019). Microtubule acetylation, characterized by stable and long-lived microtubules, is critical in tumor aggressiveness. For instance, αTAT1 overexpression in colorectal cancer has been shown to promote tumor cell invasion via Wnt1-mediated ß-catenin signaling (Oh et al., 2017). Furthermore, increased microtubule acetylation promotes the invasion of basal-like breast cancer by enhancing microtentacle formation (Boggs et al., 2015) or alleviating endoplasmic reticulum stress (Ko et al., 2021). Microtubule acetylation defects caused by α-tubulin K40R mutation (Boggs et al., 2015) and αTAT1 knockout (KO) (Kwon et al., 2020) reduce TNBC invasiveness and growth, respectively, implying involvement of this PTM in TNBC progression and potential as an effective therapeutic target. In this regard, we previously identified small-molecule inhibitors GM-90257 and GM-90631 (GM compounds) that reduce microtubule acetylation by interfering with interactions between microtubules and αTAT1. The pathway through which GM compounds induce TNBC cell-specific death is unknown at present (Kwon et al., 2020). Elucidation of the mechanisms by which microtubule acetylation is modulated to induce cell death in TNBC should provide novel insights for further development of effective targeted therapy.

Cancer cell death is induced by a variety of intracellular signaling pathways. A key molecule is c-Jun N-terminal kinase (JNK), also known as a stress-activated protein kinase, which is involved in multiple tumorigenic regulatory functions (Gkouveris and Nikitakis, 2017). After activation by stress-related signals such as DNA damage, cytoskeletal abnormalities and inflammatory cytokines (Tricker et al., 2011; Weston and Davis, 2007), JNK translocates to the nucleus to activate downstream Jun and activating transcription factor (ATF) family proteins (van Dam and Castellazzi, 2001). Phosphorylated c-Jun and ATF2 form dimeric complexes (such as c-Jun/c-Jun, c-Jun/c-Fos, and c-Jun/ATF2) that constitute the transcription factor activator protein-1 (AP-1) (Gazon et al., 2018). AP-1 is reported to promote cancer cell proliferation, death, invasion and drug resistance (Fan and Podar, 2021), with varying effects depending on the cancer cell type and initial stimulus (Gazon et al., 2018). JNK/AP-1 activation via microtubule depolymerization has been shown to promote cell cycle arrest and death in cervical, lung, and breast cancer cells (Kolomeichuk et al., 2008; Thomas et al., 2016), a common mechanism of microtubule-targeting anticancer agents (Bates and Eastman, 2017; Wang et al., 1998). Since microtubule acetylation deficiency in TNBC cells causes disruption of the microtubule structure (Kwon et al., 2020), we examined the hypothesis that targeting of microtubule acetylation could affect the signaling pathways involved in TNBC cell survival and explored the underlying mechanisms.

Data from the current study showed that GM compounds stimulate JNK signaling in TNBC cells. Treatment with GM compounds activated c-Fos and phosphorylated c-Jun in the nucleus, resulting in AP-1-mediated mitotic arrest and cell death. Based on the collective findings, we propose that JNK/AP-1 signaling axis serves as a novel pathway linking microtubule acetylation with cancer cell death in TNBC.

MATERIALS AND METHODS

Cell culture

MDA-MB-231 was kindly provided by Dr. JS Nam (Gwangju Institute of Science and Technology [GIST], Korea). αTAT1 KO MDA-MB-231 cells were generated using the CRISPR-Cas9 system as described in a recent paper (Ko et al., 2021). Briefly, Lentiviral particles expressing a guide sequence (5'-CATGAGTCTGTGCAACGCCA-3') targeting *Atat1* were produced by transfection of HEK293T cells with lentiCRISPR v2 plasmid, psPAX2, and pMD2.G using polyethylenimine for 48 h (Oh et al., 2017) MDA-MB-231 cells transduced with the lentivirus were selected with puromycin (1 μg/ml) for 2 weeks. Hs578t was purchased from the Korean Cell Line Bank. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with high glucose, 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco) in a humidified incubator at 37°C and 5% CO_2. All cell lines were confirmed not to be infected with mycoplasma using e-Myco VALiD Mycoplasma PCR Detection kit (iNtRON, Korea).

Cancer cell proliferation assay

For 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, 1 × 10^4 MDA-MB-231 cells were plated on 96 well plate for 12 h, and replaced with media containing GM compounds or dimethylsulfoxide (DMSO). After 24 h, 10 μl of 0.1% MTT (Sigma, USA) in phosphate-buffered saline (PBS) was added to cells, and incubated at 37°C for 3 h. The formazan crystal was solubilized with 100 μl DMSO and examined the absorbance at 560 nm with a reference wavelength at 670 nm. For anchorage independent growth assay, 2× DMEM growth media and 1% agarose solution were mixed in a 1:1 ratio and solidified as a base agar layer for 30 min. MDA-MB-231 (5 × 10^3 cells) in DMEM growth media were mixed with 0.6% agarose solution in a 1:1 ratio and seeded on top of the previous mixture. The cells in the soft agar were treated with 500 μl of media containing GM compounds or SP600125 or T-5224 every other day, and the colonies proliferated were observed under a light microscope after 3 weeks.

Western blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche, Switzerland). The proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Merck, Germany) and detected using antibodies against acetyl-α-tubulin (T6199; Sigma), p-JNK (4668; Cell Signaling

Technology [CST], USA), JNK (AHO1362; Invitrogen, USA), p-ERK (9106; CST), ERK (4696; CST), p-p38 (9215; CST), p38 (9212; CST), p-c-Jun (3270; CST), c-Jun (ab32137; Abcam, UK), c-Fos (2250; CST), p-ATF2 (27934; CST), ATF2 (35031; CST), Bcl2 (4223; CST), p-Histone H3 (9701; CST), p-cdc2 (4539; CST), p-Elk1 (9181; CST), α-tubulin (T6199; Sigma), and cyclin antibody sampler kit (9869; CST).

Real-time quantitative PCR

Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's protocol, and mRNA was reverse transcribed into cDNA using TOPscript RT DryMIX (Enzynomics, Korea). The expression of mRNA was quantified using TB Green Premix Ex Taq (Takara, Japan) on LightCycler 480 System (Roche). The sequences of primers used are as follows; *Ccnb1*: 5'-GACCTGTGTCAGGCTTTCTCTG-3' (forward), 5'-GGTATTTTGGTCTGACTGCTTGC-3' (reverse); *Plk1*: 5'-GCACAGTGTCAATGCCTCCAAG-3' (forward), 5'-GCCGTACTTGTCCGAATAGTCC-3' (reverse); *Ccne1*: 5'-TGTGTCCTGGATGTTGACTGCC-3' (forward), 5'-CTCTATGTCGCACCACTGATACC-3' (reverse).

Immunofluorescence staining

Washed cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% triton-X 100 in PBS, and blocked with 0.1% bovine serum albumin. Antibodies against c-Fos (CST) and p-c-Jun (CST) were used to visualize protein expression patterns, and filamentous actin and nuclei were stained using Alexa 555 conjugated phalloidin (A34055; Thermo Fisher Scientific, USA), and DAPI (9542; Sigma), respectively. Fluorescence signals were observed with confocal microscope (Olympus, Japan).

AP-1 transcription activity assay

Nuclear extracts of MDA-MB-231 cells treated with GM compounds for 9 h were harvested with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol. Nuclear extract (10 μg) was applied on TransAM AP-1 kit (Active Motif, Germany), and the absorbance at 450 nm with a reference wavelength of 655 nm was measured to quantify the DNA binding activity of AP-1 transcription factor.

Fluorescence-activated cell sorting (FACS) assay

For cell cycle analysis, 5×10^5 MDA-MB-231 cells treated with chemicals were harvested and resuspended in 400 μl PBS. Cells were fixed by vortexing thoroughly with 800 μl ice-cold ethanol and left in 4°C at least 2 h. After washing of fixed cells with PBS, 200 μl of 50 μg/ml propidium iodide (Thermo Fisher Scientific) solution containing 100 μg/ml RNase A (R4642; Sigma) was added and incubated in 37°C for 30 min. FACS analysis was performed using a filter to detect phycoerythrin on a FACSCanto II (BD Biosciences, USA) instrument.

Next-generation sequencing

Total RNAs were extracted from MDA-MB-231 cells treated with DMSO or GM-90257 for 24 h using TRIzol Reagent (Thermo Fisher Scientific), and determined purity by NanoDrop8000 spectrophotometer. RNA fragments (1 μg) were reverse-transcribed to complementary DNAs (cDNAs) using Truseq Stranded mRNA Prep kit (Illumina, USA), and enriched to construct the final cDNA library. The library was sequenced with Novaseq 6000 sequencing system (Illumina), and analyzed by Tophat (v2.0.13) and Cuffdiff (v2.2.0). Upregulated differentially expressed genes (DEGs) were selected according to the following criteria: $\log_2$[fold change] ≥ 1 and P value < 0.05. Gene Ontology (GO) term was analyzed among the DEGs.

Immunohistochemistry (IHC)

Breast tumor section harvested from xenograft model mice were deparaffinized and rehydrated with Histo-Clear (National Diagnostic, USA), and ethanol, respectively. Antigen retrieval was carried out in a humidified and heated chamber containing IHC-Tek epitope retrieval solution (IHC World, USA). The specimen was treated with antibodies against c-Fos (CST), p-c-Jun (CST), and p-cdc2 (CST), and stained using EnVision Detection Systems Peroxidase/DAB (Dako, USA) for expressed protein and Mayer's hematoxylin (Dako) for nuclei. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the kit (ab206386; Abcam) was performed according to the manufacturer's protocol. Stained sections were observed using Aperio ImageScope (Leica, Germany).

Breast tumor xenograft

NOD.Cg-*Prkd*^{scid}/J mice (#001303) were purchased from the Jackson Laboratory (USA), and maintained in a pathogen-free facility with free access to autoclaved food and water. Female filial mice aged 9 to 12 weeks were used for xenograft. MDA-MB-231 (2×10^6 cells) which were suspended in a 1:1 ratio of DMEM and Matrigel (Corning, USA) were injected into the left inguinal mammary fat pad. GM compounds in 50 μl DMSO were injected intraperitoneally every other day since the mean tumor volume reached 100 mm^3. Tumor volume was calculated by the formula: volume = (length × width^2)/2. All procedures with mice were performed with the approval of the Animal Care and Ethics Committees of the GIST (GIST-2021-102).

Statistical analysis

All experiments were performed independently in three times, and two-tailed Student's t-test was used to evaluate significant changes in experimental groups. The data in the graph were demonstrated as mean ± SD, and the significance of each P value range was indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Data availability statement

The data generated during the current study are included in the article and supplementary materials, and available from the corresponding author on reasonable request.

RESULTS

Depletion of microtubule acetylation via αTAT1 KO and GM compounds stimulates the JNK signaling pathway

Previous studies by our group showed that GM compounds,

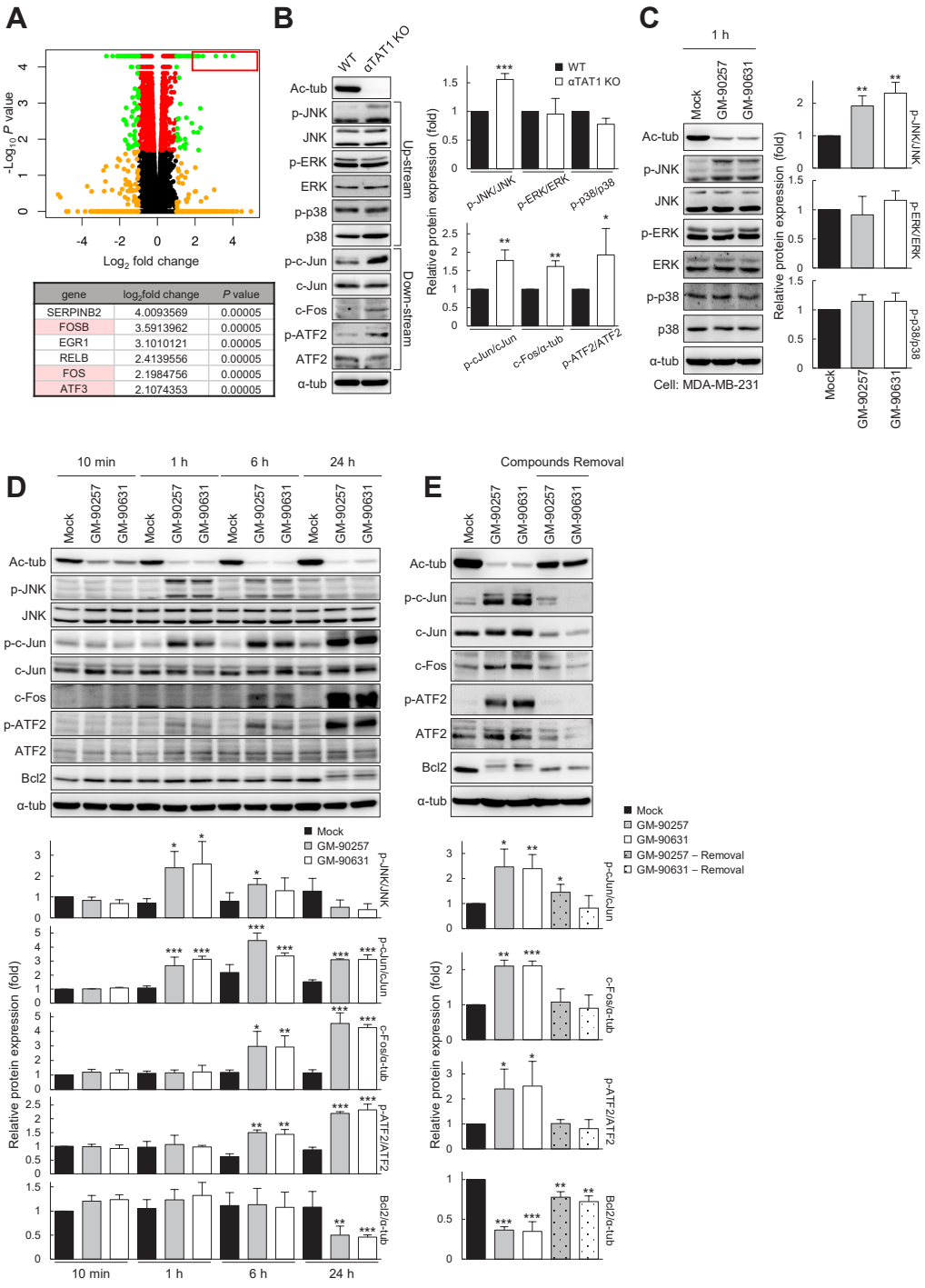


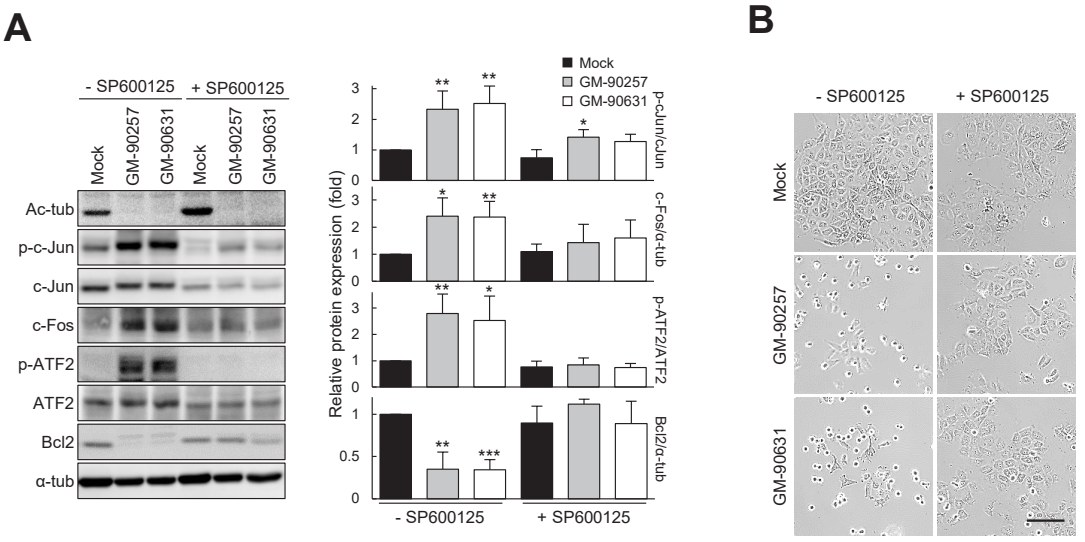
Fig. 1. JNK signaling is triggered in MDA-MB-231 cells treated with GM compounds. (A) Volcano plot obtained from RNA-seq analysis of DMSO or GM-90257-treated MDA-MB-231 cells. The table below depicts increased genes with high significance and fold changes. (B) Western blot showing expression of acetyl-α-tubulin and MAPK-related signaling in WT and αTAT1 KO MDA-MB-231 cells (n = 3). Relative protein levels were normalized as indicated in the graph. (C) Early time changes in MAPK signaling by GM compounds in MDA-MB-231 cells (n = 3). Phosphorylation levels were evaluated according to whole protein expression. (D) Time-course treatment of MDA-MB-231 with GM compounds (n = 3). Relative fold changes of each protein group were normalized to the corresponding levels in the Mock treatment group at 10 min. (E) Western blot analysis of JNK signaling after removal of GM compounds by replacing with fresh serum-free medium at 24 h. The concentration of GM compounds used for treatment was 1 μM for 24 h, unless otherwise indicated. All data are presented as mean ± SD. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ (Student's *t*-test). MAPK, mitogen-activated protein kinase; WT, wild-type; αTAT1, α-tubulin N-acetyltransferase 1; KO, knockout; Ac-tub, Acetylated tubulin; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; α-tub, α-tubulin.

which are microtubule acetylation-targeting inhibitors, cause cell death in MDA-MB-231 but not other luminal type breast cancer or normal breast cells (Kwon et al., 2020). To investigate the mechanisms by which GM compounds trigger death of MDA-MB-231 cells, we initially sequenced mRNA isolated from these cells treated for 24 h with DMSO (control) or GM-90257. Next-generation sequencing analysis revealed significant upregulation of *Fosb*, *Fos*, and *Atf3* transcripts, downstream components of the mitogen-activated protein kinase (MAPK) superfamily, in GM-90257-treated cells (Fig. 1A). To further explore the association of MAPK signaling with microtubule acetylation, we compared the activation status of MAPKs, such as JNK, extracellular signal-regulated kinase (ERK) and p38, as well as their downstream effectors in wild-type (WT) and αTAT1 KO MDA-MB-231 cells. αTAT1 KO MDA-MB-231 cells displayed increased phosphorylation of JNK and downstream effectors, including c-Jun and ATF2 (Fig. 1B). In addition, levels of c-Fos protein were significantly increased. Moreover, JNK phosphorylation was enhanced after brief treatment with GM compounds (Fig. 1C). Our collective results indicate that inhibition of microtubule acetylation promotes activation of JNK and downstream signaling in MDA-MB-231 cells.

To further confirm the effects of GM compounds, we examined the activity of JNK and related downstream effectors after treatment with the GM compounds over a time-course of 24 h. Phosphorylation of JNK was observed 1 h after inhibition of microtubule acetylation and other JNK downstream effectors were sequentially phosphorylated in a time-dependent manner until the 24 h time-point (Fig. 1D). Notably, expression of the anti-apoptotic factor Bcl2 was reduced 24 h after GM compound treatment. Activation of these signaling events was alleviated 24 h after removal of inhibitors (Fig. 1E), indicating that GM compounds trigger JNK-mediated cell death signaling along with downregulation of microtubule acetylation in MDA-MB-231 cells.

GM compounds induce TNBC cell death through JNK activation

To ascertain whether GM compound-induced JNK signaling is involved in TNBC cell death, we initially explored whether the cell death process could be reversed by the JNK inhibitor, SP600125, in the TNBC cell lines MDA-MB-231 and Hs578t. SP600125 effectively reduced phosphorylation



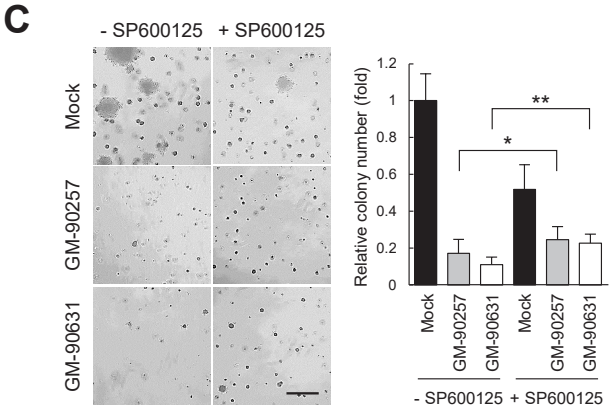


Fig. 2. JNK inhibition alleviates GM compound-induced TNBC cell death. (A) JNK inhibition via SP600125 in MDA-MB-231 cells treated with GM compounds (n = 3). Cells were co-treated with SP600125 and GM compounds in serum-free medium and fold changes calculated relative to the control group with no JNK inhibition. (B) Morphology of MDA-MB-231 cells treated with GM compounds or SP600125. Scale bar = 100 μm. (C) Anchorage-independent growth assay of MDA-MB-231 cells treated with GM compounds or SP600125 every 2 days. The number of colonies was counted using ImageJ software. Scale bar = 200 μm. The concentration of GM compounds used for treatment was 1 μM and that of SP600125 was 100 μM for 24 h, unless otherwise indicated. All data are presented as mean ± SD. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ (Student's *t*-test). JNK, c-Jun N-terminal kinase; TNBC, triple-negative breast cancer; Ac-tub, Acetylated tubulin; a-tub, α-tubulin.

of c-Jun and ATF2 directly downstream of JNK induced by GM compounds, with concomitant restoration of c-Fos and Bcl2 expression (Fig. 2A, Supplementary Fig. S1A). The shrinkage phenotype of MDA-MB-231 cells induced by GM compounds, a representative phenomenon that occurs prior to cell death, was alleviated to its original phenotype upon SP600125 treatment (Fig. 2B), indicating that cell death by GM compounds is mediated by the JNK pathway. Data from

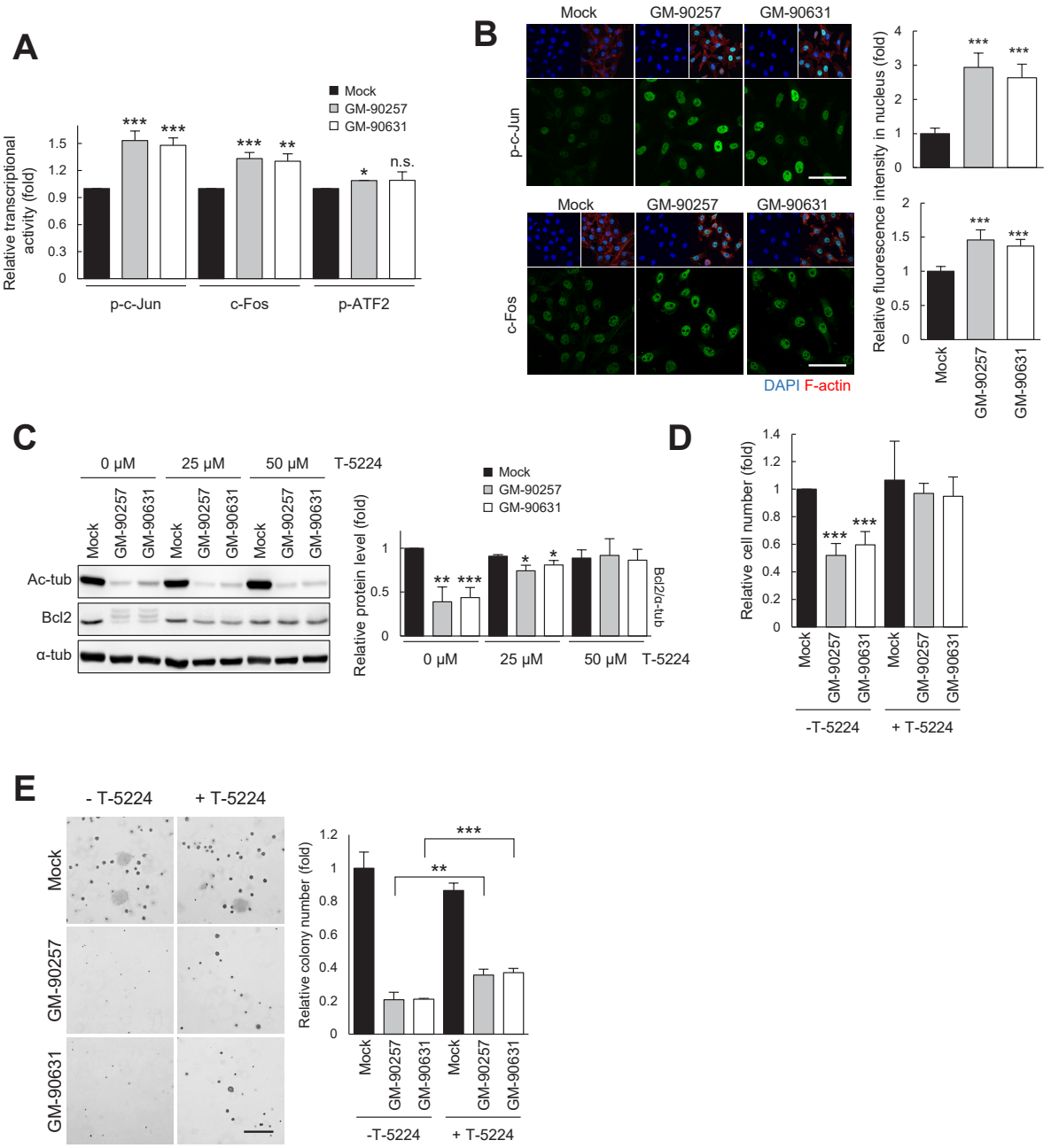


Fig. 3. MDA-MB-231 cell death induction by GM compounds occurs through the microtubule acetylation/JNK/p-c-Jun/c-Fos signaling cascade. (A) AP-1 transcription factor assay on nuclear lysates (10 μg) of MDA-MB-231 treated for 9 h with GM compounds (n = 3). (B) Confocal microscopy images of MDA-MB-231 cells treated with GM compounds for 9 h. Intensity of fluorescence in nuclear regions was measured using ImageJ software. Scale bars = 50 μm. (C) Expression of Bcl2 in MDA-MB-231 cells treated with GM compounds or T-5224, as indicated (n = 3). (D) Relative cell survival rates of MDA-MB-231 cells treated with GM compounds or T-5224 (n = 3) examined via MTT assay. (E) Anchorage-independent growth assay of MDA-MB-231 cells treated with GM compounds or T-5224. The medium containing compounds was replaced every 2 days and colonies counted using ImageJ software. Scale bar = 200 μm. GM compounds were used at a concentration of 1 μM, and T-5224 at 50 μM for 24 h, unless otherwise indicated. All data are presented as mean ± SD. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ (Student's *t*-test). AP-1, activator protein-1; Ac-tub, Acetylated tubulin; a-tub, α-tubulin.

the anchorage-independent growth assay revealed that MDA-MB-231 cells treated with GM compounds displayed a ~80% decrease in colony formation whereas cells treated with a combination of GM compounds and SP600125 showed a significant increase in the number of colonies relative to treatment with GM compound alone (Fig. 2C). Moreover, GM compounds caused little cell death in αTAT1 KO MDA-MB-231 (Supplementary Fig. S1B), therefore the collective findings indicate that activation of JNK signaling induced by GM compound-mediated inhibition of microtubule acetylation is required for death of MDA-MB-231 cells.

GM compounds promote AP-1 transcription factor formation via stimulatory effects on c-Jun and c-Fos, leading to AP-1-mediated cancer cell death

Next, we investigated whether the AP-1 complex, a downstream effector of JNK linked to cell death, is required for the anti-cancer activity of GM compounds (Shaulian and Karin, 2002). An ELISA-based AP-1 transcription factor assay was performed on nuclear extracts of MDA-MB-231 cells treated with or without GM compounds. Upon exposure to GM compounds, p-c-Jun and c-Fos bound AP-1 recognition DNA elements, but not ATF2 (Fig. 3A). Immunocytochemistry experiments confirmed the nuclear localization of p-c-Jun and c-Fos following treatment with GM compounds (Fig. 3B). The results suggest that GM compounds activate the AP-1 transcription factor in the nucleus of MDA-MB-231 cells.

To determine whether AP-1 complex signaling downstream of JNK is necessary for cell death induced by GM compounds, cell death yield was evaluated using a p-c-Jun/c-Fos AP-1-specific inhibitor, T-5224 (Ishida et al., 2015). Bcl2 suppressed by GM compounds was recovered in a concentration-dependent manner following T-5224 treatment (Fig. 3C). T-5224 prevented cell death and inhibition of colony formation by GM compounds (Figs. 3D and 3E). Overall, these findings support the hypothesis that the microtubule acetylation/JNK/AP-1 signaling axis serves as a mechanism underlying cell death induced by GM compounds.

Activated AP-1 by GM compounds arrests the cell cycle at the G_2/M in MDA-MB-231 cells

Microtubule targeting agents exert antitumor effects, such as cancer cell death and cell cycle arrest, by inhibiting microtubule dynamics (Loong and Yeo, 2014). Since GM compounds disrupt the microtubule structure in MDA-MB-231 cells through reducing acetylation, we examined their potential effects on the cell cycle. GO term analysis of RNA-seq data obtained from MDA-MB-231 cells treated with GM-90257 revealed reduced expression of gene groups related to mitosis, in particular, M-phase (Fig. 4A). Expression of cyclin D3, E1, and A2 with roles in G_0/G_1 phase progression (Fan et al., 2017), G_1-S transition (Ohtsubo et al., 1995), and mitotic entry (Loukil et al., 2015) was gradually decreased with increasing concentrations of GM-90257 (Fig. 4B). In contrast, the level of cyclin B1 involved in G_2/M transition (Androic et al., 2008) increased with progressive doses of GM-90257 and was eventually saturated (Fig. 4B). Live images of MDA-MB-231 in the cell division stage showed that mitosis was not completely terminated, even after 120 min, following GM-90257 treatment (Fig. 4C). Based on the results, we propose that GM compounds not only activate the JNK/AP-1 signaling axis but also contribute to mitotic arrest at the G_2/M phase.

FACS analysis disclosed that GM compounds promote accumulation at the G_2/M phase whereas T-5224 restores the G_0/G_1 and S phases of MDA-MB-231 cells in a concentration-dependent manner (Fig. 4D). To clarify whether GM compounds induce G_2/M phase arrest through the JNK/AP-1 complex pathway, we examined expression patterns of the representative markers of G_2/M arrest. Phosphorylation of histone H3 and cdc2 and mRNA expression of *Ccnb1*, *Plk1* and *Ccne1* were restored following treatment with T-5224 (Figs. 4E and 4F), supporting the theory that GM compounds specifically block mitosis through activation of the AP-1 transcription factor.

GM compounds attenuate cancer cell growth and metastasis by inducing cancer cell death and mitotic arrest *in vivo*

We performed xenograft experiments in mice using MDA-MB-231 cells to validate the significance of JNK/AP-1 signaling in actions of GM compounds during cancer progression *in vivo*. Both tumor growth tendency and tumor weight were significantly reduced in GM compound-injected groups compared to the control group (Figs. 5A and 5B). Immunostaining of tumor specimens revealed a significant increase in staining intensity of p-c-Jun and c-Fos in cancer tissues of the GM compound injected group, particularly within the nucleus (Fig. 5C). Furthermore, immunostaining of phosphorylated cdc2 and data from the TUNEL assay revealed that mitotic-arrested cells at the G_2/M phase and apoptotic cells were more prevalent in cancer tissues from the GM compound-injected groups (Fig. 5D). Data obtained from xenograft experiments clearly support *in vitro* data showing that GM compounds activate c-Jun and c-Fos, resulting in cell cycle arrest and death.

We proceeded to treat MDA-MB-231 xenograft mice with GM compounds for an extended period of time to further examine their effectiveness as anticancer agents against TNBC. In the experimental group injected with GM compounds, lung metastasis was suppressed, along with a considerable delay in cancer-related death (Figs. 5E and 5F). Taken together, our results indicate that GM compounds effectively reduce TNBC cancer growth and metastasis, and consequently, mortality.

DISCUSSION

While overall breast cancer survival has steadily increased, with a reported rate of 91% by 2020 (Kim and Kim, 2022; Viale, 2020), TNBC, the most aggressive and difficult-to-treat subtype, has a 5-year survival rate of 77% (Giaquinto et al., 2022). Hormone therapy and HER2 targeting are ineffective for TNBC and chemotherapy is the main treatment modality, particularly at the metastatic stage (Wahba and El-Hadaad, 2015). However, chemotherapeutic regimens, such as doxorubicin and paclitaxel, recommended for preoperative systemic treatment, target highly proliferative cells, causing a range of side-effects, such as hair loss and damage to digestive mucosa (Foa et al., 1994; Gewirtz, 1999). Specific

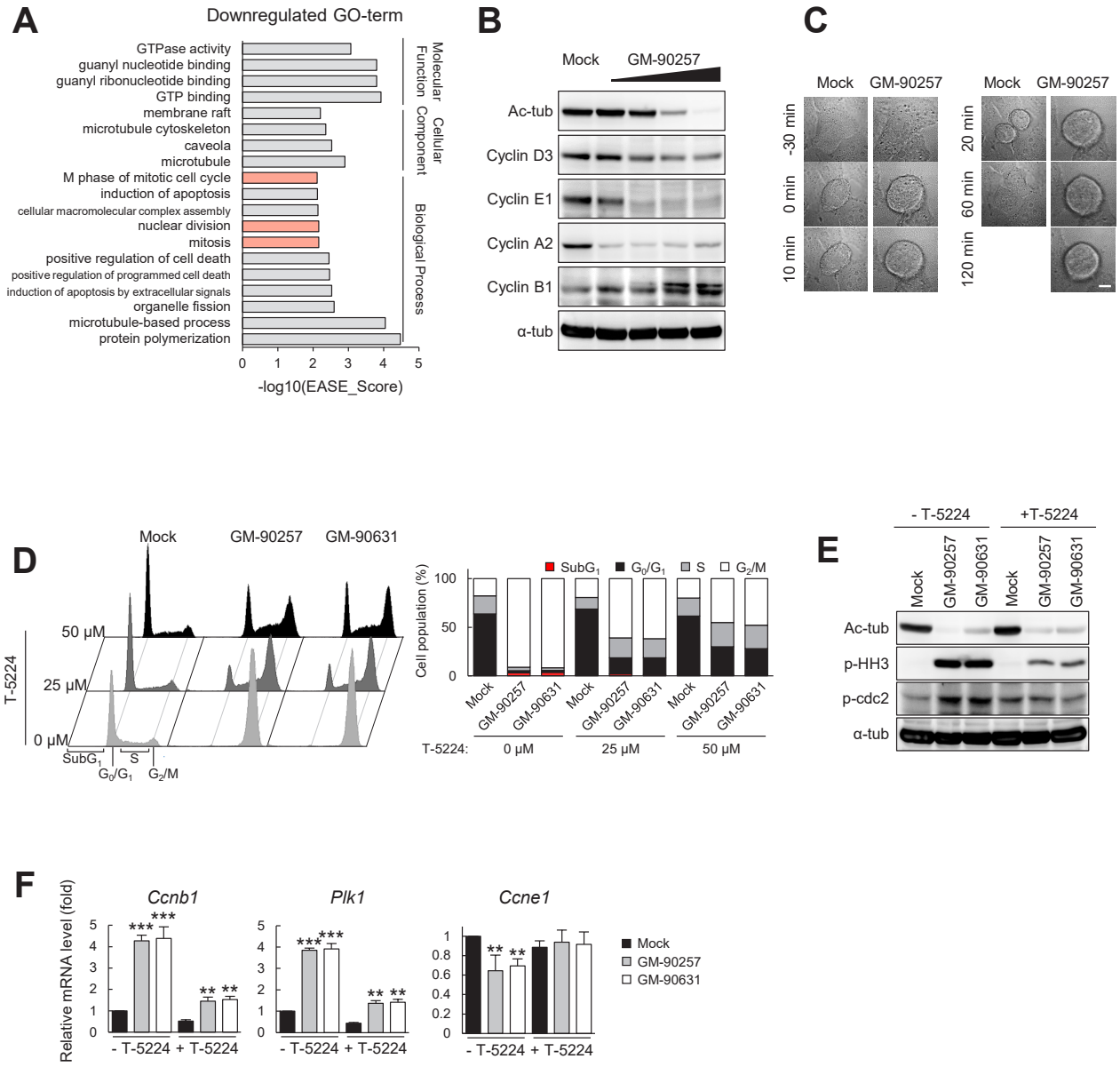


Fig. 4. GM compounds induce cell cycle arrest of MDA-MB-231 at the G_2/M phase through p-c-Jun/c-Fos activation. (A) Gene ontology term analysis of GM-90257- or DMSO-treated MDA-MB-231 cells. Downregulated gene families were classified according to their respective functions. Red boxes indicate the mitosis-related gene group. (B) Western blot analysis of expression of cyclin at increasing GM-90257 concentrations (0.1, 0.25, 0.5, 1 μM). (C) Live imaging of MDA-MB-231 cells treated with 0.5 μM GM-90257 (treatment time-point: 0 min) at the beginning of cell division. Scale bar = 10 μm. (D) Cell cycle analysis of MDA-MB-231 cells treated with GM compounds or T-5224 via propidium iodide staining-FACS. Individual cell cycle stages were classified according to the indicated criteria. (E) Representative G_2/M arrest marker expression in MDA-MB-231 cells treated with GM compounds or T-5224 examined via western blot. (F) Real time PCR assay for mRNA expression of cell cycle-related markers. Relative gene expression was normalized to that of *Gapdh*. GM compounds were used at a concentration of 1 μM and T-5224 at 50 μM for 24 h, unless otherwise indicated. All data are presented as mean ± SD. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ (Student's *t*-test). GO, Gene Ontology; GTPase, guanosine triphosphatase; Ac-tub, Acetylated tubulin; a-tub, α-tubulin.

targets that can overcome the limitations of current TNBC treatments are therefore an urgent clinical requirement. Microtubule acetylation, a proposed marker of basal-like TNBC, is upregulated in 72% TNBC patients (Boggs et al., 2015). Furthermore, acetylation of microtubules increases with cancer stage progression (Boggs et al., 2015), potentially presenting a powerful target for advanced TNBC cases that no longer responds to standard anticancer agents. Novel microtubule acetylation-specific agents, known as GM compounds, have been shown to exert no significant effects on

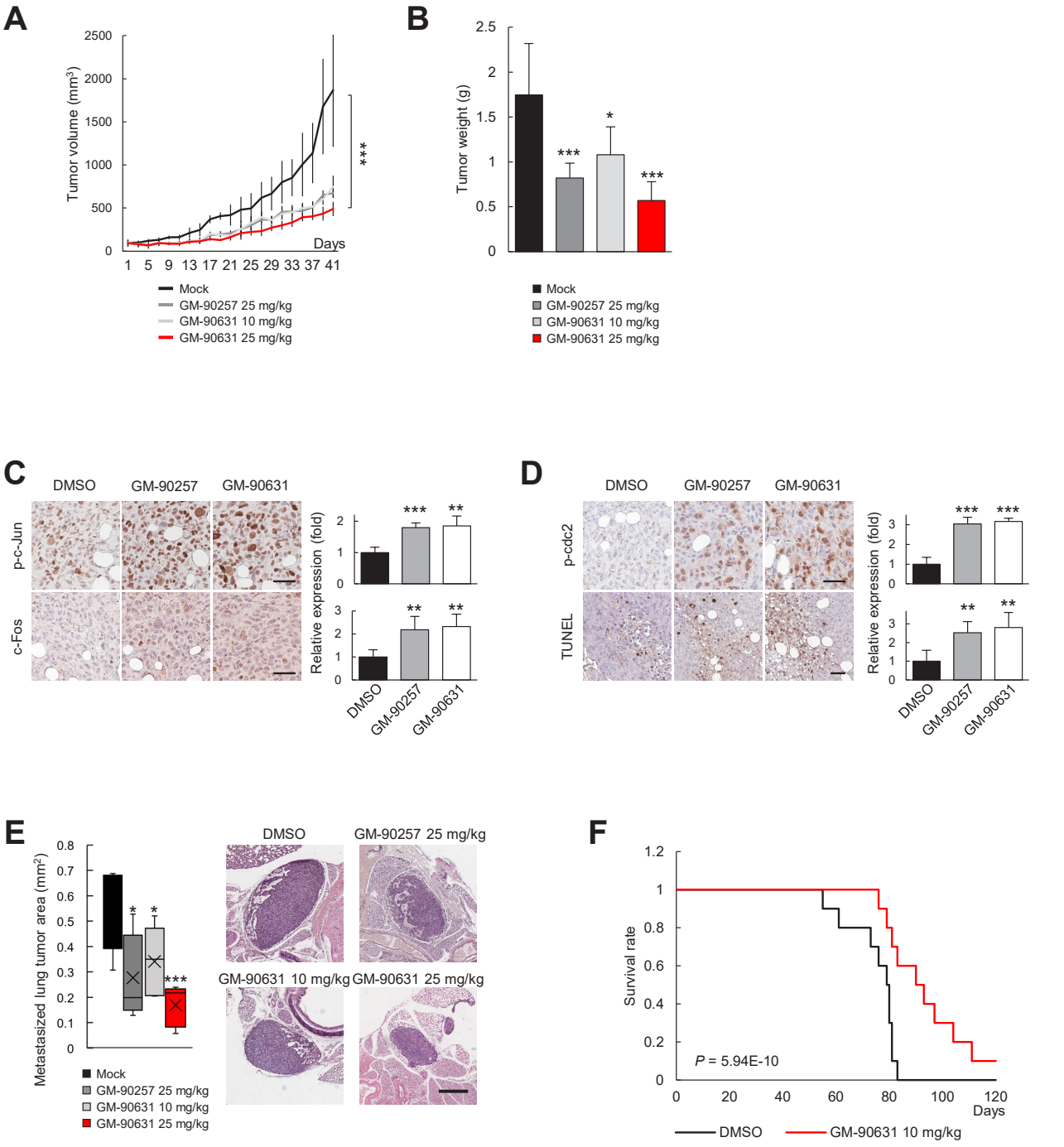


Fig. 5. GM compounds activate c-Jun and c-Fos and attenuate cancer growth, metastasis, and mortality *in vivo*. (A) Tumor growth following injection of GM compounds at a tumor volume of 100 mm^3 (n = 5). Significant differences were evaluated between Mock control and GM compound treatment groups. (B) Weights of collected tumors after sacrifice (n = 5). (C) Representative immunohistochemistry images of tumor specimens derived from mice injected with 25 mg/kg GM compounds. Evaluation of the relative expression levels of p-c-Jun and c-Fos from randomly selected areas using ImageScope software. Scale bar = 50 μm. (D) Immunostaining of representative markers of G_2/M arrest and apoptotic cells. Staining intensity was quantified using ImageScope software. Scale bars = 50 μm. (E) H&E staining (n = 5) of areas of lung metastasis. Representative images of cross-sections of metastasized tumors analyzed using ImageScope software. Scale bar = 300 μm. (F) Kaplan-Meier plot of MDA-MB-231-xenografted mice injected with DMSO or 10 mg/kg GM-90631 every 2 days (n = 10). All data are presented as mean ± SD. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ (Student's *t*-test).

survival of non-TNBC cells (Kwon et al., 2020), hair loss (data not shown) or weight loss (Supplementary Fig. S1C) in mice. In-depth knowledge of the mechanisms by which GM compounds induce TNBC-specific death may provide valuable insights that facilitate development of strategies to improve TNBC targeted therapy.

Since microtubule acetylation in centrioles and mitotic spindles during cell cycle phases facilitates precise control of spindle development and alignment of segregated chromosomes (Nagai et al., 2013; Nekooki-Machida et al., 2018), we initially hypothesized that cell death caused by inhibition of microtubule acetylation in response to GM compounds is attributable to abnormal control of spindle development or chromosome segregation. αTAT1 KO mouse embryogenesis, on the other hand, was not lethal or abnormal in terms of phenotype (Kalebic et al., 2013), implying that alternatives to microtubule acetylation during cell division may contribute to microtubule stability (Rasamizafy et al., 2021). In view of this finding, we hypothesized that GM compounds inhibit TNBC cell survival through mechanisms other than mitotic spindle disruption. Since PTMs of microtubules play important roles in intracellular signaling propagation, we focused on the signaling pathway changes that occur upon reduction of microtubule acetylation, resulting in cell death.

GM compounds rupture the highly acetylated microtubules in MDA-MB-231 cells, therefore the determination of microtubule disruption-related events could aid in clarifying subsequent signaling events. Microtubules actively interact with microtubule-associated proteins (MAPs), which not only control the behavior and stability of microtubules but also act as linkers to other signaling complexes (Bodakuntla et al., 2019). Guanine nucleotide exchange factor-H1 (GEF-H1), a MAP that activates Rho guanosine triphosphatase (GTPase), is reported to stimulate JNK activity. Specifically, GEF-H1 released from unstable microtubules activates RhoA-mediated MAPK kinase 4 (MKK4)/JNK signaling (Kashyap et al., 2019). Our preliminary data showed accumulation of RhoA-GTP and p-MKK4 following short-term exposure to GM compounds (data not shown). Further research is required to establish the relationship between the activities of GM compounds and GEF-H1.

Activation of c-Jun, ATF2 and c-Fos via the JNK pathway is implicated in GM compound-induced cell death. JNK, on the other hand, phosphorylates and activates c-Jun and ATF2, but is not directly involved in c-Fos activation (van Dam et al., 1993). Rather than JNK, c-Fos has been identified as a direct downstream target of other MAP kinases, ERK and p38 (Price et al., 1996), which were not activated by both αTAT1 KO and GM compounds (Figs. 1B and 1C). Furthermore, the increase in c-Fos occurred later than c-Jun phosphorylation after JNK activation (Fig. 1D), indicating that c-Fos is upregulated by an indirect signaling pathway involving JNK. Numerous studies have demonstrated that phosphorylation of ETS like-1 protein (Elk-1) directs induction of c-Fos transcription via association with the serum response element of the DNA promoter of the *c-fos* gene (Cavigelli et al., 1995; Deng and Karin, 1994; Li et al., 2001). Our results demonstrate that GM compounds induce JNK-dependent Elk-1 phosphorylation, which has the potential to promoting transcription of c-Fos (Supplementary Fig. S1D). Accordingly, we propose that GM compounds increase c-Fos through this signaling pathway for activity as a transcription factor.

We performed a series of experiments to determine whether c-Fos and c-Jun heterodimerize to form functional AP-1 transcription factors following GM compound treatment. c-Jun forms homodimers or heterodimers with leucine-zipper containing proteins whereas c-Fos can only form heterodimers, particularly with c-Jun, with high stability and transcriptional activity (Halazonetis et al., 1988; O'Shea et al., 1992). Our results indicate that the AP-1 complex induced by GM compounds is composed of c-Fos/c-Jun heterodimers, which recognize DNA sequences containing the 12-O-tetradecanoylphorbol-13-acetate-responsive element (Fig. 3A) and initiate subsequent gene transcription. Although c-Jun and c-Fos are known to induce upregulation of cell proliferation-related oncogenes, evidence for a converse role of c-Jun in tumor suppression has recently been reported (Garces de Los Fayos Alonso et al., 2018). The distinct role of the AP-1 complex as an on-off regulator of tumor progression may therefore be dependent on the specific tumor context (Eferl and Wagner, 2003). In our study, the AP-1 complex induced by GM compounds showed activity as a tumor suppressor by influencing the expression of genes involved in cell death and mitotic arrest (Figs. 3 and 4), which was further confirmed in MDA-MB-231 xenograft mice injected with GM compounds (Figs. 5C and 5D).

While this study focused primarily on microtubule acetylation in TNBC in terms of regulation of cell survival, microtubule acetylation is widely known to promote breast cancer cell migration and invasion. We additionally showed that GM compounds have the potential to inhibit metastasis in the lung samples of breast cancer model mice (Fig. 5E). As mentioned earlier, chemotherapy is recommended for advanced TNBC cases to reduce the frequency of metastasis (Chen et al., 2020). While the precise targets of GM compounds in the context of TNBC metastatic regulation are yet to be validated, RNA-seq analysis revealed downregulation of matrix metallopeptidase genes such as *Mmp1* and *Mmp3*, which contribute to TNBC invasion through proteolysis of the extracellular matrix (Wang et al., 2019). Based on these preliminary results, further research into the roles and mechanisms of action of GM compounds in metastasis should contribute to the development of novel strategies to control TNBC progression.

The current study revealed a novel mechanism by which GM compounds inhibit microtubule acetylation to exert anticancer effects against TNBC. The JNK/AP-1 pathway was markedly activated following treatment with GM compounds, accompanied by cancer cell death and mitotic arrest. Cancer cell death caused by GM compounds was found to be specifically associated with microtubule acetylation, which is common in TNBC patients. Our collective findings support the potential utility of GM compounds as targeted chemotherapy and highlight a critical role of JNK/AP-1 signaling in TNBC tumor suppression.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

S.A. conceived and performed experiments, and wrote the manuscript, A.K. conceived and performed experiments. Y.O. performed experiments. S.R. and W.K.S. secured funding, provided expertise and feedback.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Suyeon Ahn	https://orcid.org/0000-0001-6992-3442
Ahreum Kwon	https://orcid.org/0000-0002-2424-8372
Youngsoo Oh	https://orcid.org/0000-0003-3912-2374
Sangmyung Rhee	https://orcid.org/0000-0003-2733-2147
Woo Keun Song	https://orcid.org/0000-0002-2786-9836

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