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Polypeptide antibiotic actinomycin D induces Mcl-1 uncanonical downregulation in lung cancer cell apoptosis

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ABSTRACT

Aims: Actinomycin (Act) D, a polypeptide antibiotic, is used clinically to inhibit the growth of malignant tumors. Act D binds to DNA at the transcription initiation complex to prevent the elongation of RNA. Act D causes DNA damage, growth inhibition, and cell death. Myeloid cell leukemia (Mcl-1) is an anti-apoptotic Bcl-2 family member protein, and the present study explored the effects and molecular mechanism of Act D-induced Mcl-1 downregulation.

Main methods: Human adenocarcinoma A549 cells were used to check the cytotoxic signaling pathways of Act D, particularly in apoptotic mechanism, in a cell-based study approach. Specific blockers targeting the apoptotic factors were examined for their possible roles.

Key findings: We found that Act D caused cell growth inhibition and apoptosis. Propidium iodide-based flow cytometric analysis and immunostaining confirmed cell apoptosis. Treatment with Act D caused DNA damage, followed by p53-independent cell death. Western blotting showed a significant decrease in Mcl-1 expression, mitochondrial transmembrane potential loss, and caspase-9/caspase-3 cascade activation. The proteasome inhibitor MG132 reversed Act D-induced Mcl-1 downregulation. However, pharmacological inhibition of glycogen synthase kinase-3, p53 expression, ER stress, autophagy, and vesicle acidification, which are Mcl-1-regulating signaling pathways, did not rescue these effects. Notably, Cullin-Ring E3 ligase partially mediated Mcl-1 downregulation. Administration of transforming growth factor-β induced mesenchymal cell differentiation, but Act D still decreased Mcl-1 and caused cell apoptosis.

Significance: All of these data show a potential pro-apoptotic effect for Act D by facilitating Mcl-1 uncanonical downregulation.

1. Introduction

Actinomycin (Act) D, a chromopeptide composed of a chromophore group and two pentapeptide chains, binds and intercalates in GC rich DNA and inhibits RNA synthesis [1]. Act D was originally used as an antibiotic compound derived from *Streptomyces parvulus*. Act D is also clinically used to treat many cancers, including Ewing's sarcoma, testicular cancer, and rhabdomyosarcoma [2], however, no further clinical use in treating lung cancers although many reports showed its synergistic anticancer effects combined with other anticancer agents against lung cancer cells in the experimental models [3,4] and a simple anticancer action by triggering RNase L-regulated DNA damage [5]. Act D induces cell death marker and tumor suppressor p53 expression, reactive oxygen species (ROS) production and cell cycle G_2/M arrest in cancer cells, likely via an RNase L-dependent manner [5,6]. Act D decreased anti-apoptosis Bcl-2 family protein expression and increased pro-apoptosis Bcl-2 family protein in lung cancer cells [7,8]. Notably, Act D-induced cell apoptosis in chronic lymphocytic leukemia was independent of p53, and treatment with Act D targeted the survival proteins Mcl-1 and Bcl-2 [9]. In addition to the blockade of transcriptional

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maintenance, the mechanism involving the downregulation of anti-apoptotic Mcl-1 in a post-modification manner is not explored for its anticancer activity.

Bcl-2 family proteins regulate mitochondrial transmembrane potential and programmed cell death [10]. The C-terminal transmembrane domains of Bcl-2 family proteins are inserted in the outer membrane of mitochondria. If cancer cells are stimulated with chemotherapy or radiotherapy, pro-apoptotic Bcl-2 family proteins determine mitochondrial outer membrane permeabilization [11]. Loss of the anti-apoptotic Bcl-2 family reduced protection of the outer mitochondrial membrane. Mcl-1 is an anti-apoptosis member of the Bcl-2 family, and it shares a structural homology to Bcl-2 and Bcl-xL because it contains three BH domains. The phosphorylated residues of Mcl-1 are Thr92, Ser121, Ser155, Ser121, Thr163, and Ser64. Cyclin-dependent kinase (CDK) 1 and mitogen-activated protein kinase target Ser64, Thr92, and Ser121 to sustain the stability of Mcl-1. However, glycogen synthase kinase (GSK)-3β, extracellular signal-regulated kinase, and c-Jun N-terminal kinase target Ser155, Ser159, and Thr163, which causes Mcl-1 destabilization via protein ubiquitination [12,13]. E3 ligases, including RING domain-containing E3 ligases and HECT domain-containing E3 ligases [14], regulate Mcl-1 protein downregulation. Several E3 ligases, such as APC/C, β-TrCP, Fbw7, Mule, and Trim17, are regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK-3β, MAPKs, p53, and CDK1 pathways [15–17] and control Mcl-1 stability. In contrast, USP9X is a deubiquitinase that reduces Mcl-1 protein ubiquitination [18].

Chemotherapeutic resistance has developed in most types of cancers and transformed mesenchymal cells in fibrosis, but this failure is generally due to activation of the DNA repair system and anti-apoptotic responses in cancer cells that restrain the efficacy of pro-apoptotic chemotherapeutics, which is likely caused by the tumor microenvironment [19]. Transforming growth factor (TGF)-β is an immunosuppressive cytokine [20] that promotes epithelial-mesenchymal transition (EMT) and increases cell survival, and it induced anti-apoptotic Bcl-2 family mRNA and protein expression in lung cancer cells [21,22]. However, the effects of Act D treatment in response to EMT are poorly investigated. The current project investigated the possible effects of Act D on cell death induction in lung cancer cells in vitro and its molecular regulation of Mcl-1 downregulation in response to EMT induction.

2. Materials and methods

2.1. Cell cultures, antibodies, and reagents

Human A549 cells (CCL185, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Rockville, MD). H1299 (CRL-5803, ATCC) were grown in RPMI 1640 medium (Invitrogen Life Technologies). WI-38 cells (CCL75, ATCC) were cultured in Eagle's minimum essential medium (EMEM). The following reagents and antibodies were used in these studies: Act D (Sigma-Aldrich, St. Louis, MO); proteasome inhibitor MG132, GSK-3β inhibitor Bio, ROS inhibitor NAC, CDK1 inhibitor Ro-3306, autophagy inhibitor 3-MA and CQ, acidification inhibitor niclosamide and bafilomycin A1, JNK inhibitor SP600125, MEK inhibitor PD98059, p38 inhibitor SB203580, E3 ligase inhibitors suramin and heclin, recombinant human TGF-β (PreproTech, Rocky Hill, NJ); 4,6-diamidino-2-phenylindole (DAPI), dimethyl cyanide (DMSO), rhodamine 123, propidium iodide (PI) solution (Sigma-Aldrich, St. Louis, MO); CM-H_2DCFDA and MitoSOX Red (Thermo Fisher Scientific, Waltham, MA); RNase, and antibodies against PARP, GSK-3α/β, lamin A/C, ubiquitin, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); antibodies against γH2AX, p53, Mcl-1, Bcl-2, Bcl-xL, Akt Ser473, Akt, GSK-3α/β Ser21/9, ERK1/2 Thr202/Tyr204, ERK1/2, JNK Thr183/Tyr185, JNK, p38 MAPK Thr180/Tyr182, p38 MAPK, caspase-9, caspase-3, and GRP78 (Cell Signaling Technology, Beverly, MA); antibodies against E-cadherin and α-SMA (GeneTex, San Antonio, TX); antibodies against polyclonal anti-rabbit Atg8 (LC3) I/II and p62 (MBL international, Nagoya, Japan); rabbit anti-mouse IgG conjugated with HRP (Abcam, Cambridge, MA); and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA).

2.2. Cytotoxicity assay

Cytotoxicity Detection Kit assays (Roche Diagnostics, Lewes, UK) were used to assess cell cytotoxicity according to the manufacturer's instructions.

2.3. Western blotting

Cell lysates were prepared using a lysis buffer containing 1 % Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02 % NaN_3, and a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). After a freeze-thaw cycle, the cell lysates were centrifuged at 12,000 rpm at 4 °C for 20 min. Protein was separated following a SDS-polyacrylamide gel electrophoresis and then immediately transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Following a blocking process with 5 % skim milk in TBST, blots were immunoprobed with the specific antibodies and developed using enhanced chemiluminescence (Pierce, Rockford, IL). The relative protein expression intensity was quantified using ImageJ software (vers. 1.41o; W. Rasband, National Institutes of Health, Bethesda, MD). Changes in the ratio of proteins compared to the indicated protein were also determined.

2.4. Annexin V/propidium iodide (PI) staining

To assay cell apoptosis, an Annexin V/PI staining kit and PI-based cell cycle analysis were performed in this study. Annexin V Apoptosis Detection Kit (BioLegend San Diego, CA) with a counterstaining dye PI was used to identify apoptotic and necrotic cells as measured by flow cytometry (Accuri C6 Plus; BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR). For cell cycle assay, the cells were fixed with 70 % ethanol in PBS for 30 min, stained with PI solution (40 μg/mL PI and 100 μg/mL RNase (Calbiochem) in PBS) in the dark at room temperature, and analyzed using flow cytometry. The emission signals were collected using an FL-2 detector (565–610 nm). The levels of apoptosis are reported as percentages of sub-G_1 cells or fold-increases relative to the untreated control.

2.5. Immunostaining

Treated cells were fixed with PBS buffer containing 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100 solution, and washed twice with ice-cold PBS. The cells were probed with anti-lamin A/C then probed with Alexa 488-conjugated goat anti-rabbit IgG. A counter dye DAPI (5 μg/mL) was used for nuclear staining. The cells were visualized under a fluorescence microscope (ZEISS).

2.6. Mitochondrial transmembrane potential (MTP) assay

Collected cells were incubated with rhodamine 123 (Sigma–Aldrich) for 30 min in the dark at room temperature, washed once with PBS, and analyzed using flow cytometry. The emission signals were collected using the FL-1 detector.

2.7. ROS detection assay

ROS generation was determined using a general ROS indicator CM-H_2DCFDA and a mitochondrial superoxide indicator MitoSOX Red. The cells were incubated with 10 μM CM-H_2DCFDA and 5 μM MitoSOX Red for 1 h according to the manufacturer's instructions (Thermo Fisher Scientific), washed with PBS, and immediately measured using flow cytometry with excitation at 492–495 nm and emission at 517–527 and

635 nm, respectively.

2.8. Statistical analysis

Data obtained from three independent tests are presented as the means ± standard deviation (SD). All sets of data were analyzed using an unpaired Student's *t*-test and one-way ANOVA with Tukey's multiple-comparison test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Act D induces apoptosis in lung adenocarcinoma cells

Act D is a polypeptide antibiotic that causes cell apoptosis and cell cycle arrest in cancer cells [7,8]. Act D may be a good therapeutic strategy against lung adenocarcinoma cells. A549 cells were treated with 0.15 μg/mL of Act D for inhibiting cellular transcription according to our previous works [23] for 48 h. The drug dose used in this study falls

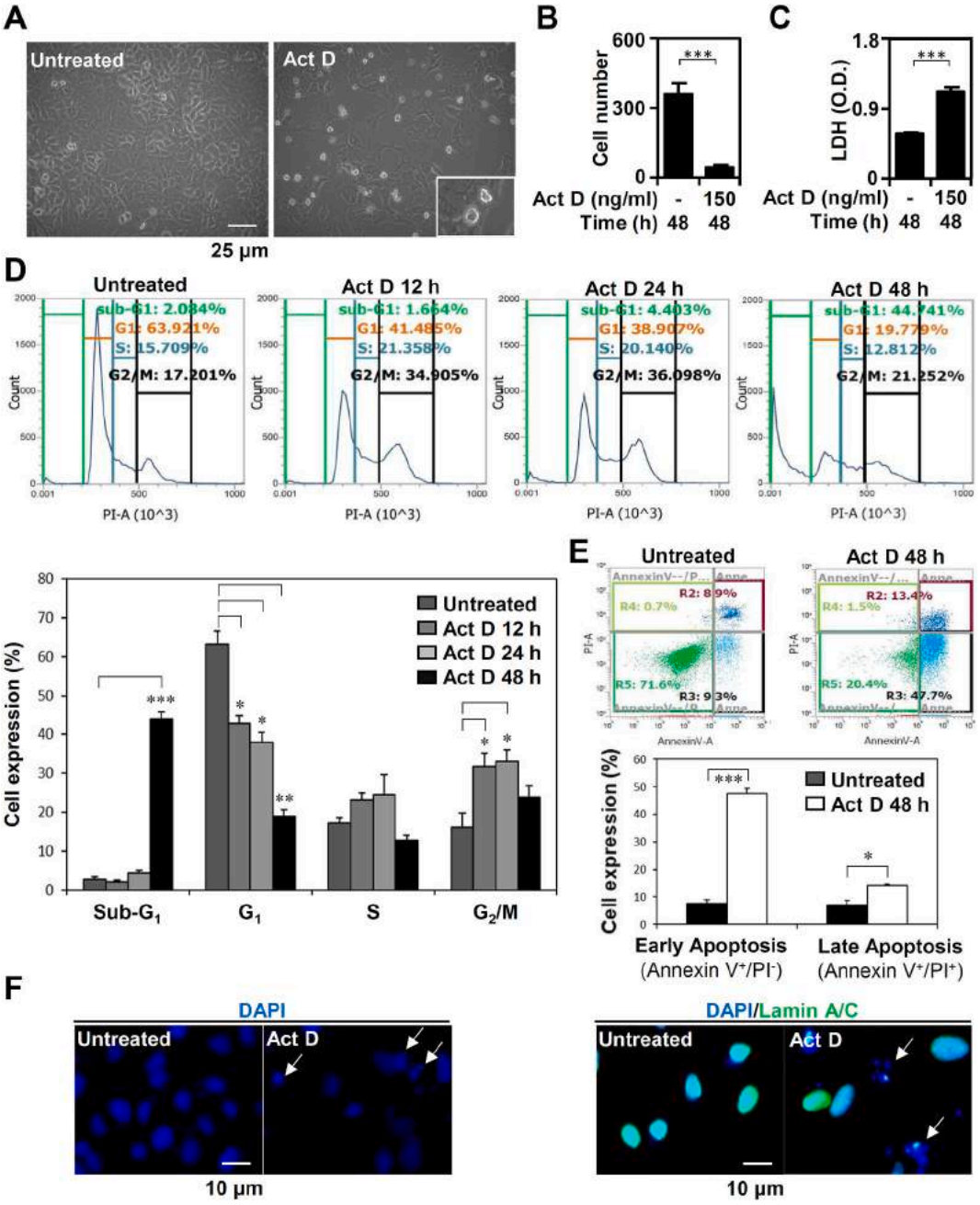

Fig. 1. Actinomycin (Act) D induced apoptosis, DNA damage, and G_2/M phase arrest in lung adenocarcinoma A549 cells. (A) Phase image showed cell rounding and detachment 2 days post-stimulation. (B) Quantification of cell number. The cell count was extrapolated from a single image and the cell number is mean ± SD obtained from three culture wells. ***$p < 0.001$. (C) LDH assay, as shown by the optical density (O.D.), revealed cytotoxicity. The results are the mean ± SD obtained from three experiments. ***$p < 0.001$. (D) PI-based flow cytometry analysis determined the percentage of cell cycle (G_1, S, G_2/M) and cell apoptosis (sub-G_1). All results are a representative set obtained from triplicate experiments and the results are a percentage of the mean ± SD obtained from three experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (E) Annexin V/PI-based flow cytometry analysis determined the percentage of cell apoptosis at early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) stages. All results are a representative set obtained from triplicate experiments and the results are a percentage of the mean ± SD obtained from three experiments. *$p < 0.05$ and ***$p < 0.001$. (F) Immunostaining and subsequent fluorescence microscopy showed lamin A/C degradation and DNA condensation (arrowed). DAPI was used for nuclear staining. All results are a representative set obtained from triplicate experiments.

within the therapeutic dose range used clinically [24]. The cell morphology showed that Act D caused cell shrinkage (Fig. 1A). Treatment with Act D significantly ($P < 0.001$) inhibited cell growth (Fig. 1B) and caused cell cytotoxicity (Fig. 1C). PI staining followed by flow cytometric analysis showed Act D significantly ($P < 0.05$) induced cell cycle arrest at the G_2/M phase (12 h post-treatment) in A549 cells followed by apoptosis (48 h post-treatment) (Fig. 1D). Annexin V/PI staining confirmed the induction of cell apoptosis caused by Act D 48 h post-treatment (Fig. 1E). Act D induced cell nuclei condensation, and immunostaining with lamin A/C antibodies demonstrated that Act D caused mitotic arrest due to the presence of lamin A/C degradation (Fig. 1F). All of these studies demonstrate that Act D induces cell apoptosis and G_2/M phase arrest in lung adenocarcinoma cells.

3.2. Act D induces DNA damage and cell apoptosis in p53-positive A549 cells and p53-negative H1299 cells

Tumor suppressive p53 causes cell cycle arrest to allow DNA repair and apoptosis under serious DNA damage [25]. Compared to p53-positive A549 cells, we used p53-negative H1299 cells to investigate the role of p53 in Act D-induced cell apoptosis. Western blotting showed that Act D significantly ($P < 0.05$) induced expression of the DNA damage marker γH2AX in A549 and H1299 cells, which indicates p53-independent DNA damage in H1299 cells (Fig. 2A). The cell morphology showed that Act D also caused cell shrinkage for 48 h in H1299 cells (Fig. 2B), which was accompanied by significant cell growth inhibition (Fig. 2C) and cell cytotoxicity (Fig. 2D) ($P < 0.001$). Treatment with Act D also induced apoptosis in H1299 cells by measuring the increased sub-G_1 cells (Fig. 2E) and Annnexin V staining (Fig. 2F). All of these studies demonstrate that Act D induces DNA damage, cell growth inhibition, and apoptosis via p53- and non-p53-regulated pathways.

3.3. Act D treatment induces mitochondrial transmembrane potential (MTP) loss, Mcl-1 decrease, and caspase-9/caspase-3 cascade activation

Because treatment with Act D decreases Mcl-1 expression in lung cancer cells [8], Act D may induce anti-apoptosis protein downregulation that causes MTP loss in lung adenocarcinoma cells. A549 and H1299 cells were treated with Act D for 48 h. Rhodamine 123 staining followed by flow cytometric analysis showed that Act D induced significant MTP loss in A549 and H1299 cells ($P < 0.05$) (Fig. 3A). Western blotting showed Act D induced cleavage of PARP and caspase-9/caspase-3 in A549 and H1299 cells (Fig. 3B). Further results showed that Act D reduced Mcl-1, Bcl-xL, and Bcl-2 expression in A549 and H1299 cells (Fig. 3C). Act D induced Mcl-1 downregulation in a time-kinetic manner and it caused significant reduction 12 h post-stimulation (Fig. 3D). All of these studies demonstrate that Act D downregulates anti-apoptosis Bcl-2 protein expression and MTP loss in lung cancer cells.

3.4. Proteasome, but not glycogen synthase kinase-3 (GSK-3), mediates Mcl-1 downregulation following Act D treatment

The proteasome regulates protein degradation in the cytosol of eukaryotic cells [26]. Act D may induce proteasome-mediated Mcl-1 downregulation. To investigate the possible mechanism of actinomycin D-induced Mcl-1 downregulation followed by cell apoptosis, we next examined the regulation of Mcl-1 decrease 12 h post-stimulation according to the results of Fig. 3D. A549 cells were co-treated with the proteasome inhibitor MG132 and Act D for 12 h, and the results showed that MG132 treatment reversed Mcl-1 expression (Fig. 4A). Because activation of GSK-3 leads to Mcl-1 destabilization, and Akt inhibits GSK-3 to stabilize Mcl-1 [13], Act D may induce GSK-3 activation to cause Mcl-1 downregulation. Western blotting showed that Act D did not induce Akt downregulation or GSK-3 activation in A549 cells (Fig. 4B). Treatment with the GSK-3 inhibitor BIO did not reverse Act D-induced Mcl-1 downregulation (Fig. 4C). All of these studies demonstrate proteasome-dependent, but GSK-3-independent, Act D-induced Mcl-1 downregulation in A549 cells.

3.5. Act D triggers Mcl-1 downregulation via a non-conventional route

Act D may cause reactive oxygen species (ROS) production in cancer cells [5,6,27]. Act D treatment may cause ROS production to downregulate Mcl-1. DCFDA-based staining followed by flow cytometric analysis showed that Act D did not cause ROS production in A549 cells (Fig. S1A). A MitoSOX red staining further confirmed the findings that Act D did not caused mitochondrial ROS generation in A549 cells (Fig. S1B). A549 cells were co-treated with Act D and the ROS inhibitor NAC for 12 h. However, inhibition of ROS did not reverse Mcl-1 downregulation (Fig. S1C). CDK1-mediated cyclin B1 activation regulates the instability of Mcl-1 [28], but treatment with the CDK1 inhibitor Ro-3306 did not inhibit Act D-mediated Mcl-1 downregulation in A549 cells (Fig. S1D). All of these studies demonstrate that ROS and CDK1 are not involved in Act D-induced Mcl-1 downregulation in A549 cells.

The regulation of protein degradation occurs via the ubiquitin–proteasome system and the autophagic-lysosomal pathway [29]. Autophagy and acidification may cause Act D-mediated Mcl-1 downregulation in A549 cells. The results showed no changes in the expression of autophagy markers in A549 cells treated with Act D (Fig. S2A). We found that the autophagy inhibitors CQ and 3-MA did not reverse Act D-induced Mcl-1 downregulation (Fig. S2B). Treatment with the acidification inhibitors niclosamide and bafilomycin A1 did not reverse Act D-induced Mcl-1 downregulation (Fig. S2C). All of these studies demonstrate that autophagy and acidification did not contribute to Act D-induced Mcl-1 downregulation in A549 cells.

The accumulation of misfolded proteins occurs from ER stress [30], and ER stress activates MAPK signaling pathways [31]. Because MAPK may induce Mcl-1 destabilization and protein degradation [16], we hypothesized that Act D-induced ER stress facilitated Act D-mediated Mcl-1 downregulation in A549 cells. Western blotting showed no increase in GRP-78 expression (Fig. S3A). Notably, Act D significantly induced the activation of all MAPKs, including ERK, JNK, and p38 MAPK, in A549 cells (Fig. S3B). However, treatment with MAPK inhibitors (SP600125, SB203580, and PD98059) did not reverse Act D-induced Mcl-1 downregulation (Fig. S3C). These studies demonstrate that Act D did not cause ER stress, and activated MAPKs are not involved in Act D-induced Mcl-1 downregulation in A549 cells.

3.6. Act D induces cullin-ring domain-containing E3 ligase-mediated Mcl-1 downregulation

The degradation of Mcl-1 is regulated by many E3 ligases, including FBW7, APC/C, β-TrCP, and Trim17 [15]. Therefore, Act D may induce E3 ligase-mediated Mcl-1 downregulation in A549 cells. A549 cells were co-treated with Act D and the Cullin-Ring E3 ligase inhibitor suramin and HECT E3 ligase inhibitor heclin for 12 h. Western blotting showed that the Cullin-Ring E3 ligase inhibitor partially reversed Mcl-1 expression, but the HECT E3 ubiquitin ligase inhibitor did not affect Mcl-1 expression (Fig. 5). All of these studies demonstrate that Mcl-1 downregulation is partially mediated via a Cullin-Ring E3-ligase in Act D-treated lung epithelial A549 cells.

3.7. Act D treatment causes cell apoptosis in lung mesenchymal and myofibroblast cells

Epithelial-mesenchymal transition (EMT) is essential in the metastasis and chemoresistance of cancer cells [32]. TGF-β is an immunosuppressive cytokine that promotes EMT and fibrosis [33,34] and induces anti-apoptosis Bcl-2 family mRNA and protein expression [21]. Act D may also reduce Mcl-1 downregulation in lung mesenchymal cells. A549 epithelial cells and WI-38 fibroblasts were treated with Act D for 48 h following a 48-h pre-treatment with TGF-β. The cell morphology

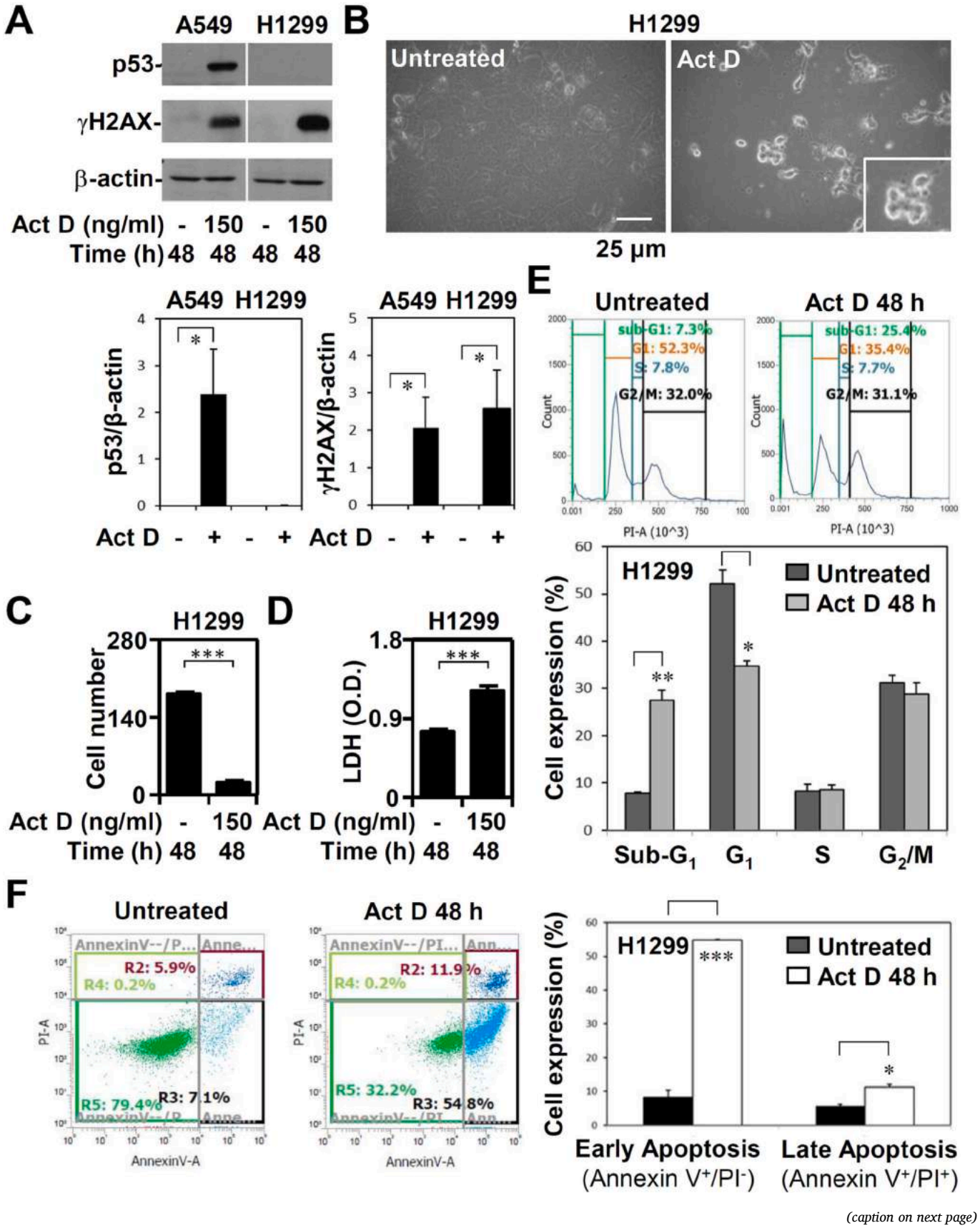

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Fig. 2. Actinomycin (Act) D induced apoptosis in p53-positive and p53-negative lung adenocarcinoma cells. (A) Western blotting showed the expression of a tumor suppressor gene (p53) and a DNA damage marker (γH2AX) in p53-positive A549 and p53-negative H1299 cells. The relative protein expression quantitated in a ratio value is mean ± SD obtained from three experiments. *$p < 0.05$. (B) Phase image showing cell apoptosis 2 days post-stimulation in H1299 cells. (C) Quantification of cell number in H1299 cells following 2 days post-stimulation. The cell count was extrapolated from a single image and the cell number is mean ± SD obtained from three culture wells. ***$p < 0.001$. (D) LDH assay, as shown by the optical density (O.D.), revealed cytotoxicity in H1299 cells following 2 days post-stimulation. The results are the mean ± SD obtained from three experiments. ***$p < 0.001$. (E) PI-based flow cytometry analysis determined the percentage of cell cycle (G_1, S, G_2/M) and cell apoptosis (sub-G_1) in H1299 cells following 2 days post-stimulation. All results are a representative set obtained from triplicate experiments and the results are the mean ± SD obtained from three experiments. *$p < 0.05$ and **$p < 0.01$. (F) Annexin V/PI-based flow cytometry analysis determined the percentage of H1299 cell apoptosis at early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) stages. All results are a representative set obtained from triplicate experiments and the results are a percentage of the mean ± SD obtained from three experiments. *$p < 0.05$ and ***$p < 0.001$.

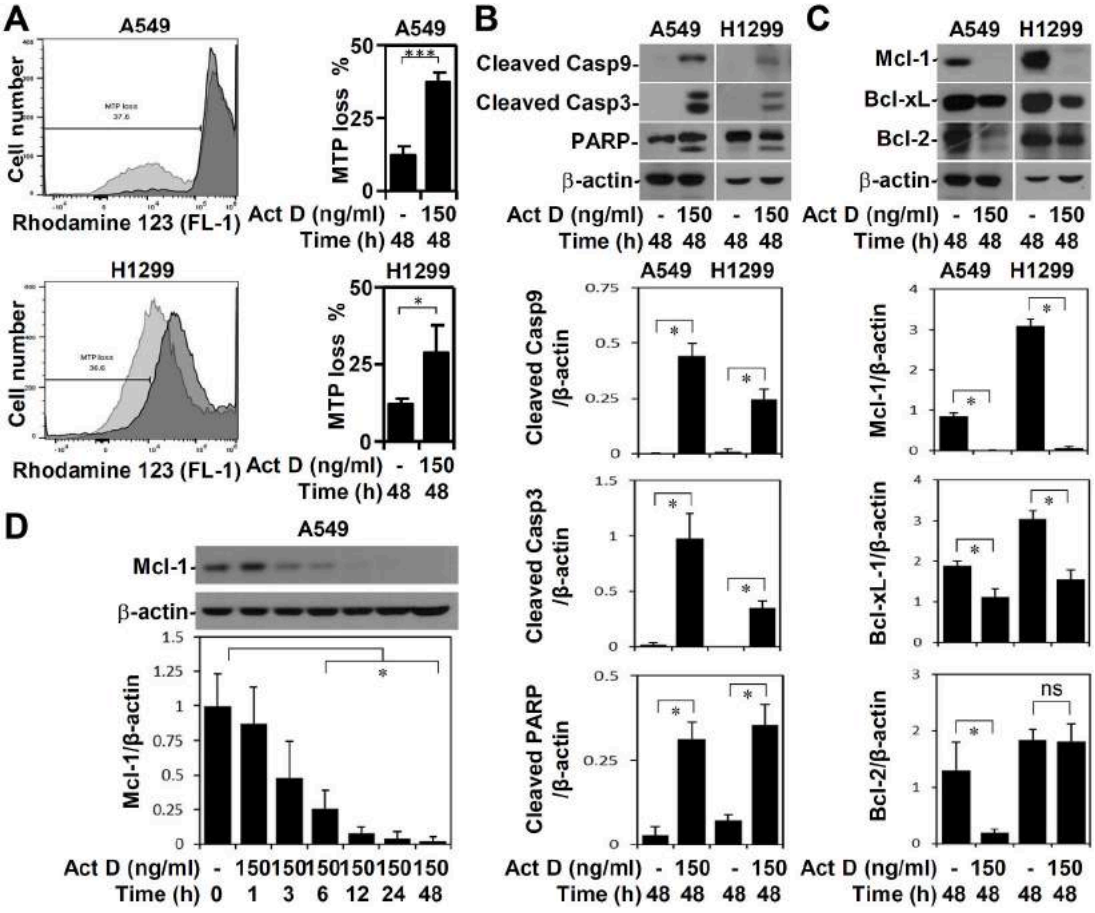

Fig. 3. Actinomycin (Act) D reduced the expression of Bcl-2 family member proteins followed by MTP loss. (A) Rhodamine 123 staining, followed by flow cytometric analysis, showed MTP loss in A549 and H1299 cells. The results are a percentage of the mean ± SD obtained from three experiments. *$p < 0.05$ and ***$p < 0.001$. (B) Western blotting showed cell death markers, including cleaved caspase (Casp) 9, cleaved Casp3, and PARP. (C) Western blotting showed anti-apoptotic Bcl-2 family member protein expression. (D) Western blotting showed Mcl-1 expression in a time-kinetic manner. All results are a representative set obtained from triplicate experiments. For Western blot analysis, the relative protein expression quantitated in a ratio value is mean ± SD obtained from three experiments. *$p <$ 0.05. ns, not significant.

showed that Act D still induced cell condensation in TGF-β-treated lung mesenchymal cells and lung myofibroblasts (Fig. 6A). The LDH assay revealed that Act D caused cell cytotoxicity for 48 h in TGF-β-treated lung mesenchymal cells and myofibroblasts (Fig. 6B), and Western blotting showed that Act D downregulated Mcl-1 expression in all TGF-β-differentiated cells (Fig. 6C). All of these studies demonstrate that Act D reduces Mcl-1 expression and causes cytotoxicity in TGF-β-treated lung mesenchymal cells and myofibroblasts.

4. Discussion

Our study demonstrated that Act D treatment facilitated MTP loss and the intrinsic apoptotic pathway involving caspase-9/caspase-3 cascade activation. For anticancer effects, TRAIL treatment induces cell apoptosis via death receptor signaling pathways in cancer cells. However, many lung cancer cells are resistant to TRAIL treatment. Cotreatment with Act D and TRAIL upregulated the death receptor signaling pathway [35], and the possible actions of Act D enhance the sensitivity of cell apoptosis in lung cancer cells [4]. Combined with our findings, these findings suggest that altering Mcl-1 expression is one of the pro-apoptotic effects of Act D, which facilitates the cytotoxic effects of anticancer treatment.

High levels of ROS cause DNA damage and p53 expression. The present study found that Act D induced DNA damage and p53 upregulation. However, no further ROS generation was found and the blockade of ROS did not decrease Act D-induced Mcl-1 downregulation. The present study excluded the involvement of ROS in regulating Act D-induced Mcl-1 downregulation and cell apoptosis in lung

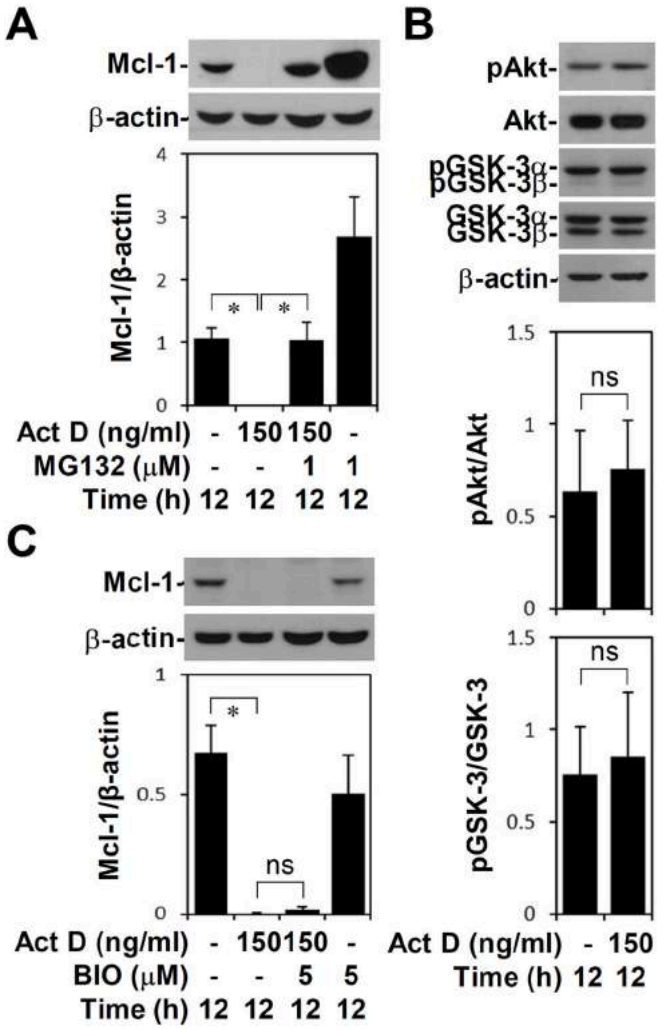

Fig. 4. Pharmacological inhibition of the proteasome, but not GSK-3β, reversed actinomycin (Act) D-induced Mcl-1 downregulation. Western blotting showed (A) Mcl-1 expression in Act D-stimulated A549 cells with or without the proteasome inhibitor (MG132) 12 h post-treatment, (B) the expression of phospho-Akt/Akt and phospho-GSK-3α/β/GSK-3α/β in Act D-stimulated A549 cells, and (C) Mcl-1 expression in GSK-3 inhibitor (Bio)-treated A549 cells. All results are a representative set obtained from triplicate experiments. The relative protein expression quantitated in a ratio value is mean ± SD obtained from three experiments. *$p < 0.05$. ns, not significant.

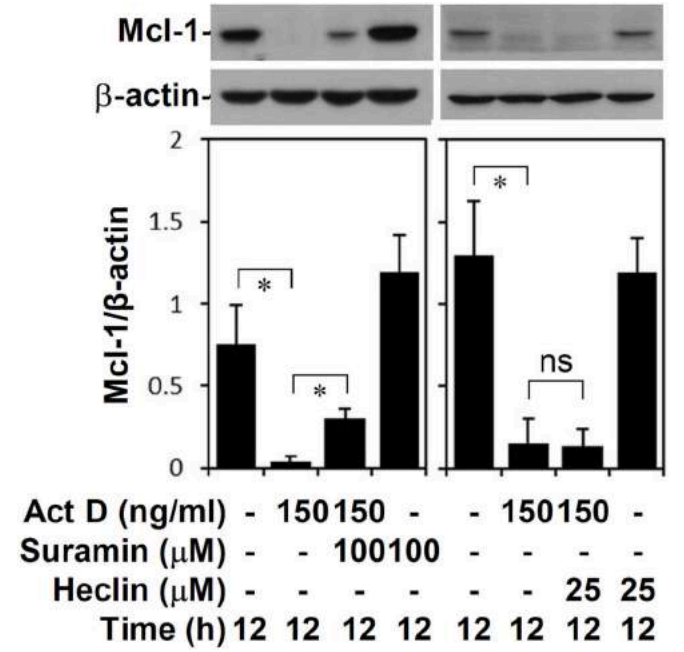

Fig. 5. Actinomycin (Act) D induced E3 ligase-mediated Mcl-1 downregulation. Western blotting showed Mcl-1 expression in A549 cells co-treated with or without E3 ligase inhibitors (suramin and heclin). All results are a representative set obtained from triplicate experiments. The relative protein expression quantitated in a ratio value is mean ± SD obtained from three experiments. *$p < 0.05$. ns, not significant.

adenocarcinoma cells. However, Act D treatment causes ROS generation in breast cancer cells and neuroblastoma cells [27,36]. Act D treatment may cause ROS-dependent and/or ROS-independent regulation over a short or long time course to trigger DNA damage and p53 expression.

Chaperone-mediated autophagy and macroautophagy clear damaged organelles and remove misfolded and aggregated proteins [37]. Unfolded proteins cause p62-mediated autolysosome acidification in the autophagic-lysosomal pathway [38]. Act D induces autophagy and inhibits cell cycle gene expression in A549 cells [39]. However, we determined that Act D induced G_2/M cell cycle arrest but did not induce autophagy in A549 cells. As similar with the findings that Act D arrests cell cycle of hepatocellular carcinoma cells and induces p53-dependent cell death [40] and Act V, an analogue of Act D, caused lung cancer cell apoptosis following a p53-regulated G_2/M cell cycle arrest [41], we further demonstrated the dual effects of Act D on p53-dependent and p53-independent cell death in lung adenocarcinoma cells. It is speculated the involvement of post-G_2/M arrest synergistically cooperated with Mcl-1 downregulation in facilitating Act D-induced lung cancer cell apoptosis. Because Act D is a transcriptional inhibitor, we speculate that Act D inhibits RNA synthesis of autophagy-related gene expression in A549 cells. ER stress may activate the downstream signaling pathways of PERK, IRE1α, and ATF6. Protein degradation, in addition to proteasome-mediated ERAD, may be executed with autophagy [31]. However, no further ER stress was identified in Act D-treated A549 cells in this study.

Many studies reported that E3 ligases, such as β-TrCP, APC/C, Fbw7, Mule, and Trim17, mediated Mcl-1 downregulation. We confirmed that Act D partially caused Cullin-Ring ubiquitin E3 ligase-mediated Mcl-1 downregulation. However, the specific Cullin-Ring ubiquitin E3 ligase needs further validation. Mule is a HECT-domain ubiquitin E3 ligase that regulates Mcl-1 downregulation [42]. Treatment with the HECT ubiquitin E3 ligase inhibitor heclin did not produce this effect in the present study. E3 ligases are regulated by CDK1-, p53-, GSK-3β-, and MAPK-regulated pathways in Mcl-1 downregulation [15,18]. Although we found that Act D induced MAPK and p53 activation, none of the identified pathways were required for Mcl-1 downregulation in Act D-treated cells.

TGF-β induces the expression of anti-apoptotic Bcl-2 family proteins in cancer cells [21]. According to our findings, TGF-β treatment increased Mcl-1 protein expression, but treatment with Act D downregulated Mcl-1 protein levels in differentiated cells. Many factors regulate Mcl-1 activation, including transcriptional activation and overexpression of deubiquitinase [18]. USP9X is a deubiquitinase that eliminates polyubiquitin chains from Mcl-1. Many transcription factors also regulate Mcl-1 expression, such as STAT3, NF-κB, HIF-1, ATF-6, and CREB. TGF-β induces the EMT process in a JAK/STAT3-regulated signaling pathway in lung cancer cells [43]. The present study demonstrated that Act D treatment effectively reduced Mcl-1 expression, which supports the application of Act D as an anticancer agent.

Furthermore, as established in TGF-β-induced lung mesenchymal and myofibroblast cell models, Act D treatment confers the anti-fibrotic effects on TGF-β differentiated cells in a post-treatment manner accordingly. As an inhibitor of transcription, pre-treatment of Act D may

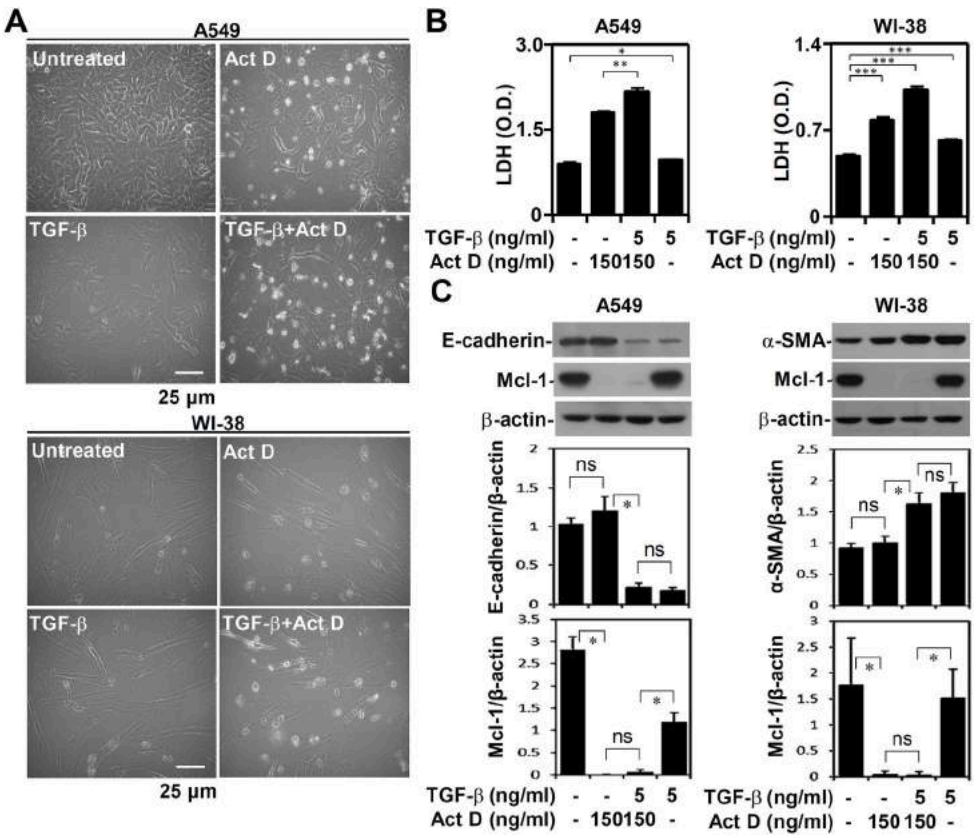

Fig. 6. Actinomycin (Act) D reduced Mcl-1 expression in TGF-β-treated lung mesenchymal cells and myofibroblasts. (A) Phase images show cell morphology in TGF-β-induced lung A549-differentiated mesenchymal cells and WI-38-differentiated myofibroblasts with Act D treatment 48 h post-treatment. (B) LDH assay revealed the cytotoxicity, and (C) Western blotting showed the expression of E-cadherin, α-SMA, and Mcl-1. All results are a representative set obtained from triplicate experiments. For LDH, the results, as shown by the optical density (O.D.), are the mean ± SD obtained from three experiments. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. For Western blot analysis, the relative protein expression quantitated in a ratio value is mean ± SD obtained from three experiments. $*p < 0.05$. ns, not significant.

affect TGF-β-induced gene expression leading to defects on cell differentiation and growth. Furthermore, under pre- and co-treatment, the direct antagonizing effects of Act D on TGF-β may need further investigation in a computational modeling test or an in vitro molecule interaction assay.

5. Conclusion

We explored the possible molecular mechanisms of Mcl-1 downregulation in Act D-treated lung adenocarcinoma cells. We identified that Mcl-1 downregulation was targeted by suramin ubiquitin-E3 ligase followed by the induction of the proteasome pathway. Act D caused Mcl-1 downregulation in TGF-β-induced EMT lung mesenchymal and myofibroblast cells. The identified mechanism of the Act D-induced Mcl-1 decrease, together with the previous findings that synergistically blocking the WNT/β-catenin signaling followed by facilitating cell apoptosis [3,4] and that causing a RNase L-regulated DNA damage in lung cancer cells [5], may assist the therapeutic strategy in treating lung cancers.

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CRediT authorship contribution statement

Chia-Ling Chen: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing. **Po-Chun Tseng:** Validation, Investigation, Writing – original draft. **Yen-Po Chao:** Validation, Investigation, Writing – original draft. **Ting-Jing Shen:** Validation, Investigation, Methodology. **Ming-Kai Jhan:** Validation, Investigation, Methodology. **Yung-Ting Wang:** Investigation, Methodology, Project administration. **Thi Thuy Nguyen:** Investigation, Methodology. **Chiou-Feng Lin:** Conceptualization, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2023.121615.

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