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Original Articles

HS-173 as a novel inducer of RIP3-dependent necroptosis in lung cancer

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ABSTRACT

Necroptosis is a form of regulated necrotic cell death mediated by receptor-interacting kinase 3 (RIP3). Recently, necroptosis has gained attention as a novel alternative therapy to target cancer cells. In this study, we screened several chemotherapeutics used in preclinical and clinical studies, and identified a drug HS-173 that induces RIP3-mediated necroptosis. HS-173 decreased the cell survival in a dose-dependent manner in RIP3-expressing lung cancer cells, compared to the cells lacking RIP3. Also, the cell death induced by HS-173 was rescued by specific necroptosis inhibitors such as necrostatin-1 and dabrafenib. Additionally, HS-173 increased the phosphorylation of RIP3 and MLKL, which was decreased by necroptosis inhibitors, indicating that HS-173 activates RIP3/MLKL signaling in lung cancer cells. HS-173 increased the necroptotic events, as observed by the increased levels of HMGB1 and necroptotic morphological features. Furthermore, HS-173 inhibited the tumor growth by stimulation of necroptosis in mouse xenograft models. Our findings offer new insights into the role of HS-173 in inducing necroptosis by enhancing RIP3 expression and activating the RIP3/MLKL signaling pathway in lung cancer cells.

1. Introduction

Lung cancer presents as a metastatic disease in majority of the cases, and it is the most common cause of cancer-related deaths in the world. It is estimated that lung cancer contributes to about 1.61 million new cases and 1.38 million deaths worldwide [1]. There are two major types of lung cancer based on the histological type: non-small cell lung cancer (NSCLC) and small cell lung cancer. Considerable advances in targeted chemotherapy have been made in the treatment of patients with NSCLC harboring specific gene alterations, such as epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) translocations [2,3], but cytotoxic chemotherapy still remains the key therapy for NSCLC patients without specific gene alterations. At present, platinum-based conventional cytotoxic chemotherapy is widely used as the first-line of treatment for NSCLC [4]. Gemcitabine, docetaxel, paclitaxel, and pemetrexed coupled with platinum are the standard chemotherapeutic agents [5–7]. However, nearly all patients develop resistance to these drugs in addition to their adverse effects, thereby limiting the efficacy. Therefore, more effective alternative therapy is required to improve the treatment of lung cancer patients.

Necrosis, a type of cell death resulting from disease and injury of the organ or tissue, was originally considered to be an accidental and unregulated cell death [8]. However, it has recently been recognized that necroptosis, a programmable form of necrosis, may be regulated via defined signal transduction pathways [9]. Necroptosis is distinguished from apoptosis, in that caspase activation is dispensable for cell death. Unlike apoptosis, necroptosis results in plasma membrane rupture, thus spilling the contents of the cell and triggering the immune system and inflammation [10,11]. In this unique type of cell death, RIP3 and MLKL play a role as critical kinases responsible for mediating necroptosis [12]. The activation of RIP3 induces the phosphorylation of MLKL, which translocates to the plasma membrane and induces membrane disruption, thus acting as a direct executioner of necroptosis [13–16]. Therefore, necroptosis is being targeted to develop alternative therapeutics to kill cancer cells.

In this study, we screened several chemotherapeutics used in

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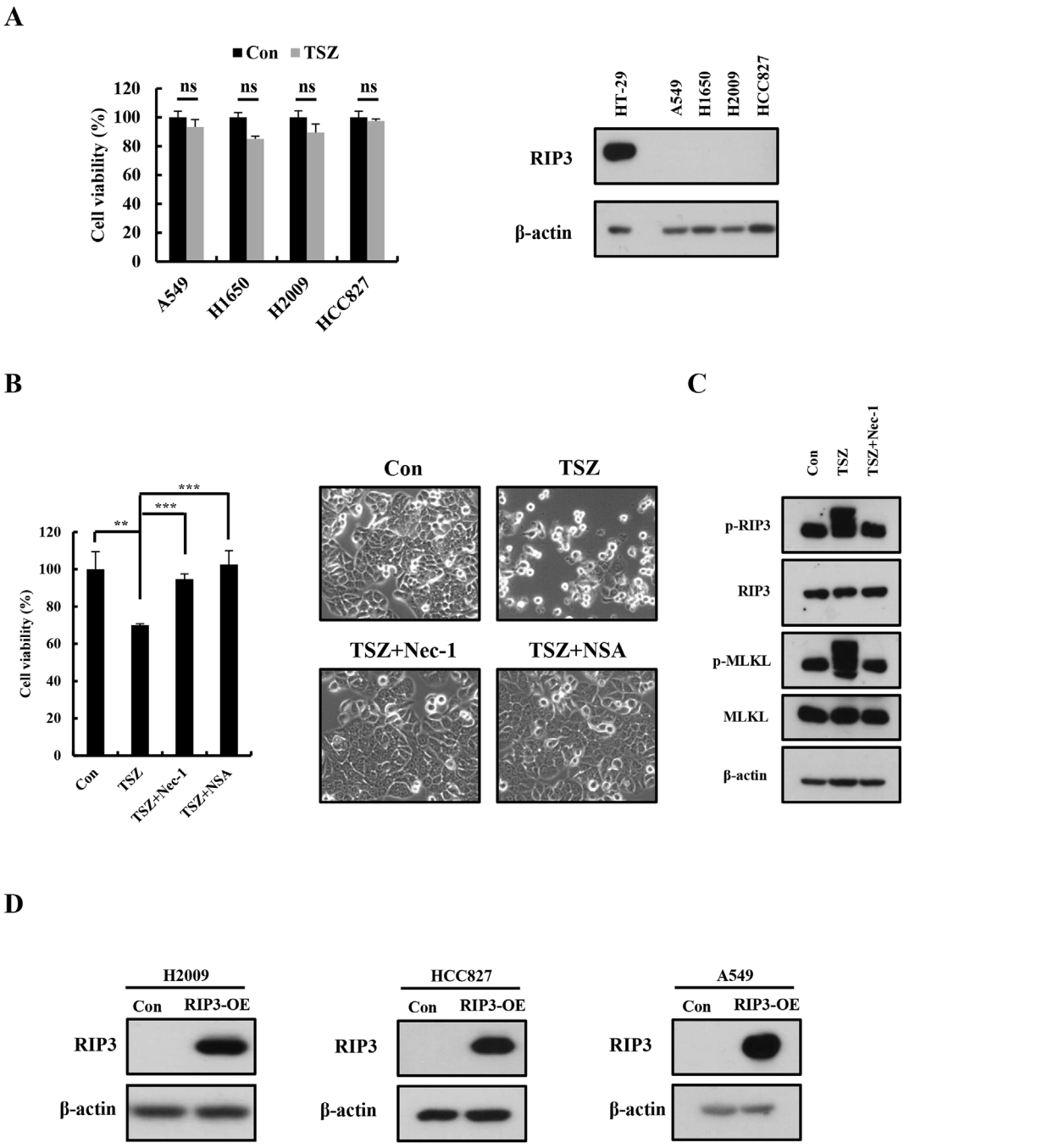

Fig. 1. Necroptosis sensitivity of RIP3 expressing lung cancer cells. (A) Lung cancer cells including H2009, HCC827, A549 and H1650 were treated with TSZ (30 ng/ml TNF-α, 200 nM Smac mimetics, 20 μM zVAD). After 24 h of TSZ treatment, the cell viability was measured using MTT assay. RIP3 levels were assessed by western blotting. (B) HT-29 RIP3-positive cells were treated with Nec-1 (40 μM) and dabrafenib (10 μM) for 1 h and then treated with TSZ. After 24 h of TSZ treatment, the cell viability was measured using MTT assay and the cell morphology was observed using a microscope. (C) HT-29 cells were treated with Nec-1 for 1 h, and then treated with TSZ. After 4 h, cell lysates were prepared and analyzed by western blotting for p-RIP3 and p-MLKL. (D) RIP3 levels were assessed by western blotting in both control and RIP3-OE lung cancer cell lines (H2009, HCC827, and A549). Data are expressed as means ± SD. from three independent experiments (**$P < 0.01$ and ***$P < 0.001$).

preclinical and clinical studies, and identified HS-173, a phosphoinositide 3-kinase (PI3K) inhibitor that induces RIP3-mediated necroptosis. The PI3K signaling pathway plays an important role in the regulation of cell proliferation, cell cycle, survival, and metastasis [17]. Aberrant activation of the PI3K/AKT/mTOR signaling pathway induces the pathogenesis of several cancers including lung cancer [18]. Activation of the PI3K/AKT/mTOR pathway may be involved in the resistance to chemotherapeutic and targeted drugs in different cancers [19]. Recently, some studies have reported that PI3K/AKT/mTOR pathway is associated with necroptotic cell death in drug treatment. AKT regulates the activation of RIP1 and its downstream during necroptosis [20]. Further, PI3K inhibitor significantly increased the necroptotic cell death, and mTOR inhibitor induced RIP-dependent necroptosis [21,22]. Given that the PI3K/AKT signaling is associated with necroptosis, we hypothesized that targeting the PI3K/AKT signaling pathway may increase the necroptotic cell death through RIP3 activation. Therefore, we explored whether HS-173, a novel PI3K inhibitor might enhance the killing of lung cancer cells by stimulation of necroptosis.

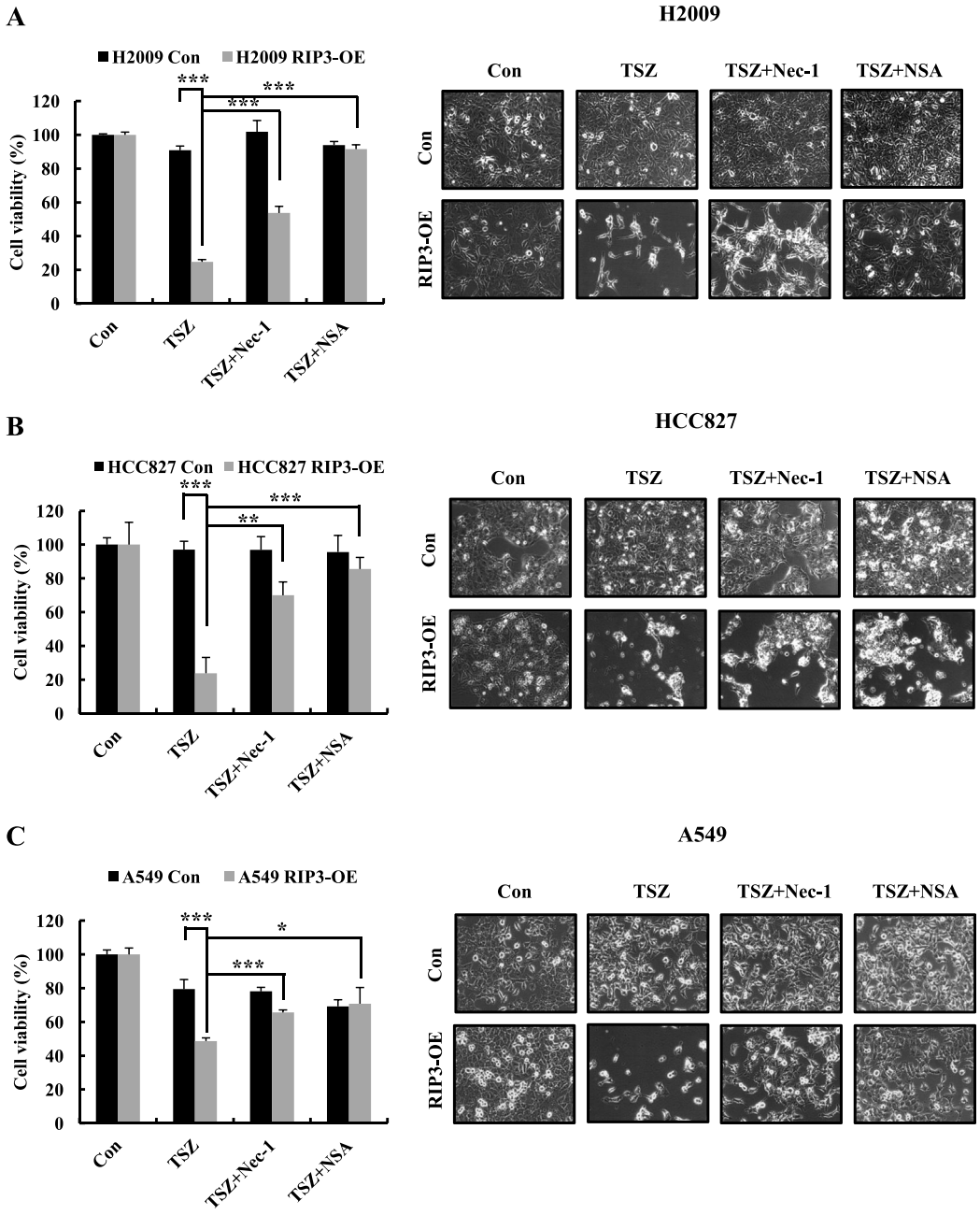

Fig. 2. Induction of necroptotic cell death in RIP3-OE lung cancer cells. (A, B and C) both control and RIP3-OE lung cancer cells (H2009, HCC827, and A549) were treated with Nec-1 (40 μM) and NSA (1 μM) for 1 h and then treated with TSZ. After 48 h of TSZ treatment, the cell viability was measured using MTT assay. Morphologic features were observed using a microscope. Data are expressed as means ± SD. from three independent experiments ($*P < 0.05$ and $***P < 0.001$).

2. Materials and methods

2.1. Cell culture

The human lung cancer cells H2009, HCC827, H1650 and A549, and the colon cancer cell line, HT-29, were purchased from the American Type Culture Collection (AATC, Manassas, VA). H2009, HCC827, H1650, A549 and HT-29 cells were cultured in Roswell Park Memorial Institute Media 1640 (RPMI-1640), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell culture media, FBS, penicillin-streptomycin, and other supplementary reagents were purchased from GIBCO (Uxbridge, UK). All the cell lines were maintained at 37 °C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2.

2.2. Establishment of RIP3 over-expressed lung cancer cell lines

The pBABE vector employs a promoter to drive high level expression of RIP3, and it contains a puromycin resistance marker to select the transfected cells. HEK293T cells were transfected with the recombinant vectors and packing mix. The viral particles were harvested from the growth media. To create stable RIP3-expressing lung cancer cells, the viral particles containing the recombinant vectors were transfected into lung cancer cell lines (H2009, HCC827 and A549) using Lipofectamine 2000 reagent (Invitrogen), and the cells were grown in the medium supplemented with puromycin for 48 h. RIP3 overexpression was confirmed by western blotting.

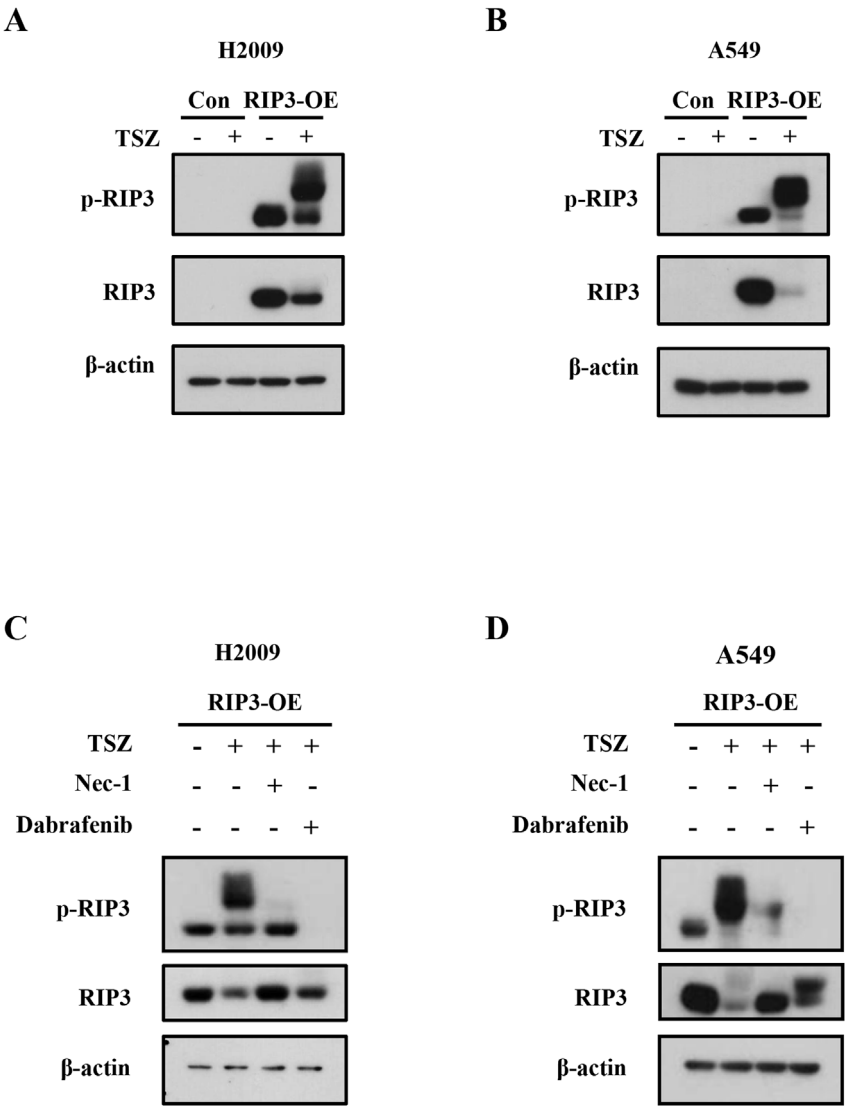

Fig. 3. Induction of necroptosis *via* RIP3 upregulation in RIP3-OE lung cancer cells. (A and B) The indicated both control and RIP3-OE lung cancer cells (H2009 and A549) were treated with TSZ for 6 h. Cell lysates were collected and assessed by western blotting for p-RIP3 levels. (C and D) H2009 and A549 RIP3-OE cells were treated with Nec-1 (40 μM) and dabrafenib (10 μM) for 1 h and then treated with TSZ for 6 h. The levels of p-RIP3 were determined by western blotting analysis.

2.3. Lentiviral shRNA experiments

The short-hairpin RNA (shRNA) plasmids targeting hRIP3 mRNA (NM_006871) were obtained from Sigma-Aldrich. Lentiviral plasmids were transfected into 293 TN cells (System Biosciences, LV900A-1) using Lipofectamine 2000 (Invitrogen). Pseudoviral particles were collected 2 days after lentiviral plasmid transfection and were used to infect the cells in the presence of polybrene (8 μg/ml). Cells were selected with puromycin 2 days after the infection, and RIP3 knockdown was confirmed by western blotting.

2.4. Preparation of HS-173

The imidazopyridine derivative, ethyl 6-(5-(phenylsulfonamido) pyridin-3-yl)imidazo[1,2-a]pyridine-3-carboxylate (HS-173), is a novel PI3Kα inhibitor that was synthesized as described in our previous study [23–27]. For all the studies, HS-173 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM before use.

2.5. MTT assay

Cells were treated with a combination of TNF-alpha (T), Smac mimetics (S), and z-VAD (Z) (TSZ) or either DMSO as a control or various concentrations (0.1–10 μM) of HS-173. After the cells were incubated for 48 h, and then 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (2 mg/ml) was added to each well, and the plate was incubated for another 4 h at 37 °C. The medium was then removed, the formazan crystals formed were dissolved in DMSO (100 μl/well) with constant shaking for 5 min. The absorbance of the solution was then measured by a microplate reader at 540 nm. This assay was conducted in triplicate.

2.6. Western blotting

Cells were washed with ice-cold PBS before lysis using RIPA buffer containing protease and phosphatase inhibitor cocktails (GenDEPOT, Barker, TX). Equal amounts of protein were separated using 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. The protein transfer was checked by

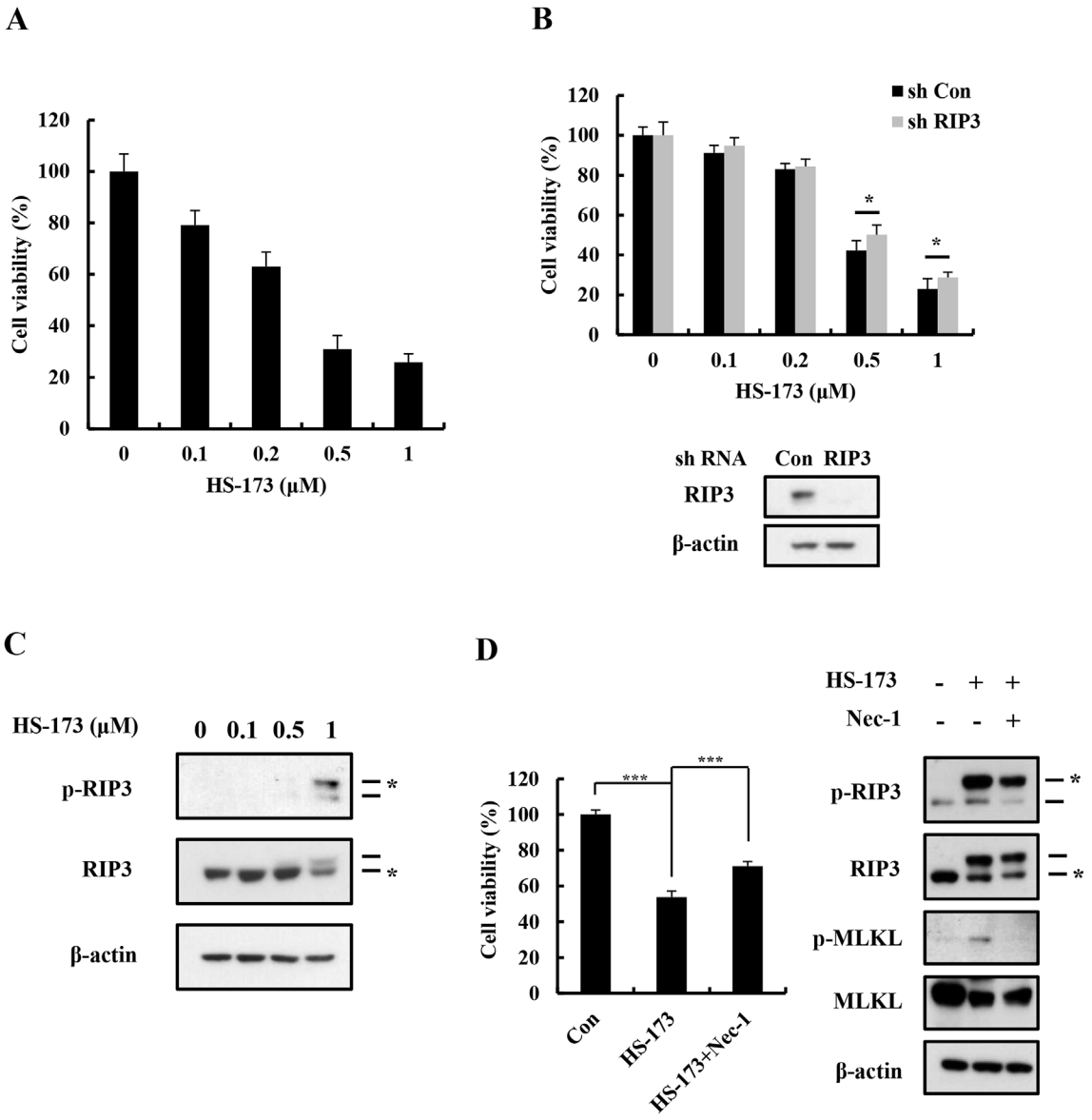

Fig. 4. Induction of necroptosis by HS-173 in HT-29 cells. (A) HT-29 cells were treated with various concentrations of HS-173 (0.1–1 μM) and the cell viability was measured using MTT assay. (B) HT-29 cells with stable expression of shRNAs against RIP3 were treated with HS-173 (0.1–1 μM) and the cell viability was measured using MTT assay. (C) The p-RIP3 levels in HT-29 cells treated with HS-173 (1 μM) were determined by western blotting. (D) HT-29 cells were treated with Nec-1 (40 μM) for 1 h and then treated with HS-173 (1 μM). After 24 h, cell viability was measured using MTT assay. The level of p-RIP3 and p-MLKL were assessed by western blotting. Data are expressed as means ± SD. from three independent experiments ($^*P < 0.05$).

staining with Ponceau S solution (Sigma–Aldrich). Immunostaining was carried out by incubating the blots with the primary antibodies. After washing three times, the blots were incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) and detected using enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Primary antibodies were purchased as follows: RIP3 (Cell Signaling Technology, MA, US), p-RIP3, p-MLKL (Abcam, Cambridge, UK) and MLKL, β-actin (Sigma-Aldrich, Ohio). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX).

2.7. Immunofluorescence

After treating with HS-173, cells were washed twice with PBS, and fixed by incubating in an acetic acid: ethanol solution (1:2) for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated in blocking buffer for 1 h at room temperature. Then the cells were incubated overnight with the primary antibody (p-MLKL or HMGB1; abcam, Cambridge, UK) at 4 °C. After washing several times with PBS, cells were incubated with rhodamine isothiocyanate (RITC)-conjugated rabbit secondary antibody (1:100; Dianova, Hamburg) for 1 h at room temperature, and then counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Slides contacting the immunostained cells were washed twice with PBS, covered with the anti-fading agent, 1,4-diazobicyclo (2,2,2)-octane (DABCO; Sigma-Aldrich), and observed and analyzed using a confocal laser-scanning microscope (Olympus) at 488 and 568 nm.

2.8. Transmission electron microscopy

The samples were suspended in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h, dehydrated, and infiltrated with Spurr resin. 90-nm ultra-thin sections were stained with uranyl acetate and analyzed through an electron microscope (FEI/Philips EM 208S/Morgagni) at 80 kV.

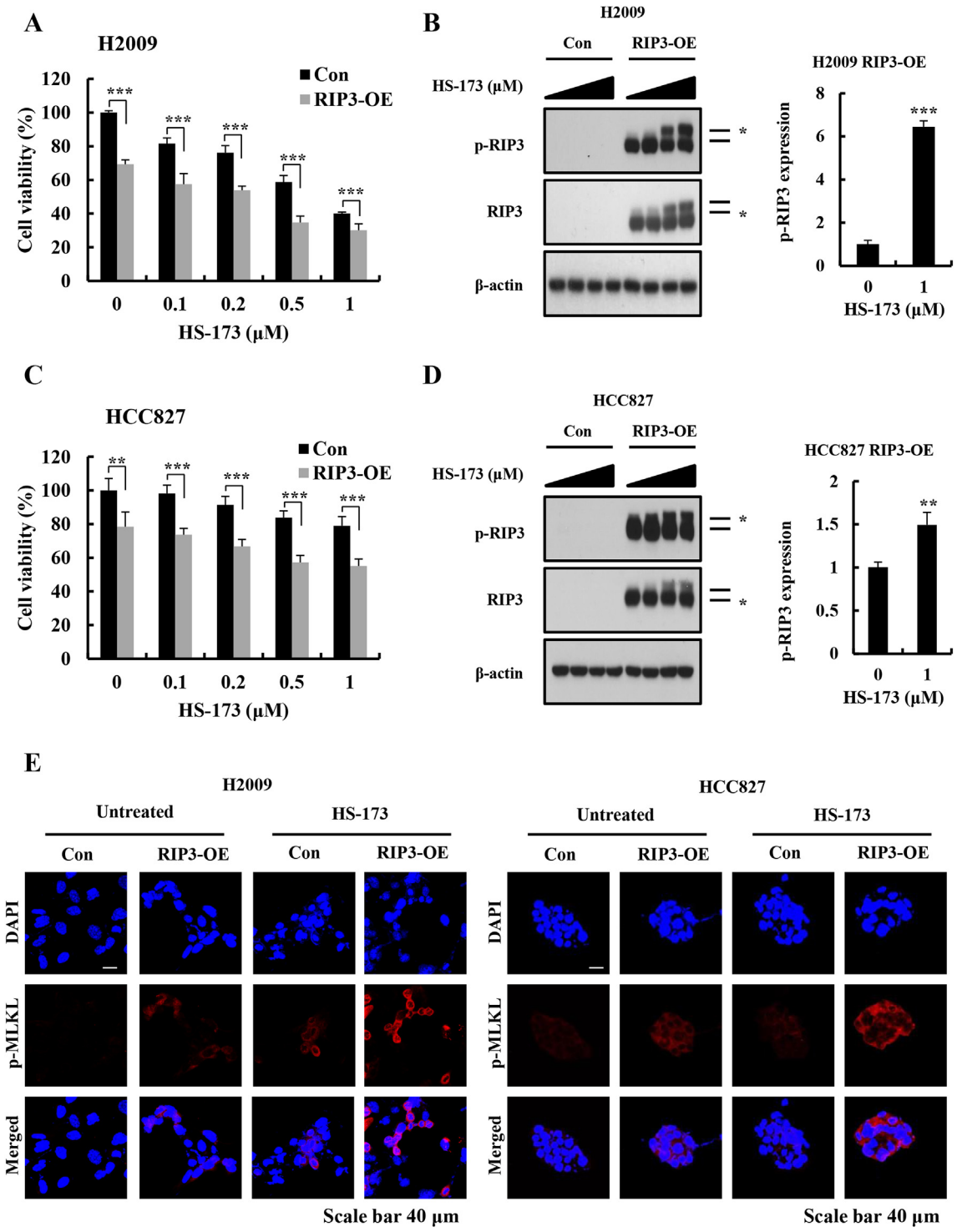

Fig. 5. Induction of necroptosis by HS-173 via upregulation of RIP3 in RIP3-OE lung cancer cells. (A) The both control and RIP3-OE H2009 cells were treated with HS-173 (0, 0.1, 0.2, 0.5, 1 μM) for 72 h and the cell viability was measured using MTT assay. (B) Both control and RIP3-OE H2009 cells were treated with HS-173 (0, 0.1, 0.5, 1 μM) for 24 h. The levels of p-RIP3 were analyzed by western blotting. (C) Cell viability of the both control and RIP3-OE HCC827 cells treated with HS-173 (0, 0.1, 0.2, 0.5, 1 μM) for 72 h was estimated by MTT assay. (D) The both control and RIP3-OE HCC827 cells were treated with HS-173 (0, 0.1, 0.5, 1 μM) for 24 h. The levels of p-RIP3 were analyzed by western blotting. (E) The both control and RIP3-OE lung cancer cells (H2009 and HCC827) were treated with or without HS-173 (0.5 μM) for 24 h. The level of p-MLKL was determined by immunofluorescence. Data are expressed as means ± SD. from three independent experiments (**$P < 0.01$ and ***$P < 0.001$).

2.9. Animals

Male BALB/c nude mice were purchased from Orient Bio Animal Inc. (Gyeonggido, Korea). All animal experiments were performed in accordance with the guidelines of the INHA Institutional Animal Care and Use Committee (INHA IACUC) at the Medical School of Inha University, under the authority of project number INHA 170217–480. Animals were fed with standard chow and tap water *ad libitum*, and maintained under a 12 h dark/light cycle at 21 °C under specific pathogen-free conditions.

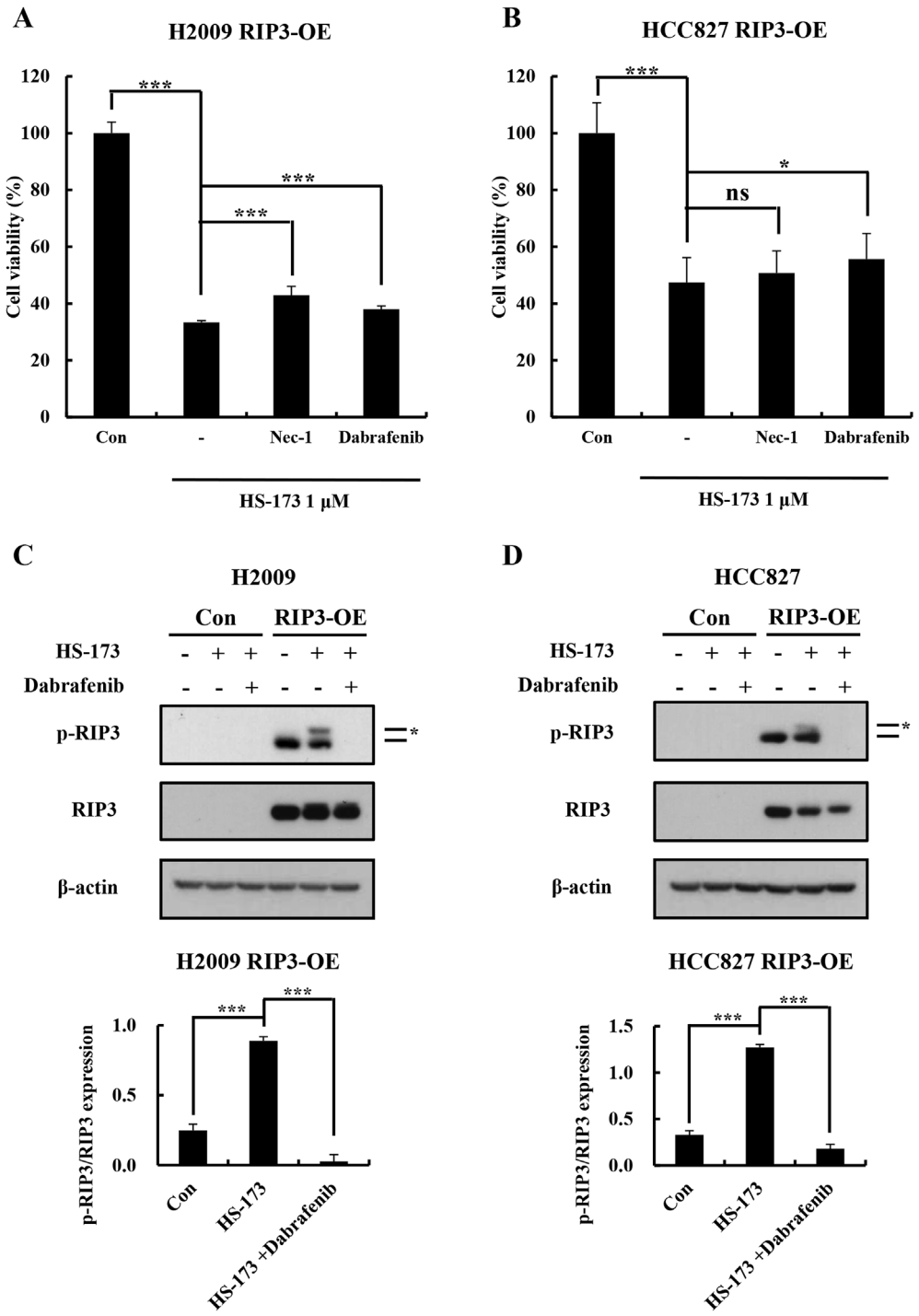

Fig. 6. Requirement of RIP3 for necroptosis by HS-173. (A and B) The RIP3-OE H2009 cells were treated with Nec-1 (40 μM) and dabrafenib (10 μM) for 1 h and then treated with HS-173 (1 μM). After HS-173 treatment for 72 h, the cell viability was measured using MTT assay. (C) The both control and RIP3-OE lung cancer cells (H2009 and HCC827) were treated with dabrafenib (10 μM) for 1 h and then treated with HS-173 (1 μM) for 24 h. The levels of p-RIP3 were analyzed by western blotting. Data are expressed as means ± SD. from three independent experiments (*$P < 0.05$ and ***$P < 0.001$).

2.10. Tumor xenograft studies

Male BALB/c nude mice (4 weeks old, weighing 18–20 g) were injected in the flank with 2×10^6 HT-29 cells. When the tumor size reached approximately 10–50 mm^3, treatment groups were administered with HS-173 (30 mg/kg) by intraperitoneal injections thrice a week. Tumor size and body weight were measured every 2 days per week, and tumor volume was calculated using the formula, $0.5 \times \text{length} \times \text{width}^2$ using Vernier calipers.

2.11. Statistical analysis

Data are expressed as means ± standard deviation (SD), and the statistical significance was calculated using analysis of variance (ANOVA) or unpaired Student's t-test, as appropriate. A P-value ≤ 0.05 was considered statistically significant.

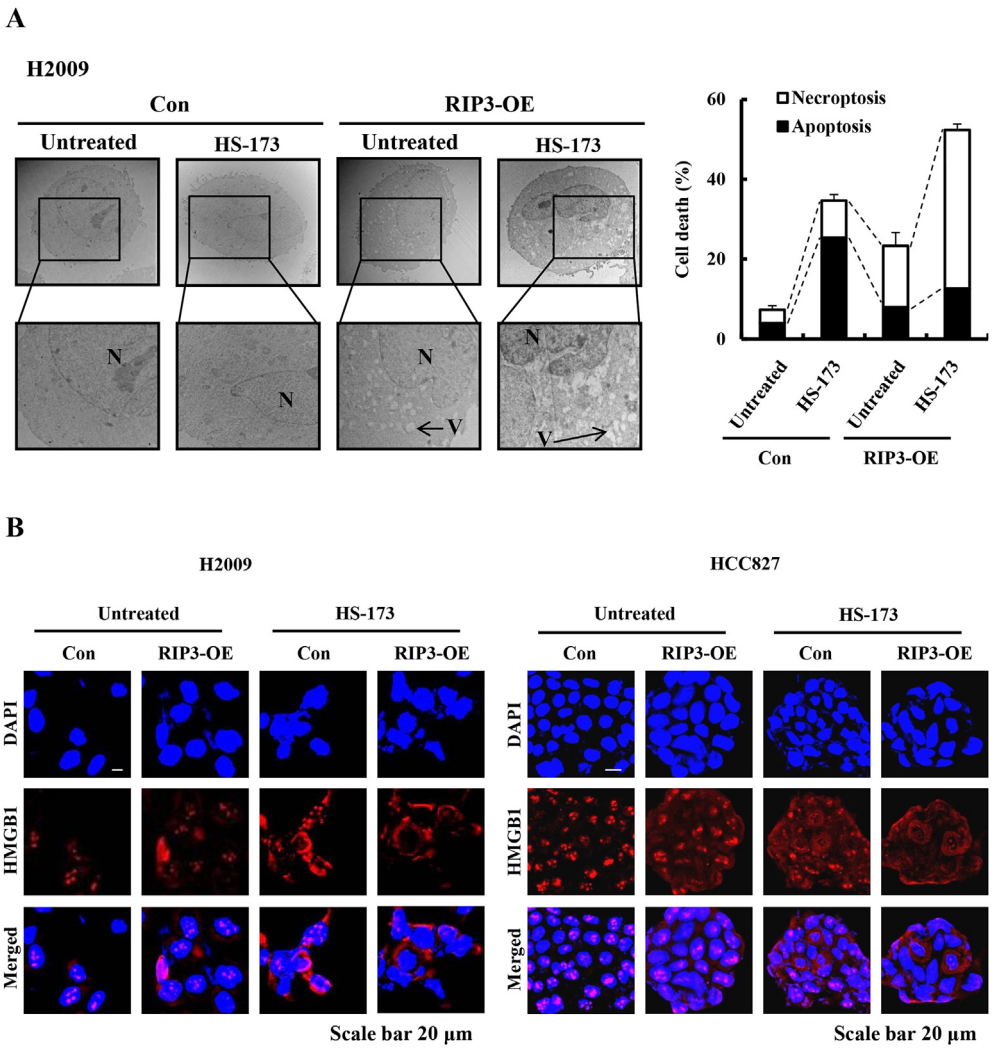

Fig. 7. Necroptosis-mediated morphological and physiological changes induced by HS-173 treatment in RIP3-OE lung cancer cells. (A) Representative TEM pictures of both control and RIP3-OE H2009 cells treated with HS-173 (0.5 μM) for 48 h. Black arrowheads indicate vacuoles. (N: nucleus, V: vacuoles) (B) Both control and RIP3-OE lung cancer cell lines (H2009 and HCC827) were treated for 24 h with or without HS-173 (0.5 μM). HMGB1 levels were determined by immunofluorescence.

3. Results

3.1. Necroptosis sensitivity on RIP3 expression in lung cancer cells

To investigate whether lung cancer cells are sensitive to necroptosis, several lung cancer cell lines, H2009, HCC827, A549, and H1650, and a positive colon cancer cell line, HT-29, were treated with necroptosis inducer TSZ (T:TNF-alpha, S:Smac mimetics, Z:z-VAD). The cells lacking RIP3 expression, which include H2009, HCC827, A549, and H1650 cell lines, did not show any cell death upon TSZ treatment (Fig. 1A). This observation is consistent with the previous reports suggesting that cells without RIP3 are completely resistant to the prototypical necroptosis stimuli. However, TSZ significantly induced cell death in HT-29 cells that have endogenous RIP3 expression. This cell death was inhibited by treatment with two necroptosis inhibitors, necropstatin-1(Nec-1, RIP1 inhibitor) and necrosulfonamide (NSA, MLKL inhibitor) [14,15] (Fig. 1B). Furthermore, TSZ treatment induced the phosphorylation of RIP3 in HT-29 cells, which was inhibited by the treatment of these cells with necroptosis inhibitor Nec-1 (Fig. 1C). To further identify the function and sensitivity of necroptosis mediated by RIP3, we established a retroviral vector-mediated RIP3 over-expressed (RIP3-OE) stable lung cancer cell lines using H2009, HCC827, and A549 cells (Fig. 1D).

3.2. Induction of necroptosis in RIP3-OE lung cancer cells

Since cell viability upon TSZ treatment was unaffected in RIP3-lacking lung cancer cells, we checked whether TSZ could induce necroptotic cell death in RIP3-OE lung cancer cells. As shown in Fig. 2A, H2009 RIP3-OE cells showed a significantly higher necroptotic cell death upon TSZ treatment, compared to RIP3-lacking H2009 cells. Also, Nec-1 and NSA significantly inhibited TSZ-mediated necroptosis in H2009 RIP3-OE cells. Likewise, these results were confirmed in HCC827 and A549 RIP3-OE lung cancer cells (Fig. 2B and C). Since RIP3 phosphorylation is an important event in TSZ-induced necroptosis, we examined the changes in RIP-mediated necroptosis signaling during the TSZ-induced necroptotic cell death [28]. As shown in Fig. 3A and B, TSZ induced RIP3 phosphorylation in H2009 and A549 RIP3-OE lung cancer cells. Additionally, the induction of RIP3 phosphorylation by TSZ was inhibited by treatment with either Nec-1 or RIP3 phosphorylation inhibitor, dabrafenib. These results show that presence of RIP3 determines the sensitivity of these cancer cells to necroptosis (Fig. 3C and D).

3.3. Induction of necroptosis by HS-173 in HT-29 cells

To further determine whether HS-173 induces necroptotic cell

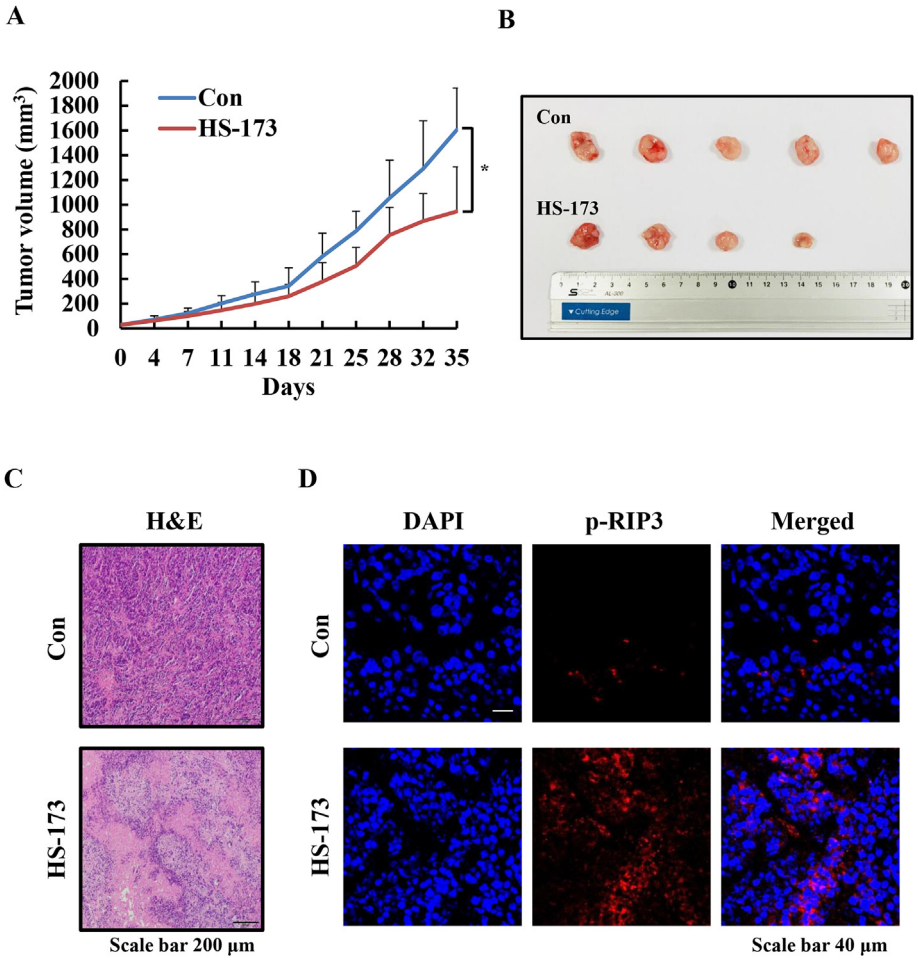

Fig. 8. Inhibition of tumor growth by HS-173 *via* necroptosis in HT-29 mouse xenograft model. (A and B) HT-29 xenograft mice were intraperitoneally injected with the vehicle alone or HS-173 (30 mg/kg, 3 times per week). Tumor volume was measured twice a week in HT-29 xenograft models. After 35 days, tumors were excised. (C) Isolated tumors were sectioned and immunostained with p-RIP3 antibody. Data are presented as means ± SD. (*$P < 0.05$).

death, we assessed the cell viability of RIP3-positive HT-29 cells after treatment with various concentrations of HS-173 for 48 h. We found that HS-173 decreased the cell viability of HT-29 cells in a dose-dependent manner (Fig. 4A). However, in HT-29 cells, where RIP3 was knocked down by shRNAs, HS-173 did not decrease the cell viability as compared to that in the RIP3-positive HT-29 control cells (Fig. 4B), indicating that cell death induced by HS-173 is mediated by necroptosis. Also, HS-173 effectively induced cell death and the phosphorylation of RIP3 and MLKL in HT-29 cells in a dose-dependent manner, which was blocked by treatment with Nec-1 (Fig. 4C and D). These results imply that RIP3 is required for HS-173-induced necroptotic cell death.

3.4. Induction of necroptosis by HS-173 via upregulation of RIP3 in RIP3-OE lung cancer cells

Next, to address whether HS-173-induced cell death is dependent on RIP3 even in lung cancer cells, we investigated the cell viability and levels of RIP3 phosphorylation in RIP3-OE lung cancer cells upon treatment with HS-173. HS-173 significantly increased the cell death and RIP3 phosphorylation in H2009 RIP3-OE cells as compared to that in H2009 RIP3-lacking cells in a dose dependent manner (Fig. 5A and B). HS-173 also showed similar effects in HCC827 RIP3-OE cells (Fig. 5C and D). RIP3 activates MLKL that translocates to the plasma membrane, resulting in necroptotic cell death by disrupting the integrity of the membrane [29]. Therefore, we investigated whether HS-173 increased the MLKL translocation into plasma membrane using immunofluorescence. HS-173 treatment increased the MLKL phosphorylation and translocation to the plasma membrane in H2009 and HCC827 RIP3-OE lung cancer cells (Fig. 5E). To clarify the role of RIP3 in HS-173-induced cell death, H2009 and HCC827 RIP3-OE lung cancer cells were treated with Nec-1 and dabrafenib. We found that Nec-1 and dabrafenib treatment significantly prevented HS-173-induced increase in the cell death of RIP3-OE lung cancer cells (Fig. 6A and B). The upregulation of RIP3 caused by HS-173 was markedly inhibited when the cells were pretreated with dabrafenib in both H2009 and HCC827 RIP3-OE lung cancer cells as seen by western blotting (Fig. 6C and D).

3.5. Necroptosis-mediated morphological and physiological changes by HS-173 in RIP3 RIP3-OE lung cancer cells

A more accurate morphological analysis using transmission electron microscopy (TEM) was performed to determine the type of cell death induced by HS-173 in H2009 RIP3-OE lung cancer cells. As shown in Fig. 7A, the H2009 control cells showed normal morphology of the cell components and cell membrane. Treatment of cells with HS-173 led to slight morphological changes in the H2009 control cells. However, H2009 RIP3-OE cells exhibited a necrosis-like phenotype changes such as vacuoles. Additionally, in H2009 RIP3-OE cells, treatment of HS-173 presented extensive organelle and cell swelling and cytoplasmic vacuolation. Recent studies have shown that RIP3-mediated necroptosis regulates the release of HMGB1 to the cytosol from the nucleus, which is suppressed by Nec-1 [30,31]. Therefore, we tested whether HS-173 treatment induced the release of HMGB-1 into the cytoplasm in H2009

RIP3-OE cells. As a result, there was a significant increase in the releases of HMGB1 into cytoplasm upon HS-173 treatment in RIP3-OE cells as compared to the control cells (Fig. 7B).

3.6. Inhibition of tumor growth by HS-173 via necroptosis in HT-29 mouse xenograft model

To evaluate the anti-tumor effect of HS-173 via necroptosis *in vivo*, xenograft model was established using HT-29 RIP3 high-expressing cancer cells. The mice were injected with HS-173 (30 mg/kg) three times per week for 35 days. The general condition of the mice (alertness and physical activity) was observed to be normal during the whole experiment in both the control and HS-173-trested groups. The mice were euthanized one day after the last treatment. We found that the tumor size of HS-173-treated group was smaller than the control group (Fig. 8A and B). In addition, HS-173 increased the necrotic regions in tumors as determined by hematoxylin and eosin (H&E) staining (Fig. 8C). Additionally, immunofluorescent studies revealed that HS-173 treatment resulted in significant activation of RIP3 (Fig. 8D).

4. Discussion

The poor prognosis of patients with advanced lung cancer has led to the development of increasingly innovative approaches to treat the disease. Although 70–80% of the cancers respond to initial chemotherapy, recurrence of the recalcitrant disease is quite common. Until now, a lot of previously used stratagems have focused on restoring the ability of cancer cells to undergo apoptosis. However, drug resistance due to the evasion and defection of apoptosis often leads to the failure of conventional chemotherapy [32]. Therefore, approaches that overcome the resistance to apoptosis would be beneficial in cancer therapy, and can allow previously developed chemotherapeutic drugs to be used more effectively. Recent studies suggest that chemotherapeutics can induce RIP3-mediated necroptosis to kill cancer cells that are resistant to apoptosis [33]. In our study, we showed a novel role of HS-173 in inducing necroptosis in lung cancer cells. HS-173-induced necroptosis was confirmed by several approaches, including necroptotic morphology of cells upon HS-173 treatment, and the rescue of the observed effects by treatment with necroptosis inhibitor Nec-1 or dabrafenib. The high activation of RIP3 was further found to be a molecular switch for HS-173 to enhance necroptosis.

Like other forms of programmed cell death, necroptosis plays an important role in preventing cancer, particularly the tumor progression. Accumulating evidence suggests that necroptosis functions against cancer by inhibiting its initiation, growth, and metastasis [34,35]. In addition, necroptosis is a mechanism used by several chemotherapeutics to eliminate cancer cells, including obatoclax, dexamethasone, bromopyruvate, and shikonin analogs [36–38]. These drugs, however, lack specificity and selectivity, and the anticancer effect is weak, and the mechanisms by which they induce necroptosis are not fully elucidated. In this study, HS-173, a novel PI3K inhibitor was revealed to effectively induce necroptosis in lung cancer cells. Depending on the key function of RIP3/MLKL signaling in TNF-induced necroptosis [39], we provided up-regulated expression of RIP3 and plasma membrane translocation of MLKL in RIP3-expressing lung cancer cells, but not in RIP3-lacking cells. As necroptosis induction by TSZ in RIP3-positive HT-29 cells led to marked increase in the activation of RIP3, we observed similar results upon HS-173 treatment. This was further confirmed by treatment with necroptosis inhibitor (Nec-1 and dabrafenib), which inhibited the HS-173-induced cell death and expression of p-RIP3. This results indicate that HS-173 is involved in the activation of RIP3/MLKL signaling in lung cancer cells. Additionally, we found that the activation of RIP3 in tumor tissues was increased in HS-173 treated HT-29 xenograft mice as compared to the control mice *in vivo*. Thus, our results established that HS-173 has an anti-tumor effect *in vivo* probably by inducing necroptosis *via* RIP3-mediated signaling, thereby corroborating with our *in vitro* cell culture studies. Although HS-173 is generally considered as an apoptosis inducer, which was supported by our previous studies on the effect of HS-173 [40,41], we believe that the necroptosis induction by HS-173 will be also a promising mechanism for targeting cancer cells.

Recent studies have shown some interesting connections between necroptosis and PI3K/AKT signaling pathway. PI3K/AKT signaling controls necroptosis through downstream targeting of mTORC1. Activation of AKT is mediated in part through mTORC1, and links the regulation of necroptosis *via* RIP and autocrine production of TNFα [20]. Also, Liu et al. have reported that necroptotic cell death was preceded by RIP-AKT complex assembly in the neuron [42]. Chemotherapetic-agents, such as FTY720, induced multiple cell death including necroptosis in human glioblastoma cells by inhibition of PI3K/AKT/mTOR pathway, which is in accordance to our results with HS-173 [43]. Given that PI3K/AKT signaling could regulate necroptosis, the necroptotic effect of HS-173 may be mediated by the integration of AKT-RIP pathway. However, this needs to be confirmed by further investigation.

In conclusion, our study has found for the first time that HS-173 induces necroptosis in lung cancer cells by enhancing the expression of RIP3 and activating RIP3/MLKL signaling pathway. This study has opened the avenues to new individualized strategies based on RIP3 expression to overcome chemo-resistance in lung cancer therapy.

Conflicts of interest

None.

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