ERK-driven autophagy enhances synergy of eribulin and cisplatin in triple-negative breast cancer

HYEMI KO[1,2], MYUNGSUN LEE[3], EUNYOUNG CHA[3] and JINSUN LEE[1,2]

[1]Department of Surgery, Chungnam National University Hospital, Daejeon 35015, Republic of Korea;
[2]Department of Surgery, Chungnam National University, School of Medicine, Daejeon 35015, Republic of Korea;
[3]Department of Biomedical Research, Chungnam National University Hospital, Daejeon 35015, Republic of Korea

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Abstract. Triple-negative breast cancer (TNBC) remains one of the most aggressive subtypes of breast cancer with limited therapeutic options, especially in resource-limited settings. The present study investigated the mechanistic synergy of eribulin and cisplatin in TNBC, with a focus on ERK-driven autophagy. MDA-MB-231 TNBC cells were treated with eribulin and cisplatin. Viability, apoptosis, autophagy and ERK activation were assessed using Cell Counting Kit-8 assays, flow cytometry, western blotting and fluorescence microscopy. The drug combination enhanced ERK activation and induced autophagy, significantly increasing cell death. ERK inhibition reversed these effects, confirming its role in mediating synergy. The present findings provide a mechanistic rationale for an affordable combination therapy that may enhance TNBC treatment efficacy, particularly in resource-limited settings.

Introduction

Breast cancer remains one of the most prevalent malignancies among women globally, with triple-negative breast cancer (TNBC) representing one of its most aggressive and therapeutically challenging subtypes (1). TNBC accounts for approximately 15-20% of breast cancer cases and is defined by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (2,3). This subtype is more commonly observed in younger women, particularly those under 40 years of age, and is frequently associated with early recurrence and high metastatic potential (3,4).

Patients with TNBC often face significant physical and psychological burdens during treatment, including fatigue, hepatic dysfunction, anxiety, and depressive symptoms. Supportive strategies, such as the administration of melatonin and silymarin, have shown beneficial effects in reducing chemotherapy-related fatigue and toxicity (5,6). Psychological support with agents like crocin has also been effective in mitigating distress associated with treatment (7). In parallel, biological markers such as cytokeratin 18 (CK18) are under investigation as indicators of therapeutic response, helping to personalize treatment regimens (8).

Eribulin mesylate (Halaven®), a synthetic analog of the natural product halichondrin B, functions as a microtubule dynamics inhibitor and has been approved for use in patients with advanced breast cancer (9,10). Pivotal trials such as EMBRACE (11) and Study 301 (12) have demonstrated its clinical benefit in metastatic breast cancer, particularly among patients previously treated with anthracycline and taxane-based regimens.

Notably, its effectiveness may be improved when used in combination with other agents. Its efficacy may improve when combined with synergistic agents.

Cisplatin, a platinum-based compound, induces cytotoxicity primarily through DNA crosslinking and inhibition of DNA repair, resulting in tumor cell apoptosis. Although it is an established agent in TNBC therapy, its use is often constrained by its dose-limiting toxicities (13,14). Nevertheless, platinum-based regimens continue to hold relevance in TNBC treatment, with recent clinical guidelines recommending carboplatin-taxane combinations as a neoadjuvant option for HER2-negative and TNBC cases (15,16).

In our previous investigation (17), we demonstrated the synergistic cytotoxic potential of eribulin combined with cisplatin in TNBC models. Building upon those findings, the present study explores the mechanistic basis of this synergy, particularly its relationship with autophagy-a regulated cellular process increasingly implicated in tumor progression and therapeutic resistance. Given the therapeutic promise of targeting autophagy in TNBC, we hypothesized that dual treatment with eribulin and cisplatin could potentiate antitumor effects by modulating this pathway.

Moreover, in light of global disparities in access to advanced cancer therapies, particularly in low- and middle-income countries (LMICs), there is a pressing need to develop cost-effective treatment strategies. The use of existing, clinically approved drugs with known safety profiles, such as eribulin and cisplatin,

Correspondence to: Professor Jinsun Lee, Department of Surgery, Chungnam National University, School of Medicine, 282 Munhwa-ro, Jung-gu, Daejeon 35015, Republic of Korea
E-mail: leejin123@cnu.ac.kr

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offers a pragmatic avenue for broadening treatment access and improving outcomes in resource-constrained settings (18).

Taken together, these considerations highlight the urgent need for treatment strategies that combine mechanistic efficacy with cost-effectiveness, particularly in the management of TNBC.

Materials and methods

Reagents and antibodies. All cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin, were sourced from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Trypsin-EDTA was acquired from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

Eribulin mesylate (1 mg/vial) was generously provided by Eisai Co., Ltd. (Tokyo, Japan), and cisplatin was obtained from JW Pharmaceutical (Seoul, Korea). The half-maximal inhibitory concentration (IC_{50}) of eribulin in MDA-MB-231 cells was determined via CCK-8 assay after 72 h of treatment and found to be 40.12 μM. Based on this, a concentration of 60 μM-approximately 1.5 times the IC_{50}- was selected for subsequent combination experiments. This supra-physiological dosing strategy aligns with previous mechanistic synergy studies, such as Ko *et al* (17) in TNBC cells combining eribulin and cisplatin and Swami *et al* (19) demonstrating similar effects in SK-BR-3 cells, as well as the *in vitro*/*in vivo* work by Terashima *et al* (20) on EMT/MET modulation in TNBC treated with eribulin plus S-1.

PD98059 (a MAPK inhibitor) and crystal violet dye were procured from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies against LC3-I/II (cat. no. 4108), phospho-ERK1/2 (Thr202/Tyr204; cat. no. 9101), and β-actin (cat. no. 4967) were purchased from Cell Signaling Technology. Additional antibodies against ERK (SC-94) and p62 (sc-48389) were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Secondary antibodies conjugated with horseradish peroxidase (anti-mouse: cat. no. 7076; anti-rabbit: cat. no. 7074) were also from Cell Signaling Technology. Chemiluminescence reagents (Super Signal® West Pico) and DAPI stain were provided by Thermo Fisher. CCK-8 kits were sourced from Dojindo Molecular Technologies (Japan), and autophagy assays (ab139484) were performed using kits from Abcam (Cambridge, MA, USA). Annexin V-FITC apoptosis detection kits were obtained from Koma Biotech (Seoul, Korea).

Cell culture. MDA-MB-231, a human breast cancer cell line, was obtained from the Korean Cell Line Bank. Cells were grown in DMEM supplemented with 10% FBS, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), sodium pyruvate (1 mM), sodium bicarbonate (1.5 g/l), and glucose (4.5 g/l). Cultures were maintained in a humidified 5% CO_2 incubator at 37˚C.

Cell viability assay. To evaluate cytotoxicity, cells were plated at 5,000 cells per well in 96-well plates and allowed to adhere overnight. Treatments with test compounds were applied for 72 h. After incubation, CCK-8 reagent (100 μl) was added, and absorbance at 450 nm was measured after a 2-h incubation using a microplate reader.

Colony formation assay. Colony formation assays were conducted to evaluate long-term proliferative capacity. MDA-MB-231 cells were seeded at low density in 6-well plates and treated with eribulin, cisplatin, or their combination for 14 days. Colonies were fixed with methanol and stained with 0.5% crystal violet.

Colonies were defined as discrete clusters containing ≥50 cells. Due to the dispersed and small-sized colonies formed by MDA-MB-231 cells even after 14 days, manual quantification under a microscope was technically unfeasible. Thus, colony numbers and areas were quantified using ImageJ software (NIH, USA) following standard image analysis steps: conversion to 8-bit grayscale, threshold adjustment, watershed segmentation, and automated particle counting via the 'Analyze Particles' function.

Western blot analysis. Cells at approximately 80% confluency were treated and harvested. Cell lysis was performed using buffer containing Tris, NaCl, EDTA, Triton X-100, NP-40, PI, DTT, and PMSF. Lysates were centrifuged, and protein concentrations were measured using a BCA protein assay kit. Protein (20 μg/lane) was resolved by SDS-PAGE and transferred onto PVDF membranes. Blots were blocked with 5% milk in PBST and incubated with primary antibodies overnight. HRP-conjugated secondary antibodies were applied, and bands were visualized with enhanced chemiluminescence. Band intensities were quantified and normalized to the respective loading controls.

Annexin V/propidium iodide staining. Apoptosis was quantified using an Annexin V-FITC/PI kit as per the manufacturer's instructions. Cells treated for 72 h were collected, stained with Annexin V-FITC and PI, and analyzed via flow cytometry. Histogram analysis was done using Kaluza software.

Autophagy detection assay. Autophagy flux was examined using Abcam's fluorescent dye-based assay (ab139484), which selectively labels autophagic compartments. Cells were cultured in 8-well chamber slides and treated with the indicated compounds (100 μM) for the specified time, fixed with paraformaldehyde, and stained with autophagy-specific dyes according to the manufacturer's instructions. Fluorescent signals were observed using a confocal microscope and quantified with ImageJ.

Combination index. To assess drug synergy, the CI was computed using the Chou-Talalay method with CompuSyn software (version 1.0, Combosyn Inc., Paramus, NJ, USA). CI values were interpreted as follows: CI <1 (synergy), CI=1 (additivity), and CI >1 (antagonism).

Statistical analysis. All experiments were independently repeated at least three times. All statistical analyses were performed using SPSS version 29.0 for Windows (IBM Corp.). One-way ANOVA was used with Tukey's Honestly Significant Difference post hoc analysis. Data are shown as the

Table I. CI value for the combination of cisplatin and eribulin in MDA-MB-231 cells.

Cisplatin, μM	Eribulin, μM	CI	DRI (cisplatin)	DRI (eribulin)
20	10	2.35	0.74	0.99
40	20	3.37	0.39	1.14
60	60	0.71	1.39	3.62
140	140	0.59	1.68	2.62

The combination is synergistic at CI <1, additive at CI=1 and antagonistic at CI >1. CI, combination index; DRI, dose reduction index.

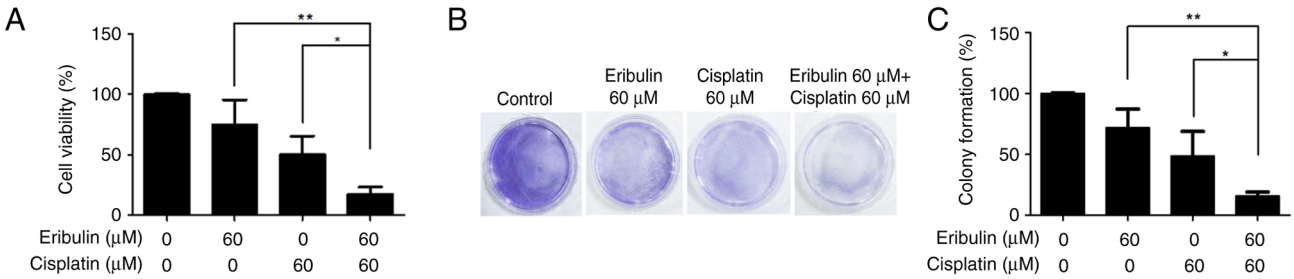

Figure 1. Combined effects of eribulin and cisplatin on growth inhibition in MDA-MB-231 cells. (A) CCK-8 assay showing the effects of eribulin combined with cisplatin vs. control on cell viability. Cell viability was measured using a CCK-8 assay. The experiments were performed in triplicate and the data are presented as the mean ± SD. *P<0.05, **P<0.005. (B) Representative images of the colony formation assay showing the effect of eribulin + cisplatin on the colony formation of MDA-MB-231 cells. (C) Quantitative results of the colony formation assay were obtained in triplicate and the data are presented as the mean ± SD. *P<0.05, **P<0.05. CCK-8, Cell Counting Kit-8.

mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of eribulin and cisplatin on cell viability and clonogenic growth. Cell viability was assessed using the CCK-8 assay (Fig. 1A), and the synergistic efficacy of the drug combination was further confirmed by colony formation assays (Fig. 1B and C). Table I summarizes the CI values derived for the two drugs in this cell model. Single treatment with eribulin mesylate (60 μM) decreased cell viability to 75.11±0.41% after 72 h of exposure. Cisplatin (60 μM) single treatment showed a reduction of 50.57±0.20%. The combination of these drugs significantly inhibited cell viability to 17.15±0.10% (Fig. 1A). Colony-forming assays were performed to test the ability of single cells to grow into colonies. This drug combination synergistically inhibited colony formation by MDA-MB-231 cells (Fig. 1B and C).

Synergistic activation of ERK1/2 by drug combination. We observed that the ERK phosphorylation level increased more than 5.5-fold in the experimental cells compared to that in the control cells after the administration of 60 μM eribulin for 72 h (Fig. 2A and B). Moreover, 60 μM cisplatin increased ERK phosphorylation 6.0-fold (Fig. 2A and B). In addition, when cisplatin was added to eribulin, ERK activation increased by 14.8 times. When cisplatin was added to eribulin, ERK activation was elevated 14.8-fold compared to the control, reflecting a 2.7- and 2.5-fold increase relative to eribulin and cisplatin treatment alone, respectively. These findings confirmed that ERK1/2 activation increased synergistically with eribulin-cisplatin combination.

Induction of autophagy by eribulin and cisplatin. To evaluate autophagy induction, the LC3-I/II ratio and p62 expression were measured. As autophagy markers, the microtubule-associated protein LC3-I/II ratio and p62 expression were determined using western blot analysis (Fig. 3A). The LC3-I/II level increased 4.3-fold in the eribulin group compared to that in the control group. Cisplatin alone increased the expression of these parameters by 6.1-fold; the corresponding value for eribulin-cisplatin combination treatment was 13.9-fold, indicating a synergistic effect (Fig. 3B). A significant downregulation of p62 was observed following co-treatment with eribulin and cisplatin, suggesting promoted autophagic degradation (Fig. 3C). The corresponding values for eribulin alone and eribulin-cisplatin combination were 9.0 and 23.6%, respectively, indicating a significant increase in autophagic activity in the combination group compared with the eribulin-only group (Fig. 3D and E). Autophagic vacuole staining showed a slight increase in autophagic activity with either eribulin or cisplatin alone, whereas their combination resulted in a marked enhancement, indicating cumulative autophagic activation.

Apoptosis induced by drug combination and its dependence on autophagy. The apoptotic activity of the combination of eribulin and cisplatin was analyzed by flow cytometry after double staining with annexin V and propidium iodide (Fig. 4A). The apoptotic activity levels were 28.61, 64.77, and 99.26%

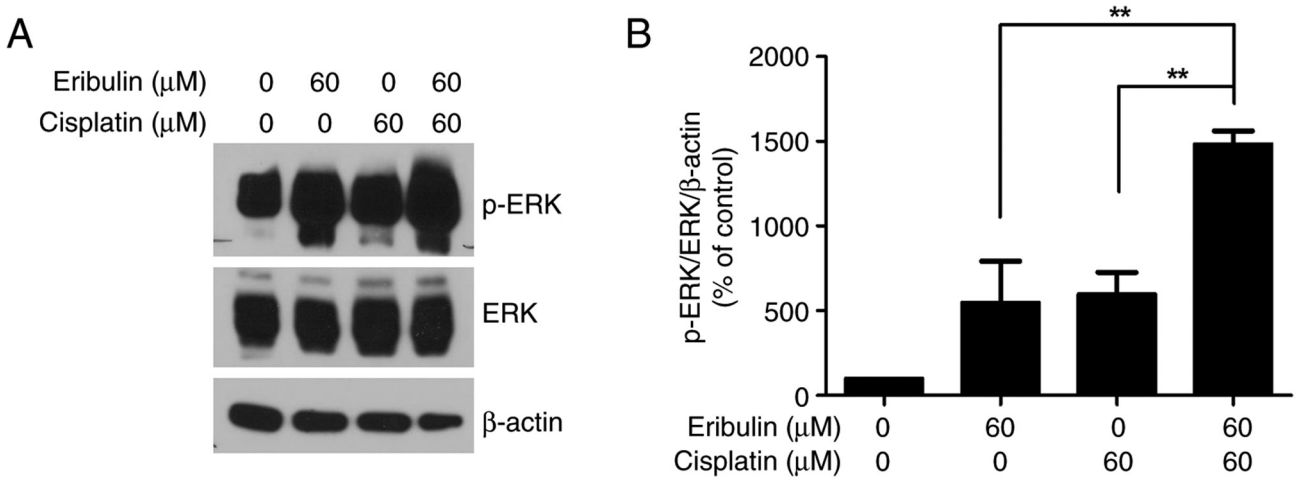

Figure 2. Enhanced ERK activation by eribulin and cisplatin. (A) MDA-MB-231 cells were treated with cisplatin alone or in combination with eribulin. Cell lysates were collected and subjected to western blotting using anti-P-ERK1/2 and anti-ERK1/2 antibodies. β-Actin was used as the internal control. (B) Semi-quantitative results of western blotting performed in triplicate. Data are presented as the mean ± SD. **P<0.005. P-, phosphorylated.

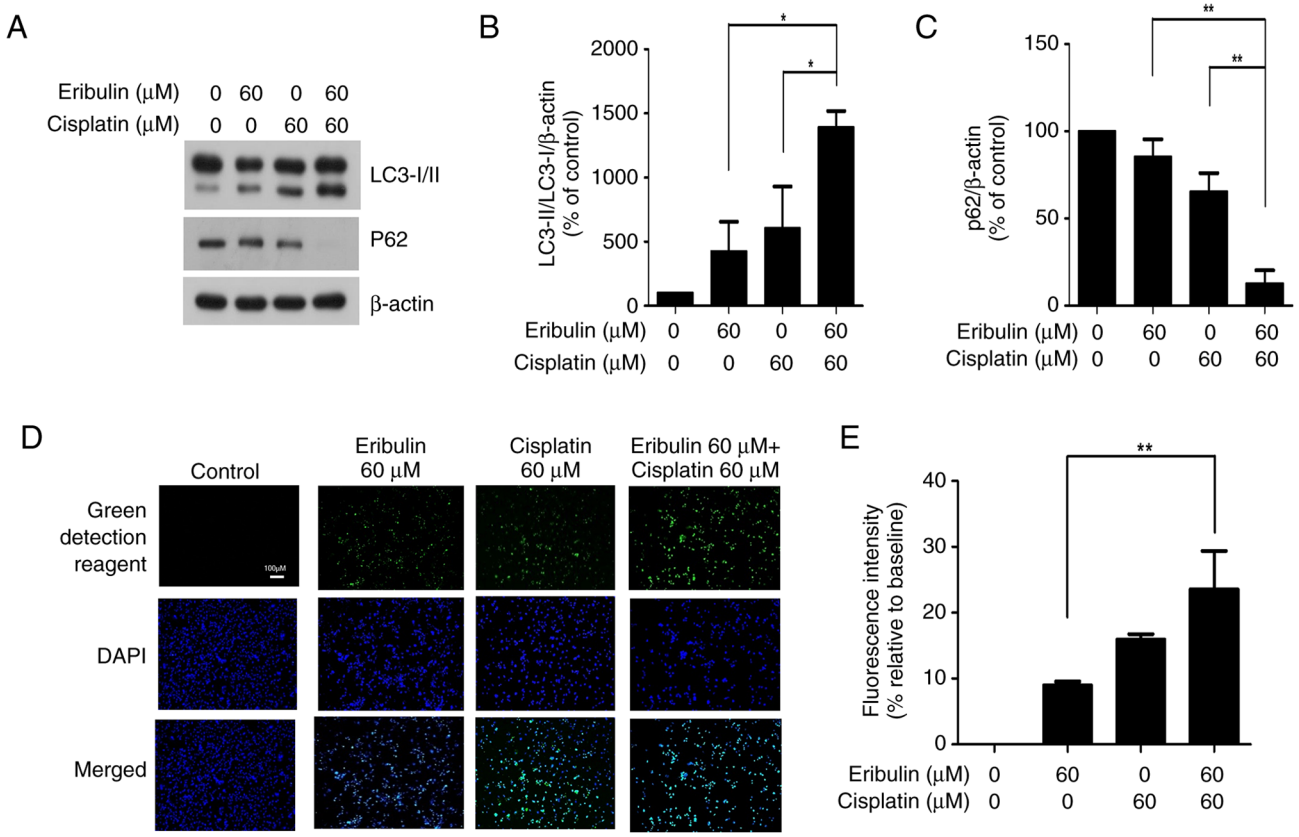

Figure 3. Effects of eribulin and cisplatin on autophagy in MDA-MB-231 cells. (A) Western blot analysis of autophagy-related proteins LC3-I/II and p62. β-actin was used as a loading control. The levels of autophagy-associated proteins (B) LC3-I/II and (C) p62 were semi-quantified. B-Actin was used as a loading control. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.005. (D) Autophagy was measured using an autophagy assay kit. Cells were treated with the indicated compounds (60 μM), labeled and analyzed by fluorescence microscopy. Green staining indicates autophagosomes and blue represents DAPI nuclear staining. Scale bar, 100 μm. (E) Quantification of the mean fluorescence intensity using ImageJ software. Data are presented as the mean ± SD. **P<0.005.

when the cells were treated with eribulin alone, cisplatin alone, or a combination thereof, respectively. Upon co-treatment with 3-methyladenine, a known autophagy inhibitor, the apoptotic rate induced by the eribulin-cisplatin combination significantly decreased, suggesting that the observed cytotoxicity is dependent on autophagic mechanisms. When 3-methyladenine samples were treated with a combination of eribulin and cisplatin, the apoptotic cell volume was reduced to 13.68% compared with 99.26% in the eribulin-cisplatin combination group (Fig. 4A and B).

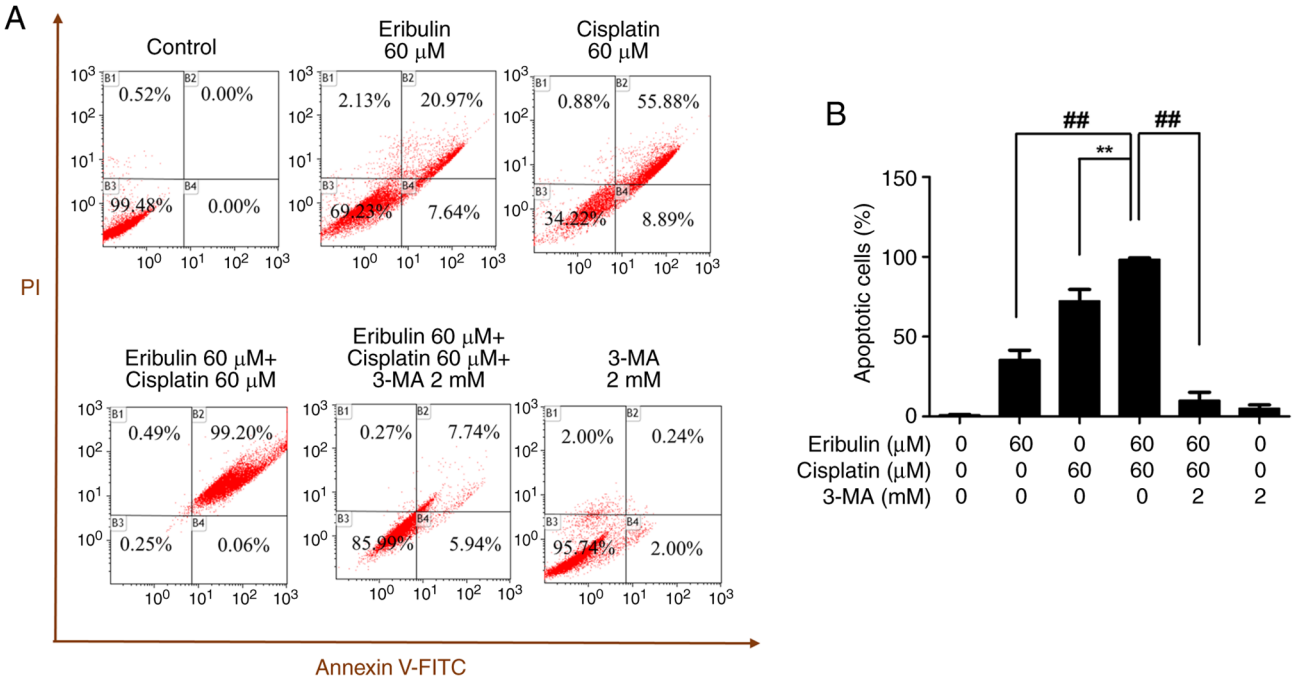

Figure 4. Effects of eribulin and cisplatin on apoptosis in MDA-MB-231 cells. (A) The apoptotic cell population was evaluated by flow cytometry following double staining with annexin V and PI. (B) Quantitative results of annexin-PI flow cytometry. The percentage of apoptotic cells is presented as the mean ± SD. **P<0.005, $^{\#\#}$P<0.005. 3-MA, 3-methyladenine.

Impact of ERK inhibition on viability, apoptosis, and autophagy. To assess the impact of ERK inhibition, cells were pretreated with PD98059 prior to exposure to eribulin and cisplatin, followed by a viability assessment using the CCK-8 assay. When PD98059 was combined with the two-drug combination, the cell viability increased significantly from 33.63 to 53.37% (Fig. 5A). Flow cytometry was used to quantify apoptotic cells following dual labeling with Annexin V and propidium iodide (Fig. 5B). Compared with the eribulin and cisplatin combination group, the apoptosis rate decreased from 92.72 to 18.01% when PD98059 was used (Fig. 5C).

Using the control value as the reference (100%), LC3-I/II expression was markedly increased in the eribulin plus cisplatin group (824.99%) but was reduced to 119.30% when PD98059 was additionally administered, whereas p62 expression increased from 38.70 to 72.59% with PD98059 treatment (Fig. 5D-F). Thus, ERK inhibition affected the expression of autophagy-related proteins.

An autophagy assay (ab139484, Abcam) confirmed that the levels of autophagosomes decreased when PD98059 was used in combination with eribulin and cisplatin (Fig. 5G). Quantitative analysis of fluorescence intensity showed consistent results, decreasing from 15.59 to 2.92% (P<0.05; Fig. 5H).

Discussion

In this study, MDA-MB-231 cells were used to investigate the cytotoxic effect of a combination of eribulin and cisplatin, both widely utilized in TNBC treatment. After 72 h of treatment, significant cancer cell death was observed, mediated by autophagy-dependent mechanisms involving ERK pathway activation. These findings reveal a novel therapeutic vulnerability in TNBC.

Eribulin, a microtubule-targeting agent derived from the marine sponge *Halichondria okadai*, has demonstrated clinical efficacy in breast cancer, particularly in taxane- and anthracycline-resistant cases (21,22). The EMBRACE trial notably supported its use in metastatic breast cancer, with further analyses confirming its benefit in TNBC (11). Previous studies have shown that eribulin can synergize with various agents, including HDAC inhibitors and RAF/MEK inhibitors, primarily through ERK pathway inactivation (23,24). However, our study uniquely reveals that eribulin induces cell death via ERK pathway activation, representing, to our knowledge, the first report of this mechanism in TNBC.

Cisplatin, a platinum-based chemotherapeutic, also activates ERK and induces apoptosis across multiple carcinoma types including cervical cancer (25), hepatocellular carcinoma (26), human glioma (27), and mouse proximal tubule cancer (28). Consistent with prior reports, our study confirms that ERK activation persists in MDA-MB-231 cells following cisplatin treatment, contributing to cell death. Remarkably, the combination of eribulin and cisplatin further amplified this ERK-driven autophagic response, leading to significantly enhanced cytotoxic effects against TNBC cells.

TNBC is molecularly defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 overexpression, rendering it unresponsive to endocrine or HER2-targeted therapies. This receptor-negative profile contributes to poor prognosis and compels TNBC cells to rely on alternative survival pathways such as the MAPK/ERK axis (29,30). Our finding that ERK activation mediates autophagy-dependent cell death in response to the combination therapy suggests that this pathway-typically associated with cellular adaptation and

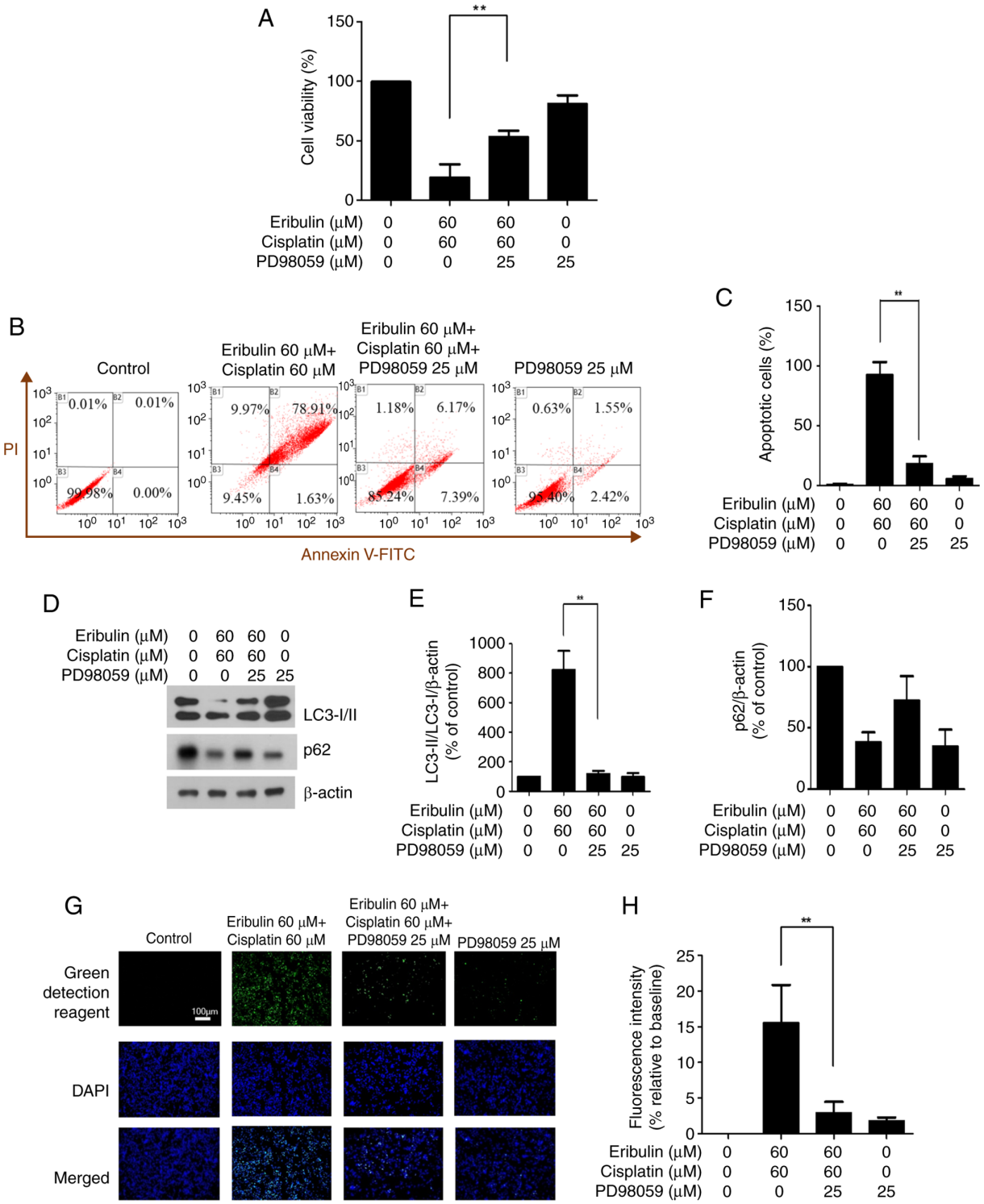

Figure 5. Effects of ERK inhibition on eribulin and cisplatin-induced changes in cell viability, colony formation, apoptosis and autophagy. (A) Following pre-incubation with PD98059 before eribulin and cisplatin treatment, cell viability was determined using the Cell Counting Kit-8 assay. Each assay was performed in triplicate. Data are presented as the mean ± SD. **P<0.005. (B) The apoptotic cell population was evaluated by flow cytometry following double staining with annexin V and PI. (C) Quantitative results of annexin-PI flow cytometry. The percentage of apoptotic cells is presented as the mean ± SD. **P<0.005. (D) Western blot analysis of autophagy-related proteins. Levels of autophagy-associated proteins (E) LC3-I/II and (F) p62 were semi-quantified. β-Actin was used as a loading control. Data are presented as the mean ± SD of three independent experiments. **P<0.005. (G) Autophagy was measured using an autophagy assay kit (ab139484; Abcam). Cells were treated with the indicated compounds (60 μM eribulin, 60 μM cisplatin and 25 μM PD98059), labeled and analyzed using fluorescence microscopy. Green staining indicates autophagosomes and blue represents DAPI nuclear staining. Scale bar, 100 μm. (H) Quantification of the mean fluorescence intensity using ImageJ software. Data are presented as the mean ± SD. **P<0.005 vs. eribulin + cisplatin group.

drug resistance-may instead represent a vulnerability in TNBC (31). Furthermore, high basal autophagic activity, often driven by MAPK/ERK signaling, has been implicated in TNBC progression and response to therapy, indicating that modulation of this pathway could sensitize cells to cytotoxic agents (32,33).

Autophagy, which often intersects with apoptotic mechanisms, is increasingly recognized as a modulator of cancer therapy response (34,35). Our data indicate that ERK activation is not merely associated with autophagic flux but is a functional mediator of combination-induced cell death. This positions ERK-mediated autophagy as a viable target for combination therapy in TNBC, particularly in the context of eribulin and cisplatin co-treatment (36,37).

Importantly, the clinical relevance of this dual-agent strategy extends beyond mechanistic insights. Although targeted therapies have revolutionized TNBC management in high-income countries, their high cost continues to restrict access in low- and middle-income countries (LMICs) (38). Given that both eribulin and cisplatin are already approved and relatively affordable, their combination may offer a scalable and cost-effective solution to mitigate global disparities in breast cancer treatment (11,17,18). This approach is consistent with current global oncology efforts aimed at expanding access to effective therapies in resource-limited settings.

In LMICs, financial barriers significantly limit access to novel targeted therapies (39). The monthly cost of immune checkpoint inhibitors or PARP inhibitors can exceed $5,000 USD, making them unattainable for most patients (40). In contrast, cisplatin and eribulin-being off-patent or comparatively affordable-are frequently listed in public health formularies. For instance, in South Korea, the cost of eribulin treatment under the national insurance system is approximately $1,500-$2,000 USD per cycle, substantially lower than newer biologics [HIRA (Health Insurance Review and Assessment Service, Korea's national health technology assessment body)]. These economic factors reinforce the feasibility of the eribulin-cisplatin combination, especially in regions striving to deliver cost-effective cancer care (17,18).

In summary, this is the first report demonstrating that eribulin alone can activate the ERK pathway to induce autophagy-mediated cytotoxicity in TNBC. These findings highlight ERK-mediated autophagy as a promising therapeutic target and underscore the clinical feasibility of the eribulin-cisplatin combination in both advanced and resource-limited settings.

Nevertheless, this study has limitations. As an *in vitro* investigation, it cannot fully recapitulate the complex tumor microenvironment. *In vivo* validation using xenograft models will be necessary to confirm the translational potential of the eribulin-cisplatin combination, particularly its impact on ERK-mediated autophagic cell death. However, the recent NCCN guidelines supporting platinum-based neoadjuvant regimens in TNBC provide a clinical rationale for future studies (18).

In conclusion, our findings demonstrate that ERK-driven autophagy mediates the synergistic cytotoxicity of eribulin and cisplatin in TNBC. This mechanism highlights a clinically feasible and cost-effective therapeutic strategy, warranting further *in vivo* validation and clinical development.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HK and JL conceived and designed the study. HK, ML, EC and JL contributed to the design of the methodology and performed experiments. Data analysis and validation were conducted by HK and JL. Software tool use and visualization were performed by HK. The experiments were performed by ML, EC and JL, with resources provided by ML and EC. Data curation was performed by HK, ML and EC. HK drafted the original manuscript, and JL performed critical review and editing. Supervision and project administration were led by JL. Funding for the project was acquired by JL. HK and JL confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

ORCID IDs: HK, 0000-0002-6621-8761; JL, 0000-0002-9345-724X; ML, 0000-0003-1732-3001; EC, 0000-0002-7281-3698.

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