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ORIGINAL ARTICLE

Radiosensitizing effect of a novel CTSS inhibitor by enhancing BRCA1 protein stability in triple-negative breast cancer cells

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Abstract

Triple-negative breast cancer (TNBC) patients harboring wild-type breast cancer susceptibility gene 1 (BRCA1) account for most TNBC patients but lack adequate targeted therapeutic options. Although radiotherapy (RT) is the primary treatment modality for TNBC patients, radioresistance is one of the major challenges. RT-induced increase in cathepsin S (CTSS) causes radioresistance through suppressing BRCA1-mediated apoptosis of tumor cells, which was induced by CTSS-mediated degradation of BRCA1. Targeting CTSS may provide a novel therapeutic opportunity for TNBC patients. Publicly available data and human tissue microarray slides were analyzed to investigate the relationship between CTSS and BRCA1 in breast cancer patients. A CTSS enzyme assay and in silico docking analysis were conducted to identify a novel CTSS inhibitor. RO5461111 was used first to confirm the concept of targeting CTSS for radiosensitizing effects. The MDA-MB-231 TNBC cell line was used for in vitro and in vivo assays. Western blotting, promoter assay, cell death assay, clonogenic survival assay, and immunohistochemistry staining were conducted to evaluate novel CTSS inhibitors. CTSS inhibitors were further evaluated for their additional benefit of inhibiting cell migration. A novel CTSS inhibitor, TS-24, increased BRCA1 protein levels and showed radiosensitization in TNBC cells with wild-type BRCA1 and in vivo in a TNBC xenograft mouse model. These effects were attributed by BRCA1-mediated apoptosis facilitated by TS-24. Furthermore, TS-24 demonstrated the additional effect of inhibiting cell migration. Our study suggests that employing CTSS inhibitors for the functional restoration of BRCA1 to enhance RT-induced apoptosis may provide a novel therapeutic opportunity for TNBC patients harboring wild-type BRCA1.

KEYWORDS
apoptosis, BRCA1 stability, CTSS inhibitors, radiosensitizer, TNBC

Abbreviations: BRCA1, breast cancer susceptibility gene 1; BRCT, BRCA1 C-terminus; CTSS, cathepsin S; GADD45, growth arrest and DNA damage 45; IR, ionizing radiation; PARP1, poly[ADP-ribose] polymerase 1; PR, progesterone receptor; RT, radiotherapy; TNBC, triple negative breast cancer.

Eun Choi and Kyung-Hwa Jeon contributed equally to this work.

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1 | INTRODUCTION

Radiotherapy (RT) is the primary established systemic treatment for triple-negative breast cancer (TNBC) patients. However, it is inconclusive whether TNBC is RT-sensitive, but its recurrence indicates the presence of RT resistance.[1] Recent studies have reported several genes related to RT resistance and therapeutic strategies to overcome this resistance. One study showed that deletion and overexpression of various gene targets in TNBC eventually activate the PI3K/Akt pathway to regulate RT resistance.[2,3] TNBC is also likely to benefit from anti-EGFR (Epihermal Growth Factor Receptor) therapy since EGFR overexpression and amplification are present in 15%–76% of TNBC.[4–7] Poly (ADP-ribose) polymerase (PARP) inhibitors have shown to inhibit the repair of damaged DNA, enhance the cytotoxicity of radiation (IR), and produce a synthetic lethal effect in TNBC patients with high BRCA mutations.[8]

Breast cancer susceptibility gene 1 (BRCA1) deficiency, due to its mutations or epigenetic inactivation, leads to an increased risk of breast cancer development; however, harmful BRCA1 gene mutations are relatively rare (<5%) in the general population, and only a minority of TNBC patients with a BRCA1 deficiency carry BRCA1 mutations.[9] Currently, researchers focus more on the downregulation of BRCA1 expression or epigenetic inactivation in sporadic tumors as a prognosis tool for chemotherapy response.[10] A recent study also suggests that the reduction of BRCA1 levels by ubiquitin proteolysis is a risk factor for BRCA1-mediated cancer development and survival.[11]

BRCA1 in the nucleus is involved in genomic stability by regulating DNA repair, cell-cycle checkpoints, and E3 ubiquitin ligase activity, which are the main functions of BRCA1 as a tumor suppressor gene.[12] However, besides its many functions in the nucleus, BRCA1-mediated apoptosis is suggested to mainly occur in the cytoplasm.[13] The specific pathways of BRCA1-mediated apoptosis in the cytoplasm are (1) the p53-dependent pathway, (2) the growth arrest and DNA damage 45 (GADD45)-mediated MEKK4/JNK (Mitogen-Activated Protein Kinase Kinase 4/Jun N-terminal Kinase) signaling pathway via the nuclear export of BRCA1, (3) the Ras/MEKK4/JNK (Rat Sarcoma virus/MEKK4/JNK) and Fas (Fas Cell Surface Death Receptor)-dependent pathways, (4) modulation of ERα (Estrogen Receptor alpha), (5) apoptotic calcium release via the binding of BRCA1 to IP_3Rs, and (6) cytosolic BRCA1-mediated proteasomal degradation of anti-apoptotic Bcl2.[14] Therefore, the restoration of cytoplasmic BRCA1 function may be a good strategy for radiosensitization by inducing tumor apoptosis.

The cysteine protease cathepsin S (CTSS), which belongs to a family of 11 cysteine cathepsin proteases, has been linked to a variety of pathologies, including cancer.[15] CTSS has been detected in multiple cancer types, including prostate,[16] gastric,[17] hepatocellular,[18] and breast carcinomas.[19] Moreover, increased CTSS expression has been shown to hold prognostic value in various cancers, where it is associated with a poor outcome.[16,18,20–23] CTSS is a unique protease from other members of the cathepsin family in that CTSS expression is restricted to specific cells and tissues under physiological conditions, and its activity is maintained at a neutral and moderately alkaline pH.[20] Collectively, these observations have attracted interest in the therapeutic potential of CTSS in cancer. Inhibition or depletion of CTSS reduced tumor invasion, burden, proliferation, and vascularization, as well as increased apoptosis.[21]

In our previous study, CTSS, induced by radiation (IR), enhanced the degradation of BRCA1, which was associated with IR resistance in breast cancer cells. The recognition of the BRCA1 C-terminus (BRCT) domain of BRCA1 by CTSS induced ubiquitination-mediated degradation of BRCA1, which eventually resulted in tumorigenesis and tumor cell survival.[22] Therefore, improving the stability of BRCA1 by inhibiting CTSS may be a good therapeutic strategy for TNBC patients. In this study, we identified a novel CTSS inhibitor, TS-24, which increased BRCA1 stability and radiosensitization in in vitro and in vivo systems.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analyses

Publicly available datasets and databases were utilized for bioinformatic analyses. An online database (http://kmplot.com/analysis[23]) and a dataset by Tang et al.[24] were used to determine the relevance of individual CTSS protein expression to the overall survival (OS). Hazard ratios (HRs) and 95% confidence intervals (CIs), as well as log-rank P were calculated and displayed on the webpage. The GSE25055 dataset, used for therapeutic response rate analysis, was obtained from the National Center for Biotechnology Information gene expression omnibus database. Fifty-four breast cancer specimens from the TCGA (The Cancer Genome Atlas) set (Breast Invasive Carcinoma [TCGA, Firehose Legacy]) were analyzed using cBioPortal (http://www.cbioportal.org[25,26]). Datasets reporting mRNA expression *z*-scores (log RNA Seq V2 RSEM) and protein level *z*-scores (mass spectrometry by CPTAC) were retrieved for the analysis.

2.2 | Cell culture

The human TNBC MDA-MB-231 (wild-type BRCA1) cell line was cultured in RPMI-1640 medium (Gibco) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Gibco) and antibiotics. The human TNBC MDA-MB-436 (mutant BRCA1) cell line was cultured in Dulbecco's minimal essential medium (Welgene, Korea) supplemented with heat-inactivated 10% FBS, 0.1 mM glutamine, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and antibiotics at 37°C in a 5% CO_2 humidified incubator.

2.3 | CTSS activity assay

CTSS protease activity was measured using a fluorescent peptidyl substrate, Z-VVR-AMC (#sc-471150; Santa Cruz Biotechnology).

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The principle relies on the ability of cathepsins to cleave the synthetic AFC (Amino-4-trifluoromethyl coumarin)-based peptide substrate to release AFC, which can be measured using a fluorometer. CTSS protease activity using a cell system was measured with assay kits for CTSS (#ab65307) purchased from Abcam. The enzymatic assay itself was conducted according to the manufacturer's instructions. The relative fluorescent unit values were analyzed using the Infinite F200 PRO microplate multi-reader (Tecan, equipped at the Ewha Drug Development Research Core Center). The specific CTSS inhibitor, RO5461111 (RO), was provided by Hoffmann La Roche and used as a positive control.

2.4 | *Gadd45* promoter assay

Gadd45 WT-luc plasmid was purchased from Addgene. *Gadd45* promoter activity was measured using a luciferase assay system kit (#E4030; Promega) according to the manufacturer's instructions.

2.5 | Flow cytometric analysis of PE Annexin V staining

Cells were treated with compounds for 6 h prior to exposure to 10 Gy irradiation. The collected cells were incubated with PE Annexin V in a buffer containing 7-amino-actionomycin and analyzed by flow cytometry. The PE Annexin V Apoptosis Detection Kit I was purchased from BD Bioscience. Flow cytometric analysis was performed using a Novocyte 2060R FACS (Fluorescence-acivated cell sorting) flow cytometer (Acea Biosciences, equipped at the Ewha Drug Development Research Core Center). FACS analysis was conducted in accordance with the manufacturer's instructions.

2.6 | Colony formation assay

Cells were treated with compounds for 6 h prior to exposure to 1 Gy irradiation. After 7 days of incubation with or without compounds, the cells were fixed with 100% methanol for 1 h and stained with trypan blue solution (1% [w/v] in PBS (Phosphate-buffered saline)). Images were taken using a ChemiDoc bio-image analyzer (Bio-Rad) and quantified using ImageJ software (NIH; The National Institutes of Health).

2.7 | Western blot

Protein band intensity was visualized by ChemiDoc (Bio-Rad) and quantified using Image J software 1.45 (National Institutes of Health, Bethesda, MD, USA). The results are presented as a fold increase or an average fold increase in the level of each protein normalized to β-actin. Information on the antibodies used for western blotting is provided in Table S1.

2.8 | Tumor xenograft experiment using NOD-SCID mice

A single-cell suspension of MDA-MB-231 cells (1×10^7 cells) was injected subcutaneously into the hind legs of 5-week-old female NOD-SCID (Nonobese diabetic/ severe combined immunodeficiency) (Koatech). A single dose of 3 or 4 Gy was delivered with a local, regional application using a ^{137}Cs irradiator (Biobeam8000, Gamma Service Medical, equipped at the Korea Institute of Radiological and Medical Science). A day after the irradiation, NOD-SCID mice were treated with 1 mg/kg RO every day or 1–2 mg/kg TS-24 three times a week. All drugs were administered intraperitoneally for 4–6 weeks. Tumor growth was monitored two or three times per week to assess treatment efficacy starting 2 weeks after the MDA-MB-231 cells were injected. The long diameter (LD) and short diameter were measured to calculate tumor volume: volume$=(LD\times SD^2)/2$. According to the Institutional Animal Care and Use Committee (IACUC), tumor size must not exceed 20 mm at the largest diameter in mice, and the maximal tumor size in this study was not exceeded. The animal studies were approved by the IACUC of Ewha Womans University [IACUC 21–027].

2.9 | Human tissue analysis

Human breast cancer tissue slides were purchased from US Biomax. For antigen retrieval, slides were placed in 10 mM citric acid buffer (pH 6.0) and heated at 100°C for 10 min. Slides were incubated overnight at 4°C with antibodies. Quantification of images was measured using an image analyzer (Image J; NIH). All statistical analyses of images were performed using GraphPad Prism software 5.0.

2.10 | Statistical analysis

Values are displayed as mean plus or minus the standard deviation or the standard error of the mean. Statistical significance was determined by ANOVA (Analysis of variance) or a two-tailed Student's *t*-test. Differences were considered significant if $P<0.05$.

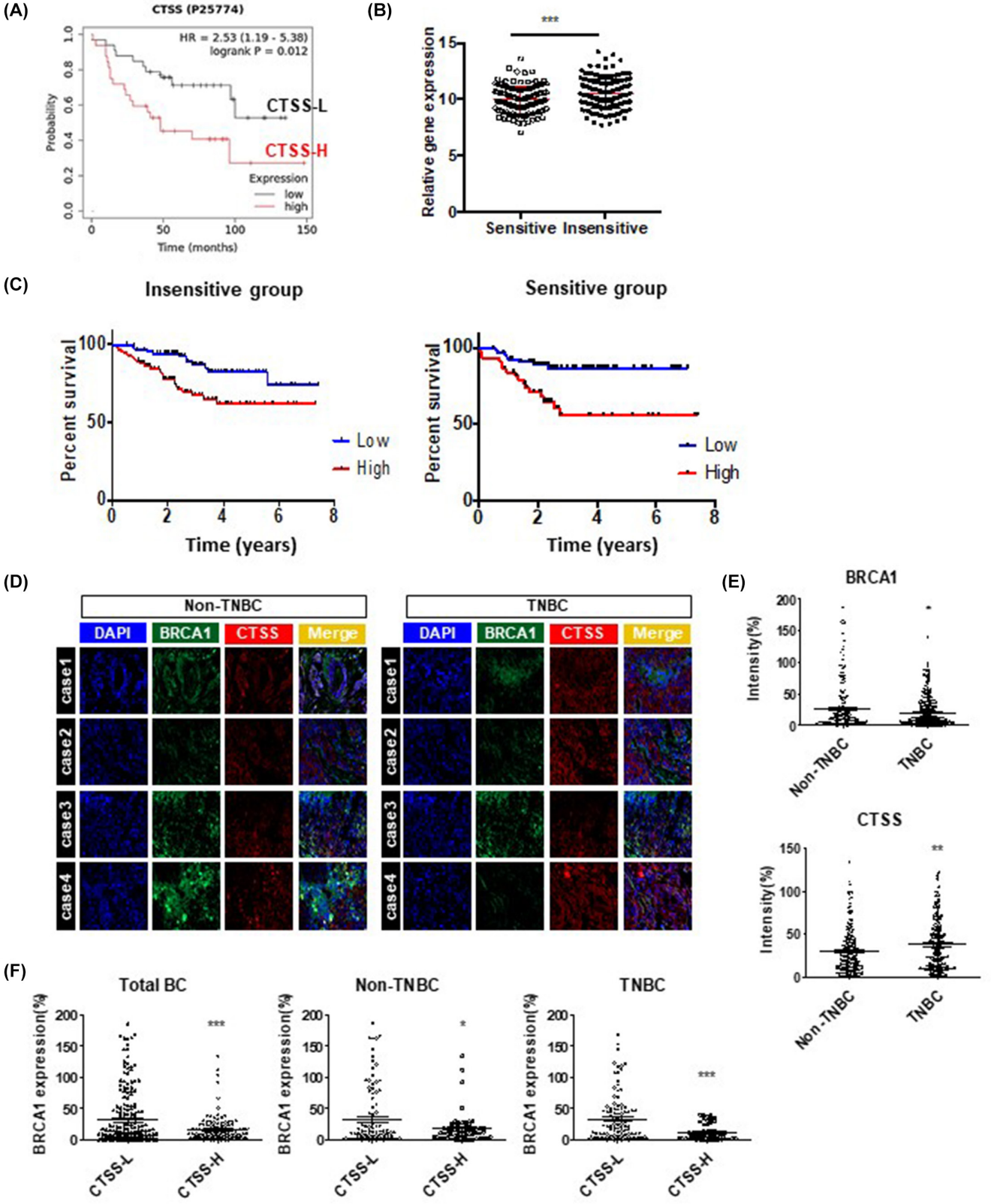
See additional information on material and methods in the Supporting Information.

3 | RESULTS

3.1 | CTSS overexpression predicted poor clinical outcome in breast cancer patients

To assess the prognostic value of CTSS, we investigated a liquid chromatography–mass spectrometry (LC-MS)-based proteomic dataset from a cohort of 65 breast cancer patients provided by Tang et al.[24] The OS analysis revealed that a higher expression of CTSS was strongly associated with reduced survival ($P=0.012$) (Figure 1a).

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A neoadjuvant study of 310 human epidermal growth factor receptor 2-negative breast cancer cases (GSE25055) was examined to identify the relationship between CTSS expression and the response to chemotherapy. The response to taxane-anthracycline chemotherapy was also well correlated with CTSS expression, where a higher expression of CTSS was found in breast cancer patients ($n = 197$) with taxane-anthracycline insensitive responses and a lower expression of CTSS ($n = 113$) was found in patients with sensitive responses (Figure 1b). An OS analysis on both the insensitive and sensitive groups demonstrated that higher expression of CTSS was associated

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FIGURE 1 Cathepsin S (CTSS) overexpression predicts poor outcome in breast cancer patients and exhibits low breast cancer susceptibility gene 1 (BRCA1) levels, especially in triple-negative breast cancer (TNBC). (A) Kaplan–Meier plot on the overall survival of breast cancer patients with high (CTSS-H) or low (CTSS-L) CTSS expression. (B) Relative CTSS expression in HER-2 negative breast cancer patients treated with the neoadjuvant treatment of taxane-anthracycline chemotherapy or endocrine therapy ($n = 310$, GSE25055 dataset). (C) Kaplan–Meier plot on the overall survival of breast cancer patients with high or low CTSS expression in human epidermal growth factor receptor 2-negative breast cancer patients grouped by their responses to the neoadjuvant treatment. (D) Immunofluorescence for BRCA1 and CTSS using a human mammary cancer tissue microarray ($n = 120$; BRCA1, green; CTSS, red). (E) The expression intensity of BRCA1 and CTSS in non-TNBC and TNBC patients. (F) Relative BRCA1 expression in the CTSS-H and CTSS-L groups. BC (Breast Cancer), DAPI (4',6-diamidino-2-phenylindole)

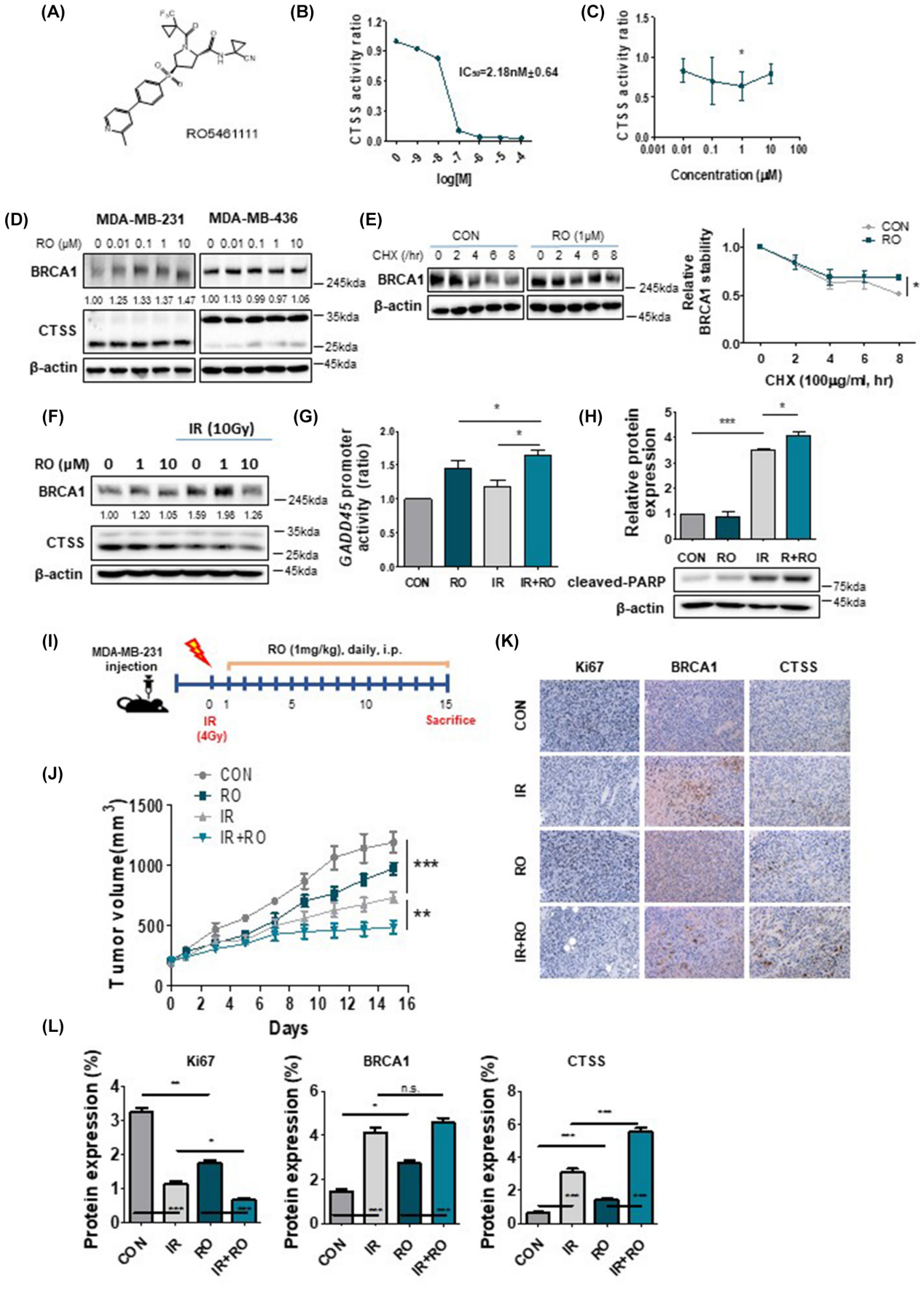
with poor prognosis (Figure 1c). Additionally, the sensitive group was particularly more influenced by the expression level of CTSS on their prognostic outcomes. We examined expression patterns of CTSS and BRCA1 in tissues of breast cancer patients ($n = 116$, each tissue with five different measures) using immunofluorescence and found the inverse correlation of these two proteins, where BRCA1 expression was dominantly induced in the CTSS negative area (Figure 1d). Moreover, TNBC patients showed relatively lower expression of BRCA1 associated with higher expression of CTSS compared to non-TNBC patients (Figure 1e). The overall tendency reflects that higher expression of CTSS was accompanied by lower expression of BRCA1 in both non-TNBC and TNBC patients. It is also assumable that a higher tendency is found in TNBC patients that a higher expression of CTSS was associated with a lower expression of BRCA1 (Figure 1f).

3.2 | CTSS inhibition induced radiosensitization in both in vitro and in vivo xenograft tumor systems, accompanied by increased levels of BRCA1 protein

A previous study suggested that CTSS binds to the BRCT domain of BRCA1 and induces ubiquitin-proteasome degradation.[22] To investigate whether a CTSS inhibitor can repress BRCA1 degradation, a specific CTSS inhibitor, RO, was used (Figure 2a). RO inhibited CTSS enzyme activity at a nanomolar concentration ($IC_{50} = 2.18 \pm 0.64$) without inhibiting other cysteine proteases such as CTSB and μ- and m-calpain activity (Figure 2b and Table S2). CTSS inhibition was also observed in the MDA-MB-231 cell line (Figure 2c). To clarify the inhibitory effect of a CTSS inhibitor via interaction with the BRCT domain, two different cell lines were used to compare the effect on BRCA1 expression, MDA-MB-231 (wild-type of BRCT domain, WT) and MDA-MB-436 (mutant type of BRCT domain, MT) cells. A dose-dependent increase in protein stability of BRCA1 was detected in the WT cells, while no change was observed in the MT cells (Figure 2d) without altering the mRNA levels of BRCA1 in the WT cells (Figure S1A). The protein half-life of BRCA1 was examined after the treatment with cycloheximide (CHX), and a slightly increased BRCA1 protein stability was observed with RO treatment (Figure 2e). CTSS has also been reported to be associated with extracellular matrix degradation, thus promoting cell migration and invasion,[27] but whether CTSS regulates other intracellular mechanisms during metastasis remains unknown. To investigate whether CTSS inhibitors can suppress migration of TNBC cell lines, RO was treated with the wound scratch. RO repressed migration of WT cell in a dose-dependent manner (Figure S1b). RO did not exhibit any toxic effects based on the fact that the IC_{50} value of cell viability was greater than 100 μM. To explore the potential mechanism associated with the involvement of BRCA1, siRNAs (Small interfering RNA) were transfected to the MDA-MB-231 cell line. CTSS knockdown inhibited cell migration and the absence of BRCA1 promoted cell migration, thus BRCA1 may also regulate cell motility and prevent migration (Figure S2A,B). However, to clearly identify the effect of BRCA1-WT on the migration, BRCA1 constructs of BRCA1-WT and BRCA1-ΔBRCT (BRCT deletion) forms were transfected to the HCC1937 lacking endogenous WT BRCA1. BRCA1 WT significantly reduced the migration of HCC1937, but BRCA1-ΔBRCT did not (Figure S2C,D), therefore BRCA1-WT-mediated regulation of cell migration may be associated with its BRCT domain. However, further investigation is needed to understand the exact mechanism. The in vivo xenograft of WT cells revealed that an intraperitoneal injection of 1 mg/kg RO every day significantly induced delayed tumor growth (Figure 1C,D). The immunohistochemistry (IHC) analysis of Ki67 and BRCA1 indicated the significant inhibition of tumor cell proliferation and increased BRCA1 protein stability with RO treatment in tumor tissues (Figure S1e).

A previous study suggests that IR activates CTSS and increases its protein expression, which leads to the increased proteasomal degradation of BRCA1 initiated by the cleavage of the BRCT domain by CTSS. BRCA1 is known for its involvement in apoptosis and the sensitization of tumor cells in combination with IR.[22] After the exposure to IR, the level of BRCA1 increased within 24 h, and BRCA1 level reached the highest enhancement with a co-treatment concentration of 1 μM RO (Figure 2f). RO exhibited stronger inhibition at a concentration of 1 μM, but declined at a concentration of 10 μM (Figure 2c). Similarly, its impact on the restoration of BRCA1 also demonstrated a corresponding dose–response relationship. IR exposure increased the BRCA1-mediated promoter activity of *GADD45*, and a co-treatment with RO amplified the promoter activity (Figure 2g). PARP1 cleavage after IR and IR-induced tumor cell death were also increased by a co-treatment with RO (Figures 3h and S1f). When the long-term effect was examined using a clonogenic survival assay, radiosensitization by RO was still effective (Figure S1g). The in vivo xenograft of WT cells revealed that intraperitoneal injection of 1 mg/kg RO every day in combination with 4 Gy IR induced significant tumor cell growth delays compared to IR alone (Figure 2i,j). The IHC data of Ki67 and BRCA1 indicated the inhibition of tumor cell proliferation and slightly increased BRCA1

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FIGURE 2 RO5461111 (RO), a specific cathepsin S (CTSS) inhibitor, upregulates breast cancer susceptibility gene 1 (BRCA1) stability and exhibits a radiosensitization effect by enhancing BRCA1-mediated apoptosis. (A) A structure of RO. Inhibitory activities of RO versus (B) the recombinant CTSS enzyme and (C) the intracellular CTSS enzyme in MDA-MB-231 cells. (D) Dose-dependent level of BRCA1 at 24 h after RO treatment in MDA-MB-231 cells (WT-BRCA1) and MDA-MB-436 cells (MT-BRCA1). (E) Western blot analysis in cells treated with 100 μg/mL cycloheximide (CHX) for various lengths of time. MDA-MB-231 cells were exposed to radiation (IR, 10 Gy) after pre-treatment with RO. (F) Western blot analysis of BRCA1 restoration. (G) Relative *GADD45* promoter activity. (H) Western blot analysis of cleaved-PARP1 (Poly[ADP-ribose] polymerase 1) induction. (I) An experimental scheme of the tumor xenograft model. (J) Changes in the tumor volume in xenografted NOD/SCID mice ($n = 4–7$ for each group). (K) Representative images of IHC analysis and (L) quantifications of Ki67, BRCA1, and CTSS positive areas. Results are the means and standard error of the mean (*$P < 0.05$, Student's *t*-test or ANOVA). i.p. (Intra-peritoneal).

protein stability with the combination of RO and IR. IR exposure significantly increased CTSS levels as expected, and the combination of IR and RO also significantly increased CTSS levels (Figure 2k,l).

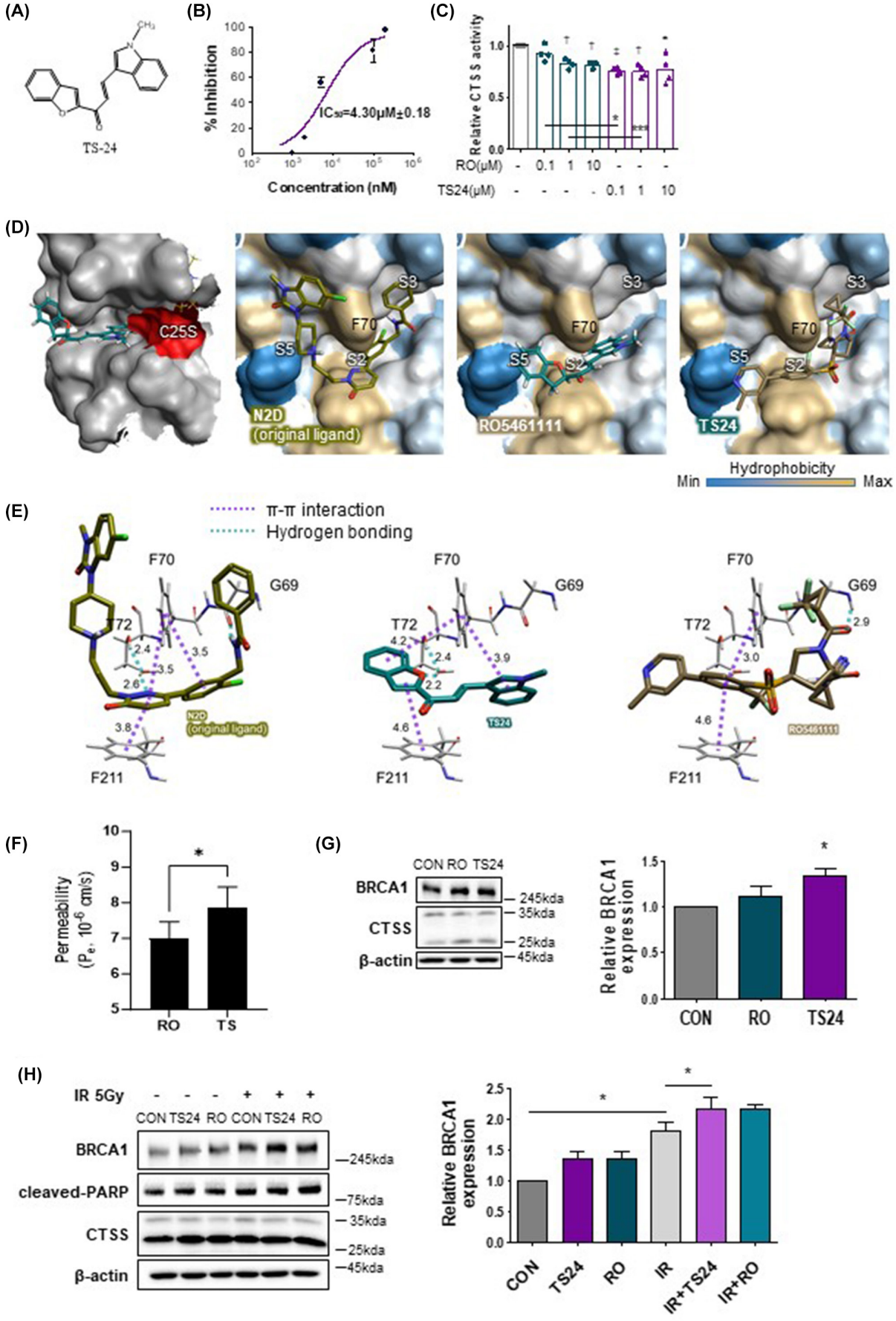
3.3 | A novel CTSS inhibitor, TS-24, possessed more druggable properties than RO

There are various CTSS inhibitors currently in phase I and II clinical trials for the treatment of rheumatoid arthritis, neuropathic pain, psoriasis, and pulmonary fibrosis,[28] but there are only a few promising CTSS inhibitors under discovery. To identify a novel CTSS inhibitor, a CTSS enzyme assay using a fluorescent peptidyl substrate, Z-VVR-AMC, was performed, and the activity of CTSS was calculated by measuring the fluorescence value at the corresponding wavelength. TS-24 was selected from a total of 188 TS series compounds tested for CTSS activity (Figure 3a). The IC_{50} value of TS-24 was 4.30 ± 0.18 μM (Figure 3b), which inhibited CTSS activity 4.1–9.6 times more strongly than other cysteine proteases (Table S2). Although the inhibitory activity of TS-24 on cell-free CTSS enzymatic activity was less potent than that of RO, TS-24 exhibited stronger inhibition in CTSS-overexpressed HEK-293T (Human embryonic kidney 293) cells and exhibited better druggable features in various assessments (Figure 3c). In the molecular docking analysis, TS-24 perfectly fitted into the catalytic cleft of CTSS. The dihydrobenzofuran ring of TS-24 fitted into the S2 and S5 pockets through π–π interactions between aromatic rings in the benzofuran ring and Phe70. A water molecule generated a network of bridging interactions between the oxygen of the dihydro-benzofuran ring and the side chain hydroxyl group of Thr72. This binding mode is similar to that of the original ligand in the crystal structure. The original ligand, N2D, showed not only π–π interactions with Phe70 but also water bridge-mediated interactions with Thr72. Thanks to the interaction with Phe211 and the two π–π interactions with Phe70, which are captured by benzofuran and indole rings, respectively, the S2 pocket can be completely blocked by TS-24. The S2 pocket occupation is important as this region is responsible for the substrate specificity of the cysteine proteases. RO fits well into the pocket occupying the S2 pocket. RO shows no water cross-linked interactions with Thr72 but interacts closely with the active site via two π–π interactions and a hydrogen bond (Figures 3d,e and S3). Characterizing druggability and bioavailability is important in drug discovery. For this reason, the molecular properties of TS-24 were predicted in silico (Table S3). TS-24 possessed more druggable properties compared to RO. TS-24, with a smaller molecular weight and smaller topological polar surface area (TPSA), exhibited improved membrane permeability, cell permeability, and intestinal absorption. All five different in silico druggability assessments predicted TS-24 as a druggable compound, while only two predicted RO as a druggable compound. The better membrane permeability of TS-24 than RO was also confirmed in the parallel artificial membrane permeability assay (Figure 3f). TS-24 also enhanced BRCA1 stability similar to or greater than RO both in single treatment and in co-treatment with IR (Figure 3g,h). Therefore, TS-24 may be a more promising compound, overcoming its poor IC_{50} value from the cell-free assay.

3.4 | TS-24 increased BRCA1 protein stability and exerted radiosensitization

To investigate whether the novel CTSS inhibitor, TS-24, with better druggability, can inhibit BRCA1 degradation, WT cells were treated with TS-24. A dose- and time-dependent increase of protein stability of BRCA1 was detected with TS-24 treatment (Figure 4a,b) without any alteration of *BRCA1* mRNA levels (Figure 4c). To assess the involvement of CTSS in TS-24-induced BRCA1 elevation, CTSS shRNAs or siRNAs were employed. In cellular models with a low level of the active form of CTSS (25 kDa), TS-24 did not induce any increase in BRCA1 protein levels (Figure 4d,e), suggesting that TS-24-mediated BRCA1 expression is CTSS dependent. The protein half-life of BRCA1 was examined after treatment of CHX, and increased BRCA1 protein stability was observed with TS-24 treatment (Figure 4f). To investigate whether a novel CTSS inhibitor can suppress migration of TNBC cell lines, TS-24 was evaluated for the wound healing assay using WT cells. TS-24 showed strong inhibition of cell migration (Figure 4g). TS-24 did not exhibit any toxicity since the IC_{50} value was greater than 100 μM. The in vivo xenograft of WT cells revealed that intraperitoneal injection of 1 mg/kg TS-24 three times a week significantly induced tumor cell growth delay (Figure 4h,i). The IHC of Ki67 and BRCA1 indicated the significant inhibition of tumor cell proliferation and increased BRCA1 protein stability with TS-24 (Figure 4j). The radiosensitization effect of TS-24 was also analyzed in vitro and in vivo. IR alone caused the increase in BRCA1 level, but TS-24 co-treatment strongly enhanced the level of BRCA1 at 24 h after the IR exposure (Figure 5a). IR exposure also increased the BRCA1-mediated promoter activity of *GADD45*, and co-treatment with TS-24 further potentiated the promoter activity (Figure 5b).

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FIGURE 3 TS-24, a novel cathepsin S (CTSS) inhibitor, exhibits stronger suppression of intracellular CTSS activity and possesses better binding capability compared to RO5461111 (RO). (A) Structure of TS-24. (B) Inhibitory activities of TS-24 versus the recombinant CTSS enzyme. (C) Inhibitory activities of TS-24 compared to RO in CTSS-overexpressed HEK-293T cells. Docking analysis of TS-24 in the CTSS crystal structure (PDB, 5QBV). (D) Binding mode of TS-24 compared to that of the original ligand, N2D. (E) Molecular interactions between the compound and the active site of CTSS were compared to those of the original ligand, N2D, in the crystal structure. The interactions are represented as dashed lines and the distances are indicated in Å. (F) A parallel artificial membrane permeability assay (PAMPA) of TS-24 and RO. (G) Western blot analysis of BRCA1 levels at 24 h after exposure to compounds. (H) Western blot analysis and quantification of BRCA1 levels at 24 h after exposure to radiation (IR, 5 Gy) after pre-treatment with compounds.

PARP1 cleavage after IR was augmented by co-treatment with TS-24 (Figure 5c) and IR-induced tumor cell death was also increased by co-treatment with TS-24 (Figure 5d). When the long-term effect was examined using a clonogenic survival assay, radiosensitization by TS-24 was still observed (Figure 5e). The effect of TS-24 in restoring BRCA1 in a dose-dependent manner and in radiosensitization was additionally observed in other cell lines, such as a murine EO771 cell line (TNBC alike type) and a human HCC70 cell line (TNBC), both harboring WT BRCA1 (Figure S4). The in vivo xenograft of WT cells revealed that intraperitoneal injection of 2 mg/kg TS-24 three times a week in combination with 3 Gy IR significantly induced tumor cell growth delays when compared to IR alone (Figure 5f,g). The IHC data of Ki67 and BRCA1 indicated the significant inhibition of tumor cell proliferation and increased BRCA1 protein stability with the combination of TS-24 and IR. As expected, IR exposure significantly increased CTSS expression, while TS-24 treatment slightly increased CTSS expression, but not significantly (Figure 5h,i). Additionally, we noted the localization of BRCA1 and CTSS through immunofluorescence (IF) analysis. Within the slide, regions exhibiting high CTSS expression correlate to lower levels of BRCA1 (Figure 5j), suggesting a potential inverse relationship, indicating that cells with elevated CTSS expression may exhibit reduced BRCA1 expression.

4 | DISCUSSION

The association between CTSS expression and the severity or prognosis of TNBC has not been clearly established yet. Our analysis of public patient data has suggested that high expression of CTSS might contribute to poor prognosis and chemo-resistance. Our IF analysis on the tissue microarray provided more profound evidence that BRCA1 expression was decreased in the CTSS positive area, and a more dominant inverse correlation was observed in TNBC patient tissues. Supportive of our findings, current studies have also demonstrated that among various breast cancer subtypes, TNBC showed the highest expression levels of CTSS,[29] suggesting that targeting CTSS may be a good therapeutic strategy for TNBC.

We applied CTSS inhibitors to restore BRCA1 protein and exhibit radiosensitization (Figure 6). To confirm our hypothesis, RO, a specific CTSS inhibitor developed by Roche, was used. RO enhanced BRCA1 stability and induced radiosensitization. To develop a novel CTSS inhibitor with better druggability than RO, TS-24 was identified by screening with a CTSS enzyme assay and an in silico docking assay. Although the CTSS inhibition IC_{50} of TS-24 was not better than RO in a cell-free system, TS-24 showed similar or better inhibition in a cell system with modest selectivity against other cysteine proteases. The S2 pocket of CTSS is composed of Phe70, Gly137, Val162, Gly165, and Phe211, and these residues make space for ligand binding.[30] The docking analysis of TS-24 revealed that TS-24 can interact with Phe70 and Phe211 via π–π interactions and with Val162 through hydrophobic interactions. As the S2 pocket occupation is known to address selective inhibition,[31] the binding mode of TS-24 might be responsible for the poor activity against other cysteine proteases, cathepsin B (IC_{50} 27.99 ± 4.26 μM) and calpains (IC_{50} 17.96 ± 8.64 μM for μ-calpain and 41.34 ± 17.83 μM for m-calpain).

Through a druggability assessment, TS-24 was verified to possess promising drug-likeness as it passed five different drug-like rules without violation. Notably, TS-24 has a much smaller TPSA for better membrane permeability and a higher score of intestinal absorption than RO. Considering the molecular properties of the conventional CTSS inhibitor, where RO has too many H-bonding acceptors, TS-24 has the potential to be developed as a clinically usable CTSS inhibitor in the aspect of druggability. Also, TS-24 showed greater BRCA1 recovery function than RO and better radiosensitization effects in TNBC cells in vitro and in TNBC xenograft mice in vivo.

Although we have mainly focused on the ability of BRCA1 to induce tumor apoptosis among its various functions, BRCA1 has long been recognized for its functions in DNA repair pathways, especially homologous recombination repair, and replication fork protection. Therefore, BRCA1-deficient individuals are exposed to a higher risk of developing breast cancer, including TNBC, but ironically are more susceptible to DNA damaging agents, such as platinum-based drugs or PARP inhibitors. However, several studies have reported that BRCA1 depletion in animal models resulted in resistance to these DNA-damaging agents.[10] Considering the complexity of BRCA1 and its various biological functions, restoring BRCA1 function may be more beneficial, considering its role in inducing tumor apoptosis and inhibiting tumorigeneses.

The limitation of this study is that although TS-24 showed better druggability than RO and demonstrated improved BRCA1 recovery ability and radiosensitization effects, TS-24 itself did not exhibit a significant radiosensitization effect. However, the consistent results of this study highlight that CTSS inhibitors can effectively induce radiosensitization by inhibiting the degradation of BRCA1. It is therefore crucial to continue the ongoing drug development targeting CTSS in order to enhance BRCA1 stability and promote

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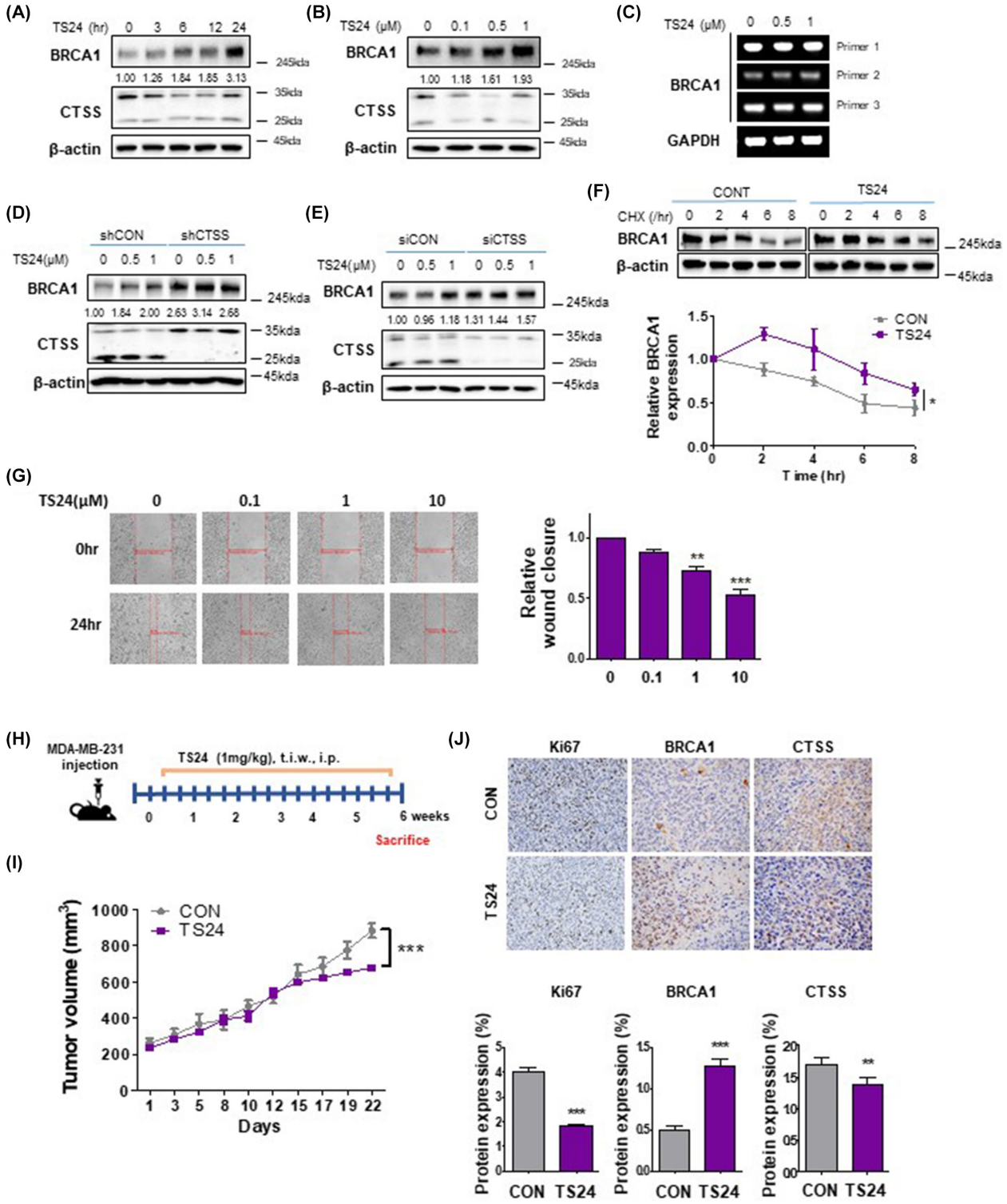


FIGURE 4 TS-24 upregulates breast cancer susceptibility gene 1 (BRCA1) stability and delays tumor growth. (A) Time-dependent levels of BRCA1 on 1 μM TS-24 treatment and (B) dose-dependent levels of BRCA1 at 24 h after TS-24 treatment. (C) Dose-dependent mRNA levels of BRCA1 at 24 h after TS-24 treatment in MDA-MB-231 cells detected with three different primers. (D) Dose-dependent levels of BRCA1 detected in MDA-MB-231 cells with stably transfected sh*CTSS* and (E) with transiently transfected si*CTSS*. (F) Western blot analysis in cells treated with 100 μg/mL cycloheximide (CHX) for various lengths of time. (G) Representative light microscope images and quantification of relative wound closure evaluated by measuring the cell-free distance and expressed as a ratio to the initial cell-free distance in MDA-MB-231 cells treated with TS-24 to evaluate migration 24 h after the initial wound. (H) An experimental scheme of the tumor xenograft model and treatment schedule. (I) Changes in the tumor volume in xenografted NOD/SCID mice ($n = 4$ for each group). (J) Representative images of IHC analysis and quantifications of Ki67, BRCA1, and CTSS positive areas. Results are the means and standard error of the mean (*$P < 0.05$, Student's *t*-test or ANOVA). t.i.w (three times a week); i.p. (Intra-peritoneal).

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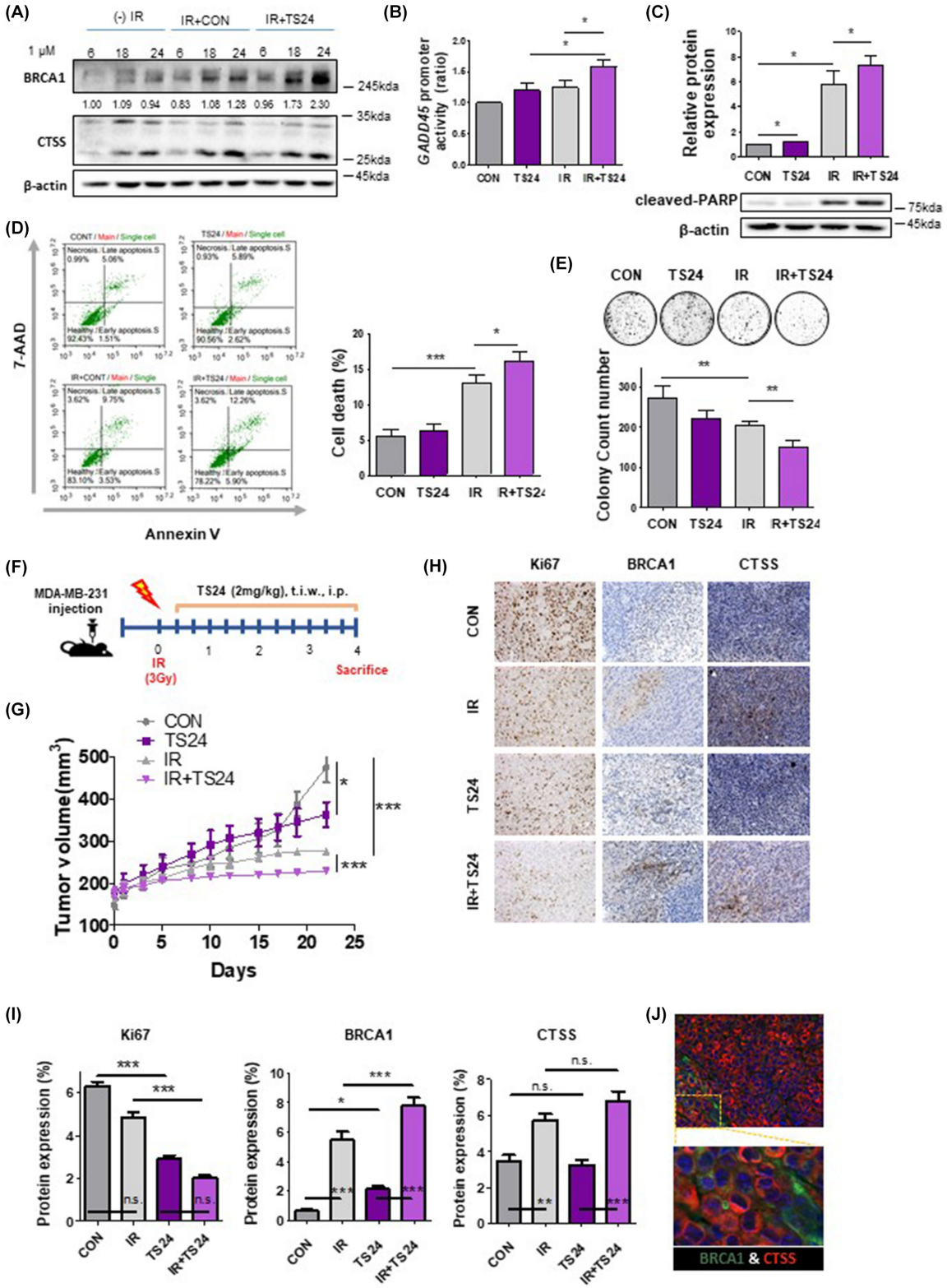


FIGURE 5 TS-24 exhibits a radiosensitization effect by enhancing breast cancer susceptibility gene 1 (BRCA1)-mediated apoptosis. MDA-MB-231 cells were exposed to radiation (IR, 10 Gy) after pretreatment with TS-24. (A) Western blot analysis of BRCA1 restoration. (B) Relative *GADD45* promoter activity. (C) Western blot analysis of cleaved-PARP induction. (D) Cell death was evaluated with 7AAD/PE-annexin V co-staining. (E) A colony forming assay was evaluated with trypan-blue staining. (F) An experimental scheme of the tumor xenograft model. (G) Changes in the tumor volume in xenografted NOD/SCID mice ($n = 3–4$ for each group). (H) Representative images of IHC analysis and (I) quantifications of Ki67, BRCA1, and cathepsin S (CTSS) positive areas. (J) immunofluorescence analysis of irradiated MDA-MB-231 cells (green, BRCA1; red, CTSS). Results are the means and standard error of the mean (*$P < 0.05$, Student's *t*-test or ANOVA). t.i.w (three times a week); i.p. (Intra-peritoneal).

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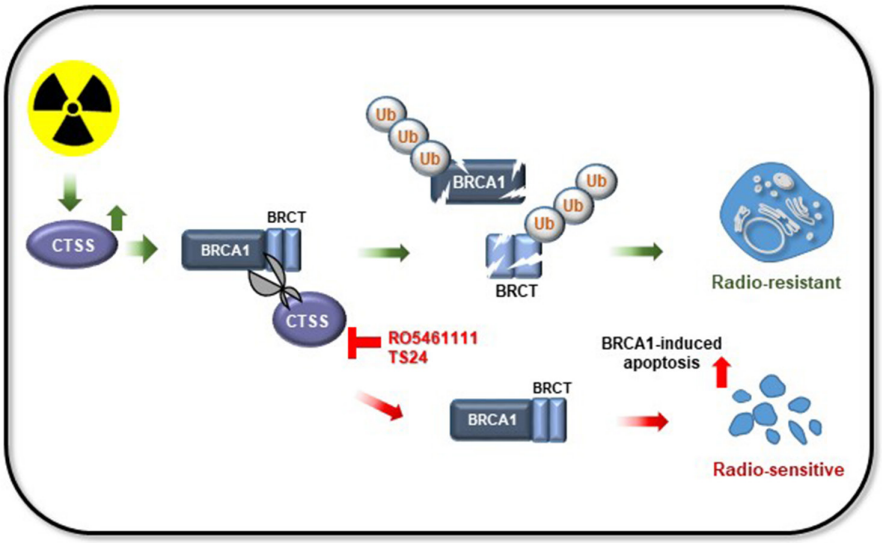


FIGURE 6 Schematic illustration of molecular mechanisms of cathepsin S (CTSS) inhibitors for radiosensitization via inhibition of breast cancer susceptibility gene 1 (BRCA1) degradation. Radiotherapy-induced CTSS accelerates cleavage of the BRCT domain of BRCA1, which leads to the ubiquitin-mediated degradation of BRCA1. Increased proteolytic degradation of BRCA1 suppresses BRCA1-mediated apoptosis and shows radioresistance. Inhibition of CTSS may provide a novel therapeutic strategy of radiosensitization through increased BRCA1-induced apoptosis of cancer cells.

radiosensitization. This study holds significance as the first attempt toward achieving this objective.

Additionally, considering the frequent occurrence of metastasis in TNBC, it would be more beneficial for TNBC patients if treatments also had the advantage of inhibiting migration. It is known that the overexpression of BRCA1 suppresses migration,[32] while CTSS has been observed to promote migration. Therefore, strategies that inhibit CTSS to enhance the protein stability of BRCA1 are proposed as a solution to address the limitation in TNBC treatment related to migration. In this context, TS-24 may exhibit an advantage.

In conclusion, although our study has some limitations, a strategy for radiosensitization by induction of BRCA1-mediated apoptosis and inhibition of migration through the functional restoration of BRCA1 induced by a CTSS inhibitor in TNBC may help improve current treatment strategies for TNBC patients who lack adequate therapy.

AUTHOR CONTRIBUTIONS

Eun Choi: Conceptualization; data curation; formal analysis; methodology; visualization; writing – original draft. **Kyung-Hwa Jeon:** Conceptualization; data curation; formal analysis; methodology; visualization; writing – original draft. **Hanhee Lee:** Methodology. **Gil-Im Mun:** Methodology. **Jeong-Ahn Kim:** Methodology. **Jae-Ho Shin:** Methodology. **Youngjoo Kwon:** Conceptualization; writing – review and editing. **Younghwa Na:** Funding acquisition; methodology; project administration; resources; writing – review and editing. **Yun-Sil Lee:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENTS

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Ewha Women's University [IACUC 21–027] and all animal experiments were conducted following the Animal Care and Handling Guidelines.

DISCLOSURE

The authors confirm that the data supporting the findings of this study are available within the article and/or its Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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