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Original Research

Piperine: An Anticancer and Senostatic Drug

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Abstract

Background: Cancer is a representative geriatric disease closely related to senescent cells and cell aging in tissues. Senescent cells that surround cancer tissues reduce the effects of various cancer treatments and induce cancer recurrence through senescence-associated secretory phenotype (SASP) secretion. Thus, for good therapeutic effect, candidate drugs should be selective for both cancer and senescent cells. In this study, we investigated the selective effect of piperine as a potential senostatic agent as well as an anticancer drug. **Methods**: The effect of piperine on cytotoxicity and cell proliferation was tested by lactate dehydrogenase (LDH) or water-soluble tetrazolium salt (WST) assay. The levels of $p16^{INK4a}$ and p21, mitogen-activated protein kinases (MAPKs), and mammalian target of rapamycin (mTOR) were analyzed by Western blot analysis. The rejuvenation effects of piperine on the senescent cells were investigated by senescence-associated beta-galactosidase (SA-β-Gal) stain, mitochondria membrane potential (MMP) and reactive oxygen species (ROS) levels, and senescence-associated secretory phenotype (SASP) secretion after treatment with piperine in senescent cells. **Results**: While piperine induced high cytotoxicity in various cancer cell lines, it led to proliferating of premature senescent cells similar with nicotinamide (NA), which is known as a rejuvenating drug of senescent cells. Piperine differently affected cancer cells and premature senescent cells due to the different responses of intracellular signaling pathways and also reversed premature senescence phenotypes and modulated SASP secretion in premature senescent cells. **Conclusions**: From these results, we propose piperine as an effective cancer treatment that can simultaneously induce senostatic effects and the removal of cancer cells, not as an adjuvant to the existing senostatics for cancer treatment.

Keywords: senescence; senostatic; anticancer; senescence-associated secretory phenotype; piperine; human diploid fibroblasts

1. Introduction

Senescent cells secrete a senescence-associated secretory phenotype (SASP) that leads to chronic inflammation, playing a crucial role in age-related functional decline and senile diseases [1]. Thus, the development of senotherapeutic interventions to remove senescent cells (senolytic drugs) or to modulate the SASP (senostatic drugs) could extend a person's healthspan or treat various diseases [2]. Several senolytics target the prosurvival pathway, such as kinase inhibitors (e.g., dasatinib), flavonoids (e.g., quercetin and fisetin), BCL-2/BCL-xL inhibitors (e.g., navitoclax), and BCL-xL inhibitors (e.g., A1331852 and A1155463) [3].

Cancer is a representative geriatric disease and is closely related to senescent cells constituting tissues. Cellular senescence itself suppresses cancer development despite the accumulation of various genetic mutations, but the SASP secreted from senescent cells promotes the development of surrounding cancer cells [4]. Therefore, to develop effective therapeutic agents to remove cancer, it is crucial to identify candidate substances that can selectively act only on cancer cells. Additionally, SASP secreted from senescent cells that surround cancer tissues reduce the effects of various cancer treatments and induce cancer recurrence through SASP secretion [5–7]. Therefore, increasing attention is being paid to the development of cancer therapeutics using senotherapies that remove senescent cells or suppress SASP secretion. Senolytics, such as quercetin, navitoclax, and fisetin, are being studied as potential cancer treatments in nonclinical or initial clinical trials [8–10]. However, for good therapeutic effect, candidate drugs should be selective for both cancer and senescent cells.

Piperine is a bioactive phenolic component that has been isolated from plants of the *Piper* species, such as black pepper (*Piper nigrum*) and long pepper (*Piper longum*) [11], and has attracted attention as a dietary phytochemical and medicine [12]. Various pharmacological properties of piperine have been suggested, including antioxidant activity [13], anti-inflammatory activity [14] and biologi-

cal enhancement [15]. Piperine also exerts a chemopreventive effect [16] and causes cellular toxicity in cancer cells by inducing various effector proteins involved in apoptosis [17–19]. Piperine was reported to suppress tumor development and metastasis in mouse models [17]. In cancer cells, piperine triggers both cell cycle arrest by activating p21 and apoptosis by activating caspase [20]. Interestingly, a combination therapeutic model of piperine with curcumin, a yellow pigment in the Indian spice turmeric (*Curcuma longa*), demonstrated neurotrophic and neuroprotective effects in a D-galactose-induced brain aging model [21], preventing the progress of aging induced by D-galactose as well as reversing hippocampal memory function due to antioxidant activity [22]. Curcumin is a well-known, promising antiaging intervention that is easy to add to one's diet. Curcumin was reported to induce an extended lifespan in various models, including fruit flies, nematodes, and mice [23–26]. While curcumin has demonstrated a direct antiaging effect, the antiaging effect of piperine has only been attributed to its antioxidant effect on brain aging in combination with curcumin. Like piperine, piperlongumine is a natural product found in various *Piper* species, and its analogs have also been suggested as senolytic agents through activation of the caspase pathway in senescent cells [27].

We previously screened single natural compounds that acted differently on cancer cells compared with premature senescent cells. Most substances showed similar effects on cancer and senescent cells, but piperine induced toxicity in cancer cells only. This study investigates the selective effect of piperine as a potential senostatic agent as well as an anticancer drug.

2. Materials and Methods

2.1 Reagents and Cells

Piperine was kindly provided by Prof. WK Oh (Seoul National University, Korea) and purchased (Merck, NY, USA). CT26 (mouse colon carcinoma, CRL-2638), T98G (human glioblastoma, CRL-1690), A431 (human skin carcinoma, CRL-1555), MCF7 (human breast adenocarcinoma, HTB-22), HepG2 (human hepatocellular carcinoma, HB-8065), and HeLa (human cervix adenocarcinoma, CCL-2) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cancer cell lines, except MCF7, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C in a 5% CO_2 incubator. The MCF7 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% FBS and 1% P/S at 37 °C in a 5% CO_2 incubator. Human diploid fibroblasts (HDFs) were cultured as previously described [28]. Briefly, the cells were maintained in 10-cm cell dishes containing DMEM supplemented with 10% FBS and 1% P/S. The cells were continuously subcultured at a ratio of 1:4. Nonsenescent HDFs (NS-HDFs) were defined as HDFs resulting from fewer than 30 population doublings and premature senescent HDFs (S-HDFs) as HDFs resulting from more than 70 population doublings. Premature senescent cells were confirmed by senescent-associated β-galactosidase (SA-β-gal) staining. All cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA).

2.2 Piperine Treatment

We investigated the selectivity of piperine by observing its effect on the growth of cancer and normal cells. Cancer and normal cells were cultured in complete medium at 37 °C in a 5% CO_2 incubator and treated once or twice with piperine (70 μM) at 2-day intervals. Thereafter, we analyzed cytotoxicity using a lactate dehydrogenase (LDH) assay or performed a visual cell count to analyze cell growth. We also treated S-HDFs in complete medium at 37 °C in a 5% CO_2 incubator with piperine (70 μM) three times at 2-day intervals to test the senomorphic effect of piperine on S-HDFs.

2.3 Cytotoxicity Assay

Cytotoxicity was assessed using an LDH assay kit (DG-LDH500; DoGen Bio, Seoul, Korea) according to the manufacturer's protocol. Briefly, cancer cells (2×10^5) were seeded in triplicate in six-well plates and then treated once or twice with piperine (70 μM) at 2-day intervals. Normal cells, such as NS-HDFs (6×10^4) and S-HDFs (3×10^4), were seeded at triplicate in six-well plates and treated two to three times with piperine (70 μM) at 2-day intervals. After the piperine treatment, the culture supernatants were collected and placed in triplicates of 96-well plates, followed by incubation with LDH solution at room temperature (RT) in the dark for 30 min. Finally, the optical density was read at 450 nm using a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA). To identify nuclear morphology, cells were fixed and stained with the nucleic acid stain 4′,6′-diamidino-2-phenylindole (DAPI) after once or twice treatment of piperine. The stained cells were analyzed with the Zeiss LSM 700 confocal microscope.

2.4 Analysis of Viable Cells

To count the viable cells, we seeded various cancer cell lines (2×10^5) in six-well plates containing complete medium and treated them with piperine (70 μM) for 24 or 48 h. We also seeded S-HDFs (3×10^4) in 6-well plates containing complete medium and treated them with piperine (70 μM) or 5 mM nicotinamide (NA, positive control) one to three times at 2-day intervals. Subsequently, these cells were harvested, resuspended in medium, and stained with trypan blue solution. The viable cells were counted using a hemocytometer.

2.5 Cell Proliferation Assay

Cell proliferation assay was assessed using an EZ-Cytox water-soluble tetrazolium salt (WST) cell proliferation assay kit (EZ-3000; DoGen Bio) according to the manufacturer's protocol. Briefly, S-HDFs (8×10^3) were seeded in triplicate in 24-well plates containing complete medium, and the cells were treated one to three times with piperine (70 μM) at 2-day intervals. Subsequently, the cells were incubated with EZ-Cytox solution, which contains a WST, for 4 h at 37 °C in a 5% CO_2 incubator. Cell proliferation was determined by measuring the absorbance at 450 nm using a microplate reader.

2.6 SA-β-Gal Staining

SA-β-gal staining was performed as previously described [28]. Briefly, S-HDFs were treated three times with piperine (70 μM) at 2-day intervals. Thereafter, the cells were fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min at RT. Then, the cells were washed twice with 1 $\times$ PBS (pH 6.0) for 5 min each and incubated in staining solution (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride) at 37 °C for 16 h. The next day, we confirmed the presence of stained cells under an inverted bright-field microscope and captured images of the cells.

2.7 Quantitative Analysis of SA-β-Gal-Stained Cells

We used MATLAB software (MathWorks Inc., Natick, MA, USA) to quantify the SA-β-gal-stained cells. First, the captured images were inverted after conversion to grayscale. Next, the image noise was removed via cutoff from 8 bit-images using the selected value (155). The sum of the total intensity was obtained by adding all the pixels with a value greater than 0. The average intensity per pixel was calculated by dividing the total intensity by the number of pixels with a value greater than 0. The equation used was as follows: average intensity = total intensity/number of pixels/number of cells. The SA-β-gal-stained cells were quantified using five different image fields.

2.8 Western Blot Analysis

Western blot analysis was performed as previously described [28]. Briefly, total proteins were extracted from the cells using radioimmunoprecipitation assay buffer (Biosesang, Seongnam, Korea) containing Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO, USA) and Phosphatase Inhibitor Cocktail I and II (Sigma-Aldrich). The protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were incubated with primary antibodies at 4 °C overnight. The antibodies used were anti-$p16^{INK4a}$ (MA5-1742) from Invitrogen (Carlsbad, CA, USA); anti-p21 (sc-397) and anti-β-actin (sc-47778) from Santa Cruz Biotechnology (Delaware, CA, USA); and anti-p38 (#8690), anti-phospho-p38 MAPK (Thr180/Tyr182) (#9211), anti-p44/42 MAPK (Erk1/2), (#9102), anti-phospho-p44/42 (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb (#4370), anti-SAPK/JNK (#9252), and anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9251) from Cell Signaling Technology (Danvers, MA, USA). The following day, the membranes were washed three times and incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology) at RT for 1 h. Protein expression was visualized using an enhanced chemiluminescence solution (DoGen) and analyzed using Image J software (V 1.8.0) (National Institutes of Health, Bethesda, MD, USA).

2.9 Measurement of Mitochondria Membrane Potential (MMP) and Reactive Oxygen Species (ROS)

We used tetramethylrhodamine (TMRM) (I34361; Invitrogen) to analyze MMP and dihydroethidium (DHE) (D23107; Molecular Probes, Eugene, OR, USA) to quantify the levels of cellular ROS. Briefly, S-HDFs were treated with piperine (70 μM) or 5 mM NA (positive control) three times at 2-day intervals. After the piperine treatment, the cells were stained with TMRM (100 nM) or DHE (5 μM) at 37 °C in the dark for 30 min. Subsequently, the cells were harvested, washed with 1 $\times$ PBS, and analyzed for TMRM or DHE fluorescence by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA). We counted 10,000 events for each sample, and the results were presented as mean fluorescence intensity using a bar graph.

2.10 Analysis of SASP Production

S-HDFs were treated three times with piperine (70 μM) at 2-day intervals. Following treatment, the culture supernatants were collected and placed in triplicates of 96-well plates. Then, the levels of the SASP secretion, including interleukin (IL)-6, IL-8, and tumor growth factor (TGF)-β1, in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols. The absorbance was read at 450 nm using a microplate reader.

2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (V 8.0) (GraphPad Software, San Diego, CA, USA). Data were presented as the mean $\pm$ standard error of the mean of at least three independent experiments. The differences between the experimental groups were analyzed for statistical significance using the nonparametric Mann–Whitney U test. p values < 0.05 were considered significant.

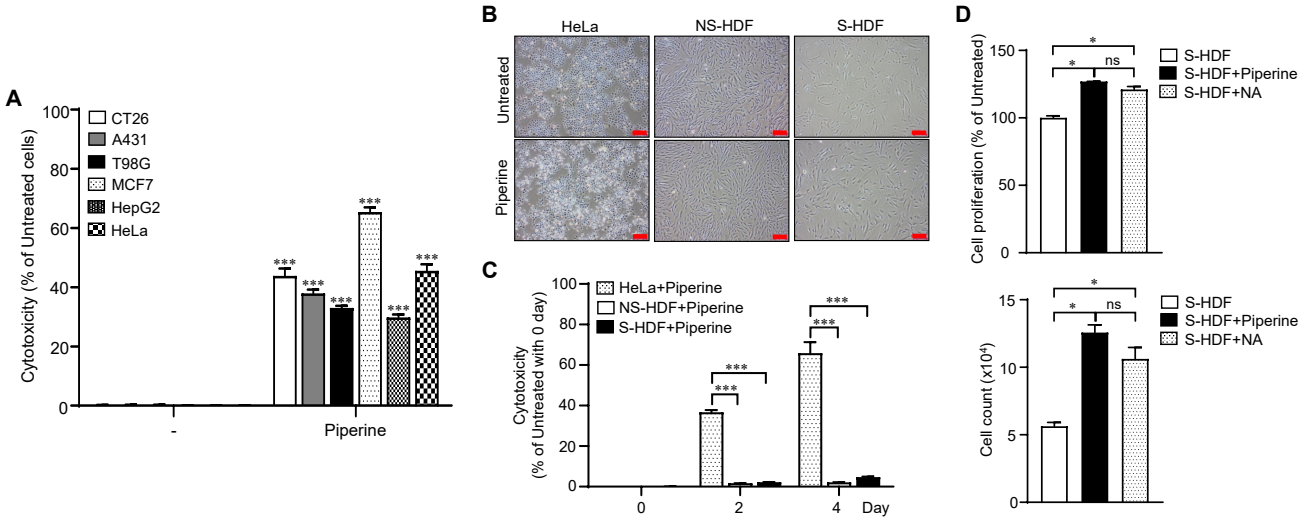

Fig. 1. Selective inhibition of cancer cell proliferation by piperine. Various cancer cells were treated with piperine (70 μM) for once. (A) Cytotoxicity was measured by lactate dehydrogenase (LDH) assay in various cells. HeLa and normal cells (NS-HDF and S-HDF) were treated twice with piperine (70 μM) at 2-day intervals. (B) Morphological changes and (C) Cytotoxicity between HeLa and normal cells was observed after treating piperine. S-HDFs were treated three times with piperine (70 μM) or 5 mM NA (positive control) at 2-day intervals. (D) Cell proliferation was measured by using a water-soluble tetrazolium salt (WST) cell proliferation assay, and the number of viable cells was counted using a hemocytometer. Data are based on three independent experiments, and statistical significance between the experimental groups was analyzed using the Mann–Whitney U test. *$p < 0.05$ compared with untreated and piperine- or NA-treated S-HDF; ***$p < 0.001$ compared with untreated and piperine-treated cancer cells (A) or compared with piperine-treated HeLa and piperine-treated NS-HDF or S-HDF (C); ns, not significant compared with piperine- and NA-treated S-HDF (D). NS-HDF, nonsenescent human diploid fibroblast; S-HDF, senescent human diploid fibroblast; CT26, mouse colon carcinoma cells; T98G, human glioblastoma cells; A431, human skin carcinoma cells; MCF7, human breast adenocarcinoma cells; HepG2, human hepatocellular carcinoma cells; HeLa, human cervix adenocarcinoma cells; NA, nicotinamide. Scale bar, 200 μm.

3. Results

3.1 Piperine Selectively Inhibits the Proliferation of Cancer Cells and Induces Senescent Cell Growth

To confirm the effect of piperine on cancer cells, we treated various cancer cell lines with piperine (70 μM) and analyzed cellular proliferation and cytotoxicity. The cancer cells showed cytotoxicity and their growth was significantly inhibited by the piperine treatment (Fig. 1A and **Supplementary Fig. 1**). Next, we compared the effect of piperine on HeLa cells, cervical cancer cells, NS-HDFs, and S-HDFs by treating each cell type twice with piperine (70 μM) at 2-day intervals. Piperine selectively induced cytotoxicity in HeLa cells but not in NS-HDFs and S-HDFs (Fig. 1B,C and **Supplementary Fig. 2**). Interestingly, continuous treatment of S-HDFs with piperine induced cell growth (Fig. 1D). These results indicate that piperine affected cancer cells differently than S-HDFs, suggesting the application of piperidine as both a cancer cell-specific therapeutic agent and a senomorphic agent to improve senescent cell function.

3.2 Piperine Induces Senescent Cell Proliferation

We extended the piperine treatment period to three times every 2 days to observe the senomorphic effects of piperine on senescent cells (Fig. 2A). Cytotoxicity was not observed in the S-HDFs after the cells had been treated with piperine three times (Fig. 2B). The effect of piperine treatment was compared with that of nicotinamide (NA), which induces senescent cell proliferation [29], to confirm the effect of piperine treatment on S-HDF growth. Then, we performed a WST cell proliferation assay and counted the number of viable cells. Interestingly, piperine induced a higher rate of cell proliferation than NA in S-HDFs (Fig. 2C,D). The expression of $p16^{INK4a}$ and p21, markers of senescent cells and cell cycle checkpoints, respectively, was also significantly reduced in S-HDFs following piperine treatment (Fig. 2E). These results show a novel effect of piperine on senescent cells, suggesting that it induces cell division in senescent cells as opposed to inducing cytotoxicity in cancer cells.

3.3 Piperine Induces Extracellular Signal-Regulated Kinase (Erk1/2) and c-Jun N-Terminal Kinase (JNK) Phosphorylation in Senescent Cells

Mitogen-activated protein kinases (MAPKs), including Erk1/2, p38, and JNK, have been implicated in senescence phenotypes such as growth arrest [30,31], apoptosis resistance [32], and the SASP secretion [33,34]. We examined whether piperine regulates MAPK pathways in

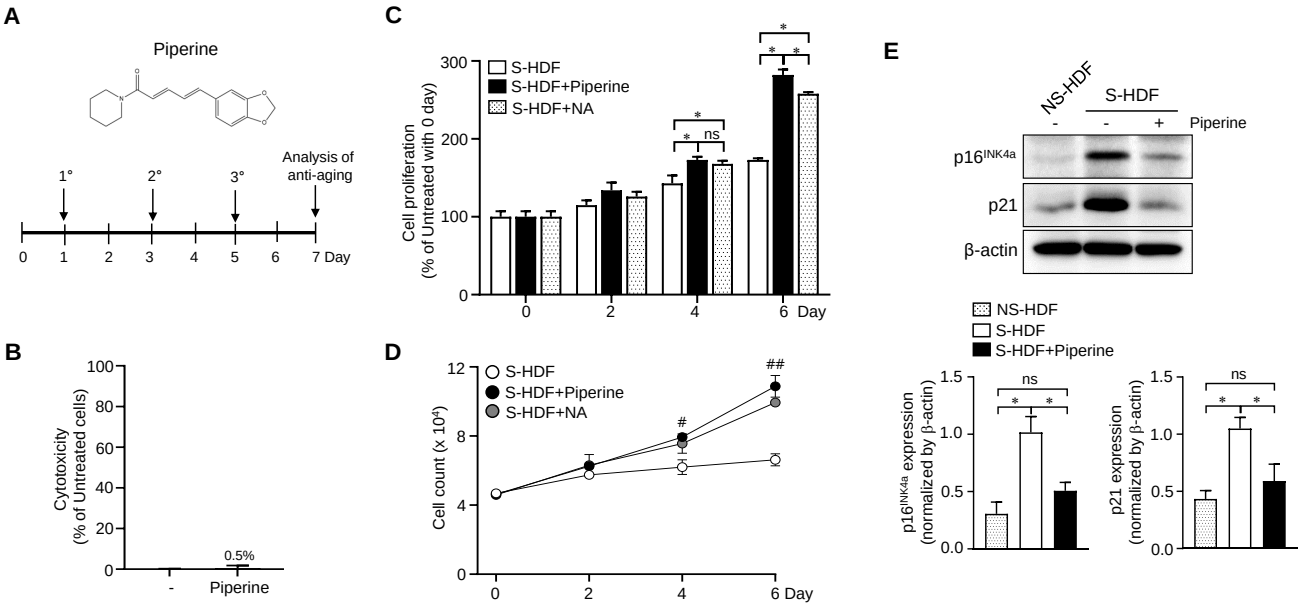

Fig. 2. Effects of piperine on the proliferation of senescent cells. (A) The chemical structural of piperine (top panel) and the experimental scheme of piperine treatment in senescent cells (bottom panel). S-HDFs were treated three times with piperine (70 μM) at 2-day intervals. (B) Cytotoxicity was measured using an LDH assay. (C) Cell proliferation was measured by treatment of piperine (70 μM) or 5 mM NA (positive control) using a WST cell proliferation assay and (D) counted using a hemocytometer on the indicated days. (E) The expression of $p16^{INK4a}$ and p21 in piperine-treated S-HDFs was analyzed by Western blot using specific antibodies. β-actin was used as a loading control. Data are based on three independent experiments, and statistical significance between the experimental groups was analyzed using the Mann–Whitney U test. $^{*}p < 0.05$ compared with untreated and piperine- or NA-treated S-HDF, compared with piperine- and NA-treated S-HDF (C), and compared with NS-HDF and S-HDF or compared with untreated and piperine-treated S-HDF (E); ns, not significant compared with piperine- and NA-treated S-HDF (C) or compared with NS-HDF and piperine-treated S-HDF (E). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with untreated and piperine-treated S-HDF (D).

S-HDFs in an effort to elucidate the mechanism of cell division S-HDFs following piperine treatment. Although p38 phosphorylation was unaffected in piperine-treated S-HDFs, Erk1/2 and JNK phosphorylation were remarkably increased (Fig. 3A). We also investigated the involvement of various signaling pathways, such as the 5' adenosine monophosphate-activated protein kinase pathway, mammalian target of rapamycin (mTOR) pathway, and autophagy, in piperine-treated S-HDFs. Piperine treatment did not affect signaling in S-HDFs (Fig. 3A, data not shown). We also investigated these signaling pathways in HeLa cells. Piperine treatment increased the phosphorylation of JNK, p38, and mTOR but not Erk1/2 (Fig. 3B). These results suggest that the signaling mechanism of piperine in senescent cells differs from that in cancer cells and imply that piperine activates Erk1/2 and JNK signaling in senescent cells, leading to the reduction of cell cycle inhibitors $p16^{INK4a}$ and p21, thereby inducing the division of senescent cells.

3.4 Piperine Reverses Senescence Phenotypes with Modulating SASP Secretion

To further determine whether piperine rescues cellular senescence phenotypes, we examined the SA-β-gal activity of S-HDFs following piperine treatment (70 μM) under the same experimental conditions. SA-β-gal activity was markedly decreased in piperine-treated S-HDFs, but the morphology of S-HDFs remained unchanged (Fig. 4A). Senescent cells are characterized by a decreased MMP [35, 36] and increased production of intracellular ROS [37,38]. Furthermore, mitochondrial dysfunction and ROS accumulation are associated with age-related diseases [38–40]. Thus, we determined the MMP and cytoplasmic ROS levels to confirm the effect of the piperine on mitochondrial function in senescent cells. Because NA leads to MMP recovery [41] and reduces ROS levels in senescent cells [42], we used NA as a control for these experiments. After treating S-HDFs with piperine (70 μM) or 5 mM NA three times at 2-day intervals, the cells were stained with TMRM to measure MMP or DHE to analyze intracellular ROS levels. We found that piperine induced MMP (Fig. 4B) while reducing intracellular ROS levels (Fig. 4C) in S-DHFs, which was similar to the effect of NA treatment. These results suggest that the piperine not only induces the division of senescent cells but also restores their functions. SASP secretion includes high levels of IL-6, IL-8, and TGF-β1 [43–46]. Thus, controlling (modulating) the secretion of SASP is crucial for the development of senotherapeutic agents. To

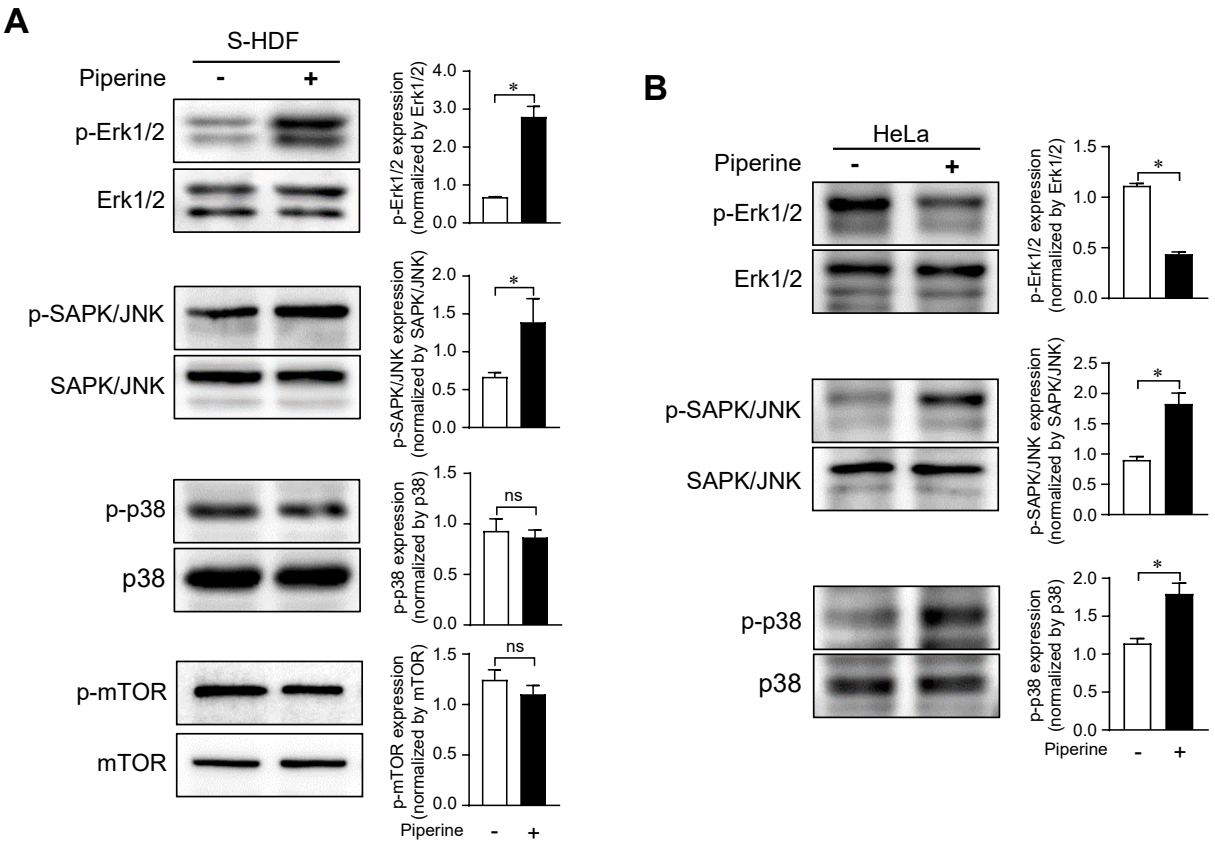

Fig. 3. Differential regulation of signaling in senescent cells and cancer cells following piperine treatment. S-HDFs (A) and HeLa cells (B) were treated with piperine (70 μM) for 16 h. The proteins associated with various signaling pathways, including the MAPKs and mTOR, were analyzed by western blot using specific antibodies. Data are based on three independent experiments, and statistical significance between the experimental groups was analyzed by the Mann–Whitney U test. *$p < 0.05$ and ns, not significant compared with untreated and piperine-treated cells. Erk, extracellular signal-regulated kinase; SAPK/JNK, stress-associated protein kinase/c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin.

investigate the effects of piperine on SASP secretion from senescent cells, we used ELISAs to determine the secretion levels of IL-6, IL-8, and TGF-β1 in cultured senescent cells following piperine treatment. The SASP secretion from S-HDFs was remarkably reduced by piperine treatment (Fig. 4D). We also examined whether piperine affected IL-6 secretion in HeLa cells. As expected, piperine treatment significantly induced IL-6 secretion (Fig. 4E). These findings implied that piperine treatment restored the functions of senescent cells and suggest that piperine is a novel senotherapeutic agent capable of suppressing SASP secretion, which affects surrounding tissues.

4. Discussion

Cellular senescence is not only closely related to the occurrence and promotion of cancer but also affects its treatment and recurrence. Although chemotherapy kills cancer cells, it also provides an environment in which cancer can recur by inducing senescence of the surrounding cells [47,48]. When cyclophosphamide was administered in an animal model with myc-induced lymphoma, cancer cell senescence was induced by $p16^{INK4a}$ and p53. The induction of cancer cell senescence has been proposed as an effective cancer treatment method with low toxicity. However, there are concerns that senescence may induce resistance to potential cancer treatments [49]. Radiation therapy is also a cancer treatment that damages DNA in cancer cells, and the subsequent ROS generation affects cancer cells [49]. Severe DNA damage causes cell death, but slight DNA damage induces cell senescence, and this phenomenon induces radiation resistance [50]. In particular, the generation of senescent cells by radiation therapy is a major cause of recurrence in glioblastoma. Therefore, drugs that directly destroy senescent cells and inhibit SASP can be effective adjuvants for cancer treatment.

Piperine inhibits the proliferation and survival of various cancer cell lines by regulating the cell cycle and activating apoptosis-related signaling within cells [51]. This compound directly modifies functions involved in the activity of various enzymes and transcription factors involved in cancer cell invasion, metastasis, and angiogenesis.

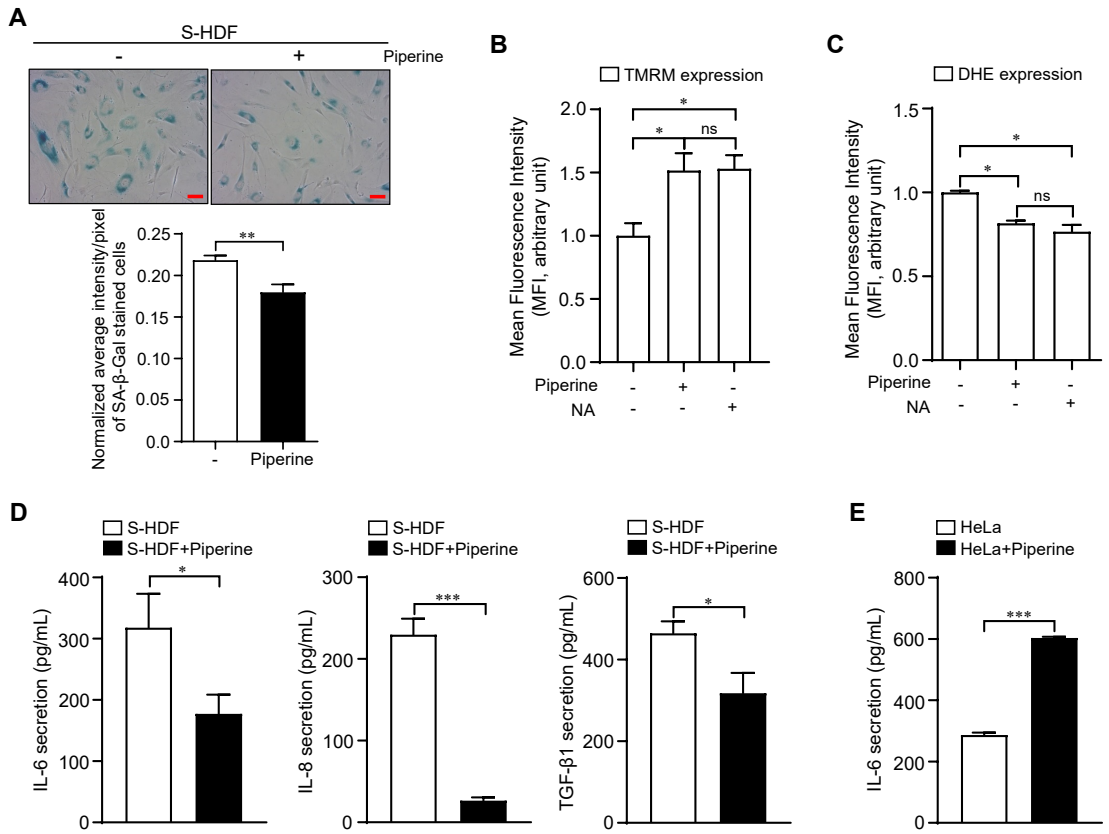

Fig. 4. Rejuvenation effects of piperine on senescent phenotypes. S-HDFs were treated three times with piperine (70 μM) at 2-day intervals. (A) SA-β-gal activity was examined in piperine-treated S-HDFs. (B) MMP function was determined by flow cytometry using the fluorescent dye TMRM. (C) ROS levels were determined by flow cytometry using the fluorescent dye DHE. The positive control was 5 mM NA. S-HDFs (D) and HeLa cells (E) were treated with piperine (70 μM) three times at 2-day intervals. Then, the supernatants were collected and analyzed by ELISA for IL-6, IL-8, and TGF-β1 secretion. Data are based on three independent experiments, and statistical significance between the experimental groups was analyzed using the Mann–Whitney U test. *$p < 0.05$ compared with untreated and NA-treated S-HDF (B) or compared with untreated and piperine-treated cells (D); **$p < 0.01$ compared with untreated with untreated and piperine-treated S-HDF; ***$p < 0.001$ compared with untreated and piperine-treated cells (Fig. 2D,E); ns, not significant compared with piperine- and NA-treated cells (Fig. 2B,C). SA-β-gal, senescent-associated β-galactosidase; ROS, reactive oxygen species; TMRM, tetramethylrhodamine; DHE, dihydroethidium; SASP, senescence-associated secretory phenotype; IL, interleukin; TGF, tumor growth factor.

Similar to previous reports, piperine induced high cytotoxicity in various cancer cell lines was not toxic to normal and senescent cells (Fig. 1). Additionally, piperine stimulated cell division, decreased SA-β-gal activity, recovered MMP, and reduced ROS generation in senescent cells, similar to the effect of NA, which has been reported to restore the function of senescent cells [29] (Figs. 2,4). We found that piperine differently affected cancer cells and senescent cells due to the different responses of intracellular signaling pathways. In senescent cells, piperine promoted Erk1/2 phosphorylation, which is involved in cell growth, whereas in HeLa cells, a cancer cell line, Erk1/2 phosphorylation was decreased and JNK and p38 phosphorylation were increased (Fig. 3). Interestingly, piperine induced different responses in senescent and cancer cells, not only in cell signaling but also in SASP secretion. Piperine increased SASP secretion in cancer cells while significantly decreasing the secretion of three SASP factors (IL-6, IL-8, and TGF-β1) in senescent cells (Fig. 4). Because SASP might contribute to several side effects after treatment with a cancer drug, these results provide piperine as the safe and effective drug for cancer treatment.

From recent studies, the specific removal effects of senolytics on senescent cancer cells have been demonstrated. The specific inhibitor of the BCL-2 family (ABT263) successfully remove a range of senescent cancer cells and *in vivo* study, ABT263 suppresses cancer recurrence and metastasis by eliminating chemotherapy-induced senescent cells [52]. However, dasatinib+quercetin, another senolytic cocktail drug, did not kill senescent hepatocellular carcinoma (HCC) cells and reduce the growth of HCC [53]. Senostatics is also effective cancer therapy by

synergistic effects. Metformin leads reduction of prostate cancer cells cultured with media from metformin-treated senescent cells by suppressing SASP [52].

5. Conclusions

It is difficult to predict the various side effects of cancer treatment substances because most cell models or animal models show the death of cancer cells but do not prove the effect on surrounding normal cells. Piperine showed the effect of inducing cancer cell-specific toxicity that does not affect normal cells, and further showed the effect of restoring the function of senescent cells that may exist around cancer cells. Therefore, we propose piperine as an effective cancer treatment that can simultaneously induce senostatic effects and the removal of cancer cells, not as an adjuvant to the existing senostatics for cancer treatment.

Author Contributions

KAC and WKO designed the research study. JSL, DYL and JHL performed the research. JTP and SCP advised experimental design. KAC and SCP analyzed the data. KAC and JSL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2704137.

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