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Quercetin induces senolysis of doxorubicin-induced senescent fibroblasts by reducing autophagy, preventing their pro-tumour effect on osteosarcoma cells

Elisa Bientinesi, Sara Ristori, Matteo Lulli, Daniela Monti *

Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence 50134, Italy

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ABSTRACT

Cellular senescence contributes to ageing and age-related diseases, and multiple therapeutic strategies are being developed to counteract it. Senolytic drugs are being tested in clinical trials to eliminate senescent cells selectively, but their effects and mechanisms are still unclear. Several studies reveal that the upregulation of senescence-associated secretory phenotype (SASP) factors in senescent cells is accompanied by increased autophagic activity to counteract the endoplasmic reticulum (ER) stress. Our study shows that Doxo-induced senescent fibroblasts yield several SASP factors and exhibit increased autophagy. Interestingly, Quercetin, a bioactive flavonoid, reduces autophagy, increases ER stress, and partially triggers senescent fibroblast death. Given the role of senescent cells in cancer progression, we tested the effect of conditioned media from untreated and quercetin-treated senescent fibroblasts on osteosarcoma cells to determine whether senolytic treatment affected tumour cell behaviour. We report that the partial senescent fibroblast clearance, achieved by quercetin, reduced osteosarcoma cell invasiveness, curbing the pro-tumour effects of senescent cells. The reduction of cell autophagic activity and increased ER stress, an undescribed effect of quercetin, emerges as a new vulnerability of Doxo-induced senescent fibroblasts and may provide a potential therapeutic target for cancer treatment, suggesting novel drug combinations as a promising strategy against the tumour.

1. Introduction

Ageing is a natural and universal feature of most living organisms. In humans, it has become a medical and social priority due to increasing life expectancy and the consequently rising prevalence of age-related disorders (ARDs). Ageing is characterized by degenerative processes resulting from accumulating unrepaired or harmful cellular changes and adaptive mechanisms impairing cell and tissue functions (Ostan et al., 2008). Therefore, it is the foremost critical risk factor for several chronic diseases, including cardiovascular and neurological disorders, diabetes, and cancer (Franceschi et al., 2018). Cellular senescence, a process characterized by permanent cell cycle arrest and the senescence-associated secretory phenotype (SASP), is one of the hallmarks of ageing and contributes to ARD development (López-Otín et al., 2022). The SASP is characterized by a large set of pro-inflammatory cytokines, chemokines, growth factors, and proteases, which affect the complex crosstalk that senescent cells engage with neighbouring cells (Birch and Gil, 2020; Coppé et al., 2010). Hence, the effects of cellular senescence in various physiological and pathological processes may be explained not only by proliferative arrest but also, and even predominantly, by the secretion of such factors and by cell-cell interactions that affect surrounding cells and compartments (Perez-Mancera et al., 2014). As such, cellular senescence has become the target of several therapeutic strategies, of which senolytic drugs are the most promising. The term "senolytic", coined by Zhu et al. in 2015 (Zhu et al., 2015), indicates the ability of a substance to target and kill senescent cells selectively, thereby restoring tissue function and reducing the adverse effects of senescence. Despite the limited number of available senolytic therapies, trials of several preclinical disease models have provided important results. Effects included enhanced healthspan and lifespan (Xu et al., 2018), reduced frailty and bone loss (Zhu et al., 2015), improved lung (Schafer et al., 2017) and kidney (Baar et al., 2017) function, amelioration of osteoarthritis (Jeon et al., 2017) and hepatic steatosis (Ogrodnik et al., 2017), and reduced stem cell dysfunction (Chang et al., 2016).

However, knowledge of the mechanisms underlying the actions of

* Correspondence to: Viale G.B. Morgagni, 50, Firenze 50134, Italy.
E-mail addresses: elisa.bientinesi@unifi.it (E. Bientinesi), sara.ristori@unifi.it (S. Ristori), matteo.lulli@unifi.it (M. Lulli), daniela.monti@unifi.it (D. Monti).

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senolytics is limited. It is, therefore, essential to gain further insight into the varied features of senolysis, to ensure that senolytic drugs are employed correctly, and, if possible, to discover new ones (López-Otín et al., 2022). In this framework, we studied the senolytic activity of quercetin on doxorubicin (Doxo)-induced senescent WI-38 fibroblasts and identified a previously undescribed mechanism of action that resulted in senescent cell clearance. Quercetin is a bioflavonoid and one of the earliest senolytic drugs studied, but its efficacy is frequently cell-type-specific. Here, we show for the first time that quercetin cleared a fraction of Doxo-induced senescent fibroblasts by selectively reducing autophagic activity, thereby exposing senescent cells to endoplasmic reticulum (ER) stress caused by SASP upregulation. Moreover, given the role of senescent cells in aiding cancer progression (Bientinesi et al., 2022; Schmitt et al., 2022), we tested the effect of conditioned media (CM) from untreated and quercetin-treated senescent fibroblasts on osteosarcoma cells to determine whether senolytic treatment affected tumour cell behaviour. We report that the partial clearance of senescent fibroblasts, achieved by quercetin, effectively reduced osteosarcoma cell invasiveness, thereby curbing the pro-tumour effects of senescent cells.

2. Materials and method

2.1. Reagents

Doxorubicin (#5927 S; Cell Signaling Technology, Beverly, MA, USA) was dissolved in dimethyl sulfoxide (DMSO; D8418, Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 10 mM and stocked at −20°C until use. Quercetin powder (purity ≥ 95 %, Q4951; Sigma-Aldrich, St. Louis, MO, USA) was resuspended in DMSO to a concentration of 30 mg mL^{-1} (99 mM). PP242 hydrate (purity ≥ 98 %, P0037; Sigma-Aldrich, St. Louis, MO, USA) powder was dissolved in DMSO to a concentration of 16.2 mM and stocked at −20°C until use. Sodium phenylbutyrate (4-PBA; purity ≥ 98 %, SML0309; Sigma-Aldrich, St. Louis, MO, USA) powder was dissolved in complete medium to a concentration of 5 mM and then diluted to a working concentration. 5-Bromo-4-chloro-3-indolyl B-D-*galactop B4252 was purchased from Sigma-Aldrich, St. Louis, MO, USA. Fluo-4 AM dye (ThermoFisher Scientific, Waltham, MA, USA) was dissolved in DMSO to a concentration of 10 μg μL^{-1} and diluted in DMEM High glucose (without Ca^{2+}) to 0.005 μg μL^{-1} for a working solution.

2.2. Cell lines and culture conditions

Human foetal lung fibroblasts WI-38 (AG06814-N, Coriell Institute for Medical Research, Camden, NJ, USA) and the human osteosarcoma cell line U2OS (pRB +/+, p53+/+) (HTB-93, ATCC, Manassas, VA, USA) were grown in complete medium (DMEM High glucose supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B, and 2 mM L-glutamine) at 37°C in a 5 % CO_2 humidified incubator. We used cells with a population doubling level <30 to avoid replicative senescence.

2.3. Induction of senescence and quercetin treatment

Briefly, to induce senescence, 24 h after seeding, WI-38 cells were treated with Doxo 50 nM for 48 h and then cultured in fresh complete medium for three days. The control was represented by cells incubated for 48 h in complete medium with 0.0005 % of DMSO (used as a diluent for Doxo) and then maintained in fresh complete medium for three days. The appearance of the senescence phenotype was confirmed by analysing several senescence markers. At this time, Quercetin 40 μM was added to the cultures, which were analysed at different time points up to three days. Control samples, proliferating and senescent cells, were treated with fresh complete medium and DMSO (used as a diluent for quercetin). To verify the autophagy hypothesis, a mammalian target of rapamycin (mTOR) inhibitor, PP242 (2.5 μM), and an inhibitor of endoplasmic reticulum stress, 4-PBA (1 mM), were separately added in combination with quercetin for indicated times.

2.4. Preparation of conditioned medium and treatment of U2OS cells

After the senolytic treatment (three days), WI-38 cells were cultured for four days with fresh complete medium. The CM was then collected, centrifuged, and used to treat U2OS cells.

2.5. Cell proliferation

Cell growth and vitality were determined with the trypan blue exclusion assay. Briefly, WI-38 and U2OS cells were trypsinised and stained with trypan blue. Then, live and dead cells were counted under a light microscope using a Bürker chamber.

2.6. Senescence-associated β-galactosidase staining

Staining for senescence-associated β-galactosidase (SA-β-Gal) was performed as described previously (Dimri et al., 1995). WI-38 cells were plated 8×10^3 cells/cm^2 and assessed for SA-β-Gal activity three days after the treatment with quercetin alone or combined with PP242 and 4-PBA, separately. At least 300 cells *per* group were counted. Positive (blue) cells were expressed as a percentage of the total cell number.

2.7. Flow-cytometric analysis of the cell cycle

Cell cycle analysis was performed three days after quercetin treatment with propidium iodide (PI) staining. Briefly, WI-38 cells were trypsinised, counted, and aliquoted in FACS tubes. They were washed in phosphate-buffered saline (PBS) 1X and then incubated in a hypotonic solution of PI (0.1 % w/v sodium citrate tribasic, 0.1 % Triton X-100, and 50 μg mL^{-1} PI) at 4°C for 30 min. The cell cycle was analysed using FACS CANTO II and BD FACSDIVA software (Becton, Dickinson & Company, Franklin Lakes, NJ, USA). The data were analysed using FlowJo Software (Becton, Dickinson & Company, Franklin Lakes, NJ, USA).

2.8. Western blotting

The whole-cell lysate was obtained using Laemmli buffer, whereas the nuclear-cytoplasmic fractions were obtained using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's instruction. Protein concentration was determined using the BCA Protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). 50 μg of protein *per* sample were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a nitrocellulose membrane (Amersham Protran, GE Healthcare Life Science) by electroblotting. After blocking with bovine serum albumin (BSA) 5 %, the membrane was incubated with primary antibody against phospho(Ser807/811)-RB (#8516), SQSTM1/p62 (#5114), beclin-1 (#3738), BcL-XL (#2764), NF-κB p65 (#4764), all from Cell Signaling Technology, Beverly, MA, USA; lamin B1 (ab16048, Abcam, Cambridge, UK), p21 (sc-817), p16 (sc-56330), fibrillarin (sc-166001), α-tubulin (sc-32293), all from Santa Cruz Biotechnology; LC3B (NBP100–2220 Novus Biologicals, Centennial, CO, USA), at 4°C overnight. Washed membranes were incubated with IRDye800CW–conjugated or IRDye680–conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) for one hour at room temperature. Antibody-coated bands were visualized by Odyssey Infrared Imaging System Densitometry (LI-COR Biosciences, Lincoln, NE, USA). Protein band intensity (p-RB, SQSTM1/p62, beclin-1, BcL-XL, lamin B1, p21, p16) was normalized to α-tubulin and expressed in relation to control cells. NF-κB p65 band intensity in the cytosolic and nuclear fraction was normalized to α-tubulin and fibrillarin,

respectively, and expressed as nuclear/cytosolic ratio.

2.9. Immunostaining

WI-38 cells were plated on sterile coverslips and placed in 12-well plates, and the treatments were performed as described above. At the end of the experiments, cells were fixed in ice-cold methanol 100 % at −20°C for 15 min, or paraformaldehyde 4 % for 10 min at room temperature, permeabilized with 0.2 % Triton X-100 for 5 min, quenched with sodium borohydride, washed, and blocked for one hour with 1 % BSA 10 % horse serum at room temperature. Slides were incubated overnight at 4°C with primary antibodies in BSA 1 % (lamin B1, #68591 and SQSTM1/p62, #5114 from Cell Signaling Technology, Beverly, MA, USA; Histone Macro H2A.1, ABE215, Merck Millipore; LC3B, NBP100–2220; Novus Biologicals, Novus Biologicals, Centennial, CO, USA). Slides were washed three times and incubated at room temperature for 40 min with secondary antibodies conjugated with Alexa Fluor 488 or Cy3 (goat anti-rabbit and goat anti-mouse, respectively, IgG (H+L) Cross-Adsorbed Secondary Antibody, ThermoFisher Scientific, Waltham, MA, USA). Nuclear DNA was stained with ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher Scientific, Waltham, MA, USA). Slides were then visualized using a Leica SP8 Confocal Microscope (Leica Microsystems, Wetzlar, Germany). ImageJ software (National Institutes of Health, Bethesda, MA, USA) was used for immunofluorescence analysis. In particular, we used JACoP plugin to calculate Pearson's correlation coefficient as the colocalization quantitative index (+1 for perfect correlation, 0 for no correlation, and −1 for perfect anticorrelation).

2.10. Apoptosis evaluation by cytofluorimetric analysis

Cell death was evaluated using Annexin V Binding Buffer 10X (#556454), BV421 Annexin V (#563973), and 7AAD (#559925; all from Becton, Dickinson and & Company, Franklin Lakes, NJ, USA). Briefly, detached cells were incubated in FACS tubes with a working solution (Binding Buffer 1X, BV421 Annexin V, and 7-AAD) for 15 min at room temperature in the dark. Subsequently, cells were analysed with FACS CANTO II and BD FACSDIVA software and data were evaluated using FlowJo Software (Becton, Dickinson and & Company, Franklin Lakes, NJ, USA). Total dead fibroblasts percentage was obtained by adding the rates of cells in early apoptosis (7-AAD$^-$/annexin V$^+$), late apoptosis (7-AAD$^+$/annexin V$^+$), and necrosis (7-AAD$^+$/annexin V$^-$).

2.11. DNA extraction and DNA ladder assay

The DNA ladder assay was used to evaluate the senolytic activity of quercetin. Apoptosis can be visualised as a 180–200 bp ladder pattern in gel electrophoresis due to double-stranded DNA cleavage by nuclear endonuclease activation. After four-hour quercetin treatment, we collected the whole culture fibroblast media, including the floating apoptotic cells, centrifuged them, and added them to the pellet of detached cells. DNA extraction was performed as follows. First, cell pellets were incubated with extraction buffer (proteinase K 0.5 mg/mL, EDTA 0.01 мpH 8, Tris 0.05 мpH 8, Triton X-100 0.5 %) for 45 min at 50°C, then with DNAse free RNAse solution (RNase Cocktail™ #2288 Ambion, Tris 1 мpH 7.6, NaCl 3 м) at 50°C for the next 45 min. Finally, after adding loading buffer (EDTA 10 mм pH8, bromophenol blue 0.25 %, sucrose 40 % w/v), samples were loaded in 2 % agarose gel stained with Eurosafe – Fluorescent Nucleic Acid Stain (Euroclone) and visualized with a UVITEC transilluminator (Cambridge).

2.12. Intracellular calcium evaluation

WI-38 cells were plated in 12-well plates, and the senescence state was induced as described above. They were treated with quercetin, alone or in combination with 4-PBA, for four hours and then labelled with Fluo-4 AM probe (ThermoFisher Scientific, Waltham, MA, USA) in DMEM without Ca^{2+} (Gibco, #21068028, ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 37°C in the dark. Subsequently, cells were detached with trypsin-EDTA, washed two times with PBS (without Ca^{2+}), and resuspended in DMEM without Ca^{2+}. The analysis was performed with FACS CANTO II and BD FACSDIVA software, and data were evaluated using FlowJo software (Becton, Dickinson and & Company, Franklin Lakes, NJ, USA).

2.13. RNA extraction and quantitative real-time PCR

Following the manufacturer's instruction, total RNA extraction was performed three days after quercetin treatment using Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada). cDNA synthesis was performed using ImProm-IITM Reverse Transcription System, and quantitative real-time PCR (qPCR) was performed using GoTaq® qPCR Master Mix (all from Promega Corporation, Madison, WI, USA). Primer sequences are reported in Table 1. qRT-PCR analysis was performed in triplicate using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). mRNA was quantified with the ΔΔCt method, and mRNA levels were normalized to GAPDH as the endogenous control. To study XBP1 expression, RNA extraction was carried out after four-hour quercetin treatment. cDNA synthesis and qRT-PCR were performed as described above. PCR products were loaded on 3.5 % agarose gel stained with Eurosafe – Fluorescent Nucleic Acid Stain and visualized with the UVITEC transilluminator. The relative spliced (sXBP1)/unspliced (uXBP1) transcription level was calculated normalizing the expression of the two forms to GAPDH.

2.14. Wound healing assay

The wound healing assay was performed to evaluate U2OS cell migration under the effect of CM from proliferating and senescent fibroblasts treated or not with quercetin. U2OS cells were plated on 24-well plates 24 h before treatment. A vertical line was scratched in the centre of the adherent cell monolayers with a sterile 200 μl micropipette tip. Then, cells were washed with PBS 1X and incubated with CM. Wound closure was monitored at 6, 24, and 48 h. The scratch closure area was measured using ImageJ software.

2.15. Invasion assay

U2OS cell invasiveness was evaluated using a Boyden chamber assay. The method is based on the passage of cells across 8μm pore size polyvinyl pyrrolidone-free polycarbonate filters (Neuro Probe, Inc., USA) precoated with matrigel (50 μg/filter), placed between the two wells of the Boyden chamber. CM from proliferating and senescent fibroblasts treated or not- with quercetin were placed in the bottom wells of the chamber. U2OS cells resuspended in complete DMEM High glucose supplemented with 2 % FBS were seeded in the upper chamber (2×10^4 cells/well) and incubated overnight. The filters were removed and fixed in methanol for one hour. Non-migrated cells attached to the upper surface of the filter were scraped, whereas migrated cells adhering to the lower filter surface were stained with crystal violet 0.01 % and counted under a light microscope (40x magnification). Each sample was analysed in triplicate. The mean values of migrated cells *per* sample were calculated and expressed relative to the control.

2.16. Colony formation assay

To evaluate colony formation, 2×10^3 U2OS cells were seeded in a 60 mm cell culture dish in complete medium and three days later, treated with different CM for seven days. Then, cells were washed with PBS 1X and stained with 0.1 % Crystal violet for 10 min. After washing twice with water, the dishes were left to dry overnight. The next day, a picture of the whole plates was taken, and the number and area of

Table 1
Primer sequences used for qRT-PCR evaluation of gene expression.

Gene	Forward primer	Reverse primer
IL-6	5'-AGACAGCCACTCACCTCTTCAG-3′	5'-TTCTGCCAGTGCCTCTTTGCTG-3′
IL-8	5'-GAGAGTGATTGAGAGTGGACCAC-3'	5'-CACAACCCTCTGCACCCAGTTT-3′
TGFβ1	5'-TACCTGAACCCGTGTTGCTCTC-3′	5'-GTTGCTGAGGTATCGCCAGGAA-3′
IL-1α	5'-TGTATGTGACTGCCCAAGATGAAG-3′	5'-AGAGGAGGTTGGTCTCACTACC-3′
MMP2	5'-AGCGAGTGGATGCCGCCTTTAA-3′	5'-CATTCCAGGCATCTGCGATGAG-3′
MMP3	5'-CACTCACAGACCTGACTCGGTT −3′	5'-AAGCAGGATCACAGTTGGCTGG-3′
CXCL12	5'-CTCAACACTCCAAACTGTGCCC-3′	5'-CTCCAGGTACTCCTGAATCCAC-3′
VEGF	5'-TTGCCTTGCTGCTCTACCTCCA-3′	5'-GATGGCAGTAGCTGCGCTGATA-3′
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3′	5'-ACCACCCTGTTGCTGTAGCCAA-3′
XBP1	5'-GGAACAGCAAGTGGTAGA-3′	5'- CTGGAGGGGTGACAAC-3′

colonies were evaluated by ImageJ software.

2.17. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± Standard Deviation (SD) of at least three independent experiments. Data were analysed using unpaired t-test to compare differences between two groups and one-way analysis of variance (ANOVA) with Turkey's post hoc to perform multiple comparison tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Quercetin exerted a senolytic effect on Doxo-induced senescent fibroblasts

WI-38 fibroblasts were treated with Doxo (50 nM) for two days to induce senescence and then incubated in fresh medium for three days (Bientinesi et al., 2022). After senescence induction, we treated proliferating (cycling) and senescent cells with quercetin 40 μM for up to three days (Fig. 1**A**). Finally, we evaluated cell viability and proliferation, cell cycle arrest, and the presence of the senescence markers. As shown in Fig. 1**B,** quercetin did not affect the growth or viability of proliferating cells, whereas it significantly reduced senescent cells by about 30 % (range, $p<0.05$ - $p<0.001$). The reduction of SA-β-Gal activity, a widely used senescence marker (Fig. 1**C**), the upregulation of pRB and lamin B1 protein expression, and the reduction of p21 and p16 levels in treated compared to untreated senescent fibroblasts (Fig. 1**D**) confirmed that quercetin affected only the senescent cell population.

In turn, cell cycle analysis showed that quercetin treatment reduced both the percentage of cells blocked in the G2/M phase, *i.e.* senescent cells (Figure S1A), and of those simultaneously negative for lamin B1 (usually absent in senescent cells) and positive for macroH2A.1, a major component of senescence-associated heterochromatin foci (SAHF) (Figure S1B). The SASP is another critical feature of senescent cells involved in ageing and ARD development. Since NF-κB is one of the main transcription factors involved in SASP modulation, we investigated whether quercetin treatment also affected NF-κB activation and the expression of SASP factors. As shown in Fig. 2**A,** NF-κB p65 nuclear translocation increased significantly in senescent fibroblasts ($p<0.05$), whereas the senolytic effect of quercetin induced a significant reduction in its nuclear expression ($p<0.05$). The expression of crucial SASP factors such as IL-6, IL-8, MMP3, MMP2, VEGF, CXCL12, IL-1α, and TGFβ1 was also significantly enhanced in untreated senescent fibroblasts and significantly reduced in those treated with quercetin (both $p<0.05$) (Fig. 2**B**).

3.2. Quercetin, by reducing autophagy, increased endoplasmic reticulum stress and induced senescent cell death

The production of SASP factors in senescent cells increases the consumption of the translational machinery and may result in ER stress (Kwon et al., 2017). Autophagy is related to senescence and seems to contribute to the SASP by supplying amino acids for rapid protein turnover (Herranz and Gil, 2018). The upregulation of SASP factors in senescent fibroblasts suggested to us that the senolytic effects of quercetin might also be mediated by autophagy inhibition exposing senescent cells with a high SASP profile to ER stress, ultimately resulting in their death. As shown in Fig. 3**A**, heightened beclin-1 expression and LC3 I to II conversion and decreased p62 levels suggested an increased autophagic activity in senescent cells. Conversely, treatment with quercetin enhanced p62 expression and reduced beclin-1 and LC3 II levels, reflecting a reduction of autophagic activity. Concurrently, confocal microscopy (Fig. 3**B**) documented a significantly greater amount of LC3 fluorescent puncta and significantly greater p62/LC3 colocalization, reflecting autophagosome formation, in untreated compared to quercetin-treated senescent cells ($p<0.05$; Pearson's correlation analysis). Altogether, these data indicate that quercetin can reduce autophagy markers and vacuole formation in senescent cells.

Furthermore, quercetin treatment induced ER stress due to the reduced autophagy activity, as evidenced by the heightened rate of XBP1 mRNA splicing, indicative of constitutive unfolded protein response (UPR) (Fig. 4**A**). Notably, quercetin treatment also downregulated the expression of BcL-XL, a critical anti-apoptotic protein, which was increased in untreated senescent cells (Fig. 4**A**). The adverse effect of quercetin on senescent cells was confirmed by the measurement of intracellular calcium (Ca^{2+}) release, another possible consequence of ER stress and cell death. As shown in Fig. 4**B**, intracellular Ca^{2+} was significantly higher in senescent cells treated with quercetin than in untreated senescent cells and proliferating cells (both $p<0.001$). At the same time point, the cytofluorimetric analysis highlighted a significantly higher percentage of dead cells in the senescent treated than the untreated cell population ($p<0.001$) (Fig. 4**C**). Lastly, the DNA ladder assay confirmed that quercetin induced apoptosis and selectively cleared senescent cells (Fig. 4**D**).

To establish the crucial role of autophagy reduction in quercetin senolysis, we employed a mammalian target of rapamycin (mTOR) inhibitor - PP242 - to trigger autophagy in proliferating and senescent cells. Autophagy was similarly enhanced by PP242 in both proliferating and senescent cells, as determined by the augment of LC3 II, beclin-1, and the decrease of p62 (Figure S2A). Interestingly, the combined treatment of quercetin and PP242 eliminated ER stress (Fig. 5**A**), as determined by the lack of XBP1 spliced form in the senescent treated cells, compared to those treated only with quercetin ($p<0.05$), highlighted the importance of autophagy as a mechanism to counteract ER stress in senescent cells. Indeed, the combination of PP242 and quercetin significantly increased the number of live senescent cells and decreased the rate of apoptosis (Fig. 5**B** and **C**), restoring the amount of SA-β-Gal positive cells (Fig. 5**D**), compared to cells treated with quercetin alone ($p<0.05$), eliminating its senolytic effect. These results proved that the reduction of autophagy by quercetin in senescent cells was necessary to induce senolysis.

Finally, we treated proliferating and senescent cells with 4-PBA to

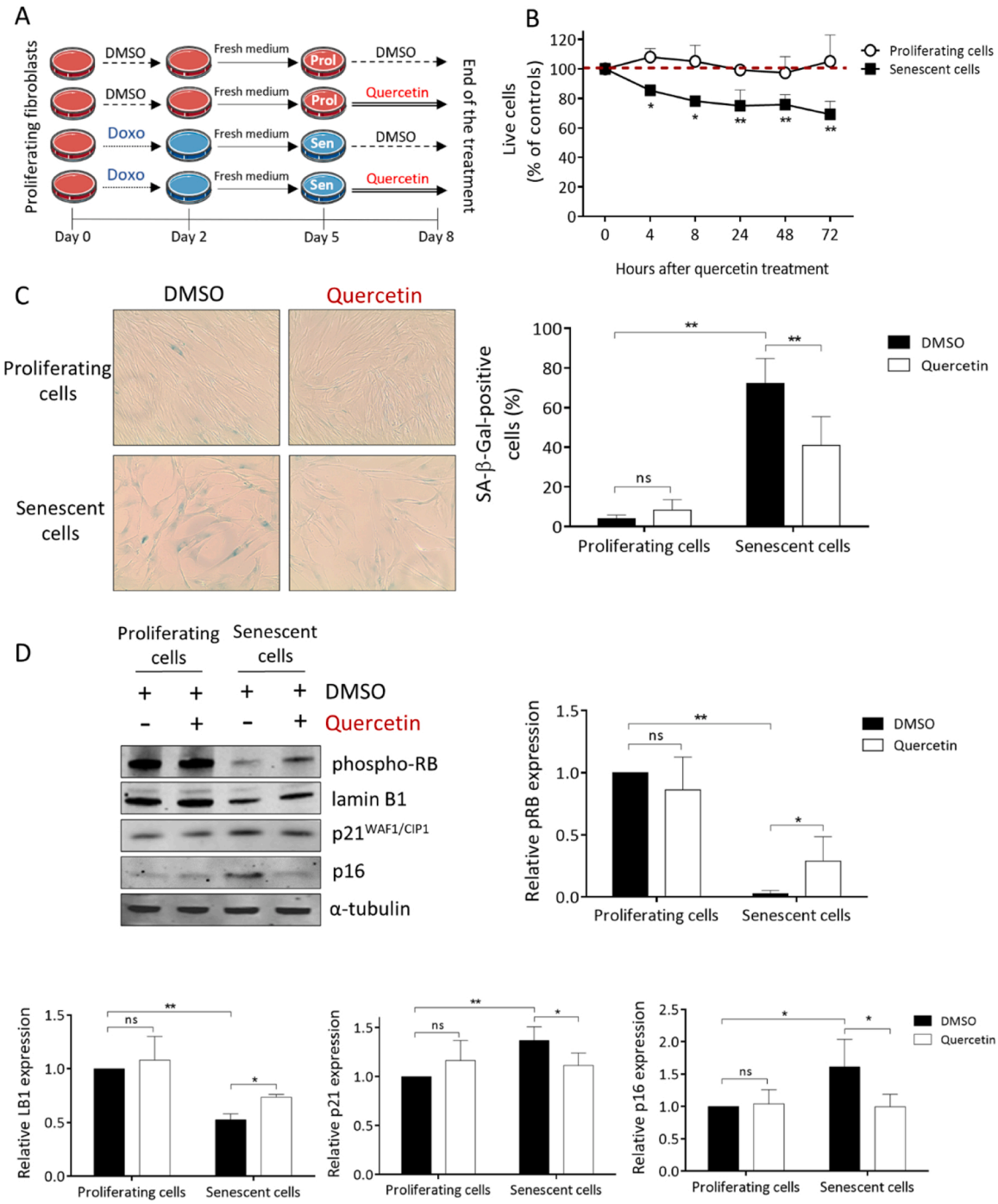

Fig. 1. Quercetin reduces the number of doxorubicin-induced senescent fibroblasts. (A) Experimental scheme. Senescence was induced by Doxo treatment for two days, followed by three days in fresh medium. Then, senescent and proliferating fibroblasts were treated with quercetin for three days. **(B)** The trypan blue exclusion assay was performed at five-time points during treatment. Live cell number was expressed as percentage of untreated proliferating and senescent cells, respectively, assumed as 100 % (red dotted line). Data are mean ± SD of at least four independent experiments. **(C)** Representative images of senescence-associated β-galactosidase (SA-β-Gal) activity (left), measured after three days of quercetin treatment. The percentage of SA-β-Gal-positive cells (blue) out of the total number of cells/well (right) was evaluated in at least four independent experiments. The results are reported as mean ± SD. **(D)** Representative immunoblot and quantification of the expression (right and bottom) of phospho-RB, lamin B1, p21, and p16. Lysates were collected after three-day quercetin treatment. Protein band intensity was normalized to α-tubulin and to control cells (Proliferating cells-DMSO). Data are mean ± SD of at least four independent experiments. *p<0.05 and **p<0.001.

alleviate ER stress and investigate its involvement in senescent cell death. The inhibition of ER stress, confirmed by the absence of XBP1 spliced form (Figure S2B), eliminated the increase of intracellular Ca^{2+}, caused by quercetin, resulting in increased live senescent cells and a reduced apoptosis rate (Fig. 6**A, B and C**). Moreover, the number of SA-β-Gal positive cells after the combined treatment of quercetin and 4-PBA did not differ from the untreated senescent cells (Fig. 6**D**). Quercetin's senolytic effect is achieved by reducing autophagy, which exposes senescent fibroblasts to ER stress.

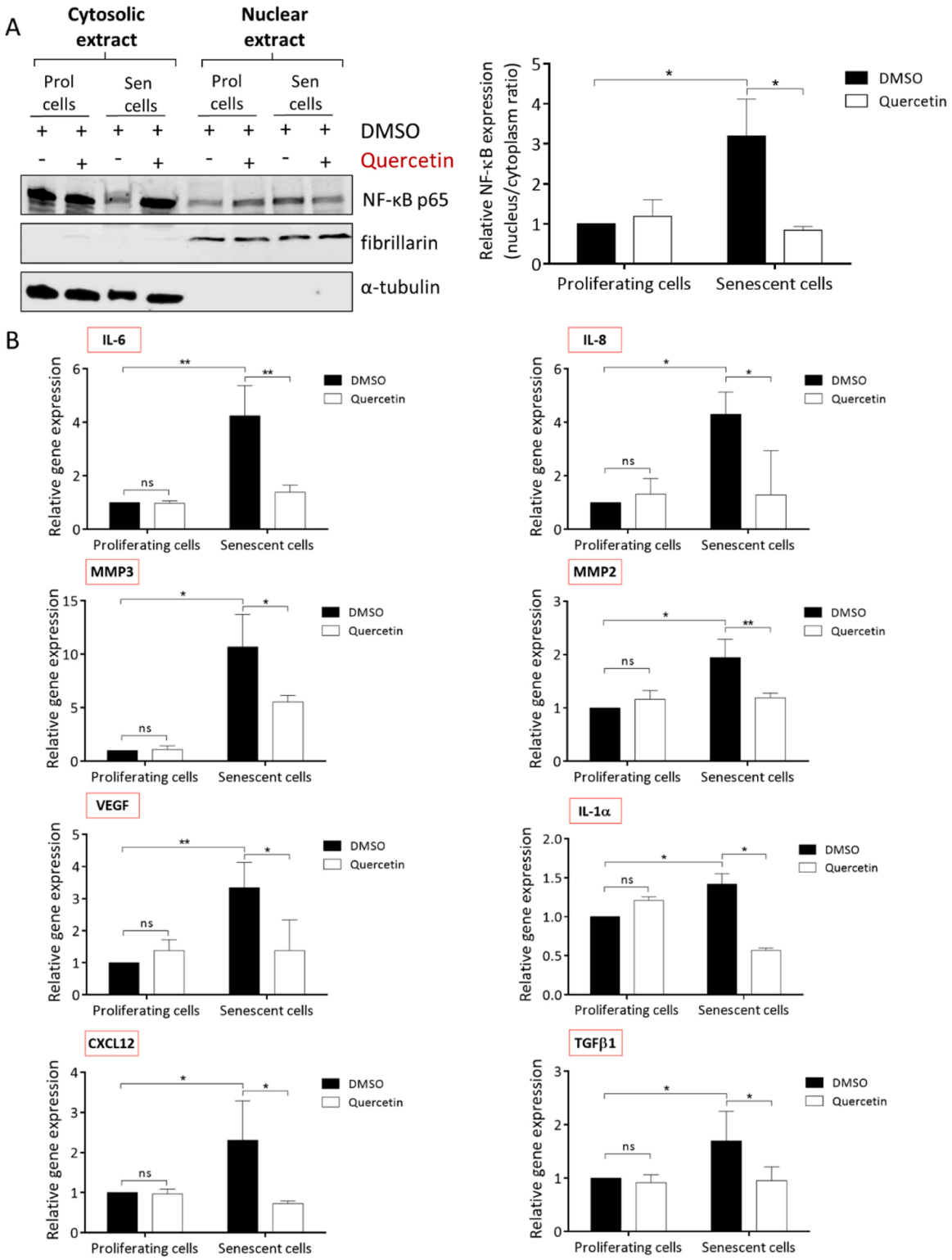

Fig. 2.. Quercetin reduces NF-κB nuclear translocation and senescence-associated secretory phenotype (SASP) factors gene expression. (A) Representatives immunoblot (left) and quantification of NF-κB p65 expression. Protein band intensity in the cytosolic and nuclear fraction was normalized to α-tubulin and fibrillarin, respectively, and expressed as nuclear/cytosolic ratio in relation to control cells (Prol cells-DMSO). Data are mean ± SD of three independent experiments. **(B)** Gene expression was analyzed after three days of quercetin treatment. GAPDH was the housekeeping gene, and data were normalized to proliferating cells treated with DMSO (assumed as value 1). Data are mean ± SD of triplicate experiments. *p<0.05 and **p<0.001. *Prol cells*: Proliferating cells; *Sen cells*: Senescent cells.

3.3. Quercetin-treated senescent cell conditioned medium reduced osteosarcoma cell growth and invasiveness

After establishing that quercetin cleared about 30 % of senescent cells, we investigated whether the reduction was sufficient to curb some of the adverse effects of senescent fibroblasts and their ability to promote tumour cell growth and invasiveness. To do this, we treated U2OS cells, an osteosarcoma cell line, with CM from proliferating and

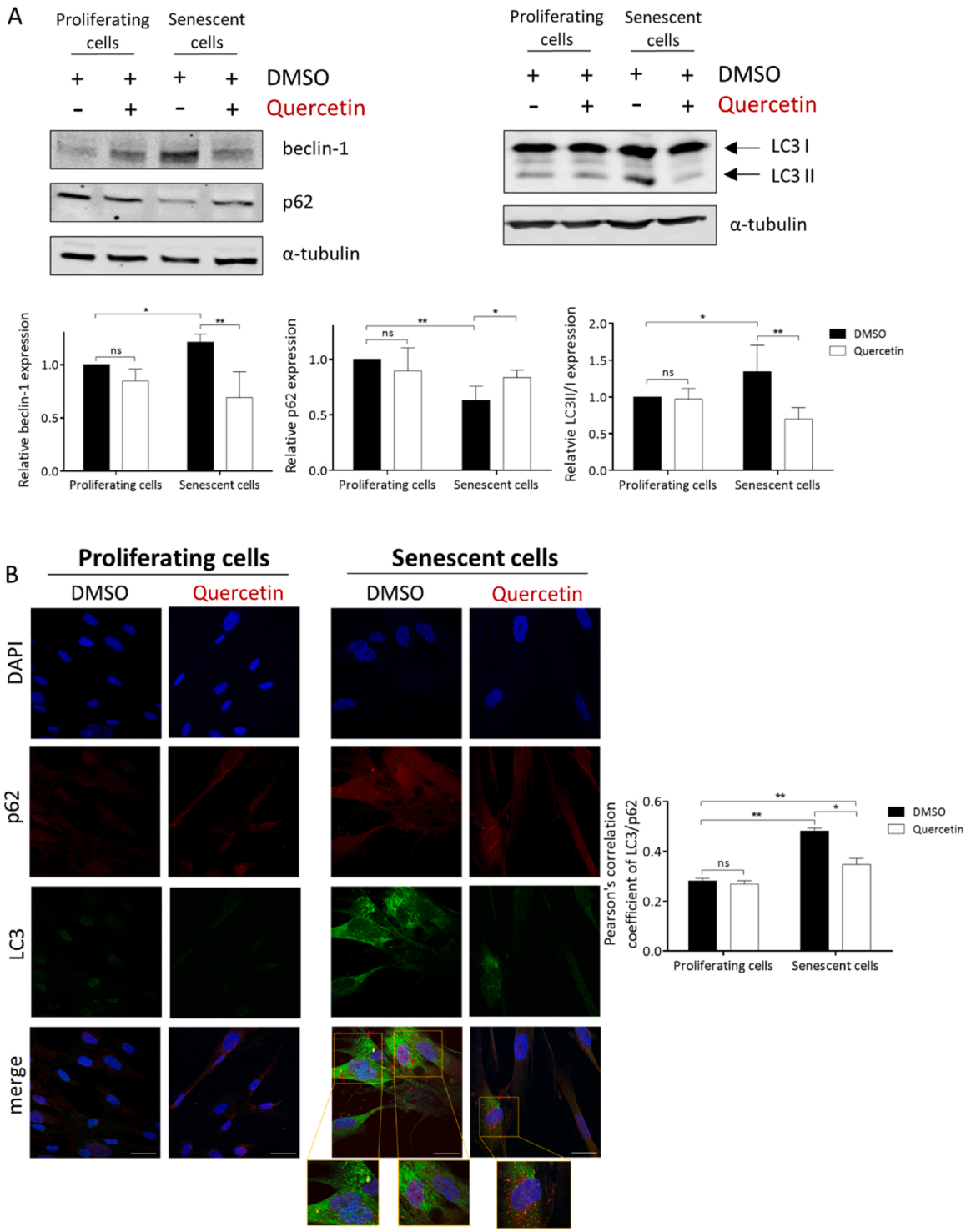

Fig. 3. Quercetin reduces autophagy in Doxo-induced senescent fibroblasts. (A) Representatives immunoblot and quantification of the expression (bottom) of beclin-1, p62/SQSTM1, and LC3 I/II. Lysates were collected after four-hour quercetin treatment. Protein band intensity was normalized to α-tubulin and to control (Proliferating cells-DMSO). Data are mean ± SD of three independent experiments. **(B)** Confocal microscopy images (left) of indirect immunofluorescence of LC3 (green) and p62/SQSTM1 (red) were performed four-hour after quercetin treatment. Red arrows indicate colocalization of LC3/p62 puncta. DNA was counterstained with DAPI (blue). Scale bars: 25 μm. Colocalization was analyzed with Pearson's correlation coefficient (right). Data are mean ± SD of three independent experiments. *$p<0.05$ and **$p<0.001$.

senescent fibroblasts, obtained as illustrated in Fig. 7**A**, and tested them for growth and invasion ability changes. As shown in Fig. 7**B-D**, CM from senescent fibroblasts (Sen-CM) significantly enhanced cell proliferation ($p<0.05$) as well as colony numbers and area compared to CM from proliferating fibroblasts treated (Prol-Q-CM) or not treated (Prol-CM) with quercetin (both $p<0.001$). In contrast, CM from quercetin-treated senescent cells (Sen-Q-CM) did not affect U2OS cell growth.

Moreover, osteosarcoma cell invasion and migration ability were enhanced by Sen-CM ($p<0.05$) and unaffected by Sen-Q-CM and Prol-CM (Fig. 8).

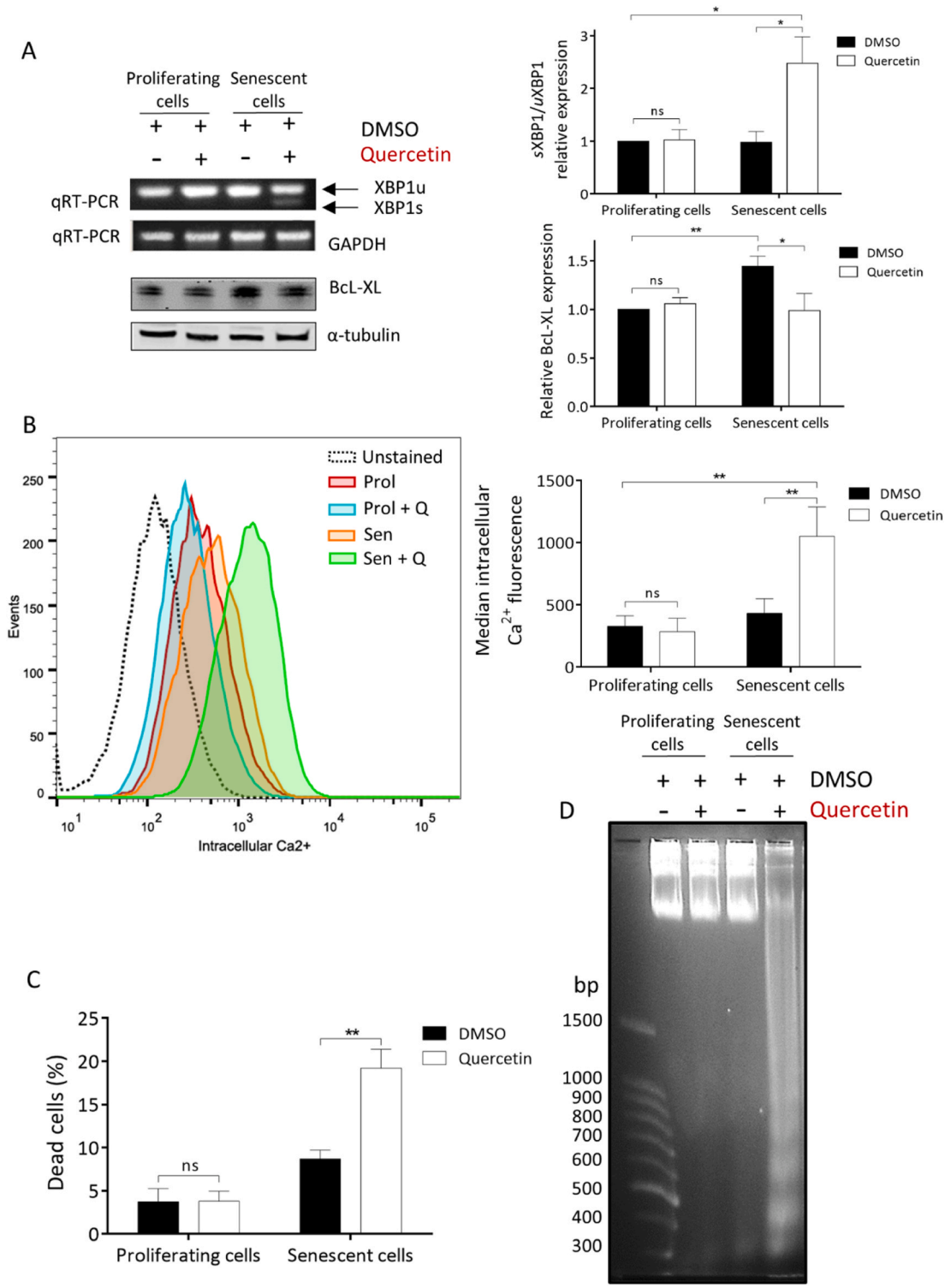

Fig. 4. Quercetin selectively induced endoplasmic reticulum (ER)-stress-mediated cell death in senescent fibroblasts. (A) qRT-PCR analysis of XBP1 transcription factor expression. The products of PCR amplification were loaded on 3.5 % agarose gel to separate unspliced (u) from the spliced (s) XBP1 forms; note that the lower band (XBP1s) indicates UPR activation related to ER stress. The relative sXBP1/uXBP1 transcription level (on the right) was calculated normalizing the expression of the two forms to GAPDH, showed below. BcL-XL expression was analysed by immunoblotting (quantification on the right). Data are mean ± SD of three independent experiments. **(B)** Cytofluorimetric analysis of Ca^{2+} with Fluo-4 AM labeling in quercetin-treated proliferating fibroblasts (Prol + Q) and senescent fibroblasts treated (Sen + Q) and not treated (Sen) with quercetin. Data from proliferating cells stained with the dye (Prol) are shown as control. Ca^{2+} levels, expressed as median fluorescence intensity in the bar graph to the right, are mean ± SD of three independent experiments. **(C)** Comparative quantification of the percentage of total dead fibroblasts (7-AAD^{+}/annexin V^{+}, 7-AAD^{-}/annexin V^{+}, and 7-AAD^{+}/annexin V^{-}) in proliferating and senescent cells treated for four hours with quercetin or not treated, obtained by cytofluorimetric analysis after labelling cells with annexin V- BV421 and 7-AAD. Data are mean ± SD of three independent experiments. **(D)** Effect of quercetin treatment on DNA fragmentation. After four-hour quercetin treatment, DNA was isolated and loaded on 2 % agarose gel stained with Eurosafe–Fluorescent Nucleic Acid Stain (Euroclone) and visualized under UV light. DNA fragmentation typical of apoptotic cells was only detected in quercetin-treated senescent fibroblasts. *$p<0.05$ and **$p<0.001$.

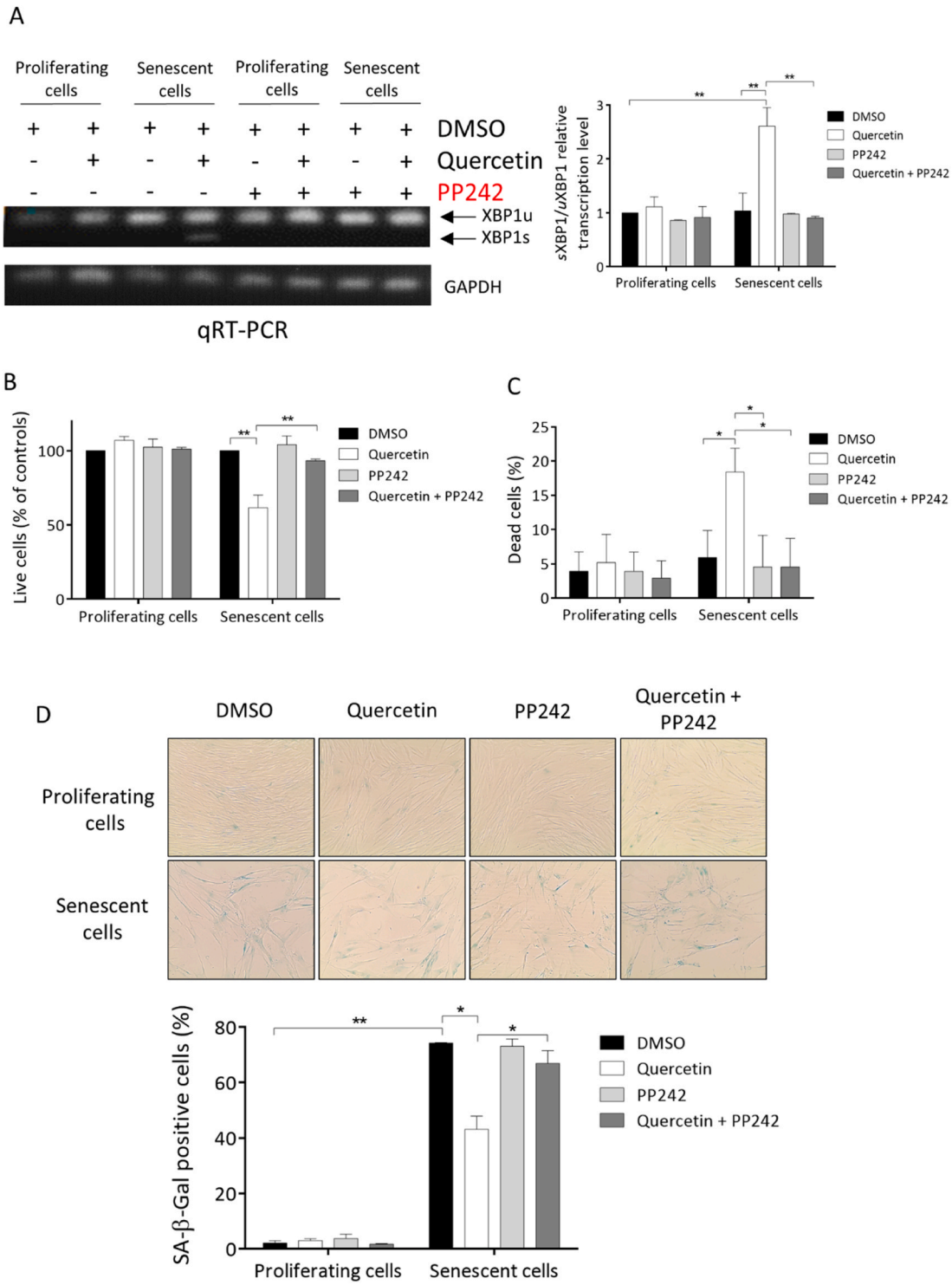

Fig. 5. The increase of autophagy mediated by PP242 in quercetin-treated senescent cells eliminated ER stress and quercetin senolytic effect. (A) qRT-PCR analysis of XBP1 transcription factor expression. The products of PCR amplification were loaded on 3.5 % agarose gel to separate unspliced (u) from the spliced (s) XBP1 forms; note that the lower band (XBP1s) indicates UPR activation related to ER stress. The relative sXBP1/uXBP1 transcription level (on the right) was calculated normalizing the expression of the two forms to GAPDH, showed below. **(B)** The trypan blue exclusion assay was performed 3 days after quercetin and PP242 treatment. Live cell number was expressed as percentage of untreated proliferating and senescent cells, respectively, assumed as 100 %. Data are mean ± SD of at least three independent experiments. **(C)** Comparative quantification of the percentage of total dead fibroblasts (7-AAD$^+$/annexin V$^+$, 7-AAD$^-$/annexin V$^+$, and 7-AAD$^+$/annexin V$^-$) in proliferating and senescent cells treated for four hours with quercetin, PP242, or the combination of two, obtained by cytofluorimetric analysis after labelling cells with annexin V- BV421 and 7-AAD. Data are mean ± SD of three independent experiments. **(D)** Representative images of senescence-associated β-galactosidase (SA-β-Gal) activity (below), measured after three days of quercetin and PP242 treatment. The percentage of SA-β-Gal-positive cells (blue) out of the total number of cells/well (below) was evaluated in at least three independent experiments. The results are reported as mean ± SD. *$p<0.05$ and **$p<0.001$.

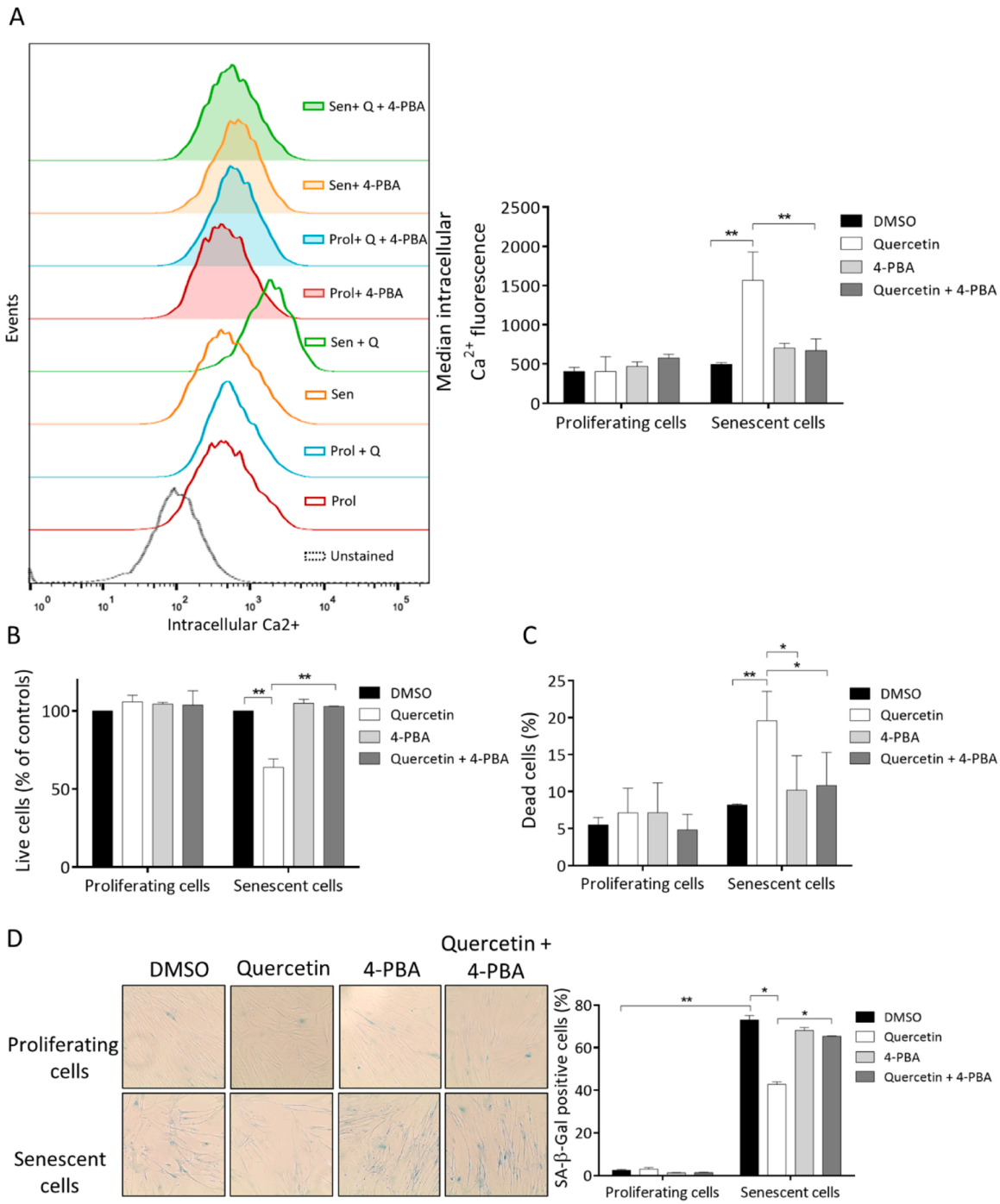

Fig. 6. The inhibition of ER stress by 4-PBA eliminated the senolytic effect of quercetin. (A) Cytofluorimetric analysis of Ca^{2+} with Fluo-4 AM labeling in proliferating (Prol) and senescent (Sen) cells treated with quercetin, 4-PBA, or the combination of two. Data from proliferating cells stained with the dye (Prol) are shown as control. Ca^{2+} levels, expressed as median fluorescence intensity in the bar graph to the right, are mean ± SD of three independent experiments. **(B)** The trypan blue exclusion assay was performed 3 days after quercetin and 4-PBA treatment. Live cell number was expressed as percentage of untreated proliferating and senescent cells, respectively, assumed as 100 %. Data are mean ± SD of at least three independent experiments. **(C)** Comparative quantification of the percentage of total dead fibroblasts (7-AAD$^+$/annexin V$^+$, 7-AAD$^-$/annexin V$^+$, and 7-AAD$^+$/annexin V$^-$) in proliferating and senescent cells treated for four hours with quercetin, 4-PBA, or the combination of two, obtained by cytofluorimetric analysis after labelling cells with annexin V- BV421 and 7-AAD. Data are mean ± SD of three independent experiments. **(D)** Representative images of senescence-associated β-galactosidase (SA-β-Gal) activity (left), measured after three days of quercetin and 4-PBA treatment. The percentage of SA-β-Gal-positive cells (blue) out of the total number of cells/well (right) was evaluated in at least three independent experiments. The results are reported as mean ± SD. *$p<0.05$ and **$p<0.001$. *Prol*: Proliferating cells; *Sen*: Senescent cells.

These data suggest that even partial clearance of the senescent cell population can curb their adverse effects, including the promotion of cancer growth and invasiveness.

4. Discussion

Ageing is an inevitable biological process that induces a progressive functional decline in essentially all organisms and represents the leading risk factor for ARDs. The chief aim of Geroscience is to gain insight into

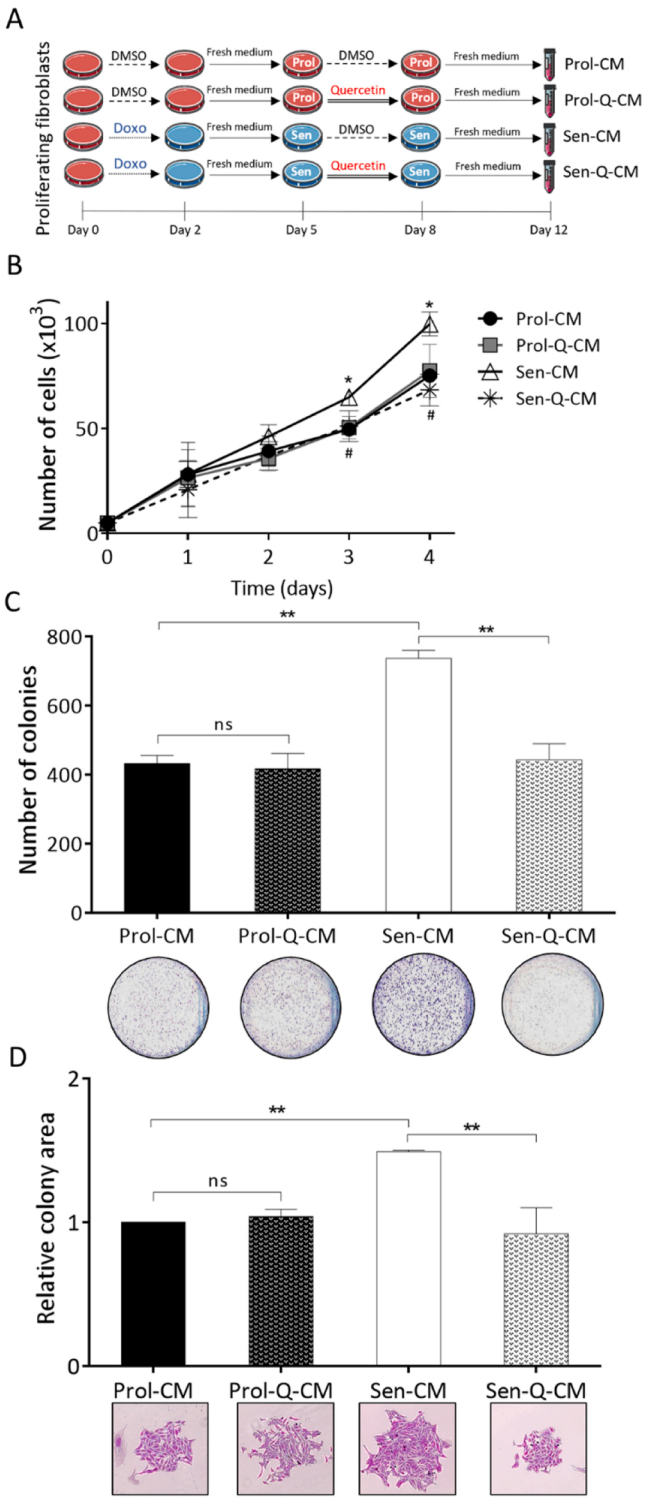

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Fig. 7. Conditioned media from quercetin-treated senescent fibroblasts reduced osteosarcoma cell growth. (A) Experimental scheme. After senolytic treatment with quercetin, fibroblasts were incubated in fresh medium for four days. Then, conditioned media (CM) were collected, centrifuged, and used to treat U2OS cells. **(B)** Trypan blue exclusion assay on U2OS cells treated with CM from control (Prol-CM), quercetin-treated young cells (Prol-Q-CM), Doxo-induced senescent fibroblasts (Sen-CM), and Doxo-induced senescent fibroblasts treated with quercetin (Sen-Q-CM). Data are mean ± SD of three independent experiments. *$p<0.05$ respect to Prol-CM; # $p<0.05$ expresses the comparison between Sen-Q-CM and Sen-CM. **(C)** Representative images (bottom) and number of U2OS colonies (top) formed after seven days of treatment with CM. Data are the mean of three independent experiments ± SD. **(D)** Colony area (representative images below) was calculated with Image J Software. Data are expressed as relative to control (Prol) and are mean ± SD of three independent experiments. *$p<0.05$ and **$p<0.001$. *Prol-CM*: control fibroblasts CM; *Prol-Q-CM*: CM from proliferating cells treated with quercetin; *Sen-CM*: CM from senescent fibroblast; *Sen-Q-CM*: CM from senescent fibroblasts treated with quercetin.

the relationship between ageing and ARDs and to improve strategies to prevent, delay, or treat them (Franceschi et al., 2018; Olivieri et al., 2018). Cellular senescence, a state of permanent growth arrest accompanied by high secretome activity, has emerged among the selected and interconnected biological processes that can drive ageing and ARDs (López-Otín et al., 2022) and is considered a hallmark of ageing (He and Sharpless, 2017) since senescent cells progressively accumulate in tissues (Amor et al., 2020). Interestingly, clearance of senescent cells has been found to extend lifespan and healthspan in aged and progeroid mice and to curb ARDs (Baker et al., 2016, 2011; Farr et al., 2017; Ogrodnik et al., 2017). The search for substances capable of selective removal of senescent cells with minimal impact on the organism has intensified since Dörr et al., (Dörr et al., (2013) described the first such drugs in 2013. However, the mechanisms underlying senolysis have not been fully elucidated, and data on the efficacy of senolytic drugs are conflicting. Therefore, it is crucial to gain further insight into their mode of action and find the vulnerabilities of senescent cells and SASP regulation. In particular, the chief aims of senolytic research are: i) to determine the amount of senescent cell clearance that will curb their adverse effects and ii) to preserve the beneficial actions of senescence, which affect a variety of physiological processes such as embryo development (Muñoz-Espín and Serrano, 2014), tissue remodelling and repair (Yun et al., 2015), wound healing (Demaria et al., 2014), and tumour suppression (Campisi, 2001; Faget et al., 2019). This study describes the efficacy of quercetin in selectively clearing Doxo-induced senescent fibroblasts through a novel mechanism of action and shows that clearance of a fraction of these cells can curb their pro-tumour effects on osteosarcoma cells. Using a model of Doxo-induced senescence developed in WI-38 fibroblasts (Bientinesi et al., 2022), we were able to demonstrate that three-day quercetin treatment reduced the number of live senescent fibroblasts and SA-β-Gal-positive cells, as also reported for other models of cell senescence (Zhu et al., 2015). The selectivity of quercetin was also demonstrated by the reduced percentage of fibroblasts positive for macroH2A.1, a major SAHF component (González-Gualda et al., 2021), and by changes in the expression of the proteins involved in cell cycle inhibition, such as p-RB, p21, and p16.

Furthermore, senescent fibroblasts exhibited significantly greater NF-κB nuclear translocation and SASP factor expression than proliferating cells, in line with earlier studies employing other senescence-inducing stimuli (Alimbetov et al., 2016; Bientinesi et al., 2022; Coppé et al., 2006; Laberge et al., 2015; Menicacci et al., 2017; Nelson et al., 2012). Quercetin significantly reduced both NF-κB translocation and SASP factor expression in senescent fibroblasts. The SASP factors analysed included pro-inflammatory cytokines such as IL-1α, IL-6, and IL-8 (Rodier and Campisi, 2011; Roger et al., 2021; Salvioli et al., 2013). These cytokines are hallmarks of inflammaging, a chronic, sterile, low-grade inflammatory state linked to ageing and several ARDs

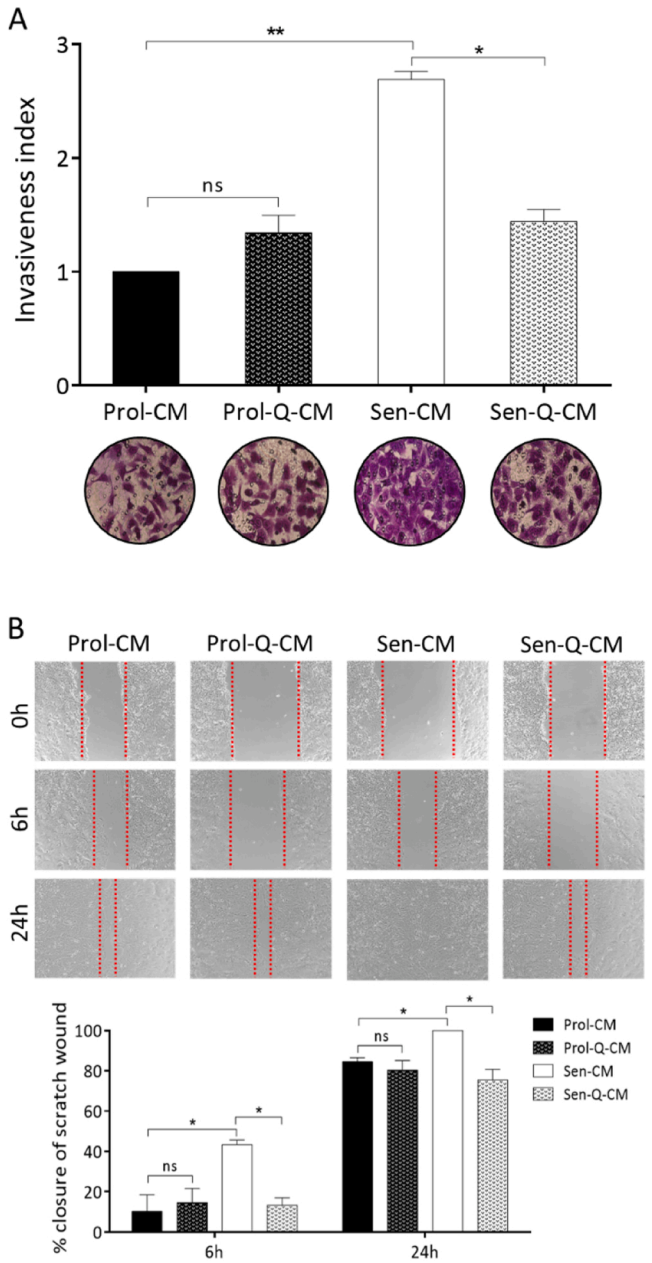

Fig. 8. Conditioned media from quercetin-treated senescent fibroblasts reduced osteosarcoma cell migration and invasiveness. (A) Boyden chamber assay on U2OS cells. Conditioned media from control (Prol-CM), proliferating cells treated with quercetin (Prol-Q-CM), Doxo-induced senescent fibroblasts (Sen-CM), and Doxo-induced senescent fibroblasts treated with quercetin (Sen-Q-CM), were placed in the bottom well of the chamber. Barr graph represents the number of cells that invaded the matrigel compared to control. Data are mean ± SD of three independent experiments. Representative images of invasive cells are reported under the graph. **(B)** Representative images (top) and percentage of scratch wound area (bottom) in Wound Healing Assay of U2OS cells treated with conditioned media. Data are the mean percentage ± SD of three independent experiments of wound closure area compared to the initial scratch. *p<0.05 and **p<0.001.: *Prol-CM*: control fibroblasts CM; *Prol-Q-CM*: CM from proliferating cells treated with quercetin; *Sen-CM*: senescent fibroblasts CM; *Sen-Q-CM*: CM from senescent fibroblasts treated with quercetin.

(Cevenini et al., 2013; Franceschi and Campisi, 2014; Olivieri et al., 2018), including cancer (Franceschi et al., 2018; Zinger et al., 2017). We also assessed the metalloproteases MMP2 and MMP3; CXCL12, which is involved in cancer cell invasion activity (Coppé et al., 2010); VEGF (Oubaha et al., 2016), which promotes angiogenesis; and TGFβ1, which stimulates cell growth and migration (Akhurst and Hata, 2012). Albeit partial, the reduction of the senescent population was sufficient to downregulate some SASP factors that promote processes such as tumour growth and progression (Birch and Gil, 2020; Faget et al., 2019).

Notably, the SASP is associated with upregulation of the translational machinery, which can induce ER proteotoxic stress (Kwon et al., 2017), ultimately resulting in increased senescent cell autophagy (Guo et al., 2012; Herranz and Gil, 2018; Lee et al., 2021). Autophagy and senescence are closely related (Wiley and Campisi, 2021). Although autophagy was initially considered an anti-senescence mechanism (García-Prat et al., 2016; Gewirtz, 2013), it is now viewed as a critical senescence mechanism due to its ability to facilitate SASP protein synthesis (Kwon et al., 2017; Lee et al., 2021; Young et al., 2009). Interestingly, Doxo-induced senescent fibroblasts showed an increase in autophagy markers and autophagosome formation, as described in other senescence models (Dörr et al., 2013; Gamerdinger et al., 2009; Guo et al., 2012; L'Hôte et al., 2021; Narita et al., 2011; Rovira et al., 2022; Young et al., 2009), likely a response to the proteotoxic stress induced by SASP factor upregulation. In contrast, four hours of quercetin treatment downregulated beclin-1 and LC3 II expression. It also reduced autophagosome formation, suggesting reduced autophagic activity in cells with a high SASP profile. At variance with our work, some studies on cancer cell lines have reported that quercetin-induced apoptosis while at the same time increasing autophagy as a protective mechanism and that inhibition of the autophagic flux enhanced quercetin-induced cell death (Kim et al., 2013; Wang et al., 2011, 2016). These findings may be explained by the fact that such studies involved cancer cell lines, which can react to quercetin treatment by increasing autophagy to resist apoptosis. Our data document for the first time that the quercetin-induced reduction of autophagic activity triggered ER stress, as reflected by the presence of XBP1 spliced mRNA, suggesting UPR activation in treated senescent cells. Indeed, findings from other cell models suggest that the XBP1 spliced form, a major mediator of UPR activation (Szegezdi et al., 2006), could be related to high SASP protein synthesis (Dörr et al., 2013; L'Hôte et al., 2021, 2022). Moreover, after incubating senescent fibroblasts in Ca^{2+}-free medium, we measured increased intracellular Ca^{2+} only in those treated with quercetin. This suggests Ca^{2+} release by the ER due to proteotoxic stress, ultimately leading to senescent cell death.

Interestingly, our data also documented that BcL-XL, an anti-apoptotic protein which seems to protect against lethal ER stress (Morishima et al., 2004; Murakami et al., 2007; Szegezdi et al., 2006), was overexpressed in senescent fibroblasts and reverted to normal levels after quercetin treatment. To further investigate the role of autophagy and ER stress in quercetin-induced senolysis, we used PP242 (Lamming et al., 2013) as an autophagy inducer to counteract the reduction of the autophagy process caused by quercetin in senescent cells. The combined treatment of PP242 and quercetin eliminated ER stress and, consequently, the senolytic effect. These findings suggest that autophagy plays a role in the senescence process and can be targeted for therapeutic interventions. Similarly, inhibiting ER stress with 4-PBA (Ayala et al., 2012) blocked the removal of senescent cells, implying ER stress was necessary for quercetin-mediated senolysis. Notably, quercetin cleared only about 30 % of senescent fibroblasts. This can be explained by the decrease of autophagic activity and the restoration of normal BcL-XL levels, which affected a pool of senescent cells with a high SASP profile. These cells underwent ER stress-mediated apoptosis. Intriguingly, the partial clearance could curb the pro-tumour effects of the senescent fibroblast CM on osteosarcoma cells. We selected these cells because osteosarcoma shows a second peak of incidence in subjects aged 75–79 years and can thus be considered an ARD (Nie and Peng, 2018). Its high mortality rate, especially among older patients, is due to the toxicity of the chemotherapy drugs, such as Doxo, and to metastasis formation (Miller et al., 2013; Smrke et al., 2021). The finding that quercetin treatment reduced the invasiveness of osteosarcoma cells, promoted by

CM from the senescent fibroblasts, suggests that quercetin could be harnessed to clear cancer-associated senescent cells from the tumour microenvironment, thus strengthening the effect of chemotherapy. This would be especially useful in elderly patients who harbour abundant senescent cells in tissues and organs. Our previous study found that pre-treatment with quercetin before Doxo administration can enhance fibroblasts' antioxidant mechanisms, reduce oxidative stress, and partially prevent the onset of senescence, resulting in a reduced number of senescent fibroblasts, which in turn decrease the production of SASP factors and the pro-tumour effect of CM (Bientinesi et al., 2022). These data suggest that quercetin could be used as an adjuvant for Doxo chemotherapy to prevent off-target senescence induction. Moreover, our current study demonstrates the effectiveness of quercetin when the senescence state is already induced, such as in patients who have already undergone chemotherapy. Therefore, quercetin shows potential in either preventing senescence induction or promoting the senolysis of Doxo-induced senescent fibroblasts, which could help reduce osteosarcoma metastasis or recurrence, potentially stemming from the support of senescent cells to the tumour. The two mechanisms of action of quercetin will also need to be demonstrated in other models of cellular senescence to determine whether they are common or specific to Doxo-induced senescence. A replicative senescence model could be particularly advantageous in verifying the effects of quercetin and understanding its potential role in preventing or reducing the ageing process and delaying the onset of age-related diseases. Some studies have reported the role of quercetin in alleviating the onset of replicative senescence, demonstrating a rejuvenating effect on tissues (Chondrogianni et al., 2010; Geng et al., 2019). However, limited data about the senolysis of replicative senescent fibroblasts are available (Hohmann et al., 2019). If these two identified mechanisms of action of quercetin are also confirmed in replicative senescence, our understanding of the effects of quercetin could be expanded. It may also serve as a beneficial strategy for young people to prevent age-associated senescence and for older individuals who have accumulated senescent cells in their tissues.

Several studies have found that a relatively small reduction (30 %) of senescent cells can prevent ARDs and extend healthspan (Baker et al., 2011; Ogrodnik et al., 2017; Pignolo et al., 2021; Roos et al., 2016; Xu et al., 2015). According to the Threshold Theory of Senescent Cell Burden, the accumulation of a certain amount/percentage of senescent cells is necessary to trigger adverse effects and dysfunction (Kirkland and Tchkonia, 2020). Nevertheless, senescence also plays several beneficial roles (Burton and Krizhanovsky, 2014). Thus, eliminating too many senescent cells may have adverse effects (Born et al., 2022; Grosse et al., 2020; Martin et al., 2021). Accordingly, senotherapeutic strategies need to strike a balance between senolysis (i.e. clearance of a sufficient number of senescent cells) and preservation of the physiological functions of senescence.

In conclusion, our report demonstrates for the first time that quercetin exerts a significant senolytic effect on Doxo-induced senescent fibroblasts; it describes a new mechanism of action of the substance and, importantly, documents that partial clearance of senescent cells is sufficient to curb their adverse effects.

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Authors' contribution

All authors contributed to the manuscript based on their expertise. Original proposal and writing: EB, DM; experimental work: EB, SR; data analysis: EB; project supervision: DM; funding acquisition: DM; figure preparation: EB, SR; text improvement: EB, DM, SR; special expertise: ML.

CRediT authorship contribution statement

Matteo Lulli: Methodology, Investigation. **Sara Ristori:** Methodology, Investigation. **Daniela Monti:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Elisa Bientinesi:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation.

Conflict of Interest

The authors declare no conflicts of interest.

Data availability

The data that has been used is confidential.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mad.2024.111957.

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