COL-3 enhances the anti-proliferative and pro-apoptotic effects of paclitaxel in breast cancer cells

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Abstract. Paclitaxel, a chemotherapeutic agent used in the treatment of breast cancer and other solid tumor types, including ovarian and lung, causes a dose-dependent neuropathic pain, which limits its use. Chemically modified tetracycline-3 (COL-3) has anticancer properties and was previously reported to inhibit neuroinflammation and protect against paclitaxel-induced neuropathic pain (PINP) in mice models. However, it is not known whether it affects the anticancer activities of paclitaxel. Thus, the aim of the present study was to evaluate the effect of COL-3 on the anticancer activity of paclitaxel on the breast cancer cell lines MCF-7 (estrogen receptor-positive), pII [estrogen receptor-negative (ER-ve)] and MDA-MB-231 (ER-ve). Cell proliferation, apoptosis and cell cycle stage were determined using an MTT assay, Annexin V/7-aminoactinomycin D and flow cytometry. The expression of various signaling molecules was determined with ELISA-based proteome profiling and western blotting. Additionally, the degree of cell invasion was determined with a Matrigel assay and caspase-3 activity was determined with a colorimetric assay. Treatment with paclitaxel or COL-3 alone inhibited cell proliferation in a concentration-dependent manner in all cell lines. The anti-proliferative effects of paclitaxel and COL-3 in combination varied from synergism against MDA-MB-231 and pII cells to notably additive and slight antagonism against MCF-7 cells. In the highly proliferative and invasive pII cells, the observed synergistic anti-proliferative effect was partially through the induction of apoptosis via modulation of caspase-3 levels and activity, and P70S6K phosphorylation, but not cell cycle arrest. COL-3 inhibited the invasion of pII cells in a concentration-dependent manner partially through inhibiting total matrix metalloproteinase activity. The combination regimen significantly inhibited the expression of two proteases, ADAM metallopeptidase with thrombospondin type 1 motif 1 and proteinase 3. In conclusion, the combination of paclitaxel and COL-3 indicated additive to synergistic anti-proliferative effects on breast cancer cells mediated partially via the induction of apoptosis. The combination regimen could further inhibit invasion and metastasis. Thus, COL-3 could be a beneficial adjunct to a paclitaxel-based anticancer regimen to improve therapeutic outcome and reduce the adverse effects of paclitaxel, primarily PINP.

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Introduction

Breast cancer is among the most frequently diagnosed cancer types and the leading cause of cancer mortalities among females based on the estimates of cancer incidence and mortality globally for 36 cancer types in 185 countries, which was produced by the International Agency for Research on Cancer in 2018 (1). In 2012, 1.7 million females were diagnosed with breast cancer, and there were 6.3 million females alive who had been diagnosed with breast cancer in the previous five years globally (2). Surgery is the most common primary therapy for cancerous lesions of the breast, followed by radiotherapy or chemotherapy if metastasis to lymph nodes or other organs is detected (3). Adjuvant hormone therapy, also termed endocrine therapy, is considered a standard therapy for ~75% of patients with tumors expressing estrogen receptor (ER) and progesterone receptor (PR) (4). However, approximately half of females with ER-positive (ER+ve) cancer exhibit intrinsic resistance (*de novo*) to endocrine therapy and the majority of patients who initially respond develop acquired resistance during the course of treatment with the anti-estrogen agents (5). These refractive patients, together with the patients with ER-negative (ER-ve) cancer, form a large population, which has poor clinical outcomes and survival rates, and require other forms of treatment, primarily chemotherapy.

Chemotherapeutic regimens primarily consist of a combination of ≥2 cytotoxic drugs (6). Although they have various side effects, treatment with chemotherapeutic drugs has improved patient survival and reduced annual relative risk of relapse and mortality in North America in 2013 (3,7). Drugs in the taxane class, including paclitaxel, are among the most frequently used agents in the treatment of various cancer types, including breast cancer (8). Paclitaxel (Taxol)

exerts its anticancer activity through binding to specific pockets within β-tubulin, thus stabilizing microtubules and preventing their depolymerization (7). This results in inhibition of cellular processes that are dependent on microtubule turnover, including inhibition of the cellular transition from G_0 to G_1, arresting the cell in the G_2/M phase, inhibition of mitosis and eventually induction of apoptosis (8-10). Paclitaxel also interferes with mitosis by inducing mitotic block at the metaphase/anaphase boundary, and forming an incomplete metaphase plate of chromosomes and an abnormal organization of spindle microtubules (11). Microtubules of different statuses, including cytoskeletal microtubules and mitotic spindles, may have different sensitivities to paclitaxel; thus, the concentration of paclitaxel may be the major determinant of its apoptogenic mechanisms (12). Paclitaxel may also exert its killing activity through a gene-directed process, which is a pathway completely independent of microtubules (11).

The use of paclitaxel is associated with a number of serious dose-dependent side effects, including paclitaxel-induced peripheral neuropathy (PIPN), which may necessitate dose reduction or withdrawal during the course of chemotherapy (13,14). However, there are no clinically-proven drugs for the prevention of PIPN, and the best available data support a moderate recommendation for duloxetine to treat PIPN (14,15). Therefore, further research is warranted to determine agents that can relieve PIPN or assist with reducing the doses of paclitaxel necessary to treat cancer, and thus indirectly reducing the risk of developing dose-limiting side effects, including PIPN. We previously observed that co-administration of paclitaxel with chemically modified tetracycline-3 (COL-3) inhibited the development of paclitaxel-induced thermal hyperalgesia in mice, indicating that COL-3 can be used for the prevention of PIPN (16). COL-3 has matrix metalloproteinase (MMP) inhibitory properties (17,18), which resulted in a number of researchers investigating its anticancer potential in a variety of cancer types, including melanoma, and lung, breast and prostate cancer (19,20). Taking this into consideration, it is plausible that the combination of COL-3 with paclitaxel may have additive or synergistic activity against a number of cancer types, which would demonstrate a double advantage of reducing side effects and increasing antitumor efficacy. Thus, the aim of the present study was to evaluate the impact of COL-3 on the anticancer activity of paclitaxel using various ER+ve and ER-ve breast cancer cell lines *in vitro*.

Materials and methods

Cell lines. The human breast carcinoma cell lines MCF-7 (ER+ve) and MDA-MB-231 (ER-ve; *de novo* resistant form) were obtained from the American Type Culture Collection (Manassas, VA, USA). The ER downregulated cell line pII (acquired resistant form) was established in Professor Yunus Luqmani's and Khajah's lab (Kuwait University, Safat, Kuwait) by transfection of MCF-7 cells with the ER directed shRNA plasmid, as described previously (21,22). Cells were maintained in culture medium containing Advanced Dulbecco's modified Eagle's medium (Advanced DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 5% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific), 6 ml/500 ml penicillin-streptomycin (10,000 U/ml penicillin and 10,000 μg/ml streptomycin), 6 ml/500 ml (200 mM) L-glutamine and 6 ml/500 ml non-essential amino acids (Invitrogen; Thermo Fisher Scientific, Inc.). All cells were cultured as monolayers in an incubator at 37˚C in an atmosphere containing 5% CO_2 and 95% humidity.

Drugs. COL-3 (purchased from Galderma, Research and Development SNC, Les Templier, France) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 1 mM and stored at -20˚C in aliquots. Paclitaxel, purchased from Tocris Bioscience (Bristol, UK), was dissolved in DMSO to a stock concentration of 1 mM and stored at -20˚C in aliquots. All experimental incubation with drugs was conducted at 37˚C, 5% CO_2 and 95% humidity in an incubator. The control vehicle used was 0.01% DMSO.

Proliferation assay. The effect of various concentrations of paclitaxel (1, 2.5, 5, 10, 25, 50, 100, and 1,000 nM), COL-3 (50, 100, 1,000, 2,500, 5,000, 10,000, and 20,000 nM) or their combination on cell proliferation was examined using a colorimetric MTT assay (Promega Corporation, Madison, WI, USA), as previously described (23,24). In brief, ~$1x10^4$ cells were seeded in triplicate wells and incubated overnight at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the medium was removed and the cells were treated with the vehicle (control), or various concentrations of paclitaxel, COL-3 or their combination. The growth was assessed after 72 h of incubation at 37˚C in an atmosphere containing 5% CO_2.

Apoptosis assay. The effect of COL-3, paclitaxel or their combination on pII cell apoptosis was measured using Annexin V/7-aminoactinomycin D apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA), as previously described (24).

Cell cycle assay. Subsequently, ~$1x10^4$ pII cells were seeded in triplicate wells and incubated overnight at 37˚C in an atmosphere containing 5% CO_2 and 95% humidity. The cells were then treated with the vehicle or various concentrations of paclitaxel, COL-3 or their combination. After 72 h of incubation at 37˚C in an atmosphere containing 5% CO_2, cells were trypsinized and washed once with ice-cold PBS. The pellet was then re-suspended with PBS and fixed by adding ice-cold 70% ethanol while vortexing at 14,000 x g for 20 sec at 4˚C. The samples were then stored at -20˚C overnight. The following day, samples were centrifuged at 66 x g for 15 min at room temperature and washed once with PBS. Pellets were treated with RNase, incubated for 15 min at 37˚C and 200 μl propidium iodide solution (DNA Prep stain kit; Beckman Coulter, Inc., Brea, CA, USA) was added. The samples were analyzed using a Cytomics FC500 flow cytometer with a maximum emission of 605 nm. The DNA content of cell duplicates during the S phase of the cell cycle, and the relative amount of cells in the G_0 and G_1 phases, in the S phase, and in the G_2 and M phases were determined utilizing the fluorescence of cells in the G_2/M phase, which were twice as high as that of cells in the G_0/G_1 phase. This was obtained using CXP Software, version 2 (Beckman Coulter, Inc.).

Matrigel invasion assay. The degree of pII cell invasion through the basement membrane matrix was determined using the Cultrex® 24-Well basement membrane extract (BME) Cell Invasion assay kit (Trevigen, Haithersburg, MD, USA). All procedures and reagent preparations were conducted according to the manufacturer's protocols. Briefly, insert membranes were coated with 1X BME and incubated at 37˚C overnight. pII cells, that had been serum-starved overnight, were re-suspended at $1x10^6$ cells/ml in Advanced DMEM with or without drugs (100, 1,000, 5,000 and 10,000 nM), and 100 μl ($1x10^5$ cells) was loaded into the upper chambers. To the bottom chamber, 500 μl DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS was added as a chemoattractant. After 24 h of incubation at 37˚C, the media in the top chambers as well as the bottom chambers were aspirated followed by washing with 1X wash buffer. Subsequently, 500 μl Cell Dissociation Solution/Calcein-acetomethylester (AM) complex was added to the bottom chamber of each well and incubated at 37˚C in an atmosphere containing CO_2 for 60 min. Cells internalize Calcein-AM and intracellular esterases cleave AM moiety generating free calcein, which fluoresces. The degree of cell invasion was determined by recording the fluorescence emission using a microplate reader (Luminometer) with a filter set of excitation/emission at 485/535 nm.

Proteome profiler analysis. A total of three different proteome profiler array kits, Human protease array kit, Human phospho-kinase array kit and Human apoptosis array kit (R&D Systems, Inc., Minneapolis, MN, USA), were used to determine the expression levels of a number of groups of proteins in cell extracts/lysates following the manufacturer's protocols.

Subsequently, ~$1x10^6$ pII cells/well were cultured for 24 h at 37˚C in an atmosphere containing 5% CO_2 under the following treatment conditions: Vehicle (control); 2.5 nM paclitaxel; 5 μM COL-3; or 2.5 nM paclitaxel and 5 μM COL-3. Upon removal of the advanced DMEM, the cell monolayers were washed once with ice-cold PBS and then lysed using lysis buffer supplemented with protease inhibitors [1 μg/ml leupeptin, 1 μg/ml aprotinin and 10 μg/ml phenylmethylsulfonyl fluoride (PMSF); Sigma-Aldrich; Merck KGaA, Darmstadt, Germany]. Cells were harvested using a sterile disposable rubber cell scraper and transferred into Eppendorf® tubes. The cell lysates were then centrifuged at 14,000 x g for 10 min at 4˚C, and the supernatant was transferred to new Eppendorf tubes for subsequent protein analysis or stored at -80˚C for later analysis. Total protein concentration in the cell lysates was determined with a standard Bradford assay.

The relative phosphorylated/expression levels of 45 kinases (cat. no. ARY003B), 35 apoptosis-associated proteins (cat. no. ARY009) and 35 proteases (cat. no. ARY021B) (R&D Systems, Inc.) were detected using Proteome Profiler™ Human Protease Array kits aforementioned, according to the manufacturer's protocols.

MMP activity. The general activity of MMPs was determined using the MMP activity assay kit from Abcam (cat. no. ab112146; Cambridge, UK), as previously described (25). pII cells were seeded ($0.1x10^6$ cells) into 6-well plates in culture medium at 37˚C and allowed to grow to 80% confluence. Cells were serum starved overnight at 37˚C in an atmosphere containing 5% CO_2, and then left untreated, or treated with 2.5 nM paclitaxel, 5 μM COL-3 or 2.5 nM paclitaxel and 5 μM COL-3 for 24 h followed by epidermal growth factor (EGF) stimulation (100 ng/ml for 30 min) at 37˚C in an atmosphere containing 5% CO_2. Subsequently, 25 μl media was removed and added to 25 μl 2 mM APMA working solution, and then incubated for 15 min at 25˚C, followed by the addition of 50 μl green substrate solution. MMP activity was measured at 10 min intervals for 1 h at 37˚C by recording fluorescence emission using a microplate reader with a filter set of excitation/emission at 485/535 nm.

DNA fragmentation assay. Cells were seeded ($0.1x10^6$ cells) in 6-well plates to 80% confluence, and then incubated with 2.5 nM paclitaxel, 5 μM COL-3 or a combination regimen (2.5 nM paclitaxel + 5 μM COL-3) for 48 h at 37˚C in an atmosphere containing 5% CO_2. Cells were then trypsinized, pelleted at 66 x g for 15 min at room temperature and DNA was isolated from the cells using a DNA isolation kit (Qiagen, Inc., Gaithersburg, MD, USA), according to the manufacture's protocol. Subsequently, 1 μg DNA was run on 2% agarose gel stained with ethidium bromide at 50 V, and the gel was examined under an UV trans-illuminator. Additionally, a 1 μg DNA ladder was also included in the experiment (λ DNA/*Hin*dIII marker; cat. no. SM0102; Thermo Fisher Scientific, Inc.). Densitometric analysis of the bands intensity was calculated using ImageJ software, version k 1.45 (National Institutes of Health, Bethesda, MD, USA).

Western blotting. Cells were cultured ($0.1x10^6$ cells; at 37˚C in an atmosphere containing 5% CO_2) in 6-well plates to an 80-90% confluence, and treated with vehicle (control), 2.5 nM paclitaxel, 5 μM COL-3 or combination regimen poly(ADP-ribose) polymerase (2.5 nM paclitaxel + 5 μM COL-3) for 48 h. The medium was subsequently aspirated off, and cell monolayers were harvested by scraping and re-suspension into 300 μl lysis buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% Triton X, 100 μg/ml PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin, and then stored at -80˚C. Protein concentration was determined with a Bradford assay using bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) as the standard, and 3 μg protein lysate was mixed with an equal volume of 2X SDS and incubated at 90˚C for 10 min. Samples were then loaded onto 10% SDS-PAGE and electrophoresed at 150 V for 1 h. Subsequently, proteins were transferred to a nitrocellulose membrane and blocked with 2% BSA for 1 h at room temperature prior to being incubated overnight at 4˚C with primary antibodies (prepared in 2% BSA) against β-actin (loading control; 1:1,000 dilution), or phospho- or total- B-cell lymphoma-2 (BCL-2)-associated X (BAX), BCL-2 associated agonist of cell death (BAD), BCL-2 antagonist/killer (BAK), BCL-2, cytochrome *c*, AKT, extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-associated protein kinase (MAPK), apoptotic peptidase activating factor 1 (APAF-1), and heat shock transcription factor-1 (HSF-1), and un-cleaved or cleaved-caspase-3, -8, -9 and PARP (all from Cell Signaling Technology, Inc., Danvers, MA, USA; 1:100 dilution). The membrane was then washed 3 times 5 min each with TBS with Tween-20 (20%) and incubated with anti-rabbit horseradish

peroxidase (HRP)-conjugated secondary antibody (1:500 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature and then developed with Super Signal enhanced chemiluminescent (Pierce; Thermo Fisher Scientific, Inc.) and visualized with Kodak X-ray film. The catalogue numbers of the used antibodies are as follows: β-actin, 4970; phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), 4370; p44/42 MAPK (Erk1/2), 9102, AKT, 9272; phospho-(Ser/Thr) AKT, 9611; p38 MAPK, 9212; phospho-p38 MAPK (Thr180/Tyr182), 9215; Apaf-1, 8723; Cytochrome *c*, 11940; HSF1, 4356, BAK, 12105; BAX, 2772; BAD, 9292; BCL-2, 4223; PARP, 9532; cleaved PARP (Asp214), 5625; caspase-9, 9502; Caspace-8, 4790; cleaved caspase-8 (Asp391), 9496; caspase-3, 9662; cleaved caspase-3 (Asp175), 9579; and anti-rabbit IgG, HRP-conjugated, 7074.

Caspase-3 activity assay. Cells ($1x10^6$) were treated with paclitaxel (2.5 nM), COL-3 (5 μM) or a combination regimen (2.5 nM paclitaxel + 5 μM COL-3) for 48 h at 37˚C in an atmosphere containing 5% CO_2 and then collected by trypsinization and washed twice with ice-cold PBS. Subsequently, the cells were centrifuged at 42 x g for 5 min at 4˚C and the pellet was washed with ice-cold lysis buffer. The protein concentration was calculated using a Bradford assay. Caspase-3 activity in the lysate of the different treatment conditions was determined using a caspase-3 colorimetric assay kit from GenScript (cat. no. L00289; Piscataway, NJ, USA), and was expressed as caspase-3 activity/mg of protein.

Data analysis. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's (for comparing between treatment conditions) or Bonferroni's (for comparing within treatment conditions) Multiple Comparison Test using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference. Using the GraphPad Prism software, the concentration of paclitaxel or COL-3 that produced the half-maximal response (IC_{50}) was calculated using non-linear regression analysis. The data were fitted to a dose-response-inhibition equation [log (inhibitor) vs. normalized response curve]. Results were expressed as the mean ± standard error of the mean.

To test for synergism, summation or antagonism, the combination index (CI) was calculated by adapting the Chou and Talalay equation for mutually non-exclusive drugs: CI = (D)1(Dx)1+(D)2(Dx)2+(D)1 (D)2(Dx)1 (Dx)2, where CI<1 indicates synergism, CI=1 indicates summation and CI>1 indicates antagonism (25,26). (D)1 and (D)2 represent the concentrations of paclitaxel and COL-3 in the combination that also inhibits cell growth by x%, respectively. (Dx)1 and (Dx)2 represent the corresponding individual concentrations of paclitaxel and COL-3 that produced the same x% growth inhibition as the combination regimen, respectively. Since a portion of the obtained data were negative, such as slightly enhanced growth instead of inhibiting it at reduced concentrations, the CompuSyn software developed by Chou and Talalay (26,27), which only accepts values between 0-1, could not be used. Thus, the individual concentration of paclitaxel or COL-3 that would produce the equivalent effect as the combined concentrations were calculated using the identical dose-response-inhibition equation used to calculate the IC_{50} using GraphPad Prism software (version 5.0).

Results

Effects of monotherapy with paclitaxel or COL-3 on breast cancer cell proliferation. Paclitaxel inhibited cell proliferation in all of the tested cell lines in a concentration-dependent manner (Fig. 1A-C). Additionally, paclitaxel had similar inhibitory effects (60-70%) in all cell lines at the highest concentrations (50 nM-1 μM); however, the degree of sensitivity of the cell lines to paclitaxel varied. The IC_{50} of paclitaxel for MCF-7 cells was 36 nM (95% confidence interval, 22.96-56.39 nM), for pII cells it was 8.6 nM (95% confidence interval, 6.188-11.97 nM) and for MDA-MB-231 cells it was 6.5 nM (95% confidence interval, 4.777-8.930 nM). The slight increase in cell viability observed at the highest doses of paclitaxel (25-100 nM) in MDA-MB-231 cells was not statistically significant ($P>0.05$), compared with the 10 nM dose, and the effects of paclitaxel may have reached a plateau phase at a concentration of 10 nM. Notably, the IC_{50} of the ER^+ cell line MCF-7 was 4-5.5 times increased, compared with the ER^- cell lines (pII and MDA-MB-231). Thus, the order of sensitivity of the cell lines to paclitaxel from the most to the least sensitive was MDA-MB-231, pII and then MCF-7.

COL-3 inhibited cell proliferation in all of the tested cell lines in a concentration-dependent manner (Fig. 1D-F). It also demonstrated similar anti-proliferative effects (60-70%) for all the cell lines at the highest concentrations (10-20 μM). However, the degree of sensitivity of the cell lines to COL-3 varied. The IC_{50} of COL-3 for MCF-7 cells was 4 μM (95% confidence interval, 3.456-4.755 μM), for pII cells it was 10 μM (95% confidence interval, 8.196-12.428 μM) and for MDA-MB-231 cells it was 4.4 μM (95% confidence interval, 3.854-5.052 μM). Notably, the IC_{50} of pII cells was ~2 times increased, compared with MDA-MB-231 and MCF-7 cells. Thus, the order of sensitivity of the cell lines to COL-3 from the most to the least sensitive was MCF-7, MDA-MB-231 and then pII. Additionally, it is notable that the IC_{50} for COL-3 was 100-1,100 times increased, compared with paclitaxel, thus indicating that the latter drug is more potent at inhibiting cell proliferation.

Effects of combination regimens of COL-3 and paclitaxel on cell proliferation. The effects of various combination regimens on MCF-7 cell proliferation varied from notably additive to a slight antagonism. Combining 2.5 nM paclitaxel with 5 μM COL-3 resulted in a notably additive effect (CI=0.99) although the combination regimen did not significantly inhibit proliferation, compared with treatment with COL-3 alone (62 vs. 58%, respectively; $P>0.05$; Fig. 2A). The combination of 5 nM paclitaxel with 2.5 μM COL-3 resulted in a slight antagonistic effect (CI=1.17; Table I). However, combination of 10 nM paclitaxel with 2.5 μM COL-3 resulted in a notably additive inhibitory effect (CI=1.09). This combination regimen resulted in 49% inhibition, which is significant ($P<0.01$), compared with treatment with each drug individually, which achieved 30-32% inhibition (Fig. 2A). Additionally, the calculated individual concentrations of paclitaxel and COL-3 that would produce the identical inhibitory effect as the combination regimen

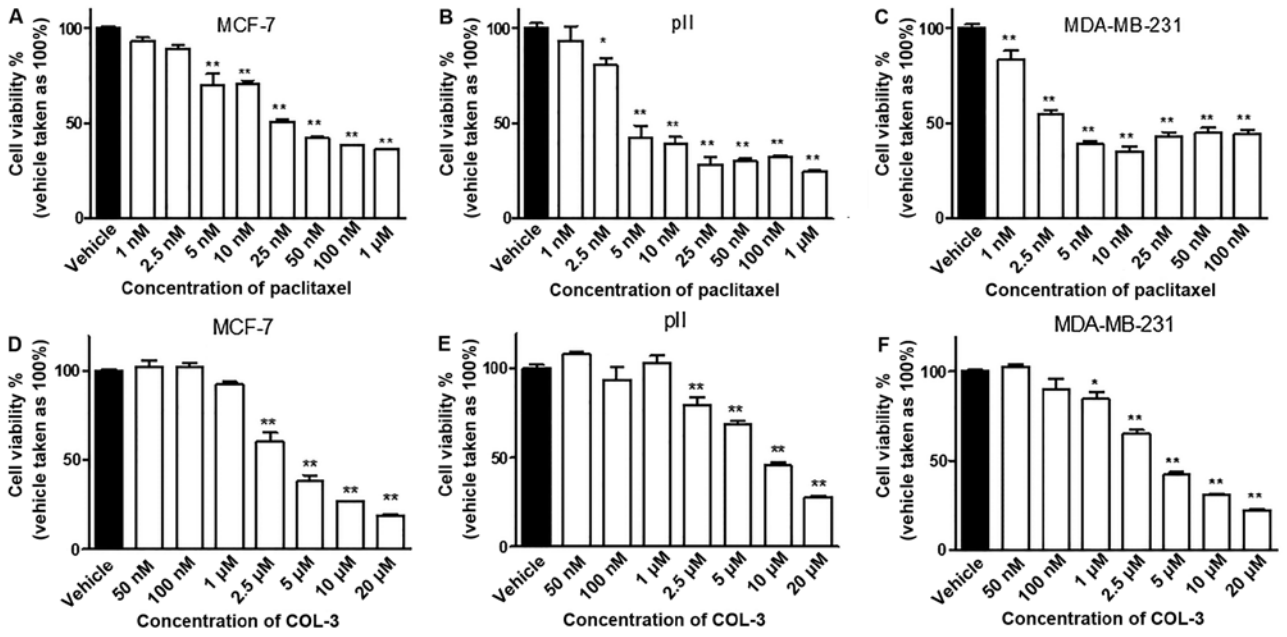

Figure 1. Effects of monotherapy with paclitaxel or COL-3 on cell viability. Growth was assessed with an MTT assay after 72 h of incubation with drugs or their vehicle. (A) MCF-7 cell viability following treatment with vehicle and different concentrations of paclitaxel. Each bar represents the mean ± standard error of the mean of 3-8 determinations. (B) pII cell viability following treatment with vehicle and different concentrations of paclitaxel. Each bar represents the mean ± standard error of the mean of 3-8 determinations. (C) MDA-MB-231 cell viability following treatment with vehicle and different concentrations of paclitaxel. Each bar represents the mean ± standard error of the mean of 12 determinations. (D) MCF-7 cell viability following treatment with vehicle and different concentrations of COL-3. Each bar represents the mean ± standard error of the mean of 6-10 determinations. (E) pII cell viability following treatment with vehicle and different concentrations of COL-3. Each bar represents the mean ± standard error of the mean of 4-13 determinations. (F) MDA-MB-231 cell viability following treatment with vehicle and different concentrations of COL-3. Each bar represents the mean ± standard error of the mean of 9 determinations. *P<0.05 and **P<0.01, compared with the vehicle-treated group. COL-3, chemically modified tetracycline-3.

Table I. Comparison of the anti-proliferative effects of combined concentrations of pac and COL-3 with the calculated individual concentrations of pac or COL-3 that would produce equivalent effect as combination for all the cell lines.

			Calculated individual concentration that produces equivalent % inhibition of cell viability as the combination regimen			Folds of reduction of the drug concentration in the combination regimen vs. individual drug treatment	
Cell lines	Combined concentrations	% inhibition of cell viability of the combination regimen	Pac (nM)	COL-3 (μM)	CI	Pac (nM)	COL-3 (μM)
MCF-7	2.5 nM pac + 5 μM COL-3	62.1	58.8	6.6	0.99	23.5	1.32
	5 nM pac + 2.5 μM COL-3	43	27.2	3	1.17	5.44	1.2
	10 nM pac + 2.5 μM COL-3	49	34.6	4	1.09	3.5	1.6
pII	2.5 nM pac + 5 μM COL-3	63.1	14.7	17.2	0.51	6	3.4
	5 nM pac + 5 μM COL-3	67.8	18.1	21.3	0.58	4	4.3
	10 nM pac + 5 μM COL-3	71	21.2	24.9	0.767	2.1	4.98
MDA-MB-231	2.5 nM pac + 1 μM COL-3	59.5	9.6	6.5	0.45	3.8	6.5
	2.5 nM pac + 2.5 μM COL-3	56.7	8.5	5.8	0.85	3.4	2.3

CI, combination index; COL-3, chemically modified tetracycline-3; pac, paclitaxel.

were 34.6 nM and 4 μM, respectively. Thus, by combining the drugs, the concentration of paclitaxel and COL-3 would be reduced by 3.5- and 1.6-fold, respectively (Table I).

For pII cells, the combination regimen resulted in synergistic inhibitory effects on cell proliferation. Combination of 2.5 nM paclitaxel with 5 μM COL-3 resulted in a synergistic effect

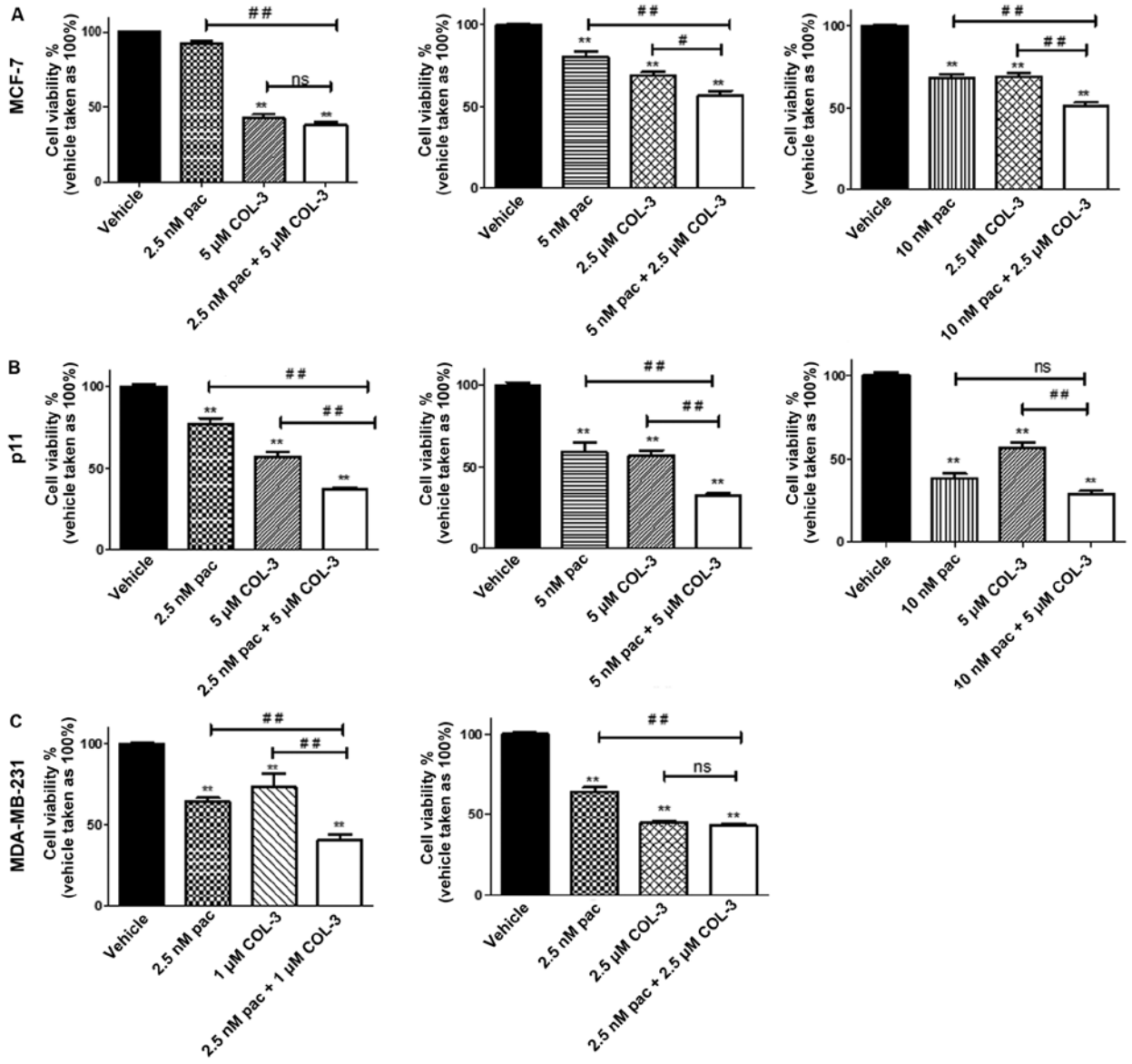

Figure 2. Effect of combination regiments on cell viability. Growth was assessed with an MTT assay after 72 h of incubation with drugs or their vehicle. (A) MCF-7 cell viability following treatment with vehicle and different concentrations of paclitaxel and COL-3 alone and in combination. Each bar represents the mean ± standard error of the mean of 12-20 independent determinations. (B) pII cell viability following treatment with vehicle and different concentrations of paclitaxel and COL-3 alone and in combination. Each bar represents the mean ± standard error of the mean of 9-12 independent determinations. (C) MDA-MB-231 cell viability following treatment with vehicle and different concentrations of paclitaxel and COL-3 alone and in combination. Each bar represents the mean ± standard error of the mean of 9-21 independent determinations. **P<0.01, compared with the vehicle-treated group. $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with the monotherapy groups. COL-3, chemically modified tetracycline-3; pac, paclitaxel; ns, not significant.

(CI=0.51; Table I). It also significantly inhibited cell proliferation by 63%, compared with treatment with individual drugs, which achieved 30-40% inhibition (P<0.01; Fig. 2B). Additionally, the calculated individual concentrations of paclitaxel and COL-3 that would produce the identical inhibitory effect as the combination regimen were 14.7 nM and 17.2 μM, respectively. Thus, by combining the drugs, the concentration of paclitaxel and COL-3 would be reduced by 5.9- and 3.4-fold, respectively (Table I). Similarly, combination of the highest concentration of paclitaxel (5 nM) with the highest concentration of COL-3 (5 μM) resulted in a synergistic inhibitory effect on cell proliferation (CI=0.58). It also significantly inhibited cell proliferation by 67.8%, compared with treatment with individual drugs, which achieved 40-50% inhibition (P<0.01; Fig. 2B). Furthermore, the calculated individual concentrations of paclitaxel and COL-3 that would produce the identical inhibitory effect as the combination regimen were 18.1 nM and 21.3 μM, respectively. Thus, by combining an increased concentration of paclitaxel with an increased concentration of COL-3, the concentration of paclitaxel and COL-3 would be reduced by 3.6- and 4.3-fold, respectively (Table I). However, combining 10 nM paclitaxel, which caused >50% inhibition, with the identical concentration of COL-3 (5 μM) resulted in moderate synergism due to it having a slightly increased inhibitory effect, compared with paclitaxel alone (70 vs. 60% reduction, respectively; P>0.05; Fig. 2B).

With regards to MDA-MB-231 cells, the combination regimen also resulted in synergistic inhibitory effects on cell proliferation. Synergism was achieved when combining 2.5 nM paclitaxel with 1 μM COL-3 (CI=0.45; Table I), which also significantly inhibited cell proliferation by 60%, compared

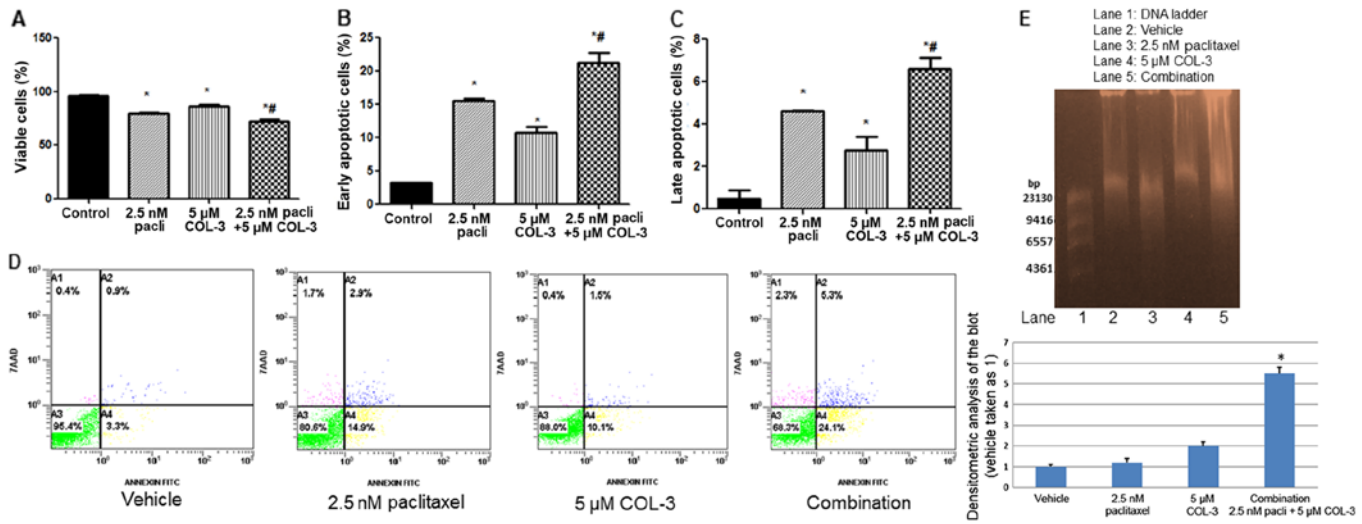

Figure 3. Effect of combination regimens on pII cell apoptosis. Apoptosis was determined using PE Annexin V/7-AAD. Samples were analyzed by flow cytometry after 72 h of incubation with the vehicle, 2.5 nM paclitaxel, 5 μM COL-3 or their combination. (A) Cell viability following treatment with vehicle or drugs. (B) Early apoptotic cells following treatment with vehicle or drugs. (C) Late apoptotic cells following treatment with vehicle or drugs. Each bar represents the mean ± standard error of the mean of 6 independent determinations. (D) Quadrant graphs of the Annexin V/7-AAD data depicted in panels A-C. (E) DNA fragmentation image for the different treatment groups along with the densitometric analysis (using ImageJ software). Using a DNA ladder, it was observed that the DNA fragments produced by the combination regimen are >23,130 bp. Each bar represents the mean ± standard error of the mean of 3 independent determinations. *P<0.05, compared with the vehicle-treated group. #P<0.05, compared with combination and individual drugs. COL-3, chemically modified tetracycline-3; pac, paclitaxel; 7-AAD, 7-aminoactinomycin D.

with treatment with individual drugs, which achieved 30-40% inhibition (P<0.01, Fig. 2C). The calculated individual concentrations of paclitaxel and COL-3 that would produce the identical inhibitory effect as the combination regimen were 9.6 nM and 6.5 μM, respectively. Thus, by combining the drugs, the concentration of paclitaxel and COL-3 would be reduced by 3.8- and 6.5-fold, respectively (Table I). Combining 2.5 nM paclitaxel with 2.5 μM COL-3 resulted in a slight synergistic inhibitory effects on cell proliferation, although the combination did not have a significant inhibitory effect, compared with COL-3 alone (57 vs. 55%, respectively; P>0.05. Fig. 2C).

Notably, the ER-ve cell lines were more responsive to the combination regimens, which resulted in synergistic inhibitory effects on cell proliferation, compared with the ER+ve cell line.

Effects of paclitaxel, COL-3 or their combination on pII cell apoptosis and cell cycle. The rational for using different concentrations of COL-3 or paclitaxel was due to the different sensitivities of the cell lines to the effects of the drugs. A concentration, which inhibited cell proliferation by ~50% in the combination regiments, was selected for the subsequent experiments.

At the tested concentrations, treatment with 2.5 nM paclitaxel or 5 μM COL-3 alone significantly decreased viable cells and increased early/late apoptotic cells, compared with treatment with vehicle (P<0.05; Fig. 3A-C). The combination regimen resulted in an enhanced effect, a significantly decreased number of viable cells and an increased percentage of early/late apoptotic cells, compared with monotherapy (P<0.05; Fig. 3A-C). Fig. 3D depicts representative examples of the dot-blot for the FACS data presented in Fig. 3A-C. In the DNA fragmentation analysis, slight DNA fragmentation was observed in vehicle treated cells (48 h) and with monotherapy with COL-3 or paclitaxel in agreement with Annexin-V/7AAD data (Fig. 3D). The combination regimen resulted in a significantly increased percentage of apoptotic cells and DNA fragmentation, compared with vehicle or monotherapy with COL-3 or paclitaxel (P<0.05), as depicted in Fig. 3D and E.

Treatment with paclitaxel at a concentration of 100 nM, significantly decreased (P<0.01) the cells in the G_0/G_1 phase, from 75.3±1.2% to 8.4±1.9%, and increased (P<0.05) cells in the G_2/M phase, from 12.0±0.5% to 27.8±1.1%, compared with the vehicle treatment; thus, it arrested the cell cycle at the G_2/M phase (Fig. 4A). Treatment with COL-3 at a concentration of 10 μM, but not 5 μM, significantly decreased cells in the S and G_2/M phases, from 9.9±1.2 to 5.8±0.7% and from 16.3±2.7 to 6.9±0.6%, respectively, while it increased cells in the G_0/G_1 phase, from 71±3 to 82±1%, compared with the vehicle treatment; thus, it arrested the cell cycle at the G_0/G_1 phase (P<0.05; Fig. 4B). Treatment with a reduced concentration of paclitaxel 2.5 nM also decreased the cells in G_0/G_1 phase, but to a lesser extent, compared with the 100 nM concentration (Fig. 4C). Paclitaxel 2.5 nM, but not COL-3 5 μM, significantly decreased the cells in the G_0/G_1 phase, compared with vehicle (P<0.05; Fig. 4C). The combination of paclitaxel and COL-3 had similar effects on the cell cycle to paclitaxel alone but resulted in a synergistic effect in enhancing the number of cells in the apoptotic phase, confirming the data presented in Fig. 3. Fig. 4D depicts the flow cytometry graphs for each treatment condition.

Effects of paclitaxel, COL-3 or their combination on the expression level of phosphokinases and apoptosis-associated proteins. No statistically significant effects on the expression levels of phosphokinases or apoptosis-associated proteins were observed following treatment with paclitaxel, COL-3 or their combination, which may be due to variation in the expression of phosphokinases or proteins between the samples analyzed (Fig. 5A and B). However, there was an increased

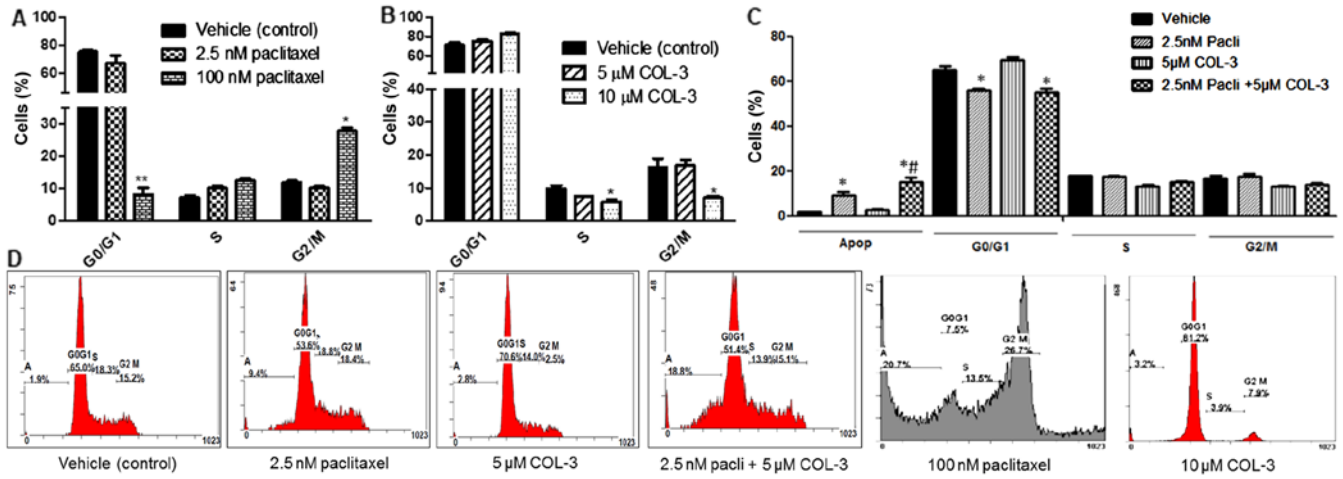

Figure 4. Effect of combination regimens on pII cell cycle. Cells were seeded in a 24-well plate, allowed to attach overnight and treated after 24 h with vehicle or different concentrations of (A) paclitaxel (2.5 and 100 nM), (B) COL-3 (5 and 10 μM) or (C) their combination (2.5 nM paclitaxel and 5 μM COL-3). Samples were then analyzed by flow cytometer after 72 h of incubation with the drugs. Each bar represents the mean ± standard error of the mean of 6 independent determinations. *P<0.05 and **P<0.01, compared with the vehicle-treated group. (D) Flow cytometry images for the various treatment groups. COL-3, chemically modified tetracycline-3.

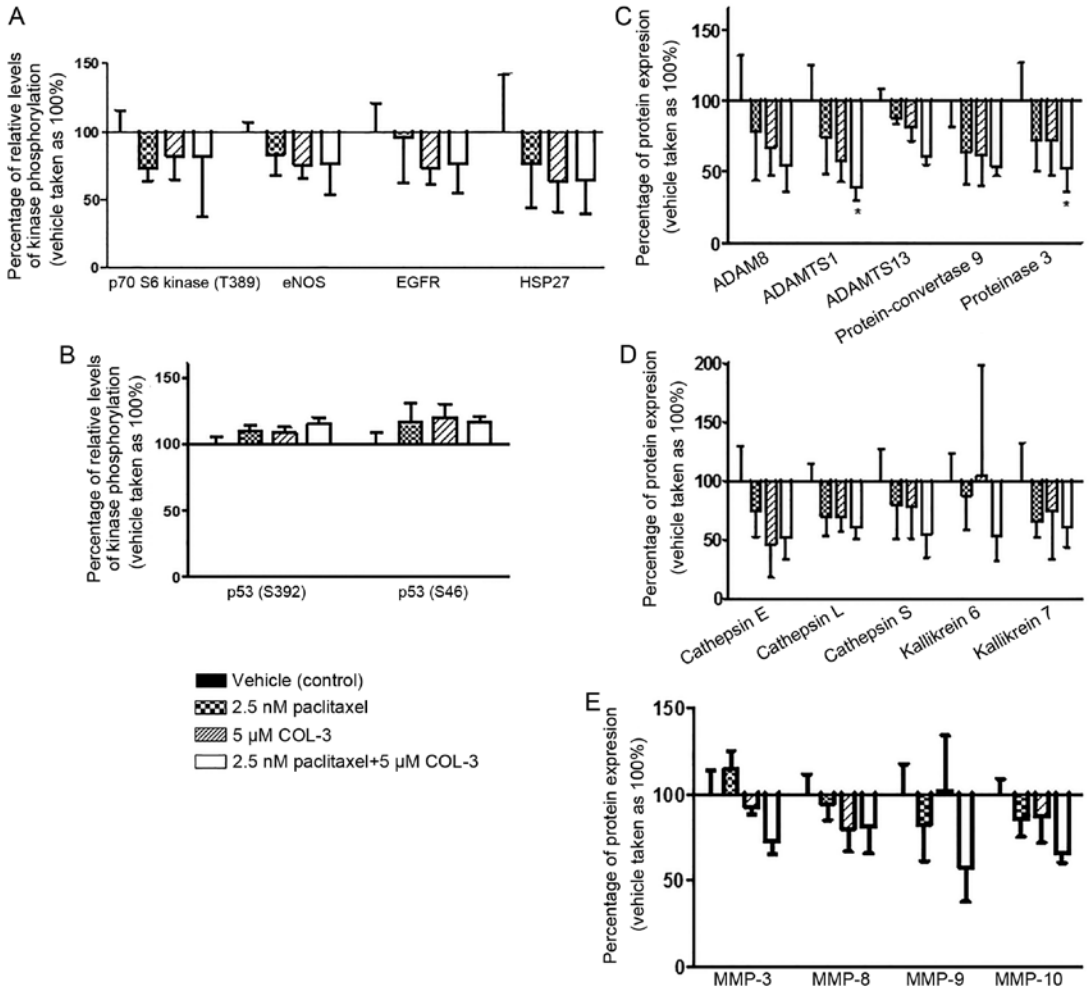

Figure 5. Effect of combination regimens on the phosphorylation levels of tested kinases and apoptosis-associated proteins in pII cells. Lysates of cells were collected after 24 h of incubation with the tested drugs. Densitometric values are means normalized to the mean of the reference spots on each blot, and the percentage of relative expression levels of selected phosphokinases and apoptosis-associated proteins was determined (vehicle set as 100%) (A) Expression levels of p70S6 kinase (T389), eNOS, EGFR and HSP27. (B) Expression levels of p53 at S392 and S46. (C) Expression levels of ADAM8, ADAMTS1, ADAMTS13, protein-convertase 9 and proteinase 3. (D) Expression levels of cathepsins E, L and S, and kallikreins 6 and 7. (E) Expression levels of MMPs -3, -8, -9 and -10. Each bar represents the mean ± standard error of the mean of 4 independent experiments. *P<0.05, compared with the vehicle treated group. eNOS, epithelial nitric oxide synthase; EGFR, epidermal growth factor receptor; HSP27, heat shock protein 27; COL-3, chemically modified tetracycline-3; MMP, matrix metalloproteinase; ADAMTS1, ADAM metallopeptidase with thrombospondin type 1 motif 1.

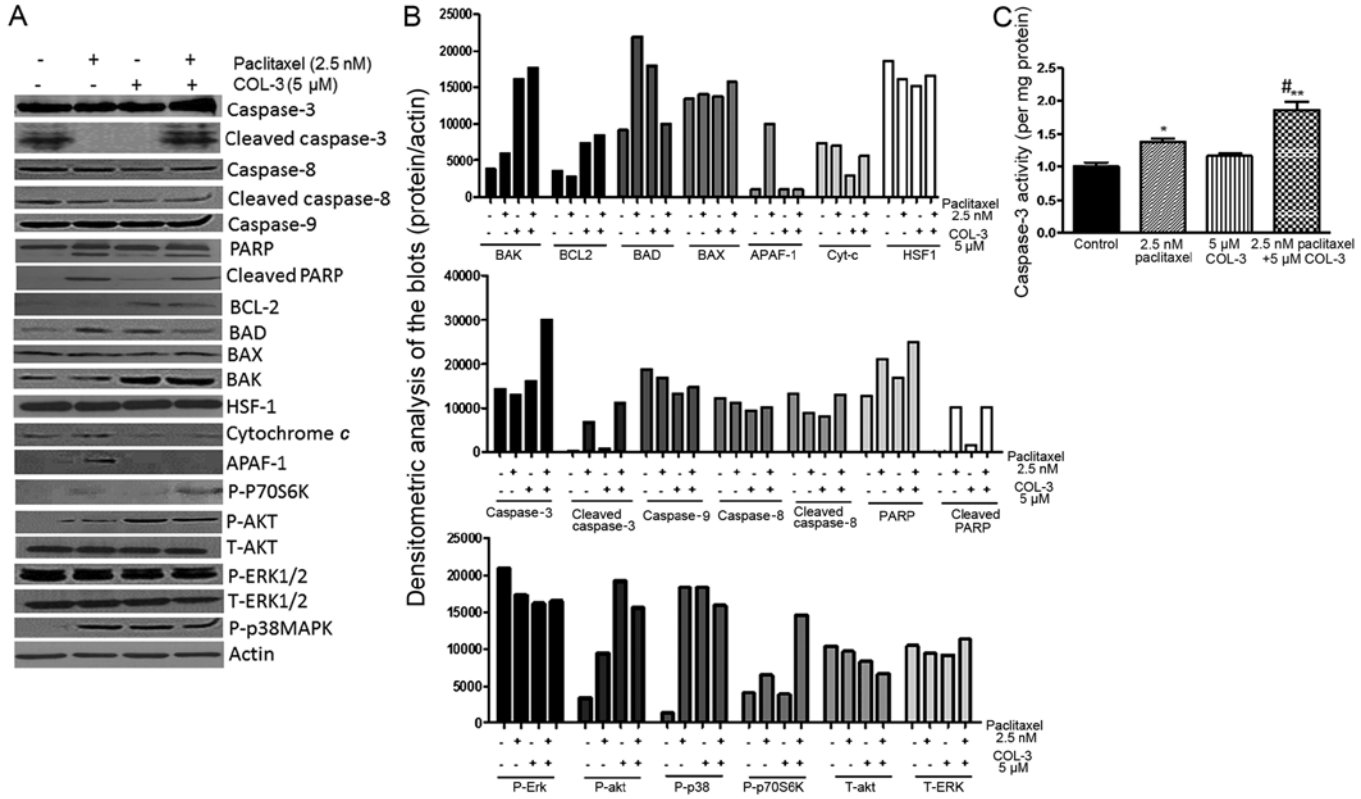

Figure 6. Effect of drug treatment on the expression and phosphorylated levels of various signaling molecules and caspase-3 activity. (A) Cells were treated with paclitaxel, COL-3 or combination regimen for 48 h. Total protein lysate (3 μg) was electrophoresed on 10% SDS-PAGE, blotted onto nitrocellulose membrane and probed with antisera to P- or T-BAX, BAD, BAK, BCL-2, cytochrome *c*, AKT, ERK1/2, p38 MAPK, APAF-1, HSF-1, and un-cleaved or cleaved-caspase-3, -8, -9, and PARP, and β-actin (loading control). This blot represents 1/3 experiments. (B) Densitometric analysis of the tested markers (normalized to β-actin). (C) Caspase-3 activity for pII cells treated with paclitaxel, COL-3 or combination regimen for 48 h. Histobars represent the mean ± standard error of the mean of 3 independent determinations, *P<0.05 and **P<0.01, compared with the UT group. $^{\#}$P<0.05, compared with the paclitaxel-treated group. p-, phospho-; t-, total-; UT, untreated; PARP, poly(ADP-ribose) polymerase; BCL-2, B-cell lymphoma-2; BAD, BCL-2 associated agonist of cell death; BAX, BCL-2-associated X; BAK, BCL-2 antagonist/killer; HSF-1, heat shock transcription factor-1; APAF-1, apoptotic peptidase activating factor 1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-associated protein kinase; COL-3, chemically modified tetracycline-3; pac, paclitaxel.

phosphorylation level of p53 at S392 and S46 in cells treated with monotherapy or with the combination regimen, compared with the vehicle-treated cells (Fig. 5B). Treatment with paclitaxel or COL-3 alone increased phosphorylated p53 at S392 by 10% and at S46 by 20%, while the combination regimen increased it by 15.5 and 17%, respectively. However, a trend of decreased phosphorylation level of heat shock protein 27 (S78/S82), p70S6 kinase (T389), epithelial nitric oxide synthase (S1177) and EGFR (Y1086) was observed in all of the treatment regimens, compared with the vehicle-treated cells (Fig. 5A).

Among the 35 tested proteases, the expression of the proteases ADAM metallopeptidase with thrombospondin type 1 motif 1 (ADAMTS1) and proteinase 3 (PR3) was significantly reduced by the combination regimen, compared with the vehicle-treated cells (61 and 48% respectively; P<0.05; Fig. 5C), but this difference was not significant with paclitaxel or COL-3 monotherapy. However, a trend towards decreased expression was observed among 12 other proteases in cells treated with monotherapy and/or the combination regimen, compared with the vehicle-treated cells. These proteases include ADAM8, ADAMTS13, cathepsin E, cathepsin L, cathepsin S, kallikrein 6, kallikrein 7, MMP-3, MMP-8, MMP-9, MMP-10 and protein-convertase 9 (Fig. 5C-E).

Effect of paclitaxel, COL-3 or their combination on the expression/phosphorylated levels of various signaling molecules. Fig. 6A and B depicts western blot analysis for the expression/phosphorylated/cleaved levels of various downstream signaling molecules important for cell survival, proliferation and motility/invasion. The combination regimen enhanced the expression of caspase-3, cleaved caspase-3 and phosphorylated P70S6K, which is a downstream target of mammalian target of rapamycin (mTOR) (28). However, COL-3 treatment (alone or in combination with paclitaxel) enhanced the expression levels of BCL-2 and BAK, which are important in cell apoptosis (29), as well as the phosphorylated AKT, the downstream target of phosphoinositide 3-kinase (28). The expression levels of cytochrome *c* and APAF-1 were decreased with COL-3 treatment, compared with paclitaxel monotherapy. The expression levels of BAD and phosphorylated p38 MAPK were increased with all treatment regiments, compared with untreated cells. However, the expression/phosphorylated/cleaved levels of caspase-8 and -9, BAX, ERK1/2, and HSF-1 were not modulated in all the tested groups. The expression profile of the total and the cleaved form of PARP was enhanced by paclitaxel, but not COL-3, treatment as well as with the combination regimen. Notably, the absence of detection of the total levels of P70S6K and p38 MAPK is a limitation of the present study. However, the total levels of ERK1/2 and AKT were not modulated,

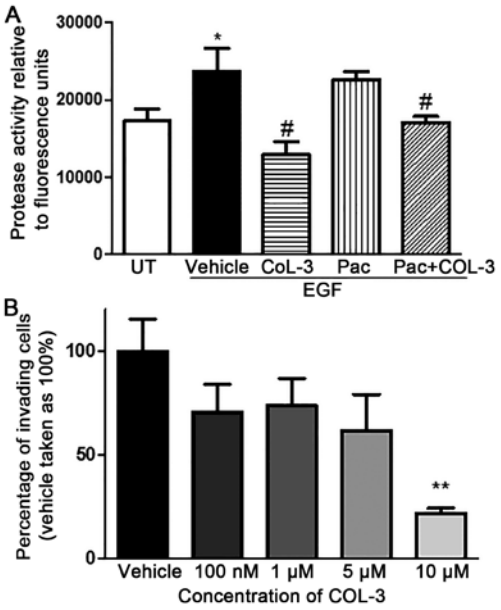

Figure 7. Effect of drug treatment in total MMP activity and invasion of pII cells. (A) Cells were left untreated (UT) or stimulated with EGF (100 ng/ml) with or without pre-treatment with individual drugs or combination regiment, and total MMP activity was determined in the supernatant. Histobars represent the mean ± SEM of 6 independent determinations, *P<0.05, compared with the UT group. $^{\#}$P<0.05, compared with the EGF/vehicle treated group. (B) Cells were left untreated (vehicle) or exposed to various concentrations of COL-3. The total number of pII cells penetrating through a basement membrane extract towards serum components was determined by fluorometric analysis. Histobars represent the mean ± SEM of 6 independent determinations. **P<0.01, compared with the vehicle-treated group. SEM, standard error of the mean; COL-3, chemically modified tetracycline-3; pac, paclitaxel; MMP, matrix metalloproteinase; UT, untreated; EGF, epidermal growth factor.

although the phosphorylated AKT level was modulated, and also the β-actin bands indicate equal loading. This indicated that the phosphorylated levels of a number of the tested molecules are modulated without affecting their total protein levels. These data demonstrated that the observed synergistic effect of the combination regimen is partially through increased total and cleaved caspase-3 levels, and P70S6K phosphorylation in pII cells.

Effect of paclitaxel, COL-3 or their combination on caspase-3 activity. Treatment with paclitaxel, but not COL-3, significantly increased caspase-3 activity in pII cells, compared with vehicle treatment (P<0.05). The combination regimen resulted in an enhanced effect and significantly increased caspase-3 activity, compared with monotherapy (P<0.05; Fig. 6C).

Effect of paclitaxel, COL-3 or their combination on total MMP activity. EGF stimulation of pII cells significantly increased the total MMP activity, compared with vehicle treatment (P<0.05; Fig. 7A). COL-3, but not paclitaxel, treatment blocked the EGF-induced MMP activity to baseline levels, and the combination regimen had similar effects to COL-3 monotherapy.

Effects of COL-3 on pII cell invasion. COL-3 produced a concentration-dependent inhibitory effect on pII cell invasion in the Cultrex® 24-well invasion assay and reached statistical significance at 10 μM, compared with vehicle (78% inhibition; P<0.01; Fig. 7B).

Discussion

The present study demonstrated that the chemically modified tetracycline COL-3 enhances the anticancer effects of paclitaxel against ER+ve and ER-ve breast cancer cell lines. COL-3 on its own had anti-proliferative effects against ER+ve and ER-ve breast cancer cell lines, although it was less potent than paclitaxel. There was synergistic anti-proliferative activity between COL-3 and paclitaxel against ER-ve breast cancer cell lines, and the anti-proliferative effects varied from notably additive to slight antagonism against the ER+ve cell line. Reduced concentrations of the drugs in combination produced similar anti-proliferative activity, compared with high concentrations of paclitaxel or COL-3 alone. The synergistic anti-proliferative activity of the drug combination against the highly invasive and proliferative ER-ve breast cancer cell line pII may be due to the enhanced anti-apoptotic activity, but not cell cycle arrest. Additionally, COL-3 inhibited pII cell invasion and MMP activity *in vitro*. The combination of paclitaxel and COL-3 also significantly increased the expression and activity of caspase-3 and phosphorylated P70S6K, a downstream target of the mTOR pathway (28). The combination inhibited the expression of two proteases, ADAMTS1 and PR3.

Although paclitaxel is considered an integral agent in the treatment of breast cancer and other solid tumor types, the dose-dependent side effects associated with the development of peripheral neuropathy (PIPN) limits its use and may render the treatment ineffective (14,30). Identification of agents that prevent PIPN while preserving the anticancer effectiveness of paclitaxel would allow for the treatment of aggressive cancer cases that require high chemotherapeutic doses, and improve the quality of life for patients with cancer and survivors of cancer who are affected by chemotherapy-induced neuropathy. Taking into consideration the indicated role of MMPs and proteinase-activated receptors in the development of paclitaxel-induced neuropathy (31,32), as well as the previously observed protective effect of the potent MMP inhibitor COL-3 against paclitaxel-induced thermal hyperalgesia in mice (16), and its indicated potential as an anticancer drug (20,33), the present study evaluated whether COL-3 affects the anticancer activity of paclitaxel using various breast cancer cell lines.

Paclitaxel significantly inhibited cell proliferation in all the tested cell lines in a concentration-dependent manner, which is consistent with previous *in vitro* reports, where it was reported to have concentration-dependent cytotoxicity on a variety of human tumor cell lines, including those derived from the breast carcinoma, lung adenocarcinoma, cervical carcinoma and colon carcinoma (34-37). Additionally, no further increase in cell killing was observed at paclitaxel concentrations >50 nM, similar to what has been reported previously (34). Furthermore, the degree of sensitivity of the cell lines to paclitaxel was variable. Paclitaxel was more effective against the ER-ve cell lines, compared with the ER+ve cell line, which may be due to the increased proliferative rate of the ER-ve cells, compared with the ER+ve cells. The present data are in agreement with previous studies that demonstrated that MDA-MB-231 and other ERα-silenced cells (derived

from MCF-7 cells) were more sensitive to the anti-proliferative effects of paclitaxel, compared with the ER+ve cells (38,39). Similarly, COL-3 significantly inhibited cell proliferation in all the tested cell lines in a concentration-dependent manner. Previous studies with COL-3 have also indicated that it has a strong ability to inhibit the proliferation of various cancer cells, including those from the prostate, colon, cervical and breast, in a concentration-dependent manner (40-44). However, the degree of sensitivity of the tested cell lines to COL-3 was variable. Unlike the case for paclitaxel, the ER status may not affect the sensitivity to COL-3. The present data are in contrast with previous studies that determined that COL-3 was not cytotoxic at concentrations up to 50 μM or 20 μg/ml against the breast cancer cell lines MDA-MB-468 and MMP-9-overexpressing sub-clone (E10) of MDA-MB-231, respectively (40,43). This difference could be due to the different cell lines used, the reduced period of incubation (48 h) used in the previous studies (40,43) or different experimental designs, including cells being plated onto extracellular matrix (ECM)-coated wells (40).

The combination regimens of paclitaxel and COL-3 had synergistic anti-proliferative effects against ER-ve cell lines and the anti-proliferative effects varied from notably additive to slightly antagonism on the ER+ve cell line. The synergistic anti-proliferative effect observed against the ER-ve pII cells was partially via the induction of apoptosis rather than inducing cell cycle arrest. Additionally, it was possible to reduce the concentrations of paclitaxel as well as COL-3 when the drugs were used in combination, while producing the identical degree of inhibition in cell proliferation as increased concentrations of each drug alone. Thus, the combination could assist in reducing the risk of developing dose-dependent side effects, including PIPN. It may also be possible to reduce the risk of developing cutaneous photo-toxicity, which is the dose-limiting side effect of COL-3 (34). A previous study demonstrated that the combination of paclitaxel with the novel epigenetic agent phenethyl isothiocyanate (PEITC) had a synergistic effect on the inhibition of growth of breast cancer cells (MCF-7 and MDA-MB-231), as well as increasing apoptosis and cell cycle arrest at the G_2/M phase (35). This indicates that it is possible to reduce the dosage of paclitaxel when it is used in combination with other drugs, which could reduce the dose-dependent side effects, while maintaining its clinical efficacy against breast cancer cells and other solid tumor types. In that study, they also observed an antagonistic effect when combining a number of doses, including 1 nM paclitaxel with 5 or 10 μM PEITC (35). Another previous study demonstrated that paclitaxel combined with a cannabidiol produced an additive to a synergistic inhibition of breast cancer cell viability (45). Cannabidiol was also indicated by the identical research group to prevent the onset of paclitaxel-induced mechanical and thermal sensitivity in rodents (46). However, the concentrations of paclitaxel that were used ranged from 2.5-10 μM, which are 1,000-fold increased, compared with the concentrations used in the present study (2.5-10 nM).

Numerous studies demonstrated that paclitaxel kills cancer cells through the induction of apoptosis (11,47-50). Previous studies have also indicated that COL-3 induces apoptosis as a mechanism of its cytotoxicity. It was also reported that COL-3 causes caspase-dependent and -independent apoptosis (42,44,51). In the present study, it was determined that the tested concentration of paclitaxel (2.5 nM) had pro-apoptotic effects on pII cells, which differs from what was reported by Jordan *et al* (11) on cervical cancer HeLa cells using a concentration of 3 nM. COL-3 treatment at increased concentration (5 μM) also significantly decreased viable cells and increased early/late apoptotic cells similar to what was reported in prostate, colon and cervical cancer cells using the identical concentration range (42,44,51). Notably, the combination regimen enhanced the percentage of early/late apoptotic cells. This indicated that the synergistic inhibitory effect on cell proliferation is partially due to the induction of cell apoptosis.

It is considered that stabilization of microtubules of paclitaxel induces mitotic arrest at the G_2/M phase of the cell cycle (47,52). However, it was reported that at low concentrations, paclitaxel increases cells in the sub-G_1 and G_1 phases, indicating G_1 arrest (52). Additionally, studies using bromodeoxyuridine for DNA labeling indicated that cells were not arrested in the G_1 phase directly (47,52). Instead, G_1 cells went through the S phase and then divided to generate 2C DNA cells, indicating that G_1 arrest does not occur in the first cell cycle, and did not prevent cells from passing through S phase and entering mitosis, but rather in the second or third cell cycle (52). However, COL-3 was determined to cause cell cycle arrest at the G_1/S phase transition in prostate and cervical cancer cells, indicating that COL-3 inhibits mitogenic signaling (42,44). The exact mechanism by which it arrests the cell cycle remains unclear due to limited studies investigating its cytotoxic effects. In agreement with previous reports, the cell cycle arrest at the G_2/M phase was observed in cells treated with increased concentration of paclitaxel (100 nM) with a significant decrease in G_0/G_1 cells, whereas the cell cycle arrest at G_0/G_1 was observed at the increased concentration of COL-3 (10 μM). The reduced concentration of COL-3 (5 μM) did not have a significant effect on the cell cycle in the G_1 or G_2/M phases. However, the reduced concentration of paclitaxel (2.5 nM) and the combination of these concentrations, which induced apoptosis as aforementioned, similarly reduced cells in the G_0/G_1 phases of the cell cycle. Previously, it was reported that mitotic arrest is not only responsible for the efficacy of paclitaxel, but also for chromosome mis-segregation on highly abnormal, multi-polar spindles that results in cell death, which may be also the case in the present study (37). Thus, performing DNA labeling should be considered in future studies to examine the effect of the reduced concentrations of paclitaxel, COL-3 or their combination on the cell cycle.

COL-3 treatment significantly reduced cell invasion partially through inhibiting total MMP activity. CMTs have been demonstrated to inhibit the enzymatic activities of gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), collagenases (MMP-1, MMP-8 and MMP-13) as well as non-collagenolytic proteases (53-55). COL-3 has been demonstrated to significantly reduce cancer cell invasion and migration *in vitro* by inhibiting MMP activity and/or expression and upregulating the expression of E-cadherins (41-43,56). The majority studies have reported its specificity towards gelatinases not only by inhibiting their activity but also their expression levels (41-43). In addition to its anti-MMP function, COL-3 directly inhibits the amidolytic (proteolytic) activity of human leukocyte elastase (HLE), a serine proteinase also

termed neutrophil elastase, and the extracellular matrix degradation mediated by HLE (19). Thus, the pleiotropic properties of CMT-3, which includes, but is not limited to, the inhibition of serine proteinases (19), MMPs (19,20) and cytokines (57), provide therapeutic potential to reduce excessive connective tissue breakdown during various pathologic processes, including inflammatory diseases, cancer metastasis and metabolic bone diseases. Previous studies using the MDA-MB-468 cells demonstrated a concentration-dependent inhibition of invasion, with 67% inhibition observed with 10 μg/ml (30 μM) COL-3 (43,47,52). Additionally, culturing E-10 cells, a transfected subclone of MDA-MB-231 cells, in 10 or 20 μM COL-3 diminished secreted MMP-9 levels by 45 or 60%, respectively, as well as inhibited their ECM degradative ability by 20-30% (43). Consistent with the previous studies, COL-3 demonstrated a concentration-dependent inhibition of pII cell invasion with a significant inhibition observed at 10 μM. Consequently, the effects of COL-3 as well as paclitaxel and their combination on the expression level of various proteases were further evaluated. Among the 35 tested proteases, the expression of ADAMTS1 and PR3 were significantly decreased in cells treated with the combination of COL-3 and paclitaxel.

In conclusion, the present study indicates that COL-3 potentiates the anticancer activity of paclitaxel by enhancing its inhibitory effects on cell proliferation, inducing apoptosis as well as inhibiting invasiveness. The molecular mechanism may involve the modulation of the expression of proteases, including ADAMTS1 and PR3, caspase-3 expression/activity and P70S6K phosphorylation. Furthermore, the combination regimen would also offer opportunities for the reduction in the effective dose of paclitaxel, which in turn would have further beneficial effects for the reduction of the dose-dependent side effects, including PIPN. This indicates that the combination of paclitaxel and COL-3 in addition to reducing the development of PIPN, as recently reported (16), would also have enhanced anticancer activity against breast cancer. Therefore, the potential of the combination against breast cancer growth and metastasis *in vivo* warrants further research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAK and WM conceived and designed the experiments. RMET, PM and MAK performed the experiments. RMET, MAK, PM and WM analyzed the data. WM and MAK contributed reagents, materials and analysis tools. RMET, MAK and WM wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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