Piperlongumine-loaded nanoparticles inhibit the growth, migration and invasion and epithelial-to-mesenchymal transition of triple-negative breast cancer cells

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Received July 15, 2020; Accepted October 30, 2020

DOI: 10.3892/ijfn.2020.11

Abstract. Metastasis and disease relapse are the major causes of morbidity and mortality among patients with triple-negative breast cancer (TNBC). Novel therapeutics that interfere with the process of metastasis, including epithelial-to-mesenchymal transition (EMT), are thus urgently required. Piperlongumine (PL) is a component of the fruits of the long pepper plant (*Piper longum*), which are used as a spice and in traditional medicine. The present study compared the anti-metastatic potential of free PL and PL-loaded nanoparticles (PL-NPs) in TNBC cells. PL was loaded into biodegradable methoxy poly(ethylene glycol)-poly-(lactide-co-glycolic) acid copolymer NPs by thin-film hydration. The effects of free PL and PL-NPs on TNBC cells were compared using colorimetric MTT assays for cell growth/viability, Transwell assays for migration/invasiveness, and western blot analysis and reverse transcription-quantitative polymerase chain reaction for expression of EMT-associated proteins and DNA methyltransferase-1. PL-NPs reduced MDA-MB-231, MDA-MB-468, and BT-549 TNBC cell growth/viability to the same extent as free PL. Treatment of the MDA-MB-231 cells with both PL-NPs and free PL inhibited migration/invasiveness, reduced the expression of matrix metalloproteinase 2 and EMT-promoting Slug, ZEB1, N-cadherin, β-catenin and Smad3, promoted E-cadherin and anti-metastatic n-Myc downstream regulated gene 1 expression, and inhibited the expression of oncogenic DNA methyltransferase-1. On the whole, the present study demonstrated that PL-NPs inhibited the metastasis-promoting activities of TNBC cells to the same extent as free PL, highlighting the feasibility of employing NPs for the delivery of PL to prevent or reduce TNBC metastasis.

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Key words: breast cancer, epithelial-to-mesenchymal transition, metastasis, nanoparticles, piperlongumine

Introduction

Breast cancer is classified into 5 distinct molecular subgroups: Normal-like, basal-like, luminal A, luminal B and human epidermal growth factor receptor (HER) 2-enriched (1-3). The majority of basal-like breast cancer cells fail to express estrogen receptor, progesterone receptor and HER2, and are therefore termed triple-negative breast cancer (TNBC) (3,4). Patients with TNBC do not benefit from hormonal (tamoxifen) and anti-HER2 antibody (trastuzumab) therapies (1,5). The lack of targeted therapies together with the high chance of lymph node involvement, high rates of metastasis, high tumor grade/size at the time of diagnosis, and high rates of relapse leads to a poor prognosis for TNBC (5).

Despite advances in treatment strategies and early detection, metastases remain as the primary cause of cancer-related mortality (5). Metastasis is a complex multi-step process involving genetic and/or epigenetic alterations that allow individual cancer cells to leave the primary tumor site, degrade and migrate through the extracellular matrix (ECM), intravasate into nearby blood and lymphatic vessels, then exit from the circulation into the parenchyma of distant tissue to form a metastatic lesion (6). Breast cancer primarily metastasizes to bone, brain, lung and liver (7). Metastasis is promoted by matrix metalloproteinases (MMPs) that degrade the ECM (8). Epithelial-to mesenchymal transition (EMT) also plays a central role in metastasis by allowing cancer cells to acquire a motile mesenchymal phenotype (9,10). The Wnt/β-catenin and transforming growth factor (TGF) β/Smad signaling pathways initiate the expression of a cascade of EMT-promoting transcription factors that include Snail, Slug, Twist1 and 2, and ZEB1 and 2 (9,10). Although E-cadherin was originally described as a tumor suppressor and the decreased expression of E-cadherin is a hallmark of EMT, recent findings indicate that E-cadherin both suppresses and promotes cancer (11). The hypermethylation of DNA by DNA methyltransferase 1 (DNMT1) results in the dysregulation of genes involved in TNBC tumorigenesis and progression, including EMT-promoting and metastasis-associated genes (12). Metastasis suppressors, such as n-Myc downstream regulated gene 1 (NDRG1), are involved in the inhibition of TGF-β/Smad and Wnt/β-catenin pathways, thereby suppressing EMT (13,14). Novel therapeutics that

target EMT and the metastatic process are required to reduce TNBC-associated mortality.

Certain bioactive dietary phytochemicals have attracted interest over the past few decades due to their potential for use in cancer prevention and treatment (15). Piperlongumine (PL), also known as piplartine, is an amide alkaloid found in long pepper plant (*Piper longum*) fruits that has a long history of use as a culinary spice, as well as in Ayurvedic medicine for the treatment of a variety of illnesses, including cancer (16,17). Laboratory studies have demonstrated that micromolar concentrations of PL are cytotoxic for multiple cancer cell types, including breast cancer cells (18-23). In TNBC cells, PL has been reported to induce apoptosis via the suppression of signal transducer and activator of transcription (STAT) 3 activation and the inhibition of phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling (22,23). Biocompatible nanoparticle (NP)-based drug delivery systems increase the effectiveness of anticancer drugs (24). NP delivery has the potential to increase the aqueous solubility and bioavailability of PL, as well as to further decrease its already low toxicity in preclinical models and clinical application (25,26). In this regard, the poly(ethylene glycol)-poly(lacti de-co-glycolic) acid (PEG-PLGA) polymer-based NP delivery of luteolin, a flavonoid with potent anticancer activity but poor pharmacokinetics, has been shown to result in more potent tumor growth inhibitory effects than the administration of free luteolin in a preclinical model of head and neck cancer (27). In addition, the authors have recently demonstrated that piperine, a major alkaloid of black pepper that is structurally similar to PL, retains its cytotoxic effect on TNBC cells when encapsulated in biocompatible methoxy poly(ethylene glycol)-poly(lactide-co-glycolic) acid (mPEG-PLGA) NPs (28).

The present study compared the effects of free PL and PL-loaded mPEG-PLGA NPs (PL-NPs) on the *in vitro* growth of 3 different TNBC cell lines, as well as the metastasis-promoting activities of MDA-MB-231 TNBC cells. Cell growth/viability, migration/invasion, and the expression levels of MMP2, NDRG1, EMT-associated molecules, and the epigenetic regulator, DNMT1, associated with cancer progression were determined by western blot analysis and/or reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Free PL and PL-NPs exerted a similar inhibitory effect on the growth and metastatic activities of TNBC cells, supporting the feasibility of the use of NPs to deliver PL for prevention or reduction of TNBC metastasis.

Materials and methods

Chemicals and reagents. Dimethyl sulfoxide (DMSO), 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride, Triton X-100, sodium deoxycholate, aprotinin, leupeptin, sodium fluoride, pepstatin A, dithiothreitol, polyamine oxidase, dichloromethane and gelatin were purchased from Sigma-Aldrich; Merck KGaA. L-glutamine, 10,000 units/ml penicillin/10,000 μg/ml streptomycin solution, 1M N-2 hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer solution, fetal bovine serum (FBS), and Dulbecco's modified Eagle medium (DMEM) were from Invitrogen; Thermo Fisher Scientific, Inc. mPEG-PLGA (5-10 kDa) was from Akina Inc. Acrylamide/bis-acrylamide (29: 1, 30% solution), ammonium persulfate, sodium dodecyl sulfate, Tris-base, Tween-20, tetramethylethelyenediamide, ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) were purchased from BioShop Canada Inc. Bio-Rad protein assay dye reagent concentrate and SsoFast EvaGreen™ Supermix® were from Bio-Rad Laboratories, Inc. PL, fibronectin and sodium orthovanadate were from EMD Millipore (Etobicoke, ON). ARP 100 was from Santa Cruz Biotechnology Inc..

Antibodies. Horseradish peroxidase (HRP)-conjugated rabbit anti-human β-actin monoclonal antibodies (Abs; cat. no. 12620), and rabbit monoclonal Abs against human β-catenin (cat. no. 8480), Slug (cat. no. 9585), ZEB1 (cat. no. 3396), pan-cadherin (cat. no. 4073), Smad3 (cat. no. 9520), NDRG1 (cat. no. 9485), and DNMT-1 (cat. no. 5032), were purchased from Cell Signaling Technology, Inc. Donkey anti-rabbit HRP-conjugated Abs (cat. no. sc-2313) were from Santa Cruz Biotechnology Inc. All Abs were diluted in 5% w/v fat-free milk or 5% w/v BSA, in Tween-TBS [20 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.05% Tween-20].

Cell lines and culture conditions. MDA-MB-231 human breast adenocarcinoma cells were kindly provided by Dr S. Drover (Memorial University of Newfoundland, St. John's, NL, Canada). MDA-MB-468 human breast adenocarcinoma cells were a generous gift from Dr P. Lee (Dalhousie University, Halifax, NS, Canada). BT-549 human breast ductal carcinoma cells were kindly provided by Dr P. Marcato (Dalhousie University, Halifax, NS, Canada). All breast cancer cell lines were free of mycoplasma contamination and were authenticated by the American Type Culture Collection (ATCC) using the short tandem repeat method. Breast cancer cells were cultured in DMEM that was supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM of L-glutamine, and 5 mM of HEPES; henceforth, known as complete DMEM. Cells were maintained at 37˚C in a humidified 10% CO_2 incubator.

NP formulation. NPs were prepared from mPEG-PLGA polymers using the thin-film hydration method, as previously described (28). In brief, 5 mg PL and 45 mg mPEG-PLGA were co-dissolved in 5 ml dichloromethane and transferred to a 250 ml round-bottom flask. The mixture was evaporated under vacuum using a rotary evaporator (Büchi Labortechnik) at 60˚C. The co-evaporation of PL and mPEG-PLGA resulted in a homogenous mixture in a form of a thin film coating the inner surface of the flask. The thin-film material was re-dissolved in 5 ml 0.9% w/v NaCl solution and stirred at 60˚C to allow the self-assembly of polymers into PL-containing micelles. The mixture was placed in a 14 kDa cut-off dialysis membrane and dialyzed against 0.9% NaCl solution at room temperature to remove any encapsulated PL. The 0.9% NaCl solution was replaced after 30 min, 2 and 4 h. The PL-NP preparation was then flash-frozen in liquid nitrogen and lyophilized. PL-NPs were reconstituted in sterile water and sonicated for 5 min using a Q125 ultrasonic probe at 50W output (QSonica L.L.C.) to obtain NPs of the desired size and further improve PL entrapment.

A JEM 1230 transmission electron microscope (JEOL Ltd.) and AMT Image Capture Engine (version 7.0; AMT Imaging) was used to image and measure 90 random particles, yielding an average NP size of 52.8±1.2 nm. Prior to use, the PL-NPs were filter-sterilized using a 0.20 μm syringe filter, which also removed polymer aggregates and any remaining PL crystals. The encapsulation efficiency was 20%, as determined by spectrophotometric analysis and a standard curve based on the absorbance of PL at 346 nm.

MTT assay. MDA-MB-231, MDA-MB-468 and BT549 cells were seeded into quadruplicate wells of a 96-well flat bottom cell culture plate at a concentration of $5x10^3$ cells/well and incubated overnight to allow cell attachment. Cells were then cultured for 48 h in the presence of medium alone, vehicle (DMSO) alone, 2.5-10 μM free PL (dissolved in DMSO) or PL-NPs, or empty NPs. MTT solution was added to each well to a final concentration of 0.5 μg/ml. Cell-free supernatant was removed and formazan crystals were solubilized in DMSO. The absorbance was measured at 570 nm using an Expert 96 microplate reader (Biochrom ASYS) and the percentage metabolic activity was determined.

Transwell migration and invasion assays. MDA-MB-231 cell monolayers were cultured for 36 h in the presence of the vehicle (DMSO) alone, 2.5 μM of free PL or PL-NPs, or empty NPs. The cells were then serum-starved for 12 h, harvested and resuspended at $1x10^6$ cells/ml in 1 ml of appropriate treatment made in serum-free DMEM. A 50 μl aliquot of the cell suspension was loaded into the upper chamber of a Transwell migration apparatus. The cells migrated through an 8 μm porous membrane that was uncoated for migration assays or coated with fibronectin (0.05% w/v) or gelatin (0.01% w/v) for invasion assays. Growth medium containing 10% FBS was used as a chemoattractant. Migrated cells were stained for 45 sec at room temperature with a Diff-Quik™ staining kit (Siemens Inc.), photographed using a Nikon Eclipse TS 100 phase contrast microscope and Infinity 1 camera (Nikon Canada Inc.), and quantified using ImageJ software (version 1.51; National Institutes of Health).

Western blot analysis. MDA-MB-231 cell monolayers were cultured for 48 h in the presence of the vehicle (DMSO) alone, 2.5 or 5 μM of free PL or PL-NPs, or empty NPs. Cells were collected and resuspended in 50 μl of cold lysis buffer [0.1% v/v NP-40, 0.25% w/v sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA pH 7.5] with a mixture of 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml apotinin, 100 μM sodium orthovanadate, 1 mM dithiothreitol, 10 mM sodium fluoride and 10 μM polyamine oxidase. Following 15 min of incubation at 4˚C, debris was removed by centrifugation at 14,000 x g for 10 min at 4˚C. The supernatant containing total cellular proteins was collected and quantified by Bradford protein assay. Protein samples (30 μg) were loaded into wells of a 10% sodium dodecyl sulfate-polyacrylamide gel and proteins were separated by electrophoresis. Proteins were transferred to a nitrocellulose membrane and blocked with 5% fat-free milk for 1 h at room temperature to avoid non-specific binding. Blots were incubated overnight at 4˚C with primary Abs (anti-Slug, anti-ZEB1, and anti-pan-cadherin, 1:500; anti-β-catenin, anti-Smad3, anti-NDRG1, anti-DMNT1, anti-β-actin, 1:1,000). The membranes were thoroughly washed for 30 min with Tweet-TBS, and then incubated for 2 h at room temperature with the HRP-conjugated secondary Ab (1:1,000), followed by washing with Tween-TBS. Equal protein loading was confirmed by probing for β-actin expression. Protein bands were visualized with chemiluminescent HRP substrate Luminata™ (Merck KGaA) and Amersham high performance chemiluminescence film (GE Healthcare). Image Lab software (version 5.2; Bio-Rad) was used for densitometric analysis.

RT-qPCR. MDA-MB-231 cell monolayers were cultured for 48 h in the presence of the vehicle (DMSO) alone, the indicated concentrations of free PL or PL-NPs, or empty NPs. Total RNA was isolated from cells using the RNeasy mini kit (Qiagen Inc.) according to the manufacturer's instructions. RNA quantity and purity were determined by spectrophotometric analysis. Approximately 500 ng of RNA were reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. Appropriately diluted cDNA samples were combined with primer mix (10 μM forward and reverse primers), nuclease-free water, and SsoFast EvaGreen™ Supermix® (Bio-Rad Laboratories, Inc.) or SYBR®-Green PCR master mix (Qiagen Inc.) at a 1: 1: 3: 5 ratio, respectively. Samples were transferred to a Multiplate™ 96-well unskirted polypropylene PCR plate in triplicates and placed in the CFX Connect™ RT-PCR detection system (B Bio-Rad Laboratories, Inc.). The reaction steps were as follows: 10 min of activation at 95˚C, 40 cycles of 10 sec of denaturation at 95˚C, and 20 sec at the primer-specific annealing temperature. β-actin was also amplified at the same time and used as a reference gene. Data obtained from the RT-qPCR reaction were analyzed using CFX Manager software (version 3.1, Bio-Rad Laboratories, Inc.). The sequences of the primers were as follows: MMP2 (63˚C) forward, 5'-TGGCAAGTACGGCTTCTGTC-3' and reverse, 5'-TTCTTGTCGCGGTCGTAGTC-3'; E-cadherin (64˚C) forward, 5'-CAGCCACAGACGCGGACGAT-3' and reverse, 5'-CTCTCGGTCCAGCCCAGTGGT-3'; and β-actin forward, 5'-AAGATCAAGATCATTGCTCCTC-3' and reverse, 5'-CAACTAAGTCATAGTCCGCC-3'.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey-Kramer or Bonferroni multiple comparisons post hoc test, where appropriate, using GraphPad Prism analysis software (version 5.0, GraphPad Software Inc.). Differences were considered statistically significant at $P<0.05$.

Results

PL and PL-NPs are equally cytotoxic to TNBC cells. The cytotoxicity of free PL and PL-NPs was compared using 3 different TNBC cell lines (Fig. 1). Following culture for 48 h in the presence of various concentrations of free PL (2.5-10 μM) or equivalent concentrations of PL-NPs, MTT assays revealed a similar concentration-dependent decrease in the number of TNBC cells (MDA-MB-231, MDA-MB-468 and BT-549) following treatment with PL and PL-NPs. Since

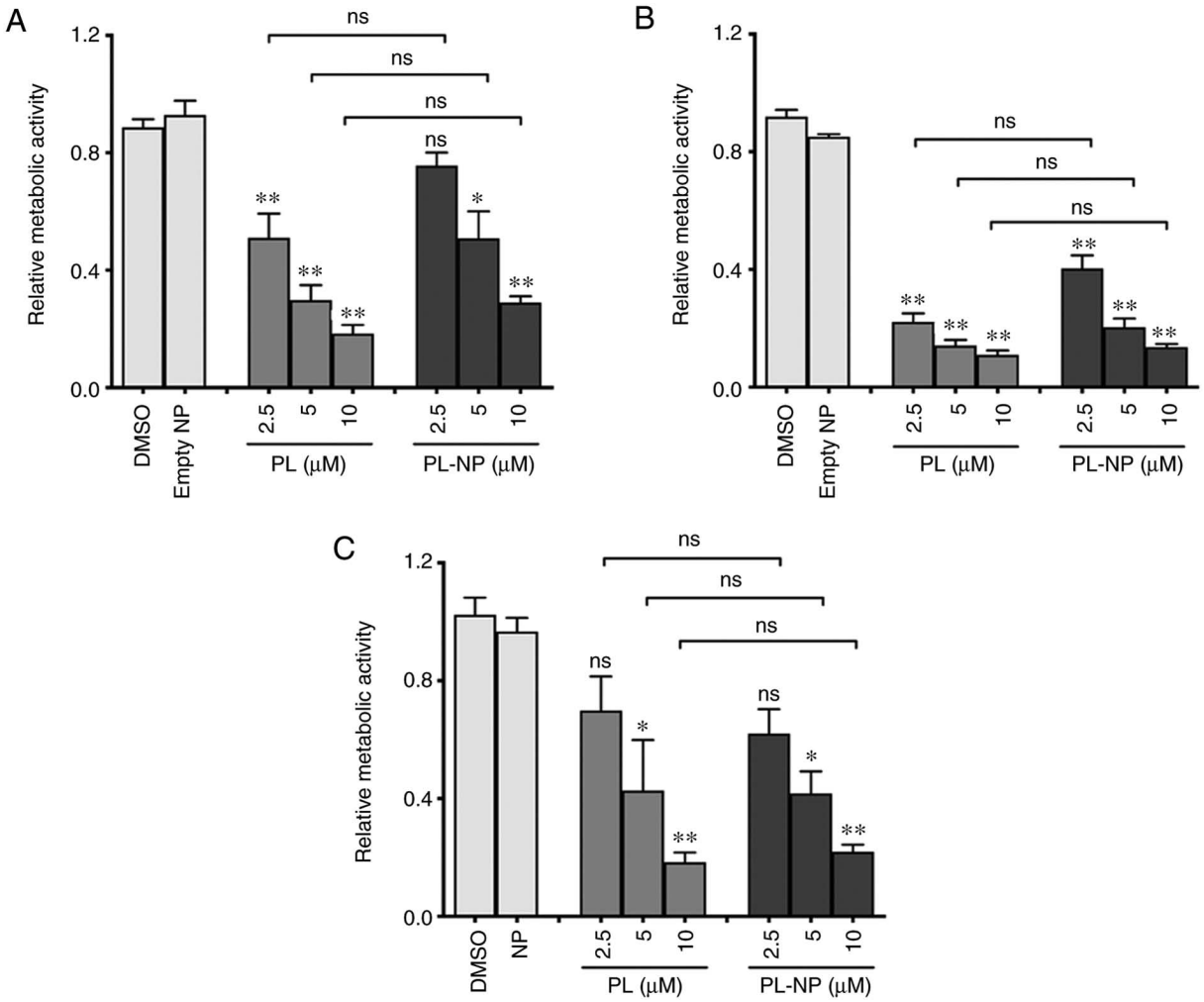

Figure 1. TNBC cell growth inhibition by PL and PL-NPs. (A) MDA-MB-231, (B) MDA-MB-468, and (C) BT-549 TNBC cells were seeded into 96-well flat bottom culture plates and cultured for 48 h in the presence of the vehicle (DMSO), empty NPs, or the indicated concentrations of free PL or PL-NPs. Cytotoxicity/growth inhibition was evaluated by the MTT assay. Data represent mean values ± standard error of the mean (SEM) of 3 independent trials. Statistical significance was determined using one-way ANOVA with the Tukey multiple comparisons post hoc test; *P<0.01, **P<0.001; ns, not significant; TNBC, triple-negative breast cancer; PL, piperlongumine; NPs, nanoparticles.

the inhibitory effects of 2.5 and 5 μM of PL in PL-NPs on the growth of MDA-MB-231 cells did not differ significantly and approximated the IC_{50}, these concentrations of PL-NPs and free PL were used in all subsequent experiments with MDA-MB-231 TNBC cells.

PL-NPs and free PL are effective inhibitors of TNBC cell migration and invasion. Transwell assays revealed that 2.5 μM of free PL or an equivalent concentration of PL-NPs significantly reduced the chemoattractant-induced migration of MDA-MB-231 TNBC cells through an uncoated membrane (Fig. 2A). Free PL and PL-NPs exerted a similar inhibitory effect on the ability of MDA-MB-231 to migrate through fibronectin- and gelatin-coated membranes (Fig. 2B and C, respectively) used to assess tumor cell invasiveness, suggesting the capacity to suppress the degradation of ECM components during metastasis. The results of RT-qPCR analysis revealed that the mRNA expression of ECM-degrading MMP2 in MDA-MB-231 cells was suppressed by approximately 70% in the presence of free PL or PL-NPs (Fig. 2D). MMP2 expression was determined by RT-qPCR as we were not able to identify a good anti-MMP2 Ab for western blot analysis. In addition, MDA-MB-231 cell migration through a gelatin-coated membrane was also markedly decreased in the presence of the selective MMP2 inhibitor, ARP 100 (Fig. S1), suggesting that the inhibitory effect of free PL and PL-NPs on MDA-MB-231 cell invasiveness was at least in part due to reduced MMP-2 expression.

Free PL and PL-NPs downregulate the expression of mesenchymal markers and upregulate E-cadherin expression in TNBC cells. Western blot analysis revealed that culture in the presence of 2.5 μM free PL or an equivalent concentration of PL-NPs significantly reduced the MDA-MB-231 TNBC cell expression of the EMT-promoting transcription factor, Slug (Fig. 3A). The expression of ZEB1, another EMT-promoting transcription factor, was also reduced in the MDA-MB-231 cells following treatment with 2.5 μM free PL or an equivalent concentration of PL-NPs (Fig. S2). Consistent with the inhibition of EMT, exposure to free PL and PL-NPs reduced the expression of the mesenchymal markers, β-catenin (Fig. 3A) and N-cadherin (Fig. 3B). By contrast, RT-qPCR revealed

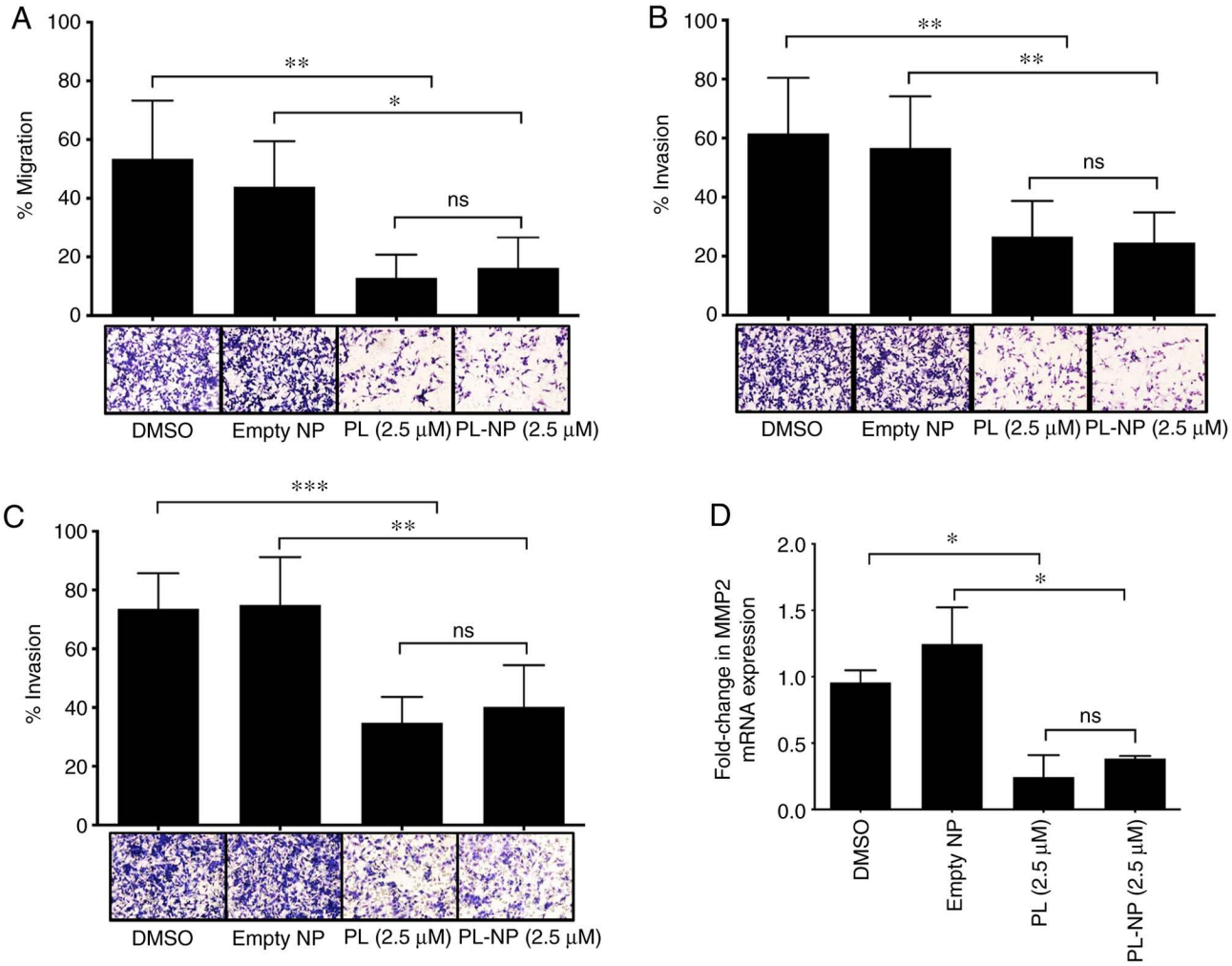

Figure 2. PL and PL-NPs inhibit the migration and invasiveness of MDA-MB-231 TNBC cells and their expression of MMP2. MDA-MB-231 cells were cultured in the presence of vehicle (DMSO), empty NPs, or 2.5 μM free PL or PL-NPs in serum-supplemented complete DMEM for 36 h followed by washing and culture for 12 h in serum-free complete DMEM. Cells were loaded into the upper chamber of a Transwell migration apparatus. Cells that moved through (A) uncoated 8 μm porous membranes used to assess migration, or (B) fibronectin-coated, and (C) gelatin-coated 8 μm porous membranes used to assess invasiveness, were stained and membranes were photographed at x20 magnification. Data shown are the mean number of migrating cells ± SEM of 3 (uncoated), 5 (fibronectin-coated) and 4 (gelatin-coated) independent experiments. (D) MDA-MB-231 cells were cultured for 48 h in the presence of vehicle (DMSO), empty NPs, or 2.5 μM free PL or PL-NPs. Total RNA was isolated from cells and mRNA was converted to cDNA. MMP2 mRNA expression was determined by RT-qPCR. β-actin was used as the reference gene. Data shown are mean MMP2 mRNA expression ± SEM of 3 independent experiments. (A-D) Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons post-test; *P<0.05, **P<0.01, ***P<0.001; ns, not significant. TNBC, triple-negative breast cancer; PL, piperlongumine; NPs, nanoparticles.

that expression of mRNA coding for the epithelial marker, E-cadherin, was increased 4-fold in the presence of free PL or PL-NPs (Fig. 3C). E-cadherin expression was determined by RT-qPCR as we were not able to identify a good anti-E-cadherin Ab for western blot analysis. Taken together, these findings indicate an equivalent inhibitory effect of free PL and PL-NPs on EMT of TNBC cells.

Free PL and PL-NPs inhibit Smad3 expression and increase NDRG1 expression in TNBC cells. The effects of free PL and PL-NPs on the expression of TGFβ/Smad signaling pathway-associated Smad-3 and anti-metastatic NDRG1 in MDA-MB-231 cells were then determined. MDA-MB-231 cells that were cultured in the presence of 5 μM free PL or an equivalent concentration of PL-NPs exhibited decreased Smad3 expression (Fig. 4A) and an increased expression of NDRG1 (Fig. 4B).

TNBC cell expression of DNMT1 is inhibited by free PL and PL-NPs. Subsequently, the expression of DNMT1 was examined following treatment of the MDA-MB-231 TNBC cells with free PL or PL-NPs to determine whether there may be an effect on the hypermethylation of TNBC cell DNA. As shown in Fig. 5, the DNMT1 levels were markedly reduced in the presence of 5 μM free PL or an equivalent concentration of PL-NPs, suggesting that PL has the potential to affect the epigenetic regulation of EMT.

Discussion

PL has been shown to exert potent cytotoxic effects on TNBC cells (22,23); however, the anti-metastatic effects of PL on TNBC cells have not yet been fully elucidated. Moreover, the feasibility of using biodegradable NPs to deliver PL to TNBCs has not yet been demonstrated. NP delivery enhances the

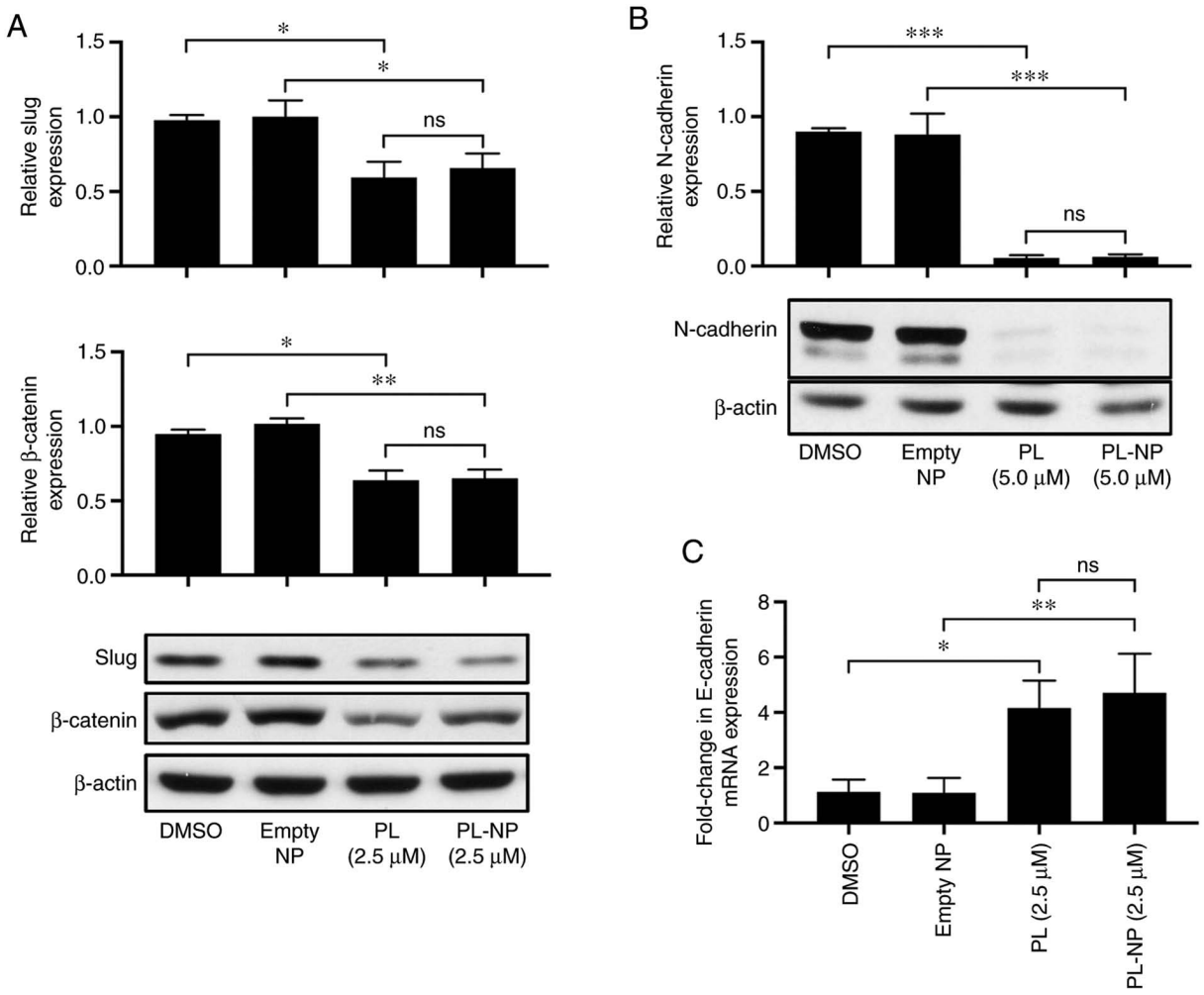

Figure 3. PL and PL-NPs suppress MDA-MB-231 TNBC cell expression of mesenchymal markers but upregulate E-cadherin expression. MDA-MB-231 cells were cultured for 48 h in the presence of the vehicle (DMSO), empty NPs, or the indicated concentrations of free PL or PL-NPs. (A and B) Total protein was isolated from lysed cells and subjected to western blot analysis. Equal protein loading was confirmed by probing for β-actin. Data shown are mean (A) Slug and β-catenin, and (B) N-cadherin expression ± SEM of 3 independent experiments. Representative blots are shown. (C) Total RNA was isolated from the cells and mRNA was converted to cDNA. E-cadherin mRNA expression was determined by RT-qPCR; β-actin was used as the reference gene. Data shown are mean E-cadherin mRNA expression ± SEM of 3 independent experiments. (A-C) Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons post-test; *P<0.05, **P<0.01, ***P<0.001; ns, not significant. TNBC, triple-negative breast cancer; PL, piperlongumine; NPs, nanoparticles.

bioefficacy of phytochemicals, such as PL, by overcoming the barriers posed by low solubility and poor bioavailability, as well as reducing the potential for undesirable toxicity to healthy tissues (25,26). To the best of our knowledge, the present study demonstrates for the first time that PL encapsulated in biocompatible NPs formed from mPEG-PLGA was as effective as free PL for the inhibition of TNBC (MDA-MB-231, MDA-MB-468 and BT-549) cell growth in monolayer cultures. Neoplastic cells take up PEG-PLGA NPs by endocytosis, after which the NPs release their cargo within the acidic environment of lysosomes (29). The present findings are in line with those of other studies showing the reduced *in vitro* growth of other cancer cell types following the NP-based delivery of other tumoricidal phytochemicals (27,28,30).

Tumor cell migration and invasion through the basement membrane and ECM are essential components of tumor metastasis (31,32). Drugs that interfere with these processes may therefore be successful in preventing or reducing metastasis. In the present study, both free PL and PL-NP inhibited the chemoattractant-directed migration of MDA-MB-231 TNBC cells across cell-permeable membranes, including membranes that were coated with the ECM components gelatin and fibronectin. The reduced migration of PL-treated TNBC cells across membranes coated with ECM components could, at least in part, result from the decreased expression of ECM-degrading MMPs that are involved in metastasis (8). Indeed, the present study demonstrated that free PL and PL-NPs downregulated the MDA-MB-231 cell expression of MMP2, which degrades both fibronectin and gelatin (33). MMP2 inhibition by ARP 100, a selective inhibitor of MMP2, also decreased the invasiveness of MDA-MB-231 cells. Taken together, these findings suggest that the decreased expression of MMP2 in the presence of PL contributed to the reduced invasiveness of PL-treated MDA-MB-231 cells. The reduced expression of MMP2 in the presence of PL was most likely due to the PL-mediated inhibition of phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling (23), as this signaling pathway is known to regulate MMP2 expression in malignant gliomas (34).

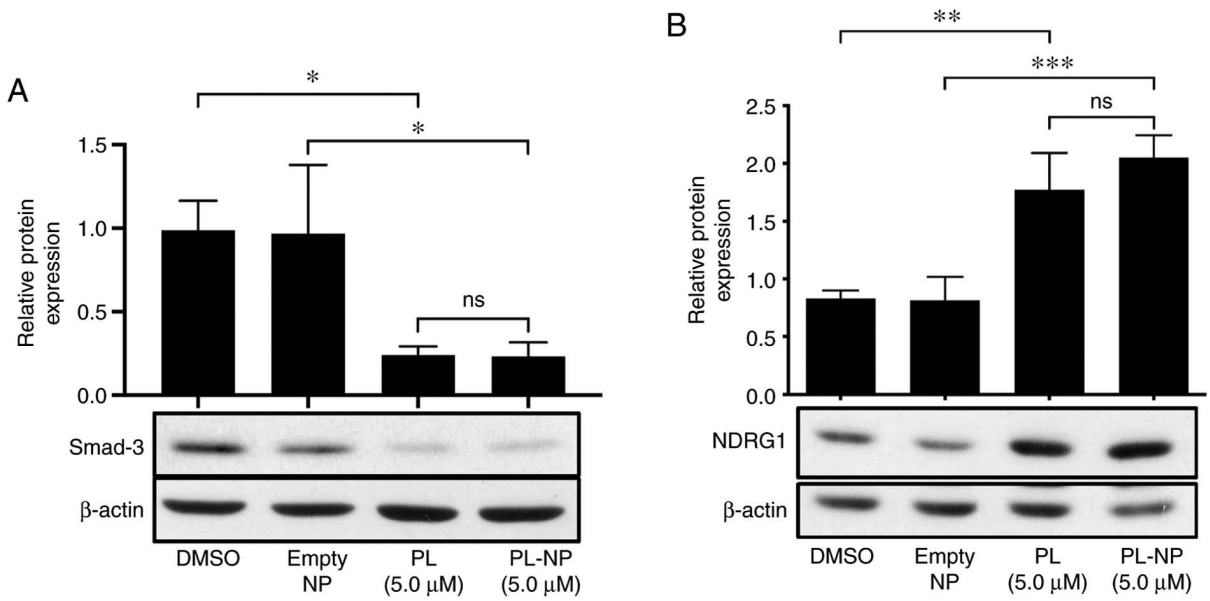

Figure 4. PL and PL-NPs downregulate Smad-3 expression and upregulate NDRG1 expression by MDA-MB-231 TNBC cells. MDA-MB-231 cells were cultured for 48 h in the presence of the vehicle (DMSO), empty NPs, or 5 μM free PL or PL-NPs. Total protein was isolated from lysed cells and subjected to western blot analysis. Equal protein loading was confirmed by probing for β-actin. Data shown are mean (A) Smad-3 and (B) NDRG1 expression ± SEM of 3 independent experiments. Representative blots are shown. Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons post-test; *P<0.05, **P<0.01, ***P<0.001; ns, not significant. TNBC, triple-negative breast cancer; PL, piperlongumine; NPs, nanoparticles.

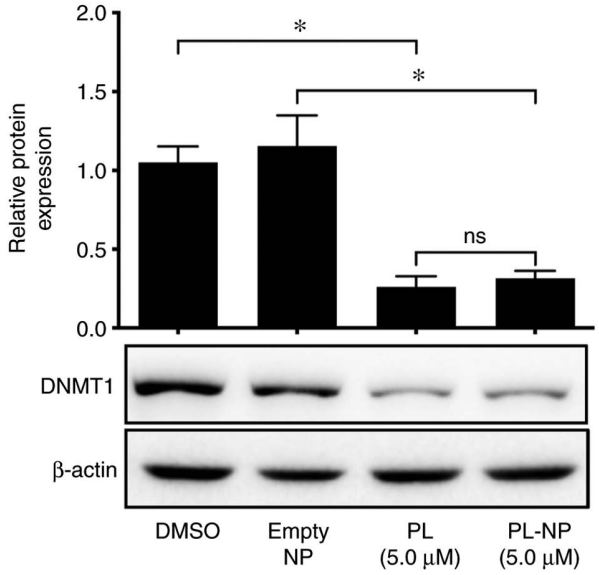

Figure 5. PL and PL-NPs inhibit DNMT-1 expression by MDA-MB-231 TNBC cells. MDA-MB-231 cells were cultured for 48 h in the presence of the vehicle (DMSO), empty NPs, or 5 μM free PL or PL-NPs. Total protein was isolated from lysed cells and subjected to western blot analysis. Equal protein loading was confirmed by probing for β-actin. Data shown are mean DNMT1 expression ± SEM of 3 independent experiments. A representative blot is shown. Relative protein expression level were compared using one-way ANOVA with the Bonferroni multiple comparisons post-test; *P<0.01; ns, not significant. TNBC, triple-negative breast cancer; PL, piperlongumine; NPs, nanoparticles.

The present study also demonstrated that free PL and PL-NPs interfered with MDA-MB-231 TNBC cell expression of Slug and ZEB1, which are EMT-promoting transcription factors (9,10). The expression of β-catenin by MDA-MB-231 cells was also suppressed in the presence of PL, suggesting the inhibition of the Wnt/β-catenin signaling pathway. This is consistent with the PL-induced downregulation of Slug expression, since Wnt/β-catenin signaling promotes Slug expression by breast cancer cells (35). The inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling pathway by PL (23) may also account for the observed decrease in β-catenin expression, since Akt inhibition blocks the nuclear localization of β-catenin, leading to its phosphorylation and proteosomal degradation (36). In addition, epithelial and mesenchymal marker expression was altered following the PL and PL-NP treatment of MDA-MB-231 cells. PL upregulated the expression of the epithelial marker, E-cadherin, and downregulated the expression of the mesenchymal marker, N-cadherin, which is associated with an invasive phenotype of breast cancer cells (37). Interestingly, ectopic expression of E-cadherin by MDA-MB-231 cells causes a shift from mesenchymal-like to epithelial-like morphology (38). E-cadherin re-expression and N-cadherin suppression following the PL and PL-NP treatment of MDA-MB-231 cells may be the result of the decreased Slug expression, since E-cadherin expression by MDA-MB-231 cells is suppressed by Slug via the upregulation of miR-221 (39), and there is an inverse association between E-cadherin and N-cadherin expression (40).

In the present study, the MDA-MB-231 TNBC cells treated with free PL or PL-NPs exhibited a reduced expression of Smad3, which is a key component of the TGFβ/Smad signaling pathway involved in the initiation of EMT (9,10). Although the present study did not determine the effects of PL treatment on Smad3 phosphorylation, it is likely that a decrease in available Smad3 would have a negative effect on Smad3 signaling, as it has been shown in colon cancer cells following Smad3 knockdown, that also resulted in decreased overall levels of Smad3 phosphorylation (41). The expression

of NDRG1, which is a tumor metastasis suppressor that abrogates the TGFβ/Smad-induced upregulation of Slug and other EMT-promoting transcription factors (13), was upregulated in the present study when MDA-MB-231 cells were treated with PL. NDRG1 also inhibits the Wnt/β-catenin signaling pathway (14); thus, the increased NDRG1 expression would be expected to suppress Wnt/β-catenin signaling. Taken together, these findings suggest that PL may promote the acquisition of an epithelial phenotype by mesenchymal-like MDA-MB-231 cells at least in part by modulating TGFβ/Smad and Wnt/β-catenin signaling pathways. At this time, the mechanism through which exposure to PL downregulates Smad3 expression and upregulates NDRG1 expression remains to be elucidated. However, PL has been reported to inhibit extracellular signal-regulated kinase 1/2 activation in colorectal cancer cells (42). A similar effect in MDA-MB-231 cells may account for the reduced Smad3 expression, since the inhibition of extracellular signal-regulated kinase signaling suppresses Smad3 expression in epithelial cells (43). The PL-induced downregulation of DNMT1 expression in MDA-MB-231 cells may account for the increased NDRG1 expression, since the inhibition of DNA methylation upregulates NDRG1 expression (44), and epigenetic silencing of NDRG1 in breast cancer cells is the result of DNA hypermethylation (45).

EMT and the metastasis of TNBC cells is associated with the DNMT1-mediated hypermethylation of DNA (12). For example, E-cadherin expression by breast cancer cells is silenced by DNMT1-mediated DNA hypermethylation (46,47). The present study demonstrates that free PL and PL-NPs inhibits DNMT1 expression in MDA-MB-231 cells, which may account for the re-expression of E-cadherin by PL-treated MDA-MB-231 cells. Although, to the best of our knowledge, this is the first study to investigate the effects of PL on the epigenetic machinery of TNBC cells, previous studies have demonstrated the effects of other dietary phytochemicals on the epigenome. For example, epigallocatechin gallate has been shown to inhibit DNMT1 activity in human esophageal, colon and prostate cancer cells, resulting in the re-expression of several tumor suppressor genes (48).

In conclusion, the present study demonstrates, for the first time, to the best of our knowledge, that NPs formed from biocompatible mPEG-PGLA can deliver PL to cultures of TNBC cells without any loss of efficacy in comparison to free PL. In this regard, the growth of TNBC cells in monolayer cultures was inhibited by PL-NPs to the same extent as free PL. In addition, TNBC migration/invasion and the expression of EMT-promoting proteins was markedly decreased in the presence of PL-NPs. By contrast, TNBC cell expression of the tumor suppressor, NDRG1, and E-cadherin, which is associated with a less invasive epithelial phenotype, was upregulated by PL-NP treatment. Moreover, PL-NPs have the potential to prevent the hypermethylation of DNA via the PL-mediated inhibition of DNMT1 expression. Further analysis of the effects of PL-NPs on the epigenome is important, considering the interest in compounds that block the epigenetic modification of DNA as chemotherapeutic agents (49). Collectively, these findings reveal an inhibitory effect of PL-NPs on the metastatic potential of TNBC cells that warrant further investigation in preclinical models of TNBC.

Acknowledgements

The authors would like to thank Ms. Mary Ann Trevors (Faculty of Medicine Electron Microscopy Core Facility, Dalhousie University) for providing assistance with NP characterization.

Funding

The present study was funded by a grant (no. 314347) to DWH from the Canadian Cancer Society. JGR was funded by a Nova Scotia Graduate Scholarship and a Cancer Research Training program award.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

JGR performed all the assays. JGR and WF performed data analysis. JGR and WF drafted the manuscript. DWH designed the research. DWH and WF revised the manuscript. 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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