Enhancing chemosensitivity of breast cancer stem cells by down regulating SOX2 and ABCG2 using Wedelolactone-encapsulated nanoparticles

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Running title
Drug nanoformulation enhances chemosensitivity of breast CSC

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Abstract

A major caveat in the treatment of breast cancer is disease recurrence after therapeutic regime at both local and distal sites. Tumor relapse is attributed to the persistence of chemoresistant cancer stem cells (CSCs), which need to be obliterated along with conventional chemotherapy. Wedelolactone (Wdl), a naturally occurring coumestan, demonstrates anticancer effects in different cancer cells, though with several limitations, and is mostly ineffective against CSCs. In order to enhance its biological activity in cancer cells and additionally target the CSCs, Wdl-encapsulated PLGA nanoparticles (nWdl) were formulated. Initial results indicated that nanoformulation of Wdl not only increased its uptake in breast cancer cells and the CSC population, it enhanced drug retention and sustained release within the cells. Enhanced drug retention was achieved by down regulation of SOX2 and ABCG2, both of which contribute to drug resistance of the CSCs. In addition, nWdl prevented epithelial-to-mesenchymal transition, suppressed cell migration and invasion, and reduced the percentage of breast cancer stem cells (brCSCs) in MDA-MB-231 cells. When administered in combination with paclitaxel, which is known to be ineffective against brCSCs, nWdl sensitized the cells to the effects of paclitaxel and reduced the percentage of ALDH+ brCSCs and mammospheres. Further nWdl suppressed growth of solid tumors in mice and also reduced CD44+/CD24low population. Taken together, our data implies that nWdl decreased metastatic potential of breast cancer stem cells, enhanced chemosensitivity through co-ordinated regulation of pluripotent and efflux genes and thereby provides an insight into effective drug delivery specifically for obliterating breast cancer stem cells.
Introduction:

Prevalence of triple negative breast cancers (TNBC), the most aggressive form of breast cancer, has increased substantially (1). Although different treatment strategies have been implemented, it remains a challenge in breast cancer management today because of the high occurrence of disease relapse (2). Tumor recurrence has been implicated to acquired chemoresistance and enhanced metastasis due to the existence of the cancer stem cells (CSCs; 2). CSCs retain the capacity for survival, self renewal and differentiation into tumorigenic cancer cells and are substantially insensitive to most conventional anticancer therapies (3), thereby suggesting that removal of CSCs is crucial for effective and more comprehensive cancer therapy. In addition, epithelial-to-mesenchymal transition (EMT) can induce enrichment and differentiation of cancer cells into CSC phenotypes (4), and plays an important role in invasion and metastasis (5) by modulating markers such as E-cadherin, cytokeratins, N-cadherin and vimentin (6). Multiple signaling pathways govern these transitions by regulating the expression of crucial EMT-related transcription factors, such as Snail, Slug and Twist (7). Thus, if cancers have to be eradicated, new selective treatments specifically targeting CSCs should be developed.

According to the World Health Organization (WHO), 80% people across the globe use medicinal plants for the treatment of cancer, since they are easily accessible, cost effective, and have less toxic side effects (8). Wedelolactone (Wdl; 7-Methoxy-5, 11, 12-trihydroxycoumestan, mol wt. 314.2), the principle active polyphenolic compound found in extracts of Wedelia calandulacea and Eclipta prostrata (9) is known for treatment of liver diseases, viral infections, human bronchial epithelial cell injury and snake bites (10). Wdl also regulates osteoclastogenesis in breast cancer (11-13), inhibits androgen-independent prostate cancer (14), and endometrial and ovarian cancer cell growth (15). Wdl holds great promise for development as an effective anticancer drug since it is widely used for prevention of inflammation and is mostly non-toxic to humans (16), although its effects on obliterating the cancer stem cell population has not been investigated till date. However, shortcomings such as poor solubility and bioavailability prevent clinical application of Wdl. Therefore, synthesized polymeric nanoparticles with efficient biodegradation and biocompatibility properties, and low antigenicity, are currently being tested to overcome these drawbacks and facilitate efficient delivery and effective functioning of the compound, specifically in the cancer stem cells (17, 18). Poly-lactide-co-glycolide (PLGA) is one of the most effectively used polymers in nanomedicine, known to effectively deliver drugs into cells because of its biodegradable and biocompatible properties, and drug products containing PLGA have been approved for parenteral use by regulatory authorities (19). Based on this,
we hypothesized that encapsulation of Wdl in PLGA nanoparticles will improve its efficacy by enhancing the anticancer effects, reducing toxic side effects and eventually result in clinically favorable outcome (20). The process of nano-encapsulation not only protects poorly soluble and unstable payloads from the biological milieu but also is minute enough for capillary penetration, internalization and endosomal escape (21). In addition, these particles have controlled release properties owing to pH and temperature sensitivity (22). Overall, PLGA-coated nanoparticles have significant advances over metal and other non-biodegradable nanoparticles since they have been proven to be safe in clinical studies, are surface-tunable and control the rate of polymer degradation and drug release (22, 23). This study describes the synthesis of Wdl-loaded nanoparticles (nWdl) for obliterating breast cancer stem cells, with reduced side effects, improved pharmacokinetics, modified biodistribution and enhanced functional efficacy, both in vitro and in vivo.

Materials and Methods:

Cell culture

Human breast cancer cell line MDA-MB-231 was obtained from National Centre for Cell Sciences, Pune, India. They were initially validated by immunohistochemistry. Cells were maintained in complete DMEM supplemented with 10% FBS and 1% penicillin–streptomycin–neomycin and incubated at 37°C in 5% CO₂. Cells were passaged every 72 h and cells in log phase were used for subsequent experiments.

Synthesis of Wdl loaded nanoparticles

The solvent displacement method was applied to prepare bioactive PLGA-encapsulated nano-wedelolactone (nWdl). Briefly, 50 mg of PLGA and 10 mg of Wdl was dissolved in 3 ml of acetone. The mixture was injected drop-wise into 20 ml of aqueous solution containing Pluronic non-ionic surfactant F68 and stirred continuously at room temperature until the organic solvent evaporated. After removing the redundant F68 from the nanoparticles, the pellet was resuspended in Milli-Q water. Nanoparticle-loaded suspensions were stored at 4°C. For blank nanoparticles, similar method was deployed without the addition of Wdl.

Preparation of FITC-tagged-PLGA-loaded Wdl nanoparticles

For synthesis of FITC-tagged PLGA-loaded Wdl nanoparticles (FITC-nWdl), 50 mg PLGA and 2 mg of FITC (in DMSO) were dissolved in 5 ml of acetone. FITC-PLGA blank nanoparticles were centrifuged and the precipitate was dried under vacuum for 24 h before determining the weight. A volume of 300 ml acetone was then added to completely dissolve the precipitate. Fluorescence intensity of FITC was measured and the standard calibration
curve of FITC was used to quantify and measure the concentration of FITC in 1 ml of FITC-PLGA blank nanoparticle and FITC-nWdl solution (24).

**Physico-chemical characterization of nWdl**

DLS was carried out using a Zetasizer, Nano-ZS instrument (Malvern Instruments, UK). The intensity of scattered light was detected at $90^\circ$ to an incident beam. The data was analyzed in the automatic mode (25). Measured size was presented as the average value of 20 runs, with triplicate measurements within each run. The size and shape of nanoparticles were analyzed by transmission electron microscopy (JEM-2100 HR-TEM, JEOL, Japan). The chemical properties of nWdl were characterized by Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer, USA) with samples as KBr pellets.

**Drug encapsulation efficiency of Wdl**

2 mg Wdl was dissolved in 2 ml acetone for complete extraction of Wdl into acetone for the loading and encapsulation estimations. The Wdl concentrations were determined at 351 nm (26). A standard plot of Wdl (0-100 µM) was prepared under identical conditions. The loading content and encapsulation efficiency (EE) of Wdl were calculated according to the following formula: EE (%) = [(drug fed - drug loss) / (drug fed)] × 100.

**In vitro cellular uptake and release profile of nWdl**

The release kinetics of nWdl was determined by dispersing 50 mg of nWdl in 10 ml phosphate buffer saline (PBS) following which nWdl release profile was determined (24). For **in vitro** cellular uptake studies, nWdl solution was added to MDA-MB-231 cells and incubated for 30 min to 8 h, after which the nanoparticles present in the medium were removed (27). Cells were co-stained with propidium iodide and harvested. Samples were analyzed by flow cytometry (BD Accuri C6, USA) or subjected to fluorescence imaging using FV 12000 (Olympus, Japan) at 330 nm, viewed with the Fluoview software (Japan).

**In vitro release rate of Wdl in serum**

To determine the release rate of nWdl in normal physiological conditions, and to resolve if proteins would impede drug release from the nanoparticle, the release kinetics was performed in 50% serum diluted in PBS. Release profile was compared at different time points (30 min – 72 h) and the release percentage was calculated based on O.D. 351nm (24).
**Cell viability assay**

Cell viability was determined MTT assay. In brief, 5,000 cells/well were seeded and treated either with Wdl or nWdl for 24, 48 and 72 h followed by addition of MTT. After incubation, 100 µl of detergent reagent was added to each well and absorbance intensity was recorded at 570 nm (SpectraMax 190 device microplate reader, Molecular Devices, USA).

**Wound healing assay**

MDA-MB-231 cells were grown in 6-well plates. A linear wound was gently created in the monolayer. Cells were incubated for 24 h in fresh media containing Wdl and nWdl. The wound closure was documented using a Axiovision microscope (Carl Zeiss Germany) at 20X magnification and captured from five randomly selected fields in each sample, at 0 and 24 h. The migration rate of control cells was taken as 100% and healing rates of treated cells were compared with respect to control cells. Images were captured at 0 and 24 h and the wound areas were calculated by NIH ImageJ software (28). The distance between the opposing edges of the wound was measured in micrometers (29).

**Transwell migration assay**

2X10^5 cells were seeded onto the porous membranes of BioCoat Matrigel invasion chambers (8 mm pore size). Treatment commenced after 24 h with indicated concentrations of Wdl and nWdl. After 24 h, the non-migrated cells in the upper compartment were wiped off gently. The cells in the insert were washed, fixed with 100% methanol and the number of migrated cells in four quadrants was observed. Quantification was done in triplicates.

**In vitro mammosphere assay**

MDA-MB-231 cells were seeded in ultra-low attachment plates (Corning Inc, USA) in serum-free DMEM/F12 supplemented with 5 µg/ml bovine insulin, 1X B27, 20 ng/ml EGF, 10 µg/ml heparin, 1% antibiotic-antimycotic solution and 100 µg/ml gentamicin (30). They were treated without or with Wdl and nWdl till the appearance of primary spheres (P1) for 6 days. Mammospheres with diameter ≥ 50 μm was counted manually. To assess sphere numbers during secondary (P2) passages, mammospheres (P1) were collected on Day 6, dissociated with 0.05% trypsin, filtered using a 40 μM sieve, and replated in ultra-low attachment plates, with no additional treatments (31). Treatments carried out in quadruplicates were determined from at least three independent experiments.
**Drug efflux assay**

For each condition, 10^6 cells were suspended in DMEM supplemented with 5% FBS. After addition of rhodamine123 (Rh123) with or without the inhibitor verapamil, cells were incubated in the dark for 30 min at 37°C. IC_{50} concentrations of nWdl were used for the assay. Cells were allowed to efflux in substrate-free media for 1 h, centrifuged, resuspended in ice-cold phosphate-buffered saline, and kept on ice until further analysis. Cells were gated for forward versus side scatter and the geometric mean of fluorescence intensity (cellular uptake of fluorescent substrate) was recorded for a total of 10,000 cells using a flow cytometer (BD Biosciences, San Jose, CA) under the green excitation emission wavelengths. All data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**RNA extraction and quantitative real-time polymerase chain reaction**

Total RNA was prepared from adherent MDA-MB-231 cells and mammospheres collected on Day 6 (P1), and converted into cDNA. After initial denaturation at 95°C for 1 min, PCR was performed for 40 cycles (15 sec at 95°C and 45 sec at 60°C) using KAPA SYBR FAST Universal qPCR kit (Kapa Biosystems, USA; 31). The primers used for gene expression analyses are presented in Supplementary Table 1. 18S mRNA was used for normalizing RNA and fold change was calculated based on vehicle-treated normalized values for each transcript.

**Western blot analysis**

Monolayer cultures of MDA-MB-231 cells, 6-day mammospheres and tissue samples were lysed using 100 μl of radioimmunoprecipitation assay (RIPA) buffer. After resolving by PAGE and transfer, the membranes were blocked with 5% bovine serum albumin. Next, they were incubated overnight with primary antibodies at 1:1000 dilution at 4°C followed by secondary antibodies for 1 h at room temperature. Protein band intensities were visualized by an enhanced chemiluminescence detection system (Optiblot ECL Detect Kit, Abcam®, UK) in BioRad ChemiDoc and relative quantification was done using the ImageJ software (31).

**Cell sorting and mammospheres culture**

To enrich the CSCs, 1x10^7 cells/ml were resuspended in Hank’s Balanced Salt Solution (HBSS) containing 2% FBS and 100 mM HEPES and stained with primary antibodies (CD24-FITC and CD44-PE; 1:100 dilution) or isotype controls for 15 min at room temperature. Cells with CD44^+/CD24^-/low phenotype were plated for mammosphere formation.
at a seeding density of 10,000 cells per well. P1 mammospheres were collected for gene expression analyses by qRT-PCR (31).

**Detection of ALDH-positive population by flow cytometry**

1x10^6 cells/ml cells were suspended in aldefluor assay buffer containing ALDH substrate (bodipy-aminocetaldehyde) and incubated for 45 min at 37°C. As a reference control, the cells were incubated in the presence of diethylaminobenzaldehyde (DEAB), a specific ALDH1A1 enzyme inhibitor. The brightly fluorescent ALDH1A1-expressing cells (ALDH1A1<sup>high</sup>) were detected in the green fluorescence channel (520–540 nm) of FACS Aria™ III (BD Biosciences, USA) and ALDH1A1<sup>high</sup> populations were sorted out (32).

**Immunophenotyping with CD24 and CD44**

The untreated and treated MDA-MB-231 cells (10^6 cells/ml) were resuspended in wash buffer and incubated in the presence of antibodies against CD44-PE, CD24-FITC, and their corresponding isotype controls at 4°C in the dark for 40 min. Subsequently, the cells were washed, resuspended in FACS buffer and processed using BD FACS Accuri C6 (31). The results were analyzed using BD Accuri C6 software (BD Biosciences, USA).

**In vivo evaluation of Wdl nanoparticles**

**Animals**

All animal experiments were conducted as per the approval and guidelines of the Institutional Animal Ethical Committee, Government of India (Registration Number 885/ac/05/CPCSEA). Female Swiss Albino mice, body weight 20-22 g, from Central Research Institute, (Kolkata, India) were housed in polypropylene cages under standard laboratory conditions of 50 ± 10% relative humidity, 22 ± 2°C temperature and 12/12 light-dark cycle for 10 days prior to commencement of experiments. Mice were fasted overnight and water was given ad libitum before experimentation. Food was restored after injections but withdrawn 6 h prior to sacrifice and analysis.

**Development of solid tumors in mice and treatment strategies**

Solid tumors were developed in animals (n=5) except in the normal group (Group I) or normal mice treated with nWdl (5 mg/kg body weight; Group II) by intraperitoneal (i.p.) injection of 2 x 10^6 viable cancer cells (0.2 ml) in the mammary fat pad of mice. Palpable tumors were allowed to develop for 14 days. Same set of experiment was repeated in the left flank of mice and tumor was developed for 14 days. Mice were subsequently divided into
three groups, each containing five animals, viz., (1) untreated tumor control (no drug treatment; Group III) (2), Wdl treated tumor-bearing mice (5 mg/kg body weight; Group IV); and (3) nWdl treated tumor-bearing mice (5 mg/kg body weight; Group V). Groups II, IV and V animals were subjected to a treatment schedule for 7 days. Nanoparticle (nWdl) toxicity was assessed in normal animals (Group II) and results of other groups were normalized to the values of those observed in Group I. After 7 days, the tumors were excised out, weighed in a pan balance, measured with slide calipers and processed for western blot analysis.

Estimation of drug toxicity, plasma drug levels and biodistribution

The immediate toxic impact of nanoparticles was elucidated from interactions with different blood cells in vivo (33), such as red blood cells (RBC), white blood cells (WBC, total and differential), and hemoglobin content, of normal mice and mice with tumors. Plasma drug levels were estimated by UV Spectral Scan (300-400 nm). For in vivo retention and biodistribution of nanoparticles, animals were injected with 5 mg/kg FITC-tagged nWdl on day 0 and sacrificed after days 3, 5 and 7. Tumors and livers were collected, perfused in PBS and subjected to flow cytometric analysis for presence of FITC-tagged nanoparticles. Further, tumors were processed for aldefluor assay and immunophenotyping to determine any changes in the cancer stem cell population due to drug retention.

Statistical Analyses

Values are shown as standard error of mean (SEM). Data were analyzed; when appropriate, significance of the differences between mean values was determined by using Students’ t-test and one-way ANOVA by post hoc testing. The software used for the analysis was SPSS 14.0. Results were considered significant at a P value of not more than 0.05. For Student’s t-test, *p<0.05, **p<0.01 and ***p<0.001 were considered significant. For sample size less than 10, non-parametric Mann Whitney U Test was performed.

Results

Formulation of nWdl revealed stable drug-loaded biopolymers

Wdl-encapsulated PLGA nanoparticles (nWdl) were formulated by varying the ratio of polyglycolic acid (PGA) and poly-lactic acid (PLA), and best composition was found to be 50:50 ratio of each. Results of DLS studies showed that nWdl had a mean hydrodynamic diameter of 95 ± 0.34 nm (Figure 1A) with polydispersity index (PDI) 0.77 ± 0.065 and zeta potential value -8.5 ± 2.35 mV (Figure 1B). We observed a uniformly narrow distribution of PDI value and negative zeta potential, which prevents particle aggregation. Transmission electron microscopy revealed spherical structures coated with clearly distinguishable PLGA
biopolymer. The average size of the nanoparticles was 60 to 80 nm (Figure 1C). nWdl was stable at pH 6 to 7 (Figure 1D). The nanoparticles showed 83.3 ± 2.15% encapsulation efficiency which indicated that majority of Wdl was entrapped into the PLGA-coated nanoparticles during synthesis. FTIR spectra, shown in Figure 1E, indicated strong peaks in the range of 1000-1500 cm⁻¹ and showed the stretching mode of Wdl which had prominently disappeared in the spectrum of polymer (nWdl), thus confirming polymerization.

Nanoformulation of Wdl reduced cellular toxicity, increased cellular uptake and enhanced in vitro drug release

Cells were seen to take up the nanoparticles as early as 30 min (Figure 2A) and significant time-dependent increase in cellular uptake was observed up to 2 h (Figure 2B). Additionally, cytosolic accumulation of nWdl was seen within 30 min whereas nuclear translocation was observed to be significant by 60 min. Uptake within both compartments was maximum by 8 h (Figure 2C), after which no further uptake was observed.

Sustained release of Wdl from PLGA was observed over a period of 3 days at pH 7.4 and 5.0, where 72.75% and 89.74% of Wdl was released, respectively (Figure 2D). The pH-sensitivity during drug release by the nanoparticles implicated efficiency in reducing their side effects while in circulation and enhancing specific release in tumors which usually have an acidic pH. In vitro drug release was also estimated in PBS containing 50% serum. The kinetics in PBS-containing serum indicated a similar profile of drug release as in PBS only, further confirming that presence of serum proteins did not impede or alter drug release from nanoparticles (Supplementary Figure S1).

Cell viability assays indicated that Wdl exhibited an IC₅₀ value of 80 ± 3.59 µg/ml, whereas the nano-encapsulated form showed an IC₅₀ value of 20 ± 1.94 µg/ml after 24 h of incubation (Figure 2Ea), which did not change significantly after 48 h and 72 h of incubation (Figures 2Eb and c). Effects of nanoparticles assessed in two other breast cancer cell lines, MDA-MB-468 and MDA-MB-435, indicated insignificant differences in the IC₅₀ doses between different cell lines (Supplementary Figure S2).

nWdl retarded migration and invasion of MDA-MB-231 cells and prevented epithelial-to-mesenchymal transition

To reduce interference in anti-invasive evaluation, all the assays were performed with half IC₅₀ concentrations (40 µg/ml for Wdl and 10 µg/ml for nWdl) for 24 h. Untreated MDA-MB-231 cells exhibited prominent wound closure activity whereas Wdl interfered with invasion of
MDA-MB-231 cells (p<0.001; Figure 3A). However, compared to the free drug, nWdl was more effective (distance=130 ± 0.125nm; p<0.001) in retarding invasion of the cells at a much lower concentration than Wdl (distance=197 ± 0.82nm; p<0.001). To further confirm our results, transwell migration assays revealed that significantly fewer number of cells migrated to the lower surface of the transwell inserts when treated with nWdl (61.7% ± 2.94; p=0.002) compared to Wdl treatment (81.4% ± 0.73; p=0.012; Figure 3B).

Analysis of cell death revealed that nWdl more effectively up regulated expression of pro-apoptotic markers and down-regulated expression of anti-apoptotic markers (Figure 3C). Furthermore, in contrast to Wdl, nWdl significantly down regulated protein levels of mesenchymal markers, like N-cadherin, vimentin, Twist, Snail and Slug and up regulated the epithelial markers E-cadherin and cytokeratin-19, both at the transcriptional (Figure 3D) and translational (Figure 3E) levels.

**nWdl treatment effectively reduced expression of ALDH-positive brCSCs cells and their self renewal capability in vitro**

To determine if reduction in cell migration is related to obliteration of the CSC population, we analyzed the percentage of ALDH-positive (ALDH+) cells when MDA-MB-231 cells were treated with 40 µg/ml Wdl or 10 µg/ml nWdl. The effects were compared to paclitaxel (PTX), a drug conventionally used for treatment of breast cancer patients, since earlier studies in our lab have confirmed that chemo-treatment enriched the CSC population, increasing the risk of disease relapse (31). PTX (2 nM) treatment led to an increase in ALDH+ brCSCs by 5.05-fold (32% ± 1.75; p=0.019) relative to untreated cells. However, expression of ALDH+ cells significantly decreased by 1.3-fold when treated with PTX + Wdl (24.2% ± 1.48; p=0.005) and 2.6-fold when treated with PTX + nWdl (12.1% ± 1.02; p=0.021), relative to PTX alone (Figure 4A). In support of the above, in vitro uptake studies specifically in mammospheres showed that the nanoparticles were effectively taken up by the spheres within 2 h of treatment (Figure 4B).

The above findings were subsequently validated in both primary and secondary mammospheres formed from MDA-MB-231 cells (Figure 4C). In the primary spheres, 20 µg/ml nWdl led to a significant reduction of ALDH+ cells by 3.2-fold (p=0.018) and 2.5-fold (p=0.014) relative to untreated control and Wdl treatment, respectively (Figure 4D), and preferentially reduced the CD44+/CD24-low cells by 2.1-fold (p=0.014) relative to control and 1.5-fold (p=0.006) relative to the free drug (Figure 4E). Furthermore, nWdl significantly inhibited formation of spheroids (Figure 4F), reduced the size of the mammospheres by 2.6-
fold (p=0.011) and 1.4-fold (p =0.005; (Figure 4G), and decreased the number of mammospheres by 2.9-fold (p=0.016) and 1.8-fold (p=0.006; Figure 4H) compared to control and Wdl treatment, respectively.

Concomitantly, in secondary mammospheres, nWdl reduced the percentage of ALDH$^+$ cells 4-fold (p=0.025) and 2.7-fold (p=0.014) compared to untreated and Wdl-treated cells, respectively (Figure 4I). Furthermore, cells that initially formed primary spheroids in the presence of nWdl did not form secondary spheres compared to the control (Figure 4J). In addition, nWdl reduced the size of secondary mammospheres by 2.6-fold (p=0.014) and 1.5-fold (p=0.006) (Figure 4K), and the number by 3.2-fold (p=0.017) and 2.1-fold (p=0.009) relative to control and Wdl, respectively (Figure 4L).

**nWdl enhances drug sensitivity in spheroids formed by brCSCs**

Effects of Wdl and nWdl on the viability of spheroids indicated that both Wdl and nWdl inhibited cell viability of primary (Figure 5A) and secondary mammospheres (Figure 5B). Concomitantly, the sensitivity of the spheres was considerably enhanced for nWdl (IC$_{50}$ 20 µg/ml for primary spheres, Figure 5A; and IC$_{50}$ 10 µg/ml for secondary spheres, Figure 5B) compared to Wdl (IC$_{50}$ 80 µg/ml for primary spheres, Figure 5A; and IC$_{50}$ 40 µg/ml for secondary spheres; Figure 5B). This finding was further ascertained by reduced drug efflux, as indicated by retention of rhodamine in mammospheres (Figure 5C), and attenuated expressions ABCG2 and ALDH1A1 at mRNA transcript (Figure 5D) and protein (Figure 5E) levels.

**nWdl modulates pluripotency and invasiveness in brCSCs**

Expression of the transcription factors Nanog, Sox2 and Oct4 in mammospheres indicated that nWdl inhibited their expression measured by qRT-PCR (Figure 5F) and western blot analysis (Figure 5G). Interestingly, nWdl treatment significantly reduced Sox2 level (p=0.004), whereas Wdl did not show any effect on Sox2 mRNA and protein levels. Protein expressions of Sox2, ABCG2 and ALDH1A1 were also examined in MDA-MB-468 and MDA-MB-435 cells, where reduced expression of the proteins was observed on nWdl treatment, similar to MDA-MB-231 cells (Supplementary Figure S3).

Treatment of brCSCs with nWdl resulted in significant down regulation of mesenchymal markers, concomitant with significant up regulation of the epithelial marker, E-cadherin, by 5.2-fold (p=0.0001) and 1.5-fold (p=0.003) compared to control and Wdl treatment, respectively, at transcript levels (Figure 5H) and protein levels (Figure 5I). Protein
expression of Slug was also examined in MDA-MB-468 and MDA-MB-435 cells, where reduced expression of the protein was observed on nWdl treatment, similar to MDA-MB-231 cells (Supplementary Figure S3).

The anti-invasive effects of Wdl are known to be mediated through NF-κB signaling (34). Subsequently, analysis of 20 µg/ml nWdl in brCSCs down regulated the expression of nuclear NF-κB protein in brCSCs compared to Wdl (Figure 5J), indicating that anti-invasive effects of nWdl on brCSCs are mediated by the inhibition of NF-κB.

\[ nWdl \text{ sensitizes brCSCs to the effects of PTX} \]

As observed for MDA-MB-231 adherent cells, treatment of mammospheres with 2 nM PTX in combination with 20 µg/ml nWdl significantly reduced their size by 3.1-fold (p=0.021) and 1.5-fold (p=0.003) relative to the control spheres and the PTX + Wdl-treated spheres, respectively. Similarly, where PTX treatment alone enhanced the number of mammospheres by 1.4-fold (p=0.006), nWdl reduced the number of mammospheres by 5.2-fold (p=0.026) relative to the control spheres (Figure 6A). These mammospheres barely produced any visible secondary mammospheres after drug withdrawal, compared to the control. Additionally, combination of nWdl and PTX reduced the expression of all pluripotency and chemoresistance markers relative to either the PTX-treated group, or the Wdl and PTX-treated group (Figure 6B). Furthermore, compared to control spheres, significantly higher ALDH\(^+\) cells were observed when mammospheres were treated with PTX alone whereas treatment with PTX + nWdl markedly reduced the percentage of ALDH\(^+\) population by 12.5-fold (p=0.051) relative to PTX-treated and 7.3-fold (p=0.032) relative to PTX + Wdl-treated spheres, respectively (Figure 6C).

\[ nWdl \text{ effectively reduces tumor volume and CSCs in mice bearing solid tumors} \]

Based on pilot studies for dose determination, 5 mg/kg body weight of Wdl and nWdl were used for treatment of tumor-bearing Swiss albino mice. It was observed that treatment with Wdl and nWdl significantly reduced tumor size by 1.5-fold and 2.3-fold, respectively, compared to that of the untreated mice (Figure 6D). Interestingly, tumor size and weight reduction was more prominent after treatment with nWdl (p<0.001) rather than Wdl (p<0.05; Figure 6D) relative to untreated control. Assessment of apoptosis in the tumors revealed that nWdl significantly up regulated expression of pro-apoptotic markers (Figure 6E). This clearly shows that the drug in its nanoencapsulated form was able to suppress metastasis through enhanced cell death. Concomitantly, compared to Wdl, nWdl treatment caused significant decline in the CD44\(^+\)/CD24\(^{low}\) population harbored in tumors, indicating that nWdl effectively
obliterated the brCSC population within a tumor (Figure 6F). Effects of nWdl on solid tumors developed in left flank also showed marked reduction in tumor volume, size and weight, and decline in the CD44+/CD24low population (Supplementary Figure S4).

The effect of nWdl on hematologic parameters indicated that nWdl did not affect the RBC and WBC counts or the hemoglobin (Hb) level in normal mice even after 7 days of treatment (Supplementary Table 2). The hematological parameters however significantly differed in mice bearing solid tumors, as compared to the control group. Significant decrease in the hemoglobin content (by 2.1-fold; p<0.05), RBC count (by 1.3-fold; p<0.05), lymphocyte (by 5.8-fold; p<0.05) and monocyte count (by 0.3-fold), along with a sharp increase in total WBC (by 4.2-fold; p<0.05) and neutrophil counts (by 3.6-fold; p<0.05) were observed in the mice bearing solid tumors. Treatment with Wdl could partially restore the values of hemoglobin, RBCs and WBCs, (Supplementary Table 2), whereas treatment with nWdl was more efficient in normalizing the levels of these parameters as compared to Wdl alone (p<0.05).

When mice bearing tumors were treated with nWdl and drug retention was assessed for up to 7 days, it was observed that 3.71% ± 1.49 of nWdl was present in the tumor within 2 h (Supplementary Figure S5A) and almost 8% ± 2.71 of the nano-encapsulated drug was retained within the tumor till day 7 (Figure 6G; p<0.05). Comparative drug levels in plasma assessed by absorbance spectrum with peak absorbance at 351 nm, corresponding to Wdl, indicated baseline concentrations of the released drug in the plasma at days 3, 5 and 7, as assessed by lack of absorbance peak in the samples (Supplementary Figure S5B). Retention was also assessed in liver and the results indicated insignificant changes on days 3, 5 and 7 (Supplementary Figure S5C).

A time- and day-wise comparison of obliteration of the brCSC population by nWdl in mice tumors indicated significant and progressive reduction of ALDH+ population by 1.74-fold within 12 hours, 2.02-fold with 24 hours (Figure 6H). Reduction by 7.7-fold was observed by day 3 (Figure 6I). There was no significant difference in ALDH+ cell population between days 3 and 5 but a further 4.6-fold reduction was observed on day 7 (Figure 6I).

Discussion
Drug efflux, a major caveat in the management of breast tumors often leading to treatment failures, has been implicated to CSCs which over express efflux pumps (31). Persistence of CSCs after chemotherapy mostly leads to disease recurrence, rendering these cells as major therapeutic targets. Designing novel strategies, such as drug nanoformulation, would
increase retention and sustained release of the concerned drug within the cancer stem cells and eventually better patient prognosis. Size compatibility of PLGA nanoparticles facilitates intracellular internalization and eludes lysosomal compartments, avoiding degradation of the drug and reduction in drug efficiency (35). There are three mechanisms of drug-loaded nanoparticle incorporation into CSCs: (i) caveolin-mediated endocytosis, (ii) clathrin-mediated endocytosis and (iii) passive transport (36). Anticancer drugs encapsulated in nanoparticles can eventually actively or passively target the CSCs, since sustained drug release in the cytoplasm improves therapeutic effect at the target site (35). Such modifications additionally reduce systemic toxicity of chemotherapy drugs and bypass certain forms of multidrug resistance (36). In contrast to other formulations like microparticles, hydrogels and implants, PLGA nanoparticles can be functionally modified to provide multiple efficacies including controlled drug release, cell specific targeting, and increased cellular uptake (37). This work emphasizes that spatiotemporal chemistry utilizing such functionalities can be used to treat breast cancer, so that sustained release and continuous exposure to the encapsulated drugs can effectively obliterate CSCs, and eventually lead to better patient prognosis in future.

PLGA-based nanoparticles demonstrated several advantages over other biopolymers for drug delivery because of their small size which helps them penetrate specific tissues via the fenestrations present in the tumor endothelial cells. This allows not only increased time in circulation but specific delivery and enhanced cellular uptake of the encapsulated drugs by their target tissues, thereby reducing side effects (38). This technology will generate more effective therapies capable of overcoming several biological barriers and side-effects that the body encounters during treatment with conventional anti-cancer drugs. Nanoformulation of wedelolactone not only enhanced its biodistribution and bioavailability, along with sustained release of the drug in the CSCs, it further sensitized them to the therapeutic effects of paclitaxel, possibly by down regulation of the ABCG2 drug efflux pumps. Consequently, increased stability of nWdl, due to their entry into cells through endocytosis and delayed hydrolysis, helped them escape the ABCG2 pumps, thereby overcoming drug resistance. The main advantage of these nanoparticles is that they can facilitate gradual and more effective drug release, thereby prolonging the life of drugs in circulation and increasing their half-life in plasma (39).

Metastasis is characterized by a transition from epithelial-to-mesenchymal phenotype. Circulating tumor cells in TNBC metastasis are reported to exhibit mesenchymal characteristics (40) and the gene signatures of these mesenchymal-type cells induced by
EMT are highly correlated with tumor aggressiveness and chemoresistance (41). We demonstrated that nWdl could inhibit EMT by triggering molecular reprogramming in MDA-MB-231 cells, with enhanced efficiency compared to Wdl. Additionally, nWdl modulated “cadherin switching”, by increasing the expression of E-cadherin and reducing the expression of N-cadherin (42, 43). Snail and Slug, which are known to regulate expression of E-cadherin in TNBC cells (44), were also regulated by nWdl. Overall, the processes of tumor metastasis and invasion, which appear to be inextricably linked to EMT, was effectively reduced by nWdl in human brCSCs in vitro. Additionally, a clear indication of apoptosis induced by the drug and enhanced by nanoformulation of the drug supports reduction of metastatic potential after treatment, both in vitro and in vivo.

A link between the acquisition of molecular and functional traits of CSCs and the induction of EMT has several implications in tumor progression (45). This study demonstrated for the first time that nWdl can target both the bulk tumor population, as well as the brCSCs, by inhibiting their self-renewal capacity and their ability to successfully connect to different metastatic niches. Interestingly, although PTX enriches the brCSC population (31), nWdl reduced the brCSC population when treated alone and more effectively in combination with PTX. In addition, nWdl reduced key regulators of brCSC self-renewal and pluripotency (46). We have already reported that SOX2 is significantly over expressed in most of the TNBC cases in humans (31). We have also knocked down and over-expressed SOX2 to further confirm that SOX2 played a major role in metastasis and CSC self-renewal. Silencing SOX2 could induce chemosensitivity in the breast cancer stem cells which were otherwise chemo-resistant (31). Here, a remarkable finding was that, unlike the other stemness markers, Wdl was unable to alter SOX2 expression; however, the nanoencapsulated form of the drug could significantly reduce the expression of SOX2 in mammospheres. Therefore, nWdl treatment could be considered a novel approach to sensitize the brCSCs, which were otherwise resistant to conventional chemo-drugs, like PTX, and eventually prevent initiation of secondary tumors after a chemotherapeutic regime. Additionally, NF-κB which is postulated to play a major role in EMT and invasiveness of TNBCs (47, 48) was significantly reduced by nWdl, conforming that reduced NF-κB expression possibly helps suppress invasiveness and migratory properties of CSCs present in MDA-MB-231 cells.

The effect of nWdl on solid tumors (both in the mammary fat pad and in the flank) in Swiss albino mice confirmed that drug-loaded nanoparticles did not affect the hematological parameters of normal mice, indicating minimum toxicity of these particles in vivo. However, nWdl significantly and specifically reduced tumor weight and volume, and improved
hematological parameters of tumor-bearing mice to levels observed in normal mice. Anemia and myelo-suppression are evident side-effects of cancer chemotherapy (49). Results indicated that tumor-bearing mice developed anemia due to reduced hemoglobin and concomitant hemolytic conditions. Administration of both Wdl and nWdl reinstated the hemoglobin content, along with restoration of hematological parameters towards normalcy, though nWdl was significantly more effective. Dose equivalence of nWdl in humans was calculated using the method of Reagan-Shaw et al. (50) which indicated that only 0.4 gm of nWdl will be more effective in simulating anti-carcinogenic effects compared to an equivalent dose of Wdl. That the brCSC population in solid tumors can be effectively reduced by nWdl has furthered credence to obliteration of metastasis and disease recurrence. Therefore, formulation of PLGA-based coumestan nano-therapeutics for targeting breast cancer stem cells will eventually provide a more effective and complete therapy for patients with TNBC. Lower toxicity and sustained release profile of these nanoparticles decipher significant reduction in size, number and metastatic potential of mammospheres, thereby rendering nWdl suitable for development as an effective chemotherapeutic drug in future.

Acknowledgements
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References


Figure legends

Figure 1: Characterization of PLGA-Wdl nanoparticles (nWdl). (A) Particle size distribution of nWdl showed a mean hydrodynamic diameter of 95 ± 0.34 nm. (B) Zeta potential of nWdl revealed a value of -8.5 ± 2.35 mV indicating low aggregation. (C) Transmission electron microscopic (TEM) image of nWdl showing an average size of 60 to 80 nm. (D) pH stability of nWdl was determined by measuring the zeta potential value and nanoparticles were found to be stable at pH 6 to 7. (E) FTIR spectra of free drug (Wdl), nWdl and blank PLGA showed a prominent stretching of Wdl in the range of 1000-1500 cm\(^{-1}\) and their disappearance when Wdl was encapsulated into PLGA indicating effective coating by PLGA.

Figure 2: Pharmacokinetics of PLGA-Wdl (nWdl) nanoparticles. (A) Time-dependent in vitro cellular uptake of nWdl by flow cytometric analysis showing maximum uptake after 2 h. (B) Confocal microscope images showing time-dependent uptake of FITC-tagged-nWdl in MDA-MB-231 reveals that uptake started within 30 min. Images were taken at magnification 20X and in oil immersion. Scale bar 100 µm. (C) Sub-cellular localization of nWdl demonstrated cytosolic accumulation of nWdl within 30 min and nuclear translocation within 60 min. (D) In vitro cumulative release kinetics of Wdl from PLGA-Wdl nanoparticles at pH 7.4 and pH 5 showed a biphasic release profile at pH 5. (E) Results of cell viability of MDA-MB-231 cells determined by MTT assay revealed an IC\(_{50}\) value of 80 ± 3.59 µg/ml for Wdl and 20 ± 1.94 µg/ml for nWdl after (a) 24 h, (b) 48 h and (c) 72 h of Wdl and nWdl treatments. All data were presented as mean ± SEM (n=6). *p<0.05 vs. control (blank nanoparticles) group, #p<0.05 vs. free drug (Wdl) treated group.

Figure 3: nWdl reduced migration and EMT of MDA-MB-231 cells. (A) Results of wound healing assay showing slower rate of migration of MDA-MB-231 cells after 24 h of treatment with half IC\(_{50}\) concentrations of Wdl (40 µg/ml) and nWdl (10 µg/ml). (B) Graphical representation of the percentage of reduced cell migration of MDA-MB-231 cells with half IC\(_{50}\) concentrations of Wdl and nWdl treatment for 24 h. Data are presented as mean ± SEM of four independent experiments. **p<0.01 vs. control (blank nanoparticles) in nWdl treated groups and *p<0.05 vs. control in free-drug (Wdl) treated groups. (C) Expression of apoptotic markers as determined by western blot analysis. β-tubulin was used as an internal control and respective fold changes are represented as ratio of net band pixel density to net loading control. (D) Expression of EMT markers as determined by the western blot analysis. β-tubulin was used as an internal control and respective fold changes are represented as ratio...
of net band pixel density to net loading control. (E) Effects of nWdl on mRNA expression of different EMT markers, measured by qRT-PCR. Results showed an effective down-regulation of mesenchymal markers and up-regulation of epithelial markers. Data are presented as mean ± SEM of four independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

Figure 4: nWdl enhanced chemosensitivity of breast cancer stem cells. (A) Aldefluor assay indicated treatment with PTX only showed 32% ALDH+ cells, which reduced to 24.2% in 2 nM PTX + 40 µg/ml Wdl and to 12.1% in 2 nM PTX + 10 µg/ml nWdl in MDA-MB-231 cells. (B) Time-dependent in vitro uptake of FITC-tagged nWdl in mammospheres, as assessed by flow cytometry analysis indicating significant uptake by 120 min. (C) Schematic representation of the experiments done using mammospheres formed from MDA-MB-231 cells. (D) Aldefluor assay showing reduction in ALDH+ cell populations in primary mammospheres. (E) Immunophenotype analysis showing significant decrease in CD44+/CD24−/low populations in nWdl treated primary mammospheres vs untreated spheres. (F) Images illustrating structure and size of primary mammospheres. Results showed significant inhibition of nWdl-treated spheroid formation. Scale bar 100 µm. Bar graph shows reduction in size (G) and number (H) of primary mammospheres in treated groups. (I) Aldefluor assay showing significant reduction in percentage of ALDH+ cell populations in secondary mammospheres on Wdl and nWdl treatment. (J) Images illustrating structure and size of secondary mammospheres in treated and untreated groups. Scale bar 100 µm. Histograms indicated reduction in size (K) and number (L) of secondary mammospheres in treated groups. **p<0.01 and ***p<0.001 vs control and Wdl were considered significant.

Figure 5: Reduced expression of stemness and EMT markers in response to nWdl. Increased susceptibility of (A) primary mammospheres and (B) secondary mammospheres to nWdl, as determined by MTT assay after 24 h of treatment. All data were presented as mean ± SEM (n=6). *p<0.05 vs. free drug-treated group. (C) Drug efflux assay in mammospheres showed significant inhibition of rhodamine efflux by nWdl, as verified by the standard pump inhibitor, Verapamil. (D) qRT-PCR and (E) protein expression of ABCG2 and ALDH1A1 showing significant down regulation after Wdl and nWdl-treatment of brCSCs. (F) Effects of nWdl on mRNA expression and (G) protein expression of different stemness markers showing reduced expression in Wdl and nWdl-treated brCSCs, as compared to untreated controls. (H) Effects of nWdl on mRNA expression and (I) protein expression of EMT markers showing up regulation of epithelial and down regulation of mesenchymal markers in brCSCs of MDA-MB-231 cells. (J) Reduced nuclear and increased cytosolic NF-
κB expression in brCSCs. Data are represented as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 were significantly different from Wdl. Respective fold changes have been represented as ratio of net band pixel density to net loading control.

Figure 6: Effect of nWdl on brCSCs in vitro and in vivo. (A) Synergistic effect of nWdl and PTX on mammospheres. Images illustrate significantly reduced size of primary mammospheres in PTX combined with either Wdl or nWdl when compared to untreated and PTX treated spheres. Scale 100 μm. (B) qRT-PCR demonstrated reduction in stemness markers in brCSCs treated with PTX-alone or in combination with nWdl. Data is represented as mean ± SEM of three independent experiments. *p<0.05. (C) Aldefluor assay showed the higher percentage of ALDH+ cells in PTX-treated group and marked reduction in ALDH+ cells in PTX + nWdl treated group. (D) Effect of Wdl and nWdl on solid tumors in mammary fat pad of mice. Images of solid tumor-bearing mice and excised tumors indicated marked reduction in tumor size and weight on nWdl treatment. Values are mean ± SEM of three independent experiments in each case. *p<0.05. (E) Expression of apoptotic markers as determined by the western blot analysis, indicating enhanced cell death on nWdl treatment. β-tubulin was used as an internal control (F) Immunophenotyping assay of mice tumors demonstrated significantly lower percentage of CD44+/24−/Low populations in nWdl treated groups. (G) Flow cytometric analysis for monitoring nWdl retention in mice tumors after 3, 5 and 7 days of a single-dose injection compared to untreated tumor control. (H) Aldefluor assay of mice tumors at different time points (0.5, 1, 2, 4, 8, 16, 24 h) after nWdl administration, showing a gradual decrease of ALDH+ cells from 30 min to 24 hours. (I) Decrease in ALDH+ cells in nWdl-treated mice after 3, 5 and 7 days of single-dose administration as compared to untreated tumor controls.
Figure 1

A. Size distribution by intensity

B. Zeta potential distribution

C. TEM image

D. pH vs. Zeta potential

E. Fourier transform infrared spectroscopy
Figure 3

A. 0 hr  
control  
Wdl  
nWdl  

24 hrs  
control  
Wdl  
nWdl  

B.  

<table>
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<tr>
<th></th>
<th>control</th>
<th>Wdl</th>
<th>nWdl</th>
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<tr>
<td>% cell migration</td>
<td>120</td>
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C.  

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Wdl</th>
<th>nWdl</th>
</tr>
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</table>
| BCL-2  | 1       | 0.39| 0.3  | (26 kDa)  
| BAX    | 1       | 1.34| 1.85 | (21 kDa)  
| CLEAVED CASPASE-3 | 1 | 1.57 | 1.62 | (17, 19 kDa)  
| CLEAVED PARP | 1 | 1.15 | 1.45 | (89 kDa)  
| B-TUBULIN |         |     |      | (55 kDa)  

D.  

CYTOKERATIN-19  

<table>
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<tr>
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<th>Wdl</th>
<th>nWdl</th>
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| E-CADHERIN  | 2.5  | 3.5  | ***  
| N-CADHERIN  | 1    | 0.76 | 0.47 | (135 kDa)  
| VIMENTIN    | 1    | 0.47 | 0.32 | (140 kDa)  
| TWIST       | 1    | 0.89 | 0.6  | (57 kDa)  
| SNAIL       | 1    | 0.8  | 0.7  | (26 kDa)  
| SLUG        | 1    | 0.79 | 0.7  | (29 kDa)  
| B-TUBULIN   | 1    | 0.79 | 0.7  | (30 kDa)  
| (55 kDa)    |
Figure 4

A. SSC FL1-A

Control  PTX  PTX+Wdl  PTX+nWdl
6%  32%  24.2%  12.1%

B. Count

0 min  30 min  60 min  120 min  240 min  480 min

C. MDA-MB-231 cells

↓ Dissociation

↓ Primary sphere formation

↓ 2nd passage

↓ Dissociation

↓ Secondary sphere formation

D. SSC FL1-A

Control  Wdl  nWdl
38.9%  30.9%  12.1%

E. CD44

Control  Wdl  nWdl
39.5%  28.1%  18.7%

F. FL1-A

G.

size of spheroids (μm)

control  Wdl  nWdl
p<0.001  p=0.001

H.

No. of spheroids

control  Wdl  nWdl
p<0.001  p=0.001

I. SSC

Control  Wdl  nWdl
17.2%  11.6%  4.3%

J. FL1-A

K.

size of spheroids (μm)

control  Wdl  nWdl
p<0.001  p=0.001

L.

No. of spheroids

control  Wdl  nWdl
p<0.001  p=0.001

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Figure 5

A. Bar graph showing % cell viability with dose (µg/ml) for Blank PLGA, Wdl, and nWdl.

B. Bar graph showing % cell viability with dose (µg/ml) for Blank PLGA, Wdl, and nWdl.

C. Graph showing count of Rhodamine FITC-A.

D. Relative mRNA expression of ABCG2 and ALDH1A1 with fold change over control for Wdl and nWdl.

E. Western blot showing relative protein expression of ABCG2, ALDH1A1, and β-TUBULIN with molecular weights.

F. Graph showing relative mRNA expression of SOX2, NANOG, and OCT4 with fold change over control for Wdl and nWdl.

G. Western blot showing relative protein expression of SOX2, OCT4, and β-TUBULIN with molecular weights.

H. Graph showing relative mRNA expression of N-CADHERIN, TWIST, SNAIL, and SLUG with fold change over control for Wdl and nWdl.

I. Western blot showing relative protein expression of E-CADHERIN, N-CADHERIN, TWIST, SNAIL, and β-TUBULIN with molecular weights.

J. Western blot showing relative protein expression of NFKB (nuclear) and HISTONE H1 with molecular weights.
Figure 6

A. Control, PTX, PTX+Wdl, PTX+nWdl

C. SSC

D. control, Wdl, nWdl

E. Bax, Bcl-2, Cl-Caspase-3, Cl-PARP, β-tubulin

F. control, Wdl, nWdl

G. Untreated, Day 3, Day 5, Day 7

H. Control DEAB, ALDH-FITC, 30 min, 1h, 2h, 4h, 8h, 16h, 24h

I. Untreated, Day 3, Day 5, Day 7

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