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Mesoporous silica nanoparticle-mediated delivery of doxorubicin and withaferin A suppresses tumour growth and angiogenesis in non-small cell lung cancer.

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Abstract

Non-small cell lung cancer (NSCLC) remains a leading cause of cancer-related mortality, and new therapeutic strategies are required to improve anti-tumour efficacy while reducing systemic toxicity. Doxorubicin is an effective anthracycline chemotherapeutic agent, but its clinical application is limited by non-specific biodistribution and dose-limiting toxicity. Withaferin A (WFA), a bioactive withanolide, has reported anti-cancer and anti-angiogenic properties, but its therapeutic translation is limited by poor aqueous solubility. In this study, mesoporous silica nanoparticles (MSNs) were investigated as a delivery platform for doxorubicin, WFA and combined doxorubicin/WFA treatment in NSCLC models using the NCI-H358 cell line. Cellular uptake and intracellular localisation were assessed by fluorescence microscopy, while anti-tumour activity was evaluated using resazurin-based viability assays, scratch wound migration assays, 3D spheroid models and the chick chorioallantoic membrane (CAM) tumour model. Potential mechanisms of action were explored using apoptosis- and angiogenesis-focused antibody microarrays. Doxorubicin-loaded nanoparticles showed time-dependent intracellular fluorescence and reduced H358 cell viability more effectively than free doxorubicin at higher concentrations. WFA-loaded nanoparticles reduced cell viability, although nanoparticle encapsulation did not significantly enhance this effect under the conditions tested. Combination doxorubicin/WFA-loaded nanoparticles reduced cell viability in a concentration-dependent manner and significantly impaired H358 migration. In 3D spheroid models, doxorubicin, WFA and combined doxorubicin/WFA treatments reduced spheroid size over seven days, with the greatest reduction observed following combination-loaded nanoparticle treatment. In the CAM model, doxorubicin, WFA and combined doxorubicin/WFA treatments significantly suppressed tumour growth, with nanoparticle-loaded formulations also reducing tumour area compared with nanoparticle controls. Antibody microarray analysis suggested modulation of apoptosis-associated proteins and reduced expression of angiogenesis-associated markers, particularly following combination nanoparticle treatment. Together, these findings support MSN-mediated delivery of doxorubicin and WFA as a promising multi-targeted strategy for suppressing NSCLC tumour growth, migration and angiogenesis.

Keywords: Non-small cell lung cancer; mesoporous silica nanoparticles; withaferin A; doxorubicin; angiogenesis; apoptosis; drug delivery; CAM model

Abbreviations

ATCC, American Type Culture Collection; BSA, bovine serum albumin; CAM, chick chorioallantoic membrane; CTAB, cetyltrimethylammonium bromide; DAPI, 4',6-diamidino-2-phenylindole; dH, Hydrodynamic diameter; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; Dox, doxorubicin; Dox-NPs, doxorubicin-loaded nanoparticles; EMT, epithelial–mesenchymal transition; FACS, fluorescence-activated cell sorting; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; H358, NCI-H358 non-small cell lung cancer cell line; MSNs, mesoporous silica nanoparticles; NSCLC, non-small cell lung cancer; NT, non-treated; NPs, nanoparticles; PBS, phosphate-buffered saline; PDI, Polydispersity index; PI, propidium iodide; RFU, relative fluorescence units; ROS, reactive oxygen species; WFA, withaferin A; WFA-NPs, withaferin A-loaded nanoparticles; Combo-NPs, combination doxorubicin/withaferin A-loaded nanoparticles; VEGF, vascular endothelial growth factor; uPA, urokinase-type plasminogen activator; IL-8, interleukin-8; HIF-1 α , hypoxia-inducible factor 1-alpha; TNF, tumour necrosis factor; TNFRSF, tumour necrosis factor receptor superfamily; TEOS, tetraethyl orthosilicate; CDKN1A, cyclin-dependent kinase inhibitor 1A; XIAP, X-linked inhibitor of apoptosis protein.

Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide, with an estimated 2.5 million new cases and 1.8 million deaths reported globally in 2022 [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases and comprises several histological subtypes, including adenocarcinoma, squamous cell carcinoma and large-cell carcinoma [2]. Despite major advances in molecularly targeted therapies and immune checkpoint inhibitors, many patients present with advanced-stage disease, and treatment resistance, tumour heterogeneity, metastatic progression and systemic toxicity continue to limit long-term therapeutic success [2]. There remains a need for therapeutic strategies that improve anti-tumour efficacy while reducing off-target toxicity associated with conventional chemotherapy.

Doxorubicin is a widely used anthracycline chemotherapeutic agent that induces anti-cancer effects through DNA intercalation, inhibition of topoisomerase II and generation of reactive oxygen species. However, its clinical application is limited by non-specific biodistribution, dose-dependent cardiotoxicity and the development of drug resistance [3]. Nanoparticle-based delivery systems offer a potential strategy to improve the therapeutic index of cytotoxic agents by enhancing tumour-associated accumulation, improving drug solubility, protecting therapeutic cargo and enabling controlled release within the tumour microenvironment [4,5].

Withaferin A (WFA) is a naturally occurring steroidal lactone, or withanolide, derived from *Withania somnifera* and has attracted considerable attention as a pleiotropic anti-cancer compound [6,7]. WFA has been reported to modulate multiple pathways associated with cancer cell survival and progression, including NF- κ B, STAT3, PI3K/Akt, oxidative stress responses, apoptosis, epithelial–mesenchymal transition and cytoskeletal regulation [6–9]. In lung cancer models, WFA has been identified as a potential anti-lung cancer and anti-metastatic agent, with reported effects on NSCLC cell viability, cancer stem-like phenotypes, migration and metastatic behaviour [8]. WFA has also been shown to inhibit epithelial–mesenchymal transition in NSCLC cells and to suppress proliferation while inducing apoptosis in A549 lung cancer cells, in part through modulation of PI3K/Akt signalling [9,10].

The anti-angiogenic activity of WFA is particularly relevant in NSCLC, where tumour vascularisation supports tumour expansion, nutrient supply and metastatic dissemination. WFA has been described as a potent inhibitor of angiogenesis, with effects on endothelial cell behaviour and tumour-associated vascular development [11,12]. In addition, WFA has been reported to enhance the therapeutic effect of doxorubicin in preclinical cancer models through mechanisms involving reactive oxygen species, DNA damage, autophagy and apoptosis [13]. However, the clinical translation of WFA is limited by poor aqueous solubility and bioavailability, providing a strong rationale for encapsulation within nanoparticle-based delivery systems [6,7].

Mesoporous silica nanoparticles (MSNs) have emerged as versatile nanocarrier platforms for cancer therapy because of their high surface area, tunable pore size, modifiable surface chemistry, biocompatibility and capacity to encapsulate a range of therapeutic agents [4,5,14]. These properties make MSNs particularly suitable for the delivery of poorly soluble or cytotoxic compounds and for combination approaches in which conventional chemotherapeutics are delivered alongside bioactive molecules that target complementary cancer-associated pathways. In lung cancer, where tumour growth, angiogenesis, inflammation and survival signalling are closely interconnected, co-delivery strategies may offer therapeutic advantages over single-agent treatment [5,14].

In the present study, the anti-tumour and anti-angiogenic effects of mesoporous silica nanoparticle-encapsulated doxorubicin and WFA in NSCLC models were investigated. The hypothesis proposed was that MSN-based encapsulation would provide an effective platform for the delivery of these agents and that the inclusion of WFA would enhance anti-cancer activity through complementary effects on tumour growth, survival signalling and angiogenesis. This study therefore evaluates the potential of MSN-mediated delivery of doxorubicin and WFA as a combinatorial therapeutic strategy for NSCLC.

Methods

Materials and reagents

Ethylene glycol (EG) hexadecyltrimethylammonium bromide (CTAB, ≥98%), ammonium nitrate, ethanol (EtOH) and methanol (MeOH) were purchased from Sigma Aldrich. Tetraethyl orthosilicate (TEOS, 99%) was bought from Alfa Aesar. Sodium hydroxide (NaOH) was from PanReact AppliChem. Withaferin A (WFA) and doxorubicin (Dox) were bought from Fisher Scientific (UK).

All reagents were used as bought and all solutions unless otherwise indicated, were prepared in deionized (DI) Milli Q water.

Instrumentation

UV-Vis absorption spectra were acquired on a Jasco V-650 spectrophotometer (Jasco Corporation, Tokyo, Japan); nanoparticle size distributions and zeta potential were measured using a dynamic light scattering Malvern Nano Zetasizer, with a 633 nm laser diode, from PROTEOMASS–BIOSCOPE facility (Caparica, Portugal). Pore size distribution and surface areas were determined by N₂ adsorption/desorption at 77 K, in a Micromeritics ASAP 2010 (Micromeritics Instrument Corporation, Norcross, USA) (Accelerated Surface Area and Porosimetry), at Analysis Laboratory at LAQV FCT UNL. Specific surface areas (SBET) and pore volume were estimated using Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods. Transmission electron microscopy (TEM) images were obtained in a JEOL JEM 2100 80-200 kV.

Synthesis of mesoporous silica nanoparticles (MSNs)

Mesoporous silica nanoparticles (MNs) were synthesized by an adaptation of the Stöber method, also known as the sol-gel method, already described by Marcelo *et al* [15]. Briefly, 150 mg of CTAB was dissolved in 30 mL Milli-Q H₂O at 50°C, under constant stirring. After 10 minutes, 10 mL of ethylene glycol and 700 µL of NaOH 1M were added, and the mixture was left under stirring at 80°C for 30 minutes. Afterwards, 750 µL of TEOS was added dropwise to the reactional mixture, and it was left under constant stirring, at 80°C for 2 hours, and further left under stirring at room temperature for 12 hours. The mixture was removed from stirring and washed twice with MeOH (2x10 mL). For template removal, the MSNs were resuspended in 20 mL of a 30

mg/mL ammonium nitrate methanolic solution and stirred for 1 hour at 60 °C, followed by centrifugation and washing in MeOH.

Loading trials

Two types of loading assays were conducted: single loadings, where only one drug was loaded onto the nanoparticle, and double loadings, where a combo of both drugs was loaded onto the nanoparticles.

For the single loading assays, 20 mg of MSNs (NPs) were suspended in 2 mL of water containing Dox at 0.25 mg/mL to obtain Dox-loaded nanoparticles (Dox-NPs), or in 2 mL of ethanol containing WFA at 0.25 mg/mL to obtain WFA-loaded nanoparticles (WFA-NPs). The mixtures were stirred in the dark at room temperature for 24 h. After incubation, the nanoparticles were collected by centrifugation and washed with the corresponding solvent. The resulting supernatants were collected and quantified by absorption spectroscopy.

For the combined loading assays (Combo-NPs), the procedure was performed sequentially. Briefly, 20 mg of NPs were first incubated with WFA in 2 mL of ethanol at 0.125 mg/mL, under stirring in the dark at room temperature for 24 h. After incubation, the nanoparticles were collected by centrifugation, washed with the corresponding solvent, and the supernatant was collected for WFA quantification. The recovered nanoparticles were then dried and subsequently incubated with Dox in 2 mL of water at 0.125 mg/mL, following the same loading procedure. After 24 h of incubation, the nanoparticles were collected by centrifugation, washed, and the supernatant was collected for Dox quantification.

All supernatants were collected, and the UV-vis spectra were acquired. The quantification of each drug in the supernatant was performed using the Lambert-Beer law. The molar absorptivity coefficient (ϵ) was obtained from calibration curves prepared for each drug, and the respective loading capacities (EE) ($\mu\text{g}/\text{mg}$) were determined.

Cell culture

Human NSCLC cell line, NCI-H358 (American Type Culture Collection (ATCC), Manassas, VA), was gifted by Dr Lakis Liloglou, Edge Hill University and University of Liverpool, Liverpool, UK. Cells were maintained in growth medium Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO₂ in a humidified incubator (Thermo Scientific™, Heracell 150i Cell incubator, USA).

Immunofluorescence

Cells were seeded onto 16mm glass cover slips at a density of 2×10^5 cells per well in 12-well plates and incubated for 24 hours at 37°C and 5% CO₂. Cells were treated with 5µM of doxorubicin, doxorubicin NPs, withaferin A or drug vehicle (DMSO or NPs) for different amounts of time (1-48 hours). The cells were washed three times with PBS then fixed by incubating with 100% methanol at -20 °C for 10 minutes. To block non-specific binding, cells were incubated at room temperature (20°C) with 1% bovine serum albumin in PBS for 30 minutes and then washed with PBS. The cells were incubated in 1:1000 primary anti-β-actin antibody (1mg/ml) (Sigma, UK) in 1% BSA in PBS for 45 minutes at room temperature. The cells were then washed three times in PBS for 5 minutes each before incubating with the secondary Alexa-488 anti-mouse antibody (1mg/ml) (Sigma, UK) at 1:2000 dilution in 1% BSA in PBS for 45 minutes at room temperature in the dark. The cells were washed three times with PBS for 5 minutes each in the dark followed by a 5-minute incubation at room temperature with 30nM DAPI (Sigma, UK) and then washed a further three times with PBS for 5 minutes. The coverslips were then washed with distilled water and mounted with Mowiol-4-88 (Sigma, UK). Samples were visualised at 60x magnification using a scanning disk immunofluorescent microscope (cellVivo, Olympus inverted microscope IX83. Tokyo, Japan).

Cell viability assay

96 well plates were seeded with 2.7×10^4 cells per well. The plate was then incubated for 24 hours at 37°C/5% CO₂. A range of concentrations (0.625-5µM) of doxorubicin, withaferin A, doxorubicin NPs, withaferin A NPs or combo (withaferin A and

doxorubicin) NPs were added to the cells and incubated for 48 hours. Diluted DMSO to the concentration of the drug treatment was added to selected wells instead of the drug treatments to act as a vehicle control for the NPs. Unloaded mesoporous NPs were also used as a control. Resazurin was prepared in DMEM and added at a final concentration of 160 μ M per well. Fluorescent intensity was measured using BMG Labtech (Ortenberg, Germany) FLUORstart Omega plate reader at the following settings: absorption: 544 nm, emission: 595 nm, gain: 800, No. flashes per well: 50. Measurements were taken at 0, 15, 30, 45, 60, 90, 120, 180 and 240 minutes. Fluorescent intensity was plotted over time using GraphPad and a line graph of this was created. The fold change of fluorescent intensity at 240 minutes relative to non-treated cells was calculated for each well using the formula $-1/(\text{fluorescent intensity}/\text{average fluorescent intensity for non-treated cells})$. Fold change was plotted as a bar chart and a two-way ANOVA was carried out in GraphPad Prism (version 5.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Wound healing assay

A 6 well plate was seeded with 3.3×10^5 cells per well. The plate was then incubated for 24 hours at 37°C/5% CO₂. Using a p200 pipette tip, a wound was created by scratching the surface of the well. Doxorubicin, withaferin A, doxorubicin NPs, withaferin A NPs or combo NPs were added to the cells at a final concentration of 0.625 μ M and incubated for 48 hours. Unloaded mesoporous NPs were also used as a control. Images of the wound were captured at 0hrs and 48hrs using AmScope Microscope Digital Camera (MU500, AmScope, C.A., USA) and a light microscope (LEICA, USA) under 5x objective. ImageJ (ImageJ, National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.net/ij/>, 1997-2018) was used to measure the width of the wound at 4 points and the average width was calculated for each treatment type at 0 and 48 hours. Migration rate for each cell line was calculated and plotted as a bar chart and a one-way ANOVA was carried out in GraphPad Prism (version 5.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Spheroid model

Cells were seeded at a density of 1×10^3 cells per well into a U-bottom 96 well Nunclon Sphera 3D culture plate. After 7 days the spheroids were imaged and treated with Doxorubicin, withaferin A, doxorubicin NPs, withaferin A NPs or combo NPs at varying concentrations. The spheroids were then imaged on day 7 in a light microscope (LEICA microscope) under a 5x objective. On day 7, the spheroids cells were stained using the LIVE/DEAD™ Viability/Cytotoxicity Kit Green/Deep Red (Thermofisher, Waltham, Massachusetts) and 30nM DAPI (Sigma, UK) per the manufacturer's instructions. Samples were imaged using a scanning disk immunofluorescent microscope (cellVivo, Olympus inverted microscope IX83. Tokyo, Japan).

Chick chorioallantoic membrane model

The chick chorioallantoic membrane (CAM) model was used to assess the anti-tumour activity of free and nanoparticle-encapsulated doxorubicin, withaferin A (WFA) and combination doxorubicin/WFA treatment in a vascularised *ex vivo* tumour model. Fertilised Dekalb White chicken eggs (Medeggs, UK) were incubated at 37°C under humidified conditions until embryonic day 3 (E3). On E3, approximately 5 mL of albumin was removed by piercing a small hole in the eggshell, withdrawing the albumin using a sterile syringe and sealing with a small section of parcel tape (Post Office, UK). A small window was then created in the eggshell under aseptic conditions using a Dremel tool to expose the developing CAM. The window was sealed, and eggs were returned to the incubator. On embryonic day 9 (E9), NCI-H358 cells were harvested, counted and resuspended in Matrigel (BD Biosciences) at a final density of 1×10^6 cells in 10 μ L. A suitable vessel junction was identified and a 10mm silicon ring was placed in the centre on the CAM surface. Next, the cell–Matrigel suspension was carefully applied onto the CAM surface within a defined inoculation area. Eggs were resealed and incubated for 24 h to allow tumour establishment. Following tumour establishment, CAM tumours were treated with free doxorubicin, WFA, free doxorubicin/WFA combination treatment, unloaded mesoporous silica nanoparticles, doxorubicin-loaded nanoparticles, WFA-loaded nanoparticles or combination doxorubicin/WFA-loaded nanoparticles. Treatments were applied directly to the tumour/CAM surface at a final drug concentration of 5 μ M in a total volume of 5 μ L. Untreated tumours and unloaded nanoparticle-treated tumours were included as

controls. Eggs were resealed and incubated until embryonic day 13 (E13). At E13, CAM tumours were imaged using a camera phone attached to a light microscope. Tumour area was quantified from captured images using ImageJ software. Tumour boundaries were manually outlined, and tumour area was calculated in mm². Data was plotted in GraphPad Prism and analysed using one-way ANOVA with an appropriate post hoc test. Statistical significance was defined as $p < 0.05$. All CAM experiments were terminated at E13, before hatching, and embryos were humanely culled at the experimental endpoint.

Microarray

To investigate apoptosis- and angiogenesis-associated protein expression following nanoparticle treatment, Proteome Profiler™ antibody arrays were performed using the Human Apoptosis Array Kit and Human Angiogenesis Array Kit (R&D Systems, Wiesbaden, Germany), according to the manufacturer's recommendations. In brief, cells were grown in T25 tissue culture flasks and treated with nanoparticle control, 1.25 µM doxorubicin-loaded nanoparticles (DOX-NPs), 1.25 µM withaferin A-loaded nanoparticles (WFA-NPs), or combination 1.25 µM doxorubicin/WFA-loaded nanoparticles (Combo-NPs) for 48 hours. Following treatment, samples were collected for analysis of apoptosis- and angiogenesis-associated proteins. For apoptosis array analysis, cells were washed with cold PBS and lysed in lysis buffer supplemented with protease inhibitor cocktail. Cell lysates were clarified by centrifugation, and total protein concentration was determined using a Bradford assay. Equal amounts (300ug) of total protein from each treatment group were applied to the Human Apoptosis Array. For angiogenesis array analysis, cells were washed with cold PBS and lysed in lysis buffer supplemented with protease inhibitor cocktail. Cell lysates were clarified by centrifugation, and total protein concentration was determined using a Bradford assay. Equal amounts (300ug) of total protein from each treatment group were applied to the Human Angiogenesis Array. Arrays were blocked, incubated with prepared samples, washed and developed according to the manufacturer's protocol. Chemiluminescent array images were acquired under identical exposure conditions. Spot intensities were quantified using ImageJ software (NIH, Bethesda, MD). Mean pixel density was calculated for each antibody spot, with background subtraction applied where

appropriate. Where duplicate spots were present, the average signal intensity was used for analysis. Protein expression was compared between nanoparticle control, DOX-NP, WFA-NP and Combo-NP treatment groups and expressed as relative mean pixel density or fold-change compared with the nanoparticle control.

Results

Synthesis and characterisation of mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNs) were synthesized by an adaptation of the Stöber method, also known as the sol-gel method, already described by Marcelo *et al* [15]. The synthesized MSNs were first characterized in terms of morphology, size distribution and textural properties, in order to evaluate their suitability as nanocarriers for Dox and WFA loading. As shown in Figure 1A,B, the TEM image reveals spherical nanoparticles with an average size of 377 ± 40 nm. The porosity of the MSNs was evaluated through N₂ adsorption/desorption analysis, confirming the mesoporous nature of the NPs. The isotherm shown in Figure 1C presents a profile characteristic of mesoporous materials, with a clear hysteresis loop between the adsorption and desorption branches, which can be correlated with a type IV isotherm. This type of curve is typical of mesoporous materials, once again confirming the mesoporous arrangement of the nanoparticles.

From these curves, the pore volume was also calculated, presenting a value of 2 cm³/g. The surface area of the nanomaterials was estimated from the same data by application of the BET model, yielding a value of approximately 961 m²/g. This relatively high surface area is consistent with the mesoporous nature of the nanoparticles and supports their potential application as drug nanocarriers. Such features are highly relevant for drug loading, since the interaction between the drug molecules and the silica matrix depends not only on the available pore volume, but also on the surface chemistry of the nanoparticles.

The NPs were also characterized by dynamic light scattering, and the hydrodynamic size and zeta potential were measured before and after drug loading. As expected, the hydrodynamic size values obtained by DLS were higher than those observed by TEM, suggesting aggregation of the NPs in aqueous suspension. This difference is commonly observed, since DLS measures the hydrodynamic diameter of particles in suspension, including the solvation layer and possible aggregates, whereas TEM provides information on the dry individual particles.

The loading of single and combo drugs was confirmed by UV-vis absorption analysis and by changes in zeta potential values. After Dox and WFA loading, the zeta potential changed from 7.6 ± 1.5 mV for unloaded MSNs to -8.6 ± 0.9 mV for Dox-NPs and to -29.5 ± 5.4 mV for WFA-NPs.

The inversion of zeta potential observed after Dox loading suggests that the loading process altered the surface charge distribution of the MSNs. Although Dox is expected to be positively charged under these conditions, the final negative zeta potential may indicate that Dox is preferentially accommodated within the mesoporous structure, while the external surface remains the deprotonated silanol groups. In contrast, the more negative zeta potential observed for WFA-NPs suggests a stronger modification of the external surface, which may indicate that WFA is more exposed at or near the nanoparticle surface.

The amount of loaded drug in each system was determined by UV-vis absorption spectroscopy, and the respective values are presented in Figure 1D. Overall, the morphological, textural and surface charge characterization confirms that the synthesized MSNs possess suitable properties for the incorporation of Dox and WFA, both in single and combined loading systems.

Cellular uptake and intracellular localisation of doxorubicin, doxorubicin-loaded nanoparticles and withaferin A

To assess cellular uptake and intracellular localisation, cells were exposed to free doxorubicin, doxorubicin-loaded nanoparticles (Dox-NPs) or withaferin A (WFA) and imaged over a 48 h time course (Figure 2). Nuclei were counterstained with DAPI, while the actin cytoskeleton was visualised to assess cell morphology. Compound-associated fluorescence was monitored in the relevant fluorescence channel. Free doxorubicin fluorescence was detectable from 1 h and appeared to increase over time, with a more pronounced intracellular signal observed at later time points (Figure 2A). By 24–48 h, doxorubicin-associated fluorescence was evident within the cell population and was visible in close association with DAPI-positive nuclei, consistent with intracellular accumulation of free doxorubicin. Cells treated with Dox-NPs also showed time-dependent intracellular fluorescence (Figure 2B). In contrast to free doxorubicin, Dox-NP-associated fluorescence appeared more punctate, particularly at

earlier time points, consistent with nanoparticle-associated uptake and intracellular vesicular localisation. Fluorescent puncta were visible from 1–2 h and became more apparent by 4–24 h. In the merged Dox-NP/DAPI images, discrete pink punctate signals were visible, supporting intracellular localisation of nanoparticle-delivered doxorubicin. By 24–48 h, Dox-NP fluorescence was observed within multiple cells, indicating sustained intracellular retention. WFA-NP treated cells displayed detectable compound-associated fluorescence over the time course, with signal observed within the cytoplasmic region (Figure 2C). Across the 1–48 h time points, WFA-NP treated cells also showed visible alterations in cell morphology compared with the untreated control, including changes in cell spreading and actin organisation. These observations suggest intracellular association of WFA-NPs and indicate that WFA-NPs exposure may affect cytoskeletal organisation and cell morphology.

Together, these data show that free doxorubicin, Dox-NPs and WFA-NPs are detectable within cells over time. Dox-NPs showed a punctate intracellular distribution consistent with nanoparticle-mediated uptake, while free doxorubicin showed broader intracellular and nuclear-associated fluorescence. Free doxorubicin and WFA-NPs also appeared to induce visible changes in cell morphology. These findings support the use of the nanoparticle system as a delivery platform for subsequent assessment of anti-cancer activity.

Doxorubicin-loaded nanoparticles reduce H358 cell viability

To determine whether doxorubicin-loaded nanoparticles altered H358 cell viability, cells were treated for 48 h with free doxorubicin or doxorubicin-loaded nanoparticles (Dox-NPs) across a concentration range of 5, 2.5, 1.25 and 0.625 μM . Cell viability/metabolic activity was then assessed by measuring relative fluorescence units (RFU) over time (Figure 3). Following treatment with free doxorubicin, RFU increased over the assay period in all treatment groups, although values remained lower than the untreated control (NT) across the concentration range tested (Figure 3A). This indicates that free doxorubicin reduced H358 cell viability/metabolic activity compared with untreated cells after 48 h exposure. However, the response to free doxorubicin was relatively similar across the concentrations tested, suggesting only a modest concentration-dependent effect under these conditions. In contrast, treatment with

Dox-NPs produced a more pronounced concentration-dependent reduction in RFU (Figure 3B). The highest concentrations of Dox-NPs, 5 and 2.5 μM , resulted in markedly lower RFU values compared with untreated cells, whereas the lower concentrations, 1.25 and 0.625 μM , showed RFU values closer to the untreated control. These data suggest that nanoparticle-delivered doxorubicin reduced H358 cell viability more effectively at higher concentrations. Fold-change analysis confirmed differences between free doxorubicin and Dox-NP treatments (Figure 3C). At 5 and 2.5 μM , Dox-NPs produced a greater reduction in viability/metabolic activity than free doxorubicin, indicating enhanced anti-cancer activity following nanoparticle-mediated delivery. At lower concentrations, the difference between free doxorubicin and Dox-NPs was less pronounced. Together, these results suggest that Dox-NPs enhance the inhibitory effect of doxorubicin on H358 cell viability, particularly at higher concentrations.

Withaferin A reduces H358 cell viability

To assess the effect of withaferin A (WFA) on H358 cell viability, cells were treated for 48 h with free WFA or WFA-loaded nanoparticles (WFA-NPs) at 5, 2.5, 1.25 and 0.625 μM . Cell viability/metabolic activity was then assessed by measuring relative fluorescence units (RFU) over time (Figure 4). Following treatment with free WFA, RFU increased over the assay period in all treatment groups; however, the highest WFA concentration, 5 μM , produced a clear reduction in RFU compared with the untreated and DMSO vehicle controls (Figure 4A). Lower concentrations of WFA showed RFU values closer to the control groups, indicating a more modest effect on H358 cell viability under these conditions. A similar response was observed following treatment with WFA-NPs (Figure 4B). RFU values increased over time across all treatment groups, but cells treated with 5 μM WFA-NPs showed reduced RFU compared with the untreated and nanoparticle control groups. Lower concentrations of WFA-NPs produced comparatively smaller reductions in RFU, suggesting that the inhibitory effect of WFA-NPs was most evident at the highest concentration tested. Fold-change analysis comparing free WFA with WFA-NPs showed a concentration-dependent reduction in cell viability/metabolic activity for both formulations (Figure 4C). The greatest reduction was observed at 5 μM , while lower concentrations

produced smaller decreases. However, there was no significant difference between free WFA and WFA-NPs across the concentration range tested. These findings indicate that WFA reduces H358 cell viability after 48 h exposure, but nanoparticle encapsulation did not significantly enhance the viability-reducing effect of WFA under these assay conditions.

Combination doxorubicin/WFA-loaded nanoparticles reduce H358 cell viability

To determine whether combined doxorubicin and withaferin A (WFA) treatment altered H358 cell viability, cells were treated for 48 h with combination doxorubicin/WFA-loaded nanoparticles (Combo-NPs) across a concentration range of 0.625 to 5 μ M. Cell viability/metabolic activity was then assessed by measuring relative fluorescence units (RFU) over time (Figure 5). Treatment with free doxorubicin/WFA combination reduced RFU in a concentration-dependent manner compared with the untreated and DMSO vehicle control groups (Figure 5A). The greatest reduction was observed at 5 μ M, while 2.5 μ M and 1.25 μ M produced intermediate reductions. Cells treated with 0.625 μ M retained higher RFU values, although these remained below the control groups. A concentration-dependent response was also observed following treatment with Combo-NPs (Figure 5B). The 5 μ M Combo-NP treatment produced the greatest reduction in RFU throughout the assay period. The 2.5 μ M treatment showed a more moderate reduction, whereas the 1.25 μ M and 0.625 μ M Combo-NP groups showed RFU values that were closer to the nanoparticle control group. Fold-change analysis comparing free combination treatment with nanoparticle-loaded combination treatment showed that both formulations reduced H358 cell viability/metabolic activity (Figure 5C). At the highest concentration, Combo-NPs produced a significantly greater reduction than the free drug combination. A significant difference was also observed at 1.25 μ M, whereas no significant difference was detected at 2.5 μ M or 0.625 μ M. These data suggest that nanoparticle-mediated co-delivery of doxorubicin and WFA can enhance the viability-reducing effect of the combination treatment at selected concentrations. Together, these findings indicate that combined doxorubicin/WFA treatment reduces H358 cell viability/metabolic activity in a concentration-dependent manner, with Combo-NPs producing strong inhibitory effects, particularly at the highest

concentration tested. These data support the potential of mesoporous silica nanoparticles as a co-delivery platform for doxorubicin and WFA in NSCLC models.

Doxorubicin-, WFA- and combination-loaded nanoparticles inhibit H358 cell migration at low dose

To determine whether nanoparticle-delivered doxorubicin, withaferin A (WFA), or combination treatment affected H358 cell migration at a lower drug concentration, a scratch wound assay was performed using 0.625 μ M treatment, and wound closure was assessed over 48 h (Figure 6). Representative images show the wound area at 0 and 48 h in untreated cells, nanoparticle control cells, and cells treated with free doxorubicin or doxorubicin-loaded nanoparticles (Figure 6A). Untreated cells and nanoparticle control cells showed clear migration into the wound area over 48 h, indicating active wound closure. There was no significant difference in migration rate between untreated cells and the nanoparticle control, suggesting that the nanoparticle vehicle alone did not inhibit H358 cell migration (Figure 6B). In contrast, free doxorubicin, WFA, and the drug-loaded nanoparticle formulations significantly reduced H358 cell migration compared with untreated and nanoparticle control cells. Free doxorubicin and WFA produced a moderate reduction in migration rate, while Dox-NPs produced a stronger inhibitory effect. WFA-NPs also reduced migration compared with controls, although the effect was less pronounced than that observed with Dox-NPs. Combination treatment also reduced migration; however, the migration rate observed with Combo-NPs was very similar to that observed with Dox-NPs alone. Therefore, under these low-dose conditions, the addition of WFA did not appear to further enhance the anti-migratory effect of Dox-NPs. Together, these findings indicate that nanoparticle-mediated delivery of doxorubicin significantly impairs H358 cell migratory capacity at a low 0.625 μ M dose. While WFA and WFA-NPs also reduced migration, the strongest inhibition was observed with Dox-NPs and Combo-NPs, which showed comparable effects in this assay.

Doxorubicin, WFA and combination-loaded nanoparticles reduce NSCLC spheroid growth in a 3D tumour model.

To assess treatment effects in a more physiologically relevant tumour model, NSCLC spheroids were treated with free doxorubicin, doxorubicin-loaded nanoparticles, free WFA, WFA-loaded nanoparticles, free doxorubicin/WFA combination treatment or combination-loaded nanoparticles and monitored over 7 days (Figure 7). Representative brightfield images showed compact spheroid formation across treatment groups, with visible differences in spheroid size and surrounding cellular material following treatment with free doxorubicin or doxorubicin-loaded nanoparticles (Figure 7A). Quantification of spheroid size showed that untreated control spheroids remained broadly stable between day 0 and day 7 (Figure 7B–D). Free doxorubicin reduced spheroid size after 7 days, although the response was not strictly concentration-dependent (Figure 7B). Doxorubicin-loaded nanoparticles also reduced spheroid size over the same period, with reductions observed across the concentration range tested. These findings indicate that both free and nanoparticle-delivered doxorubicin retain anti-tumour activity in a 3D NSCLC spheroid model. WFA and WFA-loaded nanoparticles also reduced spheroid size by day 7, although the effect appeared more modest than that observed with doxorubicin-based treatments (Figure 7C). WFA-loaded nanoparticles showed a broadly similar pattern to free WFA, suggesting that nanoparticle encapsulation did not markedly enhance the effect of WFA on spheroid size under these conditions. Combination doxorubicin/WFA treatment produced the greatest reduction in spheroid size after 7 days (Figure 7D). The reduction was concentration-dependent, with the strongest effect observed at the highest combination concentration. Combination-loaded nanoparticles produced a more pronounced reduction than the corresponding free combination treatments, suggesting that nanoparticle-mediated co-delivery may enhance the anti-tumour effect of doxorubicin/WFA in the 3D spheroid model. Together, these data demonstrate that doxorubicin, WFA and doxorubicin/WFA combination treatments reduce NSCLC spheroid growth over 7 days. The strongest effect was observed with combination-loaded nanoparticles, supporting the potential of MSN-mediated co-delivery as an anti-tumour strategy in 3D NSCLC models.

Doxorubicin, WFA and combination-loaded nanoparticles suppress tumour growth in the CAM model

To evaluate the anti-tumour activity of free and nanoparticle-encapsulated treatments in an *ex vivo* tumour model, NSCLC tumours were established on the chick chorioallantoic membrane (CAM) and treated with doxorubicin, withaferin A (WFA), combination doxorubicin/WFA, or their corresponding nanoparticle-loaded formulations (Figure 8). Representative CAM images showed visible tumour formation in untreated and nanoparticle control groups, with associated vascularisation around the tumour area (Figure 8A). In contrast, CAMs treated with free doxorubicin, WFA, combination treatment, or their corresponding nanoparticle-loaded formulations showed visibly smaller tumour masses. The reduction in tumour size appeared most pronounced in the doxorubicin-loaded nanoparticle and combination treatment groups. Quantification of tumour area confirmed that all drug-treated groups significantly reduced tumour size compared with the untreated or nanoparticle control groups (Figure 8B). Free doxorubicin and free combination treatment markedly reduced tumour area compared with untreated controls, while WFA alone produced a more moderate reduction. Nanoparticle-loaded formulations also significantly reduced tumour size compared with the nanoparticle control. Among these, doxorubicin-loaded nanoparticles produced the greatest reduction in tumour area, followed by nanoparticle-loaded combination treatment and WFA-loaded nanoparticles. Together, these data demonstrate that doxorubicin, WFA and doxorubicin/WFA combination treatments suppress NSCLC tumour growth in the CAM model. The strong reduction observed following nanoparticle-loaded treatment supports the potential of MSN-based delivery as an anti-tumour strategy in a vascularised tumour microenvironment.

Nanoparticle treatments modulate apoptosis- and angiogenesis-associated protein expression.

To investigate potential mechanisms underlying the anti-tumour effects of the nanoparticle treatments, apoptosis- and angiogenesis-associated protein expression was assessed using antibody microarrays (Figure 9). Mean pixel density was compared between control nanoparticles, doxorubicin-loaded nanoparticles (DOX-NPs), WFA-loaded nanoparticles (WFA-NPs) and combination doxorubicin/WFA-loaded nanoparticles (Combo-NPs). Analysis of apoptosis-associated proteins showed distinct treatment-specific expression profiles (Figure 9A). DOX-NPs

produced increased signal intensity for several apoptosis-related markers compared with the nanoparticle control, including Bad, Bax, caspase-associated proteins, Fas/TNFRSF6/CD95, HIF-1 α , HSP27, p21/CIP1/CDKN1A, survivin and TNFR1/TNFRSF1A. WFA-NPs also increased several apoptosis-associated markers, including components linked to death receptor signalling, stress response and cell-cycle regulation. Combo-NPs produced a broader but generally more moderate profile across the apoptosis array compared with DOX-NPs, with increased signal detected for selected markers including caspase-associated proteins, Fas/TNFRSF6/CD95, HSP27, p21/CIP1/CDKN1A, survivin and XIAP.

The angiogenesis antibody microarray analysis showed that nanoparticle treatment altered the expression of several angiogenesis-associated proteins (Figure 9B). Control NP-treated samples showed high levels of IL-8, uPA and VEGF, consistent with a pro-angiogenic profile. DOX-NPs reduced the signal intensity of several angiogenic markers relative to the control NP group, including amphiregulin, IL-8, thrombospondin-1, uPA and VEGF. WFA-NPs showed a mixed response, with reduced amphiregulin and IGFBP-1 but retained or increased signal for IL-8, thrombospondin-1, uPA and VEGF. In contrast, Combo-NPs showed consistently low signal intensity across the angiogenesis-associated proteins assessed, including amphiregulin, IL-8, thrombospondin-1, uPA and VEGF.

Together, these data suggest that nanoparticle-mediated delivery of doxorubicin, WFA and the doxorubicin/WFA combination alters apoptosis- and angiogenesis-associated signalling pathways. The apoptosis array indicates activation of stress, death receptor and cell-cycle-associated responses, while the angiogenesis array suggests that Combo-NPs most strongly suppress pro-angiogenic protein expression. These findings support a mechanism in which the anti-tumour effects of the nanoparticle treatments involve both induction of apoptosis-associated pathways and inhibition of angiogenesis-associated signalling.

Discussion

This study investigated the anti-tumour potential of mesoporous silica nanoparticle (MSN)-mediated delivery of doxorubicin, withaferin A (WFA), and combined doxorubicin/WFA treatment in NSCLC models. The findings demonstrate that

doxorubicin, WFA and their nanoparticle-loaded formulations are taken up by H358 cells, reduce cell viability/metabolic activity, impair migratory capacity, suppress spheroid growth and reduce tumour size in the CAM model. Mechanistic analysis further suggested that these effects are associated with modulation of apoptosis- and angiogenesis-related signalling pathways. Together, these data support the potential of MSN-based delivery as a platform for improving the anti-cancer activity of conventional and bioactive therapeutic agents in NSCLC.

Cellular uptake analysis showed time-dependent cell-associated fluorescence following treatment with free doxorubicin, doxorubicin-loaded nanoparticles and WFA. Free doxorubicin showed broader intracellular and nuclear-associated fluorescence, consistent with the known ability of doxorubicin to intercalate into DNA and accumulate in nuclei. In contrast, doxorubicin-loaded nanoparticles produced a more punctate intracellular fluorescence pattern, particularly at earlier time points, consistent with nanoparticle-associated internalisation and possible endosomal or vesicular localisation. This difference in localisation is important because nanoparticle-mediated uptake may alter intracellular drug trafficking, retention and release kinetics compared with free drug exposure. WFA-treated cells also showed detectable intracellular fluorescence and changes in cell spreading and actin organisation, consistent with previous reports that WFA can affect cytoskeletal organisation and epithelial–mesenchymal transition-associated behaviour [6–9]. These findings confirm that the treatments reached the cellular compartment and support subsequent functional assessment of their anti-cancer effects.

The viability data showed that free doxorubicin reduced H358 cell viability/metabolic activity after 48 h exposure, while doxorubicin-loaded nanoparticles produced a more marked reduction, particularly at higher concentrations. This suggests that MSN-mediated delivery may enhance the inhibitory effect of doxorubicin in H358 cells. Doxorubicin remains an effective cytotoxic agent, but its clinical use is limited by non-specific biodistribution, systemic toxicity and dose-limiting cardiotoxicity [3,17]. Nanoparticle-based delivery strategies are therefore attractive because they may improve drug solubility, increase tumour-associated delivery, protect cargo and permit more controlled release [4–5,14,17]. The increased activity observed with doxorubicin-loaded nanoparticles is consistent with this rationale and supports the use of MSNs as a delivery system for cytotoxic compounds in lung cancer models.

WFA also reduced H358 cell viability, with the strongest effect observed at 5 μ M. However, comparison of free WFA and WFA-loaded nanoparticles showed no significant difference across the concentration range tested. This suggests that, under the assay conditions used here, nanoparticle encapsulation did not substantially enhance the viability-reducing effect of WFA in H358 cells. WFA is a pleiotropic natural compound reported to influence multiple cancer-associated pathways, including NF- κ B, STAT3, PI3K/Akt, apoptosis, oxidative stress, EMT and cytoskeletal regulation [6–10,17]. However, its poor aqueous solubility and bioavailability remain barriers to therapeutic translation [6,7]. The absence of a clear enhancement with WFA-NPs in the 2D viability assay may reflect differences in WFA release kinetics, intracellular availability, assay timing or the sensitivity of the resazurin metabolic readout. It is also possible that WFA exerts stronger effects on migration, cytoskeletal organisation or angiogenesis-related pathways than on short-term metabolic activity in this model.

Combination treatment with doxorubicin and WFA produced a concentration-dependent reduction in H358 viability/metabolic activity, and nanoparticle-loaded combination treatment enhanced this effect at selected concentrations. The strongest reduction was observed at the highest doxorubicin/WFA combination concentration. These findings suggest that co-delivery of doxorubicin and WFA may provide therapeutic benefit by targeting complementary mechanisms. Doxorubicin primarily acts through DNA damage, topoisomerase II inhibition and oxidative stress, whereas WFA has been linked to modulation of survival, inflammatory, cytoskeletal and angiogenic pathways [3,6–10,18]. Previous studies have reported that WFA can enhance doxorubicin activity in preclinical cancer models through mechanisms involving reactive oxygen species, DNA damage, autophagy and apoptosis [13]. Although formal synergy analysis was not performed in the present study, the data support the concept that doxorubicin/WFA co-delivery may increase anti-cancer activity compared with selected single-agent treatments.

The scratch wound assay showed that drug-loaded nanoparticles significantly impaired H358 migration. Untreated cells and nanoparticle controls migrated efficiently into the wound area over 48 h, whereas doxorubicin-, WFA- and combination-loaded nanoparticles reduced migration rates. The most pronounced inhibition was observed with the highest concentration of combination nanoparticles. These findings are relevant because migration and invasion contribute to local tumour progression and

metastatic dissemination in NSCLC. WFA has previously been reported to inhibit EMT and metastatic behaviour in NSCLC models [8,9], and the reduced migration observed here is consistent with these reported anti-migratory effects. The strong response to combination nanoparticles suggests that co-delivery may affect not only cell viability but also migratory behaviour, potentially through effects on cytoskeletal organisation, stress signalling, survival pathways and EMT-associated processes.

The 3D spheroid model provided a more physiologically relevant system to assess treatment response. Free doxorubicin and doxorubicin-loaded nanoparticles reduced NSCLC spheroid size over 7 days, indicating that doxorubicin retained anti-tumour activity in a 3D tumour-like structure. WFA and WFA-loaded nanoparticles also reduced spheroid size, although the effect appeared more modest than that observed with doxorubicin-based treatments. In contrast, combination doxorubicin/WFA treatment produced the greatest reduction in spheroid size, with the strongest effect observed following combination-loaded nanoparticle treatment. These findings suggest that co-delivery of doxorubicin and WFA may enhance anti-tumour activity in 3D NSCLC models, potentially through complementary effects on cytotoxicity, stress signalling, cytoskeletal organisation and cell survival. Spheroids better represent aspects of solid tumour architecture, including cell–cell interactions, extracellular matrix-associated constraints, gradients in nutrients and oxygen, and reduced drug penetration compared with 2D monolayer cultures [18]. These features make spheroid models particularly useful for evaluating nanocarrier-based therapeutics, where penetration, retention and release behaviour may differ from conventional 2D assays [20].

The CAM model further demonstrated that free and nanoparticle-loaded treatments reduced NSCLC tumour size in a vascularised *ex vivo* environment. Both doxorubicin and WFA reduced tumour area, and nanoparticle-loaded formulations also significantly suppressed tumour growth compared with nanoparticle controls. The CAM model is particularly valuable because it enables assessment of tumour growth, vascularisation and treatment response within a rapidly developing vascular network [21-23]. This is relevant to NSCLC progression, where tumour vascularisation supports growth, nutrient supply and metastatic dissemination. The reduction in tumour size observed following nanoparticle-loaded treatment supports the anti-tumour potential of the MSN platform in a more complex biological system than 2D culture. Importantly, these

findings also align with the known anti-angiogenic properties of WFA, which has been reported to inhibit endothelial cell behaviour and tumour-associated vascular development [11,12].

The antibody microarray data provide preliminary mechanistic insight into the pathways associated with nanoparticle treatment. Doxorubicin-loaded nanoparticles increased signal intensity for several apoptosis-associated proteins, including Bad, Bax, caspase-associated proteins, Fas/TNFRSF6/CD95, HIF-1 α , HSP27, p21/CIP1/CDKN1A, survivin and TNF R1/TNFRSF1A. These changes suggest activation of stress, death receptor, cell-cycle and apoptosis-associated pathways. WFA-loaded nanoparticles also altered apoptosis-related markers, consistent with the reported ability of WFA to induce apoptosis and modulate survival signalling [6,7,10,18]. Combination nanoparticles produced a broader but generally moderate apoptosis-associated profile, suggesting that the combined treatment may influence multiple pathways rather than driving a single dominant apoptotic response.

The angiogenesis microarray data showed that combination nanoparticles produced low signal intensity across several angiogenesis-associated markers, including amphiregulin, IL-8, thrombospondin-1, uPA and VEGF. This is notable because angiogenesis is essential for tumour growth and dissemination, and VEGF, IL-8 and uPA are all associated with pro-angiogenic and tumour-promoting activity. The suppression of angiogenesis-associated protein signals by combination nanoparticles is consistent with the CAM tumour data and supports a mechanism involving inhibition of tumour-associated angiogenic signalling. WFA has previously been described as a potent inhibitor of angiogenesis [11,12], and the present findings suggest that its inclusion in combination nanoparticle formulations may contribute to anti-angiogenic effects in NSCLC models.

Overall, the data suggest that MSN-mediated delivery of doxorubicin and WFA suppresses NSCLC tumour-associated behaviour through multiple mechanisms, including reduced viability/metabolic activity, impaired migration, reduced spheroid growth, suppression of CAM tumour development and modulation of apoptosis- and angiogenesis-associated proteins. The findings are particularly supportive of combination nanoparticle treatment, which showed strong effects on cell viability, migration and angiogenesis-associated protein expression. This multi-targeted activity

is relevant to NSCLC, where tumour progression is driven by interconnected survival, migratory, inflammatory and angiogenic pathways.

Future studies could focus on validating the nanoparticle formulations across multiple NSCLC models, quantifying drug loading and release kinetics, confirming intracellular trafficking, and validating apoptosis and angiogenesis targets identified by the antibody arrays. Additional 3D models, including multicellular spheroids or tumour–stromal co-cultures, would provide greater insight into tumour microenvironment interactions and nanocarrier performance [19,20]. Further CAM studies could also assess vascular branching, vessel density and haemorrhagic or toxic effects in addition to tumour area [21-23]. *In vivo* studies would ultimately be required to determine biodistribution, safety, pharmacokinetics and therapeutic efficacy. Given the limitations of doxorubicin toxicity and the pleiotropic anti-cancer properties of WFA, MSN-mediated co-delivery represents a promising strategy that warrants further investigation.

In conclusion, this study demonstrates that mesoporous silica nanoparticle-mediated delivery of doxorubicin, WFA and combination doxorubicin/WFA treatment suppresses key tumour-associated behaviours in NSCLC models. Drug-loaded nanoparticles reduced cell viability/metabolic activity, impaired migration, decreased spheroid growth and suppressed tumour development in the CAM model. The combination-loaded nanoparticles showed particularly strong effects in the viability, migration and 3D spheroid assays, supporting the potential value of MSN-mediated co-delivery. Mechanistic analysis further suggests involvement of apoptosis-associated pathways and inhibition of angiogenesis-associated signalling, particularly following combination nanoparticle treatment. These findings support the potential of MSN-based co-delivery of doxorubicin and WFA as a multi-targeted therapeutic approach for NSCLC.

Figure legends

Figure 1 - Characterization of the synthesized mesoporous silica nanoparticles:

A) TEM image showing spherical MSNs with mesoporous structure; B) particle size distribution histogram; C) N₂ adsorption/desorption isotherm (D) Size, Zeta potential and encapsulation efficiency (µg/mg) of NPs.

Figure 2. Time-dependent cell-associated fluorescence following treatment with

free doxorubicin, doxorubicin-loaded and withaferin A nanoparticles. Cells were treated with 5 µM free doxorubicin, 5 µM doxorubicin-loaded nanoparticles (Dox-NPs) or 5 µM withaferin A (WFA) and imaged at 0, 1, 2, 4, 24 and 48 h. Nuclei were stained with DAPI. Actin staining was used to visualise cell morphology. **(A)** Free doxorubicin-treated cells. **(B)** Dox-NP-treated cells. **(C)** WFA-NP-treated cells. Free doxorubicin and Dox-NP-associated fluorescence were detected in the red channel, while WFA-NP associated fluorescence was detected in the green channel. Dox-NPs showed punctate intracellular fluorescence consistent with nanoparticle-associated uptake, whereas free doxorubicin showed broader intracellular and nuclear-associated fluorescence over time. WFA-NP treated cells showed detectable intracellular fluorescence and visible changes in cell morphology across the time course. Scale bars are shown on individual images.

Figure 3. Effect of free doxorubicin and doxorubicin-loaded nanoparticles on

H358 cell viability. H358 cells were treated for 48 h with free doxorubicin or doxorubicin-loaded nanoparticles (Dox-NPs) at 5, 2.5, 1.25 and 0.625 µM. Cell viability/metabolic activity was assessed by measuring relative fluorescence units (RFU) over time. **(A)** RFU measurements following treatment with free doxorubicin. **(B)** RFU measurements following treatment with Dox-NPs. **(C)** Fold-change comparison between free doxorubicin and Dox-NP treatments. Dox-NPs produced a greater reduction in viability/metabolic activity than free doxorubicin at higher concentrations, particularly 5 and 2.5 µM. Data are presented as mean ± error bars. Statistical significance is indicated on the graph.

Figure 4. Effect of free withaferin A and withaferin A-loaded nanoparticles on H358 cell viability. H358 cells were treated for 48 h with free withaferin A (WFA) or WFA-loaded nanoparticles (WFA-NPs) at 5, 2.5, 1.25 and 0.625 μ M. Cell viability/metabolic activity was assessed by measuring relative fluorescence units (RFU) over time. **(A)** RFU measurements following treatment with free WFA, including untreated and DMSO vehicle controls. **(B)** RFU measurements following treatment with WFA-NPs, including untreated and nanoparticle controls. **(C)** Fold-change comparison between free WFA and WFA-NP treatments. Both free WFA and WFA-NPs reduced H358 cell viability/metabolic activity, with the greatest reduction observed at 5 μ M. No significant difference was observed between free WFA and WFA-NP treatments across the concentration range tested. Data are presented as mean $\pm$ error bars. ns, not significant.

Figure 5. Effect of free and nanoparticle-loaded doxorubicin/WFA combination treatment on H358 cell viability. H358 cells were treated with free doxorubicin/WFA combination treatment or combination doxorubicin/WFA-loaded nanoparticles (Combo-NPs) at 0.625, 1.25, 2.5 and 5 μ M. Relative fluorescence units (RFU) were measured over a 240 min assay period. **(A)** RFU measurements following treatment with free doxorubicin/WFA combination treatment, including untreated and DMSO vehicle controls. **(B)** RFU measurements following treatment with Combo-NPs, including untreated and nanoparticle controls. **(C)** Fold-change comparison between free combination treatment and Combo-NPs at each concentration. Statistical significance is indicated on the graph; ns, not significant.

Figure 6. Effect of low-dose drug-loaded nanoparticles on H358 cell migration. H358 cell migration was assessed using a scratch wound assay over 48 h. **(A)** Representative brightfield images showing wound closure at 0 and 48 h in untreated cells, nanoparticle control cells, 0.625 μ M doxorubicin-treated cells and 0.625 μ M doxorubicin-loaded nanoparticle-treated cells. Red lines indicate the wound boundaries. Scale bar = 100 μ m. **(B)** Quantification of migration rate following treatment with nanoparticle control, free doxorubicin, free WFA, doxorubicin-loaded nanoparticles, WFA-loaded nanoparticles, free doxorubicin/WFA combination

treatment and doxorubicin/WFA combination-loaded nanoparticles at 0.625 μ M. Untreated and nanoparticle control cells showed comparable migration rates, whereas drug-loaded nanoparticle formulations significantly reduced H358 cell migration. The strongest inhibition was observed following treatment with doxorubicin-loaded nanoparticles and combination-loaded nanoparticles. Statistical analysis was performed using one-way ANOVA with multiple comparisons. ns, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Data are presented as mean $\pm$ SD (n = 4).

Figure 7. Effect of free doxorubicin, WFA, combination treatment and nanoparticle-loaded formulations on NSCLC spheroid size. NSCLC spheroids were treated with free doxorubicin, doxorubicin-loaded nanoparticles, free withaferin A (WFA), WFA-loaded nanoparticles, free doxorubicin/WFA combination treatment or combination-loaded nanoparticles and monitored over 7 days. **(A)** Representative brightfield images of spheroids following treatment with free doxorubicin or doxorubicin-loaded nanoparticles. Scale bar = 0.5 mm. **(B)** Quantification of spheroid size at day 0 and day 7 following treatment with free doxorubicin or doxorubicin-loaded nanoparticles at 0.625, 1.25, 2.5 and 5 μ M. **(C)** Quantification of spheroid size at day 0 and day 7 following treatment with free WFA or WFA-loaded nanoparticles at 0.625, 1.25, 2.5 and 5 μ M. **(D)** Quantification of spheroid size at day 0 and day 7 following treatment with free doxorubicin/WFA combination treatment or combination-loaded nanoparticles. Combination-loaded nanoparticles produced the greatest reduction in spheroid size after 7 days. Data are presented as mean $\pm$ error bars.

Figure 8. Effect of doxorubicin, WFA and combination-loaded nanoparticles on NSCLC tumour growth in the CAM model. NSCLC tumours were established on the chick chorioallantoic membrane (CAM) and treated with free doxorubicin, withaferin A (WFA), free doxorubicin/WFA combination treatment, nanoparticle control, doxorubicin-loaded nanoparticles, WFA-loaded nanoparticles or doxorubicin/WFA-loaded combination nanoparticles. **(A)** Representative images of CAM tumours following treatment. **(B)** Quantification of tumour size measured as tumour area (mm²). Free and nanoparticle-loaded drug treatments significantly reduced tumour size

compared with the relevant untreated or nanoparticle control groups. Statistical significance is indicated on the graph; **** $p < 0.0001$.

Figure 9. Effect of nanoparticle treatments on apoptosis- and angiogenesis-associated protein expression. Antibody microarrays were used to assess changes in protein expression following treatment with control nanoparticles, 1.25 μ M doxorubicin-loaded nanoparticles (DOX-NPs), 1.25 μ M withaferin A-loaded nanoparticles (WFA-NPs) and combination 1.25 μ M doxorubicin/WFA-loaded nanoparticles (Combo-NPs) for 48 hours. **(A)** Mean pixel density of apoptosis-associated proteins. DOX-NPs, WFA-NPs and Combo-NPs altered the expression of multiple apoptosis-associated markers, including proteins involved in caspase signalling, death receptor signalling, stress responses and cell-cycle regulation. **(B)** Mean pixel density of angiogenesis-associated proteins. Combo-NPs showed reduced signal intensity across the angiogenesis-associated markers assessed, including amphiregulin, IL-8, thrombospondin-1, uPA and VEGF. Data are presented as mean pixel density.

Declarations

Ethics approval and consent to participate

Not applicable. This study did not involve human participants. Experiments used the established NCI-H358 human cell line and the chick chorioallantoic membrane model. CAM experiments were terminated at embryonic day 13, before hatching, and were conducted in accordance with applicable institutional procedures and relevant regulations.

Consent for publication

Not applicable. This manuscript does not include any individual person's data in any form, including individual details, images or videos.

Availability of data and materials

The data supporting the findings of this study are available within the article and its supplementary materials. Additional data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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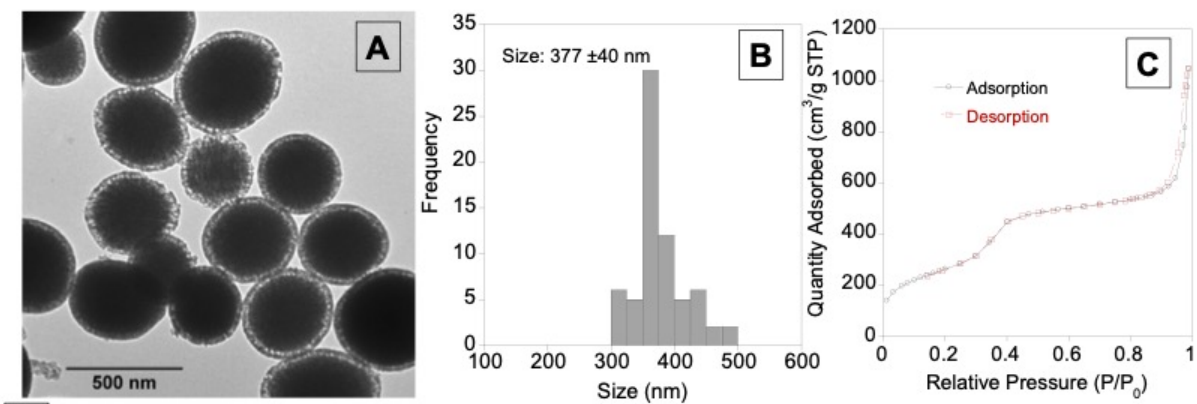
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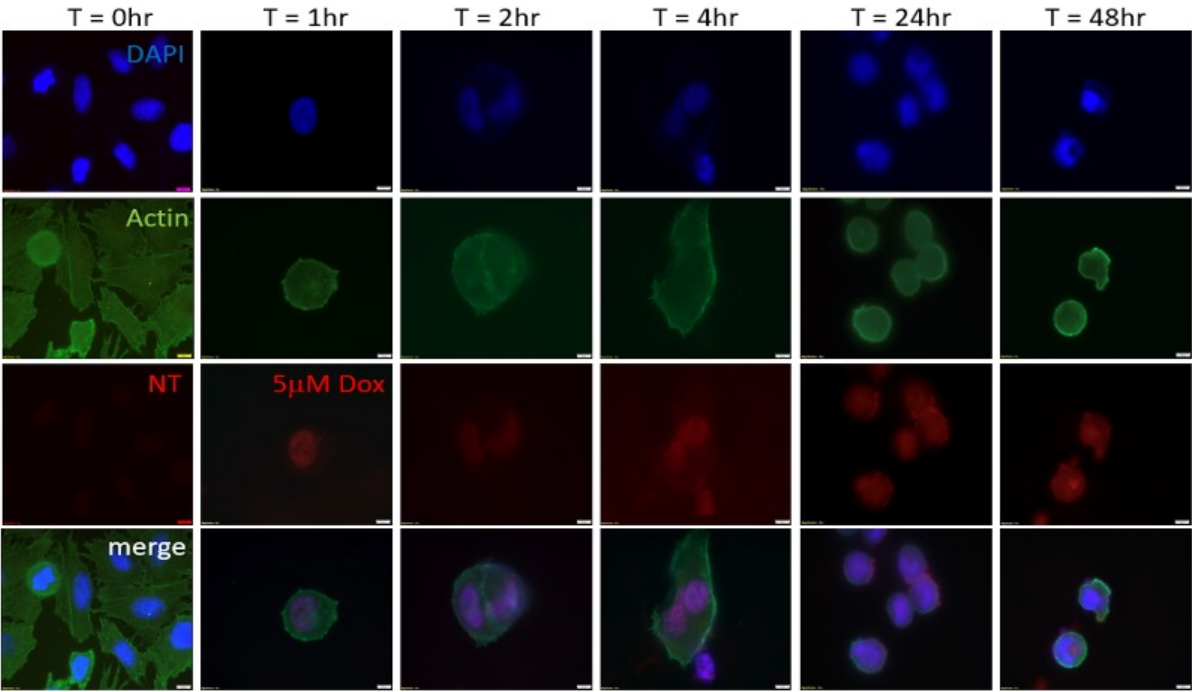
Figure 1



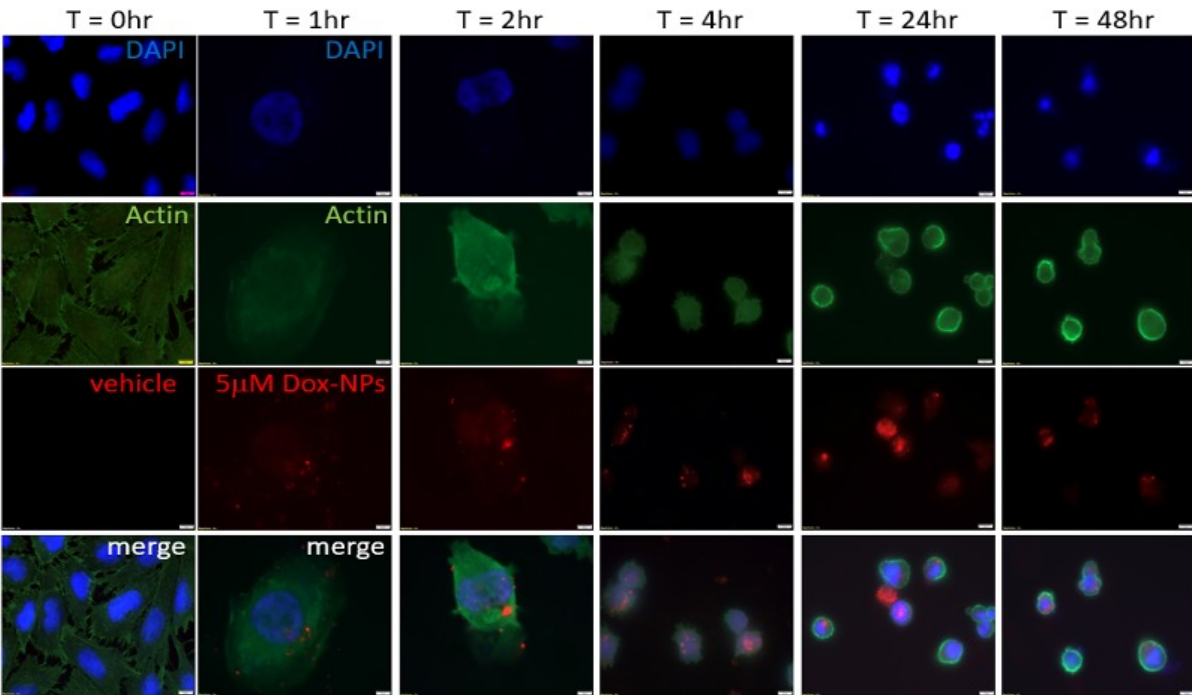
D				
Nanoparticles	MSNs (NPs)	Dox-NPs	WFA-NPs	Combo-NPs
dH(nm)	545 ± 89	685 ± 97	936 ± 28	1657 ± 81
PDI	0.745 ± 0.169	0.607 ± 0.014	0.882 ± 0.057	0.397 ± 0.080
Zeta-Potential (mV)	7.6 ± 1.5	-8.6 ± 0.9	-29.5 ± 5.4	-15.9 ± 3.2
EE (µg/mg)	-	23.1	16.1	4.6 (WFA)/13.8 (Dox)

Figure 2

A



B



C

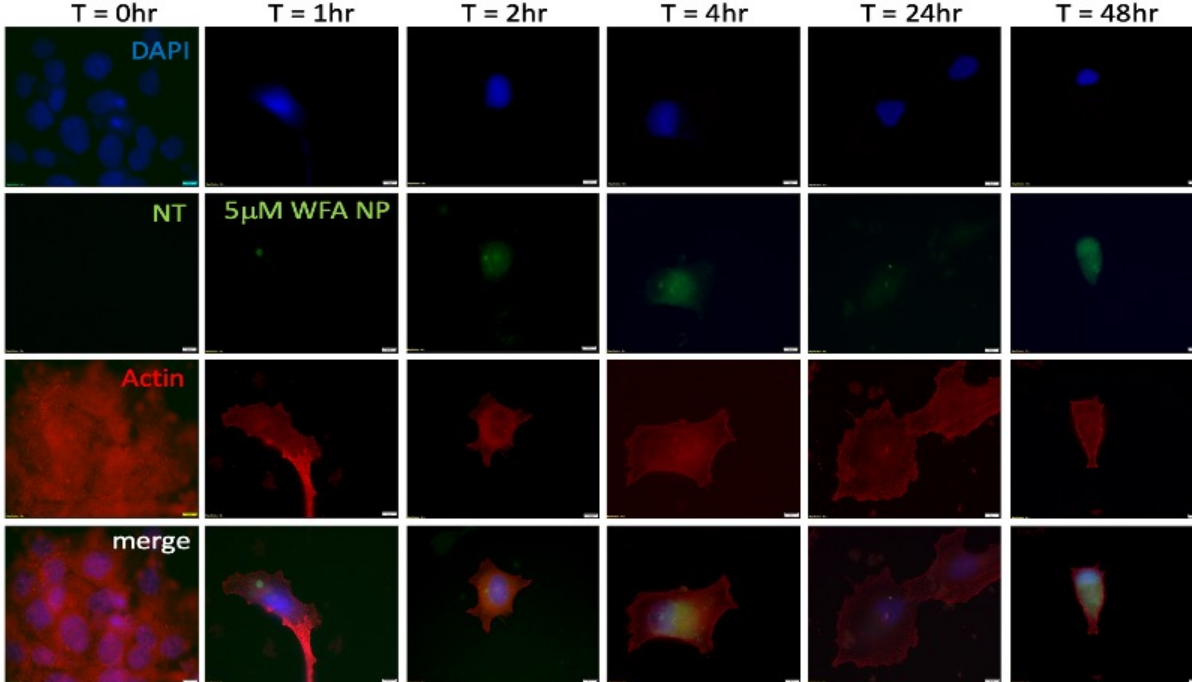


Figure 3

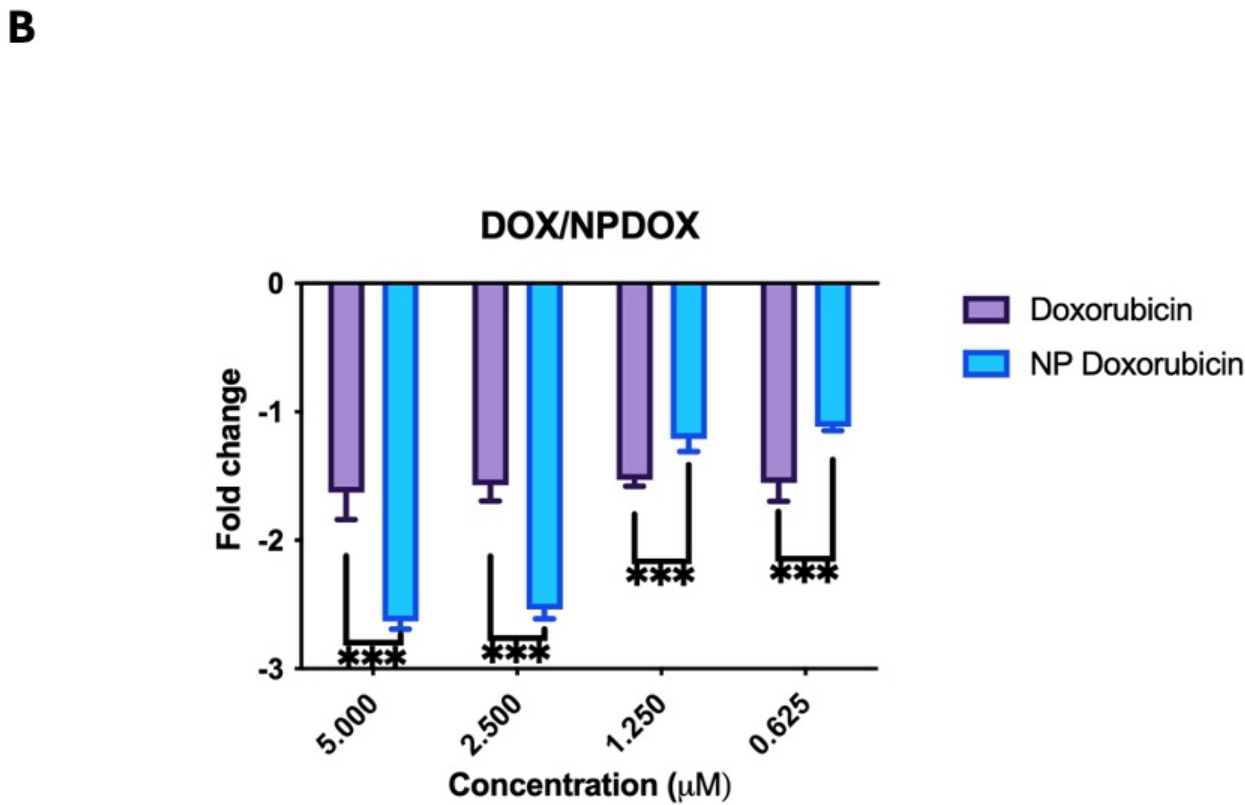
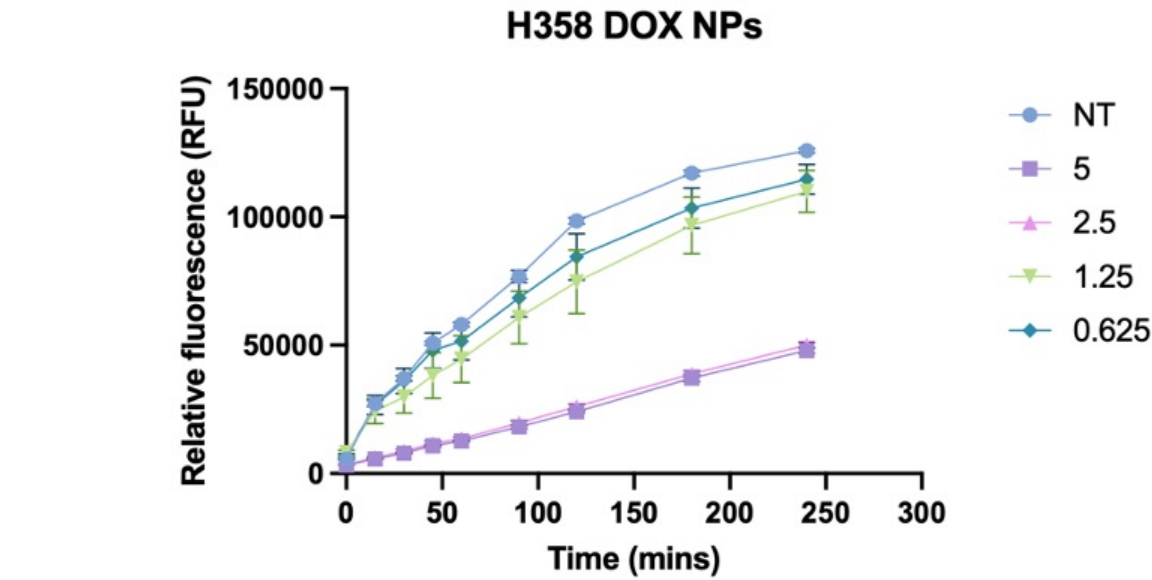
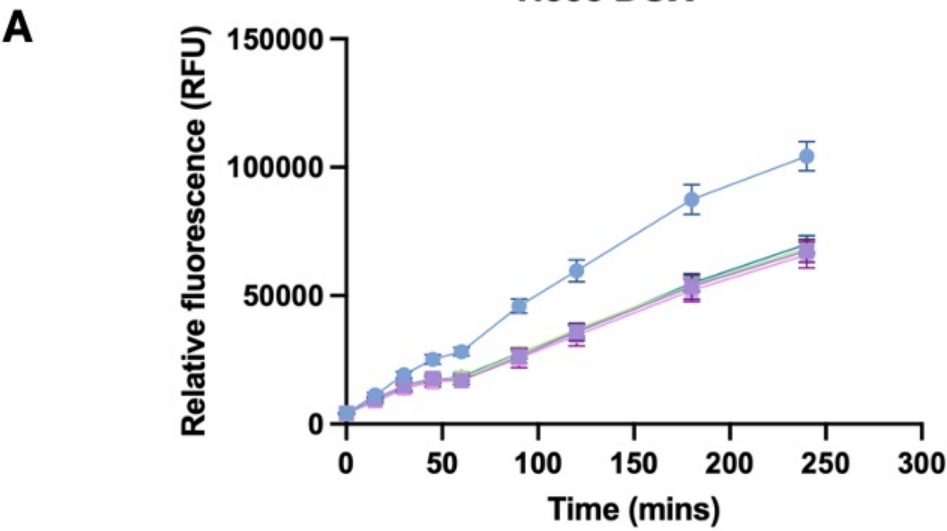
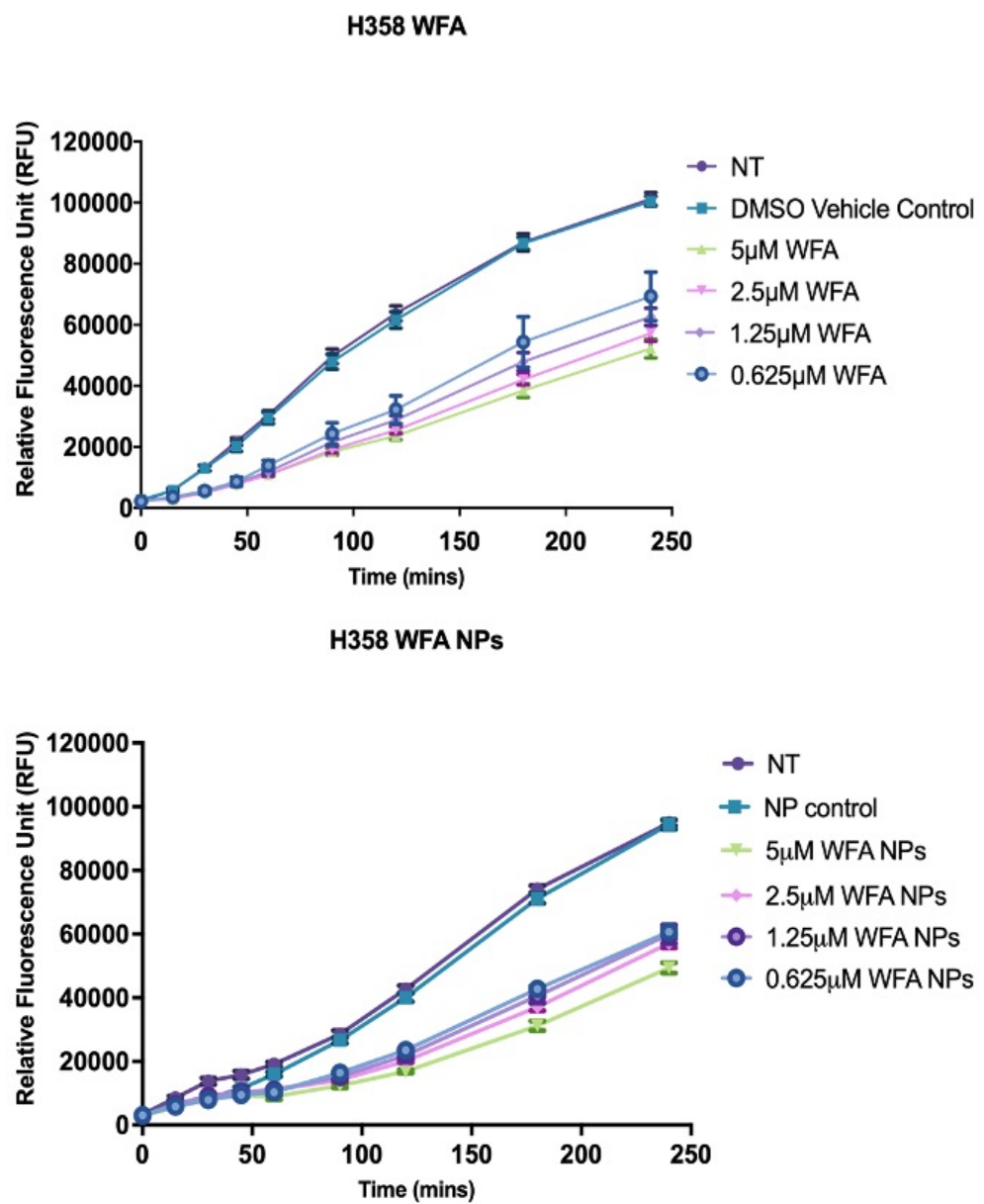


Figure 4

A



B

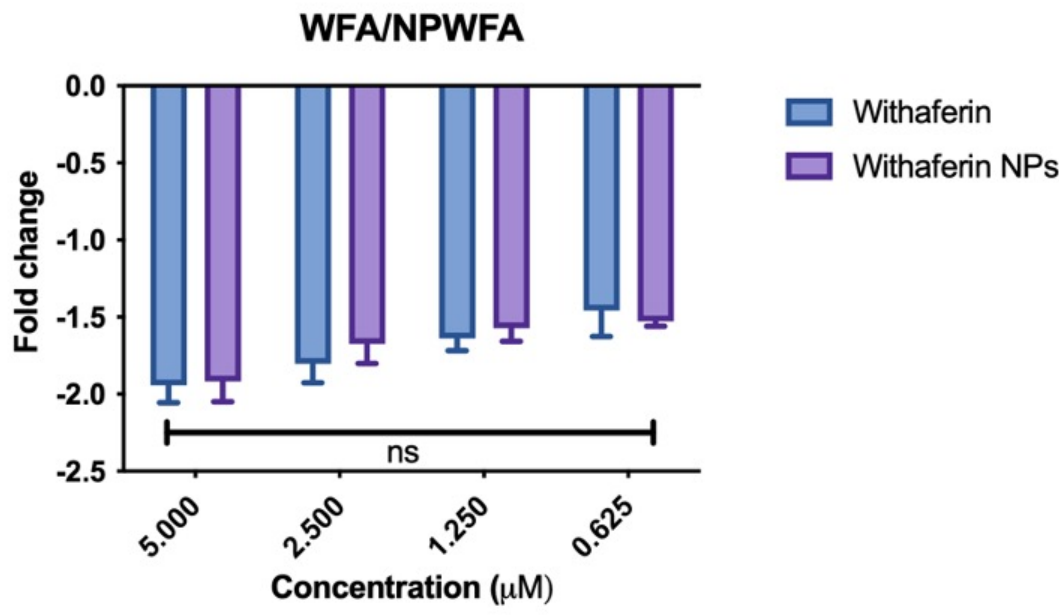


Figure 5

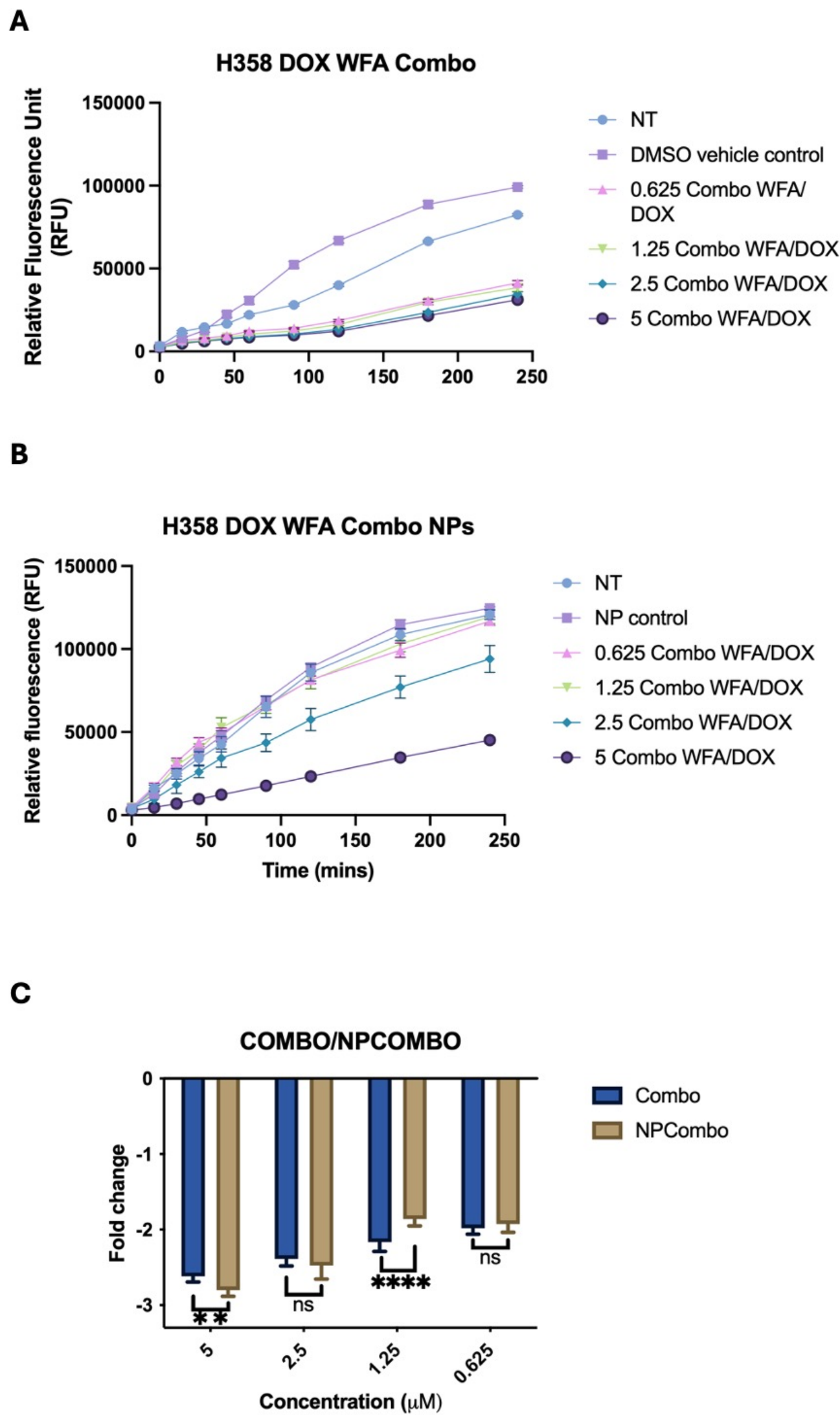


Figure 6

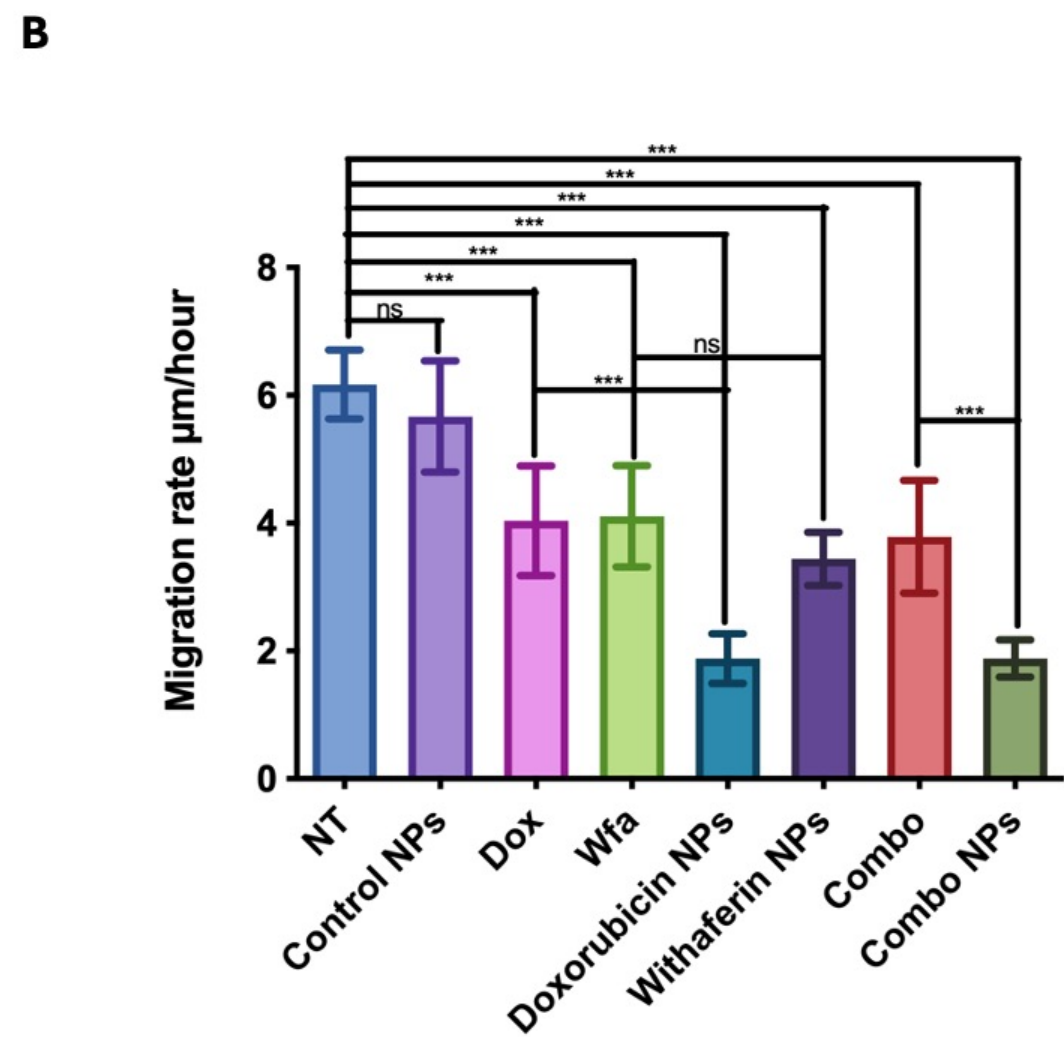
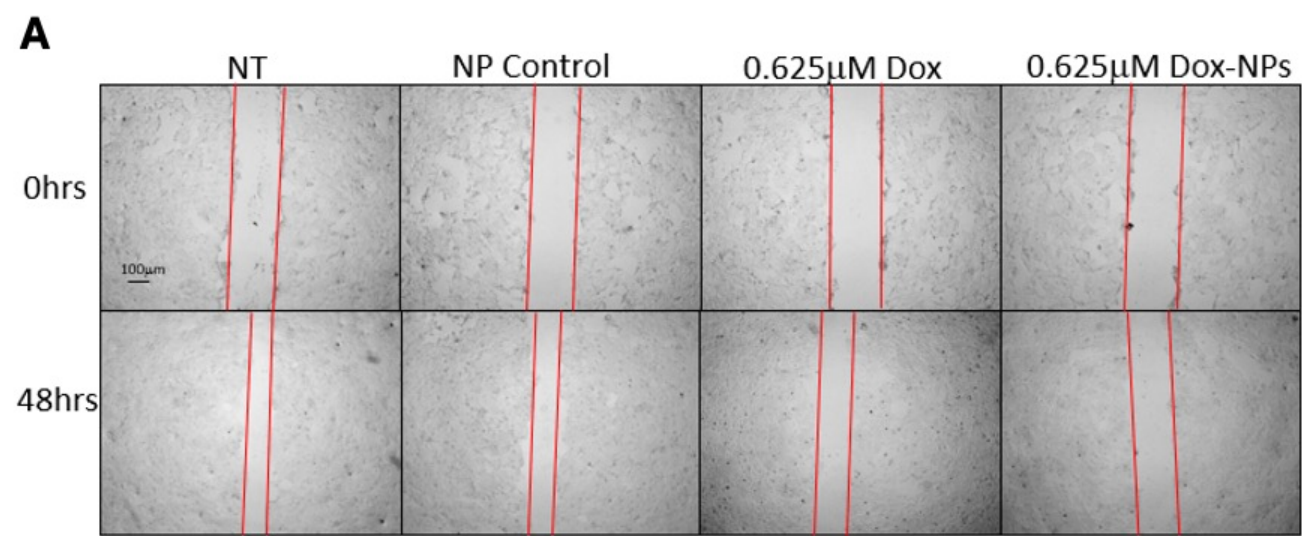


Figure 7

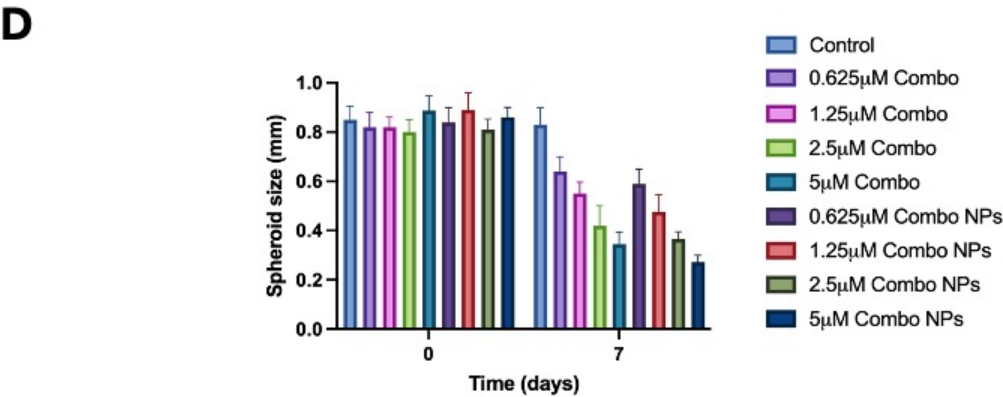
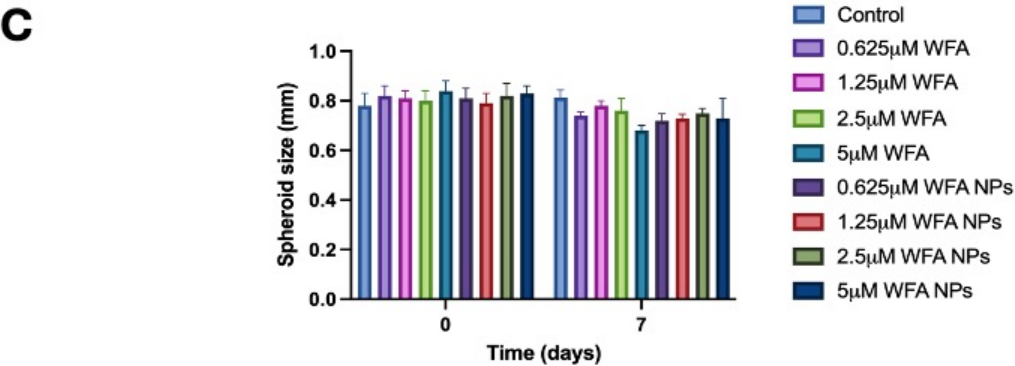
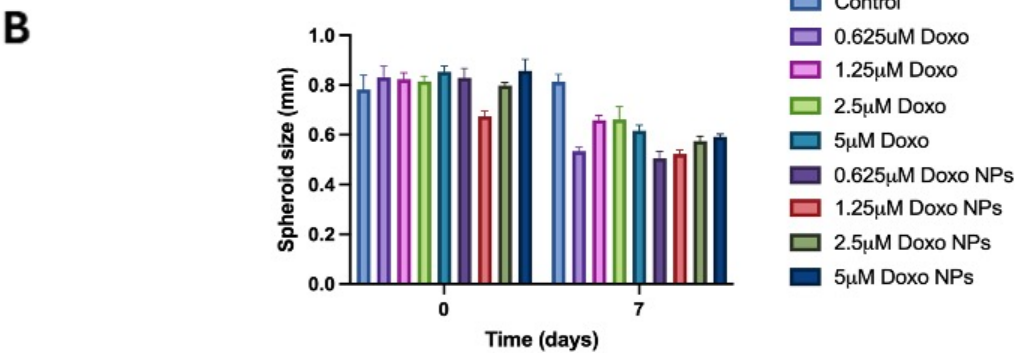
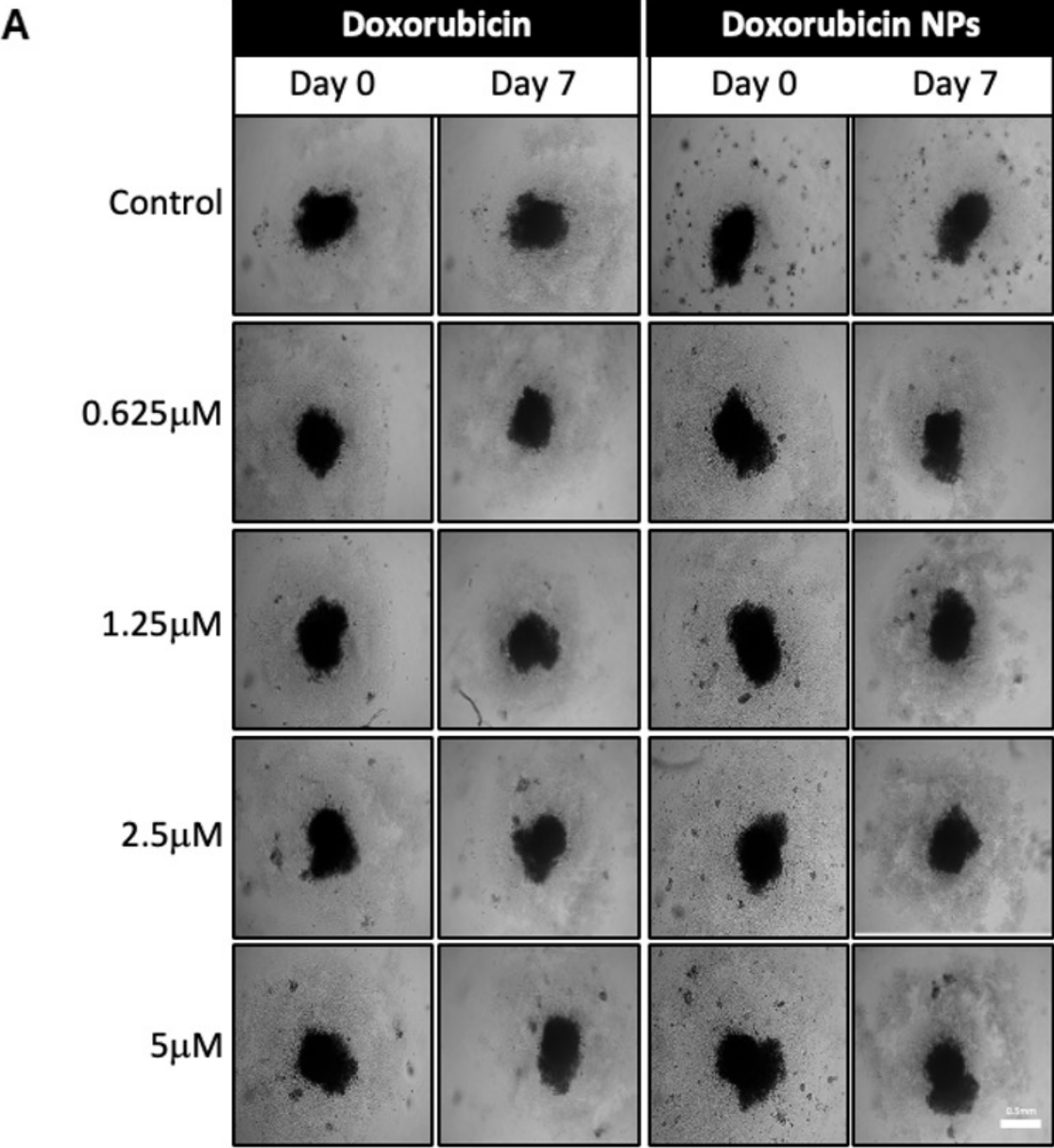
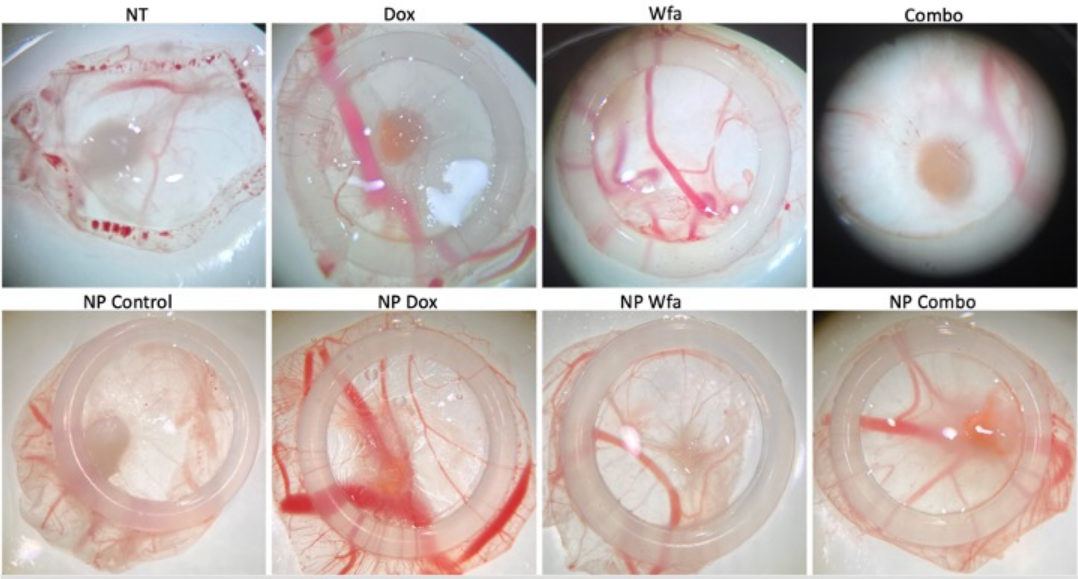


Figure 8

A



B

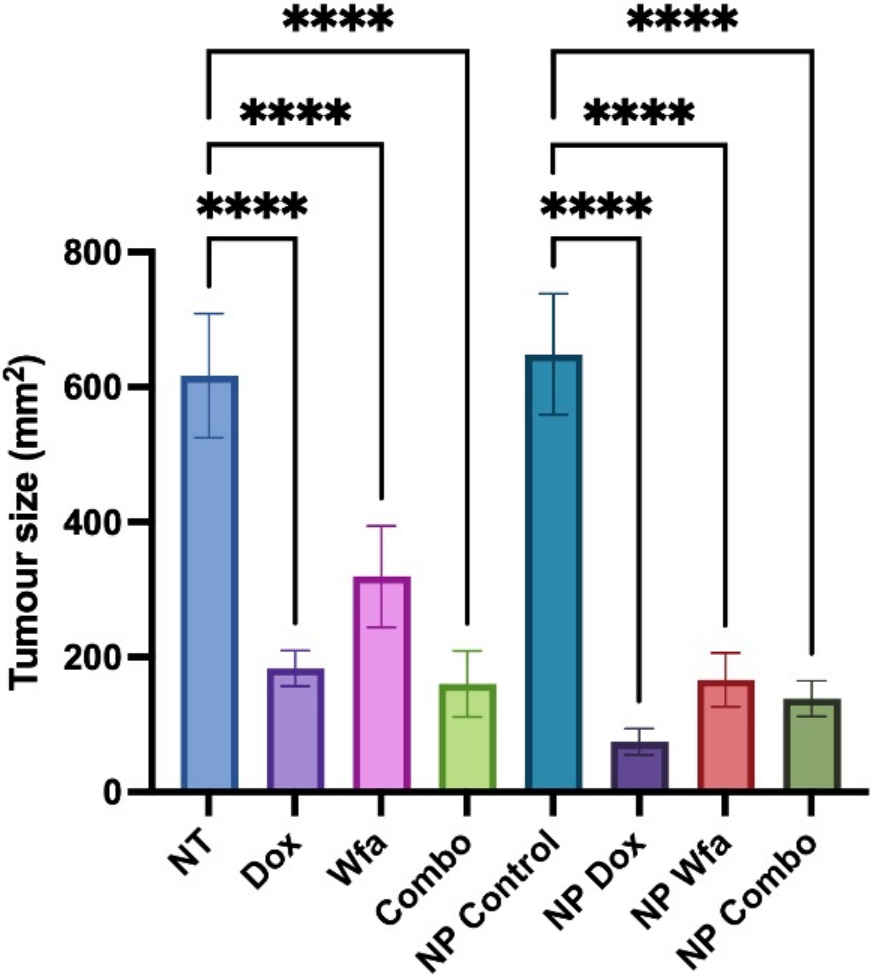
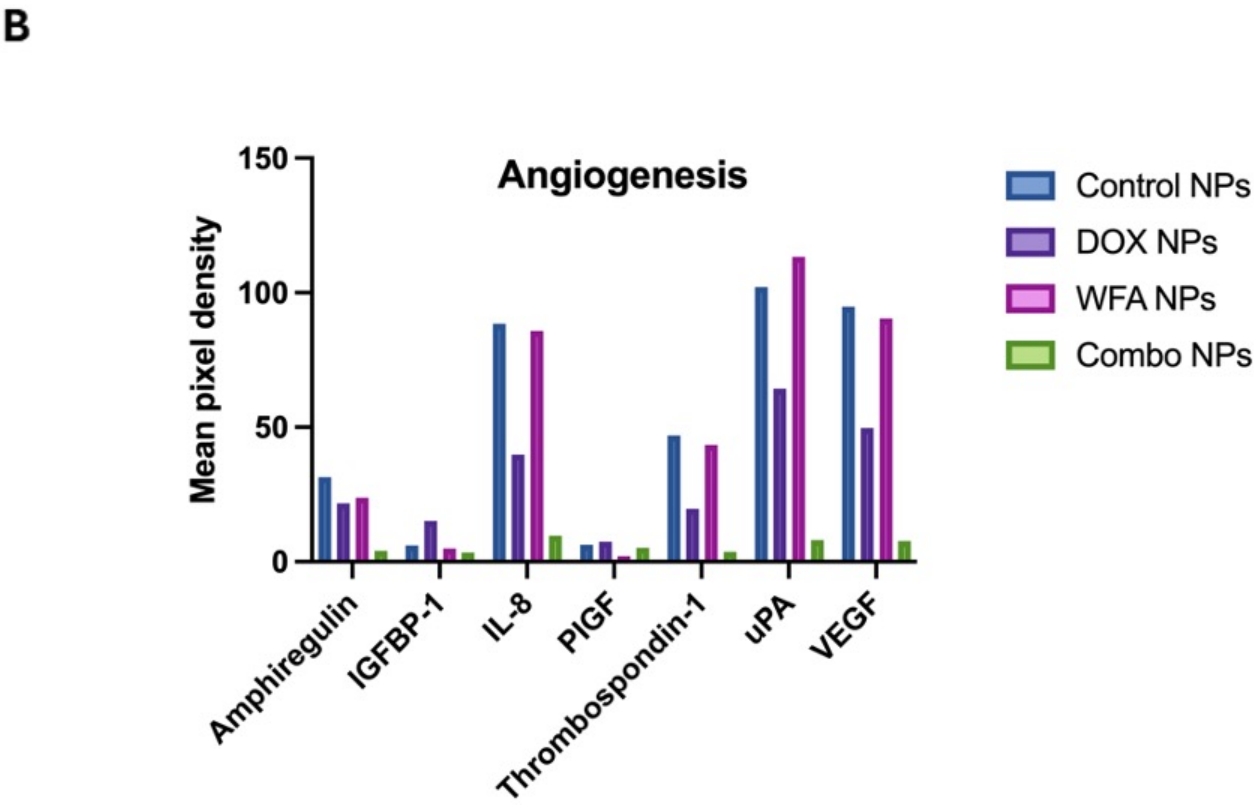
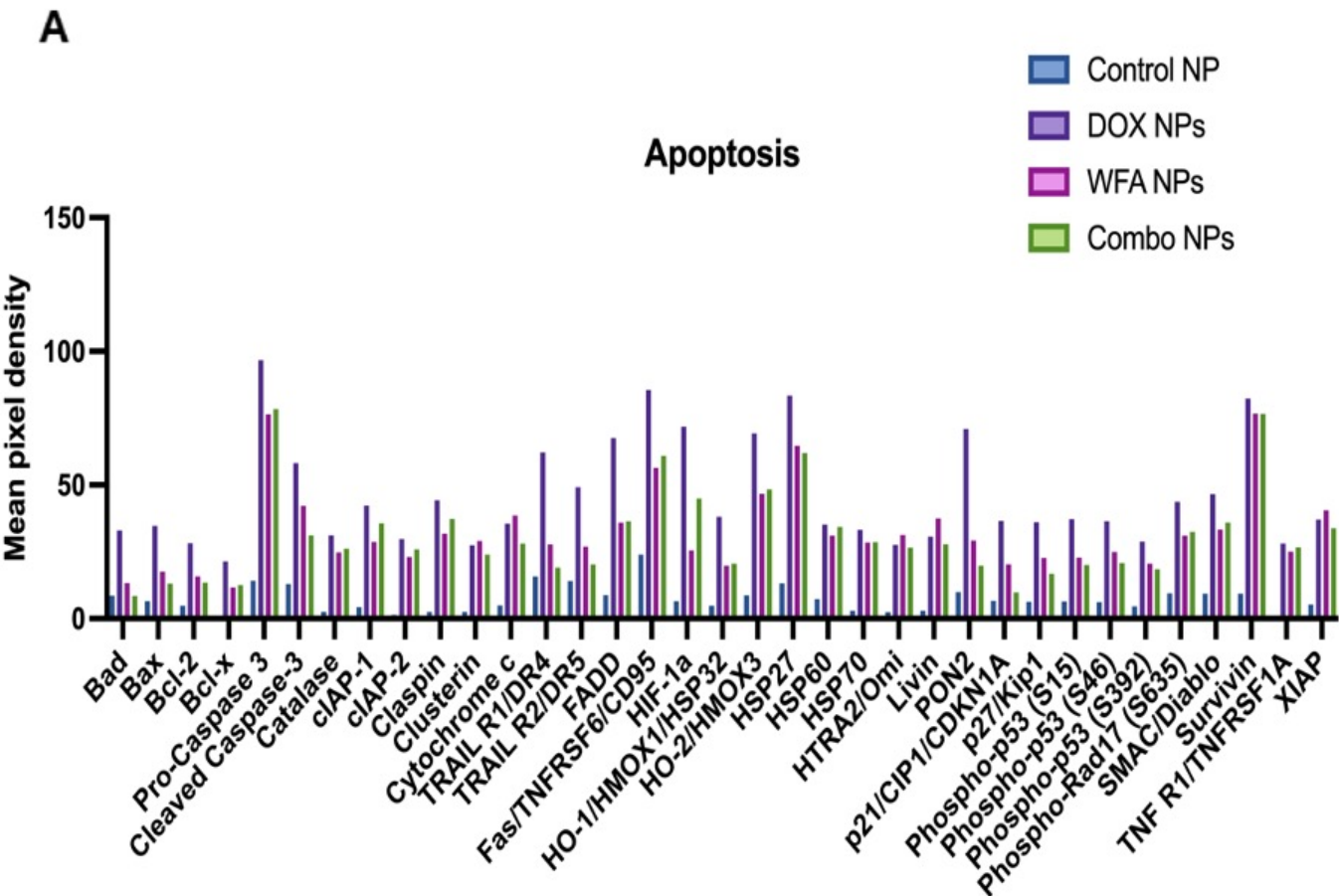


Figure 9



Supplementary Files

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