

Nanoformulated Bioactive Compounds Derived from Different Natural Products Combat Pancreatic Cancer Cell Proliferation

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Purpose: This study was designed to determine the potential effect of nanoencapsulated bioactive compounds from different natural sources on human pancreatic cancer.

Background: Pancreatic cancer carries the highest fatality rate among all human cancers because of its high metastatic potential and late presentation at the time of diagnosis. Hence there is a need for improved methods to prevent and treat it. Natural products, such as 3, 3'-diindolylmethane (DIM) and ellagic acid (EA) demonstrated anticancer efficacy against various cancer types. However, DIM is insoluble. Hence, using nanotechnology to encapsulate these compounds in combination with EA might improve their physical and chemical properties and their delivery to the cancer cells.

Methods: Human pancreatic cancer cells, namely SUIT2-luciferase transfected, were used to examine the effects of DIM or EA and their nanoformulation in poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) [PLGA-PEG] nanoparticles (NPs) on SUIT2-luciferase cell viability/proliferation over 24 hrs. Additionally, effects on tumor weight and angiogenesis were determined using the chick chorioallantoic membrane (CAM) tumor implant model.

Results: Both DIM and EA PLGA-PEG NPs resulted in rapid suppression of pancreatic cancer cell viability/proliferation within 24 hrs ($P < 0.01$), while the non-encapsulated DIM and EA did not show any significant effect on SUIT2 cancer cell viability or cell proliferation (MTT assay). In the CAM pancreatic cancer cell (SUIT2) implant model, results showed a greater suppression of tumor weight ($P < 0.01$), tumor cell viability, and tumor angiogenesis ($P < 0.01$) for DIM NPs and EA NPs and their combinations versus DIM or EA alone.

Conclusion: Nanoformulation of DIM and EA resulted in a more effective suppression of pancreatic cancer cell viability, pancreatic tumor weight, implanted cancer cell viability, and tumor angiogenesis as compared with these bioactive compounds alone.

Keywords: pancreatic cancer, 3, 3'-diindolylmethane, ellagic acid, PLGA-PEG nanoparticles, anti-angiogenesis, chick chorioallantoic membrane model

Introduction

Pancreatic cancer carries the highest fatality rate among all human cancers.¹ Despite the recent advances in pancreatic cancer care, its prognosis remains poor and the survival rate for 5 years is less than 5% because pancreatic cancer is highly aggressive. Improvements in its treatment are essential.^{2,3}

Numerous attempts have been made by researchers to find new anticancer drugs of natural origin such as thymoquinone,⁴ curcumin,⁵ and papaverine.⁶ 3, 3'-diindolylmethane (DIM), which has been isolated from broccoli, Brussels sprouts,

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cabbage, and kale and ellagic acid (EA), which is present in raspberries and blackberries, have anticancer properties, but they are insoluble or unstable. Therefore, nanoformulations have improved their bioavailability.⁷⁻⁹

Drugs' nanoformulations have been extensively developed and used for cancer therapy if approved by the Food and Drug Administration (FDA), leading to effective anticancer potential with less toxicity.¹⁰ Nanoparticles (NPs) such as carbon, ceramic, chitosan, and poly(lactic-co-glycolic acid) (PLGA) NPs have been extensively used for drug delivery enhancement.^{11,12} PLGA NPs are biocompatible, biodegradable, and stable in biological fluids, protect the loaded compounds from degradation, and provide their sustained release.¹³ PLGA NPs are taken up by fluid-phase pinocytosis and endocytosis^{14,15} and rapidly escape the endolysosomes and release the encapsulated material in the cytoplasm.¹⁴ PLGA polymer undergoes spontaneous and enzymatic hydrolysis of its ester linkages to produce lactic acid and glycolic acid. Because both lactic acid and glycolic acid are endogenous molecules, they are easily metabolized to carbon dioxide and water via the Krebs cycle.¹³ The biodegradable PLGA polymer is known to be safe in humans. The FDA and the European Medicine Agency have approved the use of PLGA NPs via the parenteral route and the use of PLGA micro-particles as implants.¹⁴ In addition, PLGA NPs are being extensively investigated as oral drug carriers.¹⁶⁻¹⁹ A major disadvantage of PLGA NPs is that they are rapidly opsonized by immunoglobulins and complement proteins and cleared by the reticuloendothelial system and may not reach target tissues.^{13,14} Modifying their surface with biocompatible polymers, such as poly(ethylene glycol) (PEG), reduces opsonization and prolongs their circulation time in the blood by several orders of magnitude.¹⁴

Experimental and limited clinical evidence of either DIM or EA alone showed potential prevention of various types of cancer, and we raised the question of whether the combinations of these different bioactive compounds in a nanoformulation could be more effective in suppressing pancreatic cancer. Therefore, SUIT2 cells were subjected to treatment with DIM, EA, combinations, and their nanoformulations (DIM NPs, EA NPs, and combinations) at concentrations ranging from 0.1 to 10 $\mu\text{g/mL}$ in the current study.

Materials and Methods

Cell Line and Reagents

Human pancreatic cancer cell line, SUIT2 expressing firefly luciferase (SUIT2-Luc), was provided by MD

Anderson Cancer Center, Houston, TX. Cell culture reagents, EA, DIM, and other common reagents were purchased from Sigma (St. Louis, MO, USA). D-Luciferin potassium salt was purchased from Caliper Life Sciences (Hopkinton, MA, USA). Matrigel was purchased from BD Bioscience (San Jose, CA, USA).

Synthesis of Nanoparticles

PLGA-PEG NPs encapsulating DIM or EA were prepared by double emulsion/solvent evaporation methods as previously described.^{9,20} Briefly, a stock solution of PLGA-PEG polymer was prepared by dispersing 80 mg/mL of this polymer in dichloromethane. A stock solution of 10 mg/mL of DIM or EA (10 $\mu\text{g/mL}$ is 40.5 μM of DIM and 33 μM of EA) was prepared by dissolving DIM or EA in dichloromethane. Five hundred μL of each stock solution was mixed together by vortexing. Then, 1 mL of this solution containing 40 mg/mL PLGA-PEG and 5 mg/mL DIM, or EA was mixed with 200 μL of PBS by intermittent sonication (2-3 times, 30 sec each time) to obtain a primary emulsion. The primary emulsion was then intermittently emulsified by sonication (30 s) in 2 mL of 1% w/v polyvinyl alcohol (PVA) solution. This water-in-oil-in-water emulsion was then added to 40 mL of 1.0% PVA solution and stirred for 30 mins under constant magnetic stirring. Immediately after, dichloromethane was evaporated at 37°C using a rotatory evaporator. The whole solution was then dialyzed using 10-12 KD cutoff dialysis membrane against distilled water for 24 hrs to remove free non-encapsulated DIM or EA. The entire solution was lyophilized and re-dispersed for further analyses.

Size Measurement of Nanoparticles by Dynamic Light Scattering

Size distribution of the EA NPs and DIM NPs in aqueous dispersion was determined with dynamic light scattering (DLS) using a Malvern zeta sizer (Malvern Instrumentation Co, Malvern, PA, USA). After the re-dispersion of the lyophilized powder in deionized water, 1 mL of the NPs solution was put in 3 mL of a 4-sided clear plastic cuvette and measured directly using the DLS.

Entrapment Efficiency

The amount of compound (DIM or EA) encapsulated in the NPs was determined by disintegrating the NPs and using UV-VIS spectroscopy to measure compound (in

this case DIM; absorbance at λ 285 nm) compared with standard curves for drug concentrations.

The entrapment efficiency was determined with the following formula:

$$\text{Entrapment efficiency (loading)} = \frac{[\text{Drug}]_f}{[\text{Drug}]_t} \times 100$$

where $[\text{Drug}]_f$ is the concentration of DIM in the NPs and $[\text{Drug}]_t$ is the theoretical concentration of the compound (meaning total amount of DIM or EA added initially).

Cell and Cell Culture

SUIT2-Luc cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Cells were cultured at 37°C with a humidifier atmosphere of 5% CO₂ to sub-confluence and treated with 0.25% (w/v) trypsin/EDTA to effect cell release from culture flask. After washing cells with culture medium, cells were suspended in DMEM (free of phenol red and fetal bovine serum) and counted.

MTT Cell Viability Assay

After 24 hrs of exposure to the compounds, cell viability in different cell treatments was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as per the manufacturer's protocol. Briefly, SUIT2 cells were seeded at a density of 10⁴ cells/well in 96-well plates for 24 hrs and then incubated with compounds (0.1 to 10 $\mu\text{g}/\text{mL}$) for 24 hrs. After treatment, MTT solution was added to each well, and plates were incubated for 4 hrs at 37°C. After aspiration of MTT containing medium, purple formazan in the living cells was solubilized by the addition of 50 μL of DMSO and incubated for 10 mins at 37°C. The optical density of each well was determined using an ELISA plate reader at an activation wavelength of 570 nm and a reference wavelength of 650 nm. The percentage of viable cells was determined by comparison with untreated control cells.

Chick Chorioallantoic Membrane (CAM) Cancer Implant Model

Tumor Growth in the CAM Cancer Implant Model

The relative potency of the different nanoformulations DIM NPs, EA NPs, or combination versus free DIM or EA in the CAM pancreatic cancer cell implant model of tumor weight and tumor angiogenesis was carried out as previously described by Marcinkiewicz et al,²¹ Deryugina and Quigley,²² and Stryker et al.²³

Seven-day-old chick embryos were purchased from Spafas, Inc. (Preston, CT, USA) and incubated at 37°C with 55% relative humidity. A hypodermic needle was used to make a small hole in the shell at the air sac and a second hole was made on the broadside of the egg, directly over an avascular portion of the embryonic membrane that was identified by candling. A false air sac was created beneath the second hole by the application of negative pressure at the first hole, causing the CAM to separate from the shell. A window, approximately 1.0 cm², was made in the shell over the dropped CAM using a small craft grinding wheel (Dermal, Division of Emerson Electric Co., Racine, WI, USA), allowing direct access to the underlying CAM.

SUIT2-Luc cells were implanted at 1 million cells per CAM in Matrigel[®] in the 7-day-old fertilized chick egg. Treatment effects on tumor weight and tumor angiogenesis were determined on day 7 after tumor cell implantation. For these studies, Matrigel was thawed overnight at 4°C and placed on ice. Cells in exponential growth phase were harvested using 0.25% trypsin-EDTA, washed, and suspended in the medium. Only suspensions of single cells with a viability exceeding 95% were used. Approximately 1 \times 10⁶ cells in 30 μL of medium mixed with the same volume (30 μL) of Matrigel were implanted on the CAM.

The treatment groups were Control – Matrigel (PBS or void NPs), Matrigel/SUIT2 (PBS or void NPs), Matrigel/SUIT2 + EA 1 $\mu\text{g}/\text{CAM}$, Matrigel/SUIT2 + DIM 1 $\mu\text{g}/\text{CAM}$, Matrigel/SUIT2 + EA + DIM 1 μg each/CAM, Matrigel/SUIT2 + EA NPs 1 $\mu\text{g}/\text{CAM}$, Matrigel/SUIT2 + DIM NPs 1 $\mu\text{g}/\text{CAM}$, and Matrigel/SUIT2 + EA NPs + DIM NPs 1 μg each/CAM.

Microscopic Analysis of CAM Sections

After incubation at 37°C with 55% relative humidity for 3 days, the CAM tissue directly beneath each filter disk was resected from control and treated CAM samples. Tissues were washed 3 times with PBS, placed in 35-mm Petri dishes (Nalge Nunc, Rochester, NY, USA) and examined under an SV6 stereomicroscope (Karl Zeiss, Thornwood, NY, USA) at 50 X magnification. Digital images of CAM sections exposed to filters were collected, using a 3-CCD color video camera system (Toshiba America, New York, NY, USA), and analyzed with Image-Pro software (Media Cybernetics, Silver Spring, MD, USA). The numbers of vessel branch points contained in a circular region equal to the area of each filter disk were counted. One image was counted in each CAM preparation, and findings from 8

CAM preparations per group were analyzed for each treatment condition. Results are presented as mean tumor weight (mg) per treatment group ($n=8$ eggs per group). The effect of these treatments was determined after 7 days of implantation.

Bioluminescent Tumor Signal Study

Ex vivo imaging was performed to confirm the signal intensity in the tumors after the termination of the study. Because the tumors were developed from SUIT2 pancreatic cancer cells expressing the luciferase gene, the bioluminescent signal intensity of the tumors was studied with an in vivo imaging system (Xenogen-IVIS Spectrum, Perkin Elmer, Akron, Ohio, USA). Excised tumors from the membrane were incubated in D-luciferin (30 mg/mL) and then imaged. Photographic and luminescence images were taken at constant exposure time. Xenogen IVIS[®] Living Image software version 3.2 was used to quantify non-saturated bioluminescence in regions of interest. Light emission between 5.5×10^6 – 7.0×10^{10} photons was assumed to be indicative of viable luciferase-labeled tumor cells and emissions below this range were considered as background. Bioluminescence was quantified as photons/second for each region of interest.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA and comparing the mean \pm SD of branch points from each experimental group with its respective control group. Statistical differences approaching $P < 0.05$ were considered statistically significant. In the CAM studies, the angiogenesis index for each treatment group was compared with the corresponding vehicle-treated control group.

Results

Characterization of DIM or EA PLGA-PEG Nanoformulations

Nanoencapsulation of either DIM or EA into PLGA-PEG NPs of an average NPs size of 180–210 nm was achieved (Figure 1). The lyophilized nanoformulation showed an average of 80% loading for DIM or EA (ie, recovering 4 mg into the dry NPs out of the 5 mg added from DIM or EA).

Effect of Nanoparticles on SUIT2-Luc Cell Viability

Using MTT cell viability assay, either DIM or EA PLGA-PEG NPs, and their combinations, resulted in

a rapid suppression of SUIT2-Luc viability within 24 hrs ($P < 0.01$), while the non-encapsulated EA or DIM and their combinations did not show any significant effect on SUIT2 cancer cell viability/cell proliferation within 24 hrs at 1 μ g/mL (Figure 2–4).

Effect of DIM or EA versus Their PLGA-PEG Nanoparticles on Pancreatic Tumor Angiogenesis and Growth

In the CAM implant model, greater suppressions of tumor angiogenesis (Figure 5) and tumor weight (Figure 6) were observed for DIM NPs or EA NPs and their combinations ($P < 0.01$) versus DIM or EA, and their combinations.

Effect of DIM or EA PLGA-PEG Nanoparticles on Bioluminescent Tumor Signal

Bioluminescence was quantified (photons/second) for the different groups as carried out using Xenogen-IVIS Spectrum for viable cancer cell image intensity. Data showed a statistically significant reduction ($P < 0.01$) of viable pancreatic cancer cell groups treated with either EA or DIM as compared with the control group (Figure 7). Furthermore, pancreatic cancer treated with DIM or EA PLGA-PEG NPs demonstrated greater suppression ($P < 0.01$) of pancreatic cancer cell viability as compared with those treated with EA or DIM (Figure 7).

Discussion

The anticancer potential of DIM has previously been investigated against numerous cancer cell lines and in vivo. Ye et al²⁴ stated that DIM targeted miR-30e-ATG5 modulating autophagy that inhibits the proliferation of gastric cancer cells (BGC-823 and SGC-7901). Also, the viability of ovarian cancer SKOV3 and A2780 cell lines treated with DIM was potently suppressed through inhibition of signal transducer and activator of transcription 3 and protein kinase B (Akt) phosphorylation. Consequently, DIM inhibited xenograft growth in nude mice implanted with those cells.²⁵ Furthermore, DIM has modulated phosphoinositide 3-kinase, Akt, mitogen-activated protein kinase, nuclear factor- κ B, and epidermal growth factor receptor/extracellular signal-regulated kinase pathways.^{26,27} DIM has also been shown to suppress tumor-derived breast cancer cells' migration and proliferation by blockade of c-Met activation by hepatocyte growth factor.²⁸ The same effect was recognized in MCF-7

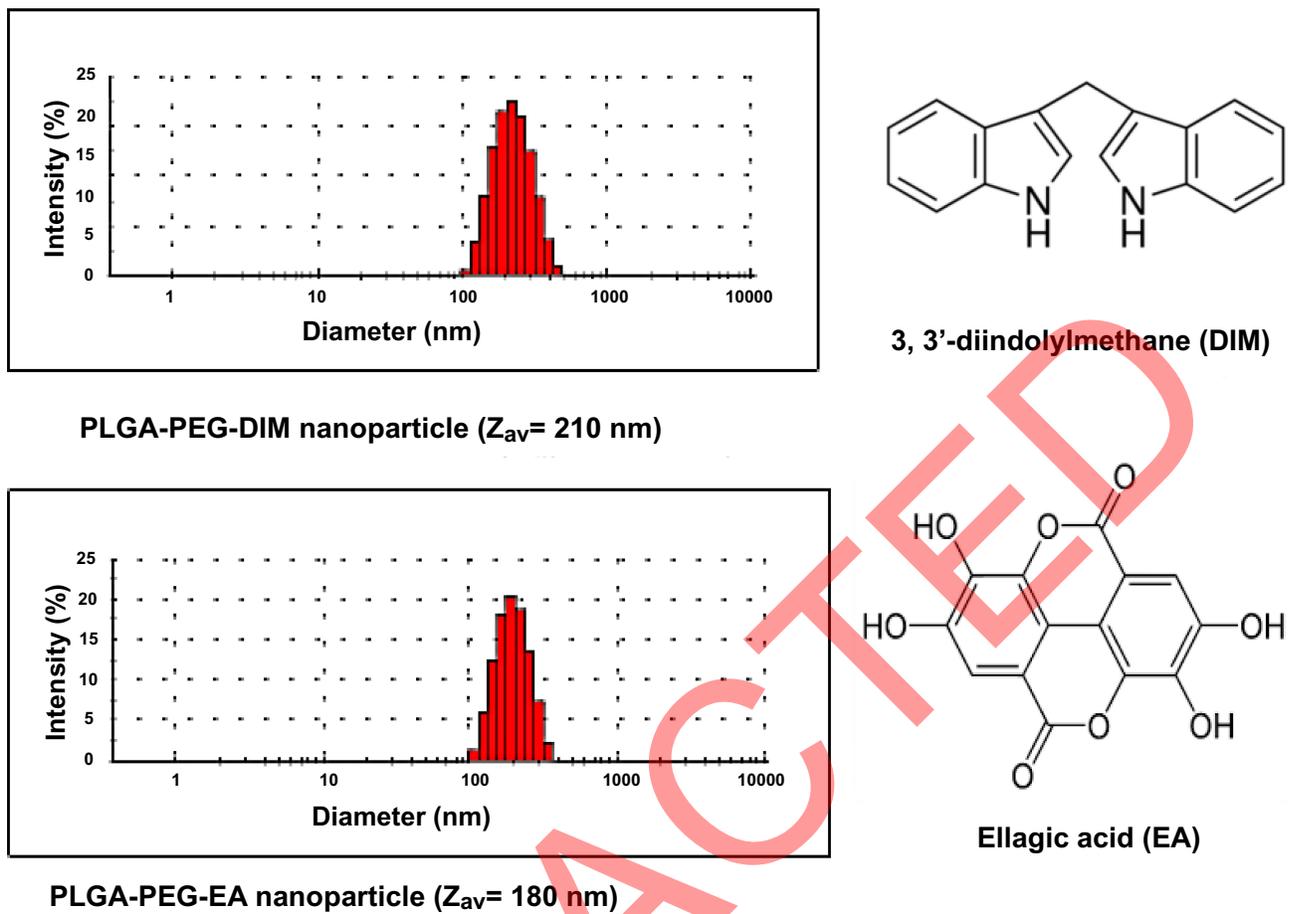


Figure 1 Nanoencapsulation of 3, 3'-diindolylmethane (DIM) or ellagic acid (EA) into PLGA-PEG nanoparticles.

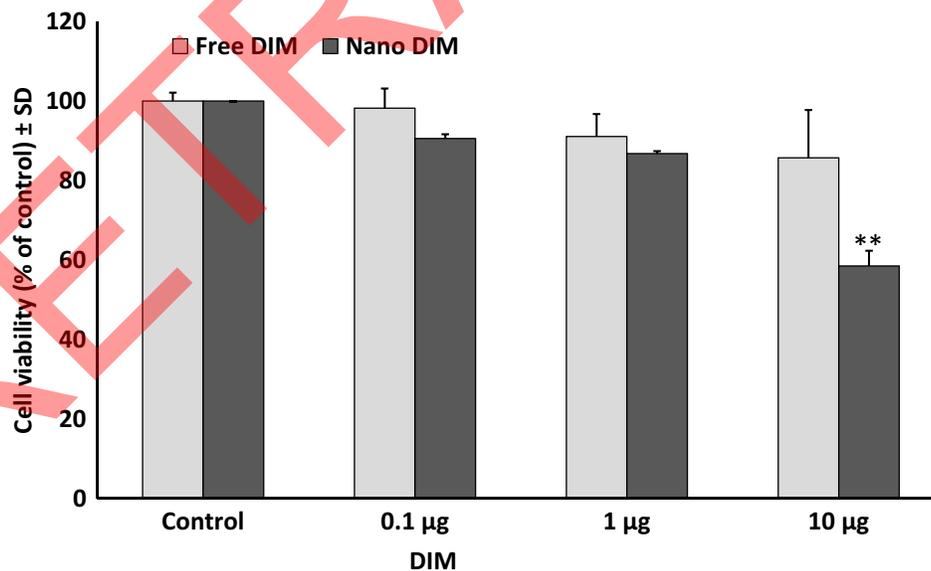


Figure 2 Effect of 3, 3'-diindolylmethane (DIM) versus DIM PLGA-PEG nanoparticles on SUIT2 cell viability after 24 hrs. Data represent mean \pm SD, n = 3, **P < 0.01.

breast cancer cells by suppressing C-X-C chemokine receptor type 4 protein expression that led to inhibition of the epithelial-mesenchymal transition.²⁹

Ellagic acid has a chemopreventive action for various cancer cells by inducing apoptosis, cell cycle arrest, and inhibiting receptor tyrosine kinases such as vascular

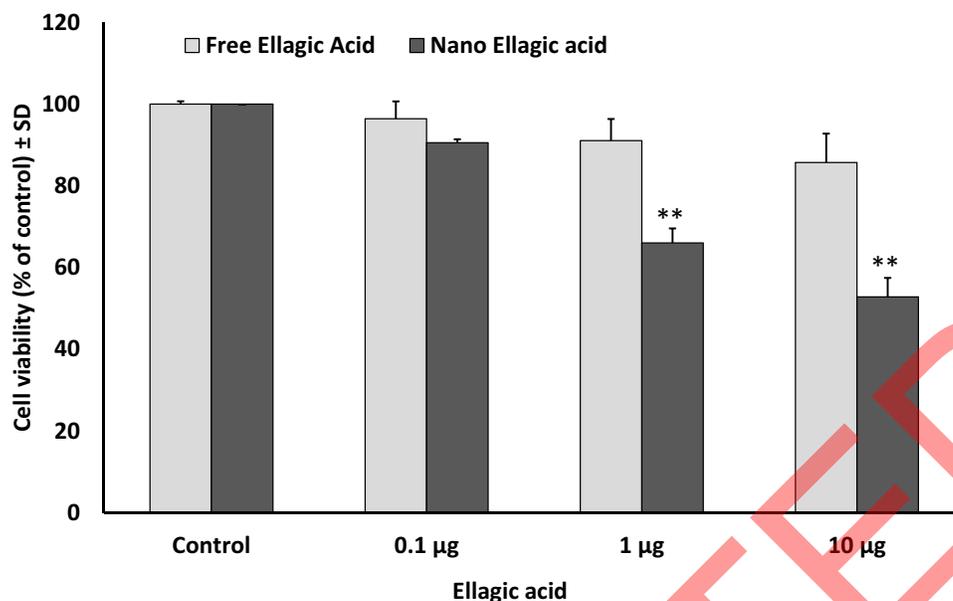


Figure 3 Effect of ellagic acid (EA) versus EA NPs on pancreatic cancer cell viability/proliferation after 24 hrs. Data represent mean \pm SD, n=3, **P < 0.01.

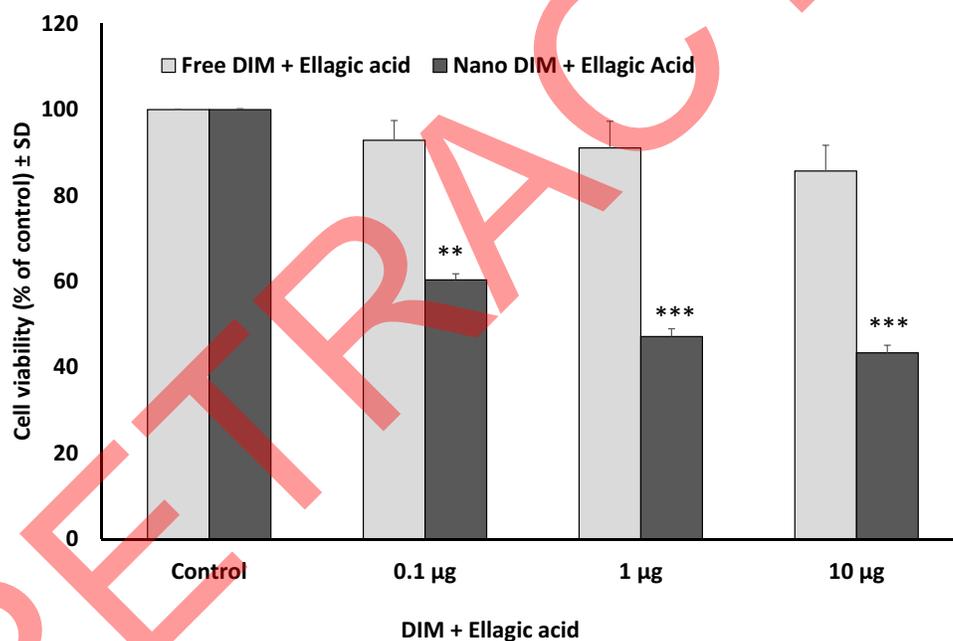


Figure 4 Effect of 3,3'-diindolylmethane (DIM) + ellagic acid (EA) versus their NPs on SUIT2 cell viability after 24 hrs. Data represent mean \pm SD, n=3, **P < 0.01, ***P < 0.001.

endothelial growth factor receptor or insulin-like growth factor receptor.^{30,31} Zhong et al³² stated that EA synergistically potentiated doxorubicin and cisplatin in suppressing HepG2, SMMC-7721, and HL-7702 cells' growth with a significant reduction in their side effects in a xenograft mouse model. Also, treatment of human prostate carcinoma PC3 cells with EA exhibited apoptosis due to downregulation of Bcl2 and upregulation of apoptotic molecules including

Bax, caspase-3, -6, -8 and -9.³³ EA inhibited PANC-1 xenografted tumor growth in nude mice that was associated with suppression of Akt, Notch, and Shh pathways.³⁴

The phytochemicals derived from plant sources provide protection against various cancer-related processes when added to cancer cells at a relatively high concentration that might not be attainable when used as a supplement. Encapsulation of EA and/or DIM into biocompatible and

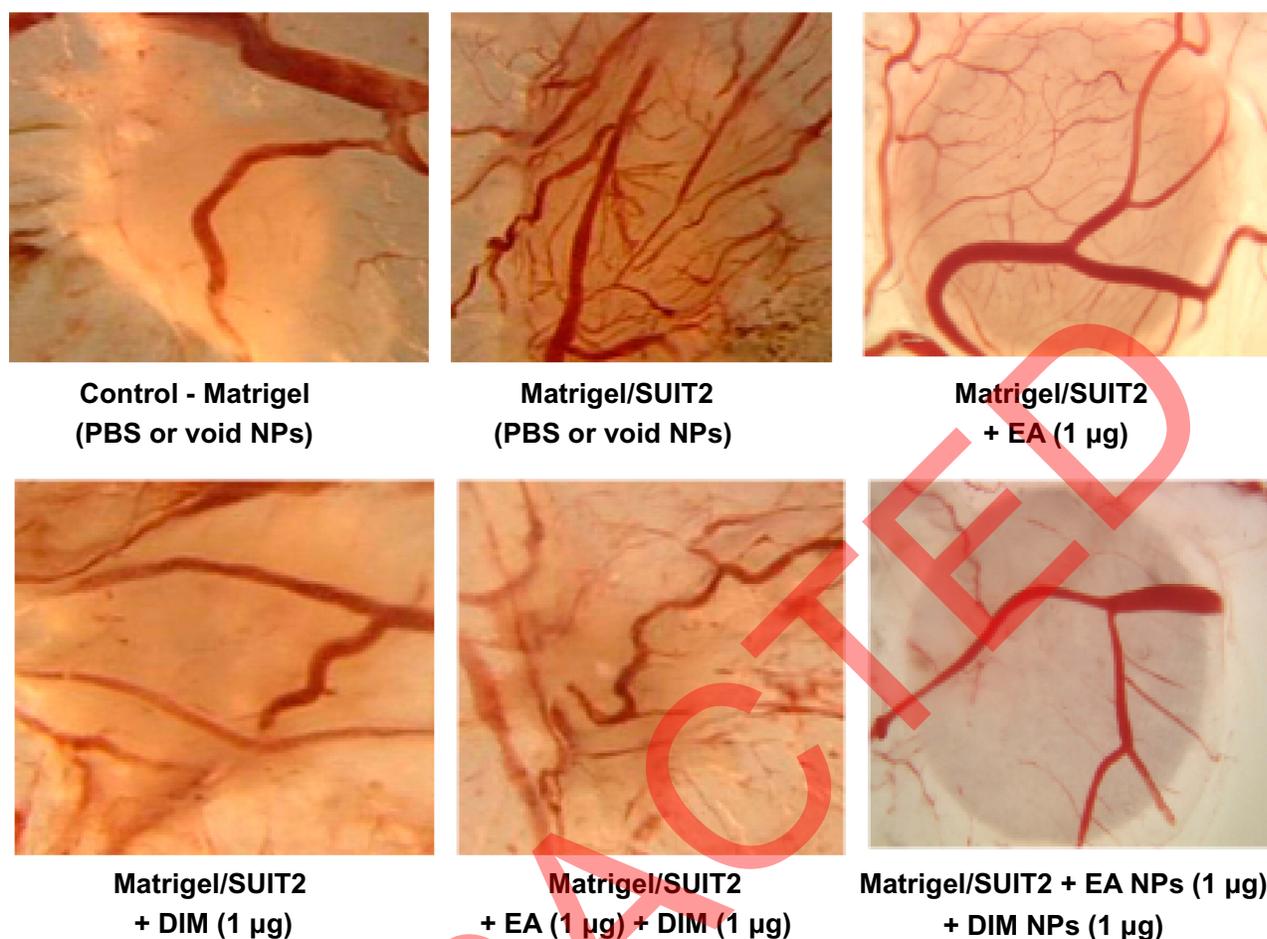


Figure 5 Effect of ellagic acid (EA), 3, 3'-diindolylmethane (DIM) versus their NPs on vascular pattern of pancreatic tumor in the chick chorioallantoic membrane (CAM) model. Representative images from the different groups. DIM NPs and EA NPs combination significantly reduced angiogenesis (** $P < 0.01$) compared with DIM and EA combination.

biodegradable PLGA-PEG NPs may overcome their susceptibility to gastrointestinal hydrolysis, poor absorption, low systemic bioavailability and a short half-life. This nanotechnology approach, initially applied to cancer therapy to decrease toxicity while increasing stability and bioavailability and allowing for selective tumor uptake of cancer drugs,³⁵ has begun to be explored in cancer prevention with dietary phytochemicals.^{36–38} As a result, a new area of investigation, nanochemoprevention, has improved the effectiveness of bioactive food compounds, such as epigallocatechin-3-gallate from green tea,^{36,39} curcumin from turmeric,¹⁶ and resveratrol from table grapes.⁴⁰

Despite limited evidence of cancer prevention for bioactive compounds derived from natural sources, there is a great potential for their utility in cancer patients. Polyphenols such as EA have poor absorption, low systemic bioavailability, and a short retention time, which limits their full chemopreventive potential.^{41–43} EA mostly accumulates in intestinal epithelial cells with limited absorption into systemic circulation.^{44–46} As a result, low

nanomolar range concentrations of free EA have been detected in human blood after consumption of pomegranate juice.^{44,47} In addition, absorbed EA has a short half-life due to rapid metabolism in the liver and excretion through the urine.⁴⁸

Generally, either DIM or EA PLGA-PEG NPs resulted in a rapid suppression of pancreatic cancer cell viability/proliferation within 24 hrs, while the non-encapsulated EA and DIM did not show any significant effect at 1 μ g. In the CAM model, a greater suppression of tumor growth and tumor angiogenesis for DIM or EA NPs and their combinations versus parent compounds were demonstrated. We hypothesized that encapsulation of either DIM or EA in PLGA-PEG NPs would increase their anticancer potential through increased cellular uptake, improved stability, and sustained release. Thus, we designed, synthesized, and characterized PLGA-PEG NPs loaded with EA or DIM or their combination and examined their effects in SUIT2 pancreatic cancer cells. We found that nanoformulations of EA, DIM, and their combination exhibited superior suppression of pancreatic cancer compared with their free counterparts.

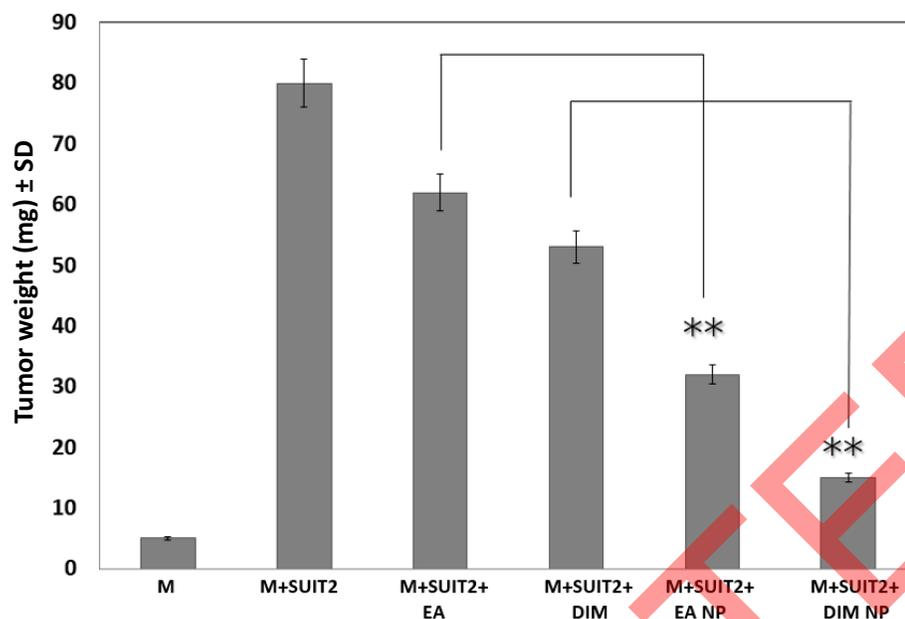


Figure 6 Effect of 3, 3'-diindolylmethane (DIM) or ellagic acid (EA) versus DIM or EA nanoparticle on pancreatic tumor growth in the CAM model. M means matrigel. Data represent mean \pm SD, n= 8 per group. ****P < 0.01.**



Figure 7 Effect of 3, 3'-diindolylmethane (DIM), ellagic acid (EA), or their nanoformulations on the luminescent signal intensity (photons/sec) of pancreatic tumors implanted in the CAM model. Data represent mean \pm SD, n= 8 per group. ****P < 0.01.**

Therefore, we can conclude that nanoformulation of DIM and EA resulted in more effective suppression of pancreatic cancer cell viability, pancreatic tumor growth, and tumor angiogenesis as compared with the parent bioactive compounds, highlighting the potential of nanoformulation of naturally driven bioactive compounds in enhancing their anticancer efficacy.

Abbreviations

MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PEG, poly(ethylene glycol); PLGA, poly(lactic-co-glycolic acid); DIM, 3, 3'-diindolylmethane; EA, Ellagic Acid; NPs, nanoparticles; EDTA, ethylenediaminetetraacetate; PBS, phosphate buffered saline; DMEM, Dulbecco's

Modified Eagle's medium; ELISA, Enzyme-Linked Immunosorbent Assay; SUIT2-Luc, pancreatic adenocarcinoma cell line transfected with luciferase; CAM model, Chick Chorioallantoic Membrane model; DLS, Dynamic Light Scattering; ANOVA, analysis of variance.

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Disclosure

Deena S Mousa, Amna A Saddiq, and Shaker S Mousa report a patent Nanoencapsulating compound a bioactive compound, a method of reducing toxicity resulting from cancer therapy. US Patent 10,251,842 B1, Issued April 9, 2019, issued to Amna Saddiq, Shaker Mousa, and Deena Mousa. The authors declare no other conflicts of interest in this work.

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