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The reduction of anti-cancer drug antagonism by the spatial protection of drugs with PLA–TPGS nanoparticles

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ABSTRACT

Docetaxel (DCL) and tamoxifen (TAM) individually are potent drugs in the fight against breast cancer. However when used in combination, they become antagonistic because of differential metabolism of both drugs. We reasoned that by spatially protecting them from metabolizing enzymes with poly (lactide)-D-α-tocopheryl polyethylene glycol succinate (PLA–TPGS) nanoparticles (NPs), we might reduce this drug antagonism. We now report that the drug antagonism between DCL and TAM in MCF7 cell line, was significantly reduced when co-delivered in PLA–TPGS NPs. In addition, this effect of NPs attenuated at high drug concentrations. To investigate the role of NPs in the reduction of drug antagonism, we quantified cellular uptake of the fluorescent model drug coumarin 6 (C6) encapsulated in a rigorous permutation of drugs-nanoparticles ratios. NPs carrying C6 exhibited enhanced cellular uptake over their free C6 counterparts at correspondingly low drug concentrations. This led us to conclude that the reduction of drug antagonism by NPs is correlated to cellular uptake and being in NPs therefore protects both drugs until they are released intracellular for therapeutic anti-cancer effect.

1. Introduction

Breast cancer affects more than 1.3 million women worldwide, kills about half a million women in the United States alone and is the most prevalent cancer diagnosed for women each year [1,2]. While drug combinations are usually synergistic, certain drug combinations are actually antagonistic [3]. Since chemotherapy usually involves more than a single drug administered at the same period, drug antagonism increases the drug dosage required for maximal anti-tumor efficacy, which escalates the toxicity to normal cells and can produce undesirable side effects. One drug can change the metabolism of the other drug and thus result in drug antagonism [4]; Metabolism in turn, can be a major source of pharmacokinetic variability [5]. The co-delivery of docetaxel and tamoxifen, which are metabolized by the CYP3A4 enzyme, could saturate the metabolic pathway—leading to incomplete drug disposition and potentially unfavorable clinical effects [6,7].

High genetic heterogeneity in breast cancer is correlated with the volume of poorly differentiated cancer cells and can account for differences in metastatic potential even within the same tumor mass [8,9]. Treatment becomes less effective because subpopulations of drug resistant cells repopulate the tumor after the 1st round of therapy [10–13]. In addition, subpopulations of cells within the tumor can have a diversity of responses to the same combination therapy. It has been shown that the same combination of docetaxel and tamoxifen can have antagonistic and synergistic effects respectively on estrogen receptor positive MCF7 and triple negative breast cancer cell lines [14,15]. Therefore, this suggests the outcome of docetaxel and tamoxifen combination therapy depends also on cancer cell types within a complicated heterogeneous tumor tissue. Further aggravating the situation is the type of drug interaction was reported to be sensitive to the drug ratio used in the combination therapy [16,17]. These observations suggest that we may have reached a threshold in treating heterogeneous breast cancer.

Nanoparticles that co-deliver therapeutic agents in combination and in pre-determined drug ratios have been intensively studied for anti-cancer applications for many reasons [18]. Nanoparticles come in many forms and strategies but generally are able to carry therapeutic agents, have appropriate sizes (100–200 nm) and surface coating (TPGS or polyethylene glycol) to prolong systemic circulation (before reaching target site) and crossing of endothelial barriers (at targeted sites), and exhibits sustained therapeutic effects intracellular [19–27]. These carrier-dependent qualities are valid regardless of drug combination and hence should increase the

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therapeutic effects in subpopulations affected by the bane of sub-optimal drug antagonisms.

There are different types of nanoparticles encapsulating docetaxel and tamoxifen, either individually or in combination with other anti-cancer agents [28–31]. Synergistic effects among the therapeutic agents were studied extensively. Docetaxel was synergistically combined with magnetic iron oxide for imaging and hyperthermia therapy, lacto bionic and folate acid for targeting, siRNA and pDNA for biological therapy [32–36]. Likewise, tamoxifen was combined with transferrin and quercetin for synergistic cytotoxic effects [37,38]. But, much less is known about the role of nanoparticles in reducing drug antagonism that is vital for the overall therapy.

Docetaxel alone is a potent anti-mitotic agent [39,40]. By itself, tamoxifen is an anti-estrogenic drug where its more active metabolite, 4-hydroxytamoxifen binds to the estrogen receptor of breast cancer cells and thus prevents estrogen ligand from exerting its proliferative action [7,41,42]. Although CYP3A4 enzyme is commonly involved in metabolizing docetaxel and tamoxifen individually, the resulting anti-tumor activity is distinctive for the respective metabolite. Significant reduction in cytotoxic property was observed when docetaxel was metabolized [43]. In contrast, tamoxifen is a pro-drug that requires CYP3A4 enzyme and can therefore occupy its reaction moiety [44,45]. We reasoned that if we can spatially present both tamoxifen and docetaxel together to CYP3A4 enzyme, tamoxifen may act as a drug decoy for CYP3A4 enzyme, thus sparing docetaxel of metabolism to its less active metabolite. We thus hypothesized that the encapsulation of docetaxel and tamoxifen in NPs could reverse free form docetaxel-tamoxifen antagonism; sacrificing tamoxifen to metabolism by CYP3A4 enzymes would spare docetaxel to exert an anti-mitotic effect and simultaneously transforming tamoxifen into its active metabolite to add on an anti-proliferative effect.

To experimentally show this interesting concept, we synthesized biodegradable polymeric nanoparticles of poly (lactide)-D-α-tocopheryl polyethylene glycol 1000 succinate as matrix material for the encapsulation of docetaxel and tamoxifen, to investigate whether these two drugs in nanoparticles have reduced drug antagonism in MCF7 cell line [15]. Various formulations of dual-drug nanoparticles, denoted as DDNPs, containing different drug ratios of docetaxel and tamoxifen were synthesized to meet the aim of this paper. The PLA–TPGS copolymer has already been used to produce nanoparticles with good levels of drug encapsulation efficiency (EE), cellular adhesion, and desirable release rate [46]. The covalent tethering of TPGS to PLA prevents desorption of TPGS from the particle surface. This step improved EE and subsequently a more consistent drug release rate [46]. Additionally, TPGS inhibits P-glycoprotein mediated multi-drug resistance and has an intrinsic toxicity on cancer cells [47,48].

2. Materials and methods

2.1. Materials

We have synthesized PLA–TPGS via a ring-opening polymerization in accordance to previous publication [49]. Docetaxel (anhydrous, 99.56% purity) was purchased from Shanghai Jinhe Bio-Technology Co. Ltd, China. Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate, $C_{33}O_5H_{54}$ $(CH_2CH_2O)_{23}$) was a procurement from Eastman Chemical Company, USA. Succinic anhydride, 4-(Dimethyl amino) pyridine (DMAP), stannous octoate ($Sn(OOCC_7H_{15})_2$), Tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), coumarin-6, phosphate buffered saline (PBS, pH 7.4), 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, trypsin ethylenediaminetetraacetic acid (EDTA), paraformaldehyde (PFA), dichloromethane (DCM) and lactide (3, 6-dimethyl-1, 4-dioxane-2, 5-dione, $C_6H_8O_4$) were all purchased from Sigma Aldrich (St. Louise, MO, USA). Ethanol was acquired from VWR Singapore Pte Ltd. Tween-80 was obtained from ICN Biomedical, Inc. (OH, USA). Triton X-100 was gotten from USB Corporation (OH, USA). Fetal bovine serum (FBS), penicillin streptomycin solution, Alexa Fluor® 647 Phalloidin and Prolong® Gold Anti fade Reagent with DAPI were made available by Invitrogen. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Thermo Scientific Hyclone (South Logan, USA). MCF7 breast cancer cells were obtained from American Type Culture Collection (ATCC). Ultrapure water was processed by the Milli-Q Plus System (Millipore Corporation, Bedford, USA).

2.2. Preparation of nanoparticles

The nanoparticles were prepared via the nanoprecipitation method [50]. Concisely, weighted amount of PLA–TPGS, docetaxel and tamoxifen (drug to polymer weight ratio 1:10) were dissolved in THF with a final polymer concentration of 10 mg/mL. The organic solution was added drop-wise using a 1 mL syringe attached with a 21G needle, into ultrapure water at an organic to water volume ratio of 1:6 under rigorous stirring in room temperature. After 3 h, the solution was washed for 3 cycles in which 1 cycle of washing involves: centrifugation of nanoparticle solution at 15,000 rpm for 20 min in 4 °C; Discard supernatant, add fresh ultrapure water to the pellet, re-suspension by vortex and sonication to obtain a homogeneous nanoparticle solution. After washing, the solution was then stored in 4 °C. The same procedure was applied to synthesize the fluorescent C6 loaded nanoparticles denoted as C6 NPs, with drug completely replaced by C6.

2.3. Characterization of nanoparticles

Nanoparticle size (Z-average) along with polydispersity was measured by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd, England). The samples were prepared by diluting the nanoparticle suspension with ultrapure water to a count rate of 200–400 kcps and sonicated for 5 min immediately before each measurement. Data were expressed as mean ± standard deviation of triplicate measurements.

Docetaxel and tamoxifen load were quantified by high performance liquid chromatography (HPLC, Agilent LC1100, Agilent, and Tokyo, Japan) at a flow rate of 1 mL/min and absorption wavelengths of 230 and 265 nm respectively, with a UV/VIS detector. A reverse-phase column (Eclipse XDB-C18, 4.6 × 250 mm, 5 mm) was used. The mobile phase used for docetaxel and tamoxifen were respectively acetonitrile/water (1:1 volume ratio) and methanol/water/triethylamine (89:11:0.11 volume ratio). In brief, nanoparticle solution was mixed by vortex and sonication to obtain homogeneous samples of 0.5 mL each, freeze-dried and dissolved in DCM to free the encapsulated drugs from the polymer matrix. After evaporating DCM in a vacuum oven, the dried sample was dissolved completely in 0.5 mL of mobile phase by vortex and sonication. The samples were filtered by 0.45 μm PVDF membrane prior to HPLC analysis. Standard curve was obtained by first plotting the absorbance against known concentrations of docetaxel and tamoxifen, followed by the insertion of a linear trend line—which was subsequently used to interpret the drug concentration in the samples. The standard curves were found to be linear with $R^2 = 0.99$. Drug load was designed to be the weight of encapsulated drugs in μg divided by the total weight of the nanoparticles in mg. Hence, the unit of drug load is μg drug/mg nanoparticles. Drug load was expressed as mean ± standard deviation of triplicate measurements.

Nanoparticle surface morphology was visualized by field emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Japan) at 50,000 magnification and an accelerating voltage of 5 kV. Samples were coated with platinum by JFC-1300 platinum coater (JEOL, Tokyo, Japan) for 30 s at 30 mA prior to imaging.

To study the in vitro drug release of nanoparticles, samples were dispersed in 1× PBS (pH 7.4) containing 0.1% v/v Tween-80, which functions to increase the drug solubility of PBS so as to simulate the sink condition. Samples were placed in a rotating water bath at 37 °C and 90 rpm. At chosen time intervals, the tubes were centrifuged at 15,000 rpm for 20 min. The supernatant was collected and the pellet was re-suspended in fresh PBS to continue the drug release study. The same procedure for the quantification of drug load was applied to measure the drug released in the supernatant.

For the in vitro colloidal stability study of nanoparticles, the nanoparticles were dispersed in 1× PBS (pH 7.4) containing 10% FBS, which simulates the in vitro conditions of nanoparticles in DMEM. Similar to in vitro drug release, samples were placed in a rotating water bath at 37 °C and 90 rpm. Nanoparticle solutions were sampled at chosen time intervals and nanoparticle size was measured via the same procedure as mentioned previously. Data represent mean ± standard deviation of triplicate measurements.

2.4. In vitro cytotoxicity studies

MCF7 breast cancer cells were cultured with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. Cells were cultured in cell culture flask and grown in an incubator at 37 °C and 5% carbon dioxide. Upon 70–90% cell confluence—which was confirmed visually under a light microscope—the DMEM was extracted and rinsed twice with 10 mL of 1× PBS (sterilized, pH 7.4) to remove all traces of serum, which would otherwise inhibit the suspension of adherent cells caused by Trypsin-EDTA. 4 mL of Trypsin-EDTA solution was added, incubated at 37 °C for 5 min and observed under light microscope for cell dispersion. Incubate long times until cell dispersion may be observed. 6 mL of DMEM was added and the cell suspension was transferred to a tube for centrifuge at 1500 rpm for 5 min. The supernatant was discarded and cell pellet was re-suspended in 6 mL DMEM. For subculture, add 0.5 mL of cell suspension to culture flask for cell growth. For cell

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