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Doxorubicin enhances curcumin's cytotoxicity in human prostate cancer cells *in vitro* by enhancing its cellular uptake

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ABSTRACT

Doxorubicin (DOX) is a widely used drug in cancer treatment. Despite its popularity, it suffers from systemic side effects and susceptibility to drug resistance. Curcumin (CURC), on the other hand, is a drug that recently gained popularity due to its wide range of biological activities, including anti-inflammatory and anti-cancer activities. Limitations to its clinical translation include its poor water solubility and the need for administration of high doses. Combinatory anti-cancer therapy has been proposed as a common approach to overcome one or more of these challenges. In this work, we propose a combinatory DOX and CURC anti-cancer therapy of prostate cancer cells *in vitro*. DOX and CURC were administered in the free drug and nanocapsule form, respectively. Cell size and complexity, cytotoxicity and apoptosis were studied by flow cytometry, MTT assay and sub-G1 quantification, respectively. Cellular uptake of CURC nanocapsules (CURC NCs) was quantified by fluorescence microscopy and high-performance liquid chromatography fluorescence detection. Results showed that *in vitro* treatment with CURC NCs in the presence of subtherapeutic concentrations of DOX, led to significant increase in prostate cancer cells (PC3) apoptosis and death. This was likely due to significantly enhanced CURC uptake by the cells. The study presents a good rationale for pursuing combinatory CURC/DOX therapy in pre-clinical tumor animal models in the near future.

1. Introduction

Cancer treatment options can be limited due to multidrug resistance and side effects. Recently, combination chemotherapy of multiple anticancer drugs has been extensively developed for overcoming these major pitfalls (Li et al., 2014). The combinational anti-cancer therapy of doxorubicin (DOX) and curcumin (CURC) represents an attractive strategy over single drug treatment to maximize the therapeutic response. DOX is a broad-spectrum chemo-therapeutic agent widely used for the treatment of several cancers including breast, ovary, cervix and prostate as reviewed recently (Tacar et al., 2013). Its effectiveness can be limited due to its high toxicity and side effects, including myelosuppression, alopecia, acute nausea, vomiting, stomatitis, cumulative cardiotoxicity (Hortobagyi, 1997), and multidrug resistance resulting, after repeated administration (Shen et al., 2008). Interestingly, a phase II trial showed that liposomal doxorubicin led to only modest anticancer activity for the treatment of hormone-refractory prostate cancer and it was suggested to include this agent in combination chemotherapy (Harris et al., 2002). In clinic, DOX has been used in multi-drug regimens with other anti-cancer agents, such as cyclophosphamide, 5-fluorouracil, docetaxel, vinblastine, and bleomycin (Hernandez and Perez, 1996; Itoh et al., 2000; Martin et al., 2003). Recent studies have reported that curcumin (CURC) can reduce DOX's adverse reactions (Sadzuka et al., 2012).

CURC has been reported in many studies to delay tumor growth *via* modulating different signalling pathways. Its limitations include poor water solubility and low potency, requiring administration of high CURC doses, in a solubilized drug form (Anand et al., 2007). We have recently reported the preparation of CURC NCs and confirmed their therapeutic efficacy in colon-bearing mice, following intravenous administration (Klippstein et al., 2015).

In this study, we propose a combinatory anticancer approach with free form and nanoformulation of DOX and curcumin, respectively. We hypothesize that co-treatment of human prostate cancer cells with CURC NCs and subtherapeutic doses of DOX can improve CURC's uptake in cells improving its therapeutic efficacy.

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In vitro cellular uptake and drug quantification studies, followed by cytotoxicity and apoptotic assays were carried out to test our hypothesis.

2. Material and methods

2.1. Material

75/25 DL-lactide/glycolide conjugate (PLGA), Mw ≈ 18,000, was a gift from Purac Biomaterials. CURC was purchased from Santa Cruz Biotechnology (UK). Snake Skin dialysis tubing (MWCO 10,000 Da) was purchased from Thermo-fisher (USA). Soybean lecithin (Epikuron 140 V) was a kind gift from Cargill Pharmaceuticals. Castor oil, Tween® 80, acetone and absolute ethanol were obtained from Sigma-Aldrich (UK). RPMI-1640 media, foetal bovine serum (FBS), penicillin/streptomycin, trypsin/EDTA, and phosphate buffered saline (PBS) were obtained from Gibco, Invitrogen (UK).

2.1.1. List of chemical compounds

PLGA (PubChem CID: 23111554); Soybean lecithin (PubChem CID: 57369748); CURC (PubChem CID: 969516); castor oil (PubChem CID: 14030006); Tween® 80 (PubChem CID: 5281955); ethanol (PubChem CID: 702); acetone (PubChem CID: 120).

2.2. Cell culture

The human prostate carcinoma cells DU145 (ATCC® HTB-81™) and PC3, kindly provided by Dr. M. Japon; Department of Endocrine Tumorigenesis and Hormonal Regulation of Cancer, Biomedicine Institute of Seville, IBIS, CSIC-University of Seville, Spain, were cultured in Advanced RPMI media supplemented with 10% FBS, 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 1% L-glutamine, at 37 °C in 5% CO_2. Cells were routinely grown in 75 cm^2 canted-neck tissue culture flasks and passaged twice a week using Trypsin/EDTA at 80% confluency.

2.3. Formulation of the NCs

CURC NCs were prepared using the nanoprecipitation technique as described in one of our previous studies (Klippstein et al., 2015). Briefly, CURC (5 mg), soybean lecithin (25 mg), and PLGA polymer (25 mg), were mixed at 1:2:0.1 molar ratio with 300 μL of castor oil dissolved in 5 mL of acetone/ethanol (60:40 v/v) mixture. This organic phase was added dropwise into the aqueous phase (10 mL) containing Tween® 80 (0.2%) as a hydrophilic surfactant; the mixture was maintained under magnetic stirring in the chemical hood for 30 min to allow solvent to diffuse and form NCs. Organic solvents were then eliminated by evaporation under reduced pressure using a Buchi rotavap. The final volume of the colloidal suspension was adjusted to 10 mL.

2.4. Uptake studies in vitro by flow cytometry

Cells were seeded at a density of 5×10^4 in 24-well plates, allowed to attach overnight and then treated with 20 μM of CURC NCs and 1 μM of DOX. After treatment, the cells were washed twice with PBS, trypsinized and centrifuged at 1500 rpm for 5 min and the cell pellet was resuspended in 250 μL of PBS. The internalization of CURC and DOX was studied on 10,000 gated cells by detecting the fluorescence using FL2 channel detector and BD FACS Calibur flow cytometer (BD Biosciences). The measurements were done in triplicate and presented as mean ± SD.

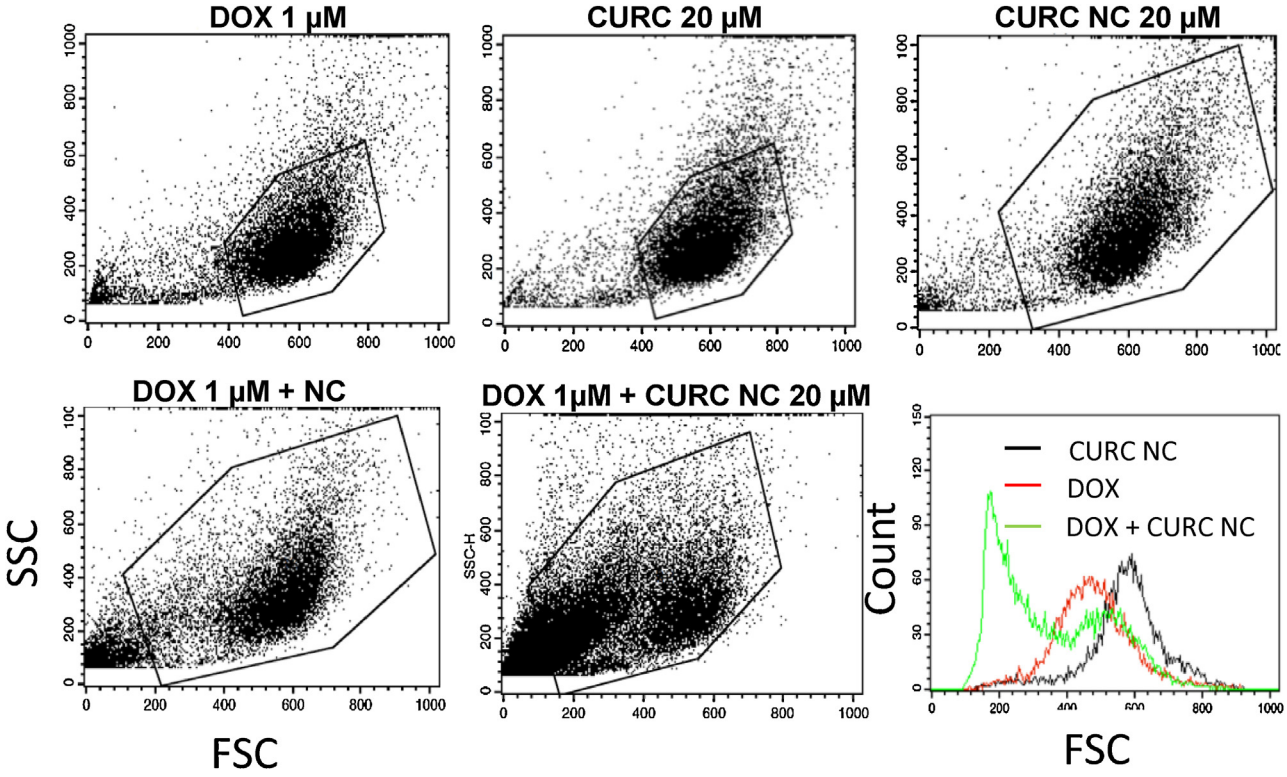

Fig. 1. Cell size and granularity of PC3 prostate cancer cells, after intracellular uptake of doxorubicin (DOX), empty NC (NC), curcumin-loaded NC (CURC NC) or their combinations. Changes in cell size (FSC detector) and granularity (SSC detector) were assessed after 4 h of incubation by flow cytometry. The combination of DOX and CURC NC but not any of the individual treatments lead to reduction in cell size, reflecting enhanced overall cytotoxicity of the combinatory treatment.