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Low-dose doxorubicin with carotenoids selectively alters redox status and upregulates oxidative stress-mediated apoptosis in breast cancer cells

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ABSTRACT

The combination of carotenoids and doxorubicin (DOX) selectively alters oxidative stress-mediated apoptosis in breast cancer cells. Primarily, cytotoxic efficiency of carotenoids (β-carotene, BC; lutein, LUT; astaxanthin, AST; or fucoxanthin, FUCO) either with or without a minimal cytotoxic dose of DOX was evaluated in MCF-7 (0.12 μM) and MDA-MB-231 cells (0.28 μM). The higher cell growth inhibition of BC and/or LUT with DOX was selected for testing in further cell-based assays. Low-dose DOX significantly enhanced cytotoxicity in carotenoid (< 5 μM)-treated cells compared to high-dose DOX (> 1 μM) or carotenoid (20 μM) treatment alone. Depleted glutathione, increased lipid peroxides and increased ROS levels in cells confirmed the cytotoxic effect. Furthermore, mitochondrial dysfunction, cell growth arrest at G0/G1 phase and caspase cascades as well as up- and down-regulated expression levels of related proteins (p21, p27, Bax, p53, Bcl-2, and cyclin D1) revealed the synergistic effect of carotenoid and DOX treatment on ROS-mediated apoptosis. These observations demonstrated increased apoptosis in BC + DOX/LUT + DOX-treated cells due to the pronounced pro-oxidant action. Interestingly, normal breast epithelial cells (MCF 10A) exposed to similar treatments resulted in non-significant cytotoxicity. These newly observed mechanistic differences of anticancer drugs on the mitigation of toxicity with carotenoids may provide insight into the targeting of cancer therapy.

1. Introduction

Currently, natural compounds are extensively used as an alternative treatment strategy to cure various chronic diseases, including cancers, with fewer side effects. However, the impact of natural compounds on effective control of tumour proliferation is not well established. Among natural compounds, carotenoids have been demonstrated to influence biochemical and molecular events of cancer cell death (Niranjana et al., 2015). Epidemiological and clinical trials have suggested that carotenoids present in rich green leafy vegetables, fruits, edible marine seaweeds and their supplements are associated with reduced risk of certain cancer and chronic diseases (Amin et al., 2009; Bakker et al., 2016; Block et al., 1992; Eliassen et al., 2015; Fergusona et al., 2015). Moreover, studies have revealed the role of β-carotene (lung and breast) (Cui et al., 2007; Gloria et al., 2014), lycopene (prostate) (Arathi et al., 2016) and lutein (mammary) (Chew et al., 2003) in the inhibition of specific cancer cell proliferation. Subsequently, fucoxanthin and astaxanthin have been demonstrated to possess bioactivity against acute inflammation and tumour growth (Kaulmann and Bohn, 2014). The chemical and structural features of carotenoids are thought to be involved in antioxidant defence mechanisms and cell signalling. In addition, others have demonstrated that β-carotene, lycopene, and canthaxanthin are involved in the inhibition of cancer cells by exhibiting pro-oxidant effects at higher cellular oxygen (O_2) tension

Abbreviations: AST, Astaxanthin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; BC, β-Carotene; BCA, Bicinchoninic acid; BHT, Butylatedhydroxytoluene; DCF-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; DMSO, Dimethyl sulphoxide; DOX, Doxorubicin; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid; FBS, Foetal bovine serum; FUCO, Fucoxanthin; GR, Glutathione reductase; GSH, Glutathione; LDH, Lactate dehydrogenase; LUT, Lutein; LPx, Lipid peroxide-malondialdehyde; MCF-7, MDA-MB-231 Human breast adenocarcinoma cell lines; MEM, Minimum essential medium eagle; MTT, 3-[4, 5-Dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide; NADPH, Nicotinamide adenine dinucleotide phosphate; p21, Cyclin-dependent kinase inhibitor 1; p27, Cyclin-dependent kinase inhibitor 1B; p53, Phosphoprotein 53; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; THF, Tetrahydrofuran

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(Agamey et al., 2004; Burton and Ingold, 1984). Furthermore, many reports have shown the cytotoxic influence of carotenoids in a dose-dependent manner (Sowmya et al., 2017).

In cancer treatments, radiation with chemotherapy is an effective measure used to manage tumour growth and proliferation of cells. In general, the cytotoxic killing of cancer cells by anticancer drugs, particularly anthracyclines (doxorubicin, taxol and paclitaxel), is mediated through the following two distinct pathways: inhibition of topoisomerase II and elevation of intracellular H_2O_2 (Conklin, 2004; Wang et al., 2004). Natural compounds, such as carotenoids, have been shown to be involved in cancer chemoprevention. However, the interaction of the natural compounds with the cellular environment along with potent anticancer drugs has not been studied (Amin et al., 2009; Conklin, 2004). The anticancer activity of the anthracycline drug, doxorubicin (DOX), is associated with enhanced intracellular ROS, and DOX has been shown to cause acute secondary toxicity in normal cells (Wang et al., 2004). To overcome these problems, a strategy for designing a permissible dose of an anticancer drug for selective cancer cell killing is most warranted to explore its potential. Further, application of DOX remains limited due to its dose-dependent secondary toxicity in non-cancerous cells. Although carotenoids are positively correlated with the reduction of cancer incidence, their synergistic role with a known anticancer drug, such as DOX, on selective cancer cell inhibition has not been addressed. Previously, carotenoids have been shown to modulate apoptotic gene expression as a result of synergistically interacting with one or more carotenoid or other phytochemicals (Linnewiel-Hermoni et al., 2015; Sowmya et al., 2017). Interestingly, Miranda-Vilela et al. (2014) observed that carotenoids reduce DOX-induced damage to normal cells in mice fed with an oil rich in carotenoids compared to vitamin C and E supplements. Tang et al. (2011) demonstrated an enhanced growth inhibitory effect of docetaxel through expression of insulin-like growth factor receptor in prostate cancer cells when co-treated with lycopene. Carotenoids may play a dual role as an antioxidant and pro-oxidant in cells subjected to variable O_2 tension. In this context, we have postulated the possibility of lutein and lycopene oxidation products acting as antioxidants or pro-oxidants to inhibit proliferation of cancer cells (Arathi et al., 2016; Lakshminarayana et al., 2013; Sowmya et al., 2017) Formation of carotenoid radicals resulting from ROS detoxification and subsequent addition of oxygen to carotenoid radicals may generate peroxy radicals and hydroperoxides at higher oxygen tension (Agamey et al., 2004; Arathi et al., 2016; Lakshminarayana et al., 2013; Siems et al., 2002). Carotenoid interaction with free radicals and oxygen species leads to the generation of their cleaved products, which possess deleterious or beneficial activity in biological systems. In addition, the structure and concentration of carotenoids affect biological membranes, which may influence cell permeability to toxins, molecular oxygen or radicals (Agamey et al., 2004). The investigation of this unusual action of carotenoids with a known anticancer drug on the selective chemoprevention of cancer may provide insight into nutritional therapy for controlling cancer complications. Although various natural compounds have been shown to possess antitumour activities, treatment is still being practised with synthetic anticancer drugs due to lack of evidence on the efficiency of natural compounds compared to chemically derived drugs (Chegaev et al., 2013). In this study, we investigated the effects of an optimal dose of an anticancer drug (DOX) with carotenoids on selective redox status and related pathways of cytotoxicity in normal and breast cancer cells. Moreover, identifying the role of the natural antioxidant, carotenoid (Fig. 1), along with a potent anticancer drug to minimize side effects is essential for the acceptance of nutritional therapy to manage cancer.

2. Materials and methods

2.1. Chemicals

Standard lutein (99%), β-carotene (98%), astaxanthin (99%), fucoxanthin (98%) bovine serum albumin, 4,6-diamidino-2-phenylindole dilactate (DAPI), propidium iodide (PI), poly-D-lysine, acridine orange (AO), ethidium bromide (EtBr), doxorubicin, and cell culture grade dimethyl sulphoxide (DMSO) and tetrahydrofuran (THF) (stabilized with 0.25% BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical and HPLC grades purchased from Sisco Research Laboratories (Mumbai, India). Minimum Essential Medium Eagle, foetal bovine serum, antibiotic-antimycotic solution, calcium and magnesium free phosphate buffer saline, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypan blue, cell culture consumables and neutral aluminium oxide (particle size: 70–230 mesh) were obtained from Hi-Media Chemical Laboratories (Mumbai, India). PE Annexin-V Apoptosis Detection (Batch: 6179925) and JC-1 Mitochondrial Membrane Potential (Batch: 6201963) assay kits were purchased from BD Pharmingen (BD Bioscience, San Diego, CA). BCA assay reagents were purchased from Thermo Scientific (USA). β-actin, Bcl-2, Bax, Cyclin D1, p21, p27, p53, Caspase-3, Caspase-8 and Caspase-9 primary antibodies were purchased from Cell Signalling Technology, Inc. (USA). Goat anti-rabbit/mouse IgG-HRP secondary antibodies and the western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CLX-posure™ film (8 × 10 inches) from Thermo Scientific (USA) was also purchased for use in this study.

2.2. Extraction, purification and analysis of carotenoids

BC and LUT were isolated from green leafy vegetables (spinach) according to a previous procedure with slight modifications (Lakshminarayana et al., 2005). Fresh leafy vegetables (50 g each) were ground with sodium sulphate (5 g) and 0.1% BHT using a mortar and pestle. Carotenoids were repeatedly extracted from the ground leafy sample using ice-cold acetone until the residue became colourless. The crude acetone extract of leafy sample was then subjected to saponification in methanolic KOH (30%) in the dark at room temperature for 3 h. The volume of carotenoid extract and alkali solution of KOH was maintained in the ratio of 1:3 (v/v). After incubation, total carotenoids were isolated by phase separation using hexane in a separatory funnel. The extraction procedure was repeated thrice, and the pooled hexane extract was washed with deionized water and filtered through Whatman No. 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator (Buchi, Switzerland) at 30–35 °C and re-constituted in a known volume of hexane. An aliquot of hexane extract was used for purification of BC and LUT by column chromatography. Likewise, AST from shrimp (*Penaeus mondon*) and FUCO from brown seaweeds (*Padina tetrasomatica*) were isolated as per the standardized methods (Sangeetha et al., 2010; Sowmya et al., 2017). In brief, shrimp meat (100 g) was ground to obtain a fine paste using a mixer grinder. A homogenized sample (10 g) was soaked in ice-cold acetone (30 mL) for 5 min, and carotenoids were extracted until sample became colourless. The pooled acetone extract was filtered through Whatman No. 1 paper and subjected to saponification to obtain free AST. Because shrimp are comprised of mono- and di-esters of AST, a mild saponification (0.2% NaOH for 16 h in a ratio of 5:1, v/v) procedure was adopted for the hydrolysis of AST esters. The saponified shrimp extract was partitioned by adding an equal volume of 10% sodium sulphate, and the upper phase of carotenoids was collected by repeated extraction using acetone. The separated pooled extract was dried under N_2 gas and re-dissolved in a known volume of acetone for purification using column chromatography. Similarly, FUCO was extracted from brown algae with acetone/methanol (7:3, v/v) repeatedly until the sample became colourless. The crude solvent extract was evaporated using N_2 gas and re-