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Induction of G_2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels

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Abstract

Carnosol and carnosic acid, two antioxidant polyphenols present in *Rosmarinus officinalis* (rosemary), were investigated for their antiproliferative properties toward Caco-2 cells. Twenty hours of treatment with both carnosol and carnosic acid inhibited ^{3}H-thymidine incorporation in a dose-dependent manner, with a 50% inhibitory concentration of 23 μM and significantly increased the doubling time of Caco-2 cells from 29.5 to 140 and 120 h, respectively. These effects were associated with accumulation of treated cells in the G_2/M phase of the cell cycle. Carnosol was found to exert its major cell cycle effect after prometaphase, and caused an increase in cyclin B1 protein levels whereas carnosic acid arrested cells prior to prometaphase, and caused a reduction in cyclin A levels. These structurally related phytochemicals, therefore, appear to arrest cells at different phases of the cell cycle possibly through influencing the levels of different cyclin proteins.

Keywords: Carnosol; Carnosic acid; Phenolic diterpenes; Cell cycle; Cyclins

1. Introduction

There is little doubt that the composition of the human diet represents one of the biggest acquired influences on cancer risk, and that numerous dietary components are already known to exert both positive and negative influences on cancer risk [1]. Chemoprevention is the long-term pharmacological management of disease risk, and several plant-derived dietary chemicals have been investigated for their potential anticancer properties [2]. Carnosol and Carnosic Acid (CA) are antioxidant phenolic diterpenes [3,4] from *Rosmarinus officinalis* (rosemary), which together comprise about 5% of the dry weight of rosemary leaves [5]. Several studies have shown that these two phytochemicals attenuate tumor initiation by chemical carcinogens in vivo. For example, topical application of carnosol inhibited tumor initiation by benzo(a)pyrene and 12-*O*-tetradecanoylphorbol-13-acetate in mouse epidermis [5], and dietary rosemary extract was found to reduce

Abbreviations: APC, anaphase promoting complex; CA, carnosic acid; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; SE, standard error of the mean.

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the incidence of dimethylbenz(a)anthracene-induced mammary tumors in rats [6]. Carnosol may attenuate chemical carcinogenesis by modulating the activities of phase II detoxifying enzymes such as glutathione *S*-transferase [7,8] and by suppressing cyclo-oxygenase-2 transcriptional activation [9].

Despite these studies, little is known about the influence of carnosol and CA on cellular proliferation. One study found that treatment of HL-60G cells with 10 μM CA for 168 h had no significant effect on cell proliferation or cell cycle distribution [10], although in another study, 10 μM CA inhibited cell proliferation in HL60 cells and caused transient G_0/G_1 phase cell cycle arrest [11]. Carnosol has been shown to induce apoptosis with downregulation of Bcl-2 in cell lines established from patients with acute lymphocytic leukaemia, but not in normal peripheral blood mononucleocytes [12], although there have been no other studies to date of the effects of carnosol or CA on proliferation on proliferation or cell cycle distribution.

Here, we demonstrate for the first time that carnosol and CA inhibit proliferation of the human colonic adenocarcinoma cell line Caco-2, and cause cell cycle arrest predominantly at G_2/M phase. Further investigation revealed that cell cycle arrest occurred after prometaphase in response to carnosol, with an associated increase in cellular cyclin B1 protein levels, but prior to prometaphase in response to CA, with a reduction in cyclin A protein levels. This study is the first demonstration that carnosol and CA can exert an antiproliferative effect through the induction of G_2/M phase cell cycle arrest, and that arrest occurs during different stages of G_2/M phase, corresponding to the different effects of carnosol and CA on cyclin levels.

2. Materials and methods

2.1. Materials

Carnosol and CA were purchased from Alexis Biochemicals (Nottingham, UK). Nocodazole, aprotinin, pepstatin and leupeptin were purchased from Calbiochem (Nottingham, UK). Antibodies to cyclins A and B1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad (Hemel Hempstead, UK). Tissue culture media and supplements were purchased from Invitrogen (Paisley, UK). All other reagents were purchased from Sigma (Poole, UK) unless stated otherwise.

2.2. Cell culture, proliferation, and cytotoxicity assays

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50,000 units/l penicillin G, 50 μg/ml streptomycin and 1× non-essential amino acids at 37 °C, 5% CO_2. Cells were subcultured every 7–10 days using 0.25% trypsin, 0.2% EDTA in Phosphate Buffered Saline (PBS). For DNA synthesis assays, cells were seeded in 12-well tissue culture plates and used at ~60% confluence. Cells were treated for either 3 or 21 h with 1–50 μM carnosol or CA in culture medium. One-microCi [^{3}H] thymidine (Amersham Pharmacia Biotech, Amersham, UK) was added to each well for the final 3 h. Each treatment was performed in triplicate and data are shown as mean ± Standard Error (SE) for three or more independent experiments. For cell proliferation assays, cells were seeded in 25 cm^2 tissue culture flasks and incubated for 24 h prior to exposure to 50 μM carnosol or CA in culture medium. Cells were harvested from treated and untreated flasks every 24 h and cell number measured using a hemacytometer. Culture media and treatments were replaced at 48 h. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13]. Briefly, Caco-2 cells were cultured to confluence in 96-well plates and treated with carnosol or CA for 24 or 48 h. MTT was added to each well to a final concentration of 0.2 mg/ml and the cells incubated for a further 5 h, over which period incorporation of MTT by untreated cells was found to be linear. Cell culture supernatants were aspirated, the cells solubilised in 200 μl dimethyl sulfoxide and optical density at 570 nm measured.

2.3. Flow cytometric determination of cell cycle phase

Cells were treated for up to 48 h with 50 μM carnosol or CA, harvested using trypsin, and fixed in