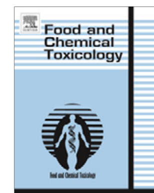
Food and Chemical Toxicology 52 (2013) 61–68

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Synergistic anti-cancer effects of resveratrol and chemotherapeutic agent clofarabine against human malignant mesothelioma MSTO-211H cells

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ARTICLE INFO

Article history:
Received 3 August 2012
Accepted 31 October 2012
Available online 9 November 2012

Keywords:
Resveratrol
Clofarabine
Mesothelioma
Specificity protein 1
Akt
c-Met

ABSTRACT

Dietary phytochemicals as adjuvants have been suggested to play important roles in enhancing chemotherapeutic potential owing to multitargeted chemopreventive properties and lack of substantial toxicity. Here, we investigated the efficacy of the combined treatment of various phytochemicals with the anticancer drug clofarabine in malignant mesothelioma MSTO-211H cells and normal mesothelial MeT-5A cells. The combined treatment of resveratrol and clofarabine produced a synergistic antiproliferative effect in MSTO-211H cells, but not in MeT-5A cells. In MSTO-211H cells, the nuclear accumulation of Sp1 and the levels of p-Akt, Sp1, c-Met, cyclin D1, and p21 were effectively decreased by the combined treatment of them. In combination with clofarabine, the ability of resveratrol to reduce the contents of Sp1 and its target gene products was also evident in a time- and dose-dependent experiment. The inhibition of phosphoinositide 3-kinase using Ly294002 augmented a decrease in the p21 level induced by their combination, but it showed no significant effects on expression of Sp1 and cyclin D1. Taken together, the data provide evidence that the synergistic antiproliferative effect of resveratrol and clofarabine is linked to the inhibition of Akt and Sp1 activities, and suggest that this combination may have therapeutic value in treatment of malignant mesothelioma.

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1. Introduction

Malignant mesothelioma (MM), a relatively rare tumor arising from surface serosal cells of the lung and pleura or the peritoneum, has a high resistance to chemotherapy. Epidemiologic studies have shown a clear connection between exposure to amphibole asbestos fibers and the subsequent development of MM (Spirtas et al., 1994), but many cases of MM are idiopathic, while some are associated with radiation exposure, exposure to simian-virus 40, chronic inflammation, and genetic factors (Fuhrer and Lazarus, 2011). Surgery, chemotherapy, and radiation therapy are options for some MM patients with limited disease extension. However, responses to current therapeutic approaches are not effective and remain largely unsuccessful due to poor responses of cancer cells to treatment and tumor re-growth after therapy. Therefore, a better understanding of the molecular and cellular mechanism underlying chemoresistance in MM will lead to the identification of more effective therapeutic targets and novel agents.

Development of multi-drug resistance, high toxicity, and related side-effects to chemotherapeutic agents remains a major obstacle in the successful treatment of cancer. A large number of dietary phytochemicals has been demonstrated to exhibit anticancer activities by interfering with multiple signaling pathways, resulting in inhibiting survival proteins or activating proapoptotic mediators. In addition, a number of dietary phytochemicals exhibit synergistic effects with conventional chemotherapy and radiotherapy (Kannaiyan et al., 2011; Quan et al., 2008). Thus, naturally-derived phytochemicals could play important roles in cancer therapy owing to multitargeted mechanistic action and lack of substantial toxicity. Of those, resveratrol has been identified as an effective candidate for overcoming chemoresistance in tumor cells by modulating cell survival proteins (Zhao et al., 2010) and the tumor suppressor gene p53 (Gatouillat et al., 2010), decreasing

Abbreviations: MM, malignant mesothelioma; NF-κB, nuclear factor-kappa B; STAT-3, signal transducer and activator of transcription 3; Sp1, specificity protein 1; SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; ECL, enhanced chemiluminescence; CI, combination index; PI3K, phosphoinositol 3-kinase; p-Akt, phosphor-Akt.

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http://dx.doi.org/10.1016/j.fct.2012.10.060

the activity of drug transporters (Nabekura et al., 2005), and down-regulating proteins involved in cell proliferation (Gatouillat et al., 2010). Resveratrol inhibits cell proliferation and overcomes chemoresistance by inhibiting the nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT-3) pathways and down-regulating the genes involved in cell survival (Bhardwaj et al., 2007). The combined treatment of resveratrol and chemotherapeutic agents is also associated with an increase in cell cycle arrest and apoptosis. For example, in chemoresistant B16 melanoma, resveratrol at a subtoxic dose (25 μM) enhances doxorubicin-induced cytotoxicity that is accompanied by G_1 phase arrest and the induction of apoptosis (Gatouillat et al., 2010).

Specificity protein 1 (Sp1) is a nuclear transcription factor that recognizes a 5′-GGGCGG-3′ motif or related GC-rich sequences, and modulates the expression of many constitutive housekeeping genes as well as inducible genes in response to various stimuli (Gidoni et al., 1984). Sp1 target gene products, including androgen receptor, transforming growth factor-β, c-Met, fatty acid synthase, matrix metalloprotein, α-integrin, and p21, have been implicated in the control of a diverse array of cellular processes, such as cell cycle control, angiogenesis, apoptosis, cell proliferation, and metastasis (Sankpal et al., 2011; Tvrdík et al., 2006). The Sp1 protein is frequently up-regulated in many cancers and is highly correlated with the stage and poor prognosis of the cancers (Guan et al., 2012). Inhibition or knockdown Sp1 usually suppresses the tumor formation, growth, and metastasis (Yuan et al., 2007; Lou et al., 2005). As Sp1 contributes to the proliferative and metastatic tumor phenotypes and is considered a negative prognostic factor for survival in various cancers, it represents an attractive target for enhancing the effects of common cancer treatments. Therefore, identification of specific compounds that target the Sp1-dependent protective response as adjuvants of chemotherapeutic drugs can be one of the ways to maximize cancer cell death and to enhance the therapeutic potential.

The goals of the present study were to identify the efficacy of various phytochemicals as a chemo-potentiator in combination with the chemotherapeutic agent clofarabine in human malignant mesothelioma MSTO-211H cells and to identify factors that are involved in this process. In the study, we show that the combined treatment of resveratrol and clofarabine synergistically inhibited mesothelioma cell proliferation but had little toxicity in normal mesothelial cells. Furthermore, we show that this synergism is associated with the inhibition of Sp1 proteins, several Sp1-regulated gene products including c-Met, cyclin D1, and p21, as well as Akt activation. Possible roles of these proteins in cancer cells were also discussed.

2. Materials and methods

2.1. Reagents and cell culture

Resveratrol, quercetin, cafestol, sulforaphane, clofarabine, sodium dodecyl sulfate (SDS), dimethylsulfoxide (DMSO), and antibody to β-actin were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Ly294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] was purchased from Calbiochem (La Jolla, CA, USA). Anti-human Sp1, p21, c-Met, and cyclin D1 antibodies, HRP-tagged secondary antibodies, and enhanced chemiluminescence (ECL) kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human Akt and phospho-Akt (p-Akt) antibodies were from Cell Signaling Technologies (Beverly, MA, USA). Cell culture media and reagents were purchased from Hyclone Laboratories Inc. (South Logan, Utah, USA). The human mesothelioma cell line MSTO-211H and human mesothelial cell line MeT-5A were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MSTO-211H cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Met-5A cells were maintained in M-199 (Welgene, Daegu, Korea) medium supplemented with 3.3 nM epidermal growth factor, 10% fetal bovine serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml.

Cells were grown to 70% confluence in a monolayer culture in this medium for 24 h before treatment. Except for resveratrol, other compounds were dissolved in DMSO. The final concentration of DMSO in culture medium was 0.1% (v/v).

2.2. Cell viability assay

Cell viability was measured using the Cell Proliferation kit II (XTT) according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN, USA). Briefly, cells (10^4 cells/well) were seeded in 96-well microtiter plates and then treated with clofarabine or other phytochemicals at various concentrations for the indicated times. After incubation, 50 μL of the XTT labeling mixture was added to each well and incubated for an additional 4 h. The formazan dye that formed was measured spectrophotometrically at 450 nM using a Glomax multi detection system (Promega, Madison, WI, USA). The results were expressed as a percentage, based on the ratio of the absorbance of treated cells to that of controls (100%).

2.3. Combination effect

The combination effect of two compounds was evaluated using the combination index (CI), which is defined as follows: $CI = D_1/D_{1a} + D_2/D_{2a} + (D_1 \times D_2)/(D_{1a} \times D_{2a})$, where: D_1 is the dose of compound 1 required to produce an *X*% cell viability in combination with compound 2; D_{1a} is the dose of compound 1 alone required to produce the same *X*% cell viability; D_2 is the dose of compound 2 required to produce an *X*% cell viability in combination with compound 1; and D_{2a} is the dose of compound 2 alone required to produce the same *X*% cell viability. The combination effect is defined as follows: CI < 1 is a synergistic effect; CI = 1 is an additive effect; and CI > 1 is an antagonistic effect.

2.4. Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were prepared according to the instructions provided in the NE-PER® nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA). Briefly, cells were resuspended in 10 vol of CER I solution, after which they were incubated in a CER II solution on ice for 1 min and homogenized. Nuclei were recovered by centrifugation at 14,000 rpm for 5 min, and the supernatant was kept as the cytoplasmic extract. The nuclear fraction was extracted for 40 min on ice in NER solution. The insoluble pellet was removed by centrifugation at 14,000 rpm for 10 min. The supernatant was used as the nuclear extract.

2.5. Western blot analysis

Whole cell lysates were prepared using RIPA buffer (1× PBS PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethanesulfonylfluoride). Briefly, proteins (40 μg per lane) were separated on NuPAGE 4–12% bis–tris polyacrylamide gels (Invitrogen) and then electrophoretically transferred to Immuno-Blot PVDF membranes. The membranes were incubated for 2 h at room temperature with a 1:500 dilution of anti-Sp1, anti-p21, anti-c-Met, and anti-p-Akt antibodies. Next, HRP-conjugated secondary antibody was applied at a dilution of 1:5000 and the signal was visualized using an ECL detection kit. The blots were then stripped using a stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7) and re-probed with anti-Akt and anti-β-actin antibodies as loading controls.

2.6. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc correction for multiple comparisons using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± SEM. Significant differences were considered with values of $p < 0.05$.

3. Results

3.1. Combination treatment of resveratrol and clofarabine synergistically inhibits cell proliferation

To determine effective doses for the treatment of phytochemicals, a cell proliferation assay was carried out using the XTT assay in MSTO-211H cells. The subtoxic dose of each phytochemical was determined based on individual IC_{10} values after 48 h treatment. All four of the tested compounds inhibited cell growth in a dose- and time-dependent manner (Fig. 1A). Resveratrol treatment, in particular, produced the most cytotoxic effect with an average IC_{10} of 10 μM, followed by quercetin (IC_{10} = 18 μM), sulforaphane (IC_{10} = 22 μM), and cafestol (IC_{10} = 30 μM). Next, the cytotoxicity of the chemotherapeutic agent clofarabine was determined against MSTO-211H cells by employing the XTT assay. Clofarabine up to

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