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Synergistic anti-proliferative effect of resveratrol and etoposide on human hepatocellular and colon cancer cell lines

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ABSTRACT

Resveratrol is an active component of grape, which has been shown to inhibit proliferation of a wide variety of tumor cells. The ability of resveratrol to enhance anti-proliferative effects of etoposide in wild type p53 liver carcinoma (HepG2) and colon cancer (HCT-116) cells was investigated with focusing on p53 activation. HepG2 cells and HCT-116 cells were treated with resveratrol and/or etoposide in a time- and dose-dependent manner and their proliferative response was evaluated by XTT assay. The expression of p53 protein was assessed using Western blot. Resveratrol exerted anti-proliferative activity on both cell types in a dose (25–100 μM)- and time (24–72 h)-dependent manner. Interestingly in HepG2 cells, resveratrol exhibited the same levels of cytotoxicity as etoposide (10 μM) when the cells treated with $\geq$25 μM for 48–72 h. In contrast to HepG2, resveratrol significantly enhanced anti-proliferative effects of etoposide in HCT-116 cells. P53 expression was up-regulated by resveratrol and etoposide and pre-incubation of both cells with resveratrol increased levels of etoposide-induced p53 expression. In line with cytotoxicity effect, combination therapy showed stronger activation of p53 in HCT-116 compared to HepG2. It seems that resveratrol exerts differential synergistic effect with etoposide on proliferation of cancer cells from different origin which is mainly accompanied by p53 activation. Our data represent a future strategy to provide much safer and more effective treatment for colon cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is a frequent malignancy worldwide with high prevalence (Blechacz and Mishra, 2013). Although surgery and non-surgical therapeutic modalities have been employed for treatment of HCC, such treatments are rarely curative and have marginal effect on survival rate. Therefore, development of more effective approaches is of great interest.

Although not as fatal as HCC, colorectal cancer (CRC) is one of the leading causes of death in men and women and globally ranks among the third most common cancers (Pan et al., 2011). The prevalence of CRC is increasing steadily despite deep understanding of its pathogenesis. It has been reported that almost 50% of patients with CRC will develop recurrent disease, indicating that currently available treatment regimens are not able to control this disease and there is an imperative need for improved therapies (Pan et al., 2011).

The most common modalities for cancer therapy include surgery, radiotherapy and chemotherapy. Although, chemotherapy is often used as a main regimen in the treatment of most cancers, chemoresistance represents a major obstacle in cancer therapy. Chemosensitization is among the strategies that are currently proposed for overcoming chemoresistance. It is based on the use of one drug to enhance activity of another one by modulating one or more mechanisms of resistance. Over the years, natural products have been discovered to be more effective than cancer drugs because of their multi-targeting property, low cost, low toxicity and immediate availability (Gupta et al., 2011). Phytochemicals are among the most promising chemopreventive and treatment options for the management of cancer. In this regard, resveratrol (*trans*-3, 4′, 5-trihydroxystilbene, $C_{14}H_{12}O_3$) (RSVL) could be viewed as an ideal molecule for

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cancer therapy due to its relatively low toxicity and capacity to target multiple signaling pathways responsible for cancer cell survival, tumor growth, inflammation, angiogenesis and metastasis (Aluyen et al., 2012; Borriello et al., 2013). RSVL is a naturally occurring product found in grapes, peanut, blueberries, raspberries and mulberries and is produced by the action of enzyme, stilbene synthase, in response to environmental stress (Athar et al., 2009; Borriello et al., 2013). Discovered in 1997 as inhibitor of initiation, promotion and progression of tumorigenesis (Jang et al., 1997), RSVL possesses a wide range of pharmacological properties including inhibition of inflammation and oxidative stress which may explain its anti-tumor activity (Aluyen et al., 2012). Thereafter, many studies have verified anti-cancer properties of RSVL in various human cancers from different origin including skin (Lee et al., 2012), stomach (Wang et al., 2012b), lung (Wang et al., 2013), breast (Su et al., 2013), prostate (Li et al., 2013), liver (Su et al., 2013; Wang et al., 2012a), pancreas (Mo et al., 2011), and colon (Vanamala et al., 2010).

The topoisomerase II inhibitor, etoposide (ETO), is an anti-neoplastic drug that has been extensively used to couple DNA damage to apoptosis in a variety of cell types (Karpinich et al., 2002, 2006; Mizumoto et al., 1994).

Here we investigated whether RSVL at different time intervals and doses could enhance chemosensitivity of wild type p53 liver carcinoma (HepG2) and colon cancer (HCT-116) cells in response to ETO. The effect of such co-treatment on p53 expression which is the main checkpoint of cell fate was then examined.

2. Materials and methods

2.1. Chemicals and reagents:

RSVL and ETO were purchased from Sigma Aldrich (St. Louis, Mo). Drugs were reconstituted in absolute ethanol and preserved at −20 °C and 4 °C, respectively. Dulbecco's Modified Eagle's Medium F-12 (DMEM-F12), fetal bovine serum (FBS) and Trypsin/EDTA were purchased from the Gibco (Pittsburgh, PA). All other cell culture additives and sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium (XTT) were purchased from Sigma. Anti-p53 (DO1) and RIPA buffer were from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein assay kit was obtained from Pierce (Rockford, IL). PVDF membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Skim milk and enhanced chemi-luminescence detection system (ECL) prepared from Amersham Pharmacia Biotech (Upsala, Sweden). Horse radish peroxidase-conjugated anti-mouse IgG was prepared from Avicenna Research Institute (Tehran, Iran).

2.2. Cell culture

The HepG2 (human liver cancer cell) and HCT-116 (human colon cancer cell) lines were obtained from national cell bank of Iran (Pasteur Institute, Iran). Cells were maintained at 37 °C incubator with 5% CO_2 in DMEM-F12 medium supplemented with antibiotics (100 U/ml penicillin, 100 g/ml streptomycin) and 10% (v/v) heat-inactivated FBS and passaged every 4–5 days.

2.3. Assessing cytotoxic activity

XTT assay was performed according to the protocol we published recently (Nikoo et al., 2012). Optimum seeding density of each cell line was first evaluated in XTT assay by examining different cell numbers starting from 2.5 to 160×10^3. From the standard curve plotted, 1×10^4 cells/well was found to be optimum cell number in both cell types. Cells were cultured in 96 well plates in 100 μl phenol red free DMEM containing 10% FBS. After the overnight incubation, they were treated with different concentrations of RSVL (12.5, 25, 50 and 100 μM) or ETO (0.5, 1, 10 and 50 μM) in the final 50 μl per well. In co-treatments, cells were pre-incubated first with aforesaid concentrations of RSVL for 60 min followed by ETO treatment (1 and 10 μM). Anti-proliferative properties of RSVL and ETO were evaluated after 24, 48 and 72 h. XTT was prepared at 1 mg/ml in pre warmed (37 °C) phenol red free DMEM. PMS was prepared at 5 mM (1.53 mg/ml) in PBS. Fresh XTT and PMS were mixed together at the appropriate concentrations. For a 0.025 mM PMS-XTT solution, 25 μl of the stock 5 mM PMS were added per 5 ml of XTT (1 mg/ml). 50 μl of this mixture (final concentration, 50 μg of XTT and 0.38 μg of PMS per well) were added to each well and incubated at 37 °C, 5% CO_2 for 4 h, the plates were mixed on a mechanical plate shaker; thereafter, the OD values were read at 450 nm. Each experiment was carried out in triplicate and the data were the average results of three independent experiments. Three types of XTT controls including negative control (culture medium + solvent without cells), solvent control (culture medium + solvent + cells) and cell growth control (culture medium + cells) were tested. The mean OD value was calculated for every test sample concentration and the relative inhibition activity calculated by the following formula:

% inhibition = 100 − (corrected mean OD sample × 100/corrected mean OD solvent controls), where: corrected sample OD = OD sample − OD negative control and corrected solvent OD = OD solvent control − OD negative control.

IC50 of each drug, the concentration necessary to produce 50% inhibition of cell growth, was calculated from linear equation ($Y = mX + b$), where $Y = 50$, m = slope, X = IC50, b = the y-intercept.

2.4. Western blotting analysis

Western blotting (WB) was performed to quantify protein expression of p53. Cells treated with RSVL (50 μM), ETO (10 μM) or combination of both drugs was lysed in RIPA buffer and the protein concentration of cell lysates were determined using BCA protein assay. 40–50 μg of protein were mixed with sample buffer and boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels at 120 V for 2 h and the bands were subsequently transferred to PVDF membranes at 100 V for 60 min. Membranes were blocked with 5% skim milk for 1 h at room temperature. The membranes were sequentially incubated with mouse monoclonal anti-p53 (dilution:1:10,000) in 3% BSA at 4 °C overnight and rabbit anti-mouse IgG HRP (1:10,000) for 1.5 h at room temperature. All washing steps were performed with Tris buffered salin (TBS) containing 0.1% Tween-20. The bands were detected using the enhanced chemi-luminescence detection system according to the manufacturer's protocol. β-actin western blotting was employed as loading control. To this end, after completion of p53 WB, membranes were re-probed by stripping buffer and incubated with rabbit anti-human β-actin polyclonal antibody and proceed as above.

2.5. Densitometry

Densitometric analysis of WB bands was performed by Alpha Ease software according to the protocol we published elsewhere (Shahbazi et al., 2011). In brief, photographs of WB membranes were prepared and contrast was adjusted so that black and white colors corresponded to 250 and 0, respectively. Bands were selected and their density was quantified in reference background density of negative control. Relative expression of p53 was presented as the percent of p53/β-actin density ratio.