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The effect of resveratrol, its naturally occurring derivatives and tannic acid on the induction of cell cycle arrest and apoptosis in rat C6 and human T98G glioma cell lines

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ABSTRACT

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a potent chemopreventive and potentially cancer therapeutic agent. Since rapid metabolism limits resveratrol bioavailability, derivatives less prone to metabolic transformation are being sought and tested.

We evaluated the effect of resveratrol, and its analogs (pterostilbene and 3,5,4′-trimethoxystilbene) along with tannic acid, on cell cycle and apoptosis in rat C6 and human T98G glioma cells. At concentration ranges both lower and higher than IC_{50} calculated based on MTT assay, all these polyphenols affected the cell cycle distribution. However, resveratrol and pterostilbene increased the percentage of the cells in S phase, while trimethoxystilbene (TMS) caused a massive accumulation of cells at the G2/M phase of the cell cycle. Tannic acid had no effect on cell cycle distribution in C6 cells, but increased the number of dead cells in both glioma cell lines. The ability to induce apoptosis by tannic acid and stilbenes was confirmed by phosphatidylserine externalization, the loss of mitochondrial membrane potential and the level of cleaved caspase-3. The apoptosis rate was most significantly increased by TMS and this was related to p53 induction.

These results indicate that methoxylated stilbenes are efficient inhibitors of glioma cell proliferation and apoptosis inducers and might be considered adjuvants in glioma therapy.

1. Introduction

Malignant gliomas are the most common central nervous system tumors and carry the worst clinical prognosis in both adults and children. Although current therapeutic protocols comprise a combination of surgical operation with irradiation and adjuvant chemotherapy, the prognosis for malignant gliomas remains very poor due to their highly aggressive biological behavior and frequent recurrence rate (Gagliano et al., 2010; Van Meir et al., 2010). Thus, the development of new agents able to reactivate cell cycle or cell death programs, particularly apoptosis, is important for the introduction of new therapeutic and/or chemopreventive strategies for malignant gliomas.

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a naturally occurring polyphenol, is assumed to possess cancer-preventive and cancer-therapeutic properties (Kuršvietienė et al., 2016). Resveratrol suppresses the proliferation of a variety of human cancer cells in vitro, including glioma cells (Gagliano et al., 2010).

In C6 rat glioma cells, resveratrol has been shown to inhibit cell growth and induce apoptosis by increasing caspase-3 mRNA level and enzyme activation (Zhang et al., 2007). However, Michels et al. (2006) demonstrated that resveratrol is not metabolized in C6 rat glioma cells, and accumulates to concentrations that drive the cell to necrosis. Induction of apoptosis by resveratrol has also been reported in human glioma U251, U87 and T98G cells (Jiang et al., 2005; Lin et al., 2011). Metabolism plays an important role in resveratrol anticancer activity.

While resveratrol is the most active, its sulfo- and glucuronide conjugates are quickly excreted and are much less effective (Sun et al., 2013; Wang et al., 2005).

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolo-carbocyanine iodide; MTP, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PS, phosphatidylserine; RNase A, ribonuclease A; TMS, 3,5,4′-trimethoxystilbene

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In view of the limited bioavailability of resveratrol, its naturally occurring and synthetic analogs are the subjects of intensive research and development. In this regard, naturally occurring methoxylated stilbenes, such as 3,5-dimethoxy-4′-hydroxy-*trans*-stilbene (pterostilbene) and 3,5,4′-trimethoxy-*trans*-stilbene, have been shown to be more efficient in eliciting chemopreventive and/or therapeutic effects than resveratrol (Kapetanovic et al., 2011; Lin et al., 2009; Lin and Ho, 2009; Aldawsari and Velázquez-Martínez, 2015).

Tannic acid, a water-soluble tannin, is, similarly to resveratrol, a common ingredient of grapes and others berry fruits. Thus, their synergic action may be expected.

Our earlier studies have shown that although both polyphenols reduced binding of carcinogenic aromatic hydrocarbons to DNA, tannic acid was a much more potent inhibitor of specific DNA adduct formation (Ignatowicz et al., 2003; Baer-Dubowska and Szaefer, 2013). Moreover, neutrophil modulation of ROS production ultimately leading to cell apoptosis has also been shown in humans (Zielińska-Przyjemska et al., 2015).

While the role of neutrophils infiltrating tumors has been neglected for some time, a significant amount of evidence has recently been gathered on their importance in experimental models as well as in human cancers (Moses and Brandau, 2016). Fossati et al. (1999) showed that most human glioma samples analyzed had significant neutrophil infiltration. Thus, compounds such as polyphenols may target both cancer tissue and infiltrated neutrophils.

In the present study, we compared the ability of resveratrol and its two natural derivatives (pterostilbene and 3,5,4′-trimethoxystilbene) and tannic acid to induce growth inhibition and apoptosis in rat C6 and human T98G glioma cells. An attempt to explain the mechanism of pro-apoptotic activity of these compounds was also undertaken.

2. Materials and methods

2.1. Chemicals

Table 1 shows the chemical structure of the examined polyphenols. Resveratrol, tannic acid (purity GC, TLC ≥ 99%), camptothecin, antibiotic solution (10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL), dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), glutamine, propidium iodide (PI), ribonuclease A (RNase A) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), non-essential amino acids (NEAA), sodium pyruvate (NaP) and all other compounds were provided by Sigma–Aldrich Co. (St Louis, MO, USA). 3,5,4′-Trimethoxystilbene was purchased from BIOMOL International, LP (Plymouth Meeting, USA). Pterostilbene was a generous gift from Dr. Agnes Rimando (Natural Products Utilization Research Unit ARS, U.S. Department of Agriculture). Its isolation protocol and purity was described by Mikstacka et al. (2007). Suppliers of antibodies and reagent kits used in this study are indicated in the respective methods descriptions.

2.2. Cell cultures and treatments

The rat C6 glioma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS and 1% antibiotics solution. The human T98G glioblastoma cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in EMEM supplemented with 10% FBS, 1% antibiotics solution, 2 mM glutamine, 1% non-essential amino acids and 1% sodium pyruvate. Cells were incubated at 37 °C in an atmosphere consisting of 95% air and 5% CO_2 in a humidified incubator until they reached 70% confluency. A total of 1×10^6 cells were seeded in ø 40 mm culture dishes. After 24 h of preincubation in culture medium containing 5% of FBS, the cells were treated with resveratrol, its analogs or tannic acid. The incubation was continued for a further 24 h to assess cell cycle distribution, apoptosis or p53 induction. Control cells were treated with a vehicle (DMSO). Cells treated with 50 nM of camptothecin were used as a positive control. The concentration of DMSO in culture medium did not exceed 0.1%.

2.3. MTT assay for cell viability

The effect of tested polyphenols on cell viability was assessed with an MTT assay according to the standard protocol (Zielińska-Przyjemska et al., 2015). Briefly, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µL of growth medium. They were allowed to attach overnight and resveratrol, its appropriate analog or tannic acid was then added to the culture medium in various concentrations (0 to 200 µM) for 24 and 48 h at 37 °C. The cells were subsequently incubated with MTT (0.5 mg/mL) solution for another 4 h. The water insoluble formazan crystals were solubilized in acidic isopropanol before the measurement of absorbance using a microplate reader (TECAN Infinite M200) at 570 nm. All the experiments were repeated three times, with at least three measurements per assay.

2.4. Flow cytometric cell cycle analysis

Cell cycle distribution was evaluated by flow cytometric analysis. After treatment, C6 or T98G cells were collected and fixed in 70% ethanol at 4 °C for 30 min. Since then, the cells were washed twice in PBS and resuspended in 250 µL of PBS containing 50 µg/mL propidium iodide (PI) and 100 µg/mL RNase A. After incubation in the dark at 37 °C for 30 min, the fluorescence of cells was analyzed with a FACSCanto flow cytometer (Becton Dickinson, USA). Data analysis and acquisition was performed using FACS Diva software (Becton Dickinson, USA).

2.5. FITC Annexin V/propidium iodide double staining assay

Apoptosis was assessed using an FITC Annexin V Apoptosis Detection kit (Becton Dickinson, San Jose, Ca, USA) according to the manufacturer's instructions. The cells treated with polyphenols for 24 h were stained with 5 µL of FITC Annexin V labeling reagent and 5 µL of PI for 15 min in the dark. Finally, samples were assessed with a FACSCanto flow cytometer (Becton Dickinson, USA), using FACS Diva software (Becton Dickinson, USA).

The fluorescence of cell surface Annexin V or DNA-bound PI markers was analyzed with flow cytometry at 488 nm excitation wavelength, emission 518 nm and 617 nm for Annexin V and PI, respectively. PI negative and Annexin V positive cells were considered early apoptotic; PI and Annexin V positive cells were considered to be in late apoptosis; PI positive and Annexin V negative cells were considered mechanically injured during the experiment; PI and Annexin V negative cells were considered normal.

2.6. Mitochondrial transmembrane potential (MTP, $\Delta\psi_m$)

The change in MTP was evaluated using a Mitochondrial Staining Kit (Sigma–Aldrich Co., St Louis, MO, USA). Briefly, rat C6 cells after treatment with polyphenols were harvested and washed with PBS. Since then, the cells were incubated with JC-1 dye (2.5 µg/mL) at 37 °C in darkness for 20 min. After washing with PBS, fluorescence emission was determined with a FACSCanto flow cytometer (Becton Dickinson, USA) using a 448 nm band pass filter for JC-1 aggregates (red fluorescence) indicating high or normal MTP, and a 525 nm band pass filter for JC-1 monomers (green fluorescence), which represents disrupted mitochondria.