Food Chemistry 250 (2018) 213–220

Contents lists available at ScienceDirect

Food Chemistry

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

ROS-driven and preferential killing of HepG2 over L-02 cells by a short-term cooperation of Cu(II) and a catechol-type resveratrol analog

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ARTICLE INFO

Keywords:
Cancer chemoprevention
Catechol
Copper
Reactive oxygen species
Resveratrol

ABSTRACT

This study was aimed at understanding why dietary polyphenols with a catechol skeleton tend to exhibit cancer chemopreventive activity by using a catechol-type stilbene (3,4-DHS) as a model molecule. Only a short-term cooperation of 3,4-DHS and exogenous Cu(II) exhibited a strong preferential ability to kill HepG2 cells over normal L02 cells. Mechanism studies reveal that this 3,4-DHS/Cu(II) system could produce extracellularly reactive oxygen species (ROS) and *o*-quinone through two sequential proton loss electron transfer followed by diffusion of ROS into cells, leading to higher intracellular accumulation of ROS, preferential disruption of redox homeostasis and more effective mitochondria-dependent apoptosis as well as necrosis of HepG2 cells than L-02 cells. This work provides further evidence that dietary catechol-type molecules show chemopreventive activity by virtue of their copper-dependent prooxidant action.

1. Introduction

Cancer chemoprevention is defined as the use of multiple intervention strategies by natural, synthetic or biological agents to reverse, suppress or prevent the progression of carcinogenic process at various stages, including tumor initiation, promotion and progression, and has emerged as a viable alternative strategy for preventing carcinogenesis (Steward & Brown, 2013; Wattenberg, 1985). Accumulating evidence suggests that modifying dietary habits by increasing consumption of fruits, vegetables and teas rich in polyphenols may decrease the risk of cancer (Khan, Afaq, & Mukhtar, 2008). Various dietary polyphenols with the catechol skeleton, such as piceatannol, green tea polyphenols, quercetin, caffeic acid and delphinidin, hydroxytyrosol, indeed show considerable promise in cancer chemoprevention. Since reactive oxygen species (ROS) or free radicals play an important role in the development of carcinogenesis, promoting cell survival, proliferation, angiogenesis and metastasis (Hussain, Hofseth, & Harris, 2003), their antioxidant activity is usually suggested to be linked to low cancer rates (Khan et al., 2008). However, under certain conditions, such as high concentrations and presence of redox-active transition metals, antioxidative polyphenols, especially those with the catechol skeleton, can be easily converted into prooxidants to generate ROS (Fan et al., 2009; León-González, Auger, & Schini-kerth, 2015). Accumulating studies have also revealed that a few polyphenols (León-González et al., 2015) and a classic antioxidant, vitamin C (Chen et al., 2007, 2008; Yun et al., 2015), work as prooxidants instead of antioxidants to kill cancer cells. Therefore, the mechanisms, by which the catechol-type molecules tend to show cancer chemopreventive activity, remain to be elucidated.

In fact, cancer cells compared with normal cells exhibit altered redox status, including increased levels of reactive oxygen species (ROS) and copper to maintain their malignant phenotypes, and thereby are more vulnerable to further ROS production (prooxidant action) (Gorrini, Harris, & Mak, 2013; Gupte & Mumper, 2009; Trachootham, Alexandre, & Huang, 2009). Hadi and co-workers have previously proposed a mechanism to explain the selective cytotoxicity of polyphenols targeting cancer cells that involves mobilization of endogenous copper and the consequent prooxidative DNA damage (Hadi et al., 2007; Khan et al., 2014). In this regard, we have also reported the prooxidation scenario in chemical detail for the Cu(II)-mediated reactions of polyphenols, including cinnamic acids (Fan et al., 2009), stilbenes (Zheng et al., 2006), stilbene-chroman hybrids (Liu et al., 2012) and chalcones (Wang et al., 2013). Among them, these molecules with the catechol skeleton have been identified as more effective prooxidants to mediate DNA damage and death of cancer cell through a sequential proton loss electron transfer-based reduction of Cu(II), followed by production of ROS and *o*-quinone. From a chemical viewpoint, this could be easily understood because catechols, compared with monophenols and other polyphenols, often facilitate chelation with cupric ions, making electron transfer between them energetically favourable. Especially, we have recently found that among resveratrol and its hydroxylated analogs, a catechol-type stilbene (3,4-dihydroxy-*trans*-stilbene, 3,4-DHS, Fig. 1A) is the only one which can effectively make use

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https://doi.org/10.1016/j.foodchem.2018.01.069
Received 5 August 2017; Received in revised form 8 November 2017; Accepted 8 January 2018
Available online 09 January 2018
0308-8146/

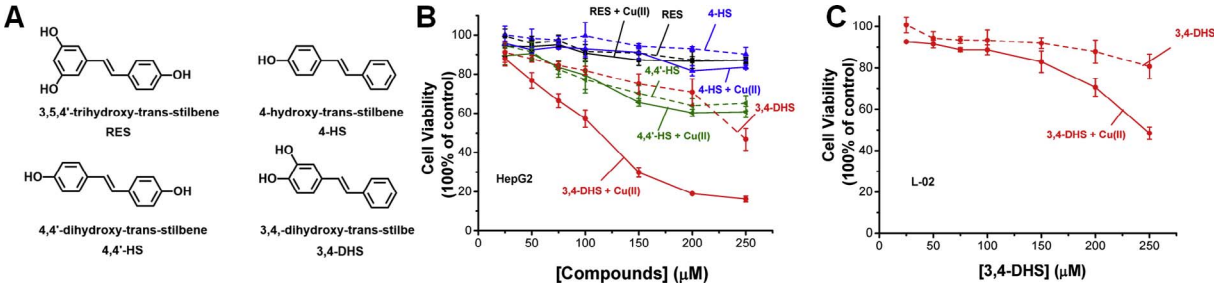

Fig. 1. Molecular structures of RES and its hydroxylated analogs (A) and their cytotoxicity against HepG2 cells (B) and L-02 cells (C) in the presence or absence of Cu(II). Cells were exposed to the test molecules at different concentrations, 300 μM Cu(II) or their combination for 3 h, followed by their removal and incubation with fresh medium for another 45 h. All data represent the mean ± SD of three independent experiments.

of endogenous copper ions to construct an efficient prooxidant system, resulting in activation of nuclear factor erythroid-2 related factor 2 (Nrf2), an important target for cancer chemoprevention (Lin, Dai, Sun, & Zhou, 2015). This has prompted us to suppose that the catechol-type molecules manifest cancer chemopreventive activity by virtue of their copper-dependent prooxidant action. However, more research is needed for confirmation of this hypothesis. For example, for inhibiting cancer progression by inducing cell death, one of the first issues is whether this prooxidant system could selectively target cancer cells over normal cells by ROS generation.

Stilbenes are a small family of plant secondary metabolites, as exemplified by the most extensively studied resveratrol (Baur & Sinclair, 2016), pterostilbene (McCormack & McFadden, 2012) and piceatannol (Piotrowska, Kucinska, & Murias, 2012), with pleiotropic health benefits, including cancer chemoprevention. Considering structural simplicity of stilbenes, we selected 3,4-DHS as a model of dietary catechol-type molecules to confirm the above hypothesis based on the following reasons: (1) it bears the same catechol moiety as natural piceatannol, but exhibits increased stability than the latter due to the absence of electron-donating 4′-OH, thereby facilitating investigation on its oxidation mechanism by Cu(II); (2) it has been identified as an ideal precursor of ROS to induce DNA damage in the presence of exogenous Cu(II) (Zheng et al., 2006), one of the most redox-active ions present in cells; and (3) as outlined above, we have used this molecule as a model of dietary catechol-type molecules to verify its copper-dependent Nrf2 activation and final cytoprotection (Lin et al., 2015). Additionally, the other hydroxylated stilbenes without the catechol moiety, that is resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RES), 4-hydroxy-*trans*-stilbene (4-HS) and 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), were used as the controls (Fig. 1A).

2. Materials and methods

2.1. Chemicals and antibodies

RES and its analogs (4-HS, 4,4′-DHS and 3,4-DHS) were synthesized by the Wittig-Horner reaction, as described in our previous paper (Shang et al., 2009). RPMI 1640, $CuCl_2{\cdot}2H_2O$, sulforhodamine B (SRB), N-acetylcysteine (NAC), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine 123, sodiumdodecyl sulfate (SDS), L-glutathione reduced (GSH), L-glutathione oxidized (GSSG) and 5,5′-dithiobis-2-nitrobenzoicacid (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Superoxide dismutase (SOD), catalase (CAT) and bicinchoninic acid (BCA) protein assay kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Primary antibodies against Bax, cytochrome *c*, caspase-3 and -9 were from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against B-cell lymphoma 2 (Bcl-2), poly(ADP-ribose) polymerase-1 (PARP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from GeneTex Inc. (Irvine, CA, USA). HRP-labeled secondary antibody was obtained from TransGen Biotech Co., Ltd. (Beijing, China).

2.2. Cell culture

HepG2 (human hepatoma cells) and L-02 (human normal liver cells) were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 kU/l penicillin, 100 kU/l streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell viability assay

Cells were seeded in 96-well plates at 1×10^4 cells/well and exposed to RES and its analogs at different concentrations (50, 75, 100, 150, 200 and 250 μM), Cu(II) (300 μM), or their combination for 3 h, followed by their removal and incubation with fresh medium for another 45 h. Cell viability was assayed using the SRB assay as detailed in our previous paper (Dai et al., 2017). In order to evaluate the effects of various redox modulators on the synergistic cytotoxicity induced by 3,4-DHS and Cu (II), the cells were pretreated with SOD, CAT or NAC at a non-toxic concentration for 1 h before the test compounds were added. SOD (≥3000 units/mg protein, 5 mg/ml), CAT (2000–5000 units/mg protein, 5 mg/ml) and NAC (50 mM) were dissolved with sterile double distilled water to generate a stock solution with the final concentrations used being 0.5 mg/ml, 0.5 mg/ml and 5 mM, respectively.

2.4. Determination of intracellular ROS levels

Intracellular ROS levels were measured by flow cytometry using an oxidation-sensitive probe, DCFH-DA (Sentürker, Tschirret-Guth, Morrow, Levine, & Shacter, 2002). In brief, cells (2×10^5 cells/well) were exposed to 3,4-DHS, Cu(II) or their combination for 3 h, followed by their removal and incubation with fresh medium for another 6 h, and then were harvested and centrifuged at 200*g* for 10 min. DCFH-DA at 2 μM was co-incubated with the cells for 30 min at 37 °C in the dark and then removed. Cells were washed twice with ice-cold PBS and kept on ice for an immediate detection of DCFH fluorescence intensity by a FACSCanto flow cytometer with excitation and emission settings of 488 and 530 nm, respectively.

2.5. UV/vis spectral measurements

A TU-1901 UV/Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) was used to monitor the Cu (II) (25 μM)-mediated spectral changes of 3,4-DHS (50 μM) in PBS (pH 7.4) and NaAc/HAc buffer solution (pH 6.0) at ambient temperature. All the spectra were run against blanks containing the buffer and Cu(II), and recorded every appointed time after addition of Cu(II).