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Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells

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Abstract

This study demonstrates cytotoxic and genotoxic potential of juglone, a chief constituent of walnut, and its underlying mechanisms against melanoma cells. MTT assay and clonogenic assay were used to study cytotoxicity, micronucleus assay to assess genotoxicity, glutathione (GSH) assay and 2′,7′-dicholorofluorescein diacetate (DCFH-DA) assay to evaluate the oxidative stress induction. Apoptosis/necrosis induction was analysed by flow cytometry. We observed a concentration-dependent decrease in cell survival with a corresponding increase in the lactate dehydrogenase levels. A dose-dependent increase in the frequency of micronucleated binucleate cells indicated the potential of juglone to induce cytogenetic damage in melanoma tumor cells. Moreover, results of the micronuclei study indicated division delay in the proliferating cell population by showing decrease in the cytokinesis blocked proliferation index. Further, juglone-induced apoptosis and necrosis could be demonstrated by oligonucleosomal ladder formation, microscopic analysis, increase in the hypodiploid fraction (sub Go peak in DNA histogram), as well as an increased percentage of AnnexinV(+)/PI(+) cells detected by flow cytometry. A significant concentration-dependent decrease in the glutathione levels and increase in dichlorofluorescein (DCF) fluorescence after juglone treatment confirmed the ability of juglone to generate intracellular reactive oxygen species. The cytotoxic effect of juglone can be attributed to mechanisms including the induction of oxidative stress, cell membrane damage, and a clastogenic action leading to cell death by both apoptosis and necrosis.

Keywords: Juglone; Melanoma; Genotoxicity; Reactive oxygen species; Apoptosis; Necrosis

1. Introduction

Quinones represent a broad category of widely distributed quinoid compounds in nature. Many quinones have been associated with a range of biological activities, including anticancer activity (Babula et al., 2007). Although, there are many clinically important agents containing a quinone nucleus with excellent anticancer activity (e.g. anthracycline, mitoxantrones and saintopin), many other quinones require testing for their anticancer activity (Kim et al., 2006). Although, DNA represents the main target of quinoids, the exact contribution of the quinone moiety to the cytotoxic effect is not clear. However, most of them belong to the groups of DNA intercalating and/or alkylating agents, and/or topoisomerase inhibitors (Rowley and Halliwell, 1983; Yamashita et al., 1991). In general, quinone toxicity has been attributed to the ability to undergo reversible oxidation-reduction reactions, as well as to its electrophilic nature leading to the formation of free radicals (Bachur et al., 1979; Giulivi and Cadenas, 1994).

Plants possess diverse principles, which are of immense nutritional and medicinal value. Plant-derived quinones are among the compounds that are extensively studied for their potential as cytotoxic/anticancer agents. Plumbagin (5-hydroxy-2-methyl-1, 4-napthoquinone), a compound derived from the

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roots of *Plumbago zeylanica*, is among the most extensively studied quinones. Our previous studies gave some insight into the anticancer and radiosensitizing properties of plumbagin (Naresh et al., 1996; Prasad et al., 1996; Devi et al., 1998; Tiwari et al., 2002). Other natural napthoquinones, such as lapachone and shikonin, also possess promising cytotoxic/anticancer properties (Wu et al., 2004; Reinicke et al., 2005).

Juglone (5-hydroxy-1, 4-napthoquinone), a structural analogue of plumbagin is a pigment that occurs as a natural product in the roots, leaves, nut-hulls, bark and wood of black walnut (*Juglans nigra* L.), European walnut (*Juglans regia* L.) and butternut (*Juglans cinerea* L.) of the Family Juglandaceae (Botanical Dermatology Database, 1999). Herbal preparations of walnut have been extensively used in folk medicine for the treatment of acne, inflammatory diseases, ringworm, bacterial, viral, fungal infections as well as cancer (Duke and Ayensu, 1985; Blumenthal, 1998). Walnut contains juglone as the principal component, with other constituents such as alpha-hydrojuglone (1,4,5-trihydroxynaphthalene) and its glycoside beta-hydrojuglone, along with caffeic acid, ellagic acid, hyperin, and kaempferol. Herbal preparations of walnut are reported to suppress the growth of spontaneous mammary adenocarcinoma in swiss albino mice (Bhargava and Westfall, 1968). Sugie and coworkers (1998) found that juglone reduced the formation of azoxymethane induced intestinal tumors in F344 rats and concluded that juglone could be a promising chemopreventive agent. Although few groups have studied the *in vitro* cytotoxic activity of juglone against cancer cell lines (Segura-Aguilar et al., 1992; Cenas et al., 2006), the exact mechanism remains doubtful. Therefore, we investigated the cytotoxic potential of juglone and its underlying mechanisms using a relatively chemo-resistant cell line (B16F1 melanoma) growing *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

Juglone purchased from Sigma (St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) diluted with Eagle's minimum essential medium (MEM) to the required concentration (the final concentration of DMSO was $<0.02\%$). All drug solutions were prepared freshly before use due to the instability of juglone in medium. 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 2′,7′-dicholorofluorescein diacetate (DCFH-DA), annexin-V-FITC apoptosis detection kit, RNase A, Proteinase-K, Nonidet P40, sodium dodecyl sulphate (SDS), Cytochalasin-B, glutathione, MEM, L-glutamine, gentamycin sulfate, fetal calf serum and DMSO were obtained from Sigma (St. Louis, MO, USA). Cytochalasin-B was dissolved in DMSO at 1 mg/ml, stored at −80 °C and diluted with PBS immediately before use.

2.2. Cell line and culture

B16F1 melanoma cell line obtained from the National Centre for Cell Science (Pune, India) was grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine and 80 μg/ml gentamycin sulfate. Cells were cultured in 75 cm^2 flasks with loosened caps and incubated in 5% CO_2 in humidified air at 37 °C (NuAire, Plymouth, MN, USA).

2.3. MTT assay for assessment of cytotoxicity

The cytotoxic potential of juglone was quantified using MTT assay. Briefly, 5×10^3 cells per well were seeded in 96-well plates and incubated. Twenty-four hours later, cells were treated with juglone within a range of 0–20 μM for 1, 24 and 48 h. After treatment, cells were washed with PBS and incubated a further 4 h with 100 μl (1 mg/ml) of MTT. The formazan crystals formed were dissolved in DMSO (100 μl) and absorbance measured at 540 nm using a microplate spectrophotometer system (Biotek ELx 800, USA). Each treatment was completed in quadruplicate (with each experiment being repeated at least twice) and IC_{50} (concentration of drug that inhibits cell growth by 50%) values were determined from the concentration versus percent viability curve.

2.4. Lactate dehydrogenase (LDH) leakage assay

LDH released in the medium was measured according to the procedure of Wroblewski and Ladue (1955). Briefly, cells were treated with juglone (0–20 μM) for 1 h and the medium from cell culture flasks of control and treated groups was collected. The media were centrifuged and 50 μl of the supernatant transferred to individual tubes containing Tris–EDTA–NADH buffer, followed by 10 min incubation at 37 °C and the addition of pyruvate solution. Absorbance was read at 339 nm using a UV–Vis spectrophotometer (UV-260, Shimadzu Corp, Tokyo, Japan) and the data were expressed as units/liter (U/l).

2.5. Clonogenic assay

Clonogenic cell death was measured using colony-forming assay according to the method of Puck and Marcus (1955). Briefly, exponentially growing cells were treated with juglone (0–20 μM) for 1 h. After treatment, appropriate number of cells was seeded into culture petri dishes in triplicate and left undisturbed for 10–12 days for colony formation. After fixation, the colonies were stained with crystal violet and the viable colonies containing 50+ cells were counted, from which the fraction surviving was calculated (Satish Rao et al., 2009).

2.6. Micronucleus assay for assessment of genotoxicity

Micronucleus assay was performed as by Fenech and Morley (1985), with minor modifications (Rao et al., 2006). Briefly, exponentially growing cells were treated with juglone for 1 h, after which the medium was replaced with fresh medium containing cytochalasin-B (final concentration 3 μg/ml). After incubation for 36 h, the cells were harvested with mild trypsin treatment (0.1%) and centrifuged (1000 rpm for 5 min). The supernatant was discarded and the resuspended