Chemico-Biological Interactions 279 (2018) 1–9

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Galangin induces cell death by modulating the expression of glyoxalase-1 and Nrf-2 in HeLa cells

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ARTICLE INFO

Keywords:
Glyoxalase-1
Nrf-2
Galangin
Apoptosis

ABSTRACT

The present study was designed to understand the anticancer property and molecular mechanisms associated with chemo preventive effects of galangin. The anticancer effect was evaluated *in vitro* using human cervical cancer cell line (HeLa). Galangin was found to be effective in inducing cell death and inhibiting proliferation & migration significantly. The inhibitory effect of galangin could be correlated with the increase in ROS production & induction of apoptosis. Besides this the activity of glyoxalase-1, an enzyme important for the detoxification of cytotoxic metabolite methy glyoxal and Nrf-2 (a trascription factor), involved in redox signalling were found to be decreased. We concluded that galangin exerts its chemo preventive effect via redox signalling by inhibiting glyoxalase-1 & increasing oxidative & carbonyl stress.

1. Introduction

A number of studies have shown that glyoxalase system can be an important molecular target for cancer prevention and treatment as elevated level of glyoxalase-1 has been observed in many tumor tissues and cancer cells [1–6]. Glyoxalase system is a defence pathway against dicarbonyl stress produced by methylglyoxal (MG) in actively dividing cells. The detoxification system consists of two enzymes, glyoxalase-1 and glyoxalase-2 and catalytic amount of cofactor, reduced glutathione (GSH). It converts toxic methylglyoxal, produced by the spontaneous degradation of triose phosphate intermediate in glycolysis, to nontoxic D-lactate [7]. Under conditions of high glycolytic flux, a condition encountered by all cancer cells, the formation of MG is increased which is counter balanced by Glyoxalase system & gives these cells an adaptive advantage [3]. Over expression of glyoxalase-1has been associated with resistance to various therapeutic treatments also. Although pharmacological inhibition of glyoxalase system can be an important strategy in cancer therapy yet due to toxicity of chemical inhibitors their clinical use has been elusive [8–10]. Phyto chemicals present in various plants especially the ones which are consumed by humans as part of their diet have been gaining attention for the past few decades as sources for various drugs. The important features being time tested usage and low toxicity. Therefore, in the present study we have evaluated the chemo preventive effect of Galangin, an active principle of galangal,a spice used in Asia & an important ingredient of many traditional medicines. It is an important flavone present in honey, propolis, *Helichrysum* & *Alpinia officinarium*. It has been reported to have many health promoting benefits ranging from free radicals scavenging to potential cancer preventive effects [11,12]. *In vitro* and *in vivo* data indicate that galangin has antioxidant properties and is capable of suppressing the genotoxicity of chemicals by modulating the activity of a number of enzymes [13]. It has also been shown to produce synergestics effects with other anticancer drugs to induce apoptosis and inhibit proliferation in various cancer cells [14].

Despite various biological effects galangin is one of the less explored flavonoids and mechanism of action has not been studied in detail. In the present work we have tried to explore the role of glyoxalase-1 in cell death induced by this phytochemical.

2. Methods and materials

2.1. Chemicals and antibodies

RPMI-1640, FBS and antibiotics were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai- 400086, India. Galangin, Bovine Serum Albumin (BSA), Ethidium bromide (EtBr), Acridine orange (AO), Triton X-100, and 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Primary mice β-actin, primary rabbit Nrf-2, primary rabbit Glo-1 and secondary anti-mice Ig-G-HRP and anti-rabbit Ig-G-HRP were purchased from Santa Cruz Biotechnology, Texas, USA. All other chemicals used were of molecular biology grade.

Human cervical cancer cell line (HeLa) were grown as monolayer in RPMI-1640 medium, supplemented with 10% (v/v) FBS, antibiotics

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https://doi.org/10.1016/j.cbi.2017.11.001
Received 12 September 2017; Received in revised form 11 October 2017; Accepted 1 November 2017
Available online 04 November 2017
0009-2797/

(penicillin 100 U/mL, streptomycin 10 μg/mL) and 1 mmol/L sodium pyruvate under standard conditions, temperature (37 °C) in a controlled humidified atmosphere containing 5% CO_2.

2.2. Cell proliferation

For evaluation of cell proliferation MTT assay was used. In brief, HeLa cells (5000 cells/well) were seeded in a 96-well plate and kept overnight. The very next day fresh medium having different concentrations of galangin (25–100 μM) was added to the wells. The cells were allowed to grow for another 24 h. Four hours before completion of incubation, 20 μl of MTT (5 mg/ml) was added in each well. Media was removed after completion of incubation and 200 μl of DMSO was added to each well. The cells were again incubated for another 5–10 min at room temperature. The purple colour thus developed was measured at 595 nm using Bio-Rad micro plate reader. The % of viability was calculated using following formula:

$$\% \text{ viability} = \frac{(Mean\ OD\ of\ treated\ cells)}{Mean\ OD\ of\ untreated\ cells\ (control)} \times 100$$

2.3. Morphological changes

To study changes in morphology 5×10^5 cells/well were seeded in the 6 well plates and kept overnight. After attachment the cells were treated with different concentrations of galangin (25–100 μM). 24 hr post galangin treatment, the images of cells were captured under the microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.4. Clonogenic assay

Fixed numbers of cells (1000 cells/well) were seeded in six well plates having 2 ml of complete RPMI- 1640 media containing different concentrations of galangin. The plates were then incubated in CO_2 incubator at 37 °C for one week and media was changed twice a week. After one week the cells were washed twice with PBS and then fixed and stained with crystal violet solution. The excess stain was washed and colonies were observed with naked eye.

2.5. Wound healing assay

5×10^5 cells were seeded in 6 well plate and when cells became 80% confluent a scratch was made with 200 μl tip in each well of plate and media replaced with the galangin (25–100 μM) containing media. The changes in the scratched portion were noted at 0 h, 12 h and 24 h under microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.6. Micro drop migration assay

Cells were trypsinized, washed, counted and suspended in media (RPMI having 0.3% agrose). 40000 cells/2 μl were added to each well in duplicate in 96 well plate and then 100 μl of fresh media having different concentration of galangin was added. After 24 h of incubation the images were captured under microscope (Nikon ECLIPSE Ti-S, Tokyo, Japan), at 10X magnification.

2.7. Total carbonyl content

Total carbonyl content was measured as per Dalle-Donne et al. [22], with some modification. In brief cells were trypsinized after 24 h of galangin treatment, washed and suspended in PBS (1×10^6 cells/100 μl in PBS). Suspended cells were sonicated (Barson) for 40 s at 25% amplitude. After sonication the proteins were precipitated with cold trichloroacetic acid (TCA, 15% final concentration). The precipitate was collected by centrifugation. A solution of 10 mM DNPH (Dinitrophenyl hydrazine) in 2 N HCl was added to the protein pellet of each sample. 2 N HCl was added to corresponding sample aliquot reagent blanks. Samples were allowed to stand in the dark at room temperature for 1 h with vortexing every 10 min; they were then precipitated with 15% TCA (final concentration) and centrifuged for 5 min. The supernatants were discarded; the protein pellets washed once more with 15% TCA, and further washed three times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Samples were then suspended in 6 M guanidine hydrochloride (dissolved in 2 N HCl) at 370 °C for 15 min with vortex mixing. Carbonyl contents was determined at 366 nm spectrophotometarically.

2.8. Detection of intracellular ROS levels

The intracellular ROS (Reactive oxygen species) generated by galangin in HeLa cells was measured using the 2′,7′-dichlorofluorescein-diacetate (DCFHDA) dye. For measuring the intracellular ROS in individual cells, 5×10^5 cells were seeded on cover-slips in a 6-well plate and incubated overnight for attachment. Next day cells were treated with fresh medium containing (25–100 μM) galangin. The cells were further incubated at 37 °C for 4 h. At the end of incubation, cover-slip was removed from the culture plate and stained with 40 μM 2′,7′-dichlorofluorescein-diacetate (DCFHDA) dye in PBS for 30 min at RT. Excess dye was removed by washing with 1× PBS. Cover-slip was mounted on glass slide and observed under a fluorescence microscope (Nikon ECLIPSE TiE, Tokyo, Japan) at 10 X magnification.

2.9. Apoptosis assay

Apoptosis in HeLa cells was detected by EtBr/AO staining solution. In brief cell suspension was mixed with EtBr/AO staining (1 mg/ml of AO in PBS + 1 mg/ml of EtBr in PBS) for 2 min. The cells were visualized under fluorescence microscope (Nikon ECLIPSE TiE, Tokyo, Japan) at 10 X magnification. The live, apoptotic and dead cells were differentiated on the basis of emitted fluorescence in merged image, that was green, orange and red respectively.

2.10. Glyoxalase-1 activity

Cytosolic fractions were prepared by lysing the cells in phoaphate buffer containing 1 mM EDTA by freez/thawing and sonication, This was followed by centrifugation at 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction. The glyoxalase-1 assay was performed using 0.2% MG and 2% glutathione in 0.1 M phosphate buffer (pH 6.8). Change in absorption was recorded at 240 nm for 2 min attributable to the formation of S-D-lactoylglutathione.

2.11. Immunofluorescence

For immunofluorescence studies the HeLa cells were grown on coverslips in 6 well plate with galangin for 24 h. Cells were then fixed with 4% formaldehyde in PBS for 15 min. After being washed with PBS and blocked with 1% BSA in 0.1% PBST, the cells were incubated with antibodies at 4 °C overnight. After being washed three times with PBST, the cells were incubated with Alexa Fluor 488–conjugated secondary antibodies for 1 h at room temperature and counterstained with DAPI. Antibody–antigen complexes were detected under a fluorescence microscope (Nikon ECLIPSE Ti, Tokyo, Japan), using a 60X objective lens.

2.12. Western blotting

Equal no of cells were seeded in 100 mm plates, when cells reached 80% confluence; they were treated with different concentrations of galangin. After 24 h of treatment, cells were trypsinized and washed with PBS and protein was extracted in RIPA (radioimmuno