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Carnosic acid induces proteasomal degradation of Cyclin B1, RB and SOX2 along with cell growth arrest and apoptosis in GBM cells

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ABSTRACT

Background: Carnosic acid (CA) is a diterpenoid found in *Rosmarinus officinalis* L. and *Salvia officinalis* L. as well as in many other *Lamiaceae*. This compound is reported to have antioxidant and antimicrobial properties. In addition, a number of reports showed that CA has a cytotoxic activity toward several cancer cell lines.

Purpose: The aim of this study was to establish whether CA has any specific antiproliferative effect toward human glioblastoma (GBM) cells and to analyze the molecular mechanisms involved.

Methods: We evaluated cell survival by MTT assay, apoptosis and DNA content by flow cytometry, protein expression and phosphorylation by immunoblot analyses.

Results: Our results showed that CA inhibited cell survival on both normal astrocytes and GBM cells. In GBM cells, in particular, CA caused an early G2 block, a reduction in the percentage of cells expressing Ki67, an enhanced expression of $p21^{WAF}$ and induced apoptosis. Furthermore, we showed that CA promoted proteasomal degradation of several substrate proteins, including Cyclin B1, retinoblastoma (RB), SOX2, and glial fibrillary acid protein (GFAP), whereas MYC levels were not modified. In addition, CA dramatically reduced the activity of CDKs.

Conclusion: In conclusion, our findings strongly suggest that CA promotes a profound deregulation of cell cycle control and reduces the survival of GBM cells via proteasome-mediated degradation of Cyclin B1, RB and SOX2.

Introduction

Carnosic acid (CA) is a bioactive molecule belonging to the natural diterpenes (Birtic et al. 2015). It is found in the *Lamiaceae* plants and in particular in the rosemary (*Rosmarinus officinalis*) and common sage (*Salvia officinalis*) dried leaves, in which its concentration reaches 2.5%. CA is increasingly used as dietary antioxidant and food preservative as well as in cosmetic preparations (Birtic et al. 2015). However, CA exhibit *in vitro* and *in vivo* growth inhibitory and/or cytotoxic activities toward several cancer cell lines derived from multiple solid tumors and leukemic cells (Barni et al. 2012; Einbond et al. 2012; Kar et al. 2012; Min et al. 2014; Wang et al. 2015; Yesil-Celiktas et al. 2010). Therefore, some reports indicated CA as a powerful chemotherapeutic agent for cancer prevention (Ngo et al. 2011) and treatment (Barni et al. 2012). CA has the ability to modulate several molecular pathways in different cell types. Indeed, in kidney cancer cells CA increased the production of reactive oxygen species (ROS) thus promoting ER stress and enhancement of TRAIL-mediated apoptosis (Min et al. 2014). In addition, CA induced autophagy in hepatocarcinoma cells through inhibition of the AKT/mTOR pathway while in prostate carcinoma cells it induced apoptosis by modulating the Akt/IKK/NF-kB signaling (Kar et al. 2012). CA also modulated the AKT signaling in myeloid leukemia cells where it induced P27 expression and a block in the G1 phase of the cell cycle (Steiner et al. 2001). In colon adenocarcinoma cells, CA reduced leptin signaling through the inhibition of the phosphorylation of AKT, ERK and IR, which caused a reduced expression of both BCL-XL and Cyclin D1 resulting in cell cycle arrest (Kim et al. 2014).

Abbreviations: CA, carnosic Acid; CDKs, cyclin dependent kinases; CHX, cycloheximide; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GBM, glioblastoma; LCC, lactacystin; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NA, normal astrocytes; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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To our knowledge, there is no information on the effects of CA on glioma or glioblastoma (GBM) cells. The purpose of our study was to investigate whether CA has any preferential cytotoxic effect on GBM cells over normal astrocytes and which molecular mechanism/s this compound activates.

Materials and methods

Cell cultures

Long-term GBM cultures were established as previously reported (Gangemi et al. 2009) from surgical samples of tumors provided by the Neurosurgery Department of the San Martino Hospital in Genoa. A written informed consent was obtained from all patients before surgery, as prescribed by the Hospital Ethic Board.

Cells were grown as a monolayer on Matrigel (BD Bioscience, Franklin Lakes, NJ) coated dishes as previously described (Gangemi et al. 2009) or in suspension conditions in regular petri dishes.

The U251MG GBM cell line was obtained from the IRCCS AOU San Martino – IST Cell factory. These cells were grown in adherent condition on regular cell culture petri dishes using the medium MEM with Earle's Balanced Salt Solution (EBSS) + 10% FBS + 2 mM L-Glutamine + 1% non-essential amino acids + 1 mM Na pyruvate (Life Technologies Ltd).

Normal human astrocytes (NA) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured following the manufacturer's instructions.

Chemicals

CA was obtained from the dichloromethane rinse extract of the fresh aerial parts of *Salvia officinalis* (1.15 kg) following a procedure previously described for other *Salvia* species (Bisio et al. 2015). Details of the procedure are described in S1 text. Dimethyl sulfoxide (DMSO), cycloheximide (CHX) was acquired from Sigma–Aldrich while lactacystin (LCC) was purchased from Calbiochem. Control cultures were exposed to growth medium containing an amount of DMSO equal to the experimental ones.

Cell survival

Cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as previously reported (Denizot and Lang 1986).

Flow cytometric analysis

Cells were harvested with their media to ensure collection of floating cells along with adherent cells and centrifuged at 980 g for 5 min.

To measure the DNA content in cell nuclei, each sample was stained with DAPI as described before (Monticone et al. 2009). The extent of apoptosis was also performed as previously reported (Monticone et al. 2009)

Immunoblot analysis

Protein cell extracts and SDS polyacrylamide gel electrophoresis were performed using standard protocols (Harlow and Lane 2003). Horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse IgGs and the SuperSignal west Pico Chemiluminescent substrate (Thermo Scientific) were used for immunodetection. Chemiluminescent signals were either collected by exposure of an X-ray film or by using C-Digit blot scanner (LI-COR Biosciences Inc., Lincoln, NE). Blot image and signal intensity were generated and measured by using the GS-800 Calibrated Imaging Densitometer and the Quantity One Software (BioRad, Hercules, CA) and the Image Studio software (LI-COR Biosciences Inc.), respectively.

Immunoblots were stripped with the Restore™ Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's instruction and absence of signal was assessed before proceeding to further antibody incubation of the membrane.

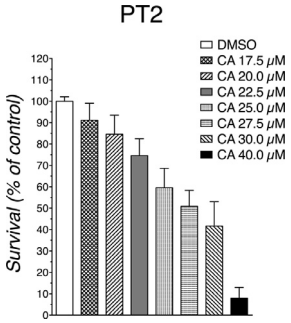

Fig. 1. CA induces a concentration-dependent decrease of cell survival in GBM cells. GBM cells obtained from patient 2 (PT2) were incubated for 48 h in growth medium containing CA at the final concentration indicated. The *Y* axis shows the fraction (in percentage, %) of cell survived after exposure to CA compared to DMSO control cultures. Mean values and standard deviation (indicated as vertical bars) from three independent experiments are shown.

Immunofluorescence analysis

Cells grown as monolayer were fixed, permeabilized, and incubated with primary and secondary antibody using protocols detailed elsewhere (Tonachini et al. 2004).

Antibodies

Table S1 shows the list of the antibodies used in this study.

RNA extraction and real-time quantitative RT-PCR (qPCR) analysis

These methods are described in the S1 Text.

Statistical analysis

All parameters measured are presented as mean ± standard deviation and were analyzed with the Student's *t*-test using a two-tailed distribution. P values < 0.05 were considered statistically significant.

Results

CA inhibits GBM and normal astrocytes cells growth in a dose-dependent fashion

Cytotoxic activity of CA in terms of cell death of glioblastoma (GBM) cells (obtained from patient 2, PT2 cells), was analyzed by MTT assay. We incubated these cultures with doses of CA ranging between 17.5 and 40 μM or with DMSO (used as diluent control) for 48 h. CA reduced the number of PT2 cells present in cultures in a concentration-dependent manner (Fig. 1) with 50% inhibition of cell survival at a CA concentration of 27.5 μM for both PT2 and NA cells (Figs. 1 and S1). Similar results were obtained with GBM cells obtained from other two patients and U251 MG cell line (data not shown). Based on these results, we used GBM PT2 cells as cell model system and 27.5 μM CA for 48 h, for all subsequent experiments, unless otherwise stated.