

Artichoke compound cynarin differentially affects the survival, growth, and stress response of normal, immortalized, and cancerous human cells

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Abstract: Cynarin (CYN) is the main derivative of caffeoylquinic acid, found in leaves and heads of artichoke. It may have hepatoprotective, antiatherosclerotic, antioxidative, choleric, and cholesterol-lowering effects. We tested the effects of various doses of CYN on the proliferative potential, survival, morphology, and stress response (SR) markers heme oxygenase-1 (HO-1) and heat shock protein-70 (HSP70) in normal human skin fibroblasts (FSF-1), telomerase-immortalized mesenchymal stem cells (hTERT-MSC), and cervical cancer cells (HeLa). The effects of CYN on cell proliferation and morphology were dose- and cell type-dependent, with 500 µM CYN as the upper limit for all cell types. While the growth and proliferation of cells decreased after exposure to 75 µM CYN for 3 days, overall survival of FSF-1 and hTERT-MSC was higher than that of HeLa cells. Furthermore, CYN induced the oxidative SR marker HO-1 in both fibroblasts and stem cells in a biphasic manner. A slight induction of HSP70 was observed only in the stem cells. Thus, CYN may be useful for protection against the growth and survival of potentially cancerous cells and may promote longevity of normal cells by inducing SR proteins. Further advanced research related to CYN and artichoke is recommended.

Key words: Cynarin, artichoke, skin fibroblasts, bone marrow, mesenchymal stem cells, HeLa, stress response

1. Introduction

A healthy lifestyle incorporating a balanced diet is a cornerstone for the maintenance and improvement of health and longevity, and for the prevention of various diseases. Several phytochemicals of fruits, spices, vegetables, and other plants have been identified as modulators of lifestyle-related diseases due to their beneficial health effects (Argyropoulou et al., 2013; Özkan and Erdoğan, 2013). Artichoke (*Cynara scolymus*) is widely consumed in the Mediterranean diet and may have hepatoprotective, choleric, cholesterol-lowering, bile-expelling, diuretic, immunomodulating, antiatherosclerotic, anti-HIV, antibacterial, anticarcinogenic, and antioxidative properties (Ernst and Pittler, 2000; Barnes et al., 2007; Ebadi, 2007). The therapeutic effects of artichoke are often ascribed to one of its constituents, cynarin (CYN) (Alonso et al., 2006; Lattanzio et al., 2009; Gouveia and Castilho, 2012). CYN is a dicaffeoylquinic acid derivative, 1,3-O-dicaffeoylquinic acid, found in both leaves and heads of artichoke and in methanolic extracts of artichoke at about the 1.5% level (Adzet and Puigmacia, 1985; Lattanzio et al., 2009; Negro et al., 2012).

In the present study we tested the effects of various doses of CYN on the proliferative potential, survival, and

morphological changes in normal human skin fibroblasts, mesenchymal stem cells, and cervical cancer cells in order to find out whether CYN can suppress proliferation of cancer cells with or without harmful effects on normal cells. In addition, we also investigated whether CYN's mode of action involves stress-induced activation of antioxidative defense pathways in the cells. This is because various phytochemicals containing polyphenols and flavonoids are known to act as mild stressors that stimulate kinases and transcription factors, which then induce the expression of numerous genes that encode antioxidant enzymes, phase-2 enzymes, and protein chaperones (Son et al., 2008; Lee et al., 2014).

Stress response is defined as the response of cells, tissues, and organisms to any physical, chemical, or biological factor, initiating a series of biological events that facilitate and promote counteraction, adaptation, and survival. Whereas an acute stress response can be harmful, a regulated stress response to low levels of stress can enhance survival and longevity, a phenomenon known as hormesis (Rattan, 2005, 2008; Rattan et al., 2013). Among the different types of hormetic agents or hormetins, nutritional hormetins have drawn much attention for their beneficial health effects (Rattan, 2013). Several dietary

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components such as resveratrol, curcumin, capsaicin, epicatechins, isothiocyanates, and some vitamins and minerals are reported to be potential nutritional hormetins (Hayes, 2007; Mattson, 2008; Son et al., 2008). In the present study we investigated whether CYN is a potential hormetin that induces an oxidative stress response by stimulating the synthesis of the stress response proteins heme oxygenase-1 (HO-1) and heat shock protein-70 (HSP70 or HSP1A).

2. Materials and methods

2.1. Chemicals

The following chemicals were used in the study: bovine serum albumin (BSA; Sigma #A4503), β -actin (Sigma-Aldrich A5441), cynarin (Sigma-Aldrich #SLBB9809V), Dulbecco's minimum essential medium (DMEM; BioWhittaker #BE12-604F), DMSO (BioWhittaker #BE17-512F), Dulbecco's phosphate without Ca and Mg (DPBS; BioWhittaker #BE17-512F), ethanol (CCS Healthcare AB #1680643), fetal bovine serum (FBS; Thermo Scientific #SV30143.03), Giemsa's azure eosin methylene blue solution (Bie&Berntsen #1.09204.0500), HO-1 (Enzo LifeSciences #OSA-110), HSP70 (Stressgen #SPA-810), mouse-HRP (Dako #P0447), methanol (VWR #20837.320), thiazolyl blue tetrazolium bromide) (MTT; Sigma-Aldrich #M2128-1G), penicillin/streptomycin (BioWhittaker #DE17-602E), phenylmethylsulfonyl fluoride (PMSF; Sigma #P7626), rabbit-HRP (Dako #P0399), and Triton X-100 (MP Biomedicals #194834).

2.2. Cell lines

We used 3 types of human cells in this study: 1) a normal diploid human facial skin fibroblast cell strain, designated FSF-1, derived from an eyelid reduction of a healthy middle-aged French woman (Jørgensen et al., 2014); 2) normal human mesenchymal stem cells isolated from bone marrow and immortalized with a catalytic subunit of telomerase with reverse transcriptase activity, designated hTERT-MSC (Simonsen et al., 2002); and 3) human cervical cancer cell line HeLa from an aggressive adenocarcinoma of the cervix (Lucey et al., 2009).

2.3. Cell culturing

All cell types were grown in T75 (75 m²) plastic tissue culture flasks with 12.5 mL of complete DMEM containing L-glutamine and glucose and supplemented with 10% FBS and 100 U/mL penicillin and streptomycin. The cells were incubated at 37 °C, 5% CO₂, 95% atmospheric air, and 95% humidity.

2.4. Cynarin solution

CYN (1,3-O-dicaffeoylquinic acid) was bought from Sigma-Aldrich as 5 mg of powder and stored at 4 °C. The stock solution of CYN (9.86 mM) was prepared by dissolving 5 mg of CYN in 1 mL of methanol followed

by appropriate dilution in the culture medium. CYN was added to the cell culture medium at various concentrations as described below.

2.5. Cell proliferative potential and survival

Cells were seeded in 6-well plates with 70,000 cells per well (~7400 cells/cm²) in normal medium and allowed to attach for 24 h, following which various concentrations of CYN were added. The effects of different concentrations of CYN on cellular growth rate and survival were recorded for 3 days by counting the number of cells, using a Countess automated cell counter. Cells were counted at least twice and the mean value of the readings was calculated.

2.6. MTT assay for toxicity

In order to determine the toxicity of CYN, the mitochondrial activity was measured by an MTT assay as an indicator of cell viability. The cytotoxicity index was calculated according to the following formula (Atasever et al., 2003):

$$\text{Cytotoxicity index (\%)} = \frac{(1 - \text{mean value of related concentration absorbance})}{\text{Mean value of control absorbance}} \times 100$$

2.7. Giemsa staining for morphological analysis

Cells were seeded in 6-well plates with 70,000 cells per well (~7400 cells/cm²). Following 24 h of attachment, the medium was treated with various doses of CYN. After 3 days of treatment, the cells were washed twice with DPBS, fixed with 100% ice-cold methanol, and incubated for 20 min at -18 °C. The ethanol was then removed and 1 mL of Giemsa stain solution (pH 6.1-7.0) was added per well. The cells were incubated for 30 min at room temperature and washed 6 times with dH₂O, and the plates were then left to dry overnight.

2.8. Microphotography

The morphology of Giemsa-stained cells and living cells was examined and photographed with a phase-contrast microscope (Zeiss Axiovert 25). Pictures were taken with either a 2.5 \times objective or a 10 \times objective, and the phase-contrast settings were manually manipulated to enhance visibility. In addition, all pictures were taken with the highest possible resolution (1300 \times 1030 pixels) and scale bars were added for each magnification.

2.9. Western blotting

Cells were treated with different concentrations of CYN for the indicated times and then were lysed in ice-cold lysis buffer (Tris HCl, PMSF, Triton X-100, protease inhibitor). Bradford analysis was performed to measure the protein content of the samples using

BSA standards (0.05-0.5 mg/mL). For western blot, 20 μ L of protein sample was loaded in 12% Bis-Tris SDS polyacrylamide gel, electrophoresis was conducted, and the gel was transferred to an equilibrated nitrocellulose

membrane (Bio-Rad). The membrane was then blocked with PBS containing 4% weight/volume nonfat dry milk, washed in PBS, and incubated with primary antibody and secondary antibody conjugated with IgG horseradish peroxidase. Immunoreactive bands were detected using the chemiluminescent compound ECL (GE-Healthcare). The film (Kodak Medical Film) was developed in a dark room with an AGFA Curix 60. β -Actin was used as a loading control. The results were quantified using the image-analysis program ImageJ.

2.10. Statistical analysis

Statistical differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test or Kruskal-Wallis test when appropriate. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. CYN modulates cellular morphology

The treatment of FSF-1, hTERT-MSC, and HeLa cells with various concentrations of CYN resulted in subtle changes in morphology depending on the dose (Figure 1). Normal cells generally maintained their morphological integrity at almost all concentrations of CYN during the 3-day period of exposure. However, cancerous HeLa cells started to become enlarged from about 25 μM onwards, with clear-cut morphological alterations at higher doses. This was an early indication of the differential effects of CYN on normal and cancerous cells. Therefore, this effect was further investigated by determining the extent of proliferation and mitochondrial activity as an indicator of toxicity (Kasımoğulları et al., 2014).

3.2. Effects of CYN on proliferation, survival, and toxicity

Both FSF-1 and hTERT-MSC cells showed a biphasic dose response to CYN with respect to growth and survival. Figure 2 shows that for the first 3 days, the growth of FSF-1 cells was: 1) stimulated by CYN concentrations between 1 μM and 50 μM ; 2) similar to the controls until 250 μM ; and 3) almost completely inhibited at 500 μM ($P < 0.05$). A similar biphasic pattern of the effects of CYN on the growth of FSF-1 cells was seen over a 6-day period, but the inhibitory effects of CYN at concentrations above 125 μM became more obvious and significant ($P < 0.05$). Figure 3 shows the effects of CYN on the growth of hTERT-MSC cells, which was either maintained or stimulated as compared with the controls until about 75 μM , following which it became progressively inhibitory at higher concentrations during the 5-day period of treatment. Thus, there was a difference between normal aging cells and immortalized nonaging cells with respect to their tolerance of CYN: whereas normal human cells could tolerate higher concentrations of CYN, immortal cells were more sensitive to the inhibitory effects of CYN.

In the case of cancerous HeLa cells, CYN concentrations above 50 μM were growth-inhibitory even within 3 days of treatment (Figure 4). The toxic effects of CYN on HeLa cells were further confirmed by MTT-based mitochondrial activity, which generally supported the above observations (data not shown).

3.3. Stress response induction

We tested 2 stress response pathways, antioxidant response and heat shock response, following CYN treatment of FSF-1 cells and hTERT-MSC. Figures 5 and 6 show the western blot pictures and the quantitative data derived from the immunoblots for the levels of HO-1. Whereas FSF-1 cells treated with 100 μM CYN had about 50% higher levels of HO-1 (Figure 5), the same extent of increase in HO-1 levels in hTERT-MSC was observed at 50 μM (Figure 6). This reconfirms the observation that immortalized cells are more sensitive to CYN as compared with normal cells. We also checked the effects of CYN on heat shock response by determining the levels of HSP70 expression. However, there was no induction of HSP70 in FSF-1 cells and only a slight, insignificant induction in hTERT-MSC (data not shown).

4. Discussion

The effects of CYN on cell proliferation were related to dose and cell type. While the growth and proliferation of all cell types were affected from 75 μM onwards, the survival of normal FSF-1 cells and hTERT-MSC was relatively higher than that of cancerous HeLa cells. This implies that normal human cells are able to tolerate CYN better than cancer cells, which were more sensitive to the growth inhibitory effects of CYN. Atasever et al. (2003) reported that CYN had different cytotoxic effects on leukemic cell lines and blasts of patients with acute lymphoblastic leukemia, and the cytotoxicity index of patients with acute lymphoblastic leukemia was $20.45 \pm 12.3\%$. In our study, the cytotoxicity index of CYN on HeLa cells was $65.46 \pm 19.3\%$. Therefore, CYN has more cytotoxic effects on HeLa cells compared with leukemic cells, and the effects of CYN depend on the cell type. However, 500 μM CYN concentrations inhibited the cell proliferation and survival of all cells. Slanina et al. (2001) indicated that CYN did not have cytotoxic effects on HeLa cells until 400 μM , while 250 μM inhibited the growth of MT-2 (human T-cell leukemia) cells. Dong et al. (2009) reported that CYN did not have cytotoxic effects on Jurkat T cells until 1000 μM .

Previous studies have shown that several phytochemicals and endogenous mediators can upregulate HO-1 expression (Son et al., 2013). Our present study has also shown that the artichoke phytochemical CYN induces HO-1 expression in normal FSF-1 cells and in hTERT-MSC cells in a biphasic dose response. Other studies have shown that resveratrol upregulates HO-1 in PC12 rat adrenal

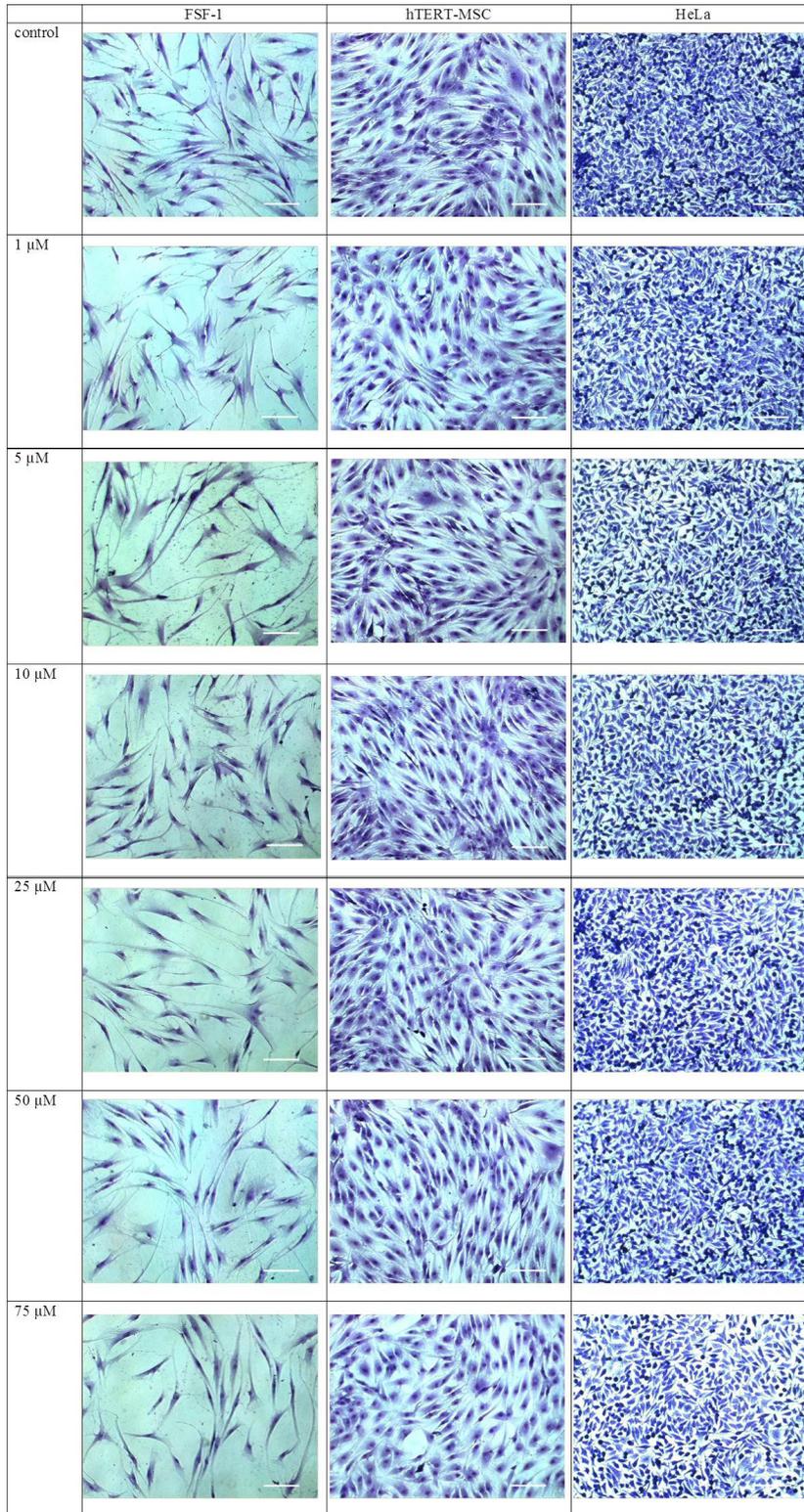


Figure 1. Effects of different concentrations of CYN on the morphology of FSF-1, hTERT-MSC, and HeLa cells following 3 days of treatment (microscopy pictures were taken using a 10 \times objective).

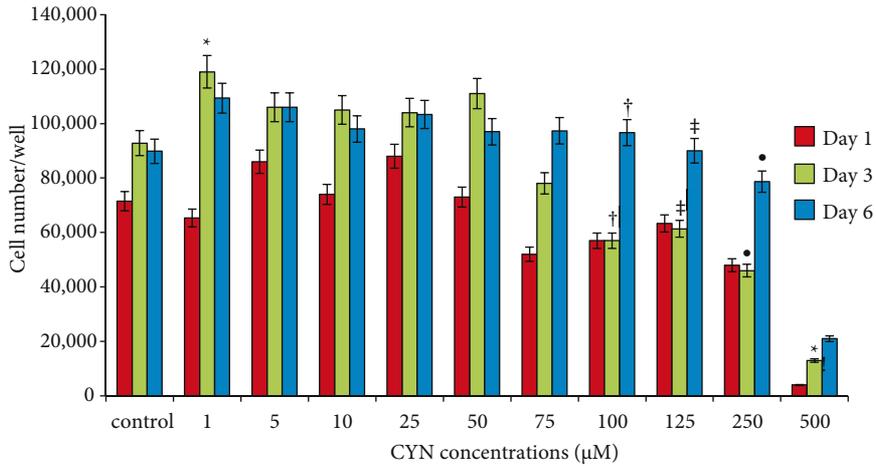


Figure 2. Effects of different concentrations of CYN on the number of normal human skin fibroblasts, FSF-1, during 6 days of treatment (*, †, ‡, •: P < 0.05).

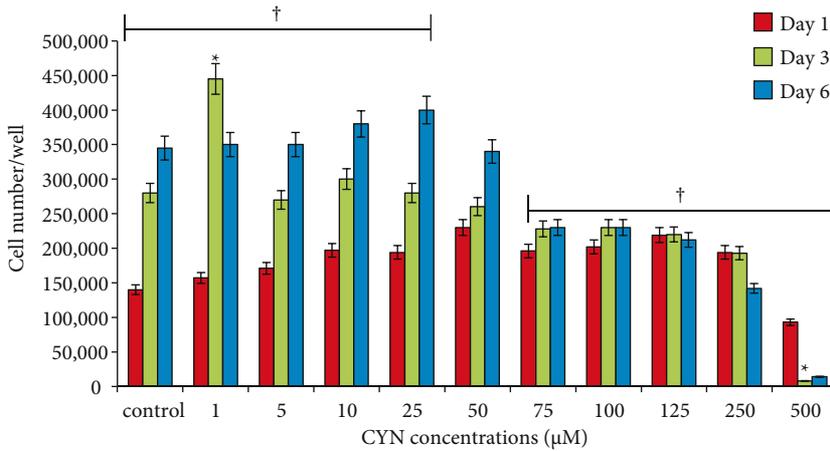


Figure 3. Effects of different concentrations of CYN on the number of telomerase-immortalized bone marrow stem cells, hTERT-MSC, during 5 days of treatment (*, †: P < 0.05).

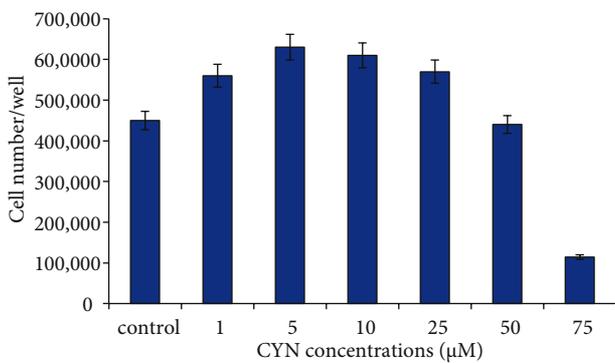


Figure 4. Effects of different concentrations of CYN on the number of cancerous HeLa cells following 3 days of treatment.

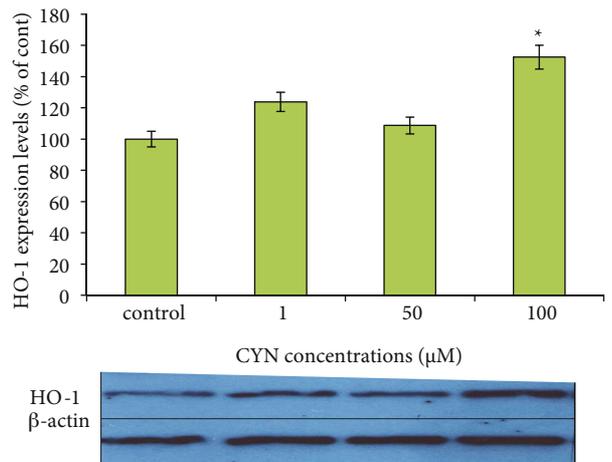


Figure 5. HO-1 expression levels in FSF-1 cells on the 3rd day of CYN treatment (*: P < 0.05).

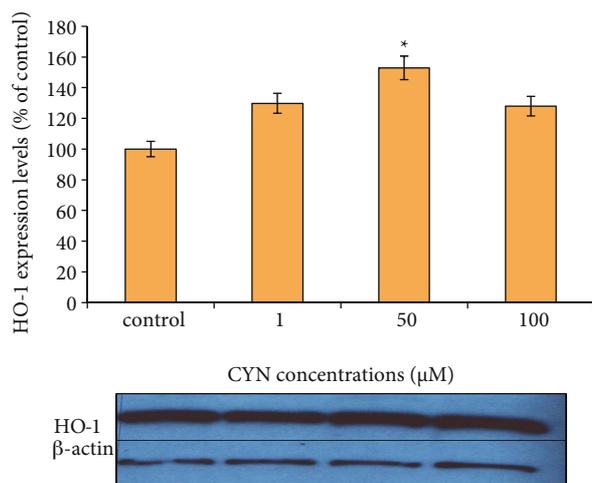


Figure 6. HO-1 expression levels in hTERT-MSC cells on the 3rd day of CYN treatment (*: $P < 0.05$).

medulla cells and human aortic smooth muscle cells in a concentration-dependent manner (Chen et al., 2005; Juan et al., 2005). Moreover, while both epigallocatechin-gallate and epigallocatechin-3-gallate induce the expression of HO-1 in endothelial cells, epigallocatechin-3-gallate upregulates HO-1 in a concentration- and time-dependent manner (Wu et al., 2006; Pullikotil et al., 2012). Additionally, curcumin stimulates the expression of HO-1 in a time- and dose-dependent manner in human skin fibroblasts, astrocytes, and endothelial cells (Scapagnini et al., 2002; Balogun et al., 2003; Rattan et al., 2009).

Some dietary phytochemicals also induce the expression of heat shock proteins. For instance, resveratrol upregulates HSP70 in cell lines and in human peripheral lymphocytes and can synergize with mild to moderate

heat shock to prevent severe heat stress damage (Putics et al., 2008). Curcumin stimulates the expression of the main inducible HSP70 in HeLa cells in a concentration- and time-dependent manner. Additionally, while curcumin cannot induce the expression of HSP70 by itself, it has a synergistic effect with mild heat shock stress in normal human skin fibroblasts and telomerase-immortalized bone marrow stem cells (Dunsmore et al., 2001; Rattan and Ali, 2007; Demirovic and Rattan, 2011). Our study showed that CYN did not induce HSP70 synthesis in FSF-1 cells, but there was a slight induction in hTERT-MSC cells. This requires further investigation.

In conclusion, the effects of CYN on cell proliferation and morphology are dose- and cell type-dependent. The growth and proliferation of normal, immortal, and cancerous cell types were affected differentially by CYN treatment. Whereas normal skin fibroblasts and telomerase-immortalized human mesenchymal stem cells could tolerate CYN without significant changes in morphology and growth, cancerous cells HeLa were strongly inhibited by this treatment. CYN appears to act by inducing the antioxidative HO-1-dependent pathway. Thus, the regular consumption of artichoke, and the presence of CYN in it, may protect against the growth and survival of potentially cancerous cells in the body at an earlier stage. Advanced molecular, epidemiological, and clinical research related to CYN is needed for more specific dietary recommendations.

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