ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 05, pp.16417-16421, May, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

DIETARY ISOTHIOCYANATES AS APOPTOTIC INDUCER FOR HUMAN COLORECTAL CANCER (CACO-2) CELLS

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ARTICLE INFO

Article History:
Received 15th February, 2015
Received in revised form
23rd March, 2015
Accepted 19th April, 2015
Published online 31st May, 2015

Key words:

Isothiocyanates, Apoptosis,
p53, Bcl-2, Caspase-3.

ABSTRACT

Isothiocyanates are naturally occurring small molecules that are formed from glucosinolate precursors of cruciferous vegetables. Many isothiocyanates, both natural and synthetic, display anticarcinogenic activity because they reduce activation of carcinogens and increase their detoxification. Recent studies show that they exhibit anti-tumor activity. Moreover, this work focused on the mitochondrial pathway. Cells were incubated with isothiocyanates concentrations, 50µM/L, 70µM/L, 90µM/L and 120µM/L for 72h at 37°C and 5%CO_2. Isothiocyanates induced cells death in a dose-dependent manner. Isothiocyanates treated cells had an antiproliferative effect, additionally, a typical characteristics of apoptosis as: DNA fragmentation, caspase-3 over expression and fall of mitochondrial membrane potential by Bcl-2 expression down regulating, p53 activation as signal for cell cycle arrest and cytological alterations. Future study will may deal with further investigations of isothiocyanates possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colonrectal cancer type.

***Citation*:** Entissar S. Al-Suhaibani and Nermin A. El-Morshedi, 2015. "Dietary isothiocyanates as apoptotic inducer for human colorectal cancer (CACO-2) cells", *International Journal of Current Research,* 7, (5), 16417-16421.

INTRODUCTION

Numerous studies support the fact that phytochemicals found in certain food substances protect against cancer. Cruciferous vegetables have been widely accepted as potential diet components that may reduce the risk of cancer. Isothiocyanates are abundant in cruciferous vegetables such as broccoli, watercress, Brussels sprouts, cabbage, Japanese radish and cauliflower, and they significantly contribute to the cancer chemo preventive activity of these vegetables (Gamet-Payrastre and Lumeau, 2000). Some isothiocyanates derived from cruciferous vegetables, such as sulforaphane (SFN), phenethyl isothiocyanate (PEITC), and benzyl isothiocyanate (BITC), are highly effective in preventing or reducing the risk of cancer induced by carcinogens in animal models (Ambrosone and Tang, 2009). They also inhibit the growth of various types of cancer cells. These anti-tumorigenic features of isothiocyanates warrant further investigation. The capacity of organic isothiocyanates to block chemical carcinogenesis was first recognized more than 30 years ago with α-naphthyl isothiocyanates. Since then, approximately 20 natural and synthetic isothiocyanates have been shown to inhibit chemically induced carcinogenesis (Traka *et al*., 2008). Over the past decade, there is continuous increase in colorectal carcinoma in the world as the most common malignant diseases. Colorectal cancer is the cause of more than 1/2 million deaths worldwide, and it was ranked as the third leading cause of cancer-related death after lung cancer and stomach cancer (Mayer, 2009). Epidemiological studies have shown strong evidence that diet and lifestyle play an important role in preventing cancer. In particular, an increased consumption of fruits and vegetables is associated with decreasing in cancer onset and mortality (Murillo *et al*., 2008). Programmed cell death or apoptosis plays an important role in normal development and is impaired in many types of cancer. Apoptosis occurs under a variety of physiological and pathological conditions that control the development and homeostasis of multicellular organisms (Kidd, 1998). The major apoptotic pathways can be divided into caspase- and mitochondria- dependent pathways, according to caspase-3 activation which is generally considered to be a key hallmark of apoptosis (Boada *et al*., 2000). Apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by the cysteine protease family called caspase-3 (Sarin *et al*, 1998).

Previously, it was showed that isothiocyanates, altered cell cycle and induced apoptosis against the cells which exposed to

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an oxidative stress (Lynn *et al*., 2006). In order to know if these obtained results could be extended to do anti-tumor activity against colorectal cancer cells, we tested this molecule on colorectal cancer (Caco-2) cells. Moreover, to gain further insight into the mechanisms by which isothiocyanates induces apoptosis in human colorectal cancer cells, we examined in this study the functional status of caspase-3 on apoptosis rate and the effect of isothiocyanates on mitochondrial integrity in human colon cancer (Caco-2).

MATERIALS AND METHODS

Chemical reagents

Isothiocyanates, MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx & E) stain, rabbit polyclonal antibodies against cleaved caspase-3, primary monoclonal antibody against Bcl-2 and against p53, AB reagent, biotinylated immunoglobulin secondary antibody and Tween 20 were purchased from Sigma-Aldrich, Egypt.

Cell line and cell culture

Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as mono-layer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5%CO_2. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

Methods

Cell Viability Assay

In vitro evaluation of antiproliferation effect growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble form azans (Mosmann, 1983). Viable cell number/well is directly proportional to formazans production. 8.25×10^3 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing isothiocyanates at concentrations: 50µM/L, 70µM/L, 90µM/L and 120µM/L for 72h at 37ºC in an incubator with 5%CO_2. After incubation, isothiocyanates modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5%CO_2). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted form azan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage= [1–(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against isothiocyanates concentrations.

Determination of DNA fragmentation by DNA laddering assay

Cells were seeded in 60-mm petri dishes at density $4x10^5$ cells/plate (treated cells by IC_{50} concentration of isothiocyanates or positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, Triton-X100 0.5%) and kept at 4ºC/10min then, lysate was centrifuged at 25.000×g/20min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15min. After drying, DNA was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH 8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide (Zhang, 2003).

Cytological changes investigation: Detached and trypsinized cells (IC_{50} concentration of isothiocyanates treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 10µL of suspension were smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5min before proceeding by Hx & E stain and examined under light microscope (John and Abraham, 1991).

Immunocytochemical investigations

By detection of Bcl-2, p53 and Caspase-3 by immunocytochemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC_{50} concentration of isothiocyanates treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with rabbit polyclonal antibodies against cleaved caspase-3 at 4°C, then AB reagent and substrate-chromogen mixture (30min). Between each step, the slide was washed with washing buffer (PBS) with 0.1% Tween 20). Cells were incubated overnight with primary monoclonal antibody against Bcl-2 and against p53 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used (Yoon *et al*., 2004). The slides were then mounted and examined under light microscope.

Statistical analysis: Results were presented as mean±standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at $P<0.05$ and high significance was set at $P\leq 0.01$ (Snedecor and Cochran, 1980).

RESULTS

Cell viability assay: *In vitro* evaluation of antiproliferation effect

Cytotoxic effect of isothiocyanates concentrations (50µM, 70µM, 90µM and 120µM)/72h on Caco-2 cells was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with isothiocyanates concentrations in a dose dependent manner. All concentrations were found to be high significantly different (P≤0.01) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with isothiocyanates concentration increasing and 95% of cell inhibition was observed in treated cells with 120µM/72h. Cell proliferation reduced about 25% and 35% when cells were treated with 50µM and 70µM for 72h, respectively. Cells proliferation decreased to 55% as treated with concentration 90µM/72h.

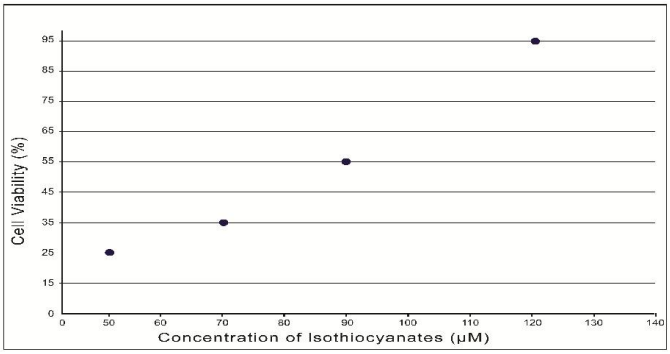

Figure 1. Effect of isothiocyanates with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean±SD, n (for each concentration) = 4]

Determination of DNA fragmentation by DNA laddering assay

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with isothiocyanates concentration at 90µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that isothiocyanates induced DNA fragmentation which was caused by apoptosis.

Cytological changes investigation

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by isothiocyanates concentration at 90µM/72h, apoptotic cells were identified by a series morphological changes as an important experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

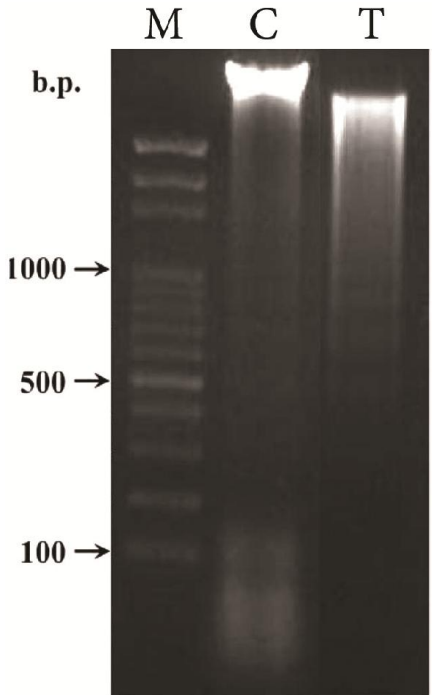

Figure 2. DNA fragmentation by DNA laddering assay of extracted DNA from isothiocyanates treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker

Immunocytochemical investigation

After Coca-2 cells treatment by isothiocyanates concentration at 90µM/72h, the reaction of caspase-3 protein was considered positive (over expression of caspase-3 protein) when over 50% of treated tumor cells had a clear brown cytoplasm staining, with slight degrading in the intensity in the same field (Figures 3f). Specially those fields that had necrotic or apoptotic nucleus as sign for isothiocyanates treatment effect, but fields of positive control cells have negative reaction of caspase-3 (cytoplasm did not show the brownish reaction stain) (Figure 3e). On the other hand regarding to the positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 55% of cells had nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane brown staining, with slight intensity degrading in the same field (Figures 3g). After Coca-2 cells treatment by isothiocyanates concentration at 90µM/72h, those fields that had necrotic or apoptotic nucleus as sign for isothiocyanates apoptotic effect with Bcl-2 negative reaction (faint to non-brown stain) (Figure 3h). Also, when applying p53 stain, p53 protein reaction in the positive control Coca-2 cells, was showed negative reaction (no brown stain) (Figure 3j). Treated Coca-2 cells, those fields had necrotic or apoptotic nucleus for isothiocyanates effect showed p53 positive reaction (over expression of p53 protein) when over 55% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3i).

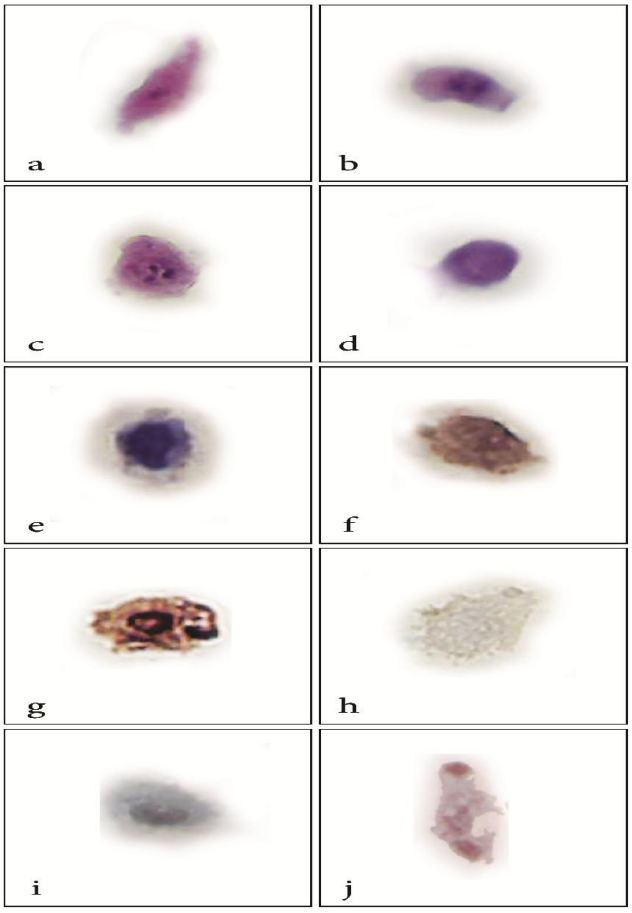

Figure 3. Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (b). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (c). Complete apoptotic cell (d). Immunocytochemistry of caspase-3 protein. Control positive cell showing cytoplasm negative reaction for caspase-3 protein (e). Treated cell showing cytoplasm positive reaction for cytoplasmic caspase-3 protein (f). Control positive cell showing Bcl-2 protein nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane showing brownish positive reaction (g). Treated cell showing negative reaction of apoptotic cell apoptosis (h). Treated cell showing p53 protein nuclear positive reaction (i). Control positive cell showing nuclear negative reaction (j)

DISCUSSION

Consumption of cruciferous vegetables has been associated with a reduced risk in development various types of cancer. This has been attributed to the bioactive hydrolysis products that are derived from these vegetables namely isothiocyanates (Higdon *et al*., 2007). A growing body of evidences from cell and animal models indicates several molecular mechanism of chemoprevention by isothiocyanates, that include modulation of detoxification, regulation of cell growth by induction of apoptosis and cell cycle arrest, induction of ROS-mechanism and regulation androgen receptor pathways (Giovannucci *et al*., 2003). Tumor suppressor protein p53 is a principal factor in regulation of growth arrest as well as apoptosis. Many apoptotic signals are mediated to the cell death machinery via p53. It interacts with other proteins or functions as a transcription factor (Jeffers *et al.,* 2003). Indeed, in response to various types of stress, p53 becomes activated and, as a consequence, cells can undergo marked phenotype changes, ranging from increased DNA repair to senescence and apoptosis (Polyak *et al*., 1997) as that was observed in recent study. It is of great importance to understand the mechanisms of apoptosis in cancer cells, as apoptosis is believed to be one of the major consequences of anticancer drug treatment against malignancies (Vousden, 2002). P53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest and differentiation responses that prevent further proliferation of stressed or damaged cells and so protect from the outgrowth of cells harboring malignant alterations (Polyak *et al*., 1996). P53 has been described as 'The Guardian of the genome', referring to its role in conserving stability by preventing genome mutation (Vogelstein *et al*., 2000).

Upon genotoxic and other stress, p53 protein levels increase. Activated p53 releases signal to cells to undergo growth arrest, cell differentiation or apoptosis (Contente *et al*., 2002). Mitochondria are involved in a variety of key events, including release of caspase-3 activators, changes in electron transport, loss of mitochondrial membrane potential, and participation of both pro- and anti-apoptotic Bcl-2 protein (Adams and Cory, 1998). Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis. Bcl-2 itself is an anti-apoptotic gene that prevents initiation steps of apoptosis and programmed cell death (An *et al*., 2004). Our results showed that isothiocyanates strongly inhibited proliferation of Caco-2 cells and blocked the cell cycle as previously described (Kristal and Lamp, 2002). Caco-2 cells which were treated with isothiocyanates exhibited increased levels of p53 expression at concentration of 90µM/72h, which suggested that p53 involved in isothiocyanates-induced Caco-2 cell death. Bcl-2 family of proteins serves as critical regulators of pathways involved in apoptosis as anti-apoptotic (Adams and Cory, 1998).

Caco-2 cells which was treated with isothiocyanates exhibited reduced levels of Bcl-2 expression. These results suggested that the mitochondrial pathway was involved in isothiocyanates-induced Caco-2 cell death. It is well known that a family of cysteinyl proteases, caspases, is involved in the apoptotic cell death. Caspase-3, one of the active executioners, promotes apoptosis by cleaving cellular substrates such as ICAD (Enari *et al*., 1998). That was agreed in the recent study by caspase-3 expression inhibition. We found that isothiocyanates induced caspase-3 activity in Caco-2 cancer cell line.

Apoptosis, as programmed cell death, is a highly organized cell death process characterized by an early obvious condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of nucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies (Boada *et al*., 2000). DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor (Hengartner, 2000). Apoptosis has specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process (Zamzami *et al*., 1996). That was agreed with the results of recent study after treatment

by isothiocyanates. Isothiocyanates decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells. Therefore, we may presume that as primary mechanism involved in isothiocyanates growth-inhibitory effects as it considered main apoptotic signals. In response to apoptogenic stimuli, the mitochondrial protein apoptosis-inducing factor (AIF) translocates through the outer mitochondrial membrane to the cytosol and to the nucleus, resulting in the induction of nuclear chromatin condensation and large DNA fragmentation in a caspase-independent manner (Daugas *et al*., 2000).

Conclusion

In summary, we demonstrated that isothiocyanates caused an inhibition of cell growth with apoptosis induction by DNA fragmentation and p53 activation in human colon (Caco-2) cancer cell line. Moreover, a large part of our study essentially focused on the mitochondrial pathway and we investigated that isothiocyanates's action was caspase-3 dependent according to its inhibitory effect on Bcl-2 expression. There was also noticeable cytological alterations. These new findings suggest that isothiocyanates -induced effects may have novel therapeutic applications for the treatment of different cancer types. Future *in vitro* and *in vivo* study will may deal with further investigations of the possible usages of isothiocyanates as a new alternative chemotherapeutic agent.

Acknowledgments

We are grateful for all of Nile center for experimental researches team specially Miss. Noha T. Badawy (Department of cell culture) for her kindly support in the cell culture and drug induction stage.

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