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Effect of Sulforaphane and Methotrexate combined treatment on Histone Deacetylase Activity in Solid Ehrlich Carcinoma

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Abstract

Histone acetylation is one of the posttranslational modification that plays a role in the regulation of gene expression, also modulation of acetylation with histone deacetylase inhibitors (HDACi) had been considered a novel strategy for cancer chemoprevention. This study was aimed to investigate the activity of histone deacetylase enzyme (HDAC) in tumor cell model Solid Ehrlich carcinoma under the effect of the antitumor drug methotrexate in combination with sulforaphane (SFN) which is a natural compound found in cruciferous vegetables. In the present study, sulforaphane was extracted from cabbage outer leaves. The HDAC activity significantly increased in tumor-bearing mice, non-significantly decreased after treatment with methotrexate, and significantly decreased after treatment with methotrexate with oral supplement by sulforaphane. However, The DNA damage decreased significantly in tumor tissue of tumor-bearing mice after treatment with methotrexate and increased significantly in tumor tissue of tumor-bearing mice treated with oral sulforaphane in combination with methotrexate treatment after carcinogenesis. In conclusion, sulforaphane has an antitumor effect via its pro-oxidant activity and affects the HDAC activity via its metabolites in an inhibitory manner that enhanced the effect of methotrexate.

Keywords: histone deacetylase (HDAC), sulforaphane (SFN), DNA damage.

1. Introduction

According to recent estimates, cancer continues to remain the second leading cause of death and is becoming the leading one in old age (Donnellan et al., 2017). It is projected that by 2030 the number of new cancer cases will increase by 70% worldwide due to demographic changes alone (Bao et al., 2017). Experimental tumors have great importance for modeling, and one of the commonest is the Ehrlich ascitic tumor, which is derived from a spontaneous

murine mammary adenocarcinoma (Ceylan et al., 2017). Mutation studies have suggested that chronic oxidative stress, particularly from chronic inflammation, is associated with carcinogenesis (Choudhury et al., 2017). In addition to inducing DNA, lipid, and protein damage, oxidative damage to protein-coding or -non-coding RNA may potentially cause errors in protein synthesis or dysregulation of gene expression. This has been proposed as an underlying mechanism of several human diseases (Nunomura, 2016). When the pro-oxidant/anti-oxidant equilibrium is lost, oxidative stress is generated, altering, and damaging many intracellular molecules, including DNA, RNA, lipids, and proteins (Margaritelis et al., 2016). Upon exposure to oxidants or oxidative stress-inducing agents, a common adaptive response induced in mammalian cells is the upregulation of stress-response genes, many of which encode antioxidant defense enzymes. While high levels of ROS production may lead to the induction of apoptosis or necrosis, increasing evidence demonstrates that low or transient ROS exposure increases cell proliferation, likely through altered expression of growth factors and proto-oncogenes (Tharmalingam et al., 2017).

There are two protein families with HDAC activity; Sirtuins and the classical HDAC family (Hsieh et al., 2016, Serrano-Gomez et al., 2016). HDACi function by blocking access to the active site (reversible or irreversible) of HDAC (Lee et al., 2017), later it was discovered that HDACis have potent proliferation-inhibitory properties with cancer cells (Shao et al., 2017).

Isothiocyanates (ITCs) are synthesized from glucosinolates (GSs) stored in plants, upon catalytic breakdown by myrosinase, a thioglucoside glucohydrolase present mainly in the crucifers and released during stress or damage and to some extent in the microflora of the intestinal tract (Li et al., 2017); ITCs rapidly accumulates in the cells, and such accumulation levels may reach 100- to 200-fold over extracellular levels (Sosa et al., 2013). Sulforaphane (SFN) was first isolated from broccoli in the early 1990s as an inducer of phase 2 enzymes (Xenobiotic metabolism) and since then numerous studies have proposed various antineoplastic pharmacological aspects of SFN, thereby suggesting its potential as a promising candidate in cancer chemoprevention (Zhang et al., 2007).

This study was aimed to investigate the activity of histone deacetylase enzyme (HDAC) in tumor cell model Solid Ehrlich carcinoma comparing normal tissue and tumor tissue, under treatment with the anti-tumor drug methotrexate and the natural isothiocyanate sulforaphane alone and in combination with methotrexate.

2. Materials and Methods

2.1. Extraction of Sulforaphane:

Crucifer aqueous extracts were prepared according to the method of Bertelli et al and Dandan Han et al (Bertelli et al., 1998, Han et al., 2011) with few modifications. Briefly, plant material was ground to a fine homogenous powder then 0.5 gram of powdered material was left to autolyze in 7ml HCl pH 3.0 (0.001 N) at 37 C^o overnight to allow complete hydrolysis of the sulphur glycosides. The supernatant was filtrated through a piece of gauze then extracted with 5ml Dichloromethane three times then the dichloromethane fraction was salted with sodium sulphate anhydrous and dehydrated using a rotary evaporator at 30^{o}C. The residue was dissolved in 2ml of 20% v/v acetonitrile and was filtered and injected to solid-phase extraction silica column“10 cm × 2.5 cm silica” that was previously euqilibrated with dichloromethane, the solid-phase extraction silica column then washed with ethyl acetate as a washing solvent that will not remove sulforaphane but the other unnecessary compounds, then dichloromethane was used as the elution solvent in subsequent steps where the largest relative amount of sulforaphane was washed out. The concentration of sulforaphane was determined using UV1102 spectrophotometer. The maximum wave length (λmax) which is unique for sulforaphane is 240 nm and its molar attenuation coefficient ε which is 950 M^{-1} cm^{-1}(Tao et al.). Sulforaphane was detected by GC-MS using Perkin Elmer Clarus

580 Gas chromatograph according to the method described by Matusheski et al (Matusheski et al., 2001), followed by checking the mass spectrum of the extracted sulforaphane in MAINLIB library. More quantities were then extracted, dehydrated by rotary evaporation and dissolved in saline for further animal oral treatment.

2.2. Animals

In this study, 60 albino female mice weighing about 20–25 g were housed in wire mesh cages and were fed standard rat chow and allowed free access to water. They were kept under constant environmental conditions [temperature (23 ±2 ^{o}C), relative humidity (80 ± 5%) and light (12 h light/dark cycles)].

All experiments were carried out according to the guidelines of the ethical committee of the Faculty of Science, Tanta University.

2.3. Solid Ehrlich Carcinoma (SEC) Tumor Model

A model of SEC was used, where 1×10^{6} of the Ehrlich carcinoma cells (ECC) obtained from the Pharmacology and Experimental Oncology Unit of the NCI, Cairo University, Egypt were implanted subcutaneously into the right thigh of the hind limb of mice. A palpable solid tumor mass (about 100mm3) was developed within 12 days.

MST (Median survival time) was monitored by recording the mortality daily for 4 weeks and % ILS (increase in life span percent) was calculated (Tanongkankit et al., 2013).

2.4. Experimental design:

Mice were randomly equally divided into the following groups:

- Group I (Negative control group): This group included 10 untreated mice.

The remaining mice were subjected to Ehrlich tumor cells which were implanted subcutaneously into the right thigh of the hind limb of mice and divided as follow:

- Group II: Tumor-bearing group "Positive control group".
- Group III: Methotrexate "MTX" provided from Sanofi Aventis was intraperitoneally injected once each 3 days of a dose of 1.25 mg/Kg for one month.
- Group IV: 1.5 mg of SFN dissolved in 0.5 ml saline "50 mg/Kg", delivered by gastric tube once daily one day before Ehrlich cells implantation and continued for one month.
- Group V: 1.5 mg of SFN dissolved in saline, delivered by gastric tube once daily one day after Ehrlich cells implantation and continued for one month.
- Group VI (Methotrexate and sulforaphane treated group): 1.5 mg of SFN dissolved in 0.5 ml saline, delivered by gastric tube once daily with intraperitoneal MTX once every 3 days of a dose 1.25 mg/Kg were given one day after subcutaneous implantation of Ehrlich cells and continued for one month.

2.5. Tissue Sampling:

After scarification, Tumors were dissected carefully and washed three times with ice-cold saline to remove extraneous materials, the tumor was chilled on ice and divided into four pieces that were wrapped in aluminum foil and stored at -80 ^{o}C till used for the preparation of tissue homogenates and nuclear extracts (Chastre et al., 1984).

Muscles from the right thigh of the normal group were dissected and washed three times with ice-cold saline, the tissue was chilled on ice and divided into four pieces that were wrapped in aluminum foil and stored at -80 ^{o}C till used for the preparation of tissue homogenates and nuclear extracts (Chastre et al., 1984).

2.6. Preparation of Tumor Nuclear Protein Extracts:

for estimation of total protein content and HDAC activity by using membrane, nuclear, and cytoplasmic protein extraction kit supplied by Biobasic INC. Canada(Ouyang et al., 2009).

2.7. Biochemical assays:

Estimation of protein content in the nuclear extract of tumor tissue according to the method of Lowry et al. (1951) (Lowry et al., 1951); Histone deacetylase activity was determined using colorimetric HDAC activity assay kit supplied by BioVision USA, Inc.; assessment of DNA damage using the diphenylamine method (Zhu et al., 1998); determination of Lipid Peroxide "Malondialdehyde (MDA)" using Lipid peroxide colorimetric assay kit supplied by BioDiagnostic Egypt and determination of total antioxidant capacity using total antioxidant capacity colorimetric assay kit supplied by BioDiagnostic Egypt.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences among groups and the Tuckey test was used to compare all groups with each other and showed the significant effect of treatment.

3. Results and Discussion

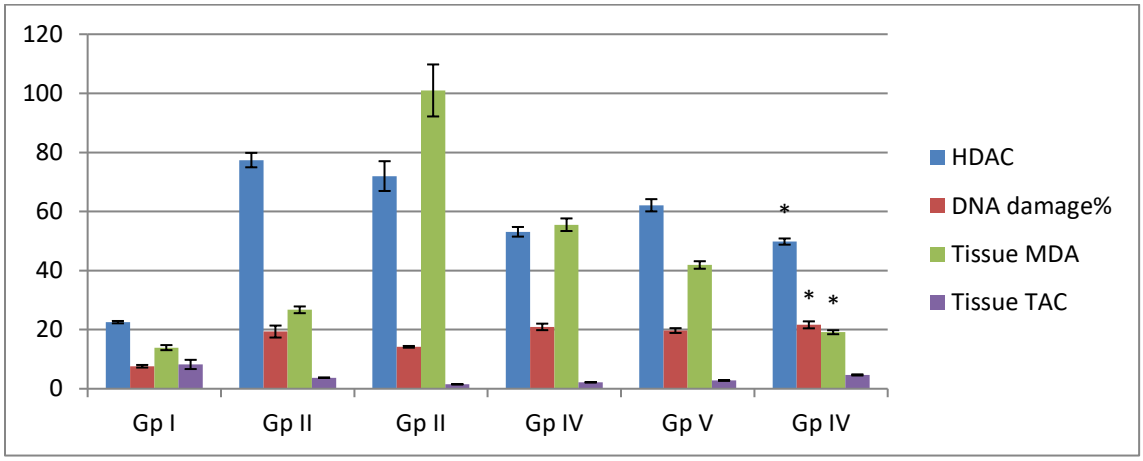

Figure 1: HDAC expressed as µM deacetylated lysine per µg extracted nuclear protein, DNA damage expressed as Percent, Tumor MDA expressed as nmol/10g tissue, Tissue TAC expressed as mM/g tissue

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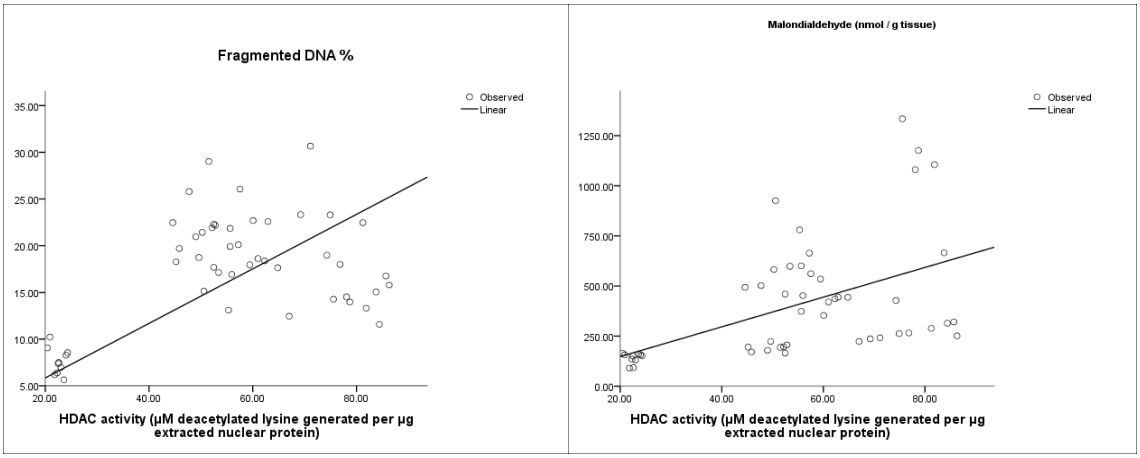

Figure 2: Forward linear correlation between the HDAC activities and fragmented DNA and MDA.

In the present work the naturally isothiocyanate sulforaphane was extracted from outer leaves of green cabbage (Brassica oleracea) in a concentration of 83.3 µg/g fresh weight (Figure 3). Farag and Motaal extracted SFN from cabbage at a concentration of 540 µg/g (Farag et al., 2010). The differences in sulforaphane content are associated with the variability of plant cultivars and multiple environmental factors, e.g. daily temperatures, soil fertility, pathogen challenge, wounding, and plant growth regulators (Fernández-León et al., 2017, Francisco et al., 2017).

HDACs were found to be overexpressed in numerous types of cancer (Spehalski et al., 2017). In the current study, all mice bearing Ehrlich tumors showed a significant increase in HDAC activity in tumor tissue, comparing normal mice muscle tissue. A significant decrease in HDAC activity was observed after treatment with sulforaphane alone or in combination with the antitumor drug methotrexate.

In agreement with the present data, Ho et al. demonstrated that sulforaphane inhibits HDAC activity in human colorectal and prostate cancer cells causing enhanced histone acetylation, derepression of P21 and Bax, and induction of cell cycle arrest/apoptosis, leading to cancer prevention (Ho et al., 2009, Johnson et al., 2017).

In the present work, there was a significant increase in DNA damage in tumor tissue comparing normal tissue, but there was a significant decrease in DNA damage in tumor tissue of mice treated only with methotrexate while the percent of DNA damage increased non-significantly after treating the mice bearing tumors with sulforaphane alone or in combination with methotrexate treatment.

Ferreira de Oliveira et al (2014) also reported that the MG-63 osteosarcoma cell line was exposed to SFN at a dietary concentration (5 µM) which resulted in genomic instability as confirmed by an increased number of DNA breaks (Ferreira de Oliveira et al., 2014, Coutinho et al., 2017).

Malondialdehyde (MDA) is the principal and most studied product of polyunsaturated fatty acid peroxidation. This aldehyde is a highly toxic molecule and should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been referred to as potentially mutagenic and atherogenic (Carrillo et al., 2017).

In the present work, the MDA level increased significantly in all tumor tissue and non-significantly increased in tumor tissue treated with both methotrexate and sulforaphane.

Likewise, De Oliveira et al observed that the increase in intracellular ROS was not gradual

between the 5 and 10 mM SFN exposure, and a sharp increase was observed particularly for the 48-h exposure period. Moreover, for 10 mM SFN, longer exposure times resulted in a larger accumulation of intracellular ROS (Ferreira de Oliveira et al., 2014, Coutinho et al., 2017).

The present work recorded a positive significant correlation between HDAC activity and fragmented DNA percentage. In agreement with the data reported by Shan et al. that class I HDACs, HDAC2 expression was dramatically correlated with expression of a set of DNA damage repair (DDR) genes, including RAD51, in breast cancer. Treatment with the HDAC inhibitor mocetinostat in two basal-like breast cancer cell lines, SUM149, and HCC1937, decreased expression of RAD51 at both mRNA and protein levels, indicating a possible mechanism by which HDAC2 regulates RAD51 expression in breast cancer (Shan et al., 2017). Robert and Rehman also previously reported that HDACi can inhibit DNA repair responses in a few cell lines, which might increase the sensitivity of tumor cells to chemotherapy and radiotherapy by leading to increased DNA damage by these treatments (Robert et al., 2016, Rehman et al., 2016).

The present study recorded a positive significant correlation between HDAC activity and tumor MDA level. Consistently Portakal et al. have found that MDA content in cancerous tissues of the breast was higher than in the corresponding noncancerous tissues (Portakal et al., 2000).

4. Conclusion

SFN is a naturally ITC found in cabbage outer leaves and showed an inhibitory effect to HDAC in Ehrlich solid tumor tissue when given orally. SFN also showed a pro-oxidant activity in tumor tissue that enhanced the tumor treatment with MTX.

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