Diallyl Disulphide Protects against Colon Cancer in vitro, of HT-29 Cells and in Male Rabbits of Colon Cancer Model: An Analysis of Genetic and Epigenetic Variations

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Abstract

Diet and dietary habits are currently accused of being among cancer causing agents. The present study was carried out in a trial to point at the beneficial anti-cancer properties of one of the most Egyptian traditional food components (Garlic). We studied the anti-cancerous properties of Diallyl disulphide (DADS), a major organosulfur compound in garlic oil, on HT29 colon cancer cell line and in vivo of male rabbits (Oryctolagus cuniculus) as an animal model of colon cancer. DADS showed differential effect on the expression of a group of genes, as it down-regulated the expression of oncogenes (e.g., CTNNB1, CCND1, BIRC5, MYC and AKT), while up-regulated the expression of tumour suppressor gene (TP53) and apoptosis regulator gene (BAX). DADS' apoptotic effect was also seen via inducing the expression of cytochrome c and activation of caspase-3. Moreover, DADS induced chromatin configuration changes through increasing histone acetylation of histone-3 and -4. Examination of 1,2 dimethyl hydrazine (DMH) induced cancer in vivo model (O. cuniculus) showed histological changes characteristic for colon tumorigenesis such as, hyperplastic intraepithelial lesions, neoplastic changes and lymphocytes infiltration, which were strongly attenuated in animals co-injected with both DADS and DMH and were not observed in the animals that received DADS prior to DMH treatment. This study suggested the protective properties of DADS against colon cancer in vitro and in vivo.

Keywords: Colon cancer; Diallyl disulphide; Dimethyl hydrazine; Histone acetylation; Apoptosis

Abbreviations: DADS: Diallyl Disulphide; FBS: Foetal Bovine Serum; PBS: Phosphate Buffered Saline; RIPA: Radio-Immuno Precipitation Assay; RT: Room Temperature

Introduction

In Egypt, the recent number of cancer patients per year was estimated to be 70,000. The highest percentage of these cases were recorded in Upper Egypt [1]; a matter needs to be urgently and seriously investigated. The causes of these elevated numbers of daily discovered cases cannot be simply grouped under the title of heredity factors, because cancer is a multi-factorial disease [2], and there are globally growing scientific evidences suggest that it could be controlled through modifying the life style of the susceptible individuals [3,4]. Among those factors that were strongly abused by their involvement in inducing cancer are pollution, in air, water and soil. The repetitive exposure to high concentrations of such pollutants was correlated with cancer incidence [5-7], particularly colorectal cancers [8].

Because dietary habits have pronounced impacts on human health/disease status, the correlation between cancer incidence and diet concerned the researchers for the last few decades, to the extent that Doll and Peto (1981) made to state that 35% of cancer deaths may be related to dietary factors [9]; Abdulla and Gruber, (2000), Sabate and Ang, (2009), who reported that ~ 40 % of cancers are directly linked to the diet [10,11]. Adversely, balanced and healthy foods, showed protective activities displayed by certain dietary components against cancer. These beneficial components have attracted the scientists' attention (e.g., Apple polyphenol extract), which prevents intestinal polyps formation in rats [12], fish oil, reduces DNA adduct formation in the rat colon [13], germinated barley, treats colitis and reduces the risk of colitic cancer [14]. Recently, one of the traditional ingredients in the Egyptian meals (Garlic), particularly DADS [15] showed strong anti-cancerous properties manifested the induction of cell cycle arrest and microtubules formation disruption in human breast adenocarcinoma cells (MDA-MB-435) [16], induced apoptosis in human leukaemia cells (HL-60) [17] and ceased human neuroblastoma cellular proliferation [18]. DADS also showed anti-invasive activities in human prostate carcinoma cells (LNCaP) through tightening the tight junctions and inhibition of matrix metalloproteinase activities [19]. These studies clearly suggested that diet is closely related with both causation and prevention of cancer.

In this work, we studied the differential impact of DADS on the expression of a group of genes known to be involved in regulation of colon tumorigenesis in vitro, in the human colon cancer cell-model (HT-29). Then we investigated both prophylactic and heeling impacts of DADS in DMH colon cancer animal model [20], using laboratory male rabbit (O. cuniculus). Our results demonstrated that DADS differentially repressed oncogenes but induced the expression of tumor suppressor genes in vitro and increased the resistance of O. cuniculus to develop colon cancer after receiving multiple doses of DMH.

Materials and Methods

Cell culture and treatments

HT-29 cells (American Type Culture Collection), were propagated in Dulbecco's modified Eagle's minimum essential medium (DMEM, Life technologies, catalogue number: 11320-033) supplemented with...
10% heat-inactivated (60°C, 30 min) fetal bovine serum (FBS; Gibco®, catalogue number: 12484-010) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, Sigma, catalogue number: P4333). Cells were incubated at 37°C in a 5% CO₂ atmosphere. For the experiment the cells were sub cultured in 6-well plates at a density of 10⁶ cells/well for 48 h and serum starved overnight before treatment. Cells were treated with vehicle control (DMSO, Sigma, catalogue number: W387509), or DADS (Sigma-Aldrich, Catalogue number 317691) supplemented in the culturing medium to a final concentration of 100 µM for 8 h.

**Real-time reverse transcription quantitative PCR (RT-qPCR)**

RNA was prepared using TRizol reagent and the Pure Link RNA mini kit (Life Technologies, catalogue number: 12183018) following the kit’s instruction. RNA was reverse-transcribed and qPCR was performed by PCR as the following: Total RNA (1 µg) was reverse transcribed using the high-capacity cDNA reverse transcription kit (Life Technologies, Catalog number: 4368813) according to the manufacture instructions. cDNA samples were diluted 1:5. RT-PCR reaction mixture was prepared by adding 4 µL cDNA, 10 µL Fast SYBR Green PCR Master Mix (Life Technologies, catalogue number: 4385612), 0.6 µl of each primer of 10 µM working concentration and 4.8 µL H₂O. Amplification conditions were initial one cycle for denaturation and enzyme activation at 95°C for 20 sec., followed by 40 cycles of 95°C for 3 sec, 60°C, 30 sec. The obtained C<sub>T</sub> values were normalized to the RPL19 (60S ribosomal protein L19). Expression levels for different conditions were obtained by comparing the mean C<sub>T</sub> value for each gene relative to the mean RPL19 C<sub>T</sub> value. For repressed genes (i.e., a ΔΔ C<sub>T</sub> value < 0.0), relative fold change is depicted graphically as - (2<sup>-ΔΔCt</sup>). 

Data were generally analyzed in biologic triplicate and technical duplicate and are expressed as mean ± SE. We used the two-tailed (or paired) Student’s t-test, using Microsoft Excel, to determine the significant differences with probability values. (≤ 0.05 were considered significant). Primers with specific sequence used in the experiment were purchased from Macrogen, Seoul, Korea.

**CTNNB1** primer pairs:
[F] 5'-CTCTCAGAGTTGCTGCTATTG-3';
[R] 5'-CCTTCCATCCCTGCTTTAG-3'.

**CYCLIN-D1** primer pairs:
[F] 5'-CACACACACACACAAACC-3';
[R] 5'-CCTCCCTTCAACACTTCCTAAA-3'.

**SURVIVIN** primer pairs:
[F] 5'-GCACCACCTCCAGGTTTAT-3';
[R] 5'-CAGACCGTTCCTATACCTCATTC-3'.

**CMYC** primer pairs:
[F] 5'-GCTTGAGTAAATTCCACGGAG-3';
[R] 5'-GAGTCGATGTGCGATGCTAGT-3'.

**AKT** primer pairs:
[F] 5'-CTCCTCCTCTCCTCGATTG-3';
[R] 5'-GCCCGAAGTCTGATGATCTTAAT-3'.

**USP28** primer pairs:
[F] 5'-CCTGACATCTTCTCGGTTC-3';
[R] 5'-CTCTTCTCCCACCTCTCTACT-3'.

**Western blot**

Following treatment, DADS or vehicle control, cellular lysates were prepared from HT-29 cells by washing the plates with ice cold phosphate buffer saline (PBS) followed by scraping the cells with plastic scraper and collecting the cells suspension in pre-chilled Falcon tubes. Cells were pelleted by centrifugation (250 x g) for 5 min and the pellets were resuspended in radio immunoprecipitation assay buffer (RIPA) [150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0] containing Halt™ Protease Inhibitor Cocktail, Life Technologies, catalog number: 78437 (1 X, final concentration). Total protein concentration were determined via protein assay kit (BioRad, Cat. No. 500-0006) and 50 µg protein were separated by SDS polyacrylamide gel electrophoreses (PAGE).

Protein bands were transferred to Polyvinylidene fluoride membrane (PVDF, Amersham, Cat. No. 10600023), then, the membrane were blocked overnight in the blocking buffer (20 mM Tris HCl, 500 mM NaCl, pH 7.5) containing 5% non-fat dry milk (BioRad, Cat. No. 170-640XTU). Desired protein bands were detected by incubating the membranes for 2 h at room temperature in the blocking buffer containing 3% non-fat dry milk and first antibody in a manufacturing recommended dilutions. We used antibodies to detect beta catenin, cyclin D1, surviving, c-myc, AKT, pAKT, P53, Bak, Bax, Ace-H3 [acetyl K27], Ace-H4 [acetyl K91], cytchrome c, caspase-3 [full length protein], and β-actin; corresponding catalogue numbers respectively are ab6302, ab16663, ab182132, ab32072, ab32505, ab66138, ab179477, ab32371, ab7977, ab4729, ab4627, ab135304, ab13847 and ab6276, all were purchased from ABCAM; USP28 antibody was purchased from Cell signaling (Cat. No. 4217). Immuno-detected band visualization was carried out using ECL plus Western Blotting Substrate (Thermo, Cat. No. PI32106).

**Animal model**

The experimental animal we used was O. cuniculus, four weeks old, obtained from the animal house of Sohag University, and were housed in polypropylene cages and maintained at controlled conditions of temperature = 28°C with a 12 h light and 12 h dark cycles and fed commercial pellet diet. Animals were divided into five groups, four animals each, and treated for four weeks as the following: group (A) received vehicle control only (distilled water containing 1 mM EDTA) via subcutaneous injection, group (B) received DMH only [20 mg/kg dissolved in the vehicle control], group (C) received DADS only (60 mg/kg, via intragastric intubation), group (D) received DADS and DMH, and finally group (E) received DADS for the first 4 weeks followed by DMH for extra 4 weeks. All treatments were received three times a
week for the entire experiment time. All experimental procedures were conducted according to the ethical standards of Sohag University for animal experimentation.

**Histopathology**

At the end of the experiment, animals were anesthetized using ether inhalation, sacrificed, carefully dissected, and the colon region from each animal were fixed in 10% formalin and imbedded in paraffin wax to prepare paraffin blocks. Paraffin sections (7 μm thickness) were then deparaffinized in xylene and hydrated in descending series of ethyl alcohol and stained with Hematoxylin and Eosin (H and E). Stained-samples Slides were mounted in DPX medium and observed under light microscope (Axio Lab. A1, Carl ZEISS, Germany) equipped with AxioscamERCS camera.

**Results**

**DADS differentially regulated gene expression in HT-29 cells**

DADS was reported to having anti-cancer properties and in a step to understand its' mechanism action, we proposed that it might modulate the expression of the gene machinery that controls carcinogenesis in the colonic cells. Therefore, we established a cell culture system using HT-29 cells (colon cancer cell model) and measured the mRNA levels by Real-Time PCR of a group of genes that are implicated in colorectal carcinogenesis (CTNNB1, CCND1, BIRC5, MYC, AKT and USP28), and in the same time we determined the mRNA levels of the tumor suppressor TP53 gene, as well as BAK1 and BAX genes, which are involved in controlling the apoptotic pathway.

Normalized C\textsubscript{T} values to the RPL19 (ribosomal protein L19) demonstrated that DADS significantly down regulated the expression of CTNNB1, CCND1, BIRC5, MYC, AKT but did not affect the mRNA level of USP28 gene. While mRNA levels of TP53 were significantly increased post DADS treatment compared to its levels in the cells treated with vehicle control. DADS also significantly increased the mRNA levels of BAX but did not affect the BAK1 mRNA (Figure 1 and Table 1).

Gene expression process passes through multiple molecular steps within the cell, starts with mRNA transcription followed by post transcription modifications and functional protein synthesis. The impact of DADS on mRNA, we seen above, may or may not extend to the following steps and affect the protein levels. Thus, we measured the levels of functional proteins of the above mentioned genes before and after DADS treatment using Western Blot (W.B.) analysis.

The protein data showed similar pattern to the mRNA levels indicative of the extended impact of DADS in differentially modulating not only mRNA message, but also the levels of the functional proteins; as compared to levels of the same proteins in the cells treated only with the vehicle control. DADS decreased the protein levels of CTNNB1, CYCLIN D1, SURVIVIN, C-MYC and AKT. HT-29 cells showed lower level of AKT activation (phosphorylated pAKT), suggesting that DADS treatment decreased the pro-survival signaling within the colon cancer cell. On the other hand HT-29 cells showed an increase in P53 and BAX proteins, while spared the USP28 and BAK protein levels (Figure 2).

We also studied the changes in the levels of acetylated histone-3 (Ac-H3, acetyl K27) and -4 (Ac-H4, acetyl K91) in the cellular lysates of HT-29 cells that were treated with DADS or vehicle control. As expected, DADS significantly increased the levels of acetylated forms of both H3 and H4 (Figure 3). This might explain the alteration in gene expression and protein levels, which was observed by RT-PCR.

![Figure 1](image1.png)

**Figure 1**: Differential impact of DADS on gene expression in H292 cells. 80% confluent cells were treated with vehicle control (DMSO) or DADS for 8 h. Bars graphs indicate mean C\textsubscript{T} values of four independent biological replicates normalized to RPL19 C\textsubscript{T} values and relative to vehicle control ± S.D. *Significant (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>% Fold change</th>
<th>S.D.</th>
<th>P value ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTNNB1</td>
<td>-31.22</td>
<td>± 0.1373</td>
<td>0.004221 *</td>
</tr>
<tr>
<td>CCND1</td>
<td>-62.02</td>
<td>± 0.2311</td>
<td>0.006642 *</td>
</tr>
<tr>
<td>BIRC5</td>
<td>-20.83</td>
<td>± 0.0702</td>
<td>0.004805 *</td>
</tr>
<tr>
<td>MYC</td>
<td>-26.64</td>
<td>± 0.1661</td>
<td>0.002741 *</td>
</tr>
<tr>
<td>AKT</td>
<td>-10.68</td>
<td>± 0.1235</td>
<td>0.004991 *</td>
</tr>
<tr>
<td>USP28</td>
<td>-3.87</td>
<td>± 0.0852</td>
<td>0.008038</td>
</tr>
<tr>
<td>TP53</td>
<td>15.02</td>
<td>± 0.1092</td>
<td>0.00297 *</td>
</tr>
<tr>
<td>BAK1</td>
<td>01.70</td>
<td>± 0.1245</td>
<td>0.063341</td>
</tr>
<tr>
<td>BAX</td>
<td>39.12</td>
<td>± 0.1846</td>
<td>0.002741 *</td>
</tr>
</tbody>
</table>

**Table 1**: RT-PCR data analysis of DADS impact on the gene expression in HT-29 cells. CT values were normalized to those of RPL19 and relative to vehicle control. Data are mean of four independent experiments (n=4), SD=standard deviation, *p ≤ 0.05 considered significant.

![Figure 2](image2.png)

**Figure 2**: DADS differential modulation of the protein levels in HT-29 cells. (A) Cellular lysates of vehicle control and DADS treated cells (100 μM, 8 h) were denatured and 50 µg/ well were separated by SDS-PAGE and trans-blotted into PVDF membrane and immunoprobed following the standard Western blot protocol. (B) Bands intensity was determined using TotalLabTM gel analysis software and expressed as arbitrary scan units after normalization to β-actin values. The reported results are mean ± SE of three independent experiments; *p ≤ 0.05.
and W.B., respectively, after DADS treatment. In addition to this, we measured the cytochrome c and caspase-3 (pro- and active forms) by W.B. analysis in the cellular lysates of HT-29 cells treated with either DADS or vehicle control (Figure 3).

An increase in the level of cytochrome c and decrease in the level of pro-caspase-3 were observed accompanied with the appearance of active caspase-3 protein bands (12-17kDa) in the cellular lysates of DADS treated cells (Figure 3).

For further investigation we generated an in vivo model for colorectal cancer by injecting the laboratory male rabbit (O. cuniculus) with DMH as mentioned in the materials and method section. Animals were divided into five groups: group (A) received vehicle control only, group (B) received DMH only, group (C) received DADS only, group (D) received DADS and DMH and group (E) received DADS first for 4 weeks followed by DMH treatment. Microscopic examination of colonic tissues of O. cuniculus showed normal organization of histological layers with flat mucosa and numerous straight tubular crypts that extend down to the muscularis mucosa (Figure 4A, 4F and 4K).

While the colonic sections prepared from animals treated with DMH (Figure 4B, 4G and 4L) displayed histological and cytological changes characteristic for hyperplastic intraepithelial lesions, such as crowded nuclei without stratification (L, the red arrow), elongation of the crypts (G, red arrows). Moreover, DMH displayed cytological features characteristic for neoplasia, such as hypercellularity accompanied with hyperchromatic nuclei and high nuclear/cytoplasmic ratio (Figure 4L, red arrow). DMH also caused lymphocytes infiltration (Figure 4B and 4G, black arrows). Such kind of histological and cytological apparitions were not seen in the DADS group (Figure 4C, 4H and 4M) which looked similar to the control group, and were strongly attenuated in the DMH+DADS group (Figure 4D, 4I and 4N), and strongly enhanced the resistance against DMH carcinogenicity in the group that received DMH after 4 weeks of DADS treatment, as most of the above described histological apparitions were not seen (Figure 4E, 4J and 4O).

Discussion

In the present study, we aimed to determine the positive role of DADS in protecting against colon cancer; particularly with the current
acccusation to diet and dietary habits of being among cancer causing agents [21-23] and in a country like Egypt facing serious problem of yearly elevated numbers of cancer patients. The rationale behind choosing DADS for studying its anti-cancerous activities against colon cancer was: 1- DADS is a major organosulfur compound in garlic oil [15], and 2- Garlic is one of the most consumable Egyptian traditional foods.

The cell line we used, HT-29, which was isolated from a human primary colon adenocarcinoma tissue in 1964 by Fogh [24]. This cell line is being extensively used as an in vitro model to test the sensitivity of colon cancers towards chemotherapeutic drugs [25]. In a previous study, we showed that after 24 hr DADS reduced viability and proliferation of colon adenocarcinoma cells (HT-29) in a dose dependent way. In the present work, the cultured HT-29 cells were treated with DADS and monitored the expression of the genes that are known to be involved in the carcinogenesis process in the colon, at both levels mRNA and protein.

The results demonstrated that DADS differentially modulated the expression of such genes through enhancing the expression of tumour suppressor genes and concomitantly repressing the expression of oncogenes. RT-PCR and WB. data showed that the mRNA and protein levels of CTNNB1 (β-catenin), CCND1 (cyclin d1), BIRC5 (survivin), MYC (c-myc) and AKT were significantly repressed after DADS treatments compared to their levels in the cells treated only with the vehicle control. These genes are known to control the carcinogenesis process as β-catenin is important in forming the adherens junction in epithelial cells, it also controls cell growth and differentiation during both normal development and tumorgenesis [26-28]. Cyclin d1 function is to regulate CDK kinases (Cyclin-dependent kinases), which are involved in regulating transcription, mRNA processing and cell cycle control. Over-expression of this gene, as seen in a variety of tumors, alters cell cycle progression and may contribute to tumorigenesis [29,30]. Survivin inhibits the progression of programmed cell death (apoptosis) by inhibiting caspase family of protease enzymes and thereby promotes cellular sustainability and tumour growth [31]. C-myc itself is a transcription factor and if it is over expressed it alters the expression of many other genes involved in cell proliferation resulting in cancer progression [32]. AKT is involved in protein synthesis pathways, and if its level is up normally elevated within the cell, it leads to cellular hypertrophy [33,34]. It also works as survival factor, upon activation (phosphorylation) through inhibiting apoptosis [35]. On the other hand, DADS up regulated the expression of P53 and BAX mRNA and protein. P53 is an important cell cycle regulator protein, it activates apoptosis in case of irreversible DNA damage [36], P53 deficiency mediates tumour formation in mammary glands [37] and elicit chemotherapeutic resistance in colon cancer cells via inducing the survival pathway [38]. BAX is a member of BCL-2 family, which regulates apoptosis via controlling mitochondrial cytochrome c release and caspases family activation [39].

DADS’ induction of the expression of such genes is indicative of its protective properties against cancer. Observed apoptotic impact of DADS comes consistent with the results obtained from the study performed on other cancer cells, such as human leukaemia HL-60 cells and MDA-MB-435 breast cancer cells, where DADS arrested the mitotic division and activated apoptosis [16,40]. In addition, chaperone protein family, Hsp70 and Hsp90 are over-expressed in a wide range of tumor types (both solid tumors and hematological malignancies), and play essential roles in apoptosis, cell proliferation, metastases, angiogenesis, and invasion pathways in cancer cell metabolism. They provide stabilization; regulation and maintenance of oncogenic client proteins (Her-2, Cdk-4, Akt, Raf-1), thus promote cancer cell survival [41]. Some approaches being used or proposed in cancer therapy based on the in inhibition of Hsp90, Hsp70 and Hsp27 [42].

In a previous study, we proved that the apoptotic effect of DADS in breast cancer cells MCF-7 might be due to the upstream alterations that happened in the chromatin conformation post DADS treatment, which might lead to changes in gene expression [43]. Therefore, DADS ability was tested to activate H3 and H4 acetylation and thereby initiation of apoptosis by measuring the levels of acetylated forms of H3 (Ace-H3, acetyl K27) and H4 (Ace-H4, acetyl K91) beside two of the well-known apoptotic molecular markers, cytochrome c and active caspase-3 [44,45]. A significant increase was observed in the acetylated form of histone-3 (Ace-H3) and -4 (Ace-H4) and in the level of cytochrome c, along with the appearance of active caspase-3 protein bands, compared to vehicle treated cells.

Induction of histone acetylation was correlated in many studies with the initiation of the programmed cell death or apoptosis, particularly in cancer cells [46,47]. These data clearly suggest that DADS provoked apoptosis in HT-29 cells and this may be caused by the chromatin changes (H3 and H4 acetylation) that altered gene expression and caused an elevation in the levels of apoptotic genes machinery (BAX, cytochrome c) and ultimately led to caspase-3 activation in HT29 cells. Then, one question could be asked: Does DADS exert a similar effect in vivo? To answer this question we designed an animal model for colon cancer (DMH induced colorectal cancer in laboratory male rabbit) [20].

Histological and cytological changes characteristic for colon tumorigenesis was seen in the histological sections prepared from O. cuniculus colonic region post DMH treatments. Such changes were hyperplastic intraepithelial lesions, indicated by crowded nuclei without stratification, disappearance of the mitotic figures on the surface of crypts and elongation of the crypts. In addition to neoplastic changes (e.g., hypercellularity, hyperchromatic nuclei and high nuclear/cytoplasmatic ratio), lymphocytes infiltration was detected.

In the animals treated only with DADS, the colonic sections looked like the controls. When DMH was injected along with DADS, the above observations were strongly attenuated and were not observed in animals that received DADS prior to DMH treatment. Altogether, suggest the protective properties of DADS, in vitro and in vivo. This still needs further work to reveal the molecular mechanisms of DADS with anti-cancerous properties.

Conclusion

DADS (a major organosulfur compound in garlic oil) anti-cancerous properties were studied in vitro and in vivo. RT-PCR and western blot data showed that DADS differentially modulates the gene expression in HT29 colon cancer cells. It represses the oncogenes and induces the tumour suppressor genes. DADS also attenuates and protects against the carcinogenic impact of DMH in the animal model. We suggest that DADS has a beneficial impact that may be due to its ability to induce histone acetylation and initiate apoptosis in cancer cells.

References


