

Diallyl disulfide-induced apoptosis in a breast-cancer cell line (MCF-7) may be caused by inhibition of histone de-acetylation

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1 **Diallyl disulfide-induced apoptosis in a breast-cancer cell line (MCF-7)**
2 **may be caused by inhibition of histone de-acetylation**

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4
5 Mohammed O. Altonsy^a, Tito N. Habib^a and Simon C. Andrews^{*, b}

6 ^aMolecular Biology Lab., Zoology Department, Faculty of Science, Sohag University,
7 285224, Egypt.

8 ^bThe School of Biological Sciences, University of Reading, Reading, RG6 6AJ, UK

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14 *Corresponding author

1
2
3 19 **Abstract**
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5 20 The health benefits of garlic have been proven by epidemiological and experimental studies.
6
7 21 Diallyl disulphide (DADS), the major organosulfur compound found in garlic oil, is known to
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9 22 lower the incidence of breast cancer both *in vitro* and *in vivo*. The studies reported here
10
11 23 demonstrate that DADS induces apoptosis in the MCF-7 breast-cancer cell line through
12
13 24 interfering with cell-cycle growth phases in a way that increases the sub-G₀ population and
14
15 25 substantially halts DNA synthesis. DADS also induces phosphatidylserine (PS) translocation
16
17 26 from the inner to the outer leaflet of the plasma membrane and activates caspase-3. Further
18
19 27 studies revealed that DADS modulates the cellular levels of Bax, Bcl-2, Bcl-xL and Bcl-w in
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21 28 a dose-dependent manner, suggesting the involvement of Bcl-2 family proteins in DADS
22
23 29 induced apoptosis. Histone deacetylation inhibitors (HDACi) are known to suppress cancer
24
25 30 growth and induce apoptosis in cancer cells. Here it is shown that DADS has HDACi
26
27 31 properties in MCF-7 cells as it lowers the removal of an acetyl group from an acetylated
28
29 32 substrate and induces histone-4 (H4) hyper-acetylation. The data thus indicate that the
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31 33 HDACi properties of DADS may be responsible for the induction of apoptosis in breast
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33 34 cancer cells.
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39 35 **Keywords**
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41 36 Breast cancer, Diallyl disulphide, Apoptosis, Histone deacetylation inhibitor.
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38 Introduction

39 Worldwide, breast cancer comprises 22.9% of all non-melanoma skin cancers and 13.7% of
40 cancer deaths in women (1). Some of the primary risk factors for female breast cancer
41 include age (2), lack of childbearing or breastfeeding (3), high hormone levels (*e.g.* estrogen)
42 (4) and ethnicity (5). However, diet is another important factor that contributes to the
43 development of breast cancer (6-8). The impact of diet on the incidence of breast cancer is
44 indicated by geographic differences in the occurrence of the disease (9), and by the many
45 dietary components experimentally proven to act as anti-breast cancer agents. One such
46 dietary factors is diallyl disulphide (DADS), which is a major (~60%) organosulfur
47 compound found in garlic oil (10). DADS inhibits the growth of breast cancer cell lines
48 (MDA-MB-231, KPL-1, MKL-F and MCF-7) (11) and reduces the incidence of *N*-methyl-*N*-
49 nitrosourea (MNU) and 2-amino-1-methyl-6-phenylimidazo-4-5-*b*-pyridine (PhIP)-induced
50 mammary tumours (12, 13). The anti-cancerous effect of DADS is often attributed to its
51 ability to induce apoptosis in cancer cells via cell-cycle arrest and modulation of the activity
52 of Bcl-2 family proteins (14-16).

53 The difference between normal and cancerous cells is that the later lack the ability to die. In
54 normal tissue, a balance between cell proliferation and cell death must be maintained,
55 otherwise cells display uncontrolled growth and thereby cancer arises (17). Cellular apoptosis
56 or programmed cell death is the key process in maintaining this balance. Apoptosis naturally
57 occurs in mammalian tissues and is regulated by a cascade of cellular proteins, such as pro-
58 and anti-apoptotic molecules. Prominent among such factors are the Bcl-2 family proteins
59 which act as signalling molecules in cellular apoptosis and survival pathways. Bcl-2 family
60 proteins include both pro-apoptotic (Bax, Bak, Bok, Bad, Bid, Bim and Bmf) and anti-
61 apoptotic members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and Bfl-1/A1) (18, 19). Such pro-apoptotic

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3 62 proteins function by promoting mitochondrial permeability, resulting in the release of
4
5 63 apoptogenic factors (e.g. cytochrome *c* and apoptosis inducing factor [AIF]) from the
6
7 64 mitochondrial inter-membrane space into the cytosol (20). The presence of such factors in the
8
9 65 cytosolic compartment induces the activation of protease activators (caspases) that ultimately
10
11 66 lead to apoptosis. Either increasing levels of pro-apoptotic Bcl-2 proteins or decreasing levels
12
13 67 of anti-apoptotic Bcl-2 proteins (or a combination of both) can result in activation of the
14
15 68 apoptotic machinery, thereby initiating apoptotic cell death. In this context, DADS up-
16
17 69 regulates pro-apoptotic Bcl-2 proteins in many types of cancerous cells. For example, DADS-
18
19 70 induces Bax expression and triggers the apoptotic mitochondrial pathway in MCF-7 human
20
21 71 breast cancer cells (14), and is known to induce apoptosis through down-regulation of anti-
22
23 72 apoptotic Bcl-2 in A549 human lung adenocarcinoma cells (21). DADS also caused down-
24
25 73 regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL in a human colon cancer cell line
26
27 74 (COLO 205), whereas it induced up-regulation of the pro-apoptotic Bak and Bax proteins
28
29 75 (22). Furthermore, DADS treatment of a human prostate cancer cell line (PC-3) resulted in
30
31 76 increased cellular levels of pro-apoptotic BAX and BAD, but decreased Bcl-2 levels (23). In
32
33 77 a similar study by Arunkumar *et al.* (2007), the apoptotic impact of DADS in PC-3 cells were
34
35 78 accompanied by histone (H3 and H4) hyper acetylation (24).

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41 79 Aberrant gene transcription is common in malignant cells, resulting in activation of some
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43 80 genes and silencing of others (25, 26). Such changes in transcriptional status are often
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45 81 correlated with alterations in histone acetylation with hyper-acetylation mediating activation
46
47 82 whilst de-acetylation promotes gene silencing (27, 28). Histone acetylation controls
48
49 83 transcription by facilitating or restraining the recruitment of transcription factors on to
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51 84 associated promoter (29, 30). Histone acetylation has received considerable interest of late
52
53 85 because of its link to the pathogenesis of cancer. The progression of carcinogenesis involves
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55 86 the transcriptional activation of 'inappropriate' genes (e.g. oncogenes, RAS, WNT, MYC,
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3 87 ERK, TRK and Bcr-Abl) at the expense of tumour suppressor genes (e.g. TP53, CDKN1B,
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5 88 HNPCC, MEN1, APC, CD95, ST5, ST7, ST14 and BRCA) and pro-apoptotic genes (e.g.
6
7 89 pro-apoptotic Bcl-2 proteins) (31-35). Since aberrant transcription of Bcl-2 family genes is
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10 90 often observed in breast cancer cells (36, 37), this raises the possibility that the modulation of
11
12 91 expression of such genes by DADS is mediated by DADS-induced histone deacetylation
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14 92 inhibition (HDACi).

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17 93 This possibility raised above is addressed here through the investigation of the effect of
18
19 94 DADS on the acetylation status of histones in the breast-cancer cell line, MCF-7. The data
20
21 95 confirm that DADS strongly induces apoptosis in MCF-7 cells, which is apparently caspase
22
23 96 dependent. DADS also enhances histone hyper-acetylation in MCF-7 cells, through its
24
25 97 HDACi activity, and promotes pro-apoptotic Bax but depresses anti-apoptotic Bcl-2, Bcl-xL
26
27 98 and Bcl-w. Thus, the anti-cancerous activity of DADS could arise from its HDACi effect.

28 29 30 31 99 **Materials and Methods**

32 33 34 100 **Cell line and treatment**

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37 101 The human mammary gland adenocarcinoma cell line (MCF-7) (38) was kindly provided by
38
39 102 Professor A El-Tayeb (Assiut University) and cells were used following 43–51 passages.
40
41 103 Cells were cultured in Dulbecco's modified Eagles's minimum essential medium
42
43 104 (CAMBREX) supplemented with 10% heat-inactivated (60 °C, 30 min) fetal bovine serum
44
45 105 (FBS, CAMBREX) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), in six-well
46
47 106 tissue culture plates in a concentration of 10^5 cells/ml at 37 °C in a 5% CO₂/95% humidified
48
49 107 atmosphere. Culturing media were supplemented with DADS (Sigma, purity ≤ 80%)
50
51 108 dissolved in dimethylsulfoxide [(DMSO), Aldrich, purity ≤ 95.5%]] in final concentrations of
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53 109 1, 10 or 100 µM DADS. The DMSO final concentration did not exceed 0.1% in all treatments
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3 including control cultures. Trichostatin A (TSA), at 1 μ M final concentration, was used to
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5 inhibit histone deacetylase activities in positive control samples (39)
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8 **Cell cycle analysis**

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10 MCF-7 cells, treated and control, were harvested by trypsinization. The cells were washed
11
12 with phosphate buffered saline (PBS). Cells were then fixed by pipetting 1 ml of cell
13
14 suspension (approximately 10^6 cells/ml) was on to 4 ml of absolute ethanol at -20 °C in a
15
16 Falcon tube while vortexing at top speed. Fixed cells were re-hydrated in 5 ml PBS for 15
17
18 min at room temperature (RT). Finally, 3 mM propidium iodide (PI) in staining buffer (100
19
20 mM Tris; pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% NP-40) was added and
21
22 mixed by gentle pipetting. Pancreatic RNAase (50 μ l of 10 μ g/ml stock; Sigma) was added
23
24 and the cells were incubated for 2 h at 4 °C. Cell cycle analysis was performed using a
25
26 FACScan Flow Cytometer (Becton Dickson) according to the manufacturer's protocol.
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31 **Annexin V/PI test**

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33 MCF-7 cells were tested for apoptosis induction by DADS using Annexin V-FITC/PI double
34
35 staining. Briefly, approximately 10^6 cells were washed in cold PBS followed by two washes
36
37 in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂,
38
39 pH 7.4). Cells were then re-suspended in 100 μ l Annexin V-FITC (4 μ g/ml in binding buffer)
40
41 and incubated on ice for 10 min. An additional 400 μ l of binding buffer, containing PI
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43 (2 μ g/ml), were added and the cellular suspension was incubated on ice for a further 15 min.
44
45 Cells thus treated were then analysed by flow cytometry.
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50 **Caspase-3 activation**

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52 MCF-7 cells were seeded in 96-well plates at a density of 10^5 cells/well. Plates were then
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54 incubated overnight at 37°C in a '5% CO₂/95% humidity' atmosphere. DADS was added at
55
56 final concentrations of 1, 10 and 100 μ M and cells were incubated for an additional 24 h. For
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3 134 positive controls, cells were treated with staurosporine (3 µg/ml) for 4 h (40) prior to
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5 135 performing the assay. Caspase-3 activation upon DADS treatment was measured using the
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7 136 Caspase-3 Fluorescence Assay Kit (Cayman chemicals) according to the manufacturer's
8
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10 137 instructions. In order to test assay specificity, negative control samples were prepared by
11
12 138 adding 10 µl/well of caspase-3 inhibitor solution (provided with the kit). The plates were read
13
14 139 on a FLUOstar OPTIMA fluorescent plate reader at 485 nm excitation and 535 nm emission
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16 140 wavelengths.

19 141 **Cellular lysate preparation and Western blot**

20
21 142 Cells were lysed with Radio-Immuno Precipitation Assay (RIPA) lysis buffer (50 mM Tris-
22
23 143 HCl, pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml PMSF
24
25 144 [phenylmethylsulphonyl fluoride], 1 µg/ml aprotinin, 1% NP-40) for 30 min on ice. The
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27 145 cellular lysate was then centrifuged at 12,000×g for 2 min at 4 °C. The supernatant was then
28
29 146 transferred to a fresh Eppendorf tube, and total protein concentrations were determined using
30
31 147 the BioRad protein assay (41). Cellular lysates were then denatured with an equal volume of
32
33 148 loading buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02%
34
35 149 bromophenol blue, pH 6.8) at 100 °C for 10 min. Protein samples (50 µg) were then
36
37 150 immediately subjected to SDS-PAGE (BioRad Mini Protein II Electrophoresis gel) and trans-
38
39 151 blotted onto a nitrocellulose membrane (Amersham). The membranes were blocked in 5%
40
41 152 non-fat dry milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) overnight at 4 °C. The
42
43 153 membranes were immuno-stained for pro-apoptotic (Bax, Bak and Bid) and anti-apoptotic
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45 154 Bcl-2 proteins (Bcl-2 and Bcl-xL [rabbit polyclonal, Abcam] and Bcl-w [Cell Signaling
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47 155 Technology®]). β-Actin primary antibody (mouse monoclonal, Abcam) was used as a
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49 156 loading control. Immuno-detected band visualisation was carried out using the
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51 157 chemiluminescent alkaline phosphatase substrate (Immobilom™ Western).
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158 Histone extraction and electrophoresis

159 Histone extraction was performed as previously published (42). Approximately 10^7 cells,
160 treated and control, were resuspended in 1 ml nuclear extraction buffer (60 mM KCl, 15 mM
161 NaCl, 3 mM MgCl₂, 15 mM piperazine- N/N' bis [2 ethanesulfonic acid]; pH 6.5, 0.1% NP-
162 40, 0.5 mM phenyl methyl sulfonyl fluoride, 1 mM tetrathionate) and kept on ice for 20 min,
163 and then centrifuged at 1300 xg and 4 °C for 10 min. The resulting nuclear pellet was
164 suspended in H₂SO₄ (0.2 M final concentration) for 2 h and centrifuged at 10,000 xg and 4 °C
165 for 10 min. The supernatants containing the histones were removed, and dissolved histones
166 were precipitated with absolute alcohol at -20 °C. The precipitant was suspended in water and
167 quantified by Bio-Rad protein assay (41). A 1 ml volume of sample buffer was prepared by
168 mixing 7.7 mg dithiothreitol (DTT), 900 µl of 8 M urea, 50 µl phenolphthalein [1% w/v in
169 95% ethanol] and 50 µl of 30% NH₄OH. The sample buffer was then added to the dissolved
170 histones in a 1:1 v/v ratio, and kept for 5 min at RT. Samples were then acidified by adding
171 5 µl of glacial acetic acid. Histones were electrophoresed in a Triton-Aced-Urea (TAU) gel as
172 described previously (43).

173 Nuclear extract preparation and DADS-HDACi activity analysis

174 Nuclear extracts were prepared as described before (44). Approximately 10^7 cells were lysed
175 in 1 ml of ice cold lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 15 mM MgCl₂, 250 mM
176 sucrose, 0.1 mM ethylene glycol bis-[beta-aminoethylether] N,N,N[1]-tetraacetic acid
177 [EGTA], 0.5% NP-40; pH 7.5), and kept on ice for 15 min. The suspension was then
178 carefully pipetted over a 4 ml layer of sucrose buffer (30% sucrose, 10 mM Tris-HCl, 10 mM
179 NaCl, 3 mM MgCl₂, pH 7.5), and centrifuged at 1300 xg and 4 °C for 10 min. The pellet was
180 resuspended in Tris-HCl buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.5) and centrifuged at
181 1300 xg, 4 °C for 10 min. The pellet (nuclear fraction) was re-suspended in extraction buffer
182 (50 mM HEPES, 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, 10% glycerol, pH 7.5) and

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3 183 ultrasonicated for 30 s and kept on ice for 30 min. The mixture was then centrifuged at
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5 184 10,000 $\times g$ and 4 °C for 10 min. The supernatant (nuclear extract) was collected and kept at -
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7 185 80 °C for HDAC activity analysis. Different concentrations of DADS were tested for HDACi
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9 186 activity, using the fluor de lysTM fluorescent assay system (Drug discovery kit-AK-500;
10
11 187 BIOMOL) according to the manufacture instructions. The kit includes deacetylases inhibitor
12
13 188 (Trichostatin A [TSA]) for use as positive control. The samples were quantified on a
14
15 189 FLUOstar OPTIMA fluorescent plate reader at 360 nm excitation and 460 nm emission
16
17 190 wavelengths.

20 21 191 **Statistical Analysis**

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23 192 All data were obtained from three independent experiments, and all results are expressed as
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25 193 mean \pm SE. We employed the two-tailed (or paired) Student's *t*-test, using Microsoft Excel, to
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27 194 determine significant differences. In all analysis, differences with probability values ≤ 0.05
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29 195 were considered significant.

31 32 196 33 34 197 35 198 **Results**

36 37 38 199 **DADS induce cell-cycle arrest and apoptosis in MCF-7 cells**

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41 200 Cell-cycle analysis of MCF-7 cells treated with 0-100 μ M DADS showed that such treatment
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43 201 causes changes in the occupation of the various stages of the cell cycle (Fig. 1). DADS
44
45 202 stimulated a concentration-dependent increase in the sub-G₀ population (representing
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47 203 apoptotic bodies and/or cells with fragmented DNA) combined with a corresponding
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49 204 decrease in the S-phase population. Thus, 100 μ M DADS induces a 56.9% ($p \leq 0.05$)
50
51 205 reduction in the proportion of cells engaging in DNA synthesis (the S-phase population)
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53 206 coupled with more than a tenfold increase (from 3.16 to 31.1%; $p \leq 0.05$) in the number of
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55 207 apoptotic, sub-G₀ cells (Fig. 1). In contrast, the G₀/G₁ and G₂/M populations were little
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3 208 affected by DADS (Fig. 1). The DADS induction of apoptosis was confirmed by Annexin
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5 209 V/PI double staining which detects both early and late apoptotic cells (AV and PI stained
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7 210 cells, respectively). Annexin V binds to phosphatidylserine (PS) translocated from the inner
8
9 211 leaflet of the plasma membrane (considered a hallmark of early apoptosis (45)). DADS
10
11 212 increased the number of early apoptotic cells from 11.0% (in untreated cells) to 18.2 ($p \leq$
12
13 213 0.05), 23.2 ($p \leq 0.05$) and 34.8% ($p \leq 0.05$) in 1, 10 and 100 μM treated cells, respectively.
14
15
16 214 Late apoptotic cells numbers were also increased in a dose-dependent manner from 7.17% in
17
18 215 untreated cells to 11.2 ($p \leq 0.05$), 16.3 ($p \leq 0.05$) and 24.9% ($p \leq 0.05$) in 1, 10 and 100 μM
19
20 216 DADS treated cells (Fig. 2). Thus, the double-staining analysis indicates that 100 μM DADS
21
22 217 increased total apoptotic cell numbers by more than threefold from 18.2 to 59.7% ($p \leq 0.05$)
23
24
25 218 (Fig. 2), a result which closely matches that obtained by single labeling (Fig. 1).

219 **DADS increases caspase-3 activity in MCF-7 cells**

220 In order to determine whether activation of caspase-3 might be involved in the observed
221 DADS-induced apoptosis of MCF-7 cells, such cells were tested for caspase-3 activity
222 following DADS treatment, by fluorescence assay. The results show that caspase-3 activity
223 increased by $80.8\% \pm 24.9$ and $270\% \pm 13.6$ in cells treated with 10 and 100 μM DADS,
224 respectively, compared to untreated cells (Fig. 3). Indeed, the caspase-3 induction achieved
225 by the highest dose of DADS (370%) was close to that obtained by staurosporine (500%) (the
226 positive control). However, low concentrations (1 μM) of DADS had no significant impact
227 on caspase-3 activation. 'Caspase-3 inhibitor' eliminated the effects of DADS on caspase-3
228 activity indicating that the increases in activity observed in the absence of inhibitor were
229 indeed caspase-3 specific (Fig. 3). These results show that DADS elicits a dose-dependent
230 stimulation of caspase-3 activity in MCF-7 cells (Fig. 3) which suggests that caspase-3
231 activation is a component of the DADS-dependent induction of apoptosis in MCF-7 cells.

232 DADS modulates the expression of Bcl-2 proteins in MCF-7 cells

233 In order to further confirm that DADS induces apoptosis in breast cancer cells and to
234 determine the likely regulatory pathway involved, Western blotting was used to examine the
235 effect of DADS on the levels of three pro-apoptotic proteins (Bax, Bak and Bid) in MCF-7
236 cells. The results show that cellular levels of Bax were raised by $23.0\% \pm 8.15$ and $32.4\% \pm$
237 7.83 , respectively, by treatment with 10 and 100 μM DADS, although levels were not notably
238 affected by DADS at 1 μM (Fig. 4). In contrast, DADS failed to significantly increase the
239 levels of either Bak or Bid in MCF-7 cells (Fig. 4). However, all three of the anti-apoptotic
240 Bcl-2 proteins examined here were reduced by DADS treatment: 10 and 100 μM DADS
241 decreased Bcl-2 cellular levels by $42.2\% \pm 9.13$ and $64.4\% \pm 9.7$; Bcl-xL cellular levels were
242 decreased by $33.3\% \pm 8.7$ and $57.7\% \pm 7.7$; and Bcl-w cellular levels were decreased by
243 $38.1\% \pm 8.4$ and $68.6\% \pm 7.4$, respectively (Fig. 4). Thus, DADS at 10-100 μM raises the
244 MCF-7 cellular levels of at least one pro-apoptotic Bcl-2 family protein (Bax) and decreases
245 those of at least three anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL and Bcl-w). These effects
246 are again fully consistent with the DADS-induced apoptosis reported above, and roles for
247 Bcl-2 proteins in modulating DADS-stimulated apoptosis in MCF-7 cells.

248

**249 DADS induces H₄ acetylation and inhibits the deacetylation activity of MCF-7 nuclear
250 extract**

251 The above studies show that the apoptosis effect generated by DADS in MCF-7 cells
252 correlates with appropriate changes in the levels Bcl-2 family proteins. Previous work
253 suggests that such changes in protein levels may arise from alterations in expression caused
254 by modifications of histone acetylation status (46, 47). To determine whether the effects of
255 DADS might arise through alterations in histone acetylation, global changes in overall

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3 256 histone acetylation levels were assayed using TAU gel electrophoresis (Fig.5 I). This analysis
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5 257 showed that histone 4 is primarily in the unacetylated and mono-acetylated forms in untreated
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7 258 MCF-7 cells. However, following treatment with 1 μ M DADS, the di-acetylated form of
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10 259 histone 4 became apparent as a minor species. As the DADS concentrations were raised more
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12 260 so, the acetylation profiles were further altered as tri- and tetra-acetylated histone 4 became
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14 261 increasingly apparent (Fig. 5I). Thus, DADS treatment raises the histone-4 acetylation status
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16 262 of MCF-7 nuclear DNA.

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18
19 263 To determine whether the increased histone acetylation levels are caused by DADS-mediated
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21 264 inhibition of deacetylase activity, the effect of DADS on the ability of MCF-7 nuclear
22
23 265 extracts to remove acetyl groups from an acetylated substrate was investigated (Fig.5 II). The
24
25 266 addition of DADS, at 10 and 100 μ M, to MCF-7 nuclear extracts diminished deacetylation
26
27 267 activities to $85.7\% \pm 17.6$ and $64.2\% \pm 12.9$ of that of controls, although 1 μ M DADS had no
28
29 268 significant effect. TSA, a potent deacetylases inhibitor (39), reduced deacetylation to 24.77%
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31 269 ± 7.6 of that of the control, showing that the assay was effective in reporting deacetylation
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33 270 inhibition. The results therefore suggest that the DADS-induced increase in acetylation of
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35 271 histone 4 is caused by the ability of DADS to act as a direct HDAC inhibitor.

36 37 38 39 40 272 **Discussion**

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42
43 273 The health benefits and medicinal properties of garlic have long been known. Records dating
44
45 274 back to 1550 BC describe garlic as a "wonder drug" for a variety of diseases (48).
46
47 275 Epidemiological studies report that high consumption of garlic decreases the risk of breast
48
49 276 cancer (49, 50). This effect has been attributed to DADS, which is present in garlic cloves at
50
51 277 ~ 140 mM as the major organosulfur compound of garlic oil (51), since it inhibits the growth
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53 278 of breast cancer cells *in vitro* through induction of apoptosis (14, 16). The effects of DADS
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55 279 are not confined to cancerous cells; it has been shown that DADS suppresses hepatic P450
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3 280 2E1 protein expression and *N*-nitrosodimethylamine demethylase activity (52, 53). It is
4
5 281 suggested that this may affect the metabolic activation of procarcinogens and the clearance
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7 282 rate of toxic metabolites (54).
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9

10 283 It should be stressed that human studies on garlic consumption and cancer risk provide
11
12 284 limited evidence of any relationship between garlic intake and protection against cancer (55-
13
14 285 58). However, the value of such human studies is often limited by variability in the garlic
15
16 286 preparations taken and uncertainty concerning the amounts of garlic consumed (58), and it is
17
18 287 suggested that further human trials are required in order to establish whether dietary garlic
19
20 288 exhibits any anti-cancerous effects (59). Important factors affecting DADS availability in
21
22 289 food include alkalinity and processing, which highlights the need to consider various aspects
23
24 290 of diet, as well as the composition of the garlic, when assessing any health benefit of garlic in
25
26 291 clinical trials (60-63). Although the direct exposure of human breast cancer cells to DADS
27
28 292 (as performed here) fails to replicate the complexity of the whole human system, such studies
29
30 293 have the advantage of avoiding the problems raised above that are associated with diet
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32 294 composition effects.
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38 295 The studies reported here confirm that DADS is able to induce apoptosis in the breast cancer
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40 296 cell line, MCF-7, by interfering with the cell cycle. DADS increased the sub-G₀, apoptotic
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42 297 population in a dose-dependent manner and at the same time decreased the occupancy of the
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44 298 S phase of the cycle, indicating an inhibition of DNA synthesis had occurred. These findings
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46 299 are consistent with those reported previously (11, 16, 64-67) where the growth inhibitory
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48 300 properties of DADS were also attributed to its induction of apoptosis and reduction in DNA
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50 301 synthesis in KPL-1, MCF-7, COLO 205, HCT-116 and B16F-10 cells. Examination of both
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52 302 early (*e.g.* PS translocation) and late (*e.g.* caspase-3 activation) molecular events
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54 303 characteristic of apoptosis confirmed the ability of DADS, at physiologically relevant
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56 304 concentration (10–100 μ M), to induce apoptosis in MCF-7 cells.
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3 305 Bcl-2 family proteins play an important role in the progression of apoptosis through
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5 306 controlling the release of apoptogenic factors, *e.g.* cytochrome *c* and ‘apoptosis-inducing
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7 307 factor’ (AIF), from the mitochondrial inter-membrane space into the cytosol (20, 68).
8
9 308 Released cytochrome *c* binds to cytosolic Apaf-1 to form the ‘apoptosome’ in a reaction that
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11 309 eventually leads to the autoactivation of pro-caspase-9 (69) which in turn activates the
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13 310 effector caspases (-3, -6 and -7) leading to apoptosis (70). Released AIF is translocated to the
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15 311 nucleus where it induces DNA fragmentation (71). The balance between the levels of pro-
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17 312 and anti-apoptotic proteins within the cell is crucial in determining whether apoptosis
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19 313 progresses (72). The involvement of Bcl-2 family proteins in DADS-induced apoptosis in
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21 314 MCF-7 cells was suggested (Fig. 4) by the DADS-dependent increase of the cellular levels of
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23 315 pro-apoptotic Bax and concomitant decreases of anti-apoptotic Bcl-2, Bcl-xL and Bcl-w. The
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25 316 results therefore suggest the involvement of Bcl-2 family proteins in the progression of
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27 317 DADS-induced caspase-dependent apoptosis in MCF-7 cells. The results reported here are in
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29 318 accordance with previous work (14), where DADS was shown to cause caspase-dependent
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31 319 apoptosis in human breast cancer cells MCF-7 through the Bax-triggered mitochondrial
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33 320 pathway. The involvement of Bcl-2 family proteins in DADS-induced apoptosis was also
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35 321 shown in human lung adenocarcinoma A549 cells (21), where a decrease in the expression of
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37 322 Bcl-2 (but no change of Bax levels) was elicited by treatment with 200 μ M DADS for 24 h.
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43 323 Interest in HDAC inhibitors has risen recently because of their anticancer potential; various
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45 324 HDACi have the ability to suppress cancer growth and induce apoptosis *in vitro* in cancer cell
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47 325 cultures and *in vivo* in tumor bearing animal models (73-76). In this study, it was shown that
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49 326 DADS has HDACi properties when presented at relatively low concentrations (10-100 μ M)
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51 327 that are likely to be well within dietary concentrations ranges (51). Low concentrations of
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53 328 DADS were found to inhibit the removal of the acetyl group from an acetylated substrate
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55 329 through reducing the HDAC enzymatic activity of MCF-7 nuclear extracts by up to 35.8%.
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3 330 Furthermore, the appearance of di-, tri- and tetra-acetylated H4 in DADS-treated cells
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5 331 demonstrated the induction of histone hyper-acetylation by DADS. Induction of cellular
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7 332 changes characteristic of apoptosis, such as cell cycle arrest, cellular senescence, and
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9 333 activation of cell death in cancerous cells upon the treatment with a variety of HDAC
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11 334 inhibitors, has been confirmed in many studies, although the underlying mechanisms are not
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13 335 entirely clear (77-79). Such apoptotic changes were clearly seen here in DADS-exposed
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15 336 MCF-7 cells. Previous studies have shown that modulation of gene expression occurs upon
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17 337 alteration of the acetylation status of associated histones. It is thought that histone hyper-
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19 338 acetylation promotes localised chromatin de-condensation (80) which thereby facilitates
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21 339 binding of transcriptional factors at cognate promoters, leading to induction of gene
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23 340 expression. DADS is likely to operate similarly by inducing histone hyper-acetylation
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25 341 through its HDACi activity resulting in alterations in the expression of various apoptosis
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27 342 factors, such as the pro- and anti-apoptotic Bcl-2 family proteins as observed here. However,
28
29 343 the precise mechanism by which Bcl-2 family protein expression is modulated and apoptosis
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31 344 is induced by DADS remains unclear. Similar effects were obtained when a renal tubular cell
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33 345 line (RPTC) was treated with a histone deacetylase inhibitor (suberoylanilide hydroxamic
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35 346 acid). This caused a decrease in Bcl-xL levels, while the expression levels of Bax and Bak
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37 347 remained relatively constant (81). It is unlikely that all of the expression changes observed
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39 348 here are a direct effect of histone acetylation status, since levels of Bcl-2, Bcl-xL and Bcl-w
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41 349 were decreased (rather than increased) by DADS. Thus, further work is required to decipher
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43 350 the precise manner in which DADS promotes apoptosis in MCF-7 breast cancer cells.
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50 **Acknowledgments**
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7 354 kits necessary to accomplish this study.
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12 356 **Abbreviations**

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16 357 DADS Diallyl disulphide

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19 358 DMSO Dimethylsulfoxide

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22 359 FBS Fetal bovine serum

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25 360 HDAC Histone deacetylation

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28 361 HDACi Histone deacetylation inhibitor

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31 362 PBS Phosphate buffered saline

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34 363 PI Propidium iodide

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37 364 PS phosphatidylserine

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40 365 RIPA Radio-Immuno Precipitation Assay

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43 366 RT Room temperature

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46 367 TSA Trichostatin A

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369 **References**

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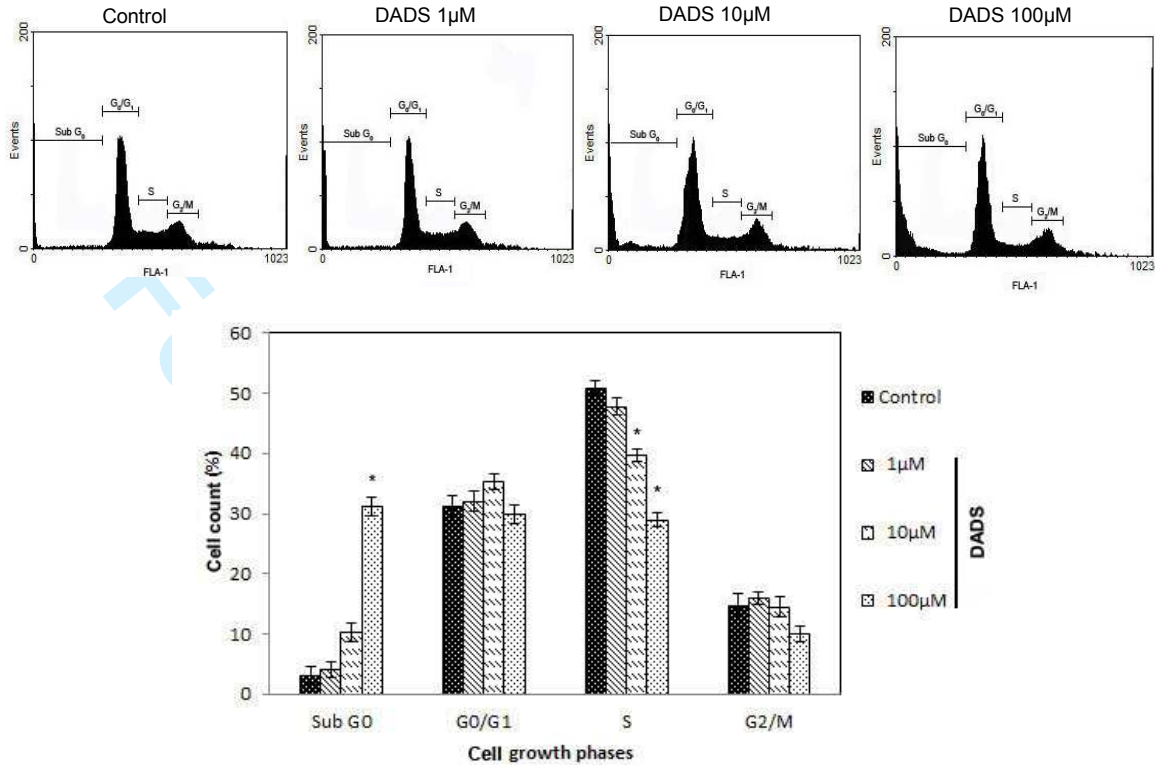


Figure 1: Cell-cycle analysis of PI labeled MCF-7 cells following treatment with 1, 10 and 100 µM DADS for 24 h. The results are reported as mean ± SE of three independent experiments. *, significance ($p \leq 0.05$).

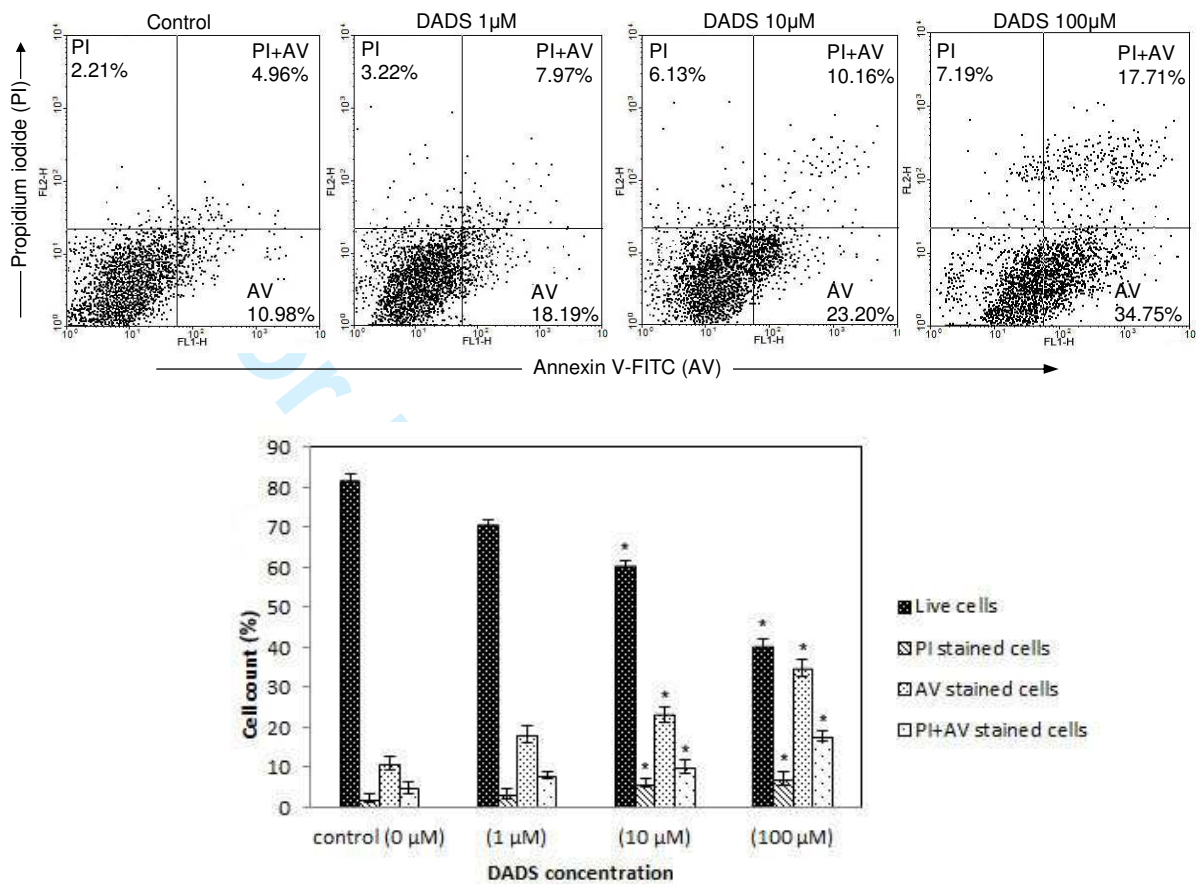


Figure 2: Estimation of the effect of DADS on the proportion of MCF-7 cells in late and early apoptotic states. The analysis was performed by flow-cytometry of Annexin V/PI-labeled cells following DADS (1, 10 and 100 μM) treatment for 24 h. The results are reported as mean ± SE of three independent experiments. *, significant ($p \leq 0.05$).

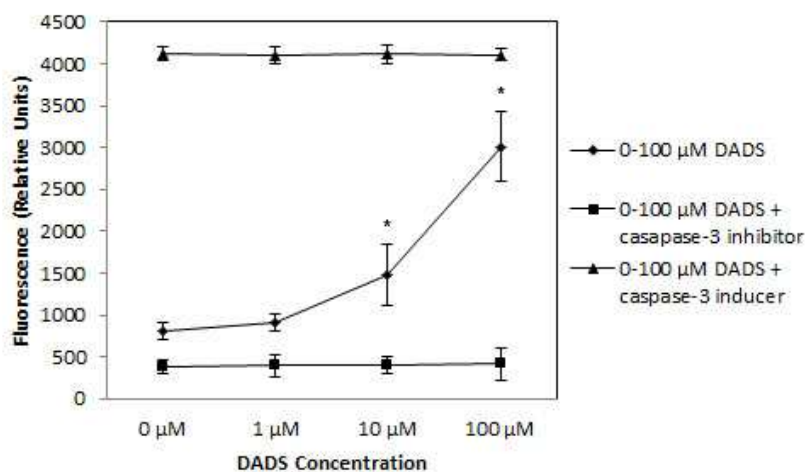


Figure 3: Fluorescence assay of caspase-3 activity in MCF-7 cells following DADS (1, 10 and 100 μM) treatment for 24 h. The positive control consists of MCF-7 cells treated as above except for the inclusion of the caspase-3 inducer, staurosporine (3 $\mu\text{g}/\text{ml}$), for the final 4 h. Negative controls were also obtained as above except that the inducer was replaced by a caspase-3 inhibitor solution (10 μl ; see Methods) which was present for the entire 24 h growth period. The results are reported as mean \pm SE of three independent experiments. *, significant ($p \leq 0.05$).

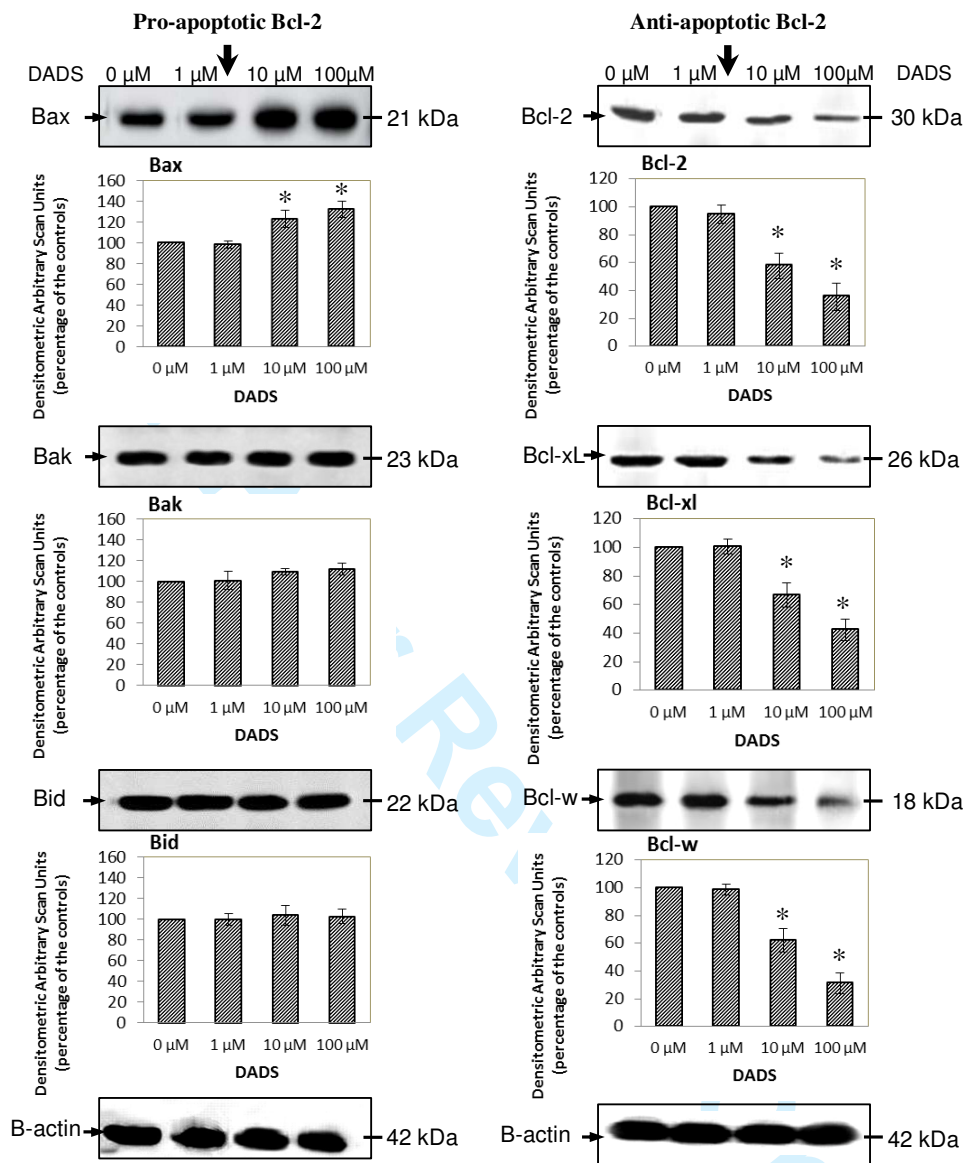


Figure 4: Western blot and densitometric analysis showing the effect of DADS on the levels of pro- and anti-apoptotic Bcl-2 proteins in MCF-7 cells. Cell cultures were treated with DADS for 24 h. Loading control, β -actin detection. Densitometric analysis was performed using TotalLab™ software. The results are reported as mean \pm SE of three independent experiments. *, significant ($p < 0.05$).

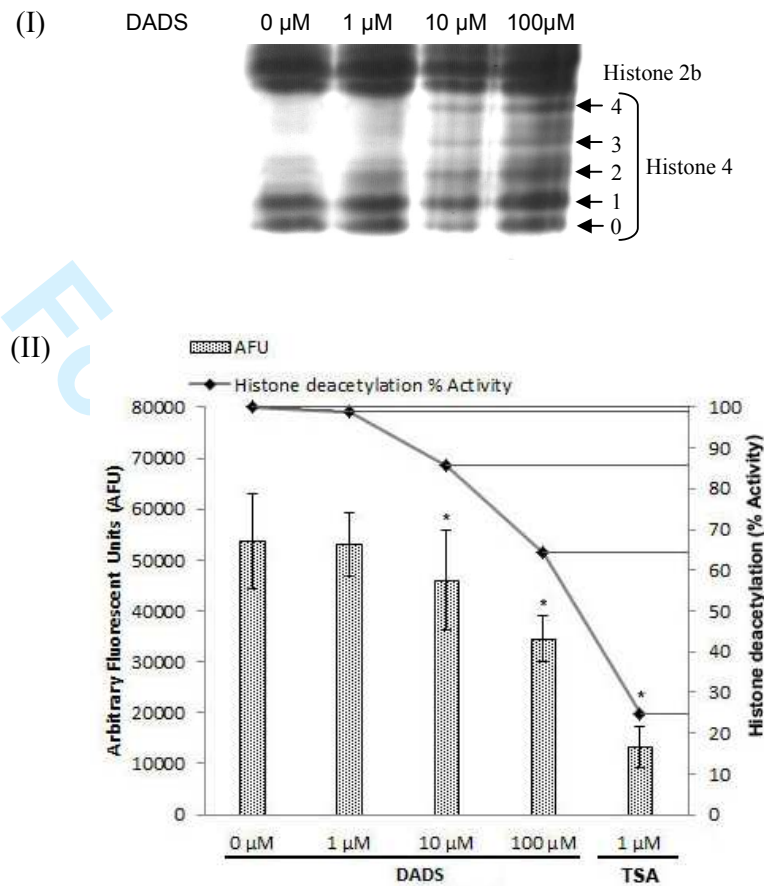


Figure 5: Effect of DADS on histone acetylation in MCF-7 cells. **(I)** DADS induction (24 h) of histone 4 hyper-acetylation in MCF-7 cells, assessed by TAU gel electrophoresis as in materials and methods. **(II)** DADS inhibition of histone deacetylation in MCF-7 nuclear extracts as determined using the Fluor de Lys substrate (Fluorimetric Assay/Drug Discovery Kit; BIOMOL); data is represented as arbitrary fluorescent units and measured as a percentage of the fluorescence signal obtained with no addition of DADS or TSA to the reaction mixture. The results are reported as mean \pm SE of three independent experiments. *, significant ($p \leq 0.05$).