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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

19   **Abstract**

20   The health benefits of garlic have been proven by epidemiological and experimental studies.

21   Diallyl disulphide (DADS), the major organosulfur compound found in garlic oil, is known to

22   lower the incidence of breast cancer both *in vitro* and *in vivo*. The studies reported here

23   demonstrate that DADS induces apoptosis in the MCF-7 breast-cancer cell line through

24   interfering with cell-cycle growth phases in a way that increases the sub-G_0 population and

25   substantially halts DNA synthesis. DADS also induces phosphatidylserine (PS) translocation

26   from the inner to the outer leaflet of the plasma membrane and activates caspase-3. Further

27   studies revealed that DADS modulates the cellular levels of Bax, Bcl-2, Bcl-xL and Bcl-w in

28   a dose-dependent manner, suggesting the involvement of Bcl-2 family proteins in DADS

29   induced apoptosis. Histone deacetylation inhibitors (HDACi) are known to suppress cancer

30   growth and induce apoptosis in cancer cells. Here it is shown that DADS has HDACi

31   properties in MCF-7 cells as it lowers the removal of an acetyl group from an acetylated

32   substrate and induces histone-4 (H4) hyper-acetylation. The data thus indicate that the

33   HDACi properties of DADS may be responsible for the induction of apoptosis in breast

34   cancer cells.

35   **Keywords**

36   Breast cancer, Diallyl disulphide, Apoptosis, Histone deacetylation inhibitor.

37

Introduction

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

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

3

62 proteins function by promoting mitochondrial permeability, resulting in the release of

63 apoptogenic factors (*e.g.* cytochrome *c* and apoptosis inducing factor [AIF]) from the

64 mitochondrial inter-membrane space into the cytosol (20). The presence of such factors in the

65 cytosolic compartment induces the activation of protease activators (caspases) that ultimately

66 lead to apoptosis. Either increasing levels of pro-apoptotic Bcl-2 proteins or decreasing levels

67 of anti-apoptotic Bcl-2 proteins (or a combination of both) can result in activation of the

68 apoptotic machinery, thereby initiating apoptotic cell death. In this context, DADS up-

69 regulates pro-apoptotic Bcl-2 proteins in many types of cancerous cells. For example, DADS-

70 induces Bax expression and triggers the apoptotic mitochondrial pathway in MCF-7 human

71 breast cancer cells (14), and is known to induce apoptosis through down-regulation of anti-

72 apoptotic Bcl-2 in A549 human lung adenocarcinoma cells (21). DADS also caused down-

73 regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL in a human colon cancer cell line

74 (COLO 205), whereas it induced up-regulation of the pro-apoptotic Bak and Bax proteins

75 (22). Furthermore, DADS treatment of a human prostate cancer cell line (PC-3) resulted in

76 increased cellular levels of pro-apoptotic BAX and BAD, but decreased Bcl-2 levels (23). In

77 a similar study by Arunkumar *et al*. (2007), the apoptotic impact of DADS in PC-3 cells were

78 accompanied by histone (H3 and H4) hyper acetylation (24).

79 Aberrant gene transcription is common in malignant cells, resulting in activation of some

80 genes and silencing of others (25, 26). Such changes in transcriptional status are often

81 correlated with alterations in histone acetylation with hyper-acetylation mediating activation

82 whilst de-acetylation promotes gene silencing (27, 28). Histone acetylation controls

83 transcription by facilitating or restraining the recruitment of transcription factors on to

84 associated promoter (29, 30). Histone acetylation has received considerable interest of late

85 because of its link to the pathogenesis of cancer. The progression of carcinogenesis involves

86 the transcriptional activation of 'inappropriate' genes (e.g. oncogenes, RAS, WNT, MYC,

4

87   ERK, TRK and Bcr-Abl) at the expense of tumour suppressor genes (e.g. TP53, CDKN1B,

88   HNPCC, MEN1, APC, CD95, ST5, ST7, ST14 and BRCA) and pro-apoptotic genes (e.g.

89   pro-apoptotic Bcl-2 proteins) (31-35). Since aberrant transcription of Bcl-2 family genes is

90   often observed in breast cancer cells (36, 37), this raises the possibility that the modulation of

91   expression of such genes by DADS is mediated by DADS-induced histone deacetylation

92   inhibition (HDACi).

93   This possibility raised above is addressed here through the investigation of the effect of

94   DADS on the acetylation status of histones in the breast-cancer cell line, MCF-7. The data

95   confirm that DADS strongly induces apoptosis in MCF-7 cells, which is apparently caspase

96   dependent. DADS also enhances histone hyper-acetylation in MCF-7 cells, through its

97   HDACi activity, and promotes pro-apoptotic Bax but depresses anti-apoptotic Bcl-2, Bcl-xL

98   and Bcl-w. Thus, the anti-cancerous activity of DADS could arise from its HDACi effect.

99   **Materials and Methods**

100   **Cell line and treatment**

101   The human mammary gland adenocarcinoma cell line (MCF-7) (38) was kindly provided by

102   Professor A El-Tayeb (Assiut University) and cells were used following 43–51 passages.

103   Cells were cultured in Dulbecco's modified Eagles's minimum essential medium

104   (CAMBREX) supplemented with 10% heat-inactivated (60 °C, 30 min) fetal bovine serum

105   (FBS, CAMBREX) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), in six-well

106   tissue culture plates in a concentration of 10^5 cells/ml at 37 ˚C in a 5% CO_2/95% humidified

107   atmosphere. Culturing media were supplemented with DADS (Sigma, purity ≤ 80%)

108   dissolved in dimethylsulfoxide [(DMSO), Aldrich, purity ≤ 95.5%)] in final concentrations of

109   1, 10 or 100 µM DADS. The DMSO final concentration did not exceed 0.1% in all treatments

5

110 including control cultures. Trichostatin A (TSA), at 1 μM final concentration, was used to

111 inhibit histone deacetylase activities in positive control samples (39)

Cell cycle analysis

113 MCF-7 cells, treated and control, were harvested by trypsinization. The cells were washed

114 with phosphate buffered saline (PBS). Cells were then fixed by pipetting 1 ml of cell

115 suspension (approximately 10^6 cells/ml) was on to 4 ml of absolute ethanol at -20 °C in a

116 Falcon tube while vortexing at top speed. Fixed cells were re-hydrated in 5 ml PBS for 15

117 min at room temperature (RT). Finally, 3 mM propidium iodide (PI) in staining buffer (100

118 mM Tris; pH 7.4, 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2, 0.1% NP-40) was added and

119 mixed by gentle pipetting. Pancreatic RNAase (50 µl of 10 µg/ml stock; Sigma) was added

120 and the cells were incubated for 2 h at 4 °C. Cell cycle analysis was performed using a

121 FACScan Flow Cytometer (Becton Dickson) according to the manufacturer's protocol.

Annexin V/PI test

123 MCF-7 cells were tested for apoptosis induction by DADS using Annexin V-FITC/PI double

124 staining. Briefly, approximately 10^6 cells were washed in cold PBS followed by two washes

125 in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$,

126 pH 7.4). Cells were then re-suspended in 100 μl Annexin V-FITC (4 μg/ml in binding buffer)

127 and incubated on ice for 10 min. An additional 400 ml of binding buffer, containing PI

128 (2 μg/ml), were added and the cellular suspension was incubated on ice for a further 15 min.

129 Cells thus treated were then analysed by flow cytometry.

Caspase-3 activation

131 MCF-7 cells were seeded in 96-well plates at a density of 10^5 cells/well. Plates were then

132 incubated overnight at 37˚C in a '5% CO_2/95% humidity' atmosphere. DADS was added at

133 final concentrations of 1, 10 and 100 μM and cells were incubated for an additional 24 h. For

6

134   positive controls, cells were treated with staurosporine (3 μg/ml) for 4 h (40) prior to

135   performing the assay. Caspase-3 activation upon DADS treatment was measured using the

136   Caspase-3 Fluorescence Assay Kit (Cayman chemicals) according to the manufacturer's

137   instructions. In order to test assay specificity, negative control samples were prepared by

138   adding 10 μl/well of caspase-3 inhibitor solution (provided with the kit). The plates were read

139   on a FLUOstar OPTIMA fluorescent plate reader at 485 nm excitation and 535 nm emission

140   wavelengths.

141   **Cellular lysate preparation and Western blot**

142   Cells were lysed with Radio-Immuno Precipitation Assay (RIPA) lysis buffer (50 mM Tris–

143   HCl, pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF

144   [phenylmethylsulphonyl fluoride], 1 μg/ml aprotinin, 1% NP-40) for 30 min on ice. The

145   cellular lysate was then centrifuged at 12,000×g for 2 min at 4 °C. The supernatant was then

146   transferred to a fresh Eppendorf tube, and total protein concentrations were determined using

147   the BioRad protein assay (41). Cellular lysates were then denatured with an equal volume of

148   loading buffer (0.125 M Tris–HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02%

149   bromophenol blue, pH 6.8) at 100 °C for 10 min. Protein samples (50 μg) were then

150   immediately subjected to SDS-PAGE (BioRad Mini Protein II Electrophoresis gel) and trans-

151   blotted onto a nitrocellulose membrane (Amersham). The membranes were blocked in 5%

152   non-fat dry milk in TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) overnight at 4 °C. The

153   membranes were immuno-stained for pro-apoptotic (Bax, Bak and Bid) and anti-apoptotic

154   Bcl-2 proteins (Bcl-2 and Bcl-xL [rabbit polyclonal, Abcam] and Bcl-w [Cell Signaling

155   Technology®]). β-Actin primary antibody (mouse monoclonal, Abcam) was used as a

156   loading control. Immuno-detected band visualisation was carried out using the

157   chemiluminescent alkaline phosphatase substrate (Immobilom™ Western).

7

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 $MgCl_2$, 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 x*g* and 4 °C for 10 min. The resulting nuclear pellet was

164   suspended in H_2SO_4 (0.2 M final concentration) for 2 h and centrifuged at 10,000 x*g* 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% NH_4OH. 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 $MgCl_2$, 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 $MgCl_2$, pH 7.5), and centrifuged at 1300 x*g* 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 x*g*, 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

8

183   ultrasonicated for 30 s and kept on ice for 30 min. The mixture was then centrifuged at

184   10,000 x*g* and 4 °C for 10 min. The supernatant (nuclear extract) was collected and kept at -

185   80 °C for HDAC activity analysis. Different concentrations of DADS were tested for HDACi

186   activity, using the fluor de lys[TM] fluorescent assay system (Drug discovery kit-AK-500;

187   BIOMOL) according to the manufacture instructions. The kit includes deacetylases inhibitor

188   (Trichostatin A [TSA]) for use as positive control. The samples were quantified on a

189   FLUOstar OPTIMA fluorescent plate reader at 360 nm excitation and 460 nm emission

190   wavelengths.

191   **Statistical Analysis**

192   All data were obtained from three independent experiments, and all results are expressed as

193   mean±SE. We employed the two-tailed (or paired) Student's *t*-test, using Microsoft Excel, to

194   determine significant differences. In all analysis, differences with probability values ≤ 0.05

195   were considered significant.

196

197
198   **Results**

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

200   Cell-cycle analysis of MCF-7 cells treated with 0-100 μM DADS showed that such treatment

201   causes changes in the occupation of the various stages of the cell cycle (Fig. 1). DADS

202   stimulated a concentration-dependent increase in the sub-G_0 population (representing

203   apoptotic bodies and/or cells with fragmented DNA) combined with a corresponding

204   decrease in the S-phase population.   Thus, 100 μM DADS induces a 56.9% ($p \leq 0.05$)

205   reduction in the proportion of cells engaging in DNA synthesis (the S-phase population)

206   coupled with more than a tenfold increase (from 3.16 to 31.1%; $p \leq 0.05$) in the number of

207   apoptotic, sub-G_0 cells (Fig. 1).   In contrast, the G_0/G_1 and G_2/M populations were little

9

208 affected by DADS (Fig. 1). The DADS induction of apoptosis was confirmed by Annexin

209 V/PI double staining which detects both early and late apoptotic cells (AV and PI stained

210 cells, respectively). Annexin V binds to phosphatidylserine (PS) translocated from the inner

211 leaflet of the plasma membrane (considered a hallmark of early apoptosis (45)). DADS

212 increased the number of early apoptotic cells from 11.0% (in untreated cells) to 18.2 ($p \leq$

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.

214 Late apoptotic cells numbers were also increased in a dose-dependent manner from 7.17% in

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

216 DADS treated cells (Fig. 2). Thus, the double-staining analysis indicates that 100 µM DADS

217 increased total apoptotic cell numbers by more than threefold from 18.2 to 59.7% ($p \leq 0.05$)

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% ± 24.9 and 270% ± 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.

10

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% ± 8.15 and 32.4% ±

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% ± 9.13 and 64.4% ± 9.7; Bcl-xL cellular levels were

242   decreased by 33.3% ± 8.7 and 57.7% ± 7.7; and Bcl-w cellular levels were decreased by

243   38.1% ± 8.4 and 68.6% ± 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 H$_4$ 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

11

256 histone acetylation levels were assayed using TAU gel electrophoresis (Fig.5 I). This analysis

257 showed that histone 4 is primarily in the unacetylated and mono-acetylated forms in untreated

258 MCF-7 cells. However, following treatment with 1 μM DADS, the di-acetylated form of

259 histone 4 became apparent as a minor species. As the DADS concentrations were raised more

260 so, the acetylation profiles were further altered as tri- and tetra-acetylated histone 4 became

261 increasingly apparent (Fig. 5I). Thus, DADS treatment raises the histone-4 acetylation status

262 of MCF-7 nuclear DNA.

263 To determine whether the increased histone acetylation levels are caused by DADS-mediated

264 inhibition of deacetylase activity, the effect of DADS on the ability of MCF-7 nuclear

265 extracts to remove acetyl groups from an acetylated substrate was investigated (Fig.5 II). The

266 addition of DADS, at 10 and 100 μM, to MCF-7 nuclear extracts diminished deacetylation

267 activities to $85.7\% \pm 17.6$ and $64.2\% \pm 12.9$ of that of controls, although 1 μM DADS had no

268 significant effect. TSA, a potent deacetylases inhibitor (39), reduced deacetylation to 24.77%

269 ± 7.6 of that of the control, showing that the assay was effective in reporting deacetylation

270 inhibition. The results therefore suggest that the DADS-induced increase in acetylation of

271 histone 4 is caused by the ability of DADS to act as a direct HDAC inibitor.

272 **Discussion**

273 The health benefits and medicinal properties of garlic have long been known. Records dating

274 back to 1550 BC describe garlic as a "wonder drug" for a variety of diseases (48).

275 Epidemiological studies report that high consumption of garlic decreases the risk of breast

276 cancer (49, 50). This effect has been attributed to DADS, which is present in garlic cloves at

277 ~140 mM as the major organosulfur compound of garlic oil (51), since it inhibits the growth

278 of breast cancer cells *in vitro* through induction of apoptosis (14, 16). The effects of DADS

279 are not confined to cancerous cells; it has been shown that DADS suppresses hepatic P450

12

280   2E1 protein expression and *N*-nitrosodimethylamine demethylase activity (52, 53). It is

281   suggested that this may affect the metabolic activation of procarcinogens and the clearance

282   rate of toxic metabolites (54).

283   It should be stressed that human studies on garlic consumption and cancer risk provide

284   limited evidence of any relationship between garlic intake and protection against cancer (55-

285   58). However, the value of such human studies is often limited by variability in the garlic

286   preparations taken and uncertainty concerning the amounts of garlic consumed (58), and it is

287   suggested that further human trials are required in order to establish whether dietary garlic

288   exhibits any anti-cancerous effects (59). Important factors affecting DADS availability in

289   food include alkalinity and processing, which highlights the need to consider various aspects

290   of diet, as well as the composition of the garlic, when assessing any health benefit of garlic in

291   clinical trials (60-63). Although the direct exposure of human breast cancer cells to DADS

292   (as performed here) fails to replicate the complexity of the whole human system, such studies

293   have the advantage of avoiding the problems raised above that are associated with diet

294   composition effects.

295   The studies reported here confirm that DADS is able to induce apoptosis in the breast cancer

296   cell line, MCF-7, by interfering with the cell cycle. DADS increased the sub-G_0, apoptotic

297   population in a dose-dependent manner and at the same time decreased the occupancy of the

298   S phase of the cycle, indicating an inhibition of DNA synthesis had occurred. These findings

299   are consistent with those reported previously (11, 16, 64-67) where the growth inhibitory

300   properties of DADS were also attributed to its induction of apoptosis and reduction in DNA

301   synthesis in KPL-1, MCF-7, COLO 205, HCT-116 and B16F-10 cells. Examination of both

302   early (*e.g.* PS translocation) and late (*e.g.* caspase-3 activation) molecular events

303   characteristic of apoptosis confirmed the ability of DADS, at physiologically relevant

304   concentration (10–100 μM), to induce apoptosis in MCF-7 cells.

13

305   Bcl-2 family proteins play an important role in the progression of apoptosis through

306   controlling the release of apoptogenic factors, *e.g.* cytochrome *c* and 'apoptosis-inducing

307   factor' (AIF), from the mitochondrial inter-membrane space into the cytosol (20, 68).

308   Released cytochrome *c* binds to cytosolic Apaf-1 to form the 'apoptosome' in a reaction that

309   eventually leads to the autoactivation of pro-caspase-9 (69) which in turn activates the

310   effector caspases (-3, -6 and -7) leading to apoptosis (70). Released AIF is translocated to the

311   nucleus where it induces DNA fragmentation (71). The balance between the levels of pro-

312   and anti-apoptotic proteins within the cell is crucial in determining whether apoptosis

313   progresses (72). The involvement of Bcl-2 family proteins in DADS-induced apoptosis in

314   MCF-7 cells was suggested (Fig. 4) by the DADS-dependent increase of the cellular levels of

315   pro-apoptotic Bax and concomitant decreases of anti-apoptotic Bcl-2, Bcl-xL and Bcl-w. The

316   results therefore suggest the involvement of Bcl-2 family proteins in the progression of

317   DADS-induced caspase-dependent apoptosis in MCF-7 cells. The results reported here are in

318   accordance with previous work (14), where DADS was shown to cause caspase-dependent

319   apoptosis in human breast cancer cells MCF-7 through the Bax-triggered mitochondrial

320   pathway. The involvement of Bcl-2 family proteins in DADS-induced apoptosis was also

321   shown in human lung adenocarcinoma A549 cells (21), where a decrease in the expression of

322   Bcl-2 (but no change of Bax levels) was elicited by treatment with 200 μM DADS for 24 h.

323   Interest in HDAC inhibitors has risen recently because of their anticancer potential; various

324   HDACi have the ability to suppress cancer growth and induce apoptosis *in vitro* in cancer cell

325   cultures and *in vivo* in tumor bearing animal models (73-76). In this study, it was shown that

326   DADS has HDACi properties when presented at relatively low concentrations (10-100 μM)

327   that are likely to be well within dietary concentrations ranges (51). Low concentrations of

328   DADS were found to inhibit the removal of the acetyl group from an acetylated substrate

329   through reducing the HDAC enzymatic activity of MCF-7 nuclear extracts by up to 35.8%.

14

330   Furthermore, the appearance of di-, tri- and tetra-acetylated H4 in DADS-treated cells

331   demonstrated the induction of histone hyper-acetylation by DADS. Induction of cellular

332   changes characteristic of apoptosis, such as cell cycle arrest, cellular senescence, and

333   activation of cell death in cancerous cells upon the treatment with a variety of HDAC

334   inhibitors, has been confirmed in many studies, although the underlying mechanisms are not

335   entirely clear (77-79). Such apoptotic changes were clearly seen here in DADS-exposed

336   MCF-7 cells. Previous studies have shown that modulation of gene expression occurs upon

337   alteration of the acetylation status of associated histones. It is thought that histone hyper-

338   acetylation promotes localised chromatin de-condensation (80) which thereby facilitates

339   binding of transcriptional factors at cognate promoters, leading to induction of gene

340   expression. DADS is likely to operate similarly by inducing histone hyper-acetylation

341   through its HDACi activity resulting in alterations in the expression of various apoptosis

342   factors, such as the pro- and anti-apoptotic Bcl-2 family proteins as observed here. However,

343   the precise mechanism by which Bcl-2 family protein expression is modulated and apoptosis

344   is induced by DADS remains unclear. Similar effects were obtained when a renal tubular cell

345   line (RPTC) was treated with a histone deacetylase inhibitor (suberoylanilide hydroxamic

346   acid). This caused a decrease in Bcl-xL levels, while the expression levels of Bax and Bak

347   remained relatively constant (81). It is unlikely that all of the expression changes observed

348   here are a direct effect of histone acetylation status, since levels of Bcl-2, Bcl-xL and Bcl-w

349   were decreased (rather than increased) by DADS. Thus, further work is required to decipher

350   the precise manner in which DADS promotes apoptosis in MCF-7 breast cancer cells.

351   **Acknowledgments**

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355

356   **Abbreviations**

357   DADS   Diallyl disulphide

358   DMSO   Dimethylsulfoxide

359   FBS   Fetal bovine serum

360   HDAC   Histone deacetylation

361   HDACi   Histone deacetylation inhibitor

362   PBS   Phosphate buffered saline

363   PI   Propidium iodide

364   PS   phosphatidylserine

365   RIPA   Radio-Immuno Precipitation Assay

366   RT   Room temperature

367   TSA   Trichostatin A

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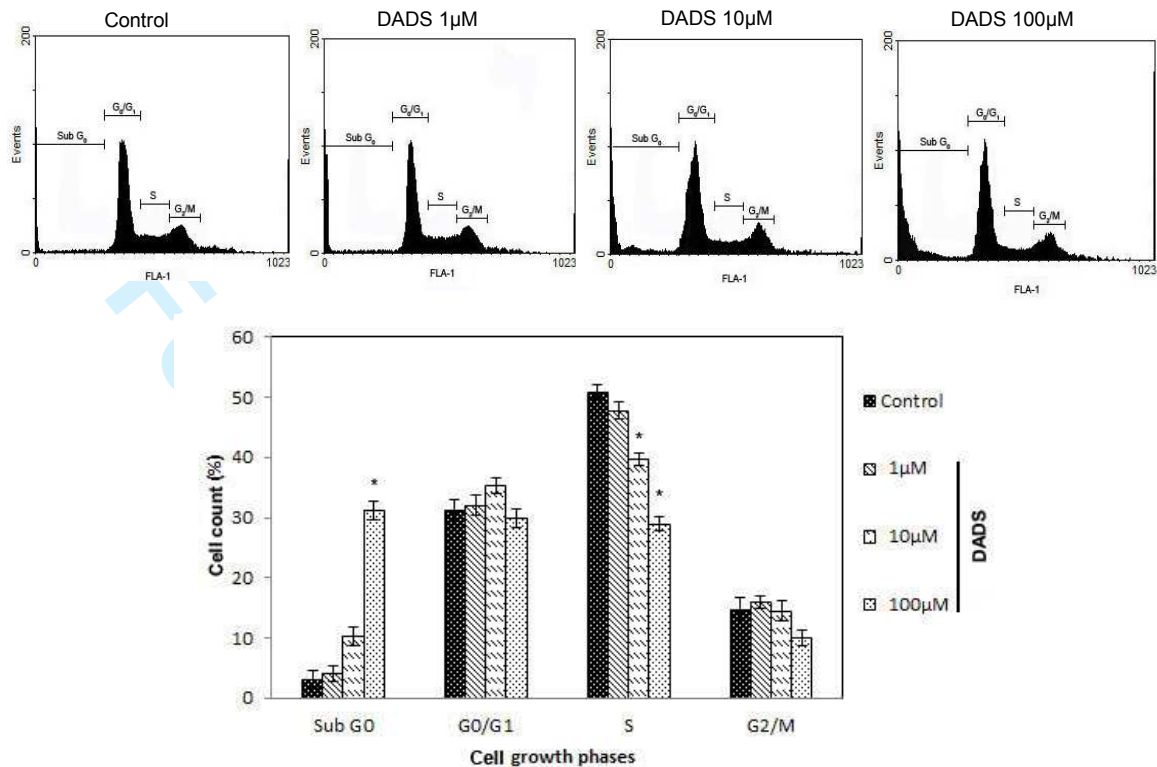589   *Pharmacol Exp Ther*. 2008; 325: 978-84.
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Figure 1: Cell-cycle analysis of PI labeled MCF-7 cells following treatment with 1, 10 and 100 μM DADS for 24 h. The results are reported as mean ± SE of three independent experiments. *, significance ($p \leq 0.05$).
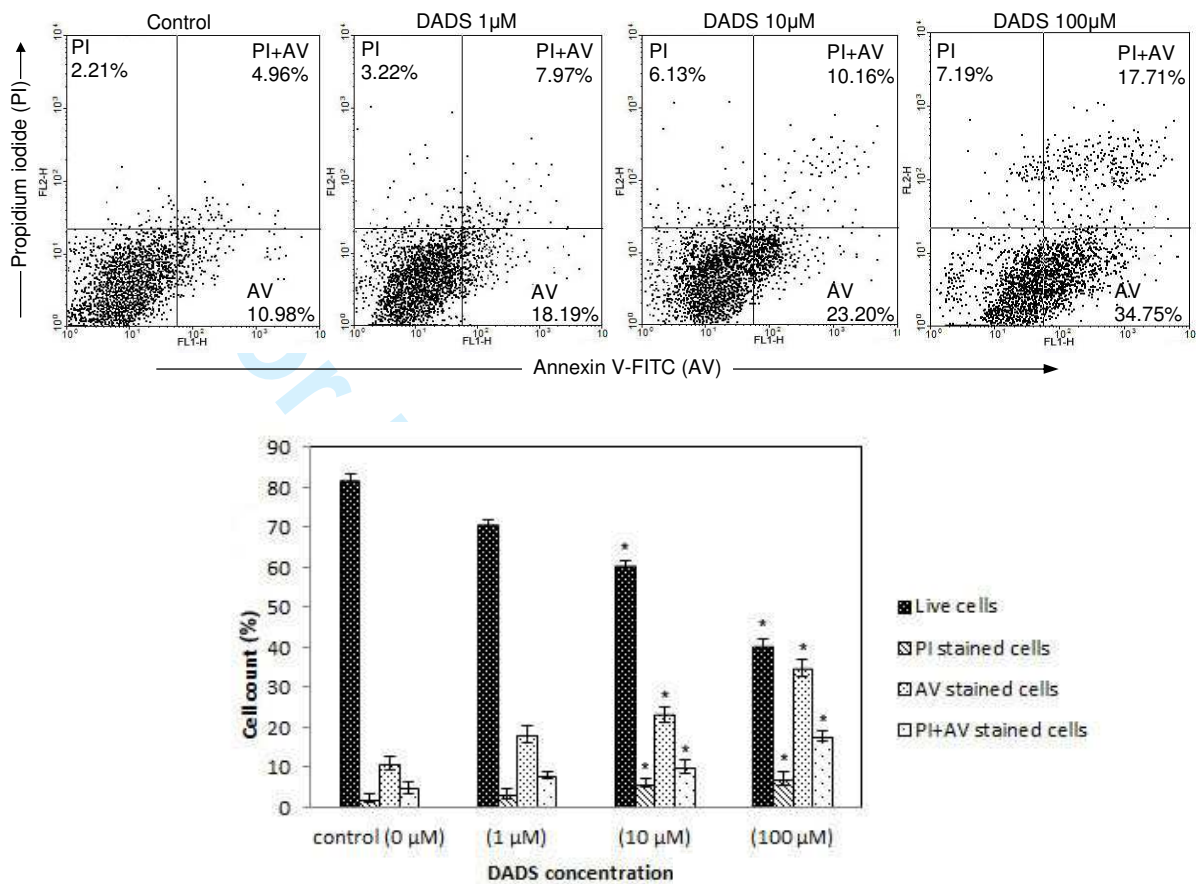
Figure 2: Estimation of the effect of DADS on the proportion of MCF-7 cells in late and early apoptotic states. The analysis was performed by flow-cytometry of Annexin V/PI-labeled cells following DADS (1, 10 and 100 μM) treatment for 24 h. The results are reported as mean ± SE of three independent experiments. *, significant ($p \leq 0.05$).
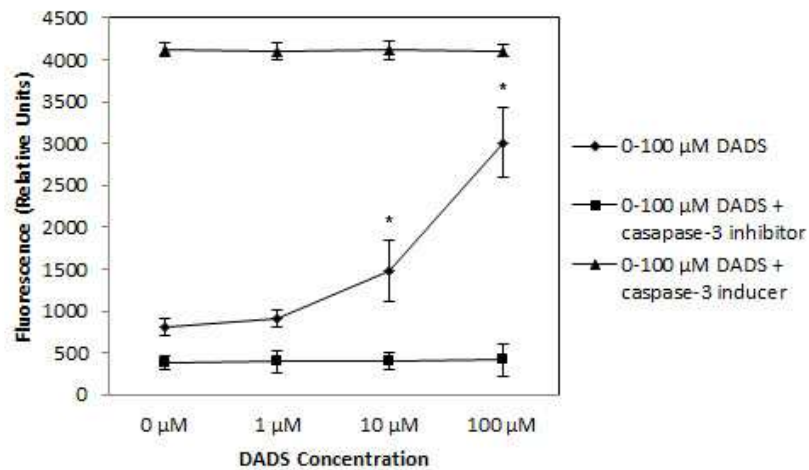
Figure 3: Fluorescence assay of caspase-3 activity in MCF-7 cells following DADS (1, 10 and 100 μM) treatment for 24 h. The positive control consists of MCF-7 cells treated as above except for the inclusion of the caspase-3 inducer, staurosporine (3 μg/ml), for the final 4 h. Negative controls were also 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 ± SE of three independent experiments. *, significant ($p \leq 0.05$).
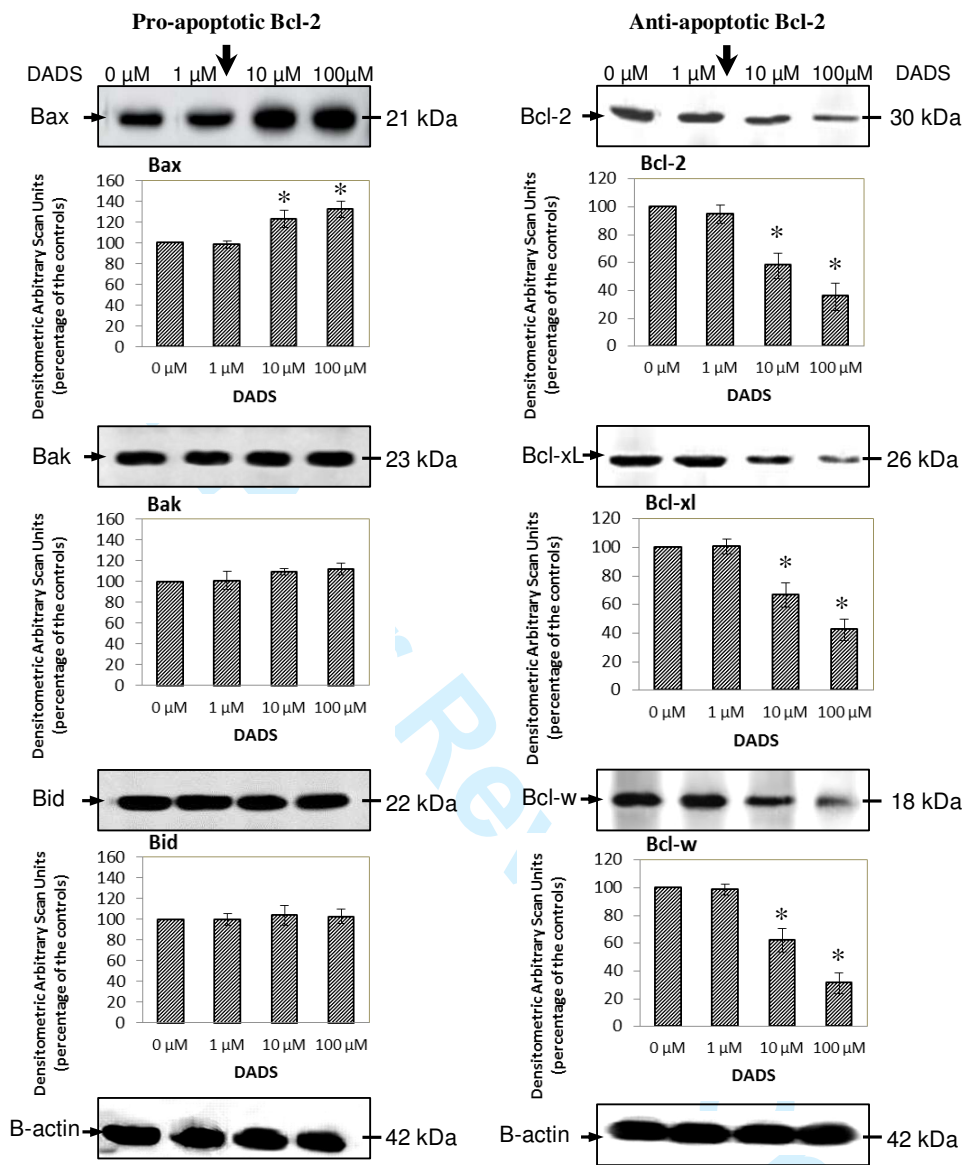
Figure 4: Western blot and densitometric analysis showing the effect of DADS on the levels of pro- and anti-apoptotic Bcl-2 proteins in MCF-7 cells. Cell cultures were treated with DADS for 24 h. Loading control, β-actin detection. Densitometric analysis was performed using TotaLabTM software. The results are reported as mean ± SE of three independent experiments. *, significant ($p \leq 0.05$).
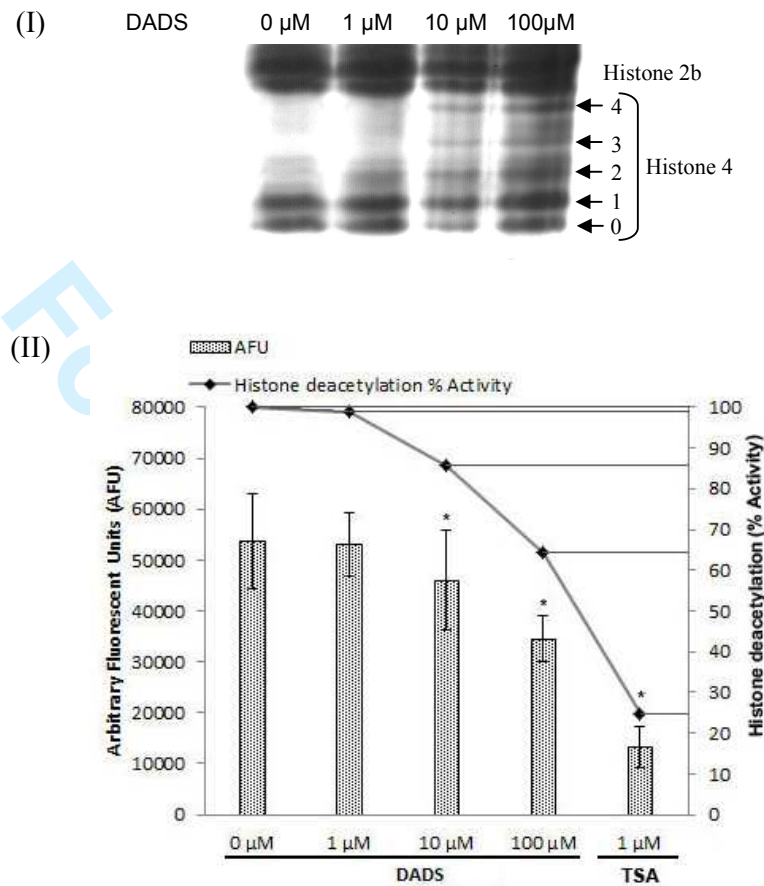
Figure 5: Effect of DADS on histone acetylation in Mcf-7 cells. **(I)** DADS induction (24 h) of histone 4 hyper-acetylation in MCF-7 cells, assessed by TAU gel electrophoresis as in materials and methods. **(II)** DADS inhibition of histone deacetylation in MCF-7 nuclear extracts as determined using 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 ± SE of three independent experiments. *, significant ($p \leq 0.05$).