Growth inhibitory effects of diallyl disulfide on human breast cancer cell lines

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Diallyl disulfide (DADS) is an oil-soluble organosulfur compound found in garlic. The effect of synthetic DADS on the growth of estrogen receptor (ER)-positive (KPL-1 and MCF-7) and -negative (MDA-MB-231 and MKL-F) human breast cancer cell lines was examined. In an in vitro MTT assay, regardless of ER status, DADS at an IC50 of 1.8–18.1 μM after 72 h incubation caused inhibition of growth in all four cell lines examined. Growth inhibition was due to apoptosis as seen by the appearance of a sub G1 fraction. In MDA-MB-231 cells, the apoptosis cascade comprised up-regulation of Bax protein (142%), down-regulation of Bcl-xL protein (38%) and activation of caspase-3 (438%) compared with controls. In an in vivo assay by orthotopic (right thoracic mammary fat pad) transplantation of KPL-1 cells in female nude mice, intraperitoneal injection of 1 or 2 mg DADS three times a week from the day of tumor cell inoculation until the end of the experiment (after 35 days) caused growth retardation and 43% reductions in primary tumor weight, respectively, compared with DADS-untreated mice without apparent side effects. Cell proliferation as evaluated by proliferating cell nuclear antigen (PCNA)-labeling in transplanted tumor of DADS-untreated mice was 59.6%, and 1 and 2 mg DADS-treated mice was 44.6 and 44.5%, respectively. In MDA-MB-231 cells, DADS antagonized the effect of lonicoleic acid (LA), a potent breast cancer cell stimulator (at DADS = 1.8 μM and LA > 6.5 x 10-3 μM concentration), and synergized the effect of eicosapentaenoic acid (EPA), a potent breast cancer cell suppressor (at DADS > 3 x 10-3 μM and EPA > 6.3 x 10-1 μM concentration). Thus, DADS could be a promising anticancer agent for both hormone-dependent and -independent breast cancers, and may harmonize with polyunsaturated fatty acids known as modulators of breast cancer cell growth.

Introduction

Geographical differences in the incidence of cancer indicate that diet plays a critical role in carcinogenesis (1,2); diet is an important factor in the development of almost 40% of all human neoplasias (3). Epidemiological studies indicate that dietary factors influence the development of breast cancer, and experimental analysis suggests that natural or synthetic constituents of diet can act as anticancer agents to inhibit breast cancer. Among dietary factors, certain phytochemicals particularly those in the daily diet, have marked cancer chemopreventive properties (4). For example, genistein, a flavonoid abundant in soy, shows anticancer activity against human breast cancer cells (5). Resveratrol, a naturally occurring product found in grapes and wine, is another dietary phytochemical that inhibits human breast cancer cell growth (6,7).

Garlic (Allium sativum L.), fresh garlic extracts, garlic compounds or synthetically prepared substitutes may be another food item that significantly affects human health. Egyptian records dating to about 1550 BC mention garlic as a remedy for a variety of diseases (8). Garlic has been demonstrated to possess antibiotic, fungicidic, anti-helmentic, anti-thrombotic, as well as anti-carcinogenetic properties (9,10). Not all epidemiologic studies support the theory that consumption of garlic reduces the risk of cancer (11,12). Breast cancer risk was shown to decrease as consumption of garlic increased (13,14). However, another study showed garlic supplement was not associated with breast cancer incidence (15). In contrast to epidemiologic studies, laboratory investigations have provided convincing evidence that garlic-containing substances inhibit a variety of chemically-induced tumors in animals (12,16,17). Garlic powder inhibits the incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors (18). Garlic contains a complex mixture of oil- and water-soluble organosulfur compounds (8). Oil-soluble diallyl disulfide (DADS) was effective in reducing the incidence and delaying the onset of N-methyl-N-nitrosourea (MNU)-induced mammary tumors (19), while water-soluble S-allyl cysteine was less effective or ineffective (19,20). DADS also reduced the multiplicity of 2-amino-1-methyl-6-phenylimidazo-4,5-b-pyridine (PhIP)-induced mammary tumors (21).

In in vitro culture experiments, oil-soluble compounds such as DADS, diallyl sulfide and diallyl trisulfide were effective as antiproliferating agents against CMT-13 canine mammary tumor cells, while the water-soluble compounds S-allyl cysteine, S-ethyl cysteine and S-propyl cysteine had little or no inhibitory effect (22); S-allylmercapto cysteine found at a low concentration in aged garlic extract was effective in suppressing MCF-7 human breast cancer cell growth (23). Essential oils of garlic are known to contain ~60% DADS (9).

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The maintenance of homeostasis in normal mammalian tissues reflects a critical balance between cell proliferation and cell loss. Apoptosis plays an essential role as a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have been improperly produced. The antiproliferative effect of DADS was attributed to suppression of the rate of cell division and induction of apoptosis (24,25). However, the molecular pathway by which DADS induces apoptosis is not well understood. The second aim was to clarify the apoptotic cascade evoked by DADS.

Any anticancerogenic agent identified by in vitro screening should be further evaluated by in vivo screening (26). It remains to be determined if DADS retards the growth of human breast cancer cells in vivo without apparent side effects. So thirdly, the growth inhibitory effect on human breast cancer cells was examined utilizing the nude mouse system. Effects of DADS in combination with other dietary factors require further evaluation. A well-known nutrient substance associated with breast cancer risk is dietary fat. Among dietary fats, n-6 polyunsaturated fatty acids (PUFAs) accelerate breast cancer cell growth, while n-3 PUFAs suppress it (27,28). Thus finally, DADS in combination with n-6 PUFA (linoleic acid, LA) and n-3 PUFA (eicosapentaenoic acid, EPA), modulators of breast cancer cell growth, was examined.

Materials and methods

Cell lines and culture conditions

KPL-1 is a human breast cancer cell line established from the malignant effusion of a breast cancer patient (29). This cell line possesses the estrogen receptor (ER) but not the progesterone receptor. MCF-7 is a transfectant of MCF-7 with the gene of a potent angiogenic growth factor, fibroblast growth factor 4, and estrogen independent (31). MDA-MB-231 is a human breast cancer cell line, established from the pleural effusion of a breast cancer patient who had received combined chemotherapy before this fluid was drawn, and behaves clinically in a hormone-independent manner (32). All cell lines were maintained in Dulbecco’s modified Eagle’s minimum essential medium (DMEM D/6046) (Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY) and grown at 37°C in 5% CO2/95% humidified air.

Chemicals

DADS was purchased from Tokyo Kasei Chemical (Tokyo). The purity was 84.3% as analyzed by gas chromatography. Oil-soluble DADS was solubilized in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto) at a concentration of 100 mg/ml and stored at –4°C in the dark. LA-ethyl ester was purchased from Wako Pure Chemicals (Osaka). The purity was 96.3%. An ethyl ester-refined EPA was kindly provided by Nissin Flour Milling (Tokyo). The purity was 95.0%. Each fatty acid ethyl ester was reconstituted in 100% ethanol at a concentration of 100 mg/ml immediately before use.

MTT assay

Viable cells exposed to DADS were enumerated by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (3). Initially, cells were seeded at a plating density of 3.0×104/well and cultured for 24 h to allow them to adhere to the plate. After pre-incubation, the culture medium was changed to the experimental medium supplemented with DADS. We studied five concentrations of DADS, covering a 4-log range (10^-2 to 10^-6 M) chosen to span the 50% inhibitory concentration (IC50) (the concentration of drug that produced a 50% reduction in cell number) as determined by preliminary assays. The final concentration of DMSO in the culture medium was found to have no antiproliferative effect on either cell line. Following culture with DADS for 24, 48 and 72 h, MTT (Sigma) was added, and then the plate samples were read at 540 nm on a scanning multiwell spectrophotometer. Data points represent the means of eight wells. Additional controls consisted of culture medium alone. The percentages of surviving cells to untreated controls were calculated.

Flow cytometry

Cells were exposed to DADS at their IC50 for 72 h in DMEM supplemented with 10% FBS. The attached and floating cells were mixed and washed in phosphate buffered saline (PBS(-)), centrifuged and fixed in 70% ethanol. After further centrifugation, the cells were treated with RNase and diluted at 0.01% in PBS(-), then treated with an application of propidium iodide (30 µg/ml) in PBS(-). Cells were analyzed by FACSscan (Becton Dickinson, Mountain View, CA) using Lysis II software. CellFIT software with a doublet discrimination module was used to eliminate the possibility of multiples of G1 cells with ordinary G2 cells.

Bax and Bcl-x expression detected by western blotting

MDA-MB-231 cells were examined for the expression of Bax and Bcl-x proteins. Cells were exposed to DADS at their IC50 for 72 h. Immunoblotting of protein was performed as previously described (5). Briefly, cell lysates were prepared and aliquots of each (containing 100 µg protein) were run on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose membrane (Bio-Rad, Richmond, CA). Membranes were probed with rabbit anti-Bax antisemur (PharMingen, San Diego, CA) and rabbit anti-Bcl-x antisemur (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Next, the membrane was treated with peroxidase conjugated-goat anti-rabbit IgG antisemur (Dako, Grostrup, Denmark). The detection of immobilized antigen was performed using ECL western blotting detection reagents and hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer’s instructions. The intensity of bands was quantified with the NIH Image 1.47 Processing and Analysis program for use with Macintosh computers.

CPTP32 (casepase-3) protease assay

Protease activity was assayed in MDA-MB-231 cells using the CPTP32 Protease Assay Kit (MBL, Nagoya, Japan). This kit utilizes a synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with p-nitroanilide (p-NA). Briefly, 50 µg cell lysate protein was incubated with DEVD-p-NA for 1 h at 37°C in a reaction buffer according to the manufacturer’s instructions and then the sample was read on a Hitachi-557 spectrophotometer (Hitachi, Tokyo) at a wavelength of 405 nm. The percentage of OD values for the samples to those for control samples indicated the percentage of protease activity.

KPL-1 tumor growth in nude mice

Thirty 6-week-old female BALB/c nude mice purchased from Charles-River Japan (Kyoto) were used as host animals. During the experiment, the animals were housed five per cage in plastic cages with sterilized white pine chips as bedding. The animal room was kept specific pathogen-free and controlled for temperature (22 ± 2°C), light (12 h light/dark cycle) and humidity (60 ± 10%). At 7 weeks of age, 2×10^3 viable KPL-1 cells/0.25 ml DMEM supplemented with 10% FCS was injected into the right thoracic mammary fat pad with a 26 gauge needle. Mice were randomly divided into three groups (1 mg DADS-treated group, 2 mg DADS-treated group and DADS-untreated group, respectively), 10 mice per group. On the same day, intraperitoneal injections of DADS dissolved in DMSO or DMSO alone were started. Three injections, three times a week, were continued until the termination of the experiment. The mice were weighed and locally growing tumors were checked once a week. Tumor volume was calculated using the standard formula, width×length×height×0.5 (33). Experiments were terminated when tumors of DADS-untreated mice started to become necrotic. At the termination of the experiment, all mice were weighed and then killed by cervical dislocation. The size and weight of the locally growing tumor were then determined. At autopsy, all organs were examined macroscopically. The primary tumors and regional axillary lymph nodes were examined histologically. Tissues were fixed in 10% neutral buffered formalin. Part of each primary tumor was fixed in methacarn. Paraform-embedded tissue sections were stained with hematoxylin and eosin. The effect of treatments are represented by T/C% = (mean tumor weight of treated group)/mean tumor weight of control group)×100. The experiments were carried out in accordance with the Guidelines for Animal Experimentation, Kansai Medical University.

PCNA immunohistochemistry and TUNEL

The cell kinetics, cell proliferation and cell death in primary tumors were evaluated. Cell proliferation was evaluated by proliferating cell nuclear antigen (PCNA) immunohistochemistry using anti-PCNA antibody (clone PC10, Novocastra, Newcastle upon Tyne, UK) on methacarn-fixed samples, and TdT-mediated dUTP-digoxigenin nick end-labeling (TUNEL) was carried out using Apop Tag (Onco, Gutersburg, MD) on formalin-fixed samples. For the proliferating index, >100 cells were counted per section and values calculated from the percentage of cells scored positive at >200 magnification (27). Apoptosis was not evaluated quantitatively due to massive karyorrhexis.

Interaction between DADS and PUFAs in MDA-MB-231 cells

The interaction between DADS and LA was evaluated in vitro as in our previous report (7). In brief, DADS at the IC50 for 72 h (1.8 µM) was added to five concentrations of LA (3.2×10^-1 to 1.3×10^-3 M) spanning a 4-log range. After 72 h incubation, the number of cells in the medium supplemented with
LA plus 1.8 µM DADS was compared with that in the medium supplemented with LA alone (100%). The interaction between DADS and EPA was determined using median-effect analysis as previously described (3,4). In this mathematical method, \( f(f_2) = (Dx)_m^{m} \) was evaluated, where \( f_2 \) is the fraction of cells affected, \( f \) the fraction unaffected, \( D \) the dose of the drug, \( D_m \) the dose causing the median effect, and \( m \) the slope of the median effect curve. The combination index (CI) was defined as

\[
CI = \frac{(D_d)_1}{(D_x)_1} + \frac{(D_d)_2}{(D_x)_2} - \frac{\alpha(D_d)_1(D_d)_2}{(D_x)_1(D_x)_2}
\]

where \( \alpha = 0 \) for drugs with mutually exclusive and \( \alpha = 1 \) for drugs with non-mutually exclusive mechanisms of action. \( (D_d)_1 \) is the dose of drug 1, \( (D_d)_2 \) is the dose of drug 2 and \( (D_x)_x \) is the dose required to produce a median effect. The computer program designed by Chou (Calcusyn analysis program for dose–effect analysis, Windows Software; Biosoft, Cambridge, UK) was used (35,36). In this program, CI values of <1, =1 and >1 indicate synergism, additivity and antagonism of drugs, respectively. DADS and EPA were combined at the ratio of their IC50 values for 72 h as determined by the MTT assay (1.8 and 620.2 µM, respectively). The combination was compared with each drug alone in every experiment.

**Data analysis**

All results are expressed as the mean \( \pm \) SE. In all in vitro experiments, the significance of differences was determined using the two-tailed, independent Student’s t-test for unpaired samples after assuring homogeneity of variances. In in vivo experiments, the significance of differences was analyzed by Mann–Whitney’s U-test to compare body weight, tumor volume, tumor weight and PCNA-labeling, and Fisher’s exact test to compare frequency of metastasis among groups. In all analyses, differences with probability values <0.05 were considered significant.

**Results**

**Effect of DADS on ER-positive and -negative cell lines in vitro**

DADS decreased the number of viable cells in all ER-positive (KPL-1 and MCF-7) and -negative (MDA-MB-231 and MKL-F) human breast cancer cell lines tested (Figure 1a–d). Dose- and time-dependent cytotoxicity (reduction in cell number to below the initial plating density) was seen in all cell lines. Among cell lines tested, time-dependent cytotoxicity was notable in KPL-1 cells, while marked cytotoxicity within the first 24 h was seen in MDA-MB-231 cells. The IC50 of DADS for 72 h treatment ranged from 1.8 to 18.1 µM.

**Mode of cell death**

To determine whether the growth inhibitory activity of DADS was related to the induction of apoptosis, the effect of DADS on the cell cycle was analyzed by flow cytometry. Representative MDA-MB-231 data are shown in Figure 2. At the IC50 for 72 h (1.8 µM), DADS induced the appearance of a sub G1 fraction, the fraction characteristic of apoptosis.

**Apoptosis cascade**

For examining the apoptosis cascade, MDA-MB-231 cells were used due to their sensitivity to DADS. In MDA-MB-231 cells, DADS at 1.8 µM (the IC50 at 72 h) significantly increased the protein level of Bax (Figure 3a) and significantly decreased that of Bcl-xL (Figure 3b), while Bcl-xl was undetectable. At 48 h after treatment, the change in Bax and Bcl-xL was maximum, 142 and 38%, respectively, compared with controls (P < 0.05, respectively). The activity of caspase-3 gradually increased and was significant at 24, 48 and 72 h, respectively, compared with controls (P < 0.01), and was maximum at 72 h (438%) (Figure 4).

**KPL-1 cell growth and metastasis in nude mice**

For in vivo experiments, KPL-1 cells were selected due to their metastatic potential to invade regional axillary lymph nodes. There was no evidence of gross toxicity resulting from DADS treatment such as altered growth of the host, and no
death occurred. According to body weight change (from 7 to 12 weeks of age), no significant difference was detected among groups (Figure 5a). However, the growth of KPL-1 cells as evaluated by tumor volume was significantly reduced in 1 and 2 mg DADS-treated mice compared with DADS-untreated controls (P < 0.05) (Figure 5b); the effect of 1 and 2 mg DADS did not differ significantly. The tumor weight and T/C% at the termination of the experiment are summarized in Table I. The mean tumor weight was significantly reduced in 2 mg DADS-treated mice compared with controls (P < 0.05), while the 1 mg DADS group and 2 mg DADS group did not differ significantly. As evaluated from T/C%, compared with the untreated control, a 43% reduction in tumor weight was observed in both 1 and 2 mg DADS-treated mice. In all primary tumors, scattered PCNA- and TUNEL-positive cells were seen. PCNA-labeling in control, 1 and 2 mg DADS-treated tumors was 59.6 ± 4.8, 44.6 ± 7.5 and 44.5 ± 4.9%, respectively (Figure 6). DADS treatment lowered PCNA-labeling (control versus 2 mg DADS, P < 0.05). In control, and human (MCF-7 and T47D) (37) breast cancer cell growth after culture with LA was compared with that in medium supplemented with the IC$_{50}$ of DADS for 72 h (Table II), cell growth suppression was found to be significant (at LA concentration = 6.5×10$^{-2}$ µM). Thus, DADS mitigated the growth stimulatory effect of LA. Next, the IC$_{50}$ of DADS (1.8 µM) and EPA (620.2 µM) at 72 h for MDA-MB-231 cells was used. As shown in Figure 8, analysis of the median effect revealed that DADS plus EPA at higher doses (DADS > 3×10$^{-3}$ µM and EPA > 6.3×10$^{-1}$ µM) had a synergistic effect (CI < 1).

**Discussion**

DADS, an oil-soluble organosulfur compound found in processed garlic, effectively inhibited the growth of human breast cancer cells in culture. DADS inhibits canine (CMT-13) (22) and human (MCF-7 and T47D) (37) breast cancer cell growth in vitro. In the present study, DADS was proved to be an effective inhibitor of both ER-positive and -negative human...
DADS and human breast cancer cell lines

Table II. Comparison between MDA-MB-231 cells cultured with linoleic acid (LA) alone or together with diallyl disulfide (DADS) at the IC\textsubscript{50} for 72 h (1.8 \textmu M)

<table>
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<tr>
<th>DADS (\textmu M)</th>
<th>LA concentration (\textmu M)</th>
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<tr>
<td></td>
<td>3.2\times10^0</td>
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<td>1.8</td>
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Growth suppression (*P < 0.05) compared with LA alone at the same concentration. Mean \pm SE of three experiments.

Breast cancer cells. The toxic effects of high dose of garlic in various forms have been cited (9). It is essential to determine if DADS suppresses human breast cancer cell growth \textit{in vivo} without marked side effects. DADS retarded the growth of human colon cancer cell (HCT-15) xenografts in nude mice without gross toxicity (38). The present study clearly demonstrated that intraperitoneal administration of 1 or 2 mg DADS three times a week was effective in inhibiting the growth of KPL-1 cells without adverse side effects. DADS at 400 mg/kg body weight is toxic but a dose of 200 mg/kg is well tolerated by rats (37); the present maximum dose applied to mice was approximately 143 mg/kg. KPL-1 is characterized by its potential to metastasize to regional lymph nodes when inoculated orthotopically into the mammary fat pad of nude mice (27,29,39). However, in the present study, metastatic potential was not suppressed by DADS treatment.

DADS caused apoptosis as determined from morphological changes and DNA fragmentation in HCT-15 human colon tumor cells (24). In the present study, the growth inhibitory properties of DADS were attributed to its induction of apoptotic cell death as indicated by the increased proportion of cells in the sub G\textsubscript{1} population. Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. A number of pathways can lead to apoptosis. The Bcl-2 family of proteins and caspase-3 are important regulators of apoptosis (40,41). In the present study, the Bcl-2 proteins were associated

Fig. 6. Effect of diallyl disulfide (DADS) on the PCNA labeling index in KPL-1 tumors in the thoracic mammary fat pad of female BALB/c nude mice. Mean \pm SE (n = 10 per group). *Indicates significantly different from control (P < 0.05).

Fig. 7. Growth curves of MDA-MB-231 cells after treatment with linoleic acid (LA) alone (O) and LA plus 1.8 \textmu M DADS (●) for 72 h. Mean \pm SE of three experiments. *P < 0.05.

Fig. 8. Median effect analysis on MDA-MB-231 cells treated with DADS plus EPA for 72 h. DADS and EPA were combined at the ratio of their IC\textsubscript{50} values for 72 h (1.8 and 620.2 \textmu M, respectively). A value of CI < 1 indicates synergism.
with up-regulation of apoptotic Bax, and down-regulation of anti-apoptotic Bcl-xL. Moreover, DADS-induced apoptosis was mediated via activation of caspase-3. Tumor growth is a balance between cell loss and cell proliferation. DADS also suppressed cell proliferation as evaluated by PCNA-labeling in in vivo experiments. Thus the tumor growth inhibition by DADS was due to promotion of cell death and suppression of cell proliferation. DADS also causes the differentiation of DS19 mouse erythroleukemic cells (37) probably mediated through induction of histone acetylation (42).

DMBA-induced increase in mammary DNA adduct levels was significantly attenuated by garlic as the concentration of corn oil in the diet increased (43). DADS is known to suppress PhIP-induced mammary carcinoma in animals fed a high-corn oil diet (21,44). LA found in corn oil is well recognized as a potent breast cancer growth stimulator. Similar to another phytochemical resveratrol (7), DADS mitigated the effect of LA. EPA, abundant in fish oil, is known to suppress breast cancer growth (27,28). EPA shows synergistic action in combination with phytochemical genistein (5) or with angiogenesis inhibitor TNP-470 (34). Synergistic effect in combination with EPA and TNP-470 was due to an acceleration of apoptosis (34). Again, synergism was observed on the use of DADS in combination with EPA. It has been reported that the co-administration of garlic with fish oil is an effective regimen for ameliorating hyperlipidemia in hypercholesterolemic men (45). In rats, after 4 weeks ingestion of either high LA or EPA diet, serum concentration of LA and EPA was 1313 and 1228 µM, respectively, with no toxicity (46). In humans, serum concentration of LA was 3555 µM (47), and EPA after 4 weeks of oral administration of EPA-rich fish oil concentrate was 722 µM (48). Thus the present in vitro dose for LA and EPA (650–1300 and >6.3×10⁻¹ µM, respectively) may be relevant to events taking place in vivo. In humans, maximum tolerable dose of fresh aqueous garlic extract is 25 ml (49). Amounts greater than this causes severe burning sensations in the esophagus and stomach as well as vomiting. DADS similarly irritates the oral mucosa. However, the maximum tolerable DADS concentration in the serum was not available at this time point.

In conclusion, DADS significantly inhibited the growth of human breast cancer cells in vitro and in vivo without marked side effects. The cancer-suppressing mechanism involved cell loss by apoptosis and a lowering of the rate of cell proliferation. It was also evident that DADS was effective in suppressing breast cancer cell growth in combination with PUFA, a well-known modulator of breast cancer growth. These results suggest that DADS may be a useful anticancer agent for management of human breast cancer.

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