

Diallyl disulfide induces apoptosis of human colon tumor cells

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The present studies compared the effects of various oil-soluble compounds containing allyl and disulfide groups on the proliferation of cultured human colon tumor cells (HCT-15). Diallyl disulfide (DADS) was more effective in inhibiting the growth of HCT-15 cells than isomolar concentrations of *S*-allyl cysteine, dipropyl disulfide (DPDS), allyl chloride, allyl glycidyl ether and allyl alcohol. These studies clearly demonstrate the importance of both the diallyl and the disulfide groups in DADS. Treatment of HCT-15 cells with 100 μ M DADS increased the intracellular calcium levels by 40%, while DPDS caused only a 12% increase in intracellular calcium. Exposure to 100 μ M DADS or more, but not DPDS, caused the cells to undergo apoptosis as determined by morphological changes and DNA fragmentation. A positive correlation ($r = 0.944$) was found between DADS-induced DNA fragmentation and its ability to increase intracellular free calcium levels. The widespread effectiveness of DADS was evident by its ability to inhibit the growth of human colon (HCT-15), skin (SK MEL-2) and lung (A549) tumor cell lines.

Introduction

Epidemiological investigations provide strong evidence that environmental factors are modifiers of the occurrence of some human cancers (1–3). Within these environmental factors, dietary habits are suggested to be an important factor influencing cancer risk (4–6). Recent epidemiological studies suggest garlic and related allium foods may protect against some types of cancer (7–9). However, inconsistencies in the protection provided by these foods are evident (10–11). The reason for these inconsistencies remain to be determined.

Laboratory investigations provide convincing evidence that garlic and associated sulfur compounds reduce experimentally induced colon, esophageal, pulmonary, skin, forestomach and breast cancer (12–18). Thus, the protection provided by garlic and related compounds is not limited to a single tissue or to a particular type of carcinogen. Allyl sulfur compounds present in garlic probably account for much of its anticancer effects. These allyl sulfur compounds have been shown to be effective in both the initiation and promotion phases of the cancer process (15,18).

Previous studies from our laboratory revealed that while the oil-soluble allyl sulfur compound diallyl disulfide was effective as an antiproliferative agent, a similar response was not observed with the water-soluble allyl sulfur compound *S*-allyl

***Abbreviations:** SAC, *S*-allyl cysteine; DADS, diallyl disulfide; DPDS, dipropyl disulfide; AGE, allyl glycidyl ether; AC, allyl chloride; AA, allyl alcohol.

cysteine (SAC*) in cultures of either canine mammary tumor cells (19) or human colon, skin and lung tumor cells (20). Addition of diallyl disulfide (DADS) at concentrations >100 μ M resulted in a marked depression in cell growth.

A sustained elevation of intracellular calcium and a depression in the activity of Ca^{2+} -ATPase enzyme accompanied the ability of DADS to induce growth inhibition and cell death (20). Such sustained intracellular calcium overload has been shown to trigger several lethal processes, including apoptosis (21,22). Recent research by Matsubara *et al.* (23) has established, using a calcium ionophore, the role of calcium-dependent degradative enzymes in causing cell death.

The present studies were designed to investigate the uniqueness of the DADS molecule in blocking the proliferation of human neoplastic cells and to examine if increased intracellular calcium induced by DADS was associated with programmed cell death.

Materials and methods

Chemicals

DADS, dipropyl disulfide (DPDS) and allyl glycidyl ether (AGE) were purchased from Fluka Chemika (Ronkonkoma, NY). Allyl chloride (AC) and allyl alcohol (AA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). SAC was provided by Wakunaga of American Co. Ltd (Mission Viejo, CA). DMSO was used to solubilize the oil-soluble compounds before adding into the cultures. Control cultures received DMSO only.

Culture conditions and cell proliferation

These studies were performed with human tumor cell lines HCT-15 (colon), A549 (lung) and SK MEL-2 (skin). All cell lines were purchased from American Type Culture Collection (ATCC). These cells were chosen because of their tissue origin, their differing growth rates and their ability to be grown under identical conditions. Additional justification for their use comes from their inclusion in screening cultures recommended by the National Cancer Institute for testing of potential antineoplastic agents (24).

In all experiments, cells were plated at $4 \times 10^5/\text{cm}^2$ from a single pellet into tissue culture flasks and grown at 37°C under a humidified, 5% CO_2 atmosphere in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% fetal bovine serum and 2 mM glutamine (GIBCO BRL, Grand Island, NY), 1% penicillin/streptomycin (10 000 U penicillin/ml and 10 mg/ml streptomycin) and 1 μ g insulin/ml (19). Cultures were plated for 24 h before the initiation of experimental procedures.

In all studies cells were harvested by trypsinization (0.025% trypsin-EDTA), diluted with RPMI-1640, removed by gentle scraping, and centrifuged at 500 g and used for specific measurements as indicated below. The cell pellet was suspended in PBS (pH 7.0) and used for cell count. Viable cells were detected using trypan blue exclusion and counted on a hemocytometer. Growth inhibition (% control) was determined using the following equation, where the original count is defined as the mean cell count at the time of addition of the test compounds (24 hours after plating): % growth inhibition = $\{1 - [(\text{final count}_{+\text{allyl/disulfide}} - \text{original count}) / (\text{final count}_{-\text{allyl/disulfide}} - \text{original count})]\} \times 100$.

Experiment 1 determined the growth of HCT-15 after a 48 h exposure to 100 μ M DADS, DPDS, AC, AA, AGE and SAC. Experiment 2 examined the impact of 100 μ M DADS, DPDS, AC, AA, AGE and SAC on the *in vitro* growth of the human tumor cell lines A549 (lung) and SK MEL-2 (skin).

Intracellular free calcium concentrations

Experiment 3 examined the impact of 100 μ M DADS and its non-allyl counterpart, DPDS, on the intracellular calcium levels. Intracellular free calcium in individual cells was examined using the Ca^{2+} -sensitive dye, Indo-1/AM, by ACAS 570 interactive laser cytometry (Meridian Instruments, Okemos,

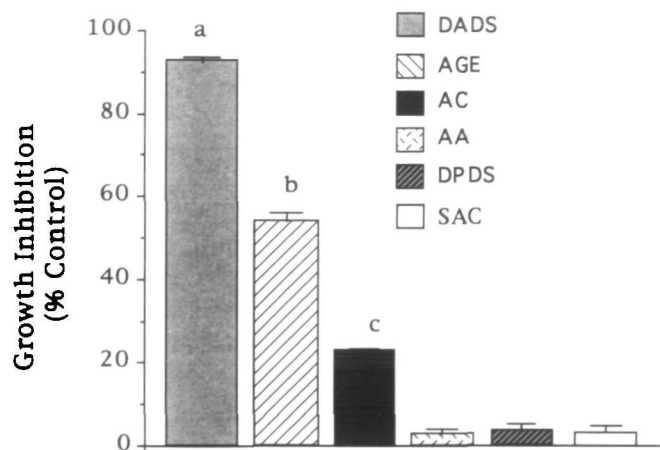


Fig. 1. Effect of adding DADS, AGE, AC, AA, DPDS and SAC on the growth of HCT-15 cells. Cells initially plated at 4×10^3 cells/cm² in tissue culture flasks were allowed to grow for 24 h before addition of 100 μ M allyl/disulfide compounds to the culture medium. Each bar represents the mean percent inhibition after 48 h of treatment. Values are the means \pm SEM ($n = 3$). Bars not sharing a common letter differ significantly ($P < 0.05$).

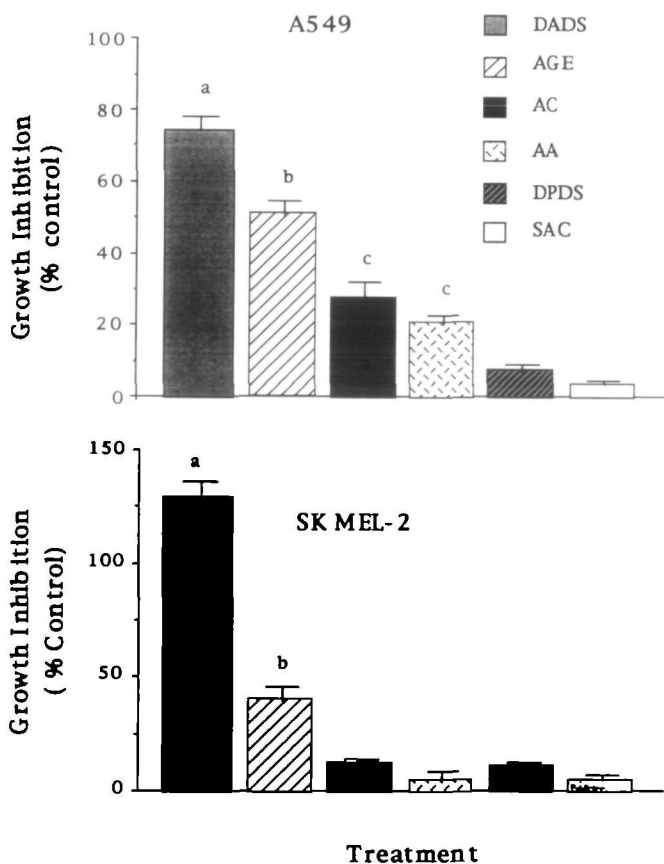


Fig. 2. Effect of adding DADS, AGE, AC, AA, DPDS and SAC on the growth of human tumor cell lines A549 (lung) and SK MEL-2 (skin). Cells initially plated at 4×10^3 cells/cm² in tissue culture flasks were allowed to grow for 24 h before addition of 100 μ M allyl/disulfide compounds to the culture medium. Each bar represents the mean percent inhibition after 48 h of treatment. Values are the means \pm SEM ($n = 3$). Bars not sharing a common letter within a cell line differ significantly ($P < 0.05$).

MI) as described by Dyer *et al.* (25). HCT-15 cultures were loaded with 2 μ M Indo-1/AM in Indo loading buffer for 20 min at 37°C. Selected cells were then image-scanned with fluorescence optics to determine the distribution of Indo-1 in cells. Indo-1 was excited at 388 nm and Ca²⁺-bound Indo-1 was

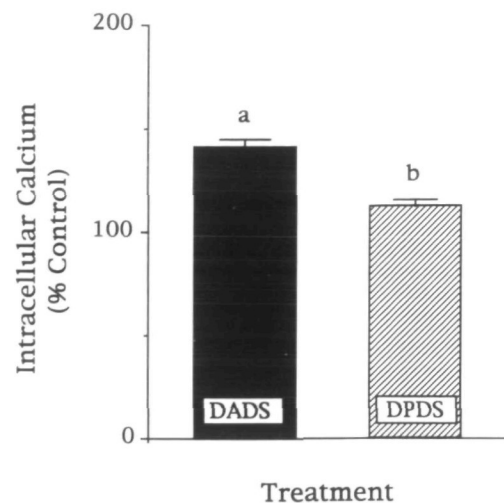


Fig. 3. Effect of DADS/DPDS on the intracellular free calcium levels in HCT-15 cells. Cells were incubated with 100 μ M DADS/DPDS for 3 h and analyzed for intracellular free calcium content. Values are the means \pm SEM of measurements for 15–18 cells from each of three cultures per treatment.

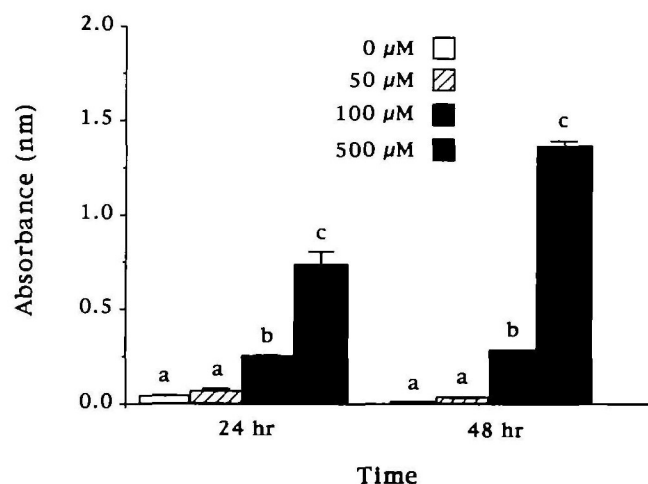


Fig. 4. Effect of varying quantities of DADS on of HCT-15 cell apoptosis. Cells were plated as described in Materials and methods and at the times indicated, 100 μ l cell lysate were tested for DNA fragmentation. Each bar represents the mean absorbance \pm SEM ($n = 3$). Bars not sharing a common letter differ significantly ($P < 0.05$).

monitored at 405 nm and unbound at 485 nm. The ratio of the fluorescence values taken at 405 and 485 nm during each scan were normalized against basal Ca²⁺ concentrations and are reported as percentage of controls. The impact of increasing doses of DADS on the intracellular calcium was also determined.

Determination of apoptotic cells and quantification of DNA fragmentation

Experiment 4 determined the fragmentation of DNA in cells treated with DADS or DPDS using an ELISA kit (Boehringer-Mannheim, Indianapolis, IN). In this study, cells were treated with DADS or DPDS for up to 48 h and lysed by the addition of the lysis buffer for 30 min. The fragmented DNA was then separated by centrifugation and the supernatant was analyzed using an antibody to DNA. The amount of DNA fragmentation per sample is expressed as mean absorbance values. Cells were treated with DADS or DPDS for 48 h and examined for apoptosis using electron microscopy in experiment 5. The electron microscopic examination was performed as described previously (23).

Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Data were analyzed using a one-way analysis of variance, followed

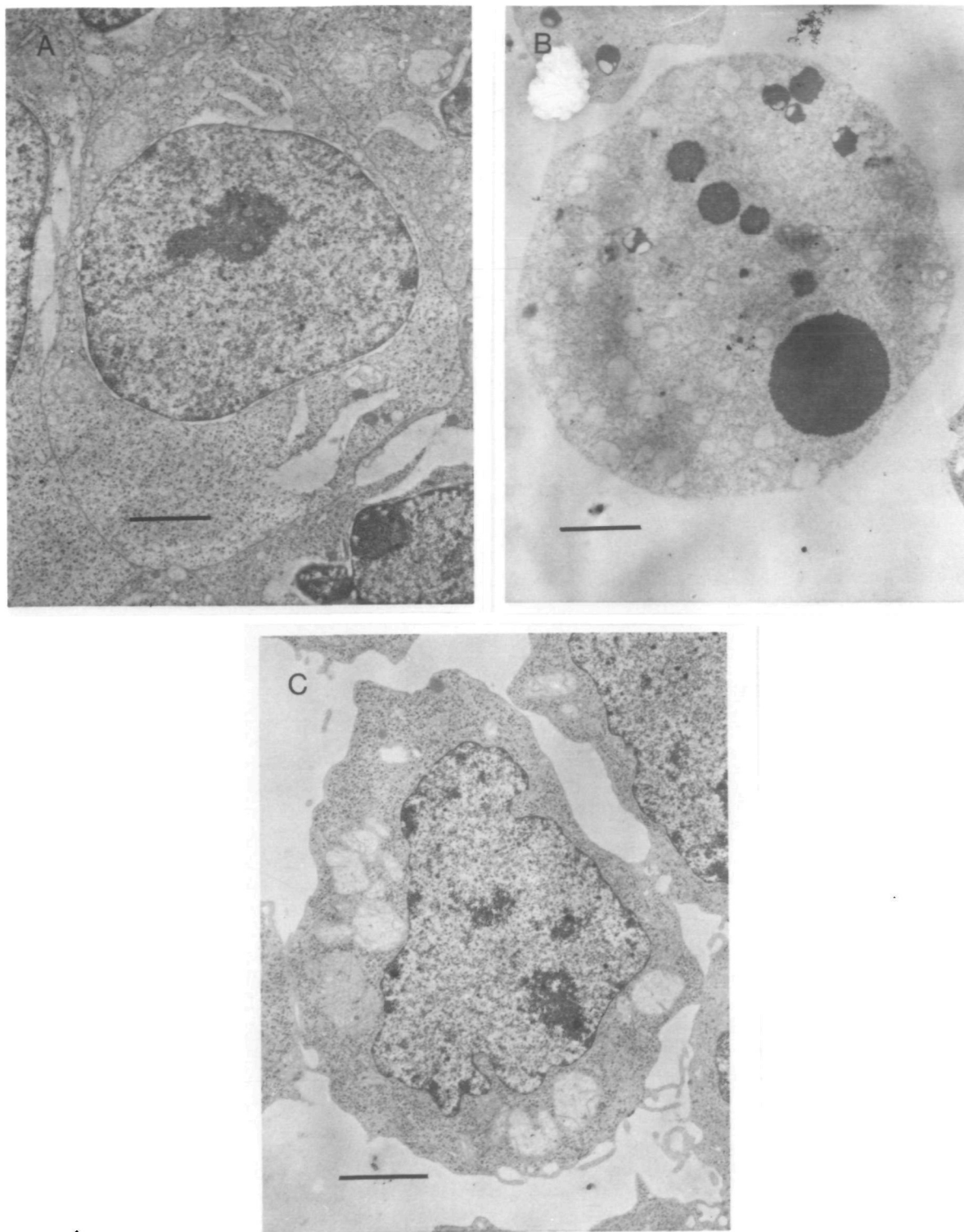


Fig. 5. Electron microscope examination of HCT-15 cells after 48 h of treatment. (A) Control; (B) 500 μM DADS; (C) 500 μM DPDS. Cells were plated and processed as described in the Materials and methods section. Bar indicates 2 μm .

by Fisher's least significant difference test (26) (Statview 512+; Abacus Concepts, Inc., Calabasas, CA).

Results

A comparison of effects of various oil-soluble sulfur compounds containing allyl or disulfide groups on the proliferation of HCT-15 cells is shown in Figure 1. Treatment of HCT-15 cells with 100 μM DADS depressed growth by almost 90%,

while isomolar concentrations of its non-allyl counterpart, DPDS, did not cause a growth depression. Addition of AGE and AC resulted in 50 and 20% growth inhibition respectively. DADS was more effective in inhibiting the growth of cells than the other allyl or disulfide compounds examined.

Similar to results with HCT-15 cells, the *in vitro* treatment of both A549 and SK MEL-2 tumor cell lines with 100 μM DADS was accompanied by marked growth inhibition (Figure

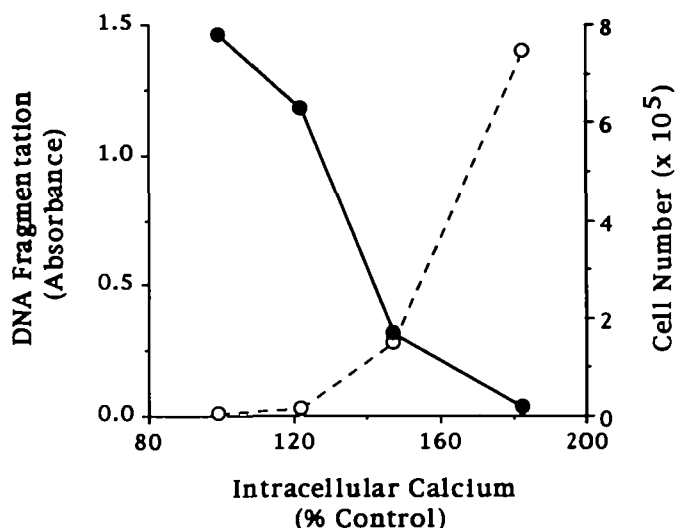


Fig. 6. Relationship between intracellular calcium levels, DNA fragmentation and cell growth observed in DADS-treated HCT-15 cells. The equation and significance of the correlation between intracellular calcium levels and DNA fragmentation (○) are $y = 0.015x - 1.71$, $r = 0.944$. The equation and significance of the correlation between intracellular calcium levels and cell growth (●) are $y = 15.44 - 0.09x$, $r = -0.924$.

2). SK MEL-2 cells were slightly more sensitive to DADS than other cell lines since some cell death was observed at this concentration. A549 cells were slightly less sensitive to 100 μM DADS than HCT-15 cells since growth was inhibited by only 75%. Some subtle differences in sensitivity to AC among the different cell lines were noted. A549 cells were more sensitive to AC than either HCT-15 or SK MEL-2 cells. However, both lung and skin tumor cell lines were not sensitive to the addition of 100 μM SAC or DPDS (Figure 2).

Figure 3 shows the effect of DADS and DPDS on the intracellular calcium concentrations in HCT-15 cells. Treatment of cells with 100 μM DADS for 3 h caused a 41% increase in the intracellular calcium, while DPDS treatment resulted in only a 12% increase.

Addition of increasing quantities of DADS resulted in a progressive depression in the growth of HCT-15 cells. The influence of DADS treatment on cellular apoptosis is shown in Figure 4. Addition of 50 μM DADS did not trigger apoptosis, while 100 μM DADS increased DNA fragmentation by 5-fold. Increasing DADS to 500 μM resulted in an almost 5-fold increase in DNA fragmentation beyond that caused by 100 μM . Based on the growth inhibition observed with 50 μM DADS, we estimate that ~70% of the inhibition observed with 100 μM DADS treatment may relate to apoptosis. Treating cells with 100 or 500 μM DPDS did not result in any significant apoptosis. The mean absorbance values of cells treated with 100 and 500 μM DPDS for 48 h were 0.011 and 0.016 respectively, compared to control values of 0.011.

Electron microscopy revealed that morphological changes characteristic of apoptosis occurred in cells treated with DADS (Figure 5). DADS-treated cells exhibit a dense staining pattern with no distinct nuclear membrane or nucleus. Control cells did not exhibit similar nuclear changes. Treatment of cells with DPDS did not result in any detectable changes that were characteristic of apoptosis.

Figure 6 illustrates the interrelationship between the intracellular calcium concentrations and the amount of DNA fragmentation following DADS treatment in HCT-15 cells. A

linear relationship between the intracellular calcium concentration and DNA fragmentation was observed with a correlation coefficient of 0.944.

Discussion

The present studies reveal that DADS is effective in reducing the growth of human tumor cells originating from colon, lung and skin. Almost a complete cessation of cell growth was observed in all three tumor cell lines following 100 μM DADS treatment. However, treatment with an equimolar amount of DPDS did not cause a suppression in cell growth. Consistent with these findings are data from Munday and Manns (27), who suggested that DADS was a more powerful hemolytic agent than DPDS. Other oil-soluble allyl compounds, including AC and AGE, were only moderately effective in inhibiting tumor cell growth. These data suggest that the efficacy of DADS depends on the presence of both the diallyl and disulfide groups. Data from other laboratories also indicate that allyl compounds differ in their capacity to inhibit cell growth (28). Shoji *et al.* showed that DADS was more effective than AA or its esters in suppressing the proliferation of T-cell lines, MT-2 and MT-4. Their study also indicated that the efficacy of DADS and AA could vary with different cell types since AA was more effective in inhibiting the proliferation of HIV-infected cells than DADS. However, a similar effect was not observed in the present studies since DADS was consistently the most effective compound examined in all three tumor cell lines.

While the mechanism by which DADS causes growth inhibition remains unknown, our previous studies suggest it may be calcium dependent (20). DADS was found to cause a sustained and dose-dependent increase in the intracellular calcium in HCT-15 cells (20). The present studies reveal that increased intracellular calcium is dependent on the type of disulfide added to the culture. Clearly, an equimolar concentration of DPDS caused only about one-quarter the increase in intracellular calcium caused by DADS. Similar to these results, Jatoe *et al.* (29) reported that disulfiram caused a greater increase in intracellular calcium than thiobenzamide disulfide or cystamine. Consistent with differences in the ability of disulfides to inhibit cellular proliferation in the present studies, they also observed that the capability of disulfides to elevate intracellular calcium correlated with their capacity to inhibit hepatocyte proliferation.

Excessive intracellular calcium is frequently associated with the activation of Ca^{2+} -dependent endonucleases. Activation of these enzymes is known to lead to apoptosis in several *in vitro* models (23,30–32). A variety of antineoplastic agents, including thapsigargin, cisplatin, VP-16 and etoposide, are recognized to induce calcium-dependent DNA fragmentation, which is considered to be the hallmark of apoptotic cell death (30–32). The propensity of DADS to bring about an elevation in calcium in the present studies and subsequent apoptosis of tumor cells is consistent with earlier correlations between these intracellular events.

The present studies and those of Oshimi (34) suggest a critical concentration of calcium must be achieved to cause a growth inhibition or apoptosis. While treatment with 50 μM DADS increased intracellular calcium by 25% and inhibited growth of HCT-15 cells by 28%, it did not induce apoptosis. Likewise a 12% increase in intracellular calcium caused by 100 μM DPDS was not accompanied by either an inhibition

of growth or apoptosis. Nevertheless, treatment with 100 μM DADS caused a 41% increase in intracellular calcium and apoptosis. Oshimi (34) reported a sustained elevation of calcium ~40% above normal concentrations was critical for DNA fragmentation. Collectively, these data suggest a critical concentration intracellular calcium concentration ~40–50% greater than the basal level is required to induce apoptosis.

In summary, the present studies show that, on an equimolar basis, DADS is considerably more effective than other allyl or disulfide compounds in inhibiting tumor cell proliferation. Furthermore these studies demonstrate the antiproliferative property of DADS is not limited to a specific single type of tissue since a comparable suppression occurred in colon, lung and skin tumor cells. Intracellular calcium-induced apoptosis accounts for part of the ability of DADS to retard the growth of tumor cells in culture. While DADS is an effective antiproliferative agent, its remains to be determined if the complex matrices of compounds existing within garlic modify its ability to retard tumor proliferation. Additional studies are needed to clarify the ability of dietary garlic and/or isolated constituents to modify the biological behavior of human neoplasms.

Acknowledgement

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