ALLYL SULFIDES MODIFY CELL GROWTH

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SUMMARY

Extensive evidence points to the ability of allyl sulfides from garlic to suppress tumor proliferation both *in vitro* and *in vivo*. This anti-neoplastic effect is generally greater for lipid-soluble than water-soluble allyl sulfides. Both concentration and duration of exposure can increase the antiproliferative effects of lipid- and water-soluble allyl sulfides. Part of their antiproliferative effects may relate to an increase in membrane fluidity and a suppression of integrin glycoprotein IIb-IIIa mediated adhesion. Alterations in cholesterol, arachidonic acid, phospholipids and/or thiols may account for these changes in membrane function. Allyl sulfides are also recognized for their ability to suppress cellular proliferation by blocking cells in the G_2/M phase and by the induction of apoptosis. This increase in the G_2/M and apoptotic cell populations correlates with depressed $p34^{cdc2}$ kinase activity, increased histone acetylation, increased intracellular calcium and elevated cellular peroxide production. While impressive pre-clinical data exist about the antineoplastic effects of allyl sulfur compounds, considerably more attention needs to be given to their effects in humans. The composition of the entire diet and a host of genetic/epigenetic factors will likely determine the true benefits that might arise from allyl sulfur compounds from garlic and other *Allium* foods.

KEY WORDS

allyl sulfide, garlic, proliferation, tumor, adhesion, membrane fluidity, cell cycle, apoptosis

1. INTRODUCTION

Scientific advances during the past decade indicate that increased fruit and vegetable consumption is linked to a depression in the risk for developing a variety of diseases /1,2/. While micronutrients including vitamins C, E, and selenium may account for some of this protection /1,3/, other food components also appear to be involved. A

Abbreviations: DAS, diallyl sulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide; SAC, S-allyl cysteine; SAMC, S-allylmercaptocysteine; DPDS, dipropyl disulfide.

host of non-essential, but biologically active, compounds have surfaced which appear to be involved in promoting disease resistance /4/.

Garlic (*Allium sativum*) is one vegetable that has long been proclaimed to have medicinal properties /5/. Chemically, garlic is composed of water (65%), carbohydrate (28%), protein (1.8%), sulfur compounds (2.3%), fiber (1.5%) and lipids (0.15%) /6/. The unusually high concentration of sulfated compounds present within garlic sets it apart from other vegetables /5/. In addition to being responsible for its characteristic odor, these sulfur compounds likely provide much of the biological and medicinal benefits ascribed to garlic.

Accumulating evidence suggests that allyl sulfides found in processed garlic account for many of its physiological benefits /5/. Specifically, allyl sulfides appear to possess anti-atherosclerotic, antithrombotic, antibiotic and anticancer properties /7,8/. The broad nature of their protective effects suggests allyl sulfides may modify common cellular events involved in the initiation and progression of several diseases. Identifying these mechanisms should help define which diseases will be most responsive to intervention strategies with garlic or selected sulfur compounds.

During the past decade considerable attention has been given to uncovering the cellular benefits of allyl sulfides in relation to cancer. Overall, these investigations reveal that changes in cellular proliferation, differentiation and/or death are associated with the ability of allyl sulfides to modify tumor and platelet activities. Each of these topics is briefly reviewed in this paper.

2. FORMATION OF BIOLOGICALLY ACTIVE ALLYL SULFIDES

A number of biologically active lipid- and water-soluble allyl sulfur compounds have been identified (Fig. 1). Relatively few, if any, of these are found in whole garlic /9/. Instead, they are formed from γ-glutamylcysteine and cysteine sulfoxide compounds during the processing and preparation of garlic (Fig. 2). It is generally believed that the antiproliferative effects of garlic result from breakdown products of alliin /10-13/. Both the type and concentration of lipid- and water-soluble allyl sulfides formed vary depending on the way garlic is prepared /9/.

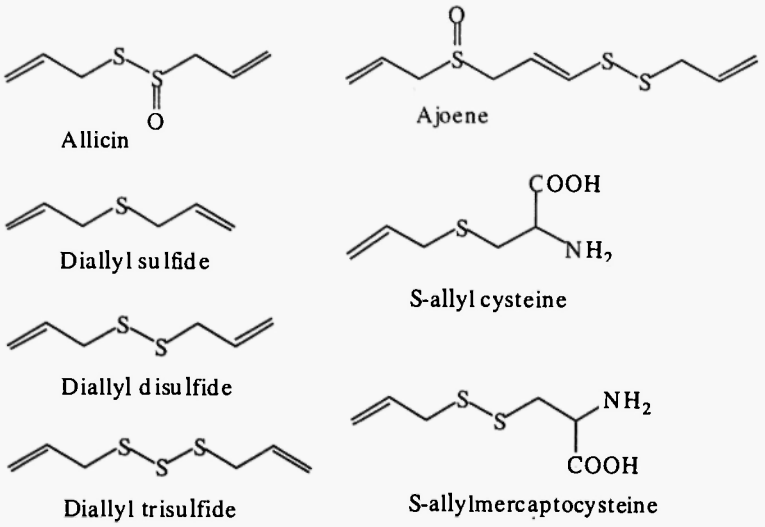

Fig. 1: Structures of biologically active allyl sulfides.

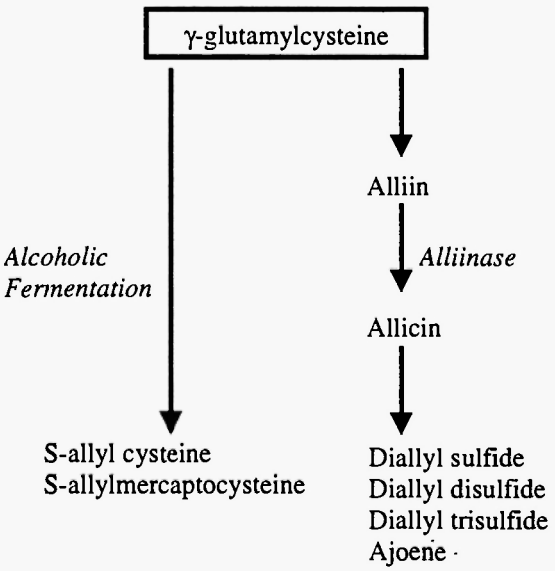

Fig. 2: Formation of biologically active allyl sulfides.

Lipid-soluble allyl sulfide formation occurs when garlic is crushed or chopped /9/. In essence, disruption of the bulb releases the enzyme alliinase from its storage in mesophyll cells and promotes its conversion of the sulfoxide alliin (S-allyl-L-cysteine sulfoxide) to its active form, allicin (dialkyl thiosulfinate). Although allicin may possess biological activity, studies reveal that it is relatively unstable and is quickly converted to other lipid-soluble sulfides such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and ajoene. Consequently, these lipid-soluble allyl sulfides provide most of the medicinal characteristics associated with crushed garlic.

Water-soluble allyl sulfides serve as the main ingredients found in aged garlic extracts /6,14/. Unlike the lipid soluble compounds, water-soluble allyl sulfur formation is not a typical byproduct of garlic cooking. Instead, alcoholic fermentation of whole garlic is used to convert γ-glutamyl-S-allyl cysteine to S-allyl cysteine (SAC) and S-allylmercaptocysteine (SAMC) /15/. SAC appears to be the main product of this reaction and provides much of the biological activity associated with these preparations.

Lipid- and water-soluble allyl sulfides are both recognized for their ability to modify the development of a number of diseases /7,8/. The characterization and isolation of these compounds have paved the way for examining their individual effects on disease development. Since uncontrolled cellular proliferation is a common feature among many diseases, considerable research has focused on the impact of allyl sulfides on cell growth and differentiation.

3. CELL PROLIFERATION

Cancer is characterized by rapid cell proliferation /16,17/. Laboratory investigations provide evidence that allyl sulfides offer an effective strategy for suppressing the growth of neoplastic cells while having a minimal effect on non-neoplastic cells /11,12,18-33/. Specifically, allyl sulfides have been shown to suppress the proliferation of a number of cultured tumor cells including human prostate, colon, skin, breast, lung, lymphoma, erythroleukemia and lymphocyte (Table 1). This widespread depression in the growth of cells originating from different tissues suggests a common antiproliferative mechanism of action.

TABLE 1

Allyl sulfides suppress human tumor growth

Compound	Cell Type	Reference
Allicin	Lymphoma	/23/
Diallyl sulfide	Prostate	/26/
	Promyelocytic leukemia	/33/
Diallyl disulfide	Lung	/12,20,25/
	Colon	/12,20,27/
	Skin	/12,20/
	Erythroleukemia	/31/
	Prostate	/26/
	Breast	/31/
Diallyl trisulfide	Lung	/25/
Ajoene	Lymphoma	/23,24/
	Promyelocytic leukemia	/32/
	Colon	/32/
	Neuroblastoma	/32/
S-Allyl cysteine	Neuroblastoma	/18/
	Breast	/29/
	Melanoma	/30/
S-Allylmercaptocysteine	Prostate	/21,26/
	Breast	/21,29/
	Colon	/13/
	Erythroleukemia	/21/

The ability of allyl sulfides to modify tumor proliferation is not limited to cells grown in culture /34-38/. Animal studies reveal that intraperitoneal/intragastric DADS and intravenous allicin administration both suppress the growth of transplanted tumor cells into palpable tumors in mice /34-36/. Since intragastric DADS was substantially less effective than intraperitoneal treatment, considerable tissue uptake and detoxification may have occurred /34/. The suppression in tumor formation observed by Weisenberger and Pensky /36/ occurred regardless of whether or not the sarcoma 180 cells were exposed to allicin (0.5 μM/5x10^6 cells) prior to or following their transplantation. Collectively, these studies highlight the increasing belief that allyl sulfides can be effective antitumorigenic agents both *in vitro* and *in vivo*.

Studies from Sundaram and Milner /12/ reveal that the allyl group is important in mediating the growth inhibitory effects associated with garlic sulfur compounds. While 100 μM DADS inhibited human colon tumor (HCT-15) growth by approximately 90%, its saturated analog dipropyl disulfide (DPDS) failed to modify proliferation. Similarly, skin and lung tumor cell growth was not affected by DPDS treatment /12/. Pinto *et al.* /26/ also report that lack of an active allyl moiety accounts for the inability of cysteine to modify prostate tumor (LNCaP) growth when compared to SAC and SAMC. Collectively, these studies suggest that the presence of the allyl group is required for these sulfides to elicit maximum growth inhibition.

Accumulating evidence indicates that not all allyl sulfides are equally effective in their ability to modify tumor cell growth /11-13, 26/. Studies by Sundaram and Milner /11/ demonstrated that DAS, DADS and DATS are more effective at inhibiting proliferation than water-soluble SAC. Similarly, studies with human lung, colon and skin tumor cells have established that while DADS (100 μM) almost completely inhibits cell growth, SAC (500 μM) is relatively ineffective /12,20/. Knowles and Milner /13/ also report that DADS (100 μM) is twice as effective at inhibiting HCT-15 tumor growth than water-soluble SAMC. Consequently, SAC and SAMC are both less effective at suppressing tumor proliferation than lipid-soluble DADS.

Studies from our laboratory provide evidence that the number of sulfur atoms present in the compounds also influences their ability to modify proliferation /11,25/. Using canine mammary tumor cells (CMT-13), the antiproliferative effects of lipid-soluble allyl sulfides

were shown to increase as the number of sulfur atoms in the compounds increased /11/. Likewise, A549 lung tumor cells were shown to be about 2.5 times more sensitive to the antitumorigenic effects of DATS than DADS /25/. Other studies reveal that the presence of the disulfide in SAMC also enhances its antiproliferative effects when compared to SAC /13,21,26,39/. Sigounas *et al.* /21/ report that while 0.25 mM SAMC completely suppresses erythroleukemia tumor proliferation, similar modifications in growth are not observed with 5 mM SAC.

The ability of allyl sulfides to modify tumor growth varies with the exposure time. Scharfenberg *et al.* /24/ showed that while a 2-day exposure to ajoene (150 fmol/cell) only suppressed BJA-B Burkitt lymphoma cell growth by ~17%, increasing the exposure to 5 days completely inhibited growth. Similarly, suppression of LNCaP prostate tumor growth by SAMC (~260 μM) increased by 55% when exposure was extended from 3 to 5 days /26/. The reason for this variation in response is unknown, but may reflect the rate of allyl sulfide catabolism by the tumor cells or subtle metabolic changes that require time for phenotypic changes to be observed. Such a delay might be expected for compounds that induce apoptotic events. Knowles and Milner /27/ reported that cells can recover from allyl sulfide exposure. In their studies, while a 24-h exposure to 50 μM DADS reduced the growth of human colon tumor cells (HCT-15) by 43%, refeeding with complete medium resulted in a return to near normal patterns of growth. At lower exposures, the antiproliferative effects of allyl sulfides are dependent on their continued presence within the culture medium. Thus, variation in the growth depression observed among tumor cells will depend not only on the quantity provided but also on the cell's ability to metabolize/detoxify the allyl sulfide.

Allyl sulfides have been shown to preferentially suppress neoplastic over non-neoplastic cell proliferation. Moreover, Sakamoto *et al.* /25/ and Scharfenberg *et al.* /23/ found that A549 lung and BJA-B Burkitt lymphoma cells are twofold more sensitive to the antiproliferative effects of DATS and ajoene, respectively, compared to non-neoplastic MRC-5 lung and FS4/BHK fibroblasts cells. Human umbilical vein endothelial cells (HUVEC) were also found to be less responsive to SAMC than erythroleukemia, breast and prostate tumor cells /21/. This decreased sensitivity of normal cells to DATS, ajoene

and SAMC suggests that allyl sulfides can be used to selectively inhibit tumor growth, while minimizing toxicity to non-neoplastic cells. However, some evidence suggests that the antiproliferative effects of allyl sulfides are not limited to tumor cells *per se*. Lee and associates /39/ reveal that SAMC is capable of suppressing normal smooth muscle cell (SMC) proliferation at concentrations (~250 μM) equivalent to those previously shown to suppress tumor growth. SMCs were also 2.5-fold more sensitive to SAMC (250 μM) than HUVECs /39/. A suppression in SMC may not always be detrimental since enhanced proliferation is a characteristic of intermediate and advanced lesion formation during atherosclerosis /40/. Since allyl sulfur compounds are consumed by reproducing women and children it becomes exceedingly important to clarify which cells are most sensitive to the effects of allyl sulfur compounds and under what circumstances. Recent evidence by Kuttan /41/ reveals that DAS and DADS treatment can in some cases promote cellularity in selected tissues. Specifically they found that DADS treatment of mice resulted in enhanced spleen and thymus weight, and an increase in bone-marrow cellularity. It remains to be determined whether these are associated with positive health implications or whether they are linked to compensatory mechanisms to counteract toxicity associated with DADS.

Active proliferation appears to be a key factor in facilitating the growth inhibitory affects ascribed to allyl sulfides /21,42/. Studies by Sigounas *et al.* /21/ showed that the antiproliferative effects of SAMC (0.25 mM) decrease when cells progress from a non-confluent dividing state towards a confluent non-dividing state. Likewise, other studies found that the ability of ajoene to suppress peripheral blood mononuclear cells (PBMC) increased by 49% and 94% when growth was stimulated by phytohemagglutinin (2 μg/ml) and phorbol myristate acetate (5 ng/ml), respectively /42/. These suppressions in growth were not apparent in unstimulated PBMCs exposed to ajoene (50 μM). Consequently, slowly dividing or non-dividing cells appear to be immune to both SAMC and ajoene.

4. CELLULAR ADHESION

Cellular proliferation is governed by the transfer of information from the plasma membrane to the nucleus in response to extracellular stimuli /43/. In tumor and atherosclerotic lesion formation, prolifer-

ation is induced by the association of circulating tumor and platelet cells with endothelial, fibroblast and SMCs /44,45/. Suppression of cellular adhesion has been shown to inhibit both tumor metastasis and platelet aggregation /44/. Several mechanisms may be involved in the ability of allyl sulfur compounds to depress cellular adhesion (Fig. 3). Increases in Phase 2 enzyme activities and induction of apoptosis have been found to coincide with changes in the adhesion of HT29 colon tumor cells following allyl sulfide treatment (50 µmol/l) /46/. Similarly, Tatarintsev *et al.* /47/ reported that ajoene (50 nmol/l) disrupted the normal clustering of H9 and Jurkat cells in culture.

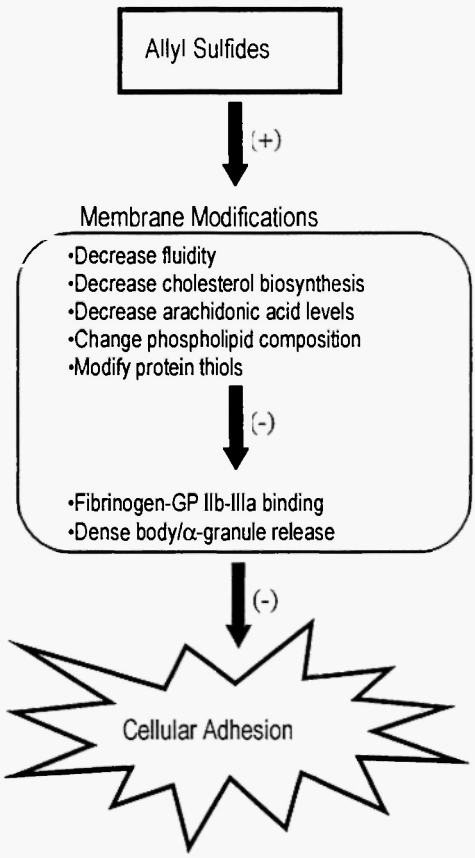

Fig. 3: Theoretical scheme of the mechanisms governing allyl sulfide inhibition of cellular adhesion.

Finally, Sigounas *et al.* /21/ revealed that adherent cells are more sensitive to the antiproliferative effects of SAMC than cells growing in suspension.

Insight on how allyl sulfides may modify tumor cell adhesion comes from platelet aggregation studies. Ajoene is one allyl sulfide highly recognized for its ability to improve blood fluidity by suppressing platelet aggregation *in vivo* /48-50/. *In vitro*, ajoene has been shown to inhibit the induction of platelet aggregation by ADP, collagen, thrombin, ionophore A-23187, and arachidonic acid /47,51-58/. Specifically, Apitz-Castro *et al.* /54/ found that treatment of platelet rich-plasma with 58 μM ajoene for 6-8 min completely inhibited collagen (1 μg/ml)-induced aggregation. These anti-aggregatory effects were not permanent, as demonstrated by the recovery of collagen-induced aggregation following ajoene removal from the medium /54/.

Studies by Block and associates /53/ revealed that the disulfide group is important in mediating ajoene's suppression of platelet aggregation. Removal of the sulfide from position 4 of ajoene decreased its ability to suppress collagen-induced aggregation twofold. ADP-induced aggregation was also less responsive to ajoene exposure following the sulfide's removal /53/. Interactions between the disulfide group and membrane sulfhydryls are thought to account for ajoene's enhanced suppression of platelet aggregation. The impact of allyl sulfides on cellular thiol status is discussed below.

Although most studies have focused on the antiplatelet activities of ajoene, evidence exists that other allyl sulfides are also capable of modifying platelet aggregation /59-61/. In particular, allicin treatment has been shown to cause a dose dependent inhibition of collagen-induced aggregation /59/. When added at 2.72 mg/ml, allicin reduced platelet aggregation by 47%. Makheja *et al.* /60/ also found that allicin was responsible for approximately 29% of the antiplatelet activity associated with garlic. Paraffinic polysulfides including DATS also depressed platelet activity by 25% /60/. Platelet adhesion to fibrinogen has also been reported to be reduced by approximately 30% in subjects taking deodorized garlic preparations containing SAC compared to a placebo supplement /62/. Thus, both water- and oil-soluble allyl sulfur compounds appear to be able to alter platelet aggregation.

Agonist activation of platelets results in a series of biochemical and morphological changes that culminate in enhanced cell-cell

communication /63,64/. This adhesion occurs through the attachment of cells to extracellular matrix proteins such as fibronectin, vitronectin, fibrinogen, thrombin, laminin and collagen /65/. Some antiplatelet compounds suppress aggregation by blocking platelet binding to ECM protein /66/. Radioisotope studies reveal that 90 s exposure to ajoene suppressed adhesion by inhibiting ^{125}I-fibrinogen binding to ADP-stimulated platelets /56/. In these studies, 98% inhibition of binding was observed in platelets at ^{125}I-fibrinogen and ajoene concentrations of 0.3 and 5 μM, respectively. These suppressions in fibrinogen binding increased as the concentration of ajoene or ^{125}I-fibrinogen in the reaction mixture increased. Providing ajoene after agonist activation, however, eliminated its ability to suppress ^{125}I-fibrinogen binding /56/. Consequently, once bound, fibrinogen was not displaced by ajoene.

Glycoprotein (GP) IIb-IIIa is an integrin complex expressed by platelets and the megakaryocytes from which they originate /67/. This complex functions as a receptor for fibrinogen and other ligands during platelet aggregation /63,67,68/. Fibrinogen binding to GP IIb-IIIa is therefore recognized as a crucial step in the induction of platelet aggregation. Studies by Apitz-Castro *et al.* /56/ demonstrated that ajoene interacts directly with GP IIb-IIIa. Using intrinsic tryptophan fluorescence as a measure of unbound GP IIb-IIIa, ajoene (10 μM) decreased fluorescent intensity by about 50%. The kinetic patterns derived from ajoene suppression of unbound GP IIb-IIIa revealed that it likely binds to a different site on the receptor than fibrinogen. The inability of ajoene to suppress monoclonal AP-2 and AP-3 antibody specificity for GP IIb-IIIa supports these findings by demonstrating that the allyl sulfide does not directly suppress fibrinogen-GP IIb-IIIa interactions /54/. As a result, these studies suggest that ajoene likely depresses GP IIb-IIIa expression and not fibrinogen affinity for the receptor.

While ajoene clearly modifies fibrinogen-GP IIb-IIIa interactions, it does not appear to interact with all ligand-receptors. For example, studies found that binding of [^{3}H]rauwolscine (α-yohimbine) to α_2 adrenergic receptors was similar between ajoene (30 μM) and untreated platelets /54/. Similarly, ajoene had no effect on WM-23 antibody binding to the thrombin receptor's GP Ib binding site /54/. Collagen and ADP primary receptor interactions were also unaffected by ajoene /54,56/. Together, these studies imply that ajoene's sup-

pression of fibrinogen binding to integrin GP IIb-IIIa is highly selective.

Since GP IIb-IIIa is expressed by many tumors /45/, ajoene may also elicit its antitumorigenic effects by suppressing this receptor. However, GP IIb-IIIa may not be the only integrin receptor modified by ajoene. Ajoene is recognized for its ability to inhibit both leukocyte and neutrophil aggregation and viral infection of T-cells /47,69,70/. LFA-1, VLA-4 and VLA-5 are the primary integrin receptors involved in the adhesion of these cells /47,71/. Given their structural and functional similarities to GP IIb-IIIa, inactivation of these receptors may also contribute to ajoene's ability to suppress neutrophil, leukocyte and T-cell adhesion. Additional research is needed to clarify the impact of ajoene on LFA-1, VLA-4 and VLA-5 integrin receptor function.

Although integrin-mediated adhesion is clearly an important modifier of aggregation, it is not the only mechanism involved in cell growth. Platelet aggregation and tumor growth are also controlled by the release of adhesive glycoproteins, growth factors, coagulation factors and arachidonic acid metabolites from stores within the cell itself /44,72/. Thrombin studies have found that ajoene (100 μM) treatment decreases the ability of platelets to release serotonin from dense bodies and β-thromboglobulin from α-granules by over 60% /52/. Depressions in thrombin-induced ATP release also parallel ajoene's suppression of platelet aggregation /54/. This suppression of dense body and α-granule release appears to be unrelated to ajoene's suppression of fibrinogen-GP IIb-IIIa adhesion since inhibition of the release reaction is not observed by other inhibitors of fibrinogen binding. Apitz-Castro *et al.* /54/ propose that perturbations in plasma membrane structure may account for the decrease in both fibrinogen-GP IIb-IIIa binding and release reactions.

5. PLASMA MEMBRANE MODIFICATIONS

Considerable evidence indicates that cells regulate both the type and quantity of protein expressed on their surface by modulating membrane fluidity /73/. Studies by Rendu *et al.* /52/ and Debouzy *et al.* /74/ provide evidence that modifications in internal plasma membrane microviscosity accompany ajoene exposure. Using electron spin resonance as an indicator of plasma membrane microviscosity, these

studies found that ajoene treatment increased the mobility of inner membrane fatty acids labeled with 16 nitroxide stearate. This increase in fatty acid mobility was associated with an increase in inner membrane fluidity and a loss of membrane cohesion. Similar changes in outer membrane mobility were not observed following ajoene exposure /52,74/. Consequently, ajoene appears to modify inner membrane dynamics without disrupting the main structure of the lipid bilayer. Debouzy and associates /74/ suggest that ajoene's localization near the inner layer following absorption accounts for its specificity for modifying inner membrane dynamics. It remains to be determined whether other allyl sulfur compounds also influence membrane microviscosity.

A number of cellular processes, including adhesion, carrier-mediated transport, endocytosis, membrane-bound enzyme activation, eicosanoid biosynthesis and DNA synthesis, are affected by changes in membrane fluidity /75-77/. Cells regulate these changes by modifying the cholesterol, phospholipid and/or fatty acid composition of the lipid bilayer /75,78/. Substantial evidence shows that decreases in membrane cholesterol composition lead to an increase in membrane fluidity /78/. Since ajoene (200 μM) has been shown to reduce primary rat hepatocyte cholesterol levels by ~70% /79/, suppression of cholesterol biosynthesis may account for its ability to increase membrane fluidity. Recently, Liu and Yeh /80/ showed that DAS, DADS, DATS, SAC and SAMC also suppressed hepatocyte cholesterol biosynthesis. Gebhardt *et al.* /79/ and Kumar *et al.* /81/ suggest that reductions in 3-hydroxy-3-methylglutaryl CoA reductase activity account for this decline in hepatocyte cholesterol biosynthesis. Since 79% reduction in 3-hydroxy-3-methylglutaryl CoA reductase activity has been observed in H-ras oncogene transformed tumors exposed to DADS (33 μmol) /35/, it is likely that suppression of cholesterol biosynthesis accompanies the growth inhibitory effects ascribed to garlic allyl sulfur compounds.

Allyl sulfide modification of membrane fluidity may not be limited to changes in cholesterol biosynthesis. Studies with fungi reveal that decreased phosphatidylcholine and increased phosphatidylethanolamine biosynthesis also accompany ajoene exposure /82,83/. In platelets, evidence exists that endogenous membrane phosphoinositide levels are reduced by ajoene /52/. Rendu *et al.* /52/ showed that while the initial hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)

and phosphatidylinositol 4-phosphate (PIP) following thrombin stimulation was similar in control and ajoene (100 μM)-treated cultures, the subsequent resynthesis of these phosphoinositides was greatly curtailed (30-40%) by the allyl sulfide. Whether these reductions in PIP_2 and PIP expression influence subsequent phosphoinositide second messenger formation remains to be determined. Clearly, additional research will need to clarify the role changes in membrane phospholipid composition may have on the ability of cells to survive allyl sulfide exposure.

Laboratory investigations provide evidence that allyl sulfides also reduce membrane arachidonic acid (AA) levels /55,58,61/. In platelets, ajoene (175-250 μM) has been shown to decrease AA incorporation into membrane phospholipids by 35-50% /55,58/. Similarly, suppressions (46% and 18%) in AA incorporation have also been observed in platelets exposed to DADS (685 μM) and DATS (337 μM), respectively /61/. Interestingly, this suppression in AA incorporation is not the only step in the AA cascade modified by allyl sulfides. DADS and DATS also inhibit AA release from membrane phospholipids and its subsequent metabolism to thromboxane B_2 (TXB_2) and 12-hydroxyeicosatetraenoic acid (12-HETE) /61/. Likewise, Srivastava and associates /58/ found that ajoene also suppressed TXB_2 and 12-HETE formation by 73% and 40%, respectively. Since increases in cytoplasmic AA levels accompanied DADS, DATS and ajoene exposure /58,61/, these reductions in TXB_2 and 12-HETE formation likely result from suppression of cyclooxygenase and lipoxygenase enzyme activities and not from lack of available AA /84/. Other studies support this observation by showing garlic ether extracts can suppress TXB_2 and 12-HETE formation at concentrations 10 times lower than that needed to suppress phospholipid deacylation (200 μg/500 μl) /85/. Consequently, the ability of allyl sulfides to suppress AA incorporation and release from membrane phospholipids likely occurs secondary to changes in eicosanoid biosynthesis.

It is becoming increasingly apparent that allyl sulfides possess a unique structure that enables them to regulate thiol-containing proteins by promoting thiol-disulfide exchange reactions /53,86,87/. For example, studies by Miron *et al.* /87/ show that allicin reduces the levels of glutathione (GSH) and 2-nitro-5-thiobenzoate present in small unilamellar phospholipid vesicles (SUV) within 5 min of exposure. Likewise, red blood cell (RBC) intracellular thiol concen-

trations also decreased by about 65% within 2 min after allicin (10 μM) exposure. Since intracellular S-allylmercaptoglutathione (GSSA) formation paralleled allicin exposure, this decrease in thiol status was not due to the release of GSH from the SUVs or RBCs. Furthermore, the rate of GSSA formation was relatively similar regardless of whether GSH was provided in solution or entrapped in SUVs. Thus, allicin appears to rapidly diffuse through the lipid bilayer and immediately interacts with cellular thiols.

Cellular thiols have a critical role in regulating division and protection from free radical damage /88,89/. Consequently, reductions in GSH concentrations are implicated in cell damage and depressed cell growth. Sundaram and Milner /11/ demonstrated that a suppression in cellular GSH levels paralleled DADS's ability to suppress canine mammary tumor growth. Similarly, Scharfenberger *et al.* /24/ found that ajoene (385 fmol) decreased GSH levels by about 80% in BJA-B Burkitt lymphoma cells within 30 min of exposure. The rapid nature of this reduction suggests that change in thiol homeostasis is an early event mediating the antiproliferative effects of ajoene. Interestingly, when exposed to lower concentrations of ajoene (150 fmol), cells appear to counteract this decrease by stimulating GSH synthesis /24/. A similar effect is seen when supplemental selenium is added to cells /90/. Thus, fluctuations in GSH levels appear to be an important determinant of a cell's ability to survive toxic exposures to various compounds including allyl sulfides.

6. CELL DIVISION

Cell growth is regulated by a series of checkpoints that control the order and timing of cell cycle transitions /43,91/. These checkpoints monitor DNA replication and chromosomal segregation to ensure that cells replicate with high fidelity. Increasing evidence shows that antiproliferative compounds can block cell cycle progression by stimulating checkpoint function /43,91/. Studies by Knowles and Milner /27/ found that the antiproliferative effects of DADS relate to a block in the progression of cells from the G_2 to the M phase of the cell cycle. These shifts in the distribution of cells were observed within 4 h of DADS (50 μM) exposure, while cell numbers were not modified until 8 h. Additional observations found that DADS induced an approximate fourfold increase in the percentage of cells in the G_2/M phase by

12 h when compared to controls (49.7 vs 12.7). This increase, however, was not permanent, as demonstrated by the return of DADS-treated cells towards a normal cell cycle by 24 h. Since similar fluctuations in the G_2/M phase population were also observed when cells were exposed to a second DADS treatment, this return towards a normal cell cycle is likely controlled by the rate of DADS metabolism and detoxification.

DADS is not the only allyl sulfide known to modify G_2/M progression. Zheng *et al.* /33/ reported that DAS exposure leads to a dose dependent increase in the proportion of promyelocytic and leukemia cells blocked in the G_2/M phase. Similarly, increases in the G_2/M population have also been observed in promyelocytic leukemia and erythroleukemia cells exposed to ajoene and SAMC, respectively /22,32/. Suppression in cellular thiol status may account for the ability of allyl sulfides to arrest cells in the G_2/M phase of the cell cycle. Studies by Poot *et al.* /92/ revealed that reductions in cellular GSH levels block cell division by impairing G_2/M progression.

$p34^{cdc2}$ kinase is a complex whose activation controls G_2/M progression by promoting chromosomal condensation and cytoskeletal reorganization /93/. Suppression of $p34^{cdc2}$ kinase activity blocks cells in the G_2/M phase. Studies by Knowles and Milner /27/ revealed that depressions in $p34^{cdc2}$ kinase activity coincide with the block in G_2/M progression caused by DADS exposure. Specifically, DADS (50 μM) decreased $p34^{cdc2}$ kinase activity by 60% in synchronized HCT-15 cells. Recent studies indicate that DADS does not directly inhibit $p34^{cdc2}$ kinase activity, but instead modifies factors involved in $p34^{cdc2}$/cyclin B_1 complex formation and activation /28/ (Fig. 4).

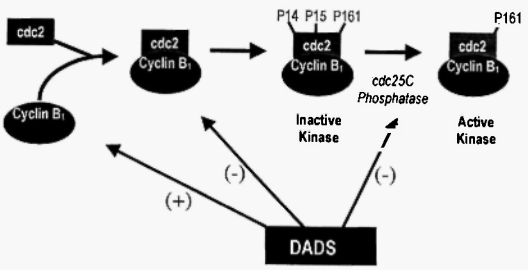

Fig. 4: Illustration of the $p34^{cdc2}$ kinase complex and the role of DADS in inhibiting kinase activity. Reprinted from Knowles and Milner /28/, with permission from Oxford University Press.

$p34^{cdc2}$ kinase activation is controlled by the association of the $p34^{cdc2}$ catalytic subunit with the cyclin B_1 regulatory unit /94/. Knowles and Milner /28/ revealed that the ability of DADS (50 μM) to suppress $p34^{cdc2}$ kinase activity is associated with 26% reduction in $p34^{cdc2}$/cyclin B_1 complex expression /28/. This suppression of complex formation was not associated with changes in $p34^{cdc2}$ or cyclin B_1 synthesis since DADS did not alter the expression of either protein. Whether a depression in $p34^{cdc2}$ and cyclin B_1 binding or an increase in complex dissociation accounts for this suppression in $p34^{cdc2}$/cyclin B_1 complex expression remains to be determined.

Phosphorylation of $p34^{cdc2}$ kinase is also a key step in regulating its activity. This kinase is known to be phosphorylated on Thr14, Tyr15 and Thr161 to form an inactive hyperphosphorylated complex /94/. Removal of Thr14 and Tyr15 by a cdc25C phosphatase converts this inactive hyperphosphorylated complex to it active hypophosphorylated form /95/. Studies by Lee *et al.* /39/ found that SAMC (100 and 500 μM) increased $p34^{cdc2}$ phosphorylation in SMCs by 45% and 219%, respectively, compared to controls. Knowles and Milner /28/ also suggest that changes in $p34^{cdc2}$ phosphorylation accompany DADS exposure. A 15% increase in $p34^{cdc2}$ phosphorylation was observed in synchronized HCT-15 cells exposed to 100 μM. A 46% suppression of cdc25C expression was observed when 50 μM DADS was added to unsynchronized cells in culture. Consequently, suppression of cdc25C expression likely accounts for the ability of allyl sulfides to induce $p34^{cdc2}$ hyperphosphorylation.

Active $p34^{cdc2}$ is recognized for its ability to phosphorylate histone H1 and promote chromatin destabilization /43,93/. Increasing data show that histone acetylation also plays an important role in destabilizing chromatin structure and facilitating transcriptional activation /96/. Some inhibitors of histone deacetylase have been shown to be potent inducers of cell differentiation and/or apoptosis /97/. Recent studies by Lea *et al.* /19/ found that DAS and DADS increased the levels of mono-, di- and tri-acetylated H4 in mouse erythroleukemia (DS19) and human leukemia (K562) cells while decreasing the levels of unacetylated H4 histone. Decreases of 31% and 8% in histone deacetylase activity accompanied the increase in histone acetylation observed in k562 cells following 100 μM DADS and DAS exposure, respectively. Additional studies provided evidence that allyl mercaptan (100 μM), a major metabolite of DADS, inhibited histone

deacetylase activity by about 63%. Collectively, these studies reveal that an inhibition in histone deacetylase activity may be a critical event in mediating the growth inhibitory effects of allyl sulfides.

7. APOPTOSIS

Apoptosis represents a form of physiological cell loss that is crucial for maintaining normal tissue function /98/. This cell death is identified by a series of morphological changes that include cell shrinkage, membrane blebbing, chromosomal condensation and DNA fragmentation /99/. Increasing evidence suggests that a number of human diseases are associated with defects in the apoptotic pathway /100/. Compounds that induce apoptosis therefore offer an effective strategy for decreasing the incidence and severity of these diseases. SAMC, DADS, DATS and ajoene are all recognized for their ability to induce DNA fragmentation /12,22,25,32/. Studies by Sundaram and Milner /12/ show that although high concentrations of DADS (100 μM) induced DNA fragmentation (fivefold increase), apoptosis did not occur at lower concentrations (50 μM). These studies along with those of Knowles and Milner /27/ suggest that changes in cell division account for the growth suppression observed using lower concentrations (50 μM). However, it is conceivable that prolonged exposure to DADS and other allyl sulfur compounds does lead to an increase in apoptosis even at lower concentrations.

The ability of DADS and DATS to induce apoptosis is associated with an increase in intracellular Ca^{2+} levels /12,25/. Studies by Sakamoto *et al.* /25/ show that intracellular Ca^{2+} levels progressively increased when exposure to DATS was extended from 1 to 4 h. An 88% increase in intracellular Ca^{2+} was observed in A549 cells 4 h following DATS (10 μM) exposure. This increase was not permanent as demonstrated by the return of intracellular Ca^{2+} levels to control levels once the cells were refed complete medium. Studies by Sundaram and Milner /20/ showed that exposure to DADS resulted in dose dependent suppression in Ca^{2+}-ATPase activity. Since this enzyme normally functions to extrude Ca^{2+} from the cell /101/, this depression in Ca^{2+}-ATPase may account for the increase in intracellular Ca^{2+} observed following DADS and DATS exposure.

Severe oxidative stress can induce cell damage and lead to apoptosis /102/. Dirsch *et al.* /32/ showed that ajoene induces a dose

and time dependent increase in peroxide production. This induction is rapid as demonstrated by 35% increase in peroxide production within 5 min of ajoene (5 μM) exposure. Furthermore, induction of NF-κB nuclear translocation was also observed following 3 h ajoene (10 μM) exposure. Collectively, these studies suggest that reactive oxygen species production plays an important role in the ability of ajoene to induce apoptosis. Additional studies will need to clarify whether the increase in peroxide production and NF-κB activation by ajoene is functionally associated. Likewise, additional studies are needed to determine whether other sulfur compounds arising from garlic are also capable of altering NF-κB.

8. CONCLUSIONS

While historical evidence pointed to garlic as an effective anti-cancer agent, only during the past decade have sufficient data emerged to support such claims. Overall, allyl sulfur compounds appear to be responsible for the antineoplastic effects of garlic in model systems. Several mechanisms appear to account for the antitumorigenic properties of allyl sulfur compounds (Fig. 5). By increasing the under-

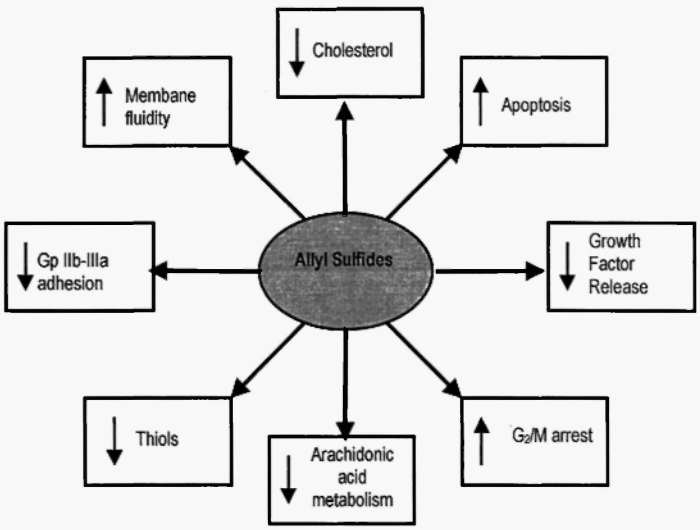

Fig. 5: Allyl sulfides modify a number of factors involved in tumor proliferation.

standing of the molecular targets for these compounds it may be possible to identify who might benefit most from exaggerated intakes of garlic and associated sulfur compounds. The ability of these compounds to preferentially suppress the growth of neoplastic over non-neoplastic cells suggests exciting possibilities may exist for developing effective intervention strategies in humans. Additional studies are needed in humans that document the biological effects of allyl sulfur compounds on biomarkers for cancer risk. This information is vital to understand the true antitumorigenic properties of garlic and related allyl sulfur constituents.

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