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Molecular Mechanisms and Targets of Cancer Chemoprevention by Garlic-derived Bioactive Compound Diallyl Trisulfide

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Abstract

Medicinal benefits of *Allium* vegetables, including garlic, have been documented throughout the written history. Health benefits of garlic and other *Allium* vegetables (e.g., onions), such as lipid lowering and anticancer effects, are credited to metabolic byproducts, including diallyl trisulfide (DATS). Evidence for anticancer effects of garlic derives from both population-based case-control studies, and clinical and laboratory investigations using purified garlic constituents such as DATS. Studies have shown that DATS can offer protection against chemically-induced neoplasia as well as oncogene-driven spontaneous cancer development in experimental rodents. Mechanisms underlying cancer chemopreventive effects of DATS are not completely understood, but known pharmacological responses to this natural product include alteration in carcinogen-metabolizing enzymes, cell cycle arrest, induction of apoptotic cell death, suppression of oncogenic signal transduction pathways, and inhibition of neoangiogenesis. This article reviews mechanisms and targets of cancer chemoprevention by DATS.

Keywords

Garlic; Diallyl Trisulfide; Apoptosis; Chemoprevention

Introduction

Historical perspective on medicinal uses of garlic has been elegantly reviewed by Rivlin[1] and Rahman[2]. Scientific literature is also packed with convincing experimental evidence demonstrating efficacy of *Allium* vegetables and/or their metabolic byproducts against a number of chronic diseases, including cardiovascular problems, diabetes, infections, and cancer[3-6]. Health benefits of garlic and other *Allium* vegetables are attributed to sulfur-containing compounds, which are generated upon processing (cutting or chewing) of these edible plants[7]. Evidence for anticancer effects of *Allium* vegetables derives from both population-based case-control studies[8-12] and laboratory findings[13-15]. For example, You et al[8] studied the association of *Allium* vegetable intake with the risk of gastric cancer in a population-based case-control study involving 564 patients and more than 1100 normal healthy subjects. This study concluded that the subjects with *Allium* vegetable intake were at a significantly lower risk of developing stomach cancer compared with low intake[8]. Similar epidemiological associations have been noted for esophageal cancer[9], prostate cancer[10], pancreatic cancer[11], and endometrial cancer[12] to name a few. These epidemiological observations have undoubtedly sparked interest among biologists to identify bioactive anticancer constituents from *Allium* vegetables. Major findings pertaining to cancer

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chemopreventive pharmacology of one such naturally-occurring compound, diallyl trisulfide (DATS), are summarized in this article.

Biochemistry of DATS Production

Biochemical synthesis of DATS (CH_2=CH-CH_2-S-S-S-CH_2-CH=CH_2) begins with γ-glutamyl-*S*-alk(en)yl-L-cysteine, which is hydrolyzed and oxidized to produce alliin (Figure 1)[7]. Alliin is the odorless precursor of DATS (also referred as allitridi in some publications). Processing of garlic (cutting or chewing) generates a vacuolar enzyme (allinase), which acts upon alliin to give rise to allicin and other alkyl alkane-thiosulfinates[7]. Allicin and related thiosulfinates are decomposed to yield various sulfur compounds including DATS. It has been estimated that one gram of fresh garlic may contain 900-1100 μg of DATS[15].

In Vivo Evidence for Anticancer/Chemopreventive Activity of DATS in Experimental Rodents

Belman and colleagues[16] were the first to show inhibition of chemically-induced skin carcinogenesis in mice by garlic oil. Published results documenting efficacy of DATS against cancer in experimental rodents are summarized in Table 1. Treatment of female A/J mice p.o. with 20 μmol DATS 96- and 48-hour prior to administration of 2 mg oral benzo[a]pyrene (BP), an environmental carcinogen abundant in cigarette smoke and barbecued food, resulted in 85% decrease in forestomach tumor multiplicity[17]. On the other hand, the number of pulmonary adenoma resulting from BP administration was not significantly reduced by DATS administration[17]. Oral administration of 25 μmol DATS, twice 48 hour apart, significantly inhibited forestomach cancer multiplicity induced by BP[18]. Gavage of 6 μmol DATS thrice weekly to male athymic mice subcutaneously implanted with PC-3 human prostate cancer cells caused retardation of xenograft growth without causing weight loss[19]. For example, twenty days after PC-3 cell injection the average tumor volume in vehicle-treated control mice (565 ± 112 mm^3) was about 3-fold higher compared with DATS-treated mice[19]. The DATS treatment in this study was started on the day of tumor cell implantation[19]. Shankar et al[20] also reported growth inhibitory effect of oral DATS administration (40 mg/kg, 5 times/week) against PC-3 cells orthotopically implanted in male BALB/c nude mice. Interestingly, co-treatment of PC-3 xenograft bearing mice with DATS (40 mg/kg, 5 times/week) and TRAIL (15 mg/kg administered intravenously on day 2, 8, 15, and 22) was more effective in inhibiting prostate tumor growth than either agent alone[20]. Intravenous administration of polybutylcyanoacrylate nanoparticle of DATS (1.5 mg/kg every alternate day for 14 days) significantly retarded the growth of orthotopically implanted HepG2 cells in nude mice[21]. Interestingly, DATS alone was not effective in this hepatocellular carcinoma xenograft model[21]. Intraperitoneal administration of 50 mg DATS/kg body weight to BALB/c nude mice with CT-26 murine colon cancer allograft significantly inhibited tumor growth[22]. Our group used a transgenic mouse model (Transgenic Adenocarcinoma of Mouse Prostate mice; commonly abbreviated as TRAMP mice) to determine efficacy of DATS for prevention of prostate cancer[23]. Incidence of poorly-differentiated carcinoma in the dorsolateral prostate of mice treated with 2 mg DATS/mouse (thrice/week) was lower by 41% (P= 0.035) in comparison with control mice. Moreover, the area occupied by the poorly-differentiated prostate cancer in mice administered with 2 mg DATS was lower by about 76% (P= 0.0189) compared with control mice[23]. Topical application of DATS at 5 or 25 μmol dose significantly inhibited incidence (22% inhibition by 25 μmol DATS) and multiplicity (25.6% and 71.1% inhibition by 5 and 25 μmol DATS, respectively) of skin papilloma in a two-stage chemically-induced skin model in female ICR mice[24]. Interestingly, in a medium-term bioassay in rats involving single i.p. injection of 200 mg diethylnitrosamine followed by two-third partial hepatectomy at week 3, gavage of 150 mg DATS/kg body weight two

weeks after carcinogen challenge caused a significant increase, not reduction, in number and area of GST-P positive foci in the liver[25]. This single study suggests that DATS may have tumor promoting effects in some models of chemically-induced cancer[25].

Pharmacokinetics of DATS

Pharmacokinetic parameters for DATS have been measured in Wistar rats after a single injection of 10 mg DATS administered *via* a jugular vein cannula[26]. Blood DATS concentration-time curves were analyzed using two-compartment analysis. The maximum blood concentration (C_{max}) of DATS was 5516.9 μg/L (~31 μM)[26] but peaked rapidly with a T_{max} (time to reach C_{max}) of about 1 min. Further studies are needed to determine the pharmacokinetic behavior and oral bioavailability of DATS in humans because this knowledge is integral for dose and schedule optimization in future clinical investigations.

Clinical Investigation of DATS

A double-blinded placebo-controlled interventional study was conducted in China using large doses of DATS in combination with selenium[27]. Inclusion criteria included at least one of the following: medical history of stomach disorder, family history of tumor, or smoking and/or alcohol consumption. A total of 2,526 and 2,507 individuals were randomly enrolled into the interventional and control arms, respectively, from villages of Shandong Province, China[27]. The interventional group of subjects was administered orally with 200 mg of synthetic DATS every day plus 100 μg of selenium every other day for one month of each year during November 1989 to December 1991. The control subjects received 2 placebo capsules. Large doses of DATS were well tolerated by all subjects without any harmful side effects. In the first five year follow-up between 1992 and 1997 after stopping the treatment, a decline in cancer morbidity rates was observed for the interventional group[27]. The relative risk, after adjustment for age, gender, and other confounders, for all tumors and gastric cancer were 0.67 (95% confidence level 0.43 - 1.03) and 0.48 (95% confidence level 0.21 - 1.06), respectively[27]. For male subjects, the relative risk for all tumors and gastric cancer were 0.51 (95% confidence level 0.30 - 0.85) and 0.36 (95% confidence level 0.14 - 0.92), respectively. However, a similar association was not evident in the female sub-group. This study not only established safety of large doses of DATS administration in humans but also demonstrated its cancer chemopreventive effect[27].

Effect of DATS on Drug Metabolizing Enzymes

Suppression of cytochrome P450-dependent monooxygenases (collectively referred to as Phase 1 drug-metabolizing enzymes), which are responsible for metabolic activation of chemical carcinogens, and/or induction of Phase 2 carcinogen detoxifying enzymes (*e.g.*, glutathione *S*-transferase, NADPH:quinone oxidoreductase) is considered major mechanism for cancer chemoprevention by naturally-occurring dietary bioactive compounds. Female A/J mice treated p.o. with 25 μmol DATS twice, 48-hour apart, and sacrificed 48 hours after the second administration exhibited a modest yet statistically significant decrease in activity of epoxide hydrolase in the forestomach[28] compared with control mice. Interestingly, hepatic epoxide hydrolase activity was about 2.6-fold higher in DATS-treated mice compared with control mice[28]. DATS administration had no effect on Phase 1 ethoxyresorufin *O*-deethylase activity in either liver or lung[28]. Glutathione *S*-transferase (GST) activity towards ultimate carcinogenic metabolite of BP was significantly increased by DATS administration in liver and forestomach, but not in the lung[28]. However, quantitation of individual GST subunit protein levels from the lung of control and DATS-treated mice revealed induction of Pi class GST subunit by about 1.8-fold[29]. Interestingly, compared with control mice, DATS-treated mice exhibited a robust increase in protein levels of other classes of GST subunits in addition to Pi class GST subunit[30]. For example,

the levels of hepatic Alpha class GST subunit α1 (mGSTA1 according to revised nomenclature), α3 (mGSTA3), and α4 (mGSTA4) were increased by about 1.7-, 8.0-, and 2.2-fold, respectively, upon DATS administration compared with control[30]. Even more robust induction of mGSTA1 and mGSTA2 by DATS administration was observed in the forestomach, a target organ for BP-induced cancer in A/J mice[30]. Noticeably, a dimer consisting of mGSTA1 and mGSTA2 subunit is exceptionally efficient in catalyzing glutathione conjugation and hence detoxification of the ultimate carcinogenic metabolite of BP compared with other classes of GSTs[31]. A 1.4- to 2.1-fold increase in protein levels of Mu class GST subunits was also evident in the liver of DATS-treated mice in comparison with control[30]. These studies provided evidence for DATS-mediated induction of carcinogen detoxifying GST subunits in the liver as well as in target organs in mice[29,30]. DATS treatment also resulted in a significant increase in NADPH:quinone oxidoreductase activity in the forestomach and lung of female A/J mice compared with control, and upregulation of its protein levels in the forestomach[32]. Five-day feeding of rats with 89 mg DATS/kg/day resulted in a robust increase in activity of NAD(P)H:quinone oxidoreductase in the liver (2.7-fold increase), kidney (5.5-fold increase), spleen (3.1-fold increase), lung (6.5-fold increase), forestomach (2.8-fold increase), and heart (2.5-fold increase) compared with those of control animals[33]. DATS-mediated increase in GST activity in rats was much less pronounced (*e.g.*, only 1.4-fold increase in the liver) compared with the effect observed on NADPH:quinone oxidoreductase activity[33]. Together, these observations point towards species-related differences (mice *versus* rats) in DATS-mediated induction of Phase 2 enzymes[29,30,32,33]. NADPH:quinone oxidoreductase and heme oxygenase mRNA levels were increased by more than 4.5-fold upon 6-hour treatment with 100 μM DATS in HepG2 cells. There was also an increase in luciferase reporter activity mediated by antioxidant response element, and the protein levels of transcription factor Nrf2[34]. DATS-mediated increase in luciferase activity was significantly attenuated by ectopic expression of a dominant negative Nrf2 and Keap1 and antioxidants[34]. In a study using rat primary hepatocytes, 24- and 48-hour exposure to 25 and/or 50 μM DATS caused an increase in activities of glutathione peroxidase and glutathione reductase[35]. Intracellular levels of reduced glutathione were also increased in rat hepatocytes after treatment with DATS[35]. On the other hand, rat hepatoma 4HIIE cells failed to show elevation of GST or NADPH:quionone oxidoreductase activity after 48-hour treatment with 50 or 100 μM DATS[36]. DATS was shown to inhibit (a) nuclear RNA synthesis in isolated rat liver nuclei, (b) 17β-estradiol binding to calf thymus DNA after liver microsome activation, and (c) RNA polymerase activity in the presence of a versatile epoxide-forming oxidant, dimethyldioxirane[37]. In a study involving HepG2 cells, DATS treatment inhibited BP-induced ethoxyresorufin *O*-deethylase activity, a marker enzyme for CYP1, by 70-95% at 100-1,000 μM concentrations[38]. Immunoblotting studies revealed DATS-mediated suppression of BP-inducible CYP1A2 protein[38]. Accordingly, levels of BP-7,8-diol were significantly reduced in DATS-treated microsomes[38]. These results suggest that the protective mechanism of DATS on BP-induced carcinogenesis is possibly also related to suppression of CYP1-mediated bioactivation of this carcinogen.

DATS-Mediated Inhibition of Cancer Cell Proliferation and Cell Cycle Arrest

Milner and colleagues were the first to demonstrate anti-proliferative effect of DATS against cancer cells[39]. DATS-mediated suppression of cancer cell proliferation is associated with cell cycle arrest[40-53], which has been reported in human liver cancer cells[40], gastric cancer cells[41], colon cancer cells[42], prostate cancer cells[43-48], lung cancer cells[50,51], bladder cancer cells[52], and skin cancer cells[53]. Most of these studies have revealed G_2/M phase or mitotic arrest upon DATS treatment, but the mechanisms underlying blockade of cell cycle progression are better characterized in prostate cancer cells[43-48]. DATS-mediated G_2/M phase cell cycle arrest in prostate cancer cells was associated with reactive oxygen species

(ROS)-dependent hyperphosphorylation and destruction of the cell division cycle 25C phosphatase[43]. Notably, DATS-mediated G_2/M phase cell cycle arrest occurred selectively in cancerous cells because a normal prostate epithelial cell line (PrEC) was resistant to cell cycle arrest by DATS[43]. Follow-up studies revealed that the ROS generation by DATS treatment in prostate cancer cells was caused by an increase in the level of labile iron due to c-Jun N-terminal kinase (JNK)-mediated degradation of the iron storage protein ferritin[45]. Further investigation indicated that DATS-treated prostate cancer cells were also arrested in prometaphase partly due to checkpoint kinase 1-dependent inactivation of the anaphase promoting complex/cyclosome[44,46,47]. A mechanistic model for DATS-induced cell cycle arrest in prostate cancer cells is schematically depicted in Figure 2. DATS-mediated cell cycle arrest in J5 human liver cancer cells was accompanied by accumulation of cyclin B1 and down-regulation of cyclin-dependent kinase (Cdk)7[40]. Even though mitotic markers were not examined in this study, accumulation of cyclin B1 is suggestive of mitotic arrest by DATS in J5 cells[40]. In H358 human lung cancer cells, DATS treatment resulted in induction of cyclin B1, and down-regulation of total and Tyr15 phosphorylated Cdk1 (inactive kinase) and cell division cycle 25C phosphatase[50].

Using HCT-15 and DLD-1 human colon cancer cells as a model[42], it was shown that DATS treatment disrupted microtubule network formation. DATS treatment also inhibited tubulin polymerization in an in vitro cell-free system[42]. Peptide mass-mapping by liquid chromatography-tandem mass spectrometry of DATS-treated tubulin revealed modification of cysteine residues Cys-12β and Cys354β[42]. DATS-mediated disruption of microtubule network formation has also been observed in HT-29 human colon cancer cell line[49]. Because DATS-mediated mitotic arrest was irreversible[46] and a fraction of cells arrested in mitosis were driven to apoptosis[47], it is reasonable to conclude that cell cycle arrest is an important event in suppression of cancer cell proliferation by DATS.

Molecular Mechanisms of DATS-Induced Apoptosis

Numerous publications have concluded that apoptosis induction is an important mechanism for anticancer activity of DATS. The first report on DATS-induced apoptosis was published by Milner and colleagues who observed DNA fragmentation in DATS-treated A549 human lung cancer cells[39]. Elucidation of the mechanism(s) underlying DATS-induced apoptosis has been the topic of intense research in the last decade. Table 2 summarizes studies delineating mechanism of DTS-induced apoptosis with some functional experiments to test validity of the observed molecular changes. Most studies implicate involvement of Bcl-2 family proteins in regulation of DATS-mediated apoptosis. For example, work from our group has revealed that DATS-induced apoptosis in PC-3 and DU145 human prostate cancer cells, which are androgen-independent and lack functional wild-type p53, is associated with a decrease in Bcl-2 protein level as well as its hyperphosphorylation leading to reduced Bcl-2:Bax interaction and activation of caspase-9 and caspase-3[54]. DATS-mediated hyperphosphorylation of Bcl-2 in PC-3 and DU145 cells is mediated by ROS-dependent activation of JNK, and to a smaller extent by activation of extracellular signal-regulated kinase 1/2[54]. DATS treatment decreased Bcl-2 and Bcl-xL protein levels and caused induction of proapoptotic multidomain protein Bak in LNCaP human prostate cancer cell line[55], which is androgen-responsive and expresses wild-type p53. While ectopic expression of Bcl-2 or Bcl-xL did not confer any protection of cell death resulting from DATS exposure in the LNCaP cells[55], partial protection against cell death was discernible in the PC-3 cell line[54]. These observations point towards cell line-specific differences in DATS-induced apoptotic mechanisms. RNA interference of Bax and Bak proteins also conferred partial but significant protection against DATS-induced apoptosis in LNCaP cells[55]. Similar to PC-3 and DU145 cells[54], DATS-induced apoptosis in LNCaP cells was accompanied by ROS generation. In LNCaP cells, both ROS generation and apoptosis resulting from DATS

treatment were significantly attenuated in the presence of *N*-acetylcysteine[55]. Collectively, these studies indicated that DATS-induced apoptosis was not influenced by the androgen-receptor or the p53 status[54,55]. In the context of androgen-receptor signaling, DATS treatment has been shown to cause transcriptional repression and inhibition of nuclear translocation of androgen-receptor in LNCaP cells, its androgen-independent variant LNCaP-C4-2, and in a cell line derived from spontaneously developing prostate tumor of a TRAMP mouse (TRAMP-C1 cells)[56]. Moreover, DATS-mediated prevention of prostate cancer development in TRAMP mice was associated with a significant decrease in expression of androgen-receptor in the poorly-differentiated cancer[56]. Thus, suppression of androgen receptor signaling by DATS probably contributes to its anticancer effect in prostate cancer.

It is intriguing to note that DATS treatment causes only a modest increase in protein levels of Bax and Bak in LNCaP cells and no increase in Bax level in the PC-3 cells yet knockdown of these proteins confers statistically significant protection against DATS-induced apoptosis[54,55]. Even though the mechanism(s) by which Bax and Bak regulate DATS-induced cell death are not fully elucidated, it is possible that DATS induces a conformational change and oligomerization of Bax/Bak resulting in their translocation to the mitochondria. This speculation is partially supported by the following correlative observations: (a) certain apoptotic stimuli cause Bax activation in an ROS-dependent manner and DATS treatment causes ROS generation[51,55]; (b) microtubule damaging agents induce Bax activation, and DATS treatment is shown to disrupt tubulin network[42].

In PC-3 and DU145 cells, DATS treatment resulted in suppression of P-Akt$^{Ser473/Thr308}$, P-GSK3α/β$^{Ser21/9}$, P-BAD^{Ser155}, total IGF-R protein level, and total PI3K protein level[57]. Net outcome of these alterations was reduced interaction between 14-3-3β and BAD leading to mitochondrial translocation of BAD[57]. Furthermore, DATS-induced apoptosis in DU145 cells was significantly attenuated by ectopic expression of constitutively active Akt[57]. DATS-mediated activation of caspase-3 and apoptosis were also inhibited in the presence of a pan-caspase inhibitor (zVAD-fmk) and a caspase-9 specific inhibitor (zLEHD-fmk)[57].

Using LNCaP and DU145 cells as models, we have investigated the role of signal transducer and activator of transcription 3 (STAT3)[58], which is activated in prostate cancer, in regulation of DATS-induced apoptosis. DATS treatment resulted in suppression of constitutive (DU145) as well as IL-6-induced (LNCaP) phosphorylation of STAT3^{Tyr705}, which correlated with inhibition of Janus-activated kinase 2 phosphorylation[58]. Constitutive and/or IL-6-induced nuclear translocation of P-STAT3 and STAT3 dimerization was also inhibited markedly on treatment with DATS in LNCaP and DU145 cell lines[58]. Inhibition of prostate cancer development in TRAMP mice by DATS correlated with a visible decrease in the levels of P-STAT3[58]. Interestingly, IL-6-mediated activation of STAT3 largely failed to confer any protection against proapoptotic response to DATS in both cells. Likewise, DATS-mediated inhibition of cell migration was either not affected or minimally reversed by the IL-6 treatment or ectopic expression of constitutively active STAT3[58]. These findings indicate that activation of STAT3 is largely dispensable for proapoptotic response to DATS, which should be viewed as a therapeutic advantage for this chemopreventive agent.

We have systematically studied the role of inhibitor of apoptosis family proteins in regulation of DATS-induced apoptosis using PC-3, LNCaP or DU145 cells[59]. Level of X-linked inhibitor of apoptosis (XIAP) protein was decreased upon 8-hour treatment with DATS[59]. In contrast, DATS-treated PC-3 and LNCaP cells exhibited marked induction of survivin and cIAP1 proteins[59]. Dorsolateral prostates from DATS-treated TRAMP mice exhibited statistically significant down-regulation of XIAP and induction of survivin protein compared with those of control mice[59]. Ectopic expression of XIAP conferred significant

protection against DATS-induced apoptosis[59]. On the other hand, DATS-induced apoptosis was only marginally affected by RNA interference of survivin or cIAP1[59]. These results indicate that DATS-induced apoptosis in prostate cancer cells is mediated by suppression of XIAP protein expression, and that XIAP represents a viable biomarker of DATS response in future clinical investigations.

Similar to the prostate cancer cells, DATS-induced apoptosis in H358 and H460 human lung cancer cell lines was associated with downregulation of Bcl-2 and Bcl-xL, and up-regulation of proapoptotic Bax, Bak, and BID protein expression[50]. BID protein was dispensable for DATS-induced apoptosis as evidenced by comparable sensitivity of SV40-immortalized mouse embryonic fibroblasts derived from wild-type and BID knockout mice. RNA interference of Bax and/or Bak significantly protected against DATS-induced apoptosis[50]. In human lung A549 adenocarcinoma cell line, DATS-induced apoptosis was accompanied by a marked and progressive increase in intracellular Ca^{2+}level[39]. In another study, DATS treatment caused downregulation of Bcl-2 (but not Bcl-xL), increased activation of JNK (but not p38), caused induction of p53 and survivin (but not Bax or Fas), and ROS generation in A549 cells[51]. Similar to the prostate cancer cells[54,55], DATS-induced apoptosis in A549 cells was significantly reduced in the presence of JNK inhibitor and *N*-acetylcysteine[51].

DATS was shown to cause apoptosis in HCT-15 and DLD-1 human colon cancer cells but the mechanism was not studied[42]. In BGC823 human gastric cancer cell line, DATS-induced apoptosis was associated with suppression of Bcl-2 expression and caspase-3 activation, but no change in Bax expression[60]. Functional studies to determine the role of Bcl-2 were not carried out by these investigators[60]. Caspase-3 activation by DATS treatment was shown in MGC803 gastric cancer cell line[61]. DATS treatment caused up-regulation of protein of programmed cell death 5 in gastric cancer cells[62]. A few studies have used proteomics and gene expression techniques to identify targets of DATS in gastric cancer cells. In DATS-treated BGC823 cells, a total of 41 unique proteins were detected with significant changes in their expression levels, including GST-Pi, voltage-dependent anion channel-1, Annexin I, Galectin, and S100A11[63]. Noticeably, nearly 50% of the DATS-modulated proteins (19/41) are associated with apoptotic pathways. DATS-inducible differentially expressed genes were identified in BGC823 gastric cancer cells using a highly specific subtractive hybridization of cDNA representational difference analysis[64]. A total of 14 cDNA fragments (11 upregulated and 3 downregulated by DATS treatment) were isolated and confirmed by reverse Northern blot analysis[64]. A proteomic study of DATS-treated Saos-2 osteosarcoma cells revealed alterations in 27 unique proteins (18 downregulated and 9 upregulated), of which 13 are related to either cell cycle or apoptosis[65]. However, functional studies to confirm the role of altered proteins in DATS-mediated cell cycle arrest or apoptosis are lacking. Similar to the prostate cancer cells[54], DATS was much more effective against Caco-2 and HT-29 human colon cancer cells compared with diallyl disulfide or diallyl sulfide[66]. Iitsuka et al[67] studied the relationship between lipophilicity and inhibitory activity against HT-29 colon cancer cell growth of natural and synthetic trisulfides. Compounds with 3-carbon chains were found to be stronger in terms of growth inhibition[67].

In T24 bladder cancer cells, correlative studies revealed suppression of P-Akt^{Ser473} and P-PDK1^{Ser241} phosphorylation, downregulation of Bcl-2. and Bax induction upon treatment with DATS[52]. DATS-induced apoptosis in A375 and BCC skin cancer cells was accompanied by ROS generation, collapse of mitochondrial membrane potential, and caspase-3 and caspase-9 cleavage[53]. DATS treatment caused apoptosis in MCF-7[68] and MDA-MB-231 human breast cancer cells[69]. Correlative studies showed induction of p53 and Bax mRNA and protein, and upregulation of Fas and downregulation of Akt and Bcl-2 mRNA in DATS-treated MCF-7 cells[68]. In MDA-MB-231 cells, DATS treatment resulted

in ROS production, which was detected through glutaredoxin (GRX), a redox-sensing molecule, and subsequently GRX was dissociated from apoptosis signal-regulating kinase 1 (ASK1). Dissociation of GRX from ASK1 resulted in the activation of ASK1 and JNK-Bim pathway. A JNK inhibitor blocked DATS-induced Bim phosphorylation and protected cells from DATS-induced cytotoxicity[69]. Caspase-8 and p38 mitogen-activated protein kinase were implicated in DATS-induced apoptosis in CNE2 human nasopharyngeal cells[70]. In T98G and U87MG human glioblastoma cells, DATS treatment caused apoptosis in association with ROS generation and caspase-3 activation[71].

Evidence for DATS-Mediated Apoptosis In Vivo

A few studies have looked at DATS-induced apoptosis *in vivo*. Our laboratory was the first to show that DATS administration (6 μmol DATS, which equates to ~1 mg DATS/mouse or roughly 50 mg DATS/kg body weight, three times/week for 20 days) to PC-3 tumor bearing mice resulted in increased number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive apoptotic bodies[19]. DATS-induced apoptosis *in vivo* in PC-3 tumor xenografts correlated with statistically significant increase in protein levels of Bax and Bak in the tumor[19]. Unlike cellular data[54,55], however, the levels of Bcl-2, Bcl-xL, or BID were not altered by DATS administration in PC-3 xenografts *in vivo*[19]. In an orthotopic PC-3 xenograft model, five weekly injection of 40 mg DATS/kg body weight for 4 weeks resulted in increased TUNEL-positive apoptotic cells[20]. DATS-mediated downregulation of Bcl-2 and Bcl-xL, upregulation of death receptor 4, death receptor 5, Bax, and Bak, and activation of caspase-8 were also reported in the orthotopic PC-3 xenografts[20]. Reasons behind discrepancy in DATS effects in subcutaneous[19] *versus* orthotopic PC-3 xenografts[20] in the context of Bcl-2 and Bcl-xL expression are not clear but likely relate to route and frequency of DATS administration. Administration of a hepatic-targeted polybutylcyanoacrylate nanoparticles of DATS (1.5 mg/kg, every other day for 14 days) to orthotopic HepG2 xenograft bearing BALB/c mice resulted in increased TUNEL-positive apoptotic bodies in the experimental tumors compared with control tumors[21]. Level of Bcl-2 protein was significantly lower in HepG2 tumors from DATS nanoparticle group compared with those of control mice, but there were no significant differences in the expression of Fas, Fas ligand, and Bax[21].

To our surprise, prevention of prostate cancer development in TRAMP mice by DATS administration (1 or 2 mg DATS/day, thrice/week for 13 weeks) was not associated with increased apoptosis as judged by TUNEL-assay[23]. Several possibilities exist to explain discrepancies in the results between cultured human prostate cancer cells[54,55] and TRAMP model[23]. One possibility relates to the frequency and dose of DATS administration. A more intensive dosing regimen, such as higher dose and/or daily administration of DATS, may be required to elicit apoptotic response in the dorsolateral prostate of TRAMP mice *in vivo*. Likewise, the possibility that earlier treatment with DATS (*e.g.*, starting at 4 weeks of age) leads to increased apoptosis as well as even greater protection against prostate carcinogenesis in TRAMP mice cannot be ignored. Additional work is needed to systematically explore these possibilities.

Inhibition of Angiogenesis and Other Effects

DATS treatment inhibited capillary-like tube formation and migration of human umbilical vain endothelial cells[72]. Anti-angiogenic effect of DATS correlated with suppression of VEGF secretion, down-regulation of VEGF receptor-2 protein level and inactivation of Akt[72]. Even though DATS treatment inhibited migration of PC-3 cells, formation of new blood vessels was comparable in PC-3 tumor xenografts from control and DATS-treated mice as judged by immunohistochemical analysis of CD31[19]. Interestingly, DATS treatment

resulted in suppression of neoangiogenesis (based on CD31 and Factor VIII staining) coupled with reduction in levels of VEGF in the orthotopic PC-3 xenografts[20]. However, similar to subcutaneous PC-3 tumor xenograft study[19], DATS treatment did not inhibit angiogenesis (CD31 staining) in the TRAMP study[23].

Among other noticeable effects, DATS has been shown to: (a) augment activation of T cells and enhance anti-tumor function of macrophages[73], (b) reduce lipopolysaccaride-induced expression of inducible nitric oxide synthase, nitric oxide production, and activation of nuclear factor-κB transcription factor in RAW 264.7 cells[74], (c) modify membrane rigidity in tumor cells and platelet membrane[75], and (d) reverse cancer chemotherapy drug resistance in a osteosarcoma cell line by lowering level of P-glycoprotein[76]. To the contrary, DATS-mediated inhibition of P-glycoprotein function was not evident in another study[77].

Because age is a known risk factor for some cancers (*e.g.*, prostate cancer), we raised the question of whether DATS treatment affects lifespan[78]. We addressed this question using *C. elegans* as a model[78]. Treatment of worms with 5-10 μM DATS increased worm mean lifespan even when treatment was started during young adulthood. DATS administration increased the lifespan of daf-2 and daf-16 mutants, but not the eat-2 mutants[78]. Microarray experiments demonstrated that a number of genes regulated by oxidative stress and the skn-1 transcription factor were altered by DATS treatment[78]. Consistently, DATS treatment caused induction of the skn-1 target gene gst-4, and this induction was dependent on skn-1[78]. We also found that the effect of DATS on worm lifespan depended on skn-1 activity in both in the intestine and neurons. Together these results indicate that DATS increases *C. elegans* lifespan by enhancing the function of the pro-longevity transcription factor skn-1[78]. Further studies are needed to determine the anti-aging effect of DATS in other experimental systems prior to its promotion as a pro-longevity remedy in humans.

Concluding Remarks and Future Directions

Research over the years has revealed that DATS targets multiple pathways to inhibit cancer development, including potentiation of carcinogen detoxification, cell cycle arrest, induction of apoptosis, suppression of oncogenic signaling, and inhibition of angiogenesis. Because DATS exhibits other pharmacological effects, such as cardiovascular and anti-microbial effects, this compound can be classified as a promiscuous agent. This property is not unique to DATS because many other promising dietary cancer chemopreventive agents (e.g., cruciferous vegetable constituent sulforaphane) function similarly[79,80]. However, promiscuity may be an advantage for cancer chemopreventive agents because pathogenesis of cancer is complex involving abnormalities in multiple checkpoints and signaling pathways. Future research on DATS should focus on pharmacokinetics, bioavailability, and clinical investigations of DATS. Because DATS targets multiple signal transduction pathways, it is also plausible that this agent may prove useful in combination chemoprevention regimens involving mechanistically distinct agents.

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Abbreviations

DATS	Diallyl trisulfide
TRAMP	Transgenic Adenocarcinoma of Mouse Prostate

GST	Glutathione *S*-transferase
BP	benzo[a]pyrene
ROS	reactive oxygen species
JNK	c-Jun N-terminal kinase
Cdk	cyclin-dependent kinase
STAT3	signal transducer and activator of transcription 3
XIAP	X-linked inhibitor of apoptosis
GRX	glutaredoxin
ASK1	apoptosis signal-regulating kinase 1
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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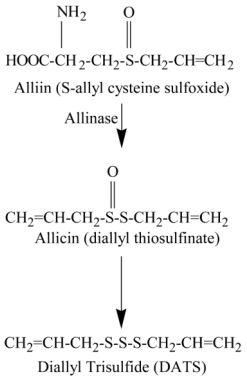

Figure 1.
Biochemical synthesis and chemical structure of diallyl trisulfide.

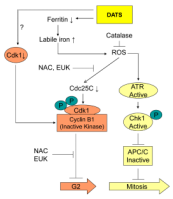

Figure 2.

Molecular mechanisms of DATS-induced cell cycle arrest in human prostate cancer cells[43-48]. The DATS treatment causes degradation of ferritin to cause an increase in levels of labile iron leading to ROS generation. The DATS-induced ROS generation results in down-regulation of Cdc25C, which is attenuated in the presence of antioxidants such as *N*-acetylcysteine and superoxide dismutase and catalase mimetic EUK134. The DATS treatment down-regulates Cdk1 expression in prostate cancer cells, but the mechanism of this effect is not yet clear. Mitotic arrest resulting from DATS exposure, characterized by accumulation of cyclin B1 and securin, is caused by checkpoint kinase 1-dependent inactivation of anaphase-promoting complex/cyclosome.

Table 1

In Vivo Efficacy of diallyl trisulfide against cancer in experimental rodents.

Species (sex)	Model	DATS dose/route	Outcome	Ref.
A/J mice (female)	BP[1]-induced	20 μmol DATS[2], oral	Forestomach cancer- 85% inhibition	17
			Lung adenoma- no effect	17
A/J mice (female)	BP-induced	25 μmol DATS, oral	Forestomach cancer- 86% inhibition	18
BALB/c nude mice (male)	PC-3 xenograft (subcutaneous)	6 μmol DATS, oral, three times/wk	Prostate tumor growth inhibition	19
BALB/c nude mice (male)	PC-3 xenograft (orthotopic)	40 mg/kg DATS, oral, five times/wk	Prostate tumor growth inhibition and potentiation of TRAIL efficacy	20
BALB/c nude mice (male)	HepG2 xenograft (orthotopic)	1.5 mg/kg, i.v, DATS-PBCA-NP[3]	Hepatocellular cancer growth inhibition	21
BALB/c nude mice (female)	CT26 allograft	50 mg/kg DATS, i.p., every fourth day	Colon cancer growth inhibition	22
TRAMP[4] mice (male)	Spontaneous	1 or 2 mg DATS/mouse, oral, thrice/wk for 13 wk	Inhibition of incidence and burden of poorly-differentiated prostate cancer	23
ICR mice (female)	two-stage skin papilloma model (DMBA[5] + TPA[6])	5 or 25 μmol DATS, topical, twice/wk for 20 wk	Inhibition of skin papilloma incidence (25 μmol DATS) and multiplicity (5 and 25 μmol DATS)	24
F344 Rats (male)	DEN[7] + PH[8] liver model	150 mg/kg DATS, gavage five times/wk for 8 wk	Increase in number and area of GST-P positive foci in the liver	25

[1] **Abbreviations:** BP, benzo[a]pyrene;

[2] DATS, diallyl trisulfide;

[3] DATS-PBCA-NP, polybutylcyanoacrylate nanoparticle of DATS;

[4] TRAMP, Transgenic Adenocarcinoma of Mouse Prostate;

[5] DMBA, 7,12-dimethylbenz(*a*)anthracene;

[6] TPA, 12-*O*-tetradecanoylphorbol-13-acetate;

[7] DEN, diethylnitrosamine;

[8] PH, partial hepatectomy.

Table 2

Molecular mechanisms of diallyl trisulfide (DATS)-induced apoptosis in cancer cells.

Tumor Type[Ref.]	Cell Line	DATS Dose	Observed Changes	Functional Studies and Outcome
Prostate[54]	PC-3, DU145	20-40 μM	↑ROS,↑P-JNK,↑P-Bcl-2, ↓Bcl-2:Bax Interaction	Protection against apoptosis by Bcl-2 and catalase overexpression
Prostate[55]	LNCaP	10-40 μM	↓Bcl-2, ↓Bcl-xL, ↑Bax, ↑Bak, ↑ROS	No effect on apoptosis by Bcl-2 or Bcl-xL overexpression, but protection by Bax/Bak siRNA
Prostate[57]	PC-3, DU145	40-80 μM	↓P-Akt, ↓GSK3-α/β, ↓P-BAD, ↓IGF-1R, ↓PI3K ↓14-3-3β:BAD interaction	Apoptosis inhibition by over-expression of constitutively active Akt and caspase inhibitors
Prostate[58]	LNCaP, DU145	20-40 μM	↓P-JAK2→↓P-STAT3 ↓STAT3 dimer formation	No effect on apoptosis by IL-6 mediated activation of STAT3
Prostate[59]	PC-3, LNCaP	20-40 μM	↓XIAP, ↑survivin, ↑cIAP1	Inhibition of apoptosis by ectopic expression of XIAP but modest effect of survivin or cIAP1 siRNA
Lung[50]	H358, H460	10-40 μM	↓Bcl-2, ↓Bcl-xL, ↑Bax ↑Bak, ↑BID	Apoptosis inhibition by Bax and/or Bak siRNA, but no effect of BID knockdown
Lung[51]	A549	12.5-100 μM	↓Bcl-2, ↑P-JNK, ↓P-ERK ↑p53, ↑survivin, ↑ROS	Inhibition of apoptosis by JNK inhibitor and antioxidants
Breast[69]	MDA-MB-231	10-100 μM	↑ROS, ↑P-ASK1, ↑JNK, ↑P-Bim	Protection against cytotoxicity by JNK inhibitor