Molecular mechanisms and targets of cancer chemoprevention by garlic-derived bioactive compound diallyl trisulfide

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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:** Apoptosis, Chemoprevention, Diallyl trisulfide, Garlic

Biochemistry of DATS production

Biochemical synthesis of DATS (CH$_2$=CH-CH$_2$-S-S-CH$_3$CH=CH$_3$) begins with $\gamma$-glutamyl-S-(en)yl-L-cysteine, which is hydrolyzed and oxidized to produce alliin (Fig. 1). Alliin is the odorless precursor of DATS. Alliinase, which acts upon alliin to give rise to allicin and related alkyl alkane-thiosulfinates. Allicin and related thiosulfimates are decomposed to yield various sulfur-containing compounds, which are generated upon processing (cutting or chewing) of these edible plants.

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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.
compounds including DATS. It has been estimated that one gram of fresh garlic may contain 900-1100 µg of DATS\textsuperscript{15}.

**In vivo evidence for anticancer/chemopreventive activity of DATS in experimental rodents**

Belman and colleagues\textsuperscript{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 for 96- and 48-h 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\textsuperscript{17}. On the other hand, the number of pulmonary adenoma resulting from BP administration was not significantly reduced by DATS administration\textsuperscript{17}. Oral administration of DATS (25 µmol), twice at an interval of 48 h, significantly inhibited forestomach cancer multiplicity induced by BP\textsuperscript{18}. Gavage of DATS (6 µmol) thrice weekly to male athymic mice subcutaneously implanted with PC-3 human prostate cancer cells caused retardation of xenograft growth without causing weight loss\textsuperscript{19}. For example, twenty days after PC-3 cell injection the average tumor volume in vehicle-treated control mice (565 ± 112 mm\textsuperscript{3}) was about 3-fold higher compared with DATS-treated mice\textsuperscript{19}. The DATS treatment in this study was started on the day of tumor cell implantation\textsuperscript{19}. Shankar et al\textsuperscript{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\textsuperscript{20}.

Intravenous administration of polybutylcyanoacrylate

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**Table 1—*In vivo* efficacy of diallyl trisulfide against cancer in experimental rodents**

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

Abbreviations: \textsuperscript{1}BP- Benzo[a]pyrene; \textsuperscript{2}DATS- Diallyl trisulfide; \textsuperscript{3}DATS-PBCA-NP- Polybutylcyanoacrylate nanoparticle of DATS; \textsuperscript{4}TRAMP- Transgenic adenocarcinoma of mouse prostate; \textsuperscript{5}DMBA- 7,12-Dimethylbenz(a)anthracene; \textsuperscript{6}TPA- 12-O-Tetradecanoylphorbol-13-acetate; \textsuperscript{7}DEN- Diethylnitrosamine; \textsuperscript{8}PH- Partial hepatectomy
nanoparticle of DATS (1.5 mg/kg every alternate day for 14 days) significantly retarded the growth of orthotopically implanted HepG2 cells in nude mice23. Interestingly, DATS alone was not effective in this hepatocellular carcinoma xenograft model23. Intraperitoneal administration of 50 µg DATS/kg body weight to BALB/c nude mice with CT-26 murine colon cancer allograft significantly inhibited tumor growth26. Our group used a transgenic mouse model (Transgenic Adenocarcinoma of Mouse Prostate mice; TRAMP mice) to determine efficacy of DATS for prevention of prostate cancer23. Incidence of poorly-differentiated carcinoma in the dorsolateral prostate of mice treated with 2 mg of 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 DATS (2 mg) was lower by about 76% (P= 0.0189) compared with control mice23. 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 of DATS, respectively) of skin papilloma in a two-stage chemically-induced skin model in female ICR mice23. Interestingly, in a medium-term bioassay in rats involving single injection (ip) of diethyl nitrosamine (200 mg) 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 liver23. This single study suggested that DATS might have tumor promoting effects in some models of chemically-induced cancer23.

**Pharmacokinetics of DATS**

Pharmacokinetic parameters for DATS have been measured in Wistar rats after a single injection of DATS (10 mg) administered via a jugular vein cannula20. Blood DATS concentration-time curves were analyzed using two-compartment analysis. The maximum blood concentration (C\text{max}) of DATS was 5516.9 µg/L (~31 μM)26 but peaked rapidly with a T\text{max} (time to reach C\text{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 selenium27. 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, China27. 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 rate was observed for the interventional group27. 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), respectively27. 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 effect27.

**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, at an interval of 48 h, and sacrificed after 48 h of the second administration exhibited a modest yet statistically significant decrease in activity of epoxide hydrolase in the forestomach28 compared with control mice. Interestingly, hepatic epoxide hydrolase activity was about 2.6-fold higher in DATS-treated mice compared with control mice28. DATS administration had no effect on Phase 1 ethoxyresorufin O-deethylase activity in liver or lung28. 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. 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. 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. 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. 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. 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. 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. These studies provided evidence for DATS-mediated induction of carcinogen detoxifying GST subunits in the liver as well as in target organs in mice. 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. 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. 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. Together, these observations point towards species-related differences (mice versus rats) in DATS-mediated induction of Phase 2 enzymes.

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. DATS-mediated suppression of cancer cell proliferation is associated with cell cycle arrest, which has been reported in human liver cancer cells, gastric cancer cells, colon cancer cells, prostate cancer cells, lung cancer cells, bladder cancer cells, and skin cancer cells. Most of these studies revealed G2/M phase or mitotic arrest upon DATS treatment, but the mechanisms underlying blockade of cell cycle progression were better characterized in prostate cancer cells. DATS-mediated G2/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. Notably,
DATS-mediated G2/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. Follow-up studies revealed that 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. 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. 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. Even though mitotic markers were not examined in this study, accumulation of cyclin B1 was suggestive of mitotic arrest by DATS in J5 cells. 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.

Using HCT-15 and DLD-1 human colon cancer cells as a model, it was shown that DATS treatment disrupted microtubule network formation. DATS treatment also inhibited tubulin polymerization in an in vitro cell-free system. Peptide mass-mapping by liquid chromatography-tandem mass spectrometry of DATS-treated tubulin revealed modification of cysteine residues Cys-12β and Cys354β. DATS-mediated disruption of microtubule network formation has also been observed in HT-29 human colon cancer cell line. Because DATS-mediated mitotic arrest was irreversible and a fraction of cells arrested in mitosis were driven to apoptosis, it was reasonable to conclude that cell cycle arrest was 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. Elucidation of the mechanism(s) underlying DATS-induced apoptosis had been the topic of intense research in the last decade. Table 2 summarizes studies delineating mechanism of DATS-induced apoptosis with some functional experiments to test validity of the observed molecular changes. Most studies implicated involvement of Bcl-2 family proteins in regulation of DATS-mediated apoptosis. For example, work from our group had revealed that DATS-induced apoptosis in PC-3 and DU145 human prostate cancer cells, which were androgen-independent and lack functional wild-type p53, was 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. DATS-mediated hyperphosphorylation of Bcl-2 in PC-3 and DU145 cells was mediated by ROS-dependent activation of JNK, and to a smaller extent by activation of extracellular signal-regulated kinase 1/2. DATS treatment decreased Bcl-2 and
Moreover, DATS-mediated prevention of prostate tumor of a TRAMP mouse (TRAMP-C1 cells) derived from spontaneously developing prostate androgen-receptor in LNCaP cells, its androgen-repression and inhibition of nuclear translocation of signaling, DATS treatment caused transcriptional status not influenced by the androgen-receptor or the p53 studies indicated that DATS-induced apoptosis was presence of ROS generation. In LNCaP cells, both ROS 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. Collectively, these studies indicated that DATS-induced apoptosis was not influenced by the androgen-receptor or the p53 status. In the context of androgen-receptor signaling, DATS treatment caused 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). 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. Thus, suppression of androgen receptor signaling by DATS probably contributed to its anticancer effect in prostate cancer.

It was intriguing to note that DATS treatment caused 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 conferred statistically significant protection against DATS-induced apoptosis. Even though the mechanism(s) by which Bax and Bak regulate DATS-induced cell death are not fully elucidated, it is possible that DATS induced a conformational change and oligomerization of Bax/Bak resulting in their translocation to the mitochondria. This speculation was partially supported by the following correlative observations: (a) certain apoptotic stimuli cause Bax activation in an ROS-dependent manner and DATS observations: (a) certain apoptotic stimuli cause Bax activation, and DATS treatment was shown to disrupt tubulin network.

In PC-3 and DU145 cells, DATS treatment resulted in suppression of P-Akt, P-JNK, P-Bcl-2, Bcl-2:Bax Interaction. Protection against apoptosis by Bcl-2 and catalase overexpression.

No effect on apoptosis by Bcl-2 or Bcl-xl overexpression, but protection by Bax/Bak siRNA

Apoptosis inhibition by over-expression of constitutively active Akt and caspase inhibitors

Inhibition of apoptosis by ectopic expression of XIAP but modest effect of survivin or cIAP1 siRNA

Inhibition of apoptosis by Jak and/or Bak siRNA, but no effect of BID knockout

Inhibition of apoptosis by JNK inhibitor and antioxidants

Protection against cytotoxicity by JNK inhibitor

Bcl-xL protein levels and caused induction of proapoptotic multidomain protein Bak in LNCaP human prostate cancer cell line, which was androgen-responsive and expressed 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, partial protection against cell death was discernible in the PC-3 cell line. These observations point towards cell line-specific differences in DATS-induced apoptotic mechanisms. RNA interference of Bak and Bak proteins also conferred partial, but significant protection against DATS-induced apoptosis in LNCaP cells. Similar to PC-3 and DU145 cells, 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. Collectively, these studies indicated that DATS-induced apoptosis was not influenced by the androgen-receptor or the p53 status. In the context of androgen-receptor signaling, DATS treatment caused 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). 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. Thus, suppression of androgen receptor signaling by DATS probably contributed to its anticancer effect in prostate cancer.

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In PC-3 and DU145 cells, DATS treatment resulted in suppression of P-Akt, P-GSK3α/β, P-BAD, P-IGF-1R, P-STAT3, P-JAK2→P-STAT3 dimer formation. Protection against apoptosis by IL-6 mediated activation of STAT3

Inhibition of apoptosis by over-expression of constitutively active Akt and caspase inhibitors

Inhibition of apoptosis by Bcl and/or Bak siRNA, but no effect of BID knockout

Inhibition of apoptosis by JNK inhibitor and antioxidants

Protection against cytotoxicity by JNK inhibitor

Table 2—Molecular mechanisms of diallyl trisulfide (DATS)-induced apoptosis in cancer cells

<table>
<thead>
<tr>
<th>Tumor Type&lt;sup&gt;Ref&lt;/sup&gt;</th>
<th>Cell line</th>
<th>DATS dose (µM)</th>
<th>Observed changes</th>
<th>Functional studies and outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate&lt;sup&gt;54&lt;/sup&gt;</td>
<td>PC-3, DU145</td>
<td>20-40</td>
<td>↑ROS, ↑P-JNK, ↑P-Bcl-2, ↓Bcl-2:Bax Interaction</td>
<td>Protection against apoptosis by Bcl-2 and catalase overexpression</td>
</tr>
<tr>
<td>Prostate&lt;sup&gt;55&lt;/sup&gt;</td>
<td>LNCaP</td>
<td>10-40</td>
<td>↑Bcl-2, ↑Bcl-xl, ↑Bax, ↑Bak, ↑ROS</td>
<td>No effect on apoptosis by Bcl-2 or Bcl-xl overexpression, but protection by Bax/Bak siRNA</td>
</tr>
<tr>
<td>Prostate&lt;sup&gt;57&lt;/sup&gt;</td>
<td>PC-3, DU145</td>
<td>40-80</td>
<td>↑P-Akt, ↑GSK3α/β, ↑P-BAD, ↑IGF-1R, ↑P-STAT3, 14-3-3β:BAD interaction</td>
<td>Apoptosis inhibition by over-expression of constitutively active Akt and caspase inhibitors</td>
</tr>
<tr>
<td>Prostate&lt;sup&gt;58&lt;/sup&gt;</td>
<td>LNCaP, DU145</td>
<td>20-40</td>
<td>↓P-JAK2→P-STAT3, ↓STAT3 dimer formation</td>
<td>No effect on apoptosis by IL-6 mediated activation of STAT3</td>
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<tr>
<td>Prostate&lt;sup&gt;59&lt;/sup&gt;</td>
<td>PC-3, LNCaP</td>
<td>20-40</td>
<td>↑XIAP, ↑survivin, ↑cIAP1</td>
<td>Inhibition of apoptosis by ectopic expression of XIAP but modest effect of survivin or cIAP1 siRNA</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;50&lt;/sup&gt;</td>
<td>H358, H460</td>
<td>10-40</td>
<td>↑Bcl-2, ↑Bcl-xl, ↑Bax, ↑Bak, ↑BID</td>
<td>Apoptosis inhibition by Bax and/or Bak siRNA, but no effect of BID knockout</td>
</tr>
<tr>
<td>Lung&lt;sup&gt;51&lt;/sup&gt;</td>
<td>A549</td>
<td>12.5-100</td>
<td>↑Bcl-2, ↑P-JNK, ↑P-ERK, ↑p53, ↑survivin, ↑ROS</td>
<td>Inhibition of apoptosis by JNK inhibitor and antioxidants</td>
</tr>
<tr>
<td>Breast&lt;sup&gt;59&lt;/sup&gt;</td>
<td>MDA-MB-231</td>
<td>10-100</td>
<td>↑ROS, ↑P-ASK1, ↑JNK, ↑P-BimEL</td>
<td>Protection against cytotoxicity by JNK inhibitor</td>
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In PC-3 and DU145 cells, DATS treatment resulted in suppression of P-Akt<sub>Ser473/Thr308</sub>, P-GSK3α/β<sub>Ser21/9</sub>, P-BAD<sub>Ser155</sub>, total IGF-R protein level, and total PI3K protein level. Net outcome of these alterations was reduced interaction between 14-3-3β and BAD leading to mitochondrial translocation of BAD. Furthermore,
DATS-induced apoptosis in DU145 cells was significantly attenuated by ectopic expression of constitutively active Akt. 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).

Using LNCaP and DU145 cells as models, we investigated the role of signal transducer and activator of transcription 3 (STAT3), which was 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 STAT3Tyr705, which correlated with inhibition of Janus-activated kinase 2 phosphorylation. 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. Inhibition of prostate cancer development in TRAMP mice by DATS correlated with a visible decrease in the levels of P-STAT3. 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. These findings indicated that activation of STAT3 was largely dispensable for proapoptotic response to DATS, which should be viewed as a therapeutic advantage for this chemopreventive agent.

We systematically studied the role of inhibitor of apoptosis family proteins in regulation of DATS-induced apoptosis using PC-3, LNCaP or DU145 cells. Level of X-linked inhibitor of apoptosis (XIAP) protein was decreased upon 8 h of treatment with DATS. In contrast, DATS-treated PC-3 and LNCaP cells exhibited marked induction of survivin and cIAP1. 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. Ectopic expression of XIAP conferred significant protection against DATS-induced apoptosis. On the other hand, DATS-induced apoptosis was only marginally affected by RNA interference of survivin or cIAP1. These results indicated that DATS-induced apoptosis in prostate cancer cells was mediated by suppression of XIAP protein expression, and that XIAP represented 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. 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. In human lung A549 adenocarcinoma cell line, DATS-induced apoptosis was accompanied by a marked and progressive increase in intracellular Ca2+ level. 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. Similar to the prostate cancer cells, DATS-mediated activation of STAT3 largely failed to confer any protection against proapoptotic response to DATS, which should be viewed as a therapeutic advantage for this chemopreventive agent.
in 27 unique proteins (18 downregulated and 9 upregulated), of which 13 were related to either cell cycle or apoptosis. However, functional studies to confirm the role of altered proteins in DATS-mediated cell cycle arrest or apoptosis were lacking. Similar to the prostate cancer cells, DATS was more effective against Caco-2 and HT-29 human colon cancer cells compared with diallyl disulfide or diallyl sulfide. Itsuka et al. 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.

In T24 bladder cancer cells, correlatives studies revealed suppression of P-AktSer473 and P-PDK1Ser241 phosphorylation, downregulation of Bcl-2, and Bax induction upon treatment with DATS. 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. DATS treatment caused apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells. Correlatives 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. 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. 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. Caspase-8 and p38 mitogen-activated protein kinase were implicated in DATS-induced apoptosis in CNE2 human nasopharyngeal cells. In T98G and U87MG human glioblastoma cells, DATS treatment caused apoptosis in association with ROS generation and caspase-3 activation.

Evidence for DATS-mediated apoptosis in vivo

Few studies were conducted on 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. 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. Unlike cellular data, however, the levels of Bcl-2, Bcl-xL, or BID were not altered by DATS administration in PC-3 xenografts in vivo. 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. 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. Reasons behind discrepancy in DATS effects in subcutaneous versus orthotopic PC-3 xenografts in the context of Bcl-2 and Bcl-xL expression were not clear, but likely related to route and frequency of DATS administration. Administration of a hepatic-targeted polybutyl-cyanoacrylate 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. 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.

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. Several possibilities exist to explain discrepancies in the results between cultured human prostate cancer cells and TRAMP model. 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, might 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) led 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 vein endothelial cells. Anti-angiogenic effect of DATS...
Correlated with suppression of VEGF secretion, down-regulation of VEGF receptor-2 protein level and inactivation of Akt27. Even though DATS treatment inhibited migration of PC-3 cells, formation of new blood vessels was comparable in PC-3 tumor xenographs from control and DATS-treated mice as judged by immunohistochemical analysis of CD3119. 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 xenographs20. However, similar to subcutaneous PC-3 tumor xenograph study19, DATS treatment did not inhibit angiogenesis (CD31 staining) in the TRAMP study23.

Among other noticeable effects, DATS was shown to: (a) augment activation of T cells and enhance anti-tumor function of macrophages75, (b) reduce lipopolysaccharide-induced expression of inducible nitric oxide synthase, nitric oxide production, and activation of nuclear factor-κB transcription factor in RAW 264.7 cells74, (c) modify membrane rigidity in tumor cells and platelet membrane75, and (d) reverse cancer chemotherapy drug resistance in a osteosarcoma cell line by lowering level of P-glycoprotein76. To the contrary, DATS-mediated inhibition of P-glycoprotein function was not evident in another study77.

Because age is a known risk factor for some cancers (e.g., prostate cancer), we raised the question of whether DATS treatment affects lifespan78. We addressed this question using C. elegans as a model79. Treatment of worms with 5-10 μM of 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 mutants78. Microarray experiments demonstrated that a number of genes regulated by oxidative stress and the skn-1 transcription factor were altered by DATS treatment78. Consistently, DATS treatment caused induction of the skn-1 target gene gst-4, and this induction was dependent on skn-178. 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 indicated that DATS increased C. elegans lifespan by enhancing the function of the pro-longevity transcription factor skn-178. 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 approaches
Research over the years revealed that DATS targeted 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 exhibited 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 similarly79,80. 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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