Published in final edited form as:

Breast Cancer Res Treat. 2013 February ; 138(1): 69–79. doi:10.1007/s10549-013-2440-2.

Critical role for reactive oxygen species in apoptosis induction and cell migration inhibition by diallyl trisulfide, a cancer chemopreventive component of garlic

Kumar Chandra-Kuntal, Joomin Lee, and Shivendra V. Singh

Department of Pharmacology & Chemical Biology, and University of Pittsburgh Cancer, Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Abstract

Diallyl trisulfide (DATS) is a structurally simple but biologically active constituent of processed garlic with *in vivo* activity against chemically-induced as well as oncogene-driven cancer in experimental rodents. The present study offers novel insights into the mechanisms underlying anticancer effects of DATS using human breast cancer cells as a model. Exposure of human breast cancer cells (MCF-7 and MDA-MB-231, respectively) and a cell line derived from spontaneously developing mammary tumor of a transgenic mouse (BRI-JM04) to DATS resulted in a dose-dependent inhibition of cell viability that was accompanied by apoptosis induction. A non-tumorigenic normal human mammary cell line (MCF-10A) was resistant to growth inhibition and apoptosis induction by DATS. The DATS-induced apoptosis in MDA-MB-231, MCF-7, and BRI-JM04 cells was associated with reactive oxygen species (ROS) production as evidenced by fluorescence microscopy and flow cytometry using a chemical probe (MitoSOX Red). Overexpression of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) as well as Mn-SOD conferred significant protection against DATS-induced ROS production and apoptotic cell death in MDA-MB-231 and MCF-7 cells. Activation of Bak, but not Bax, resulting from DATS treatment was markedly suppressed by overexpression of Mn-SOD. The DATS treatment caused ROS generation, but not activation of Bax or Bak, in MCF-10A cells. Furthermore, the DATS-mediated inhibition of cell migration was partially but significantly attenuated by Cu,Zn-SOD and Mn-SOD overexpression in association with changes in levels of proteins involved in epithelial-mesenchymal transition. The DATS-mediated induction of heme oxygenase-1 was partially attenuated by overexpression of Mn-SOD. These results provide novel mechanistic insights indicating a critical role for ROS in anticancer effects of DATS.

Keywords

diallyl trisulfide; reactive oxygen species; apoptosis; chemoprevention

Correspondence: Shivendra V. Singh, 2.32A Hillman Cancer Center Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213, USA. Phone: 412-623-3263; Fax: 412-623-7828; singhs@upmc.edu.

Conflict of interests

KC-K, JL, and SVS declare no conflict of interest.

Financial disclosure:

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under award number R01 CA113363-07 (to SVS). This research project used the Flow Cytometry Facility that was supported in part by a grant from the National Cancer Institute at the National Institutes of Health under award number P30 CA047904.

Introduction

Epidemiological studies have revealed an inverse association between dietary intake of *Allium* vegetables (eg, garlic and onion) and cancer risk [1, 2]. Water-soluble as well as lipid-soluble organosulfur compounds (OSCs) with anticancer activity have now been identified from *Allium* vegetables [3, 4]. It has been shown that even a subtle change in the structure of lipid-soluble OSCs can profoundly affect their anticancer activity *in vitro* (eg, inhibition of cancer cell proliferation and apoptosis induction) [5]. For example, diallyl trisulfide (DATS) is a much more potent inducer of apoptotic cell death compared with diallyl sulfide or diallyl disulfide in human prostate and breast cancer cells [5, 6]. Likewise, structure-activity relationship studies have established a critical role for the allyl group in anticancer effects of lipid-soluble OSCs as the compounds with saturated groups flanking the sulfur atoms (eg, propyl groups) are inactive regardless of the number of sulfur atoms [5].

Anticancer effect of lipid-soluble OSCs has been extended to *in vivo* models [6–12]. For example, we were the first to demonstrate that oral administration of diallyl disulfide inhibited growth of H-RAS oncogene transformed cells subcutaneously implanted in athymic mice in association with inhibition of p21^{H-ras} processing in the tumor [7]. Likewise, gavage with 6 μ mol DATS three times per week to PC-3 human prostate cancer bearing male athymic mice resulted in retardation of the xenograft growth [9]. Tumor volume for MCF-7 human breast cancer xenografts was significantly lower compared with control after oral treatment with 5 μ mol/kg DATS twice per week for 1 month in female Balb/c nude mice [6]. The incidence of poorly-differentiated carcinoma in the dorsolateral prostate of mice treated with 2 mg DATS/mouse (three times per week) was lower by 41% ($P=0.035$) in comparison with control mice [11]. In addition, DATS treatment inhibited angiogenic features in human umbilical vein endothelial cells [13].

The mechanisms underlying anticancer effects of DATS are not fully understood, but known cellular responses to this agent in cultured cancer cells include G₂ phase and mitotic arrest [14–16] and apoptosis induction [5, 6, 17, 18]. Previous studies, including those from our laboratory, have also revealed that the cell cycle arrest and apoptosis induction resulting from DATS exposure are associated with production of reactive oxygen species (ROS) [5, 6, 14, 17]. However, the mechanisms downstream of ROS production in DATS-induced apoptosis are not fully understood. The present study provides novel insights into the mechanism by which ROS regulates anticancer effects of DATS.

Materials and methods

Reagents

DATS (purity >98%) was purchased from LKT Laboratories (St. Paul, MN). Cell culture reagents were purchased from Invitrogen-Life Technologies (Carlsbad, CA); 4',6-diamidino-2-phenylindole (DAPI), eosin, and anti-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); antibodies against total and active Bak, Mn-superoxide dismutase (Mn-SOD) and Cu,Zn-SOD were from Calbiochem-EMD Millipore (Billerica, MA); the antibody against active Bax (clone 6A7) and E-cadherin were purchased from BD Transduction Laboratories-BD Biosciences (San Jose, CA). An antibody used against vimentin was procured from Santa Cruz Biotechnology (Santa Cruz, CA). A kit to quantify histone-associated DNA fragment release into the cytosol was from Roche Diagnostics (Mannheim, Germany).

Cell lines

The MCF-7, MDA-MB-231 and MCF-10A cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described by us previously [19]. The BRI-JM04 cell line (a generous gift from Dr. Anne Lenferink, Biotechnology Research Institute, Montreal, Canada) was maintained in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Each of these cell lines was authenticated and found to be of human (MCF-7, MDA-MB-231, and MCF-10A) or mouse (BRI-JM04) origin.

Cell viability and apoptosis assays

Stock solution of DATS was prepared in dimethyl sulfoxide (DMSO) and diluted with complete medium before experimental procedure. An equal volume of DMSO (final concentration <0.2%) was added to controls. Desired cell line was treated for 24 h with either DMSO (control) or specified concentrations of DATS. The effect of DATS treatment on cell viability was determined by trypan blue dye exclusion assay as described by us previously [5]. Apoptosis induction resulting from DATS treatment (24 h exposure) was assessed by analysis of histone-associated DNA fragment release into the cytosol.

Measurement of ROS production

ROS generation was determined by fluorescence microscopy or by flow cytometry using a chemical probe (MitoSOX Red) as described previously [20].

Stable overexpression of Mn-SOD and Cu,Zn-SOD

MCF-7 and MDA-MB-231 cells were stably transfected with pcDNA3.1 empty vector or the same vector encoding for Mn-SOD or Cu, Zn-SOD using Fugene6. Cells were selected by culture in the presence of 800 µg/mL of G418. Overexpression of the desired protein was confirmed by western blotting.

Immunocytochemical analysis

Desired cells (4×10^4 cells/mL) were cultured on coverslips and allowed to attach by overnight incubation. The cells were treated with DMSO or DATS for specified time at 37°C. Immunocytochemical analysis for active Bax or active Bak was performed as described previously [20, 26]. The stained cells were examined under a Leica DC300F microscope at $\times 100$ objective magnification.

Cell migration assay

Cell migration was determined using Transwell Boyden chamber (Corning, Acton, MA) containing a polycarbonate filter with a pore size of 8 µm as described previously [21]. The motile cells on the bottom face of the membrane were fixed with 90% ethanol and stained with eosin. Four randomly selected fields were examined under a microscope at 10 magnification.

Immunoblotting

Details of cell lysate preparation and western blotting have been described by us previously [22, 23]. The immunoreactive bands were detected using enhanced chemiluminescence method. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

Statistical analysis

Statistical significance of difference in measured variables between control and DATS treated groups was determined by one-way ANOVA followed by either Dunnett's test (for dose-response studies) or Bonferroni's test (for multiple comparisons). Difference was considered significant at $P < 0.05$.

Results

DATS inhibited viability of breast cancer cells in association with apoptosis induction

During the preparation of this manuscript, Na et al [6] showed inhibition of MCF-7 cell viability in the presence of DATS. Because MCF-7 cell line is estrogen-sensitive and expresses wild-type p53, it was of interest to determine if DATS-mediated inhibition of cell viability was affected by estrogen-receptor, p53 or human epidermal growth factor receptor-2 (HER-2) status. We addressed this question using MDA-MB-231 cells (estrogen-independent cell line with mutant p53 and normal HER-2) and BRI-JM04 (estrogen-receptor negative mouse mammary tumor cell line with HER-2 overexpression). As shown in Figure 1a, viability of MDA-MB-231, MCF-7, and BRI-JM04 cells was inhibited in the presence of DATS in a concentration dependent manner. Moreover, each tested cell line exhibited more or less similar sensitivity to DATS (Figure 1a). Interestingly, the viability of MCF-10A cell line (a non-tumorigenic cell line originally isolated from a fibrocystic breast disease and spontaneously immortalized) was not affected in the presence of DATS (Figure 1a). Consistent with previous observations in MCF-7 cells [6], the DATS-mediated inhibition of MDA-MB-231 and BRI-JM04 cell viability was accompanied by apoptosis induction as judged by analysis of histone-associated DNA fragment release into the cytosol (Figure 1b). Similar to cell viability data, the MCF-10A cell line was resistant to DATS-induced apoptosis (Figure 1b).

DATS treatment caused ROS generation in cancerous and normal breast cells

ROS have been implicated in apoptosis induction by several cancer chemopreventive phytochemicals, including withaferin A and isothiocyanates [19, 20, 24, 25]. Moreover, the DATS-induced apoptosis in MCF-7 cells was associated with ROS production [6]. We questioned if the ROS-dependence of DATS-induced apoptosis was unique to the MCF-7 cell line. The present study indicated that the ROS production upon exposure to DATS was a generalized phenomenon as evidenced by the data in MDA-MB-231 and BRI-JM04 cells using a chemical probe of ROS detection (MitoSOX Red). The MitoSOX Red is a cell permeable and mitochondria-targeting chemical probe. As shown in Figure 2a, MitoSOX Red fluorescence in the DMSO-treated control MDA-MB-231 and MCF-7 cells was weak and diffuse. On the other hand, treatment of both MDA-MB-231 and MCF-7 cell with DATS (3 h) resulted in enrichment of MitoSOX Red fluorescence that co-localized with the mitochondria as evidenced by appearance of yellow-orange color due to the merging of MitoSOX Red-associated red fluorescence and MitoTracker green-associated green fluorescence (Figure 2a). The basal MitoSOX Red fluorescence was relatively higher in the non-tumorigenic MCF-10A cell line than in either MDA-MB-231 or MCF-7. However, similar to breast cancer cells, DATS treatment caused an increase in MitoSOX Red fluorescence in MCF-10A cells as well (Figure 2a). The DATS-mediated ROS production in MDA-MB-231, MCF-7, BRI-JM04, and MCF-10A cells was confirmed by flow cytometry (Figure 2b). However, cell line-specific differences in the kinetics of DATS-induced ROS production were also observed. For example, ROS generation by DATS treatment was sustainable for up to 6 h in MDA-MB-231 and BRI-JM04 cells. On the other hand, enrichment of MitoSOX Red fluorescence over DMSO treated control peaked between 3 and 6 h in MCF-7 cells (Figure 2b). The DATS-mediated ROS production was modest but

highly variable or statistically insignificant at the 1- or 2-hour time points at least in MCF-7 cells (results not shown).

Overexpression of SOD conferred protection against DATS-induced apoptosis

Previous studies have utilized small molecule antioxidant *N*-acetylcysteine to determine the role for ROS in DATS-induced apoptosis [6]. In the present study we used a genetic approach involving overexpression of SOD to firmly establish the contribution of ROS in apoptotic cell death by DATS. A genetic method is preferred over a chemical approach involving small molecules due to the possibility of off-target effects. As expected, overexpression of both Cu,Zn-SOD and Mn-SOD (Figure 3a) resulted in a significant decrease in DATS-mediated ROS production in MDA-MB-231 and MCF-7 cells (Figure 3b). Inhibition of cell viability (Figure 3c) as well as apoptosis induction (Figure 3d) resulting from DATS exposure (24 h treatment) was partially but statistically significantly attenuated by overexpression of SOD (both Cu,Zn-SOD and Mn-SOD) in MDA-MB-231 and MCF-7 cells. Notably, the extent of DATS-induced apoptosis in vector transfected control MDA-MB-231 cells (Figure 3) was different from that observed in the untransfected cells (Figure 1). The discrepancy likely results from variability inherent to the assay for histone-associated DNA fragment release into the cytosol and/or cellular alterations associated with transfection.

Overexpression of SOD attenuated DATS-mediated activation of Bak in breast cancer cells

The results shown thus far indicated that ROS generation was critical for apoptosis induction by DATS but did not offer any insight into the mechanisms downstream of ROS generation in apoptotic cell death. The ROS-dependent activation of Bax and/or Bak is one possible mechanism underlying DATS-induced apoptosis. The ROS-dependent activation of Bax and/or Bak has been demonstrated previously in some models of apoptosis [26, 27]. We therefore determined the effect of DATS treatment on activation of Bax and Bak by immunofluorescence microscopy using antibodies specific for detection of activated proteins. As shown in Figure 4a,b the DATS treatment resulted in an increase in fluorescence associated with the active Bax and Bak in empty vector transfected MCF-7 cells in comparison with DMSO-treated control. Overexpression of Mn-SOD did not show any protection with respect to enrichment of activated Bax fluorescence (Figure 4a). In contrast, Mn-SOD overexpression protected against DATS-mediated activation of Bak (Figure 4b). These results indicated that overexpression of Mn-SOD attenuated DATS-mediated activation of Bak, but not Bax, in MCF-7 cells. Unlike MCF-7 cells, DATS-mediated activation of Bax or Bak was not readily evident in the MCF-10A cell line (Figure 4c). These results explain resistance of MCF-10A cells to DATS-induced apoptosis despite ROS production.

SOD overexpression attenuated DATS-mediated inhibition of MDA-MB-231 cell migration

An important component of tumor progression is the propensity of cancer cells to migrate and invade [28]. As shown in Figure 5a, DATS treatment inhibited migration of MDA-MB-231 cells. Because MCF-7 cells are not invasive, this analysis utilized MDA-MB-231 cells only. The DATS-mediated inhibition of cell migration was partially but statistically significantly attenuated by overexpression of Cu,Zn-SOD as well as Mn-SOD in MDA-MB-231 cells (Figure 5b). These results indicated that DATS-mediated inhibition of breast cancer cell migration was partially dependent on ROS production.

DATS treatment up-regulated E-cadherin in MDA-MB-231 breast cancer cells

The epithelial-mesenchymal transition (EMT) is critical for migration of cancer cells [29]. Suppression of E-cadherin coupled with induction of mesenchymal marker proteins (eg,

vimentin) is a biochemical hallmark of EMT [29]. We raised the question of whether DATS treatment inhibited EMT and whether this effect was related to ROS production. This analysis was restricted to MDA-MB-231 cells because MCF-7 is an epithelial-type cell line. Immunoblotting experiments revealed modest induction of E-cadherin and suppression of vimentin in DATS-treated MDA-MB-231 cells (Figure 6a). The DATS-mediated induction of E-cadherin was confirmed by immunofluorescence microscopy using empty vector transfected MDA-MB-231 cells (Figure 6b). Interestingly, overexpression of Mn-SOD alone resulted in induction of E-cadherin, which is consistent with tumor suppressor role for Mn-SOD [30–32]. However, overexpression of Mn-SOD markedly attenuated DATS-mediated induction of E-cadherin (Figure 6b). The empty vector transfected MDA-MB-231 cells exhibited suppression of vimentin protein level after 24 h treatment with DATS. On the other hand, the DATS-mediated suppression of vimentin protein expression was not observed in Mn-SOD overexpressing MDA-MB-231 cells. These results not only indicated redox-sensitive regulation of E-cadherin and vimentin protein expression, but also suggested that the Mn-SOD-mediated protection against DATS-induced cell migration inhibition may, at least in part, be related to modulation of the EMT.

Mn-SOD overexpression partially protected DATS-mediated induction of heme oxygenase-1 (HO-1)

Induction of detoxifying enzymes is believed to contribute to cancer chemoprevention by DATS [33]. We addressed the question of whether DATS-mediated ROS generation was responsible for induction of detoxifying enzymes. As shown in Figure 6d, induction of a prototypical detoxification protein hemoxygenase-1 (HO-1) was only partially attenuated by overexpression of SOD especially at the higher doses (Figure 6d).

Discussion

The DATS is a naturally-occurring cancer chemopreventive agent that has been tested for its *in vivo* efficacy in rodent cancer models [6,8–12] and in humans [34]. A double-blinded and placebo-controlled interventional study in China using 200 mg of synthetic DATS every day plus 100 µg of selenium indicated that DATS was well tolerated by all subjects [34]. In the first five years of follow-up after stopping the treatment, a decline in cancer morbidity rate was observed in the interventional group [34]. The relative risks, after adjustment for age, gender, and other confounders, for all tumors and gastric cancer were 0.67 (95% confidence interval 0.43 – 1.03) and 0.48 (95% confidence interval 0.21 – 1.06), respectively [34]. The present study indicates, for the first time, that the DATS treatment causes apoptosis induction in breast cancer cells regardless of the estrogen receptor, p53, and HER-2 status. Notably, inhibition of cell viability and migration and apoptosis induction resulting from DATS exposure in cultured breast cancer cells are observed at pharmacologically achievable concentration based on pharmacokinetic data in rats [35].

The mechanism by which DATS causes ROS production has been investigated previously and involves elevation of labile iron pool due to ferritin degradation in an c-jun NH₂-terminal kinase-dependent manner [36–38]. A role for the adapter protein p66Shc in DATS-induced ROS production was also demonstrated recently [37]. Another objective of the present study was to firmly establish the role of ROS in anticancer effects of DATS because previous studies have mostly relied on small molecule antioxidants to establish this association [6, 14]. Results described herein indicate that ROS generation is critical not only for DATS-induced apoptosis but also inhibition of the cell migration. The ROS-dependent activation of Bak, but not Bax, seems critical for apoptosis induction by DATS, whereas redox-sensitive changes in EMT markers is associated with inhibition of cell migration. It is important to point out that, to the best of our knowledge, the present study is the first to show modulation of EMT by DATS treatment. However, the mechanism by which DATS

treatment inhibits biochemical features of EMT (ie, induction of E-cadherin and vimentin suppression) remains elusive. Nevertheless, it is reasonable to conclude that ROS may not fully account for the induction of apoptosis as well as inhibition of cell migration by DATS as these effects are only partially attenuated by overexpression of SOD.

The relationship between DATS-mediated ROS production and activation of c-jun NH₂-terminal kinases (JNK) has been studied in the MCF-7 cell line [6]. The DATS was shown to cause JNK activation in an ROS-dependent manner in MCF-7 cells [6]. These observations are consistent with our own data in prostate cancer cells [5]. Based on these results, we conclude that ROS function upstream of JNK activation in execution of DATS-induced apoptosis.

The possibility that DATS-mediated inhibition of breast cancer cell migration is partly due to ensuing apoptosis can't be excluded because both these effects are partially abrogated by overexpression of SOD. Molecular circuitry facilitating cancer cell migration is quite complex involving alterations in cell morphology and activation of signaling mechanisms [39]. Literature data exists to suggest redox-sensitive regulation of cancer cell migration [40]. For example, members of the mitogen-activated protein kinase family are activated during cell migration, and ROS have been implicated in activation of these kinases via growth factor stimulation of receptor tyrosine kinases [41, 42]. Growth factor-stimulated ROS have been shown to transmit signals necessary for cell migration [39]. ROS can also serve to trigger cell death as is the case for DATS. It is possible that the level of oxidative stress after treatment of cancer cells with DATS is sufficient to provoke apoptosis and inhibition of cell migration as observed in the present study.

The potential adverse effect of ROS production by DATS deserves attention as oxidative stress is linked to the pathogenesis of many chronic diseases including cancer. However, the DATS-mediated ROS production may simply serve to kill cancer cells without having any harmful side effects as evidenced by the following observations: (a) DATS is abundant in vegetables consumed by humans on a daily basis, yet epidemiological data indicate an inverse association between dietary intake of *Allium* vegetables and the risk of cancer [1, 2]; (b) DATS administration is well-tolerated by experimental animals as well as humans [6, 10–12]; and (c) the DATS-mediated inhibition of cell viability and apoptosis induction is selective for cancer cells and not evident in a normal human mammary epithelial cell line.

Abbreviations

DATS	Diallyl trisulfide
OSCs	Organosulfides
ROS	Reactive oxygen species
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
PBS	Phosphate-buffered saline
SOD	Superoxide dismutase
HER-2	Human epidermal growth factor receptor-2
EMT	Epithelial-mesenchymal transition
JNK	c-Jun NH ₂ -terminal kinase
HO-1	Heme oxygenase-1

References

1. Challier B, Perarnau JM, Viel JF. Garlic, onion and cereal fiber as protective factors for breast cancer: a French case-control study. *Eur J Epidemiol.* 1998; 14:737–747. [PubMed: 9928867]
2. Fleischauer AT, Poole C, Arab L. Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. *Am J Clin Nutr.* 2000; 72:1047–1052. [PubMed: 11010950]
3. Block E. The organosulfur chemistry of the genus *Allium*: Implications for the organic chemistry of sulfur. *Angew Chem Int Ed Engl.* 1992; 31:1135–1178.
4. Antony ML, Singh SV. Molecular mechanisms and targets of cancer chemoprevention by garlic-derived bioactive compound diallyl trisulfide. *Ind J Exp Biol.* 2011; 49:805–816.
5. Xiao D, Choi S, Johnson DE, et al. Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. *Oncogene.* 2004; 23:5594–5606. [PubMed: 15184882]
6. Na HK, Kim EH, Choi MA, Park JM, Kim DH, Surh YJ. Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. *Biochem Pharmacol.* 2012; 84:1241–1250. [PubMed: 22981381]
7. Singh SV, Mohan RR, Agarwal R, et al. Novel anti-carcinogenic activity of an organosulfide from garlic: Inhibition of H-RAS oncogene transformed tumor growth in vivo by diallyl disulfide is associated with inhibition of p21^{H-ras} processing. *Biochem Biophys Res Commun.* 1996; 225:660–665. [PubMed: 8753815]
8. Sporn VL, Barany G, Wattenberg LW. Effects of organosulfur compounds from garlic and onions on benzo[*a*]pyrene-induced neoplasia and glutathione *S*-transferase activity in the mouse. *Carcinogenesis.* 1988; 9:131–134. [PubMed: 3335037]
9. Xiao D, Lew KL, Kim YA, et al. Diallyl trisulfide suppresses growth of PC-3 human prostate cancer xenograft *in vivo* in association with Bax and Bak induction. *Clin Cancer Res.* 2006; 12:6836–6843. [PubMed: 17121905]
10. Wu PP, Liu KC, Huang WW, et al. Diallyl trisulfide (DATS) inhibits mouse colon tumor in mouse CT-26 cells allograft model in vivo. *Phytomed.* 2011; 18:672–676.
11. Singh SV, Powolny AA, Stan SD, et al. Garlic constituent diallyl trisulfide prevents development of poorly differentiated prostate cancer and pulmonary metastasis multiplicity in TRAMP mice. *Cancer Res.* 2008; 68:9503–9511. [PubMed: 19010926]
12. Shrotriya S, Kundu JK, Na HK, Surh YJ. Diallyl trisulfide inhibits phorbol ester-induced tumor promotion, activation of AP-1, and expression of COX-2 in mouse skin by blocking JNK and Akt signaling. *Cancer Res.* 2010; 70:1932–1940. [PubMed: 20179211]
13. Xiao D, Li M, Herman-Antosiewicz A, et al. Diallyl trisulfide inhibits angiogenic features of human umbilical vein endothelial cells by causing Akt inactivation and down-regulation of VEGF and VEGF-R2. *Nut Cancer.* 2006; 55:94–107.
14. Xiao D, Herman-Antosiewicz A, Antosiewicz J, et al. Diallyl trisulfide-induced G2-M phase cell cycle arrest in human prostate cancer cells is caused by reactive oxygen species-dependent destruction and hyperphosphorylation of cell division cycle 25C. *Oncogene.* 2005; 24:6256–6268. [PubMed: 15940258]
15. Herman-Antosiewicz A, Singh SV. Checkpoint kinase 1 regulates diallyl trisulfide-induced mitotic arrest in human prostate cancer cells. *J Biol Chem.* 2005; 280:28519–28528. [PubMed: 15961392]
16. Herman-Antosiewicz A, Stan SD, Hahm ER, Xiao D, Singh SV. Activation of a novel ataxia-telangiectasia mutated and Rad3 related/checkpoint kinase 1-dependent prometaphase checkpoint in cancer cells by diallyl trisulfide, a promising cancer chemopreventive constituent of processed garlic. *Mol Cancer Ther.* 2007; 6:1249–1261. [PubMed: 17406033]
17. Kim YA, Xiao D, Xiao H, et al. Mitochondria-mediated apoptosis by diallyl trisulfide in human prostate cancer cells is associated with generation of reactive oxygen species and regulated by Bax/Bak. *Mol Cancer Ther.* 2007; 6:1599–1609. [PubMed: 17513609]
18. Xiao D, Singh SV. Diallyl trisulfide, a constituent of processed garlic, inactivates Akt to trigger mitochondrial translocation of BAD and caspase-mediated apoptosis in human prostate cancer cells. *Carcinogenesis.* 2006; 27:533–540. [PubMed: 16169930]

19. Xiao D, Vogel V, Singh SV. Benzyl isothiocyanate-induced apoptosis in human breast cancer cells is initiated by reactive oxygen species and regulated by Bax and Bak. *Mol Cancer Ther.* 2006; 5:2931–2945. [PubMed: 17121941]
20. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. *PLoS ONE.* 2011; 6:e23354. [PubMed: 21853114]
21. Kim SH, Sehrawat A, Sakao K, Hahm ER, Singh SV. Notch activation by phenethyl isothiocyanate attenuates its inhibitory effect on prostate cancer cell migration. *PLoS ONE.* 2011; 6:e26615. [PubMed: 22039516]
22. Xiao D, Srivastava SK, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G₂/M arrest and inducing apoptosis. *Carcinogenesis.* 2003; 24:891–897. [PubMed: 12771033]
23. Powolny AA, Bommarreddy A, Hahm ER, et al. Chemopreventative potential of the cruciferous vegetable constituent phenethyl isothiocyanate in a mouse model of prostate cancer. *J Natl Cancer Inst.* 2011; 103:571–584. [PubMed: 21330634]
24. Singh SV, Srivastava SK, Choi S, et al. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. *J Biol Chem.* 2005; 280:19911–19924. [PubMed: 15764812]
25. Xiao D, Powolny AA, Moura MB, et al. Phenethyl isothiocyanate inhibits oxidative phosphorylation to trigger reactive oxygen species-mediated death of human prostate cancer cells. *J Biol Chem.* 2010; 285:26558–26569. [PubMed: 20571029]
26. Xiao D, Powolny AA, Singh SV. Benzyl isothiocyanate targets mitochondrial respiratory chain to trigger reactive oxygen species-dependent apoptosis in human breast cancer cells. *J Biol Chem.* 2008; 283:30151–30163. [PubMed: 18768478]
27. Buccellato LJ, Tso M, Akinci OI, Chandel NS, Budinger GR. Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. *J Biol Chem.* 2004; 279:6753–6760. [PubMed: 14625274]
28. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanism. *Nat Rev Cancer.* 2003; 3:362–374. [PubMed: 12724734]
29. Tomaskovic-Crook E, Thompson EW, Thierry JP. Epithelial to mesenchymal transition and breast cancer. *Breast Cancer Res.* 2009; 11:213. [PubMed: 19909494]
30. Werts ED, Gould MN. Relationships between cellular superoxide dismutase and susceptibility to chemically induced cancer in the rat mammary gland. *Carcinogenesis.* 1986; 7:1197–1201. [PubMed: 3087649]
31. Li JJ, Oberley LW, St Clair DK, Ridnour LA, Oberley TD. Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase. *Oncogene.* 1995; 10:1989–2000. [PubMed: 7761099]
32. Tsai SM, Hou MF, Wu SH, et al. Expression of manganese superoxide dismutase in patients with breast cancer. *Kaohsiung J Med Sci.* 2011; 27:167–172. [PubMed: 21527182]
33. Chen C, Pung D, Leong V, et al. Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Rad Biol Med.* 2004; 37:1578–1590. [PubMed: 15477009]
34. Li H, Li HQ, Wang Y, et al. An intervention study to prevent gastric cancer by micro-selenium and large dose of allitridum. *Chin Med J.* 2004; 117:1155–1160. [PubMed: 15361287]
35. Sun X, Guo T, He J, et al. Simultaneous determination of diallyl trisulfide and diallyl disulfide in rat blood by gas chromatography with electron-capture detection. *Pharmazie.* 2006; 61:985–988. [PubMed: 17283653]
36. Antosiewicz J, Herman-Antosiewicz A, Marynowski SW, Singh SV. c-Jun NH₂-terminal kinase signaling axis regulates diallyl trisulfide-induced generation of reactive oxygen species and cell cycle arrest in human prostate cancer cells. *Cancer Res.* 2006; 66:5379–5386. [PubMed: 16707465]
37. Borkowska A, Sielicka-Dudzin A, Herman-Antosiewicz A, et al. Diallyl trisulfide-induced prostate cancer cell death is associated with Akt/PKB dephosphorylation mediated by P-p66shc. *Eur J Nutr.* 2012; 51:817–825. [PubMed: 22020565]

38. Sielicka-Dudzin A, Borkowska A, Herman-Antosiewicz A, et al. Impact of JNK1, JNK2, and ligase Itch on reactive oxygen species formation and survival of prostate cancer cells treated with diallyl trisulfide. *Eur J Nutr.* 2012; 51:573–581. [PubMed: 21863240]
39. Hurd TR, Degennaro M, Lehmann R. Redox regulation of cell migration and adhesion. *Trends Cell Biol.* 2011; 22:107–115. [PubMed: 22209517]
40. Tothhawng L, Deng S, Pervaiz S, Yap CT. Redox regulation of cancer cell migration and invasion. *Mitochondrion.* 2012 in press. S1567–7249(12)00203–6. 10.1016/j.mito.2012.08.002
41. Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog.* 2006; 5:14. [PubMed: 16689993]
42. Wu WS, Wu JR, Hu CT. Signal cross talks for sustained MAPK activation and cell migration: the potential role of reactive oxygen species. *Cancer Metastasis Rev.* 2008; 27:303–314. [PubMed: 18299806]

Figure 1.

DATS inhibits viability of breast cancer cells in association with apoptosis induction. **a** Effect of DATS treatment (24 h exposure) on viability of breast cancer cells (MDA-MB-231, MCF-7, and BRI-JM04) and MCF-10A cells as determined by trypan blue dye exclusion assay. **b** Effect of DATS treatment (24 h exposure) on histone-associated DNA fragment release into the cytosol, a measure of apoptotic cell death, in breast cancer cells (MDA-MB-231, MCF-7, and BRI-JM04) and MCF-10A cells. Results shown are mean $\pm$ SD ($n=3$). *Significantly different ($P < 0.05$) compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Each experiment was performed at least twice.

Figure 2.

DATS treatment caused ROS production in cancerous and normal breast cells. **a** Immunofluorescence microscopy for MitoSOX Red and MitoTracker Green fluorescence in MDA-MB-231, MCF-7, and MCF-10A cells following 3 h treatment with DMSO (control) or DATS (20 or 40 μ M). **b** Flow cytometric measurement of MitoSOX Red fluorescence in MDA-MB-231, MCF-7, BRI-JM04, and MCF-10A cells following 3 or 6 h treatment with DMSO (control) or DATS (20 or 40 μ M). Results shown are mean $\pm$ SD (n=3).

*Significantly different ($P < 0.05$) compared with corresponding DMSO-treated control by one-way ANOVA followed by Dunnett's test. Each experiment was performed at least twice and the results were consistent.

Figure 3.

Overexpression of SOD confers protection against DATS-induced apoptosis. **a** Western blotting for Cu,Zn-SOD or Mn-SOD in MDA-MB-231 or MCF-7 cells stably transfected with empty pcDNA3.1 vector (lane 1) or the same vector encoding for Cu,Zn-SOD or Mn-SOD (lane 2). The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein level. Number on top of the immunoreactive band represents change in protein level relative to empty vector transfected cells. **b** ROS generation after 3 h treatment with DMSO (control) or the indicated concentration of DATS in MDA-MB-231 or MCF-7 cells stably transfected with empty vector or corresponding SOD plasmid. **c** Cell viability after 24 h treatment with DMSO (control) or the indicated concentration of DATS in MDA-MB-231 or MCF-7 cells stably transfected with empty vector or SOD (Cu,Zn-SOD or Mn-SOD) plasmid. **d** Histone-associated DNA fragment release into the cytosol after 24 h treatment with DMSO (control) or the indicated concentration of DATS in MDA-MB-231 or MCF-7 cells stably transfected with empty vector or SOD (Cu,Zn-SOD or Mn-SOD) plasmid. Results shown are mean $\pm$ SD (n=3). Significantly different (P < 0.05) compared with * DMSO-treated control, and # between empty vector transfected cells and SOD overexpressing cells by one-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 4.

Overexpression of Mn-SOD confers protection against DATS-mediated Bak activation. **a** Immunocytochemical analysis for activated Bak in MCF-7 cells transfected with empty pcDNA3.1 vector or the same vector encoding for Mn-SOD and treated for 8 h with DMSO (control) or the indicated concentrations of DATS. **b** Immunocytochemical analysis for activated Bak in MCF-7 cells transfected with empty pcDNA3.1 vector or the same vector encoding for Mn-SOD and treated for 8 h with DMSO (control) or the indicated concentrations of DATS. **c** Immunocytochemical analysis for activated Bak and activated Bak in MCF-10A cells after 8 h treatment with DMSO or the indicated concentrations of DATS. Images were acquired at 100 objective magnification. Each experiment was repeated twice, and representative data from one such experiment are shown.

Figure 5.

Overexpression of Mn-SOD confers protection against DATS-mediated inhibition of cell migration. **a** Representative images depicting *in vitro* migration by MDA-MB-231 cells transfected with the empty pcDNA3.1 vector or the same vector encoding for Cu,Zn-SOD or Mn-SOD and treated for 24 h with DMSO (control) or the indicated concentrations of DATS ($\times 10$ objective magnification). **b** Quantitation of the results shown in panel **a**. Results shown are mean $\pm$ SD ($n=3$). Significantly different ($P < 0.05$) compared with * DMSO-treated control, and # between empty vector transfected cells and SOD overexpressing cells by one-way ANOVA followed by Bonferroni's multiple comparison test. Each experiment was performed at least twice and the results were consistent.

Figure 6.

Overexpression of Mn-SOD confers protection against DATS-mediated suppression of vimentin. **a** Immunoblotting for E-cadherin and vimentin using lysates from MDA-MB-231 cells treated for 8 h with DMSO or the indicated concentrations of DATS. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein level. Numbers on top of the immunoreactive bands represent change in protein level relative to DMSO-treated control. Immunoblotting for each protein was performed at least twice using independently prepared lysates, and representative data from one such experiment are shown. **b** Immunocytochemical analysis for E-cadherin in MDA-MB-231 cells transfected with empty pcDNA3.1 vector or the same vector encoding for Mn-SOD and treated for 8 h with DMSO (control) or the indicated concentrations of DATS. **c** Immunocytochemical analysis for vimentin in MDA-MB-231 cells transfected with empty vector or the same vector encoding for Mn-SOD and treated for 24 h with DMSO (control) or the indicated concentrations of DATS. Images were acquired at 100 objective magnification. **d** Western blotting for heme oxygenase-1 using lysates from MCF-7 cells transfected with empty pcDNA3.1 vector or the same vector encoding for Mn-SOD and treated for 24 h with DMSO (control) or the indicated concentrations of DATS. Quantitation relative to respective DMSO-treated control is shown.