INTRODUCTION
Breast cancer is the second most common cancer in American women [1]. Recent cancer statistics announced that 281,550 new cases of breast cancer are estimated to be diagnosed in American women and 43,600 women in the USA are expected to die from breast cancer [1]. In recent years, the overall mortality rates from breast cancer have decreased but the incidence rates continue to increase by about 0.5% per year [1]. As breast cancer is highly heterogeneous disease broadly grouped into three major molecular subtypes (luminal-subtype, HER2-enriched, and basal-like) with distinct gene expression signature, treatment options are subtype-dependent whilst current prevention strategy is available for luminal-type breast cancer only [2-6]. The agents used for prevention of luminal-type breast cancer include selective estrogen receptor modulators (tamoxifen and raloxifene) and aromatase inhibitors like exemestane [4-6]. However, these agents also have side effects. For example, chronic tamoxifen use is associated with the increased risk of uterine cancer, cataracts, and perimenopausal symptoms [7,8]. Therefore, a safe and inexpensive chemopreventive intervention efficacious against different subtypes of breast cancer is clinically attractive.

Epidemiological studies including the French case-control study and the Puerto Rico study published in 1998 and 2020, respectively, have shown that breast cancer risk is inversely associated with increased intake of garlic and onions [9,10]. The antitumor effect of garlic/onions is attributable to organosulfur compounds that are generated upon cutting or
chewing of these edible plants [11]. Diallyl trisulfide (DATS) is one such component of garlic that exhibits antitumor activity in breast cancer in vitro and in vivo [12-23]. The antitumor characteristics of DATS in breast cancer include inhibition of cell proliferation, apoptosis induction, cell cycle arrest, suppression of self-renewal of breast cancer-stem like cells, and inhibition of metastasis properties in vitro and/or in vivo [12-23]. Several oncogenic pathways are also inhibited by DATS treatment in breast cancer cells in vitro, and the examples include NF-κB, HIF-1α, Wnt/β-catenin, leptin, STAT3, etc. [12-23]. We have also demonstrated previously that FoxQ1 transcription factor is a novel target of DATS in suppressing self-renewal of breast cancer stem-like cells [17].

The estrogen receptor-α (ER-α) is a well-known driver of luminal-type human breast cancer [24]. We have shown previously that DATS treatment inhibits ER-α expression and activity in luminal-type human breast cancer cells [15]. Treatment of MCF-7 and T47D cells with DATS resulted in down-regulation of ER-α protein [15]. The DATS was more effective in decreasing the level of ER-α protein when compared to its mono and disulfide analogs [15]. Na et al. [13] were the first to demonstrate an in vivo inhibitory effect of DATS in a therapeutic setting (xenograft model) of luminal-type MCF-7 cells. A very mild regimen of twice weekly oral administration of about 0.9 mg DATS/kg body weight for one month exhibited remarkable inhibition (> 80%) of MCF-7 xenograft growth [13]. These observations prompted us to determine chemopreventive efficacy of DATS using an N-methyl-N-nitrosourea (MNU)-induced rat mammary tumor model, which shares molecular resemblance to luminal-type human breast cancers [22,25]. In this experiment, DATS was administered by gavage at 5 mg/kg and 25 mg/kg body weight [22]. Even though DATS treatment did not cause weight loss or any other signs of overt toxicity, the incidence of mammary cancer was not affected [22]. The primary goal of the present study was to test whether mammary cancer in the MNU-rat model is affected by oral administration of DATS at 50 mg/kg body weight. Mammary tumors from control and DATS-treated rats were also used for RNA-seq analysis.

MATERIALS AND METHODS

Reagents

The DATS (purity about 99%) was purchased from LKT Laboratories (St. Paul, MN, USA), dissolved in dimethyl sulfoxide (DMSO; 28 mM stock), and stored at –80°C prior to use. Antibodies against neurofascin (NFASC), ribosomal protein L11 (RPL11), ribosomal protein S14 (RPS14), NADH:ubiquinone oxireductase core subunit V1 (NDUFV1), and NADH:ubiquinone oxireductase core subunit S1 (NDUFS1) were purchased from Proteintech Group (Rosemont, IL, USA). The N-acetyltransferase domain containing 1 (NATD1) antibody was from Aviva Systems Biology (San Diego, CA, USA). The antibodies against p38α/β mitogen-activated protein kinase (MAPK), phospho-(Tyr 182)-p38, extracellular-signal-related kinase 1 (ERK1), and phospho-(Tyr 204)-ERK were from Santa Cruz Biotechnology (Dallas, TX, USA). The c-Jun N-terminal kinase 2 (JNK2) antibody not recognizing JNK1

![Figure 1. Effect of diallyl trisulfide (DATS) administration on N-methyl-N-nitrosourea (MNU)-induced mammary tumor development in female Sprague–Dawley rats.](http://www.jcpjournal.org)
or JNK3 but detecting endogenous JNK2 at a molecular weight of 46 and 54 kDa and phospho-(Thr183/Tyr185)-JNK antibody were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody was from Sigma-Aldrich (St. Louis, MO, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from GeneTex (Irvine, CA, USA).

Ethics statement
The use of rats for the chemoprevention study was approved by the Animal Care and Use Committee of the University of Pittsburgh (PHS Assurance Number: D16-00118).

Chemoprevention study
Twenty-one day old female Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). The MNU was freshly prepared in acidified (pH < 5.0) 0.9% sodium chloride solution in the dark prior to injection. The rats were injected intraperitoneally with 50 mg/kg body weight of MNU. One week after MNU injection, the rats were randomly divided into two groups and given GATS or vehicle 5 times a week for 10 weeks. Control rats (n = 20) were treated with 100 µL of corn oil by gavage, while the second group of rats (n = 20) received 50 mg/kg of DATS in corn oil by gavage. Body weight of the rats was recorded weekly. The rats were monitored daily for palpable tumors to compute latency, and the mammary tumor volume was measured with a caliper two times/week. One rat from the control group was euthanized 11 weeks after MNU injection. The rest of the rats were euthanized 13 days after MNU injection and thus this animal was excluded from the study. The remaining rats were euthanized 11 weeks after MNU injection.

RNA-seq analysis
Tumor weight-matched 3 mammary tumors from the control and DATS-treated rats were used for RNA-seq analysis. RNA extraction and quality control were done by Cancer Genomics Facility of the UPMC Hillman Cancer Center. The RNA-seq analysis was outsourced to Novogene (Sacramento, CA, USA). The details of RNA-seq analysis have been published elsewhere [26]. The RNA-seq data were analyzed using the gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway tools. The RNA-seq data presented in this study have been submitted to the Gene Expression Omnibus of NCBI and can be retrieved by accession number GSE189640.

Western blotting
Preparation of lysates from rat mammary tumors was done as described previously [27]. Blots were stripped and re-probed with anti-β-actin or GAPDH antibody for normalization. Immunoreactive bands were visualized by the enhanced chemiluminescence method. Quantification of protein expression was performed by use of UN-SCAN-IT gel analysis & graph digitizing software (Version 7.1; Silk Scientific Corporation, Orem, UT, USA).

Statistical analysis
GraphPad Prism (version 8.0.0; GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Statistical significance of difference was determined by two-tailed unpaired Student’s t-test.

Figure 2. Effect of DATS treatment on gene expression changes in mammary tumors of rats. (A) Venn diagram showing unique as well as common genes between control and DATS treatment groups. (B) Volcano scatter plots displaying genes upregulated or downregulated in response to DATS exposure in mammary tumors of rats. (C) Heatmap visualizing the differentially expressed genes by DATS administration. (D) Gene Set Enrichment Analysis (GSEA). DATS, diallyl trisulfide; MNU, N-methyl-N-nitrosourea; CON, control.
RESULTS

Effect of DATS administration on MNU-induced mammary tumorigenesis in rats
In our previous study, administration of 5 mg/kg and 25 mg/kg body weight DATS was unable to prevent MNU-induced mammary carcinogenesis [22]. Therefore, we escalated the dose of DATS to 50 mg/kg body weight to assess its in vivo efficacy. As can be seen in Figure 1A, the body weight of the rats was not affected by DATS administration. Mammary tumor latency was delayed by 2 weeks by DATS administration (Fig. 1B). On the other hand, DATS administration did not affect tumor incidence, burden or multiplicity (Fig. 1C).

Effect of DATS administration on gene expression in mammary tissues
Next, we performed RNA-seq analysis using mammary tumors from control and DATS-treated rats (n = 3) to gain insights into the lack of in vivo efficacy of DATS in the rat model. The total mapping rates for the tumors of the control and the DATS-treated groups were about 95.35% ± 0.62% and 95.47% ± 0.29%, respectively (Table S1). Unique mapping rates for the control and DATS-treated rats were about 88.46% ± 0.29% and 88.08% ± 0.58%, respectively (Table S1). The Venn diagram showing unique as well as common genes between mammary tumors of control and DATS-treated rats is shown in Figure 2A. The Volcano scatter plot showed that DATS administration significantly upregulated

Figure 3. The gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses. The GO enrichment analysis of upregulated (A) or downregulated (B) genes by diallyl trisulfide (DATS) administration. The KEGG pathway enrichment analysis of upregulated (C) or downregulated (D) genes by DATS treatment. Padj, adjusted P-value.
577 genes and downregulated 507 genes (Fig. 2B). The heatmap displays similar gene expression pattern among different samples within the same group (Fig. 2C). The gene set enrichment analysis (GSEA) showing upregulated or downregulated genes by DATS is presented in Figure 2D.

The GO and KEGG pathway analyses

Next, the differential expressed genes in response to DATS treatment were analyzed by GO and KEGG pathway tools (Fig. 3). The GO pathway analysis indicated upregulation of genes associated with translation, ribonucleoprotein complex, and peptide biosynthetic/metabolic processes (Fig. 3A). The downregulated genes in response to DATS treatment from the GO pathway analysis included plasma membrane, cell periphery, and cysteine-type endopeptidase inhibitor activity, and so forth (Fig. 3B). We focused on ribosomal proteins for verification of the RNA-seq data because KEGG pathway analysis also showed enrichment of genes associated with ribosome in response to DATS treatment (Fig. 3C). Ribosomal biogenesis is a target for cancer therapy [28]. A total of 33 ribosome-associated genes were upregulated by DATS treatment (Table S2). As can be seen in Figure 4A, RNA-seq showed upregulation of RPL11 and RPS14 in mammary tumors from DATS-treated rats when compared to controls. Figure 4B shows western blots for RPL11 and RPS14 proteins. The expression of RPL11 protein, but not RPS14, was significantly higher in mammary tumors of DATS-treated rats when compared to controls (Fig. 4C).

The KEGG pathway analysis showed DATS-mediated upregulation of genes associated with oxidative phosphorylation (Fig. 3C). The expression of complex I subunit NDUFV1 was significantly higher in the mammary tumors of DATS-treated rats when compared to control tumors (Figure S1A). However, the protein levels of complex I subunits NDUFV1 or NDUF1 were not affected by DATS treatment (Figure S1B and S1C).

The KEGG pathway analysis of the RNA-seq data indicated downregulation of genes associated with MAPK (Fig. 3D). Except for JNK2 protein, the expression of mRNA or protein levels of other MAPK were not affected by DATS treatment (Fig. 5).

The GSEA analysis indicate that NFASC and NATD1 are highly expressed genes in response to DATS treatment (Fig. 2D). RNA-seq analysis shows the increased expression of NFASC (> 9.9 fold) following DATS treatment (Fig. 6A). There was a trend for an increase in the protein level of NFASC in mammary tumors from DATS-treated rats, but the difference was not statistically significant (Fig. 6B and 6C). Breast cancer TCGA data set reveals a significantly low expression of NFASC in breast tumor when compared to normal mammary tissues (data not shown). Mammary tumors from the DATS-treated rats showed an increase in mRNA levels of NATD1 (Fig. 6A). Surprisingly, the protein level of NATD1 was lower in the DATS-treated group compared to control, albeit statistical insignificance due to large data scatter in the control group (Fig. 6B and 6C). It is possible that DATS treatment decreases the translation or stability of this protein. Further experimentation is necessary to explore these possibilities.

DISCUSSION

In a double-blind and placebo-controlled interventional study in gastric cancer patients, DATS was well-tolerated after daily administration at 200 mg in combination with 100 µg of selenium for one month of each year during 1989 to 1991 [29].

Figure 4. DATS administration elevated mRNA and protein expression of RPL11 in the mammary tumors of rats. (A) Quantification of RPL11 and RPS14 levels in RNA-seq data. Results shown are mean ± SD (n = 3). (B) Immunoblots for RPL11, RPS14, and β-actin in tumor lysates from rats. (C) Quantification of the expression of the proteins shown in (B). Results shown are mean ± SD (n = 6 for both groups). RPL11, ribosomal protein L11; FPKM, the expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced; DATS, diallyl trisulfide; RPS14, ribosomal protein S14. *Significantly different compared with control (P < 0.05) by unpaired Student’s t-test.
In the first five-year follow-up (1992 to 1996) after treatment termination, a marked decrease (about 50% reduction) in the mortality rate and relative risk of cancer was reported [30]. These encouraging results prompted us to study breast cancer prevention by DATS treatment.

Schaffer et al. [31] were the first to demonstrate chemoprevention of MNU-induced breast cancer in rats by dietary administration of diallyl disulfide, which is a naturally occurring analog of DATS. The in vivo efficacy of DATS in breast cancer has been evaluated using breast cancer xenograft models as well as chemically-induced breast cancer in rats. For instance, oral administration of 5 µmol DATS/kg body weight (equivalent to 0.9 mg DATS/kg body weight) twice a week exhibited dramatic reduction in tumor volume in mice xenografted with ER-positive MCF-7 cells [13]. However, daily intragastrical administration of 25 to 50 mg/kg DATS did not affect tumor burden (weight and volume) but significantly reduced lung metastasis in mice xenografted with triple-negative MDA-MB-231 cells [19,21]. In the present study, oral gavage of 50 mg DATS/kg body weight (5 times a week for 10 weeks) was still not efficacious to inhibit tumor growth indicating that much higher doses of DATS may be required to achieve chemoprevention of breast cancer.

The expression of RPL11 and its mRNA transcript was significantly upregulated in the mammary tumor after DATS administration (Fig. 4). On the other hand, the DATS-mediated
upregulation of \textit{RPS14} gene was not reflected at the protein level. The reason for this discrepancy is unclear. Evidence continues to accumulate to implicate ribosomal biogenesis in cancer [28,32-34]. As an example, increased ribosomal biogenesis was shown to attenuate the expression and activity of p53 [32]. Studies have also indicated that tumor aggressiveness is associated with alterations in ribosomal control, leading to reduced quality control of translation in cancer cells [35]. The expression of genes associated with translation was also higher in the tumors of DATS-treated rats in comparison with controls (Fig. 3A). Interestingly, low expression of ribosome-regulating genes including \textit{RPL11} was associated with a poor overall survival in triple-negative breast cancer patients [36]. Therefore, further work is necessary to determine the role of \textit{RPL11} in breast cancer and the consequences of its upregulation in anticancer effect of DATS.

Previous studies have determined the role of MAPK in anticancer effects of DATS in breast cancer cells [13,16]. Liu et al. [16] showed that the antimetastatic effect of DATS was linked to suppression of ERK. On the other hand, the DATS-mediated apoptosis was caused by reactive oxygen species (ROS)-mediated activation of JNK [13]. In this study, we found a modest increase in protein level of \textit{JNK2} only in mammary tumors of DATS-treated rats. Because the mRNA level of \textit{JNK2} was not affected by DATS administration, it is possible that stability of \textit{JNK2} protein is increased by DATS treatment. Further work is necessary to explore this possibility.

Besides ribosome-associated genes, mitochondria-related genes were also upregulated by DATS treatment. While verification of the RNA-seq data with respect to DATS-mediated upregulation of mitochondria-related genes is yet to be done, mitochondrial dysfunction promotes tumorigenesis to a more aggressive phenotype [37]. Alterations in the intracellular level of ROS are critical for the mitochondrial signaling in neoplastic transformation and cancer progression [37]. It is possible that upregulation of mitochondria-associated genes by DATS treatment contributes to the lack of its chemopreventive efficacy.

In this article, we focused on multiple target pathways for verification of the RNA-seq data, including ribosomal biogenesis proteins \textit{RPL11} and \textit{RPS14}, MAPK (ERK, JNK, and p38), and complex I subunit proteins NDUFV1 and NDUFS1 of oxidative phosphorylation. Some of these pathways are interrelated. For example, studies have shown a role for MAPK in regulation of ribosomal biogenesis [38]. However, the DATS-treated tumors did not show a change in phosphorylation of MAPK, which is a measure of their activation. Thus, it is reasonable to conclude that an increase in ribosomal biogenesis genes by DATS treatment may be independent of MAPK. The DATS-treated tumors also showed upregulation of genes associated with translation. Even though these gene expression changes are yet to be confirmed at the protein level, translation elongation factor 4 is known to regulate oxidative phosphorylation complexes and the production of ROS [39]. We have shown previously that DATS treatment causes ROS production leading to apoptosis in breast cancer cells [14]. Na et al. [13] have also shown ROS production by DATS treatment in breast cancer cells. It is possible that upregulation of genes associated with translation contributes to ROS production following DATS treatment.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2022.27.1.22.

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