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Multi-targeted DATS Prevents Tumor Progression and Promotes Apoptosis in an Animal Model of Glioblastoma via HDAC-inhibition

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Abstract

Glioblastoma, the most malignant and lethal of brain tumors, remains incurable despite aggressive chemotherapy and surgical interventions. Few new chemotherapeutics for glioblastoma therapy have been explored in preclinical models, and some agents approved for have reached the clinical setting. However success rates are not significant. Previous investigations involving diallyl trisulfide (DATS), a garlic constituent, have indicated significant anti-cancer effects *in vitro*, including: glioblastoma growth inhibition, extrinsic and intrinsic apoptotic pathway activation, and cell death. DATS has also been shown to inhibit histone deacetylase activity and impede glioblastoma tumor progression. We hypothesized that DATS would block ectopic U87MG induced tumors by inhibiting multiple pro-apoptotic pathways via HDAC. To this end, ectopic tumors were developed in SCID mice and subsequently treated with daily intraperitoneal injections of DATS. Results indicate that a range of DATS doses (10µg/kg-10mg/kg) dose-dependently reduced tumor volume and number of mitotic cells within tumors after seven days. Our histological and biochemical assays demonstrate that DATS reduces mitosis in tumors, decreases HDAC activity, increases in acetylation of H3 and H4, inhibits cell cycle progression, promotes apoptotic cascade activation (m-calpain, Bax, caspase-3) and decreases pro-survival markers (Survivin, Bcl-2, p-Akt, c-Myc, mTOR, EGFR, VEGF). Our data also demonstrates an increase in p21/WAF1 expression, which correlates with increased p53 expression and MDM2 degradation following DATS treatment. Finally, histological assessment and enzyme assays suggest that even the highest dose of DATS administered in this study did not negatively impact hepatic function. These *in vivo* findings strongly support orthotopic investigation into the therapeutic potential of DATS and further review of the epigenetic mechanisms behind its anti-cancer activities.

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Keywords

Glioblastoma; Diallyl Trisulfide; p53; Mitosis; Histone Deacetylase

1. Introduction

Glioblastoma is the most aggressive and malignant of the astrocytic tumors [1]. The current standard of care for glioblastoma often involves a multi-modal approach combining surgery, radiation, and/or chemotherapy [2]. Unfortunately, a cure for this deadly disease remains elusive. Therefore, developing an effective treatment strategy for glioblastoma is extremely important.

Garlic a members of the *allium* family contain an interesting and complex range of water-soluble and fatsoluble organosulfur compounds, some of which have been implicated as cancer chemopreventive agents [3]. Recently, Allyl derivatives from garlic were among the first compounds described to impact histone acetylation status by increased histone acetylation (H3/H4) in human cancer cells [4-6], implicating HDACs as possible targets. However, the specific role of HDAC inhibition and histone acetylation in DNA damage signaling has not been elucidated in these studies. Previous studies from our laboratory and others demonstrate that Diallyl trisulfide (DATS) inhibits *in vitro* tumor growth inhibition of multiple cancer cell lines, including glioblastoma [4-6]. Similar findings in neuroblastoma cell lines strengthen this argument [7]. Our group previously demonstrated that DATS increased calpain and Bax while reducing Bcl-2 expression to promote apoptotic cell death [4]. Though DATS treatment correlates with increased reactive oxygen species production, the fact that it does not seem to affect non-dividing cells suggests other pathways are more directly linked to DATS mediated anti-cancer activities [6]. DATS also inhibits histone deacetylase (HDAC) [8, 9], blocks G₂/M phase cell cycle progression, activate the Chk1-Chk2-Cdc25C pathway and inhibits DNA damage response proteins like p53 [6, 10]. Recent reports also supported that by suppressing oncogenic protein expression [4, 11-16] via HDAC inhibition [8, 9], DATS may restore p53 function by raising p53 and p21waf1=cip1 levels and thus predispose cellular apoptosis in aberrantly dividing cells as seen in cancer. At the same time, *in vivo* studies of ectopically grown prostate tumors revealed DATS significantly reduced tumor size without significantly altering liver enzyme levels or altering liver histological profiles [17]. Indeed, it has been suggested that high doses of DATS (up to 62mg/kg body weight) are tolerable in animal models[18]. In fact, 7-day oral treatments of 30mg/kg DATS prevents hepatotoxicity associated with elevated alkaline phosphatase (ALP) levels [19].

Based on other studies and the strength of our previous *in vitro* findings [4], we hypothesized that daily DATS treatment in ectopically grown U87MG tumors would result in reduced tumor volume, mitotic index, and oncogenic expression concurrent with increased apoptotic indicators. Indeed, DATS reduced tumor volume and mitotic index by preventing cell cycle progression and oncogene expression while disinhibiting intracellular apoptotic signaling relating to histone (H)3 and H4 acetylation.

2. Methods

2.1 U87MG cell culture and Ectopic glioblastoma Induction

U87MG cell lines were obtained from American Type Culture Collections (ATCC; Manassas, VA) and grown in 1×RPMI 1640 with L-glutamine (Cellgro; Manassas, VA), 10% FBS (BioAbChem; Ladson, SC), 1% penicillin/streptomycin (Cellgro) at 37°C and 5% CO₂. Media were changed every other day. Cells were collected for implantation by scraping and centrifugation upon achieving ~80% confluency.

Animal experiments were performed in compliance with the MUSC Institutional Animal Care and Use Committee guidelines and the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (National Institutes of Health, Bethesda, MD, USA). NOD CB17-prkdcscid/J mice were used for this experiment. Approximately 1×10⁶ cultured U87MG cells suspended in 125µL of 40% media to 60% Matrigel Basement Membrane Matrix (BD Bioscience, Sparks, MD) were implanted subcutaneously over the right rear flank of anesthetized animals. Tumors were allowed to grow for 3 weeks before beginning 7 daily treatments of DATS (LKT Laboratories, Inc., St. Paul, MN) suspended in sterile saline delivered at the following doses: 10ug/kg, 100ug/kg, and 10mg/kg. Control animals saline alone. Animals were sacrificed, and dermal tumors were excised using scalpel and forceps and weighed wet before being quick frozen in Tissue Mounting Medium (TMM; Fisher Scientific Corp., Suwanee, GA) over dry ice. Livers were collected at the same time and stored similarly.

2.2 Processing and Hematoxylin & Eosin (H&E) Staining of Tumor and Liver Tissues

Fresh frozen (-70°C) tumors and livers were cut at 8µm thickness on a cryostat and stored at -80°C prior to staining (Reichert-Jung, Leica, Deerfield, IL). Tissue was stained with Harris hematoxylin (Fisher Scientific, Kalamazoo, MI) and counterstained with eosin (Fisher Scientific, Kalamazoo, MI) to identify histopathological characteristics of ectopic tumor and liver samples. A minimum of 3 images per slice and 3 slices per sample from throughout the tumor sample were taken at 400x magnification with an Olympus fluorescent microscope (Olympus America Inc, Center Valley, PA) and mitotic bodies were counted using ImageJ cell counter software. Images were compiled and processed using Adobe Photoshop Elements.

2.3 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) and survivin/mcalpain immunofluorescent staining

TUNEL assay was performed according to kit instructions for the Promega Dead End Fluorometric TUNEL kit (Promega Corp., Madison, WI). Survivin/mcalpain immunofluorescent staining was performed using our laboratory standard protocol. We used primary rabbit monoclonal anti-human Survivin (AbCam, Cambridge, MA) and rabbit polyclonal anti-mcalpain (Santacruz Biotechnology) for immunohistochemistry. At the same time, we mounted the slides with Vectashield with DAPI nuclear stain. A minimum of 3 by 200X magnification images were captured for each section using a Zeiss fluorescent microscope (Carl Zeiss, Inc., North America, USA) made available by the Medical

University of South Carolina Storm Eye Institute. Images were compiled and processed using Adobe Photoshop Elements.

2.4 Histone extraction and HDAC activity assay

Histones were extracted from 2×10^6 cells as described previously (40). Effects of DATS on HDAC activity was assessed with the HDAC colorimetric Activity Assay kit (EMD Millipore Corporation, Billerica, MA, USA).

2.5 Western Blotting

The western blot analysis was performed by standard procedures (Das *et al.* 2006 and 2008b). Membranes were shortly incubated with enhanced chemiluminescent reagents (ECL or ECL Plus, Amersham Biosciences) and imaged on Alpha-Innotech using FluorChem FC2 Imaging System (Cell Biosciences). We stained 1 set of gel with Coomassie Blue to ensure loading of equal amounts of nuclear proteins in all lanes. Monoclonal antibody against β -actin (Sigma) was used to standardize cytosolic protein loading on SDS-PAGE gels. All primary IgG antibodies were purchased from Santa Cruz Biotech or Calbiochem (Gibbstown, NG, USA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated rabbit anti-goat IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN Biomedicals, Solon, OH, USA).

2.6 ALP Assay

Following DATS treatment we examined toxicology markers to determine any hepatotoxicity associated with drug administration per standard protocols [21-23]. Homogenates of the liver tissues were used for the assessment of ALP levels using commercially available colorimetric assay kits (Biotron Diagnostics, Hemet, CA). Therefore, hepatotoxicity was analyzed via histopathological examination of liver tissue for hallmark characteristics of drug toxicity via H&E staining [24].

2.7 Statistical Analyses

Data was gathered and processed in Microsoft Excel 2007 to determine sample means. Cell counts were made using NIH Image J cell counter module from at least 3 images per slice and 3 slices per sample ($n=3$). All statistical analyses were performed using the SPSS, Inc. PASW statistical package. Means of all data by group were compared by ANOVA with Bonferroni's post-hoc test where $**p < 0.01$ and $*p < 0.05$ was considered significant.

3. Results

3.1 DATS reduces visible tumor size, tumor mass and mitosis in tumors dose-dependently

U87MG xenografted SCID mice were treated with DATS (Figure 1). Treatment with DATS for 7 days showed more regression of U87MG tumor volume than from control xenograft (Figure A and B). Our wet tumor weights revealed a significant DATS-dependent reduction in tumor mass ranging from 20% at $10\mu\text{g/kg}$ to 90% at 10mg/kg compared to control (Figure 1B). An $\sim 50\%$ reduction was found after treatment with $100\mu\text{g/kg}$ DATS treatment. $N = 6$ for Control; $N = 3$ for $10\mu\text{g/kg}$ DATS; $N = 4$ $100\mu\text{g/kg}$ DATS; $N = 4$ for 10mg/kg DATS. To evaluate potential histological changes, tumor sections from all treatment groups were

subjected to histological evaluation by hematoxylin and eosin (H&E) staining at magnification under light microscope (Figure 1C). Examination of H&E stained tumor sections allowed for quantification of mitotically active cells from 400X images (Figure 1D). Our data demonstrates that treatments of DATS reduced the number of mitotic nuclei throughout the tumor by ~50% at 10 μ g/kg DATS ($P = 0.09$), ~70% at 100 μ g/kg DATS ($P = 0.013$), and nearly 80% at 10mg/kg ($P = 0.002$) when compared to untreated controls (Figure 1B). $N=6$ for Control; $N=3$ for 10 μ g/kg DAT; $N = 4$ for 100 μ g/kg DATS; $N = 5$ for 10mg/kg.

3.2 DATS treatment decreases HDAC activity in vivo

Earlier data demonstrated HDAC inhibition by many organosulfur compounds. Thus, we used HDAC colorimetric assay to measure HDAC activities following 7 days DATS treatment in U87MG xenograft. We found that DATS dramatically reduced HDAC activities as compared to control (Figure 2A). Our western blots showed a strong induction of histone H3 and H4 acetylation, which coincident with HDAC inhibition (Figure 2 B&C). These findings provided the first evidence that DATS act as a HDAC inhibitor in glioblastoma xenograft animal model.

3.3 DATS inhibits cell cycle progression and promotes p53-Mediated Tumor Suppression Pathways

We used Western blotting to determine the role of the cell cycle proteins and p53-mediated tumor suppression pathways in tumor reduction following 7 days DATS treatment in U87MG xenograft. We observed significant reduction of G₂/M phase checkpoint regulator cdk1 and its activator cdc25c following dose dependent DATS treatments. Whereas increased Tyr15 phosphorylation of cdk1 suggests that Wee1 activities may work in concert with decreased cdc25c to prevent the G₂/M phase transition (Figure 3A). At the same time, significant increased p21/WAF1 correlates with increased p53 expression and Mouse double minute 2 homolog (MDM2) degradation following DATS treatment (Figure 3B & D) suggesting re-activation of the G₁/S phase transitional checkpoint. U87MG cells are known to contain wild type p53 but mutated PTEN. In this experiment, PTEN levels remained unchanged (Figure 3C). These results provide a strong evidence that DATS reduce tumor growth by inhibits cell cycle progression and promotes p53-mediated tumor suppression pathways.

3.4 DATS reduced Survivin, other Oncogenes expression and increased Cell Death Markers

We used Western blotting to determine expression of survivin, other oncogenes and cell death markers following DATS treatment in U87MG xenograft. Data obtained here showed a multiple mechanism during glioblastoma reduction. DATS increased TUNEL positive staining in a dose-dependent manner with 10mg/kg DATS exhibiting the highest TUNEL expression (Figure 4A). Co-staining with survivin, a tumor survival marker, revealed moderately decreased expression correlating qualitatively with increasing doses of DATS (Figure 4A). Likewise, expression patterns of mcalpain, a protease connected to apoptosis induction, were visibly elevated in tumors exposed to higher doses of DATS (Figure 4B). However, other oncogene products (p-Akt, c-myc, and mTOR) and angiogenesis marker

VEGF were also decreased by DATS treatment (Figure 4B) suggesting that DATS treatment may be sufficient to overcome deactivating mutations involving PTEN in glioblastoma.

To further support apoptotic activation in tumor samples, various pro-apoptotic indicators were analyzed by Western blot. Increases in pro-apoptotic protein (calpain, and Bax), decrease in Bcl-2 expression, caspase-3 activation and ICAD degradation of treated samples confirmed apoptotic activation in DATS treated samples (Figure 4C). Conversely, levels of survivin decreased as apoptotic markers increased (Figure 4B).

3.5 DATS treatment does not negatively affect hepatic morphology or hepatic & renal enzyme levels

Drug-induced hepatic injury is the most common reason cited for withdrawal of an approved drug. Thus, we checked the hepatic morphology (by H&E staining) or hepatic & renal enzyme levels (colometrically) after DATS treatments for drug toxicity effects. H&E of 10µm frozen livers sections from mice revealed no morphological changes between the highest DATS doses and control samples (Figure 5A). Our results also revealed no change in the liver ALP levels in response to treatment with 10 µg-10mg/kg DATS (Figure 5B).

4. Discussion

The aim of this study was to examine whether the garlic compound DATS could effectively reduce ectopic glioblastoma tumor burden. Indeed, a 7-day treatment regimen of DATS decreased tumor mass dose dependently with significant reductions seen in 100µg/kg and 10mg/kg DATS treated animals (Figure 1). DATS also dose-dependently decreased the mitotic indexes of specimens (Figure 1). To date, no studies have shown similar effects using DATS treatment in ectopic xenograft models. The present investigation provides possible insight into this question by showing, for the first time, that DATS is an inhibitor of HDAC activity in glioblastoma xenograft model.

It is well established that DATS and organosulfur compounds can induce tumor cell death by activating apoptotic pathways including: caspase activation, Bax and Bad increases, and Bcl-2 decreases [4, 16, 25, 26]. However, the exact mechanisms by which this occurs are unclear. One mechanism may involve the production of ROS by organosulfur compounds [18]. Filomeni and others found that diallyl disulfide (DADS; which differs from DATS by one sulfur atom) caused dissociation of glutathione-S-transferase to c-Jun kinase complex (a key anti-ROS action), strongly implying DADS mediated ROS production [27]. The following year, Xiao and colleagues found DATS to be a far more potent ROS-mediated apoptosis inducer in prostate cancer cells - a finding later affirmed in glioblastoma cell lines [4, 28]. However, DATS does not appear to harm mitotically inactive cells [6] suggesting that ROS production is only one effect of apoptosis induction. When one considers that p53 was upregulated by DATS treatment, perhaps due to decreased MDM2 expression, and that p53 itself induces ROS production in response to DNA damage [29], past findings indicating DATS mediated ROS production look more like indirect effects. DATS, in effect, could selectively target cancer cells that already express DNA damage by disinhibiting normal repair or apoptotic induction mechanisms like p53. DATS also reduced cell cycle progression marker cdk1 (Figure 3). This effect could be related to decreased

cdc25c mediated cdk1 activation or to Wee1 inactivation of cdk1 as suggested by Tyr15 phosphorylation [30]. Regardless, DATS seems to reduce G₁/S and G₂/M transitional capabilities in glioblastoma cells.

Survivin expression represents one of the most important indicators of tumor growth in human patients, as previous ectopic glioblastoma models have shown a significant reduction in mitotic activity following survivin RNA knockdown [15]. Indeed, survivin expression was visibly decreased in a dose dependent manner in the current study (Figure 3). Moreover, EGFR and VEGF levels were reduced, suggesting potential applicability for DATS in glioblastoma expressing receptor tyrosine kinase mutations. DATS also appears to elevate mcalpain levels, a pro-apoptotic cysteine protease, suggesting that DATS mediates tumor size reduction via calcium dysregulation and apoptotic induction.

How pro-apoptotic cascades are activated following DATS treatment remains unknown. At least one study has suggested a role for garlic derivatives in directly manipulating cellular and organelle membrane potentials to affect tumor cell death [31]. Others have suggested an epigenetic mechanism whereby anti-apoptotic oncogenes are suppressed by DATS inhibition of HDACs [18], which may be supported by current findings (Figure 2). C-myc levels were also found to be reduced (Figure 4). This may indicate DATS, in addition to affecting HDAC inhibition, may further prevent transcription directly by decreasing c-myc expression and indirectly through c-myc mediated histone acetylation [32]. Interestingly, the current findings also suggest that DATS bypasses PTEN (Figure 3), which is inactive in U87MG cells, to affect pro-survival and pro-mitotic cascades (Figure 4) – a finding supported by other studies [5, 18, 33, 34]. Downstream of PTEN, p-Akt levels remained changed, but this was insufficient to prevent MDM2 degradation and p53 increases (Figure 3). Decreased mTOR, which is upstream of survivin, may explain DATS mediated decreases in survivin expression (Figure 4). Yet, despite these findings in tumors, unchanged ALP levels and histologically normal liver findings suggests little to no toxicity after weeklong exposure to 10mg DATS (Figure 5). These findings are in line with previous high dose DATS efficacy reports [18, 19]. Thus the signaling pathways that activate cell death induced by DATS are of crucial interest, and in this study, we provide DATS as HDACi-induced apoptosis.

The current findings demonstrate that DATS inhibits U87MG induced ectopic tumor growth without significant hepatic effect. DATS is a naturally occurring compound, is consumed by large populations with no ill effects, and can be generated at low cost to patients. Therefore, it may be swiftly translatable to clinical trials. First, more epigenetic mechanistic studies and orthotopic glioblastoma studies must be performed.

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Figure 2. DATS Reduces HDAC activities and Acetylation of H3 and H4 Dose-Dependently

(A) Determination of HDAC activity using a colorimetric assay. (B) Western blots document levels H3 and H4 acetylation after 7days DATS exposure. Densitometric analysis show percent change of optical density of the H3 and H4 acetylation from Control (C).

Figure 3. DATS Alters Cell Cycle Proteins and Reduced Tumor Growth by P53-Mediated Tumor Suppression Pathways

(A-B) Western blots to show levels of Cdk1, p-Cdk1, Cdc25C, p53, MDM-2, p21Waf1 and PTEN. (C-D) Densitometric analysis document percent change of optical density of the cdc 25c, p-Cdk-1, p53, MDM-2 and p21Waf1 following dose dependly DATS treatments.

Figure 4. Dose Dependent Treatment of DATS Changed expression of Survivin, other Oncogenes and Cell Death Markers

200X survivin (red) and TUNEL (green) co-staining in tumors (A). Separately, 200X images suggest DATS dependent m-calpain increases (B), suggesting a calpain component in DATS-mediated tumor regression. Insets represent 2X digital magnifications of representative co-labeled regions. (C-D) Western blots to show levels of Survivin, mcalpain, p-Akt, c-Myc, Mtor, EGFR, VEGF, Bax, Bcl-2, Caspase-3, and ICAD following dose dependently DATS treatments

Figure 5. DATS Does Not Interfere with Hepatic Morphology or ALP Activity

H&E of fresh frozen sections revealed no histopathological changes in liver appearance of 10mg/kg treated SCID mice compared with saline controls (A). Likewise, ALP levels (colorimetrically) were unchanged by 10µg-10mg/kg DATS (B).