Diallyl trisulfide induces apoptosis by suppressing NF-κB signaling through destabilization of TRAF6 in primary effusion lymphoma

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Abstract. The allyl sulfides, including diallyl sulfide (DAS), diallyl disulfide (DAD), and diallyl trisulfide (DAT), contained in garlic and members of the Allium family, have a variety of pharmacological activities. Therefore, allyl sulfides have been evaluated as potential novel chemotherapeutic agents. Here, we found that DAT inhibited nuclear factor-κB (NF-κB) signaling and induced apoptosis in primary effusion lymphoma (PEL), a subtype of non-Hodgkin's B-cell lymphoma caused by Kaposi's sarcoma-associated herpesvirus (KSHV). We examined the cytotoxic effects of DAS, DAD and DAT on PEL cells. DAT significantly reduced the viability of PEL cells compared with uninfected B-lymphoma cells, and induced the apoptosis of PEL cells by activating caspase-9. DAT induced apoptosis in PEL cells. In addition, DAT suppressed the production of progeny virus from PEL cells. The administration of DAT suppressed the development of PEL cells and ascites in SCID mice xenografted with PEL cells. These findings provide evidence that DAT has antitumor activity against PEL cells in vitro and in vivo, suggesting it to be a novel therapeutic agent for the treatment of PEL.

Introduction

Primary effusion lymphoma (PEL, also termed body-cavity-based lymphoma) is a malignant B-cell lymphoma caused by Kaposi's sarcoma-associated herpesvirus (KSHV, also named HHV-8) in immunosuppressed individuals, such as AIDS patients or those that have undergone organ transplantation (1,2). PEL is a subtype of non-Hodgkin's lymphoma and is characterized by lymphomatous effusions of pleural and abdominal cavities. KSHV is a rhadinovirus of the γ-herpesvirus subfamily and is closely related to herpesvirus saimiri and Epstein-Barr virus (EBV). KSHV is the causative agent of Kaposi's sarcoma and AIDS-related lymphoproliferative disorders, such as PEL and multicentric Castleman's disease (3). Similar to other herpesviruses, KSHV has two life cycles (latency and lytic replication). The KSHV genome circularizes and forms a double-stranded DNA, the episome, in the nucleus of PEL cells during latent infection. To establish a latent infection, KSHV expresses several viral genes, including latency-associated nuclear antigen (LANA), v-FLIP, v-cyclin, kaposin and microRNAs, in PEL cells. LANA is required for the replication and maintenance of viral DNA, and contributes to KSHV-associated oncogenesis through interaction with cellular molecules, such as p53, Rb and GSK-3. These viral proteins and RNA manipulate cellular signaling pathways, including nuclear factor-κB (NF-κB), Akt, Wnt and Erk, to maintain the malignant phenotype and ensure PEL cell survival (4-6). Especially, NF-κB signaling is constitutively activated in KSHV-infected PEL cells to facilitate anti-apoptosis and growth (7-11). KSHV alternates between lytic replication and latency by RTA expression. RTA, encoded by the immediate-early gene ORF50, is a critical switch molecule for initiating lytic replication.
In canonical NF-κB signaling, the NF-κB transcription factor, which is a heterodimer of p50 and p65/RelA, is retained in the cytosol by interaction with IκBα (12). IκBα is further regulated by the IκB kinase (IKK) complex, consisting of IKKα, IKKβ and IKKγ/NEMO. A stimulus, such as LPS or IL-1β, induces activation of TRAF6 through MyD88 and IRAK, and TRAF6 induces the activation of TAK1-TAB2 complex, which phosphorylates the catalytic subunit IKKβ and activates the IKK complex (13). TRAF6, an E3 ubiquitin ligase, can modify IKKγ and TRAF6 itself via K63-linked polyubiquitination, and K63-linked polyubiquitination of IKKγ and TRAF6 contributes to the activation of IKK complex (14). The activated IKK complex phosphorylates Ser32 and Ser36 of IκBα, leading to nuclear translocation of NF-κB and the NF-κB-dependent transcriptional activation.

Garlic (Allium sativum L.) is widely used in traditional herbal remedies and alternative medicine. Garlic oil from fresh garlic contains allyl sulfides, including diallyl sulfide (DAS), diallyl disulfide (DAD), diallyl trisulfide (DAT), and other allyl polysulfides (15). The proportions of allyl sulfides in garlic oil are ~6% DAS, 30% DAD, 40% DAT and 24% other analogs (16). Allyl sulfides have many biological functions, including suppression of inflammation, upregulation of detoxification, enhancement of histone acetylation, generation of reactive oxygen species (ROS), and induction of endoplasmic reticulum (ER) stress (17,18). There have been many reports of anticancer effects of these compounds against a variety of cancers, including prostate, lung, breast, and colon cancer cells (19-22). In addition, DAT directly produces ROS many reports of anticancer effects of these compounds against cancer cells (24,25). PEL is an aggressive lymphoma caused by KSHV, and is resistant to chemotherapy regimens, such as CHOP and R-CHOP (26). Therefore, the development of novel and effective drugs for PEL is required. Regarding biological properties of allyl sulfides, these could be novel compounds used with molecular target drugs for the treatment of PEL. The anticancer effect of allyl sulfides against PEL remains unknown; we therefore investigated whether allyl sulfides kill PEL cells and the underlying molecular mechanism thereof.

Materials and methods

Cells and reagents. KSHV-positive PEL cell lines (BC2, BC3, BCBL1 and HBL6) and KSHV-negative lymphoma cell lines (Ramos, BJAB and DG75) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, DAS (Wako, Osaka, Japan), DAD (Tokyo Chemical Industry, Tokyo, Japan), DAT (LKT Laboratories, St. Paul, MN, USA) were dissolved in dimethyl sulfoxide (DMSO).

Cell viability assay. Cells were seeded on 96-well plates at 2.5x10^4 cells/well in 100 µl of medium with or without various concentrations of DAS, DAD or DAT and then incubated at 37°C for 24 h. The number of viable cells was estimated using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan) as described previously (27). The optical density at 450 nm of each sample was measured using a microplate spectrophotometer (Tecan M200; Tecan, Kanagawa, Japan) and expressed as a percentage of the value in untreated cells (defined as 100%). Data are shown as the means ± SEM of three independent experiments.

Western blotting and antibodies. Western blotting was performed as described previously (28). Primary antibodies used in these experiments included those against Ser32/36-phospho-IκBα, Ser176/180-phospho-IKKα/β, caspase-3, -7, -8, -9 and PARP (Cell Signaling Technology, Danvers, MA, USA), IκBα, p65, and p21^{Waf1} (BD Biosciences, Franklin Lakes, NJ, USA), β-actin, histone H2B, K-bZIP and S-tag (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). FK2 that we established previously (29) was used to detect polyubiquitin molecules.

Caspase assay. To measure caspase activity, 1.0x10^5 BC3 PEL cells/well were added to 1 ml of medium and incubated with DAT for 3 h. Activities of caspase-8 and -9 in cell lysates were measured using a caspase-Glo assay kit (Promega, Madison, WI, USA) with luciferin-conjugated LETD or LEHD polypeptide, respectively, according to the manufacturer's instructions. Luminescence was detected using a GloMax 20/20 luminometer (Promega). The caspase activity in untreated cells was defined as 1.0 relative light unit.

Immunofluorescence (IF) analysis. Prior to IF analysis, BCBL1 cells were treated with 10 µM DAT for 18 h and fixed in methanol on glass slides followed by incubation with primary antibodies for 1 h. After washing, the cells were incubated with Alexa Fluor 594-conjugated donkey anti-mouse IgG or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). To stain the nuclei, cells were incubated in 4 ng/ml of DAPI in PBS during binding of the secondary antibody. Immunofluorescence images were obtained by fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Nuclear protein extraction. Harvested cells were incubated with hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, and 1 mM DTT on ice for 15 min, and then cells were lysed by addition of NP-40 (final concentration, 0.6%) and heavy agitation with a vortex mixer. Cell lysates were centrifuged at 15,000 rpm for 30 sec at 4°C. The nuclear pellets were rinsed with the hypotonic buffer supplemented with 0.6% NP-40. The obtained nuclear extracts were lysed in SDS-PAGE sample buffer.

Luciferase reporter assay. BC3 cells (1x10^5) were transfected with 2 µg of NF-κB-luciferase reporter (pGL4.32) and 1 µg of pSV-β-Gal (both from Promega) used as an internal control with 9 µg polyethyleneimine. Transfected cells were incubated in medium with various concentrations of DAT for 6 h. Cells were resuspended in 0.1 ml of passive lysis buffer (Promega) for luciferase assays. Luciferase activity was measured with a GloMax 20/20 luminometer. The luciferase activity of DAT-untreated cells was defined as 1.0 relative light unit.
Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR. Total RNA was purified and extracted from 1x10⁶ cells using an Illustra RNAspin Mini RNA Isolation kit (GE Healthcare, Buckinghamshire, UK). First-strand cDNA was synthesized from 20 ng of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). To quantify cDNA, PCR was performed using GoTaq Flexi DNA polymerase (Promega). The PCR products were analyzed by electrophoresis on 2% agarose gels and staining with ethidium bromide. The nucleotide sequences of oligonucleotides used for RT-PCR primers were as follows: IKKα forward, 5'-AGA CGT CAG GGA GAC TAT AGT-3' and reverse, 5'-ACT GGA TAC TAC AAG AGA GGC GGC-3'; IKKβ forward, 5'-AGG TGC CAT CCT CAC CCT GC-3' and reverse, 5'-AAT GTC CAC CTC ACT CTT CC-3'; IKKγ forward, 5'-AGT TGC AGG TGG CCT ATC ACC-3' and reverse, 5'-CTG ATG TCC TCG ATC CTG GC-3'; TRAF6 forward, 5'-CAG GGG TAT AGC TTG CCC TCA C-3' and reverse, 5'-TGG AAC GTG TGG ATT CCC AG-3'; GAPDH forward, 5'-TGA CCA CAG TCC ATG CCA TC-3' and reverse, 5'-GGG GAG ATT CAG TGT GGT GG-3'.

For quantification of cDNA, Real-time RT-PCR was performed as described previously (10). Briefly, total RNA was purified and extracted from 1x10⁶ cells and first-strand cDNA was synthesized from 20 ng of total RNA. To quantify cDNA, SYBR-Green real-time PCR was performed using the MiniOpticon real-time PCR (Bio-Rad, Hercules, CA, USA). The open reading frames (ORFs) of human ubiquitin, IKKα, IKKβ, IKKγ and TRAF6 were amplified by PCR. All constructs were verified by DNA sequencing. For eukaryotic expression, the respective PCR fragments were cloned into the pCI-neo-Flag, pCI-neo-T7, pCI-neo-Myc or pCI-neo-S-tag vector, which had been generated by inserting oligonucleotides encoding three repeats of a Flag-tag sequence, three repeats of a T7-tag sequence, a Myc-tag sequence, and two repeats of an S-tag (S-tag peptide), respectively, into the pCI-neo mammalian expression vector (Promega).

Transfection and plasmids. 293/TLR4-CD2-CD14 (293/TLR4) cells were purchased from InvivoGen (San Diego, CA, USA). The 293 or HeLa cells seeded at 2x10⁵ cells/10 cm dish were transfected using the Chen-Okayama calcium phosphate procedure (30) with 10 µg of plasmid, and harvested 48 h after transfection. The open reading frames (ORFs) of human ubiquitin, IKKα, IKKβ, IKKγ and TRAF6 were amplified by PCR. All constructs were verified by DNA sequencing. For eukaryotic expression, the respective PCR fragments were cloned into the pCI-neo-Flag, pCI-neo-T7, pCI-neo-Myc or pCI-neo-S-tag vector, which had been generated by inserting oligonucleotides encoding three repeats of a Flag-tag sequence, three repeats of a T7-tag sequence, a Myc-tag sequence, and two repeats of an S-tag (S-tag peptide), respectively, into the pCI-neo mammalian expression vector (Promega).

Immunoprecipitation. Immunoprecipitation assays to detect polyubiquitination of IκBα were performed according to the methods described by Saji et al (10). BC3 cells (1x10⁵) were lysed in 1 ml RIPA buffer. Cell lysates were incubated with 5 µg of anti-IκBα antibody. Immunoprecipitated IκBα was immunoblotted with FK2 (anti-polyubiquitin) or anti-IκBα antibody. Immunoprecipitation assays to detect the interactions of IκKα, IκKβ and IκKγ were performed, as described previously (5).

Measurement of proteasome activity. BC3 cells (1x10⁵) were lysed in 0.2 ml of buffer containing 50 mM Tris-HCl (pH 7.6), 0.1 mM MgCl₂, 0.1 mM EDTA, 1% glycerol, 1 mM DTT, 0.2 mM ATP, 0.2% NP-40, and homogenized with 27 G needles and 1 ml disposable syringes. The chymotrypsin-like activity of the proteasome in cell lysate was assessed with the fluorogenic peptide, Suc-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) (Peptide Institute, Osaka, Japan). The cell lysates (5 µl) were incubated in 95 µl of reaction buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 1 mM DTT, 2 mM ATP, 0.1 mM Suc-Leu-Leu-Val-Tyr-MCA. The fluorescence intensity owing to AMC (excitation, 380 nm; emission, 460 nm) was determined using a microplate fluorometer (Infinite M200; Tecan, Kawasaki, Japan).

Real-time PCR measurement of viral load. Real-time PCR was performed by the method described previously (10). BCBL1 cells (2x10⁵) were treated with 3 mM n-butyrate and DAT for 48 h, and the culture media were harvested. To obtain only enveloped and encapsulated viral genomes, media were incubated with DNase I, and viral DNA was purified using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). To quantify viral DNA, SYBR Green real-time PCR was performed by MiniOpticon real-time PCR (Bio-Rad). To generate a standard curve for cycle threshold versus genomic copy number, the pCIneo-KSHV ORF50/RTA plasmid was serially diluted to known concentrations in the range of 10⁵-10³ plasmid molecules/µl. Each PCR mixture contained 1 µl viral or standard DNA, 0.5 µM ORF50 primers, 10 µl SYBR Master Mix Plus (Bio-Rad), and H₂O in a total volume of 20 µl. The following primers were used to amplify a 140-bp amplicon internal to the ORF50 sequence: ORF50/RTA forward, 5'-ATA ATC CGA TCA TCC ACC AC-3' and reverse, 5'-TGG AAC GTG TGG ATT CCC AG-3'. The primer set for GAPDH forward, 5'-GAG TCA ACG CAT TGG GTC GT-3' and reverse, 5'-GAG AAC CTT CCC GTC TCT AG-3' was used as an internal control for normalization. For quantification, the expression level of each gene was normalized to that of the GAPDH gene.

Xenograft mouse model of PEL. CB17 SCID female mice aged 5 weeks were purchased from CLEA Japan (Tokyo, Japan). Mice were allowed to feed ad libitum on sterilized laboratory chow and water. Mice were injected intraperitoneally with 5x10⁵ BCBL1 cells before 1 week of DAT administration. The DAT dissolved in corn oil or corn oil alone was administered into the intraperitoneal region at a dose of 5 mg/kg body weight each day for 3 weeks. Mice were observed and body weight was measured each day for 3 weeks. All mice were sacrificed on day 21, and the ascites and organs were collected. The ascites collected from each mouse was centrifuged to determine the tumor weight. Genomic DNA of tumor cells in ascites was collected from each mouse was centrifuged to determine the tumor weight. Genomic DNA of tumor cells in ascites was collected from each mouse was centrifuged to determine the tumor weight. Genomic DNA of tumor cells in ascites was collected from each mouse was centrifuged to determine the tumor weight. Genomic DNA of tumor cells in ascites was collected from each mouse was centrifuged to determine the tumor weight.
SHIGEMI et al: DIALLYL TRISULFIDE DESTABILIZES TRAF6 AND SUPPRESSES NF-κB SIGNALING

Results

Cytotoxic effects of DAT on PEL cells. First, we examined the cytotoxic effects of allyl sulfides (DAS, DAD and DAT) on KSHV-infected PEL cell lines (BC3, BCBL1, HBL6 and BC2) and KSHV-uninfected lymphoma cell lines (Ramos and DG75). These B-lymphoma cells were cultured in the presence of DAS, DAD or DAT for 24 h, and the cytotoxicity was evaluated by analyzing the viability of allyl sulfide-treated versus untreated cells (Fig. 1). DAS and DAD treatments slightly decreased the viability of PEL cells compared to KSHV-uninfected cells (Fig. 1A and B) whereas DAT treatment selectively prevented the proliferation of PEL cells compared to KSHV-uninfected cells (Fig. 1A and B) whereas DAT treatment selectively prevented the proliferation of PEL cell lines at lower concentrations than required for KSHV-uninfected Ramos and DG75 cell lines (Fig. 1C). In particular, treatment with 20 µM DAT showed marked antiproliferative effects on PEL cell lines with no influence on KSHV-uninfected cell lines. The cytotoxic effects of DAT on B-lymphoma cells are summarized in Table I. As DAT showed strong antiproliferative activities against PEL, we focused on the cytotoxic effects of DAT and analyzed the underlying molecular machinery.

DAT induces apoptosis through the activation of caspases in PEL cells. We next investigated whether the cytotoxic effects of DAT were due to apoptotic cell death. Apoptosis is induced by the activation of executioner caspases, including caspase-3 and -7, which have been previously activated via either an intrinsic pathway (caspase-9) or an extrinsic pathway (caspase-8). We monitored the cleavage (i.e., activation) of caspase-3, -7, -8 and -9 by immunoblotting of lysates prepared from PEL and Ramos cells cultured with DAT (Fig. 2A). Active caspase-3, -7 and -9 were detected in BC3 and BCBL1 cells. Further, the amounts of cleaved PARP were increased in DAT-treated BC3 and BCBL1 cells. However, neither the cleavage of caspase-8 nor the accumulation of cleaved PARP was detected in DAT-treated Ramos cells. In addition to the activation of caspase, p21Cip1 was upregulated in DAT-treated cells. To obtain further evidence of the activation of caspase-9, we measured the peptidase activities of caspase-8 and -9 in BC3 cells pretreated with DAT (Fig. 2B). Compared to treatment with vehicle control, treatment with DAT increased caspase-8 and -9 activities by 1.2- and 1.6-fold, respectively. There was no significant difference in caspase-8 between DAT-treatment and control. These data indicate that DAT inhibited the growth of PEL cells by cell cycle arrest, leading to caspase-9-dependent apoptosis. We adopted different treatment times and drug concentrations as shown in Fig. 2. The reasons of treatment times are as follows. Activations of executioner caspases (caspase-3 and -7) and PARP cleavage occur later in the apoptosis cascade, while activations of initiator caspases (caspase-9 and -8) occur early in the apoptosis. In fact, strong activation of caspase-9 and PARP cleavage were detected in cells treated with DAT for 6 and 12 h, respectively (Fig. 2A). Regarding drug concentrations, more DAT were necessary for...
western blotting to detect cleaved molecules (i.e., activated forms) because western blot analysis has low detection sensitivity as compared with peptidase assay.

**DAT suppresses NF-κB signaling through the stabilization of IκBα.** Several signaling pathways are activated in PEL cells to maintain malignant potential and enhance cell survival. Especially, the constitutive and/or transient activation of NF-κB signaling is necessary for PEL to achieve the establishment of KSHV infection, survival of infected cells, and viral replication (4,7-11). To obtain insight into the machinery by which DAT induces apoptosis of KSHV-infected PEL cells, we analyzed the effect of DAT treatment on NF-κB signaling. PEL and KSHV-uninfected Ramos cells were incubated in medium with 10 µM DAT, expression of IκBα was elucidated by western blot analysis. DAT treatment significantly increased the expression of IκBα in PEL cells, whereas, upregulation of IκBα was not detected in Ramos cells (Fig. 3A). The long exposure image is shown, indicating the IκBα upregulation in BC3 treated with DAT for 6 and 12 h. To determine whether DAT-mediated upregulation of IκBα is due to transcriptional activation of IκBα, we measured the expression of IκBα mRNA in PEL cells treated with DAT by real-time RT-PCR (Fig. 3B). The result indicated that DAT treatment had no influence on IκBα mRNA in BCBL1, suggesting that DAT induces the stabilization of IκBα. As DAT showed increased expression of IκBα, we next examined the suppression of NF-κB nuclear translocation and the downregulation of NF-κB-dependent transcriptional activity by DAT treatment. IF analysis showed that compared to untreated controls, DAT treatment induced cytosolic localization of p65 in BCBL1 cells (Fig. 3C). The amount of p65 in the nucleus decreased in BC3 and BCBL1 cells treated with DAT for 12 and 24 h (Fig. 3D), as compared to Ramos cells, while DAT did not alter the total amount of p65 in all cell lines (Fig. 3D). In addition, we performed a reporter assay using the NF-κB reporter plasmid to confirm the suppression of NF-κB in BC3 cells by DAT-treatment. As expected, DAT treatment suppressed NF-κB transcriptional activity of BC3 cells in a concentration-dependent manner (Fig. 3E). Western blot analysis for detection of immunoprecipitated IκBα using lysates from DAT-treated BC3 and BCBL1 is also shown. Data show that DAT-treatment for 6 h significantly induced IκBα upregulation in PEL cells. These data indicated that DAT treatment suppressed NF-κB signaling through the stabilization of IκBα in PEL cells.

**DAT induces stabilization of IκBα by inhibiting phosphorylation of IκBα.** The mechanism of IκBα degradation consists of three sequential steps: first, the phosphorylation of IκBα by IKK complex; second, polyubiquitination of phosphorylated IκBα by E3 ubiquitin ligase; and third, degradation of polyubiquitinated IκBα by 26S proteasome. We attempted to determine which step is influenced by DAT treatment, leading to the stabilization of IκBα. First, we monitored polyubiquitination of IκBα (Fig. 4A). To detect polyubiquitination of IκBα, immunoprecipitated IκBα from DAT and mG132-treated BC3 cell lysate was subsequently probed by immunoblotting with an anti-polyubiquitin antibody (FK2). However, there was no difference in amount of polyubiquitination of IκBα between
DAT-treated and untreated cells. Next, to examine whether DAT inhibited the degradation of IkBα by the 26S proteasome, we measured the chymotrypsin-like activity of the proteasome. In BC3 cells treated with DAT for 24 h, the proteasome activity decreased by ~20% compared to that in untreated cells (Fig. 4B). The proteasome inhibitor MG132 was used as a positive control. We also investigated the effects of DAT on IkBα phosphorylation in PEL cells. When BC3 cells were treated with DAT, the amount of phosphorylated IkBα (p-IkBα) protein was significantly decreased compared to untreated cells (Fig. 4C). BAY11-7082, which inhibits IKK and IkB phosphorylation, was used as a positive control. We evaluated the cytotoxic effect of BAY11-7082 on PEL to confirm the contribution of IkBα phosphorylation to apoptotic activity on PEL. Treatment with BAY11-7082 markedly decreased the viability of BC3 PEL cells compared to KSHV-uninfected BJAB cells (Fig. 4D). Thus, our data demonstrated that DAT stabilized IkBα through inhibition of IkB phosphorylation. Furthermore, DAT-mediated suppression of NF-κB signaling through inhibition of IkB phosphorylation can be a trigger for apoptosis of PEL cells.

DAT suppresses the phosphorylation of IKKβ through down-regulation of TRAF6. We demonstrated that DAT stabilized IkBα through inhibition of IkB phosphorylation in PEL cells. To determine whether DAT-mediated phosphorylation of IkBα is due to quantitative or qualitative changes in IKK, we examined the mRNA expression, complex formation and
The phosphorylation state of IKK in DAT-treated PEL. The results indicated that DAT treatment did not affect the levels of IKKa, IKKβ or IKKγ mRNA in BCBL1 or Ramos cells (Fig. 5A). We next examined the complexation of IKK in DAT-treated cells. HeLa cells were transfected with Flag-IKKα, T7-IKKβ and S-tag-IKKγ expression plasmids, and treated with DAT. Cell lysates were subjected to coprecipitation assay using S protein-immobilized beads. However, there were no changes in complex formation of IKK associated with DAT treatment (Fig. 5B). Next, we examined whether DAT suppresses the phosphorylation of endogenous IKKa or IKKβ in PEL, HeLa and 293 cells. However, we could not detect the phosphorylation signal of endogenous IKKa/β, because the phosphorylation and expression levels of IKKa/β in normal cultured cells were too low. Therefore, we used 293/TLR4 cells constitutively expressing TLR4. 293/TLR4 cells transfected with either Flag-IKKα or T7-IKKβ cultured with or without DAT-containing medium in the presence of LPS to activate the NF-κB signal. When Flag-tagged IKKα-transfected cells were treated with LPS, small amounts of Ser176 in exogenous IKKα were phosphorylated, and DAT treatment suppressed this phosphorylation (Fig. 5C). On the contrary, in T7-tagged IKKβ-transfected cells, large amounts of IKKβ were phosphorylated in an LPS-independent manner, and DAT treatment strongly suppressed the phosphorylation of Ser180 at T7-IKKβ (Fig. 5D). That is, DAT suppressed the IKK complex by inhibiting the phosphorylation of IKKβ, which is the catalytic subunit of the IKK complex and directly phosphorylates IκBα for degradation. As the ubiquitin ligase, TRAF6, which is self-activated by TRAF6 polyubiquitination, induces IKKβ phosphorylation via activation of TAK1-TAB2 complex, we compared the self-polyubiquitination of TRAF6 in the presence or absence of DAT. HeLa cells transfected with T7-TRAF6 and Myc-ubiquitin were cultured with or without DAT, and cell extracts were assayed by coimmunoprecipitation assay. Immunoprecipitated T7-TRAF6 was immunoblotted with anti-T7 and anti-myc antibody to detect TRAF6 and polyubiquitinated TRAF6, respectively. DAT treatment slightly reduced the polyubiquitination of TRAF6 (Fig. 5E, left panel), but significantly reduced the amount of TRAF6 protein (Fig. 5E, right panel). Therefore, we compared the destabilization of TRAF6 in the presence or absence of

Figure 4. Inhibition of the phosphorylation of IκBα by DAT. (A) Influence of DAT on polyubiquitination of IκBα in PEL cells. BC3 cells were incubated with 10 µM DAT and 1 µM proteasome inhibitor (MG132) for 24 h. To detect polyubiquitination of IκBα, immunoprecipitated IκBα protein was subjected to immunoblotting using FK2, an anti-polyubiquitination antibody. (B) Influence of DAT on the chymotrypsin-like activity of the proteasome in PEL cells. BC3 cells were cultured with 10 µM DAT or 1 µM MG132 for 24 h. The chymotrypsin-like activities of cell lysates were evaluated by fluorometric assay using the synthetic peptide. The proteasome activity of untreated cells was defined as 100%. (C) Immunoblotting of IκBα and phospho-IκBα using DAT-treated BC3 cells. Cells were cultured with or without 10 µM DAT or 0.2 µM BAY11-7082 for 24 h. To detect phosphorylation of IκBα, cell lysates were subjected to blotting using anti-Ser32/36-phospho-IκBα antibodies. To equalize the amounts of IκBα among all samples, 40 µl of none-treated, 10 µl of DAT-treated, and 5 µl of BAY-treated cell lysates were applied to SDS-PAGE. (D) Cytotoxic effects of BAY11-7082 on BC3 and KSHV-uninfected BJAB cells. Cells were incubated with various concentrations of BAY11-7082 for 24 h. Cell viability in inhibitor-untreated cells was defined as 100%.
DAT. S-tag-TRAF6-transfected 293/TLR4 or HeLa cells were incubated with DAT, and TRAF6 was detected by immunoblotting with an anti-S-tag antibody. The results indicated that DAT treatment for 3 and 6 h induced destabilization of TRAF6 in 293/TLR4 (Fig. 5F, upper panel) and HeLa cells (Fig. 5F, lower panel). In addition, the proteasome inhibitor, mG132, overcame the DAT-induced destabilization of TRAF6. In Fig. 5A, we confirmed that DAT did not affect the expression of TRAF6 mRNA in cells. These data indicate that DAT induces degradation of TRAF6 by the proteasome, and DAT-mediated downregulation of TRAF-6 suppresses the phosphorylation of IKKβ and activation of IKK complex. Furthermore, DAT-mediated suppression of IKK induces the suppression of IxBα phosphorylation for polyubiquitination and proteasomal degradation of IxBα.

**DAT inhibits KSHV replication in PEL.** NF-κB signaling is essential for replication of KSHV (10,11). Therefore, we investigated whether DAT suppresses KSHV lytic replication in PEL cells (Fig. 6A). The results showed that DAT suppressed viral particle production at low concentrations, which did not affect BCBL1 cell growth (Fig. 1). Next, to investigate which step of viral replication is prevented by DAT we examined the effect of DAT on lytic gene expression for viral replication. DAT treatment decreased mRNA expression of RTA (immediate-early gene) and K8.1 (late gene) in BCBL1 cells (upper panel) or HeLa cells (lower panel) were analyzed by immunoblotting using an anti-S-tag rabbit polyclonal antibody.
NF-κB activity is required for both KSHV-infected cell survival and viral replication in PEL cells.

**Treatment with DAT suppresses the development of PEL in SCID mice.** As DAT showed selective cytotoxicity on PEL cell lines (Fig. 1), we next investigated whether DAT treatment exerted cytotoxic effects against xenograft PEL cells in SCID mice. BCBL1 cells were injected intraperitoneally into SCID mice 1 week prior to commencement of DAT administration. DAT or vehicle (corn oil) was injected intraperitoneally each day for 21 days. The mice with and without DAT administration showed significantly different gross appearance (Fig. 7A): the abdomen of corn oil-treated PEL-xenografted mice (control mice) showed expansion, whereas DAT-treated mice had an apparently normal body shape. Moreover, the body-weight increase of DAT-treated mice was less marked than that of control mice (Fig. 7B). Necropsy revealed that the spleens of control mice showed distention compared to those of DAT-treated mice (Fig. 7C), whereas there were no significant morphological differences in other organs between DAT-treated and -untreated mice. The weight of the spleen in the DAT-treated group was ~0.07 g, which was lower than that of the corn oil-treated group (Fig. 7D). As the average weight of the spleen in normal 6-7-week-old SCID mice is ~0.06 g (data not shown), the spleens of control mice were considerably larger than those of normal and DAT-treated mice. The weight of tumor cells in ascites in the DAT-treated group was significantly less than that of control group (Fig. 7E). Real-time PCR indicated that ascites and spleen tumor cells were infected with KSHV in corn oil-treated mice, and DAT prevented BCBL1 development in ascites.

**Discussion**

Our data showed that DAT exhibited the greatest cytotoxicity against PEL among the allyl sulfides (DAS, DAD and DAT) tested in this study (Fig. 1). DAT specifically inhibited the growth of PEL cell lines compared with KSHV-uninfected cells both *in vitro* and *in vivo* (Figs. 1C and 7). DAS, DAD and DAT have antitumor effects, but DAS has been shown to have antioxidant and protective effects against oxidative or chemical stress rather than cytotoxic effects (31). On the other hand, DAD and DAT show cytotoxic rather than cell-protective effects. In particular, DAT was shown to induce apoptosis in cancer cells by the generation of ROS and the dysregulation of signaling pathways, including Bcl2-caspase-9, Erk, Jnk and Akt signaling (32,33). By cleavage within its trisulfide bonds, DAT generates ROS, leading to ER stress and apoptotic cell effects. In particular, DAT was shown to induce apoptosis in cancer cells by the generation of ROS and the dysregulation of signaling pathways, including Bcl2-caspase-9, Erk, Jnk and Akt signaling (32,33). By cleavage within its trisulfide bonds, DAT generates ROS, leading to ER stress and apoptotic cell death (20-23,34). In addition, DAT has been reported to suppress NF-κB and to induce apoptosis, DAT reduced LPS-induced NF-κB transcriptional activation in macrophages (35), and DAT suppressed high-glucose-induced apoptosis by inhibiting NF-κB signaling via decreases in nuclear translocation of NF-κB (36). Furthermore, DAT suppressed NF-κB through an increase in IκBα in osteosarcoma cells, resulting in a decrease in P-glycoprotein (37). DAT suppresses dextran sodium sulfate-induced mouse colitis by inducing STAT3 phosphorylation and suppression of IκBα phosphorylation (24). However, the detailed mechanism by which DAT inhibits NF-κB signaling has not been determined. To our knowledge, this is the first report of the detailed mechanism of dysregulation of NF-κB signaling by DAT. Thus, DAT has the ability to interfere in cell signaling including NF-κB, while constitutive and/or transient activation of NF-κB, Akt and Erk signaling are necessary for PEL to enhance cell growth and survival (4-11). These findings indicated that xenografted BCBL1 developed in ascites of corn oil-treated control mice, and DAT prevented BCBL1 development in ascites.

**Figure 6. Effects of DAT on lytic replication in BCBL1 cells.** (A) Suppression of KSHV replication by DAT. BCBL1 cells were cultured for 24 h with DAT in RPMI medium containing 3 mM n-butyrate for induction of KSHV lytic replication. The Hsp90 inhibitor, geldanamycin (GA), was used as a positive control for inhibition of lytic replication (11). Culture medium containing virus particles was harvested and KSHV genome copy numbers were quantified by real-time PCR. The KSHV genome copy number in DAT-untreated BCBL1 cells induced by n-butyrate was defined as 1.0. (B) The effects of DAT on mRNA expression of the lytic genes, RTA/ORF50 and K8.1. BCBL1 cells were treated for 24 h with 0, 0.2 or 1.0 µM DAT in the presence of 3 mM n-butyrate. Total RNA was extracted from harvested cells and subjected to real-time RT-PCR. The levels of respective gene expression in DAT-untreated BCBL1 cells induced by n-butyrate were defined as 1.0.
may be a reason why DAT, rather than DAS and DAD, showed selective cytotoxic effect on PEL cells.

The present study revealed a novel biological function of DAT and a mechanism for DAT-mediated NF-κB suppression. We propose a model in which TRAF6 downregulation by DAT results in suppression of NF-κB signaling (Fig. 8). DAT induced the proteasomal degradation of TRAF6, and this DAT-mediated TRAF6 downregulation suppressed IKKβ phosphorylation (Fig. 5), resulting in inhibition of the IKK complex. The suppression of the IKK complex by DAT decreased the Ser32 and Ser36 phosphorylation of IκBα (Fig. 4C) followed by stabilization of IκBα in a DAT-dependent manner (Fig. 3A). DAT induced stabilization of IκBα by these molecular mechanisms, and then suppressed both the nuclear localization of p65 and the transcriptional activity of NF-κB in PEL cells (Fig. 3); in contrast, DAT did not affect the stabilization of IκBα in KSHV-uninfected cells. It has been suggested that TRAF6 induces the activation of IKK complex and TAK1-TAB2 complex via K63-linked polyubiquitin (12-14). TRAF6, an E3 ubiquitin ligase, catalyzes synthesis of K63-linked polyu-
The K63-linked polyubiquitination of IKKγ and TRAF6 itself (12). The K63-linked polyubiquitination chains function as a scaffold to recruit the TAK1-TAB2 complex and IKK complex through binding to TAB2 and IKKγ, respectively (13). Recruitment of the kinase complexes facilitates phosphorylation of the catalytic subunit IKKβ by TAK1, leading to activation of the IKK complex. The activated IKK complex induces phosphorylation of IkBα, which acts as a trigger for K48-linked polyubiquitination by SCF-type E3 ubiquitin ligase (38), and the subsequent proteasomal degradation of IkBα. TRAF6 has the RING domain for binding E2, and has been classified as a monomeric RING-type E3 ubiquitin ligase. TRAF6 interacts with Ubc13, which is one of the E2s, forms a dimer with Uev1A (also called Mms2), and mediates the K63-linked polyubiquitination of IKKγ and TRAF6 itself (13,39). The functions of TRAF6 are not aimed at targeting proteins for degradation, but to induce qualitative changes in polyubiquitinated proteins, including stabilization (40), changing localization (41), and signal cascade activation (13,14). It has been suggested that K63-linked self-polyubiquitination of TRAF6 is important for signal transduction from TRAF6 to downstream effectors, such as IKKγ or TAK1-TAB2 complex. It is demonstrated that DAT induces the proteasome-dependent degradation of TRAF6, leading to suppression of NF-κB signaling. This suggests that DAT may induce malfunction or disorder of TRAF6-Ubc13-Uev1A complex, resulting in K48-linked self-polyubiquitination of TRAF6 and subsequent TRAF6 degradation by the 26S proteasome. It is possible that additional consequences of DAT-induced TRAF6 degradation will become apparent upon further characterization of additional participants in the TRAF6 complex in the presence of DAT.

We propose that the PEL-specific antitumor activity of DAT treatment is mainly due to the DAT-induced NF-κB inhibition. Constitutive NF-κB activation is essential for the antiapoptosis and growth of PEL cells (7-9), consistent with our data, the NF-κB inhibitor BAY11-7082, decreased the viability of PEL cells compared to KSHV-uninfected cells (Fig. 4D), and other NF-κB inhibitors also have PEL-specific cytotoxic effects (10,11). In fact, KSHV targets NF-κB signaling to influence gene expression and apoptosis. KSHV-encoded v-FLIP, K15, v-GPCR, K1 and K7 activate NF-κB signaling to achieve antiapoptosis in KSHV-infected cells, including PEL cells (4,6). Therefore, DAT-mediated destabilization of TRAF6 induces stabilization of IkBα and the subsequent inhibition of NF-κB signaling, which in turn may result in apoptosis of PEL cells. NF-κB signaling is also required for KSHV replication (10,11). DAT inhibited virus production at low concentrations, such as 0.2 and 1.0 μM (Fig. 6A), which do not influence proliferation of PEL cells (Fig. 1). DAT administration suppressed the ascites in the peritoneal cavity and the development of KSHV-infected cells in ascites of PEL-xenografted SCID mice (Fig. 7). DAT significantly inhibited the growth and infiltration of PEL cells in vivo and in vitro. Taken together, our data suggest that DAT may serve as a new lead compound for the development of novel drugs against not only PEL but also KSHV infection.

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References


