

Diallyl trisulfide induces apoptosis in human primary colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is one of the most prevalent types of cancer worldwide and a common cause of morbidity and mortality in humans. The garlic-derived organosulfur compound diallyl trisulfide (DATS) has been shown to induce apoptosis in many human cancer cell lines *in vitro* and also affords significant protection against cancer in animal tumor models *in vivo*. There is no available information to show DATS-induced apoptosis *in vitro* and the molecular mechanisms of apoptosis in human primary colorectal cancer cells. In this study, we investigated the cytotoxic effects in DATS in primary colorectal cancer cells. DATS inhibited the viability of primary colorectal cancer cells in a time- and dose-dependent manner. After treatment with DATS, primary colorectal cancer cells exhibited DNA condensation by DAPI stain. DATS increased reactive oxygen species (ROS) production in primary colorectal cancer cells. The mitochondria-dependent apoptotic signaling pathway was shown to be involved as determined by increase in the levels of cytochrome c, Apaf-1, AIF and caspase-3 and caspase-9 in DATS-treated primary colorectal cancer cells. The decrease in the level of $\Delta\Psi_m$ was associated with an increase in the Bax/Bcl-2 ratio which led to activation of caspase-9 and -3. Based on our results, DATS induces apoptotic cell death in human primary colorectal cancer cells through a mitochondria-dependent signaling pathway.

Introduction

Colorectal cancer represents almost 10% of all tumors and it is the third most common form of malignancy, behind prostate and lung cancers worldwide (1,2). More than 70% of colon cancers are related to diet and lifestyle and it was suggested that changes in dietary and lifestyle patterns can reduce colon cancer rates (3). Persons with a diet high in vegetables, cereals, fruits and seeds have a lower risk of colon cancer, and polyphenols in fruit led to reduce colon cancer risk experimentally (4). Thus, the therapy of the human colon cancer, induction of apoptosis is recognized as a very useful and promising approach.

Apoptosis is a mode of programmed cell death that is important for maintaining cell number, and deregulation of apoptosis may contribute to development of neurodegenerative disorders and cancer (5). There are two protein families regulating apoptosis; one is the Bcl-2 family which is involved in the initiation phase of apoptosis, and the other is caspase family of proteases that are responsible for the execution phase (6,7). It is well-known that cytochrome c release from the mitochondrial inter-membrane space represents an important checkpoint in apoptosis (8,9). Thus, at this checkpoint, Bcl-2 family plays regulatory influence on this process (10).

Diallyl trisulfide (DATS) is one of the main activity compounds in garlic extract (11,12) and has a broad-spectrum anti-neoplastic activity such as induction of apoptosis in many human cancer cells (13-19). DATS-induced apoptosis correlates with downregulation and hyper-phosphorylation of Bcl-2 in human prostate cancer cells (20). It was reported that p38/MAPK and caspase-8 are involved in the process of DATS-induced apoptosis in human CNE2 cells and interact with each other (21). Recently, in our laboratory, we have found that DATS inhibited migration and invasion of human colon cancer colo 205 cells *in vitro* (22) and inhibited tumor growth in an allograft animal model (23).

DATS-induced apoptosis has been shown in many human cancer cells but the cytotoxic effects on human primary colorectal cancer cells have not yet been defined. Therefore, the aim of this study is to investigate the effect of DATS on

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the human primary colorectal cancer cells and to elucidate its mechanism.

Materials and methods

Chemicals and reagents. Diallyl trisulfide (DATS) (99% purity), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco-BRL/Invitrogen Corp. (Grand Island, NY, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and 3,3'-diethyloxycarbocyanine iodide (DiOC₆) were purchased from Molecular Probes (Invitrogen, Eugene, OR, USA). Antibodies to cytochrome c, Apaf-1, caspase-9 and caspase-3, Bcl-2 and Bax were purchased from Cell Signaling (USA). All other chemicals used were of analytical grade.

Isolation of human primary colorectal cancer cells. Three colorectal carcinoma specimens from three patients were obtained from 2008 to 2009 from the Department of Surgery, China Medical University Hospital, Taichung, Taiwan after approval of the experiment by the hospital's Ethics Committee, and with written, informed consent from patients (IRB NO: DMR-96-IRB-72) (24). Each specimen was dissected into 1-mm³ pieces, immersed in a 10-fold volume of 0.25% trypsin solution (Sigma-Aldrich), maintained at 4°C overnight and then incubated for 1 h at 37°C. Then the trypsin was added to the cells (each well) followed by with FBS, the solution containing released cells was collected by using centrifugation at 150 x g for 5 min. After centrifugation, cells in each tube were re-suspended with RPMI-1640 supplemented with 10% FBS, and seeded into a 10-cm culture dish. Undigested tissue from each patient was immediately immersed in collagenase solution (500 U/ml in RPMI-1640 medium with 10% serum) (Sigma-Aldrich) in a plate and incubated at 37°C for 1 h. Released cells were collected, centrifuged, re-suspended with RPMI-1640 medium supplemented with 10% FBS, and seeded into a culture flask. When primary cultures became confluent then cells were detached by trypsin (0.25%)-ethylenediaminetetraacetic acid (EDTA) (0.02%) solution (Sigma-Aldrich), examined and counted under phase-contrast microscope, then were centrifuged and re-suspended with RPMI-1640 medium supplemented with 10% FBS and seeded into new culture flasks (25-27).

Cell viability assay. Human primary colorectal cancer cells were seeded onto 96-well plates at 1x10⁴ cells/well 24 h before treatment. The cultures were then rinsed in phenol-free RPMI-1640 medium and incubated with the DATS at the final concentrations 0, 10, 20 and 40 μM in RPMI-1640 culture medium for 24 h. At the end of incubation, 20 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) was added to each well and incubated for 4 h at 37°C then the MTT solution was removed and 200 μl dimethylsulfoxide (DMSO) was added to dissolve the crystals. The absorbance of each well at 570 nm was measured by using a spectrophotometric plate reader (Bio-Rad Labora-

tories, Tokyo, Japan) (24). All values were compared to the corresponding controls. All assays were performed with 3 replicates.

DAPI staining for apoptosis. Human primary colorectal cancer cells were plated in the 12-well plates at the density of 2x10⁵ cells/well for overnight then were treated with DATS (20 μM) for 24 h, and cells in each well were then fixed in 4% paraformaldehyde for 30 min. Then cells from each treatment were added 0.1% Triton X-100 and maintained for 10 min and then incubated with 1 μg/ml of DAPI staining solution for 30 min in the dark. Apoptotic cells from each treatment and control were observed through fluorescence microscopy (Zeiss, Oberkochen, Germany) as previously described (24,27).

Measurement of reactive oxygen species (ROS) production. ROS production from DATS-treated and -untreated cells was measured by flow cytometry following staining with 500 μl of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Invitrogen). Human primary colorectal cancer cells were plated in the 12-well plate at the density of 2x10⁵ cells/well for 24 h then were treated with or without DATS (20 μM) for 0, 6 and 12 h. Then cells from each well were collected and were stained with 20 μM DCFH-DA for 30 min at 37°C and the fluorescence intensity in cells was determined using the flow cytometer (24,27).

Mitochondrial membrane potential assays. The levels of mitochondrial membrane potential ($\Delta\Psi_m$) from DATS-treated and -untreated cells was measured by flow cytometry following staining with 500 μl of DiOC₆ (1 μmol/l, Invitrogen) for $\Delta\Psi_m$. Human primary colorectal cancer cells were plated in the 12-well plate at the density of 2x10⁵ cells/well for 24 h then were treated with or without DATS (20 μM) for 0, 6 and 12 h. Then cells from each well were collected and were stained with 500 μl of DiOC₆ (1 μmol/l, Invitrogen) for $\Delta\Psi_m$ for 30 min at 37°C, and the fluorescence intensity in cells was determined using the flow cytometer (Becton-Dickinson) (24,27).

Assays of caspase-9 and caspase-3 activity. Human primary colorectal cancer cells at the density of 2x10⁵ cells/well in 10-cm culture dish were treated with 20 μM DATS and incubated for 0 and 24 h. The activities of caspase-9 and caspase-3 were assessed according to the manufacturer's instruction of caspase colorimetric kit (R&D Systems Inc.). At the end of incubation, cells in each well were harvested and lysed in 50 μl lysis buffer containing 2 mM DTT for 10 min then centrifuged, the supernatant containing 200 μg protein were incubated with caspase-9 and caspase-3 substrate (Ac-DEVD-pNA and Ac-IETD-pNA, respectively) in reaction buffer. Then all samples from DATA-treated and -untreated cells were incubated in 96-well flat bottom microplate at 37°C for 1 h. The level of released pNA was measured with ELISA reader (Anthos Reader 2001, Anthos Labtec) at 405 nm wavelength as previously described (28,29).

Western blot analysis. Human primary colorectal cancer cells at a density of 1x10⁷ cells in 75 T flasks were incubated with 20 μM DATS for 0 and 24 h for examining the protein levels

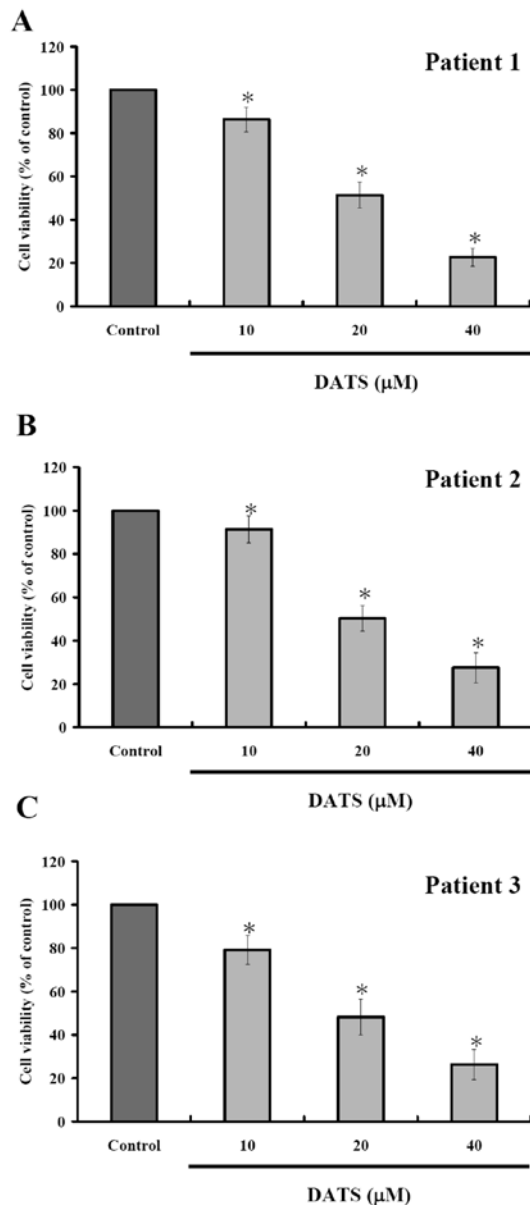


Figure 1. DATS decreases the percentage of viability in human primary colorectal cancer cells. Cells at a density of 2×10^5 cells/well in 12-well plate were treated with 0, 10, 20 and 40 μ M of DATS for 24 h then harvested for the determinations of percentage of viable cells from 3 patients (A, B and C) as described in Materials and methods. Data are presented as mean \pm SD in triplicate. * $P < 0.05$, significantly different compared between DATS and DMSO-treated groups.

correlated with apoptosis. At the end of incubation, cells from each treatment were collected, and the total protein lysate was isolated, gel electrophoresis and immunoblotting were conducted as previously described (24,26,27). The primary antibodies were anti-cytochrome c, anti-Apaf-1, anti-caspase-9 and anti-caspase-3, anti-Bcl-2 and anti-Bax. Immunoreactive proteins of all examined samples were visualized with the ECL chemiluminescent detection system (Perkin-Elmer Life Science, MA, USA) and BioMax Light Film (Eastman Kodak, New Haven, CT, USA) according to the manufacturer's instructions.

Statistical analyses. Data are presented as the mean \pm SD for the indicated number of separate experiment. Statistical

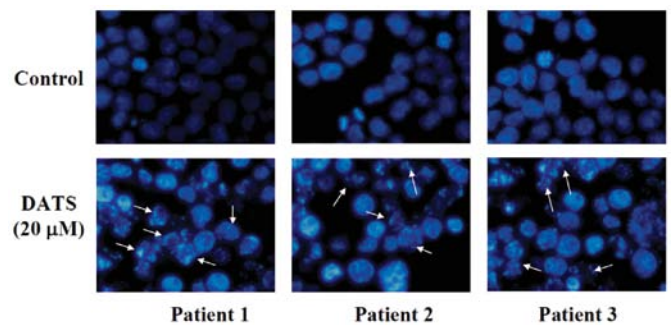


Figure 2. DATS induces apoptosis human primary colorectal cancer cells. Cells at the density of 2×10^5 cells/well in 12-well plates were maintained 24 h then were incubated with 0 and 20 μ M of DATS for 24 h, then isolated for the examination of apoptosis using DAPI staining and photographed under fluorescence microscopy.

analyses of data were performed by Student's t-test, and $P < 0.05$ was considered significant.

Results

DATS decreases the percentage of viability in human primary colorectal cancer cells. To measure DATS-mediated effects on human primary colorectal cancer cells, the cells after incubated with 10, 20 and 40 μ M DATS for 24 h were harvested and the percentage of viable cells were measured by MTT assay. Results are shown in Fig. 1, indicating that increase of DATS concentration led to decreased percentage of viable cells. As expected, 24-h incubation showed apparent stronger dose-dependent effects of DATS.

DATS induces apoptosis of human primary colorectal cancer cells. To investigate the effect of DATS on nuclear alterations, cells were stained with DAPI and results are shown in Fig. 2, demonstrating that the cells underwent remarkable nuclear changes upon treatment. In the control (untreated) cells, the nuclei were intact, round, and uniformly stained. However, after exposure to DATS, the cells manifested nuclear shrinkage/condensation and nuclear fragmentation. At 20 μ M of DATS, a number of cells exhibited nuclear shrinkage and chromatin condensation but these aberrant nuclear alterations were not seen in the control cells. These observations showed that DATS-induced apoptosis occurred in primary colorectal cancer cells.

DATS induces reactive oxygen species (ROS) production and decreases the level of mitochondrial membrane potential ($\Delta\Psi_m$) in human primary colorectal cancer cells. To investigate whether or not DATS-induced apoptosis is *via* the production of ROS, we measured the intracellular level of ROS during treatment with DATS by DCFH-DA and using a flow cytometer and results are shown in Fig. 3A. Results indicate that DATS induced ROS production in a time-dependent manner. The oxidation of DCF was dependent upon DATS treatment time (Fig. 3A). To investigate whether or not DATS-induced apoptosis is through decreasing the levels of mitochondrial membrane potential, cells were collected and stained with DiOC₆ and results are shown in Fig. 3B, indicating that DATS

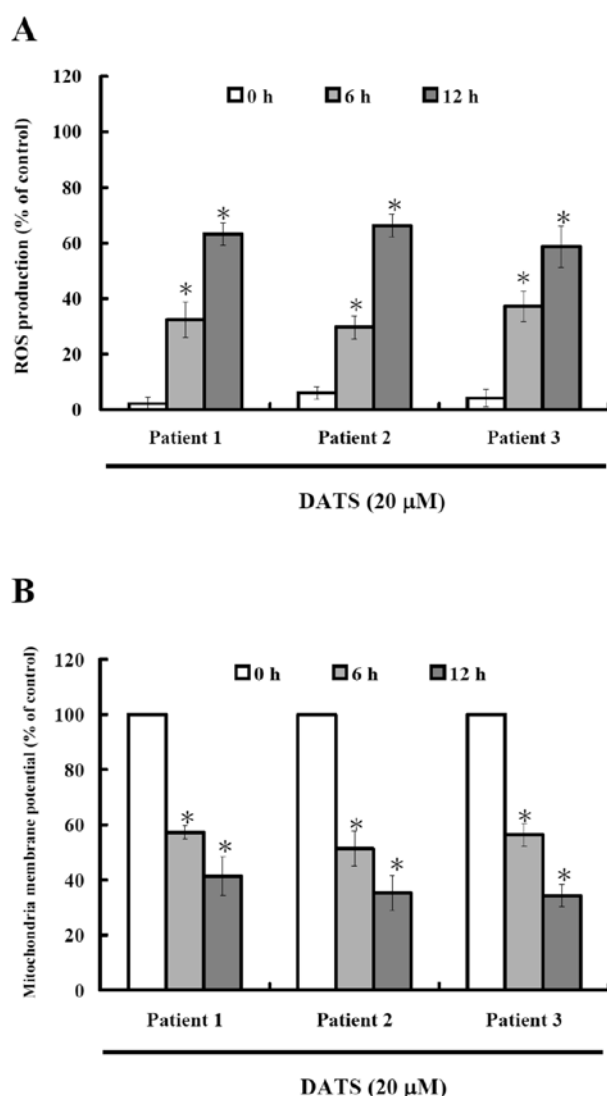


Figure 3. DATS induces reactive oxygen species (ROS) production and decreases the level of mitochondrial membrane potential ($\Delta\Psi_m$) in human primary colorectal cancer. Cells incubated in 12-well plates for 24 h were treated with or without 20 μ M of DATS for 0, 6 and 12 h. The cells were harvested and washed with PBS twice, re-suspended in 500 μ l of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μ M) for ROS (A) and 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) (1 μ mol/l) for $\Delta\Psi_m$ (B). Cells then were incubated at 37°C for 30 min and were analyzed by flow cytometry as detailed in Materials and methods.

decreased the levels of $\Delta\Psi_m$ in human primary colorectal cancer cells and these effects are time-dependent.

DATS affects caspase-9 and -3 activities in human primary colorectal cancer cells. To investigate whether or not DATS-induced apoptosis is *via* the activation of caspases, the cells after treatment with DATS were harvested and caspase-9 and -3 activities were measured and results are shown in Fig. 4. Results indicated that DATS promoted the activation of caspase-9 and caspase-3 in the examined cancer cells. Based on this observation, DATS induced apoptosis was via activation of caspase-9 and caspase-3.

DATS affects apoptosis-associated proteins in human primary colorectal cancer cells. For further examining the effects of

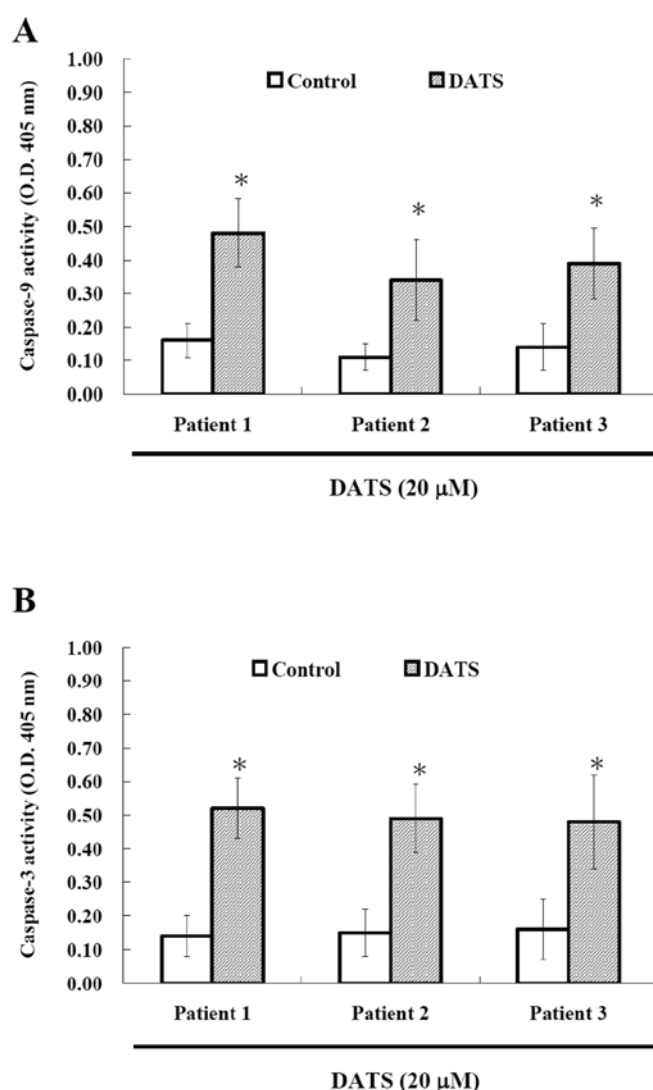


Figure 4. DATS affect caspase-9 and caspase-3 activities in human primary colorectal cancer cells. Cells (2×10^5 cells/well) cultured in 12-well plates were treated with or without 20 μ M DATS for 0 and 24 h, then cells were harvested and analyzed for caspase-9 (A) and -3 (B) activities by flow cytometric analysis as described in Materials and methods. Columns, mean ($n=3$); bars, SD. $P < 0.05$ significantly different compared with control.

DATS-induced apoptosis in the human primary colorectal cancer cells through apoptosis-associated protein levels, the cells after exposure to 20 μ M DATS for 24 h were harvested for western blotting and result are shown in Fig. 5. DATS increased the protein levels of cytochrome c, caspase-9 and caspase-3, showing that DATS promoted the release of cytochrome c from mitochondria, and then led to the activation of caspase-9 and caspase-3. Furthermore, DATS promoted the pro-apoptotic protein Bax and inhibited the anti-apoptotic protein, leading to apoptosis in the three examined human colorectal cancer cell types.

Discussion

Numerous studies have shown that DATS induces cytotoxic effects in many human cancer cells through cell cycle arrest and induction of apoptosis. However, there is no information to show DATS induced apoptosis in human primary colorectal

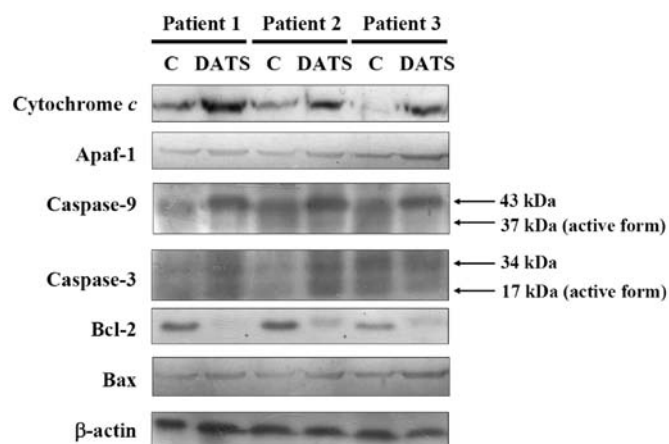


Figure 5. DATS affects apoptosis-associated proteins in human primary colorectal cancer cells. Cells (1×10^6 /well) seeded into 6-well plates were treated with $20 \mu\text{M}$ of DATS then incubated for 0 and 24 h and harvested for western blotting to examine the protein levels of cytochrome c, Apaf-1, caspase-9 and -3, Bcl-2 and Bax as described in Materials and methods.

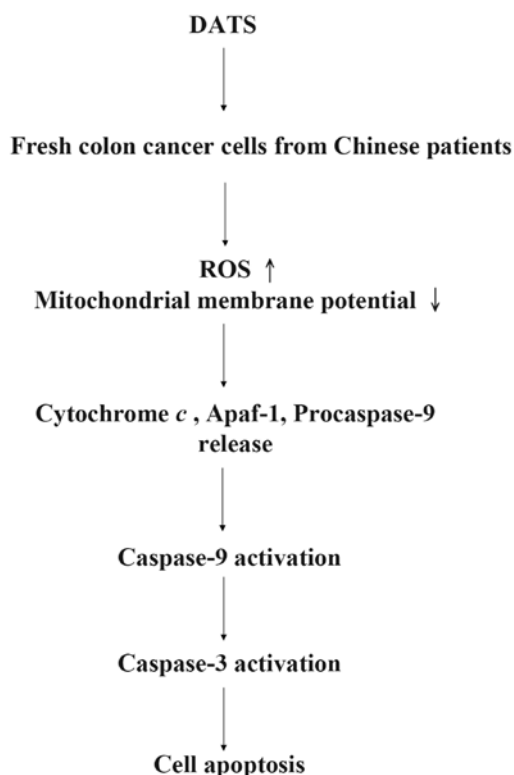


Figure 6. The possible signaling pathway for DATS-induced apoptosis in human primary colorectal cancer cells.

cancer cells. Thus, the major objective of the present study was to test anticancer responses to DATS on human primary colorectal cancer cells. DATS is a well documented highly promising cancer-chemopreventive constituent of processed garlic. Recently it was reported that DATS treatment suppresses STAT3 phosphorylation in prostate cancer cells in culture and *in vivo*, but activation of this oncogenic transcription factor is largely dispensable for cellular responses to DATS (30).

The present study revealed that DATS treatment decreased the percentage of viable cells (Fig. 1), induced apoptosis based

on DAPI staining (Fig. 2), promoted the ROS generation and decrease the levels of $\Delta\Psi_m$ (Fig. 3) and promoted the activities of caspase-9 and -3 (Fig. 4) that were analyzed by flow cytometric assay. To clarify the underlying mechanism for DATS-induced apoptosis, we used western blotting to confirm that DATS promoted the release of cytochrome c from mitochondria and promoted the activation of caspase-9 and -3, further, DATS increased the pro-apoptotic protein Bax and inhibited the anti-apoptotic protein Bcl-2 leading to apoptosis (Fig. 5).

Fig. 3 indicates that DATS induced ROS generation in the 3 isolated cancer cell types and these effects are time-dependent. This is in agreement with other recent reports indicating that DATS can be reduced in cancer cells to hydroperthiol that leads to H_2O_2 generation, thereby influencing transmission of signals regulating cell proliferation and apoptosis (31). Other reports also show that the cytotoxicity caused by DATS is mediated by the generation of ROS and subsequent activation of the ASK1-JNK-Bim signal transduction pathway in human breast carcinoma MDA-MB-231 cells (32). Reports also exist showing that *in vivo* healthy mice injected intraperitoneally with allyl sulfides for ten days. The experiment revealed that DATS as well as DADS diminished lipid peroxidation and ROS level in normal mouse liver (31). Thus, it is possible that DATS-induced elevation of the intracellular level of ROS is due to disruption of mitochondrial electron transport chain activity, as Fig. 3 clearly demonstrates that DATS decreased the levels of $\Delta\Psi_m$. This needs to be determined in future studies. It is well known that the ratio of Bax/Bcl-2 is involved at the level of $\Delta\Psi_m$ in mitochondria (10). Our results from western blotting clearly showed that DATS increased the pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 (Fig. 5). Thus, DATS affects the levels of $\Delta\Psi_m$ in mitochondria via the changes in the ratio of Bax/Bcl-2. DATS has also clearly been demonstrated to promote caspase-3 activation (31). Our data showed that DATS promoted the activities of caspase-9 and caspase-3 (Fig. 4), and also confirmed by western blotting (Fig. 5). It is well documented that apoptosis can be divided into caspase-dependent and caspase-independent pathways (33), based on our results we suggest that DATS induces apoptosis through the caspase-dependent pathway (Fig. 6).

In summary, the present study shows the cytotoxic effects of DATS via ROS generation, dysfunction of mitochondria (decreased the levels of $\Delta\Psi_m$ in mitochondria) due to the increase in the ratio of Bax/Bcl-2, promoting the activation of caspase-9 and -3 leading to apoptosis in human primary colorectal cancer cells.

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