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Diallyl Disulfide (DADS) Induces Upregulation of PTEN in Human Leukemia Cells

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Human leukemia 세포에서 diallyl disulfide(DADS)에 의한 PTEN 발현 유도

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ABSTRACT - The differentiation of leukemia cells into mature cells is a major target of the human leukemia therapy. As differentiated leukemia cells lose their proliferative and tumor-forming abilities, differentiation inducers may be useful for the treatment of leukemia. In this study, the experiments were designed to determine whether diallyl disulfide (DADS) regulates expressions of tumor suppressor protein PTEN (phosphatase and tension homologue) in HL-60 cells. DADS causes upregulation of PTEN in a time- and dose-dependent manner, which was correlated with decrease of phospho-Akt level. These results suggest that DADS induces upregulation of PTEN in human leukemia cells. These results suggest that DADS may be a useful anticancer agent for management of human leukemia.

Key words: diallyl disulfide(DADS), PTEN (phosphatase and tension homologue), human leukemia.

Fruits and vegetables are excellent sources of fiber, vitamins, and minerals, but they also contain components like polyphenols, terpenes, alkaloids, and phenolics that may provide substantial health benefits beyond basic nutrition. Research over the last decade has been shown that several micronutrients in fruits and vegetables reduce cancer. The active components of dietary phytochemicals that most often appear to be protective against cancer are cucumin, genistein, resveratrol, diallyl disulfide (DADS), allicin, capsaicin, silymarin, catechins, inositol hexophosphate, isoflavones, saponin, beta-carotene, selenium, flavonoids, and dietary fiber[1)]. These dietary agents are believed to suppress the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis.

Allium vegetables, including garlic, onions, leeks, chives, and scallions are used throughout the world for their sensory characteristics as well as their apparent health benefits. The ability of these foods to serve as antimicrobial, antithrombotic, and antitumor, antilipidemic, antiarthritic, and hyperglycemic agents has kindled widespread interest in these vegetables as medicinal foods[2)]. In particular, garlic is a plant commonly used as a food item in many different cultures of the world, and its medicinal properties have been focused since ancient times. Epidemiological studies show that enhanced garlic consumption is closely related with reduced cancer incidence[3)]. Whereas it remains to be determined which constituent of constituents within garlic is most responsible for their proposed anticancer properties, there are many evidences suggesting that organosulfur constituents are most likely involved. DADS, an lipid-soluble organosulfur compound in processed garlic, can influence a number of molecular events involved with cancer, including inhibiting mutagenesis, blocking carcinogen DNA adduct formation, scavenging free radicals, as well as blocking cell proliferation, differentiation, and angiogenesis[1,2)]. Recently it has been known that DADS induces apoptosis of human cancer cells including HL-60 cells and HCT-15 cells[4,5)].

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The novel tumor suppressor gene *PTEN/MMAC1/TEP1* on the chromosome10q23 protein product (PTEN) is a lipid phosphatase that dephosphorylates the D3 position of phosphatidylinositol3,4,5-trisphosphate (PIP_3), second messengers downstream of phosphatidylinositol 3-kinase (PI3-Kinase). Mutations or reduction of PTEN have been focused in cancers and associated with their invasiveness and metastatic properties[6-10]. In hematological malignancies, mutations and hemizygous deletions of PTEN also are found in 40% of leukemia cell lines such as HL-60 cells[11]. PTEN has been identified as novel tumor suppressor and its tumor suppressor function is critically associated with phosphatase activity of inositol-3- phosphatase[12,13]. Therefore, PTEN modulates several cellular functions of PI3-Kinase, including cellgrowth, cellmigration, cell differentiation, and cell survival[12]. Recently it has been known that HL-60 cells control the progression of its cell cycle through the activation of the PI3-Kinase signaling pathway[14,15], suggesting that HL-60 cell maintains its cell cycle through the generation of PIP_3. On the contrary, PTEN could lead to cell cycle arrest in various cancer cells, including glioma, prostate cancer and breast cancer cells[16-20]. Furthermore, PTEN induces cell differentiation in normal mammalian cells[21,22]. These results indicate that the progression of cell cycle of HL-60 cells is dependent on its PIP_3 level, the net enzymatic balance of between PI3K and PTEN. Collectively, it is likely to suggest that PTEN is an important regulator of differentiation and proliferation in HL-60 cells, and then it is to potential target of chemotherapy.

The differentiation of leukemia cells into mature cells is a major target of the human leukemia therapy. As differentiated leukemia cells lose their proliferative and tumor-forming abilities, differentiation inducers may be useful for the treatment of leukemia. So far, it is well known that various pharmacological agents can induce differentiation of HL-60 cells into granulocyte-like or monocyte/macrophage-like cells such as dimethyl sulfoxide (DMSO), tumor necrosis factor-α (TNF-α), and retinoic acid (RA)[23-29]. In fact, All- *trans*- retinoic acid (ATRA) therapy is widely used in the treatment of acute promyelocytic leukemia (APL) patients[30]. Interestingly, these differentiation inducers also cause upregulation of PTEN in HL-60 cells[20,31,32]. Taken above findings together, it is likely to suggest that these pharmacological agents induce differentiation through activation of PTEN signaling pathway in leukemia.

Our aim in this study was to determine whether DADS induces upregulation of PTEN in human HL-60 cells.

Materials and Methods

Materials

Anti-PTEN, p53, and p21 monoclonal antibody (mAb) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI1640 and fetal calf serum were from Gibco-BRL(Gaithersburg, MD, USA). Hanks' balanced salt solution (HBSS) and EDTA were from Sigma Chemical Co. (St. Louis, MO, USA). Dially disulfide (DADS), pyrrolidine dithiocarbamate (PDTC), SB203580, PD98059, and SP600125 were obtained from Calbiochem (SanDiego, CA, USA).

Cell culture

Human leukemia cell line, HL-60 cells and human breast cancer cell line, MCF-7 and MDA-MB231 cells were obtained form the American Type Culture Collection (Rockvil, MD, USA). These cells were cultured in RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, antibiotics (penicillin G 60 mg/L, streptomycin 100 mg/L, amphotericin B 50 μg/L) at a humid atmosphere (5% CO_2, 95% air).

Western blot analysis for PTEN protein expression

Human leukemia cells ($1x10^6$ cells) were seeded on 100-mm culture dishes and harvested in phosphate buffered saline (PBS). After washing with PBS, cell pellets were lysed with the lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1 μg/ml aprotinin). After incubation for 30 min at 4 ºC, cellular debris was removed by centrifugation at 100,000 × g for 30 min, and supernatants were analyzed by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bio-Rad protein assay regent (Bio-Rad Laboratories, USA). Samples (50 μg) were prepared with the four volume of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue at 95 ºC for 5 min. SDS-PAGE was performed in 10% slab gel. Proteins were transferred to nitrocellulose paper. The membrane was washed in blocking buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% fat-free milk) for 60 min at room temperature with shaking and then washed with TBST (TBS, 0.01% Tween 20). Primary antibody against PTEN was incubated at 4 ºC for 4 hr with 10 μg/ml. The secondary HRP-conjugated antibody was goat anti-mouse IgG (Santa Cruz, CA, USA). Reactive proteins were detected using enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for PTEN mRNA expression

To examine the contribution of transcriptional control in PTEN regulation, RT-PCR was performed by using RNA PCR Kit (GeneAmp, Applied Biosystem, USA). Total RNA was isolated from cells by using TRIzol reagent following the manufacture's instructions. Five microgram of total RNA was transcribed into cDNA in a 20 μl final volume of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 1 mM each dNTP) and 2.4 μM oligo-d (T)16-primer, 1 units RNase inhibitor, and 2.5 units M-MLV RNase H-reverse transcriptase by incubation for 15 minutes at 70 ºC and 50 minutes at 42 ºC. The reaction was stopped by incubation at 95 ºC for 10 minutes. PCR aliquots of the synthesized cDNA were added to a 45 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 2 units Taq DNA polymerase, and 0.4 μM of each PCR primer: sense primer, human PTEN (5'CCGGAATTCATGACAGCCATCATCAAAGA-3'), antisense primer, human PTEN (5'-CGCGGATCCTCA-GACTTTTGTAATTTGTG-3'). Amplification for PTEN was initiated with 3 minutes of denaturation at 94 ºC followed by 26 cycles at 94 ºC for 1 minute, 94 ºC for 1 minute, 55 ºC for 1 minute, and 72 ºC for 3 minutes. After the last cycle of amplification, the samples were incubated for 5 minutes at 72 ºC. β-actin PCR was performed with 2.5 μl of aliquots of synthesized cDNA using primers at a concentration of 0.15 μM: sense primer, human β-actin (5'-CCACGAAACT-ACCTTCAACTCC-3'), antisense primer (5'-TCATACT-CCTGCTGCTTGCTGATCC-3'). The obtained PCR products were analyzed on ethidium bromide-stained agarose (2%) gels.

Statistical Analysis

All experimental data are mean ± standard deviation (SD). Statistical analysis was performed using Student's test, and $p < 0.005$ was considered to be significant.

Results

DADS induces upregulation of PTEN expression in HL-60 cells

Previous study has shown that DMSO, a compound containing sulfur ion, upregulates PTEN expression in HL-60 cells[32]. Thus, I firstly examined whether expression of PTEN could be upregulated by DADS, another compound containing sulfur ion. HL-60 cells (1×10^6 cells) were treated with 1.0 μM DADS for various periods of times and used to prepare whole cell extracts for western blotting of PTEN. DADS caused an increase of PTEN protein level in a time-dependent manner (Fig. 1). PTEN level was significantly increased within 24 hr after treatment with DADS and this response was persistent till 72 hr. Cell viabilities of leukemia cells receiving DADS were declined after 72 hr treatment (data not shown). Next experiments were designed to determine whether DADS stimulates expression of PTEN in a dose dependent manner. Leukemia

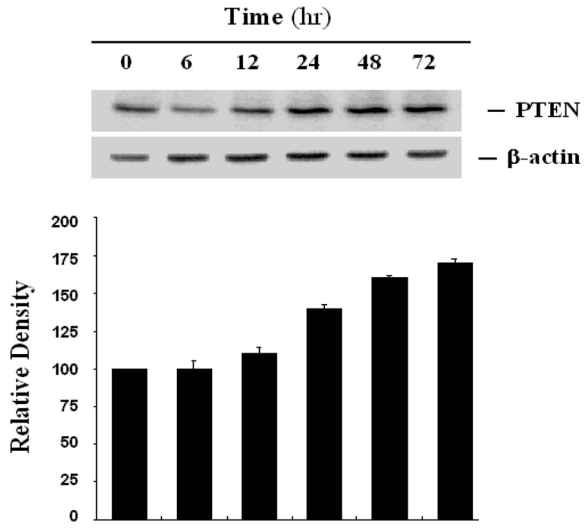

Fig. 1. Time-dependent effect of DADS on the PTEN expression in HL-60 cells. HL-60 cells (1×10^6) were stimulated with 10 μM DADS for the indicated times. PTEN protein level was analyzed by western blotting. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

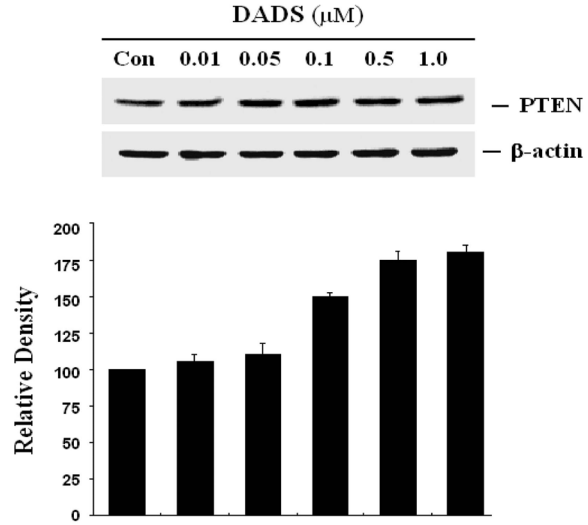

Fig. 2. Dose-dependent effect of DADS on the PTEN expression in HL-60 cells. HL-60 cells (1×10^6) were stimulated with various doses of DADS for 48h. PTEN protein level was analyzed by western blotting. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

cells (1×10^6cells) were incubated with various concentrations of DADS (0-1.0 μM) for 48 hr. DADS also dose-dependently stimulated PTEN expression (Fig. 2). PTEN protein level of leukemia cells was increased from 0.1 μM DADS, and DADS induces upregulation of PTEN in a dose-dependent manner. At the same time, I also determined the mRNA levels of PTEN in a function of DADS concentrations. As shown at Fig. 3, PTEN mRNA level in leukemia cells was also increased from 0.1 μM DADS. These findings suggest that DADS can induce PTEN expression at mRNA level, either by increasing transcription or by decreasing mRNA turnover. An increased expression of PTEN could induce the inhibition of Akt phosphorylation. Therefore, western blotting was performed to detect the phosphorylated Akt in the DADS-treated HL-60 cells (Fig. 4). This reduction in phosphorylated Akt correlated with the increase in PTEN expression, and occurred at the same time point and with the same dose.

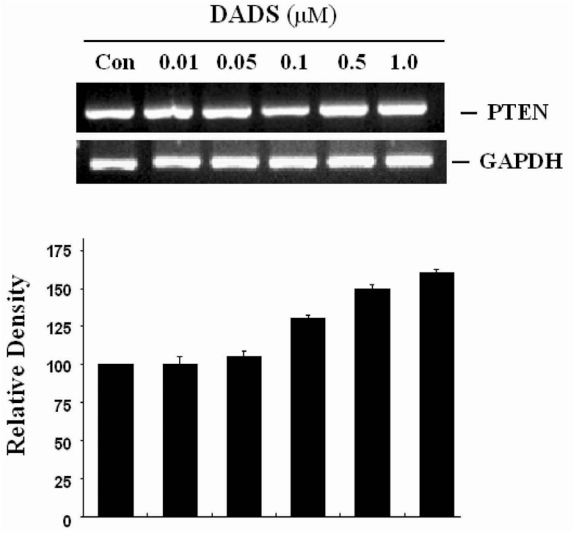

Fig. 3. Effect of DADS on mRNA level of PTEN in HL-60 cells. HL-60 cells (1×10^6) were stimulated with various doses of DADS for 48h. PTEN mRNA expression was analyzed by RT-PCR. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

DADS doesn't change p53 expression in HL-60 cells

It has been well known that PTEN controls p53 in cancer cells[36]. Thus, I secondly examined whether expression of p53 could be also changed by DADS. DADS caused an increase of PTEN protein level in a dose-dependent manner. However, p53 protein level was not changed after treatment with DADS (Fig. 5). To clearly confirm effect of DADS on p53 in HL-60 cells, I analyzed whether DADS induces expression of p21, an important downstream of p53 signaling pathways[37]. As shown in Fig. 6, DADS also does not change p21 expression in HL-60 cells.

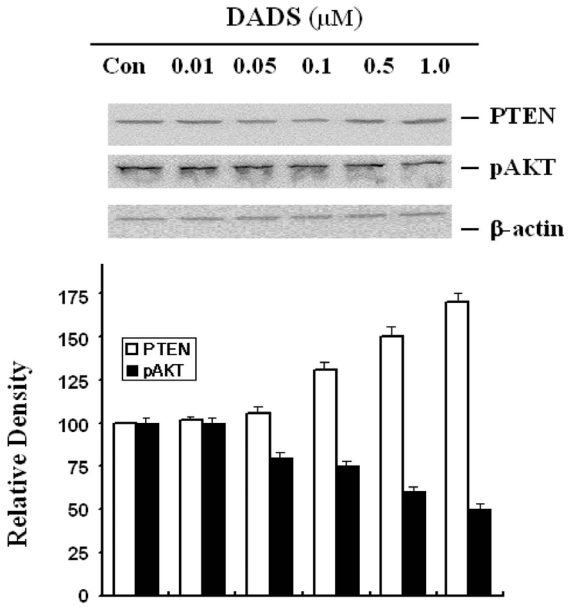

Fig. 4. Effect of DADS on phosphor-Akt level in HL-60 cells. HL-60 cells (1×10^6) were stimulated with various doses of DADS for 48h. Phospho-Akt level was analyzed by western blotting. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

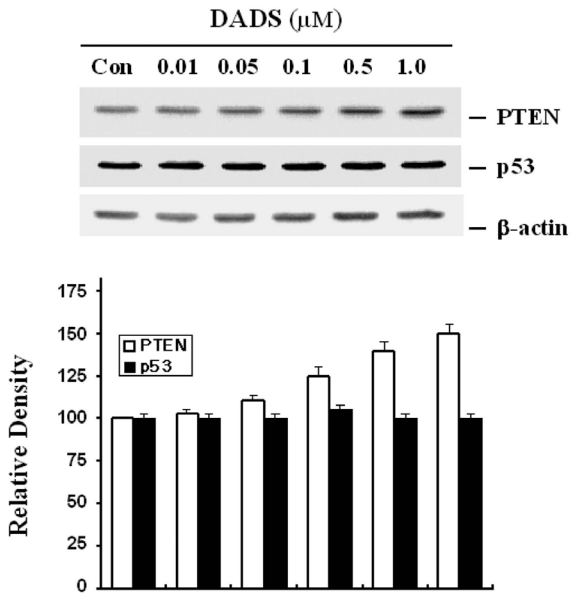

Fig. 5. Different effects of DADS on PTEN and p53 expression in leukemia cells. HL-60 cells (1×10^6) were stimulated with various doses of DADS for 48h. PTEN or p53 protein level was analyzed by western blotting. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

MAPK is required for DADS-induced upregulation of PTEN in HL-60 cells

I next examined the mechanisms for DADS-induced expression of PTEN. Muliple activated protein kinases (MAPKs), JNK, ERK, and p38 can be activated in response to a wide variety of stimuli such as inflammatory cytokines and are involved in stress responses by regulating many cellular functions. Recently, it has been known that MAPKplays a major role in stress-induced PTEN upregulation[38,39]. Furthermore, DADS activates MAPKs in cancer cells[40-42]. To determine whether or not MAPK is involved in enhancement of DADS-induced PTEN expression in HL-60 cells, HL-60 cells (1×10^6 cells) were preincubated with 10 μM MAPK inhibitors for 3 hr, prior to treatment with 1.0 μM DADS for 48 hr, and then analyzed for PTEN expression using western blotting. As shown in Fig. 7, only inhibition of ERK pathway with PD98059, an ERK inhibitor, significantly inhibited DADS-induced PTEN expression, whereas inhibition of JNK pathway and nuclear factor-B (NF-B) pathway with each specific inhibitor, SP600125, a JNK inhibitor, and PDTC, a NF-B inhibitor, respectively, did not effect on PTEN expression. These data indicate that enhancement of DADS-induced PTEN expression is mediated via activation of ERK pathway. Interestingly, inhibition of p38 pathway with SB203580, a p38 inhibitor, caused higher enhancement of DADS-induced PTEN expression than no inhibition of the pathway. This finding was similar to that inhibition of MAPK with specific inhibitors, SB203580, causes enhanced apoptosis without DADS treatment[43]. These data indicate that NF-kB pathway is not, but MAPK pathway is involved in the DADS-induced PTEN expression in HL-60 cell.

Discussion

DADS, an oil-soluble organosulfur compound found in processed garlic, effectively inhibited the growth of human cancer cells[1-5]. In the present study, DADS was proved to be an effective inducer of tumor suppressor PTEN expression in human leukemia cells.

The novel tumor PTEN has been identified as novel tumor suppressor and its tumor suppressor function is critically associated with phosphatase activity of inositol-3-phosphatase[12,13]. PTEN modulates several cellular functions of PI3-Kinase, including cell growth, cell migration, cell differentiation, and cell survival[12]. Consequently, PTEN is an important regulator of differentiation and proliferation in HL-60 cells, and then it is to potential target of chemotherapy. However, the control mechanism of PTEN expression remained largely unclear. Previous studies have shown that transcription factor peroxisome proliferator activated receptor (PPAR) and NF-B are involved in PTEN expression in cancer cells[31,32,39,46-49]. However, I here found that NF-B pathway is not, but MAPK pathway is involved in the DADS-induced PTEN expression in HL-60 cell. It has been well known that MAPK pathway is importantly involved in the DADS-mediated antineoplastic actions in various cancer cells such as apoptosis[43,50]. On the contrary, garlic metabolites fail to inhibit the activation of the transcription factor NF-B[51]. Furthermore, DADS inhibits

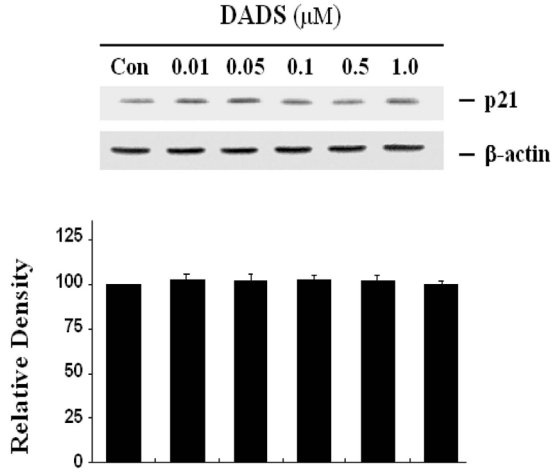

Fig. 6. Dose-dependent effect of DADS on p21 expression in HL-60 cells. HL-60 cells (1×10^6) were stimulated with various doses of DADS for 48h. p21 protein level was analyzed by western blotting. β-actin was used as a loading control. The analysis of electrophoretic band was performed with the LAS-1000 (Fujifilm, Japan).

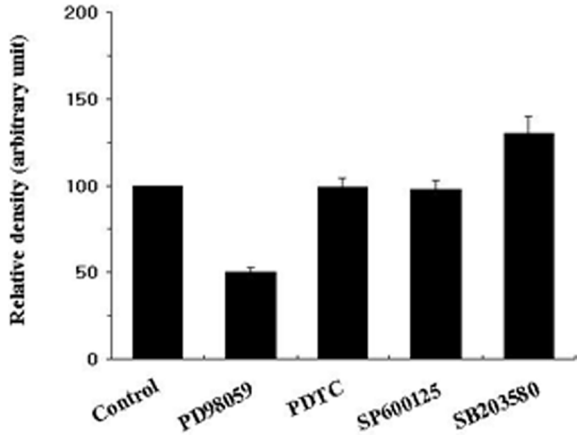

Fig. 7. Inhibition of DADS-induced PTEN expression by various inhibitors in HL-60 cells. HL-60 cells (1×10^6 cells) were pre-treated with 10 μM inhibitors and were stimulated with 1.0 μM DADS for 48 h. PTEN level was analyzed by western blot. The analysis of electrophoretic band was performed with LAS-1000 (Fujifilm, Japan).

NF-B activation in macrophages[52]. Taking all these reports together, it is likely to suggest that DADS induces PTEN expression through MAPK activation in HL-60 cells.

In conclusion, DADS was proved to be an effective inducer of tumor suppressor PTEN expression in HL-60 cells. Our results suggest the first evidence that DADS is to be strong inducer of PTEN in leukemia cells. These results suggest that DADS may be a useful anticancer agent for management of human leukemia.

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