Diallyl Disulfide Inhibits WEHI-3 Leukemia Cells *In Vivo*

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Abstract. *Enhanced garlic (Allium sativum) consumption is closely related to reduced cancer incidence, as shown in epidemiological studies. Diallyl disulfide (DADS), a component of garlic, inhibits the proliferation of human blood, colon, lung and skin cancer cells. Although DADS had been reported to induce apoptosis in human leukemia HL-60 cells, there are no reports regarding whether or not it affects leukemia cells in vivo. Therefore, the present study is focused on the in vivo effects of DADS on WEHI-3 leukemia cells. The effects of DADS on murine WEHI-3 cells were initially examined, and the results indicated that DADS induced cytotoxicity and that this effect was dose-dependent. The effects of DADS on WEHI-3 in BALB/c mice were also examined, and the results indicated that DADS decreased the percentage of MAC-3 marker, indicating that differentiation of the precursor of macrophage and T cells was inhibited. The weights of liver and spleen were also measured, and the results indicated that DADS decreased the weight of these organs. An important characteristic of WEHI-3 leukemia is the enlarged spleen in mice after i.p. injection of WEHI-3 cells. Based on pathological examination, the function of DADS was observed in the liver and spleen of mice previously injected with WEHI-3 cells. Apparently, DADS affects WEHI-3 cells both in vitro and in vivo.*

Diallyl disulfide, a thioether compound found naturally in garlic, is an effective inhibitor of growth in neoplastic CMT-13 cells (1). DADS is an effective inhibitor of the promotion phase of DMBA-induced skin tumors in mice (2). It was further reported that DADS inhibited C6 glioma through the inhibition of H-ras in Sprague-Dawley rats (3). DADS also acts as an antiproliferative agent in neuroblastoma cells by inducing apoptosis (4). It was reported that DADS-induced apoptosis is triggered by the generation of hydrogen peroxide, the activation of caspase-3, degradation of PARP and fragmentation of DNA (5). Recently, we also found that DADS inhibits N-acetyltransferase activity in HL-60 cells (6).

Many different tumor systems for *in vivo* evaluation of new anticancer agents are available. Murine host systems are often used for experimental tumor therapy due to the low cost associated with the ease with which cancer production is established and its widely accepted experimental end-points (7, 8). The murine WEHI-3 leukemia cell line was first established in 1969 and showed characteristics of myelomonocytic leukemia. It has been used to induce leukemia in syngenic Balb/c mice for evaluating the antileukemia effects of drugs. However, there is no available information regarding the effects of DADS on leukemia cells *in vivo*. Therefore, in the present study, the antitumor effects of DADS on murine WEHI-3B leukemia cells were investigated *in vitro* and *in vivo*. It was demonstrated that DADS induced cytotoxicity in murine leukemia WEHI-3 cells *in vitro* and also affected WEHI-3 cells and tumor formation *in vivo*.

Materials and Methods

Materials and reagents. The following chemicals and reagents were obtained from the companies indicated: Diallyl disulfide (DADS) was purchased from Fluka Chemika Co. (Bucha, Switzerland). RPMI 1640, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Male BALB/c mice. Male BALB/c mice, of 22-28 g in weight, were obtained at the age of 8 weeks from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan, ROC).

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Key Words: Diallyl disulfide, WEHI-3 leukemia cells, BALB/c mice, apoptosis.

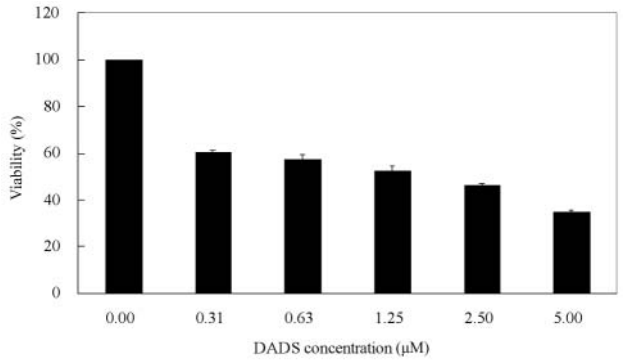


Figure 1. *The percentage of viable WEHI-3 cells after DADS treatment for 48 h. WEHI-3 cells (2x10^5 cells/well; 12 well-plates) were plated in RPMI 1640 medium + 10% FBS with different DADS concentrations for 48 h. The cells were then collected by centrifugation and the viable cells were determined by Trypan blue exclusion and flow cytometry, as described in the Materials and Methods. Each point is the mean±S.D. of 3 experiments. *p<0.05*

Murine WEHI-3 leukemia cells. The murine myelomonocytic leukemia cell line WEHI-3 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine.

In vitro studies. Assay for cytotoxic activity. About 2x10^5 cells (WEHI-3) were incubated for 48 h without (control) or with DADS at concentrations of 0, 0.31, 0.63, 1.25, 2.50 and 5.00 μM. The percentage of viable WEHI-3 cells was determined by Trypan blue exclusion and flow cytometry, as described previously (9, 10).

In vivo studies.

a) Drug treatment. These experiments were divided into two parts: Part I. The normal animals (60 BLAB/c mice) were divided into 3 groups: Group I was the control (20 animals); Group II was treated with olive oil (vehicle); while Group III was treated with DADS in olive oil. Part II. The normal animals (60 BLAB/c mice) were divided into 3 groups: Group I was injected with WEHI-3 only as the control (20 animals); Group II was injected with WEHI-3 then treated with vinblastin (25 μg/100 μL) in olive oil as the positive control; Group III was injected with WEHI-3 and then treated with DADS (25 μM/100 μL) in olive oil. All animals were orally given the above daily dose for up to 3 weeks before being weighed.

DADS (Sigma, MO, USA) was dissolved in olive oil (Sigma, MO, USA) to the treat mice. The Balb/c mice were randomly divided into 6 groups receiving different treatments.

b) Blood samples and immunofluorescence staining. Blood was collected (about 1 mL) from each animal from each group at the end of the experiments and was treated with ammonium chloride to lyse the red blood cells, followed by centrifugation for 15 minutes at 1500 rpm at 4˚C. The isolated white blood cells were examined for cell markers, such as Mac-3, CD11b, CD3, CD14

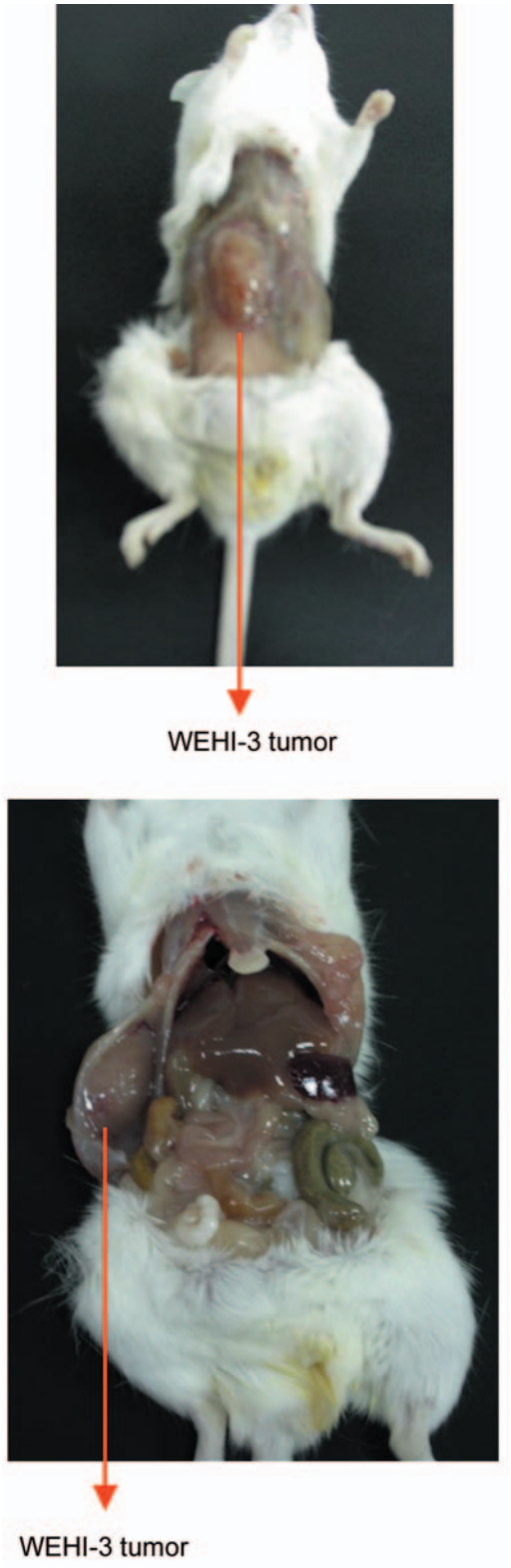


Figure 2. *Representative pictures of BLAB/c mice after injection with WEHI-3 cells over 3 weeks. After injection with WEHI-3 cells (1x10^6 cells/100 μL) in PBS for 3 weeks, blood was collected and the animals were sacrificed.*

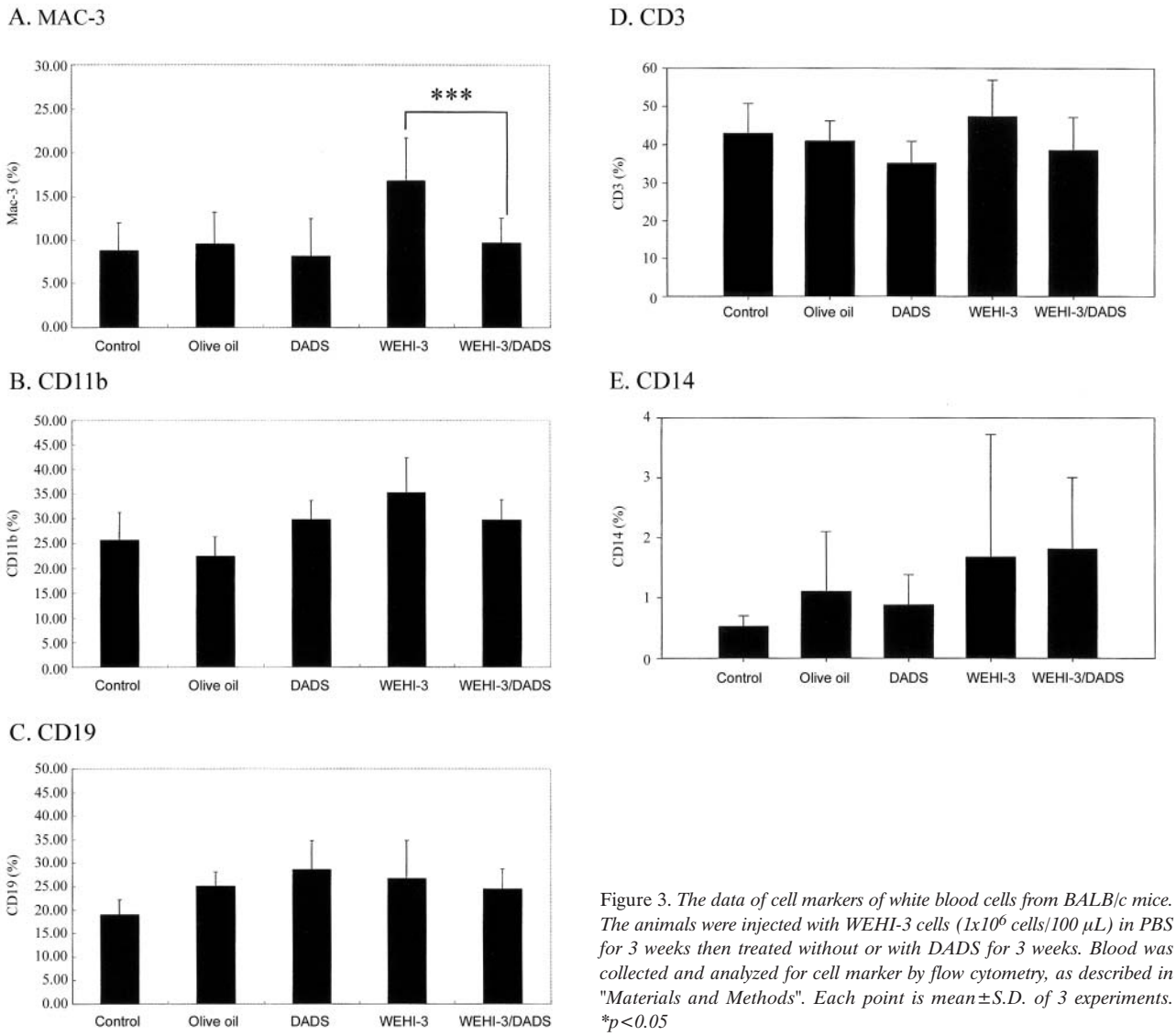


Figure 3. *The data of cell markers of white blood cells from BALB/c mice. The animals were injected with WEHI-3 cells (1x10^6 cells/100 μL) in PBS for 3 weeks then treated without or with DADS for 3 weeks. Blood was collected and analyzed for cell marker by flow cytometry, as described in "Materials and Methods". Each point is mean±S.D. of 3 experiments. *p<0.05*

and CD19, based on staining with the anti-Mac-3, CD11b, CD3, CD14 and CD19 antibodies (PharMingen). These cells were then stained with the second fluorescent antibody before being analyzed to determine the cell marker levels by flow cytometry (FACS CaliburTM, Becton Dickinson, NJ, USA), as described by Ormerod 2000 (11).

c) Tissues samples (liver and spleen). Each animal from each group was weighed before blood sampling. The liver and spleen samples were obtained, weighed individually and used for histopathology.

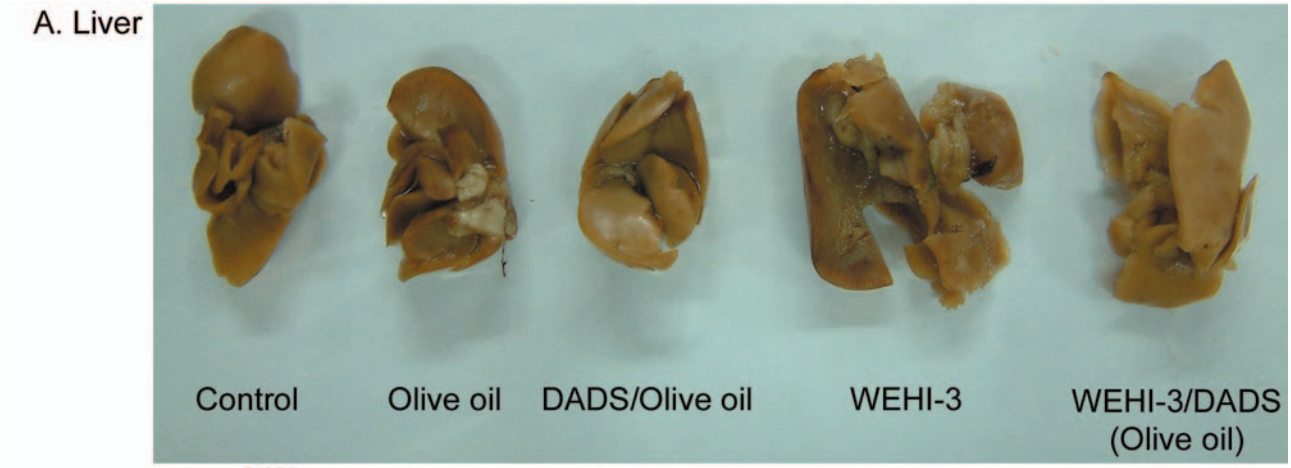
d) Histopathology. Tissue samples from the spleen and liver were fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 mm were stained with hematoxylin and eosin according to standard procedures.

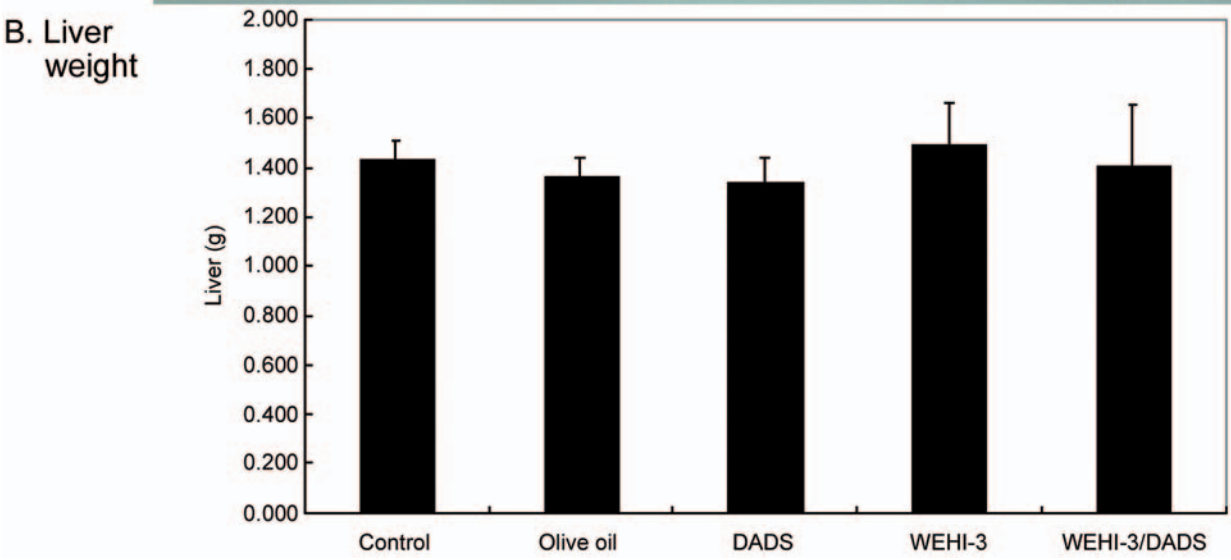
e) Statistics. The results were expressed as mean±SD and the difference between groups was analyzed by one-way ANOVA. $^*p<0.05$ was taken to be significant.

Results

DADS-induced cytotoxiciy in WEHI-3 cells. The percentage of viable cells was determined by Trypan blue exclusion and flow cytometry, and the results are presented in Figure 1. The data indicated that DADS decreased the percentage of viable cells in both examined cell lines and that these effects were dose-dependent.

WEHI-3-induced leukemia tumor in animals. Representative pictures of the animals after injection with WEHI-3 for 3 weeks, indicating the presence of leukemia tumors, are provided in Figure 2. Histopathologically, neoplastic cells mainly infiltrated the sinusoid and the portal tract of the





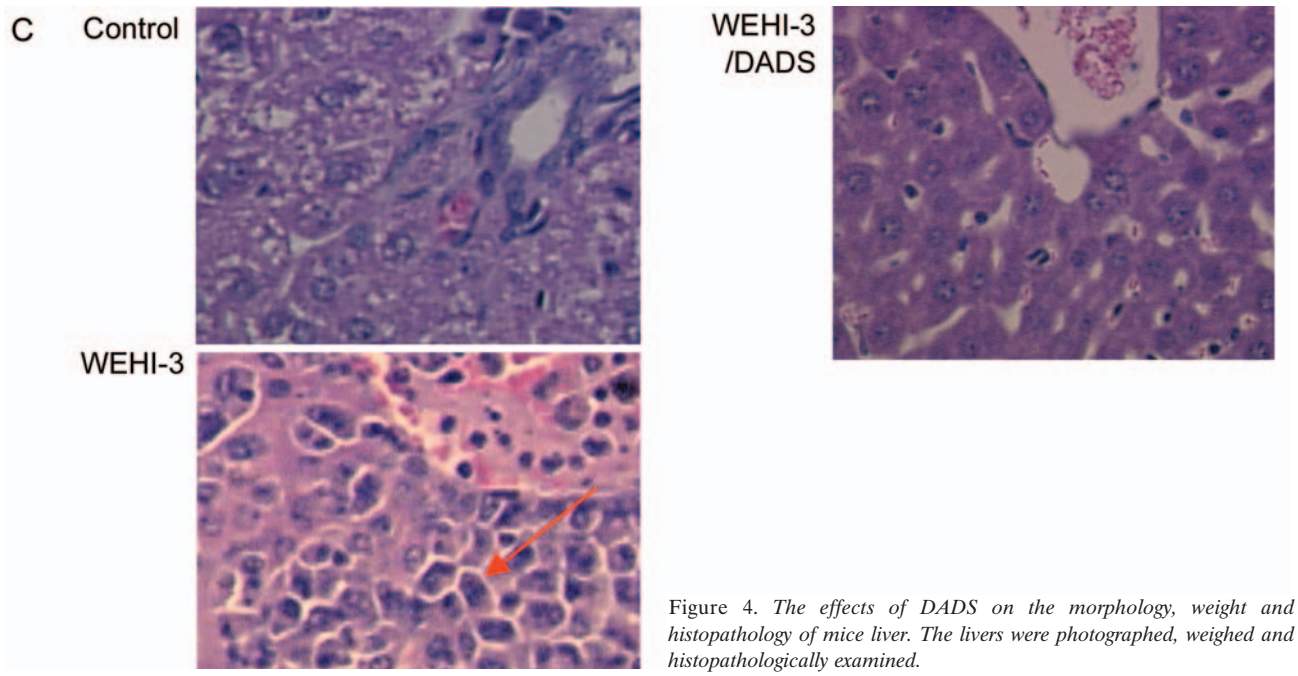
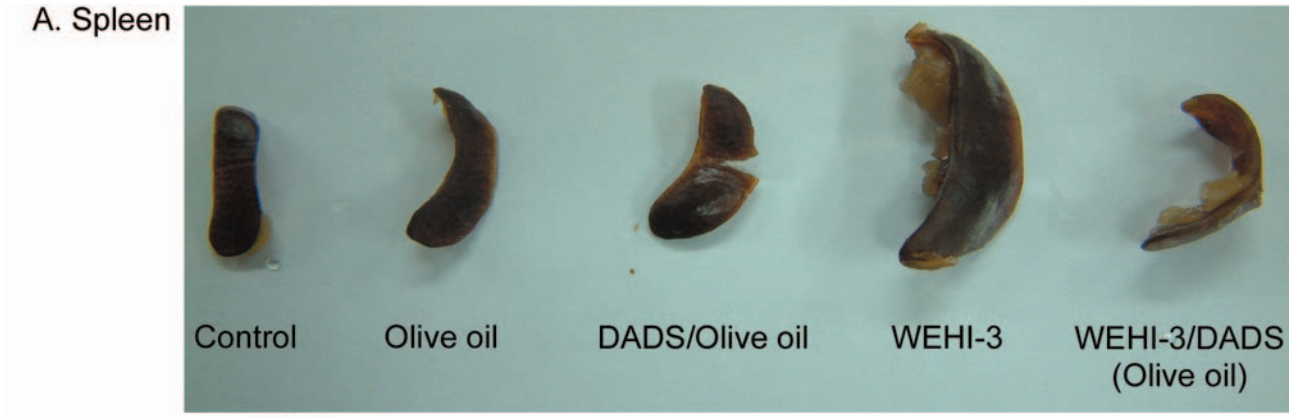
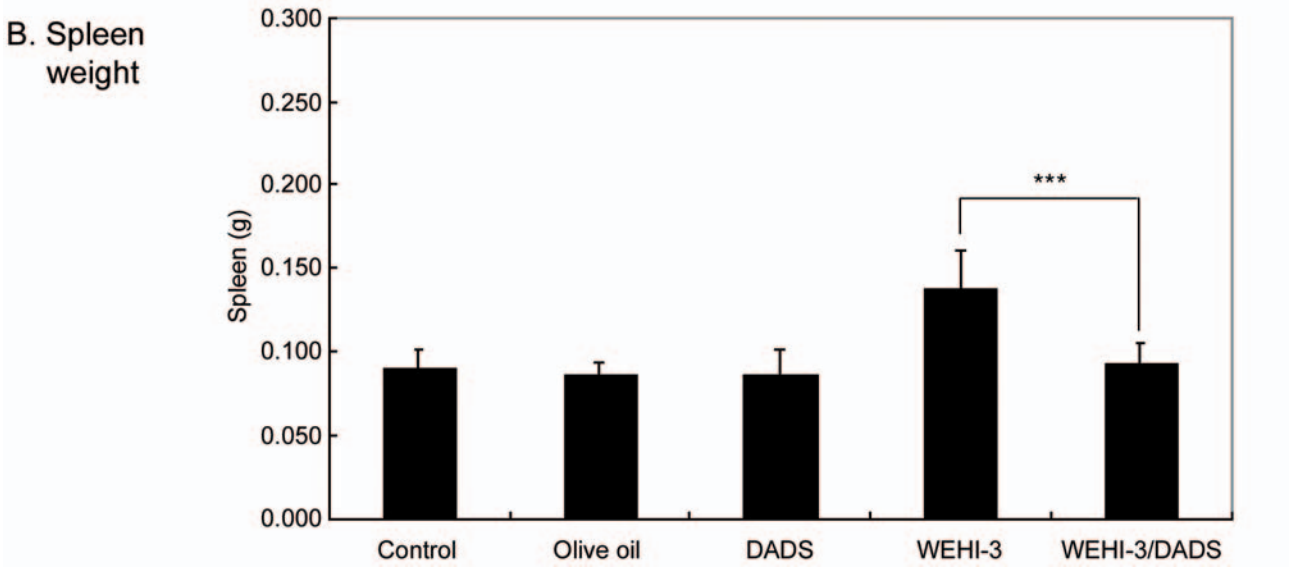


Figure 4. *The effects of DADS on the morphology, weight and histopathology of mice liver. The livers were photographed, weighed and histopathologically examined.*





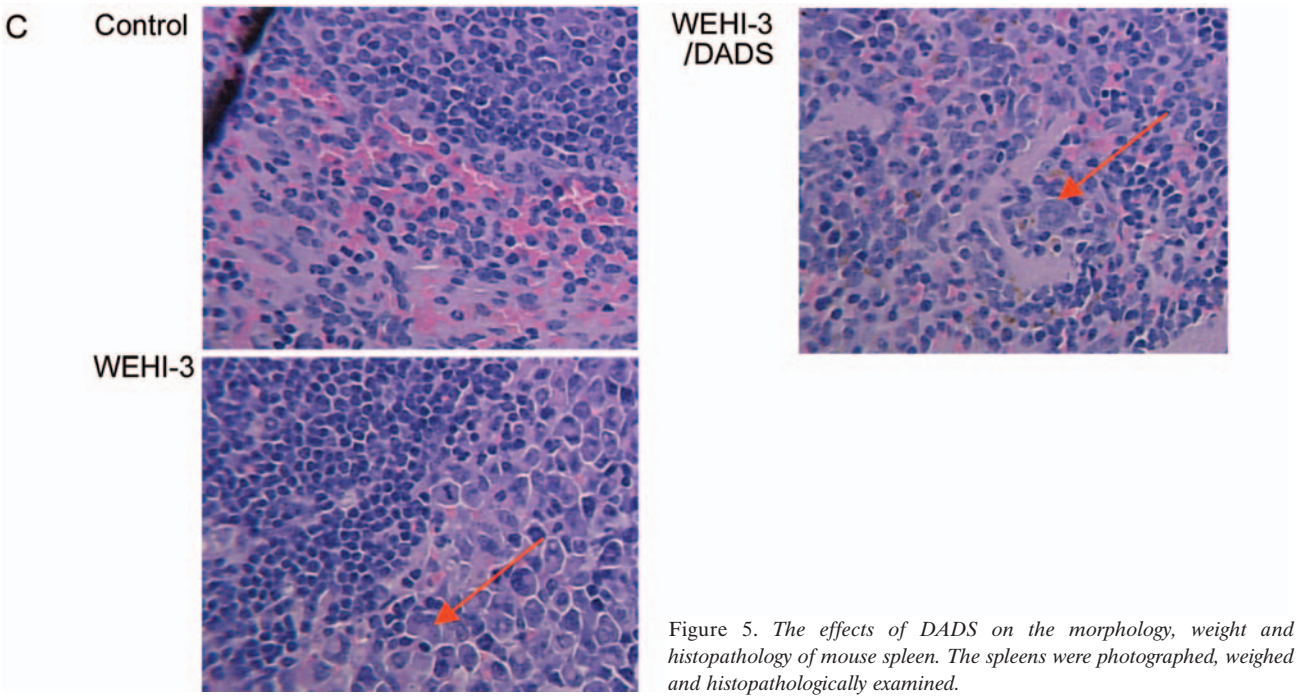


Figure 5. *The effects of DADS on the morphology, weight and histopathology of mouse spleen. The spleens were photographed, weighed and histopathologically examined.*

liver. Occasionally, neoplastic cells also destroyed the hepatic cord and tumor embolism was frequently seen. The spleens showed marked expansion of red pulp, while the white pulp showed relatively little change. The neoplastic cells contained large irregular nuclei with clumpped chromatin, prominent nucleoli and abundant clear and light eosinophilic cytoplasm. Frequent mitotic figures were also noted.

The effects of DADS on the whole blood cell surface markers after injection with WEHI-3 cells. The data for cell markers of white blood cells from BALB/c mice after treatment with or with DADS in olive oil are presented in Figure 3. It can be seen that DADS induced a significant difference of MAC-3 between WEHI-3-only treated groups ($p<0.05$).

The effects of DADS on the pharmacology, weight and histopathology of the liver. Liver tissues were isolated from animals and were photographed, weighed and histopathologically examined. Representative results are presented in Figure 4A, B and C. The results indicated that DADS affected the morphology, weight and cells of liver. The histopathological examination indicated that, after treatment with DADS, the liver demonstrated a pattern ranging from minimal histopathological change to scanty small neoplastic cell nests present in the sinusoid with apoptosis.

The effects of DADS on the pharmacology, weight and histopathology of the spleen. Spleen tissues were isolated from animals and were photographed, weighed and histopathologically examined. Representative results are presented in Figure 5A, B and C. The results indicated that DADS affected the morphology, weight and cells of the spleen. The histopathological examination indicated that, after treatment with DADS, the spleens either disclosed markedly decreased numbers of neoplastic cells or the cells were hard to find in the red pulp. Also, the number of megakaryocytes increased.

Discussion

Although DADS had been shown to inhibit the proliferation of HL-60 and cause apoptosis through caspase-3 activity (5), the data raise the possibility that DADS may have some chemopreventive value for human myeloid leukemia (5). In the present study, the cytotoxicity of DADS in WEHI-3 cells was examined, since there is no information addressing the *in vivo* effects of DADS. We focused on the fact that cell viability decreased after treatment with various concentrations of DADS in a dose-dependent manner. This is in agreement with the reports from other investigators, which showed that DADS decreased the percentage of viable cells in HL-60 *in vivo* (6).

The *in vivo* model, through mice injected *i.p.* with WEHI-3, is well established (12). Murine monomyelocytic WEHI-3 leukemia cells were originally derived from the BALB/c mouse (13) and have been demonstrated to be an ideal system for the study of potential therapeutic drugs such as ATRA, aclacinomycin A, IL-6, G-CSF and vitamin D_3 which could induced *in vitro* differentiation of WEHI-3 cells in monocytic and granulocytic lineages (14-18). We examined the effects of DADS on WEHI-3 tumor cells in BLAB/c mice *in vivo*. We first focused on DADS, in normal control animals and the results indicated that, after 4 months, examination of the sacrificed animals revealed no abnormalities. We subsequently injected WEHI-3 cells *i.p.*, followed by orogastric tube for DADS treatment before sacrificing the animals for examination. The results demonstrated that DADS statistically decreased the percentage of MAC-3 cells and T cells in the blood.

Although there are no reports addressing the effect of DADS on WEHI-3 cells *in vivo*, DADS has been reported to decrease the occurrence of colon cancer. Here, we showed that DADS inhibited spleen leukemia tumor growth in a WEHI-3B leukemia murine model. A notable characteristic of this model is the elevation of peripheral monocytes and granulocytes with immature morphology and apparently enlarged and infiltrated spleens, as compared with the normal counterpart (12). However, we observed that, in the DADS-treated groups, the size of the spleen decreased and there was a significant difference between the control and DADS-treated groups. Our data also indicated, however, that DADS decreased the percentage of viable cells. Thus, the influence of DADS on apoptosis induction, already the subject of many studies, as well as an understanding of its mechanism of action, should be further investigated in terms of signal pathway factors.

Acknowledgements

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