

Berberine Inhibits WEHI-3 Leukemia Cells *In Vivo*

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Abstract. Berberine, an isoquinoline alkaloid, has a wide range of pharmacological effects including anticancer activities, yet the exact effects on leukemia *in vivo* are unknown. Our previous studies have demonstrated that berberine induced cytotoxicity against murine leukemia WEHI-3 cells *in vitro* in a dose-dependent manner. In order to understand the berberine action against leukemia, the effect of berberine on WEHI-3 leukemia cells *in vivo* was studied. The results showed that Mac-3 and CD11b markers were reduced, indicating differentiation inhibition of the macrophages and granulocytes precursors. There was no affect on the CD14 marker but the CD19 marker that was indicating the promotion of the differentiation of the B-cells precursors. The weights of spleen samples from mice treated with berberine were found to be lower when compared to these from untreated animals.

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium), an alkaloid, has been used as an antibiotic against many bacterial species (1, 2). Berberine has recently been examined for anticancer activity, including against human cancer cells (3-6). It was also shown that berberine interacted with nucleic acids (7) especially DNA (8) *in vitro* and also induced apoptosis and cell cycle arrest in some cancer cells (6, 9). Berberine induced dose-dependent G2/M pause and apoptosis in Balb/c 3T3 cells (10). Our laboratory also demonstrated that berberine induced cell cycle

arrest and apoptosis in a human gastric cancer cell line (SNU-5) (11) and human leukemia HL-60 and mice leukemia WEHI-3 cells (12, 13). Our previous studies demonstrated that berberine inhibited N-acetyltransferase activity (14) and the distribution and metabolism of 2-aminofluorene in various tissues of Sprague-Dawley rats after oral treatment (15). However, the effects of berberine on leukemia *in vivo* are still unclear. Therefore, the purpose of the present study was to determine the effect of berberine on mice leukemia cells (WEHI3) *in vivo*.

Materials and Methods

Materials and reagents. Berberine and olive oil were obtained from Sigma (MO, USA). RPMI-1640, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Murine leukemia cell line (WEHI-3). WEHI-3 cells (murine myelomonocytic leukemia cell line) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin (10000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine and grown in 75-cm² tissue culture flasks and 37°C under a humidified 5% CO₂ atmosphere.

Male BALB/c mice. Male BALB/c mice (approximately 22-28 g) were obtained at the age of 8 weeks from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan). The animals were maintained at the Animal Center of China Medical University for 2 weeks under animal guidelines before the grouping and experiments.

Berberine treatment. Sixty BALB/c mice were split into 4 groups. Each group contained 15 animals. Group I was categorized as control, Group II treated with olive oil, Group III *i.v.* injected with 1x10⁶ cells/100 µL of WEHI-3 cells as a positive control, Group IV injected with WEHI-3 cells and treated with (200 mg/kg) berberine

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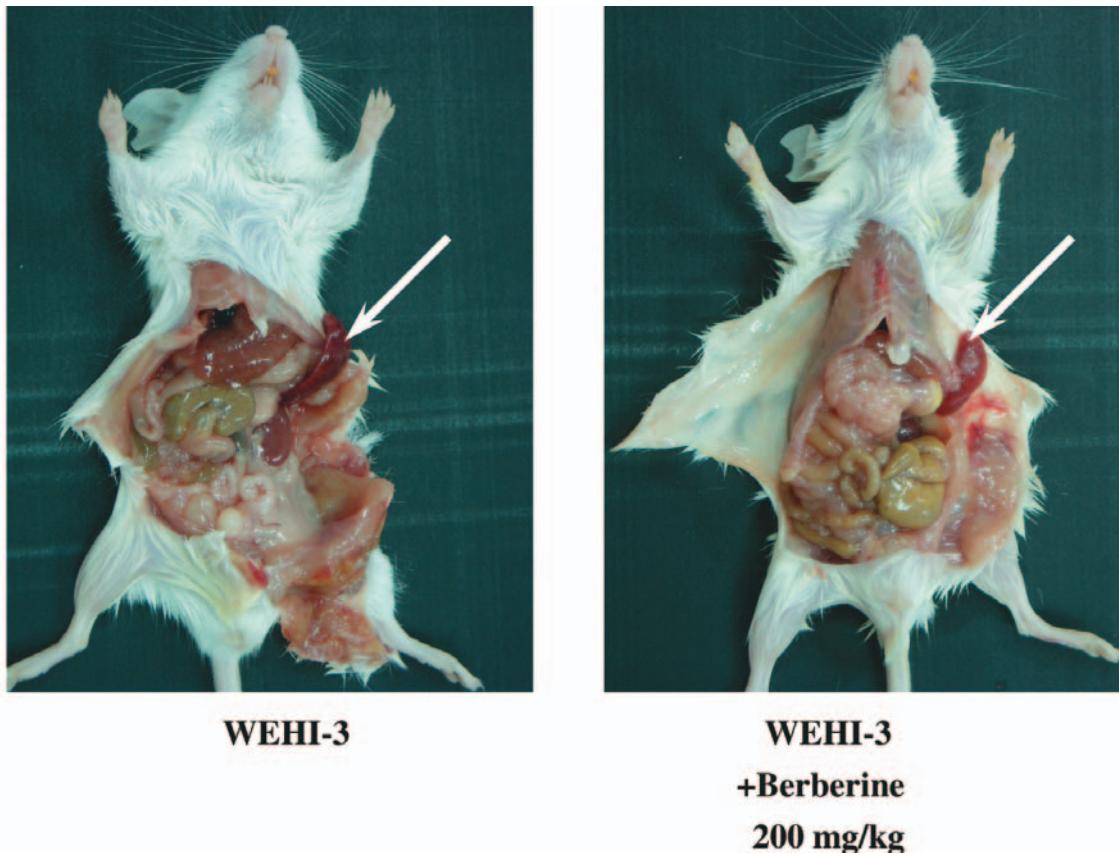


Figure 1. The enlargement of spleen in mice injected with WEHI-3 cells. The BALB/c mice injected with WEHI-3 cells were then treated with or without berberine for 3 weeks. The animals were sacrificed and the spleen is shown in panel A and B (arrow).

in olive oil. All animals were given the above doses orally and daily for 3 weeks before being weighed, sacrificed and photographed.

Blood samples and immunofluorescence staining. Blood was collected (about 1 mL) from each animal of each group at the end of the experiments. These samples were then treated with ammonium chloride to lyse the red blood cells and immediately centrifuged at 1500 rpm at 4°C for 15 minutes. The white blood cells were then isolated, counted and were stained with anti-Mac-3, CD11b, CD3, CD14 and CD19 antibodies (PharMingen, NJ, USA), followed by a second fluorescent antibody before being analyzed for cell markers using flow cytometric analysis. The cell marker levels were determined using flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA) as described elsewhere (16, 17).

Tissue samples (liver and spleen). Each individual animal was weighed before the blood specimens were sampled. The liver and spleen samples were individually obtained, weighed and used for histopathology (11, 17).

Histopathology. Tissue samples (spleen and liver) of each individual animal were fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 µm were stained with hematoxylin and eosin, following standard procedures as described elsewhere (16, 17).

Statistics. The results were expressed as mean±SD and the group differences were analyzed using one-way ANOVA. $P<0.01$ was considered as significant.

Results

Spleen size in mice injected with WEHI-3 cells. The location and size of spleen in mice after injection with WEHI-3 cells are shown in Figure 1A and that of berberine-treated mice after injection with WEHI-3 cells in Figure 1B. One location is indicated by an arrow to indicate that berberine decreased the size of spleen.

The effects of berberine on whole blood cell surface markers in mice injected with WEHI-3 cells. Flow cytometric analysis data indicating cell markers of white blood cells from BALB/c mice injected with WEHI-3 cells after treatment with or without berberine are shown in Figure 2. Berberine led to a significant reduction in the proportion of cells expression Mac-3 and CD11b in comparison to the WEHI-3-only treated groups ($p<0.01$) (Figure 2A and C). Berberine significantly increased

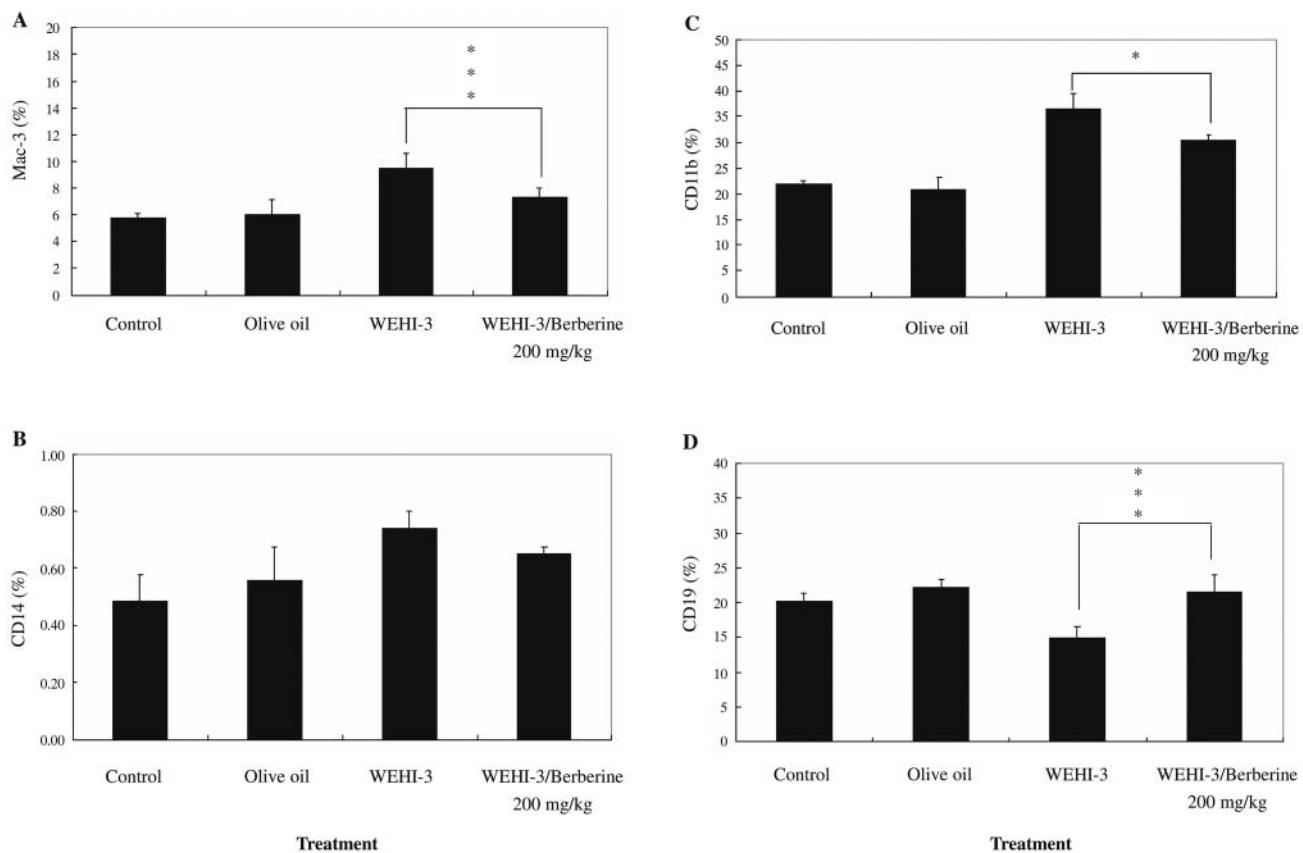


Figure 2. Cell marker levels of white blood cells from BALB/c mice injected with WEHI-3 cells after treatment with berberine. The animals were injected with WEHI-3 cells (1×10^6 cells/ $100 \mu\text{L}$) in PBS for 3 weeks and were then treated without or with berberine for 3 weeks. Blood was collected and analyzed for cell markers (A: Mac-3; B: CD14, C: CD11b; D: CD19) with flow cytometry as described in Materials and Methods. Each point is the mean \pm S.D. of three experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

the proportion of cells expression CD19 when compared to controls ($p < 0.05$) (Figure 2D). The data demonstrated that berberine promoted the levels of CD19 indicating promoted B-cell levels. However, berberine did not significantly affect CD14 levels compared to controls (Figure 2B).

The effects of berberine on the weight and histopathology of spleen. Spleen tissues were isolated from each animal group, photographed, weighed and histopathologically examined. Representative results can be seen in Figure 3. Berberine affected spleen morphology and weight reducing the size of the spleen. The results also show that spleen tissue either obviously exhibited reduced numbers of neoplastic cells or the cells were difficult to locate in the red pulp. Megakaryocyte numbers also increased with Berberine treatment, based on the data obtained from histopathological examination (data not shown).

The effects of berberine on the weight and histopathology of liver. Liver tissues were isolated from each animal group,

photographed, weighed and histopathologically examined. Representative results are shown in Figure 4. It can be seen that berberine affected liver morphology and weigh but not significantly. Furthermore, using histopathological examination, the liver demonstrated a pattern ranging from minimal histopathological change to scanty small neoplastic cell nests located in the sinusoid with apoptosis.

Discussion

The *in vivo* model of mice injected with *i.v.* with WEHI-3 cells used for anti-leukemia studies is well established (16-18). The important point for this model is that murine host systems are used for experimental tumor therapy and contain several available factors such as the low cost, the ease with which cancer production is established and the widely accepted experimental end-points (19, 20). An other point is that murine monomyelocytic WEHI-3 leukemia cells were originally derived from the BALB/c mouse (20) and other investigators' studies demonstrated that it serves

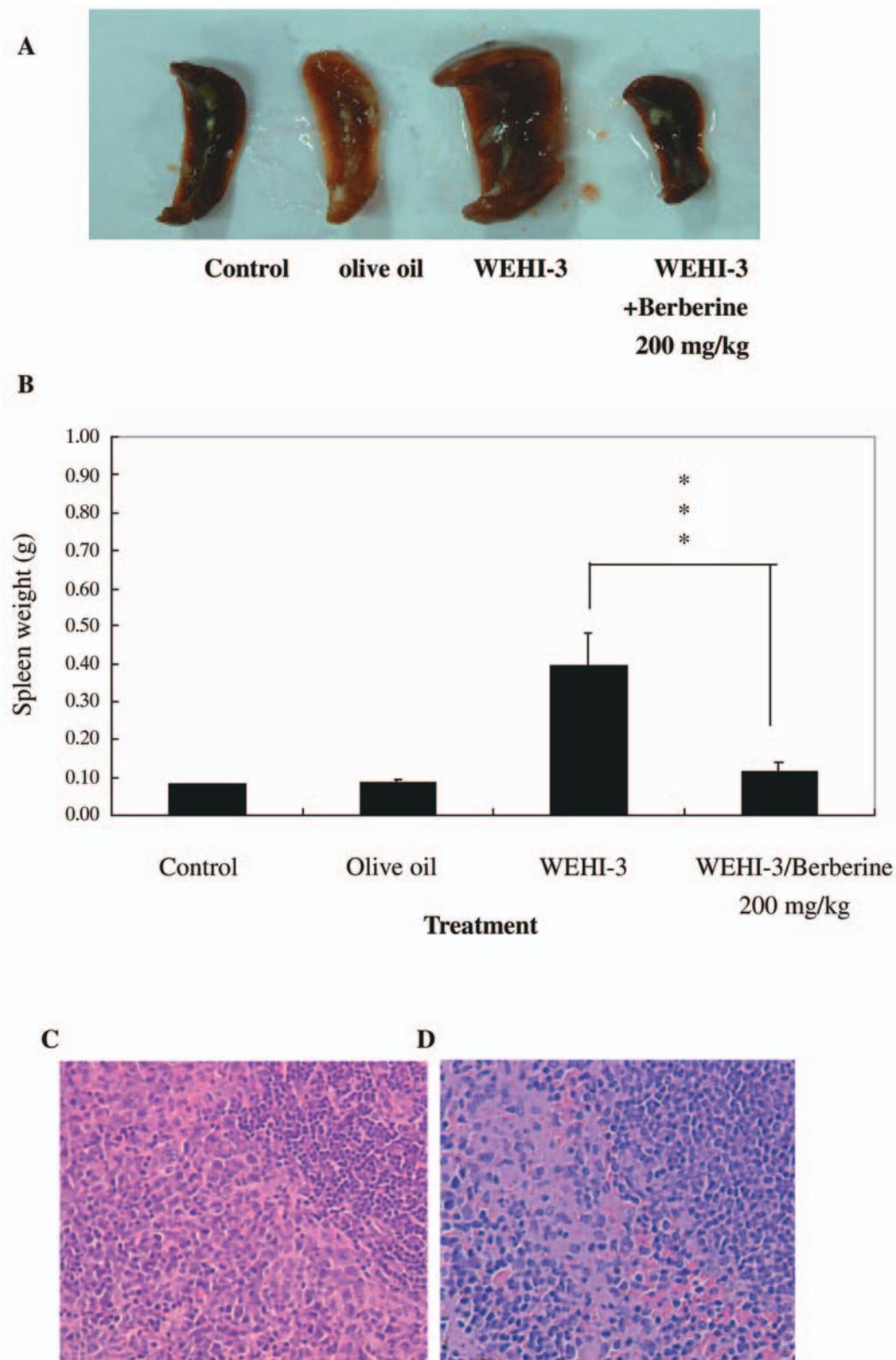


Figure 3. The effects of berberine on the morphology, weight and histopathology of the spleen from BALB/c mice injected with WEHI-3 cells after treatment with berberine. Spleens from each animal of each group were excised. (A) photographed, (B) weighed and (C) histopathologically examined as described in Materials and Methods. Each point is the mean \pm S.D. of three experiments. * $p<0.05$, ** $p<0.01$ *** $p<0.001$.

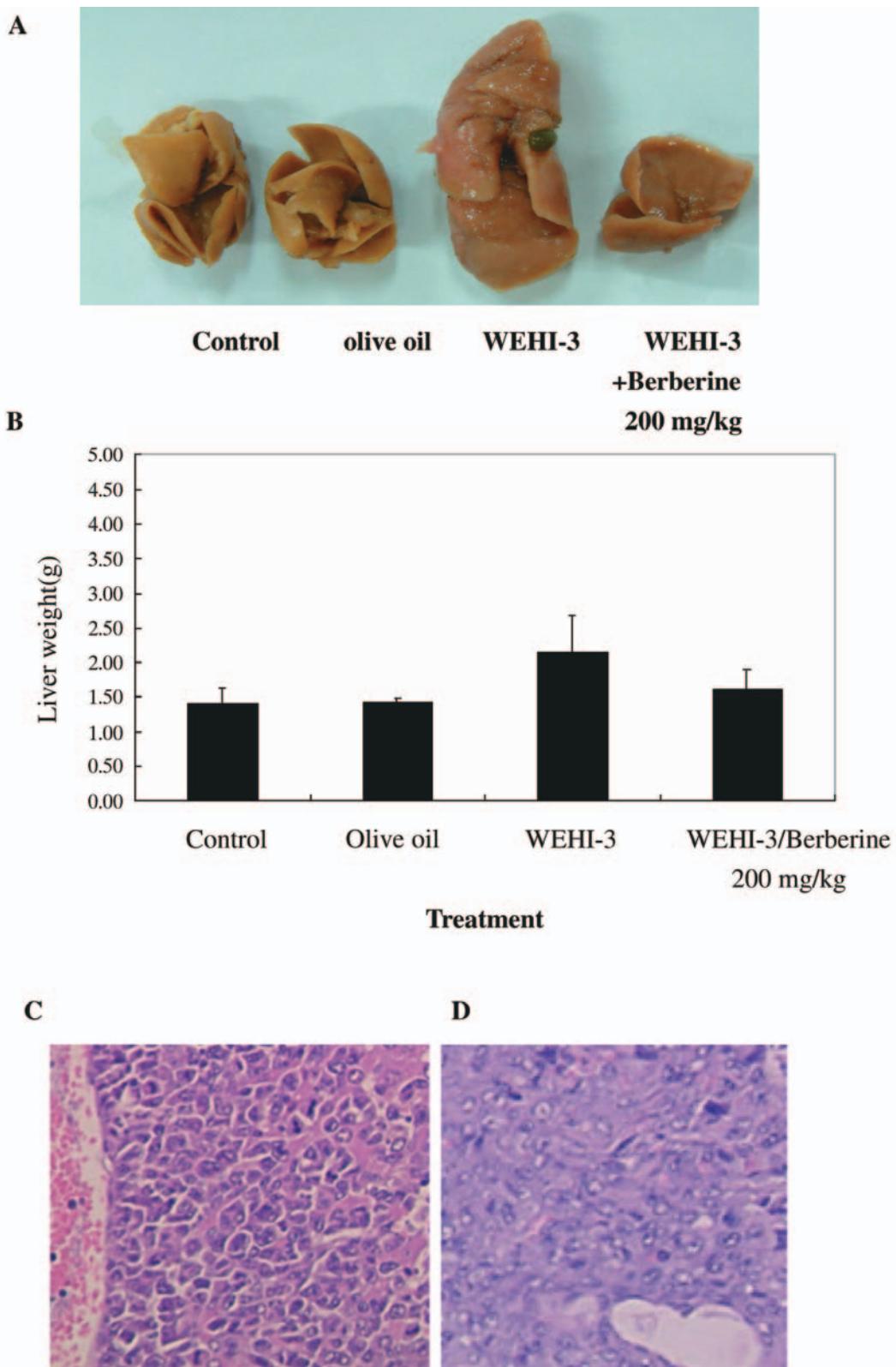


Figure 4. The effects of berberine on the morphology, weight and histopathology of the liver from BALB/c mice injected with WEHI-3 cells after treatment with berberine. Livers from each animal of each group were excised, (A) photographed, (B) weighed and (C) histopathologically examined as described in the Materials and Methods. Each point is the mean \pm S.D. of three experiments. * $p<0.05$, ** $p<0.01$ *** $p<0.001$.

as an ideal system for the study of potential therapeutic drugs such as ATRA, aclacinomycin A, IL-6, G-CSF and vitamin D3, which were able to induce *in vitro* differentiation of WEHI-3 in monocytic and granulocytic lineages (21-25). Our earlier studies also used this model to demonstrate that DADS affected leukemia cells *in vivo* (16).

Our previous studies demonstrated that berberine induced cell cycle arrest (12) and apoptosis (13) in both HL-60 and WEHI-3 cell lines, raising the possibility that berberine could affect leukemia cells *in vivo*. In the present study, we examined the effects of berberine *in vivo* in WEHI-3 tumor cells in BALB/c mice. The results demonstrate that berberine statistically significantly reduced the size and weight of spleen in these animals and also reduced the percentage of MAC-3 and CD11b cells in the blood. An interesting point is that our data showed berberine promoted the CD19 marker, indicating it may promote B-cell numbers but it did not affect the CD14 or CD3 markers (data not shown).

Our results demonstrated that berberine inhibits leukemia-related spleen growth. A notable characteristic of this model is the elevation of peripheral monocytes and granulocytes with immature morphology, as well as enlarged and infiltrated spleens compared to their normal counterparts.

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References

- Hahn FE and Ciak J: Antibiotics, Gottlieb D, Shaw PD and Cocoran JW (eds.). Springer-Verlag, New York., Vol. 3, pp. 577, 1975.
- Ghosh AK, Bhaktacharyya, F K and Ghosh DK: Amastigote inhibition and mode of action of berberine. *Exp Parasitol* 60: 404-413, 1985.
- Hano K: Pharmacological studies on metabolism of cancer tissues: pharmacological studies on carcinostatic effects of some plant components and their derivatives (1). *Gann* 48: 443-450, 1957.
- Hoshi A, Ikekawa T, Ikeda Y, Shirakawa S, Ligo M, Karetani K and Fukoka F: Antitumor activity of berberrubine derivatives. *Gann* 67: 321-325, 1976.
- Zhang RX: Laboratory studies of berberine used alone and in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea to treat malignant brain tumors. *Chinese Med J* 103: 658-665, 1990.
- Li KK, Motwani M, Tong W, Bornmann W and Schwartz GK: Huanglian, a Chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. *Mol Pharmacol* 58: 1287-1293, 2000.
- Yamagishi H: Interaction between nuclei acid and berberine sulfate. *J Cell Biol* 15: 589-592, 1962.
- Klimek M and Hnilica L: Ultraviolet and visible light absorption of berberine. *Arch Biochem Biophys* 81: 105-110, 1959.
- Kuo CL, Chou CC and Yung BYM: Berberine complexes with DNA in berberine-induced apoptosis in human leukemia HL-60 cells. *Cancer Lett* 93: 193-200, 1995.
- Yang IW, Chou CC and Yung BY: Dose-dependent effects of berberine on cell cycle pause and apoptosis in Balb/c 3T3 cells. *Naunyn Schmiedebergs Arch Pharmacol* 354: 102-108, 1996.
- Lin JP, Yang JS, Lee JH, Hsieh WT and Chung JG: Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line. *World J Gastroenterol* 12(1): 21-28, 2006.
- Lin CC, Lin SY, Chung JG, Lin JP, Chen GW and Kao ST: Down-regulation of cyclin B1 and up-regulation of Wee1 by berberine promotes entry of leukemia cells into the G2/M-phase of the cell cycle. *Anticancer Res* 26(24): 1097-1104, 2006.
- Lin CC, Kao ST, Chen GW, Ho HC and Chung JG: Apoptosis of human leukemia HL-60 cells and murine leukemia WEHI-3 cells induced by berberine through the activation of caspase-3. *Anticancer Res* 26(1A): 227-242, 2006.
- Lin CC, Kao ST, Chen GW and Chung JG: Berberine decreased N-acetylation of 2-aminofluorene through inhibition of N-acetyltransferase gene expression in human leukemia HL-60 cells. *Anticancer Res* 25(6B): 4149-4155, 2005.
- Lin CC, Kao ST, Chen GW and Chung JG: Effects of oral administration of berberine on distribution and metabolism of 2-aminofluorene in Sprague-Dawley rats. *In Vivo* 21: 321-328, 2007.
- Yang JS, Kok LF, Lin YH, Kuo TC, Yang JL, Lin CC, Chen GW, Huang WW, Ho HC and Chung JG: Diallyl disulfide inhibits WEHI-3 leukemia cells *in vivo*. *Anticancer Res* 26(1A): 219-225, 2006.
- Lu HF, Liu JY, Hsueh SC, Yang YY, Yang JS, Tan TW, Kok LF, Lu CC, Lan SH, Wu SY, Liao SS, Ip SW and Chung JG: Menthol inhibits WEHI-3 leukemia cells *in vitro* and *in vivo*. *In Vivo* 21: 285-290, 2007.
- He Q and Na XD: The effects and mechanisms of a novel 2-aminosteroid on murine WEHI-3B leukemia cells *in vitro* and *in vivo*. *Leukemia Res* 25: 455-461, 2001.
- Taghian AG and Suitt HD: Animal systems for translational research in radiation oncology. *Acta Oncol* 38: 829-838, 1999.
- Zips A, Thames HD and Baumann M: New anticancer agents: *in vitro* and *in vivo* evaluation. *In Vivo* 19: 1-8, 2005.
- Gamba-Vitalo C, Blair OC, Keys SR and Sartorelli AC: Differentiation of WEHI-3B D+ monomyelocytic leukemia cells by retinoic acid aclacinomycin A. *Cancer Res* 46: 1189-1194, 1986.
- Burgess AW and Metcalf D: Characterization of a serum factor stimulating the differentiation of myelomonocytic leukemia cells. *Intl J Cancer* 26: 647-654, 1980.
- Metcalf D: Actions and interactions of G-CSF, LIF, and IL-6 on normal and leukemic murine cells. *Leukemia* 3: 349-354, 1989.
- Li JM and Sartorelli AC: Synergistic induction of the differentiation of WEHI-3B D+ myelomonocytic leukemia cells by retinoic acid and granulocyte colony-stimulating factor. *Leukemia Res* 16: 571-576, 1992.
- Li JM, Finch RA and Sartorelli AC: Role of vitamin D3 receptor in the synergistic differentiation of WEHI-3B leukemia cells by vitamin D3 and retinoic acid. *Exp Cell Res* 249: 279-290, 1999.

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