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The quinolone derivative CHM-1 inhibits murine WEHI-3 leukemia in BALB/c mice in vivo

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The quinolone derivative CHM-1 inhibits murine WEHI-3 leukemia in BALB/c mice *in vivo*

TUNG-YUAN LAI[1,2], JAI-SING YANG[3], PING-PING WU[4,5], WEN-WEN HUANG[6], SHENG-CHU KUO[5], CHIA-YU MA[7], W. GIBSON WOOD[8] AND JING-GUNG CHUNG[6,9]

[1]School of Post-baccalaureate Chinese Medicine, [3]Department of Pharmacology, [4]School of Pharmacy, [5]Graduate Institute of Pharmaceutical Chemistry, [6]Department of Biological Science and Technology, China Medical University, Taichung 404, [2]Department of Chinese Internal Medicine, China Medical University Hospital, Taichung 404, [7]Department of Food and Beverage Management, Technology and Science Institute of Northern Taiwan, Taipei 112, [9]Department of Biotechnology, Asia University, Taichung 413, Taiwan, [8]Department of Pharmacology, University of Minnesota, School of Medicine and Geriatric Research, Education and Clinical Center, VA Medical Center, Minneapolis, MN, USA

Running title: CHM-1 inhibits WEHI-3 cells leukemia *in vivo*

Correspondence to: Dr. Jing-Gung Chung, Department of Biological Science and Technology, China Medical University. No 91, Hsueh-Shih Road, Taichung 404, Taiwan. Tel: +886-4-2205-3366 ext 2161 Fax: +886-4-2205-3764, jgchung@mail.cmu.edu.tw

Abstract

CHM-1 (2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one) is a quinolone derivative and which has been reported to induce apoptosis and inhibit invasion of cancer cells. However, there is no available information to address effects of CHM-1 on leukemia cells *in vivo*. Therefore, the present study examined effects of CHM-1 using a mouse model of leukemia. We established leukemia in mice by injecting WEHI-3 cells into BALB/c mice. Mice were then treated with or without CHM-1 (5 and 10 mg/kg). CHM-1 promoted the total survival rate of leukemia mice and these effects were dose-dependently. CHM-1 increased body weight and decreased spleen weight, but it did not affect liver weight. The levels of cell markers Mac-3 and CD11b were reduced by CHM-1, indicating that the differentiation of the precursor of macrophage cells was inhibited. Levels of CD3 and CD19 were induced by CHM-1, suggesting that the differentiation of precursors of T and B cells were promoted in PBMC. Results of the present study indicate that CHM-1 has an inhibitory effect on leukemia induced in mice *in vivo* and warrants further study as to mechanisms and effects in other types of cancer.

Keywords: CHM-1, leukemia WEHI-3 cells, leukemia murine model, *in vivo*

Introduction

Leukemia is one of the main causes of death in humans worldwide. In the U.S. approximately 3.7 individuals per 100,000 die each year from leukemia [1]. In Taiwan, about 2.1 individuals per 100,000 thousand die per year of leukemia based on reports of the Department of Health, Executive Yuan, R.O.C. (Taiwan). Currently, strategies for treatment of leukemia include radiotherapy, chemotherapy, or a combination of both but have proven unsatisfactory. Based on epidemiological and animal studies [2], increased consumption of a plant-based diet reduces the risk of cancer development [3]. Herbal based dietary supplements contain numerous phytochemicals which can be used as cancer suppressors with Taxol being a prime example.

Quinolone derivatives have been shown to be useful as antibacterial agents through the inhibition of bacterial DNA gyrase [4] and they also inhibit platelet aggregation [5, 6]. The 2-phenyl-4-quinolones induce cytotoxicity in several human cancer cell lines. Several reports also demonstrated that synthesized 2-phenyl-4-quinolone series compounds can inhibit tubulin polymerization through binding to tubulin at the colchicine-binding site and they can act as anti-mitotic agents [6]. It was reported that the synthesized 2-phenylpyrroloquinolin-4-ones inhibited hepatocellular carcinoma growth both i*n vitro* and *in vivo* [7]. Wang et al.

was first to report that 2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one (CHM-1) (Figure 1) can act as an anti-invasive agent in hepatocellular carcinoma cells [8]. It is well-documented that murine WEHI-3 leukemia cells can be used as experimental tumor therapy [9, 10] and murine WEHI-3 leukemia cells can be used for inducing leukemia in syngenic BALB/c mice for evaluating anti-leukemia effects of drugs. However, there are no reports on the effects of CHM-1 on leukemia cells *in vivo*. We investigated the effects of CHM-1 on WEHI-3 leukemia cells in a mouse model. Also, CHM-1 promoted the survival rate of WEHI-3 cells leukemia *in vivo*.

Materials and methods

Materials and reagents

CHM-1 was generously provided by Dr. Sheng-Chu Kuo (Graduate Institute of Pharmaceutical Chemistry, College of Pharmacy, China Medical University). Dimethyl sulfoxide (DMSO), potassium phosphates, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640, fetal bovine serum, penicillin-streptomycin and L-glutamine were obtained from Invitrogen/Gibco BRL (Grand Island, NY, USA).

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

Murine WEHI-3 leukemia cells-The WEHI-3 cell line (murine myelomonocytic leukemia cells) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). WEHI-3 cells were cultured into 75-cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and grown at 37°C under a humidified 5% CO_2 [9, 10, 11].

CHM-1 treatment-The experimental design is shown in Figure 2A. Forty BALB/c mice were divided into 4 groups: Group I was control; Group II was injected with WEHI-3 cells only and treated with PBS (vehicle); Group III and Group IV were injected with WEHI-3 cells and then treated with CHM-1 (5 and 10 mg/kg) in PBS. Injections WEHI-3 cells ($1x10^6$ in PBS) were *i.v.* All animals were administered CHM-1 orally each day for up to 3 weeks before being weighed [9, 10, 11].

Blood samples and immunofluorescence staining-After the end of the CHM-1

treatment, surviving animals of each group was counted and body weights determined. Blood (1 ml) was collected from each animal and ammonium chloride was added for lysing of the red blood cells, followed by centrifugation for 15 min at 1500 rpm at 4°C. The isolated white blood cells were examined for cell markers, including Mac-3, CD11b, CD3, and CD19 based on the staining with anti-Mac-3-PE, CD11b-FITC, CD3-FITC, and CD19-PE antibodies (BD PharMingen, San Diego, CA, USA). Cell marker levels were determined by flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA) as described [9, 10, 11].

Liver and spleen tissue -These samples were obtained from each animal and used for histopathology. Tissue samples from spleen were fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 mm were stained with hematoxylin and eosin according to standard procedures [9].

Statistics analysis-The results were expressed as mean ± SD and differences between control and experimental groups analyzed by Student's-*t* test. *$p<0.05$ was taken as significant.

Results

CHM-1 affected the survival rate of WEHI-3 cells leukemia mice

Leukemia mice were treated with PBS only, or 5 and 10 mg/kg of CHM-1 and the representative survival rates are shown in Figure 2B. Animals were treated with CHM-1 for 21 days and the survival rates were 25, 40, 50 and 100% in each group of without CHM-1 treatment, 5 and 10 mg/kg of CHM-1 treatment and normal untreated mice, respectively.

CHM-1 affected the WEHI-3-induced leukemia mice body, spleen and liver weights in vivo

Mice were treated with or without CHM-1 (5 and 10 mg/kg) and representative animal body weights, spleen and liver weights are shown in Figure 3A, B and C. Both doses of CHM-1 increased body weight significantly as compared to the PBS-treated group (Fig. 3A). CHM-1 treatment at both concentrations also decreased significantly the spleen weight compared to the PBS group (Fig. 3B), but it did not affect liver weights (data not shown).

CHM-1 affected spleen histopathology

Spleen tissue was isolated from BALB/c mice of each group after injection with WEHI-3 cells and treated with or without CHM-1 for 3 weeks. Representative

histopathology is presented in Figure 4. CHM-1 treatment, either markedly decreased the number of spleen neoplastic cells or the cells were not detectable in the red pulp. Also, the number of megakaryocytes increased. These results indicated that CHM-1 decreased the number of leukemia cells in the spleen.

CHM-1 altered surface markers of whole blood cells from WEHI-3 cell leukemia mice

Cell marker data of white blood cells from BALB/c mice after injection with WEHI-3 cells for 1 week then treated with or without CHM-1 (5 and 10 mg/body weight) in PBS are presented in Figure 4A, B, C and D. CHM-1 decreased levels of Mac-3 (Fig. 4A) and CD11b (Fig. 4B), suggesting that the differentiation of the precursor of macrophage cells was inhibited. However, CHM-1 increased the levels of CD3 (Fig. 3C) and CD19 (Fig. 4D) as compared to the WEHI-3 cells only treated groups ($p<0.05$) which indicates that the differentiation of the precursor of T and B cells was enhanced.

Discussion

Several reports have shown that CHM-1 affects many types of human cancer cell lines; however, the effects of CHM-1 in an *in vivo* animal system have not been

reported. We are the first to show that CHM-1 can promote the survival rate of WEHI-3 cell leukemia mice *in vivo* and it also increased the body weight, decreased the spleen weight but did not significantly affect liver weight. CHM-1 decreased the cell marker Mac-3 and CD11b but increased the cell markers CD3 and CD19. Generation of a leukemia mouse model by *i.v.* injection with WEHI-3 cells is well established [12]. The WEHI-3 cell line was murine monomyelocytic leukemia cells which were originally derived from the BALB/c mouse [13]. Furthermore, it has been demonstrated that the WEHI-3 leukemia model is an ideal system for the study of potential therapeutic drugs such as ATRA, aclacinomycin A, IL-6, G-CSF and vitamin D3 induced *in vitro* differentiation of WEHI-3 in monocytic and granulocytic lineages [14]. Our previous studies also used this leukemia animal model which showed that diallyl sulfides (DAS) [13], quercetin [9] and benzyl isothiocyanate (BITC) [11] could inhibit WEHI-3 cells *in vivo*.

CHM-1 statistically decreased the percentage of Mac-3 and CD11b and promoted the percentage of CD3 and CD19 in blood. Due to the WEHI-3 cells being murine monomyelocytic leukemia cells and originally derived from the BALB/c mouse. Therefore, the CD marker population in blood is reasonable and it showed that the number of monocyte and macrophage (Mac-3 and CD11b) are elevated in examined mice. Our observations from WEHI-3 cells injected mice indicated that

the major characteristic of leukemia animal model are the elevation of peripheral monocytes and granulocytes with immature morphology in blood samples, an enlargement of spleen tissues with infiltrated spleens as compared with normal tissue and those data are in agreement with other reports [12].

In conclusion, we showed that CHM-1 *in vivo* decreased the percentage of viable WEHI-3 cells in a mouse model of leukemia. CHM-1 may have potential as a therapeutic agent for leukemia cells.

Acknowledgements

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References

1. Jensen CD, Block G, Buffler P, Ma X, Selvin S, Month S. Maternal dietary risk factors in childhood acute lymphoblastic leukemia (United States). Cancer Causes Control. 2004; 15: 559-570.
2. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, et al. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. Carcinogenesis. 2000; 21: 921-927.
3. Mutoh M, Takahashi M, Fukuda K, Komatsu H, Enya T, Matsushima-Hibiya Y, et al. Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: structure-activity relationship. Jpn J Cancer Res. 2000; 91: 686-691; Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res. 2000; 60: 3823-3831.

4. Hooper DC. Mode of action of fluoroquinolones. Drugs. 1999; 58 Suppl 2: 6-10.
5. Kuo SC, Lee HZ, Juang JP, Lin YT, Wu TS, Chang JJ, et al. Synthesis and cytotoxicity of 1,6,7,8-substituted 2-(4'-substituted phenyl)-4-quinolones and related compounds: identification as antimitotic agents interacting with tubulin. J Med Chem. 1993; 36: 1146-1156.
6. Xia Y, Yang ZY, Xia P, Bastow KF, Tachibana Y, Kuo SC, et al. Antitumor agents. 181. Synthesis and biological evaluation of 6,7,2',3',4'-substituted-1,2,3,4-tetrahydro-2-phenyl-4-quinolones as a new class of antimitotic antitumor agents. J Med Chem. 1998; 41: 1155-1162.
7. Ferlin MG, Chiarelotto G, Gasparotto V, Dalla Via L, Pezzi V, Barzon L, et al. Synthesis and in vitro and in vivo antitumor activity of 2-phenylpyrroloquinolin-4-ones. J Med Chem. 2005; 48: 3417-3427.
8. Wang SW, Pan SL, Peng CY, Huang DY, Tsai AC, Chang YL, et al. CHM-1 inhibits hepatocyte growth factor-induced invasion of SK-Hep-1 human hepatocellular carcinoma cells by suppressing matrix metalloproteinase-9 expression. Cancer Lett. 2007; 257: 87-96.
9. Yu CS, Lai KC, Yang JS, Chiang JH, Lu CC, Wu CL, et al. Quercetin inhibited murine leukemia WEHI-3 cells in vivo and promoted immune response. Phytother Res. 2009.
10. Yang JS, Kok LF, Lin YH, Kuo TC, Yang JL, Lin CC, et al. Diallyl disulfide inhibits WEHI-3 leukemia cells in vivo. Anticancer research. 2006; 26: 219-225.
11. Tsou MF, Peng CT, Shih MC, Yang JS, Lu CC, Chiang JH, et al. Benzyl isothiocyanate inhibits murine WEHI-3 leukemia cells in vitro and promotes phagocytosis in BALB/c mice in vivo. Leukemia research. 2009; 33: 1505-1511.
12. Warner NL, Moore MA, Metcalf D. A transplantable myelomonocytic leukemia in BALB-c mice: cytology, karyotype, and muramidase content. Journal of the National Cancer Institute. 1969; 43: 963-982.
13. Astashkin EI, Til'kunova NA, Zalepugin DY, Grachev SV. Diallyl sulfide depletes the inositol trisphosphate-sensitive intracellular Ca2+ stores and activates SOC-channels in HL-60 human cells. Dokl Biol Sci. 2004; 399: 500-502.
14. He Q, Na X. The effects and mechanisms of a novel 2-aminosteroid on murine WEHI-3B leukemia cells in vitro and in vivo. Leukemia research. 2001; 25: 455-461.

Figure Legends

Figure 1 The structure of 2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one (CHM-1).

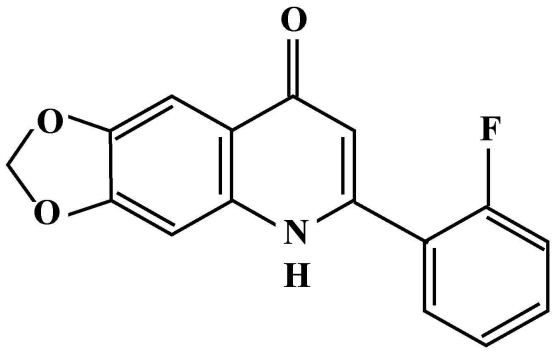
Figure 2 CHM-1 affected the survival rate in WEHI-3 cells leukemia mice. The experimental design of CHM-1 affect WEHI-3 cells leukemia animal model (A). The animal will be *i.v.* injected with WEHI-3 cells ($1x10^6$ cells/100 µL) in PBS then treated with or without CHM-1 by i.p. (5 and 10 mg/kg body weight) for 3 weeks, before whole survival rate was counted as described in Materials and Methods. "*P<0.05 is a significant difference between control (WEHI-3 cells injected only) and experimental (WEHI-3 cells injected then were treated with CHM-1 at 5 or 10 mg/kg, i.p) groups.

Figure 3 CHM-1 affected the weights of spleen and liver tissues and body weight. BLAB/c mice after injection with WEHI-3 cells ($1x10^6$ cells/100 µL) in PBS and treated with or without CHM-1 (5 and 10 mg/kg body weight) for 3 weeks. Body weight (A) and spleens (B) were weighed individually. *P<0.05 is a significant difference between control (WEHI-3 cells injected only) and experimental (WEHI-3 cells injected then were treated with CHM-1 at 5 or 10 mg/kg, i.p) groups

Figure 4 CHM-1 affected the histopathology of mice spleen. BALB/c mice were injected with WEHI-3 cells ($1x10^6$ cells/100 μL) in PBS for 3 weeks then treated with or without CHM-1 (5 and 10 mg/kg body weight) for 3 weeks. Spleens were photographed, weighed and histopathologically examined.

Figure 5 CHM-1 affected the cell markers of white blood cells from WEHI-3 leukemia BALB/c mice. BALB/c mice were injected with WEHI-3 cells ($1x10^6$ cells/100 μl) in PBS for 1 week then treated without or with CHM-1 (5 and 10 mg/kg body weight) for 3 weeks. Blood was collected and analyzed for cell marker by flow cytometry as described in Materials and Methods. A: Mac-3; B: CD11b; C: CD3; D: CD19. Each point is mean ± S.D. of three experiments. *P<0.05 is a significant difference between control (WEHI-3 cells injected only) and experimental (WEHI-3 cells injected then were treated with CHM-1 at 5 or 10 mg/kg, i.p) groups.

Figure 1



2-(2-fluorophenyl)-6,7-methylenedioxyquinolin-4-one (CHM-1)

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Figure 2

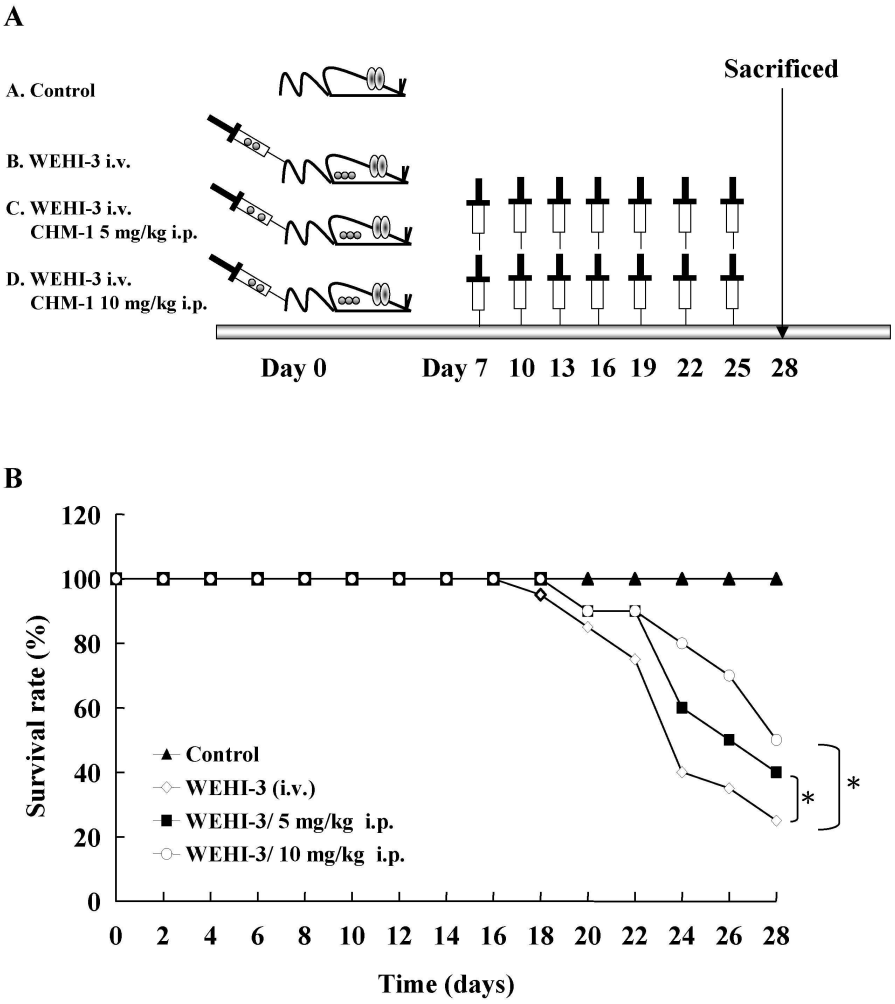

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Figure 3

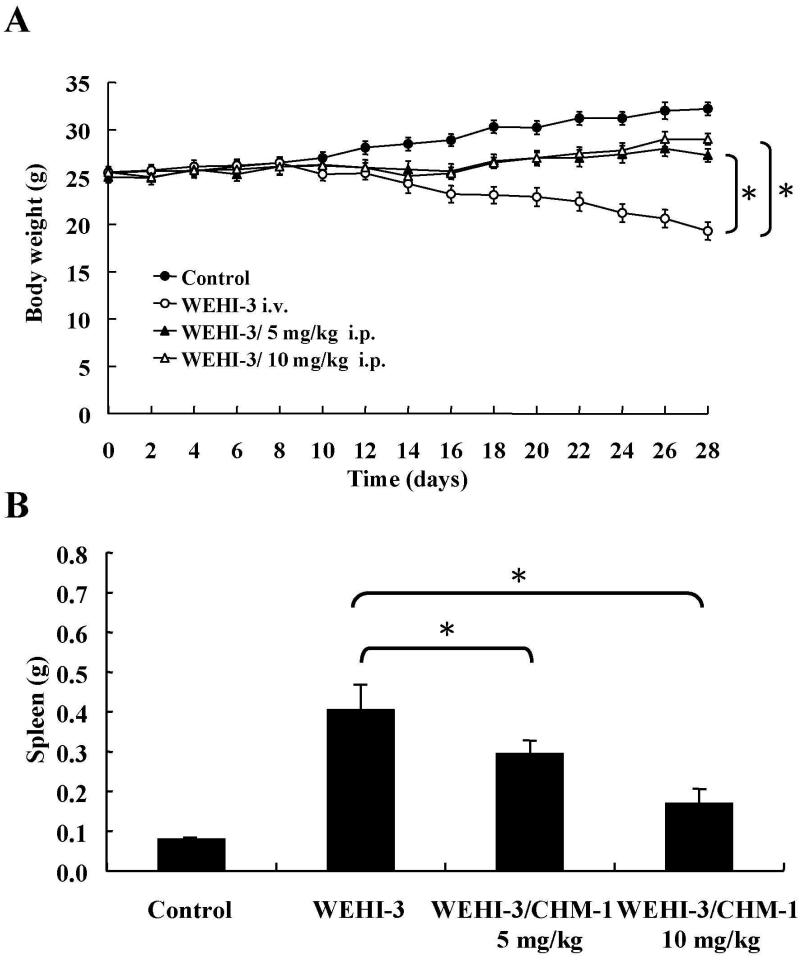

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Figure 4

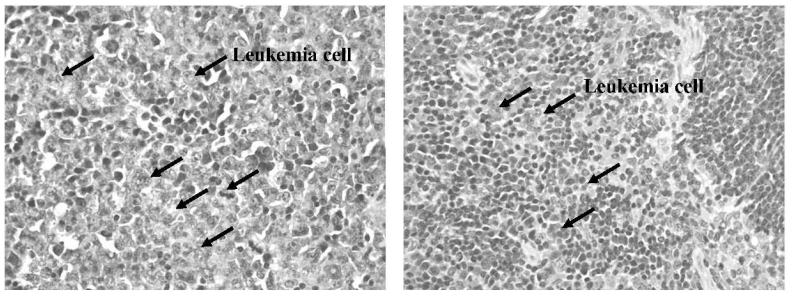

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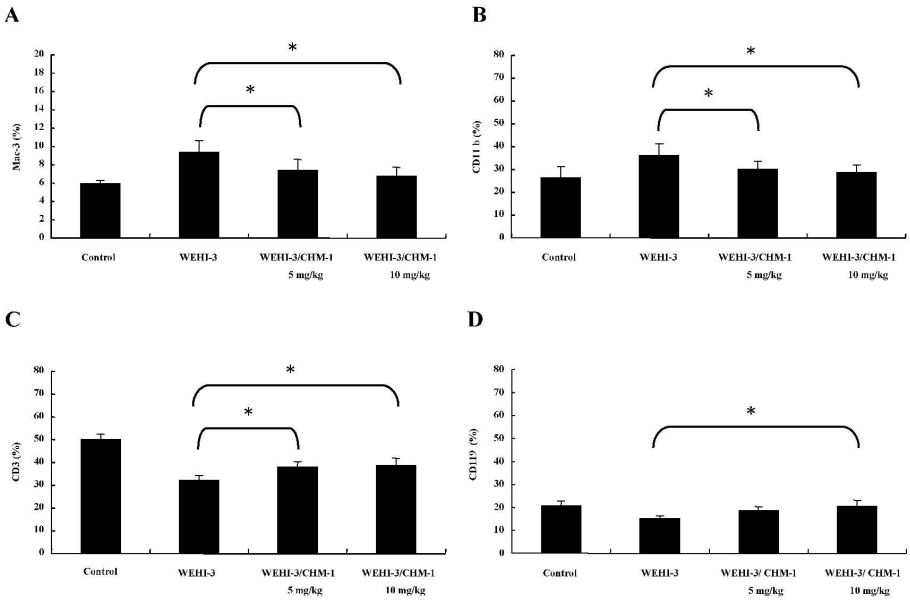
Figure 5



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