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

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Abstract. *Berberine has a wide range of biological actions that suggest it may be of use in cancer prevention. It was previously reported that berberine induced cell cycle arrest, not only at the G0/G1-phase, but also at the G2/M-phase in a dose-dependent manner. However, the mechanism of berberine-induced G2/M-phase arrest in leukemia cells is not fully understood. In the present study, the effects of the naturally occurring berberine (the major constituent of Coptis chinensis) on the cell cycle, as well as on CDK1, cyclin B1, 14-3-3σ, Wee1 and Cdc25c expressions, were investigated in the human promyelocytic leukemia HL-60 cells and in the murine myelomonocytic leukemia WEHI-3 cells. The flow cytometry assays indicated that berberine induced G2/M-phase arrest in both examined cell lines. The berberine-induced G2/M-phase arrest in both examined cell lines was accompanied by increased levels of Wee1 and 14-3-3σ, but decreased levels of Cdc25c, CDK1 and cyclin B1. However, CDK2 expression was not affected as revealed by Western blotting assay. Berberine induced G2/M arrest in both the examined cells via the inhibition of cyclin B1 and the promotion of Wee1.*

Berberine, an isoquinoline alkaloid commonly used in Chinese herbal medicine, exists in many natural plants (*Berberis aquifolium*, *Berberis vulgaris*, *Berberis aristata* and *Coptis chinensis*). Berberine has been demonstrated to display antibacterial (1), antifungal (2), antimalarial (3), anti-inflammatory (4), anticholinergic (5) and anti-arrhythmic activities (6). It was also shown that berberine exerts its anti-inflammatory effects through inhibition of cyclooxygenase-2 (COX-2) expression (7). Many studies have shown that berberine has anticancer activity, based on its antineoplastic properties (8-12). Berberine-induced apoptosis might be COX-2-dependent and was found to be related to decreased Akt phosphorylation and Mcl-1 expression (7).

The modulated expression of cell cycle regulatory molecules on cell cycle arrest in many cell types is well documented (13-15). These regulatory molecules, especially the cyclins and cyclin-dependent kinases (cdks), are evolutionarily conserved proteins essential for cell cycle control. Many other molecules can affect these regulator proteins, leading to cell cycle arrest. In murine leukemia L1210 cells treated with 10-50 μM berberine, G0/G1 cell cycle arrest was observed. Furthermore, a concentration-dependent decrease of cells in the S-phase and an increase of cells in the G2/M-phase were detected (16).

To date, the exact mechanisms of the action of berberine on cell cycle arrest in human and murine leukemia cells have not been fully investigated. In our primary screening experiments, berberine was found, by flow cytometry analysis, to induce cell cycle arrest in both cell lines. Therefore, the molecular mechanisms of berberine-induced cell cycle arrest in human and murine leukemia cell lines (HL-60 and WEHI-3) were the focus of this investigation.

Materials and Methods

Chemicals and reagents. Berberine, Tris-HCl, triton X-100, propidium iodide (PI) and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased

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Key Words: Berberine, leukemia HL-60, WEHI-3, G2/M arrest.

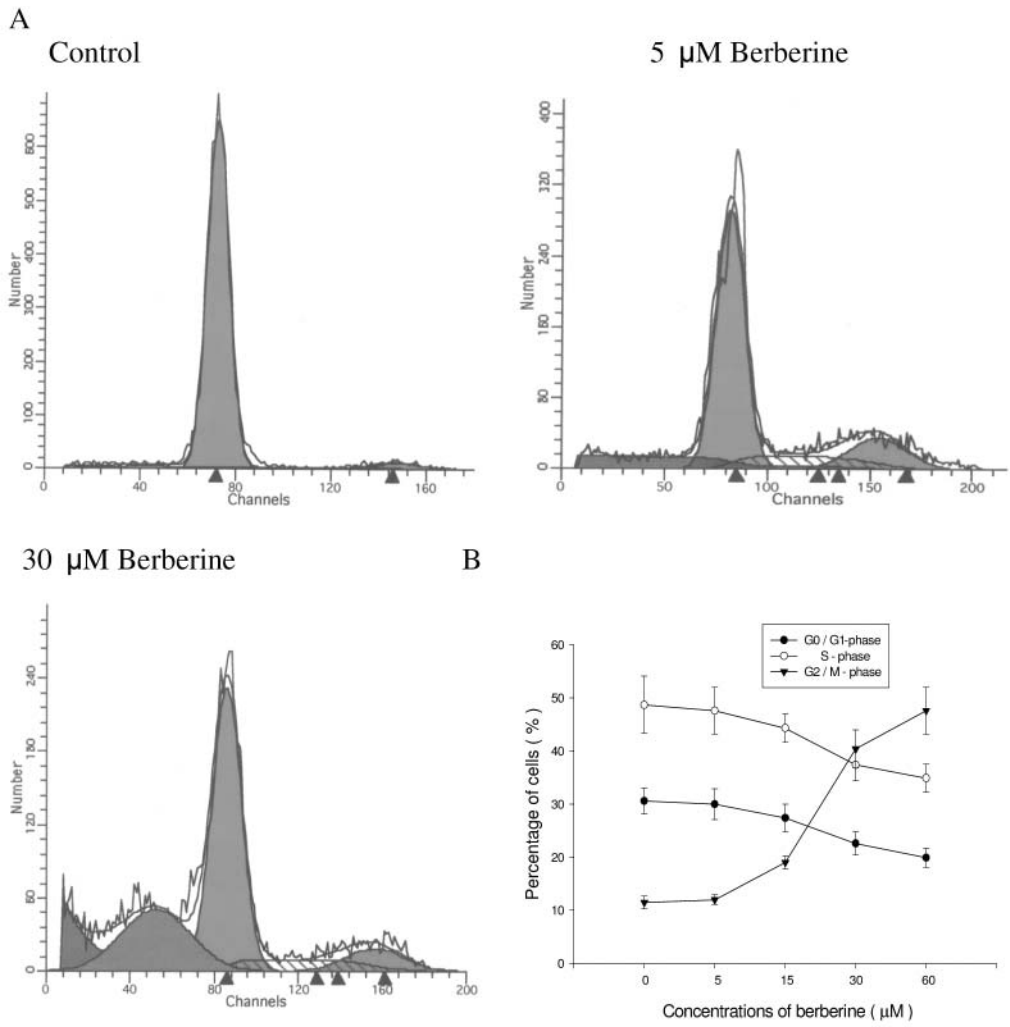

Figure 1. *Flow cytometric analysis of the effects of berberine on the cell cycle in HL-60. The HL-60 cells were exposed to various concentrations of berberine for 48 h, then harvested and analyzed by flow cytometry, as described in the Materials and Methods section. Panel A: representative profiles; panel B: the percent of cells in phase. Data represents mean±S.D. of 3 experiments. *p<0.05.*

from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The monoclonal antibodies for Western blotting were obtained from Calbiochem Biosciences Ltd. (Darmstadt, Germany) and the anti-14-3-3σ was obtained from Santa Cruz Biotechnology Inc. (CA, USA).

Leukemia cell lines. The human promyelocytic HL-60 and the murine myelomonocytic WEHI-3 leukemia cell lines were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks containing RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine and were grown at 37˚C in a humidified 5% CO_2 and 95% air atmosphere. All data presented in this report are from at least 3 independent experiments.

Flow cytometry analysis of DNA content. Approximately $3x10^5$ HL-60 or WEHI-3 cells/well in a 12-well plate were incubated with various concentrations of berberine (0, 5, 15, 30, or 60 μM) for different time-periods before the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase (Sigma) and 0.1% triton x-100. After 30 min at 37˚C in the dark, the cells were analyzed by a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm (17). The percentage of cells that had undergone G0/G1-, S- and G2/M-phase was assessed as the ratio of the fluorescence area to the total area of cells (17).

Western blotting. Approximately $3x10^6$ cells/well (HL-60 or WEHI-3) in a 12-well plate were incubated with berberine (0, 5, 15, 30, or 60 μM) for 24 h before the cells were harvested by centrifugation. The protein was extracted as previously described (18). The cyclin

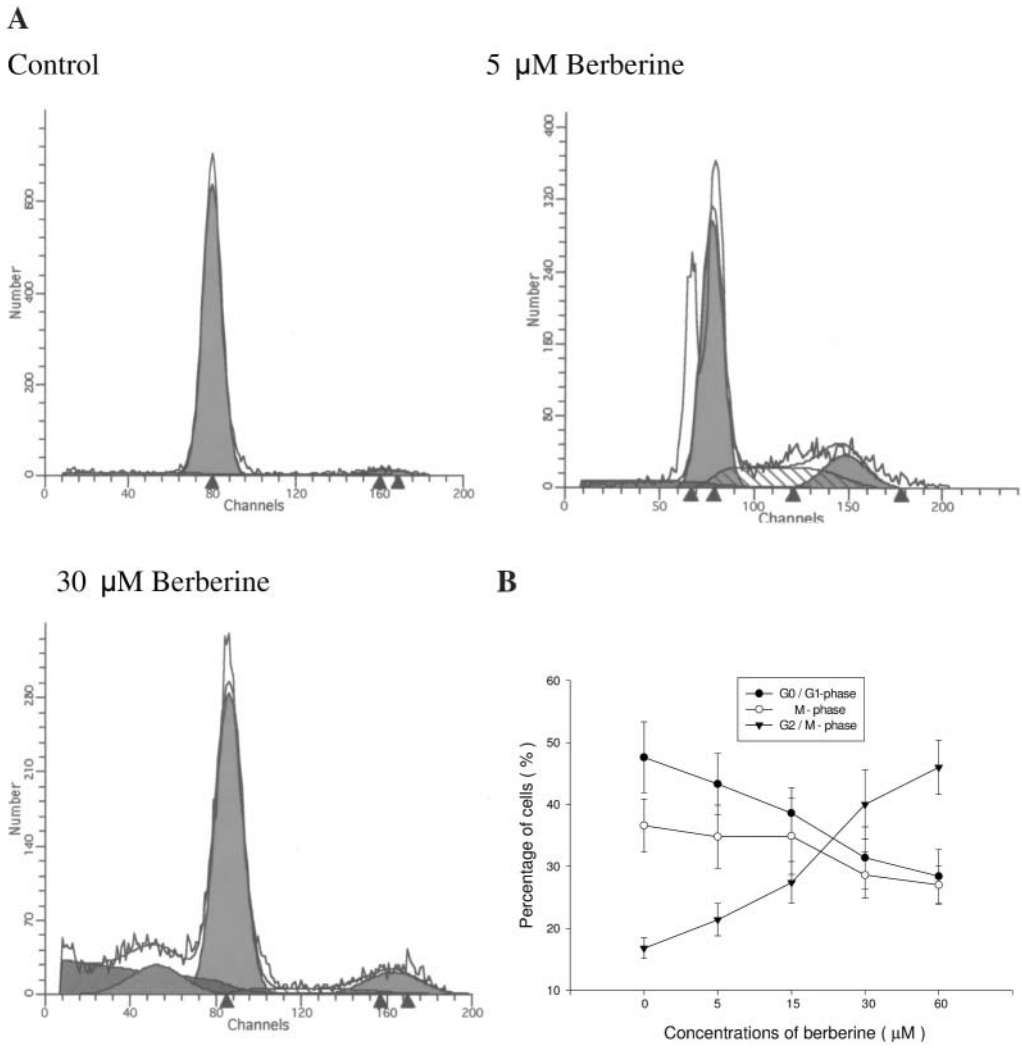

Figure 2. *Flow cytometric analysis of the effects of berberine on the cell cycle in WEHI-3. The WEHI-3 cells were exposed to various concentrations of berberine for 48 h and were the harvested and analyzed by flow cytometry, as described in the Materials and Methods section. Panel A: representative profiles; panel B: the percent of cells in phase. The data represents mean±S.D. of 3 experiments. *p<0.05.*

B1, CDK1, 14-3-3-σ, Cdc25c and Wee1 expressions were examined and measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (18).

Statistical analysis. The statistical calculations on the data were performed using an unpaired Student's *t*-test and ANOVA analysis. Statistical significance was set at $p<0.05$.

Results

Bereberine induced cell cycle arrest in human leukemia HL-60 and WEHI-3 cells. The results from the flow cytometric analysis for the cell cycle indicated that during the 48-h time-period, berberine increased the percentage of cells in G2/M (enhanced G2/M peak), and decreased the percentage of cells in G0/G1, in both the examined cell lines (Figure 1A and B; Figure 2A and 2B). A typical pattern of DNA content that reflects the G0/G1-, S- and G2/M- phases of the cell cycle, as shown in the control group, is depicted in Figures 1A and 2A. An increase of berberine concentrations led to an increase of cells in the G2/M-phase in the HL-60 (Figure 1B) and WEHI-3 (Figure 2B) cells, showing that berberine induced G2/M-phase arrest.

Western blotting to examine the effect of berberine on CDK1, cyclin B1, cyclin E, 14-3-3σ, Wee1 and cdc25c expressions in HL-60 and WEHI-3 cells. The results indicate that berberine increased the expressions of 14-3-3σ (Figure 3C) and Wee1

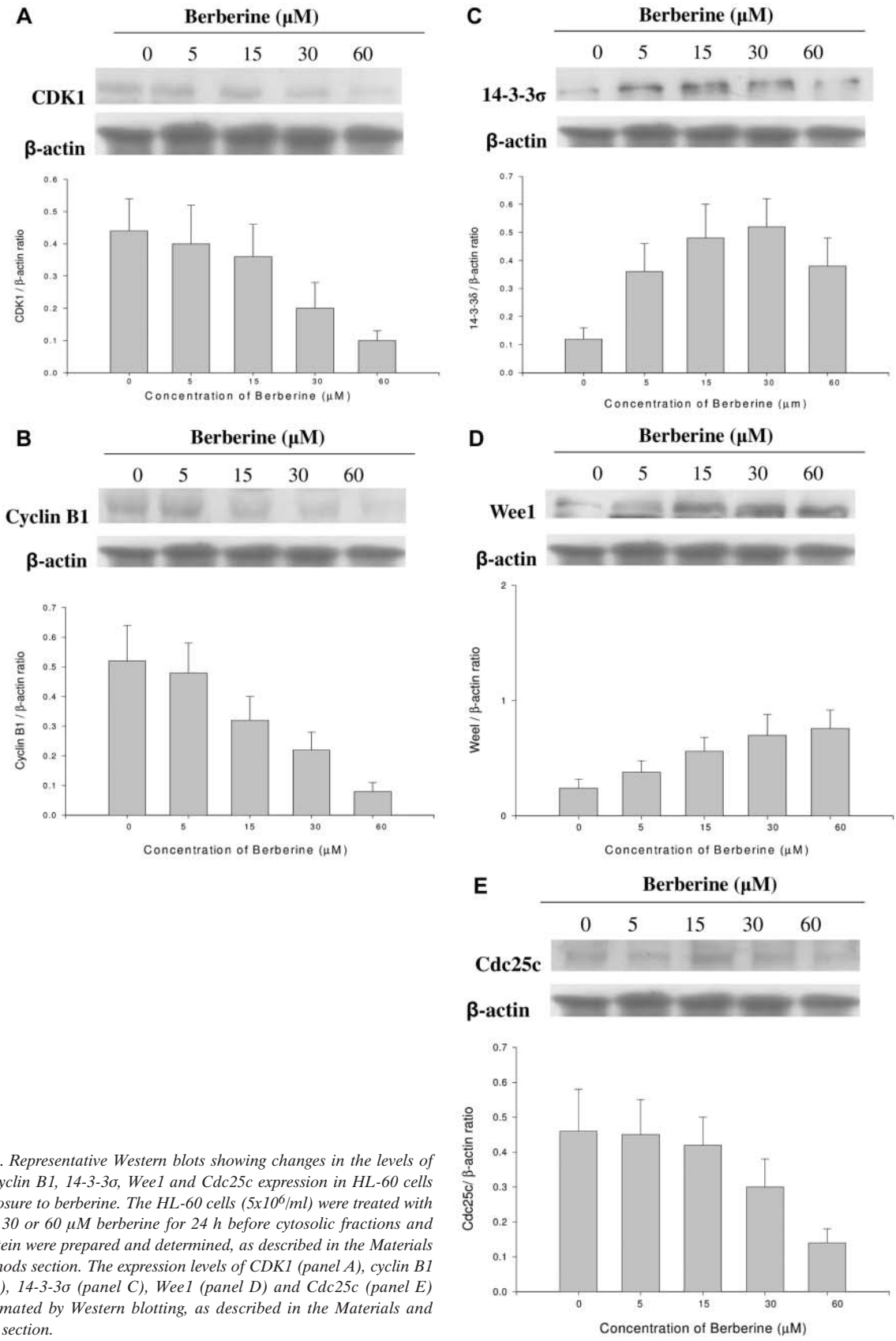

Figure 3. *Representative Western blots showing changes in the levels of CDK1, cyclin B1, 14-3-3σ, Wee1 and Cdc25c expression in HL-60 cells after exposure to berberine. The HL-60 cells (5x10^6/ml) were treated with 0, 5, 15, 30 or 60 μM berberine for 24 h before cytosolic fractions and total protein were prepared and determined, as described in the Materials and Methods section. The expression levels of CDK1 (panel A), cyclin B1 (panel B), 14-3-3σ (panel C), Wee1 (panel D) and Cdc25c (panel E) were estimated by Western blotting, as described in the Materials and Methods section.*

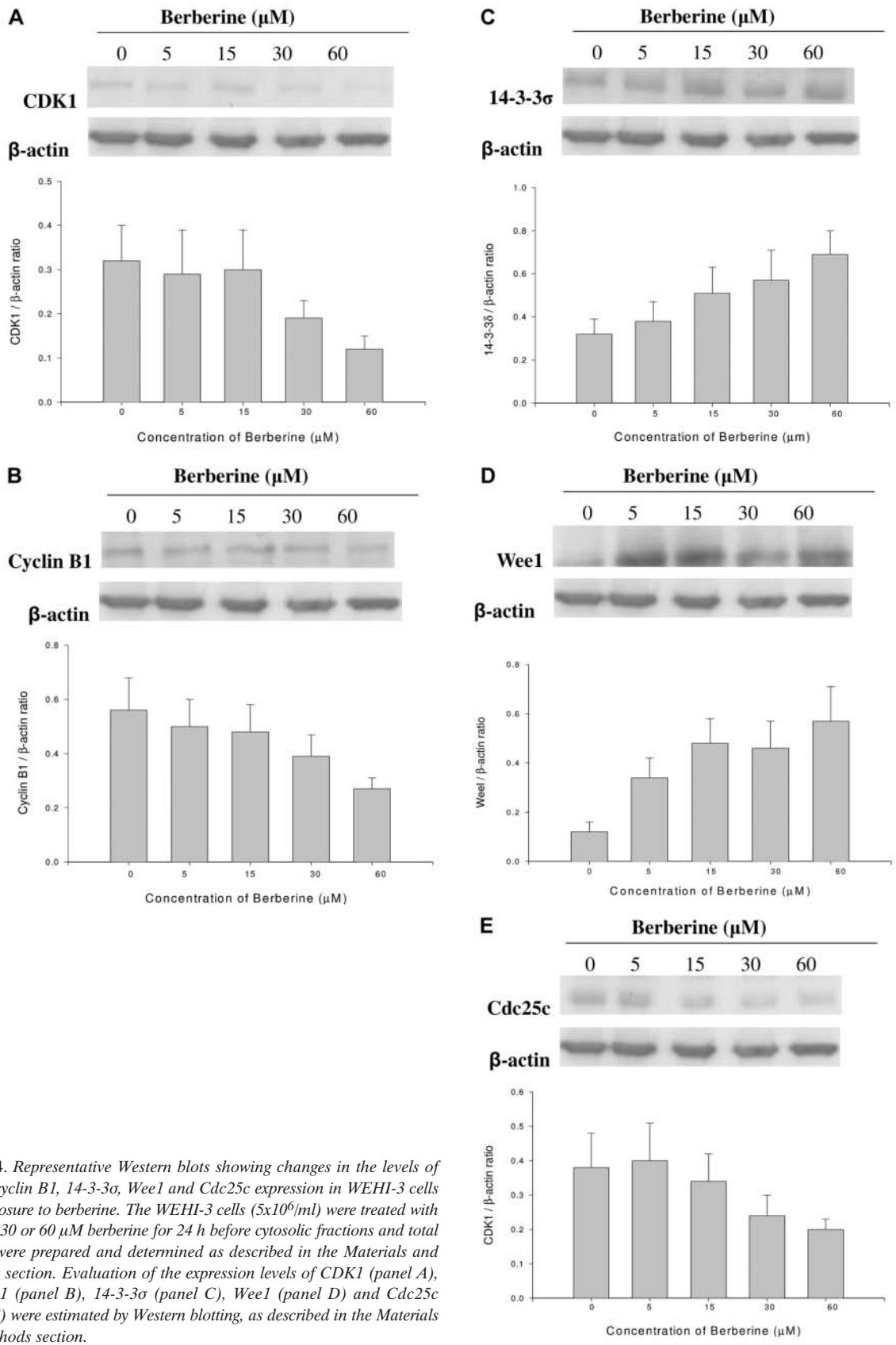

Figure 4. *Representative Western blots showing changes in the levels of CDK1, cyclin B1, 14-3-3σ, Wee1 and Cdc25c expression in WEHI-3 cells after exposure to berberine. The WEHI-3 cells ($5x10^6$/ml) were treated with 0, 5, 15, 30 or 60 μM berberine for 24 h before cytosolic fractions and total protein were prepared and determined as described in the Materials and Methods section. Evaluation of the expression levels of CDK1 (panel A), cyclin B1 (panel B), 14-3-3σ (panel C), Wee1 (panel D) and Cdc25c (panel E) were estimated by Western blotting, as described in the Materials and Methods section.*

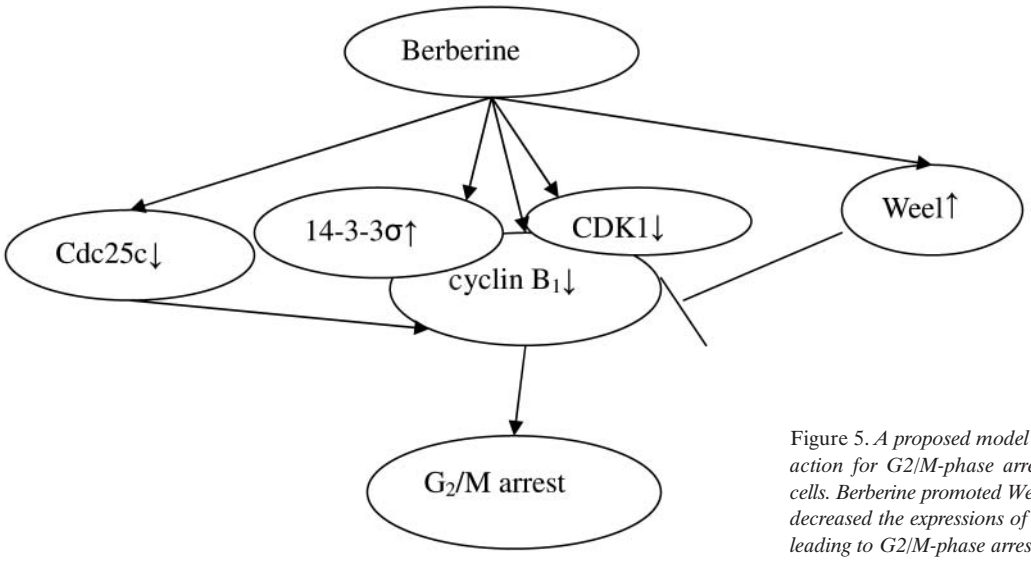

Figure 5. *A proposed model of the berberine mechanism of action for G2/M-phase arrest in the examined leukemia cells. Berberine promoted Wee1 and 14-3-3σ expressions but decreased the expressions of CDK1, cyclin B1 and Cdc25c, leading to G2/M-phase arrest in both cell lines examined.*

(Figure 3D) in HL-60 and 14-3-3σ (Figure 4C) and Wee1 (Figure 4D) in WEHI-3 cells, but decreased the CDK1 (Figure 3A), cyclin B1 (Figure 3B) and Cdc25c (Figure 3E) expressions in HL-60 and that of CDK1 (Figure 4A), cyclin B1 (Figure 4B) and Cdc25c (Figure 4E) in WEHI-3. These effects were all dose-dependent.

Discussion

Our data indicated that berberine induced G2/M arrest in both the examined leukemia cell lines and that these effects were dose- and time-dependent. The anticancer drug taxol (paciltaxel), which is isolated from plants, also had the same function on cells and was thus used as a positive control (data not shown). It was reported that cell cycle arrest in response to DNA damage is associated with the G2/M damage checkpoint, which is regulated by cyclin B1/CDK1 and initiated by DNA damage activation of the cyclin kinase inhibitor p21 (20-24). Our earlier studies indicated that berberine induced DNA damage in both HL-60 and WEHI-3 cells (25). In the present study, our results also revealed that berberine increased 14-3-3σ and Wee1 expression and decreased the expression of CDK1, cyclin B1 and Cdc25c, leading to the G2/M arrest in both examined cell lines.

The effective dose of berberine for both cell lines was 15-60 μM, which is smaller than that reported by Yang *et al.,* who demonstrated that berberine (200 μM) treatment was associated with the accumulation of numerous apoptotic cells, as identified by condensed nuclei and a decrease in cell size. The accumulation of cells in the G2/M-phase rather than the induction of apoptosis was observed after 48-72 h of 100 μM berberine treatment (26). Berberine was previously demonstrated to be a new cholesterol-lowering drug (27) and had inhibitory effects on potassium and calcium currents in isolated rat hepatocytes, which may be involved in hepatoprotection (28). It was shown that berberine could directly inhibit human umbilical vein endothelial cell (HUVEC) tube formation and migration *in vitro*. Other investigators provided molecular evidence to support berberine as a potent anti-angiogenic agent in cancer therapy (29). Therefore, berberine acts like the anticancer agent taxol and may be used as an antileukemia agent. Our laboratory is working on the effects of berberine on leukemia cells in an animal model (murine) *in vivo*.

It is well known that cell cycle checkpoints play an important role in the control mechanisms that ensure the proper execution of cell cycle events. One of these checkpoints, the G2/M checkpoint, blocks entry into mitosis when DNA is damaged (30). Our results also showed that berberine promoted p21 expression (data not shown). Although HL-60 is p53-deficient, an increase in the expression of p53 was detected in WEHI-3 cells after treatment with berberine and G2/M-phase arrest was induced in both cell lines, corroborating reports from other investigators. These reports demonstrated that p53 can regulate the G2/M transition, through the induction of p21 and of 14-3-3σ, a protein that normally sequesters cyclin B1-Cdc2 complexes in the cytoplasm (31, 32). It is well known that the p53 and p21 proteins play an important role in cell cycle regulation. p21 is an inhibitor of cyclin/cyclin-dependent kinase complexes and interacts with other regulators of signal transduction (33). The induction of p21 is mediated by both p53 and p53-independent mechanisms and is essential for the onset of both G1 and G2 cell cycle arrest in damage response and cell senescence (34).

It was reported that other proteins, such as 14-3-3σ, belong to a family of highly conserved proteins (alpha, beta, delta, sigma, tau, zeta *etc*.) with molecular weights of 25- to 30-kDa. Such proteins are expressed in all eukaryotic cells, modulate a wide variety of cellular processes and play an important role in the regulation of signal transduction pathways associated with the control of cell proliferation, differentiation and survival (35). It should be noted that 14-3-3σ is a p53-dependent inhibitor of G2/M progression and its overexpression can cause G2/M cell cycle arrest (36). 14-3-3σ is able to up-regulate Cdc2 phosphorylation *via* Wee1 and down-regulate Cdc25c, thus controlling the entry of cells into mitosis by maintaining the G2/M checkpoint (37). In the present study, berberine was found to affect the checkpoint enzymes of the cell cycle before leading to G2/M arrest. We used the PCR method to examine those associated genes that were affected by berberine (data not shown), in agreement with other reports that demonstrated that berberine binds strongly to the single-stranded poly(rA) structure by a mechanism of partial intercalation leading to its use in gene regulation in eukaryotic cells (38).

Based on the study results, we have illustrated the molecular mechanisms of berberine action (Figure 5), which induced G2/M-phase arrest through the promotion of Wee1 and 14-3-3σ expression and the inhibition of CDK1, cyclin B1 and Cdc25c expressions in both the examined cell lines.

References

1 Amin AH, Subbaiah TV and Abbasi KM: Berberine sulfate: antimicrobial activity, bioassay, and mode of action. Can J Microbiol *15*: 1067-1076, 1969.

2 Han Y and Lee JH: Berberine synergy with amphotericin B against disseminated candidiasis in mice. Biol Pharm Bull *28*: 541-544, 2005.

3 Ghosh AK, Bhattacharyya FK and Ghosh DK: *Leishmania donovani*: amastigote inhibition and mode of action of berberine. Exp Parasitol *60*: 404-413, 2000.

4 Dairou J, Atmane N, Rodrigues-Lima F and Dupret JM: Peroxynitrite irreversibly inactivates the human xenobiotic-metabolizing enzyme arylamine N-acetyltransferase 1 (NAT1) in human breast cancer cells: a cellular and mechanistic study. J Biol Chem *279*: 7708-7714, 2004.

5 Tsai CS and Ochillo RF: Pharmacological effects of berberine on the longitudinal muscle of the guinea-pig isolated ileum. Arch Int Pharmacodyn Ther *310*: 116-131, 1991.

6 Wang YX and Zheng YM: Ionic mechanism responsible for prolongation of cardiac action – potential duration by berberine. J Cardiovasc Pharmacol *30*: 214-222, 1997.

7 Kuo CL, Chi CW and Liu TY: Modulation of apoptosis by berberine through inhibition of cyclooxygenase-2 and Mcl-1 expression in oral cancer cells. In Vivo *19*: 247-252, 2005.

8 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, 2000.

9 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.

10 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.

11 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.

12 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.

13 Farhana L, Dawson M, Rishi AK, Zhang Y, Van Buren E, Trivedi C, Reichert U, Fang G, Kirschner MW and Fontana JA: Cyclin B and E2F-1 expression in prostate carcinoma cells treated with the novel retinoid CD437 are regulated by the ubiquitin-mediated pathway. Cancer Res *62*: 3842-3849, 2002.

14 Freya W, Bora A, Antje C and Jurgen S: Downregulation of the cyclin D1/cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J Nutr *131*: 2197-2203, 2001.

15 Joe AK, Liu H, Suzui M, Vural M, Xiao D and Weinstein B: Reveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res *8*: 893-903, 2002.

16 Jantova S, Cipak L, Cernakova M and Kost'alova D: Effect of berberine on proliferation, cell cycle and apoptosis in HeLa and L1210 cells. J Pharm Pharmacol *55*: 1143-1149, 2003.

17 Hsu MH, Kuo SC, Chen CJ, Chung JG, Lai YY and Huang LJ: 1-(3,4-Dimethoxyphenyl)-3,5-dodecenedione (I6) induces G1 arrest and apoptosis in human promyelocytic leukemia HL-60 cells. Leuk Res *29*: 1399-1406, 2005.

18 Chen HC, Hsieh WT, Chang WC and Chung JG: Aloe-emodin induced *in vitro* G2/M arrest of cell cycle in human promyelocytic leukemia HL-60 cells. Food Chem Toxicol *42*: 1251-1257, 2004.

19 Yu FS, Yu CS, Lin JP, Chen SC, Lai WW and Chung JG: Diallyl disulfide inhibits N-acetyltransferase activity and gene expression in human esophagus epidermoid carcinoma CE 81T/VGH cells. Food Chem Toxicol *43*: 1029-1036, 2005.

20 Porter L, Singh G and Lee J: Abundance of cyclin B1 regulates γ-radiation-induced apoptosis. Blood *95*: 2645-2650, 2000.

21 Kao G, McKenna G, Maity A, Blank K and Muschel R: Cyclin B1 is a rate limiting component of the radiation induced G2 delay in HeLa cells. Cancer Res *57*: 753-758, 1997.

22 Toyoshima F, Moriguchi T, Wada A, Fukuda M and Nishida E: Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint. EMBO J *17*: 2728-2735, 1998.

23 Jin P, Hardy S and Morgan D: Nuclear location of cyclin B1 controls mitotic entry after DNA damage. J Cell Biol *141*: 875-885, 1998.

24 Takizawa C, Weiss K and Morgan D: Ran-independent nuclear import of cyclin B1-cdc2 by important beta. Proc Natl Acad Sci USA *96*: 7938-7943, 1999.

25 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*: 227-242, 2006.

26 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.
27 Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Wang Y, Li Z, Liu J and Jiang JD: Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med *10*: 1344-1351, 2004.
28 Wang F, Zhou HY, Zhao G, Fu LY, Cheng L, Chen JG and Yao WX: Inhibitory effects of berberine on ion channels of rat hepatocytes. World J Gastroenterol *10*: 2842-2845, 2004.
29 Lin S, Tsai SC, Lee CC, Wang BW, Liou JY and Shyu KG: Berberine inhibits HIF-1alpha expression *via* enhanced proteolysis. Mol Pharmacol *66*: 612-619, 2004.
30 Taylor WR and Stark GR: Regulation of the G2/M transition by p53. Oncogene *20*: 1803-1815, 2001.
31 Hermeking H, Lengauer C, Polyak K, He T-C, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B: 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell *1*: 3-11, 1997.
32 Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B: Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282*: 1497-1501, 1998.
33 Roninson I: Oncogenic functions of tumour suppressor $p21^{Waf1/Cip1/Sdi1}$: association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett *179*: 1-14, 2002.
34 Adriaenssens E, Lemoine J, EI Yazidi-Belkoura I and Hondermarck H: Growth signaling in breast cancer cells: outcomes and promises of proteomics. Biochem Pharmacol *64*: 797-803, 2002.
35 Fu H, Subramanian RR and Masters SC: 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol *40*: 617-647, 2000.
36 Taylor WR and Stark GR: Regulation of the G2/M transition by p53. Oncogene *20*: 1803-1815, 2001.
37 Chan TA, Hermeking H, Lengauer C, Kinzler KW and Vogelstein B: 14-3-3 sigma is required to prevent mitotic catastrophe after DNA damage. Nature (Lond) *401*: 616-620, 1999.
38 Yadav RC, Kumar GS, Bhadra K, Giri P, Sinha R, Pal S and Maiti M: Berberine, a strong polyriboadenylic acid binding plant alkaloid: spectroscopic, viscometric, and thermodynamic study. Bioorg Med Chem *13*: 165-174, 2005.

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