

Full-length article

Curcumin, a potent anti-tumor reagent, is a novel histone deacetylase inhibitor regulating B-NHL cell line Raji proliferation¹

Hong-li LIU², Yan CHEN^{2,3}, Guo-hui CUI², Jian-feng ZHOU⁴

²Department of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; ⁴Department of Hematology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Key words

curcumin; histone deacetylase; acetylated-histone H4; Raji

¹ Project supported by the National Natural Science Foundation of China (No 30271672).

³ Correspondence to Prof Yan CHEN.

Phn 86-27-6604-6609.

Fax 86-27-8572-6387.

Email liuhl60@tom.com

Received 2004-10-15

Accepted 2004-12-20

doi: 10.1111/j.1745-7254.2005.00081.x

Abstract

Aim: To investigate curcumin (diferuloylmethane) induced apoptosis and its molecular mechanism of action in B-NHL cell line Raji cells. **Methods:** Raji cells were cultured in RPMI-1640 medium and treated with curcumin in different concentrations. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) assay was used to detect growth inhibition and Hoechst 33258 staining was used to detect apoptosis. Immunocytochemistry and Western blot were used to detect the expressions of histone deacetylase 1, 3, and 8 (HDAC1, HDAC3, and HDAC8) and acetylated histone H4 (Ac-histone H4) protein. **Results:** Curcumin inhibited the proliferation of B-NHL cell line Raji cells with a 36-h IC₅₀ value of 24.1±2.0 μmol/L. Hoechst 33258 staining showed that curcumin could induce Raji cell apoptosis. The expression levels of HDAC1, HDAC3, and HDAC8 proteins were downregulated following curcumin treatment in Raji cells, whereas Ac-histone H4 protein expression was upregulated after treatment with curcumin. **Conclusion:** Curcumin, as a new member of the histone deacetylase inhibitors, can inhibit the expression of class I HDACs (HDAC1, HDAC3, and HDAC8), and can increase the expression of Ac-histone H4 in Raji cells. Curcumin plays an important role in regulating B-NHL cell line Raji cell proliferation and apoptosis.

Introduction

Histone acetylation/deacetylation is a key mechanism for regulating transcription. Acetylation of the ε-amino group of specific lysine residues within the N-terminal tail of core histones results in location chromatin relaxation. In general, histone acetylase activity is correlated with transcription activation, whereas histone deacetylase activity is correlated with transcription repression^[1,2]. Significant progress has been made in the use of histone deacetylase inhibitors as antineoplastic drugs. Several reagents have been shown to be histone deacetylase inhibitors (HDACIs), including TSA (Trichostatin A), butyrate, FR90228, and sulindac^[3,4]. The mechanism of HDACIs relates to inducing cell cycle arrest and apoptotic responses, and is regulated by changes in histone acetylation and deacetylation. Emerging evidence suggests that a family of histone deacetylases may exist to

regulate diverse cellular functions, including chromatin structure, gene expression, cell cycle progression, and oncogenesis^[5].

Curcumin, the major component of the spice turmeric and the yellow pigment in curry powder, has been widely used in India and other parts of South-East Asia as a spice and a coloring agent in cooking. Many studies have shown that curcumin (diferuloylmethane) has significantly antiproliferative and apoptotic effects for cancer treatment, including pancreatic carcinoma, liver carcinoma, and leukemia^[6,7]. Experimental studies have also revealed that curcumin regulates molecules in the cell signal transduction pathways, including NF-kappaB, Akt, MAPK, p53, AR, Ras, and ER pathways^[8,9]. Research has shown that curcumin is structurally related to sulindac, and the latter is a member of HDACs. Sulindac exerts significant chemopreventive activity, which is related to cell cycle arrest and the histone acetylation/

deacetylation state^[10]. In a previous study we revealed that curcumin inhibited K562 cell proliferation by Janus kinase-signal transducer and by activating transcription and activator protein-5 signaling pathways^[11]. In the present study, we chose the B-NHL cell line as the target. We assumed that curcumin could inhibit carcinoma cell proliferation by regulating Raji cells and we explored the underlying mechanism of curcumin regulating the histone acetylation/deacetylation pathway.

Materials and methods

Drugs and reagents Curcumin was purchased from Sigma Chemical Company (St Louis, MO, USA) and initially dissolved in dimethylsulfoxide (Me₂SO), stored at -20 °C, and thawed before use. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium (MTT) was purchased from Janssen Chimica Company (New Brunswick, NJ), and RPMI-1640 medium, Hoechst 33258, and Me₂SO were purchased from Sigma. Anti-HDAC1, anti-HDAC3, anti-HDAC8, and anti-Ac-histone H4 were purchased from Santa Cruz (California, USA). Streptavidin peroxidase (SP) reagent kits were purchased from Zhongshan Company (Beijing, China). Chemiluminescence (ECL) reagent kits were purchased from Pierce Biotechnology, Inc (Rockford, IL). The Raji cell line was obtained from China Center for Typical Culture Collection (Wuhan, China). The following treatments were applied: untreated Raji cells and Raji cells treated with 6.25 μmol/L, 12.5 μmol/L, and 25 μmol/L of curcumin for 24 h. All cell groups were grown in RPMI-1640 culture medium containing 10% fetal calf serum (FCS) and 2 mmol/L *L*-glutamine at 37 °C in a 5% CO₂ incubator.

MTT assay The antiproliferative effects of curcumin against different cell groups were determined using the MTT dye uptake method. In brief, the cells (40 000 per well) were incubated in triplicate in a 96-well plate. Different concentrations of curcumin were added, and the final concentrations were 6.25, 12.5, 25, 50, and 100 μmol/L. The plates were in the presence or absence of the indicated test samples for 0, 24, 36, 48, 60, and 72 h. The largest Me₂SO dissolved concentration group acted as the control group. Thereafter, 20 μL MTT solution (5 g/L in phosphate-buffered saline [PBS]) was added to each well. After 4 h at 37 °C, the supernatant was removed and 150 μL Me₂SO was added. When the blue crystal was dissolved, the optical density (*OD*) was detected in the microplate reader at 570 nm wavelength using a 96-well multiscanner autoreader (Biotech Instruments, New York, USA). The following formula was used: Cell proliferation inhibited (%) = [1 - (*OD* of the experimental samples / *OD* of the

control)] × 100%.

Apoptosis assay Curcumin-induced apoptosis was monitored by the extent of nuclear fragmentation. Nuclear fragmentation was visualized by Hoechst 33258 staining of apoptotic nuclei. Apoptotic cells were collected by centrifugation, washed with PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. Subsequently the cells were washed and resuspended in 20 μL PBS before being deposited on poly lysine-coated coverslips and left to adhere to the cover slips for 30 min at room temperature, after which the cover slips were washed twice with PBS. The adhered cells were then incubated with 0.1% Triton X-100 for 5 min at room temperature and rinsed with PBS three times. The coverslips were treated with Hoechst 33258 at 37 °C for 30 min, rinsed with PBS, and mounted on slides with glycerol-PBS. The cells were viewed with an Olympus BH-2 fluorescence microscope (Japan).

Immunocytochemistry analysis Curcumin-treated cells were plated onto a glass slide, air dried for 1 h at room temperature, and fixed with cold acetone. After brief washing in PBS, the slides were blocked with 5% normal goat serum for 1 h and incubated with HDAC1, HDAC3, HDAC8, and Ac-histone H4 (dilution 1:100, respectively). After being left overnight at 4 °C the cells were treated with biotinylated link secondary antibody and peroxidase-labeled streptavidin followed by diaminobenzidine (DAB). The cells were viewed with an Olympus microscope and their individual *OD* values were recorded using an HPIAS 1000 Image Analysis System (High Resolution Pathological Image & Word Analysis System, Beijing, China).

Western blot analysis Lysates were prepared from 1 × 10⁷ cells by dissolving cell pellets in 100 μL of lysis buffer (Na₂PO₄ (pH 7.4) 20 mmol/L, NaCl 150 mmol/L, Triton X-100 1%, aprotinin 1%, phenylmethylsulfonyl fluoride 1 mmol/L, leupeptin 10 g/L, NaF 100 mmol/L, and Na₃VO₄ 2 mmol/L). Lysates were centrifuged at 18 000 × *g* for 15 min and the supernatant was collected. Protein content was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2 mol/L DTT) was added to the lysates. Lysates were heated to 100 °C for 5 min, and 80 μg of protein was loaded into each well of a 10% SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose and blocked with 5% non-fat milk, and the primary antibodies HDAC1, HDAC3, HDAC8, and Ac-histone H4 were added. After overnight incubation at 4 °C the blots were washed, exposed to HRP-conjugated corresponding secondary antibodies for 1 h, and finally detected by ECL.

Quantification of the bands was carried out using densitometric analysis software, Quantity One (Bio-Rad), and processed as described previously^[12].

Statistical analysis All data were expressed as mean±SD using SPSS 10.0 for windows 98. Using linear *t*-tests for statistics analysis, *P* values of less than 0.01 or 0.05 were considered to be statistically significant.

Results

Effects of curcumin on the proliferation of Raji cells by MTT Raji cells treated with different concentrations of curcumin for 0, 24, 36, 48, 60, and 72 h resulted in the inhibition of cell proliferation in a dose- and time-dependent manner. The *OD* value of curcumin-treated groups decreased significantly compared with the untreated group. Results reveal great differences between curcumin-treated groups and the untreated group (Figure 1). The *IC*₅₀ of 36 h is 24.1±2.0 μmol/L.

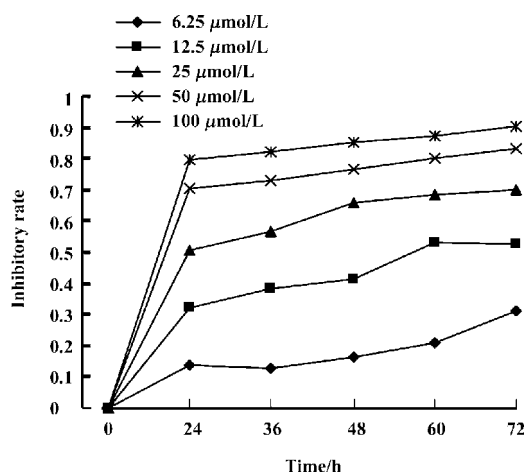


Figure 1. Antiproliferation effect of curcumin in Raji cells. Raji cells were treated with various concentrations of curcumin as indicated for 0, 24, 36, 48, 60, and 72 h. Growth inhibition was determined using a MTT assay and shown as inhibitory rate.

Nuclear damage observed using Hoechst 33258 staining Apoptotic nuclear morphology was assessed using Hoechst 33258 staining. Hoechst 33258 staining of untreated Raji cells and cells treated with 25 μmol/L curcumin for 24 h was conducted. Curcumin permeates the cell and is known to play a role in cancer chemoprevention and tumor growth suppression. Exposure of tumor cells to curcumin *in vitro* results in the inhibition of cell proliferation and the induction of apoptosis. Consistent with previous reports on other cell lines^[9,11], treatment of Raji cells with curcumin (24 h exposure to 25 μmol/L curcumin) induces apoptosis (Figure 2).

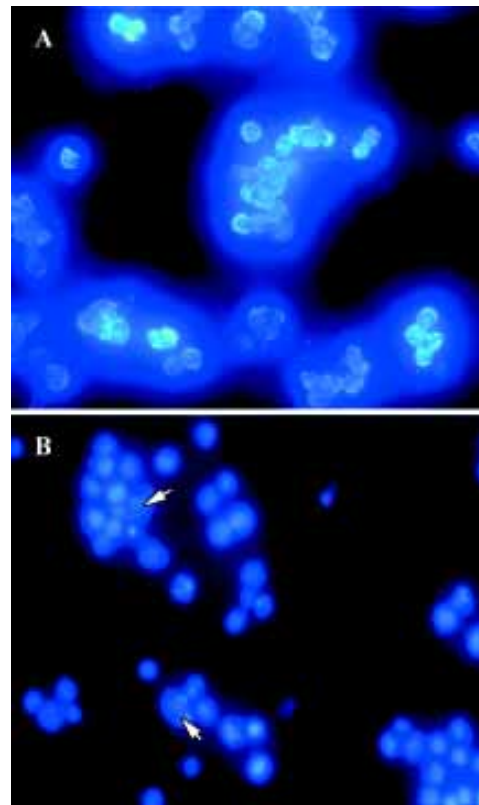


Figure 2. (A) Untreated Raji cells. (B) Cells treated with 25 μmol/L curcumin for 24 h. Photographs were taken under an Olympus BH-2 fluorescence microscope (×40). Arrows indicate apoptotic nuclear fragmentation.

Expression of HDACs and Ac-histone H4 on Raji cells and curcumin-treated cells using immunocytochemistry Our results reveal that the expression of HDAC1, HDAC3, and HDAC8 was significantly higher in Raji cells compared with curcumin-treated cells (25 μmol/L for 24 h) (*P*<0.05). The expression of Ac-histone H4 was significantly higher in curcumin-treated cells (25 μmol/L for 24 h) than in Raji cells (*P*<0.05)(Figure 3). Photos were analyzed using a HPIAS 1000 Image Analysis System and *OD* values were recorded (Figure 4).

Expression of HDAC1, HDAC3, and HDAC8 on Raji cells and curcumin-treated cells using Western blot Our results reveal that curcumin can induce antiproliferation and apoptosis in Raji cells. However, it is unclear how curcumin induces this antiproliferation and apoptosis. Cells treated with 6.25, 12.5, and 25 μmol/L of curcumin for 24 h were lysed and resolved in 10% SDS-PAGE, and Western blot analysis was carried out using anti-HDAC1, anti-HDAC3, and anti-HDAC8. Figure 5 shows considerable changes in HDAC1, HDAC3, and HDAC8 following curcumin treatment.

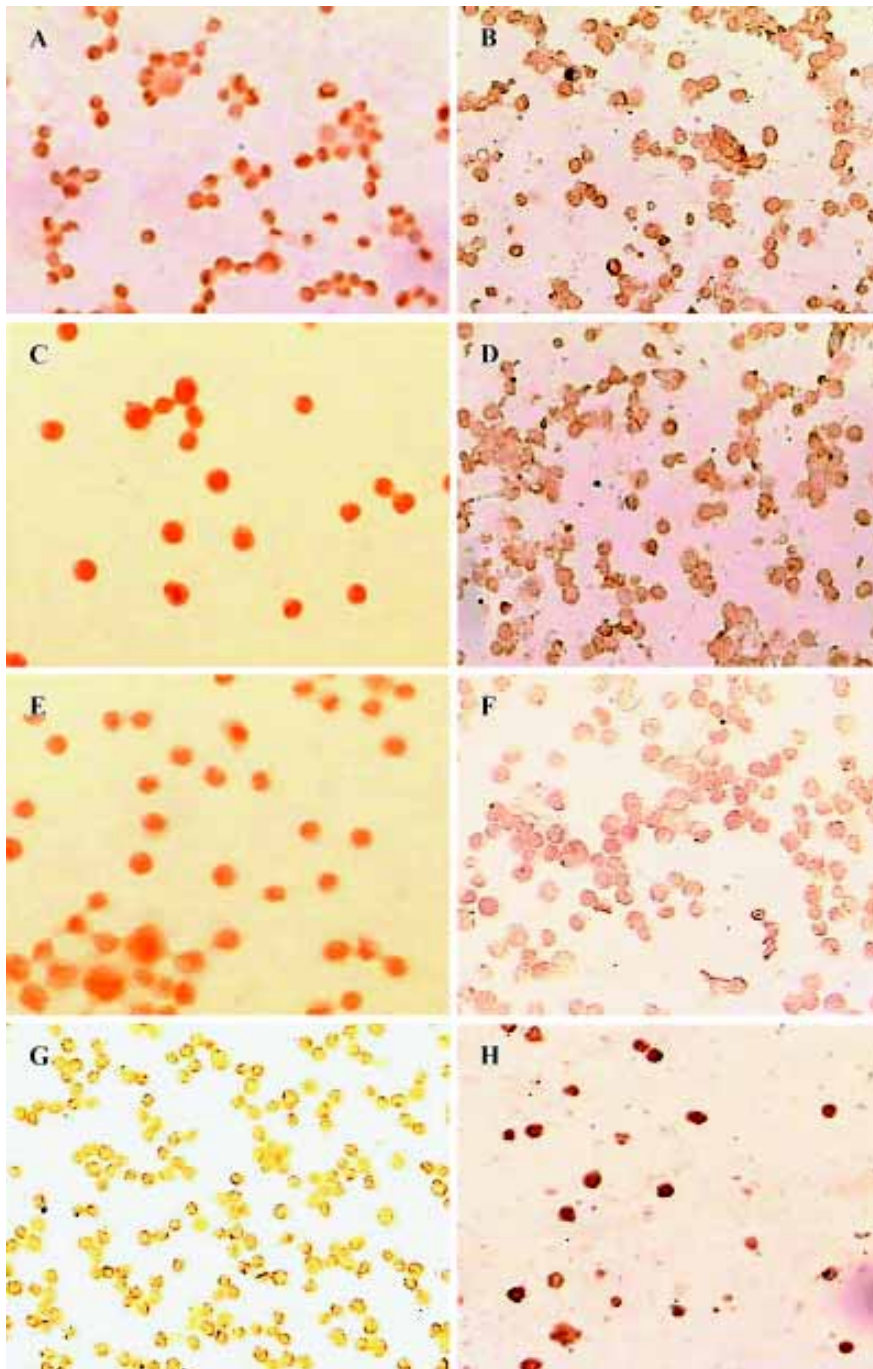


Figure 3. (A, B) Expression of HDAC1 in control and curcumin-treated cells; (C, D) Expression of HDAC3 in control and curcumin-treated cells; (E, F) Expression of HDAC8 in control and curcumin-treated cells; (G, H) expression of Ac-histone H4 in control and curcumin-treated cells.

These results indicate that HDAC1, HDAC3, and HDAC8 are related to curcumin-mediated apoptosis. The levels of HDAC1, HDAC3 and HDAC8 protein decreased in a dose-dependent manner (Figure 5).

Expression of Ac-histone H4 in Raji cells and curcumin-treated cells The expression of Ac-histone H4 was significantly greater in curcumin-treated Raji cells (6.25, 12.5, and 25 $\mu\text{mol/L}$, for 24 h) than that in Raji cells ($P < 0.05$, Figure 6).

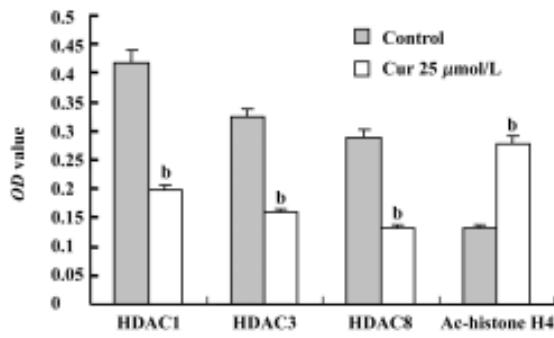


Figure 4. OD value of HDAC1, HDAC3, HDAC8, and Ac-histone H4 in Raji cells and curcumin-treated Raji cells (25 μmol/L, 24 h). ^b*P*<0.05 vs control.

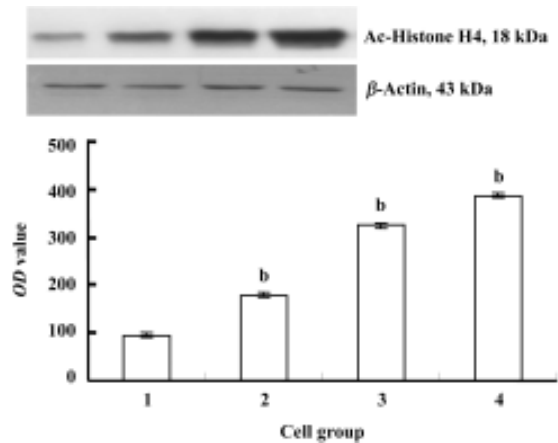


Figure 6. Effect of curcumin on the expression of Ac-histone H4 in Raji cells. Cells were treated with different concentrations of curcumin for 24 h and Ac-histone H4 expression was tested using Western blot analysis. 1, 2, 3, and 4 are the control, and curcumin 6.25, 12.5, and 25 μmol/L, respectively. *n*=3. Mean±SD. ^b*P*<0.05 compared with the control.

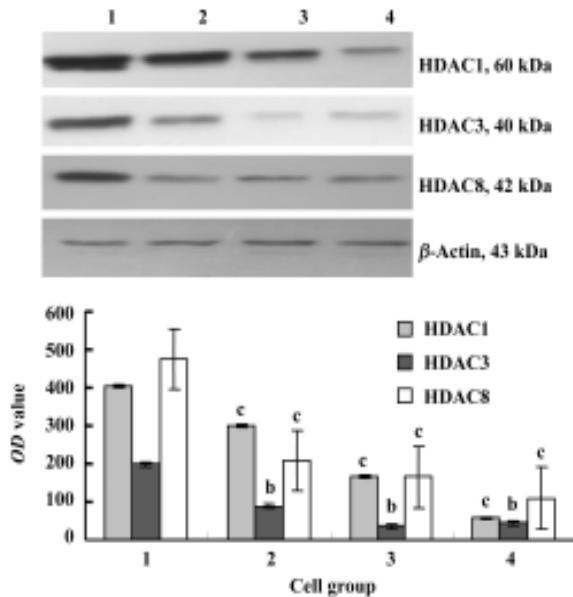


Figure 5. Effects of curcumin on the expression of HDACs in Raji cells. Cells were treated with different concentrations of curcumin for 24 h and HDACs expression was tested using Western blot analysis. 1, 2, 3, and 4 are the control, and curcumin 6.25, 12.5, and 25 μmol/L, respectively. *n*=3. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 compared with the control.

Discussion

Reversible histone acetylation occurs in the ε-amino group of the specific internal lysine residues located at the highly basic N-terminal domains of core histones. Histone acetyltransferase (HAT) and HDAC control the addition and removal of acetyl groups on proteins and maintain a dynamic balance of steady-state acetylation^[13]. A balance between the acetylation and deacetylation states of these pro-

teins forms the basis for the regulation of transcription. Research has shown that HDACs have wide ranges of effects on cell function. These effects include specific gene activation, inhibition of cell proliferation and cell cycle arrest, as well as induction of cell differentiation^[14]. Multiple forms of HDACs have been identified in mammalian cells. In humans, at least 11 HDACs have been uncovered. They are classified into three general classes: class I (HDAC1, 2, 3, 8, and 11), class II (HDAC4, 5, 6, 7, 9, and 10) and class III^[15]. Class I enzymes are smaller polypeptides of approximately 500 amino acids, whereas class II HDACs are much larger proteins with approximately 100 amino acids. Most class II HDACs shuttle between the cytoplasm and nucleus and regulate myogenesis. HDACs can react with co-repressor (Mad/Max, N-CoR, SMRT) to regulate cell proliferation and change the dynamics of chromatin structure^[16]. In general, HDAC1, HDAC3, and HDAC8 are located in the cell nucleus. Many signal transfer pathways (RAS/MAPK, JAK-STAT) and transcriptional factors related to hematopoietic stem cells are regulated by HDACs and HATs.

B-NHL plays an important role in blood system tumors. We chose the Burkitt lymphoma cell line Raji as the research target. Previous studies have revealed that lymphoma is related to the rearrangement of BCL6. Several studies have shown that BCL6 is rearranged in 30%–40% of diffuse large cell lymphoma (DLCL) and 6%–14% of follicular lymphomas (FL). In addition, the chromosomal band 3q27 affects IG gene loci that lead to lymphoma. Abnormal BCL6 can regulate cell-cycle factors (pRB, PLZF) by recruiting HDACs,

and can affect the cell cycle. As the result of an abnormal recruiting function, many transcriptional factors can suppress specific genes, which can lead to carcinogenesis. HDACs can cure cancer by inhibiting HDACs and blocking abnormal recruitment.

Chemoprevention is a rapidly growing field in cancer research that focuses on inhibiting and delaying the onset of carcinogenesis. A large number of natural products have been evaluated as potential chemopreventive agents. Numerous studies have shown that curcumin could suppress the proliferation of many cancer cells. In the present study, we found that curcumin could inhibit the proliferation of Raji cells, and that the effects were time- and dose-dependent. The 36 h IC_{50} of curcumin was $24.1 \pm 2.0 \mu\text{mol/L}$. Curcumin can lead to the apoptosis of Raji cells, and can affect cell cycles. The cell cycle was arrested in G_0/G_1 and G_2/M phases, and the S phase also decreased (data not shown). However, the mechanisms of apoptosis and cell cycle arrest are not clear. In our study, the expression of HDAC1, HDAC3, HDAC8 and Ac-histone H4 on B-NHL cell line Raji and curcumin-treated Raji cells (different concentrations for 24 h) was examined using immunocytochemistry and Western blot analysis. The expression of HDAC1, HDAC3, and HDAC8 proteins on Raji cells decreased compared with the control Raji group, whereas Ac-histone H4 expression increased compared with the control Raji group in a dose-dependent way.

Trichostatin (TSA) was the first discovered HDAC inhibitor (HDACI), followed by sodium butyrate, sulindac, MS-27-275, and FR90228^[10]. *In vitro* and *in vivo* studies examining HDACs reveal that HDACs affect many cell functions, such as cell proliferation, chromosome remodeling, and gene transcription. The mechanism of action relates to inhibiting HDACs, increasing the function of HATs, and increasing histone deacetylation. Hu *et al*^[15] found that TSA decreased the expression of HDAC1, HDAC3, and HDAC8 in SW620 in a dose-dependent way. The IC_{50} was approximately 0.1–0.3 nmol/L. In addition, TSA can increase the expression of Ac-histone H4 and lead to apoptosis, which is related to the SV40 promoter. Balasubramanyam *et al*^[13] reported that curcumin was a specific inhibitor of p300/CBP HAT activity, but not of PCAF, *in vitro* and *in vivo*. Furthermore, curcumin can also inhibit the p300-mediated acetylation of p53 *in vivo*. Curcumin specifically represses the p300/CBP HAT activity-dependent transcriptional activation from chromatin, but not from a DNA template.

Thus, we believe that curcumin, as a new member of the HDACs, can inhibit the expression of HDAC1, HDAC3, and HDAC8 in curcumin-treated Raji cells and can increase the

expression of Ac-histone H4. In addition, curcumin can inhibit cell proliferation and induce apoptosis. Dysfunction of histone acetyltransferases and histone deacetylases is often associated with the manifestation of several different types of cancer. These enzymes, therefore, are potential new targets for therapy. However, the mechanism by which the abnormal function of HDAC1, HDAC3, and HDAC8 regulates gene transcription remains to be determined. The increase in Ac-histone H4 highlights whose gene will be opened and provides a new field to examine the action mechanism of curcumin.

Acknowledgements

Thanks to the Tumour Biology Laboratory, Center of Gynaecology, Tongji Hospital, Huazhong University of Science and Technology for offering relevant experimental facilities and technical support. We wish to particularly thank Prof Jian-feng ZHOU and Yun-ping LU for their guidance and help with the experiment.

References

- 1 Secrist JP, Zhou X, Richon VM. HDAC inhibitors for the treatment of cancer. *Curr Opin Invest Drugs* 2003; 12: 1422–7.
- 2 Hu E, Dul E, Sung CM, Chen Z, Kirkpatrick R, Zhang GF, *et al*. Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J Pharmacol Exp Ther* 2003; 307: 720–8.
- 3 Buggy JJ, Sideris ML, Mak P, Lorimer DD, McIntosh B, Clark JM. Cloning and characterization of a novel human histone deacetylase, HDAC8. *Biochem J* 2000; 350: 199–205.
- 4 Murata M, Towatari M, Kosugi H. Apoptotic cytotoxic effects of a histone deacetylase inhibitor, FK228, on malignant lymphoid cells. *Jpn J Cancer Res* 2000; 91: 1154–60.
- 5 Graessle S, Loidl P, Brosch G. Histone acetylation: plants and fungi as model systems for the investigation of histone deacetylases. *Cell Mol Life Sci* 2001; 58: 704–20.
- 6 Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 2003; 23: 363–98.
- 7 Leu TH, Maa MC. The molecular mechanisms for the antitumorigenic effect of curcumin. *Curr Med Chem Anti-Cancer Agents* 2002; 2: 357–70.
- 8 Ranjan D, Chen C, Johnston TD, Jeon H, Nagabhusan M. Curcumin inhibits mitogen stimulated lymphocyte proliferation, NF κ B activation, and IL-2 signaling. *J Surg Res* 2004; 121: 171–7.
- 9 Wu L, Xu J, Wu G, Chen Y. Inhibitory effect of curcumin on proliferation of K562 cells involves down-regulation of p210bcr/abl-initiated Ras signal transduction pathway. *Acta Pharmacol Sin* 2003; 24: 1155–60.
- 10 Mariadason JM, Corner GA, Augenlicht LH. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer.

- Cancer Res 2000; 60: 4561-72.
- 11 Chen W, Chen Y, Gu J, He J. Effects of curcumin on proliferation of K562 cells involves STAT5 signal transduction pathway. *Chin J Hematol* 2004; 25: 151-3.
 - 12 Zhou N, Zhu X, Zhou J, Li M, Zhang X, Huang P, *et al.* 2-Methoxyestradiol induces cell cycle arrest and apoptosis of nasopharyngeal carcinoma cells. *Acta Pharmacol Sin* 2004; 25: 1515-20.
 - 13 Balasubramanyam K, Varier RA, Altaf M, Swaminathan V, Siddappa NB, Ranga U, *et al.* Curcumin, a novel p300/CBP specific inhibitor of acetyltransferase, represses the acetylation of histones/nonhistone proteins and HAT dependent chromatin transcription. *J Biol Chem* 2004; 279: 51163-71.
 - 14 Sullivan SA, Landsman D. Characterization of sequence variability in nucleosome core histone folds. *Proteins* 2003; 52: 454-65.
 - 15 Hu E, Chen Z, Fredrickson T, Zhu Y, Kirkpatrick R, Zhang GF, *et al.* Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. *J Biol Chem* 2000; 20: 15254-64.
 - 16 Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, *et al.* Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. *Int J Cancer* 2003; 103: 572-6.
-

SBS 11th ANNUAL CONFERENCE AND EXHIBITION
DRUG DISCOVERY: From Targets to Candidates

September 11-15, 2005

Geneva, Switzerland

Info: The Society for Biomolecular Screening

36 Tamarack Avenue, #348

Danbury, CT 06811, USA

Phn 1 (203) 743-1336

Fax 1 (203) 748-7557

E-Mail email@sbsonline.org

[Http://www.sbsonline.org/sbscon/2005/index.php](http://www.sbsonline.org/sbscon/2005/index.php)