School of Life Sciences[1], Lanzhou University, Lanzhou, China; Hematological Center of PLA[2], Department of Hematology, the General Hospital of Lanzhou, Lanzhou, China

Quercetin and trichostatin A cooperatively kill human leukemia cells

Jie Chen[2], Jiu-hong Kang[1]

Received December 21, 2004, accepted February 2, 2005

Dr. Jiu-hong Kang, School of Life Sciences, Lanzhou University, Lanzhou, 730000, China
kangjiuhong@lzu.edu.cn

Pharmazie 60: 856–860 (2005)

Quercetin (QU) and trichostatin A (TSA) are promising anticancer drugs. While QU mainly exerts its anticancer activity through scavenging reactive oxygen species (ROS), the anticancer activity of TSA was attributed to its inhibition on histone deacetylases (HDAC). In the present study it was investigated, whether the combination of QU and TSA could improve their anticancer activity against human leukemia cells (HL-60). The cytotoxicity of QU and TSA increased in a time and dose-dependent manner. QU (10, 20 and 40 μM) was able to diminish the ROS generation (indicated by the level of malondialdehyde, MDA) but showed no influence on the histone acetylation in HL-60 cells; on the contrary, TSA (20, 40, 80 and 160 nM) showed no inhibition on ROS generation but significantly increased the histone acetylation, indicating the possible role of both scavenging ROS and increasing histone acetylation in the induction of cell death in HL-60 cells. This conclusion was confirmed by the findings that the combinations of QU and TSA at different concentrations could not only diminish ROS generation, but also increase histone acetylation, and hence showed more significant cytotoxicity in HL-60 cells than either of its components. Collectively, the present data indicate that a combination of QU and TSA can cooperatively kill HL-60 cells through the combination of their activities of scavenging ROS and increasing histone acetylation.

1. Introduction

Quercetin, a common dietary flavonoid found in onions, apples, and tea (Morand et al. 1998; Hollman et al. 1997), has been found to show anticancer activity in different cancer cells (Chen et al. 2004a, 2004b; Cipak et al. 2003; Kaneuchi et al. 2003; Chan et al. 2003; Lee et al. 2002; Feng et al. 2001; Liesveld et al. 2003; Kang et al. 1997; Uddin et al. 1995; Kuo et al. 2002; Kim et al. 2000; Kawaii et al. 1999). Like most polyphenols, QU is a naturally occurring antioxidant (Morand et al. 1998; Hollman et al. 1997; Duthie et al. 2000), and this antioxidant activity is critically involved in its anticancer activity (Chen et al. 2004a, 2004b; Duthie et al. 2000; Feng et al. 2001).
Recent studies found that histone hypoacetylation plays an important role in gene silencing and carcinogenesis (Archer et al. 1999; Klochendler-Yeivin et al. 2001; Lehrmann et al. 2002). Specifically, histone acetylation contributes to the formation of a transcriptionally competent environment by 'opening' chromatin and permits access of transcription factors to DNA (Fry et al. 2002; Grunstein 1997), whereas histone deacetylation contributes to a 'closed' chromatin state and transcriptional repression. The histone acetylation-deacetylation balance favors hypoacetylation in tumor cells (Archer et al. 1999), while it is accurately maintained through a balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities in normal cells (Archer et al. 1999; Klochendler-Yeivin et al. 2001). Thus increasing the acetylation of histones in tumor cells through inhibiting the activity of HDAC has been suggested as an efficient strategy to combat cancer, and HDAC inhibitors are thought to be promising both as single anti-cancer agents and in combination therapies (Marks et al. 2000; Hendersona et al. 2003; Yamashita et al. 2003; Donadelli et al. 2003; Rosato et al. 2003; Marks et al. 2001; Kim et al. 2003). Among the HDAC inhibitors, trichostatin A (TSA) has been studied well (Yamashita et al. 2003; Donadelli et al. 2003; Rosato et al. 2003; Marks et al. 2001).
Considering that QU and TSA exert their anticancer activity through different mechanisms, the combination of them may significantly improve their cytotoxicity to neoplastic cells. To address this hypothesis, the cytotoxicity of QU, TSA and their combination was studied in human leukemia HL-60 cells, and their effects on ROS generation (as indicated by the level of malondialdehyde, MDA) and histone acetylation were also evaluated.

2. Investigations, results and discussion

2.1. *Cytotoxicity of QU and TSA in human leukemia cells*

Treating cells with QU and TSA markedly decreased cell proliferation in a concentration- (Fig. 1A) and time-dependent manner (Fig. 1B, 1C). At the same time, the trypan blue exclusion assay showed that QU and TSA treatment significantly increased the trypan blue-stained cells, indicating the loss of cell membrane integrity and cell death (Fig. 1D), this increase of cell death also exhibited a time-dependent manner (Fig. 1E, 1F).

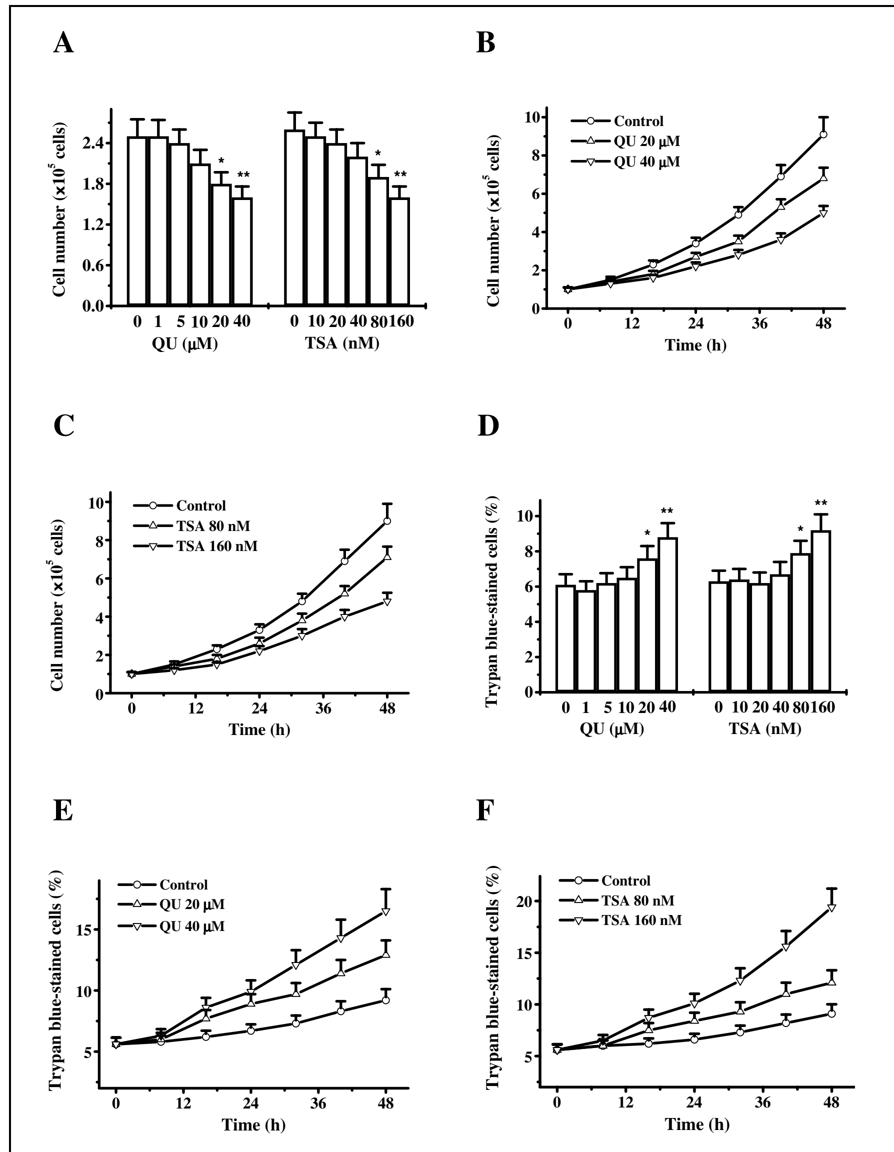

Fig. 1:
Effect of QU and TSA on the proliferation and viability of HL-60 cells. Cells were incubated with different concentrations of QU and TSA for 16 h in A, D; with 20 and 40 μM of QU or with 80 and 160 nM of TSA for the indicated times in B, C, E, F. Then cell number (A, B, C) and trypan blue-stained cells (D, E, F) were measured. Means ± SD of three parallel experiments was indicated, $n = 3 \times 3$ cultures per conditions $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group in A and D

2.2. Effect of QU and TSA on the ROS generation and histone acetylation

Since ROS and histone hypoacetylation are critically involved in the different stages of carcinogenesis (Deshpande et al. 2002; Archer et al. 1999; Klochendler-Yeivin et al. 2001; Lehrmann et al. 2002), both antioxidants and HDAC inhibitors are thought to be promising anti-cancer agents (Kang et al. 1999, 2000, 2004; Prasad et al. 1999; Drisko et al. 2003; Conklin 2000; Marks et al. 2000, 2001; Hendersona et al. 2003; Yamashita et al. 2003; Donadelli et al. 2003; Rosato et al. 2003; Kim et al. 2003). QU is a naturally occurring antioxidant (Morand et al. 1998; Hollman et al. 1997; Duthie et al. 2000), and this antioxidant activity plays important roles in its anticancer activity (Chen et al. 2004a, 2004b; Duthie et al. 2000; Feng et al. 2001), while TSA is a well-known HDAC inhibitor and has been found to exhibit cytotoxicity in

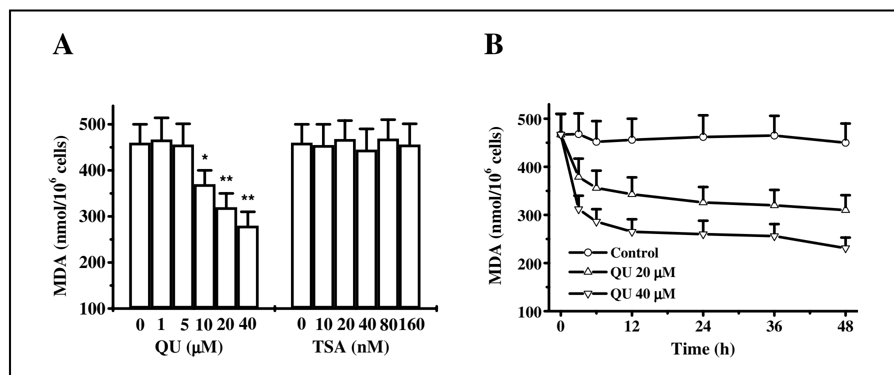

Fig. 2:
Effect of QU and TSA on lipid peroxidation in HL-60 cells. Cells were incubated with different concentrations of QU and TSA for 16 h in A, or with 20 and 40 μM of QU for the indicated times in B. Means ± SD of three parallel experiments was indicated, $n = 3 \times 3$ cultures per conditions, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control group in A

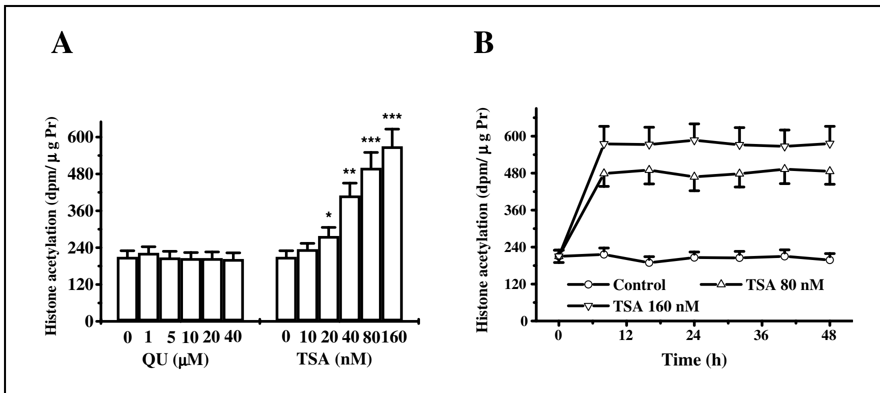

Fig. 3:
Effect of QU and TSA on histone acetylation in HL-60 cells. Cells were incubated with different concentrations of QU and TSA for 16 h in A, or with 80 and 160 nM of TSA for the indicated times in B. Means ± SD of three parallel experiments was indicated, $n = 3 \times 3$ cultures per conditions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the corresponding control group in A

different tumor cells (Yamashita et al. 2003; Donadelli et al. 2003; Rosato et al. 2003; Marks et al. 2001). Thus we detected whether QU and TSA affected the ROS generation and histone acetylation in HL-60 cells at the conditions where they exhibited cytotoxicity. For this purpose, according to Gutteridge et al. (1990), lipid peroxidation (LPO, as indicated by MDA content), resulting from the direct interaction between *in vivo* ROS and unsaturated fatty acids, was used as one representative of *in vivo* ROS accumulation. Fig. 2A shows that treating cells with gradually increased QU led to a concentration-dependent decrease in the concentration of MDA, while TSA showed no influence on ROS generation at all. Based on Fig. 2B, QU treatment caused a rapid, followed by a sustained decrease in the amount of MDA in HL-60 cells. On the contrary, when the state of histone acetylation was evaluated in differently treated cells, QU had no obvious influence on the acetylation of histones (Fig. 3A), while TSA significantly increased the histone acetylation in HL-60 cells in a dose-dependent manner (Fig. 3A). As indicated by Fig. 3B, TSA caused a rapid, followed by a sustained increase in histone acetylation. Collectively, these results suggest that QU and TSA kill HL-60 cells via different mechanisms, i.e., QU via its antioxidant activity, while TSA via its HDAC inhibition activity.

2.3. Combination of QU and TSA improved their cytotoxicity in HL-60 cells

Since QU and TSA exert their anticancer activity through different mechanisms in HL-60 cells, it was investigated whether the combination of them could cooperatively kill HL-60 cells. For this purpose, the effect of QU and TSA combinations on ROS generation and histone acetylation was determined. As expected, the combinations of QU and TSA at different concentrations not only significantly attenuated the ROS generation (Fig. 4A), but also significantly increased the histone acetylation (Fig. 4B). Specifically, the QU and TSA combinations showed similar activity in scavenging ROS with different concentrations of QU alone and similar activity in increasing histone acetylation with the TSA alone, indicating that their combinations did not weaken the ROS scavenging activity of QU or the inhibition activity of TSA on HDAC (Fig. 4A, 4B). In other words, these combinations possessed both

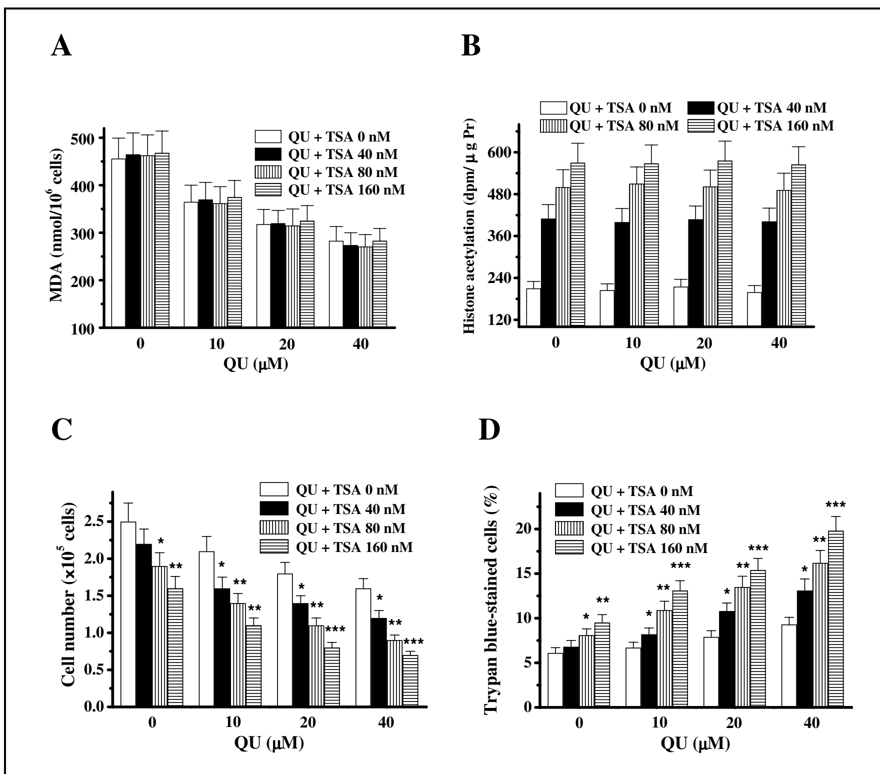

Fig. 4:
ROS scavenging, histone acetylation increasing and anticancer activity of QU and TSA combinations. Cells were treated with different concentrations of QU alone, or the combinations of QU with different concentrations of TSA for 16 h. The effect of QU and TSA combinations on ROS generation (A), histone acetylation (B), cell proliferation (C), and the trypan blue penetration (D) was examined. Means of three parallel experiments are displayed (means ± SD), $n = 3 \times 3$ cultures per conditions, *, **, *** indicates $p < 0.05$, $p < 0.01$, $p < 0.001$ as compared with the corresponding QU alone group

the characteristic of QU and the activity of TSA. Moreover, these combinations of QU and TSA significantly improved each of their effect on the proliferation arrest (Fig. 4C) and trypan-blue penetration (Fig. 4D), indicating the cooperative anticancer activity of QU and TSA.
In summary, although additional experiments are needed to clarify the detailed mechanism on the anticancer activity of QU, TSA and their combinations, our present study proved that 1), in human leukemia cells, QU and TSA exert their anticancer activity through scavenging ROS or increasing histone acetylation, respectively; 2), the combination of QU and TSA did not weaken either the ROS scavenging activity of QU or the HDAC inhibition activity of TSA; 3) combining these compounds is a promising strategy to improve the anticancer activity of QU and TSA. Given the fact that polyphenols with similar structures possess similar antioxidant activity (Wang et al. 1996), our present data may also suggest a good strategy to improve the anticancer activity of other polyphenols with structures similar to QU.

3. Experimental

3.1. Reagents

Quercetin, trichostatin A, trypsin, trypan blue, 1,1,3,3-tetramethoxypropane and thiobarbituric acid were purchased from Sigma (Sigma, St. Louis, MO), RPMI-1640 was purchased from Gibco (Gibco, Santa Clara, CA). All other reagents are of analytical grade.

3.2. Cell culture and treatment

Human leukemia cells (HL-60) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (equivalent to 100 units/ml and 100 mg/ml, respectively) at 37 °C in a humidified atmosphere containing 5% CO_2. After culturing the cells (1×10^5 cells/ml) for 24 h, the culture medium was aspirated and replaced with new medium containing quercetin (QU) and/or trichostatin A (TSA) where indicated. After that, the different cultures were replaced with fresh medium containing the corresponding reagents every 24 h.

3.3. Determination of cell proliferation and viability

Cells at a concentration of 1×10^5/ml were cultured for 24 h, then treated with QU and/or other agents at the indicated concentrations, three dishes for each of the differently treated cells were collected every 8 h in the first 2 days, the total and dead cells were counted respectively by using the trypan blue stain exclusion method under a phase-contrast microscope.

3.4. The malondialdehyde (MDA) assay

After different treatment, HL-60 cells were collected and suspended in the lysis buffer (Kang et al. 2003). And the suspension was collected after pipetting the cell mixture up and down for 20 times. MDA amount in the obtained suspension was immediately evaluated using a colorimetric assay (Ohkawa et al. 1979). All the analyses were performed in triplicate.

3.5. Histone purification and histone acetylation assay

Cells were plated at a density of 2×10^5 cells/ml, exposed to QU and/or TSA as indicated in the presence of 10 μCi/ml [^{3}H] acetate (5.0 Ci/mmol) for the indicated times. Preparation of histones from HL-60 cells was done according to Cousens et al. (1979) with the following modifications: the washed cells were suspended in lysis buffer (Cousens et al. 1979) containing TSA (100 ng/ml) and PMSF (1 mM). After pipetting up and down for 20 times, the nuclei were washed three times in the lysis buffer and once in 10 mM Tris and 13 mM EDTA (pH 7.4). The histones were extracted from the pellet in 0.4 N H_2SO_4. After centrifugation, the histones in the supernatant were collected by cold-acetone precipitation, air-dried, then suspended in 4 M urea and stored at −20 °C before use. ^{3}H-labelled histones were determined by liquid scintillation counting.

3.6. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA post-hoc Bonferroni), and $p < 0.05$, 0.01, or 0.001 were denoted as *, **, or ***, respectively.

References

Archer SY, Hodin RA (1999) Histones acetylation and cancer. Curr Opin Gene Devel 9: 171–174.
Chan MM, Fong D, Soprano KJ, Holmes WF, Heverling H (2003) Inhibition of growth and sensitization to cisplatin-mediated killing of ovarian cancer cells by polyphenolic chemopreventive agents. J Cell Physiol 194: 63–70.
Chen J, Ou YX, Da WM, Kang JH (2004a) Coadjustment of quercetin and hydrogen peroxide: the role of ROS in the cytotoxicity of quercetin. Pharmazie 59: 155–158.
Chen J, Kang JH, Da WM, Ou YX (2004b) Combination with water-soluble antioxidants increases the anticancer activity of quercetin in human leukemia cells. Pharmazie 59: 859–863.
Cipak L, Rauko P, Miadokova E, Cipakova I, Novotny L (2003) Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. Leuk Res 27: 65–72.
Conklin KA (2000) Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. Nutr Cancer 37: 1–18.
Cousens LS, Gallwitz D, Alberts BM (1979) Different accessibilities in chromatin to histone acetylase. J Biol Chem 254: 1716–1723.
Deshpande SS, Irani K (2002) Oxidant signalling in carcinogenesis: a commentary. Hum Exp Toxicol 21: 63–64.
Donadelli M, Costanzo C, Faggioli L, Scupoli MT, Moore PS, Bassi C, Scarpa A, Palmieri M (2003) Trichostatin A, an inhibitor of histone deacetylases, strongly suppresses growth of pancreatic adenocarcinoma cells. Mol Carcinogen 38: 59–69.
Drisko JA, Chapman J, Hunter VJ (2003) The use of antioxidant therapies during chemotherapy. Gynecol Oncol 88: 434–439.
Duthie GG, Duthie SJ, Kyle JAM (2000) Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr Res Rev 13: 79–106.
Feng Q, Kumagai T, Torii Y, Nakamura Y, Osawa T, Uchida K (2001) Anticarcinogenic antioxidants as inhibitors against intracellular oxidative stress. Free Radic Res 35: 779–788.
Fry CJ, Peterson CL, (2002) Unlocking the gates to gene expression. Science 295: 1847–1848.
Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389: 349–352.
Gutteridge JMC, Halliwell B (1990) The mesurement and mechanism of lipid peroxidation in biological system. Trends Biochem Sci 15: 129–135.
Hendersona C, Brancolini C (2003) Apoptotic pathways activated by histone deacetylase inhibitors: implications for the drug-resistant phenotype. Drug Resist Updates 6: 247–256
Hollman PC, Van Trijp JM, Mengelers MJ, de Vries JH, Katan MB (1997) Bioavailability of the dietary antioxidant flavonol quercetin in man. Cancer Lett 114: 139–140.
Kaneuchi M, Sasaki M, Tanaka Y, Sakuragi N, Fujimoto S, Dahiya R (2003) Quercetin regulates growth of Ishikawa cells through the suppression of EGF and cyclin D1. Int J Oncol 22: 159–164.
Kang J, Chen J, Zhang D, Da W, Ou Y (2004) Synergistic killing of human leukemia cells by antioxidants and trichostatin A. Cancer Chemother Pharmacol 54: 537–545.
Kang J, Shi Y, Zheng R (1999) Effects of ascorbic acid on human hepatoma cell proliferation and redifferentiation. Acta Pharmacol Sin 20: 1019–1024.
Kang JH, Shi YM, Zheng RL (2000) Effects of ascorbic acid and DL-alpha-tocopherol on human hepatoma cell proliferation and redifferentiation. Acta Pharmacol Sin 21: 348–352.
Kang J, Zhang Y, Chen J, Chen H, Lin C, Wang Q, Ou Y (2003) Nickel-induced histone hypoacetylation: the role of reactive oxygen species. Toxicol Sci 74: 279–286.
Kang TB, Liang NC (1997) Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. Biochem Pharmacol 54: 1013–1018.
Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M (1999) Effect of citrus flavonoids on HL-60 cell differentiation. Anticancer Res 19(2A): 1261–1269.
Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63: 7291–7300.
Kim SY, Gao JJ, Kang HK (2000) Two flavonoids from the leaves of Morus alba induce differentiation of the human promyelocytic leukemia (HL-60) cell line. Biol Pharm Bull. 23: 451–455.
Klochendler-Yeivin A, Yaniv M, (2001) Chromatin modifiers and tumor suppression. Biochim Biophys Acta 1551: M1–M10.
Kuo HM, Ho HJ, Chao PD, Chung JG (2002) Quercetin glucuronides inhibited 2-aminofluorene acetylation in human acute myeloid HL-60 leukemia cells. Phytomedicine 9: 625–631.
Lee LT, Huang YT, Hwang JJ, Lee PP, Ke FC, Nair MP, Kanadaswam C, Lee MT (2002). Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells. Anticancer Res 22: 1615–1627.

Lehrmann H Pritchard LL Harel-Bellan A (2002) Histone acetyltransferases and deacetylases in the control of cell proliferation and differentiation. Adv Cancer Res 86: 41–65.
Liesveld JL, Abboud CN, Lu C, McNair C, Menon A, Smith A, Rosell K, Rapoport AP (2003) Flavonoid effects on normal and leukemic cells. Leuk Res 27: 517–527.
Marks PA, Richon VM, Rifkind RA (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Nat Cancer Inst 92: 1210–1216.
Marks PA, Richon VM, Breslow R, Rifkind RA (2001) Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 13: 477–483
Morand C, Crespy V, Manach C, Besson C, Demigné C, Rémésy C (1998) Plasma metabolites of quercetin and their antioxidant properties. Am J Physiol 275: R212–219.
Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351–358.
Prasad KN, Kumar A, Kochupillai V, Cole WC (1999) High doses of multiple antioxidant vitamins: essential ingredients in improving the efficacy of standard cancer therapy. J Am Coll Nutr 18: 13–25.
Rosato RR, Grant S (2003) Histone deacetylase inhibitors in cancer therapy. Cancer Biol Ther 2: 30–37.
Uddin S, Choudhry MA (1995) Quercetin, a bioflavonoid, inhibits the DNA synthesis of human leukemia cells. Biochem Mol Biol Int 36: 545–550.
Wang PF, Kang JH, Zheng RL, Lu JF, Yang ZF, Jia ZJ, Gao JJ (1996) Scavenging effects of phenylpropanoid glycosides from Pedicularis on superoxide anion and hydroxyl radical by the spin trapping method. Biochem Pharmacol 51: 687–691.
Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, Sugimachi K (2003) Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 103: 572–576.