State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P. R. China

Apoptosis induction and G2/M arrest of 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone from *Rubia yunnanensis* in human cervical cancer HeLa cells

GUANG-ZHI ZENG, JUN-TING FAN, JUN-JU XU, YAN LI, NING-HUA TAN

Received September 29, 2012, accepted November 3, 2012

Ning-Hua Tan, State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, P. R. China
nhtan@mail.kib.ac.cn

Pharmazie 68: 293–299 (2013) *doi: 10.1691/ph.2013.2808*

2-Methyl-1,3,6-trihydroxy-9,10-anthraquinone (MTA), one of the major components isolated from the traditional Chinese medicine *Rubia yunnanensis*, exhibited inhibitory activity on the proliferation of several human cancer cell lines. The results from an annexin V-FITC (fluoresein-5-isothiocyanate) apoptosis assay and DNA content analysis showed that MTA exerted cytotoxicity via apoptosis induction and G2/M cell cycle arrest in human cervical carcinoma HeLa cells. Further, MTA was found to induce apoptosis of HeLa cells through the mitochondria-mediated pathway. It caused the translocation of Bax to the mitochondria and release of cytochrome c into the cytosol, which caused the cleavage of caspase and poly(ADP-ribose) polymerase and finally triggered the apoptosis. Furthermore, the p53/p21/Cdc2-cyclin B1 signaling was found related to the G2/M arrest caused by MTA. The over-expression of p21 and down-expression of cyclin B1 caused by MTA inactivated the Cdc2-cyclin B1 complex of G2/M checkpoint and finally caused the G2/M arrest in HeLa cells. This study demonstrated that MTA is a potential anti-cancer component of *R. yunnanensis*, a folk anti-cancer herb used in Yunnan, China.

1. Introduction

Cancer is one of the leading causes of death worldwide, and cervical cancer is the leading cause of cancer death in women, especially in developing nations (Maxime et al. 2007; Nour 2009; Schiffman et al. 2011). Apoptosis is an important mechanism in adult tissues for the removal of superfluous or damaged cells. However, unbalanced apoptosis contributes to a variety of pathologies, including cancer (Maxime et al. 2007). The cell cycle is an ordered set of events that occur in a dividing cell and is regulated by checkpoints that are capable of delaying or stopping the cell cycle in response to intra- and extracellular stressors (Bucher and Britten 2008). The dysregulation of the cell cycle results in uncontrolled cell proliferation, such as cancer (Collins et al. 1997). Thus, the induction of apoptosis and the cell cycle arrest are regarded as potentially effective strategies for the elimination of cancer cells (Buolamwini 2000; Blankenberg 2009; Zivny et al. 2010).

Roots from *Rubia yunnanensis* (Franch.) Diels (Rubiaceae) have been used to treat illnesses such as tuberculosis, menoxenia, rheumatism and cancer in China (Lan 1976; Fan et al. 2010; Yang 2000). Three major types of components were isolated from this plant: arboriane-type triterpenoids, quinones and Rubiaceae-type cyclopeptides (RAs). RAs are the characteristic constituents of plants of the Rubiaceae family (Fan et al. 2010). Two components from *Rubia* species have been studied as drug candidates. RA-VII from *R. cordifolia* has been used as a potential anti-cancer candidate in phase I clinical trials in Japan and the USA (Tan and Zhou 2006; Itokawa et al. 1984a, b). Rubiadate, the synthetic derivative of 2-naphthalenecarboxylic acid, a naphthoquinone from *R. cordifolia*, has been used as a leukogenic agent in China for the treatment of leucopenia caused by cancer chemotherapy (Yang and Liu 1997).

Rubiarbonol G, 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone (MTA) (Fig. 1) and RA-V are representatives of the three major components in *R. yunnanensis*. Our previous study demonstrated that these three compounds showed cytotoxicity, with RA-V exhibiting the most potent activity (Fan et al. 2011). Additionally, we elucidated the cytotoxic mechanisms of RA-V (Fan et al. 2010; Yue et al. 2011) and rubiarbonol G (data unpublished). MTA has been reported to have inhibitory activities on phosphatase of regenerating liver-3, platelet aggregation and the release of β-hexosaminidase (Wu et al. 2003; Chung et al. 1994; Tao et al. 2003; Moon et al. 2010), but there has been no report regarding its cytotoxic mechanism. Here, we provide the first report on the cytotoxic mechanism of MTA.

2. Investigations and results

2.1. MTA inhibits the growth of four cancer cell lines

Results from the cytotoxicity assay showed that MTA inhibited cell growth of these four cancer cell lines, the IC_{50} values were 45.39, 38.56, 42.27 and 59.57 μM for HeLa, A549, BEL-7402 and MDA-MB-231 cells, respectively (Fig. 1A). For

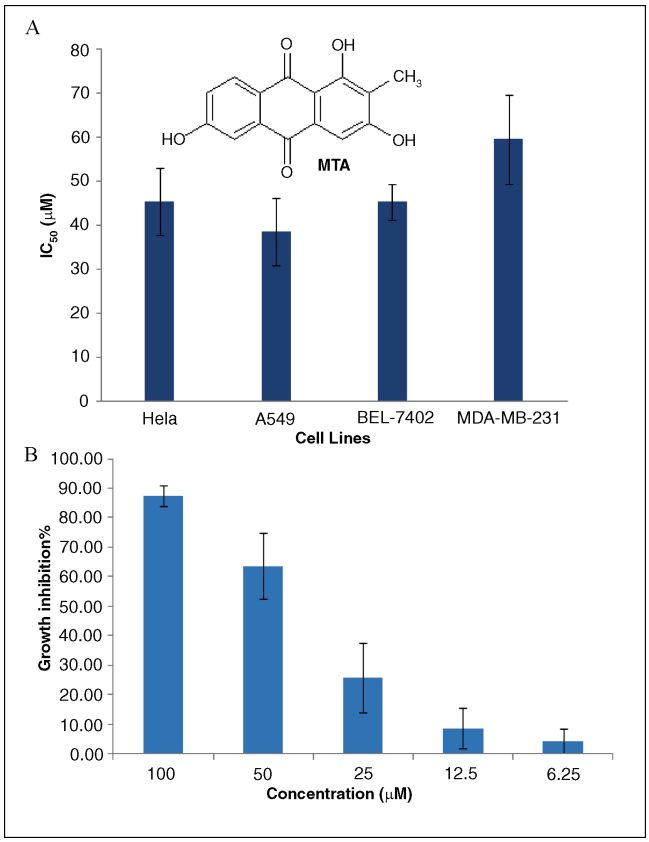

Fig. 1: Chemical structure of 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone (MTA) and the cytotoxicity results. (A) Chemical structure of MTA and its cytotoxicity on HeLa, A549, BEL-7402 and MDA-MB-231 cancer cells. (B) concentration-dependent inhibition of MTA on the proliferation of HeLa cell. Data are the mean ± SD values from at least three independent experiments

HeLa cells, the cell growth was significantly inhibited in a concentration-dependent manner between the concentration of 6.25 and 100 μM (Fig. 1B). The cytotoxic mechanism of MTA in HeLa cells was studied in the following experiments.

2.2. MTA induces apoptosis in HeLa cells

The results from the FITC annexin V apoptosis assay demonstrated that treatment with MTA for 24 h increased both the annexin V-FITC- and annexin V-FITC/PI-positive cells, indicating that MTA treatment acts in both early and late stages of apoptosis in HeLa cells (Fig. 2). MTA caused apoptosis in a concentration-dependent manner, in concentrations of 25, 50 and 100 μM. After treatment with 100 μM MTA for 24 h, the percentage of annexin V-positive and annexin V/PI-positive cells increased from 3.10% to 9.70%; and from 5.87% to 25.45%, respectively. The results indicated that MTA in high concentration significantly inhibited the growth of HeLa cells by inducing apoptosis.

2.3. MTA causes the G2/M arrest of HeLa cells

As shown in Fig. 3, after the MTA treatment, the number of cells in the G2/M phase increased in a concentration-dependent manner. Following MTA treatment of 25, 50 and 100 μM for 24 h, the percentages of the cells in the G2/M phase increased from 23.21% to 25.34%, 48.94%, 62.24%, and the cells in G0/G1 phase decreased from 45.19% to 38.16%, 28.90%, 19.87%, respectively. Moreover, treatment with 100 μM MTA led to an accumulation of sub-G1-hypodiploid cells (Fig. 3A), which is an indicator of apoptotic cells with fragmented DNA.

2.4. Effects of MTA on the expression of apoptosis-/cell cycle-related proteins

MTA significantly induced the apoptosis of HeLa cells in a dose-dependent manner (Fig. 2). To determine the potential mechanism of MTA-induced apoptosis, the expression of several apoptosis-related proteins were detected by western blotting. The results showed that the expression levels of Bcl-2 were down-regulated by MTA treatment not after 24 h (Fig. 4A-B) but after 48 h (data not shown). We further determined the expression levels of Bax and cytochrome c by extracting the mitochondrial protein and cytoplasmic protein from MTA-treated HeLa cells. As shown in Fig. 4, the levels of Bax in the mitochondria and the levels of cytochrome c in the cytosol were up-regulated after 100 μM treatment for 24 h, while no obvious changes of the total proteins were observed. Therefore, MTA-induced apoptosis may be attributed to the negative modulation of Bcl-2/Bax ratio and the transactivation of Bax in mitochondria. Additionally, after MTA treatment for 24 h, levels of caspase 3, caspase 9, and PARP expression of HeLa cells were determined. Dose-dependent proteolytic cleavage of caspase 3, caspase 9, and PARP were detected (Fig. 4C-E). These results indicate that MTA induces apoptosis in HeLa cells through the intrinsic mitochondria-dependent pathway.

MTA potently caused the G2/M arrest in HeLa cells in a dose-dependent manner (Fig. 3), the expression of cell cycle-related proteins cyclin B1, Cdc2 and Cdc25C were determined. The results indicate that the MTA treatment caused a dose-dependent reduction in the levels of cyclin B1, phospho-Cdc25C(S216) and phospho-Cdc2(Y15) (Fig. 5). Moreover, 100 μM MTA induced apparent p53 protein expression in HeLa cells, 1.7-folds of control levels at 24 h. This p53 activation was related to the increased expression of the cyclin-dependent kinase inhibitor p21 (Fig. 5). These results suggest that p53/p21/cdk1-cyclinB1 signaling may be involved in MTA-mediated G2/M arrest in HeLa cells.

3. Discussion

Natural products have been regarded as important sources of potential chemotherapeutic agents in cancer therapy. In this study, we found that MTA, a major anthraquinone in *R. yunnanensis*, exhibited cytotoxicity on four human cancer cell lines (Fig. 1A). The investigation of its cytotoxic mechanism indicated that the inhibition of HeLa cell proliferation may be attributed to the apoptosis inducement and G2/M cell cycle arrest (Fig. 2 and Fig. 3).

The Bcl-2 family of proteins comprises pivotal regulators of apoptotic cell death. The family contains two subgroups, the anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL and Mcl-1) and the pro-apoptotic proteins (e.g., Bax, Bak, and Bid) (Zinkel et al. 2006; Straten and Andersen 2010; Martinou and Youle 2011). A slight change in the dynamic balance of these proteins may result in either the inhibition or promotion of cell death (Ola et al. 2011). Bcl-2 was found to be over-expressed in many cancer cells (Straten and Andersen 2010), and Bax is demonstrated to antagonize Bcl-2 function by forming Bax/Bcl-2 heterodimers (Manion and Hockenbery 2003). Moreover, upon apoptotic stimulation, Bax translocates from the cytosol to the mitochondrial membrane, which increases the membrane's permeability and leads to the release of cytochrome c from mitochondria into cytosol. The release of cytochrome c initiates the apoptotic caspase cascade (Jurgensmeier et al. 1998; Narita et al. 1998). In this study, we evaluated the protein expression of Bcl-2 and Bax. The results show that levels of Bax expression in mitochondria increased after MTA treatment (Fig. 4A, 4F). The levels of cytochrome c were also analyzed, and the increased expres-

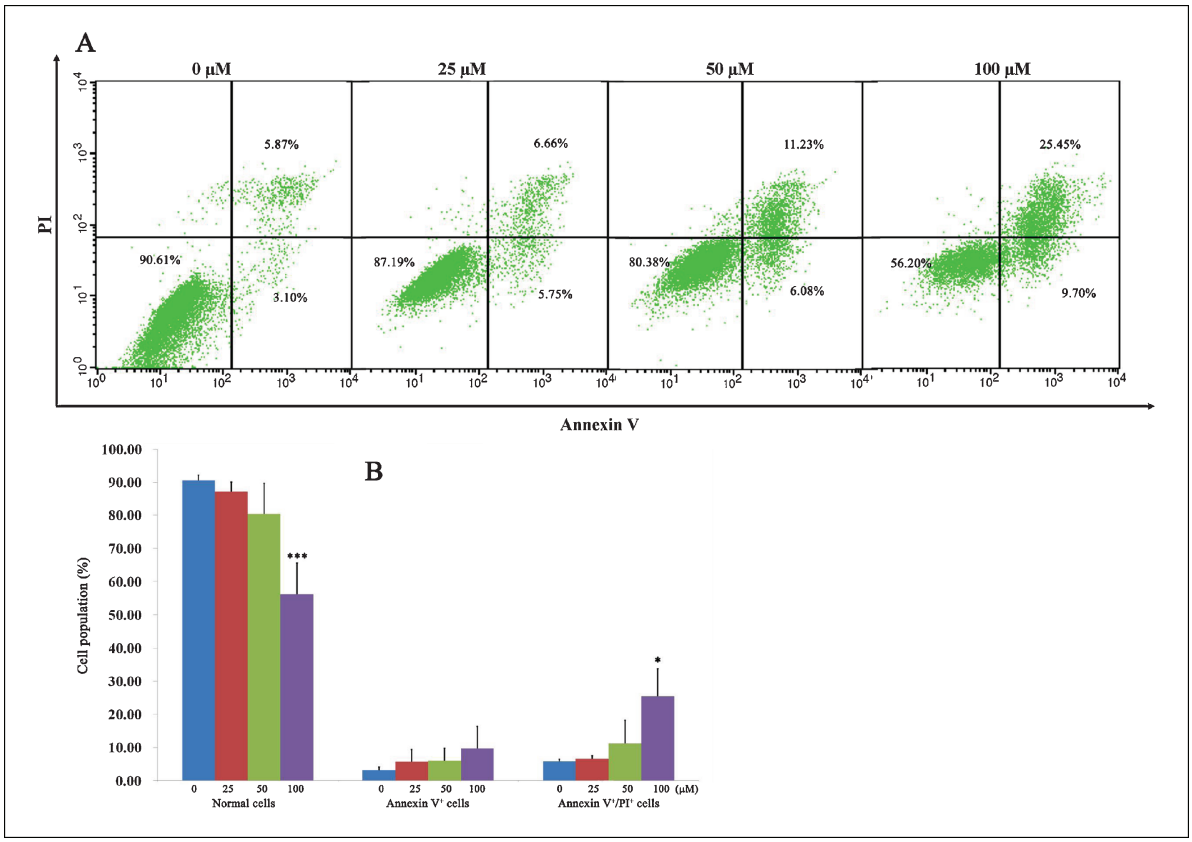

Fig. 2: The results of annexin V-FITC/PI double staining in HeLa cells after MTA treatment. (A) After treatment with 0, 25, 50 and 100 μM MTA for 24 h, the percentage of annexin V-FITC-positive and annexin V-FITC/PI-positive cells increased from 8.97% to 12.41%, 17.31% and 35.15%, respectively. (B) The change in cell populations after treatment with 0, 25, 50 and 100 μM MTA for 24 h. Data are the mean ± SD values from three independent experiments. $^{*}P < 0.05$ significantly different from the control group (0 μM MTA)

sion of cytochrome c in the cytosol was detected (Fig. 4A, 4G). Additionally, the proteolytic cleavage of caspase 3, caspase 9, and PARP were also detected after MTA treatment (Fig. 4C-E). All these results suggest that MTA changes the balance between Bax and Bcl-2 and causes Bax translocation to the mitochondria, which leads to the release of cytochrome c into the cytosol. The released cytochrome c associates with the 46KDa caspase 9 in cytosol and mediates the activation of caspase 9. The activated-caspase 9 (35KDa) initiates the caspase cascade and leads to the cleavage of poly(ADP-ribose) polymerase (PARP) which ultimately induces apoptosis in HeLa cells. All these results indicate that MTA induces apoptosis in HeLa cells through the intrinsic mitochondria-dependent pathway.

An ordered cell cycle progression is regulated by cell cycle checkpoints. The complexes formed by cyclin-dependent kinases (Cdks) and their regulatory partners, cyclins, are responsible for governing the checkpoints in a cell division cycle (Bucher and Britten 2008; Wohlbold and Fisher 2009; Collins and Garrett 2005). The Cdc2/cyclin B1 (Cdc2 also designated as Cdk1) complex is a key regulator involved in the G2/M checkpoint to control the entry into mitosis. The activation of the Cdc2/cyclin B1 complex is dependent on the phosphorylation of Thr161 and the dephosphorylation of Thr14 and Tyr15 of Cdc2, which are catalyzed by Cdk-activating kinase and Cdc25C, respectively (Aressy and Ducommun 2008; Smits and Medema 2001). In a cell cycle progression, the activated Cdc25C (dephosphorylated at Ser216) triggers the activation of Cdc2/cyclin B1 and allows the cells to enter into mitosis (Aressy and Ducommun 2008; Chu and Liu 2009; Hermeking and Benzinger 2006). In this study, MTA treatment notably caused G2/M arrest in HeLa cells (Fig. 3); the western blot results show that the protein expression levels of cyclin B1, phospho-Cdc2(Y15) and phospho-Cdc25C(S216) decreased after treatment. Meanwhile, no significant changes of total Cdc2 and Cdc25C were observed (Fig. 5A-D). We suggest that the down-regulation of cyclin B1 played a major role in the G2/M arrest caused by MTA, though Cdc2 is activated by MTA treatment. Because Cdks are generally abundant throughout the cell cycle, but cyclins are transiently expressed at the appropriate period of the cell cycle. In human cells, cyclin B is synthesized from the end of the S phase, and its level increases steadily in the G2 phase. The regulated synthesis and destruction of cyclins control the activation of the Cdk/cyclin complex (Smits and Medema 2001; Pines and Hunter 1989). Hence, it is reasonable to suppose that reduction of cyclin B1 induced by MTA blocked the cells entry into mitosis.

The p53 protein plays a central role in the regulation of apoptosis and cell cycle in response to a broad range of stresses such as oncogene activation, hypoxia, and DNA damage induced by various cytotoxic agents (Soengas et al. 1999). Upon DNA damage stimuli, p53's transcription factor activities are induced, resulting in the regulation of down-stream target genes, such as the cyclin-dependent kinase inhibitor p21 and pro-apoptotic Bcl-2 family proteins, which function in pathways of cell cycle regulation, apoptosis and DNA repair (Basu and Haldar 1998; Liu and Kulesz-Martin 2001). In this study, MTA induced the strong p53 expression in HeLa cells, which was followed by an increased expression of p21 (Fig. 5A). p21 is a universal inhibitor of cyclin-dependent kinases, it associates with Cdk/cyclin complexes and inhibits their activity and prevents cell cycle progression (Weiss 2003). The results suggest that p53/p21/Cdc2-cyclin B1 signaling may be involved in MTA induced G2/M cell cycle arrest, and the over-expression of p21 and down-expression of cyclin B1 are responsible for the arrest.

Upon DNA damage, p53 is accumulated and activated to induce the cell cycle arrest to allow for DNA repair and the induction

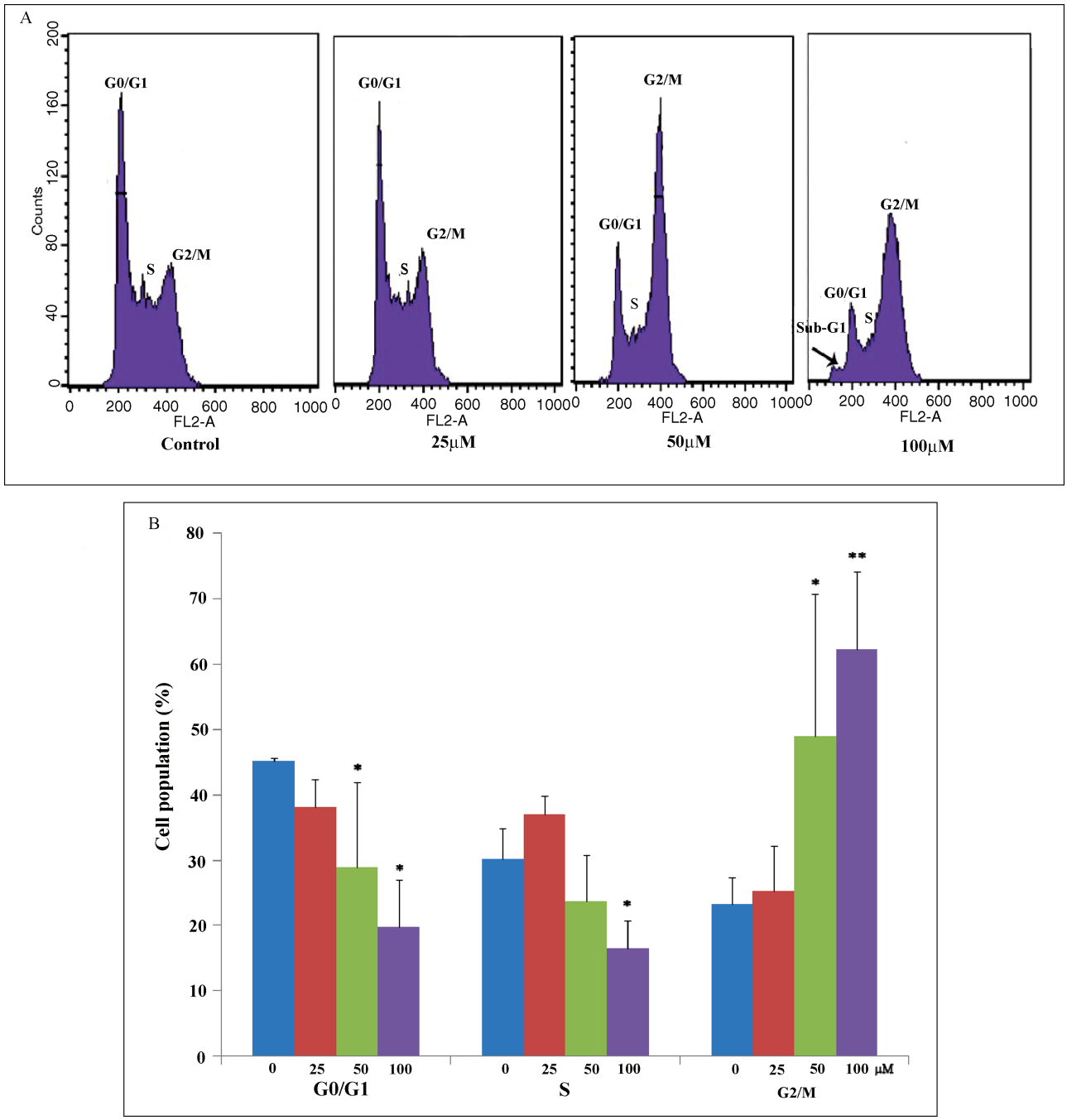

Fig. 3: MTA induced G2/M arrest in HeLa cells. (A) Results from DNA content analysis. After treatment with 0, 25, 50 and 100 μM MTA for 24 h, the percentage of G2/M cells increased from 23.21% to 25.34%, 48.95% and 62.24%, respectively. The cells in sub-G1 significantly increased after treatment with 100 μM MTA. (B) Cell cycle distributions after treatment with 0, 25, 50 and 100 μM MTA for 24 h. Data are the mean ± SD values from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the control group

of apoptosis for cases where the DNA damage is too severe to be repaired properly (Basu and Haldar 1998). In addition to the regulation of p21 for cell cycle, p53 promotes apoptosis in response to cellular stresses by regulation of the Bcl-2 proteins. It is reported that p53 could down-regulate Bcl-2 expression and transactivate Bax, which induces the release of cytochrome c, thereby activating the intrinsic apoptosis pathway (Basu and Haldar 1998; Schuler et al. 2000). In this study, MTA caused down-expression of Bcl-2, transactivation of Bax in mitochondria, and the release of cytochrome c into the cytosol, and finally the activation of caspase 9 and caspase 3 (Fig. 4). Hence, it is supposed that MTA induces apoptosis of HeLa cells in a way of p53-mediated caspase activation. It is reported that p53 can mediate the apoptosis and cell cycle arrest separately (Abrahamson et al. 1995). Thus, the tansactivation of Bax and the over-expression of p21 caused by enhanced p53 expression after MTA treatment for 24 h suggest the possibility that MTA induces apoptosis and cell cycle arrest independently.

In conclusion, this study demonstrated that the mechanism of MTA in HeLa cells is associated with the intrinsic mitochondria-dependent apoptosis inducement and p53/p21/Cdc2-cyclin B1 signaling mediated G2/M cell cycle arrest. As one of the major constituents of *R. yunnanensis*, MTA plays an important role in the clinical use of this anti-cancer herb.

4. Experimental

4.1. Plant material and extraction

Roots of *R. yunnanensis* were purchased from Yunnan Lv-Sheng Pharmaceutical Co. Ltd., in Kunming, China and identified by Prof. Su-Gong

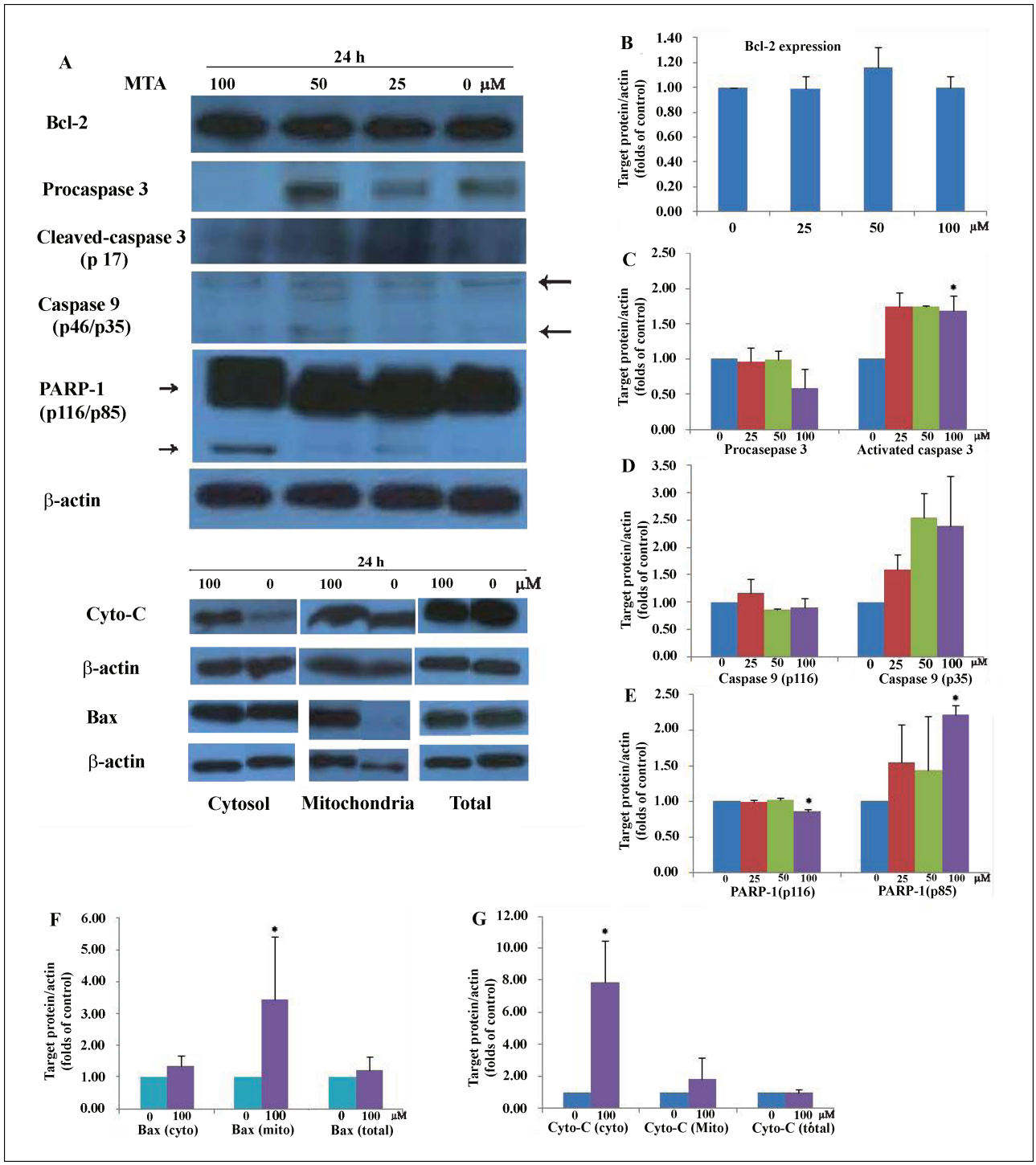

Fig. 4: Results from the western blot analysis. Cells treated with 0, 25, 50 and 100 μM MTA for 24 h were lysed, and the lysates were subjected to western blot analysis with antibodies against Bcl-2, Bax, cytochrome c, procaspase 3, activated caspase 3, caspase 9 and PARP. β-actin was used as the loading control. (A) Representative immunoblots showed the effects of MTA treatment on the protein expression of HeLa cells. Actin expression was determined to confirm equal protein loading. (B, C, D, F, G) The quantified results of protein levels, which were adjusted with corresponding β-actin protein level and expressed as fold of control. "Total" means the protein of the whole cell lysate, "mito" means the protein of the mitochondrial lysate, "cyto" means the protein of the cytosolic lysate. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the control group

Wu at the Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No.Wu20070905) was deposited at the Herbarium of Kunming Institute of Botany. The detailed extraction and identification of 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone were described in our previous publication (Fan et al. 2011).

4.2. Cell culture

The cancer cell lines, HeLa (human cervical cancer), A549 (human lung cancer), MDA-MB-231 (human breast cancer) and BEL-7402 (human liver cancer), were obtained from the Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China) and Wuhan University (Wuhan, China). Cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Tianjin Hao-Yang Biological Manufacture Co. LTD., Tianjin, China) in a humidified incubator with 5% CO_2 at 37 °C.

4.3. Cytotoxicity assay

The cancer cell lines (HeLa, A549, BEL-7402 and MDA-MB-231) were treated with five concentrations (100, 50, 25, 12.5 and 6.25 μM) of MTA for 48 or 72 h (for BEL-7402), and the percentage of cell viability was

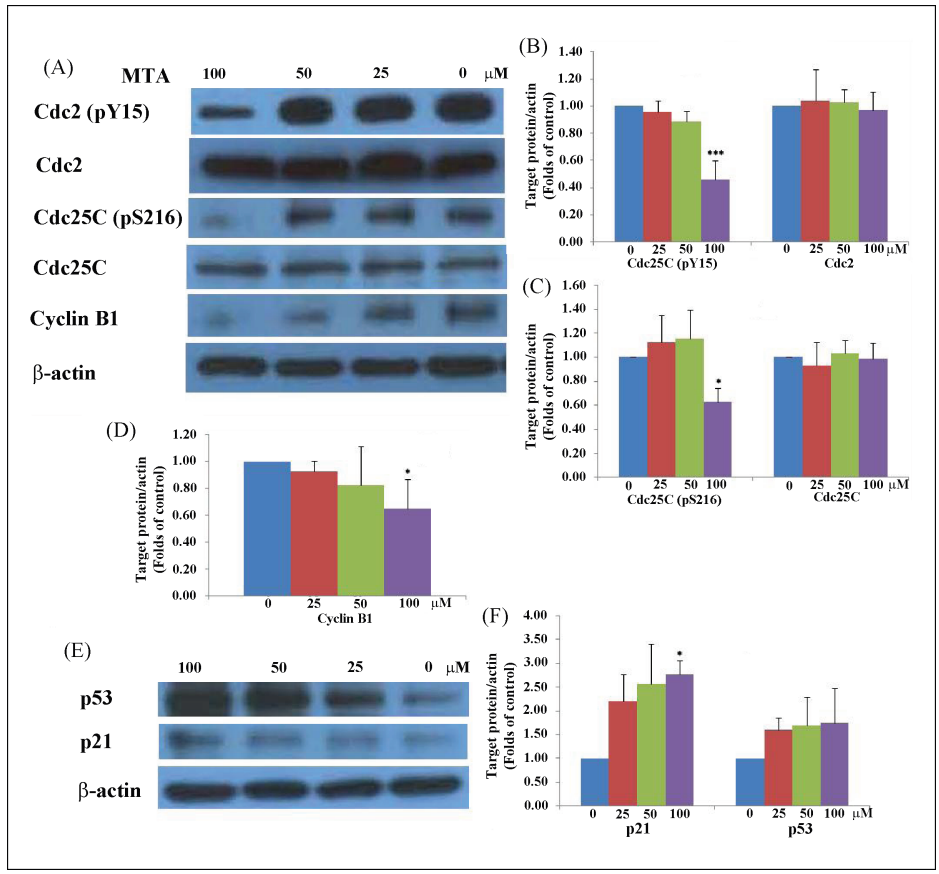

Fig. 5: Results from the western blot analysis. Cells treated with 0, 25, 50 and 100 μM MTA for 24 h were lysed, and the lysates were subjected to western blot analysis with antibodies against phospho-Cdc25C(S216), phospho-Cdc2(Y15), Cdc25C, Cdc2, cyclin B1, p53 and p21. β-actin was used as the loading control. (A, E) Representative immunoblots showed the effects of MTA treatment on the protein expression of HeLa cells. Actin expression was determined to confirm equal protein loading. (B, C, D, F) The quantified results of protein levels, which were adjusted with corresponding β-actin protein level and expressed as fold of control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ significantly different from the control group

assessed using an SRB (sulforhodamine B, Sigma, St. Louis, MO, USA) assay (Skehan et al. 1990; Zeng et al. 2009). The IC_{50} (50% inhibitory concentration) values were adopted as the cytotoxicity of the compound on the cells. Taxol isolated and identified in the lab (purity 98%) was used as a reference compound.

4.4. Apoptosis assay

The percentage of apoptotic cells was assessed with a FITC (fluorescein-5-isothiocyanate) annexin V apoptosis detection kit (BD Biosciences, San Diego, CA, USA). After treatment with MTA for 24 h, the cell suspensions were prepared and processed according to the manufacturer's instructions. The fluorescence intensity of the cells was measured by flow cytometry (BD Biosciences, FACSCalibur, Franklin Lakes, NJ, USA). Actinomycin D (Bio Basic Inc., Toronto, Canada) was used as a reference compound.

4.5. Cell cycle analysis

To determine whether MTA affects the cell cycle progression of HeLa cells, the distribution of the nuclear DNA content was assessed by PI (propidium iodide) staining. After treatment with various concentrations of MTA (25, 50 and 100 μM) for 24 h, the cells were harvested, washed with PBS and fixed overnight in 75% ethanol at −20 °C. The cells were then treated with 1% RNase A (w/v) for 30 min at 37 °C and stained with 0.5 mg/ml PI in PBS. The fluorescence intensity of the cells was measured by flow cytometry.

4.6. Western blot analysis

The expression of cellular proteins was evaluated by western blotting. After treatment for 24 h, the cells were washed twice with ice-cold PBS, and the total proteins were solubilized and extracted with the RIPA buffer (Boytime, Haimen, China) containing a protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland) for 30 min on ice. The insoluble material was removed by centrifugation at 16,000 × g for 10 min at 4 °C, and the supernatants were collected. Protein of the cytosolic and mitochondrial fractions was isolated using a mitochondria isolation kit for cultured cells (Pierce Biotechnology, Rochford, IL, USA) according to the manufacturer's instructions. The protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rochford, IL, USA). Equal amounts of protein (20–40 μg) from each sample were subjected to 6–12% SDS-PAGE. The separated proteins were transferred onto polyvinylidene difluoride membranes, followed by blocking with 5% nonfat milk powder (w/v) in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) for at least 1 h at room temperature. The membranes were probed with primary antibodies specific for Blc-2, Bax, cytochrome c (Cyto-C), activated caspase 3, caspase 9 (46/35), phospho-Cdc25C(S216), Cdc25C, Cdc2, cyclin B1, poly(ADP-ribose) polymerase (PARP 116/85), p21 (Epitomics Inc., Burlingame, CA, USA), procaspase 3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-Cdc2(Y15) (Cell Signaling Technology, Inc., Beverly, MA, USA), p53 (Anbo Biotechnology Co., San Francisco, CA, USA) and β-actin (Abcam Inc., Cambridge, MA, USA) in 2% nonfat milk and TBST at 4 °C overnight. After washing three times with TBST, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and visualized using an ECL Plus western blotting detection system (GE Healthcare Bio-sciences, Buckinghamshire, NA, UK). The blots were scanned and analyzed using ImageJ software.

4.7. Statistical analysis

All of the data are expressed as the mean ± standard deviation. Control and MTA-treated cells were compared using the One-way ANOVA and Student's *t*-test. A value of $P<0.05$ was considered statistically significant.

Acknowledgments: This work was supported by the Foundation of Chinese Academy of Sciences (West Light Program, Hundred Talents Program and KSCX2-YW-R-177), the National Natural Science Foundation of China (U1032602, 91013002, 30725048, 21102152), the National New Drug Innovation Major Project of China (2011ZX09307-002-02), the National Basic Research Program of China (2009CB522300 and 2013CB127505), and the Natural Science Foundation of Yunnan Province (2011FZ206 and 2012GA003).

References

Abrahamson JLA, Lee JM, Bernstein A (1995) Regulation of p53-mediated apoptosis and cell cycle arrest by steel factor. Mol Cell Biol 15: 6953–6960.

Aressy B, Ducommun B (2008) Cell cycle control by the Cdc25 phosphatase. Anti-Cancer Agents Med Chem 8: 818–824.

Basu A, Haldar S (1998) The relationship between Bcl2, Bax and p53: Consequences for cell cycle progression and cell death. Mol Hum Reprod 4: 1099–1109.

Blankenberg FG (2009) Apoptosis imaging: Anti-cancer agents in medicinal chemistry. Anticancer Agents Med Chem 9: 944–951.

Bucher N, Britten CD (2008) G2 checkpoint abrogation and checkpoint kinase-1 targeting in the treatment of cancer. Br J Cancer 98: 523–528.

Buolamwini JK (2000) Cell cycle molecular targets in novel anticancer drug discovery. Curr Pharm Design 6: 379–392.

Chulu JLC, Liu HJ (2009) Recent patents cell cycle regulatory proteins. Recent Patents on Biotechnol 3: 1–9.

Chung MI, Jou SJ, Cheng TH, Lin CN, Ko FN, Teng CM (1994) Antiplatelet constituents of Formosan *Rubia akane*. J Nat Prod 57: 313–316.

Collins I, Garrett MD (2005) Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. Curr Opin Pharmacol 5: 366–373.

Collins K, Jacks T, Pavletich N (1997) The cell cycle and cancer. Pro Natl Acad Sci USA 94: 2776–2778.

Fan JT, Kuang B, Zeng GZ, Zhao SM, Ji CJ, Zhang YM, Tan NH (2011) Biologically active arborinane-type triterpenoids and anthraquinones from *Rubia yunnanensis*. J Nat Prod 74: 2069–2080.

Fan JT, Su J, Peng YM, Li Y, Li J, Zhou YB, Zeng GZ, Yan H, Tan NH (2010) Rubiyunnanins C–H, cytotoxic cyclic hexapeptides from *Rubia yunnanensis* inhibiting nitric oxide production and NF-κB activation. Bioorg Med Chem 18: 8226–8234.

Hermeking H, Benzinger A (2006) 14-3-3 Proteins in cell cycle regulation. Semin Cancer Biol 16: 183–192.

Itokawa H, Takeya K, Mori N, Hamanaka T, Sonobe T, Mihara K (1984a) Isolation and antitumor activity of cyclic hexapeptides isolated from Rubiae radix. Chem Pharm Bull 32: 284–290.

Itokawa H, Takeya K, Mori N, Sonobe T, Serisawa N, Hamanaka T, Mihashi S (1984b) Studies on antitumor cyclic hexapeptides RA obtained from Rubiae Radix, Rubiaceae. III. On derivatives of RA-V and their *in vivo* activities. Chem Pharm Bull 32: 3216–3226.

Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC (1998) Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 95: 4997–5002.

Lan M (1976) Dian Nan Ben Cao, 2nd ed. Kunming, Yunnan People's Press.

Liu Y, Kulesz-Martin M (2001) p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DMA binding. Carcinogenesis 22: 851–860.

Manion MK, Hockenbery DM (2003) Targeting Bcl-2 related proteins in cancer therapy. Cancer Biol Ther 2: S105–S114.

Martinou JC, Youle RJ (2011) Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 21: 92–101.

Maxime H, Tremblay ML, Meng TC (2007) Protein tyrosine phosphatases: Emerging regulators of apoptosis. Cell Cycle 6: 2773–2781.

Moon MK, Han YM, Lee YJ, Lee LH, Yang JH, Kwon BM, Kim DK (2010) Inhibitory activities of anthraquinones from *Rubia akane* on phosphatase regenerating liver-3. Arch Pharm Res 33: 1747–1757.

Narita M, Shimizu S, Ito T, Chittenden T, Luta RJ, Matsuda H, Tsujimoto Y (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci USA 95: 14681–14686.

Nour NM (2009) Cervical cancer: A preventable death. Rev Obstet Gynecol 2: 240–244.

Ola MS, Nawaz M, Ahsan H (2011) Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 351: 41–58.

Pines J, Hunter T (1989) Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell 58: 833–846.

Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE (2011) Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst 103: 368–383.

Schuler M, Bossy-Wetzel E, Goldstein JC, Fitzgerald P, Green DR (2000) P53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J Biol Chem 275: 7337–7342.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesc H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer drug screening. J Natl Cancer Inst 82: 1107–1112.

Smits VAJ, Medema RH (2001) Checking out the G2/M transition. Biochim Biophys Acta 1519: 1–12.

Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW, Lowe SW (1999) Apaf-1 and caspase 9 in p53-dependent apoptosis and tumor inhibition. Science 284: 156–159.

Straten P, Andersen MH (2010) The anti-apoptotic members of the Bcl-2 family are attractive tumor-associated antigens. Oncotarget 1: 239–245.

Tan NH, Zhou J (2006) Plant cyclopeptides. Chem Rev 106: 840–895.

Tao J, Morikawa T, Ando S, Matsuda H, Yoshikawa M (2003) Bioactive constituents from Chinese natural medicines. XI. Inhibitors on NO production and degranulation in RBL-2H3 from *Rubia yunnanensis*: Structures of rubianosides II, III, and IV, rubianol-g, and rubianthraquinone. Chem Pharm Bull 51: 654–662.

Weiss RH (2003) $p21^{Waf1/Cip1}$ as a therapeutic target in breast and other cancers. Cancer cell 4: 425–429.

Wohlbold L, Fisher RP (2009) Behind the wheel and under the hood: Functions of cyclin-dependent kinases in response to DNA damage. DNA Repair 8: 1018–1024.

Wu TS, Lin DM, Shi LS, Damu AG, Kuo PC, Kuo YH (2003) Cytotoxic anthraquinones from the stems of *Rubia wallichiana* Decne. Chem Pharm Bull 51: 948–950.

Yang SL, Liu F (1997) *Rubia*: Pharmacological actions and applications. Int J Oriental Med 22: 157–160.

Yang Y (2000) Clinical use of *Rubia yunnanensi* (in Chinese). Chin J Ethnomed Ethnopharm 43: 119–120.

Yue GGL, Fan JT, Lee JKM, Zeng GZ, Ho TWF, Fung KP, Leung PC, Tan NH, Lau CBS (2011) Cyclopeptide RA-V inhibits angiogenesis by down-regulating extracellular signal-regulated kinase 1/2 phosphorylation in HUVEC and HMEC-1 endothelial cells. Br J Pharmacol 164: 1883–1898.

Zeng GZ, Tan NH, Ji CJ, Fan JT, Huang HQ, Han HJ, Zhou GB (2009) Apoptosis inducement of bigelovin from *Inula helianthus-aquatica* on human leukemia U937 cells. Phytother Res 23: 885–891.

Zinkel S, Gross A, Yang E (2006) Bcl-2 family in DNA damage and cell cycle control. Cell Death Differ 13: 1351–359.

Zivny J, Klener jr. P, Pytlik R, Andera L (2010) The role of apoptosis in cancer development and treatment: Focusing on the development and treatment of hematologic malignancies. Curr Pharm Design 16: 11–33.