JBUON 2014; 19(3): 650-655
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Emodin azide methyl anthraquinone derivative induced G0/G1 arrest in HER2/*neu*-overexpressing MDA-MB-453 breast cancer cells

Yan-yan Yan[1,2], Li-wu Fu[2], Wei Zhang[3], Hong-shan Ma[3], Cun-gen Ma[1], Yong-ju Liang[2], Bin-yu Liu[1], Jie-zhong Yu[1], Qiu-zhen Wu[3], Yi-min Dong[3]

[1]Medical College, Shanxi Datong University, Datong, Shanxi, People's Republic of China; [2]State Key Laboratory of Oncology in Southern China, Cancer Center, Sun Yat-sen University, Guangzhou, People's Republic of China; [3]The Third People's Hospital of Datong, Datong, Shanxi, People's Republic of China

Summary

Purpose: *Our previous data have shown that emodin azide methyl anthraquinone derivative (AMAD) triggered mitochondrial-dependent cell apoptosis involving caspase-8-mediated Bid cleavage, and induced proteasomal degradation of HER2/neu by blocking Her2/neu binding to Hsp90. In the present study, we futher investigated the effect of this compound on the cell cycle and related molecular mechanisms in HER2/neu-overexpressing MDA-MB-453 breast cancer cells.*

Methods: *The cell cycle distribution was tested by flow cytometry. The expression of cell cycle-related proteins was determined by Western blot analysis; DNA agarose gel electrophoresis was used to examine the apoptosis of MDA-MB-453 cells induced by emodin AMAD.*

Results: *After MDA-MB-453 cells were treated with different concentrations of emodin AMAD for 24 hrs, cells were arrested in G0/G1 phase, and the expression of G0/G1 related proteins c-Myc, Cyclin D1, CDK4 and p-Rb changed. DNA fragmentation appeared on the agarose gel in a concentration-dependent manner.*

Conclusion: *Emodin AMAD induced G0/G1 arrest in Her2/neu-overexpressing MDA-MB-453 cancer cells. This G0/G1 arrest was associated with decreasing protein expression of c-Myc, Cyclin D1, CDK4, and p-Rb.*

Key words: *apoptosis, cell cycle, Emodin AMAD, HER2/neu*

Introduction

It is well known that overexpression of HER2/neu is associated with poor prognosis in many malignancies [1], including cancers of the breast, ovary, lung, prostate, etc. During the last decade, HER2/neu has been targeted in order to develop novel anticancer drugs in the form of small molecules (e.g. lapatinib) or monoclonal antibodies (e.g. herceptin), which have shown promising results. However, their efficacy and long-term use in patients are quite limited because of resistance to these inhibitors or severe side effects. Therefore, novel therapeutic strategies in this field are still required.

Like doxorubicin and daunorubicin, emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a single anthracycline 1,8-dihydroxy-anthraquinone derivative and an active ingredient of various Chinese herbs including Rhubarb, Polygonum cuspidatum, Polygonum multiflorum, etc. Pharmacologic studies have indicated that emodin has immunosuppressive, antibacterial, anti-inflammatory, and antitumor properties. Recent studies clearly revealed that emodin suppressed the growth and induced apoptosis in several cancer cell lines, including breast, lung, colorectal and prostate cancer cells [2-4]. In addition, emodin could induce differentiation and enhance the sensitivity of tumor cells to chemotherapy. Recently, emodin was also found to block the phosphorylation of HER2/neu and to increase the suscepti-

Correspondence to: Yan-yan Yan, MD, PhD. Medical College, Shanxi Datong University, East Yuhe Bridge, Datong, Shanxi, 037009, People's Republic of China. Tel:+86 352 6011150, Fax:+86 352 6011150, E-mail: zwsanyan@163.com
Received: 26/04/2014; Accepted: 13/05/2014

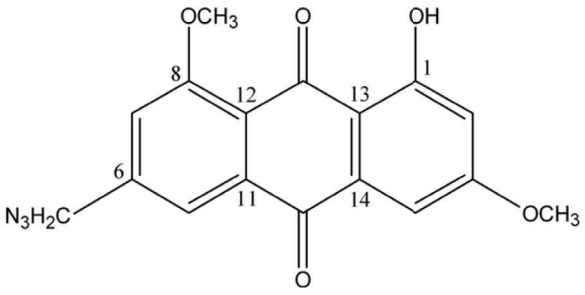


Figure 1. Chemical structure of emodin Azide Methyl Anthraquinone Derivative (AMAD).

bility of HER2/neu overexpressing cancer cells to standard cytotoxic agents. However, compared with other monomers such as taxol and camptothecin, the anticancer activity of emodin was not obvious and its side effects, such as cardiac toxicity, limited its clinical application in cancer chemotherapy. Therefore, in cooperation with the group of the School of Chemistry and Chemical Engineering, Sun Yat-sen University, we modified the structure of emodin and synthesized a series of emodin anthraquinone derivatives. Of these compounds, AMAD (6-azido-methyl-1-hydroxy-3,8-dimethoxy-9,10 anthraquinone) (Figure 1) exhibited the most potent anticancer activity in vitro and in vivo. Our previous data showed that emodin AMAD triggered mitochondrial-dependent cell apoptosis involving caspase-8-mediated Bid cleavage and induced proteasomal degradation of HER2/neu by blocking HER2/neu binding to Hsp90 [5,6], which revealed that AMAD might be a promising compound offering better anticancer treatment options. In this study, we further investigated the effect of this compound on the cell cycle and related molecular mechanisms in HER2/neu-overexpressing MDA-MB-453 cells, which might provide experimental evidence for clinical treatment.

Methods

Chemicals and reagents

Emodin AMAD with a purity of >98% was obtained from the modified structure of emodin extracted from the natural Giant Knotweed Rhizome of traditional Chinese herbs, synthesized by Lianquan Gu group (School of Chemistry and Chemical Engineering, Sun Yat-sen University). DNAzol® Reagent (Genomic DNA Isolation Reagent) was purchased from Invitrogen Co, Carlsbad, USA). Antibodies against c-Myc, Cyclin D1, CDK4, p-Rb and the enhanced PhototopeTM-HRP Detection Kit were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against GAPDH, anti-mouse IgG-HRP and anti-rabbit IgG-HRP were purchased from KangChen Biotechnology Co. (Shanghai, China). All tissue culture supplies were purchased from Gibco-BRL Co, NY, USA). Prestained Protein Marker was purchased from Fermentas (MBI) Co., Ottawa, Canada. Propidium iodide (PI) and RNase A were purchased from Sigma (St Louis, MO, USA). Other routine laboratory reagents were obtained from Whiga Biotechnology Co. (Guangzhou, China) of analytical or HPLC grade.

Cell lines and cell culture

Human breast cancer cell lines MDA-MB-453 obtained from Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China) were grown in DMEM medium, which contained 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). Cells were cultured in a humidified atmosphere incubator of 5% CO_2 and 95% air at 37 °C.

Cell cycle analysis

After MDA-MB-453 cells were treated with 2.30-9.20 μM emodin AMAD for 24 hrs, the cells were collected and washed twice with cold PBS. Then the cells were fixed in 70% ice-cold ethanol overnight. After washing twice with PBS, 5×10^5 cells were resuspended in 0.5 mL PBS containning RNase A (100 μg/ml) and PI (100 μg/ml) for 30 min at 37 °C in the dark. The DNA content of cells was then analyzed by flow cytometer [7] (Becton Dickinson, USA).

Whole-cell lysates and Western blot analysis

After MDA-MB-453 cells were exposed to 2.30-9.20 μM emodin AMAD for 24 hrs, whole cells were harvested, washed twice with ice-cold PBS, the pellet was vortexed and 1 × lysis buffer (50 mM Tris-Cl [pH 6.8], 10% glycerol, 2% sodium dodecylsulphate, 0.25‰ bromphenol blue and 0.1 M dithiothreitol) was added in 100 μL /5×10^6 cells. After being heated at 95 °C for 20 min, the lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The protein concentration was determined by nucleic acid-protein analyzer (Beckman, USA). Equal amount of lysate protein was separated on 8-12% sodium dodecylsulfate–polyacrylamide gel electrophore-

sis (SDS-PAGE) and transferred onto PVDF membrane (Pall, USA). The nonspecific binding sites were blocked with TBST buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7.4), and 0.4% (V/V) Tween 20) containing 5% nonfat dry milk for 2 hrs. The membranes were incubated overnight at 4 °C with specific primary antibodies. Then the membranes were washed three times with TBST buffer and incubated at room temperature for 1 h with HRP-conjugated secondary antibody. After thrice wash with TBST buffer, the immunoblots were visualized by the enhanced PhototopeTM-HRP Detection Kit purchased from Cell Signaling Technology (Beverly, MA) and exposed to Kodak medical X-ray processor [8] (Kodak, USA).

DNA fragmentation assay

After MDA-MB-453 cells were exposed to 2.30-18.40 μM emodin AMAD for 24 hrs, the extraction of DNA was carried out as described in the manual of DNAzol reagent (Invitrogen, USA). Briefly, $5x10^6$ cells were harvested, washed once with ice-cold PBS, and centrifuged at 1000 rpm for 5 min. The cell pellets were resuspended in 1 mL DNAzol reagent and incubated at room temperature for 2 hrs. After centrifugation at 12,000 rpm for 15 min, the supernatant was transferred to a 1.5 mL eppendorff. Subsequently, 0.5 mL ice-cold dehydrated alcohol was added to precipitate DNA, which was collected by centrifugation at 10,000 rpm for 10 min. Then the pellets were washed twice with 75% ice-cold alcohol, dried in air and dissolved in 100 mL TE buffer (100 mM Tris-Cl [pH 8.0], 10 mM EDTA). Each DNA preparation was mixed with 5 mL RNase A of 10 mM at 37 °C overnight. Before electrophoresis, every 5 mL of extracted DNA samples were added with 1 mL 6 × loading buffer. Then the samples were electrophoresed on 0.8% agarose gel stained with 1.5 mM ethidium bromide (EB) at 60 V for 45 min in 0.5 x TBE buffer (44.5 mmol/L Tris-boric acid and 1 mmol/L EDTA [pH 8.3]). Analyses and photography were performed with the gel imaging and analysis system [9] (Vilber Lourmat, France).

Statistics

Results were analysed using t-test or one-way ANOVA with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were presented as means ± SD of at least triplicate determinations. *$p < 0.05$ was indicative of significant difference, and **$p < 0.01$ was indicative of very significant difference.

Results

Emodin AMAD caused cell cycle arrest in G0/G1 phase in MDA-MB-453 cells

It is reported that many emodin derivatives caused cell cycle arrest in G0/G1 phase in some human cells. To investigate whether emodin AMAD affects the cell cycle phase distribution in tumor cells, the cell cycle of MDA-MB-453 cells was analysed by PI staining and flow cytometry analysis. The results showed that treatment of emodin AMAD caused MDA-MB-453 cells arrest in G0/G1 phase (Figure 2A and C). After MDA-MB-453 cells were treated with 2.30-9.20 μM for 24 hrs, the contents of G0/G1 phase were 49.03±3.10%, 64.77±5.32%, 82.57±0.47% and 87.97±4.80% respectively (Figure 2B).

Emodin AMAD caused changes in G0/G1 phase-associated proteins in MDA-MB-453 cells

After MDA-MB-453 cells were treated with different concentrations of Emodin AMAD for 24 h, cells were arrested in G0/G1 phase, and western blot analysis showed that the expression of G0/G1 related proteins c-Myc, Cyclin D1, CDK4 and p-Rb were downregulated in a dose-dependent manner (Figure 3).

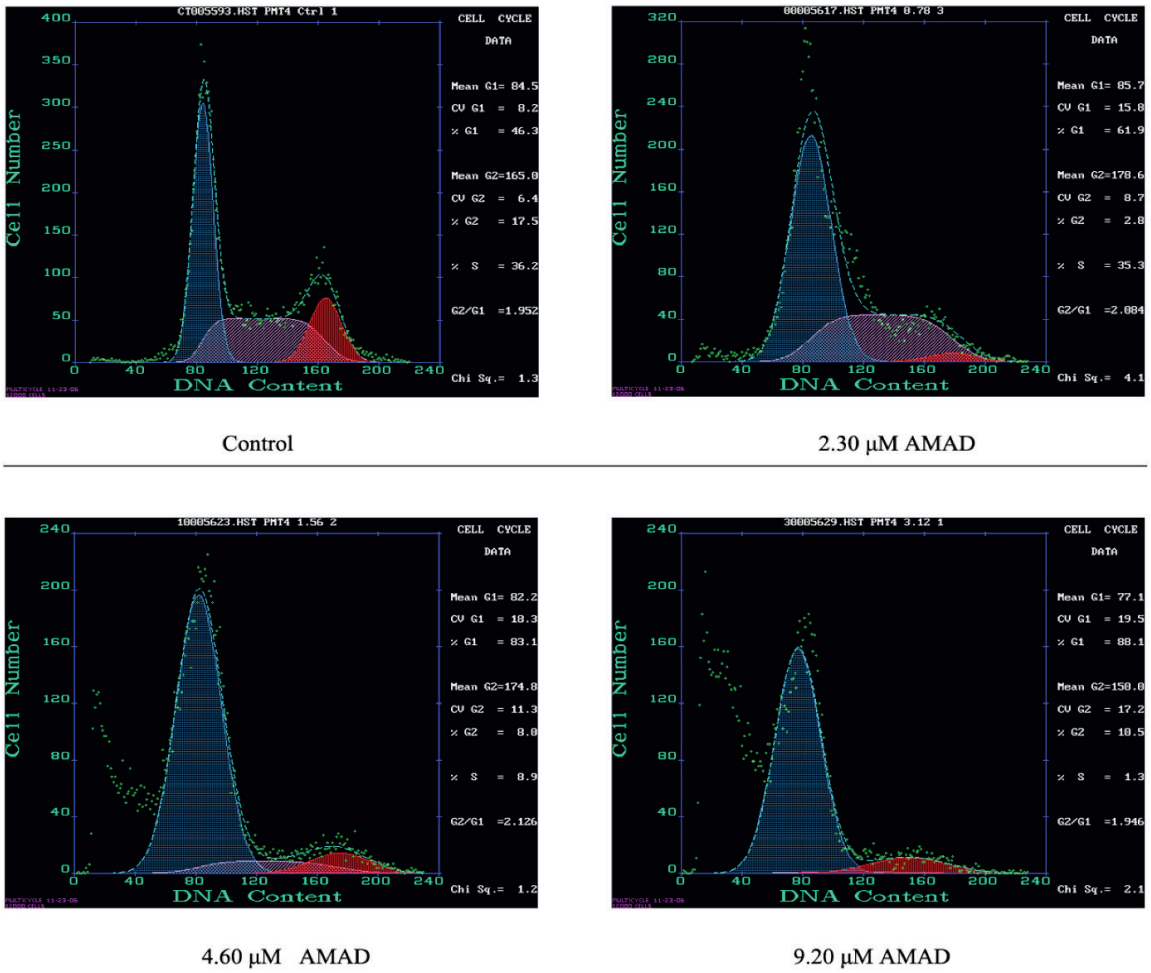
Emodin AMAD induced apoptosis characteristic DNA ladder in MDA-MB-453 cells

To further investigate cell apoptosis induced by emodin AMAD, we examined the nucleic DNA fragmentation, which is an important indicator of apoptosis. After MDA-MB-453 cells were treated with 2.30-18.40 μM emodin AMAD for 24 hrs, DNA was extracted and analyzed by electrophoresis. DNA fragmentation analysis showed a typical ladder pattern of DNA fragmentation due to nucleic DNA cleavage associated with apoptosis (Figure 4). The results confirmed that emodin AMAD induced apoptosis in MDA-MB-453 cells.

Discussion

Several studies have shown that a number of herbal extracts and isolated compounds possess antitumor activity. Emodin AMAD was obtained from structural modification of emodin extracted from nature's giant knotweed rhizome of traditional Chinese herbs. Previous studies have shown that in our laboratory this compound triggered mitochondrial-dependent apoptosis and in-

A



B

Effect of Emodin AMAD on cell cycle in MDA-MB-453

Group	Emodin AMAD (24 h/μM)			
	Control	2.30	4.60	9.20
GO/G1	49.03 ± 3.10	64.77 ± 5.32	82.57 ± 0.47	87.97 ± 4.80
S	31.23 ± 4.48	30.43 ± 6.54	10.57 ± 1.76	4.20 ± 6.18
G2/M	19.73 ± 2.07	4.83 ± 1.78	6.87 ± 1.33	7.80 ± 2.49

C

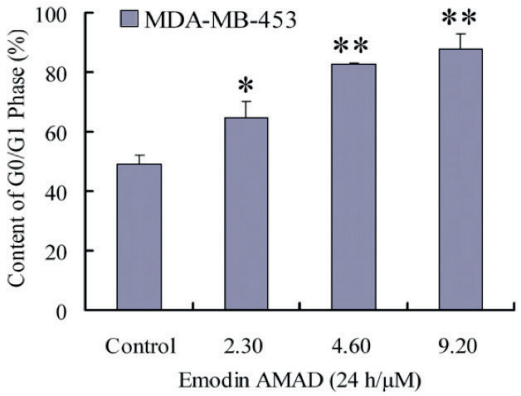


Figure 2. Emodin AMAD treatment resulted in cell cycle arrest at G0/G1. The cell cycle analysis was determined by PI staining and flow cytometry. **A:** after MDA-MB-453 cells were treated with 2.30-9.20 μM for 24 hrs, the cell cycle distribution was tested by flow cytometry; **B:** the content of G0/G1, S and G2/M phase is shown (mean±SD of at least 3 determinations); **C:** The G0/G1 content of MDA-MB-453 cells increased in a concentration-dependent manner. *p< 0.05 and **p < 0.01 vs the control.

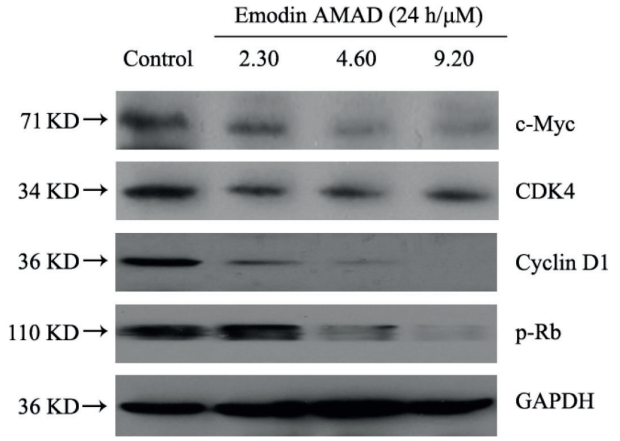


Figure 3. Emodin AMAD caused changes of G0/G1 phase associated proteins in MDA-MB-453 cells. After MDA-MB-453 cells were treated with 2.30-9.20 μM AMAD for 24 hrs, the whole-cell lysates were assayed by Western blot and the corresponding antibodies. Glyceraldehyde-3-phosphate dehydrogenase detection was used to confirm equal protein loading.

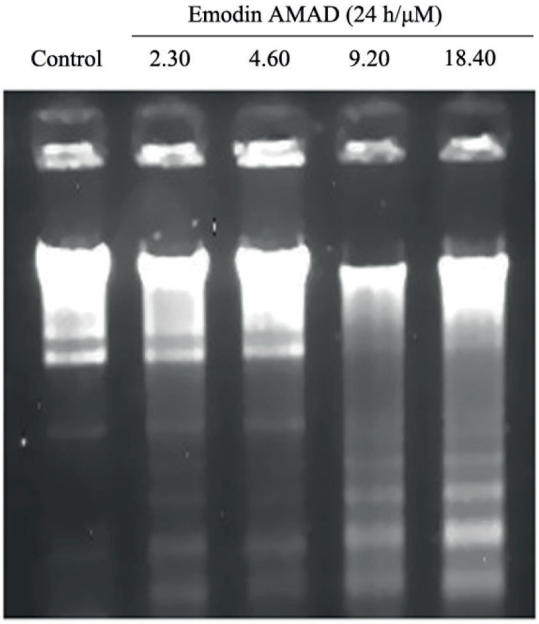


Figure 4. Emodin AMAD induced apoptosis characteristic DNA ladder in MDA-MB-453 cells. Internucleosomal DNA fragmentation in AMAD-treated MDA-MB-453 cells on agarose electrophoresis. Cells were treated with AMAD for 24 hrs in different concentrations.

duced proteasomal degradation of Her2 in Her2/neu-overexpressing cancer cells, which revealed that AMAD might be a promising compound offering better anticancer treatment options.

Cell cycle progression is precisely regulated by a series of cell cycle regulators, including cyclins, CDKS and CDK inhibitors (CDKIs) [10-12]. Progression through the G0/G1 phase and transition from G0/G1 into the S phase are regulated by cyclin D, E and their dependent kinases. The cyclin D-CDK4/6 complexes induce phosphorylation of the retinoblastoma (Rb) protein and the release of E2F, which triggers G0/G1 cell cycle progression [13,14]. Deregulation of the cell cycle in the G0/G1 phase has been implicated in tumor development and proliferation [15-18]. Several studies have demonstrated that emodin inhibits the growth of various tumor cells and have determined the mechanisms involved in inducing G1/S phase cell cycle arrest and triggering apoptosis [19,20]. In our study, we identified that emodin AMAD treatment triggers cell cycle arrest at the G0/G1 phase in MDA-MB-453 cells (Figure 2), and the expression of G0/G1 phase related proteins cyclin D1, CDK4 and p-Rb were downregulated in a dose-dependent manner (Figure 3). In addition, the downregulation of c-Myc may be associated with the regulation of the cell cycle after AMAD treatment. These partly account for MDA-MB-453 cell proliferation inhibition following AMAD treatment.

Acknowledgement

This work was supported by the National Natural Sciences Foundation of China grant (No. 81061160507), the National Science Foundation for Young Scientists of Shanxi Province, China (2014021037-6), and the Priming Scientific Research Foundation for Ph.D. in Shanxi Datong University (No. 2010-B-11).

References

1. Oshima RG, Lesperance J, Munoz V et al. Angiogenic acceleration of Neu induced mammary tumor progression and metastasis. Cancer Res 2004;64:169-179.
2. Zhang L, Lau YK, Xi L et al. Tyrosine kinase inhibitors, emodin and its derivative repress HER-2/neu-induced cellular transformation and metastasis-associated properties. Oncogene 1998; 16:2855-2863.
3. Zhang L, Lau YK, Xia W, Hortobagyi GN, Hung MC. Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing breast cancer cells in

athymic mice and sensitizes these cells to the inhibitory effect of paclitaxel. Clin Cancer Res 1999;5:343-353.

4. Lee HZ. Effects and mechanisms of emodin on cell death in human lung squamous cell carcinoma. Br J Pharmacol 2001;134:11-20.
5. Yan YY, Zheng LS, Zhang X et al. Blockade of Her2/neu binding to Hsp90 by emodin azide methyl anthraquinone derivative induces proteasomal degradation of Her2/neu. Mol Pharm 2011; 8:1687-1697.
6. Yan Y, Su X, Liang Y et al. Emodin azide methyl anthraquinone derivative triggers mitochondrial-dependent cell apoptosis involving in caspase-8-mediated Bid cleavage. Mol Cancer Ther 2008;7:1688-1697.
7. Zhang JY, Tao LY, Liang YJ et al. Secalonic acid D induced leukemia cell apoptosis and cell cycle arrest of G(1) with involvement of GSK-3beta/beta-catenin/c-Myc pathway. Cell Cycle 2009;8:2444-2450.
8. Yan YY, Guo Y, Zhang W et al. Celastrol Enhanced the Anticancer Effect of Lapatinib in Human Hepatocellular Carcinoma Cells in vitro. J BUON 2014;19:451-457.
9. Zhang JY, Wu HY, Xia XK et al. Anthracenedione derivative 1403P-3 induces apoptosis in KB and KBv200 cells via reactive oxygen species-independent mitochondrial pathway and death receptor pathway. Cancer Biol Ther 2007;6:1413-1421.
10. Cheng M, Olivier P, Diehl JA et al. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J 1999;18:1571-1583.
11. LaBaer J, Garrett MD, Stevenson LF et al. New functional activities for the p21 family of CDK inhibitors. Genes Dev 1997;11:847-862.
12. Parry D, Mahony D, Wills K, Lees E. Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. Mol Cell Biol 1999;19:1775-1783.
13. Johnson DG, Schneider-Broussard R. Role of E2F in cell cycle control and cancer. Front Biosci 1998;3:447-448.
14. Agami R, Bernards R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 2000;102:55-66.
15. Malumbres M, Pevarello P, Barbacid M, Bischoff JR. Cdk inhibitors in cancer therapy: What is next? Trends Pharmacol Sci 2008;29:16-21.
16. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 2011;11:558-572.
17. Fernandez PC, Frank SR, Wang L et al. Genomic targets of the human c-Myc protein. Genes Dev 2003;17:1115-1129.
18. Hermeking H, Rago C, Schuhmacher M et al. Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A 2000;97:2229-2234.
19. Kwak HJ, Park MJ, Park CM et al. Emodin inhibits vascular endothelial growth factor-A-induced angiogenesis by blocking receptor-2 (KDR/Flk-1) phosphorylation. Int J Cancer 2006;118:2711-2720.
20. Shao J, Zhang F, Bai Z, Wang C, Yuan Y, Wang W. Synthesis and antitumor activity of emodin quaternary ammonium salt derivatives. Eur J Med Chem 2012;56:308-319.