

RESEARCH ARTICLE

Effects of Celecoxib on Cycle Kinetics of Gastric Cancer Cells and Protein Expression of Cytochrome C and Caspase-9

Yu-Jie Wang¹, Xiao-Ping Niu^{2*}, Li Yang¹, Zhen Han², Ying-Jie Ma¹

Abstract

Objective: This investigation aimed to determine effects of celecoxib on the cell cycle kinetics of the gastric cancer cell line MGC803 and the mechanisms involved by assessing expression of cytochrome C and caspase-9 at the protein level. **Methods:** Cell proliferation of MGC803 was determined by MTT assay after treatment with celecoxib. Apoptosis was assessed using fluorescence staining and cell cycle kinetics by flow cytometry. Western blotting was used to detect the expression of caspase-9 protein and of cytochrome C protein in cell cytosol and mitochondria. **Results:** Celecoxib was able to restrain proliferation and induce apoptosis in a dose- and time-dependent manner, inducing G0/G1 cell cycle arrest, release of cytochrome C into the cytosol, and cleavage of pro-caspase-9 into its active form. **Conclusion:** Celecoxib can induce apoptosis in MGC803 cells through a mechanism involving cell cycle arrest, mitochondrial cytochrome C release and caspase activation.

Keywords: Celecoxib - MGC803 - cell cycle - cytochrome C - caspase-9 - apoptosis

Asian Pacific J Cancer Prev, **14** (4), 2343-2347

Introduction

Gastric cancer is the second most common cause of cancer-associated death in the world. The high mortality is largely attributed to the huge number of at-risk individuals as well as the delay in clinical presentation. As the majority of cases present with advanced disease, conventional therapies (surgery, chemotherapy, and radiotherapy) have limited efficacy to reduce mortality (Bazuro et al., 2008; Wu et al., 2009; Barr, 2011). Currently, the limited treatment and poor prognosis of this disease calls for more effective drug therapies. Target-protein-based cancer therapy has become available in clinical practice. Several promising molecules have been shown to target specific pathways for the cancer cell growth. Cyclooxygenase-2 (COX-2) and the constitutively expressed equivalent COX-1 are key enzymes responsible for the generation of prostaglandins from arachidonic acid. Whereas COX-1 is expressed constitutively in most tissues and is responsible for the production of prostaglandins controlling normal physiologic functions, COX-2 is induced by mitogenic and inflammatory stimuli (Smith et al., 2000). This results in the enhanced synthesis of prostaglandins in neoplastic and inflamed tissues (Coussens and Werb, 2002). Overexpression of COX-2 has been demonstrated in several adult epithelial tumors such as colon cancer (Lai et al., 2004), gastric cancer (Thiel et al., 2011) and hepatocellular carcinoma (Ogunwobi and Liu, 2011). COX-2 is linked to tumor-promoting effects, including tumor growth and metastasis, by stimulating invasiveness

and angiogenesis (Chen et al., 2009; Liu et al., 2011), inhibiting apoptosis and immune surveillance (Ohno et al., 2005), and enhancing drug resistance (Meher et al., 2008). These findings suggest that COX-2 may play a key role in carcinogenesis and makes it a potential target in cancer therapy (Ghosh et al., 2010; Khan et al., 2011).

Recent epidemiological studies revealed that prolonged treatment with non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of cancer (such as colonic, rectal, and stomach cancer). NSAIDs inhibit cell proliferation and induce apoptosis in a number of cancer cell lines in vitro and in vivo, which is considered to be an important mechanism for the anti-tumour activity of NSAIDs (Gu et al., 2005; Entezari Heravi et al., 2011; Fischer et al., 2011). However, the molecular pathways of this process are unclear. It is thought that the antineoplastic mechanism of NSAIDs involves the inhibition of COX-2 activity while the gastrointestinal complications of NSAIDs are attributed to the inhibition of COX-1. To circumvent the side effects associated with COX-1 inhibition, selective COX-2 inhibitors, such as celecoxib and Rofecoxib, were developed (Matthias et al., 2006; Xiao et al., 2008). Celecoxib is a selective COX-2 inhibitor. It has similar anti-inflammatory activities to those of traditional NSAIDs but fewer gastrointestinal side effects (Fujimura et al., 2007; Arber, 2008). In this study we investigated the effect of Celecoxib on the cell cycle kinetics of gastric cancer cell line MGC803 and the probable mechanism by examining the expressions of cytochrome C and Caspase-9 at protein level.

¹Department of Gastroenterology, Zhengzhou People's Hospital Affiliated to Southern Medical University, Zhengzhou, Henan, ²Department of Digestion Medicine, Yijishan Hospital of Wannan Medicine College, Wuhu, Anhui, China *For correspondence: xiaopingniu@126.com

Materials and Methods

Cell and culture

Human gastric cancer cell line MGC803 was purchased from Cancer Research Institute, Central South University. The cells were incubated in RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

MTT assay

Cells were seeded at a density of 5×10³ per well in 96-well plates in RPMI-1640 containing 10% FBS for 24 h, then cells were treated with celecoxib (15, 30, or 60 μmol/L respectively). After 12, 24, 48, or 72 hours incubation, 20 μl MTT (5 g/L) was added to each well and incubated for 4 h. Supernatant was then removed and 150 μl DMSO was added. It was shaken for 5 min until the crystal was dissolved. The absorbance at 570 nm was measured with a micro-ELISA reader. The negative control wells had no cells, with culture medium and DMSO only. Each assay was performed in triplicate.

Apoptosis assay

Cells were cultured in six-well plates in RPMI-1640 with 10% FBS medium and were treated with different concentrations of celecoxib (15 μmol/L, 30 μmol/L, 60 μmol/L) for 12 h, 24 h, 48 h and 72 h. The cover slips were washed three times with phosphate-buffered saline (PBS) and single cell suspensions were fixed in 1% PBS. Cells were stained with 100 μg/ml acridine orange (AO) and 100 μg/ml ethidium bromide (EB) for 1 min. Then cells were observed under fluorescence microscope. At least 200 cells were counted and the percentage of apoptotic cells was determined.

Flow cytometry

MGC803 cells were incubated with Celecoxib. Analysis of cell cycle distribution was performed by flow cytometry. Cells were treated for 48 h in the medium containing 10% FBS with 15 μmol/L, 30 μmol/L, 60 μmol/L celecoxib respectively. DMSO (sigma) was used as drug-free control. Cells were harvested by trypsinization, washed twice with PBS, fixed by cold alcohol at 4°C, dyed with propidium iodide (PI) and then were analyzed by flowcytometry. The experiments were performed in triplicates.

Western blotting analysis

Cells were cultured in six-well plates in RPMI-1640 with 10% FBS medium and were treated with different concentrations of celecoxib (15 μmol/L, 30 μmol/L, and 60 μmol/L) for 48 h. The cells were extracted with lysis buffer containing protease inhibitors (20 mmol/L Tris-HCl, pH 7.4, 50 mmol/L sodium chloride, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP-40). Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin (Sigma) as the standard. Western blotting was carried out. Briefly, an equal amount of total cell lysate (40

μg) was solubilized in sample buffer and boiled for 5 min. Twenty-five microliters of this lysate were electrophoresed on a 10% SDS-PAGE gel and then the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using transfer buffer at 400 mA for 1 h. Non-specific binding was blocked with 10 mmol/L Tris-HCl buffered saline, pH 7.6, plus 0.05% Tween-20 (TBS-T) containing 5% skimmed milk powder for 1 h at room temperature. Membranes were incubated with primary antibody overnight at 4°C. The primary antibodies used included: a polyclonal rabbit anti-human caspase-9 (pro-caspase-9 and cleaved caspase-9) antibody (1:1000) (BD Pharmingen, San Diego, CA). After washing three times with TBS-T solution and incubation with secondary antibody (1:5000–10000 dilution) for 1h at room temperature, bands were visualized with the enhanced chemiluminescence system (GE healthcare, Little Chalfont, UK). Afterwards, membranes were re-blotted with anti-β-actin antibody for normalization and equal protein loading.

Preparation of Mitochondrial and Cytosolic Fractions

Preparation of mitochondrial and cytosolic fractions was carried out as described previously (Liou et al., 2007). Briefly, MGC803 cell were treated with different concentrations of celecoxib for 48 h. Cells were gently homogenized with a Dounce homogenizer in a buffer (20 mmol/L HEPES, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml pepstatin, and 250 mmol/L sucrose). The homogenate was centrifuged at 750g for 5 min at 4°C to remove unbroken cells and nuclei. Then, the supernatant was centrifuged at 16,000g for 20 min at 4°C. The pellet from this step was saved as the mitochondrial fraction, and the supernatant was subjected to further ultracentrifugation at 100,000g for 1 h at 4°C to eliminate trace membrane contamination. The supernatant was saved as the cytoplasmic fraction. For immunoblotting, proteins of the two fractions were separated using 12% SDS-PAGE, followed by electroblotting onto a polyvinylidene difluoride membrane. Cytochrome C was detected by a mouse anti-human cytochrome C monoclonal antibody (1:1000) (BD Pharmingen). Secondary goat anti-mouse HRP-labeled antibody was detected by enhanced chemiluminescence. β-actin and COX IV were probed as the internal control of the cytosolic and mitochondrial fractions, respectively.

Experimental design and statistical analysis

All experiments were performed in triplicate and were repeated at least three times. Representative experiments and mean values ± SD are shown. Statistical differences were determined by Student's t-test. A P value of <0.05 was considered statistically significant.

Results

Celecoxib inhibit proliferation of MGC803 cells

MTT assay was used to test the cell proliferation of MGC803 after Celecoxib treatment. The result shows a

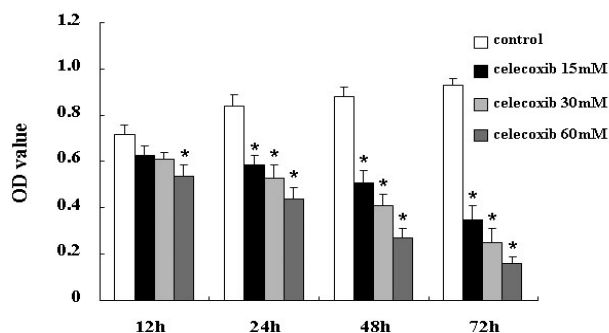


Figure 1. OD Value Change of Various Concentrations of Celecoxib on Proliferation of MGC803 Cells. The graph represents the mean \pm SD of triplicate assays. The results are from three identical experiments. (* $P < 0.05$ versus control)

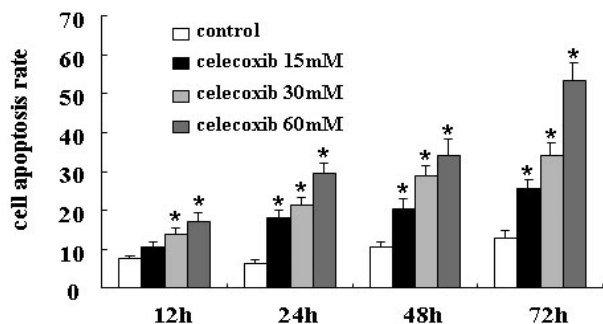


Figure 2. Effect of Various Concentrations of Celecoxib on Apoptosis of MGC803 Cells in Vitro. The graph represents the mean \pm SD of triplicate assays. The results are from three identical experiments (* $P < 0.05$ versus control)

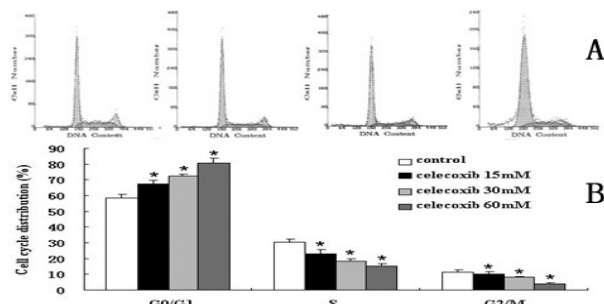


Figure 3. MGC803 Cells were Treated with Different Concentrations of Celecoxib for 48 h. Cell cycle kinetics was detected by flow cytometry. The graph represents the means \pm SD of triplicate assays. The results are from three identical experiments (* $P < 0.05$ versus control)

dose-dependent and time-dependent inhibition of cell proliferation by Celecoxib (Figure 1).

Celecoxib induce apoptosis in MGC803 cells

Acridine orange staining was used to check for apoptosis of MGC803 cells after the treatment with Celecoxib. Celecoxib was able to induce apoptosis of the cells in a dose- and time-dependent manner (Figure 2).

Effect of Celecoxib on cell cycle phase distribution

The effect of Celecoxib in cell cycle phase distribution of MGC803 was accessed by flow cytometry analysis. As shown in Figure 3, after 48h treatment, Celecoxib caused a dose-dependent alteration in the cell cycle distribution of MGC803 cells. It increased the proportion of cells in the G0/G1 phase and decreased the proportion in G2/M and S phase.

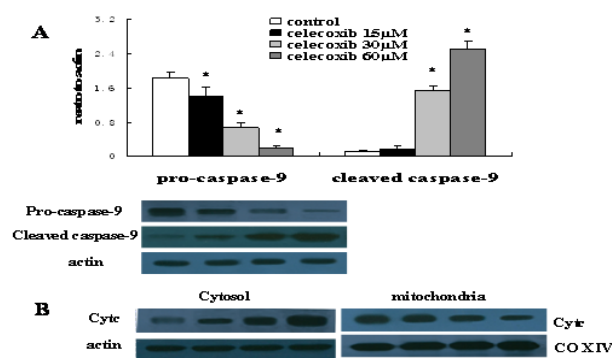


Figure 4. Cytochrome C Translocation and Caspase Activation. A. Histograms show full-length and cleaved caspase-9 levels normalized to the corresponding β -actin level, as previously described in Materials and Methods. Asterisks indicate significance, with * $P < 0.05$ (significant)

Celecoxib Causes Apoptosis through Cytochrome C Translocation and Caspase Activation

MGC803 cells were treated for 48h with celecoxib 15–60 μ mol/L. the expression of cytochrome C protein in cell plasma was increased and which in mitochondria was decreased. The expression of cleaved caspase-9 was increased. In the other hand, the expression of pro-caspase-9 was decreased. All these implied that cytochrome C was released and caspase-9 was activated (Figure 4).

Discussion

A number of studies have shown that NSAIDs can inhibit the growth of cancer cells, but the molecular mechanisms remains unclear. Celecoxib is a new COX-2 specific inhibitor for the treatment of rheumatoid arthritis, osteoarthritis and acute pain (Clemett and Goa, 2000). Celecoxib is the only NSAID that has been approved by the FDA (in December 1999) for adjuvant treatment of patients with familial adenomatous polyposis. Since the introduction of Celecoxib in 1998 and Rofecoxib in 1999, more than 3000 studies have investigated the molecular targets and clinical effects of these drugs, and discusses the anticarcinogenic molecular mechanisms associated with selective COX-2 inhibitors and their COX-independent mechanisms of action (Matthias et al., 2006; Xiao et al., 2008). In general, the anticarcinogenic mechanisms of selective COX-2 inhibitors include blocking cell cycle progression, angiogenesis, apoptosis and suppresses tumor metastasis (Li et al., 2008; Park et al., 2010; Fisher et al., 2011; Sobolewski et al., 2011). Our study shows that Celecoxib was able to restrain the proliferation and induce apoptosis of MGC803 cells in a dose- and time-dependent manner.

Previous observation has shown that cell cycle arrest might result in apoptosis due to the existence of cell cycle checkpoint and feedback control (Pietenpol et al., 2002). Several evidences have suggested that some anticancer drug induced apoptosis may occur via a signaling pathway independent of cell cycle arrest (Hsu et al., 2005; Wang et al., 2007). We have found that Celecoxib can change the distribution of human gastric cancer cell line MGC803 in cell cycle. There is an increase in the proportion of

MGC803 cells in Go/G1 phase and a relative decrease in the percentage of cells in S and G2/M phase. It suggests that apoptosis induced by Celecoxib is related to cell cycle arrest.

In the mitochondrial-dependent apoptosis pathway, the instability of mitochondria leads to the redistribution of Cyt C into the cytosol, which initiates the formation of the apoptosome and the sequential activation of caspase-9 and -3. The signal transduction pathways that are triggered by the central gate in mitochondria play a critical role in anticancer drug-induced apoptosis (Calviello et al., 2003; Zhang et al., 2006). Studies have found that celecoxib can induce apoptosis in some human cancer cell line in vitro. However, the exact mechanism of apoptosis induced by celecoxib have not been elucidated previously (Chakraborti et al., 2010). The present study found that Celecoxib was able to induce apoptosis of MGC803 cells in a dose- and time-dependent manner. We also have found Cyt C release from mitochondria into cytosol and the activation of caspase-9 in MGC803 cell after 48h of treatment with Celecoxib. All these novel findings suggest that mitochondrial-dependent pathway is involved in celecoxib-triggered MGC803 cell apoptosis.

In summary, our results in the present study demonstrate that Celecoxib can inhibit proliferation and induce of apoptosis of human gastric cancer cells. The molecular mechanism may involve its blocking of cell cycle progress, cytochrome C release and caspase activation.

Acknowledgements

This study was supported by the General Project of the Natural Science Foundation of the Department of Education, Anhui Province (No. KJ2007B064).

References

Arber N (2008). Cyclooxygenase-2 inhibitors in colorectal cancer prevention: point. *Cancer Epidemiol Biomarkers Prev*, **17**, 1852-7.

Barr H (2011). Gastrointestinal cancer: current screening strategies. *Recent Results Cancer Res*, **185**, 149-57.

Bazuro GE, Torino F, Gasparini G, Capurso L (2008). Chemoprevention in gastrointestinal adenocarcinoma: for few but not for all? *Minerva Gastroenterol Dietol*, **54**, 429-44.

Calviello G, Di Nicuolo F, Piccioni E, et al (2003). gamma-Tocopheryl quinone induces apoptosis in cancer cells via caspase-9 activation and cytochrome c release. *Carcinogenesis*, **24**, 427-33.

Chakraborti AK, Garg SK, Kumar R, Motiwala HF, Jadhav PS (2010). Progress in COX-2 inhibitors: a journey so far. *Curr Med Chem*, **17**, 1563-93.

Chen WT, Hung WC, Kang WY, et al (2009). Overexpression of cyclooxygenase-2 in urothelial carcinoma in conjunction with tumor-associated-macrophage infiltration, hypoxia-inducible factor-1alpha expression, and tumor angiogenesis. *APMIS*, **117**, 176-84.

Clemett D, Goa KL (2000). Celecoxib: a review of its use in osteoarthritis, rheumatoid arthritis and acute pain. *Drugs*, **59**, 957-80.

Coussens LM, Werb Z (2002). Inflammation and cancer. *Nature*, **420**, 860-7.

Entezari Heravi R, Hadizadeh F, Sankian M, et al (2011). Novel selective Cox-2 inhibitors induce apoptosis in Caco-2 colorectal carcinoma cell line. *Eur J Pharm Sci*, **44**, 479-86.

Fischer SM, Hawk ET, Lubet RA (2011). Coxibs and other nonsteroidal anti-inflammatory drugs in animal models of cancer chemoprevention. *Cancer Prev Res*, **4**, 1728-35.

Fisher JC, Gander JW, Haley MJ, et al (2011). Inhibition of cyclooxygenase 2 reduces tumor metastasis and inflammatory signaling during blockade of vascular endothelial growth factor. *Vasc Cell*, **3**, 22.

Fujimura T, Ohta T, Oyama K, Miyashita T, Miwa K (2007). Cyclooxygenase-2 (COX-2) in carcinogenesis and selective COX-2 inhibitors for chemoprevention in gastrointestinal cancers. *J Gastrointest Cancer*, **38**, 78-82.

Ghosh N, Chaki R, Mandal V, Mandal SC (2010). COX-2 as a target for cancer chemotherapy. *Pharmacol Rep*, **62**, 233-44.

Gu Q, Wang JD, Xia HH, et al (2005). Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. *Carcinogenesis*, **26**, 541-6.

Hsu YL, Kuo YC, Kuo PL, et al (2005). Apoptotic effects of extract from *Antrodia camphorata* fruiting bodies in human hepatocellular carcinoma cell lines. *Cancer Lett*, **221**, 77-89.

Khan Z, Khan N, Tiwari RP, et al (2011). Biology of Cox-2: an application in cancer therapeutics. *Curr Drug Targets*, **12**, 1082-93.

Lai MY, Huang JA, Liang ZH, et al (2004). Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. *Clin Cancer Res*, **10**, 8465-71.

Li S, Tong Q, Zhang W, et al (2008). Mechanism of growth inhibitory effects of cyclooxygenase-2 inhibitor-NS398 on cancer cells. *Cancer Invest*, **26**, 333-7.

Liu H, Xiao J, Yang Y, et al (2011). COX-2 expression is correlated with VEGF-C, lymphangiogenesis and lymph node metastasis in human cervical cancer. *Microvasc Res*, **82**, 131-40.

Liou JP, Hsu KS, Kuo CC, Chang CY, Chang JY (2007). A novel oral indoline-sulfonamide agent, N-[1-(4-methoxybenzenesulfonyl)-2, 3-dihydro-1H-indol-7-yl]-isonicotinamide (J30), exhibits potent activity against human cancer cells in vitro and in vivo through the disruption of microtubule. *J Pharmacol Exp Ther*, **323**, 398-405.

Matthias C, Schuster MT, Zieger S, Harr us U (2006). COX-2 inhibitors celecoxib and rofecoxib prevent oxidative DNA fragmentation. *Anticancer Res*, **26**, 2003-7.

Mehar A, Macanas-Pirard P, Mizokami A, et al (2008). The effects of cyclooxygenase-2 expression in prostate cancer cells: modulation of response to cytotoxic agents. *J Pharmacol Exp Ther*, **324**, 1181-7.

Ogunwobi OO, Liu C (2011). Hepatocyte growth factor upregulation promotes carcinogenesis and epithelial-mesenchymal transition in hepatocellular carcinoma via Akt and COX-2 pathways. *Clin Exp Metastasis*, **28**, 721-31.

Ohno Y, Ohno S, Suzuki N, et al (2005). Role of cyclooxygenase-2 in immunomodulation and prognosis of endometrial carcinoma. *Int J Cancer*, **114**, 696-701.

Park SW, Kim HS, Hah JW, et al (2010). Celecoxib inhibits cell proliferation through the activation of ERK and p38 MAPK in head and neck squamous cell carcinoma cell lines. *Anticancer Drugs*, **21**, 823-30.

Pietenpol JA, Stewart ZA (2002). Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. *Toxicology*, **181-182**, 475-81.

Smith WL, DeWitt DL, Garavito RM (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*, **69**, 145-82.

Sobolewski C, Cerella C, Dicato M, Diederich M (2011). Cox-2 inhibitors induce early c-Myc downregulation and lead to

- expression of differentiation markers in leukemia cells. *Cell Cycle*, **10**, 2978-93.
- Thiel A, Mrena J, Ristimäki A (2011). Cyclooxygenase-2 and gastric cancer. *Cancer Metastasis Rev*, **30**, 387-95.
- Wang GY, Zhang JW, Lü QH, Xu RZ, Dong QH (2007). Berbamine induces apoptosis in human hepatoma cell line SMMC7721 by loss in mitochondrial transmembrane potential and caspase activation. *J Zhejiang Univ Sci B*, **8**, 248-55.
- Wu K, Nie Y, Guo C, et al (2009). Molecular basis of therapeutic approaches to gastric cancer. *J Gastroenterol Hepatol*, **24**, 37-41.
- Xiao H, Zhang Q, Lin Y, Reddy BS, Yang CS (2008). Combination of atorvastatin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. *Int J Cancer*, **122**, 2115-24.
- Zhang CL, Wu LJ, Tashiro S, Onodera S, Ikejima T (2004). Oridonin induced A375-S2 cell apoptosis via bax-regulated caspase pathway activation, dependent on the cytochrome c/caspase-9 apoptosome. *J Asian Nat Prod Res*, **6**, 127-38.