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Anti-Proliferative Effects of Evodiamine on Human Thyroid Cancer Cell Line ARO

Meng-Ching Chen,[1] Ching-Han Yu,[1,2] Shyi-Wu Wang,[3] Hsiao-Fung Pu,[1] Shu-Fen Kan,[1] Lie-Chwen Lin,[4] Chin-Wen Chi,[5] Lary Low-Tone Ho,[5] Chen-Hsen Lee,[6] and Paulus S. Wang[1,4,7*]

[1]*Department of Physiology, School of Medicine, National Yang-Ming University, Taipei City 11221, Taiwan, Republic of China*

[2]*Department of Physiology, School of Medicine, Chung Shan Medical University, Taichung City 40201,Taiwan, Republic of China*

[3]*Department of Physiology and Pharmacology, Chang Gung University, Kwei-Shan, Taoyuan 33302, Taiwan, Republic of China*

[4]*Department of Herbal Drugs and Natural Products, National Research Institute of Chinese Medicine, Taipei City 11221, Taiwan, Republic of China*

[5]*Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei City 11217, Taiwan, Republic of China*

[6]*Department of Surgery, Taipei Veterans General Hospital, Taipei City 11217, Taiwan, Republic of China*

[7]*Department of Medical Research and Education, Taipei City Hospital, Taipei City 10341, Taiwan, Republic of China*

ABSTRACT

The incidence of thyroid cancer increases with age, and it is twice in women as common as in men. The undifferentiated thyroid cancer (UTC) is the most aggressive of all thyroid cancers. Unfortunately, there are almost no efficacious therapeutic modalities. It is important to develop some new effective therapies. Evodiamine is a chemical extracted from a kind of Chinese herb named Wu-Chu-Yu and has been demonstrated to be effective in preventing the growth of a variety of cancer cells. In the present study, the mechanism by which evodiamine inhibited the undifferentiated thyroid cancer cell line ARO was examined. Based on 3-(4,5-dimethylthiazol -2-yle)2,5-diphenyltetrazolium bromide (MTT) assay, cell proliferation rate was reduced dose-dependently by evodiamine, but not by rutaecarpine. According to the flow cytometric analysis, evodiamine treatment resulted in G2/M arrest and DNA fragmentation in ARO cells. The G2/M arrest was accompanied with an increase of the expression of cdc25C, cyclin B1, and cdc2-p161 protein, and it was also with a decrease of the expression of cdc2-p15. Furthermore, by using the TUNEL assay, evodiamine-induced apoptosis was observed at 48 h and extended to 72 h. Western blotting demonstrated that evodiamine treatment induced the activation of caspase-8, caspase-9, caspase-3, and the cleavage of poly ADP-ribose polymerase (PARP). These results suggested that evodiamine inhibited the growth of the ARO cells, arrested them at M phase, and induced apoptosis through caspases signaling. J. Cell. Biochem. 110: 1495–1503, 2010.

KEY WORDS: EVODIAMINE; THYROID CANCER CELL LINE ARO; G2/M ARREST; APOPTOSIS

Thyroid carcinoma is one of the most common malignancies in the endocrine system, and its incidence is higher in women than in men [Harris, 2002]. Papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and undifferentiated thyroid cancer (UTC) are the major type thyroid carcinomas originated from follicular epithelium, and all of them are resistant to chemotherapy [Stassi et al., 2003]. PTC and FTC are more differentiated with good prognosis and reveal good response to surgery and radioiodine treatment [Vini and Harmer, 2002], while UTC is highly aggressive and invasive. UTC exhibits worse prognosis associated with airway obstruction and distant metastasis [Pasieka, 2003; Sherman, 2003]. UTC patients usually die within 1 year after diagnosis, and

Shyi-Wu Wang and Paulus S. Wang contributed equally to this work.

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*Correspondence to: Paulus S. Wang, PhD, Department of Physiology, School of Medicine, National Yang-Ming University, Taipei City 11221, Taiwan, Republic of China. E-mail: pswang@ym.edu.tw

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combination therapies consisting of surgery, radioiodine, external radiation and chemotherapy cannot reduce the mortality [McIver et al., 2001]. Thus, the development of new effective therapeutic agents to approach UTC is extremely necessary.

Chemicals against cancer cells can be achieved by growth inhibition or apoptosis induction. The anticancer drugs, vinca alkaloids and paclitaxel, are microtubule inhibitors which block cell cycle process and trigger cell death subsequently [Blagosklonny et al., 1997; Bhalla, 2003]. There are several chemicals extracted from herbs exhibiting antiproliferative effects on cancer cells. Our previous studies demonstrated that digoxin and digitoxin, the major active ingredient of Chansu, represent inhibitory effects of proliferation in prostate cancer cells [Yeh et al., 2001, 2003]. Evodiamine and rutaecarpine are chemicals extracted from a kind of Chinese herb named Wu-Chu-Yu, which has been conventionally used as the treatment of gastrointestinal disorders and headache [Chiou et al., 1996]. The chemical structures of theses two compounds are similar, and both of them share some effects like antiallergic effect [Shin et al., 2007] and inhibition of corticosterone production in rat zona fasciculata-reticularis cells [Yu et al., 2009]. It had been reported that evodiamine exhibits several bioactive functions including vasodilation, anti-inflammation, bronchoconstriction, hypothermia and catecholamine secretion [Tsai et al., 1995; Chiou et al., 1996; Yoshizumi et al., 1997; Kobayashi et al., 2000]. In recent years, evodiamine has been demonstrated to be effective to prevent the growth of a variety of cancer cells. It was showed that evodiamine induced apoptosis through increasing the ratio of Bax/Bcl-2 expression in cervical cancer and melanoma [Wang et al., 2005; Yang et al., 2008]. The up-regulation of Bax expression and caspase-3 activity, which are known to initiate apoptosis, resulted in evodiamine treatment in leukemic T-lymphocytes [Huang et al., 2004]. We also found that evodiamine causes cell cycle arrest and apoptosis in androgen-dependent and -independent prostate cancer cells [Kan et al., 2004, 2007].

Accordingly, it was expected to know whether evodiamine affects the proliferation of UTC and to explore the underlying mechanisms. Thus our study aimed to provide a rationale for developing anticancer therapeutics against the undifferentiated thyroid cancer.

MATERIAL AND METHODS

CELLS, CULTURE MEDIUM, AND EVODIAMINE

The anaplastic thyroid cancer cell line ARO was kindly provided by Dr. Chin-Wen Chi (Department of Pharmacology, National Yang-Ming University, Taipei, Taiwan, ROC). ARO cells were propagated in RPMI 1640 Media (Gibco Laboratories, Buffalo, Grand Island, NY) with 10% fetal calf serum (FCS, KBH, Israel), 50 units/ml potassium penicillin G (Sigma Chemical Co., St. Louis, MO) and 50 units/ml streptomycin sulfate (Sigma Chemical Co.). Normal human mammary epithelial cell line H184B5H5/M10 was purchased from the Bioresource Collection and Research Center (BCRC), Taiwan, ROC. H184B5H5/M10 cells were cultured in alpha-minimum essential medium (Gibco) supplemented with 10% FCS. Both ARO and H184B5H5/M10 cells were cultured in humidified atmosphere of 5% CO_2–95% air at 37°C. Evodiamine and rutaecarpine were provided by Dr. Lie-Chwen Lin (National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC).

CELL PROLIFERATION ASSAY

The colorimetric [3-(4,5-dimethyl- thiazol-2-yle) 2,5-diphenyltetrazolium bromide] (MTT) assay was employed to quantify the cell proliferation. Briefly, cells were cultured in 96-well microplates (Falcon, Franklin Lakes, NJ) with the indicated media supplemented with 10% FCS. After 24 h, the media were removed and replaced by fresh medium containing different concentrations of drug, and the control group was incubated with drug-free medium. After 24, 48, 72, and 96 h, the media were removed and replaced by 50 μl of 1 mg/ml MTT (Sigma Chemical Co.) in serum-free media. After incubation with 5% CO_2–95% air at 37°C for 4 h, the MTT solution was removed and followed by adding 50 μl DMSO, and the plates were shaken for 3 min. The optical density of each condition was measured by the microplate reader (Dynatech Laboratories, Chantilly, VA) at a wavelength of 570 nm with a reference wavelength of 630 nm. Each experiment was repeated in triplicates.

FLOW CYTOMETRIC ANALYSIS OF THE CELL CYCLE DISTRIBUTION

Following evodiamine treatment, the ARO cells were harvested and centrifuged. Cell pellets were resuspended and fixed by 1% formaldehyde (Waco, Osaka, Japan) for 20 min. The fixed cells were mixed with 5 ml 70% cold ethanol (Sigma Chemical Co.) and incubated at −20°C for 4 h. The cells were washed and stained with 5 mg/ml propidium iodide (PI, Sigma Chemical Co.) in PBS containing 0.1% Triton X-100 solution and 10 μg/ml RNase (Sigma Chemical Co.) in the dark. The DNA content was analyzed by FACSCalibur (Becton Dickinson Co., Franklin Lakes, NJ) with CellQuest software.

TDT-MEDIATED dUTP NICK END LABELING (TUNEL) ASSAY

TUNEL assay was performed for the quantification of DNA fragmentation via an Apoptosis Detection System kit (Promega Corporation, Madison, WI). After exposure to evodiamine (1×10^{-7} to 1×10^{-5} M) for different time intervals, the ARO cells were harvested and fixed with 1% formaldehyde and 70% alcohol as previous described in the cell cycle analysis. The fixed cells (2×10^6 cells) were suspended with equilibrium buffer (200 mM potassium cocadehate, 25 mM Tris–HCl; 0.2 mM DTT, 0.25 mg/ml BSA and 2.5 mM cobalt chloride) and incubated for 5 min at room temperature. After centrifugation, the pallets were resuspended with 50 μl of TUNEL reaction mixture (containing 50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris–HCl, 1 mM EDTA and TdT enzyme, 25 U) and incubated at 37°C for 1 h in the dark. EDTA (20 mM) was added to stop the reaction. Cells were washed twice with PBS containing 0.1% Triton X-100 and 5 mg/ml BSA following centrifugation. The cells were resuspended with PBS and stained with 5 mg/ml PI (Sigma Chemical Co.) in PBS containing 0.1% Triton X-100 solution and 10 μg/ml RNase (Sigma Chemical Co.). The DNA fragmentation was determined by FACSCalibur (Becton Dickinson Co.) with an argon laser turned to wavelength 488 nm for excitation. The fluorescein-12-dUTP was measured at wavelength 520 nm.

WESTERN BLOTTING

The ARO cells treated with evodiamine were harvested and lysed in triple-detergent lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% Na-deoxycholate; 1% NP-40; 0.1% SDS). The cell lysates were centrifuged at 14,000*g* and 4°C for 15 min. Protein concentration of supernatant was determined. Equal amounts of lysate protein were separated by SDS–polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA). After being blocked with 5% nonfat skim milk in TBST [20 mM Tris–HCl (pH 7.6), 135 mM NaCl and 0.1% Tween 20), the membranes were probed with antibodies against PARP (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-caspase-3 (1:1,000, Cell Signaling Technology, MA), rabbit anti-caspase-8 (1:1,000, Cell Signaling Technology), mouse anti-caspase-9 (1:1,000, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), mouse anti-cyclin B1 (1:1,000, Upstate Biotechnology, NY), rabbit anti-cdc2 (1:1,000, BD Transduction Laboratories, San Diego, CA), rabbit anti- phospho-cdc2 (Thr 161, 1:1,000, Cell Signaling Technology), rabbit anti-phospho-cdc2 (Try 15, 1:1,000, Cell Signaling Technology), mouse anti-cdc25C (1:1,000, Santa Cruz Biotechnology), mouse anti-wee1 (1:500, Santa Cruz Biotechnology), mouse anti-C23 (1:1,000, Santa Cruz Biotechnology), or mouse anti-β-actin (1:8,000, Sigma Chemical Co.). The membranes were washed and incubated with horseradish peroxidase-labeled goat anti-mouse (1:10,000, Promega Corporation) or goat anti-rabbit (1:10,000, Promega Corporation). The signals were visualized with an enhanced chemiluminescence detection (Western blotting reagents, Amersham International Co., Bucks, UK).

IMMUNOPRECIPITATION

Treated ARO cells were lysed with the triple-detergent lysis buffer, and total protein was determined. One mg of cell lysate was incubated with 2 μg of anti-cdc2 overnight at 4°C. Subsequently, 20 μl of protein A/G-conjuagated agrose beads (Santa Cruz Biotechnology) were added and incubated for 2 h at room temperature. Immunoprecipited proteins were further analyzed by SDS–PAGE and Western blotting detection to mouse anti-cyclin B1 and rabbit anti-cdc2.

STATISTICAL ANALYSIS

The results were expressed as means ± standard error of mean (SEM) for each experiment. One-way analysis of variance (ANOVA) was performed for multiple comparisons. The difference between specific means was analyzed for significance by Duncan's multiplerange test [Steel and Torrie, 1980]. Values of P less than 0.05 were considered statistically significant.

RESULTS

EFFECTS OF EVODIAMINE AND RUTAECARPINE ON PROLIFERATION OF ARO AND H184B5H5/M10 CELLS

The effects of evodiamine (1×10^{-8} to 1×10^{-5} M) on the proliferation of ARO and H184B5H5/M10 cells were performed after incubation for 1–4 days. The cell proliferation was evaluated by MTT assay. After 24 h incubation, evodiamine at the concentration 1×10^{-5} M significantly decreased ($P < 0.01$) the proliferation of ARO cells. The anti-proliferation effect of evodiamine at 1×10^{-6} M was observed at 48 h after treatment ($P < 0.01$). The significant inhibitory effects on ARO cell growth caused by evodiamine were persisted for 96 h (Fig. 1A). But there were no similar effects caused by rutaecarpine, another major extract from Wu-Chu-Yu (Fig. 1B). The cytotoxicity of evodiamine (1×10^{-7} to 1×10^{-5} M) on ARO cells was less then 5% after 4, 8, 12, 24, or 48 h treatment (data not shown). To examine the anti-proliferative effects of evodiamine and rutaecarpine on non-malignant cells, MTT assay was performed on normal human mammary epithelial cell line H184B5H5/M10 after drugs treatment. The cell proliferation of H184B5H5/M10 cells was not influenced by rutaecarpine. The inhibition of cell proliferation by evodiamine only presented at the concentration 1×10^{-5} M on day 4 (Fig. 1C–D).

EFFECTS OF EVODIAMINE ON CELL CYCLE PROGRESSION IN ARO CELLS

To explore the cause of the anti-proliferative effects of evodiamine, the cell cycle distribution was measured after drug treatment at the indicated time period by flow cytometric analysis. Evodiamine treatment resulted in G2/M arrest of ARO cells (Fig. 2A). Administration of evodiamine (1×10^{-6} M) increased the cell population in G2/M phase by 34.8% ($P < 0.05$), 53.5% ($P < 0.01$), and 43.7% ($P < 0.01$) at 8, 12, and 18 h, respectively. A higher concentration (1×10^{-5} M) further increased the G2/M phase cells by 42.4% ($P < 0.01$), 67.1% ($P < 0.01$), and 74.2% ($P < 0.01$) at the corresponding time points. The interference of the cell cycle progression caused by evodiamine could be extended to 72 h (Fig. 2B). Furthermore, an increasing level of hypodiploid population (DNA content less than 2n) was also observed after 8 h incubation and persisted for 72 h in ARO cells (Fig. 2C).

EFFECTS OF EVODIAMINE ON THE EXPRESSION OF REGULATORS OF CELL CYCLE IN ARO CELLS

To examine the mechanism of G2/M arrest caused by evodiamine, the expressions of cell cycle-regulating molecules were studied by immunoblot analysis. There was an increasing expression of cyclin B1 after evodiamine (1×10^{-6} and 1×10^{-5} M) treatment for 12 and 18 h, and the expression of cdc2 was not altered by several doses of evodiamine during the same time period. The elevated expression of phospho-cdc2 at threonine 161 site was detected to be accompanied with a decrease in the expression of phospho-cdc2 at tyrosine 15 after evodiamine exposure for 12 and 18 h (Fig. 3A). The expression of β-actin was used as an internal control. Similar results were obtained from four separate experiments.

We further investigated the expression of wee1 and cdc25C, which were known to regulate the activities of cdc2 and cyclin B1. The data showed that the expression of wee1 was not altered after evodiamine treatment. The level of cdc25C expression elevated at 12 h after incubation with evodiamine (1×10^{-6} and 1×10^{-5} M) and extended to 18 h (Fig. 3B,C).

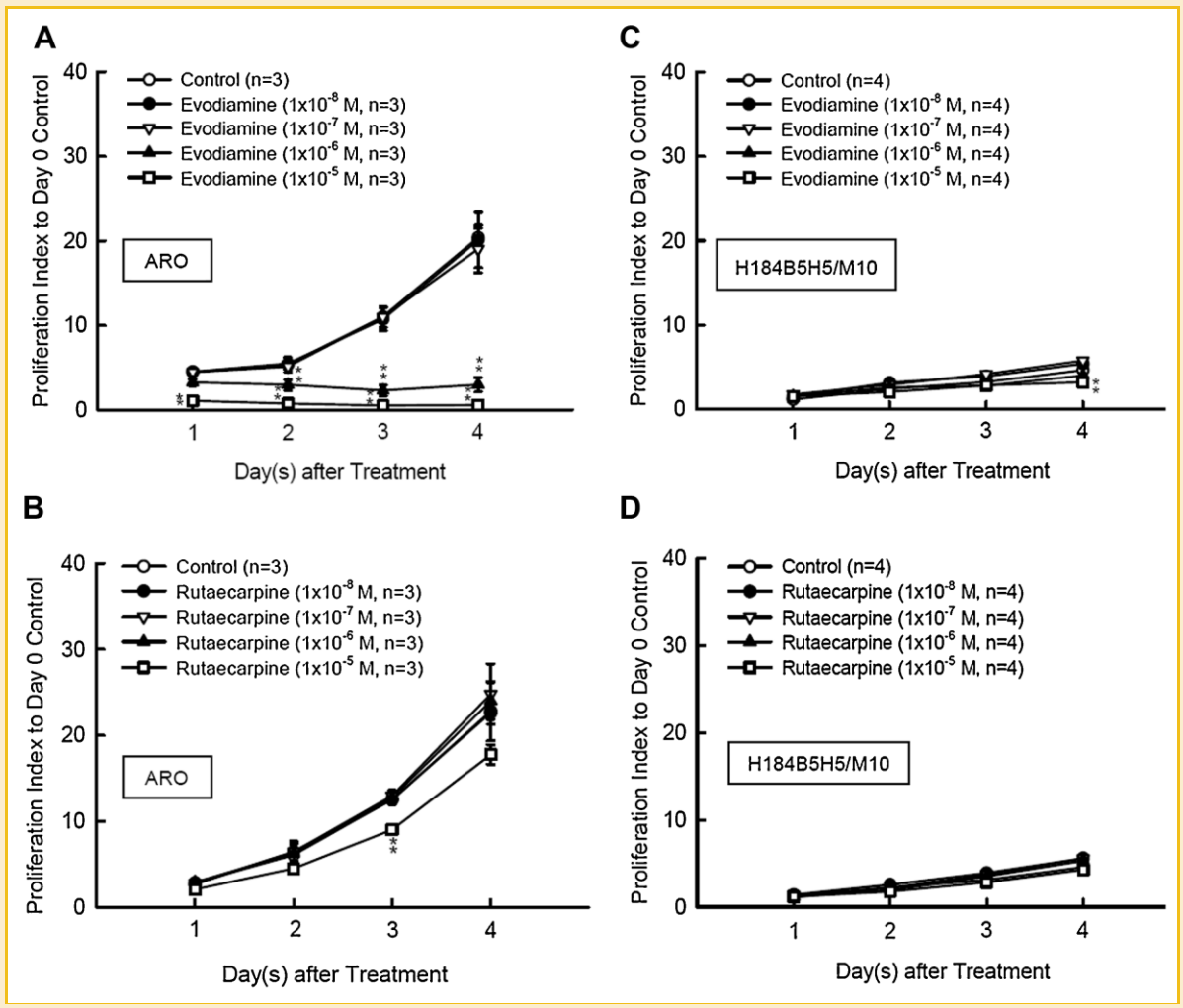

Fig. 1. Effects of evodiamine and rutaecarpine on the proliferation of ARO cells (A,B) and H184B5H5/M10 cells (C,D) after incubation for 1–4 days. Cell proliferation was measured by MTT assay. The results are expressed as means ± SEM of three or four independent experiments. $^{**}P < 0.01$ versus control.

To determine whether evodiamine induced the ARO cell cycle arrest at G2 or M phase, the expression of cdc2-cyclin B1 complex was examined. The immunoprecipited cdc2 was further separated by SDS–PAGE and analyzed by cyclin B1. In Figure 3D,E, the level of cyclin B1 expression was elevated after the administration of evodiamine at the concentration of 1×10^{-6} and 1×10^{-5} M for 12 and 18 h. These results showed that evodiamine induced the formation of cdc2-cyclin B1 complex.

EVODIAMINE-INDUCED APOPTOSIS

Based on flow cytometric analysis, evodiamine caused an elevation of hypodiploid population as well as G2/M arrest in ARO cells. The results indicated that evodiamine induced apoptosis. We further detected the quantification of DNA fragmentation via TUNEL assay after various concentrations of evodiamine treatment. The data showed that evodiamine (1×10^{-6} M) induced an increase of apoptotic cells by 8.62% ($P < 0.05$) and 6.67% ($P < 0.05$) at 48 and 72 h, respectively. After exposure to higher concentration of evodiamine (1×10^{-5} M), the numbers of TUNEL-positive cells were elevated (Fig. 4A,B). Similar results were obtained from four separate experiments.

EFFECTS OF EVODIAMINE ON CASPASE-3 ACTIVATION AND PARP CLEAVAGE

To clarify the mechanism responsible for evodiamine-induced apoptosis, caspase-3 activation and cleavage of the caspase substrate PARP were examined. ARO cells were treated with evodiamine for various periods of time, and the cell lysates were analyzed by immunoblot. Caspase-3 activation was detected after evodiamine (1×10^{-6} and 1×10^{-5} M) incubation for 48 h. After a longer period of exposure, evodiamine significantly increased the cleavage form of caspase-3 at 72 h (Fig. 5A). To demonstrate the cleavage of substrate PARP caused by evodiamine, the nuclear lysates were analyzed. Similarly, PARP cleavage was observed after evodiamine (1×10^{-6} and 1×10^{-5} M) incubation for 48 and 72 h (Fig. 5B).

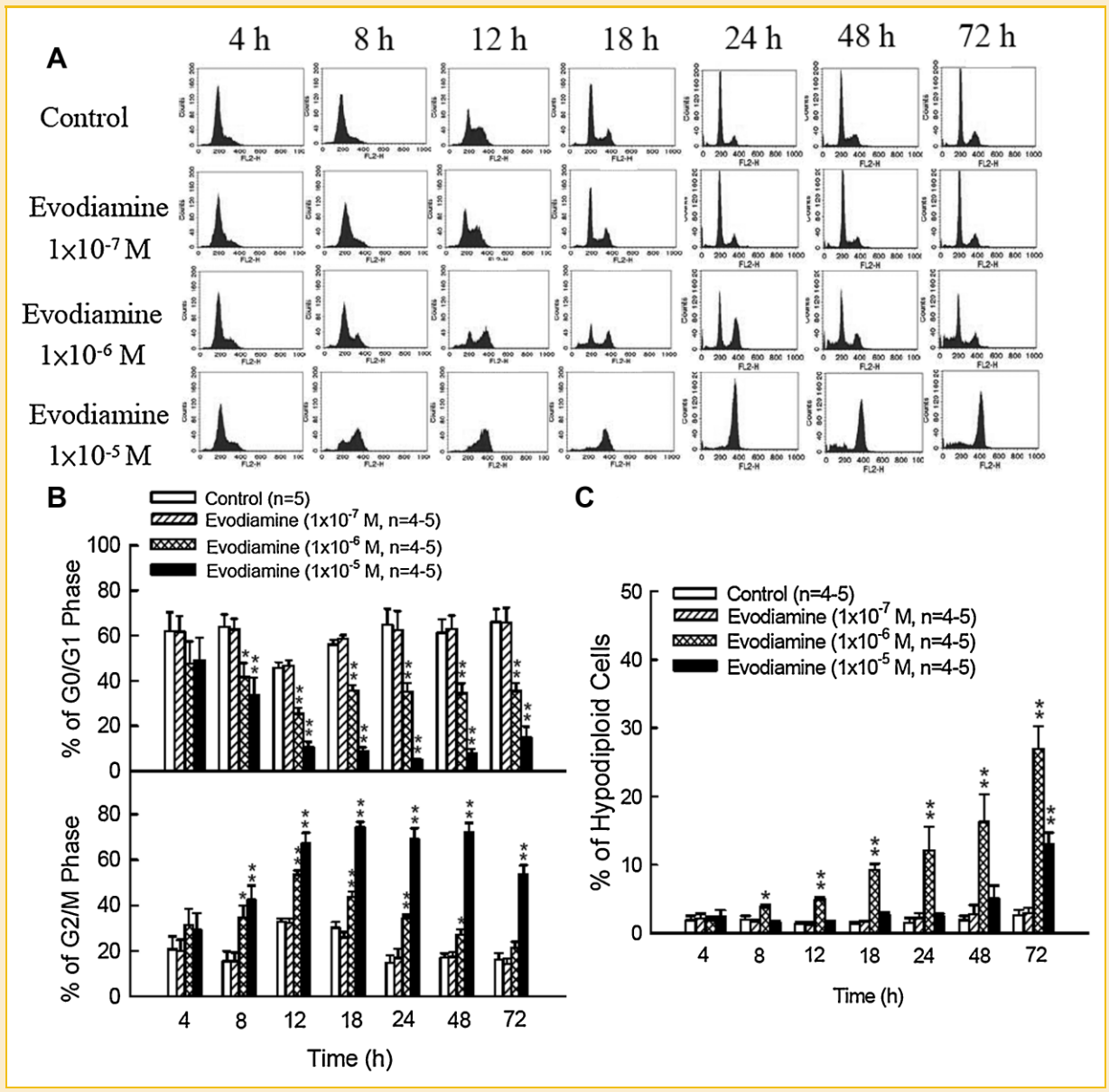

Fig. 2. A: Cell cycle distribution after evodiamine treatment in ARO cells. After exposure of evodiamine, the DNA histogram was measured by flow cytometry at the indicated time period. B: The percentage of G0/G1 and G2/M phase distribution after evodiamine treatment in ARO cells. C: The percentage of hypodiploid cells after evodiamine treatment in ARO cells. The results are expressed as means ± SEM of four or five independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

The expressions of β-actin and C23 were internal controls. Similar experiments were repeated three times.

EFFECTS OF EVODIAMINE ON CASPASES PROCESSING

Most caspases exist as inactive zymogens in normal situation and are activated by proteolytic cleavage during apoptosis. Since evodiamine promoted caspase-3 activation and cleavage of PARP, the activation of upstream caspase-8, -9 was further examined. Immunoblot was performed for the lysate of ARO cells following incubation with evodiamine for different periods. The results showed that procaspase-8 was cleaved after evodiamine treatment (1×10^{-6} and 1×10^{-5} M) for 36 h and increased significantly at 60 h (Fig. 5C). The proteolytic fragments of caspase-9 were observed at 48 h and could be extended to 72 h (Fig. 5D). The expression of β-actin was internal control. Similar results were obtained from two separate experiments.

DISCUSSION

Thyroid carcinoma is one of the most common malignant cancers in the endocrine system. There are several therapeutic modalities, but the undifferentiated thyroid cancer (UTC), the most lethal one, is hard to be approached [Gimm, 2001]. It is necessary to develop a new weapon against UTC. It has been reported that evodiamine exhibits antitumor effects on a variety of cancer cells. In this

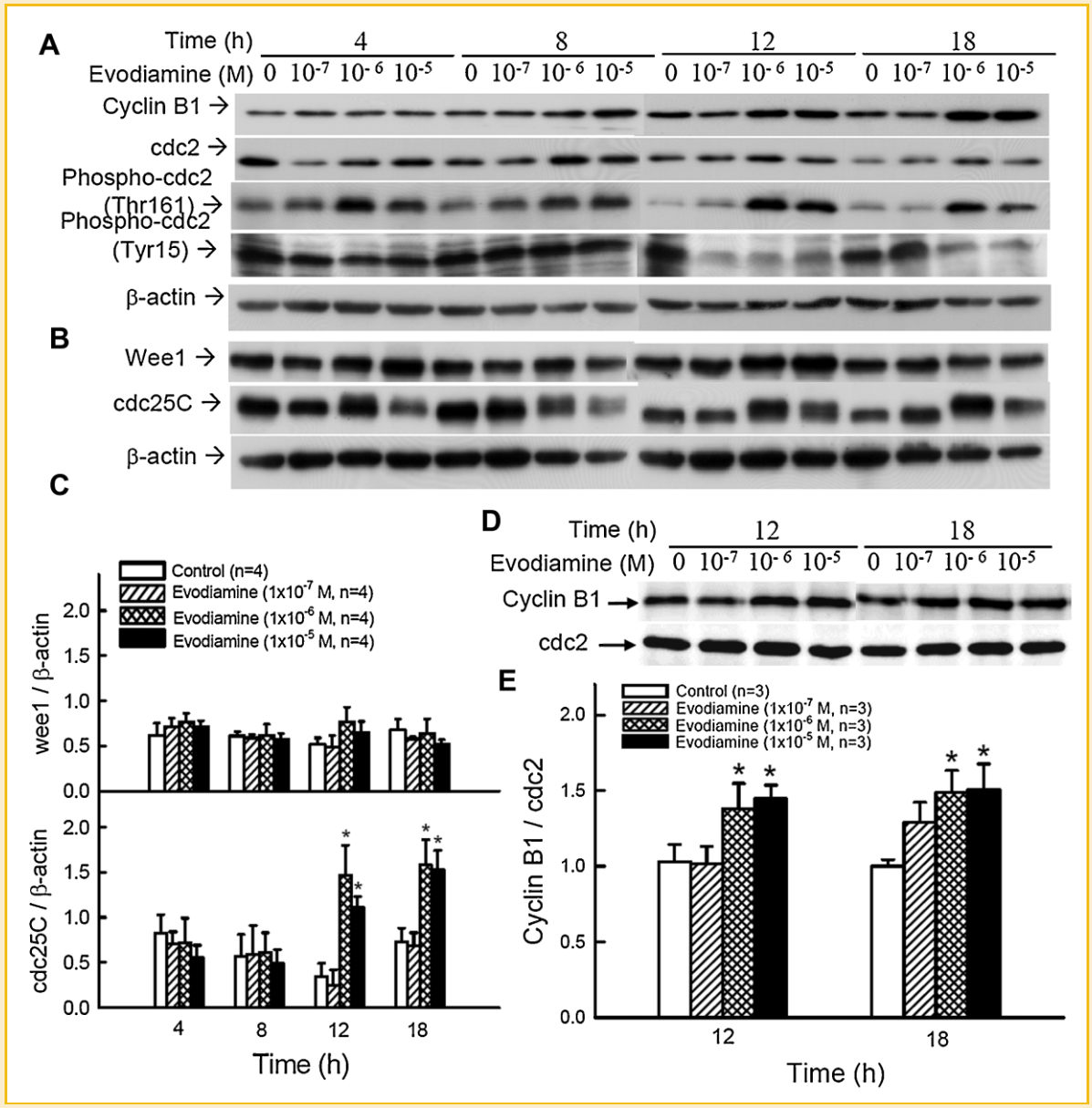

Fig. 3. A,B: Expressions of cell cycle–regulating molecules after evodiamine treatment in ARO cells. ARO cells were incubated with evodiamine for 4–18 h. The cell lysate were separated by SDS–PAGE and analyzed by Western blot. Similar results were obtained from four independent experiments. C: The results are expressed as means ± SEM of four independent experiments. D: The binding of cdc2 with cyclin B1 after the treatment of evodiamine for 12 and 18 h. Immunoprecipitation of cdc2 was performed. Samples were further analyzed by Western blotting detection of cyclin B1. E: The results were quantified as means ± SEM of three independent experiments. $^{*}P < 0.05$ versus control.

study, we demonstrated that evodiamine, the bioactive compound extracted from Wu-Chu-Yu [Lin et al., 1999], significantly inhibited the proliferation of undifferentiated thyroid cancer ARO cells, whereas rutaecarpine did not. We also found very little anti-proliferative effects of evodiamine on normal human mammary epithelial cell line H184B5H5/M10. Thus, the effective concentration of evodiamine could be used in the in vivo study.

Evodiamine has been shown to induce cell cycle arrest in prostate cancer, breast cancer, and colorectal cancer [Huang et al., 2004; Kan et al., 2004; Kan et al., 2007; Yang et al., 2009]. According to the flow cytometry analysis in the present study, the evodiamine-induced cell cycle arrest in G2/M phase in ARO cells had a dose-dependent manner. Chemicals that interrupt cell cycle in proliferating cells are candidates of new treatments for cancers [Collins and Garrett, 2005]. Camptothecin and huanglian were found to inhibit proliferation of human cancer cells by inducing G2 arrest which was associated with suppression of cdc2 kinase activity and cyclin B1 expression [Li et al., 2000; Janss et al., 2001]. Paclitaxel (Taxol), a chemotherapeutic agent, was revealed to suppress the proliferation of cancer cells by the accumulation of cells in G2/M phase and inducing apoptosis [McDaid and Horwitz, 2001]. In ARO cells, the decreased expression of phospho-p15cdc2 and increased expression of cyclin B1 and phospho-p161cdc2 caused by evodiamine were accompanied with G2/M phase arrest. The enhanced expression of

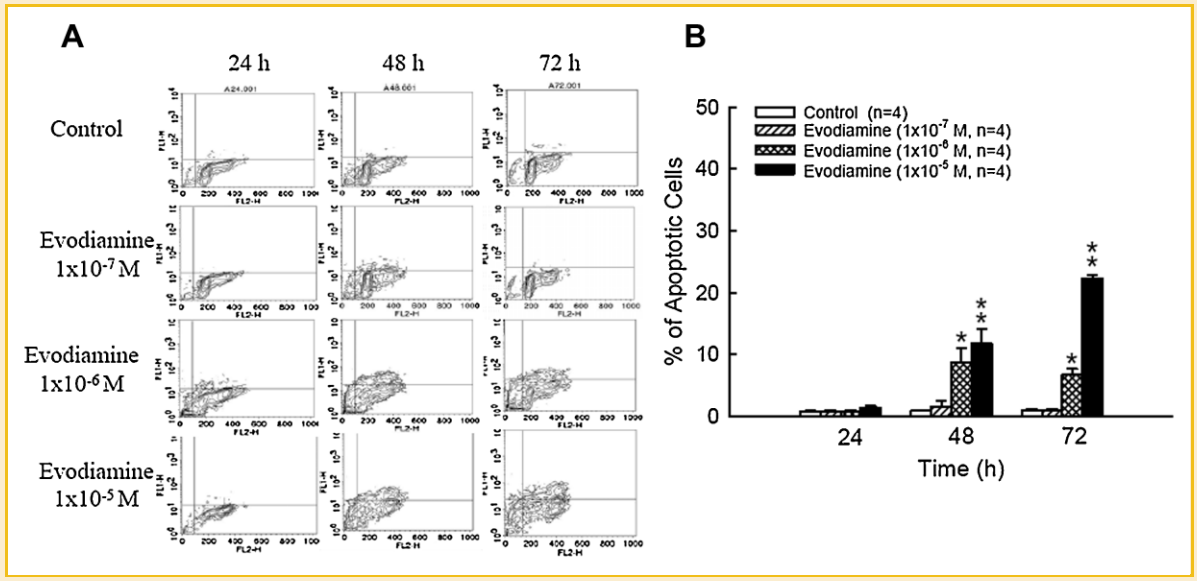

Fig. 4. A: Detection of evodiamine-induced apoptosis in ARO cells. ARO cells were incubated with evodiamine and evaluated for DNA fragmentation at the indicated duration by using the TUNEL assay. B: The percentage of evodiamine-induced apoptosis in ARO cells. The results are expressed as means ± SEM of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

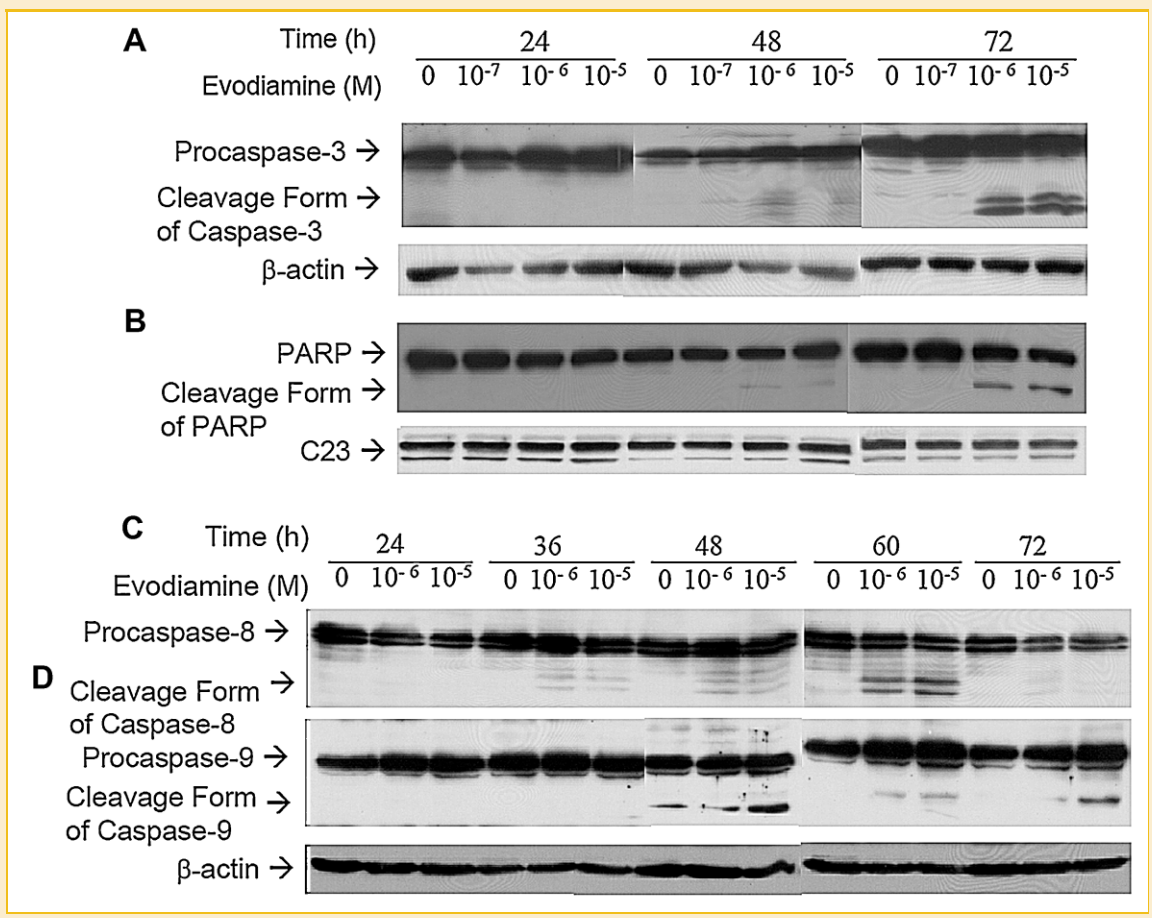

Fig. 5. Analysis of evodiamine-induced caspases processing (A,C,D) and PARP (B) expressions in ARO cells. ARO cells were incubated with evodiamine for different time intervals. The cell lysate was extracted and analyzed by Western blot. Similar results were obtained in two or three separate experiments.

cdc25C was also observed, whereas wee1 was not. Cdc2 is inactivated throughout the S and G2 phases since the wee1 protein kinase phosphorylates cdc2 on tyrosine 15. In the late G2, cdc25C dephosphorylates cdc2 on tyrosine 15, and CAK (Cdk-activating kinase) phosphorylates cdc2 on threonine 161, which results in the activation of cdc2-cyclin B1 complex [Graves et al., 2000; Castedo et al., 2004]. Cyclin B1 is destructed at the end of mitosis phase, and inactivated cdc2 is necessary for the exit from mitosis [Ciliberto et al., 2005; Pines, 1995]. Our results indicated that evodiamine indirectly activates cdc2 by cdc25C and an increased expression of cyclin B1. Immunoprecipitation showed that the level of cdc2-cyclin B1 complex was elevated after evodiamine treatment. It meant that ARO cells would pass through the G2-M checkpoint and arrest in M phase after evodiamine treatment.

An increased amount of the hypodiploid population was observed by flow cytometry after 8-h incubation with evodiamine. As a result, TUNEL assay was performed to quantify the DNA fragmentations. According to the TUNEL assay, evodiamine led to the apoptosis of ARO cells in a time- and dose-dependent manner.

Recently, the mechanisms of apoptosis caused by evodiamine in cancer cells were investigated. Liao et al. [2005] found that caspase-7 was activated after evodiamine treatment, but that caspase-3 failed to be cleaved in the multiple-drug resistant breast cancer NCI/ADR-RES cells. Other studies reported that evodiamine induced apoptosis by caspase-3 activation in human leukemic T-lymphocytes, human prostate cancer cells (LNCaP, DU145 and PC3), and human melanoma, A375-S2 [Zhang et al., 2003; Huang et al., 2004; Kan et al., 2004, 2007]. In our study, we demonstrated that caspase-3 was involved in evodiamine-induced apoptosis in ARO cells. Cleavage of PARP, which is known to be the downstream effector of caspase-3, was also observed after evodiamine treatment for 48 h. The exhibition of cleaved PARP is a marker of apoptosis [Sima and Li, 2005]. PARP plays a role in DNA repairs. Cleaved PARP would lose its ability to respond to DNA fragmentation during drug-induced apoptosis [Boulares et al., 1999]. In the light of MTT assay, evodiamine significantly decreased the proliferation of ARO cells, although the proliferation index was greater than 1 after incubation for 24 h till 48 h. These results implied that the anti-proliferation effects of evodiamine consisted of cell cycle arrest in the first 48 h after evodiamine treatment and apoptosis thereafter.

The upstream of caspase-3 activation was determined subsequently. Caspases are the central executioners in the apoptotic process. Most of them are synthesized as zymogens (procaspases) and activated by the proteolytic cleavage [Scaffidi et al., 1998]. Caspases have been divided into the initiator (caspase-8 and caspase-9) and the effector (caspase-3 and caspase-7), which is activated by the former and then initiates apoptosis [Hengartner, 2000]. Evodiamine-induced apoptosis is mediated by the activation of caspase-9 in human prostate cancer cells [Kan et al., 2004, 2007]. In the present study, the proteolytic processes of caspase-8 and -9 were induced after evodiamine treatment for 36 and 48 h, respectively. Caspase-8 is the main initiator caspase in the death-receptor pathway, and activation of caspase-9 is essential for the mitochondrial pathway [Zimmermann et al., 2001; Senderowicz, 2004]. On the other hand, caspase-8-mediated cleavage of Bid induces the release of cytochrome *c* from mitochondria, which in turn activates caspase-9 and apoptosis [Fadeel and Orrenius, 2005]. Accordingly, it needs to be determined whether cleaved caspase-3 is caused by death-receptor pathway, mitochondrial pathway, or both of them.

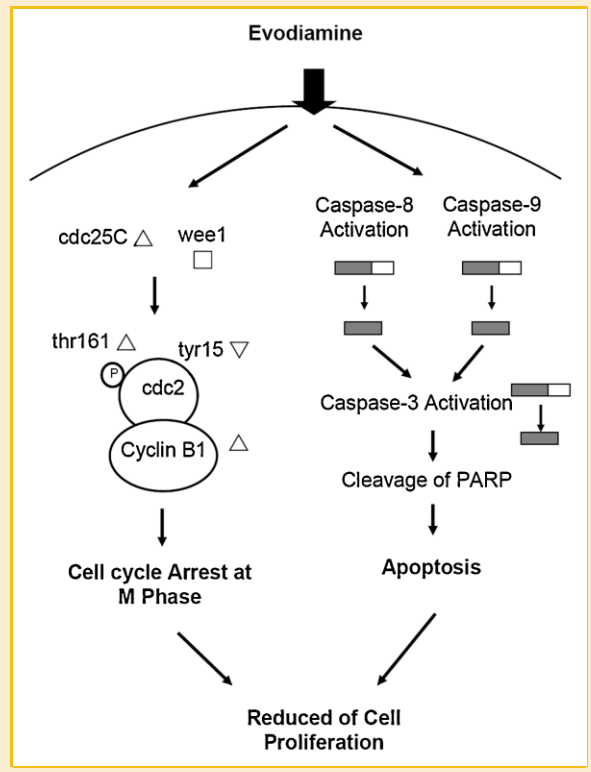

Fig. 6. Schematic model of cell cycle arrest and the apoptotic mechanism caused by evodiamine on human thyroid cancer cells. △ = Increased, ▽ = Decreased, □ = Unchanged.

Figure 6 is the schematic model of the anti-proliferation effects of evodiamine on human thyroid cancer ARO cells. In summary, our study demonstrated that evodiamine caused [1] M phase arrest which was associated with the decreased expression of phospho-p15cdc2, the increased expression of cyclin B1 and phospho-p161cdc2, and [2] apoptosis through a caspase-dependent mechanism in human thyroid cancer ARO cells. Therapeutic agents which target cell cycle and apoptosis regulation have emerged as attractive remedies for cancers [Maddika et al., 2007]. Our results indicate evodiamine as a potential anti-tumor agent for undifferentiated thyroid cancer.

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