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## Abstract

**Background:** To investigate the effects of ephedrine and anisodamine on the proliferation of human breast cancer.

**Materials and Methods:** SKBR3 cell was treated with or without ephedrine and / or anisodamine, respectively. The trypan blue exclusion assay was used to determine cell numbers. Flow cytometry was used to assess cell cycle distribution and apoptosis. The concentration of cAMP and cyclin D1 was analyzed by enzyme-linked immunosorbent assay. Western blot was used to measure PKA.

**Results:** Ephedrine and anisodamine inhibited cell proliferation and arrested SKBR3 cells at G<sub>0</sub>/G<sub>1</sub> phases. Ephedrine and anisodamine increased the level of CD1 in SKBR3 cells. Furthermore, significant change in intracellular cAMP concentration was found in SKBR3 cells treated with ephedrine and anisodamine. The phosphorylation of PKA substrate was not activated after 48 hours of treatment with ephedrine and anisodamine.

**Conclusion:** Ephedrine and anisodamine inhibit the proliferation of SKBR3 cells via a significantly change of intracellular cAMP concentration.

**Key words:** anisodamine, breast cancer, cyclic adenosine monophosphate, ephedrine, proliferation

**Abbreviations:** cAMP, cyclic adenosine monophosphate; TCM, traditional Chinese medicine; ELISA, enzyme-linked immunosorbent assay; CD1, cyclin D1; SNS, sympathetic nervous system.

## Introduction

Breast cancer is the most common malignancy in women in the United States and is second cancer as a cause of cancer death (Jemal et al. 2008). At present, the cancer treatment by chemotherapeutic agents, surgery and radiation have not been fully effective against the high incidence or low survival rate of most the cancers. The development of new therapeutic approach to breast cancer remains one of the most challenging areas in cancer research.

The current study shows that selecting cyclic adenosine monophosphate (cAMP) as a therapeutic target has been a new therapeutic strategy against cancer (Taylor et al. 2008a). cAMP is the secondly messenger to be discovered. It plays an important role in intracellular signal transduction of various stimuli and is known to be involved in metabolism, cell proliferation, differentiation and so on (Robinson-White et al. 2002, Robinson-White et al. 2003). In particular, cAMP signaling has an important role in cancer biology. Selective modulation of PKA isozymes using cAMP elevating agents can induce growth inhibition in a wide variety of cancer models through the induction of apoptosis and / or cell cycle arrest (El-Mowafy et al. 2003, Vitale et al. 2009).

The sympathetic nervous system can function in cAMP signal system (Linden 1987, Wallukat 2002). Furthermore, the sympathetic nervous system is known to affect angiogenesis, vessel permeability, immune responses and carcinogenesis. Recent findings indicate that, in addition to angiogenic and lymphangiogenic factors, tumor cells release neurotrophic factors that initiate innervation. Interactions between cytokines and sympathetic neurotransmitters, and their respective receptors expressed by the nerve, immune and tumor cells appear to influence tumor growth.

Ephedra and Henbane Seed, which have the bioactivity of warming Yang and eliminating cold in traditional Chinese medicine (TCM), are closely related to the sympathetic nervous (Chen et al. 2010). The major bioactive component of Ephedra and Henbane Seed are ephedrine and

anisodamine, which can act on the adrenergic receptor and cholinergic receptor, respectively. Together, these findings prompted us to investigate whether ephedrine and anisodamine may have effect on cAMP levels and induce growth inhibition in human breast cancer cells.

## Materials and Methods

### Materials and Cell Culture

Human breast cancer SKBR3 cells were obtained from immunology laboratory of Tianjin Medical University Cancer Hospital and were maintained in DMEM (GIBCO) supplemented with 10% FBS (Hangzhou Sijiqing Biological Engineering Materials Co) and penicillin / streptomycin in a 37°C incubator with 5% CO<sub>2</sub>. Cells were plated on 6- or 24-multiplates at  $1 \times 10^5$ /ml or  $5 \times 10^3$ /ml and allowed to grow for 1 day before being exposed to ephedrine (1µg/ml, 10µg/ml and 100µg/ml, respectively) and anisodamine (0.2µg/ml, 2µg/ml and 20µg/ml, respectively). For the control group, an equal volume of DMEM was added into the medium. Cyclic AMP enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (USA). Annexin V-FITC /7-AAD apoptosis kits were purchased from Biolegend (USA).

### Proliferation assay

SKBR3 cells were seeded into 24-well plates at a density of  $5 \times 10^3$  per well and were treated as indicated. Cells were collected after trypsin digestion and counted with a hemocytometer by trypan blue counting method.

### Flow cytometry

Cells were collected at 48 hours after adding ephedrine or anisodamine, washed three times with 1×PBS, and pelleted by low speed centrifugation. Pellet was resuspended with 70% ethanol for 30 min at 4°C. Cells were spun down and were incubated with the DNA-binding dye propidium iodide (PI) solution [0.1% sodium citrate (w/v), 0.1% Triton X-100 (v/v), and 50 mg/L PI in deionized water] for 1 hour at room temperature. Finally, cells were analyzed by flow cytometer.

### cAMP accumulation

Cells were plated on 6-well plates at a density of  $5 \times 10^3$  per well and incubated in DMEM (GIBCO) supplemented with 10% FBS for 48 hours, then washed three times with PBS. Transient assay for cyclic AMP was performed by incubation with drugs for 30 minutes and durable assay for cyclic AMP was performed by incubation with drugs for 24 hours. Assay medium was aspirated, and cells were washed three times in cold PBS. Cells were resuspend in Cell Lysis Buffer 5 (1X) to a concentration of  $1 \times 10^7$  cells / ml. Cells were freezed at  $\leq -20^\circ$  C and thawed with gentle mixing. The freeze/thaw cycle was repeated once. Then it centrifuged at 600 g for 10 minutes at 2 - 8°C to remove cellular debris. The supernate was assayed immediately using the cAMP elisa kit (R&D, USA).

### Western blot analysis

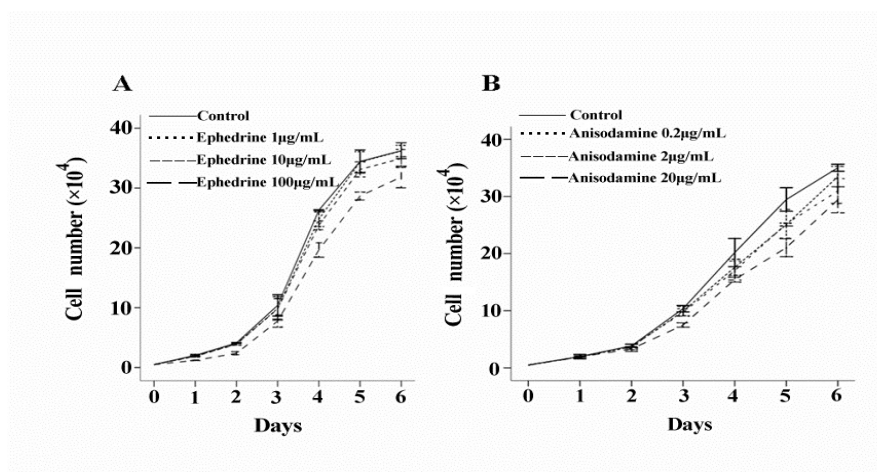
Cells were plated on 6-well plates at a density of  $5 \times 10^3$  per well and incubated in DMEM (GIBCO) supplemented with 10% FBS for 24 hours. The media were replaced with fresh media containing 100 µg/ml ephedrine, 5 µg/ml anisodamine, 20 µg/ml epinephrine, 0.15 µg/ml atropine and 1.5 µg/ml atropine, and cells were cultured for 48 hours. Then cells were lysed and subjected to western blotting. Aliquots of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Blots were incubated with mouse monoclonal anti-phospho-PKA substrate rabbit antibody (1:1000, Cell Signaling Technology), and anti-actin antibody (1:5000, Sigma) overnight. Protein expression was detected using the ECL Western Blotting detection system.

## ELISA

ELISA were performed to detect the levels of cyclin D1 (CD1) (Dakewei, China) according to the manufacturer's instructions. Cells were plated on 6-well plates at a density of  $5 \times 10^3$  per well and incubated in DMEM (GIBCO) supplemented with 10% FBS for 24 hours. The media were replaced with fresh media containing 100  $\mu\text{g/ml}$  ephedrine, 5  $\mu\text{g/ml}$  anisodamine, 20  $\mu\text{g/ml}$  epinephrine and 0.015  $\mu\text{g/ml}$  atropine, and cells were cultured for 48 hours. Then cells were lysed and the levels of CD1 were measured.

## Data Analysis and Statistics

Data are presented as the mean  $\pm$  SD ( $n=3$ ). Statistical comparisons ( $t$  tests and one - way analysis of variance) were performed, and graphics were produced using SPSS (Version: 13.0, Chicago, USA).



**Figure 1:** Effects of ephedrine and anisodamine on proliferation of SKBR3 cells

SKBR3 cells were exposed to ephedrine at different concentrations and harvested at 24, 48, 72, 96, 120 and 144 hours. Trypan blue exclusion assay was used to determine the total cell count and viable cell number. (A) Growth curves of SKBR3 cells treated with ephedrine. (B) Growth curves of SKBR3 cells treated with anisodamine. Ephedrine and anisodamine could inhibit the proliferation of SKBR3 cells and these inhibit effects are dose-dependently.

## Results

## Effects of ephedrine and anisodamine on the proliferation of SKBr3 cells

The relationship between concentration of ephedrine and anisodamine and their antiproliferation effect on SKBR3 cells was investigated by trypan blue counting method. The growth curves showed that ephedrine and anisodamine had a significant inhibitory effect on the proliferation of SKBR3 cells (Fig. 1). The maximum inhibition rates for ephedrine and anisodamine on SKBR3 were 42.88% and 28.57%, respectively. In contrast to high concentration of ephedrine (500  $\mu\text{g/ml}$ ), which caused death of SKBR3 cells within several hours, ephedrine (1  $\mu\text{g/ml}$  - 100  $\mu\text{g/ml}$ ) and anisodamine (0.2  $\mu\text{g/ml}$  - 100  $\mu\text{g/ml}$ ) did not show any impact on SKBR3 cell survival. A direct count of the percentage of cell death showed no difference between control and ephedrine / anisodamine treated SKBR3 cells (data without shown).

**Effects of ephedrine and anisodamine on apoptosis and cells cycle distribution**

We carried out flow cytometry analysis on SKBR3 cells treated with various concentrations of ephedrine and anisodamine. The percentage of G<sub>0</sub>/G<sub>1</sub> - phase cells in the group treated with 100 µg/ml ephedrine and the group treated with 20 µg/ml anisodamine was higher than that of the control group, while the percentage of S phase cells and G<sub>2</sub>/M - phase cells decreased in the treatment groups (Table 1, Table 2). Although the percentage of apoptosis was difference between ephedrine - treated group and control group ( $2.835 \pm 0.535$  VS  $0.710 \pm 0.100$ ,  $P < 0.01$ ), ephedrine and anisodamine did not induce the apoptosis of SKBR3 cell significantly (Table 1, Table 2).

**Table 1:** Effect of ephedrine on cell cycle distribution and apoptosis of SKBR3 cells

Group	G <sub>0</sub> /G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)	Apoptosis (%)
Control	70.705±0.795	20.175±1.235	9.120±0.440	0.710±0.100
ephedrine 1µg/ml	69.820±0.170	21.400±1.260	8.780±1.090	0.785±0.085
ephedrine 10µg/ml	72.350±0.610	18.900±0.150	8.750±0.760	1.575±0.135*
ephedrine 100µg/ml	84.010±0.015*	9.330±0.120*	6.655±0.105**	2.835±0.535*
epinephrine 0.05µg/ml	68.920±3.300	21.767±4.725	8.670±0.780	0.400±0.010
epinephrine 0.5µg/ml	67.990±2.070	21.935±2.575	10.075±0.505	0.565±0.005
epinephrine 5µg/ml	68.990±4.530	21.870±4.830	9.147±0.305	0.380±0.010

\* Versus control group  $P < 0.01$ , \*\* Versus control group  $P < 0.05$ .

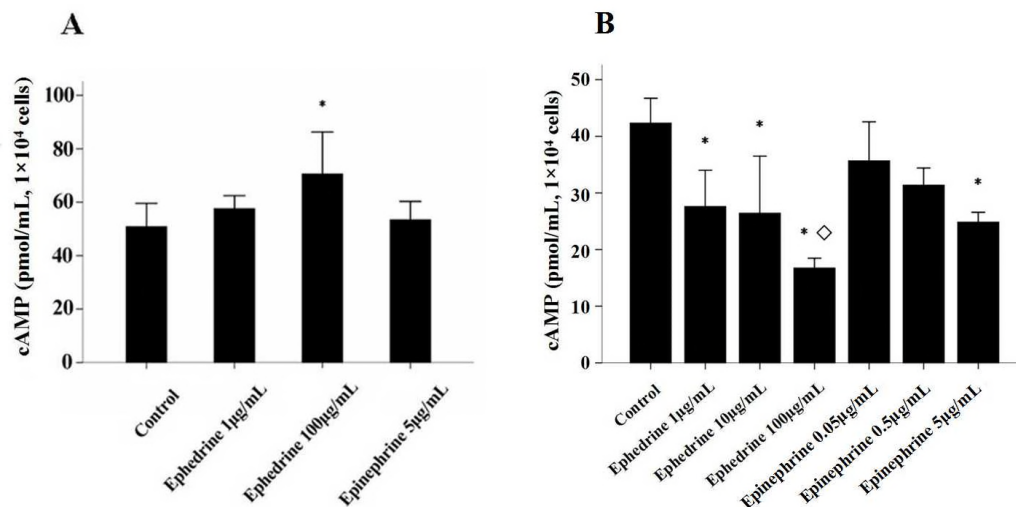
**Table 2:** Effect of anisodamine on cell cycle distribution and apoptosis of SKBR3 cells

Group	G <sub>0</sub> /G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)	Apoptosis (%)
Control	53.530±0.242	34.550±0.283	11.920±0.525	2.530±0.323
anisodamine 0.2µg/ml	55.335±0.345	33.440±0.052	11.225±0.292	1.940±0.294
anisodamine 2µg/ml	54.645±0.788	34.205±0.087	11.150±1.270	2.070±0.167
anisodamine 20µg/ml	63.305±0.632*	28.760±0.727*	7.935±0.095*	1.267±0.101
atropine 0.015µg/ml	59.630±0.341*	30.960±0.260*	9.410±0.081**	1.287±0.401
atropine 0.15µg/ml	55.885±0.303**	33.065±1.335	11.040±0.468	2.450±0.058
atropine 1.5µg/ml	55.070±0.104	32.945±0.176	11.985±0.072	1.340±0.012

\* Versus control group  $P < 0.01$ , \*\* Versus control group  $P < 0.05$ .

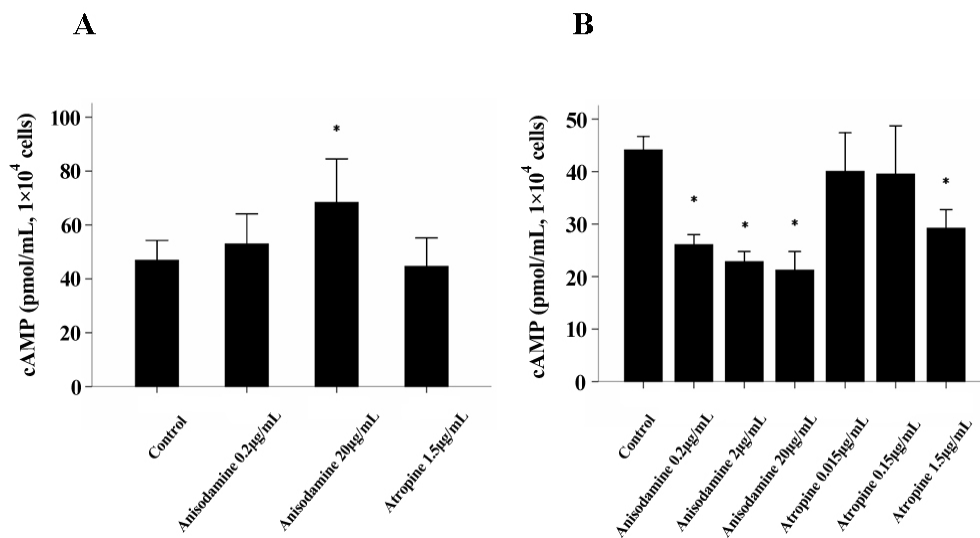
**Effects of ephedrine and anisodamine on intracellular cAMP**

To determine whether ephedrine and anisodamine effect intracellular cAMP in SKBR3 cells, we studied the concentration of cAMP at 30 minutes and 24 hours after treatment with ephedrine and anisodamine. Treatment with ephedrine 100µg/ml and anisodamine 20 µg/ml for 30 minutes resulted in a significant increase of intracellular cAMP content in SKBR3 cell, whereas the cAMP content decreased significantly at 24 hours (Fig.2, Fig. 3).



**Figure 2:** Effect of ephedrine on intracellular cAMP concentration of SKBR3 cells

(A) Effect of ephedrine on intracellular cAMP concentration at 30 minutes. (B) Effect of ephedrine on intracellular cAMP concentration at 24 hours. The intracellular cAMP content in SKBR3 cells was significantly increased at 30 minutes and decreased significantly at 24 hours. \* Versus control group  $P < 0.05$ , ◇ Versus ephedrine 10µg/ml  $P < 0.05$ .

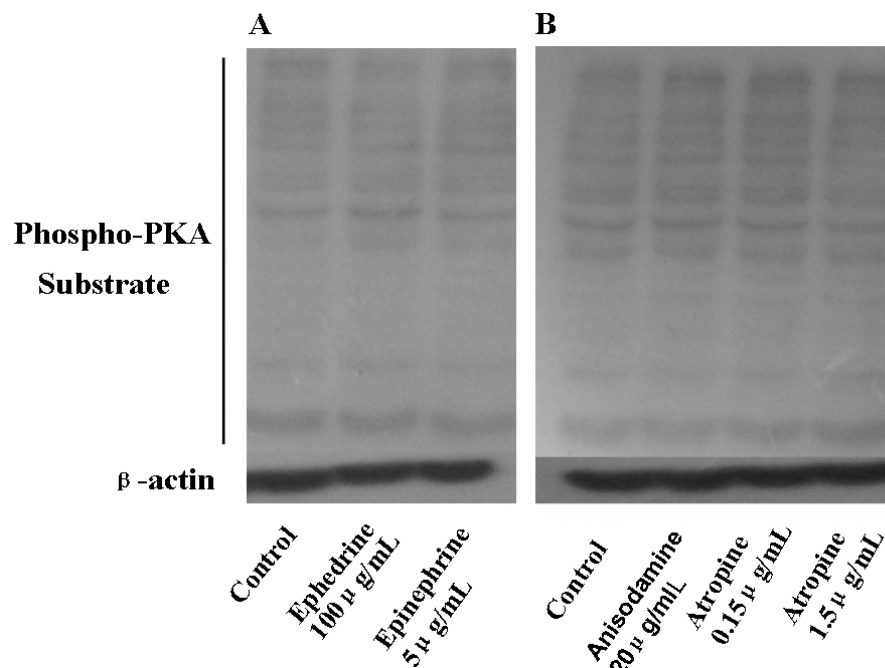


**Figure 3:** Effect of anisodamine on intracellular cAMP concentration of SKBR3 cells

(A) Effect of anisodamine on intracellular cAMP concentration at 30 minutes. (B) Effect of anisodamine on intracellular cAMP concentration at 24 hours. The intracellular cAMP content in SKBR3 cells was significantly increased at 30 minutes and decreased significantly at 24 hours. \* Versus control group  $P < 0.05$ .

**Effects of ephedrine and anisodamine on PKA**

The phosphorylation of PKA substrate of SKBR3 cells were analyzed by Western blotting. The cells were gathered in 48 hours. The results indicated that the phosphorylation of PKA substrate was not activated at 48 hours after treatment with ephedrine and anisodamine (Fig.4).

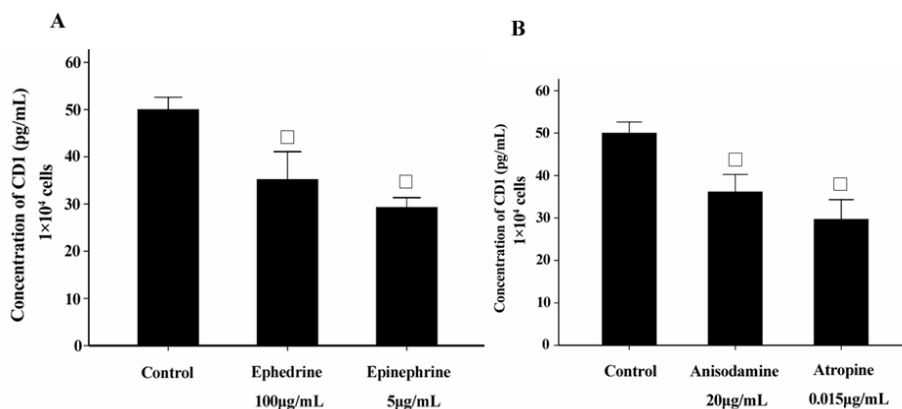


**Figure 4:** Effects of ephedrine and anisodamine on PKA of SKBR3 cells

(A) Effect of ephedrine on PKA of SKBR3 cells at 48 hours after treatment with ephedrine. (B) Effect of anisodamine on PKA of SKBR3 cells at 48 hours after treatment with anisodamine. Both ephedrine and anisodamine did not make phosphorylation of PKA substrate activated.

#### Effects of ephedrine and anisodamine on CD1

We observed the level of CD1 in SKBR3 cells after treatment with ephedrine or anisodamine for 48 hours. Ephedrine and anisodamine increased the level of CD1 in SKBR3 cells significantly. Moreover, there were no significant difference in the level of CD1 between ephedrine and epinephrine, and in the level of CD1 between anisodamine and atropine (Fig.5).



**Figure 5:** Effects of ephedrine and anisodamine on CD1 of SKBR3 cells

(A) Effect of ephedrine and epinephrine on CD1 of SKBR3 cells. (B) Effect of anisodamine and atropine on CD1 of SKBR3 cells. Both ephedrine and anisodamine increased the level of CD1 in SKBR3 cells significantly. There were no significant difference in the level of CD1 between ephedrine and epinephrine, anisodamine and atropine ( $\square P > 0.05$ ).

## Discussion

There are abundant systematic concepts of health and the wisdom of life-cultivation in TCM, not only including the connotation of modern health, but also with many of its own characteristics. One of the health concepts of TCM is the balance of Yin - Yang in the human body (Wang et al. 2010). The two types of energy, Yin and Yang, constituting a vital substance that circulates through the body, are important to health. Drug therapy has been one of the means used in TCM to keep these elements and the flow of energy in balance. Many herbs used thousands of years ago in China are the source of new pharmaceuticals in western medicine (Borchardt 2003). Ephedra and Henbane Seed, the herbs in TCM, have the effect of warming Yang and eliminating cold and are closely related to the sympathetic nervous system (SNS) (Chen et al. 2010). There major bioactive component of Ephedra and Henbane Seed are ephedrine and anisodamine, which act on the adrenergic receptor and cholinergic receptor, respectively.

Ephedrine, which is obtained from the stems of plants of *ephedra sinica*, has been popularly used as a natural product in therapy of asthma, weight loss, energy enhancement and many other areas. Ephedrine is a sympathomimetic amine. The principal mechanism of its action relies on its indirect stimulation of the adrenergic receptor system, which is part of the SNS, by increasing the activity of noradrenaline at the post-synaptic  $\alpha$ - and  $\beta$ -receptors. Anisodamine, a naturally occurring tropane alkaloid found in some plants of the Solanaceae family, also known as 7 $\beta$ -hydroxyhyoscyamine, is an anticholinergic and  $\alpha$ 1-adrenergic receptor antagonist used in the treatment of acute circulatory shock in China (Varma et al. 1986, Zhang et al. 2008).

This study shows the anti-tumor effects of ephedrine and anisodamine in human breast cancer cells. In the study, we investigated the effects of ephedrine and anisodamine on the proliferation, cell cycle distribution and apoptosis of SKBR3 human breast cancer cells. Both ephedrine and anisodamine dramatically inhibited the proliferation of SKBR3 cells and blocked cells in the G<sub>0</sub>/G<sub>1</sub> phase. However, the inhibition of tumor growth is not apparently caused by a direct cytotoxic effect or apoptosis against tumor cells. CD1 is essential for cell progression through the G<sub>1</sub> phase of the cell cycle. Dramatically, ephedrine and anisodamine increased the level of CD1 in SKBR3 cells.

Since cAMP or its analogs had been shown to be effective inducers of growth inhibition and cell cycle arrest in several cell lines (Rivas et al. 2003, Warrington et al. 2010), we hypothesized that ephedrine and anisodamine inhibit proliferation and induce cell cycle arrest by its ability to elevate intracellular cAMP in SKBR3 cells. To test this hypothesis, we analyzed the intracellular cAMP in SKBR3 cells treated with ephedrine and anisodamine. Our data indicated that intracellular cAMP concentration changed significantly in response to ephedrine and anisodamine treatment.

cAMP is the second messenger to be discovered. It plays a important role in intracellular signal transduction of various stimuli and is known to be involved in cell proliferation (Robinson-White et al. 2002, Robinson-White et al. 2003). In particular, cAMP signaling has an important role in cancer biology. So that selecting cAMP as a therapeutic target has been a new therapeutic strategy against cancer. Since the main intracellular target for cAMP in mammalian cells is the PKA, PKA is to target the synthesis and degradation of cAMP (Taylor et al. 2008b, Tasken et al. 1997, Dumaz et al. 2005, Cho-Chung et al. 1995, Stork et al. 2002). Unfortunately, the phosphorylation of PKA substrate was not activated at 48 hour after treatment with ephedrine and anisodamine.

In conclusion, the results demonstrated that ephedrine and anisodamine, the major bioactive component Ephedra and Henbane Seed, have a powerful antiproliferation by inducing cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phases. cAMP may account for the inhibitory effect of ephedrine and anisodamine on cell proliferation in SKBR3 cells. Further studies are needed to assess the anti-tumor effect of Ephedra and Henbane Seed.

## Acknowledgments

This work was supported by grants from the National Science Foundation of China (No. 81173376).

## Disclosure of Potential Conflicts of Interests

None of the authors has any competing interest to declare. The authors have no relevant financial interests to disclose.

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