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• ESOPHAGEAL CANCER •

# RRR-α-tocopheryl succinate inhibits human gastric cancer SGC-7901 cell growth by inducing apoptosis and DNA synthesis arrest?

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## Abstract

AIM: **To investigate the effects of growth inhibition of human gastric cancer SGC-7901 cell with RRR-α-tocopheryl succinate (VES), a derivative of natural Vitamin E, via inducing apoptosis and DNA synthesis arrest.**

METHODS: **Human gastric cancer SGC-7901 cells were regularly incubated in the presence of VES at 5, 10 and 20mg·L$^{-1}$ (VES was dissolved in absolute ethanol and diluted in RPMI 1640 complete condition media correspondingly to a final concentration of VES and 1mL·L$^{-1}$ ethanol), succinic acid and ethanol equivalents as vehicle (VEH) control and condition media only as untreated (UT) control. Trypan blue dye exclusion analysis and MTT assay were applied to detect the cell proliferation. 37kBq of tritiated thymidine was added to cells and [$^3$H] TdR uptake was measured to observe DNA synthesis. Apoptotic morphology was observed by electron microscopy and DAPI staining. Flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were performed to detect VES-triggered apoptosis.**

RESULTS: **VES inhibited SGC-7901 cell growth in a dose-dependent manner. The growth curve showed suppression by 24.7%, 49.2% and 68.7% following 24h of VES treatment at 5, 10 and 20 mg·L$^{-1}$, respectively, similar to the findings from MTT assay. DNA synthesis was evidently reduced by 35%, 45% and 98% after 24h VES treatment at 20 mg·L$^{-1}$ and 48h at 10 and 20 mg·L$^{-1}$, respectively. VES induced SGC-7901 cells to undergo apoptosis with typically apoptotic characteristics, including morphological changes of chromatin condensation, chromatin crescent formation/margination, nucleus fragmentation and apoptotic body formation, typical apoptotic sub-G1 peak by flow cytometry and increase of apoptotic cells by TUNEL assay in which 90% of cells underwent apoptosis after 48h of VES treatment at 20 mg·L$^{-1}$.**

CONCLUSION: **VES can inhibit human gastric cancer SGC-7901 cell growth by inducing apoptosis and DNA synthesis arrest. Inhibition of SGC-7901 cell growth by VES is dose- and time-dependent. Therefore VES can function as a potent chemotherapeutic agent against human gastric carcinogenesis.**

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## INTRODUCTION

Vitamin E is charactered as a fat-soluble membrane antioxidant[1,2]. Vitamin E succinate (RRR-α-tocopheryl succinate; VES), a derivative of natural vitamin E, however, does not possess antioxidant properties unless the succinate group is removed by a nonspecific esterase. VES has been demonstrated to be a potent growth inhibitor of various cancer cell types *in vitro* and *in vivo*[3,4]. For example, VES has been shown to inhibit the growth of human monoblastic leukemia cells *in vitro*[5], murine B-16 melanoma cells *in vitro*[6], hamster buccal pouch tumor cells *in vivo*[7], avian lymphoid cells *in vitro*[8,9], murine EL4 T lymphoma cells *in vitro*[4,10], human gastric cancer cells *in vitro* and *in vivo*[11-13], and human breast cancer cells *in vitro* and *in vivo*[14,15].

The exact mechanisms are not clearly known, but the inhibitory effect of VES on the proliferation of rapidly dividing cells can be attributed to the induction of cell cycle blockage[16], increased secretion and activation of transforming growth factor-$\beta_s$ (TGF-$\beta_s$) and TGF-β receptor II[9,14,17], and the induction of apoptosis[18-20]. In many instances, growth inhibition following terminal differentiation[21-23] or anticancer drug treatment[24-26] results in apoptosis. Apoptosis, namely, programmed cell death, is an active and physiological process characterized by a series of morphological and biological alterations including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of nucleus and extensive degradation of chromosomal DNA into oligomers of 180bp[27-30]. The exact mechanisms of apoptosis are still unclear, but our earlier studies indicated that VES can secrete and activate biologically active TGF-β and then TGF-β increases the kinase activity of c-Jun N-terminal kinase(JNK) followed by phosphorylation of c-Jun, and finally activated c-Jun triggers apoptosis in human gastric cancer SGC-7901 cells[31].

Gastric cancer is common in China[32-41]. Since VES is a potential tumor chemopreventive and chemotherapeutic agent, it is worthwhile to investigate the manner in which VES inhibits the cell growth and thereafter gain a better understanding of the molecular events involved. In this study, we demonstrate the ability of VES to inhibit cell proliferation, arrest DNA synthesis and induce human gastric cancer SGC-7901 cells to undergo apoptosis, address the involvement of certain apoptosis-related events in this process and prove that VES-triggered apoptosis is different from VES-induced DNA synthesis arrest.

## MATERIALS AND METHODS

### *Materials*

VES, DAPI (4',6-diamidine-2'-phenylindole dihydrochloride,

succinic acid, MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-dipenyltetrazolium bromide] and propidium iodide were purchased from Sigma Co. Ltd. RPMI 1640 media was obtained from Gibco, BRL. *In situ* cell death detection kit was purchased from Boehringer Mannheim, Indianapolis, Inc. $^3$H-TdR was supplied by Chinese Academy of Sciences.

### *Methods*

**Cell culture** Human gastric cancer cell lines SGC-7901 were maintained in RPMI 1640 medium supplemented with 100mL·L$^{-1}$ fetal calf serum (FCS), 100kU·L$^{-1}$ penicillin, 100mg·L$^{-1}$ streptomycin and 2mmol·L$^{-1}$ L-glutamine under 50mL·L$^{-1}$ $CO_2$ in a humidified incubator at 37℃. SGC-7901 cells were incubated for different time periods in the presence of VES at 5, 10 and 20mg·L$^{-1}$ (VES was dissolved in absolute ethanol and diluted in RPMI 1640 complete condition media correspondingly to a final concentration of VES and 1mL·L$^{-1}$ ethanol), succinic acid and ethanol equivalents as vehicle (VEH) control and condition media only as untreated (UT) control.

**Growth curve** Exponentially growing SGC-7901 cells were trypsinized and aliquoted into 24-well flat-bottomed tissue culture plates at 5×10$^4$·well$^{-1}$. The cells were allowed to attach overnight and then incubated for seven days in the presence or absence of VES. Cell number and viability were determined by trypan blue dye exclusion analysis.

**MTT assay** The cells were inoculated in a 96-well plate (5×10$^3$·well$^{-1}$) as described previously[42]. In brief, cells after 24h of incubation were treated with VES and controls and then cultured for six days. The cells in eight wells at every dose were supplemented with 0.2mL of 5g·L$^{-1}$ MTT every day. After 4h, culture media were discarded followed by addition of 0.2ml of DMSO and vibration for 10min. The absorbance (A) was measured at 570nm using a microplate reader. The percentage of viable cells was calculated as follow:(A of experimental group /A of control group)×100%. [$^3$H] Thymidine incorporation. The cells were treated with VES for 24 or 48h as described previously[43]. 37kBq of tritiated thymidine were added to cells during the last 6h of culture and harvested onto glass fiber filters. [$^3$H] TdR uptake was measured in a Beckman LS5000 TD liquid scintillation counter.

**Electron microscopy** The cells treated with VES or VEH were trypsinized and harvested after 24h and 48h, respectively. Subsequently the cells were immersed with Epon 821, imbedded in capsules and converged for 72h at 60℃, the cells were prepared into ultrathin section (60nm) and stained with uranyl acetate and lead citrate. Cell morphology was examined by transmission electron microscopy.

**DAPI staining** Treated cells were pelleted and washed three times with distilled water, and then stained with 2mg·L$^{-1}$ DAPI in 100% methanol for 15min at 37℃. Cells were viewed using a fluorescence microscope with ultraviolet (UV) excitation at 300~500nm. Cells with nuclei that contained clear condensed chromatin or cells with fragmented nuclei were scored as apoptosis.

**TUNEL assay** Apoptosis of SGC-7901 cells was analyzed by using *in situ* cell death detection kit. The method is essentially based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique, and it can detect apoptosis at very early stages. In brief, cells were treated in the presence or absence of VES and fixed overnight in 100g·L$^{-1}$ formaldehyde, treated with proteinase K and then $H_2O_2$, labeled with fluorescein dUTP in a humid box for 1h at 37℃. The cells were then combined with POD-Horseradish peroxidase, colorized with DAB (3,3-diaminobenzidine) and counterstained with methyl green. Cells were visualized with light microscope.

**Flow cytometry** Cells were incubated with various doses of VES, harvested, washed with phosphate-buffered saline (PBS) twice and fixed with 700mL·L$^{-1}$ ethanol at 4℃ overnight. Fixed cells were washed twice with PBS and stained with 800μL propidium iodide and 200μL deoxyribonulcease-free ribonuclease A in PBS. The fluorescence indensity of propidium iodide-stained nuclei was determined by a FACscan.

**Statistical analysis** Student's *t* test was used to assess statistical significance of differences. If $P<0.05$, the difference was considered significant.

## RESULTS

### *Inhibition of Human Gastric Cancer SGC-7901 Cell Proliferation by VES*

**Growth curve** VES has been previously shown to function as a growth inhibitory agent. In this study, VES inhibited SGC-7901 cell growth in a dose-dependent manner. The growth curve showed that cell growth was suppressed by 24.7%, 49.2% and 68.7% following 24h of VES treatment at 5, 10 and 20mg·L$^{-1}$, respectively. The percentage of inhibition was 100% on day 4 with the treatment of VES at 20mg·L$^{-1}$ (Figure 1).

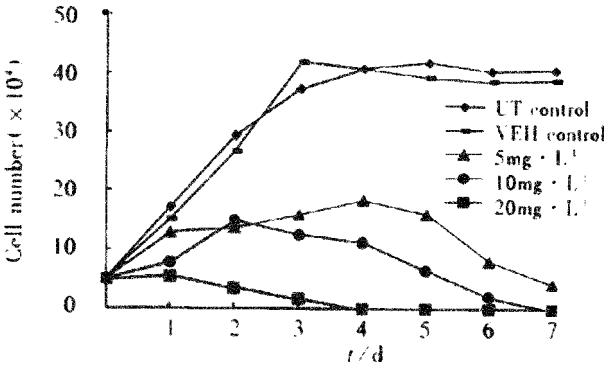


**Figure 1** Growth curve of SGC-7901 cell treated with VES.

**MTT assay** SGC-7901 cells were treated in the presence or absence of VES for six days and cell viability was daily measured by MTT proliferation analysis. VES significantly inhibited cell growth and VES at 10 and 20 mg·L$^{-1}$ had an obviously inhibitory effect by 100% on the 5th and 4th days, respectively (Table1), similar to the findings from growth curve.

**Table 1** Effect of VES on the viability of SGC-7901 cells

| Group | Rate of viable cells /% | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| UT control | 100 | 100 | 100 | 100 | 100 | 100 |
| VEH control | 105.5 | 102.4 | 92.1 | 95.3 | 105.7 | 97.5 |
| 5 mg·L$^{-1}$ VES | 94.8 | 101.2 | 91.4 | 144.3 | 91.4 | 74.6 |
| 10 mg·L$^{-1}$ VES | 91.4 | 64.6 | 20.8 | 4.2 | 0 | 0 |
| 20 mg·L$^{-1}$ VES | 53.4 | 32.9 | 4.3 | 0 | 0 | 0 |

**DNA synthesis arrest by VES**

VES treatment of SGC-7901 cells inhibited [$^3$H] thymidine

uptake with a dose-response relationship. DNA synthesis was evidently reduced by 35%, 45% and 98% after 24h VES treatment at 20 mg·L$^{-1}$ and 48h at 10 and 20 mg·L$^{-1}$, respectively, compared with UT control ($P$<0.01). On the other hand, VEH control did not affect SGC-7901 cell proliferation (Table 2).

**Table 2** Inhibitory effect of VES on DNA synthesis incorporated with $^3$H-TdR ($\bar{x}\pm s$, $n$=6)

| Group | Radioactive intensity / Bq·10$^4$cell$^{-1}$) | |
|---|---|---|
| | 24h(% of inhibition ratio) | 48h(% of inhibition ratio) |
| UT control | 64.1±10.2(0) | 143.2±13.7(0) |
| VEH control | 63.8±10.3(1) | 141.9±14.7(0) |
| 5 mg·L$^{-1}$ VES | 61.8±10.4(4) | 137.1±16.3(4) |
| 10 mg·L$^{-1}$ VES | 57.2±10.7(11) | 79.3±10.0(45)$^b$ |
| 20 mg·L$^{-1}$ VES | 41.4± 8.0(35)$^b$ | 2.2±0.3(98)$^b$ |

$^b P$<0.01, *vs* UT control.

### *VES induction of apoptosis*

**Morphological changes** Apoptotic characteristics including chromatin condensation, chromatin crescent formation/ margination, DNA fragmentation and apoptotic body formation were seen by electron microscopy (Figure 2) and by fluorescence microscopy of DAPI-labelled cells (Figure 3). The cells undergoing apoptosis evidently increased when the concentration of VES was elevated and reaction time was prolonged.

**Flow cytometry** VES-induced apoptosis was studied further in SGC-7901 cells using flow ctyometry. Cell cycle analysis after VES treatment revealed the presence of a sub-G1 apoptotic peak (Figure 4).

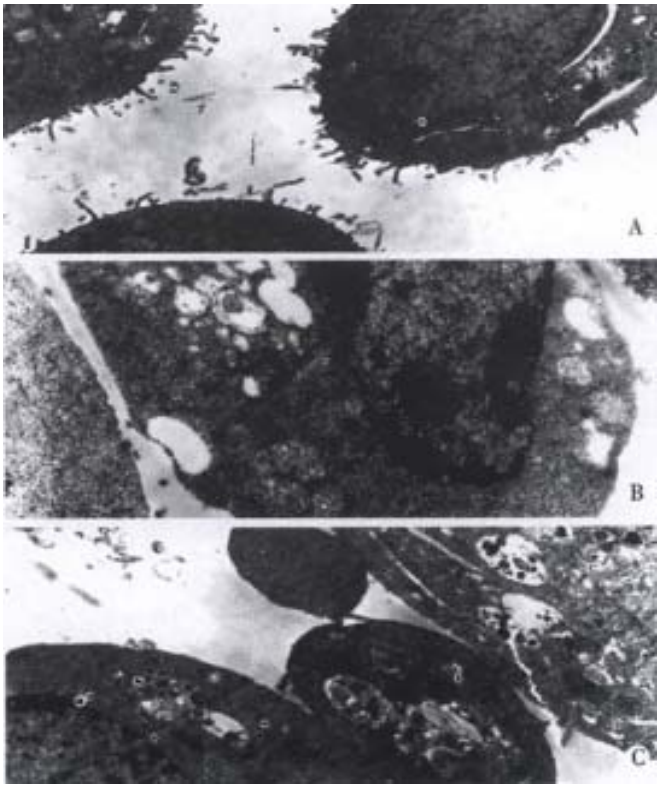


**Figure 2** VES-induced apoptosis in SGC-7901 cells with transmission electron microscope
A: Normal cell; B: Apoptotic cell with chromatin condensation, chromatin crescent formation / margination; C: Cell with apoptotic body.

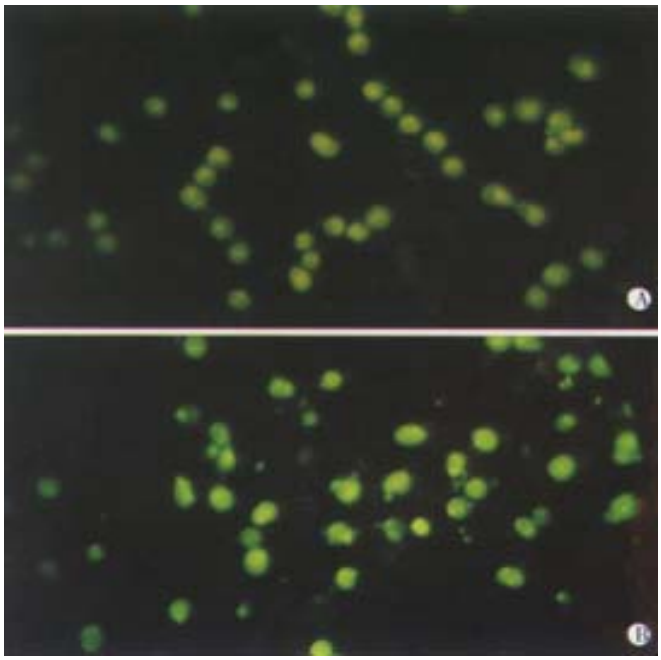

**Figure 3** VES-induced apoptosis in SGC-7901 cells with DAPI staining UT control; VES at 20 mg·L$^{-1}$

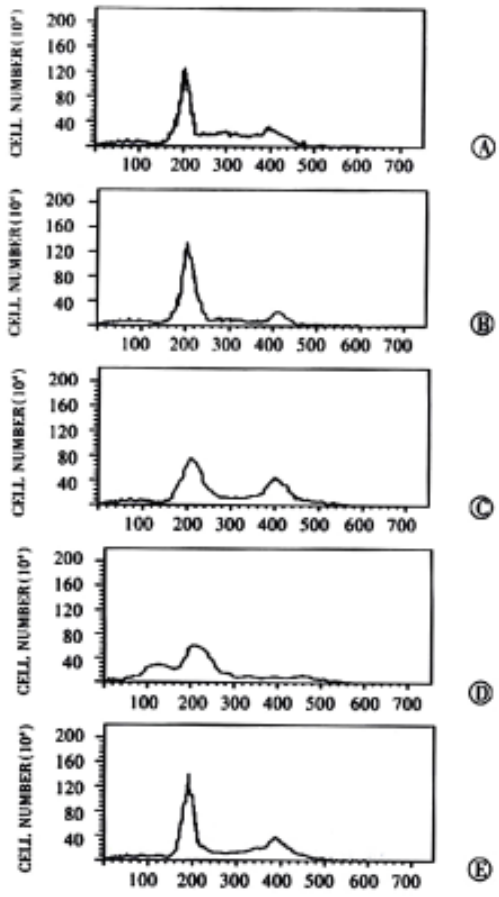


**Figure 4** VES-induced apoptosis in SGC-7901 cells with flow cytometryA: UT control; B: VEH control; C: VES at 5 mg·L$^{-1}$; D: VES at 10 mg·L$^{-1}$; E: VES at 20 mg·L$^{-1}$.

**TUNEL assay** Apoptotic cell death was determined by TUNEL assay according to the manufacturer's instructions. The results showed that apoptotic cells increased with the increase of the concentration of VES in an obviously dose-dependent manner. The percentage of apoptosis was 90% after VES treatment at 20 mg·L$^{-1}$ (Figure 5 and Table 3).

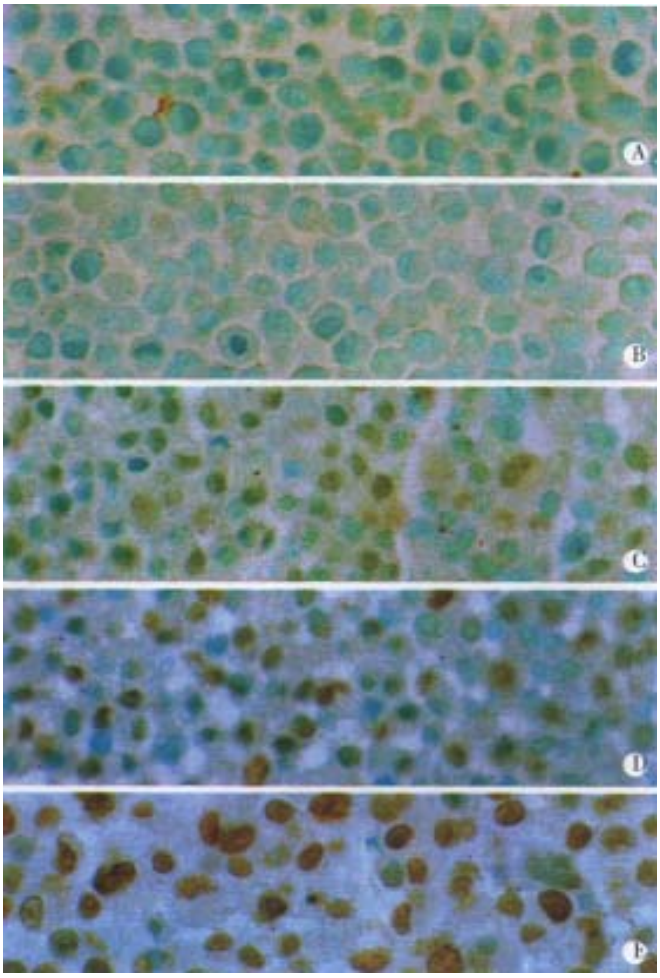

**Figure 5** VES-induced apoptosis by TUNEL assay.
A: UT control; B: VEH control; C: VES at 5 mg·L$^{-1}$; D: VES at 10 mg·L$^{-1}$; E: VES at 20 mg·L$^{-1}$.

**Table 3** Induction of apoptosis after 48h of VES treatment in SGC-7901 cells

| Group | Apoptotic cells / $10^4$ cells | Rate of apoptosis / % |
|---|---|---|
| UT control | 6 | 0.06 |
| VEH control | 28 | 0.28 |
| 5 mg·L$^{-1}$ VES | 1248$^b$ | 12.48 |
| 10 mg·L$^{-1}$ VES | 5736$^b$ | 57.63 |
| 20 mg·L$^{-1}$ VES | 8996$^b$ | 89.96 |

$^b P<0.01$, *vs* UT control.

## DISCUSSION

Cell growth is regulated by the interaction of cell proliferation and cell death. When cell growth is inhibited, the decrease of cell proliferation and increase of cell death will occur. Cell proliferation is performed by continuously proceeding into cell cycle and will be suppressed if cell cycle is disturbed[44-46]. The disorder of DNA synthesis, however, is a common reason for the disturbance of cell cycles[47,48]. Cell death is another principal reason for cell growth inhibition and is relevant to cell proliferation. Apoptotic cell death is a naturally occurring process of cell suicide which plays a crucial role in the development and homeostasis of metazons by eliminating superfluous or unwanted cells (reviewed by[49-53]).

Previous studies showed that VES can block DNA synthesis in tumor cells[43,54]. The percent of DNA synthesis arrest is more than 90% when human breast cancer cells are treated with VES at 10 mg·L$^{-1}$, similar to the rate of breast cancer cell growth inhibition which exceeds 80%[15]. A recent study shows that VES can function as an apoptosis inducer in tumor cells. In this study, VES was found to inhibit cell proliferation in human gastric cancer SGC-7901 cells by trypan blue exclusion analysis and MTT assay. Cell growth was inhibited by 100% with treatment of VES at 10 mg·L$^{-1}$ on day 7 and at 20 mg·L$^{-1}$ on day 4 in the former experiment, while on the 6$^{th}$ and 4th days in the latter one. Meanwhile, VES significantly suppressed cell growth by DNA synthesis arrest via preventing SGC-7901 cells from progressinginto S-phase, ultimately blocking the cell proliferation. In order to further elucidate the mechanisms of inhibitory effects of VES on cell growth, we determined the incidence of VES-mediated apoptosis in SGC-7901 cells by electron microscopy, DAPI staining, flow cytometry and TUNEL assay on the basis of morphological and molecular level. The results demonstrated that VES-treated SGC-7901 cells were characteristic by typical apoptotic alterations, including morphological changes by electron microscopy and DAPI staining, typical apoptotic sub-$G_1$ peak observed by flow cytometry; increase of apoptotic cells with the elevation of the concentration of VES in a clearly dose-dependent manner by TUNEL assay.The data above implicated that VES inhibits human gastric cancer SGC-7901 cell growth by inducing apoptosis and DNA synthesis arrest.

Apoptosis is a complex and programmed process which is regulated by a variety of factors[55-60]. The antiproliferative actions of VES may be due, in part, to the ability of VES to induce autocrine acting biologically active TGF-βs[9,14,17]. In addition, VES-mediated apoptosis is inhibited when c-Jun mutant is transfected into breast cancer cells[61]. JNK, mitogen-activated protein kinase (MAPK) family member, plays an important role in the course of VES-triggered apoptosis[31,61]. Although the exact mechanisms involved in VES-induced apoptosis have not been well known up to now, the ability of a compound to induce 90% apoptosis in a tumor cell population within 48h is noticeable. It is also noteworthy that VES can selectively inhibit the growth of tumor cells but not normal cells[8,62-64]. The research in our laboratory demonstrated that VES inhibited benzo (a)pyrene (B(a)P)-induced forestomach carcinogenesis in female mice[11], while the inhibition and inhibitory mechanisms of VES *in vivo* are worth further investigating.

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