Cinobufagin Promotes Cell Cycle Arrest and Apoptosis to Block Human Esophageal Squamous Cell Carcinoma Cells Growth via the p73 Signalling Pathway

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INTRODUCTION

Esophageal cancer (EC) is a common malignant tumour of the digestive system. According to the latest statistics, 572000 new cases and 309000 deaths are estimated in 2018. EC occurs frequently in certain parts of China, and its incidence rate ranks fifth worldwide, esophageal squamous cell carcinoma (ESCC) is the most common histologic subtype in China. Since EC lacks typical clinical symptoms at early stages, it is usually diagnosed at middle or advanced stage with progressive dysphagia and systemic metastasis, which leads to poor prognosis.

There are many different therapies for EC: early surgical resection is a priority, and postoperative neoadjuvant chemotherapy or radiotherapy has been proven to be helpful in EC treatment. However, chemotherapy is the most important approach for 40 to 60% of patients with advanced EC who cannot undergo surgery. The combination of cisplatin and 5-fluorouracil (5-FU) has been accepted as the common chemotherapy regimen for patients with unresectable locally advanced or metastatic EC. New protocols involving combinations with capecitabine and paclitaxel have shown significant advantages in phase II clinical trials, and the corresponding phase III trials are also underway. Unfortunately, chemotherapy-related adverse effects not only lead to a serious economic burden on patients and society but also are responsible for the incomplete treatment of these patients.

Bufo bufo gargarizans Cantor and Bufo melanostictus Schneider have been found to be a source of some Chinese medicines because their skin is rich in scorpion lactones, peptides and alkaloids. Cinobufacini (an alternative name for Huachansu) is a water-soluble extraction of dried rind. Since the 1970s, Cinobufacini has been widely used in the treatment of clinical cancer in China, and Huachansu injection, as a traditional Chinese medicine preparation approved by the China Food and Drug Administration (FDA) ISO9002, is the main type of preparation. Numerous clinical trials have confirmed repeatedly that Huachansu injection has anticancer activity against several human cancer types, such as lung cancer, gastric cancer, liver cancer, pancreatic cancer and gall-bladder cancer, regardless of its use alone or in combination with other chemotherapeutic drugs. Wei et al. and Lou et al. compared the efficacy and safety of various traditional Chinese medicine injections used in combination with radio-
therapy for EC and found that Huachansu injection can synergistically promote the efficiency of conventional therapies and significantly improve the quality of life of patients with EC.

Many studies have reported that cinobufagin is the main bioactive component of Huachansu injection, and its antitumor mechanism was also elucidated. On one hand, cinobufagin induces both apoptosis of cancer cells derived from liver cancer, lung cancer, breast cancer, colon cancer, prostate cancer and osteosarcoma\(^{21-24}\) and cell cycle arrest,\(^{25-27}\) thereby inhibiting cancer cell growth; on the other hand, Wang et al. found that cinobufagin can promote the proliferation of mouse spleen cells and peritoneal macrophages and regulate cellular immunity.\(^{28}\) However, the effects of cinobufagin on ESCC cells and the precise molecular mechanisms involved are poorly studied and understood. EC-109, Kyse-150 and Kyse-520 are typical ESCC cell lines which harbor different degrees of differentiation. Therefore, this study focused on clarifying the molecular mechanisms mediating the anticancer effects of cinobufagin on three human ESCC cell lines.

MATERIALS AND METHODS

**Cell Lines and Materials** Human ESCC cells (EC-109, Kyse-150, and Kyse-520) purchased from the cell bank of the Chinese Academy of Sciences (CCAS, Shanghai, China) were maintained in RPMI-1640 media (Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 µg/mL) and cultured at 37°C in 5% CO\(_2\). Cinobufagin (>98% purity) is a product of Nature Standard Biotechnology Co., Ltd. (Shanghai, China) and was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution at a concentration of 0.1 mol/L, stored at −20°C, and diluted in serum-free medium to each working concentration so that the final DMSO concentration in all the experiments was not >0.1%. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan); the cell cycle kit was provided by Beijing 4A Biotech Co., Ltd. (Beijing, China); HiFiScript cDNA synthesis kit and UltraSYBR mixture were obtained from ComWin Biotech Co., Ltd. (Beijing, China); RNAiso was procured from TaKaRa (Tokyo, Japan); Cell Lines and Materials

**Human ESCC cells (EC-109, Kyse-150, and Kyse-520) purchased from the cell bank of the Chinese Academy of Sciences (CCAS, Shanghai, China) were maintained in RPMI-1640 media (Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 µg/mL) and cultured at 37°C in 5% CO\(_2\). Cinobufagin (>98% purity) is a product of Nature Standard Biotechnology Co., Ltd. (Shanghai, China) and was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution at a concentration of 0.1 mol/L, stored at −20°C, and diluted in serum-free medium to each working concentration so that the final DMSO concentration in all the experiments was not >0.1%. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan); the cell cycle kit was provided by Beijing 4A Biotech Co., Ltd. (Beijing, China); HiFiScript cDNA synthesis kit and UltraSYBR mixture were obtained from ComWin Biotech Co., Ltd. (Beijing, China); RNAiso was procured from TaKaRa (Tokyo, Japan); Hoechst 33342 and materials for Western blotting were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China); monoclonal antibodies targeting p53 and p73 were purchased from Boster Biotech (Wuhan, China); specific antibodies against caspase-3, cleaved caspase-3, Cdc2, Cdc2 (tyrosine (Tyr) 15), Mdm2, Puma and Noxa were obtained from Abcam (Cambridge, MA, U.S.A.); anti-cyclin B1, anti-p21, and anti-Weel antibodies were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), and primary antibodies targeting Bax, Bel-2, β-actin as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from ProteinTech (Chicago, IL, U.S.A.).

**Cell Viability Assay** As reported previously,\(^{29}\) cell viability was measured by the CCK-8 assay. Briefly, the three ESCC cell lines in the logarithmic growth phase were seeded in 96-well plates (5 × 10\(^{3}\) cells/well) and cultured for 24 h to allow cells to attach and then incubated with 100 µL of medium containing different concentrations of cinobufagin (0.025, 0.1, 0.4, 1.6, 6.4 µmol/L) or equivalent vehicle (0.1% DMSO). A blank control group (no cells, only equal amount of medium) was also established, and there were 5 duplicate wells in each group. After treatment for 12, 24, and 48 h, 10 µL of CCK-8 solution was added to each well and incubated for 4 h at 37°C. The absorbance at 450 nm was measured by a full-wavelength microplate reader (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The experiments were carried out in triplicate.

**Cell Growth Curve** Three ESCC cell lines were inoculated into 6-well plates (1000 cells/well) and cultured for 24 h. The cells were treated with a gradient concentration of cinobufagin (0.05, 0.1, 0.2, 0.5, 1 µmol/L) or an equal amount of vehicle (0.1% DMSO) for 7 d. During this study, 5 fixed sites per well were monitored using a JuLi™ Stage Real-Time cell recorder (NanoEnTek, Seoul, Korea) at 24-h intervals, and the confluence was also measured by the system software to plot the cell growth curve. The experiments were carried out in triplicate.

**Cloning and Formation** To assess the effect of cinobufagin on cell clone formation, three ESCC cell lines were seeded in a 6-well plate at a density of 500 cells/well and incubated with medium containing cinobufagin (0.05, 0.1, 0.2, 0.5, 1 µmol/L) or vehicle (0.1% DMSO). After treatment at 37°C for 10 d, the cell colonies were fixed with 4% parafomaldehyde and stained with 0.5% crystal violet. Then, clearly visible colonies (foci >50 cells) were photographed and counted. The experiments were carried out in triplicate.

**Hoechst 33342 Staining** To clearly observe apoptotic cell death and nuclear morphology, three ESCC cell lines were stained with Hoechst 33342. Briefly, cells were seeded into 6-well plates and treated with 0.5 or 1 µmol/L cinobufagin for 24 h. Then, the cells were harvested, washed twice with phosphate buffered saline (PBS), and fixed with 75% ice-cold ethanol at −20°C overnight. Next, 4 µL of ribonuclease (RNase) A (2.5 mg/mL), 15 µL of propidium iodide (PI) staining solution (25×) and 0.4 mL of staining buffer were added and incubated for 30 min at 37°C in the dark. The cell cycle distribution was analysed on an FC500 flow cytometer (Beckman, U.S.A.). The experiments were carried out in triplicate.

**Total RNA Extraction and Quantitative (q)RT-PCR Analysis** Three ESCC cell lines incubated in 6-well plates were treated with low-dose cinobufagin (1 µmol/L), high-dose cinobufagin (0.5 µmol/L) and DMSO (0.1%) for 24 h. Then, cells were collected, total RNA was extracted by TRIzol reagent-phenol chloroform, cDNA was synthesized, and real-time quantitative (q)RT-PCR amplification was performed using a commercial kit according to the manufacturers’ instructions. Each real-time PCR was carried out in triplicate on a PikoReal Real-Time PCR instrument (Thermo Corporation, U.S.A.). In detail, the 20-µL reaction mixture for amplification of the cDNA fragments included 2 µL of cDNA, 10 µL of 2×UltraSYBR Mixture, 6.8 µL of RNase ddH\(_2\)O and 0.6 µL of two specific primers (10 µmol L\(^{-1}\)). The PCR program was

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as follows: denaturation, 1 cycle at 95°C for 10 min; amplification, 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analysed using the $2^{-\Delta\Delta C_t}$ method, and values were normalized to \(\beta\)-actin expression. The primers listed in Table 1 were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

**Western Blot Analysis** Three ESCC cell lines cultured in 10-cm dishes were treated as described above. After 24 h, total protein was extracted according to the instructions of the protein extraction reagent. The protein concentration was determined by the BCA quantitative method, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through a 10% gel, transferred, and blocked conventionally. Primary antibody diluted at 1:200–1000 was added to the membrane and incubated overnight at 4°C. Next, HRP-conjugated secondary antibody (1:5000 dilution) was incubated on the membrane for 1 h at room temperature, after which the membrane was treated for 1 min with ECL agent and exposed. The resulting protein bands were analysed with Scion Image software. The degree

| Gene    | Sequence (5’→3’)                  |
|---------|-----------------------------------|
| Cyclin B1 | FP: TCGGAGAGCATCTAAGATTGGAGAGG   |
|         | RP: TGTGGCATACTGTGCTTGACATGCC    |
| p21     | FP: ATGGACCTCAGCTTCGTCAC         |
|         | RP: GTCCACATGGTCTTCTGCTG          |
| Cdc2    | FP: AACTCAAGGTCAGTTGATGCCATAG    |
|         | RP: TCTGCATAAGACACATCTGAGCAC     |
| Wee1    | FP: ATGTGCTGCTGCTGGCTGAAACC      |
|         | RP: AGTGCAATTGCTGAAGTTCCTTC      |
| \(\beta\)-Actin | FP: TGTTTGAGACCTTACACCC   |
|         | RP: AGCAGCTGTGGCGGTACAGG         |

**Table 2. IC\(\text{so}\) (\(\mu\)M) Values of Cinobufagin in Tested Human Esophageal Cancer Cells**

| Cell line | 12 h     | 24 h   | 48 h  |
|-----------|----------|--------|-------|
| EC-109    | 1.40 ± 0.25 | 0.91 ± 0.04 | 0.13 ± 0.02 |
| Kyse-150  | 8.83 ± 0.22 | 0.66 ± 0.10 | 0.09 ± 0.04 |
| Kyse-520  | 6.00 ± 0.07 | 0.62 ± 0.03 | 0.08 ± 0.03 |

Fig. 1. Effects of Cinobufagin on the Growth of EC-109, Kyse-150, and Kyse-520 Cells

The cells were treated with a gradient concentration of cinobufagin (0.05, 0.1, 0.2, 0.5, 1 \(\mu\)mol/l) or an equal amount of vehicle (0.1% DMSO) for 7 d. (A). Cell growth curves in each group were plotted according to the data obtained from a real-time cell recorder; (B). Representative photomicrographs showing EC cells that formed visible colonies (foci > 50 cells). Bar graphs on the right side of the picture show the quantification of colonies per well. One-way ANOVA and Dunnett’s test were used to determine the differences between the cinobufagin treatment group and the vehicle (DMSO) treatment group. The data are presented as the \(x \pm s\) of experiments conducted in triplicate (**\(p < 0.01\), compared with the DMSO control group). (Color figure can be accessed in the online version.)
analysis showed that the grey ratio of the target protein to β-actin was relative expression. The experiments were carried out in triplicate.

**Statistical Analysis** The experimental data obtained from three separate experiments were expressed as $\bar{x} \pm s$, and SPSS 22.0 software was used for analysis. One-way ANOVA or two-way ANOVA where appropriate followed by Dunnett’s test was used to determine the differences among multiple comparisons, with $\alpha = 0.05$ as the test level.

**RESULTS**

**Cinobufagin Inhibits the Proliferation of ESCC Cells**

First, we used the CCK-8 assay to detect the inhibitory effect of cinobufagin on the proliferation of EC-109, Kyse-150 and Kyse-520 cells. The IC$_{50}$ of cinobufagin in three ESCC cell lines with different degrees of differentiation at 12, 24 and 48h is shown in Table 2. Then, growth curves and colony formation assays were performed to further assess the effect of cinobufagin on cell proliferation in different ESCC cell lines. Compared with the control group, cells treated with concentrations of cinobufagin ranging from 0.05 to 1 µmol/L, especially in the 0.2, 0.5 and 1 µmol/L groups, exhibited slower growth (Fig. 1A). The colony forming ability of the three ESCC cell lines exposed to different cinobufagin doses was significantly reduced compared to that of the vehicle-treated cells (Fig. 1B), and the differences between the cinobufagin treatments were statistically significant ($p < 0.001$). Clearly, cinobufagin can inhibit the proliferation of ESCC *in vitro*. These results demonstrated that cinobufagin could effectively inhibit the growth of ESCC cells in a time- and concentration-dependent manner. In addition, the IC$_{50}$ values of cinobufagin in three ESCC cell lines at 24h ranged from 0.62 to 0.99 µmol/L, so 0.5 and 1 µmol/l cinobufagin were added to the cell monolayers and incubated for 24h for subsequent experiments.

**Cinobufagin Induces G2/M Phase Arrest in ESCC Cells**

To clarify the inhibitory effect of cinobufagin, cell cycle distribution was examined after cells were treated with cinobufagin for 24h. As shown in Fig. 2, 0.5 µmol/l and 1 µmol/l cinobufagin increased the G2/M population of EC109, Kyse-150 and Kyse-520 cells to 24.14 ± 1.77% and 30.25 ± 1.86%, 26.12 ± 1.94% and 29.70 ± 2.68%, and 18.36 ± 1.42% and 22.33 ± 1.56%, respectively. By contrast, the G2/M population of the respective EC109, Kyse-150 and Kyse-520 control groups was 14.66 ± 2.36%, 15.27 ± 1.78%, and 13.84 ± 1.76%. These results indicate that cinobufagin can cause a notable G2/M phase arrest in EC-109, Kyse-150 and Kyse-520 cells.

**Cinobufagin Induces Apoptosis of ESCC Cells**

To determine whether the cinobufagin-induced inhibition of ESCC cell growth was due to cell apoptosis, EC-109, Kyse-150 and Kyse-520 cells treated with 0.5 and 1 µmol/L cinobufagin were stained with Hoechst 33342. The results (Fig. 3A) showed that after treatment with increasing concentrations of cinobufagin,
an increasing amount of nuclear condensation and number of
apoptotic bodies were observed in these ESCC cells, implying
that the inhibitory effect of cinobufagin on ESCC cell
growth was related to the induction of apoptosis. Among the
many apoptosis regulatory genes, the Bcl-2 protein family
and the caspase family are the most concerned at present.
Bax and Bcl-2 are the most important regulatory genes whose
functions are antagonistic in the process of apoptosis regula-
tion, while caspase-3 is the most critical apoptosis-executing
protease in the process of apoptosis.24,29) Therefore, Western
blotting was used to analyse the protein expression of Bax, Bcl-
2, caspase-3 and cleaved caspase-3 (B). The band densities
were normalized to that of β-actin, and the bar graph shows the ratio of Bax/Bcl-2 (C), caspase-3 (D) and cleaved caspase-3 (E).
Two-way ANOVA and Dunnett’s test were used to determine the differences between the cinobufagin treatment group and the vehicle (DMSO) treatment group. The data are presented as the x ± s of experiments conducted in triplicate (**p < 0.01, compared with the DMSO control group). (Color figure can be accessed in the online version.)
lines after cinobufagin treatment (Fig. 3E), while Bcl-2 and caspase-3 expression decreased as the cinobufagin concentration increased (Fig. 3D). The ratio of Bax/Bcl-2 was unregulated in a dose-dependent manner after cinobufagin treatment (Fig. 3C). These findings indicated that cinobufagin could induce cell apoptosis via regulation of Bax and Bcl-2 and activation of caspase-3.

**Effect of Cinobufagin on the Expression of G2/M Cell Cycle-Related Genes in ESCC Cells** To explore the mechanism by which cinobufagin induces cell cycle arrest, the expression of several G2/M cell cycle-related genes, such as p21, Wee1, cyclin B1 and Cdc2, were assessed by Western blot and qRT-PCR. The levels of p21, Wee1 and phosphorylated Cdc2 (Tyr 15) increased, and the levels of cyclin B1 and Cdc2 decreased after treatment with cinobufagin (Figs. 4E–J). Moreover, the same effects of cinobufagin on the mRNA expression were also illustrated and compared by qRT-PCR (Figs. 4A–D). These findings suggested that the cinobufagin-induced G2/M arrest was probably due to the decrease in the expression of the cyclin B-Cdc2 complex and the inactivation of Cdc2, and the increased expression of p21 and Wee1 are increased during this process.

**Effect of Cinobufagin on the p73 Signalling Pathway in ESCC Cells** Having established that cinobufagin-induced G2/M cell cycle arrest and apoptosis accompanied increases in p21 and Bax expression, we attempted to further elucidate the mechanisms by which this effect is achieved. p53 plays an important role in the regulation of the cell cycle and apoptosis, especially the p53-Mdm2 loop, but most ESCC cell lines harbour p53 mutations. In fact, our results also revealed that Mdm2 expression in EC-109, Kyse-150 and Kyse-520 cells treated with cinobufagin was downregulated at the protein level, and this treatment had nothing to do with p53 expression (Figs. 5A–C), suggesting that the induction of p21 and Bax do not contribute to the regulation of p53 expression. p73 is a member of the p53 family; therefore, we also investigated the effects of cinobufagin on p73 expression in ESCC cells using Western blotting. The results showed that the protein expression of p73 was upregulated in ESCC cells after a 24-h treatment with cinobufagin (Fig. 5C). In addition, we next detected the expression of p21, Bax, Puma and Noxa, which are common downstream molecules of p53 and p73. Higher expression of the above molecules in the three ESCC cell lines was associated with higher cinobufagin concentrations (Figs. 3B, 4J and 5D–F). Taken together, our data indicate that the inhibitory effect of cinobufagin on ESCC cells is achieved by activating p73 and its downstream molecules.

**DISCUSSION**

EC, the seventh most aggressive tumour, is now the sixth leading cause of cancer death worldwide. Data from the National Central Cancer Registry (NCCR) in China show that EC is still a great public health burden in China; there
were approximately 477,900 new cases of EC in 2015 and approximately 37,500 deaths, accounting for 11.13 and 13.33% of all morbidity and mortality, respectively. In recent years, Huachansu injection, tablets, capsules and other preparations have been marketed and widely used at oncology clinics in China, especially for the treatment of patients with various malignant tumours. It has been reported that cinobufagin, one of the major active compounds of Huachansu, can inhibit cell proliferation, induce differentiation, promote apoptosis, cause cycle arrest, decrease tumour angiogenesis, reverse multidrug resistance and regulate the immune response. Nonetheless, the anticancer effect of cinobufagin on ESCC cells has seldom been reported. In this study, in view of the possible differences in the effects of cinobufagin on human ESCC cells with different degrees of differentiation, we first detected the cytotoxicity of cinobufagin on EC-109 (highly differentiated), Kyse-150 (poorly differentiated) and Kyse-520 (moderately differentiated) cells. The results showed that cinobufagin could markedly inhibit the proliferation of these three kinds of ESCC cells in a dose- and time-dependent manner (Fig. 1). Notably, the inhibitory effect of cinobufagin on the growth of esophageal cancer cells is universal, but more studies are needed to confirm whether there is a clear correlation between the anti-tumor efficiency and the differentiation of ESCC cells.

The specific complexes formed by different CDKs and their specific cyclin targets regulate the cell cycle, and cyclin B1/Cdc2 kinase activity dominates the G2/M phase transition. Cdc2 is an evolutionarily conserved serine/threonine protein kinase that is required for the cell cycle transition from G2 to M in all eukaryotic cells. Cdc2 is inactive when phosphorylated, and activation of Cdc2 is regulated by its binding to cyclin B and reversible dephosphorylation. Wee1 phosphorylates Cdc2 and suppresses its activity, and p21 is an inhibitor of the cyclin B1/Cdc2 kinase complex. Our data demonstrated increased upregulation of p21 and Wee1 and downregulation of Cdc2 and cyclin B1 at the mRNA and protein levels during G2/M phase arrest in EC-109, Kyse-150 and Kyse-520 cells by inducing cell cycle arrest at G2/M phase. There are also many reports that cinobufagin inhibits cell growth via induction of G2/M arrest in different cancer cell lines, such as KYN-2, HepG2 and Huh-7 hepatocellular carcinoma cells and U2OS, MG63 and SaOS-2 colon cancer cells. The results of these studies were consistent with our findings. However, some studies held different views: Zhang et al. demonstrated that cinobufagin induces cell cycle arrest at G0/G1 phases in MCF-7 breast cancer cells, and Zhang et al. proved that the induced cell cycle arrest was not limited to a specific phase and varied in multiple non-small cell lung cancer cell lines.

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In recent years, the phenomenon of cell cycle-mediated apoptosis has also received increasing attention and is an appre-
ciated approach to eliminate cancer cells.\textsuperscript{38,39} In this regard, cinobufagin is a promising chemical because it is capable of selectively or preferentially killing cancer cells by inhibiting cell cycle progression and/or inducing apoptosis.\textsuperscript{21–27} This study initially revealed that cinobufagin treatment induced apoptosis of EC-109, Kyse-150 and Kyse-520 cells (Fig. 3). Cinobufagin showed the same potency on the induction of apoptosis as that on the inhibition of cell cycle progression and cell growth (Figs. 1, 2, 3A). Taken together, these results clearly imply that cinobufagin inhibits the growth of ESCC cells via induction of cell G2/M phase arrest and apoptosis.

As we all know, Bcl-2 is an antiapoptotic protein, and Bax is a proapoptotic protein. Caspase-3 is the common downstream apoptotic executioner of many apoptotic pathways, and its activation depends to a large extent on the release of cytochrome c. Bax increases the permeability of the mitochondrial membranes, which arbitrarily results in the formation of pores in mitochondria, and leads to the release of mitochondrial factors such as AIF cytochrome c and dATP to induce cell apoptosis. Bcl-2, a protein homologous to Bax, protects mitochondria against dysfunction during apoptosis by forming homodimers with Bax. Therefore, an increase in the Bax/Bcl-2 ratio triggers Bax to increase the mitochondrial membrane permeability, allowing proapoptotic factors to escape into the cytosol.\textsuperscript{29,40} Recent studies reported that Cinobufagin increased reactive oxygen species and cleaved caspase-3 in multiple non-small cell lung cancer cell lines,\textsuperscript{24} and upregulated the Bax/Bcl-2 ratio in MCF-7 breast cancer cells,\textsuperscript{27} and Bax accelerated apoptotic death after cinobufagin treatment in U2OS osteosarcoma cells and countered the death repressor activity of Bcl-2.\textsuperscript{26} Consistent with this finding, cinobufagin induced an increase in the Bax/Bcl-2 ratio and cleaved-caspase 3 in ESCC cells (Fig. 3B). This study confirmed that apoptosis was induced by cinobufagin, although further experiments are required to validate whether this induction is dependent on the mitochondrial pathway and to investigate the signalling mechanisms involved.

As an important tumour suppressor, p53 can trigger a variety of regulatory events during the cell cycle to monitor the integrity of the genome. In healthy unstressed cells, p53 activity must remain low, as inappropriate activation of p53 accelerates premature apoptosis or senescence, while disruption of p53 must be rapidly eliminated to quickly establish the p53 stress response.\textsuperscript{31} The Mdm2 protein is the core of strict control and function as the main negative regulator of p53.\textsuperscript{30} However, our study found that p53 protein expression did not increase with the decrease in Mdm2 expression after treatment with cinobufagin. Given that p53, a “sentinel” gene that responds to DNA damage, is either deleted or inactivated by mutation in many human cancer cells, resulting in uncontrolled cell proliferation,\textsuperscript{32} we hypothesized that p53 may not exert any anticancer activity in EC-109, Kyse-150 and Kyse-520 cells. In addition, p73 is a member of the p53 family, which has more than 63% identity within the DNA binding region of p53, and p73 is not as susceptible to mutations. Both p73 and p53 have a high degree of similarity in transcriptional activity, as evidenced by their large number of overlapping target genes, including p21, Bax, Puma and Noxa. p21 is a cyclin-dependent kinase inhibitor that leads to cell cycle arrest,\textsuperscript{38} and Noxa and Puma bind to anti-apoptotic protein Bcl-2 and enter caspase cascade activation to induce apoptosis.\textsuperscript{41} As a result, p73 shares some functions of p53 to induce cell cycle arrest and apoptosis.\textsuperscript{34,35} Our current study also found that in EC-109, Kyse-150 and Kyse-520 cells, the expression of p73 protein was significantly increased after treatment with cinobufagin, and its downstream target genes that are responsible for the regulation of cell cycle progression (p21) or apoptosis (Bax, Puma and Noxa) were also increased. These results suggested that the increased G2/M arrest and apoptosis in ESCC cells treated with cinobufagin may be primarily attributed to the increase in p73 protein (Fig. 6).

In conclusion, we investigated the anticancer effects of cinobufagin on human ESCC cell lines (EC-109, Kyse-150 and Kyse-520) and explored the molecular mechanisms leading to these effects. Cinobufagin induces G2/M arrest and apoptosis by regulating the expression of p21, cyclin B1, Bax, Puma and Noxa and activation of caspase 3. The suppressive effect of cinobufagin on ESCC cells is associated with activation of the...
p73 pathway and its downstream molecules.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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