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ORIGINAL ARTICLE

# Steroidal dimer by001 inhibits proliferation and migration of esophageal cancer cells via multiple mechanisms

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## Abstract

**Objective** To investigate the potential inhibitory effects of structurally novel steroidal dimer by001 in esophageal cancer in vitro.

**Methods** The cytotoxicity of by001 on esophageal, gastric, neuroblastoma and prostate cancer cells was examined MTT assay and colony formation assay. By001 induced apoptosis and production of intracellular reactive oxygen species on esophageal cancer cells Ec109, TE-1 and human normal gastric epithelial cells GES-1 was detected by flow cytometry. The effect of by001 on mitochondrial membrane potential was detected by fluorescence microscope through JC-1 staining. The level of intracellular reactive oxygen species was measured by fluorescence microscope and flow cytometry via DCFH-DA staining. The effect of by001 on members of Bcl-2 family, Fas, LC3, PARP and caspases was determined by Western blot. The effect of by001 on migration was measured by
transwell assay.

**Results** By001 effectively inhibited proliferation of esophageal, gastric, neuroblastoma and prostate cancer cells in a time- and concentration-dependent manner in vitro. By001 reduced the number and the size of colonies at low micromolar concentrations, elevated cellular ROS levels and caused mitochondrial dysfunction in esophageal cancer cells. Molecular mechanistic studies showed that by001 triggered apoptosis through regulating members of Bcl-2 family and Fas.

**Conclusions** These findings suggested that by001 may inhibited proliferation of esophageal cancer cells through mitochondria and death receptor-mediated apoptotic pathways, autophagy induction, as well as suppressed migration of esophageal cancer cells.

**Keywords** Steroidal dimer · Steroidal N-heterocycles · Anti-proliferative activity · Migration · Esophageal cancer

## Introduction

Steroids, an important class of polycyclic compounds, are essential in maintaining normal physiological functions and also play important roles in the treatment of diverse diseases. Due to the low abundance and synthetic intractability of natural steroids, many efforts have been devoted to chemical modifications of commercially available steroids, generating millions of structurally novel steroidal compounds (particularly the steroidal N-heterocycles) with interesting and diverse biological activities [1–4]; some of these steroidal derivatives have advanced into clinical trials or are currently being used for the treatment of diseases. For examples, abiraterone [5] and galeterone [6, 7] derived from dehydroepiandrosterone (DHEA) have been used in clinic for the treatment of all-stage prostate cancers. Among steroids, of particular

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interest are the steroidal dimers, which are also found in nature (e.g., cephalostatin I, ritterazines A, and crellastatin A) and show potent antiproliferative activities against human cancer cell lines [8]. Inspired by the structural novelty and promising bioactivities of steroidal dimers, a large number of new steroidal dimers have been synthesized and assessed for their biological potential [9–11]. However, their detailed modes of action are less studied. In continuation with our previous interest in identifying potent anticancer agents, we screened our in-house steroid-based molecular library using the methyl thiazolyl tetrazolium (MTT) assay [12–21], leading to the identification of a structurally symmetric [1, 2, 4] triazolo [1,5-*a*] pyrimidine-based steroidal dimer (named by001), which possessed potent anti-proliferative activity against several human cancer cell lines and showed remarkably more potent activity than its analogs previously synthesized in our group (Fig. 1). Herein, we disclose its mechanisms of inducing cell death and the ability of inhibiting migration. By001 may serve as a template for designing potent steroid-based anticancer agents. Further work will be focused on investigating the structure–activity relationships (SARs) through the structural simplification strategy considering the high molecular weight (about 803) of by001.

## Materials and methods

### Cell culture

All cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Ec109, TE-1, PC-3 and GES-1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. SH-Y5Y and MGC 803 cells were cultured in DMEM (high glucose) medium with the same concentration of fetal bovine serum, penicillin and streptomycin.

### MTT assay

Cells were seeded into the wells of the flat-bottomed 96-well culture plates in RPMI-1640 medium at a density of 3000–5000 cells per well. After an overnight attachment period, the cells were treated with medium containing 0, 0.5, 1, 2, 3, 4, 6, 8, 16, 32 μM of by001. After incubation for 72 h, 20 μL MTT (5 g/L) was added into each well, and incubation was continued for 4 h at 37 °C. Then, supernatant was removed; formazan was solubilized from cells by 150 μL DMSO. Absorbance values were measured at 570 nm after shaking for 10 min. $IC_{50}$ values were calculated using SPSS 20.0 software.

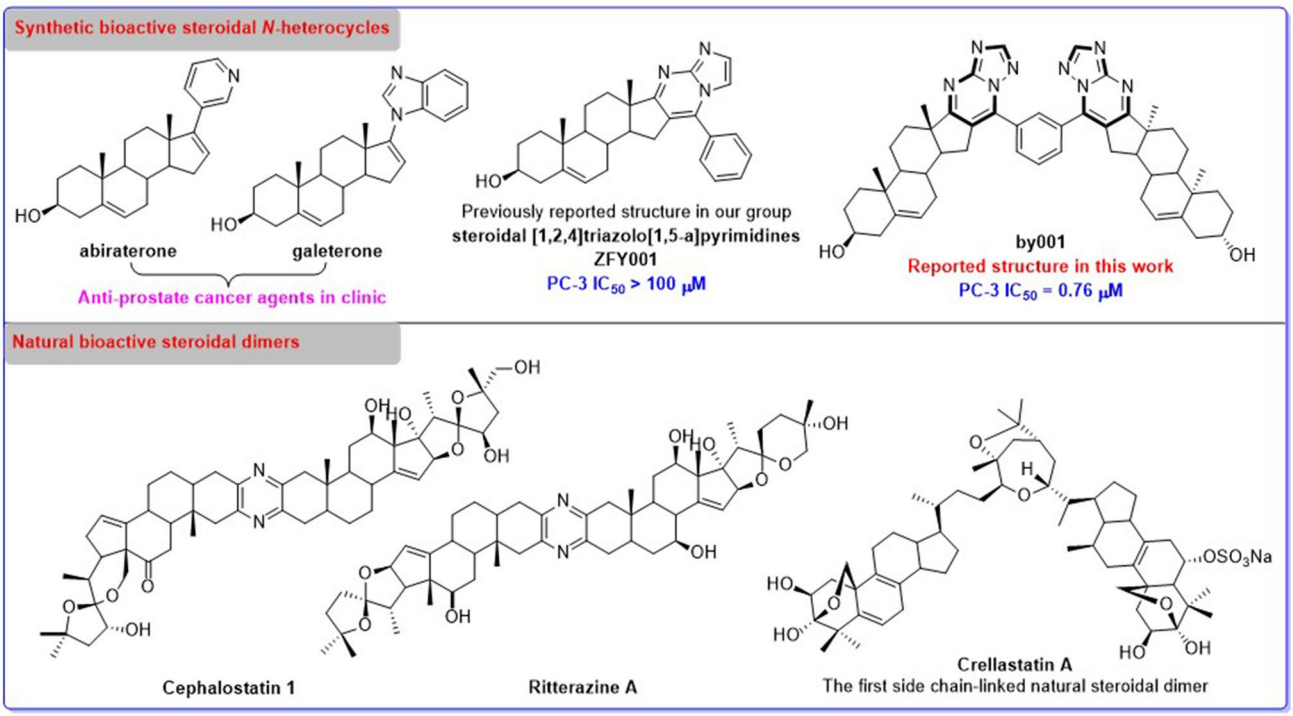


**Fig. 1** Selected bioactive steroids and by001 reported in this work

### Colony formation assay

Cells were seeded into 6-well plate with a density of 1000 cells per well. After treatment of by001 (0, 0.25, 1, 3.0 μM), cells were cultured for 7 days [22]. The colonies were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. The colonies containing 50 or more cells were numbered by Image J software. The area of a single clone was measured by Nikon NIS analysis software.

### Detection of mitochondrial membrane potential

Cells were treated by by001 (0, 5, 10, 20 μM) for 24 h before stained with JC-1 (5 μg/mL) at 37 °C for 20 min and then washed with warmed medium without fetal bovine serum. The stained cells were taken photos and analyzed with laser scanning microscope (Nikon A1, Tokyo, Japan). Quantitative analysis was performed using the NIS-Elements AR software (Nikon).

### Apoptosis assay

Apoptosis was measured using the Annexin V-Fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (KeyGEN BioTECH, Nanjing, China). Briefly, Ec109, TE-1 or GES-1 cells were seeded into 6-well plates. After exposure to the indicated concentration of by001 for 24 h, the cells were harvested, washed with PBS, and then resuspended in 500 μL binding buffer, stained with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min in the dark and finally detected and analyzed by BD LSRFortessa Cell Analyzer flow cytometer (Becton Dickinson, NJ, USA).

## ROS detection

The intracellular reactive oxygen species (ROS) was measured with 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Cells were cultured in 6-well plates under various conditions (0, 5, 10, 20 μM) for 6 h. Then the cells stained with DCFH-DA (10 μM) for 20 min, washed with phosphate buffered saline (PBS) three times. The fluorescence of DCFH was detected by high content screening system (Array Scan XTI, Thermo Fisher, Waltham, MA, USA) and flow cytometry (LSRFortessa, Becton Dickinson, NJ, USA).

### Western blot

The collected cells were lysed on ice by radioimmunoprecipitation assay (RIPA) buffer and the protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay Kit. Samples were separated by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel and then transferred onto a

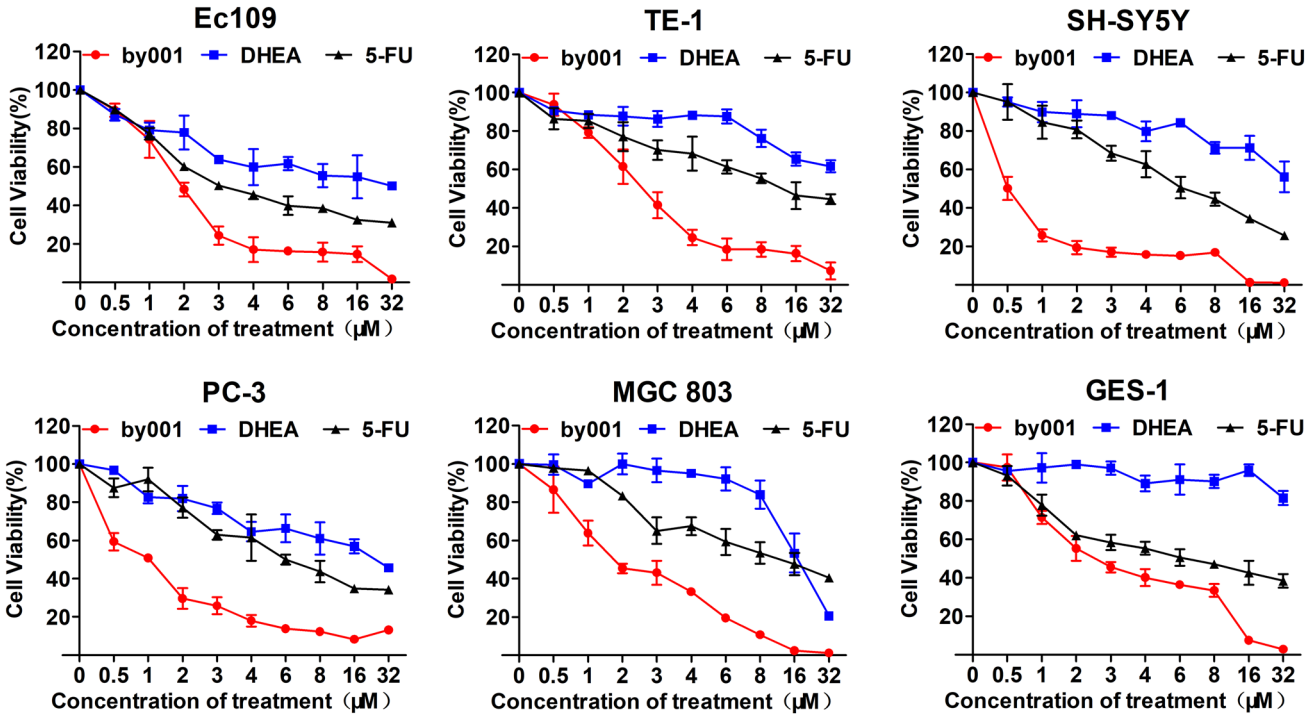


**Fig. 2** Cell viability after treatment with by001 for 72 h. Data were shown as mean ± SD from three independent experiments

**Table 1** $IC_{50}$ values of by001, DHEA, and 5-FU against human cancer cell lines and GES-1

| Cell lines | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | by001 | DHEA | 5-FU |
| Ec109 | 1.90 ± 0.73 | 24.66 ± 3.65 | 4.61 ± 0.53 |
| TE-1 | 2.71 ± 0.38 | 87.24 ± 9.12 | 15.10 ± 2.54 |
| SH-SY5Y | 0.41 ± 0.12 | 67.42 ± 2.62 | 6.93 ± 0.46 |
| PC-3 | 0.76 ± 0.16 | 20.36 ± 1.37 | 7.87 ± 1.31 |
| MGC 803 | 1.92 ± 0.38 | 17.51 ± 1.58 | 13.17 ± 1.64 |
| GES-1 | 2.76 ± 0.46 | > 150 | 7.95 ± 1.77 |

polyvinylidene fluoride (PVDF) membrane. The transferred membranes were blocked for 1 h in 10% nonfat milk in TBST (Tris-buffered saline, TBS containing 0.05% Tween-20) and incubated with primary antibodies overnight at 4 °C. The goat anti-rabbit secondary antibody incubated for 2 h at room temperature. The protein bands were visualized using the enhanced chemiluminescence (ECL) Western Detection System. Relative protein levels were quantified by Image J software. β-Actin was used as the loading control [23].

## Migration assay

Cells were seeded in the upper chamber and cultured in the medium with 2% fetal bovine serum and by001 (0.25, 1, 3 μM) for 24 h. Then, the cells that migrated to the lower chamber were fixed with 4% paraformaldehyde and stained by Hoechst 33342 and numbered using the high content screening system (Array Scan XTI, Thermo Fisher, Waltham, MA, USA).

## Statistical analysis

All data are presented as the mean ± standard deviation (SD) form three independent repeated experiments and were analyzed using the GraphPad Prism software. Statistical significance was evaluated using one-way analysis of variance (ANOVA).

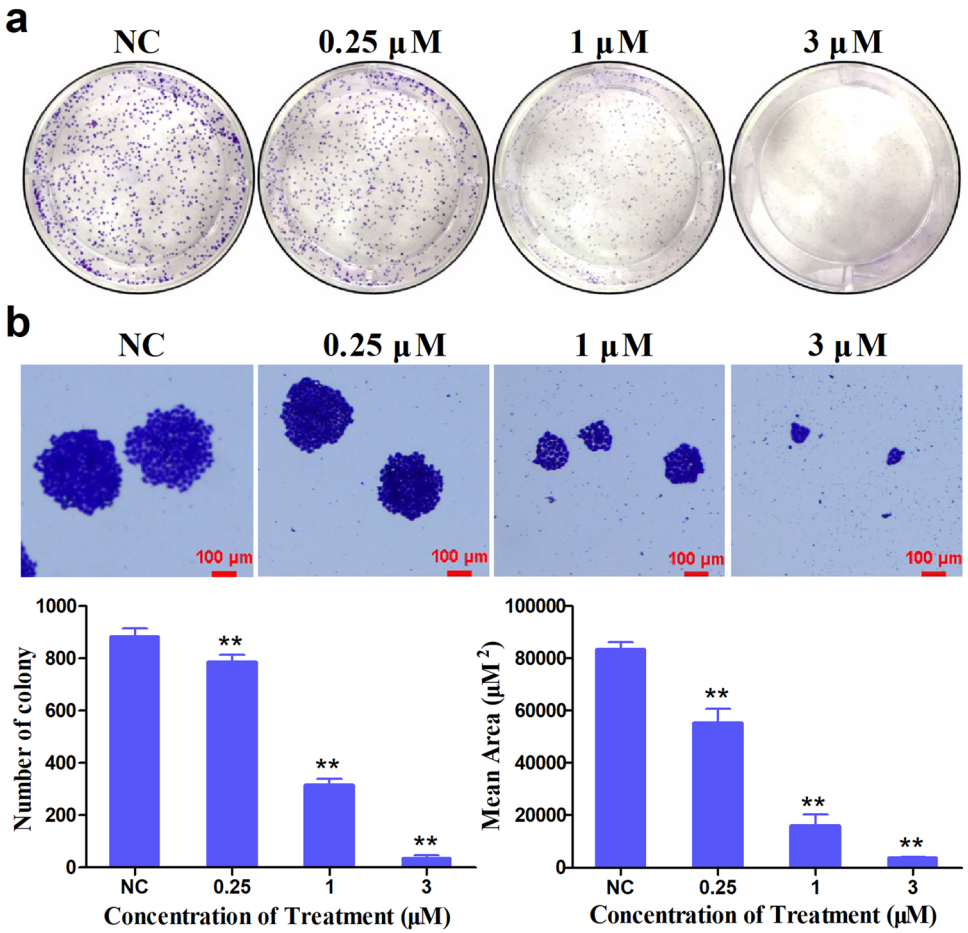


**Fig. 3** By001 inhibited colony formation of Ec109 cells. Ec109 were treated with by001 for 7 days, then the number of colonies were counted (**a**) and the size of colonies were measured (**b**). Scale bar is 100 μM. $^{**}P < 0.01$

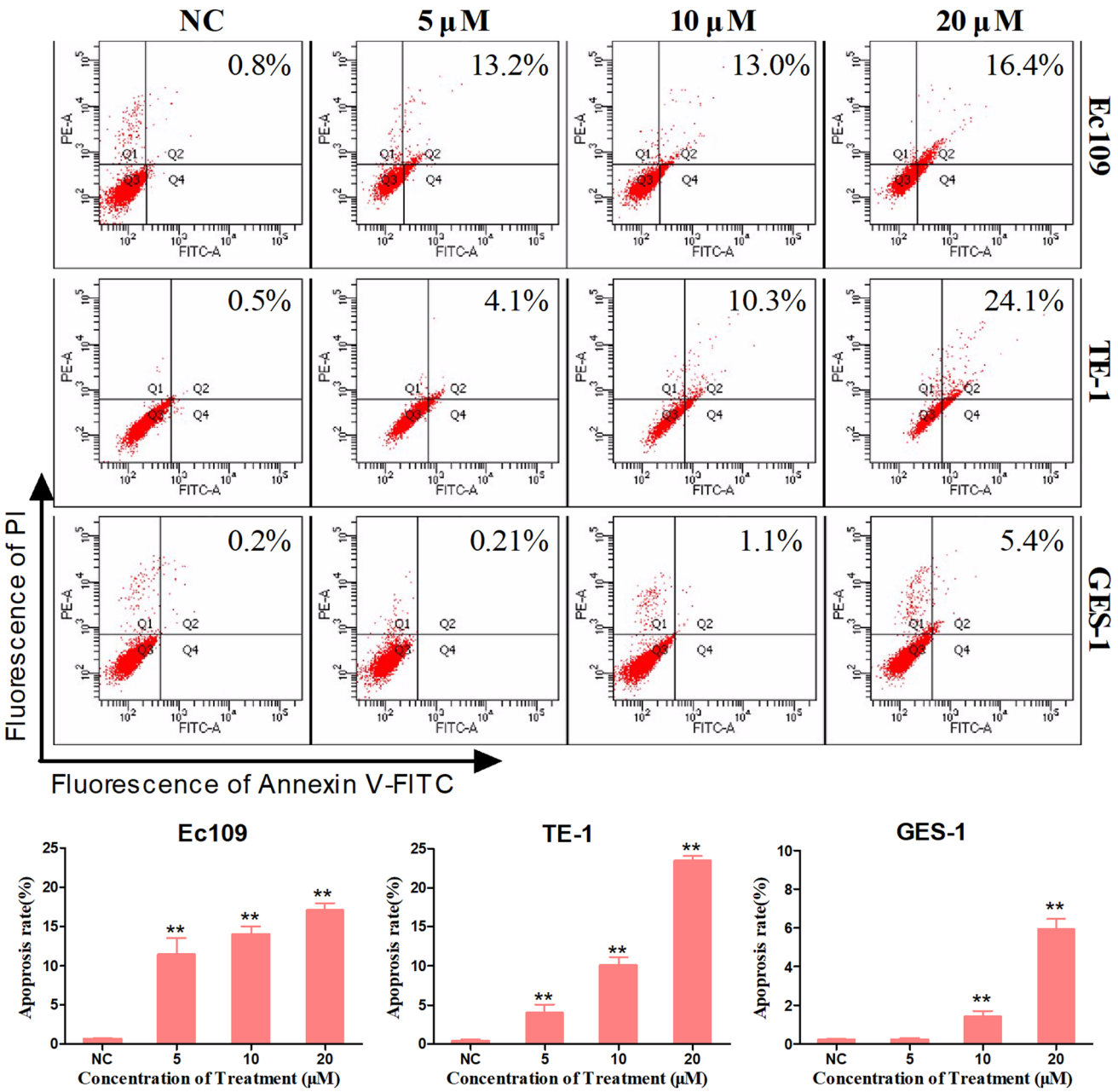


**Fig. 4** By001 triggered apoptosis in Ec109, TE-1 and GES-1 cells. Cancer cells Ec109, TE-1 and GES-1 cells were treated with by001 for 24 h and detected by flow cytometry. $^{**}P < 0.01$

## Results and discussion

### Evaluation of antiproliferative activities and SARs studies

With by001 in hand, we next evaluated the effect of by001 on the cell viability of human cancer cells of different origins including esophageal (Ec109 and TE-1), gastric (MGC 803), neuroblastoma (SH-SY5Y), prostate (PC-3) cancers using the MTT assay. Dehydroepiandrosterone (DHEA) and 5-FU were used as the control. The gastric epithelial cell line GES-1 was used to study the selectivity between the cancer cells and normal cells, as well as the potential toxicity. As shown in Fig. 2, the cell viability of several cancer cells sharply decreased in a concentration-dependent manner after treatment with by001 for 72 h, while cells treated with DHEA and 5-FU showed a gradual decline in cell viability. The $IC_{50}$ values of by001 against different cancer cells and human gastric epithelial cell line GES-1 are listed in Table 1. Evidently, by001 showed good growth inhibition against the

tested cancer cell lines ($IC_{50} < 2.8$ μM), especially against SH-SY5Y and PC-3 cells ($IC_{50} = 0.41$ and 0.76 μM, respectively). In contrast, ZFY001 (Fig. 1) displayed weak growth inhibition against PC-3 cells, highlighting the importance of the dimer scaffold. However, we can also observe that by001 also reduced survival of normal GES-1 cells potently with an $IC_{50}$ value of 2.76 μM, similar to that of MGC-803 cells ($IC_{50} = 1.92$ μM), suggesting the low selectivity of by001. Collectively, by001 is a structurally new cytotoxic agent, highly more potent than DHEA and ZYF001. These data suggest that the [1, 2, 4] triazolo [1,5-*a*] pyrimidine scaffold and the dimer skeleton are necessary for the activity.

## By001 reduced colony formation of Ec109 cells

Colony formation assay is a common in vitro cell survival assay, which can reflect the ability of a single cell growing into a colony. To study the effect of by001 on the colony formation in Ec109 cells, different concentrations of by001 were used to treat the cells for 7 days. In Fig. 3a, by001 concentration-dependently reduced the number of colony of Ec109 cells, and by001 at 3 μM inhibited nearly 95% of colony formation. Treatment of by001 also caused a sharp decrease inthe size of the colonies (Fig. 3b). The results indicate that by001 is capable of inhibiting the colony formation of Ec109 cells.

## By001 induced apoptosis in esophageal cancer cells

To study the anti-tumor mechanisms of by001, we detected apoptosis induced by by001 in Ec109, TE-1 and GES-1 cells. As can be seen in Fig. 4, in esophageal cancer Ec109 and TE-1 cells, by001 induced apoptosis in a concentration-dependent manner after treatment for 24 h. 20 μM of by001 triggered 17.13% and 23.52% apoptosis in Ec109 and TE-1 cells, respectively, while in gastric epithelial cells GES-1, 20 μM by001 only triggered 5.93% apoptosis. The results suggested that by001 reduced cell viability of Ec109 and TE-1 mainly through inducing apoptosis. In addition, compared with cancer cells, normal cells GES-1 showed a low response to by001 at 24 h.

In our previous study, we found that by001 decreased the mitochondrial membrane potential (MMP) and induced apoptosis of Ec109 cells. However, the exact mechanism of inducing cell death remains unclear. To study the underlying mechanisms, we measured the MMP of Ec109 and TE-1 cells using the JC-1 dye. The intensity of green fluorescence reflects the loss of MMP. As shown in Fig. 5, after treatment with by001 for 24 h, the green fluorescence intensity in Ec109 and TE-1 cells increased with the increasing concentration of by001, accompanied with decrease of red fluorescence intensity, indicating the loss of MMP. The results suggest that by001 caused mitochondrial dysfunction,

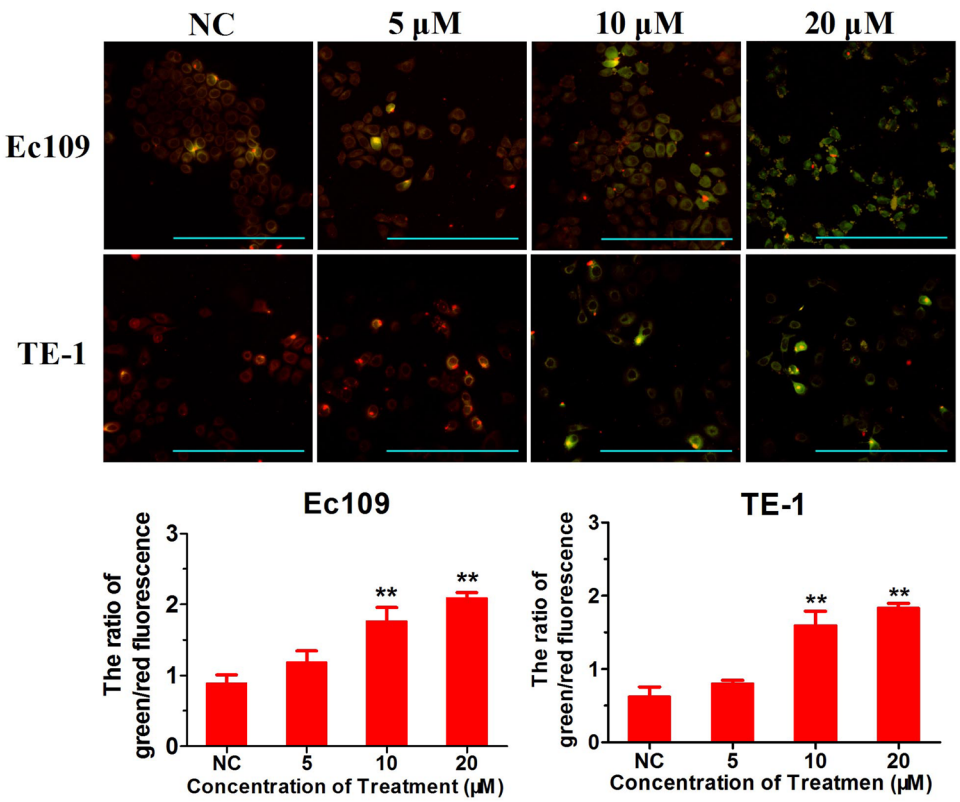


**Fig. 5** By001 induced MMP loss in Ec109 and TE-1 cells. Cells were treated with by001 for 24 h and then stained with JC-1 before detecting using the confocal laser scanning microscopy. Scale bar is 200 μm

thereby inducing apoptosis in esophageal carcinoma cells Ec109 and TE-1.

### By001 triggered cellular ROS generation in esophageal cancer cells

Compared to normal cells, cancer cells have a higher demand on the mitochondrial respiratory chain to generate more ATP for their rapid growth and differentiation, thus inevitably making cancer cells have high ROS levels. To determine whether by001 triggers ROS generation in esophageal cancer cells, the fluorescent probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect the ROS levels in TE-1 and Ec109 cells after treatment with by001. DCFH-DA is diffused into cells and then enzymatically hydrolyzed by cellular esterases to give the non-fluorescent 2′, 7′-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized by ROS to form highly fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF). After treatment with by001 for 12 h, the fluorescence of DCF was measured by the high-content screening and flow cytometry. As shown in Fig. 6a, c, by001 concentration-dependently increased the

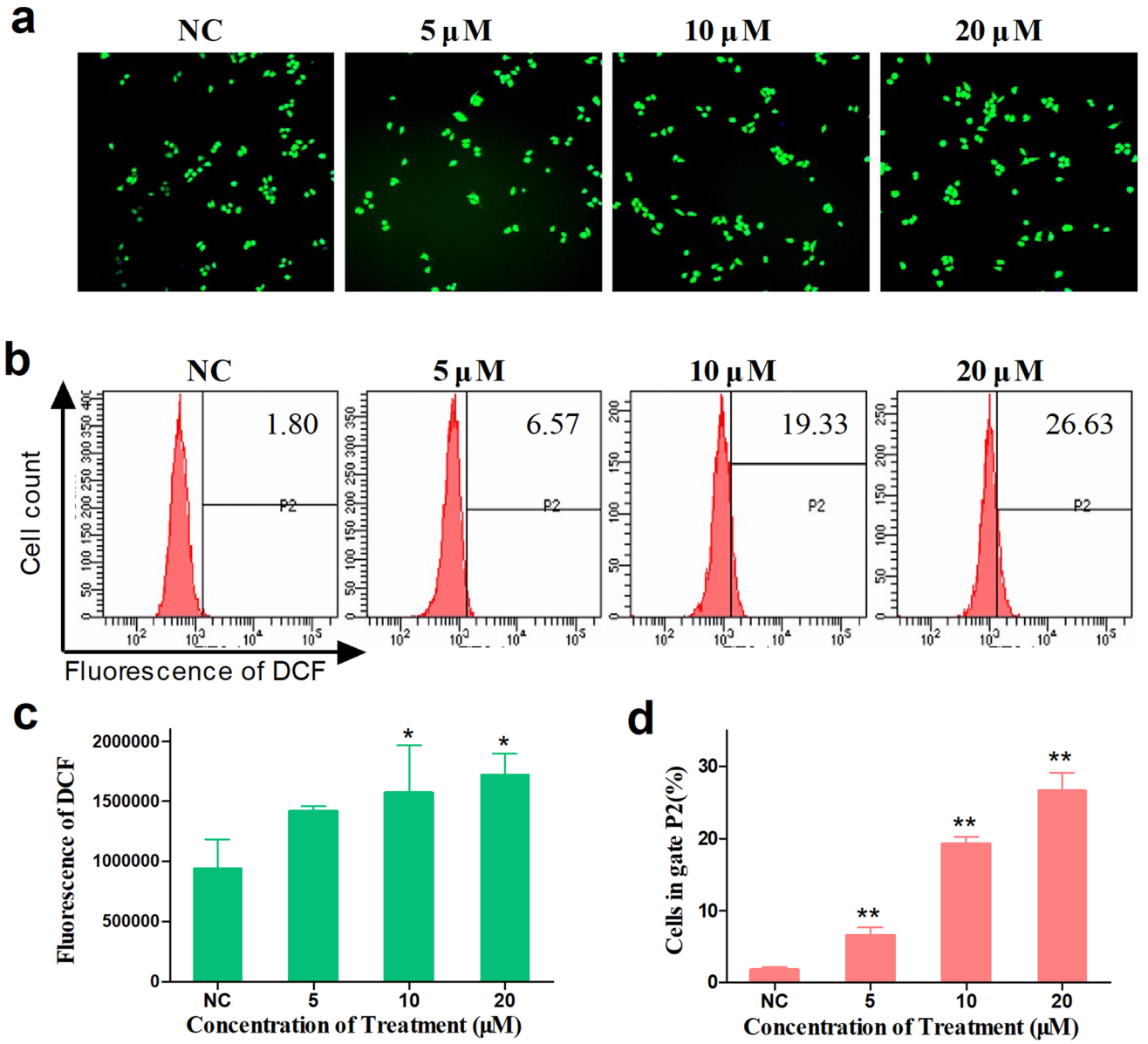


**Fig. 6** By001 elevated ROS levels in Ec109 cells. **a** ROS level measured by high-content screening system. **b** ROS level measured by flow cytometry. **c** Mean fluorescence of DCF analyzed by high-content screening system. **d** Percentage of cells in gate P2 analyzed by flow cytometry. Cancer cells Ec109 were treated with by001 for 6 h and stained with DCFH-DA, and then analyzed the high content screening system and flow cytometry. $^{*}P < 0.05$; $^{**}P < 0.01$

mean fluorescence intensity of DCF in Ec109 cells. In the meanwhile, the results from flow cytometry showed that 5, 10 and 20 μM of by001 triggered 6.6%, 19.3% and 26.6% cells with an excess of ROS (Fig. 6b, d). These results indicate that by001 may elevate cellular ROS levels, thereby leading to apoptosis of Ec109 cells.

## Mechanistic studies of by001 inducing apoptosis of esophageal cancer cells

Based on the observed potent anticancer efficacy of by001, we first examined the expression changes of apoptosis-related proteins in Ec109 cells. As shown in Fig. 7, for the Bcl-2 family proteins, the expression levels of pro-apoptotic proteins Bax and Bak were upregulated with the increasing concentration of by001, and the anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 were correspondingly down-regulated, which led to the dysfunction of mitochondria. Besides, treatment of Ec109 cells with by001 also increased the expression levels of Fas, cleaved caspase-8, and caspase-9, which then activated caspase-3 and cleaved PARP, finally leading to the DNA damage and apoptosis. Interestingly, we also found that by001 led to the cleavage of LC3I, forming LC3II, which is involved in the induction of the autophagy. Taken together, the results suggest that by001 may induce the death of Ec109 cells via triggering autophagy and apoptosis through both mitochondrial and death receptor pathways (Fig. 7). Treatment of another esophageal cancer cell line TE-1 with by001 also led to similar results (Fig. 8). The expression levels of Bax were upregulated, while the Bcl-2 was down-regulated. By001 also increased expression levels of Fas, activated caspase-8, caspase-9 and then caspase-3, finally leading to apoptosis of TE-1 cells. In this part, our results elucidated that by001 inhibited esophageal cancer cells by inducing apoptosis and autophagy, but the potential specific molecular targets of by001 remains elusive.

## By001 inhibited migration of Ec109 cancer cells

The tumor development is usually accompanied with neoplasm metastasis, which would lead to the failure of therapy, neoplasm recurrence, and even death. Herein, we determined

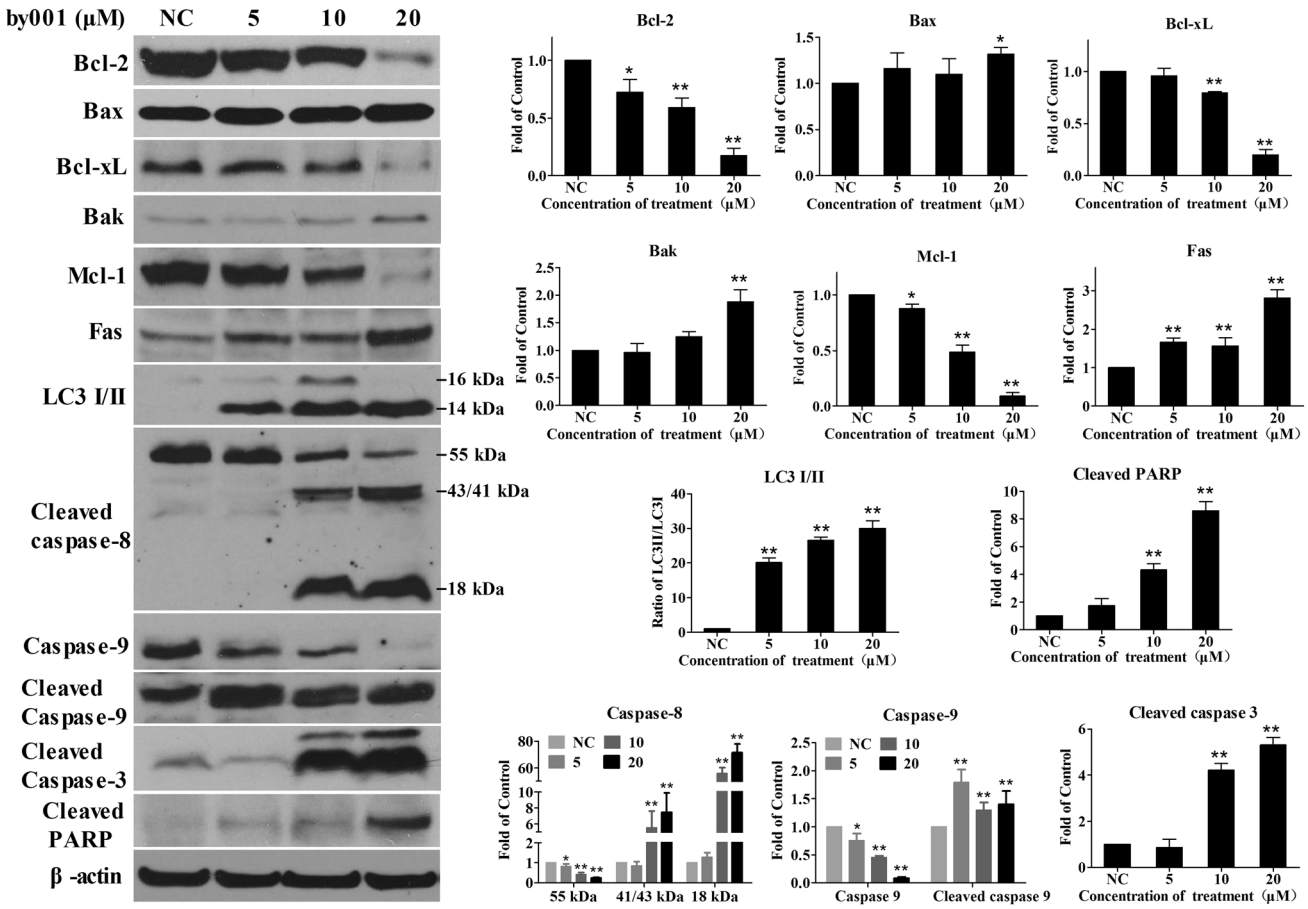


**Fig. 7** Expression changes of key proteins in Ec109 cells after treatment with by001. Ec109 cells were treated with by001 for 24 h before lysis and analysis through western blot assay. β-Actin is used as the loading control. $^{*}P<0.05$; $^{**}P<0.01$

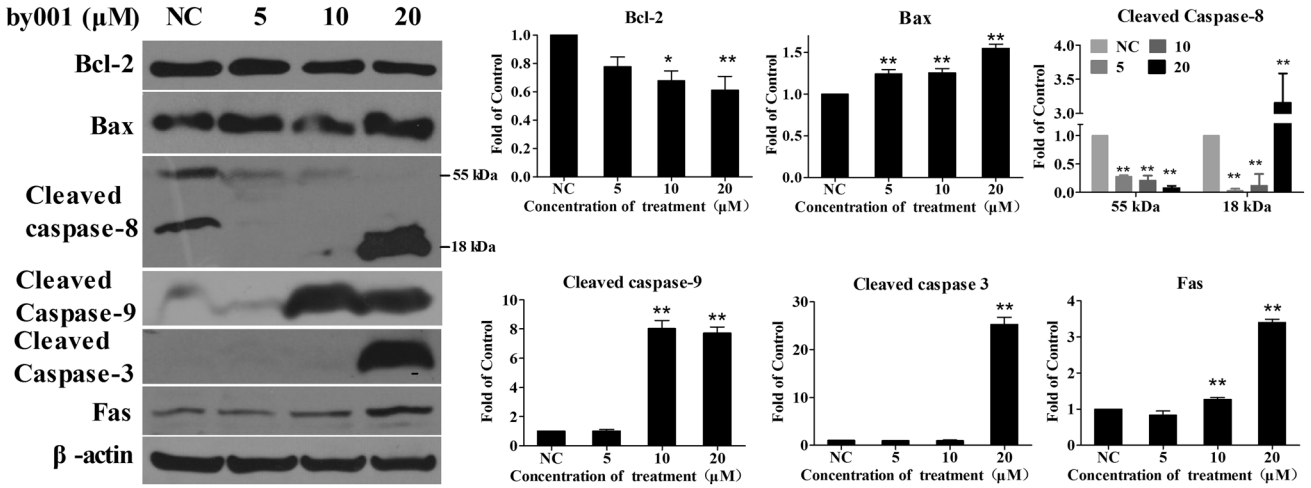


**Fig. 8** Expression changes of key proteins in TE-1 cells after treatment with by001. TE-1 cells were treated with by001 for 24 h before lysis and analysis through western blot assay. β-Actin is used as the loading control. $*P<0.05$; $**P<0.01$

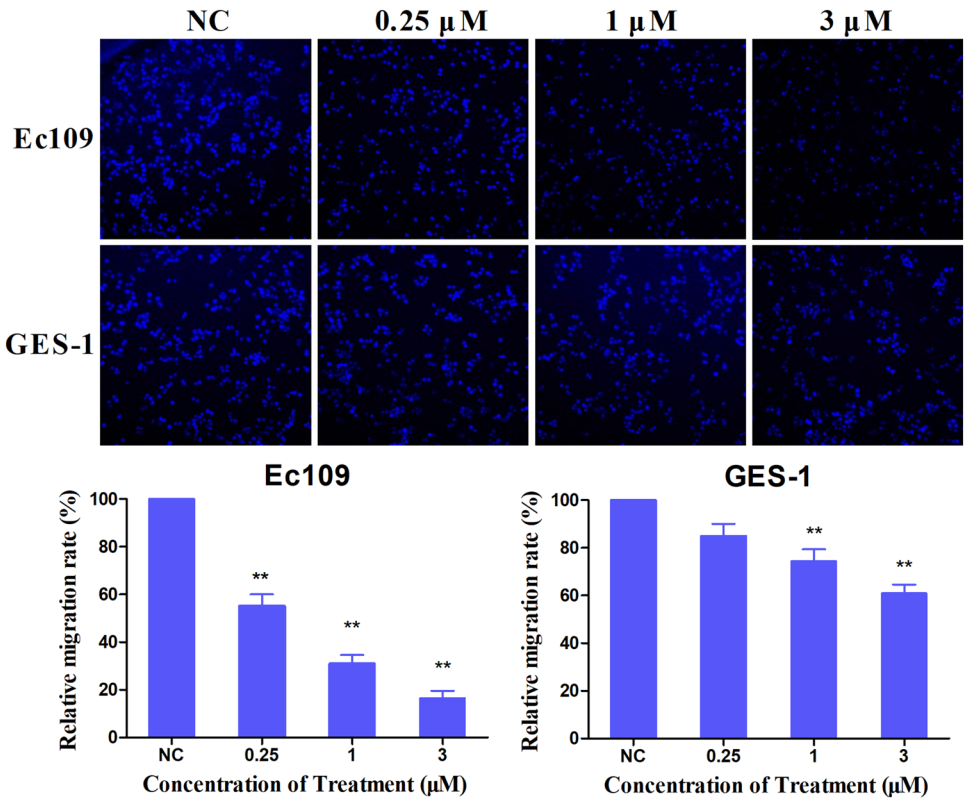


**Fig. 9** By001 suppressed migration of Ec109 and GES-1 cells. Ec109 and GES-1 cells were suspended in medium with different concentrations of by001 and seeded in the upper chamber. After incubation for 24 h, the cells that migrated to the lower chamber were stained by Hoechst 33342 and detected using the high content screening system. $**P<0.01$

the ability of by001 affecting migration of esophageal cancer cell Ec109 using the transwell assay. Ec109 cells were seeded in the upper chamber and treated with by001 for 24 h. As shown in Fig. 9, by001 concentration-dependently suppressed migration of Ec109 cells, the migration rates at 0.25, 1 and 3 μM were about 55%, 31% and 17%, respectively. In GES-1 cells, the migration rate at 0.25, 1 and 3 μM were about 85%, 74% and 61%, suggesting the ability of by001 suppressing migration of cancer cells.

Based on the above results, we can conclude that by001 inhibited colony formation, elevated cellular ROS levels, and then caused mitochondrial dysfunction. By001 induced cell death via triggering autophagy and inducing apoptosis through both the mitochondrion and death receptor-mediated

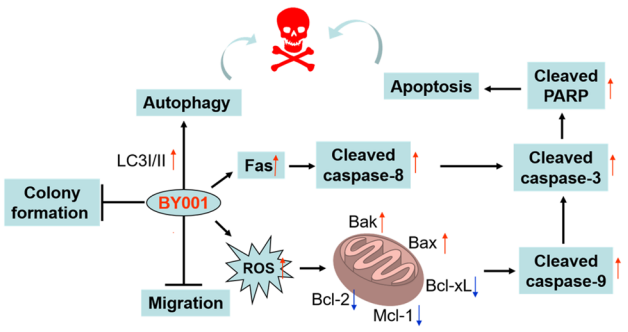


**Fig. 10** Schematic illustration of by001 inducing death of Ec109 cells

pathways, as well as inhibited migration of esophageal cancer cells (Fig. 10).

## Conclusions

Inspired by the anticancer potential of steroidal *N*-heterocycles and following our previous success in identifying steroid-based anticancer agents, we described the anti-proliferative activity of a structurally novel steroidal dimer by001 and its mechanisms of inducing death of esophageal cancer cells. By001 reduced survival of different cancer cells at low micromolar levels and colony formation, elevated cellular ROS levels and caused mitochondrial dysfunction. Mechanistic studies showed that by001 induced cell death through the mitochondrial and death receptor-mediated apoptotic pathways and autophagy induction, as well as inhibited migration.

Although by001 also has similar cytotoxicity to normal GES-1 at low micromolar levels, it may serve as a template for designing potent steroid-based anticancer agents due to its excellent anticancer potency. In addition, by001 deserves further investigation to identify its specific molecular targets in our future research as a novel chemotherapeutic strategy for patients with esophageal cancer. Our results may offer an evidence that dimeric steroidal scaffolds have improved biological activities relative to the corresponding monomers.

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## Compliance with ethical standards

**Conflict of interest** Author Sai-Qi Wang declares that she has no conflict of interest. Author Kai-Rui Zhou declares that she has no conflict of interest. Author Xiao-Li Shi declares that she has no conflict of interest. Author Hui-Fang Lv declares that she has no conflict of interest. Author Liang-Yu Bie declares that he has no conflict of interest. Author Wei-Jie Zhao declares that he has no conflict of interest. Author Xiao-Bing Chen declares that he has no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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