

# The direct anti-cancer efficacy of Sapylin on breast cancer cells in vitro and in vivo

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## Abstract

**Objective:** On the basis of our previous study in which we studied cancer cells under in vitro and in vivo hypoxia conditions, we have now investigated the anti-cancer efficacy of Sapylin on breast cancer cells in mice and human. **Materials and Methods:** We used different concentrations of Sapylin and the three kinds of breast cancer cells. We used water-soluble tetrazolium salt cell proliferation test (WST-1) to detect changes in cell proliferation and Fluorescein Iothiocyanate-Propidium Iodide (Anexin V FITC-PI) to detect changes in the rate of apoptosis by flow cytometry. We also used reverse transcription-polymerase chain reaction (RT-PCR) to detect possible changes of mRNA expression and used western blot in order to test changes related to protein expression that could lead to cell death. The anti-tumor effect was studied by locally injecting Sapylin into an animal tumor model of breast cancer. We also studied the possible postoperative adverse clinical side effects in 60 female breast cancer patients, stage II-III, aged 25-55 years. The patients underwent a modified, radical operation with smooth incisions which healed well. **Results:** Sapylin was able to inhibit by 10%-15% the proliferation of all three kinds of breast cancer cells and also to present positive correlation in vivo with some phenomena which were time and concentration dependent. After applying Sapylin for 48h, the apoptosis rate was significantly increased by 12%-20%. Apoptosis of breast cancer cells may be related to biological effects supporting cells survival, through B-cell lymphoma gene 2 (Bcl-2nd) Ki67 mRNA expression descent and Bcl-2 associated X Protein (Bax mRNA) expression. This process ultimately promotes cell death. At the same time this process also showed a significant anti-tumor effect (50%-60%) in a mice model. We found no significant adverse reactions, the patients had no significant pain and the postoperative wound was partially healed. After 5 days, the drainage was well reduced and remained so more in the study group than in the control group at a range of 20%-30% ( $P < 0.05$ ). **Conclusion:** In our research, Sapylin displayed a strong direct anti-cancer effect in breast cancer cells and supported postoperative recovery. Clinically we noticed an obvious reduction of drainage in contrast with the control group.

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## Introduction

A source for Sapylin is group A hemolytic streptococcus Su strain, obtained under penicillin treatment. Sapylin has an immunological activity and significantly activates inflammatory cells. These cells, as we know, surround, infiltrate and adhere to target cells which become degenerated and destructed. Natural killer (NK) cells are also activated and act as non-specific immune factors of treatment [1, 2]. The difference between Sapylin and other immune activators is that, in addition to activation of the immune effector cells, in order to act as a killer to tumor cells, Sapylin can still block cells DNA, RNA and protein synthesis, resulting in tumor cells degeneration and disintegration, thus acting as a direct anti-tumor factor [3]. Survivin, a protein and member of inhibitor of apoptosis family, is closely related with the tumorigenesis. Survivin does not only directly effects caspase3, inhibiting its activity to block the apoptosis process [4], but also acts on the cell cycle regulation factor CDK4, to inhibit pro-caspase3 activation and finally blocks apoptosis. Bcl-2 and Bax both belong to the Bcl-2 family proteins and promote apoptosis [5, 6] and also have close ties with the mitochondria [7, 8]. Bcl-2 and Bax function after they form a heterodimer. When the expression of Bax is high, it forms a homodimer which promotes apoptosis. P53 gene is an important tumor suppressor gene, and the mutant P53 loses its tumor suppressor function, which is easy to detect [9, 10]. P27 participates in cell cycle regulation to suppress tumor growth, which works mainly through the cell cycle-dependent kinase K (CDK) or cell cycle (CDK-cyclin) complexes [11, 12]. Ki67, indicator of cell proliferation, is

currently a more positive nuclear proliferation marker, present in all stages of cell cycle except the G0 phase. Because of its short half-life and its rapid degradation from the cell cycle, it has become the most reliable indicator of tumor cells proliferation activity [13]. Caspase-3 in the action of multiple pathways of apoptosis factors is the final factor. Its expression is closely and directly related with cell apoptosis [14]. When is activated, it can work through a variety of different signals and transduction systems to induce apoptosis and cell death [15]. Microtubule associated protein 1 light chain 3 (LC3) and beclin1 are both important genes related to autophagy, which work through the PI3K/AKT pathway [16, 17]. Beclin1, a candidate tumor suppressor gene [18], is a regulation gene of autophagy, which can increase autophagy to inhibit tumor growth.

Our previous studies showed that Sapylin exerts a direct cytotoxic effect on SGC7901 gastric cancer cells in vitro [19, 20]. In this study, three kinds of human breast cancer cells: MCF-7, SK-BR-3 and MDA-MB-453 were used as an in vitro and in vivo model in BALB/C-nude mice and at the same time patients were clinically followed as for the anti-tumor effect of Sapylin and specifically the effect on cell growth, death, gene expression, Bcl-2, caspase3 and LC3 in breast cancer cells and postoperative recovery in patients.

## Materials, Subjects and Methods

### Reagents

Sapylin was obtained from Winteam Ruia Group Company (Klinische Einheit) (1KE equivalent to 0.1mg dry streptococcus+2700U/mL penicillin). (Dulbecco's Modified Eagle Medium and Roswell Memorial Park Institute) DMEM and RPMI 1640 medium, fetal bovine serum (FBS), 0.25% Ethylene diamine tetraacetic acid (EDTA)-trypsin mixture (containing 0.25% trypsin w/v and 0.02% EDTA w/v), and antibiotics solution (containing 10000U/mL penicillin and 10mg/mL streptomycin) were purchased from Gibco (USA). Western blot and (enhanced chemi-luminescent) ECL kit color reagent were purchased from Pik-day Biotechnology Institute (USA). Primers were synthesized by the Sangon Biotech (Shanghai) Co. to code gene as follows:

GADPH 471bp	sense:5'-ACCACAGTCCATGCCATCAC-3'
	anti-sense:5'-CCACCACCCTGTTGCTGTA-3'
Ki67 126bp	sense:5'-ATCGTCCCAGGTGGAAGAGTT-3'
	anti-sense:5'-ATAGTAACCAGGCGTCTCGTGG-3'
Bcl-2 191bp	sense:5'-CAGCTGCACCTGACGCCCTT-3'
	anti-sense:5'-GCCTCCGTTATCCTGGATCC-3'
survivin 363bp	sense:5'-AGCCCTTTCTCAAGGACCAC-3'
	anti-sense:5'-GCACTTTCTTCGAGTTTCC-3'

Bax 209bp	sense:5'-GATGCGTCCACCAAGAAGC-3'
	anti-sense:5'-TGAGCACTCCCGCCACAA-3'

### Cells culture

The cells were type: MCF-7, SK-BR-3 and MDA-MB-453 and purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The MDA-MB-453 cells were maintained in RPMI1640 essential medium supplemented with 10% phosphate-buffered saline (FBS) v/v, and incubated at 37°C under 5% carbon oxide (CO<sub>2</sub>). Cells were subcultured every 3 to 4 days. MCF-7 and SK-BR-3 cells were maintained under DMEM medium with the same condition.

The effect of Sapylin on breast cancer cells' growth was examine by the WST-1 assay. Breast cancer cells MCF-7, SK-BR-3 and MDA-MB-453 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences in a logarithmic growth phase and were seeded in 96 well plates at a density of 5000 cells per well, incubated at 37 °C and under 5% CO<sub>2</sub> for 24h. Sapylin was diluted in the culture medium immediately before addition to each well at the desired final concentrations 1KE equivalent to 0.1mg dry streptococcus+2700U/mL penicillin: 0, 0.01, 0.1, 0.5 and 1KE/mL. The treatment lasted for 12h, 24h, 36h and 48h. Each concentration was placed in three parallel holes. The control group was set without Sapylin and its blank hole without cells. Ten  $\mu$ L of WST-1 at 5mg/mL, (Nanjing KeyGen Biotech, Nanjing, China) working solution was added to each well at a final concentration of 500 $\mu$ g/mL. The mixture in each well was incubated for another 2.5h and after that we used the microplate reader (Quant), Bio-Tek Services, Inc., Richmond, VA, USA) to detect the absorbance of formazan (produced by WST-1 and some dehydrogenase from mitochondria) at 450nm wave length. Dehydrogenase: A kind of oxidoreductase EC1, which can be used as a catalyst for the transfer of hydrogen or electrons from the donor to the acceptor. The dehydrogenase is named after the oxidation of the substrate (hydrogen donor compound). The relative cell viability was indirectly expressed as a percentage of the control well. The inhibition rate was calculated by the formula: cell inhibition rate=[1-absorbance of experimental group/control group absorbance] $\times$ 100%.

### The effect of Sapylin on breast cancer cells' apoptosis rate

Breast cancer cells in logarithmic growth phase were seeded in 6 well plates at a density of 105/mL, 37°C and 5% CO<sub>2</sub> for 24h. After treatment with or without a cocentration of 0.5KE/mL of Sapylin for 48h, the cells were harvested by trypsinization and washed once with phosphate-buffered saline (PBS, pH 7.4). After centrifugation, the cells were suspended in a 100 $\mu$ L binding buffer (Invitrogen CO. USA) and then stained with FITC-annexin V and propidium iodide (Invitrogen CO. USA). After they reacted under dark for 10min, the cells were resuspended by another 400 $\mu$ L binding buffer and we used the flow cytometer (Model BD LSR II, BD Bioscience, San Jose, CA, U.S.A.) to measure cell apoptosis.

### The RNA level of survivin, Bcl-2, Bax and Ki67 examined by semi-quantitative RT-PCR

After treatment with or without Sapylin for 48h, cells were harvested and total RNA was extracted by trizol (Takara Biotechnology CO., Dalian, China) and then reversed and amplified by the reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara) under 94°C for 30sec, 60°C for 30sec and 72°C for 30sec, in a total of 30 cycles. The products were detected by 1.5%-2% agarose gel (Invitrogen CO. USA) electrophoresis. The results were analyzed by Bio-rad Gel Doc™ XR+imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.).

### The protein expression level of P53 P27 caspase LC3 and Beclin1 detected by western blot

Cells were washed first, and then suspended in 100μL lysate dodecyl sulfate sodium salt+phenylmethanesulfonyl fluoride (SDS+PMSF). Total protein concentration was measured by using the bicinchoninic acid (BCA) assay via microplate reader (Bio-tek) at 562nm wavelength.

After an equal amount of proteins loaded in each lane, we used 10% SDS w/v polyacrylamide gel electrophoresis (SDS-PAGE) to separate and then electrically transfer to a polyvinylidene difluoride (PVDF) membrane. After blocking the membrane with 5% skim milk w/v, target proteins were immune-detected using specific antibodies. All primary antibodies were used in 1:1000 dilution, hatching overnight at 4°C. After mixing the antibody with the protein washed with this buffered saline with tween-20 (TBST) 3 times for 20min. The horseradish peroxidase (HRP) conjugated antibody (Wuhan Boster Biological Engineering Co. Wuhan, China) was applied as the secondary antibody, and the positive bands were detected using enhanced chemiluminescent (ECL) plus. Finally, we used Bio-Rad Chemi Doc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) to scan and analyse the bands. The P53, caspase 3, 8, 9 mouse monoclonal antibody and p27 rabbit monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA). The β-actin, LC3 and the Beclin rabbit monoclonal antibody were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

### Sapylin's anti-tumor effect on breast cancer in mice

In our study, we used six weeks old female Balb/c nu/nu mice as the experimental animals, obtained from Shanghai Animal Center (Shanghai, China). The animals were housed in sterilized cages with filtered air, under a 12h light/12 dark cycle, and had free access to sterile water and animal food. All animals were kept under the supervision and guidance of the ethics committee of Liaocheng People's Hospital. All animals were well fed. After approximately one week of acclimatization, the cells ( $5 \times 10^6$  cells, twice a week) were injected subcutaneously (s.c) into the right flanks of each mouse in order to form the tumor model. Tumors were allowed to develop for 3 weeks. The animals were randomly grouped 6 per group and received one of the following treatments: placebo (0.9% normal saline (NS), intraperitoneal (i.p.), Sapylin (20KE/kg body weight per i.p. injection,

once a week). To estimate tumor size, maximum diameters of the tumors were measured twice a week using a slide caliper. Tumor volumes were calculated by assuming a spherical shape and using the following formula:  $\text{volume} = (\text{mean of diameter})^3 \times \pi / 6$ . At the end of the experiment, the animals were euthanized with CO<sub>2</sub> overdose followed by decapitation and then tumor tissues were removed, trimmed of excess collective tissues, and weighed. Inhibition rate =  $[(\text{tumor volume in control group} - \text{experimental group}) / \text{tumor volume in control group}] \times 100\%$ .

### Clinical research

Sixty female patients who underwent modified radical mastectomy were divided into two groups: a) The control group of 30 cases who were injected intraperitoneally (i.p.) in accordance with conventional methods 500mL normal saline (NS) washing. b) The study group of 30 cases who were similarly injected with streptococcus group A subgroup 10KE mixed with 20~30mL NS spray with a multi-point injection in the area around the tumor, pectoralis major fascia and between pectoralis. Each injection point was about 0.5mL. The dose of streptococcus 10KE was injected once.

We noted the postoperative reactions of the two groups as for drainage characteristics: color, amount, character, clarity, wound healing and pain intensity and temperature changes.

### Medical ethics

As part of the experiment, nude mice were used as a model to study biological effects and were treated in accordance with relevant rules.

In the clinical study in both groups after informed consent, patients understood and agreed with the procedure of treatment agreed to join this study. The Liaocheng Hospital Ethics Committee, affiliated to Taishan Medical University, confirmed that the whole study was in accord with medical ethics.

### Statistics

Each experiment of the in vitro cell culture study was repeated at least three times. Data were presented as mean ± standard deviation of multiple independent experiments. We have obtained similar results from two independent in vivo animal experiments. Only one set of the data is shown. Statistical significance is analyzed using the one-way ANOVA and Dunnett's test (SPSS 18.0 software). P value of less than 0.05 was considered statistically significant.

## Results

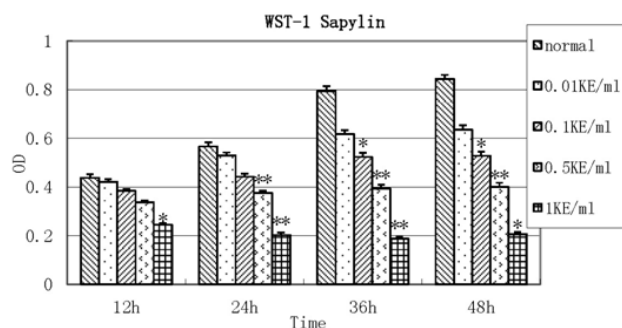
### Strong anticancer effects of Sapylin

*In vitro study.* Compared with the normal control group, the reduction in cell viability caused by Sapylin (10%-20%) was significant. We noticed a dose and time dependent phenomenon (Figure 1). Furthermore, we found that cell apoptosis rate

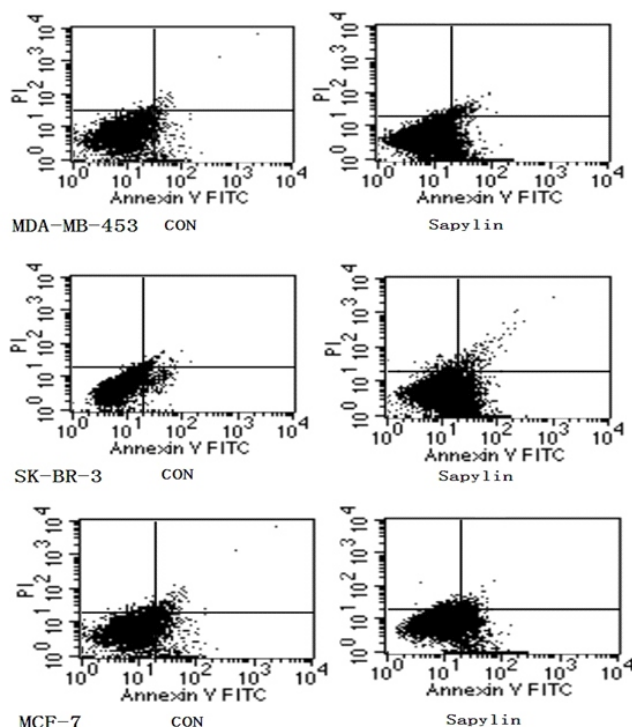


under Sapylin was significantly increased by 10%-15% (Figure 2, Table 1).

*In vivo study.* In the tumor bearing model of mice, as for the antitumor effect of Sapylin, there was no significant difference in body weight changes, among the different treatment groups. The Sapylin group showed a markedly growth inhibition of the tumor of 12%-20% (Figure 3, Table 2).



**Figure 1.** Sapylin effects on breast cancer cells' proliferation (in vitro cells). \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 2.** The effect of Sapylin on breast cancer cells' apoptosis rate.



**Figure 3.** The anti-tumor effect of Sapylin in mice.

**Table 1.** The effect of Sapylin on breast cancer cells' apoptosis rate (in vitro cells)

Item	Control (%)	Sapylin (%)	Inhibition rate (%)
MCF-7	97.4	83.35	14.42
MDA-MB-453	96.11	77.35	19.52
SK-BR-3	90.4	78.71	12.93

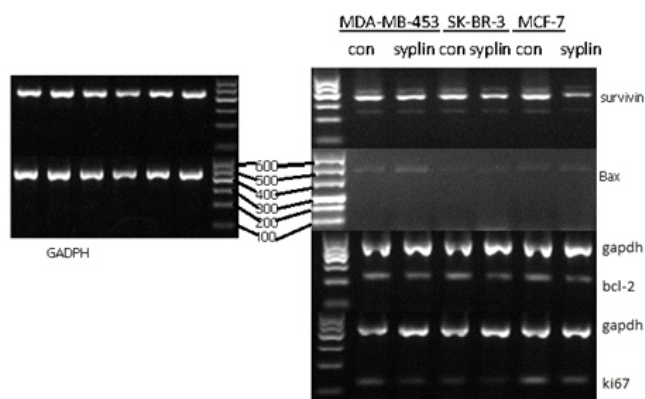
**Table 2.** The anti-tumor efficacy of Sapylin in mice

Item	Tumor volume (cm <sup>3</sup> )	Inhibition rate (%)
Control 1	2.145±0.241	
MCF-7	0.952±0.15	56.87 **
Control 2	1.88±0.133	
MDA-MB-453	0.784±0.109	60.21 **
Control 3	1.45±0.128	
SK-BR-3	0.619±0.064	57.31 **

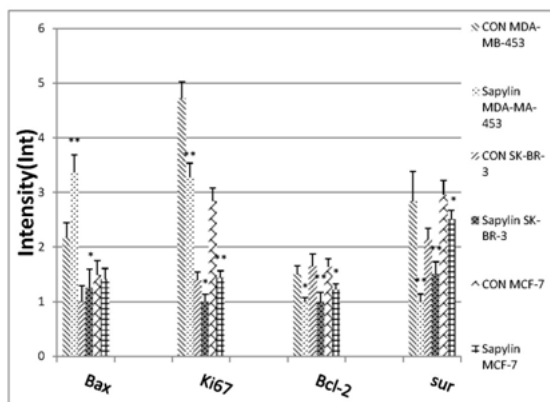
\*\* $P < 0.01$

### Mechanism of the effect of Sapylin induced cell death

Because the effect of Sapylin in inducing cell death was rather strong, we decided to study the underlying mechanisms of this effect: Modulation of Sapylin induced survivin, Bcl-2, Bax and Ki67 mRNA expression. We found that Sapylin modulated the cell death gene. The level of survivin, Bcl-2 and Ki67 mRNA expression were reduced, while the Bax mRNA expression was increased (Figure 4).



**Figure 4A.** Survivin, Bcl-2, Bax and Ki67 mRNA level after Sapylin intervention (in vitro cells).



**Figure 4B.** Analysis of survivin, Bcl-2, Bax and Ki67 mRNA level after Sapylin intervention (in vitro cells). \*P<0.05, \*\*P<0.01.

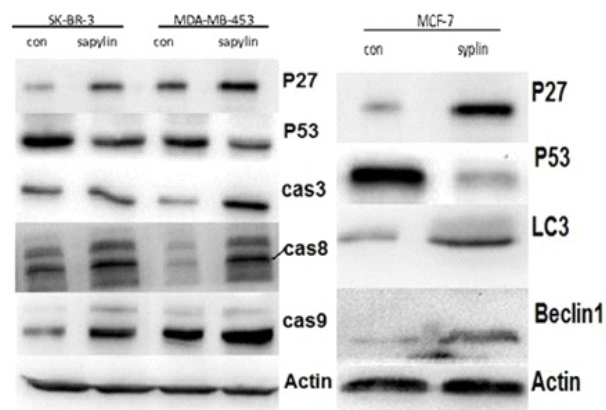
*Detection of P27, P53, caspase, LC3 and Beclin by Western blot.* Apoptosis-related protein P53 expression was reduced by 10%-12%, and P27 caspase expression was significantly increased by 22%-41% in MDA-MB-453 and by 12%-23% in the SK-BR-3 cells. Autophagy related protein LC3 and Beclin1 were significantly increased by 80%-234%. The experimental groups were statistically different from the control group (P<0.05, Figure 5).

#### Group A streptococcus injection-related effects on postoperative adverse circumstances

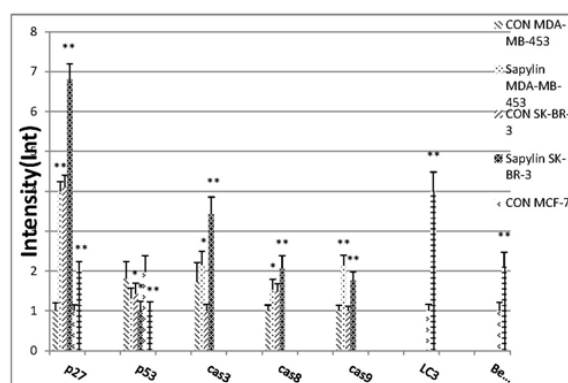
After 5 days of clinical observation the drainage was reduced in a daily process and was maintained at a lower level than in the control group (Table 3 and Table 4, P<0.05).

## Discussion

Sapilyn as an anti-tumor immune regulator is widely used to treat malignant pleural effusion. Compared with some other intracavity administered chemotherapeutic drugs and im-



**Figure 5A.** Related apoptosis and autophagy protein expression after Sapylin intervention in three kinds of breast cancer cells (in vitro cells).



**Figure 5B.** The analysis of apoptosis and autophagy protein (in vitro cells). \*P<0.05, \*\*P<0.01.

mune-stimulants, Sapylin is more effective [21, 22]. It has been reported that Sapylin can effectively prevent bladder cancer recurrence [23], and is an effective adjuvant chemotherapeutic drug for cancers of the lung, liver, stomach, lymphoma and other solid tumors [24, 25].

Our results indicate that Sapylin induces breast cancer cells death process. The specific mechanism may include: the direct effect of survivin gene on caspase, the formation of CDK/CDK-Cyrlin complex to block apoptosis and autophagy

**Table 3.** Group A Streptococcus related to surgery and adverse reactions in female patients.

	Day	Wound	Pain (Analgesics amount)	Temperature (°C)	Adverse reactions
Postoperative	1	well	Light (Analgesia)	37.4±0.52	No
	2	well	Light (Analgesia)	37.4±0.47	No
	3	well	Light	37.1±0.43	No
	4	well	Light	37.0±0.25	No
	5	well	Light	36.9±0.29	No

**Table 4.** Group A *Streptococcus* related to surgery and adverse reactions in female patients.

Item	Time	Observer group (mL)	Control group (mL)
Drainage and traits	day 1	64±36 bloody	113±44 bloody
	day 2	64±31 light bloody	164±51 bloody
	day 3	67±43 light bloody	127±42 light bloody
	day 4	54±27 yellowish	93±35 light bloody
	day 5	51±34 yellowish	70±28 light bloody

and to act on the cell cycle to decrease the proportion of G0/G1 phase and increase S phase, so that cells remain in S phase, the increase of Bax mRNA reduces Bcl-2 inhibition of cytochrome C, which indirectly increases the activation of caspase3 and reduces Ki67 to affect cell proliferation. Eventually, key factors of cell death like caspase, LC3 and Beclin increased. All is a complex process, with the participation of multi-genes which ultimately changes the cell cycle, inhibits cell proliferation, and promotes apoptosis and autophagy.

The inhibition of programmed cell death is closely related to abnormal cell proliferation and tumor development. Our results showed that Sapylin can reduce the cell proliferation, induce cells early death and reverse tumor cells death programme properties. This is a complex, multi-gene, pathway involved process, but cell death process is multi-system and multiple factors are involved in complex processes. Finally, as an immune regulator, Sapylin shows an obviously direct anti-tumor effect, and the specific mechanism of Sapylin-induced breast cancer cell death needs further experimental studies.

*In conclusion*, our results indicate that Sapylin displays a strong direct anti-cancer effect on breast cancer cells. The specific mechanism of Sapylin-induced breast cancer cells death needs further experimental studies.

*Authors' contribution:* Guimei Zhao directed the design of the study and performed the statistical analysis. Tingting Ge carried out the animal studies, participated in and drafted the manuscript. Xiangju Yang carried out the clinical studies. All authors read and approved the final manuscript.

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*The authors declare that they have no conflicts of interest.*

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Raphael's fresco. The School of Athens (1510-11). Painted in response to the remarkable ferment in Renaissance, Italy. Stanza della Segnatura, Vatican Palace, Rome. In the middle, Plato points upwards and Aristotle points at level, horizontally.