ORIGINAL RESEARCH

Core Target and Key Signal Pathway of Xiaoluo in the Treatment of Thyroid Cancer

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ABSTRACT

Context • At present, hormone therapy and surgery are the main treatments for thyroid cancer, and they have a quick effect but a high recurrence rate. Also, the side effects are significant. it's extremely urgent to explore treatments that can take into account both therapeutic benefits and side effects.

Objective • The study intended to explore whether Xiaoluo has an inhibitory effect on the proliferation of thyroid-cancer cells in vitro and to examine the core target and key signaling pathway of Xiaoluo in the treatment of thyroid cancer, using the thyroid-cancer cell line SW579.

Design • The research team performed an in-vitro study.

Setting • The study took place at the College of Pharmacy at Harbin University of Commerce in Harbin, China.

Outcome Measures • The research team used a Western blot analysis to detect the expression of apoptosis proteins—B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Caspase-3—and the activity related to the signaling pathways phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin 1 (mTORC1). The team measured optical densities and inhibition rates for the 1, 2, 5, 10, and 15 mg/mL Xiaokuo groups and for a negative control group. The research team measured apoptosis, expression of Bcl-2, Bax, and Caspase-3, and expression of P13K, AKT, and mTor for the 10 μmol/L LY294002, 10 mg/mL Xiaoluo, 100 ng/mL IGF-1, and 100 ng/mL IGF-1+10 mg/mL Xiaoluo groups and for the blank control group.

Results • The inhibition of SW579 cell proliferation increased with each increase in the Xiaoluo concentration from 1-15 mg/mL, and the inhibition rate reached 49.63% when the concentration was 15 mg/ml. The Xiaoluo group's late and total apoptosis rates were significantly higher (both $P < .01$) than those of the blank control group. The Xiaoluo group's expression of the Bcl-2 protein was significantly lower ($P < .05$), and its expressions of Bax and Caspase-3 were significantly higher (both $P < .01$) than those of the blank control group. The Xiaoluo group's expressions of P-PI3K, P-Akt, and P-MTOR were significantly lower than those of the blank group (all $P < .01$). These findings were comparable to those that occurred with use of the PI3K/ AKT/mTORC1 signaling pathway inhibitor LY294002.

Conclusions • Xiaoluo exerts its antithyroid-cancer effects through the induction of apoptosis in thyroid cancer cells through the inhibition of the PI3K/AKT/mTORC1 signaling pathway. Xiaoluo may serve as a potential therapeutic agent for the treatment of thyroid cancer. (*Altern Ther Health Med.* 2023;29(5):400-409).

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Thyroid cancer is a common malignant tumor of the endocrine system that a follicular epithelial lesion of the thyroid mainly causes. At present, hormone therapy and surgery are the main treatments for thyroid cancer, and they have a quick effect but a high recurrence rate.[1,1] Also, the side effects are significant.

Recurrence happens for up to 30% of thyroid-cancer patients, and the recurrence rate may vary depending on the type and stage of thyroid cancer.[2] This recurrence can cause adverse reactions such as swelling or lumps in the neck, difficulty swallowing or breathing, hoarseness or voice changes, and coughing up of blood.[2,3] Therefore, it's extremely urgent to explore treatments that can take into account both therapeutic benefits and side effects.

Xiaoluo

Xiaoluo is a classic, Traditional Chinese Medicine (TCM) prescription for treating thyroid diseases, with

various medicinal values. Xiaoluo is indeed a classic Traditional Chinese Medicine (TCM) prescription that has been used to treat various thyroid diseases. The prescription is made up of a combination of herbs that have been carefully selected for their medicinal properties and is believed to have a range of therapeutic effects. In terms of its medicinal values, Xiaoluo has been shown to have anti-inflammatory, anti-oxidant, and immune-modulating properties, which are thought to help reduce the symptoms of thyroid disease and improve overall thyroid function. Additionally, some studies have suggested that Xiaoluo may also have a positive impact on the body's hormonal balance, which could further contribute to its therapeutic effects. According to the basic theory of TCM, practitioners select a combination of ingredients according to the condition of returning taste to the meridian and the principle of king, minister, and assistant. In TCM, the principle of "king, minister, and assistant" refers to the concept of using a combination of herbs that work together in a hierarchy to achieve the desired therapeutic effect. The "king" herb is the primary ingredient that targets the main issue or pattern of disharmony. The "minister" herbs support and enhance the effect of the king herb, while the "assistant" herbs help to balance the formula and address any secondary symptoms or imbalances. Cheng Guopeng, in his ancient book, wrote about the Xiaoluo pill, which TCM practitioners originally prescribed for curing tuberculosis and dispersing nodules.[4]

Xiaoluo includes three ingredients: Fritillaria, Scrophularia radix, and Ostrea. Fritillaria is the principal herb in the formula, the king, and has the effect of dispelling abscesses and promoting blood circulation as well as clearing heat and resolving phlegm. It can help the body's vital energy to flow freely and prevent external invasion.

Scrophularia radix is the minister herb, which nourishes the liver and clears the lungs, nourishes yin and cools the blood, and enables the smooth flow of qi and blood. Ostrea is the assistant herb, which has the effect of dispelling wind and evil throughout the whole body, regulating the liver, and consolidating the yin to stop perspiration and astringency.

Fritillaria and Scrophularia Radix supplement each other's effects, and the qi is strong and the surface is solid. They can eliminate the pathogenic factors of wind and heat. The addition of Ostrea can indirectly nourish yin and reduce liver fire, thus achieving the effect of soothing liver qi.

Fritillaria and Scrophularia Radix are plant-based ingredients, while Ostrea is a seafood ingredient. TCM practitioners obtain Fritillaria from the bulb of the Fritillaria genus, and Scrophularia Radix is the root of Scrofularia ningpoensis. The Latin binomials for these plants are Fritillaria cirrhosa and Scrofularia ningpoensis, respectively.

The three ingredients aren't necessarily present in equal parts in a prescription, and TCM practitioners typically prepare Xiaoluo in a ratio of 2:3:10 for Fritillaria, Scrophularia Radix, and Ostrea, respectively. Xiaoluo has the clinical effects of clearing heat and removing phlegm, and softening and hardening nodules.[5,6]

Xiaoluo combines the characteristics of a TCM compound, with less flavor and a precise prescription, with that of a treatment for thyroid cancer that has multiple links, multiple targets, and multiple processes, and TCM practitioners have rapidly favored it.[7,8] It can enhance synergism, reduce toxicity, and comprehensively treat diseases.

Treatment Targets

Phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin 1 (mTORC1) pathway. The PI3K/AKT/mTORC1 pathway is the main, intracellular regulatory pathway, a signal transduction pathway, and is involved in regulating cell differentiation and proliferation, apoptosis, metabolism, and response to extracellular factors.[9,10] The PI3K/AKT signaling pathway can regulate mTORC1; activated AKT can activate mTORC1 directly or indirectly; and the phosphorylation of mTORC1 can inhibit cell proliferation and autophagy.[11,12]

Paying attention to the apoptosis process of cancer cells has become an important step in the treatment of cancer. Chen et al and Sheu et al found that Xiaoluo can inhibit the proliferation of the human acute myeloid leukemia cells KG-1A and Alzheimer's disease PC12 cells by promoting late apoptosis of cancer cells.[13,14]

Saji and Ringel and Zhang et al found that the PI3K/AKT/ mTORC1 pathway is closely related to the occurrence of thyroid cancer and that the pathway includes a variety of oncogene-encoded proteins.[15,16] Zhang et al and Vernieri et al found that those oncogene-encoded proteins and participate in the regulation of cell proliferation, apoptosis, and migration.[17,18]

B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and Caspase-3. The Bcl-2-family proteins are crucial to controlling apoptosis, and a change in Bcl-2/Bax can be a signal of subsequent apoptosis. An imbalanced expression of Bcl-2 and Bax is due to the combination of Bcl-2 and Bax into dimers, which can damage an intact mitochondrial inner membrane and promote the expression of Caspase-3, which can lead to the obstruction of apoptosis and the proliferation of cancer cells.[19]

Current Study

The research team's previous in-vivo experiments have confirmed that the PI3K/AKT/mTORC1 signaling pathway is overactivated in thyroid carcinoma.[20]

The current study intended to explore whether Xiaoluo has an inhibitory effect on the proliferation of thyroid-cancer cells in vitro and to examine the core target and key signaling pathway of Xiaoluo in the treatment of thyroid cancer, using the thyroid-cancer cell line SW579.

METHODS

Procedures

The research team performed an in-vitro study, which took place at the College of Pharmacy at Harbin University of Commerce in Harbin, China.

Materials. The research team purchased: (1) fetal bovine serum (FBS) from Sijiqing Biological (Nanjing, China); (2) Xiaoluo freeze-dried powder from Beijing Tongrentang Pharmaceutical (Beijing, China); (3) dimethyl sulfoxide (DMSO) from Meilun Biological; (4) LY294002 from Cell Signaling Technology (Danvers, MA, USA); (5) IGF-1 from R&D Systems (Minneapolis, MN, USA); (6) phosphate buffered saline (PBS) from Shandong LanDian Biological Technology Co., LTD. (Shouguang, Weifang, China); (7) 3-(4,5)-dimethylthiazol (-z-y1)-3,5-di- phenyltetrazolium bromide (MTT) Powder from Blue Sky Biological (Tianjin, China); (8) the human thyroid cancer SW579 cell line, in a freezer tube, from the School of Pharmacy at Harbin University of Commerce (Harbin, China); (9) Dulbecco's Modified Eagle Medium (DMEM) from Meilun Biological (Dalian, China), (10) trypsin from Biyuntian Biological (Shanghai, China); (11) DNA Binding Buffer from Thermo Fisher Scientific (Waltham, MA, USA); (12) an Annexin V/PI apoptosis kit from Nanjing KGI Biotechnology (Nanjing, China); (13) Xiaoluo pills (Beijing Tongrentang Co.,ltd., Beijing, China; (14) radio-immunoprecipitation assay (RIPA) from Thermo Fisher Scientific (Waltham, MA, USA); (15) standard bovine serum albumin (BSA) from Thermo Fisher Scientific (Waltham, MA, USA); (16) bicinchoninic acid (BCA) detection kit from Thermo Fisher Scientific (Waltham, MA, USA); (17) sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) from Thermo Fisher Scientific (Waltham, MA, USA); (18) 10-hydroxycamptothecin (HCPT) from Sigma-Aldrich (St. Louis, MO, USA); (19) polyvinylidene fluoride (PVDF); (20) Lichun red from Beyotime (Shanghai, China); (21) tris-buffered saline with 0.1% Tween 20 detergent (TBST) from Solarbio (Beijing, China); (22) electrochemiluminescence (ECL) kit from Tanon (Shanghai, China);

Material preparation. For the FBS, the research team: (1) placed the FBS serum in a water bath at 56°C and heated it for 30 min, fully shaking it during heating to avoid precipitation; and (2) after inactivation, filtered the serum and stored it in a refrigerator at -20°C.

For the Xiaoluo mother liquid, the team dissolved the freeze-dried powder, the alcohol extract of Xiaoluo, with DMSO to prepare a concentration of 20 mg/mL.

For the LY294002 solution, which is a potent and specific inhibitor of phosphatidylinositol 3-kinase (PI3K), the research team weighed the LY294002 and dissolved it with DMSO to prepare a concentration of 10 μmol/L.

For the insulin-like growth factor 1(IGF-1) solution, which is an activator PI3/AKT/mTORC1 pathway, the team dissolved 0.76 mg of IGF-1 in 100 mL of PBS to obtain a concentration of 100 ng/mL.

For the MTT solution, the team: (1) weighed 5 mg of MTT powder into an Eppendorf (EP) tube from Eppendorf (Hamburg, Germany) to shut out light, (2) added one mL of PBS to mix it thoroughly, and (3) then stored the solution in a dry location without light, for later use.

SW579 cancer-cell line. The research team: (1) placed the purchased SW579-cell freezer tube in a water bath preheated to 37°C for quick shaking to dissolve it; (2) after dissolving the solution, quickly transferred the cells in the freezer tube to a 4-ml EP tube and centrifuged it at 1200 rpm for 5 min; and (3) after discarding the supernatant, used 1-2 ml of DMEM complete medium to suspend cell precipitation, transferred it to a cell-culture bottle, and placed it in an incubator (Thermo Fisher Scientific ,Waltham, MA, USA).

Cell proliferation. The research team: (1) after 4-5 days of SW579 cancer-cell culture, removed the cell bottle from the incubator, placed it under an inverted microscope (Olympus, Tokyo, Japan) to observe the cell growth, and removed the cells for counting at the logarithmic growth stage; (2) poured out the discarded DMEM culture medium, suspended the cells in a cell flask, and washed them with PBS 2-3 times; (3) added 1-2 ml of trypsin, tapped the cells, and observed the free state of most of the cells under the microscope (Olympus, Tokyo, Japan); (4) sucked out the cell suspension and placed it in an EP tube; (5) after centrifugation at 1000 rpm for 5 min, discarded the supernatant, suspended the cells again with fresh culture medium, moved them to the cell flask, and placed it in the incubator (Shanghai Jinghong Experimental Equipment Co., Ltd. Shanghai,China).

Proliferation inhibition. Using the MTT assay, the research team: (1) took the logarithmic-growth cell suspension of the thyroid cancer SW579 cells and inoculated them into a 96-well plates with 5×103 cells per well, (2) established cultures of 1, 2, 5, 10, and 15 mg/mL of Xiaokuo to create the five Xiaokuo intervention groups and a culture of 100 μL of constant-volume DMEM medium to create the negative control group and placed the cultures in a cell incubator; (4) set six parallel holes in each group and cultured the cells in a cell incubator; (5) after 48 h, discarded the supernatant and added 100 μL of MTT solution; (6) after 4 h of culture in the incubator, discarded the supernatant; (7) added 150 μL of DMSO solution to each well and measured the absorbance optical density (OD) at 490 nm; and (8) calculated the inhibition rate and half inhibitory concentration (IC50) of each group. Cell inhibition rate % = (1- average OD value of the administration group/average OD value of the blank control group) × 100%

Cell apoptosis rate. Using flow cytometry, the research team: (1) using the SW579, logarithmic-growth cell suspension, adjusted the trypsin-digested cell concentration to 5×105 cells /mL; (2) placed 1 mL of the cells in a high-temperature pulp centrifuge at 1000 rpm and centrifuged them at 4°C for 10 min, (3) discarded the supernatant; (4) after adding one ml of cold PBS solution, gently shook the cells to suspend them; (5) at 1000 rpm, centrifuged them at 4°C for 10 min and discarded the supernatant, repeating the step 3-4 times; (6) added the cell suspension to 200 μL of DNA Binding Buffer: (7) added 10 μL of Annexin v-fitc and 5 μL of PI and mixed gently, with the suspension reacting at room temperature for 15 min; (8) added 300 μL of DNA Binding Buffer and carried out the detection quickly within one h.

Apoptosis-related protein-expression activities. Using the Western blot, the research team: (1) collected SW579

cancer cells at the logarithmic-growth stage; and (2) 24 h later, replaced the DMEM culture medium. The team divided the cancer cells into five bottles to create groups: (1) 10 μmol/L of LY294002, to create the 10 μmol/L LY294002 group; (2) 10 mg/mL of Xiaoluo pills, to create the 10 mg/mL Xiaoluo group; (3) 100 ng/mL of IGF-1, to create the 100 ng/mL IGF-1 group, (4) 100 ng/mL of IGF-1+10 mg/mL Xiaoluo, to create the 100 ng/mL IGF-1+10 mg/mL Xiaoluo group, and (5) 100 ng/mL of DMEM culture medium to create the blank control group.

The team: (1) incubated all bottles at 37°C in a CO_2-free incubator (Shanghai Jinghong Experimental Equipment Co., Ltd. Shanghai,China); (2) after 48 h, collected the cell suspensions and centrifuged them at 1000 r/min for 10 min; (3) discarded the supernatants, added 100 μL of total protein lysate (RIPA: PMSF= 100:1), and lysed the cultures in an ice bath for 30 min; (4) scraped the cells with a cell scraper and centrifuged them at 12 000 r/min at 4°C for 15 min, and (8) removed the supernatant and stored it at -80°C.

Determination of protein content. The research team: (1) diluted the standard bovine serum albumin (BSA) with PBS, according to the instructions of the BCA detection kit, to concentrations of 0.00, 0.25, 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 mg/mL; (2) added 20 μL of each concentration to separate wells in the 96-well plate and 200 μL of BCA working solution (BCA reagent: Cu reagent = 50:1) in the same manner and fully mixed them; (3) set three parallel samples, measured the absorbance value at a 562-nm wavelength after incubation at 37°C for 30 min, and drew a standard curve; (4) measured 10 μL of each protein sample and diluted them to 100 μL using PBS, measured 20 μL of diluted protein sample, and measured 200 μL of BCA working solution and added them all to all wells in the 96-well plate; (5) set three parallel samples again, incubated them at 37°C for 30 min, measured the absorbance value at a 562 nm wavelength, and calculated the protein content using the linear regression equation.

Sample preparation. The research team: (1) calculated the loading volume of 25 μg of protein according to the content of the protein sample; (2) mixed the protein sample solution with SDS loading buffer at a ratio of 4:1 and supplemented the volume with lysate; (3) put the solutions in a metal bath for denaturation at 100°C for 5 min, using centrifugation before and after the denaturation, and cooling the solutions to room temperature for later use.

SDS-PAGE. The research team: (1) prepared the materials and instruments and assembled a clean glass plate; (2) added double-steam water to the gap in the glass plate to check whether leakage existed; (3) after confirming that no problem existed, drained the double-steam water; (4) prepared separation glue and slowly added it to the gap between the glass plates immediately after its preparation; (5) added a small amount of isopropyl alcohol to prevent the gel from shrinking and allowed it to stand at room temperature; (6) after the separation glue completely solidified, poured off the isopropyl alcohol, rinsed the plate gently with double-steam water for 3-5 times until it was tasteless, and drained the remaining moisture with filter paper; (7) after making a 5% concentrated glue, inserted it into a 1.5-mm ten-tooth comb and let it stand at room temperature; (8) after the separation glue completely solidified, covered it with a wet towel and let it stand overnight at 4°C; and (9) assembled the electrophoresis rack, put it into the vertical electrophoresis tank, added the electrophoresis solution over the short plate of the glass plate, and gently pulled the comb out vertically upward.

The team: (1) added 10 μL of prestained protein marker to the blank group, which used HCPT, and to the 10 μmol/L LY294002, 10 mg/mL Xiaoluo, 100 ng/mL IGF-1, and IGF-1+10 mg/mL Xiaoluo groups and then added 1× sample loading buffer to the two edges; (2) after sample loading, connected the electrophoresis apparatus, set a constant pressure of 100 V, and adjusted the voltage to 140 V when the bromophenol blue reached the interface of the separating glue and concentrated glue; and (3) stopped the electrophoresis when it reached the bottom of the separating glue.

PVDF membrane pretreatment. The research team: (1) at 10 min before the end of electrophoresis, soaked the PVDF membrane in methanol for 2 min and then put it into an electroconversion solution for preparation; (2) placed filter paper, sponge, and splint in the electrotransfer solution; (3) put sponge; three layers of filter paper, PVDF film, with the upper right corner cut to distinguish between positive and negative; cut gel, and three layers of filter paper and sponge, with each layer placement using a glass rod to fully remove the bubbles; (4) carefully closed the splint, placed it vertically into the electric transfer tank, filled the tank with electric transfer liquid, and placed the whole device in a low-temperature environment; (5) connected the tank with the electrophoresis apparatus and used 100 V of power for two hours; (6) after the film transferred, washed the membranes with methanol for 2 min and dyed them with Lichun red for 2 min in a 37°C shaker; (7) according to the protein molecular-weight standard, took the PVDF membrane of the target fragment, sealed it with 5% skim milk powder in a 37°C shaker for one h; (8) after the one h, removed the PVDF membrane and cleaned it using a TBST shaker for 5 min; (9) placed the membrane in a 1:1000 primary antibody solution, diluted with sealing solution, and shook the membrane overnight at 4°C; (10) removed the film and put it in TBST three times, for 10 min each; (11) placed the membrane in a 1:5000 primary antibody solution, diluted with blocking solution, and incubated it in a TBST shaker at room temperature for 2 h; (12) after removing the film, cleaned the TBST three times, for 10 min each; and (13) removed the membrane, stained it with an ECL kit, photographed it using a protein gel imaging system (Bio-Rad, Hercules, CA, USA), and recorded and saved the results.

Outcome measures. The research team: (1) determined the dosage of Xiaoluo required to inhibit the growth of the thyroid-cancer cell line SW579, using the MTT assay; (2) detected the apoptosis cycle of the thyroid-cancer cells,

using flow cytometry; (3) reversed the inhibition effects of Xiaoluo on the PI3/AKT/mTORC1 pathway by adding the pathway activator IGF-1, to study the pro-apoptotic effects of Xiaoluo on the thyroid-cancer cell line SW579; (4) used the Western blot to explore the regulatory effects of Xiaoluo on the PI3K/AKT/mTORC1 pathway, and on the Bcl-2, Bax, and Caspase-3 proteins that are closely related to apoptosis, to study the mechanism of the effects of Xiaoluo on the thyroid-cancer SW579 cells based on the PI3K/AKT/mTORC1 pathway; and (5) used the LY294002 solution for a comparison of Xiaoluo's inhibition of the PI3K/AKT/mTORC1 signaling pathway.

Outcome Measures

Optical density and inhibition rate. The research team measured the optical densities and inhibition rates for the 1, 2, 5, 10, and 15 mg/mL Xiaokuo groups and for the negative control group. The results of these measurements can provide valuable insights into the effectiveness of the treatment and the dosage required to achieve a desired effect.

Optical density measurements reflect the amount of light absorbed by a solution, which can be used to determine the concentration of a substance. In our study, we measured the optical densities of the Xiaokuo groups and the negative control group at different concentrations, ranging from 1 to 15 mg/mL. These measurements can help to determine the optimal dosage of Xiaokuo required to achieve a therapeutic effect.

Inhibition rate measurements reflect the percentage of inhibition of a particular process or reaction. In our study, we measured the inhibition rates of the Xiaokuo groups and the negative control group, which can help to determine the effectiveness of the treatment in inhibiting tumor growth or other relevant biological processes.

The possible results of these measurements would be different depending on the specific study design and objectives. However, in general, higher optical densities and inhibition rates in the Xiaokuo groups compared to the negative control group would suggest that Xiaokuo is effective in inhibiting tumor growth or other biological processes. The optimal dosage of Xiaokuo required to achieve this effect can also be inferred from the optical density measurements.

Cell Apoptosis. The research team measured the apoptosis for the 10 μmol/L LY294002, 10 mg/mL Xiaoluo, 100 ng/mL IGF-1, and 100 ng/mL IGF-1+10 mg/mL Xiaoluo groups and for the blank control group. The results of this experiment could provide valuable insights into the effects of LY294002, Xiaoluo, and IGF-1 on cell apoptosis in the context of thyroid cancer.

Apoptosis, also known as programmed cell death, is a critical process that regulates cell growth and development. In the context of cancer, the ability to induce apoptosis is often impaired, leading to uncontrolled cell growth and proliferation.

The measurement of apoptosis in different treatment groups, including the blank control, LY294002, Xiaoluo, IGF-1, and IGF-1+Xiaoluo groups, can provide information about the effects of these treatments on cell death. For example, if the LY294002 treatment group shows an increase in apoptosis compared to the blank control group, this could indicate that LY294002 is promoting cell death and could be a potential therapeutic strategy for thyroid cancer.

On the other hand, if the Xiaoluo and/or IGF-1 treatment groups show a decrease in apoptosis compared to the blank control, this could indicate that these treatments are inhibiting apoptosis and may be contributing to the progression of thyroid cancer.

Overall, the measurement of apoptosis in different treatment groups is a crucial step in understanding the effects of potential therapeutic agents on cancer cells.

Protein expression of Bcl-2, Bax, and Caspase-3. The research team measured the expression of Bcl-2, Bax, and Caspase-3 for the 10 μmol/L LY294002, 10 mg/mL Xiaoluo, 100 ng/mL IGF-1, and 100 ng/mL IGF-1+10 mg/mL Xiaoluo groups and for the blank control group. The measurement of protein expression in these groups is crucial to understanding the potential effects of different treatments on the regulation of cell proliferation, apoptosis, and migration.

Based on the information provided, it is possible that the results of the protein expression analysis may show varying levels of Bcl-2, Bax, and Caspase-3 expression in each of the treatment groups compared to the blank control group.

For example, the LY294002 group, which is an inhibitor of the PI3K/AKT pathway, may result in decreased expression of Bcl-2 and increased expression of Bax and Caspase-3, indicating an increase in apoptosis.

On the other hand, the Xiaoluo group, which is a traditional Chinese medicine, may result in decreased expression of Bax and Caspase-3 and increased expression of Bcl-2, indicating a decrease in apoptosis.

The IGF-1 and IGF-1+Xiaoluo groups may result in increased expression of all three proteins, indicating increased cell proliferation and potential resistance to apoptosis.

However, it is important to note that these are only hypothetical scenarios, and the actual results may vary depending on the specific experimental conditions and sample characteristics. Overall, analyzing protein expression can provide valuable insights into the potential mechanisms of different treatments on thyroid cancer and help inform future research and treatment strategies.

Protein expression of PI3K, AKT, and mTOR. The research team measured the expression of P13K, AKT, and mTor for the 10 μmol/L LY294002, 10 mg/mL Xiaoluo, 100 ng/mL IGF-1, and 100 ng/mL IGF-1+10 mg/mL Xiaoluo groups and for the blank control group. The measurement of protein expression is crucial to understanding the role of these proteins in cancer development and progression.

The possible results of this experiment could include variations in the expression levels of PI3K, AKT, and mTOR between the different treatment groups and the control group. A decrease in protein expression in the LY294002 treatment group may indicate inhibition of the PI3K/AKT/

Table 1. Optical Densities and Inhibition Rates: Effects of Xiaoluo Extract on Proliferation of Thyroid-cancer SW579 cells (N = 30)

Optical Density (OD)					
Negative Control Group n = 5 Mean ± SD	Xiaoluo Dosage Groups				
	1 mg/mL n = 5 Mean ± SD	2 mg/mL n = 5 Mean	5 mg/mL n= 5 Mean ± SD	10 mg/mL n = 5 Mean ± SD	15 mg/mL n = 5 Mean ± SD
0.834 ± 0.045	0.657 ± 0.072	0.545 ± 0.039	0.496 ± 0.024	0.448 ± 0.034	0.409 ± 0.041
Comparisons Between Groups					
	Control and 1 mg/mL Groups	Control and 2 mg/mL Groups	Control and 5 mg/mL Groups	Control and 10 mg/mL Groups	Control and 15 mg/mL Groups
t	4.398	9.702	12.789	13.526	14.016
P value	<.01[a]	<.01[a]	<.01[a]	<.01[a]	<.01[a]
Inhibition Rate (IR) %					
Negative Control Group n = 5 Mean ± SD	Xiaoluo Dosage Groups				
	1 mg/mL n = 5 Mean ± SD	2 mg/mL n = 5 Mean	5 mg/mL n = 5 Mean ± SD	10 mg/mL n = 5 Mean ± SD	15 mg/mL n = 5 Mean ± SD
0.00 ± 0.00	22.33 ± 1.26	33.69 ± 1.18	41.92 ± 1.84	45.36 ± 1.37	49.63 ± 1.29
Comparisons Between Groups					
	Control and 1 mg/mL Groups	Control and 2 mg/mL Groups	Control and 5 mg/mL Groups	Control and 10 mg/mL Groups	Control and 15 mg/mL Groups
P value	<.01[a]	<.01[a]	<.01[a]	<.01[a]	<.01[a]

[a]$P < .01$, indicating that all the Xiaoluo groups' optical densities were significantly lower and their inhibition rates were significantly higher, in a concentration-dependent manner, than those of the negative control group

mTORC1 pathway, whereas an increase in expression in the IGF-1 treatment group may suggest activation of this pathway. The combined treatment group of IGF-1 and Xiaoluo may show a different pattern of protein expression compared to the individual treatment groups.

The interpretation of these results is critical for understanding the molecular mechanisms of cancer development and the potential for targeted therapies. Further research is necessary to validate these findings and to explore the therapeutic potential of these treatments.

Statistical Analysis

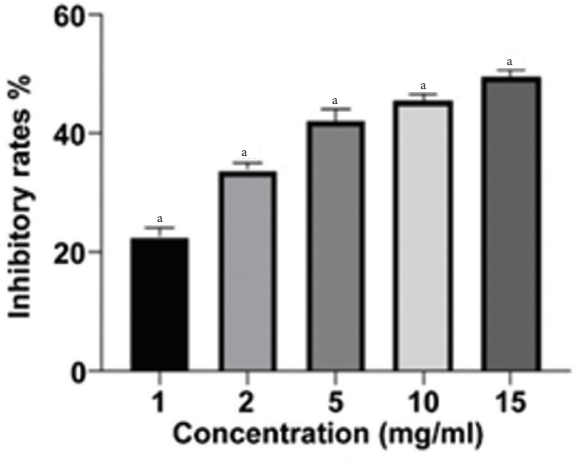
The research team analyzed the data using the Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). The team expressed the measurement data as means ± standard deviations (SDs) and used analysis of variance (ANOVA) to analyze differences between groups. $P < .05$ indicated a significant difference.

RESULTS

Xiaoluo's Inhibitory Effects

As Table 1 shows, the negative control group's optical density for the SW579 cells was 0.834 ± 0.045 and its inhibition rate was 0.00 ± 0.00%. The higher the dosage of the Xiaoluo extract, the higher the inhibition rate was (Figure 1). The inhibition of SW579 cell proliferation increased with each increase in the Xiaoluo concentration from 1-15 mg/mL, and the inhibition rate reached 49.63% when the concentration was 15 mg/ml.

Figure 1. Effects of Different Dosages of Xiaoluo on the Inhibition of SW579-Cell Proliferation. The negative control group didn't inhibit cell proliferation (0%).



[a]$P < .01$, indicating that all of the Xiaoluo dosage groups' inhibition rates were significantly higher than those of the control group, at 0.00 ± 0.00%, in a concentration-dependent manner

All of the Xiaoluo groups' optical densities were significantly lower and their inhibition rates were significantly higher, in a concentration-dependent manner, than those of the control group (all $P < .01$).

Table 2. Effects of Xiaoluo Extract and its Active Components on Late and Total Apoptosis Rates of Thyroid-cancer SW579 Cells, as Detected Using Flow Cytometry (n = 5)

Late Apoptosis Rate %				
Control Group n = 5 Mean ± SD	**LY294002 Group n = 5 Mean ± SD**	**Xiaoluo Group n = 5 Mean ± SD**	**Igf-1 Group n = 5 Mean ± SD**	**Igf-1 + Xiaoluo Group n = 5 Mean ± SD**
5.97 ± 0.21	21.73 ± 0.49	11.23 ± 0.25	2.29 ± 0.17	5.42 ± 0.23
Comparisons Between Groups				
	Control and LY294002 Groups	**Control and Xiaoluo Groups**	**Control and Igf-1 Groups**	**Control and Igf-1 + Xiaoluo Groups**
t	66.104	36.024	30.456	3.949
P value	<.01[a]	<.01[a]	<.01[a]	<.01[a]
Total Apoptosis Rate %				
Control Group n = 5 Mean ± SD	**LY294002 Group n = 5 Mean ± SD**	**Xiaoluo Group n = 5 Mean ± SD**	**Igf-1 Group n = 5 Mean ± SD**	**Igf-1 + Xiaoluo Group n = 5 Mean ± SD**
6.92 ± 0.37	23.10 ± 0.35	15.24 ± 0.20	3.21 ± 0.25	6.95 ± 0.39
Comparisons Between Groups				
	Control and LY294002 Groups	**Control and Xiaoluo Groups**	**Control and Igf-1 Groups**	**Control and Igf-1 + Xiaoluo Groups**
t	71.036	44.233	18.578	0.124
P value	<.01[a]	<.01[a]	<.01[a]	.904

[a]$P < .01$, indicating that the late and total apoptosis rates of the LY294002 and Xiaoluo groups are significantly higher and those of the Igf-1 group were significantly lower than those of the control group

Cell Apoptosis

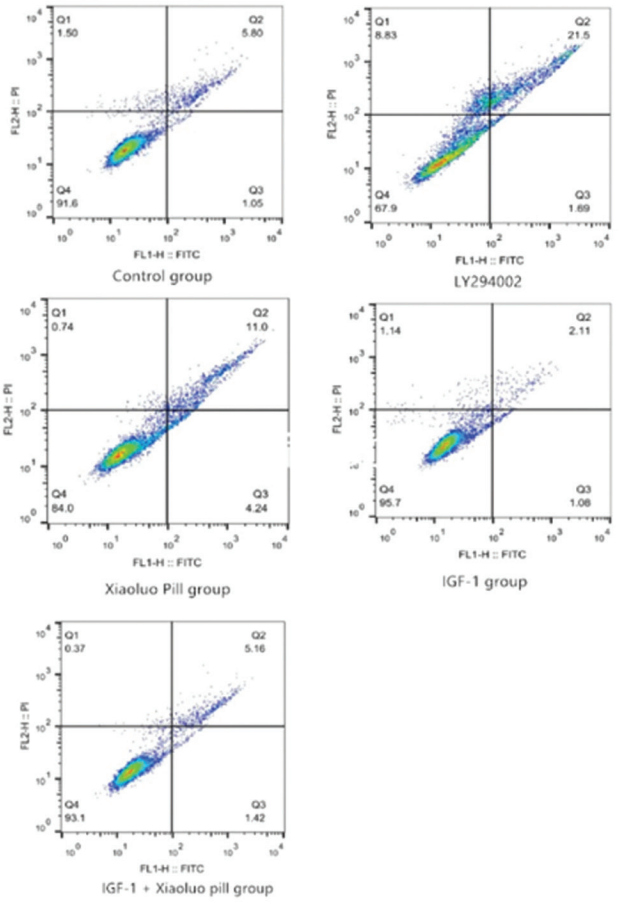
As Table 2 and Figure 2 show, apoptosis occurred in all of the groups and was concentrated in the late stage of apoptosis. The late apoptosis rate of the LY294002 group was 21.73% ± 0.49 ($P < .01$) and that of the Xiaoluo group was 11.23% ± 0.25 ($p < .01$). With the addition of the pathway activator IGF-1, the late apoptosis rate of the IGF-1 group was 2.29% ± 0.17 and that of the IGF-1 + Xiaoluo group was 5.24% ± 0.23.

The LY294002 and Xiaoluo groups' late and total apoptosis rates were significantly higher (all $P < .01$) and those of the IGF-1 group were significantly lower (both $P < .01$) than those of the control group. No significant differences existed in the total apoptosis rate between the IGF-1+ Xiaoluo group and the control group ($P > .05$).

Protein Expression of Bcl-2, Bax , and Caspase-3

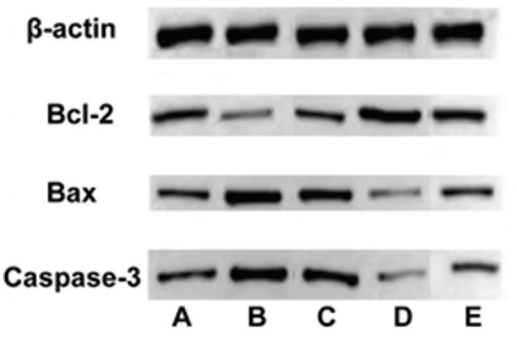
Figures 3, 4, 5, and 6 show the expression of the apoptotic proteins Bcl-2, Bax, and Caspase-3 in the control, LY294002, Xiaoluo, IGF-1, IGF-1+10 mg/mL Xiaoluo groups. The expression of Bcl-2, ,Bax, and Caspase-3 in each group changed to different degrees after treatment with thyroid-cancer SW579 cells compared with the blank control group.

Figure 2. Apoptosis Rate of Xiaoluo and its Chemical Components on Thyroid Cancer Cells SW579, as Detected Using Flow Cytometry



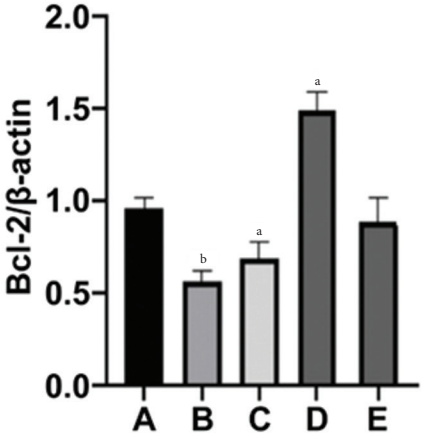
Abbreviations: FITC, fluorescein isothiocyanate; FL1-H, fluorescence intensity; IGF-1, insulin-like growth factor 1.

Figure 3. Effects of Xiaoluo on the Expression Levels of Bcl-2, Bax, and Caspase-3 Proteins in SW579 Cells, as Detected Using Western Blot Analysis. A = blank control group; B =10 μmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.

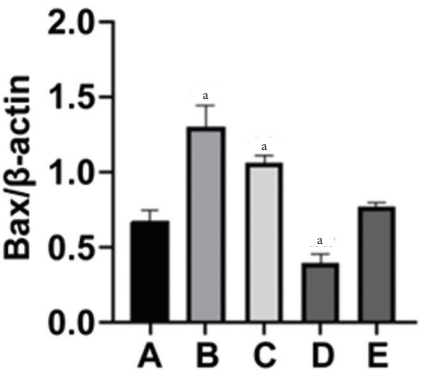
Figure 4. Bcl-2 Protein Expression, as Detected Using Western Blot Analysis (n = 3). A = control group; B = 10 μmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



[a]$P < .05$, indicating that the 10 mg/mL Xiaoluo group had significantly lower levels and the 100 ng/mL IGF-1 group had significantly higher levels of Bcl-2 than the control group did
[b]$P < .01$, indicating that that the 10 μmol/L LY294002 group had significantly lower levels of Bcl-2 than the control group did

Abbreviations: Bcl-2, B-cell lymphoma 2.

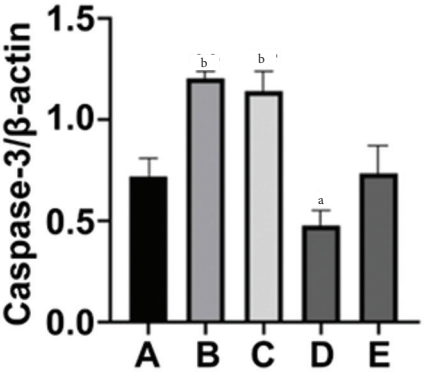
Figure 5. Expression of Bax Protein, as Detected Using Western Blot Analysis (n = 3). A = blank control group; B =10 μmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



[a]$P < .01$, indicating that that the 10 μmol/L LY294002 and 10 mg/mL Xiaoluo groups had significantly higher levels and the 100 ng/mL IGF-1 group had significantly lower levels of Bax protein than the control group did

Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.

Figure 6. Caspase-3 Protein Expression, as Detected Using Western Blot Analysis (N=3). A = blank control group; B =10 μmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



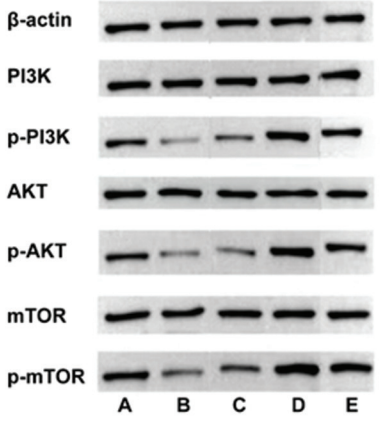
[a]$P < .05$, indicating that the 100 ng/mL IGF-1 group had significantly lower levels of Caspase-3 protein than the control group did
[b]$P < .01$, indicating that that the 10 μmol/L LY294002 and the 10 mg/mL Xiaoluo group had significantly higher levels of Caspase-3 protein than the control group did

The LY294002 ($P < .01$) and Xiaoluo ($P < .05$) groups' expression of the Bcl-2 protein was significantly lower, and their expressions of Bax and Caspase-3 were significantly higher (both $P < .01$) than those of the control group. The IGF-1 group's expression of Bcl-2 protein was significantly higher ($P < .05$) and it expressions of Bax ($P < .01$) and Caspase-3 ($P < .05$) were significantly lower than those of the control group.

Protein Expression of PI3K/AKT/mTOR Pathway

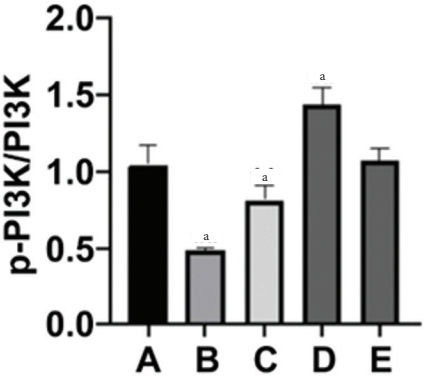
Figures 7, 8, 9, and 10 show the expression of the apoptotic proteins PI3K, AKT, and mTOR. The protein expressions of P-PI3K, P-Akt, and P-MTOR in the LY294002 and Xiaoluo groups were significantly lower than those of the blank group (all $P < .01$). The pathway activator IGF-1 group had significantly higher expressions of P-PI3K, P-Akt, and P-MTOR than those of the control group (all $P < .01$). The IGF-1 + Xiaoluo group also had significantly higher expressions of P-Akt ($P < .05$) and P-MTOR ($P < .01$) than those of the control group, but no significant difference existed between that group's P-PI3K expression and that of the control group.

Figure 7. Effects of Xiaoluo on the Expression of PI3K, AKT, and mTOR in Gastric Cancer SW579 Cells, as Detected Using Western Blot Analysis. A = blank control group; B =10 μmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



Abbreviations: AKT, protein kinase B; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase.

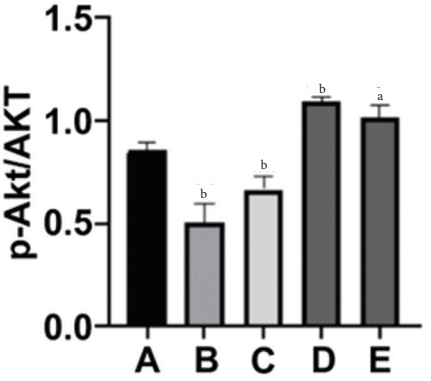
Figure 8. Expression of P-PI3K/PI3K Protein, as Detected Using Western Blot Analysis (N=3). A = blank control group; B =10 µmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



[a]$P < .01$, indicating that that the 10 µmol/L LY294002 and the 10 mg/mL Xiaoluo group had significantly lower expression and the 100 ng/mL IGF-1 group had significantly higher expression of PI3K/PI3K protein than the control group did

Abbreviations: PI3K, phosphoinositide 3-kinase.

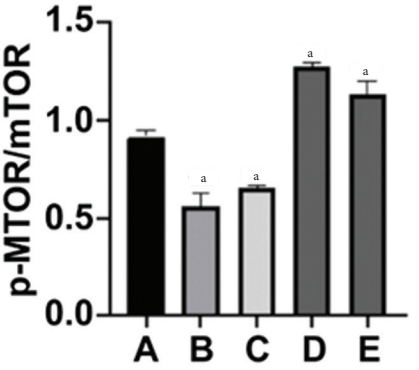
Figure 9. P-Akt/AKT Protein Expression, as Detected Using Western Blot Analysis (N=3). A = blank control group; B =10 µmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



[a]$P < .05$, indicating that the 100 ng/mL IGF-1+10 mg/mL Xiaoluo group had significantly higher expression of P-Akt/AKT protein than the control group did
[b]$P < .01$, indicating that that the 10 µmol/L LY294002 and the 10 mg/mL Xiaoluo group had significantly lower expression and the 100 ng/mL IGF-1 group had significantly higher expression of P-Akt/AKT protein than the control group did

Abbreviations: AKT, protein kinase B.

Figure 10. P-mTOR/mTOR Protein Expression, as Detected Using Western Blot Analysis (N=3). A = blank control group; B =10 µmol/L LY294002 group; C = 10 mg/mL Xiaoluo group; D = 100 ng/mL IGF-1 group; and E = 100 ng/mL IGF-1+10 mg/mL Xiaoluo group.



[a]$P < .01$, indicating that that the 10 µmol/L LY294002 and the 10 mg/mL Xiaoluo group had significantly lower expression and the 100 ng/mL IGF-1 and 100 ng/mL IGF-1+10 mg/mL Xiaoluo groups had significantly higher expression of P-mTOR/mTOR protein than the control group did

Abbreviations: mTOR, mammalian target of rapamycin.

DISCUSSION

It's difficult to elucidate the mechanism of action from one direction because of the complex composition of TCM compounds. The current study provided theoretical support for the clinical application of the Xiaoluo pill and the study of anti-thyroid cancer and provided research direction for further development of new anti-thyroid-cancer drugs.

The current study found that Xiaoluo inhibited proliferation of SW579 cancer cells in a concentration-dependent manner and that it could promote apoptosis of the SW579 cells. Also the effect was similar to that found for the group using the PI3K/AKT/mTOR pathway inhibitor LY294002. The IGF-1 group showed enhanced proliferative ability for the SW579 cells and a decrease in the apoptosis rate. The Xiaoluo group showed an increased apoptosis rate for the SW579 cells, mainly in late apoptosis. These findings are consistent with the findings of other researchers.[14,15]

The current study showed that Xiaoluo can play an anti-thyroid-cancer role by promoting the apoptosis of cancer cells and inhibiting their proliferation rate. The Xiaoluo downregulated the expression of Bcl-2, upregulated Bax protein, and decreased the ratio of Bcl-2 to Bax.

The current study also found that Xiaoluo had significant inhibitory effect on p-PI3K, P-Akt and P-MTOR protein contents, and the inhibitory effect was similar to that of PI3K/AKT/mTORC1 signaling pathway inhibitor.

The current study had some limitations, The research team conducted the study solely in vitro, using only SW579 cells as a model for thyroid cancer. It would be beneficial to further investigate the effects of Xiaoluo on other thyroid-cancer cell lines in vitro and in in-vivo animal models to validate the findings. Additionally, while the study focused on the PI3K/AKT/mTORC1 signaling pathway, it' possible that other pathways may be involved in the anti-thyroid-cancer effects of Xiaoluo. Therefore, future research should aim to explore the broader mechanism of action of Xiaoluo in the treatment of thyroid cancer.

Furthermore, the research team acknowledges that the clinical application of Xiaoluo may be limited by the availability and accessibility of the herbal ingredients used in its formulation. As such, the research team encourages further research into the identification of the active compounds within Xiaoluo so that researchers may use it to develop more accessible and standardized treatment options.

CONCLUSIONS

Xiaoluo exerts its antithyroid-cancer effects through the induction of apoptosis in thyroid-cancer cells through the inhibition of the PI3K/AKT/mTORC1 signaling pathway. Xiaoluo may serve as a potential therapeutic agent for the treatment of thyroid cancer.

AUTHORS' DISCLOSURE STATEMENT

The authors declared that they have no conflicts of interest related to the study.

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