Synthesis of *Acanthus ilicifolius* Linn alkaloid 2-
Benzoxazolinone Derivative and its effect on cervical cancer
C-33A cells

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Abstract. Cervical cancer seriously threatens women's health, it seriously harm the patient’s physical and mental health. The new derivative of *Acanthus ilicifolius* Linn alkaloid 2-benzoxazone was obtained from o-aminophenol, substituted benzaldehyde, trichloroacetic acid and isocyanate via a tandem Ugi 4CC/SN cyclization (N-cyclohexyl-2-(2-benzoxazolone -3-yI)-2-p-trifluoromethylphenylacetamide, BOABB), and the toxicity of the new compound to the cervical cancer C-33A cells was detected by CCK-8 assay, wound healing assay and apoptotic assay. The results exhibited that BOABB had obvious inhibitory effect on C-33A cells, and the calculated IC50 was 32.3 μM. Wound healing assay showed that BOABB could significantly inhibit cell migration (P<0.05). The apoptotic assay demonstrated that BOABB has induced apoptosis in C-33A cells (from 10.86% to 34.70%).

1. Introduction

*Acanthus ilicifolius* Linn has anti-inflammatory, antioxidant, hepatoprotective and tumor activities [1, 2] which is a mangrove plant distributed in tropical coastline. Benzoxazinoids are plant secondary metabolites of *Acanthus ilicifolius* Linn, which have certain biological activities, they are active factors in many gramineous plants that play the role of antifungal, insect, virus and bacteria [3, 4], it also has mutagenic activities [5], therefore, the structure has a research prospect as a lead compound in the
synthesis of new drugs. Through structural modification, substances with high stability and hydrophilicity can be obtained. At the same time, it is hopeful to improve the biological activity of the compounds and enhance the antioxidant capacity and cytotoxicity. This is a good alternative structure for the study of anticancer drugs.

Cancer is a complex and diverse group of diseases. As one of the most intractable disease in the 20th century, the number of cancers continues to rise in the 21st century, and has gradually become a difficult problem perplexing human public health [6]. Survey data show that cervical cancer is the fourth fatal disease of women all over the world which makes the global health problems face severe challenges. In 2020, there were about 604,000 new patients and 342,000 deaths of cervical cancer [7]. Cancer will become the main and only disease hindering the growth of life expectancy.

Using a tandem Ugi 4CC/S$_5$ cyclization of o-aminophenol, substituted benzaldehyde, trichloroacetic acid and isocyanate, we have synthesized a new compound (BOABB) with benzoxazolinone core structure which is one of the alkaloids in the Acanthus ilicifolius Linn. Because this kind of structure has not been studied in anti-cervical cancer cells, therefore, we investigated the effect of this compound BOABB on the proliferation and apoptosis of cervical cancer C-33A cells. We were pleasantly to find that the compound BOABB could significantly inhibit the proliferation of cervical cancer cells and induce the apoptosis.

2. Materials and methods

2.1. Basic reagent
CCK-8 was obtained from TargetMol (Boston, USA). Apoptosis analysis kit was obtained from Beyotime (Shanghai, China).

2.2. Cell cultures
Cervical cancer cell line C-33A was stemmed from experimental center of Sinopharm Dongfeng General Hospital, and maintained in DMEM (Gibco, USA) medium containing 10% fetal bovine serum (Every Green, China). The cells were cultured in an incubator at 37 °C with 5% CO$_2$.

2.3. Synthetic strategy of acanthus ilicifolius linn alkaloid 2-benzoxazolinone derivative
The specific synthetic route can refer to the published articles [8], this paper will show the schematic diagram of the synthetic route of the compound required in this experiment (Scheme 1), and the NMR data are attached to references [9].

2.4. CCK-8 assay
Cell counting kit-8 (CCK-8) assay was used to determine the toxicity of BOABB to C-33A cells. C-33A adherent cells were digested into a suspension of single cells using trypsin. Subsequently, adjust the cells to the appropriate concentration and plant them in 96 well plates, incubate in cell incubator for 24 hours. The cells were treated with 10, 20, 30, 40, 50, 60 μmol/L for 48 hours, and CCK-8 was added to the same medium for 2 hours. The absorbance of the culture medium at 450nm was measured using the multifunctional microplate reader. OD value can reflect the number of living cells.
2.5. Wound healing assay
Wound healing assay was used to detect the effect of migration on C-33A cells within treated by BOABB. Add cells in the 6-well plates, put it in an incubator at 37 °C with 5% CO₂ content overnight. The next day, make a vertical mark with the head of the spear against the ruler, wash the cells with PBS for 3 times, remove the delimited cells, and add the prepared drugs in different concentrations. Put it in an incubator (Thermo, USA) at 37 °C with 5% CO₂ for culture, take photos at 0, 24, 48, 72 hours respectively.

2.6. Apoptotic assay
The results of BOABB on apoptosis of C-33A cells was stained by Annexin V-FITC/PI apoptosis kit. Refer to the manufacturer’s instructions for specific operation steps (MULTI SCIENCES, China). The cells were planted in the 6-well plate and co-cultured with BOABB in the medium for 48 hours. The cells were digested with Accutase (MULTI SCIENCES, China), collected into the microtubes (Axygen, USA), after washing twice with PBS, centrifuge and add loading buffer to resuspend the cells, successively added with 5μL Annexin V and 10μL PI dye, incubated in dark for 5 minutes, and determined by flow cytometer (Agilent, NovoCyte).

3. Results and discussion
We used a tandem Ugi 4CC/S₈ cyclization to synthesize N-cyclohexyl-2-(2-benzoxazolone-3-yl)-2-p-trifluoromethylphenylacetamide, the synthetic route of the new structure was outlined in Scheme 1.

Scheme 1. Synthesis of N-cyclohexyl-2-(2-benzoxazolone-3-yl)-2- p-trifluoromethylphenylacetamide
3.1. Inhibitory effects of BOABB on C-33A cell viability
The results indicated that BOABB could inhibit the viability of C-33A cells in the range of 10–60 μmol/L (Table 1, Figure 1). In this study, it can be preliminarily determined that BOABB can inhibit the activity of C-33A cells. With the increase of BOABB concentration, the stronger the ability to inhibit cell proliferation. It is calculated that the IC₅₀ of BOABB is 32.3μM.

| Concentration | Mean (%) | SD (%) |
|---------------|----------|--------|
| Control       | 99.67    | 0.58   |
| 10 μM         | 98.67    | 1.53   |
| 20 μM         | 84.84    | 12.55  |
| 30 μM         | 53.84    | 2.81   |
| 40 μM         | 31.90    | 1.47   |
| 50 μM         | 32.09    | 7.02   |
| 60 μM         | 32.74    | 4.11   |

3.2. Wound healing rates of BOABB on C-33A cell
The wound healing assay was conducted to verify the effect of the BOABB on the migration ability of C-33A cells, the cells were treated with BOABB at concentrations of 20, 30 and 40 μmol/L, respectively. Take photos with an optical microscope and camera at the time set for the experiment. The wound healing assay effect is shown in the Figure 2. the results showed that BOABB could significantly inhibit cell migration (P<0.01).

3.3. Induction of apoptosis in C-33A cells
Annexin V-FITC/PI detection kit was used to detect the effect of BOABB on C-33A cells apoptosis. According to the IC₅₀ value calculated by CCK-8 assay, the 30 μM is the drug concentration to observe the effect of apoptosis. 8000 events were collected in the experimental setting. The result showed that
after 48h of treatment, the apoptosis rate of the control group was 10.86%, the 30 μM group were 34.70%. Two groups were compared with each other, the 30 μM group was significantly increased. (Figure 3).

Figure 2. The wound healing results in C-33A cells. (A. The state of scratch after BOABB acted on cells at 0h, 24h, 48h and 72h. B. Statistical data of wound healing rate. **P<0.01, ****P<0.001).

Figure 3. Effect diagram of C-33A cell apoptosis induced by BOABB.

4. Conclusion
Because of its diverse biological activities, Acanthus ilicifolius Linn can be used in many fields, especially in the field of medicine. A new type of alkaloid synthesized with Acanthus ilicifolius Linn as the basic mother nucleus, N- cyclohexyl -2-(2- benoxazolone -3- yl) -2- p-trifluoromethylphenylacetamide were synthesized via ring closing reaction after polymerization reaction and its inhibition of cervical cancer cell proliferation was investigated. Through some limited experiments, we preliminarily infer that BOABB can inhibit the activity of C-33A cells, inhibit cell
migration and induce cell apoptosis. However, further research is needed to confirm the anti-cervical cancer drug of this new compound.

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[9] N-cyclohexyl-2-(2-benzoazalone-3-yl)-2-p-trifluoromethylphenylacetamide (BOABB). White solid (0.35 g, yield: 83%). Mp: 183-184 °C. 1H NMR (CDCl₃, 400 MHz): δ 7.64-6.70 (m, 8H, Ar-H), 6.63 (s, 1H, NH), 6.15 (s, 1H, CH), 3.87-3.84 (m, 1H, CH), 2.01-1.13 (m, 10H, 5CH₂). 13C NMR (CDCl₃, 100 MHz): δ 165.2, 154.8, 142.4, 137.4, 129.3, 128.4, 128.4, 125.9, 124.1, 123.0, 112.0, 110.1, 60.2, 49.2, 32.7, 25.3, 24.7. EI-MS m/z (%): 418 (M⁺, 2), 292 (M⁺-CONHCy 67), 158 (p-CF₃C₆H₄CH⁺, 100), 126 (CONHCy⁺, 33), 134 (2-benzoazolone⁺, 78), 83 (C⁺, 21). Anal. Calcd for C₂₃H₂₅F₃N₂O₅: C, 63.15; H, 5.06; N, 6.70. Found: C, 63.10; H, 5.10; N, 6.65.