STUDY ON ANTICANCER EFFECT OF SYNTHETIC BIOGENIC SOURCE OF GERMACRENE A

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ABSTRACT

Objective: Based on obtaining germacrene A, which is the analogue of synthetic β-elemene, by means of synthetic biology, this experiment aims to explore the anti-tumor effect of germacrene A.

Methods: The cell line used in this study was bladder cancer T24 cells. The morphological changes of bladder cancer T24 cells treated with germacrene A were observed by inverted microscope; the death of cancer cells was observed by trypan blue staining; cell viability was measured by the MTT assay and the inhibition rate of the cancer cells by the germacrene A was investigated. Based on the results obtained from the experiments, the anti-cancer effect of germacrene A derived from synthetic biology was analyzed.

Results: After administration, the volume of T24 cells was significantly reduced. The shape of the T24 cells gradually changed from wedge-shaped and polygonal to round, and the T24 cells soon fell off, atrophied and disintegrated. After Tet blue staining, a large number of T24 cells after administration were observed to be dead; It was found by means of MTT assay that T24 cells were inhibited to varying degrees by the effects of different concentrations of germacrene A.

Conclusion: The above experimental results show that the synthetic biological source of germacrene A has obvious inhibitory effect on bladder cancer T24 cells, and this effect has obvious time and concentration dependence.

Keywords: Germacrene A; Synthetic drugs; Antitumor; β-elemene

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1 INTRODUCTION

1.1 Research status and significance

According to a global survey of cancer by scientists, the incidence of cancer has continued to rise in recent years, and new cancer cases in China are at the forefront. Bladder cancer is a malignant tumor that occurs on the bladder mucosa. It is the most common malignant tumor in the urinary system and one of the top ten common tumors in the body. It accounts for the first incidence of urogenital tumors in China. Bladder cancer has a high mortality rate as a common tumor in the urinary system, and its main cause of death is postoperative recurrence. It ranks fourth among common malignant tumors in the United States and ninth in the genitourinary system worldwide. Although the treatment of other cancers is progressing rapidly, the treatment of bladder cancer is relatively stagnant. The treatment of superficial bladder cancer includes a thorough transurethral resection of bladder tumor plus postoperative intravesical instillation of BCG, bladder infusion chemotherapy drug epirubicin, pirarubicin, intravesical chemotherapy and immune therapy, etc. But it is always difficult to remove all lesions and does not solve the problem of high recurrence after surgery and progressing to a higher level. Therefore, identifying new treatments and improving response to existing treatments may help improve the prognosis of patients with bladder cancer.

The national second-class anticancer drug, elemene, is an effective anti-cancer ingredient extracted from sputum, with mild side effects, clear curative effect, and many advantages. And its anti-cancer effect is better than some traditional chemotherapy drugs, and elemene shows low toxicity to normal cells. This kind of anticancer drug mainly comes from natural plant extraction, but the direct extraction production method has many uncontrollable abilities and shortcomings. Therefore, finding a new mode of production has become an inevitable trend.

In recent years, many researchers have developed new ways, such as the introduction of dehydrogenase and cytochrome P450 enzymes derived from Artemisia annua plants, so that the production of artemisinin can increase to industrial level. This idea of producing natural medicines through synthetic biology has been recognized by the scientific community.

1.2 β-elemene

Elemene is a kind of non-cytotoxic anti-tumor drug extracted from natural plant Wenyujin, which has inhibitory effects on various tumor cells. The most important active ingredient is β-elemene. The chemical name of β-elemene is 1-methyl-1-vinyl-2,4-diisopropyl cyclohexane, and its molecular formula is C13H24. The molecular weight of β-elemene is 204, β-elemene mainly exerts direct anti-tumor effects by inhibiting tumor cell proliferation, inducing apoptosis of tumor cells and inhibiting angiogenesis in tumor tissues, and it also can inhibit distant metastasis of tumors, and exert direct or indirect resistance. Meanwhile, β-elemene can be used in a variety of cancers in clinical applications. As an effective ingredient in the extraction of pure natural Chinese herbal medicines, β-elemene has excellent properties such as abundant sources and less toxic side effects, and it has broad prospects in anti-tumor therapy.

According to the current research results, β-elemene is mainly played by inhibiting tumor cell migration and invasion, inducing tumor cell apoptosis and inhibiting tumor angiogenesis. It has broad prospects for use in clinical treatment.

1.3 Synthetic biology

Synthetic biology, as a discipline of biology and engineering, is actually the application of engineering thinking in biology. Most of the plant-derived natural drugs are synthesized by the organism itself, and they are a trace amount of secondary metabolites, such as, artemisinin, glycyrrhizic acid, morphine, asiaticoside, paclitaxel, and etc. These substances are widely used in the fields of anti-cancer, anti-HIV, cosmetology, liver protection and flavoring. Direct extraction from natural plants is the main form of production of natural medicines from these plants today, and many of our well-known drugs are derived from this type of production. However, this production method directly extracted from the original plant has many shortcomings, such as, low drug content, large production difference, difficult product purification, long plant growth, and serious damage to natural resources, especially wild resources that cannot be planted. Nowadays, wild
precious Chinese herbal medicines such as ginseng and Ganoderma lucidum are endangered due to excessive utilization and destruction. Although some varieties can be supplemented by cultivation methods, due to the deterioration of varieties and other reasons, especially the problems of continuous cropping, the sustainability of resource utilization in China is facing great difficulties. Synthesizing corresponding active ingredient through biotechnology will become a new way for the protective production of Chinese herbal medicines. 

2 EXPERIMENTAL MATERIALS

2.1 Cell line

The bladder cancer T24 cell line, purchased from Shanghai Hongshun Biotechnology Co., Ltd., meets the requirements of ADCC.

2.2 Reagent

<table>
<thead>
<tr>
<th>Reagent name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germacrene A (purity above 96%)</td>
<td>Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences</td>
</tr>
<tr>
<td>0.25 Trypsin</td>
<td>American Gibco</td>
</tr>
<tr>
<td>EDTA</td>
<td>Tianjin Dingshengxin Chemical Co., Ltd.</td>
</tr>
<tr>
<td>DMSO</td>
<td>Xingchang Chemical</td>
</tr>
<tr>
<td>RPMI-1640 Medium</td>
<td>American Gibco</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>American Gibco</td>
</tr>
<tr>
<td>PBS</td>
<td>Zhejiang Hangzhou Xinfu Pharmaceutical Co., Ltd.</td>
</tr>
<tr>
<td>Thiazole Blue (MTT)</td>
<td>Shanghai Baili Biotechnology Co., Ltd.</td>
</tr>
<tr>
<td>Dimethyl sulfoxide Sigma USA</td>
<td>American Sigma</td>
</tr>
<tr>
<td>0.4% trypan blue</td>
<td>Beijing Aidelai cyclic peptide organism</td>
</tr>
</tbody>
</table>

2.3 Main instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-clean work bench</td>
<td>Changsha Qulong Instrument Equipment Co., Ltd.</td>
</tr>
<tr>
<td>Thermostat cell incubator</td>
<td>American Thermo</td>
</tr>
<tr>
<td>Frozen Multi-Function Centrifuge</td>
<td>American Beckman</td>
</tr>
<tr>
<td>Electronic balance</td>
<td>Shanghai Shenhui Electronics Co., Ltd.</td>
</tr>
<tr>
<td>Inverted microscope</td>
<td>Shanghai Zhongheng Instrument Co., Ltd.</td>
</tr>
<tr>
<td>Microplate reader</td>
<td>American BIO-TEK company</td>
</tr>
<tr>
<td>1000μL pipetting gun</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>100μL pipetting gun</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Water bath box</td>
<td>Shanghai Zhiyu Analytical Instrument Manufacturing Co., Ltd.</td>
</tr>
<tr>
<td>Liquid nitrogen tank (East Asia brand liquid nitrogen container)</td>
<td>Leshan East Asia Machine Electric Co., Ltd.</td>
</tr>
<tr>
<td>UV disinfection vehicle (SNXIN)</td>
<td>Jiangsu Shenxing Optoelectronic Medical Equipment Co., Ltd.</td>
</tr>
<tr>
<td>Electric thermostat blast drying oven (DHG-9075A)</td>
<td>Shanghai Feiyue Experimental Instrument Co., Ltd.</td>
</tr>
<tr>
<td>6-well sterile plate</td>
<td>American Costar</td>
</tr>
<tr>
<td>96-well sterile culture plate</td>
<td>BIOFIL</td>
</tr>
</tbody>
</table>
3 EXPERIMENTAL CONTENT

3.1 Solution preparation

3.1.1 Germacrene A solution

The germacrene A solution was filtered using a 0.22 μm sterile filter. The solution density was adjusted to 10 mmol/L with PBS buffer to prepare a germacrene A mother liquor. The prepared mother liquid was diluted stepwise with a serum-free medium to prepare a solution of gemasene A of 0.02, 0.01, and 0.008 mg/ml. Please use the drug solution right after it was ready.

3.1.2 Preparation of PBS buffer

Pour a packet of PBS into 2000 mL of ultrapure water. After it is fully dissolved, adjust the pH to 7.2 and dispense with a screw bottle to prevent contamination of the PBS in subsequent experiments and cause waste. Autoclave for 30 minutes, cool down to room temperature and store at 4 °C.

3.1.3 Preparation of cell culture medium

Take an appropriate amount of fetal bovine serum and medium, and arrange it in a ratio of 1:9. After mixing evenly, it is dispensed with a screw bottle to prevent contamination due to mistakes during the experimental operation, so that the culture solution is completely wasted. The preparation was completed and stored at 4 °C, and it was rewarmed at 37 °C before use.

3.1.4 Preparation of MTT (5mg/mL)

Weigh accurately 0.5 g of MTT, dissolve it in 1000 mL of PBS buffer in the dark, mix well, and store at 4 °C.

3.1.5 Preparation of 0.25% trypsin

Weigh 0.25 g of trypsin, dissolve it in 100 mL of PBS buffer, stir at low speed, filter and dispense, store at 4 °C, and use it for a short time.

3.1.6 Preparation of cell cryopreservation solution

Take appropriate amount of DMSO and FBS, mix well in a ratio of 1:9, and use it after it is ready.

3.2 Cell culture

3.2.1 Cellular resuscitation

The cryotube containing the cells was taken out and thawed at 37°C. After thawing thoroughly, the cell suspension was aspirated into a centrifuge tube, diluted with an appropriate amount of the culture solution and centrifuged (500 rpm, 3 min) in a centrifuge. Discard the supernatant and add an appropriate amount of the culture solution to the centrifuge tube until the cells are evenly distributed to prepare a suspension. The cell suspension was transferred to a culture flask, cultured in a 37°C, 5% CO₂ incubator, and the culture solution was changed in time during the culture. Pay attention to observe the cell growth under a microscope, and the cells need subculturing when they are grown to about 80% to 95% of bottle bottom.

3.2.2 Cell passaging

Remove the flask from the incubator and tighten the cap during the procedure. Place the culture flask on the ultra-clean workbench, aspirate the culture solution, discard it, rinse it with appropriate amount of PBS buffer for 2~3 times and discard the PBS. Slowly add an appropriate amount of 0.05% trypsin solution along the cell-free side and digest at room temperature for about 1 to 3 minutes. During the period, observe the cell morphology under the microscope. After the cells are completely digested, discard most of the trypsin and gently tap. The culture solution was added and pipetted into a single cell suspension. Transfer the suspension to the centrifuge tube and continue to blow. After pipetting evenly, count using a cell counting plate to adjust the concentration. The desired suspension is placed in a culture flask, and an appropriate amount of the culture solution is added, and the culture is continued in a constant temperature incubator.

3.2.3 Cell cryopreservation

The culture medium was aspirated, and the bladder cancer T24 cells in the logarithmic growth phase were digested with trypsin digestive solution containing EDTA, observed under a microscope, and after centrifugation was completed, centrifuged (1000 rpm, 5 min), the supernatant was discarded. And add appropriate amount of frozen solution (containing 10% DMSO and 90% FBS), adjust the cell density to 1 × 10^6 ~ 1 × 10^7 / ml, blow evenly and dispense into the cryotube, put in 4°C 4h; -20°C 4h; -80°C 4h conditions, the temperature was lowered, and finally store the cryotube in a liquid nitrogen tank for storage.
3.3 Grouping

Control group: only medium was added.

Experimental group: The concentration of germacrene A was set to be 0.008 mg/ml, 0.01 mg/ml, and 0.02 mg/ml, respectively.

Each of the above groups is provided with three duplicate holes.

3.4 Experimental methods

This study is based on obtaining germacrene A, which is the analogue of synthetic β-elemene, by means of synthetic biology, the following three detection methods were used to conduct experiments and investigate the effect of germacrene A on T24 bladder cancer cells:

3.4.1 Observation of cell morphology changes by inverted microscope

Human bladder cancer T24 cells in logarithmic growth phase were inoculated into 6-well cell culture plates and cultured in a 37 °C, 5% CO₂ incubator for 24 h. After the cells were attached, each group of drugs was added and cultured for 24 h, respectively. Morphological changes of human bladder cancer T24 cells were observed under a microscope.

3.4.2 Trypan blue exclusion method to detect cell death

Human bladder cancer T24 cells in logarithmic growth phase were inoculated into 6-well cell culture plates and cultured for 24 hours. The mice were divided into experimental group and control group. Different concentrations of germacrene A were added and cultured for 24 hours. The cells in the culture wells were collected and trypsinized to prepare a single cell suspension. Adjust the cell density appropriately and blow evenly. Take 0.5-1.0 ml of cell suspension into the centrifuge tube, add 0.4% trypan blue dye solution in equal amount, stain for 2-3 min, and add a small amount of cell suspension with a micropipette. Slides were used to observe cell staining. Dead cells can be stained by trypan blue, which can be seen as light blue cells under the microscope. Live cells cannot be stained, and the cells are colorless and transparent.

3.4.3 MTT assay for the effect of germacrene A on cancer cell proliferation

The log phase cells were seeded into 96-well plates, and the cells in the 96-well plates were cultured at constant temperature in a cell culture incubator. After adherence, different concentrations of germacrene A were added to the corresponding wells. 20 μL of the prepared MTT solution was added to each well after 24 hours and 48 hours of drug action, respectively. After incubating for 4 h at a constant temperature, the upper medium was discarded, 100 μL of DMSO was added, and shaken for 10 min. The treated 96-well plates were examined using a microplate reader. The absorbance A value was measured at a wavelength of 490 nm.

Cell proliferation inhibition rate (%) = (control hole OD value - drug hole OD value) / control hole OD value × 100%

The cell proliferation inhibition rate was calculated according to the above formula.

4 EXPERIMENTAL RESULTS

4.1 Cell morphology change results

The changes in the morphology of human bladder transitional cell carcinoma T24 cells by germacrene A are shown in the figure below. In the control group, the cells were wedge-shaped or long fusiform, adherent to the wall and vigorous, with high cell density, uniform growth distribution, clear contour and visible cell membrane. In the experimental group T24 cells treated with germacrene A, cell growth was inhibited and the number was significantly reduced. In the experimental group with increased concentration, the cell volume of the tumor cells was significantly reduced, the shape was gradually changed from a wedge shape to a polygonal shape, and the cells were soon fell off, atrophied and fragmented. There were many broken cell fragments in the medium. The greater the concentration of administration, the changes in cell morphology are more obvious and the number of survival cells is smaller.
4.2 Trypan blue rejection method results

The membrane structure of normal living cells is intact, and they can repel the trypan blue; The membrane permeability of cells which lose their activity or incomplete cell membrane increases, and those cells can be dyed blue by trypan blue. So living cells and dead cells can be differentiate by trypan blue. Through the experimental results, it can be found that the number of cell staining of the tumor cells in the experimental group after the treatment with germacrene A was significantly increased. In the experimental group with increased concentration, the number of cells stained was higher, and only a few were colorless and transparent cells. The morphology of germacrene A in human bladder transitional cell carcinoma T24 cells was stained with trypan blue as shown in Figure 5-8.
4.3 MTT test results

After treatment with different concentrations of germacrene A, cell growth was inhibited to varying degrees, and this effect showed time and concentration dependence, as shown in Table 1 and Figure 9. At the same time, the inhibition rate increases with the increase of the concentration; at the same concentration, the inhibition rate increases with the increase of the action time.

Table 1: Effect of in vitro Germacrene A on cell growth inhibition

<table>
<thead>
<tr>
<th>Group</th>
<th>concentration (mg/ml)</th>
<th>OD value</th>
<th>cell inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>Germacrene A</td>
<td>0.02</td>
<td>0.069±0.005</td>
<td>0.048±0.004</td>
</tr>
<tr>
<td>group</td>
<td>0.01</td>
<td>0.090±0.004</td>
<td>0.073±0.003</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.099±0.003</td>
<td>0.080±0.005</td>
</tr>
<tr>
<td>Control group</td>
<td>-</td>
<td>0.112±0.004</td>
<td>0.107±0.003</td>
</tr>
</tbody>
</table>

Figure 9: In vitro germacrene A cell inhibition rate

5 EXPERIMENTAL DISCUSSION AND PROSPECTS

T24 cells are wedge-shaped or long fusiform, adherent growth, T24 cells without drug treatment have strong growth, large cell density, uniform growth distribution, clear outline and clear cell membrane. After TMA cells treated with germacrene A, cell growth was inhibited, the number of cells was significantly reduced, and the cell volume was also significantly reduced. The shape was gradually changed from a wedge shape to a polygonal shape, as the action time lengthens, the phenomena such as shedding, shrinkage, fragmentation begin to appear. And as the concentration of the drug is increased, the change in cell morphology is more obvious, and the number of cell survival is less, showing a certain gradient.

Normal living cells, whose membrane structure are intact, can repel the trypan blue, so that the trypan blue can not get inside the cells; While the cells that lose activity or incomplete cell membrane can be dyed blue by trypan blue because the permeability of the membrane increases, It can be considered that the cell has died when cell membrane lose integrity, and it has same effect on necrotic, So living cells and dead cells can be distinguished quickly and easily.

Afer the T24 cells treated with germacrene A were stained with trypan blue, it can be found that the cells began to die and the cell density decreased when they are reacted with different concentrations of germacrene A. Compared with the viable cells, the dead cells were stained. Compared with the blank control group, the morphological effect of germacrene A on T24 cells was significant, and it became more and more significant with the increase of concentration. From this result, it can be seen that gemalene A has a significant effect on inhibiting bladder cancer T24 cells.
The principle of MTT assay is that succinate dehydrogenase in living cell mitochondria can reduce exogenous MTT to water-insoluble blue-purple crystal formamidine and deposit it in cells, while dead cells do not. Dimethyl sulfoxide can dissolve the hyperthyroidism in the cells, and its light absorption value is measured by a microplate reader at a wavelength of 490 nm. The amount of MTT crystal formation is proportional to the number of cells in a certain cell number range. The number of viable cells is judged based on the measured absorbance value.

From the result of the experiment which detects the proliferation of germacrene A on T24 cells by the MTT assay, it can be found that after different concentrations of germacrene A, the growth of cells is affected to varying degrees. With the increase of drug concentration, the cell inhibition rate begins to rise; the action time is longer, the inhibition rate is higher. This inhibition of cancer cells exhibits a dependence on time and concentration. Germacrene A has a very significant effect in inhibiting the proliferation of bladder cancer T24 cells, especially under high dose treatment. The discovery of this phenomenon is conducive to the further study of germacrene A, which can provide a certain experimental basis for the clinical trial of germacrene A.

From the above experimental results, it can be found that after different concentrations of germacrene A, cell growth was inhibited by different levels, and the inhibition was time-dependent and concentration-dependent. Germacrene A can inhibit the growth of cancer cells in a concentration-dependent manner, and high dose can significantly prevent the proliferation of cancer cells. The drug effects at different times also have different effects on tumor cells. These results demonstrate that germacrene A has a good performance in inhibiting tumor cell proliferation. This finding contributes to the experimental basis for further research and clinical trials of gemasene A.

In summary, gemalene A has obvious curative effect on anti-tumor effect. The anti-tumor mechanism of germacrene A may be through induction of apoptosis, but a clearer mechanism remains to be explored.

Nowadays, the anti-tumor mechanism of β-elemene, which has been put into clinical use, has been relatively mature, and it has made some progress in the research on the mechanism of anti-drug resistance 13-14. Further investigation and research are needed on the study of resistance of germacrene A.

Cancer is one of the leading causes of human death, and countless researchers are working on anti-tumor drugs. For the research of anti-tumor drugs, the road is still very long, and it is an inevitable trend to continuously explore new drugs. With the development of biotechnology, synthetic biology has become a green production method, which has important significance in the research of various natural medicines 15, and its development prospects are worthy of expectation.

REFERENCES


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