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Andrographolide, A Diterpene Lactone Derived from the Traditional Chinese Medicine Andrographis Paniculata, Induces Senescence in Human Esophageal Cancer Cells Via the P21/P27 Pathway

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Abstract: This paper explores the research progress of Andrographolide (AD) as a potential anticancer drug, particularly its novel discoveries in inducing tumor cell senescence, and proposes possible new strategies for the treatment of esophageal cancer based on these findings. Esophageal cancer, a malignant tumor originating from the esophageal epithelium, has a complex pathogenesis involving multiple factors such as heredity, environment, and lifestyle. As an active component extracted from the plant Andrographis paniculata, AD has been widely used in antibacterial and anti-inflammatory treatments and has garnered attention due to its high bioavailability, long-lasting effects, and low side effects. In recent years, studies have shown that AD exhibits potential in the treatment of cardiovascular, liver, and gallbladder diseases, as well as in enhancing immunity and antitumor effects. Notably, in lung cancer, AD has been found to induce cell senescence through the p53/p21 and Skp2/p27 pathways, thereby inhibiting tumor growth. Given AD's antitumor mechanisms in lung cancer, this paper postulates that AD may induce senescence in esophageal squamous cell carcinoma (e.g., TE-1 cell line) through similar pathways, offering a novel therapeutic target for esophageal cancer treatment. Further research will focus on optimizing the concentration and duration of AD treatment to explore its effectiveness in inducing senescence in TE-1 cells, laying the foundation for the development of new therapies against esophageal cancer.

Keywords: Traditional Chinese medicine, Andrographolide, esophageal cancer TE-1 Senescence, p21 and p27.

1. Introduction

Esophageal cancer is a malignant tumor occurring in the esophageal epithelium. Its pathogenesis is complex, involving many factors such as heredity, environment and lifestyle. The two main types of esophageal cancer are esophageal squamous cell carcinoma and esophageal adenocarcinoma. Esophageal cancer is caused by changes in the cells in the wall of the esophagus, specifically, esophageal cancer originates in the cells lining the esophagus. The esophagus is the tube that connects the throat to the stomach. The two most common types of esophageal cancer are squamous cell carcinoma and adenocarcinoma, with squamous cell carcinoma more common in the upper esophagus and adenocarcinoma more common in the lower esophagus.

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With the challenges posed by esophageal cancer in mind, scientific exploration has led to the discovery of compounds with potential therapeutic value. One such compound, andrographolide.

In 1911, Gorter firstly isolated a crystalline substance from *Andrographis paniculata*, identified it as a diterpene lactone compound, and named it andrographolide [1]

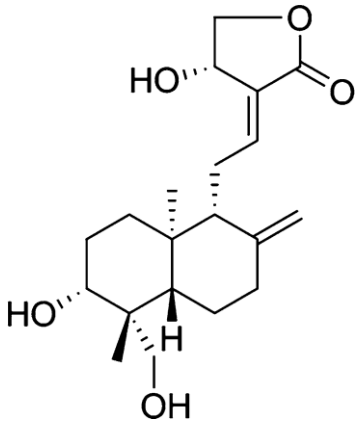

Figure 1: The structure of andrographolide

Andrographolide is widely used. It is rapidly absorbed in animals and exhibits a long efficacy and high bioavailability, with no obvious side effects. In recent years, clinical application has still focused on antibacterial and anti-inflammatory effects; however, with the advancement of research, andrographolide has demonstrated potential in the prevention and treatment of cardiovascular, liver, and gallbladder diseases, as well as enhanced immunity, anti-tumor effects, and other multiple benefits, making it a promising Chinese medicine worthy of further research and development.[2]

Andrographolide (AD) has been used to treat inflammation-related diseases. AD may also have anti-tumor effects. AD can induce apoptosis and cell cycle arrest, inhibit tumor angiogenesis and metastasis in a variety of tumor cell types. In recent studies, the primary anti-inflammatory drug AD has been found to have potential antitumor effects on lung cancer. They demonstrate for the first time in vitro and in vivo that AD induces senescence in lung adenocarcinoma via p53/p21 and Skp2/p27. Therefore, AD is a promising senescence induction therapy for the treatment of refractory human lung adenocarcinoma. [3-4]

Given the recent breakthroughs in understanding the antitumor potential of Andrographolide (AD) in lung cancer, particularly its ability to induce senescence through specific pathways, it is intriguing to consider how this knowledge might be extended to other cancer types. While AD has primarily been studied for its anti-inflammatory properties, its emerging role in cancer therapy, particularly through the modulation of cell cycle regulators like p21 and p27, opens up avenues for exploring its efficacy in treating other malignancies. This leads us to hypothesize about the potential impact of AD, or similar compounds, on esophageal cancer, specifically through enhancing the senescence of esophageal squamous cell lines like TE-1, a crucial step towards developing novel therapeutic strategies for this challenging disease.[5]

I predict that increasing concentrations and treatment durations with Andrographolide is able to induce senescence in human esophageal squamous cell line TE-1 through an increase in p21 and p27 resulting in decreased TE-1 viability.

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2. Materials and methods

2.1. Cell media and reagents

The esophageal squamous cell carcinoma TE-1 cell line was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/μl streptomycin and 100 μg/μl penicillin in a humidified incubator containing 5% CO2 at 37˚C. Andrographolide was dissolved in dimethyl sulfoxide (DMSO) and kept at − 20 ◦C before use. The purity of andrographolide is 98%.

Cells were started with 1 % DMSO or AD (1μ M, 10μ M, 100μ M) after 3, 12 and 48 hours to assess the effect of AD on different stages of cell cycle inhibitory proteins.

2.2. Animals

Male rats 4-years- of-aged weighing 20-23g were obtained from the laboratory.

2.3. SA-β-galactosidase staining

SA-β-galactosidase staining kit (Beyotime, Shanghai, China) was used to determine the expression of senescence-associated β-galactosidase (SA-β-Gal).

2.4. Western blotting

Cell or tissue lysates were prepared with RIPA lysis buffer (Beyotime, Shanghai, China). 20–50 μg proteins were loaded per lane. Proteins were transferred to a polyvinylidene fluoride membrane. After blocking with 5% nonfat milk in TBST, the membranes were incubated with the primary antibody, then incubated with secondary antibody. Immune complexes were detected using an ECL Kit (Share Bio, Shanghai, China).

2.5. Tunnel assay

2×10^3 cells were seeded in 96-well black culture plates in triplicate overnight, and then treated with DMSO or AD. A positive control (MLN4924) and a negative control (DMSO) were included in each experiment. The TdT enzyme and the marker (dUTP) were added, and in the presence of the TdT enzyme, the marker was ligated to the 3 ′ -OH end of the DNA. Was ed by fluorescence microscope using a fluorescent marker (FITC). Experimental results were observed under a microscope and recorded, and the apoptotic cells were counted and statistically analyzed. All the treatment groups are compared with the control group which does not receive any treatment.

2.6. In vivo experiment

TE-1 cells (2×10^6cells per mouse) were injected subcutaneously into the right frontal side of the mice. Mice were randomly divided into four groups, with AD or cyclodextrins serving as controls. After six days, the mice respectively intraperitoneal injected 50, 100 or 150 mg/kg AD in 20% cyclodextrin or the same dose of 20% cyclodextrin as a control every day. One month later, all mice were sacrificed and tumors were collected, photographed, and weighed. Tumors of treated mice were compared to control groups not receiving any treatment.

Statistical methods:

The collected statistical data from SA-β-galactosidase stainin, Western blot, and Tunnel assay will be analyzed using Student's T-Test.

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3. Results

Table 1: The combination of possible results

Combination Result # (CR#)					
	AD increases senescence by b-gal assay	AD increases p21 by western blot	AD increases p27 by western blot	AD decreases viability by tunnel assay	Support of hypothesis
1	+	+	+	+	Full
2	+	+	+	-	Partial
3	+	+	-	+	Partial
4	+	-	+	+	Partial
5	-	+	+	+	Partial
6	+	+	-	-	Partial
7	+	-	-	+	Partial
8	-	-	+	+	Partial
9	+	-	+	-	Partial
10	-	+	-	+	Partial
11	-	+	+	-	Partial
12	+	-	-	-	Partial
13	-	+	-	-	Partial
14	-	-	+	-	Partial
15	-	-	-	+	Partial
16	-	-	-	-	Fully Contradicts

Note: "+" indicates the same effect as positive control, "-" indicates the same effect as negative control.

Possible Result 1: In the TE-1 esophageal cancer cell line, low concentrations of AD significantly increased the senescence marker, SA-β-gal activity. Concurrently, the addition of AD led to an increase in the expression of p21 and p27 proteins, both of which are crucial negative regulators of the cell cycle G1 phase. Furthermore, AD interacted with the cell cycle G1 phase, inhibiting DNA synthesis by upregulating the expression of p21 and p27, thereby blocking the transition of cells from the G1 phase to the S phase.

Possible Result 2: After AD treatment, the abundance of p21 and p27 cell cycle inhibitors increased, and cellular senescence also increased. However, the cell viability did not decrease significantly, similar to that of the negative control DMSO.

Possible Result 3: After AD treatment, p21 abundance was higher than control and no significant change in the abundance of p27.. AD can make cells senescence and reduce activity by increasing the abundance of p21.

Possible Result 4: After AD treatment, p27 abundance was higher than control and no significant change in the abundance of p21. The p27 can inhibit cell activity and block the transition from G1 to S phase, keeping cells in G1 phase. AD can make cells senescence and reduce activity by increasing the abundance of p27.

Possible Result 5: Ability to increase the abundance of p21 / p27 and to decrease the activity of oesophageal cancer cells, but no cellular senescence was observed.

Possible Result 6: The anticancer drug AD induced cell senescence and increased p21 expression in esophageal cancer cells, but had no significant effect on p27 and did not decrease cell activity.

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Possible Result 7: There are no significant changes in P21 / p27 gene expression, and AD does not directly interact with the p21 / p27 tumor suppressor gene. However, it has shown a significant effect in inducing the senescence of cancer cells and reducing their cellular activity.

Possible Result 8: AD showed an increased effect on p27 abundance, effectively inhibiting the proliferative activity of cancer cells, revealing its key role in cell cycle regulation. However, AD did not exert a significant effect on another cell cycle inhibitory protein, p21. Moreover, no clear evidence that AD induces cancer cells into the senescent state was observed in the experiment.

Possible Result 9: AD was able to increase the abundance of p27 and induce senescence in human oesophageal cancer cells. But it had no effect on p21.

Possible Result 10: Can increase the abundance of p21 and reduce the activity of human esophageal cancer cells. But had no obvious effect on p27 and was not sufficient to make cell senescence

Possible results 11: AD is able to affect p21 / p27, but is not sufficient to trigger comprehensive anticancer effects. So cancer cells are not active or senescent.

Possible result 12: AD enables cancer cell senescence but not by increasing p21 / p27 abundance, possibly through other mechanisms. And could not observe the reduced activity of the cancer cells

Possible result 13: AD is able to increase the abundance of p21, but has no effect on the inhibition of the whole cancer.

Possible result 14: AD is able to increase the abundance of p27, but has no effect on the inhibition of the whole cancer.

Possible result 15: After treatment with AD, there was no effect for p21 / p27. However, the observed reduction of cancer cell activity indicates that it does not act on p21 / p27 and has the potential to inhibit cancer cells.

Possible result 16: There was no significant increase in p21 / p27 and no effect on cellular senescence and decreased activity. AD had no therapeutic effect on human oesophageal cancer cells.

Concentration and treatment duration results

Increasing the concentration may reduce, maintain, or enhance the inhibitory effect on cancer cells. Similarly, increasing the duration may also reduce, maintain, or enhance the inhibitory effect on cancer cells.

4. Discussion

Previous studies have demonstrated that AD has an anti-inflammatory profile and the ability to treat lung adenocarcinoma. Possible results 1,3,4,7 suggest that AD exhibits different molecular mechanisms in inducing senescence and reducing cellular activity in esophageal cancer cells. In addition to its known role by increasing cell cycle suppressor proteins p21 and p27, AD may also inhibit cancer cell proliferation by activating or upregating other CDK inhibitors (e.g., p15, p16, p57, etc.) to block cell cycle progression, especially G1/S phase transition. Although the different findings differ in the expression changes of p21 and p27, the common finding is that AD both have significant anticancer effects. Therefore, further studies of the molecular mechanisms of AD, including its interaction with other cell cycle regulators, signaling pathways as well as epigenetics, is important for the development of novel anticancer drugs.

Possible result 5,8,10, despite observing that AD significantly impacts the expression of cell cycle inhibitors such as p21 and p27, and effectively inhibits the proliferative activity of cancer cells, no clear evidence of cellular senescence was detected. This suggests that the induction of cellular senescence may be a complex and multistep process that does not solely rely on the expression changes of a single protein. Rather, it encompasses epigenetic modifications, metabolic reprogramming, autophagy and lysosomal function, intercellular communication, non-coding RNA regulation, as well as the synergistic effects of multiple signaling pathways. Furthermore, the

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potential existence of various drug resistance mechanisms in cancer cells, such as upregulation of drug efflux pumps, activation of drug metabolizing enzymes, or mutations in drug targets, may also attenuate the ability of AD to induce cellular senescence.

Possible result 2,6,9,Although AD has a significant effect in promoting cellular senescence in esophageal cancer cells, it does not directly lead to a marked decrease in the overall cellular activity. This phenomenon highlights the complexity and multi-faceted nature of cellular senescence, which encompasses not only the inhibition of proliferative capacity but also intricate interactions with metabolic status, cell-to-cell communications, and the tumor microenvironment. The metabolic reprogramming that accompanies cellular senescence, along with alterations in cell-cell interactions and the secretion of senescence-associated secretory phenotype (SASP) factors, likely contribute to a nuanced modulation of cellular activity rather than a straightforward reduction. Furthermore, the tumor microenvironment, composed of diverse cellular components and intricate signaling networks, serves as an additional layer of regulation that may attenuate the direct impact of AD-induced senescence on the overall activity of cancer cells. Therefore, our findings emphasize the importance of considering these multi-level mechanisms when evaluating the efficacy of anticancer drugs targeting cellular senescence as a means to inhibit tumor growth and progression.

Possible results 11,13,14,16, Despite AD's ability to affect the key cell cycle regulators p21 and p27, this impact has not been sufficient to trigger a comprehensive anticancer effect. Specifically, AD's performance in enhancing p21/p27 expression, inducing cellular senescence, and reducing cellular activity is limited, and it fails to effectively inhibit the overall proliferation of human esophageal cancer cells. This suggests that AD may only have a limited effect on certain aspects of cell cycle regulation while failing to effectively interfere with multiple crucial mechanisms related to cell proliferation, apoptosis, metabolism, immune evasion, and drug resistance. These mechanisms encompass the regulation of signaling pathways such as PI3K/Akt/mTOR and MAPK/ERK, alterations in epigenetic modifications, unique metabolic characteristics of cancer cells, activation of the immune system, as well as cancer cells' adaptability and resistance to drugs. Therefore, AD exhibits certain limitations in its anticancer effect, prompting the need for future exploration of multi-target, multi-pathway anticancer strategies that target these complex mechanisms to achieve more effective cancer treatment.

Possible results12,15,Andrographolide may inhibit cancer progression by regulating multiple key signaling pathways (such as PI3K/Akt/mTOR, MAPK/ERK, etc.), thereby affecting cell growth, metabolism, and stress response. Simultaneously, it plays a crucial role at the epigenetic level by modulating DNA methylation, histone modifications, and non-coding RNA expression, finely tuning the gene expression and biological behavior of cancer cells. Furthermore, andrographolide precisely targets the metabolic characteristics of cancer cells, such as enhanced glycolysis and glutamine metabolism, weakening their survival capabilities. Notably, it also acts as an activator in the immune system, promoting the activation and proliferation of immune cells while optimizing the tumor microenvironment to enhance immune killing of cancer cells. Additionally, the synergistic effects of andrographolide with other anticancer drugs add a new dimension to its anticancer efficacy. In conclusion, the anticancer mechanism of andrographolide constitutes a complex, multi-dimensional, and multi-layered network. Its comprehensive exploration will pave new avenues for cancer treatment and bring renewed hope for patient recovery.

Discussion of concentration and treatment duration results

Under the premise of keeping all other treatment conditions constant, increasing the concentration of AD may lead to three distinct outcomes: Firstly, an enhancement of its inhibitory effect on cancer cells, as higher concentrations of drug molecules may bind more effectively to target sites within the cancer cells, thereby triggering stronger biological effects. Secondly, the inhibitory effect may remain unchanged, which could occur when the concentration reaches a saturation point or when there is an

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upper limit to the cell's response to the drug. Thirdly, the inhibitory effect may diminish, possibly due to cytotoxic reactions caused by high concentrations of the drug, resulting in a conflict between cell death mechanisms and the intended anticancer mechanism, or because the drug, at specific concentrations, binds non-specifically to non-target molecules within the cell, thereby weakening its specific anticancer action.

Furthermore, extending the duration of AD administration can also yield three different effects: Firstly, as the duration of drug exposure increases, its inhibitory effect on cancer cells accumulates gradually, leading to a stronger anticancer effect. Secondly, the inhibitory effect may stabilize at a plateau after a certain point in time and no longer increase significantly with further extension of exposure, possibly because the cells have developed some degree of adaptation or resistance to the drug. Thirdly, prolonged drug exposure may trigger compensatory mechanisms or repair processes within the cells, resulting in decreased sensitivity to the drug and consequently weakening its anticancer effect.

5. Conclusion

In conclusion, the effects of AD on human esophageal cancer cells are discussed. The present findings will provide an idea of whether AD can treat esophageal cancer. The observed therapeutic effects will be able to provide suitable indications for future clinical and drug development targeting human esophageal cancer.

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