Effectiveness of 12-octadecenoic acid methyl ester, an active ingredient in Kansui, in inhibiting non-small cell lung carcinoma cells potential

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Abstract

Lung cancer is widespread in China and has many new cases and deaths. There are about 450000 new cancer patients in China every year. Non-small cell lung cancer (NSCLC) accounted for 80%. Previous studies have found that T33, a TCM formula, has the function of treating cancer by inducing autophagy of Colorectal cancer. Therefore, his study aims to determine the ability of 12-octadecenoic acid methyl ester (an active ingredient in T33) to induce autophagy of A549 non-small cell lung cancer to expand the available treatment methods for lung cancer patients. This study will measure cell killing by MTT in vitro and tumor size reduction of A549 xenografts in vivo and measure autophagy by LC3-A/B western blot. The positive control is taxol, and negative control is PBS/DMSO. In this study, by observing the size of cancer cell tissue and the number of LC3-A/B, we can determine whether 12-octadecenoic acid methyl ester can successfully induce autophagy of NSCLC and thus generate the potential for the treatment of NSCLC. The results of this study will provide some potential theoretical basis for the principle of traditional Chinese medicine in treating cancer.

Keywords: 12-octadecenoic acid methyl ester, Non-small cell lung cancer (NSCLC), Autophagy, LC3-B

1. Introduction

Lung Cancer was the most common cause of death from cancer with more than 1.38 million deaths worldwide [1]. The data from the International Agency for Research of Cancer (IARC) show that in 2020 approximately 4,560,000 people were newly diagnosed with lung cancer and 450,000 died of it in China [2]. Non-small cell lung cancer (NSCLC) accounts for the 80% of all lung cancers. Its main types are: adenocarcinoma (including BAC) 32-40%, squamous 25-30%, large cell 8-16% [3]. Traditional Chinese medicine has been widely used in the treatment of non-small cell lung cancer in China, with Solanum nigrum as the representative [4]. Traditional Chinese medicine has the advantages of less adverse reactions in the treatment of non-small cell lung cancer, but its efficiency is low.

Autophagy is a physiological process of cells, which can degrade and eliminate damaged organelles and wrong proteins. Autophagy is a process in which cells degrade part of their own components through lysosomes. Autophagy is related to many pathological processes, and inducing autophagy of cancer cells is an effective method for treating cancer at present. Recent studies have shown that the occurrence and treatment of cancer are related to autophagy [5,6]. Autophagy can be selective or non-selective, and its mechanism is controlled by a series of proteins.LC3-B is a marker in autophagy, with 125 amino acid residues. LC3-B is modified from LC3-A and is the structural protein of autophage. LC3-B/LC3-A ratio can reflect the level of autophagy.LC3-B on the inner and outer membrane of autophagosomes and has the function of binding with degradation substrate .Mature autophagosomes can combine with lysosomes to play an autophagic role.

12-octadecenoic acid methyl ester ($C_{19}H_{36}O_2$) is a biologically active substance isolated from an ingredient in Kansui. Kansui is a kind of root tuber, which is often used as a traditional Chinese medicine to treat edema and epilepsy.Traditional Chinese medicine T33,where kansui is one of the effective ingredients, has been proved to have the effect of treating cancer through inducing cancer cells autophagy[7]. Therefore, 12-octadecenoic acid methyl ester has certain anticancer potential (see Figure 1).

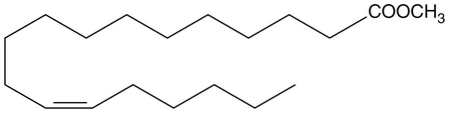

Figure 1. Cis-12-octadecenoic acid methyl ester [8]

This article aims to investigate whether 12-octadecenoic acid methyl ester could induce autophagy of cancerous cells to achieve the effect of tumor inhibition and treatment.

Hypothesis: I predict 12-octadecenoic acid methyl ester could induces autophagy in A549 non-small cell lung carcinoma cells to play an anticancer role.

2. Methods and materials

2.1 Reagents

The 12-octadecenoic acid methyl ester will be purchased commercially.<https://www.sigmaaldrich.com/US/en/product/supelco/46951u>

2.2 Cell lines

The A549 non-small cell lung cancer (NSCLC) will be purchased commercially.

2.3 Cell culture

NSCLC samples will be cultured in ACL4 which is plused 5% FBS, SCCRh 1171, R10, and HITES plus 2% FBS [9]. The cell will be kept at 8% CO_2 and 90% O_2. The temperature will be around 37℃. Culture medium will be changed three times per week.

CCD-18Co cells will be cultured with Dulbecco's modified Eagle's medium (DMEM) .The cell will be kept at 8% CO2 and 90% O2 . The temperature will be around 37℃.Culture medium will be changed three times per week.

2.4 Laboratory animals

30 male and female BALB/c Nude mice from 4 weeks old will be uesd for studies. All mice will be housed under suitable sunlight, temperature, humidity, and given regular rations of food and water. All mice will be under specific pathogen-free conditions and allowed to acclimate for ten days prior to experimentation [10]. All animal breeding and experiments will comply with the guidelines of relevant authorities.

2.5 MTT assay

5000 cells were seeded on a 96-well plate and cultured in a 5% CO2, 37°C cell culture incubator. 12-octadecenoic acid methyl ester were added and cultivated for about 30 h under the same conditions. Add 0.2 ml 0.5% MTT and incubate for 4 hours. Aspirate the culture medium in the wells, add 100-150 μl DMSO to each well, and shake on a shaker at low speed for 10 minutes. Measure the absorbance of each well at OD470nm in an enzyme-linked immunosorbent assay instrument [11].

2.6 Trans-well migration assay

Using a trans-well migration assay kit with 8.0-μm pores bought commercially. For the upper chamber, different concentrations of 12-octadecenoic acid methyl ester and 4×10^4 cells will be seeded in the serum-free medium. Fill the lower chamber with medium containing 10% FBS and cultured for 24 hours. Then, 10% neutral buffered formalin will be added to fix the migrated cells. Next, stain with 0.5% crystal violet for 15 minutes. A computerized microscope will be used to capture images. Finally, 5 different fields of view were randomly selected to observe and count, and the average value was taken. Can be repeated three times.

2.7 Immunoblotting

Antibodies related to autophagy can be detected by immunoblotting, such as antibodies against Atg5, Atg7 and Beclin-1. First, the nitrocellulose membrane was incubated with 2.5% BSA and a certain amount of antibody in PBS for 2 hours, and horse radish peroxidase (HRP)-conjugated antibody was added and incubated for another 1 hour. Finally, antigen-antibody complexes are detected with a special chemiluminescent substrate (Millipore, USA) and the blot is quantified using a densitometer.

2.8 Immunofluorescence staining

A LC3B Antibody Kit will be used for autophagy detection. Briefly, human NSCLC sample cells were cultured on coverslips and fixed with 4% paraformaldehyde. After 10 minutes of permeabilization with Triton X-100, the picks were soaked in blocking solution. After hybridization with LC3-B antibody, wait for incubation with conjugated secondary antibody and DAPI. Finally, observe and record with a fluorescence microscope.

2.9 Animals and tumor xenografts

Human NSCLC samples will be injected subcutaneously into mice to generate xenogeneic tumors. When the tumors grow to a size close to 60 mm, the mice will be randomly divided into three groups of 10 mice each. The three groups are control group, low-dose group and high-dose group, which are administered intragastrically with 1X PBS 200mg/kg 12-octadecenoic acid methyl ester and 600mg/kg 12-octadecenoic acid methyl ester respectively. The experiment will last for five weeks, and the tumor volume in all mice will be continuously measured and calculated every week. Mice were finally euthanized by carbon dioxide asphyxiation. After visual death of the mouse, continue to circulate carbon dioxide for one minute to ensure the death of the mouse. Finally, tumor size will be measured.

2.10 Statistics

All experiments will be repeated at least three times. The statistical significance of all numerical data acquired from MTT assay, Trans-well migration assay, immunoblotting, immunofluorescence staining and animal studies will be analyzed using student's T-test. A p-value less than 0.05 will be significant.

3. Results

3.1 The survival and growth of A549 cancer cells

The results that are beneficial to the treatment of 12-octadecenoic acid methyl ester will be defined as that when 12-octadecenoic acid methyl ester inhibits the survival and growth of cancer cells at the previously indicated concentration, reaching a level similar to that of paclitaxel, and the performance is significantly superior to that of PBS treatment. When 12-octadecenoic acid methyl ester cannot inhibit the migration of cancer cells, the inhibition degree is similar to that of paclitaxel, but the performance is similar to that of PBS treatment, 12-octadecenoic acid methyl ester will not be favored.

3.2 Metastasis Xenograft Model

The results beneficial to the treatment of 12-octadecenoic acid methyl ester will be defined as that when 12-octadecenoic acid methyl ester inhibits the tissue size of cancer cells at the previously indicated concentration, reaching a level similar to that of paclitaxel, and the performance is significantly better than that of PBS treatment. When 12-octadecenoic acid methyl ester cannot inhibit the migration of cancer cells, the inhibition degree is similar to that of paclitaxel, but the performance is similar to that of PBS treatment, 12-octadecenoic acid methyl ester will not help.

3.3 Expression of substances related to autophagy of A549 cancer cells

The results beneficial to the treatment of 12-octadecenoic acid methyl ester will be defined as whether the ratio of LC3-B/LC3-A and the number of Atg5, Atg7 and Beclin-1 proteins in 12-octadecenoic acid methyl ester at the previously indicated concentration increase to the same extent as that of paclitaxel, while the performance is significantly better than that of PBS treatment. When 12-octadecenoic acid methyl ester cannot inhibit the migration of cancer cells, the inhibition degree is similar to that of paclitaxel, but the performance is similar to that of PBS treatment, 12-octadecenoic acid methyl ester will not help.

3.4 Combination of Possible Results (PR)

Table 1. Possible combination of results

		Increased killing of cells by MTT	Decreased tumor size in xenografts	Increased autophagy by LC3-A/B	Support Hypothesis?
Possible Results (PR)	1	+	+	+	yes
	2	+	+	-	P
	3	+	-	+	P
	4	+	-	-	P
	5	-	+	+	P
	6	-	+	-	P
	7	-	-	+	P
	8	-	-	-	NO

Note. A "+" indicated a positive result where 12-octadecenoic acid methyl ester performs similarly to that of taxol. A "-" indicates a negative result where 12-octadecenoic acid methyl ester fails to perform similarly to taxol treatment and instead performs similarly to PBS streatment. A "P" indicates partial support where 12-octadecenoic acid methyl ester either fails to reduce invasion, reduce migration, or the metastasis of A549 lung cancer cells in vivo (see Table 1).

4. Possible Results

12-octadecenoic acid methyl ester may be favored for all models of measuring anticancer potential or may not be favored for all models of measuring anticancer potential (PR1 and PR8, respectively).

12-octadecenoic acid methyl ester may also be shown to be effective in vitro, but partially effective (PR2 and PR3) or but completely ineffective in vivo (PR4).

12-octadecenoic acid methyl ester may also be not conducive to the display of anticancer potential in vitro, but it is conducive to the treatment of cancer in vivo (PR5).

Another possible result is that 12-octadecenoic acid methyl ester is not conducive to the potential of anticancer activity in vitro and the expression of autophagy-related proteins, but can effectively inhibit the size of A549

cancer cells (PR6).

On the contrary, 12-octadecenoic acid methyl ester is not conducive to the potential of anticancer activity in vitro and can't effectively inhibit the size of A549 cancer cells, but the autophagy-related protein can be expressed (PR7).

PR1 fully supports the hypothesis that 12-octadecenoic acid methyl ester can be treated by inducing autophagy of non-small cell lung cancer. Because 12-octadecenoic acid methyl ester can effectively inhibit the survival and growth of A549 non-small cell lung cancer through MTT assay. Moreover, in vivo experiments, 12-octadecenoic acid methyl ester can reduce the volume of A549 non-small cell lung cancer cell tissue, indicating that it has a positive effect on cancer treatment. In addition, the LC3-B/LC3-A ratio can effectively reflect whether autophagy is induced. The expression of Atg5, Atg7 and Beclin-1 proteins is also related to autophagy. Therefore, the increase in the proportion of LC3-B/LC3-A and the increase in the number of proteins such as Atg5, Atg7 and Beclin-1 suggest that the inhibition of A549 non-small cell lung cancer is related to the induced autophagy.After that, p62/SQSTM1 protein can be detected for further research to further confirm the relationship between 12-octadecenoic acid methyl ester and induced autophagy.

PR2 partially proves this hypothesis. It can be seen that 12-octadecenoic acid methyl ester can inhibit the growth of A549 non-small cell lung cancer cells in vitro and also affect the size of non-small cell lung cancer cell tissue in vivo. However, the proportion of LC3-B/LC3-A Atg5, Atg7 and Beclin-1 protein quantity, these parameters related to autophagy, did not increase. This may indicate that 12-octadecenoic acid methyl ester has the potential to treat non-small cell lung cancer, although it has no ability to induce autophagy of non-small cell lung cancer cells.

PR3, PR5 and PR7 partially prove this hypothesis. These three proteins successfully expressed Atg5, Atg7 and Beclin-1 and other proteins related to autophagy. 12-octadecenoic acid methyl ester in PR3 has inhibitory effect on A549 NSMCLC in vitro, and the protein related to autophagy is indeed expressed, but it has no effect on the volume of cancer cell tissue in vivo. The 12-octadecenoic acid methyl ester in PR5 does not help inhibit the growth of non-small cell lung cancer cells in vitro, but is beneficial to inhibit the volume of non-small cell lung cancer cells in vivo. While PR7 only has the expression of autophagy-related protein, but has no effect on the activity of cells in vivo and in vitro. At this time, 2-octadecenoic acid methyl ester is not suitable for the treatment of patients with non-small cell lung cancer. This may be due to the inappropriate dose in vivo and in vitro studies, and subsequent studies can be conducted by adjusting the concentration. Then judge whether it is a qualified treatment.

PR4 and PR6 partially prove this hypothesis. Both did not increase the number of proteins related to autophagy such as Atg5, Atg7 and Beclin-1, and the proportion of LC3-B/LC3-A. 12-octadecenoic acid methyl ester in PR4 has inhibitory effect on A549 NSMCLC in vitro, but has no effect on tumor tissue volume in vivo. The 12-octadecenoic acid methyl ester in PR6 does not help inhibit the growth of non-small cell lung cancer cells in vitro, but is beneficial to inhibit the volume of non-small cell lung cancer cell tissue in vivo. This indicates that 12-octadecenoic acid methyl ester may have therapeutic function for non-small cell lung cancer. Later, it is necessary to change the concentration and other parameters to further study the effect of 12-octadecenoic acid methyl ester on non-small cell lung cancer in vivo and in vitro. And detect whether the number of p62/SQSTM1 protein is reduced to further judge the relationship with autophagy.

PR8 completely refutes the hypothesis that 12-octadecenoic acid methyl ester can achieve therapeutic purposes by inducing autophagy of non-small cell lung cancer cells, which is the least expected result of the eight combinations. Because 12-octadecenoic acid methyl ester did not inhibit the growth of non-small cell lung cancer cells in vivo and in vitro, and the number of protein expression related to autophagy did not significantly increase. This statement contradicts all assumptions.

5. Discussion

The current research has made great progress in the research of lung cancer and non-small cell lung cancer, but most of the treatment methods are based on the research of western medicine. After T33 was confirmed by recent studies to have the effect of inducing autophagy of colon cancer cells, 12-octadecenoic acid methyl ester as its active ingredient has the potential to treat non-small cell lung cancer. This study is conducive to the expansion of treatment methods for non-small cell lung cancer, and also conducive to the further study of traditional Chinese medicine.

For PR1, both in vivo and in animal experiments, 12-octadecenoic acid methyl ester has been proved to be as effective as paclitaxel in inhibiting cancer cells and inducing autophagy of cancer cells. This has achieved the purpose of this experiment. Later, we can further verify that 12-octadecenoic acid methyl ester can inhibit the expansion of cancer tissue by inducing autophagy of NSCLC cells by changing the concentration of 12-octadecenoic acid methyl ester and the type of cell line to achieve the purpose of treatment.Preclinical experiments such as pharmacological tests can also be performed

subsequently to verify its potential as a medicine. The effect of dose on its action can also be continued to be investigated.

For PR3, 5 and 7, it has been proved that they can induce autophagy of NSCLC cells, but they only partially or completely do not inhibit the growth of NSCLC cells.

This may be because the time of in vivo and in vitro tests is too short, and the number of cancer cells is not enough, so the results of in vivo and in vitro tests are inconsistent with expectations. In addition, it is also possible that cancer cells avoid being cleared by T cells through autophagy [12], which needs to be verified by subsequent tests, such as testing the concentration of cytochrome c. It is also possible to change the animals used in the subsequent experiments to investigate the results of xenografts.

For PR2, 4 and 6, 12-octadecenoic acid methyl ester shows the ability to kill NSCLC, but it is not achieved by inducing NSCLC autophagy. This may be due to the error in testing LC3-A/B, which can be eliminated by multiple tests. It can also be tested the concentration of Atg5, Atg7 and Beclin-1, which are also indicators to verify autophagy. If it is the same result, it shows that 12-octadecenoic acid methyl ester has the potential to become a drug for NSCLC lung cancer, but the mechanism of producing therapeutic effect is not as predicted. The mechanism can be verified by subsequent experiments.

For PR8, this shows that 12-octadecenoic acid methyl ester has no potential to become a drug for treating NSCLC.

In general, the presence of PR1, 3, 5 and 7 can completely indicate that 12-octadecenoic acid methyl ester can inhibit NSCLC by inducing NSCLC autophagy, and the presence of PR2, 4 and 6 indicates that 12-octadecenoic acid methyl ester has the potential to become a drug for the treatment of NSCLC, while the presence of PR8 is not the desired result.

6. Conclusion

In conclusion, this paper studied the effect of 12-octadecenoic acid methyl ester on A549 non-small cell lung cancer cells and explored the potential of 12-octadecenoic acid methyl ester to inhibit the metastasis of A549 non-small cell lung cancer cells in vitro and in vivo. The research results show that whether MART-10 can increase the expression of the ratio of LC3-B/LC3-A and the number of Atg5, Atg7 and Beclin-1 proteins, reduce the volume of non-small cell lung cancer tissue in vivo, potentially induce autophagy of non-small cell lung cancer, and act on the treatment of non-small cell lung cancer treatment. The observed anti-metastasis effect indicates the potential of 12-octadecenoic acid methyl ester in the treatment of small cell lung cancer in the future.

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