Inhibition of Lung Adenocarcinoma Growth by using Oridonin

Zhongrui Jiang

Abstract

The abnormal activity of the Notch signal pathway is a common manifestation of many cancerous cells. Therefore, blocking the notch pathway of cancer cells positively affects cancer treatment. Because oridonin directly acts on the target notch receptor 1-4 cancer cells, inhibiting the Notch signaling pathway can inhibit the growth of cancer cells.

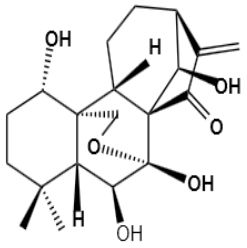
Keywords: oridonin, lung adenocarcinoma, notch pathway

1. Introduction

Notch pathway plays an important role in a variety of cancer cells. The abnormal transcription of notch1-4 receptor protein in cancer cells has been confirmed to be related to the growth regulation and metastasis of cancer cells [1]. Oridonin, as a product extracted from Rabdosia, has been reported to have positive effects on diabetes and gout [2]. It can also interact with notch1 protein in cancer cells to inhibit the growth of cells [3]. However, whether only notch1 protein is the effective target of oridonin is still unknown. In this article, we will discuss whether oridonin has a role in notch3, another receptor protein of the notch pathway.

2. Literature Review

Chinese traditional medicine has always been an important treasure of human drug sources. Some single active ingredients extracted from microorganisms, animals and plants have incredible biological characteristics and structure diversity. Extracting active components from traditional Chinese medicine has also become an effective method to discover new drugs. The 2015 Nobel Prize in Physiology or Medicine emphasized that the drug molecules extracted from natural products can produce good resistance to some of parasites [4]. Oridonin (Figure 1), molecular formula C20H28O6, is a kind of natural terpenoid with biological activity that exists in Rabdosia plants from the Labtea family. Rabdosia rubescens has the functions of clearing heat, detoxifying, activating blood circulation and relieving pain. It has been used as a herb by Chinese people for thousands of years to treat sore throat and snakebite. Oridonin has shown the potential effect of anti-inflammatory [5] treating diabetes and gout [6]. In recent years, with the deepening research on oridonin, people found that it has great value as an anti-cancer drug. At present, it is known that oridonin has a variety of anticancer activities against many common cancers, such as breast cancer, rectal cancer, gastric cancer, leukemia and many cancers [7]. But at the same time, the research on the toxicity of oridonin is still not deep enough, and there is still no accurate answer to whether oridonin will produce toxicity under certain conditions in the academia (Figure 1).



Oridonin

Figure 1: The chemical structure of Oridonin

In a modern society where resources are gradually satisfied, cancer is an important part of the cause of death of the population. Among them, lung cancer is the most common cancer. In China, lung cancer accounts for 20.3% of all cancers, and the female incidence rate reaches 12.6% [8]. Even in today's more perfect medical conditions and technology, the five-year survival rate of lung cancer patients after surgery is still at a low level. At the end of the twentieth century, the overall incidence rate of lung cancer showed a downward trend, which was related to the improvement of cigarette production technology and the awareness of early smoking cessation. Among all lung cancers, lung adenocarcinoma is undoubtedly the disease with the highest incidence rate and the highest mortality rate. It is a very common primary lung cancer, accounting for 40% of all lung cancer patients [5]. The first three stages of lung adenocarcinoma are characterized by leprosy growth, usually with tumor cells arranged on the alveolar wall. With the progress of cell fibrosis, alveolar collapse and differentiation, tumors will develop in a more solid and huge direction [9]. When dealing with early lung adenocarcinoma, surgical

resection can play a good therapeutic effect, and the specific survival rate after resection of tumors with certain characteristics can reach over 90%. Radiotherapy is also one of the rapidly developing cancer treatments in the past decade, but it cannot be denied that this treatment method will cause a lot of irreversible harm to the patient. In addition, drug also has a good effect, such as' cisplatin 'can effectively slow down the development of cancer, or even completely kill cancer cells.

Notch pathway was first discovered in the study of drosophila mutants in 1910. In the study, 'Haploinsufficiency of NOTCH' caused notches at the end of the wing of the fruit fly, and it would not cause a fatal effect on the life of the D. melanogaster mutants. notch pathway has been proved to be an ancient and highly conservative signaling pathway in organisms. In 1991, notch genes were first found to be associated with human T cell acute lymphoblastic leukemia (T-ALL). In 1997, it was found that Alagille syndrome (AGS) was associated with notch ligand JAG1 [10]. So far, people gradually have found that notch signaling pathway is widely associated with human diseases. In later studies, people found that notch pathway is associated with many cancers. At present, the research interest in the role of notch signaling system in cancer is increasing day by day. There are four receptor proteins notch1-4 that are commonly reported in cancer cells. Their corresponding genes often mutate in cancer cells. We can find changes in notch receptors in various cancers, and the activation mode of notch is different in different cancer cells. For example, notch can be activated by upstream signals or structural changes caused by its internal mutations [11,12].

3. Hypothesis

Therefore, blocking the notch pathway of cancer cells has a positive effect on cancer treatment. The treatment with increasing concentrations and for various duration with oridonin may also inhibit the growth of A549 lung adenocarcinoma that significantly decrease notch3 expression by acting on notch3 transcription. We will perform MTT assay, QRT-PCR and western blot for notch3 (also looking for notch3 cleavage by western). The positive control of cell experiment is using DAPT to block the notch pathway, and the negative control is NS (normal saline). I predict Oridonin can inhibit the growth of lung adenocarcinoma by inhibiting the notch3 mRNA transcription and notch3 expression.

3.1. *Material*

1.cell lines:LUAD cell lines A549
2.MTT Assay Kit (Cell Proliferation)
3.TRIzol reagent (Invitrogen)
4.PrimeScript RT Kit
5.SDS-PAGE
6.notch 3 protein,notch 3 protein primary antibody,notch 3 protein secondary antibody

3.2. *Cell culture*

A549 cells are grown in a 96-well microplate (200 l aliquots each well) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, and antibiotics (penicillin 100 U/ml and streptomycin 100 U/ml) at 37℃in a humidified atmosphere with 5% CO2 and 95% air incubator and subculture by harvesting with 0.25% (w/v) trypsin and 0.1% (w/v) EDTA. For following experimental procedures, each culture was generated in triplicate.

A549 cells will subject to 10ɥM, 100ɥM, 1000ɥM oridonin supplied at the beginning (0h) and culture cells for 12 hours to evaluate the influence of oridonin. Each experiment will be repeated three times at different concentrations. The positive control of cell experiment is using 5ɥM DAPT to block the notch pathway, and the negative control is NS groups.

4. Method

4.1. *MTT assay*

After 12 hours of cell culture, we dilute the cells to a certain concentration. According to the manufacturer's instructions, cells with a certain concentration are accurately inject into the kit. After three hours of culture, MTT solution is added and continue to culture for four to six hours. After that, remove all the solutions and add dimethyl sulfoxide to dissolve the sediment. Set zero adjustment hole and control hole, read the absorbance at OD 570nm to judge the cell activity [13].

4.2. *RNA isolation and quantitative reverse transcription PCR(QRT-PCR)*

Use TRIzol reagent to extract all RNA from LUAD cells (cells treated with oridonin, DAPT and normal saline), and then use PrimeScript RT kit to reverse transcribe the RNA to obtain cDNA. After PCR amplification, the system ABI7500 was used for QRT-PCR reaction to detect the relative expression level of genes [14].

4.3. *Western blot*

Break LUAD cells A549, centrifuge to obtain protein. After rough treatment, use 10% SDS-PAGE to separate them to obtain protein samples, then transfer the protein to PVDF membrane, use notch3 primary antibody and secondary antibody to label notch3 protein, and finally analyze the membrane [15].

5. Experiment Results:

5.1. *MTT assay*

Possible Result 1: decrease the proliferation of lung adenocarcinoma cells.

Oridonin has a variety of inhibition pathways to lung adenocarcinoma. One is to inhibit notch signal pathway, which interferes cancer growth and metastasis by inhibiting notch ligand protein.

Possible Result 2: Number of lung adenocarcinoma cells remain the same after treating the highest concentration of oridonin.

At the test concentration, oridonin does not affect the normal growth and reproduction of lung adenocarcinoma cells, and there is no significant difference in the number of cells between lung adenocarcinoma and the control group.

5.2. *QRT-PCR*

Possible Result 1: transcription of notch 3 mRNA between experimental group and control group has no significant difference, having little effect on the transcription of notch3 mRNA.

mRNA transcription of lung adenocarcinoma cells, which treated at the test concentration is not significantly different from that of the control group. Oridonin had no prominent effect on inhibiting the mRNA transcription of notch3 protein.

Possible Result 2: the transcription of notch3 mRNA is significantly reduced.

After treating cells at certain concentration, the decrease of mRNA transcription level indicates that the transcription of notch3 protein is reduced after treatment with oridonin, thus the notch signal pathway is significantly restricted.

5.3. *Western blot:*

Possible Result 1: the concentration of notch3 protein decreased with the increase of oridonin concentration to treat cells.

The expression of notch3 is dose-dependent on the concentration of oridonin. With the increase of drug concentration, the concentration of effective notch3 protein is lower. It can be determined that oridonin inhibits notch3 to block notch signaling pathway and regulate cell growth.

Possible Result 2: no direct relationship between the concentration of notch3 protein and the concentration of oridonin is found

Under the test concentration gradient, the change of oridonin concentration has no direct relationship with the concentration of notch3. To some extent, oridonin has little effect on inhibiting the expression of notch3, and its effect on notch signal pathway is still unknown (Table 1).

Table 1: Combination of Possible Results (CR)

Possible observations	CR 1	CR 2	CR 3	CR 4	CR5	CR6	CR7	CR8
Decreased proliferation by MTT?	+	+	+	+	-	-	-	-
Decreased transcription of notch 3 protein by qRTPCR?	+	-	+	-	+	+	-	-
Decreased cleavage of Notch by WB?	+	+	-	-	+	-	+	-
Supporting Hypothesis ?	**YES**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**NO**

NOTE: "+"represent the closer to the positive control group,far from the NC group
"-"represent the closer to the NC group,far from the positive control group

6. Discussion

In many previous studies on oridonin, it has been shown that oridonin has a dose-dependent effect on the treatment of tumors [16]. However, in current research of anti-cancer

Chinese herbal medicine, the mechanism of most herbs is still unclear. The anti-cancer mechanism of oridonin, the most effective anti-cancer component extracted from Rabdosia rubescent, is still unclear, but some scholars have also provided many hypotheses to explain its anti-cancer mechanism according to their experimental results.

CR1 shows that oridonin can block notch signal by inhibiting the expression of notch 3 to inhibit tumor cells growth. It totally supports the Hypothesis, and the experiment results are exactly what we think. On the premise that oridonin is effective, analyze the transcription level of notch 3 mRNA in oridonin treated cells and the content of notch 3 protein in actual cells can determine whether oridonin has an impact on the expression of notch 3 protein. In this ideal situation, oridonin as a small molecule may activate some other recipient to inhibit the transcription or it has its way to get in the cell to affect some process. This situation indicates the connection of oridonin and the transcription of notch3 mRNA but the mechanism of how it affects the transcription remains unknown.

In CR8, oridonin has no effect in the highest concentration. This is the exact opposite of the hypothesis. This situation may be related to the dose dependence of cancer cells. oridonin may have a better impact in the cells. The effect of oridonin increases as the concentration rises. Therefore, it can be considered to increase the dose and repeat the test.

Many documents point out that oridonin can affect the cancer cell growth in many different ways. It means we can see the decrease of living cells at least. So it is barely possible to get these data. This combination of results indicates that A549 cell lines the presence of a typical immune response towards foreign chemicals.

CR2 shows the decreased notch3 cleavage but the normal level of mRNA transcription. After the transcription, there is also a regulatory process in the protein translation. It is a complicated process requiring mRNA modification, transportation and translation. They require many other enzymes, and these proteins might form a feedback regulation to keep dynamic equilibrium between the mRNA and protein. There is a gap of time and space between mRNA transcription and notch3 expression. So, one explanation of this result is these series of reactions have not reached its' equilibrium and redundant mRNA is still degrading. It is totally reasonable if many mRNAs do not translate the matching mount of proteins.

CR3 shares a similar problem with CR2. Time and location of transcription and translation of genes are different, so it is possible that at the time of our detection, the peak mRNA has been degraded under the negative feedback regulation, but notch3 protein has not been reduced. The normal level of notch3 also indicates that notch pathway is not affected, and due to the good inhibition of oridonin, it is possible that there is another mechanism of how oridonin works or it affects other protein to block notch pathway. This result partly supports the hypothesis, but more data are needed to support the hypothesis.

Another explanation is the high concentration of oridonin causes some side effects and kills the cancer cells or the toxicity of oridonin in high concentration is fatal to cancer cells.

CR4 is the worst situation in my design. It shares some similarities with CR8. Barely any data can support the hypothesis. In this situation, oridonin has no effect on the transcription and notch3 expression but it can still inhibit the cell growth. It may indicate some defects in testing apparatus or method failure. Or there is another mechanism of how does oridonin work or it affects other proteins to block notch pathway.

CR5 and CR7 are similar that the lower cleavage of notch3 does not lead to the inhibition of cell growth. Some parts of result support the hypothesis. One possible reason is that notch pathway has compensatory mechanism to deal some fluctuation of receptor content. In this situation, low concentration of oridonin only has limited effects, but increase the concentration and retest may have a better result that support the hypothesis.

CR6 indicates that oridonin can inhibit the transcription of notch3 mRNA, but due to the reasons in CR3, notch3 proteins do not decrease. According to the hypothesis, the decreased level of notch3 lead to the inhibition of notch pathway. The result cannot tell us what will happen if notch3 decreases. So, I will take a longer time of cell culture, that may bring some difference.

7. Conclusion

In conclusion, this study explores the effect of oridonin in lung adenocarcinoma cells. The results can indicate whether oridonin can inhibit notch signaling pathway by affecting the expression of notch 3 protein, thereby inhibiting the growth of lung adenocarcinoma cells in vitro. This also provides a idea for the mechanism of oridonin in lung adenocarcinoma. Oridonin, as a new natural plant extract, undoubtedly has great potential in the direction of anti-tumor drug. The effects of oridonin observed in the experiment will provide suitable indication for the development of oridonin analogues in the future.

Reference

[1] Zhou, B., Lin, W., Long, Y., Yang, Y., Zhang, H., Wu, K., & Chu, Q. (2022). Notch signaling pathway: architecture, disease,

and therapeutics. Signal transduction and targeted therapy, 7(1), 95. https://doi.org/10.1038/s41392-022-00934-y

[2] He, H., Jiang, H., Chen, Y., Ye, J., Wang, A., Wang, C., Liu, Q., Liang, G., Deng, X., Jiang, W., & Zhou, R. (2018). Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. Nature communications, 9(1), 2550. https://doi.org/10.1038/s41467-018-04947-6

[3] Xia, S., Zhang, X., Li, C., & Guan, H. (2017). Oridonin inhibits breast cancer growth and metastasis through blocking the Notch signaling. Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society, 25(4), 638–643. https://doi.org/10.1016/j.jsps.2017.04.037

[4] Miller, L. H., & Su, X. (2011). Artemisinin: discovery from the Chinese herbal garden. Cell, 146(6), 855–858. https://doi.org/10.1016/j.cell.2011.08.024

[5] Wang, M., Xu, B., Liu, L., & Wang, D. (2022). Oridonin attenuates dextran sulfate sodiuminduced ulcerative colitis in mice via the Sirt1/NFκB/p53 pathway. Molecular medicine reports, 26(4), 312. https://doi.org/10.3892/mmr.2022.12828

[6] Mokhtari T. (2022). Targeting autophagy and neuroinflammation pathways with plant-derived natural compounds as potential antidepressant agents. Phytotherapy research : PTR, 36(9), 3470–3489. https://doi.org/10.1002/ptr.7551

[7] Li, X., Zhang, C. T., Ma, W., Xie, X., & Huang, Q. (2021). Oridonin: A Review of Its Pharmacology, Pharmacokinetics and Toxicity. Frontiers in pharmacology, 12, 645824. https://doi.org/10.3389/fphar.2021.645824

[8] Chen, W., Zheng, R., Baade, P. D., Zhang, S., Zeng, H., Bray, F., Jemal, A., Yu, X. Q., & He, J. (2016). Cancer statistics in China, 2015. CA: a cancer journal for clinicians, 66(2), 115–132. https://doi.org/10.3322/caac.21338

[9] Hutchinson, B. D., Shroff, G. S., Truong, M. T., & Ko, J. P. (2019). Spectrum of Lung Adenocarcinoma. Seminars in ultrasound, CT, and MR, 40(3), 255–264. https://doi.org/10.1053/j.sult.2018.11.009

[10] Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E., Rand, E. B., Piccoli, D. A., Hood, L., & Spinner, N. B. (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nature genetics, 16(3), 243–251. https://doi.org/10.1038/ng0797-243

[11] Zhou, B., Lin, W., Long, Y., Yang, Y., Zhang, H., Wu, K., & Chu, Q. (2022). Notch signaling pathway: architecture, disease, and therapeutics. Signal transduction and targeted therapy, 7(1), 95. https://doi.org/10.1038/s41392-022-00934-y

[12] Myers, D. J., & Wallen, J. M. (2022). Lung Adenocarcinoma. In StatPearls. StatPearls Publishing.

[13] Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of Cell Viability by the MTT Assay. Cold Spring Harbor protocols, 2018(6), 10.1101/pdb.prot095505. https://doi.org/10.1101/pdb.prot095505

[14] Zhang, M., Han, Y., Zheng, Y., Zhang, Y., Zhao, X., Gao, Z., & Liu, X. (2020). ZEB1-activated LINC01123 accelerates the malignancy in lung adenocarcinoma through NOTCH signaling pathway. Cell death & disease, 11(11), 981. https://doi.org/10.1038/s41419-020-03166-6

[15] Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. North American journal of medical sciences, 4(9), 429–434. https://doi.org/10.4103/1947-2714.100998

[16] Gui, Z., Luo, F., Yang, Y., Shen, C., Li, S., & Xu, J. (2017). Oridonin inhibition and miR200b3p/ZEB1 axis in human pancreatic cancer. International journal of oncology, 50(1), 111–120. https://doi.org/10.3892/ijo.2016.3772