The Inhibition of Human Epithelial Cells Proliferation by Oridonin through Interrupted Notch Signalling Pathway

Mingze Xu

Abstract

Since the Notch signaling pathway plays a vital role in cell proliferation, oridonin restrains cancer cell growth by limiting the Notch pathway. The study aims to investigate whether oridonin can kill normal epithelial cells similar to cancer cells in vitro. Also, the study aims to find whether the inhibition of Notch-1 by oridonin is on a transcriptional level. Methods: The study will use human epithelial cells (MCF10A) and breast cancer cells (MCF7). These two types of cells will be treated with various concentrations of oridonin (0.01mM, 0.1mM, 1mM, and 10mM) for a different amount of time (24h, 48h, 72h). The cell viability will be measured by WST-1 assay. Possible Results: There are eight possible results, three for the proliferation of epithelial cells and two for the transcriptional level of Notch-1 in epithelial cells. Normal epithelial cells experience a more significant loss of viable cells compared with cancer cells: (1) with decreased transcription and expression of Notch-1; (2) with decreased expression of Notch-1 but no decrease in transcription; (3) with a decrease in transcription but no decrease in expression of Notch-1 (4) with no decrease in expression and transcription of Notch-1. Normal epithelial cells experience a similar or less loss of viable cells compared with cancer cells, (5) with decreased transcription and expression of Notch-1; (6) with decreased expression of Notch-1 but no decrease in transcription; (7) with a decrease in transcription but no decrease in expression of Notch-1 (8) with no decrease in expression and transcription of Notch-1. Conclusion: The loss of normal cells is considered a trade-off for the anti-tumor activity of oridonin. If little or no damage to non-tumorigenic epithelial cells is detected, then oridonin may be a good cure for breast cancer with fewer side effects. Also, by investigating whether the inhibition of Notch-1 by oridonin is on a transcriptional level, we can gain more insights into the mechanism of inhibition.

Keywords: Oridonin, Epithelial cells, Breast cancer, Notch-1 receptor, Notch signalling pathway

1. Introduction

Oridonin is an active secondary metabolite found in *Rabdosia rubescens* (also known as *isodon rubescens*), a traditional Chinese medicinal plant. The structure of oridonin is shown below in figure 1. Historically, *R. rubescens* was commonly used to treat different types of inflammations, including tonsillitis and bronchitis, because of the anti-inflammatory activity of oridonin [1]. Oridonin can be obtained both from the plant and chemical synthesis [2]. In recent studies, the anticancer activity of oridonin has been investigated. It is shown that the growth of human breast cancer cells can be inhibited by oridonin by inhibiting the expression of Notch receptors 1-4 [3]. Breast cancer is one of the most common types of cancer. According to the Public Health Agency of Canada, breast cancer is the second most common cancer in Canadian women. Around 25% of new cases of cancer are breast cancer in Canada. Due to the high severity and lethality of breast cancer, researchers are currently working on possible solutions to inhibit the development and metastasis of breast cancer. Oridonin may be a potential cure for breast cancer due to its inhibition of the Notch signalling pathway. Abnormal notch signalling is related to cancer development [4]. For example, a high level of expression of the Notch1 receptor is detected in breast cancer, and the increased expression level is related to a lower survival rate of patients with breast cancer [5]. However, the Notch signalling pathway also plays a vital role in regulating cell proliferation and tissue homeostasis [6].

Using oridonin as a treatment for breast cancer may potentially interrupt the proliferation of other normal cells due to the impaired Notch signalling pathway. For example, the Notch signalling pathway plays an essential role in the fate decisions of intestinal epithelial cells. Suppressing the Notch pathway may cause abnormal cell proliferation and differentiation. As a result, the compositions of cells in intestinal epithelium will be changed, and the digestive and protective functions of epithelium will be compromised [7]. After inhibiting the Notch pathway by removing the transcription factor CSL/RBP-J, most intestinal stem cells are converted into goblet cells in mice. Due to this conversion, there is a significant loss of proliferative intestinal cells [8]. So, it is predicted that inhibiting the Notch pathway may have deleterious

effects on epithelial cell proliferation.

To date, there has been little research on the impact of oridonin on the apoptosis, proliferation and viability of normal non-tumorigenic epithelial cells. Therefore, this study aims to investigate the proliferation of human epithelial cells after the treatment of oridonin using WST-1 assay. At the same time, breast cancer cells will receive the same treatment. Their proliferation will also be measured using WST-1 assay to determine whether oridonin damages more cancer cells or normal epithelial cells. Moreover, the expression of NOTCH1 will be analyzed using RT-qPCR and western blot. If the viability of human epithelial cells and expression of NOTCH1 decreases, then it can be concluded that oridonin inhibits the proliferation of normal cells through the inhibition of Notch receptors. Also, the mRNA level of the gene encoding NOTCH 1 will be analyzed through RT-qPCR to determine whether oridonin's inhibition is on a transcriptional or translational level. It is predicted that treatment with increasing concentrations and for various durations with oridonin will reduce NOTCH1 expression and kill normal epithelial cells similar to cancer cells.

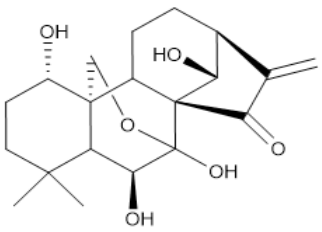

Figure 1. Structure of oridonin.

Hypothesis: It is predicted that treatment with increasing concentrations and for various durations with oridonin will reduce NOTCH1 expression and kill intestinal stem cells similar to cancer cells.

2. Materials and Methods

Materials:

- MCF10A cell line
- MCF7 cell line
- DMEM/F12
- Insulin
- Hydrocortisone
- 5% horse serum
- Cholera toxin
- Oridonin
- N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
- WST-1 Reagent
- KiCqStart™ One-Step Probe RT-qPCR ReadyMix™
- Microplate reader
- PureLink RNA Mini Kit
- RIPA buffer
- mPAGE™ 4X Sample Buffer
- DTT
- PVDF membrane
- NOTCH1 monoclonal antibody (mN1A, mouse IgG1)
- Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488

Methods:

2.1 *Growth medium for human epithelial cells and breast cancer cells*

Human epithelial cells (MCF10A) and human breast cancer cells (MCF7) are used in this experiment. MCF10A is grown in DMEM/F12 medium, with human recombinant epidermal growth factor (20ng/mL), insulin (10μg/mL), hydrocortisone (0.5μg/mL), 5% horse serum, and cholera toxin (100ng/mL). The breast cancer cells (MCF7) are grown in DMEM/F12 medium with 5% horse serum and insulin (10μg/mL)

The breast cancer cells and normal epithelial cells are divided into four experimental groups and two control groups, respectively, with 12 wells for each treatment group (60000 cells per well). For experimental groups, 0.1mL of 0.01mM, 0.1mM, 1mM, and 10mM of oridonin is added to the cell growth media. The cells will be incubated in each experimental group with oridonin for 24h, 48h, and 72h, respectively. For the positive control group, 0.1mL of 1mM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a known Notch receptor inhibitor, is added to the cell growth media. For the negative control group, 0.1mL of PBS is added. The cells are cultured at 37ºC and 5% CO_2 for 24h, 48h, and 72h, respectively. The cells are harvested after incubation, and their viability and expression level of Notch 1 is analyzed [9,10].

2.2 *Cell viability analysis: WST-1 Assay*

According to WST-1 assay protocol, cell proliferation reagent WST-1 (10μL/well) is added to every well in each treatment group. Furthermore, the cells are incubated at 37ºC, 5% CO_2, for 4 hours. Using a microplate reader, the absorbance of cell culture is measured at 440 nm. A single cell well with only the growth medium and WST-1 is used to black the microplate reader.

2.3 *Expression of Notch 1 receptor: Quantitative RT-PCR*

The total RNA of ISCs is extracted with PureLink RNA Mini Kit. Using the OligoArchitect™ program, the primer specific to the Notch 1 gene is designed. The total RNA is added to KiCqStart™ One-Step Probe RT-qPCR ReadyMix™ with the designed gene-specific primer.

Following the protocol of the one-step RT-qPCR, the mRNA of the Notch 1 receptor is reversely transcribed into cDNA, then the expression level of the Notch 1 receptor is detected.

2.4 *Expression of Notch 1 receptor: Western Blot*

Protein is extracted from cells using RIPA buffer following the manufacturer's protocol. The extracted protein is separated with SDS-PAGE (2.5μL mPAGE™ 4X Sample Buffer, 1M DTT). Notch 1 protein has a molecular weight of 270 kDa, and the part of the gel containing 270 kDa protein is cut. Target proteins are transferred onto the PVDF membrane in the 1X transfer buffer, using a sandwich of two pieces of filter paper with polyacrylamide gel and PVDF membrane in the middle. The electric field is applied to facilitate protein transfer.

The PVDF membrane containing the target protein is washed with 5% nonfat dry milk for 1 hour to avoid any non-specific binding of the antibody to the membrane. NOTCH1 monoclonal antibody (mN1A, mouse IgG1) is diluted in a blocking solution. The PVDF membrane is incubated with the antibody for 1 hour at room temperature. Secondary antibodies (Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, 5μg/mL) are added to the membrane. The Invitrogen™ Alexa Fluor 488 dye is excited with a 488nm laser line. Then, using a fluorescence microscope, the intensity of light emitted at 525nm is measured.

2.5 *Statistics*

All experiments are repeated for three times with different cells. The triplicate data gathered from WST-1 Assay, RT-qPCR and Western blot are reported as mean value and SEM. A student's t-test is applied to analyze the significance of the results ($p<0.05$).

3. Results

3.1 *The proliferation of human epithelial cells*

Possible Result 1: Oridonin causes a decrease in viable cell numbers of human epithelial cells, and the decrease is minor compared with cancer cells.

Compared with the negative control group, which receives no oridonin, the proliferation of epithelial cells is interrupted by oridonin in the experimental group. So, the numbers of viable cells in experimental groups are smaller according to WST-1 assay. However, the number of viable cancer cells experiences a more significant decrease than normal epithelial cells.

Possible Result 2: Oridonin causes a decrease in viable cell numbers of human epithelial cells, but the decrease is more significant compared with cancer cells.

Compared with the negative control group, which receives no oridonin, the proliferation of epithelial cells is interrupted by oridonin in the experimental group. So, the numbers of viable cells in experimental groups are smaller according to WST-1 assay. However, the number of viable cancer cells experiences a smaller decrease than normal epithelial cells.

Possible Result 3: Oridonin causes no change in viable cell numbers of human intestinal stem cells, and the number of cancer cells is decreased

The WST-1 assay shows no change in the number of viable cells in the experimental group compared with the negative control. Oridonin fails to inhibit the proliferation of epithelial cells, but it can inhibit the growth of cancer cells.

Possible Result 4: Oridonin causes an increase in viable cell numbers of human intestinal stem cells, and the number of cancer cells is decreased.

The WST-1 assay shows an increase in the number of viable cells in the experimental group compared with the negative control. Oridonin fails to inhibit the proliferation of epithelial cells, but it can inhibit the growth of cancer cells.

3.2 *Effect of oridonin on the transcription level of Notch-1*

Possible Result 5: Oridonin causes a decrease in Notch 1 receptor mRNA level

The data from RT-qPCR indicates a decrease in Notch 1 receptor mRNA level. Expression of the Notch 1 receptor is limited by oridonin on the transcriptional level.

Possible Result 6: Oridonin causes no change in Notch 1 receptor mRNA level

The data from RT-qPCR indicates no change in Notch 1 receptor mRNA level. Expression of the Notch 1 receptor is not limited by oridonin on the transcriptional level.

Possible Result 7: Oridonin causes an increase in Notch 1 receptor mRNA level

The data from RT-qPCR indicates an increase in Notch 1 receptor mRNA level. Expression of the Notch 1 receptor is not limited by oridonin on the transcriptional level.

3.3 *Effect of oridonin on the expression of Notch-1*

Possible Result 8: Oridonin causes a decrease in Notch 1 receptor expression level

According to Western Blot results, the Notch 1 receptor expression is decreased. It can be caused by either a transcriptional or translational level limitation due to oridonin.

Possible Result 9: Oridonin causes no change in Notch 1 receptor expression level

Oridonin fails to limit the transcription or translation of genes encoding the Notch 1 receptor. As a result, there is no change in the expression of the Notch 1 receptor.

Possible Result 10: Oridonin causes an increase in Notch 1 receptor expression level

Oridonin fails to limit the transcription or translation of genes encoding the Notch 1 receptor. As a result, there is an increase in the expression of the Notch 1 receptor.

3.4 *Effect of time and concentration of oridonin treatment*

Possible Result 11: Inhibition of cell proliferation, mRNA level and Notch-1 expression do not change with the increase of concentration and treatment time with oridonin.

Possible Result 12: Inhibition of cell proliferation, mRNA level and Notch-1 expression increase with the increase of concentration and treatment time with oridonin.

Possible Result 13: Inhibition of cell proliferation, mRNA level and Notch-1 expression decrease with the decrease of concentration and treatment time with oridonin.

3.5 Possible result combination

Possible Result Combination 1: Normal epithelial cells experience a more significant loss of viable cells compared with the cancer cell, with decreased transcription and expression of Notch-1.

Possible Result Combination 2: Normal epithelial cells experience a more significant loss of viable cells compared with cancer cell, with decreased expression of Notch-1 but no decrease in transcription.

Possible Result Combination 3: Normal epithelial cells experience a more significant loss of viable cells compared with cancer cell, with decrease in transcription level but no decrease in expression of Notch-1.

Possible Result Combination 4: Normal epithelial cells experience a more significant loss of viable cells compared with cancer cell, with no decrease in expression and transcription of Notch-1.

Possible Result Combination 5: Normal epithelial cells experience a similar or less loss of viable cells compared with cancer cells, with decreased transcription and expression of Notch-1.

Possible Result Combination 6: Normal epithelial cells experience a similar or less loss of viable cells compared with cancer cells, with decreased expression of Notch-1 but no decrease in transcription.

Possible Result Combination 7: Normal epithelial cells experience a similar or less loss of viable cells compared with cancer cells, with decrease in transcription but no decrease in expression of Notch-1.

Possible Result Combination 8: Normal epithelial cells experience a similar or less loss of viable cells compared with cancer cells, with no decrease in expression and transcription of Notch-1.

Table 1. The possible combination of results

Possible observation	1	2	3	4	5	6	7	8
normal cell proliferation similar to cancer cells by WST1?	+	+	+	+	-	-	-	-
Reduced normal cell transcription level of Notch 1 similar to cancer cells by RT-qPCR?	+	-	+	-	+	+	-	-
Reduced normal cell protein level of Notch 1 similar to cancer cells by WB?	+	+	-	-	+	-	+	-
Supporting Hypothesis?	**YES**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**Partially**	**NO**

Note. "+" means results similar to positive control (DAPT). "-" means results similar to negative control (PBS)

4. Discussion

Previous studies have shown that the inhibition of the Notch signalling pathway can influence the homeostasis of epithelial cells, including the renewal of damaged epithelial cells, epithelial cell differentiation and proliferation, and maintenance of stem cells. For example, exposure to cigarette smoke extracts, an inhibitor of Notch-1, can significantly lower the signalling activation of Notch-1 and slow down the replacement of bronchial epithelial cells. Oridonin, as a potential cure for breast

cancer, limits the development of cancer cells through inhibition of Notch 1-4 [3]. So, it is predicted that increasing amounts and treatment durations of oridonin can damage normal epithelial cells and cancer cells at the same time.

Possible Result Combinations 1 and 5 mainly focus on the trade-offs of using oridonin as an inhibitor of cancer growth. Current cancer treatment methods, including chemotherapy and radiation therapy, are facing the same problem: normal proliferative cells may be damaged as a side effect. This study compares the number of viable cells between normal epithelial cells and breast cancer cells. Because, to date, no evidence points out that oridonin has specificity towards cancer cells, it is expected that normal epithelial cells will experience the same level or even more damage than cancer cells. Cell division speed can be much faster in cancer cells, so normal epithelial cells may experience a more significant loss of viable cells compared with cancer cells, as shown in possible result combination 1. This result combination is similar to the positive control, the Notch expression is inhibited, and leads to impaired normal cell proliferation. The hypothesis is fully supported by the result combination 1.

If the possible result combination 5 is observed, the normal epithelial cells experience more negligible damage than cancer cells, hypothesis is partially supported. Then it can be concluded that oridonin may have potential preferability on inhibiting cancer cells. This result indicates that oridonin may be a potential cure for breast cancer, but further experiments on the metabolism and toxicity of oridonin in human bodies need to be carried out. Hypothesis is partially supported.

Comparing the Possible Result Combination 2 with Combination 3, these two results mainly focus on whether the inhibition of Notch-1 is on a transcriptional or translational level. The Possible Result Combination 2 reveals that the inhibition of Notch-1 by oridonin is not on a transcriptional level because the mRNA level of Notch-1 is not decreased according to the result of RT-qPCR. At the same time, the expression level of Notch-1 is decreased according to the result of the western blot. This possible result combination suggests that oridonin may inhibit Notch-1 on a translational level. The mechanism of translation inhibition is unknown. Further research needs to be done to determine the mechanism, whether the protein folding, or synthesis is restrained by oridonin. Hypothesis is partially supported.

The possible result combination 3 reveals that although the transcription of Notch-1 is inhibited, the expression remains the same in intestinal epithelial cells. This can be due to enhanced Notch-1 translation. The increase in the translation compensates the decrease in transcription of Notch-1. Also, because the expression of Notch-1 stays the same while cell proliferation is decreased, it can be concluded that the impaired cell proliferation is not due to Notch-1, hypothesis is partially supported.

According to previous research, all of Notch 1-4 are downregulated in breast cancer cells after treatment with oridonin[3]. It is unclear which Notch receptor plays a significant role in the proliferation of cancer cells. The Possible Result Combination 4 illustrates that oridonin inhibits the growth of intestinal epithelial cells but not through the Notch-1 pathway. In this possible result combination, the transcriptional level and expression of Notch-1 are not limited by oridonin. The inhibition of breast cancer cells is due to other reasons, including Notch 2-4. Hypothesis is partially supported.

The Possible Result Combination 6 and 7 investigate whether the expression of Notch-1 is on a transcriptional level. In result combination 6, mRNA level of Notch-1 is decreased while the expression remains the same. This result is similar to result combination 3, but the normal cells are not damaged. Hypothesis is partially supported. In result combination 7, the mRNA level of Notch-1 is not inhibited, but the expression of Notch-1 is decreased. It can be concluded that some other reasons lead to the improper translation or folding of Notch-1, but further research is needed to find out the exact reason. Hypothesis is partially supported.

The Possible Result Combination 8 entirely refutes the hypothesis. The proliferation of normal epithelial cells is not interrupted by oridonin, and the expression of the Notch-1 receptor is not inhibited in normal cells. Under this condition, if breast cancer cells are successfully inhibited, it can be concluded that oridonin is a possible cancer cure with no significant side effects, at least to the epithelial cells

If the duration increases and negative result stays at negative, we are confident that the negative is not a “fake negative”. If there is same cell death with increasing oridonin amount, then it can be concluded that it is something other than oridonin which kill the cells.

5. Conclusion

In conclusion, this study investigates oridonin’s interruption of non-tumorigenic epithelial cell proliferation. The loss of normal cells is considered as a trade-off for the anti-tumor activity of oridonin. So, the growth inhibition of cancer and normal cells is compared in this study to determine whether the benefits outweigh the disadvantages when considering oridonin as a potential cancer cure. Moreover, the general inhibition mechanisms of Notch-1 by oridonin are researched. The result would indicate whether this inhibition of Notch-1 is

on a transcriptional level.

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