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Investigation of the Relationship Between NADK and RSL3 and Exploration of Potential Usage of RSL3: Potential Applications of RSL3: Insights from Its Relationship with NADK

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Abstract: Lung cancer remains the leading cause of cancer-related deaths, and ferroptosis has emerged as a potential therapeutic target due to its role in tumor suppression. RSL3, a GPX4 inhibitor, and NADK knockdown both promote ferroptosis, but their relationship in lung cancer treatment remains unexplored. Cell viability was assessed using the MTT assay, ferroptosis was measured via the BIPOC assay, and NADK levels were analyzed by Western blot. Positive controls included H_2O_2 for NADK, erastin for ferroptosis, and taxol for cell viability, while PBS served as the negative control. Eight possible experimental outcomes were identified, each offering different insights into the effects of RSL3 on ferroptosis and NADK levels. The results determine whether RSL3 reduces NADK in addition to promoting ferroptosis, which could impact future combination therapy strategies. This study examines whether RSL3 reduces NADK while inducing ferroptosis in A549 cells, potentially eliminating the need for separate NADK-reducing drugs. If no direct effect on NADK is observed, a combination of RSL3 and NADK inhibitors could be considered to enhance ferroptosis-based cancer therapy.

Keywords: NAD kinase, RSL3, LUAD

1. Introduction

Lung cancer is the second most common malignant tumor in the world, and its mortality continues to rank first among all cancers. According to statistics, approximately 1.81 million people die from lung cancer each year, which is the second leading cause of cancer death. [1] Notably, the incidence of lung adenocarcinoma has surpassed that of lung squamous cell carcinoma (Roy-Chowdhuri, 2021; Ruiz-Cordero and Devine, 2020) [2,3], and exposure to air pollutants such as PM2.5 has been significantly associated with the development of lung adenocarcinoma in nonsmokers (Hill et al., 2023). [4] In view of the major threat to human health, gaining an in-depth understanding of its pathogenesis and developing new clinical intervention strategies has become a global medical problem.

Ferroptosis is a recently discovered type of programmed cell death that occurs when there is an imbalance in the cell's redox balance. Iron depletion and lipophilic free radical antioxidants such as ferrostatin 1, lipostatin 1, vitamin E, or ubiquitin inhibit the process of cell death. There are two main mechanisms. One is iron-dependent lethal mechanism, which is not completely equivalent to

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ferroptosis, and ferroptosis may be related to lysosomal toxicity. The second is the iron-independent oxidative stress mechanism. [5]

In lung cancer, FSP1 and GPX4 play crucial roles in reducing lung cancer sensitivity to ferroptosis by enhancing the cell's antioxidant defenses. GPX4 counteracts ferroptosis by reducing lipid peroxides, while FSP1 uses NAD(P)H and oxidized CoQ10 to produce NAD(P)+ and reduced CoQ10, thus preventing ferroptosis. [6]

It is already known that knockdown of NADK promotes LUAD ferroptosis via the NADPH/FSP1axis (Meng et al., 2024), which can suppress cancer cells in the lungs. [7]

RSL3 induces ferroptosis by inhibiting GPX4, thereby disrupting the cellular antioxidant system. [8,9] Although there is currently no direct evidence suggesting that RSL3 directly targets NADK. it may indirectly regulate NADK activity by altering the cellular oxidative stress environment and metabolic pathways, potentially enhancing the cell's antioxidant capacity. [10] However, no relevant studies have been published to date

As an inhibitor of GPX4, RSL3 can suppress ferroptosis but may activate the ferroptosis process in a completely different way compared to the FSP1 signaling pathway. The amount of NADK shouldn't logically change. [11] However, if we hypothesize that RSL3 can reduce the amount of NADK, achieving the same effect as knocking down NADK, then not only would RSL3 promote ferroptosis, but the amount of NADK would decrease, leading to ferroptosis that kills cancer cells. This would show that RSL3 has the effect of both promoting ferroptosis and reducing NADK, eliminating the need for doctors to treat patients with both RSL3 and NADK-reducing drugs.

If there is no direct effect of RSL3 on the amount of NADK, doctors could use both methods—RSL3 and NADK reduction—to potentially increase the overall effect through drug combination.

2. Hypothesis

I predict that increasing concentrations and treatment durations of RSL3 kills A549 cells through ferroptosis and does not to elevate NADK levels.

3. Methodology

In this study, we aimed to evaluate the effects of various treatments on NADK expression, BIPOC levels, and cell viability. To assess these effects, cells were treated with positive controls (H_2O_2 for NADK, erastin for BIPOC, and taxol for cell viability) and a negative control (PBS). H_2O_2 was used to induce an increase in NADK levels, while erastin served as a positive control for BIPOC induction, and taxol was used to decrease cell viability. The negative control, PBS, represented baseline conditions. Experimental treatments were applied in varying concentrations, and the effects on NADK expression were measured through Western blot and qPCR analysis, BIPOC levels were assessed using immunofluorescence, Western blot, or qPCR, and cell viability was determined using MTT [12], CellTiter-Glo, or Trypan blue exclusion assays.

Our sample is A549 cells, a human lung adenocarcinoma cell line. The negative control is phosphate-buffered saline (PBS), and ferroptosis inducers such as erastin as a positive control and taxol, a low-toxicity anti-cancer agent. The concentration of RSL3 ranging from 1 to 10 μM, with treatment durations varying from 6 to 48 hours.

For the MTT assay, the condition for incubating cell is 37°C, with 5% CO_2 and approximately 90% humidity.

The changes in measurements were interpreted relative to the positive and negative controls. The result indicates the measurement changes in the direction indicated in the column header similar to the positive control (H2O2 for NADK, erastin for BIPOC, taxol for viability) and the opposite to the negative control in PBS. - indicates the measurement changes in the opposite of the direction indicated

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in the column header similar to the negative control (PBS) and the opposite to the positive control ((H2O2 for NADK, erastin for BIPOC, taxol for viability). Statistical significance was determined using ANOVA or t-tests to compare the effects of various treatments. This experimental design aimed to reveal potential modulators of NADK, BIPOC, and cell viability through comparison with established control conditions.

4. Results

Table 1: Possible results of the above-mentioned comparative experiments

Combination of possible results (CR)	RSL3 decreases viability by MTT assay	RSL3 increases ferroptosis by BIPOC FACS assay	RSL3 decreases NADK levels by western	Support hypothesis
CR1	+	+	+	Fully yes
CR2	+	+	-	Partial
CR3	+	-	+	Partial
CR4	-	+	+	Partial
CR5	-	-	+	Partial
CR6	+	-	-	Partial
CR7	-	+	-	Partial
CR8	-	-	-	Fully no

+ indicates the measurement changes in the direction indicated in the column header similar to the positive control (H2O2 for NADK, erastin for BIPOC, taxol for viability) and the opposite to the negative control in PBS. - indicates the measurement changes in the opposite of the direction indicated in the column header similar to the negative control (PBS) and the opposite to the positive control ((H2O2 for NADK, erastin for BIPOC, taxol for viability).

In total, there are 8 possible results, arranged in certain sequence, I will show the first group which fully supports hypothesis, and left groups consist of partial support and fully contradict to hypothesis.

Result 1 (CR1)

*A*s the amount of RSL3 increasing, the survival rate of the A549 cells was reflected to be low. This is a significant phenomenon which can be seen in the MTT assay. Additionally, according to the BIPOC FACS assay, the rate of ferroptosis with RSL3 treatment was increasing when raising the usage of RSL3. And lastly, The Western blot analysis has demonstrated that with RSL3 treatment, there was a decreasing trend in NADK levels in A549 cells.

Other groups show different results with same experiments. I won't elaborate on all possible results further here.

RSL3 treatment on A549 cells can be influenced by both concentration and duration. If RSL3 functions solely through GPX4 inhibition without directly affecting NADK, increasing treatment concentration or duration may not significantly alter NADK levels, as observed in the Western blot analysis. However, if RSL3 indirectly regulates NADK expression, extended treatment or higher doses might lead to a measurable decrease in NADK levels, reinforcing the hypothesis that RSL3 promotes ferroptosis through both GPX4 inhibition and NADK downregulation. In some cases, prolonged exposure to RSL3 may induce cellular resistance, potentially diminishing the effectiveness of ferroptosis induction at higher treatment durations. Moreover, RSL3 may exhibit nonlinear effects, where lower concentrations produce a moderate ferroptosis response, while higher concentrations enhance this effect; however, excessively high concentrations might shift the cell death mechanism toward apoptosis or necrosis rather than ferroptosis alone.

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5. Discussion

Previous research has shown that knockdown of NADK can promote ferroptosis to kill cancer cells. At the same time, RSL3, as an inhibitor of GPX4, can also induce ferroptosis to kill cancer cells. NADK influences the production of NADPH, which is closely related to the synthesis of GSH. We are unsure if there is a relationship between the amount of NADK and the use of RSL3. If there is a proportional relationship, it would mean that RSL3 has a better effect than a single treatment for knocking down NADK. In this case, using a combination of the two drugs would maximize the effect.

As a result, this research aims to demonstrate that increasing concentrations and treatment durations of RSL3 will lead to decreased viability, as indicated by the MTT assay, an increased rate of ferroptosis, as shown by the BIPOC FACS assay, and a decrease in NADK levels, as indicated by Western blot analysis.

Combination of Results (CR1) shows that RSL3 influences tumor cells through the ferroptosis pathway by affecting GPX4, a key signal in the ferroptosis pathway. This aligns with previous studies. Furthermore, the experiment also shows that RSL3 can decrease NADK levels, promoting ferroptosis. The possible reasons for this phenomenon are RSL3-induced ferroptosis increases oxidative stress by inhibiting GPX4, leading to excessive lipid peroxidation and subsequently triggering protein degradation. Oxidative stress can result in the degradation of various proteins, including NADK, through proteasomal or lysosomal pathways. Additionally, RSL3-induced oxidative stress may activate stress-responsive transcription factors (e.g., NRF2 inhibition or p53 activation), leading to the downregulation of NADK gene expression.

In the future, scientists could test the combination of these two drugs on animal models, such as gorillas, which share a similar structure with humans. This could help assess the efficacy and determine the appropriate dosage for the combination therapy.

Combination of Results (CR2) shows that RSL3 influences tumor cells through the ferroptosis pathway by affecting GPX4, an important signal in the ferroptosis pathway. However, the experiment contradicts the hypothesis that RSL3 can decrease NADK levels, which would lead to ferroptosis. The decrease in viability observed in the MTT assay supports our first hypothesis—that RSL3 can inhibit LUAD. The increase in ferroptosis observed in the BIPOC FACS assay supports our second hypothesis—that LUAD inhibition is due to increased ferroptosis caused by RSL3. However, the increase or no change in NADK levels, observed in the Western blot analysis, contradicts our third hypothesis. This could suggest that the pathways of RSL3 and NADK do not affect each other and may even be inversely proportional. The possible reasons why this phenomenon occur are due to different cell types responding, and some may have regulatory mechanisms that maintain NADK expression or protein stability. For example, some cancer cells may rely on NADK for survival and thus actively preserve its levels. Additionally, some cells may have compensatory mechanisms to protect NADK from degradation or transcriptional downregulation. For instance, cells with high NRF2 activity may upregulate antioxidant responses, counteracting the oxidative stress.

Therefore, combining the two drugs in treatment becomes crucial to offset any negative effects on ferroptosis rates.

Combination of Results (CR3) shows that RSL3 influences tumor cells through the ferroptosis pathway by affecting GPX4, an important signal in the ferroptosis pathway. However, ferroptosis was not significantly affected by RSL3. This may be due to RSL3 not solely affecting cell growth through the induction of ferroptosis. RSL3 could also exert additional effects by inhibiting GPX4, which leads to changes in other cellular processes such as suppressing cell proliferation, disrupting the cell cycle, or initiating other forms of cell death, such as apoptosis or necrosis. These effects may obscure the role of ferroptosis under certain conditions. The experiment also showed that RSL3 can decrease NADK levels, promoting ferroptosis. The decrease in viability, as shown in the MTT assay,

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supports our first hypothesis. However, the decrease in ferroptosis, as indicated by the BIPOC FACS assay, contradicts our second hypothesis. The decrease in NADK levels, as shown by the Western blot analysis, supports our third hypothesis. In the future, it would be worth testing the combination of the two drugs on animal models to see if both drugs lead to cancer cell death in vivo, such as in gorillas, which share a similar structure with humans.

Combination of Results (CR4) shows that RSL3 cannot influence tumor cells through the ferroptosis pathway by affecting GPX4, an important signal in the ferroptosis pathway. However, it can still promote ferroptosis. This may be due to different cancer cells exhibiting varying responses to RSL3. Some cancer cells may develop resistance to RSL3-induced effects, particularly under conditions of external stress. These cells may employ alternative mechanisms to prevent ferroptosis, such as upregulating antioxidant enzymes like GPX4 or modulating iron metabolism. As a result, they may fail to exhibit the cell death typically induced by RSL3. However, ferroptosis at the cellular level could represent an independent process that can still be triggered in these cell types. The experiment also shows that RSL3 can decrease NADK levels, promoting ferroptosis. The increase in viability, as indicated by the MTT assay, contradicts our first hypothesis. The increase in ferroptosis, as shown by the BIPOC FACS assay, supports our second hypothesis. Finally, the decrease in NADK levels, as shown by the Western blot analysis, supports our third hypothesis. Future studies could change the type of cancer cells tested to see if RSL3 can promote ferroptosis in other types of cancer cells.

Combination of Results (CR5) shows that RSL3 cannot influence tumor cells through the ferroptosis pathway by affecting GPX4, an important signal in the ferroptosis pathway, due to the lack of an effect on ferroptosis. This result might be due to different types of cancer cells exhibiting varying responses to RSL3. Some cancer cells may develop resistance to RSL3-induced effects, particularly under conditions of external stress. These cells may employ alternative mechanisms to prevent ferroptosis, such as upregulating antioxidant enzymes like GPX4 or modulating iron metabolism. As a result, they may fail to exhibit the cell death typically induced by RSL3. Additionally, the experiment proves that RSL3 can decrease the amount of NADK, which could lead to ferroptosis. The increase in viability observed in the MTT assay contradicts our first hypothesis, which states that RSL3 inhibits LUAD. The decrease in ferroptosis, as indicated by the BIPOC FACS assay, contradicts our second hypothesis, which suggests that LUAD inhibition occurs through the induction of ferroptosis by RSL3. However, the decrease in NADK levels, as shown by the Western blot analysis, supports our third hypothesis. In the future, we could test the effect of the combination of the two drugs on animals, such as gorillas, which have similar structures to humans, to assess the efficacy and determine the appropriate dosage of the combination treatment.

Combination of Results (CR6) shows that RSL3 influences tumor cells through the ferroptosis pathway by affecting GPX4, an important signal in the ferroptosis pathway. However, ferroptosis was not significantly affected by RSL3. The reason for this result might be that RSL3 does not only affect cell growth through the induction of ferroptosis; it may also have additional effects, such as inhibiting GPX4, which leads to alterations in other cellular processes, including the suppression of cell proliferation, disruption of the cell cycle, or the initiation of alternative forms of cell death, such as apoptosis or necrosis. These effects could obscure the role of ferroptosis under certain conditions. This finding highlights the importance of combining both RSL3 and NADK-reducing drugs in treatment to mitigate any potential negative effects on ferroptosis rates.

Combination of Results (CR7) shows that RSL3 cannot influence tumor cells through the ferroptosis pathway by affecting GPX4. On the other hand, it can still drive for ferroptosis. Different types of cancer cells exhibiting manifold responses to RSL3 might result in this result. Some cancer cells may develop resistance to RSL3-induced effects. Alternative mechanisms might act on this cell to prevent ferroptosis, such as upregulating antioxidant enzymes like GPX4 or modulating iron

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metabolism. As a result, they may fail to exhibit the typical cell death induced by RSL3. However, ferroptosis is an independent process that can be triggered in these cell types. This further suggests that RSL3 and NADK may not interact in the same signaling pathway, which can show the potential value of combining both treatments to offset any negative effects on ferroptosis.

Combination of Results (CR8) shows that RSL3 cannot influence tumor cells through the ferroptosis pathway by affecting GPX4, a significant signal in the ferroptosis pathway, as no effect on ferroptosis was observed. Different types of cancer cells exhibiting manifold responses to RSL3 might result in this result. Some cancer cells have possibilities to develop resistance to RSL3-induced effects. In addition, alternative mechanisms might act on this cell to prevent ferroptosis, for instance, upregulating antioxidant enzymes like GPX4 or modulating iron metabolism. As a result, they may fail to exhibit the typical cell death induced by RSL3. Moreover, the experiment demonstrates that RSL3 can decrease the amount of NADK, which can drive for ferroptosis. The increase in viability observed in the MTT assay contradicts our first hypothesis, which states that RSL3 inhibits LUAD. The decrease in ferroptosis, as indicated by the BIPOC FACS assay, contradicts our second hypothesis. The increase or no change in NADK levels, as shown by the Western blot analysis, contradicts our third hypothesis, suggesting that the two substances operate within separate signaling pathways and do not affect each other. This reinforces the importance of combining both RSL3 and NADK-reducing treatments to counteract any negative effects on ferroptosis rates.

6. Conclusion

Overall, this study investigates the effect of RSL3 on A549 cells. It also examines whether RSL3 functions through the ferroptosis pathway by knocking down NADK to promote the death of cancer cells. The results may indicate whether combining RSL3 with NADK knockdown drugs is valuable. If the hypothesis is supported, RSL3 would not only promote the ferroptosis process but also decrease the amount of NADK, leading to ferroptosis and the subsequent killing of cancer cells. This would demonstrate that RSL3 has the effect of both promoting ferroptosis and reducing NADK, eliminating the need for doctors to treat patients with both RSL3 and NADK-reducing drugs.

If there is no direct effect of RSL3 on the amount of NADK, doctors could use both methods—RSL3 and NADK reduction—to potentially increase the overall effect through drug combination. In the future, scientists could test the combination of these two drugs on animal models, such as gorillas, which share a similar structure with humans. This could help assess the efficacy and determine the appropriate dosage for the combination therapy.

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