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Evaluating the Efficacy of Astragalus Polysaccharide (APS) in Treating Lung Cancer (LUAD) via Inhibition of the Wnt/β-Catenin Signaling Pathway

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Abstract: Lung cancer is the characterization of a tumor that develops in the lungs, and it is one of the leading causes of death globally. Lung Adenocarcinoma (LUAD) is one of the most prevalent lung cancers. The Wnt/β-Catenin signaling pathway controls cell proliferation, and abnormalities in the pathway have been associated with cancer development and progression. A variety of polysaccharides have been proven to contain anti-tumor effects through the indirect killing of tumor cells via cell signaling pathway regulation. Astragalus polysaccharide (APS) is the most active component of *Astragalus membranaceus*, a traditional Chinese medicine. Due to its low toxicity, APS is a prime candidate in exploring the inhibition of cellular pathways such as Wnt/β-catenin. However, the effects of APS on tumor cells are not fully understood. This study seeks to examine the effects of APS in inducing cytotoxicity and apoptosis in A549 cells from the LUAD cell line. To understand the mechanism of APS on tumor cells, various assays will be employed, such as MTT, Annexin V tracing, and western blots for the cleavage of Poly (ADP-ribose) Polymerase (PARP) protein and the presence of β-Catenin. The results of the study will determine the mechanisms of APS, and its potential application as a treatment for cancer.

Keywords: Lung Adenocarcinoma, *Astragalus membranaceus,* Wnt/β-Catenin signaling pathway

1. Introduction

Lung cancer is one of the most prevalent causes of death worldwide. In 2020, 1.8 million deaths related to lung cancer occurred, which accounted for approximately 11.4% of total cancer cases and 18.0% of total cancer deaths worldwide [1]. Due to light symptoms, only 15% of patients are diagnosed while cancer progression was at an early stage, resulting in a high mortality rate [2].

Two major forms of lung cancer are non–small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Out of these two forms, NSCLC is more prevalent, with 85% of all lung cancers being NSCLC (Herbst et al., 2008). The most prevalent NSCLC is Lung Adenocarcinoma (LUAD), which accounts for 40% of all lung cancers and occurs in both smokers and non-smokers regardless of age or gender [3]. Cancer arises from cancer stem cells (CSCs), and studies suggest that CSCs are prone to chemoresistance and recurrence in lung cancer patients [4]. Currently, there has been much research targeting CSCs. Specifically, studies have shown that targeting signaling pathways such as

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Wnt/β-catenin could potentially inhibit biomarkers correlated with CSCs, thereby preventing cancer [4].

The Wnt/β-Catenin signaling pathway is also known as the canonical Wnt pathway. Though it mainly controls cell proliferation, it also regulates tissue self-renewal in mammals [5]. It is activated when extracellular Wnt ligands bind to cell membrane receptors, which prevents the breakdown of β-catenin, and allows β-catenin to be transferred to the nucleus, ultimately causing the activation of target genes that facilitate cell proliferation and survival [6]. Studies on Wnt signaling have been steadily growing, as mutations in the pathway have been linked to various diseases. This connection suggests that the Wnt/β-Catenin signaling pathway holds strong potential as a therapeutic target for disease treatment [6].

Astragalus membranaceus is also known as 'Huangqi". It is a type of Chinese traditional medicine known for its ability to "invigorate Qi and raise Yang," and has been used in treatment for cardiovascular diseases [7]. The main component of *A. membranaceus* is Astragalus polysaccharide (APS), which possesses the potential to develop a drug that can improve or treat various diseases [8]. APS is a water-soluble heteropolysaccharide with bioactive properties, and it is extracted from the stems or dried roots of *A. membranaceus* [9]. Polysaccharides, known as natural immune regulators, are widely applied in the clinical management of tumors [10]. At present, their anti-tumor effects primarily involve two aspects: indirectly killing tumor cells by influencing cell signaling pathways, or directly affecting them by promoting differentiation or triggering apoptosis in tumor cells [11]. Due to its low toxicity, APS is a prime candidate in exploring the inhibition of cellular pathways such as Wnt/β-catenin, along with the induction of apoptosis. Currently, APS shows promising effects in binding to β-catenin and preventing its translocation into the cell, therefore preventing β-catenin build-up and inhibiting growth and proliferation of tumor cells [12].

Based on the high mortality rate of lung cancer and the potential of APS as a cancer treatment, this study would like to further explore the effects of APS inducing cytotoxicity of A549 cells from the LUAD cell line.

2. Hypothesis

This study hypothesizes that increasing concentrations and treatment durations of APS induces cytotoxicity of A549 cells through apoptosis and inhibition of Wnt/β-catenin.

3. Materials and methods

Apoptosis will be measured through Apoptosis staining, by tracing Annexin V through difference in fluorescence using Flow Cytometry Activated Cell Sorting (FACS), and by using western blot to observe cells undergoing apoptosis through analyzing the cleavage of Poly(ADP-ribose) Polymerase (PARP) protein by western blot. β-catenin activation will also be measured through a western blot for the phospho-β-Catenin (Ser675) antibody.

APS will be administered in increasing dosages in cells, starting at 10 μM and increasing by 10 μM each time, ending at 50 μM. After APS is administered, the cells will incubate at time points of 0 hours, 4 hours, 12 hours, 16 hours, and 24 hours.

3.1. MTT assay

The MTT assay is a colorimetric assay used to assess cellular metabolic activity by assessing the reduction of tetrazolium dye (MTT) to purple formazan crystals by mitochondrial dehydrogenases. mitochondrial dehydrogenases are only present when the cell is viable, so MTT will not convert to purple crystals when the cell is undergoing apoptosis. Thus, the stronger the purple color present, the

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more viable the cells are, and likewise, the gradual decrease of purple across samples with different concentrations of APS could show an increase in cytotoxicity levels.

To conduct the assay, a MTT Cell Proliferation Assay Kit can be used. First, cells need to be plated in 96-well plates. APS will be added to columns of wells in increasing concentration, with columns of positive control, taxol, and negative control, PBS, administered to cell wells without the presence of APS. Then, MTT will be added to each well. The plate will be incubated in a dark environment due to MTT's light sensitivity for 4 hours. After incubation, MTT should be converted into purple formazan crystals in viable cells. To dissolve the crystals, Dimethyl sulfoxide (DMSO) will be added to each well, which will spread the purple color evenly in the well. Finally, data can be generated from a plate reader that will characterize the concentration of purple color in each well. As taxol induces apoptosis, the positive control wells containing taxol should be clear, whereas the negative control wells containing PBS, which will do nothing to cells, should have a purple color. If purple color lightens as APS concentrations increase, the assay would support the hypothesis, as the color would show that cell viability decreases.

3.2. Annexin V staining

To determine whether necrotic or apoptotic cells are present in a sample, Annexin V staining can be employed. The human vascular anticoagulant annexin V is a phospholipid-binding protein with a high affinity for a phospholipid named phosphatidylserine (PS). PS is normally located in the inner leaflet of the plasma membrane, however, during apoptosis, it is transferred to the extracellular side of the plasma membrane to facilitate cell recognition and phagocytosis by macrophages. In order to determine necrotic cells in a sample and the progression of apoptosis, both annexin V and Propidium Iodide (PI) can be monitored. Annexin V can bind to PS throughout apoptosis, but ceases to bind to PS as the cell membrane destabilizes in a necrotic cell. PI is a fluorescent stain that possesses the capability to enter late-stage apoptotic and necrotic cells.

To conduct the study, the Dead Cell Apoptosis Kit with Annexin V FITC and PI can be used. The kit uses recombinant annexin V linked to green-fluorescent FITC dye and PI that will stain late-stage apoptotic/necrotic cells with red fluorescence. Thus, after the kit is administered, cells beginning apoptosis will show green fluorescence, cells at late-stage apoptosis will show both red and green fluorescence, necrotic cells will show red fluorescence, and live cells will show little or no fluorescence.

To administer the study, first, a plate of A549 cells from the LUAD cell line need to be cultured. At the beginning of the study, the cell culture media should be removed from all wells on the plate, and cells should be washed with phosphate buffered saline (PBS). Three groups should be established: the positive control group, the negative control group, and the experimental group. In the positive control group, taxol will be administered, as it promotes apoptosis, which means the annexin V assay will definitely be positive. In the negative control group, PBS will be added, which means the cells won't be stained, and annexin V assay will be negative. In the experimental group, APS will be added in increasing dosages. Afterwards, annexin V and PI will be administered to all wells in the plate. To allow binding, the plate will be incubated in the dark for 15 minutes. Finally, fluorescence will be tested using FACS, which will generate a graph showing the amount of apoptotic, necrotic, and healthy cells in the plate. If apoptosis increases with increased concentrations of APS, the hypothesis will be supported.

3.3. Western blot for PARP cleavage

The poly (ADP-ribose) polymerase (PARP) is a 113 kDa nuclear enzyme, and during apoptosis, it is cleaved in fragments of 89 and 24 kDa by lysosomal proteases. To characterize the amount of cells

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undergoing apoptosis in a sample, western blotting could be utilized, where specific antibodies will bind to cleaved PARP fragments.

To conduct this assay, first, the protein samples need to be prepared. Three groups of samples should be used: the positive control with taxol, the negative control with PBS, and the experimental group with increasing concentrations of APS. The cells in each group need to be lysed and denatured, through detergent and high heat, so that all the proteins in the cells will be unfolded and individual instead of in complexes. Loading dye will also be added to visualize the protein samples. Next, protein samples should be loaded into wells, and gel electrophoresis should be conducted. Afterwards, the gel will be taken out, and the proteins will be pulled from the gel and into a membrane, where the protein can be detected through antibodies. A primary antibody will be administered, which will stick to PARP fragments of 89 and 24 kDa. Then, a secondary antibody will be administered, and it will attach to the primary antibody with a fluorophore on the other end. By accounting for the amount of luminescence, PARP fragments will be accounted for. The positive control should yield a high level of luminescence, as taxol promotes apoptosis. The negative control should contain no luminescence, because PBS does not change the samples when administered. In the experimental group, if luminescence increases as APS concentration increases, the hypothesis will be supported.

3.4. Western blot for the phospho-β-Catenin (Ser675) antibody

The phospho-β-Catenin (Ser675) antibody detects levels of β-Catenin in a sample. β-Catenin is an important component of the Wnt/β-Catenin signaling pathway, as it acts as the key signal transducer that translocates to the nucleus to regulate gene expression. Without β-Catenin, the Wnt/β-Catenin signaling pathway would not be able to activate genes in charge of cell proliferation. Thus, by determining the amount of β-Catenin in samples with increasing concentrations of APS, it can be determined as to whether APS truly inhibited the Wnt/β-Catenin signaling pathway through destabilizing β-Catenin.

To conduct this assay, first, the protein samples need to be prepared. Three groups of samples should be used: the positive control with LiCl, the negative control with PBS, and the experimental group with increasing concentrations of APS. The cells in each group need to be lysed and denatured, through detergent and high heat, so that all the proteins in the cells will be unfolded and individual instead of in complexes. Loading dye will also be added to visualize the protein samples. Next, protein samples should be loaded into wells, and gel electrophoresis should be conducted. Afterwards, the gel will be taken out, and the proteins will be pulled from the gel and into a membrane, where the protein can be detected through antibodies. A primary antibody, the phospho-β-Catenin (Ser675) antibody, will be administered. Then, a secondary antibody will be administered, and it will attach to the primary antibody with a fluorophore on the other end. By accounting for the amount of luminescence, β-Catenin quantity will be accounted for. The positive control should contain no luminescence, because PBS does not change the samples when administered, and without LiCl prompting the accumulation of β-Catenin, β-Catenin will be degraded. The negative control should yield a high level of luminescence, as LiCl prevents the degradation of β-Catenin. In the experimental group, if luminescence/β-Catenin levels decrease as APS concentration increases, the hypothesis will be supported.

Experiments will be repeated 5 times for each assay. Statistical significance for all numerical data from MTT and Western Blot assays will be determined through the student's t-test, with $P < 0.05$.

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4. Results

Table 1: Changes in measurement with combination of possible results

Combination of possible results (CR)	APS decrease viability by MTT assay	APS increases apoptosis by AnnexinV/PI FACS	APS increases PARP cleavage by western blot	APS decrease phospho-b-catenin by western blot	Support hypothesis
CR1	+	+	+	+	Full
CR2	+	+	+	-	Partial
CR3	+	+	-	+	Partial
CR4	+	-	+	+	Partial
CR5	-	+	+	+	Partial
CR6	+	+	-	-	Partial
CR7	+	-	+	-	Partial
CR8	+	-	-	+	Partial
CR9	-	+	+	-	Partial
CR10	-	+	-	+	Partial
CR11	-	-	+	+	Partial
CR12	+	-	-	-	Partial
CR13	-	+	-	-	Partial
CR14	-	-	+	-	Partial
CR15	-	-	-	+	Partial
CR16	-	-	-	-	Fully contradicts

+ indicates the measurement changes in the direction indicated in the column header similar to the positive control (taxol for MTT, PARP, annexinV/PI, PBS for β-Catenin) and the opposite to the negative control. - indicates the measurement changes in the opposite of the direction indicated in the column header similar to the negative control (PBS, LiCl for β-Catenin) and the opposite to the positive control (taxol for MTT, PARP, annexinV/PI, PBS for β-Catenin).

4.1. Combination of result 1 (CR1)

According to Table 1, this result fully supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, detection of β-Catenin through western blot decreases.

4.2. Combination of result 2 (CR2)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

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4.3. Combination of result 3 (CR3)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.4. Combination of result 4 (CR4)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.5. Combination of result 5 (CR5)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.6. Combination of result 6 (CR6)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

4.7. Combination of result 7 (CR7)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

4.8. Combination of result 8 (CR8)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through

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western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.9. Combination of result 9 (CR9)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

4.10. Combination of result 10 (CR10)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.11. Combination of result 11 (CR11)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.12. Combination of result 12 (CR12)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay decreases. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

4.13. Combination of result 13 (CR13)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining increases. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

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4.14. Combination of result 14 (CR14)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot increases. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

4.15. Combination of result 15 (CR15)

According to Table 1, this result partially supports the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot decreases.

4.16. Combination of result 16 (CR16)

According to Table 1, this result fully contradicts the hypothesis. As APS concentration and treatment duration increase, cell viability shown through MTT assay does not change significantly. As APS concentration and treatment duration increase, cell apoptosis measured by Annexin V staining does not change significantly. As APS concentration and treatment duration increase, PARP cleavage shown through western blot does not change significantly. As APS concentration and treatment duration increase, β-Catenin levels detected through western blot do not change significantly.

5. Discussion

Combination of result 1 (CR1) shows that the hypothesis is fully supported. The MTT assay shows that increasing APS concentration and treatment duration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining supports the results of the MTT assay and shows that most of the cells are in late-stage apoptosis or necrosis by showing an increasing amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This proves that increasing concentration and treatment duration of APS could inhibit cell proliferation. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell apoptosis. This shows that APS is functioning to prevent the repair of DNA, thus inducing apoptosis in cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. Future experiments can be conducted to determine ideal treatment duration and APS concentration by testing samples with different durations and higher or lower concentration with the same assays above. Tests for ferroptosis, necrosis, and

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other assays for cell proliferation can be done to ascertain results and determine whether APS only causes cell death through apoptosis, or other mechanisms.

Combination of result 2 (CR2) demonstrates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining supports the results of the MTT assay and shows that most of the cells are in late-stage apoptosis by showing an increasing amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This proves that increasing concentrations and treatment duration of APS could cause apoptosis. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell apoptosis. This shows that APS is inducing apoptosis in cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentrations and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that although APS may induce cytotoxicity and apoptosis, it does not do so through degrading β-Catenin to impact the Wnt/β-Catenin signaling pathway. Further experiments can be conducted to test for APS inhibition of other proliferation pathways, or determine if APS is causing a factor other than β-Catenin to impair the function of the Wnt/β-Catenin signaling pathway.

Combination of result 3 (CR3) indicates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining supports the results of the MTT assay and shows that most of the cells are in late-stage apoptosis or necrosis by showing an increasing amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This proves that increasing concentration and treatment duration of APS could inhibit cell proliferation. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells. Since PARP cleavage is usually synonymous with apoptosis, it is possible that APS presented a mutation that caused the inactivation of caspase-3, which prevented the cleavage of PARP while the cell underwent apoptosis. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations and treatment duration increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. Future assays can be conducted to detect whether APS could inhibit caspase-3 functioning.

Combination of result 4 (CR4) shows that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity. Annexin V staining shows that most of the cells are not undergoing apoptosis by showing a low amount of green

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and red fluorescence. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell death. This shows that APS is functioning to prevent the repair of DNA in cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations and treatment duration increase. These results demonstrate that APS has a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. The reason why cells experience cytotoxicity and gain more PARP fragments may be due to the cells undergoing necrosis instead of apoptosis due to high concentrations and treatment duration of APS. Future tests can measure necrosis in tumor cells to verify this hypothesis.

Combination of result 5 (CR5) demonstrates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining shows an increasing amount of green and red fluorescence in cells with increasing concentration and treatment duration of APS. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell apoptosis. This shows that APS is functioning to prevent the repair of DNA, thus inducing apoptosis in cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. Even without experiencing cytotoxicity, the cell could still undergo apoptosis and PARP cleavage through the inhibition of β-Catenin by APS. Future tests can be conducted to verify whether APS has a cytotoxic effect using other assays such as CCK8.

Combination of result 6 (CR6) indicates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining supports the results of the MTT assay and shows that most of the cells are in late-stage apoptosis or necrosis by showing an increasing amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This proves that increasing concentration and treatment duration of APS could inhibit cell proliferation. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells. Since PARP cleavage is usually synonymous with apoptosis, it is possible that APS presented a mutation that caused the inactivation of caspase-3, which prevented the cleavage of PARP. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that though APS may induce cytotoxicity, it does

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not do so through degrading β-Catenin. Further experiments can be conducted to test for caspase-3 concentration in cells to verify whether caspase-3 was inactivated by APS.

Combination of result 7 (CR7) shows that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining shows that most of the cells are not undergoing cell death by showing a low amount of green and red fluorescence. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell death. This shows that APS is functioning to prevent the repair of DNA, thus inducing cell death in cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that though APS may induce cytotoxicity, it does not do so through degrading β-Catenin. APS induces cytotoxicity, but instead of inducing apoptosis, it induces necrosis, which results in PARP cleavage. Further tests can be done to measure necrosis in cells administered with high concentrations of APS.

Combination of result 8 (CR8) demonstrates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining shows that most of the cells are not undergoing cell death by showing a low amount of green and red fluorescence. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change with an increasing concentration and treatment duration of APS. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. The uncleaved PARP and absence of apoptosis in cells may be due to APS inducing ferroptosis instead of apoptosis, as ferroptosis does not induce cleaved PARP. Further experiments can be done to measure for ferroptosis in cancer cells administered with high APS concentrations.

Combination of result 9 (CR9) indicates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining shows an increasing amount of green and red fluorescence in cells with increasing concentration and treatment duration of APS. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample. Since uncleaved PARP functions to repair DNA, large amounts of PARP fragments are a sign of cell apoptosis. This shows that APS is functioning to prevent the repair of DNA, thus inducing apoptosis in cancer cells. The western blot for the phospho-β-Catenin

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(Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. Thus, while APS does not induce cytotoxicity, it does induce apoptosis and PARP cleavage. Further tests can be done on the effects of longer treatment durations of APS and its impact on cell apoptosis.

Combination of result 10 (CR10) shows that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining shows an increasing amount of green and red fluorescence in cells with increasing concentration and treatment duration of APS. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This result, coupled with the MTT assay demonstrating cell viability, seems to show that APS had an effect on disturbing membrane stability in viable cells. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. It is possible that APS does not induce cytotoxicity and instead functions to inhibit caspase-3, resulting in a lack of PARP cleavage despite apoptosis. Further tests can be done to measure caspase-3 levels in the cell.

Combination of result 11 (CR11) demonstrates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining supports this result, as it shows that most of the cells are not undergoing cell death from observing a low amount of green and red fluorescence. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample, showing that APS may function to promote caspase cleavage of PARP. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling pathway. The reason why PARP is cleaved despite the absence of apoptosis may be due to APS functioning to induce necrosis, which also results in PARP cleavage. Future tests can be done to measure necrosis in cells.

Combination of result 12 (CR12) indicates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration will show a decrease in viability, as the wells will be lighter in color, which means that less MTT is being converted to purple formazan crystals by mitochondrial dehydrogenases. Mitochondrial dehydrogenases are only present when the cell is viable, so a decrease in conversion means that the cell is experiencing cytotoxicity due to increasing concentration and treatment duration of APS. Annexin V staining shows that most of the cells are not experiencing cell death by showing a low amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that flow into the cell due to membrane instability. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. The presence of cytotoxicity despite the absence of apoptosis may be because APS is functioning to induce

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ferroptosis instead, which may explain why there is an absence of PARP cleavage. Further experiments can be done to measure ferroptosis in cells.

Combination of result 13 (CR13) shows that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining shows an increasing amount of green and red fluorescence in cells with increasing concentration and treatment duration of APS. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that were able to flow into the cell due to membrane instability. This result, coupled with the MTT assay demonstrating cell viability, seems to show that APS had an effect on disturbing membrane stability in viable cells. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells. Since PARP cleavage is usually synonymous with apoptosis, it is possible that APS presented a mutation that caused the inactivation of caspase-3, which prevented the cleavage of PARP. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that though APS may induce cytotoxicity, it does not do so through degrading β-Catenin. Further experiments can be done to test for caspase-3 levels to verify caspase-3 inhibition or mutation in cells.

Combination of result 14 (CR14) demonstrates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining supports this result, as it shows that most of the cells are viable from observing a low amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that flow into the cell due to membrane instability. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes in preparation for apoptosis. The western blot for PARP cleavage shows that there is a positive correlation between the amount of APS added to cells and the amount of PARP fragments obtained from the sample, showing that APS may function to promote caspase cleavage of PARP. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that though APS may induce cytotoxicity, it does not do so through degrading β-Catenin. The presence of cleaved PARP fragments may be because APS induced cell necrosis instead of apoptosis, resulting in PARP cleavage. Future assays can be conducted on necrosis levels in cells.

Combination of result 15 (CR15) indicates that the hypothesis is not fully supported. The MTT assay shows that increasing APS concentration and treatment duration will not result in any changes in viability of tumor cells. Annexin V staining supports this result, as it shows that most of the cells are viable from observing a low amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that flow into the cell due to membrane instability. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes in preparation for apoptosis. It can be seen that APS was not able to disturb membrane stability even in increasing concentration and treatment duration. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells, which is expected as apoptosis was not induced by APS. The western blot for the phospho-β-Catenin (Ser675) antibody shows decreasing levels of luminescence in samples with increasing concentration and treatment duration of APS, which means that β-Catenin levels decrease as APS concentrations increase. These results demonstrate that APS had a direct effect in degrading β-Catenin, preventing the functioning of the Wnt/β-Catenin signaling

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pathway. The lack of cell death despite the inhibition of β-Catenin may be because the cell utilized other cell proliferation mechanisms. Future tests can be conducted to measure the activity of other signaling pathways related to cell proliferation.

Combination of result 16 (CR16) shows that the hypothesis is not supported. The MTT assay shows that increasing APS concentration will show no change in viability, as the wells will remain the same color in spite of changes in concentration and duration. Annexin V staining supports this result, as it shows that most of the cells are viable from observing a low amount of green and red fluorescence. Green fluorescence is from the binding of annexin V to PS on the membrane, and red fluorescence is from the PI particles that flow into the cell due to membrane instability. The low levels of fluorescence shows that cancer cell membranes are stable, and PS was not moved to the outer membrane for cell recognition purposes in preparation for apoptosis. The western blot for PARP cleavage shows that the amount of PARP fragments in the sample did not change when an increasing concentration and treatment duration of APS was added to cancer cells, which is expected as cells are not undergoing apoptosis. The western blot for the phospho-β-Catenin (Ser675) antibody shows that increasing concentration and treatment duration of APS has no impact on the amount of β-Catenin in cancer cells. These results demonstrate that APS has no effect on inducing cytotoxicity, apoptosis, or inhibiting the Wnt/β-Catenin pathway in cancer cells. Future tests can be done to measure whether APS may function as a carcinogen instead.

6. Conclusion

Overall, this study investigates the effects of APS on inducing cytotoxicity of A549 cells from LUAD cell line. It also explores whether APS functions through inhibition of the Wnt/β-Catenin signaling pathway. The results would indicate whether APS can be used as treatment for patients with LUAD. If the hypothesis is supported, APS will be proven to have an effect in destabilizing β-Catenin, thereby causing the inhibition of the Wnt/β-catenin signaling pathway and cell proliferation. The results from this research is crucial in determining the mechanisms of APS, and its potential application as a treatment to not only LUAD, but also other forms of cancer, as the Wnt/β-catenin signaling pathway is common in all cells.

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