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Andrographolide Protects Against Liver Cancer by Suppressing the AKT-mTOR Pathway & GPX4 Protein to Induce Ferroptosis

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Abstract. Andrographolide is a potential drug to alleviate liver cancer by inducing ferroptosis in the cancer cells. In this article, a series of experiments would be conducted to test whether it has positive results on cancer killing. In addition, we would find out the mechanism between Andrographolide and ferroptosis. Furthermore, methods would also be used to identify the target site of Andrographolide inside the human body. Those possible outcomes in this article could help to a creative understanding of how Andrographolide inhibits hepatoma.

Keywords: Andrographolide, ferroptosis, liver cancer, virtual experiment

1. Introduction

Liver cancer is one of the most serious worldwide health issues [1]. According to numerical data collected from SEER, from January to July in 2025, the number of newly diagnosed cases of liver cancer was 42,240, which accounted for more than 2% of all the new cancer patients in 2025. Notably, the death rate is extremely high in liver cancer, which accounts for approximately 5% of the death rate among all cancer-caused deaths. In recent decades, both the estimated new cases and death rate have been steadily increasing [2]. With a low survival rate of liver cancer, there is an eagerness to find out possible therapies to alleviate this disease [3]. Using traditional Chinese medicine (TCM) to alleviate liver cancer has a long and profound history. TCM demonstrated unique clinical benefit in specified scientific practice, including multi-targeting, reducing side effects, and positive affecting [4]. Ferroptosis is a new type of apoptosis that is iron-dependent and could be detected by excessive lipid peroxidation [5]. It is now proven that ferroptosis has great potential in alleviating different types of cancer cells, such as liver cancer and lung cancer [6]. The most well-known mechanism for ferroptosis includes over-accumulation of iron-dependent lipid-Reactive Oxygen Species and the inhibition of Glutathione peroxidase 4 [7]. GPX4 is an essential inhibitor of ferroptosis, which plays an important role in shifting toxic lipid hydroperoxides into non-toxic lipid alcohols by reducing glutathione (the amino acid) to reduce lipid peroxidation and protect cells from ferroptosis [8]. One of the most significant pathways of ferroptosis is AKT-mTOR. The inhibition of AKT would suppress the AKT-mTOR pathway. This signaling creates a stable environment in liver cancer cells(HepG2) by reducing the synthesis of lipid materials [9],

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which suppresses the expression of oncogenic signals, as well as suppressing GPX4 to induce lipid peroxidation, then activating the ferroptosis pathway [10]. Andrographolide (AP; C20H30O5), a newly discovered diterpene lactone molecule extracted from a traditional Chinese medical herb Andrographis paniculata [11]. It demonstrates a wide range of medicinal effects, including anti-oxidant, anti-inflammatory, and so on. Nowadays, it is also proven to be an effective molecule to inhibit the AKT-mTOR pathway [12]. Based on all the theoretical mechanisms of Andrographolide, it is predicted to have a wide potential pharmacological activity against many kinds of cancers [13]. Giving Andrographolide to liver cancer patients, could possibly induce ferroptosis inside liver cancer cells, showing a potential therapy for this disease.

2. Hypothesis

It is predicted that increasing concentrations and durations of Andrographolide turn down both AKT-mTOR activation and the expression of GPX4 protein, thereby triggering ferroptosis in liver cancer and eliminating HepG2 cells.

3. Methods and materials

In this experiment, several experiments would be used. Including MTT experiment, BODIPY tests, and Western blot tests to prove the hypothesis. Different ranges of concentration of Andrographolide would be set to test whether there is a relationship between cell killing and drugs concentration. In addition, the data of the test would be tested by GraphPad Prism for statistic discrepancy.

The primary isolated HepG2 liver cancer cells will be purchased from Sun-Yat-Sen University. The cells will be cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), together with 1% penicillin-streptomycin, and will be incubated in an incubator with a temperature of 37 °C and 5% CO2.

4. Experiment design

4.1. MTT assay tests

HepG2 cells would be plated in 96-well plates at a density of 7×10^3 cells per milliliter for 24 hours. After that, the HepG2 cells were treated with multi-step concentrations (0, 1, 2, 5, 10, 20, 40, 80, 200μM) of Andrographolide for 0h, 1h, 3h, 6h, 12h, and 24h. The OD value of each single well was measured utilizing an MTT assay kit. A formula would be used to quantify the relative viability of the cell. Cell viability (%) = [(OD value of the Andrographolide group - OD value of the blank group)/(OD value of the RSL3 group - OD value of the blank group)] × 100% [14]. The duration test would be designed to reduce the possibility of false negative results, and it may help find whether there is a linear correlation between cell elimination and time.

PBS or pure DMEM would serve as the negative control group, and Sorafinib would serve as the positive control group.

4.2. BODYPI assay tests

HepG2 cells would be planted in a 6-well plate with a concentration of 7,000 cells per well, and cultured at 37℃, 5% CO2 overnight. Inducing ferroptosis with RSL3 (10μM) as the positive control group, and adding PBS in a well as the control group. Add concentrations of 1, 2, 5, 10μM of Andro in the other 4 wells. Incubating the cells for 0, 1, 3, 6, 12, and 24 hours. Add BODIPY working

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solution (10μM as final concentration), incubate 30 minutes at 37°C in the dark. Using 100μL PBS to wash each well sufficiently to remove unbound dye [15].

The detection would require the use of Fluorescence Microscopy. By checking the intensity of the fluorescence that reflects the amount of ROS inside the cells, the dose-effect relationship between Andrographolide and ferroptosis could be figured out. Also, whether Andrographolide induced ferroptosis inside HepG2 cells could be tested.

PBS or pure DMEM would serve as the negative control group, and Sorafinib would serve as the positive control group.

4.3. Western blot for (AKT, p-S6, GPX4)

Harvested HepG2 cells would be broken down by lysate; the whole production should be operated on ice throughout the whole experiment. Then, cell tissues would be separated by spinning at 15,000rpm for 15min at 4°C in a centrifugal machine. Each sample was loaded with a total amount of 25 μg of target (phospho-AKT/phospho-S6/phospho-GPX4) in a 4–20 % SDS-PAGE gel and transferred to a 0.45 μm PVDF membrane after electrophoresis. The membrane was blocked with skim milk for 2.5h and then incubated with the primary antibody at 4 °C for 12 hours. Use TBS buffer to wash the membrane after incubation on a shaking bed for 10 minutes each time, repeat washing 3 times. Then, the incubation of secondary antibody from rats or rabbits would require a soaking on a shaking bed. Then repeat the washing on the last step 3 times. At last, incubation of the dye should take place in a dark room for 45 minutes. Finally, use chemiluminescence imaging systems to detect the intensity of chemiluminescent signals so as to quantify proteins [16].

In the Western blot experiment, HepG2 cells incubated in PBS would serve as the blank control group. By observing whether the expression of p-AKT, p-S6, and GPX4 would drop while increasing the concentration of Andrographolide. Also, unphosphorylated AKT, S6, and GPX4 would be set as the negative control group. Sorafenib would serve as the positive control group to compare whether the reduction in the Andrographolide group has statistical significance. ($p<0.01$) The whole experiment would be repeated 10 times to avoid fake negative results.

5. Statistical analysis

For the MTT assay test, three repeated wells are set for each concentration, and every test is repeated 10 times. The mean of the OD value from the Ultraviolet spectrophotometer would be collected in a sheet. The Standard deviation would be calculated by GraphPad Prism. Statistics would be run by One-way Anova for difference examination and Student's t-test to verify the credibility of the data. ($P<0.01$)

For the WB test, each specific test would be repeated 5 times to confirm whether the trend is the same as the hypothesis. The quantity of proteins in the test should present a negative correlation with concentration. Linear tests would be applicated to create a fit curve, the square of the coefficient of association should be larger than 0.99.

According to table 1, all possible combinations of the results from designed experiments are listed. By combining and analyzing those results, the relationship between ferroptosis and Andrographolide could be tested out. Furthermore, the most possible target site where Andrographolide induces ferroptosis would also be tested.

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Table 1. Possible combinations of results from the experiment above

Combination Result # (CR#)	Increasing Andro decreases viability (MTT)	Increasing Andro increases ferroptosis (BODIPY)	Increasing Andro decreases GPX4 by WB	Increasing Andro decreases p-akt(WB	Increasing Andro decreases p-S6(WB)	Support of 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	-	-	+	+	+	Partial
CR17	+	+	-	-	-	Partial
CR18	+	-	+	-	-	Partial
CR19	-	+	+	-	-	Partial
CR20	+	-	-	+	-	Partial
CR21	-	+	-	+	-	Partial
CR22	-	-	+	+	-	Partial
CR23	+	-	-	-	+	Partial
CR24	-	+	-	-	+	Partial
CR25	-	-	+	-	+	Partial
CR26	-	-	-	+	+	Partial
CR27	+	-	-	-	-	Partial
CR28	-	+	-	-	-	Partial
CR29	-	-	+	-	-	Partial
CR30	-	-	-	+	-	Partial
CR31	-	-	-	-	+	Partial
CR32	-	-	-	-	-	Fully Contradicts

The positive sign of MTT test means that Andro could kill liver cancer cells. The positive sign of BODIPY test means that Andro induced ferroptosis inside the HepG2 cells. The positive result of

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the WB experiments means that Andro would suppress the expression of those proteins. All the positive sign indicates that the result is matched with the hypothesis. The negative sign means the result of the experiment demonstrate an opposite result of the hypothesis.

Results:

CR1: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The result of BODIPY shows green fluorescence. The WB test results indicate that all three potential proteins are suppressed compared to the negative group.

CR2: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The WB experiment demonstrates a decrease in AKT and GPX4, but the expression of mTOR did not decrease.

CR3: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The WB test demonstrates a decrease in GPX4 and mTOR, but the expression of AKT did not decrease.

CR4: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The WB test demonstrates a decrease in AKT and mTOR, but the expression of GPX4 did not decrease.

CR5: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that the expression of GPX4, AKT, and mTOR is all decreased.

CR6: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that the expression of GPX4, AKT and mTOR is all decreased.

CR7: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that the expression of only GPX4 is decreased; neither AKT nor mTOR has decreased.

CR8: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence.. The result from the WB test shows that the expression of only AKT is decreased, while mTOR and GPX4 did not decrease compared to the control group.

CR9: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that both the expression of GPX4 and AKT are decreased, while mTOR did not decrease compared to the control group.

CR10: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that both the expression of GPX4 and AKT are decreased.

CR11: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that only mTOR is decreased, while AKT and GPX4 did not decrease compared to the control group.

CR12: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that both the expression of GPX4 and mTOR are decreased, while AKT did not decrease compared to the control group.

CR13: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that

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both the expression of GPX4 and mTOR are decreased, while AKT did not decrease compared to the control group.

CR14: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that both the expression of AKT and mTOR are decreased, while GPX4 did not decrease compared to the control group.

CR15: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence.. The result from the WB test shows that both the expression of AKT and mTOR are decreased, while GPX4 did not decrease compared to the control group.

CR16: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not demonstrate green fluorescence but red fluorescence instead. The result from the WB test shows that the expression of AKT, mTOR, and GPX4 is all decreased.

CR17: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that none of AKT and mTOR, GPX4 is decreased compared to the control group.

CR18: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of GPX4 is decreased, while AKT and mTOR did not decrease compared to the control group.

CR19: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that only the expression of GPX4 is decreased.

CR20: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of AKT is decreased, while GPX4 and mTOR did not decrease compared to the control group.

CR21: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that only the expression of AKT is decreased.

CR22: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that the expression of AKT, mTOR, and GPX4 is decreased.

CR23: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of mTOR is decreased, while AKT and GPX4 did not decrease compared to the control group.

CR24: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that only the expression of mTOR is decreased.

CR25: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that both the expression of mTOR and GPX4 are decreased.

CR26: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The

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result from the WB test shows that both the expression of mTOR and AKT are decreased.

CR27: After exposure to Andro, the viability of HepG2 cells decreases compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that none of the expression of those three proteins has decreased compared to the control group.

CR28: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test shows green fluorescence. The result from the WB test shows that none of the expression of those three proteins has decreased compared to the control group.

CR29: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of GPX4 is decreased.

CR30: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of AKT is decreased.

CR31: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that only the expression of mTOR is decreased.

CR32: After exposure to Andro, the viability of HepG2 cells remains compared to the negative control group. The BODIPY test did not show green fluorescence but red fluorescence instead. The result from the WB test shows that none of the expression of those three proteins is decreased.

Possible results for the variables of concentration and treatment duration

According to table 2,there would be four different results as the concentration of Andro increase or decrease as the table below. Also, the duration test of Andro is also listed.

Table 2. Potential results

MTT/result	positive	negative	remains	random
concentration	+/-	+/-	+/-	+/-
duration	+/-	+/-	+/-	+/-

1. Concentration test:

①If the viability increased as the concentration increased linearly, it means the drug could induce cell division, therefore increasing cell viability.

② If the viability decreases as the concentration increases, it means the drug could inhibit cell viability. And there is a linear correlation between drug concentration and the death rate for cells.

③If the viability remains the same as the concentration increases, it means the drug does nothing about cell viability.

④ If the viability changes randomly as the concentration increases, it means the interaction between drugs and cells is also affected by other issues such as the amount of receptors or enzymes.

2. Duration test

① If the viability increased linearly as the reacting time increased, it means the drug does not affect cell viability. If it increased sharply, it should be considered that the drug would promote cell viability.

② If the viability decreases as the reaction time increases, it means the drug could inhibit cell viability. And there is a linear correlation between drug concentration and the death rate for cells.

③ If the viability remains the same as the time increases, it means the drug does nothing about cell viability.

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④ If the viability changes randomly as the concentration increases, it means the interaction between drugs and cells is also affected by other issues, such as the amount of receptors or enzymes. In addition, drugs may need time to activate the ferroptosis pathway.

6. Discussion

The purpose of the experiment is to investigate the mechanism between Andrographolide and ferroptosis in liver cancer cells, which is evaluated by measuring cell viability, whether Andrographolide induces ferroptosis, and AKT, mTOR, and GPX4 expression. Thirty-two combinations of possible results are listed in the table to be discussed.

CR1: The result from CR1 is fully matched with the hypothesis. The positive result for BODIPY suggests that Andro eliminates the cell specifically by inducing ferroptosis. It has been inferred that Andrographolide is a specific ferroptosis inducer that works specifically through the AKT-mTOR-GPX4 pathway to eliminate liver cancer cells functionally.

CR2: The positive result for BODIPY suggests that Andro eliminates the cell specifically by inducing ferroptosis. The WB result indicates that Andro induces ferroptosis by majorly suppressing GPX4. It is partially matched with the hypothesis. Results indicated that Andro reduced AKT but not mTOR. It might suggest that Andro induces ferroptosis via the PI3K-AKT pathway instead of the AKT-mTOR pathway [17].

CR3: The positive result for BODIPY suggests that Andro eliminates the cell specifically by inducing ferroptosis. The result from the WB test shows that both GPX4 and mTOR are decreased, while AKT increased compared to the control group. It indicates that Andrographolide induces ferroptosis by specifically acting on mTOR individually, and therefore reduces the expression of GPX4 at the downstream [8].

CR4: The result from CR4 indicates that Andro eliminates HepG2 cells by inducing ferroptosis specifically by the AKT-mTOR pathway. The positive result for BODIPY suggests that Andro eliminates the cell specifically by inducing ferroptosis. The result from the WB test shows that the expression of GPX4 remains the same with the negative control group, while both AKT and mTOR decreased.

CR5: The result from CR5 indicates that Andro might induce other kinds of apoptosis by the AKT-mTOR-GPX4 pathway. The result from BODIPY suggests that Andro eliminates the cell without inducing ferroptosis.

CR6: The positive result for BODIPY suggests that Andro has induced ferroptosis inside the cells, though the cell viability did not decrease as the concentration of Andro increase in CR6. It might indicate that Andro has induced a slight ferroptosis that eliminated cells more slowly than the proliferation of HepG2. That could probably explain the reason why ferroptosis-induced cells have an increase in cell viability.

CR7: The positive result for BODIPY from CR7 suggests that Andro eliminates the cell by inducing ferroptosis. The WB result indicates that Andro induces ferroptosis by specifically suppressing the GPX4 protein, as a result, ferroptosis is induced inside HepG2 cells.

CR8: The positive result for BODIPY from CR8 suggests that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce ferroptosis in other pathways, such as AKT-NRF2, which is different from the cascade reaction of the AKT-mTOR-GPX4 pathway [18].

CR9: The negative result for BODIPY from CR9 suggests that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce other kinds of apoptosis or simply overexpress ROS to eliminate HepG2 cells.

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CR10: The positive result for BODIPY from CR10 suggests that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis that eliminates cells slower than the proliferation of HepG2, or it may increase mTOR and save cells by other pathways. Further experiments should be required to raise the concentration of Andrographolide in order to test the correlation between cell viability and concentration.

CR11: The positive result for BODIPY from CR11 suggests that Andro eliminates the cell specifically by inducing ferroptosis. It might indicate that Andro induces ferroptosis by acting individually on mTOR; there might be another downstream protein to activate ferroptosis instead of GPX4, such as SQLE/cholesterol/mTOR signaling pathway [19].

CR12: The positive result from the MTT test and the negative result for BODIPY from CR12 suggest that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce other kinds of apoptosis, such as cuproptosis or simply overexpress ROS to eliminate HepG2 cells.

CR13: The positive result for MTT and BODIPY experiments from CR13 suggests that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis that eliminates cells slower than the proliferation of HepG2, or it may increase AKT and save cells by other pathways, such as AKT-NRF2 [18].

CR14: The positive result from the MTT test and the negative result for BODIPY from CR14 suggest that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce lysosomal cell death or other kinds of apoptosis by inhibiting the PI3K/AKT/mTOR pathway to eliminate HepG2 cells [20].

CR15: The negative result from the MTT test and the positive result for BODIPY from CR15 suggest that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis that eliminates cells slower than the proliferation of HepG2, or it may increase the expression of GPX4 to reduce lipid peroxidation inside HepG2 cells.

CR16: The negative results for both the MTT assay test and BODIPY from CR16 suggest that Andro did not inhibit the viability of HepG2 nor induce ferroptosis inside the cells. It indicates that there is no statistically significant between Andro and ferroptosis. Andro may suppress the AKT-mTOR-GPX4 pathway as a side effect when acting on its major pathway, which does no harm to HepG2 cells.

CR17: The positive result for both the MTT assay test and BODIPY from CR17 suggests that Andro eliminates the cell specifically by inducing ferroptosis. It indicates that Andro induces ferroptosis by acting on other pathways. For example, the NF-κB pathway, which lacks correlation to the AKT-mTOR-GPX4 pathway [21].

CR18: The positive result for MTT assay tests and the negative result for BODIPY from The positive result for MTT assay tests and the negative result for BODIPY from CR18 suggest that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce other kinds of apoptosis or inhibit the TrX1-GPX4 pathway to eliminate HepG2 cells [22].

CR19: The negative result from the MTT assay test and the positive result for BODIPY from CR19 suggest that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis by suppressing GPX4, which eliminates cells more slowly than the proliferation of HepG2.

CR20: The positive result from the MTT assay test and the negative result for BODIPY from CR20 suggest that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might induce other kinds of apoptosis or cell-killing mechanisms by inhibiting AKT to eliminate HepG2 cells.

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CR21: The positive result for BODIPY from CR21 suggests that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis by suppressing AKT, which eliminates cells more slowly than the proliferation of HepG2. Since it did not activate the whole pathway, it is foreseeable that the intensity of ferroptosis would be lower.

CR22: The negative results for both the MTT assay and BODIPY test from CR22 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may suppress the AKT and GPX4 proteins separately, which could not activate the cascade reaction for ferroptosis.

CR23:The positive result for the MTT assay test and the negative result for BODIPY from CR23 suggests that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro might reduce the activity of mTORC1, which allows autophagy to restore cellular homeostasis and eliminate HepG2 cells [23].

CR24:The negative result for the MTT assay test and the positive result for BODIPY from CR24 suggest that Andro has induced ferroptosis inside the cells. It indicates that Andro might induce a slight ferroptosis by suppressing mTOR, which eliminates cells more slowly than the proliferation of HepG2. Since it did not activate the whole pathway, it is foreseeable that the intensity of ferroptosis would be lower.

CR25: The negative results for both the MTT assay test and BODIPY from CR25 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may suppress the mTOR and GPX4 proteins separately, which could not activate the cascade reaction for ferroptosis.

CR26: The negative results for both the MTT assay test and BODIPY from CR26 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may suppress the AKT-mTOR pathway, then the cascade reaction for other biological activities, instead of ferroptosis.

CR27: The positive result from the MTT assay test and the negative result for BODIPY from CR27 suggest that Andro eliminates the cell without inducing ferroptosis. It indicates that Andro eliminates liver cancer cells without activating the ferroptosis pathway. It might reduce the cell viability by toxicity of its own or another form of apoptosis.

CR28: The negative result for the MTT assay test and the positive result for BODIPY from CR28 suggests that Andro has induced ferroptosis inside the cells. It indicates that Andro induces ferroptosis through other pathways. Furthermore, the intensity of ferroptosis induced by Andro may be lower than the proliferation of HepG2 cells.

CR29: The negative results for both the MTT assay test and BODIPY from CR29 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may specifically suppress the GPX4 protein, then the cascade reaction for other biological activity, instead of ferroptosis.

CR30: The negative results for both the MTT assay test and BODIPY from CR30 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may specifically suppress the AKT protein, then the cascade reaction for other biological activity, instead of ferroptosis.

CR31: The negative results for both the MTT assay test and BODIPY from CR31 suggest that Andro did not induce ferroptosis inside the cells. It indicates that there is no correlation between Andro and ferroptosis; it may specifically suppress the mTOR protein, then the cascade reaction for other biological activities, instead of ferroptosis.

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CR32: The result from CR32 fully contradicts the hypothesis. All the indices demonstrate negative results against the hypothesis. It indicates that Andro could not eliminate HepG2 cells, and there is no correlation between Andro and ferroptosis. It also lacks activity in activating the cascade activation with the AKT-mTOR-GPX4 pathway.

7. Conclusion

This study investigates the effects of Andrographolide on HepG2 liver cancer cells, focusing on cell viability, ferroptosis of cells, and AKT-mTOR-GPX4 expression. Multiple combinations of possible results highlight the potential activity for Andro to induce ferroptosis. Further researches are required to elucidate the underlying mechanisms of the total pathway for ferroptosis. As efforts in paper, experiments have indicated several possible results of the mechanism for Andrographolide to alleviate liver cancer. It is creative to consider the relationship between Andro and ferroptosis since researchers have done few experiments about this pathway with Andrographolide. It may discover a potential liver cancer therapy and even provide a supplement to the database for natural ferroptosis inducers. This discovery would also improve the advances in the field of computer-associated drug design. If the mechanism between Andrographolide and liver cancer inhibition is confirmed, artificial intelligence could imitate the structure of Andrographolide. After that, it may present similar molecules that also alleviate liver cancer. Further experiments about the toxicity of healthy liver cells are also essential for the whole experiment, since it directly affects the compliance of the medication.

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