

The Effect of Cyldindropermopsin on KIR3DL3 receptors' Activity, and Colon Cancer Development

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Abstract. To explore the efficacy of astragaloside in the treatment of colon cancer and the cytotoxic mechanism of colon cancer cells. This paper mainly focuses on the inhibition of HHLA2-KIR3DL3 pathway to explore and study the possible mechanism of astragaloside. The physiological effects of astragaloside on colon cancer cells in vitro and in vivo should be tested through cell culture and animal experiments. At the same time, the influence of astragaloside on the physiological function of normal cells should be confirmed by MTT assay, and the binding effect of astragaloside with KIR3DL3 should be determined by SPR. At the same time, under the premise that the experimental results support the hypothesis, the optimal concentration and the most timely time of astragaloside to kill colon cancer cells were investigated. The reduction of tumor size in the astragaloside treatment group can prove that astragaloside can inhibit the growth of colon cancer cells. The combination of astragaloside and KIR3DL3 can further speculate that the inhibition of colon cancer cells may be related to the inhibitory function of KIR3DL3.

Keywords: Astragaloside, Colon Cancer, KIR3DL3 Receptor, HHLA2-KIR3DL3 Pathway.

1. Introduction

Colon cancer is one of the most common malignant tumors in the world at present, and the inhibition and treatment of colon cancer have become one of the hot spots in the current research [1]. Based on data from the last five years (2012-2016), the age-standardized incidence of colon cancer was 27.6 cases per 100,000 people, and the age-standardized incidence was significantly higher than that of rectal cancer, with a mortality rate of 13.9 cases per 100,000 people [2]. In recent years, immune checkpoint inhibitors have made significant progress as drugs for the treatment of colon cancer. However, according to existing experimental data, the clinical response rate of the method of inhibiting the PD-1 pathway through immune checkpoints is less than 20%. So we need to develop new immune checkpoints to find more effective treatments for colon cancer.

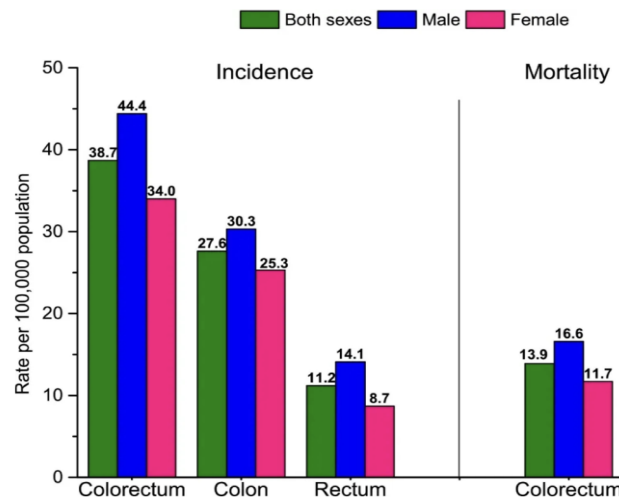


Figure 1. From left to right, colorectal cancer, colon cancer, colorectal cancer incidence, and colorectal cancer mortality in the United States over the last 5 years. Green - total data for both sexes, blue - male, red - female. (Image credit: Resources [2])

In recent years, immune checkpoint inhibitors have made significant progress as drugs for the treatment of colon cancer. However, according to existing experimental data, the clinical response rate of the method of inhibiting the PD-1 pathway through immune checkpoints is less than 20% [5]. Studies have shown that this is due to the complex ligand interactions involved in PD-1 pathway, which has potential feedback regulation or restriction on immune system signal transduction [3]. So we need to develop new immune checkpoints to find more effective treatments for colon cancer.

According to the study, HHLA2-KIR3DL3 pathway (Figure 2. HHLA2 Immune pathway Diagram [3]) is a relatively simple immune regulatory signaling pathway found so far. The HHLA2 protein in members of the B7 family is reported to be less expressed in normal tissues and more expressed in human cancer cells [3,5]. KIR3DL3, a member of the KIR suppressor receptor family, is specifically expressed in primates and suppresses immune function by being expressed on the surface of T and NK cells. Therefore, designing antibodies against KIR3DL3 or finding natural substances that inhibit the binding of KIR3DL3 to HHLA2 is a potential treatment for colon cancer [4,5].

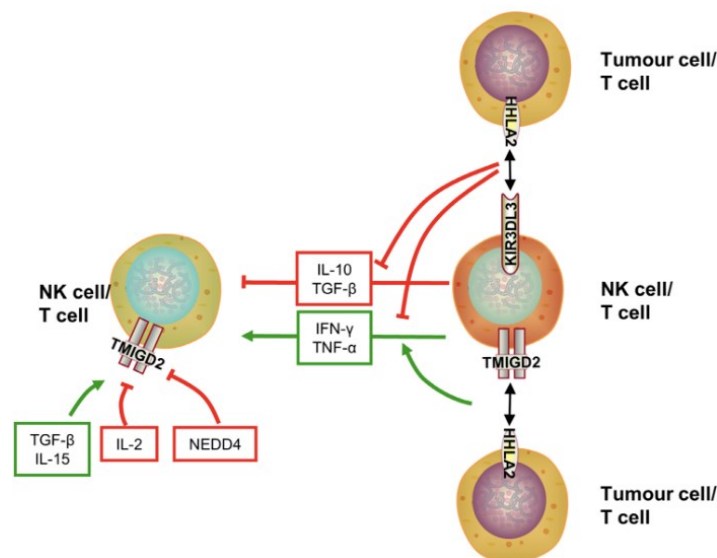


Figure 2. HHLA2 Immune pathway Diagram[3]

Studies have shown that astragaloside has certain pharmacological effects on anti-tumor and immune regulation. Astragaloside regulates the expression of inflammatory factors by regulating TLR4/NF- κ B and STAT1 signaling pathways, thereby improving the level of inflammation and exerting its immune effect. However, whether it can act on HHLA2-KIR3DL3 pathway has not been mentioned in the study. Therefore, this paper focuses on exploring the interaction of astragaloside with HHLA2-KIR3DL3 pathway and further research on astragaloside for the treatment of colon cancer [6].

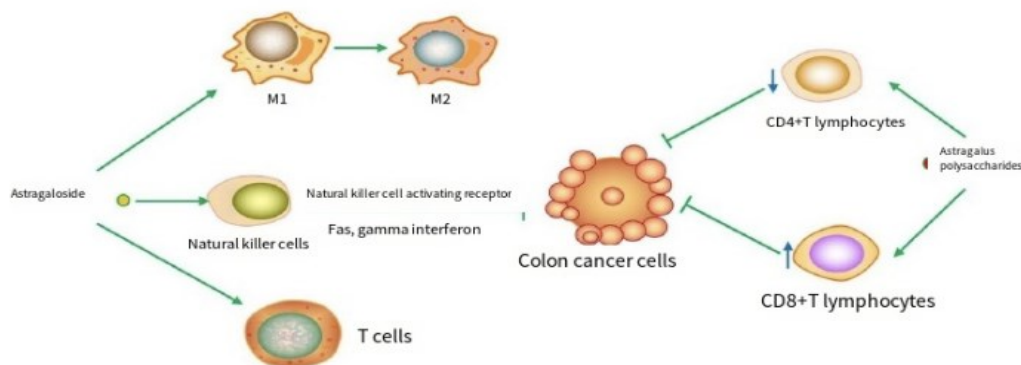


Figure 3. Effects of Astragalus active ingredients on immune regulation [6]

As to whether astragaloside will affect other functions of the body on the basis of inhibiting KIR3DL3 function, a series of animal experiments should be conducted to obtain data for evaluation. In addition, targeted molecules can be designed on the surface of astragaloside to reduce the potential harm and influence of the drug on other tissues of the body.

Hypothesis: I predict that Astragaloside will bind to the KIR3DL3 receptor on the cell surface, reduce the viability of HCT116 colon cancer cells in vitro, and shrink the HCT116 graft tumor. At the same time, the proliferation inhibition of colon cancer cells was tissue-specific.

Measure viability by MTT assay and binding between astragaloside and KIR3DL3 by SPR, and tumor size by weight. Positive control is taxol for the killing assays and a known ligand for KIR3DL3 for the binding assay and negative control is DMSO/PBS.

2. Material and Methods

2.1. MTT assay

Logarithmic cells were collected, the concentration of cell suspension was adjusted, 100ul was added to each hole, and the density of cells to be measured was adjusted to 1000-10000 holes by laying the plate (the edge holes were filled with sterile PBS). 5% CO₂, incubation at 37 °C, until the cell single layer is fully covered with the bottom of the hole (96 holes flat floor), adding the concentration gradient of the drug, in principle, the cell can be added after the wall, or two hours, or half a day, but we often lay the board in the afternoon of the day before, the next morning. Generally 5-7 gradients, each hole 100ul, set 3-5 multiple holes. It is recommended to set 5, otherwise it is difficult to reflect the real situation. Incubate with 5% CO₂ at 37°C for 16-48 hours and observe under an inverted microscope. Add 20ul MTT solution (5mg/ml, i.e. 0.5% MTT) to each well and continue to culture for 4h. If the drug can react with MTT, the culture medium can be centrifuged first and then discarded, and the culture medium containing MTT can be added after careful washing with PBS 2-3 times. Stop the culture and carefully remove the culture liquid from the hole. Add 150ul dimethyl sulfoxide to each well and oscillate in a shaker for 10min at a low speed to fully dissolve the crystal. The light absorption values of each hole were measured at OD490nm by ELISA. At the same time, zero holes (medium, MTT, DSO) and control holes (cells, dissolving media of the same concentration, culture medium, MTT, DSO) were set up[7].

2.2. Surface plasmon resonance

SPR chip was used to detect the state of molecules in the solution. 0.1mol/L KIR3DL3 solution and 0.1mol/L Astragaloside solution were fully mixed for 15 minutes and then detected by SPR instrument.

1.1 Animal treatment protocol

NSG male mice aged 6 weeks were purchased from X Company and 2×10^6 Rko- vectorKO-HHLA2-WT and RKO-HHLA2-8NQ cells were injected subcutaneously into NSG mice. Tumor size was recorded every two days starting from day 10 after vaccination. In addition, in order to evaluate the killing effect of astragaloside on colon cancer tumor tissue of mice, a certain concentration of astragaloside solution was injected into the experimental mice every two days, and the control mice were injected with the same amount of PBS under the same living conditions. Tumor size was recorded every two days.

2.3. Statistical Analysis

The experiments in this study should be repeated three or more times to ensure statistical significance. Firstly, the MTT method was employed to assess the impact of low concentrations of astragaloside on normal tissue cells and monitor changes in tumor size over a two-day interval after treatment with Astragaloside solution. Additionally, the SPR method was utilized to investigate the binding status between KIR3DL3 and astragaloside, providing joint verification for the hypothesis.

Before determining the optimal concentration and timing of astragaloside, it is crucial to confirm if the experimental results align with the hypothesis. If proven true, a preliminary experiment on normal tissue cells must be conducted first to determine both concentration and time when astragaloside exhibits its cytotoxic effect on normal tissue. Subsequently, subsequent experiments should focus on identifying an optimal concentration and treatment duration that effectively kills colon cancer cells while sparing normal tissue.

When establishing the optimum concentration of astragaloside and determining experimental timing, it is essential to arrange various experimental groups with specific concentration gradients and treatment time gradients simultaneously. Each condition should undergo no less than three repetitions to achieve statistically significant results.

3. Results

3.1. Possible Results Table

Table 1. Experimental prediction of Astragaloside mechanism verification

Combination Result # (CR#)				
	Astragaloside reduces normal tissue viability by MTT	Astragaloside shrinks xenograft tumor	Astragaloside interacts with KIR3DL3 by SPR	Support of hypothesis
1	+	+	+	Partial
2	+	+	-	Partial
3	+	-	+	Partial
4	-	+	+	Full
5	-	-	+	Partial
6	-	+	-	Partial
7	-	-	-	Partial
8	+	-	-	Fully Contradicts

Table legend: "+" indicates results accord with the expectation, "-" indicates results do not accord with the expectation.

CR1: If I obtain this result I would see Astragaloside reduces cells viability and shrinks the volume of tumor. Furthermore, Astragaloside can interact with KIR3DL3.

CR2: If I obtain this result I would see Astragaloside reduces cells viability and shrinks the volume of tumor, but Astragaloside cannot interact with KIR3DL3.

CR3: If I obtain this result I would see Astragaloside reduces cells viability but do not shrink the volume of tumor. Furthermore, Astragaloside can interact with KIR3DL3.

CR4: If I obtain this result I would see Astragaloside do not reduce cells viability but shrinks the volume of tumor. Furthermore, Astragaloside can interact with KIR3DL3.

CR5: If I obtain this result I would see Astragaloside not only cannot reduce cells viability but also do not shrink the volume of tumor, but Astragaloside can interact with KIR3DL3.

CR6: If I obtain this result I would see Astragaloside do not reduce cells viability but shrinks the volume of tumor, but Astragaloside cannot interact with KIR3DL3.

CR7: If I obtain this result I would see Astragaloside not only do not reduce cells viability but also do not shrink the volume of tumor. Furthermore, Astragaloside can interact with KIR3DL3.

CR8: If I obtain this result I would see Astragaloside reduce cells viability but do not shrink the volume of tumor. Furthermore, Astragaloside can interact with KIR3DL3.

3.2. Possible results for the variables of concentration and treatment duration

Too long treatment time or too high concentration may lead to the cytotoxicity of astragaloside to normal cells, and may also change the conformation of astragaloside to produce a domain that can bind KIR3DL3. Short treatment time will lead to no obvious cytotoxicity of astragaloside to normal cells and colon cancer cells, and will also lead to insufficient binding time of KIR3DL3 and astragaloside, resulting in errors in experimental results.

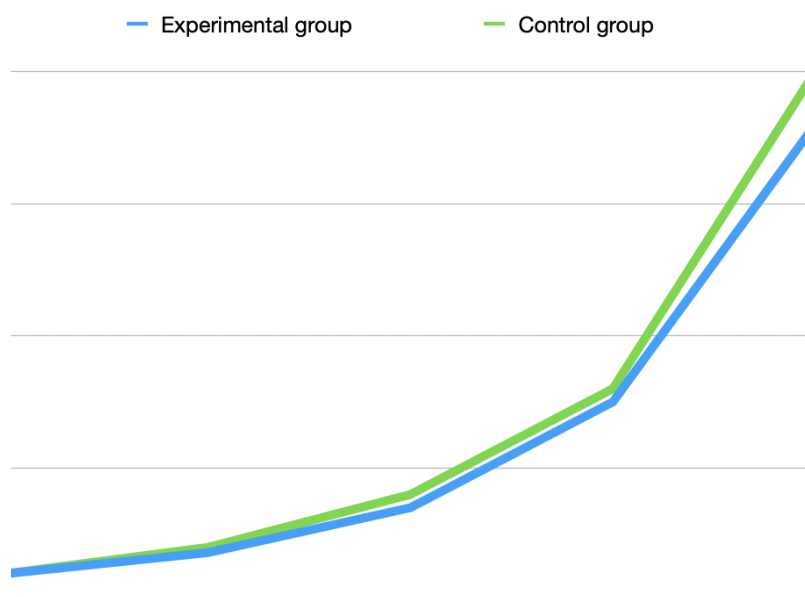


Figure 4. low concentration or short treatment duration

Figure 4 illustrates the simulated tumor size/concentration or tumor size/treatment time curve based on theoretical predictions after appropriate treatment of colon cancer cells with a low concentration of astragaloside or a short treatment time with an appropriate concentration.

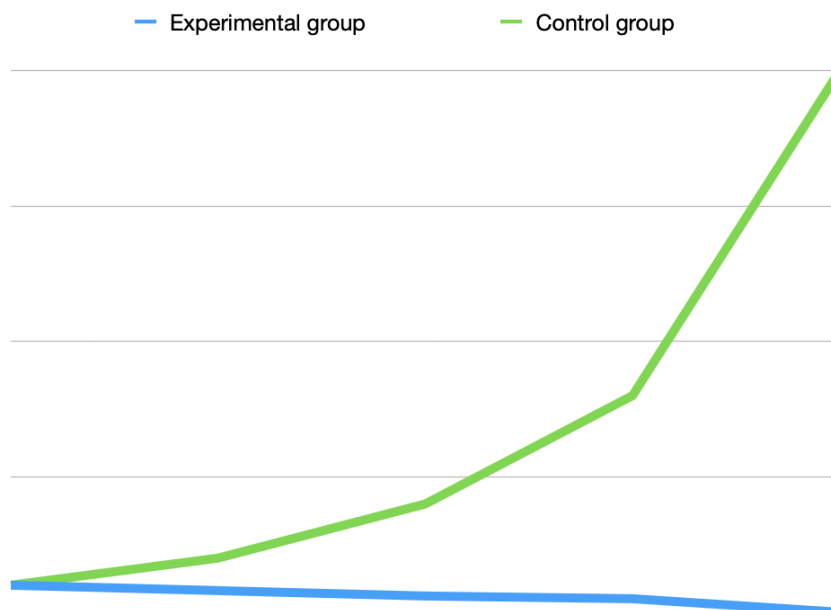


Figure 5. proper concentration or proper treatment duration

Figure 5 shows the tumor size/treatment time curve or tumor size/concentration curve simulated by theoretical prediction after Astragaloside IV solution with appropriate concentration treated colon cancer tissue for different time or with different concentration of Astragaloside IV solution within appropriate time.

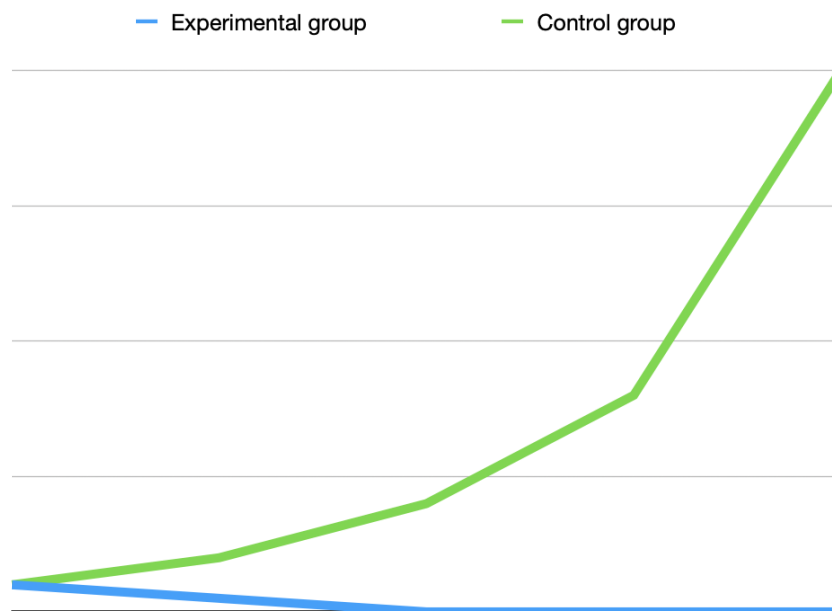


Figure 6. high concentration and long treatment duration

Figure 6 shows the tumor size/treatment time curve or tumor size/concentration curve simulated by theoretical prediction after Astragaloside IV solution with high concentration treated colon cancer tissue for different time or with different concentration of Astragaloside IV solution within long time.

4. Discussion

CR1: I think certain concentrations of astragaloside is lethal to both normal tissues and colon cancer cells, so it is necessary to introduce drug targeting molecules in practical application. At the same time, astragaloside can bind to KIR3DL3 molecules, indicating that it may ensure the activity of NK cells by inhibiting the inhibition of KIR3DL3 receptor on colon cancer tissues in vivo.

CR2: I think certain concentrations of astragaloside is lethal to both normal tissues and colon cancer cells, so it is necessary to introduce drug targeting molecules in practical application. However, astragaloside cannot bind to KIR3DL3 molecule, indicating that Astragaloside may not inhibit the inhibition of KIR3DL3 receptor on NK cells through binding with KIR3DL3 to ensure the activity of NK cells and make them clear colon cancer tissues in vivo, but may have other ways or simply kill cells and tissues.

CR3: I think certain concentrations of astragaloside is lethal to normal tissue, but not to colon cancer tissue. these results indicate that astragaloside cannot be developed as a specific drug for colon cancer alone, and may need to be combined with other drugs to achieve the desired effect.

CR4: I think this result is completely consistent with the expected result of this hypothesis, which can be used as the basis for the next step of normal cell tissue killing pre-experiment and the experiment to determine the optimal concentration and the optimal treatment time of astragaloside.

CR5: I think the results showed that certain concentrations of astragaloside can bind to KIR3DL3 but has no harm to normal tissue cells and colon cancer cells, indicating that KIR3DL3 may be inhibited in the follow-up in vivo experiments of astragaloside to ensure the activity of NK cells to eliminate colon cancer cells, and in this case, it also has great potential for the treatment of colon cancer. It is also significant for its regulatory mechanism of regulating cellular immunity.

CR6: I think the results showed that astragaloside at a specific concentration could neither bind to KIR3DL3 nor kill normal tissues, but could kill colon cancer cells, indicating that astragaloside solution at this concentration may have specific killing effects on colon cancer cells or there are other mechanisms of action.

CR7: I think the results showed that astragaloside had no effect on the above three effects. Under the premise that the experimental process was correct, it may be due to inadequate concentration or the physiological effects of astragaloside need to be played in the body environment, and it has no effect on the treatment of individual cell populations.

CR8: I think the experimental results are completely contrary to the hypothesis, indicating that astragaloside has some regulatory mechanism or physiological effect on normal tissues, and finally achieves the regulation of other physiological functions in the body to control the cancer process.

Discussion of the possible results for the variables of concentration and treatment duration

The initial concentration gradient range of astragaloside IV was designed to be 0.1mg/L-1mg/L, the treatment time was 30 minutes, and the tumor size was measured every 2 days. Two months later, a series of tumor size/solution concentration curves were drawn for different treatment times, and the lowest peak interval of the curve was found for further concentration gradient setting, and the optimal treatment concentration was finally determined.

When the concentration is below the optimal level or the treatment time is shorter than the optimal duration, tumors in the experimental group are predicted to continue growing, albeit at a slower rate compared to the control group. Overall, these results demonstrate an inhibitory effect on colon cancer tumor growth. This suggests that either the concentration range or treatment duration is too low or short. (Figure 4)

When the concentration is appropriate and the treatment time is appropriate, it is predicted that the tumor size will gradually disappear within the observation time (half a month), rather than the immediate death of all. These results indicate that astragaloside solution can effectively inhibit the growth of colon cancer without harming tissue cells at proper concentration and treatment time. This suggests that either the concentration range or treatment duration is appropriate. (Figure 5)

When the treatment duration exceeded the optimal time or the concentration surpassed the recommended level, Astragaloside solution exhibited non-specific cytotoxicity towards cells. In such

instances, it was anticipated that a significant number of tumor cells would have undergone cell death during the initial or subsequent assessments, while a substantial loss of normal cells occurred during pre-experimental stages. This suggests that either the concentration range or treatment duration is excessively high or prolonged. (Figure 6)

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