Cover Page

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Title of Project: Study of the Anti-Cancer and Anti-Mitotic Activities of Acetylcorynoline Purified from *Corydalis* longicalcarata Rhizomes

Title: Study of the Anti-Cancer and Anti-Mitotic Activities of Acetylcorynoline Purified from *Corydalis* longicalcarata Rhizomes

Abstract

Introduction: My research is based on acetylcorynoline, which was extracted from *Corydalis* by methods of purification and bioassay-guided isolation along with corynoline. By experimenting—immunofluorescent staining, testing IC50, and time lapse— on RPE MBC cell, acetylcorynoline showed the ability of arresting mitosis and inducing polyploidy. Hence, I believe that acetylcorynoline might be able to conduct anticancer activity, which was never reported before.

Objective: In order to check whether acetylcorynoline has anticancer activity, I aimed my focus on the arrangement of spindle poles and kinetochores; I also focused on whether acetylcorynoline was able to induce polyploidy. In theory, if acetylcorynoline has anticancer activity, instead of forming regular spindles and order arrangement kinetochores, random arrangement of spindles and kinetochores would be resulted.

Method: In order to observe spindles and kinetochores in a cell, I have conducted immunofluorescent staining. Antibody Histone H3Ser10P was added first to identify cells in early mitosis and ß-tubulin to locate spindles and spindles poles. To provide image of kinetochores, antibody CREST is added to the cells. Later, a study of time lapse is conducted in order to observe the failure of cytokinesis.

Results: IC50 values were tested when acetylcorynoline was added in cancer cells. The results showed that the concentrations that acetylcorynoline could inhibit 50% of the cancer cells are majorly arranged from 1μM to 31μM, which indicate that acetylcorynoline was effective. The experiment was followed by double staining of Histone H3ser10P and ß-tubulin. When cells were treated with 10μM, ones that were in prometaphase showed the formation of monopolar spindle pole instead of bipolar poles. When cells were treated with 20μM, the cells showed multipolar spindle poles. Cells treated with the drug after 24 hours showed the induction of polyploidy. Antibody CREST showed a different result. When 10μM of acetylcorynoline was added to cells, no major change happened in either 6 hours or 24 hours, which is forming a line in the middle of the cells. However, when the cells were treated with 20μM, kinetochores were scattered, locating in every corner

of the cells. Cells treated with the drug after 24 hours also showed the induction of polyploidy. The final experiment Time Lapse showed that prophase in cells after treatment was significantly longer than the control group cells, hence the entire mitosis of treated cells was two times longer than the control. The Time Lapse also showed failure of cytokinesis—as cells started to separate into two cells, they stopped the separation and became a big cell again.

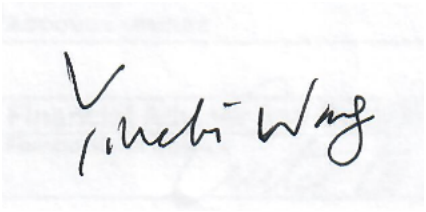
Conclusion: Acetylcorynoline has the ability to arrest mitosis and form polyploidy, hence having anticancer activity.

Key Word: Mitosis, Mitosis Arrest, Cytokinesis, Polyploidy, Monopolar, Multipolar, RPE series, CREST, DAPI, Histone H3serP, ß-tubulin, Immunofluorescent Staining, Time Lapse, Spindle, Spindle Pole, Kinetochore, Acetylcorynoline, IC50, Chromosomes, Anticancer Activity.

Declaration

I state that the submitted manuscript was the research work and the research results obtained under the guidance of my supervisor, Ziqi Yan at the J. Michael Bishop Institute of Cancer Research in Chengdu, China and instructed by Betty Wang from IvyMind in Delaware, USA. As far as I aware, except for the reference, the manuscript does not contain research results that others have published or written. If there is anything wrong, I am wiling to bear all relevant responsibilities.

Signature:



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Study of the Anti-Cancer and Anti-Mitotic Activities of Acetylcorynoline Purified from *Corydalis* longicalcarata Rhizomes

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1. Introduction

Rhizomes of *Corydalis* (Papaveraceae) was previously extracted by methanol extract when we were checking the crude extracts from the herbal library that were able to activate the spindle checkpoint and arrest cells in mitosis. After conducting bioassay-guided isolation and purification, we resulted in acquiring corynoline and acetylcorynoline. From previous studies, acetylcorynoline was identified with anti-inflammation activity[1], anti-fungal activity[2], and anthelminthic activity[3]. However, there was no report about anti-mitotic activity. Acetylcorynoline was find with the ability of arresting mitosis in prometaphase, and ultimately forming polyploidy in response of preventing cytokinesis. The results from experiments proved that acetylcorynoline has a huge potential for development of anticancer therapeutics.

The experiments were conducted on RPE series, which were derived from human retinal pigment epithelium. It is then stably transected with a hTERT expression construct for immortalization. The resulting cell line was then engineered to overexpress the MYC and BCL2 oncogenes, generating the RPE MBC cell line. The cell line mimics cancer cells that overexpress MYC and BCL2, and readily form viable polyploidy cells when cytokinesis is prevented.

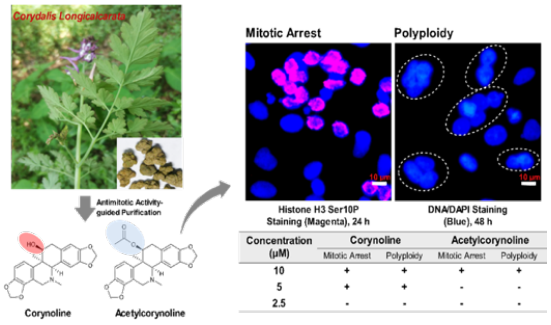
Polyploidy is when a cell contains more than two homogenous sets of chromosomes, which can be identified as a failure of mitosis. Mitosis undergoes five crucial stages: prophase, prometaphase, metaphase, anaphase, and telophase. During mitosis, the duplicated chromosomes are first condensed, followed by attachment of kinetochore microtubules, also known as spindle fibers. The spindle fibers come from the centrosomes that replicated themselves during G1 stage of interphase, a resting phase prior to mitosis. During prometaphase, spindle fibers will be projected from the centrosomes and connect with the aligned chromosomes. Microtubules, containing α- and ß-tubulin, play an important rule, such as regulation to cell movement, cytoplasmic transportation, and chromosome alignment, However, when microtubules are disrupted, monopolar and multipolar spindles will be produced, causing mitotic catastrophe.

In a series of experiments conducted by many reseachers, MTT method for IC50 was conducted in a variety of human cancer cell lines to test the survival rate of the cells after acetylcorynoline treatment, which could give us information about effectiveness. Later, we performed immunofluorescence of Histone H3 Ser 10 Phosphorylation (Histone H3ser10P) and ß-tubulin double staining, which revealed multipolar spindles. We also have preformed immunofluorescent staining with a human autoserum CREST, which revealed the erratic arrangement of kinetochore. Through observing in Time Lapse of cells treated with the phytochemical, we were able to see the failure of cytokinesis. All these resulted might suggest that acetylcorynoline is able to arrest cells in early mitosis, prevent cytokinesis, and lead to the formation of polyploidy.

2. Results and Discussion

2.1 Extraction of acetylcorynoline from *Corydalis* longicalcarata Rhizomes

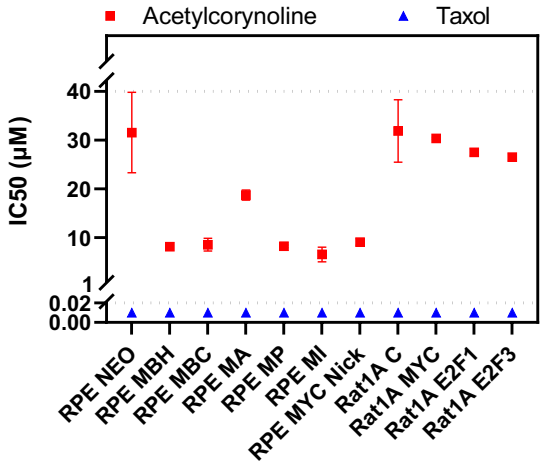
Figure 1. Acetylcorynoline is extracted from a natural product and shows the ability to arrest mitosis.



The researchers in the laboratory first screened the natural product library and found an extract that has anti-mitotic activity. Two compounds were later purified from *Corydalis* Longicalcarata, introducing corynoline and acetylcorynoline. The researchers furthered their experiment and conducted DAPI staining and Histone H3Ser10P staining, finding that two compounds can arrest mitosis within 24 hours after the treatment and induce polyploidy after 48 hours.

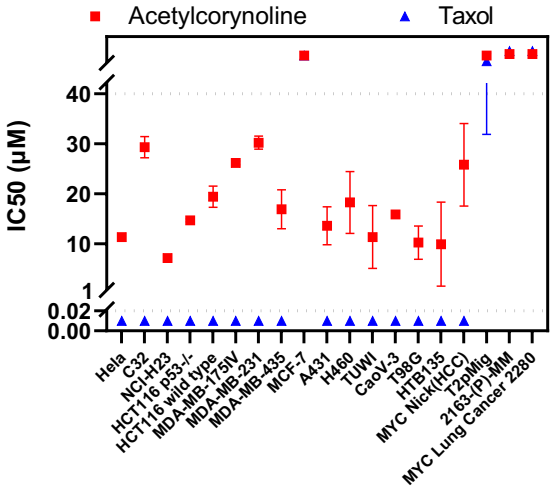
2.2 Study of effectiveness of acetylcorynoline

Figure 2. IC50 of acetylcorynoline in RPE cell line.



IC50 represents the concentration of drug that can inhibit a specific biological component by 50%. The results are compared with a positive control group Taxol, which is a medicine in the market that can kill fifty percent of the cells under 0.02 μM in all cells.

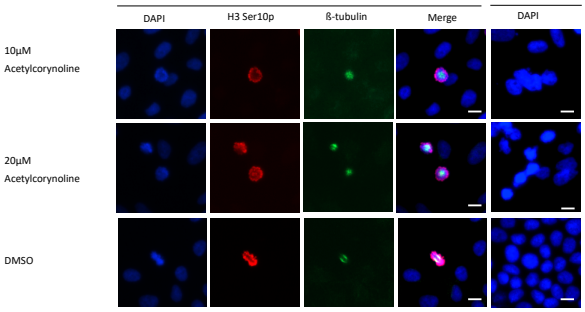
Figure 3. IC50 of acetylcorynoline in human cancer cell line.



In this experiment, the range of concentration is from 0.02μM to 40μM. As shown in both Figure 2 and Figure 3, the majority of the concentration for acetylcorynoline is between 1μM and 31μM. In Figure 2, RPE MBC cell line has its corresponding to 8μM. Even though the results show that acetylcorynoline are not as good as those of Taxol, the concentrations in Figure 2 and Figure 3 show the potential of this drug and is worthy to keep the research.

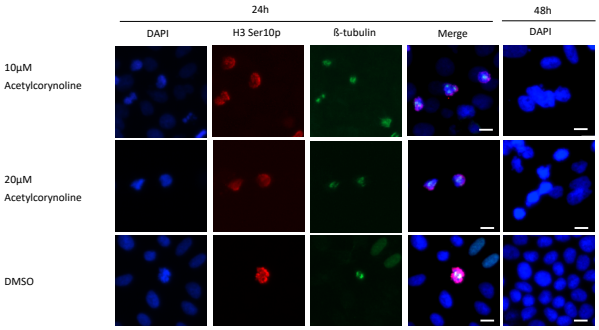
2.3 Double Staining of Histone H3ser10P and ß-tubulin

Figure 4. Effects of 6 hours after treatment with DAPI of 48 hours.



In this experiment, we can see the abnormal localization of spindles in early mitosis. Different staining has different functions. Histone H3ser10P (red on the diagrams) was used to indicate cells that were undergoing early mitosis. This includes prometaphase and metaphase. ß-tubulin (green on the diagrams) was used to determine the spindle fibers of the cells.

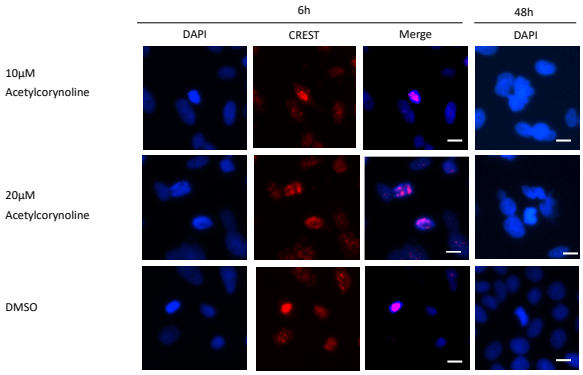
Figure 5. Effects of 24 hours after treatment and DAPI staining after 48 hours.



In Figure 4, after using Histone H3ser10P, we have located the cells in early mitosis. In DMSO (the control group), spindles were located regularly and correctly, as shown in ß-tubulin. This is when two poles (centrosomes) were located on the opposite sides of the cell, spurring microtubules to connect with kinetochores. However, compared to the DMSO, the cell that was treated with 10μM acetylcorynoline had irregular arrangement of spindles. Shown in the green diagram in the first row, there was only one pole, known as monopolar spindles, and the spindles were located all over the cell instead of arranging on the opposite sides. Similar result was shown in the cells after treated with 20μM acetylcorynoline. The spindles of both cells were located disorderly, forming two monopolar spindles. In Figure 5, the concentrations of the drug were still the same, but the cells were treated for 24 hours. In the DMSO group, it was obvious to see the bipolar arrangement of the spindles. Yet, after adding 10μM of acetylcorynoline, through ß-tubulin, multiple poles of spindles—multipolar spindles—were formed as a result. Similar effect performed on cells that were treated with 20μM of the drug. The spindles were arrayed disorderly, resulting in multipolar spindles. From the same figure, polyploidies were formed after 48 hours of treatment with different concentrations of the drug, meaning that the drug had effect on arresting mitosis. This experiment, first, directly shows the ability of arresting mitosis, which is an important element for anticancer activity aforementioned. Secondly, acetylcorynoline is identified with the ability of disordering spindles: monopolar and multipolar spindles. These aspects may result in failure of splitting cells or irregular splitting, which can result in polyploidy,

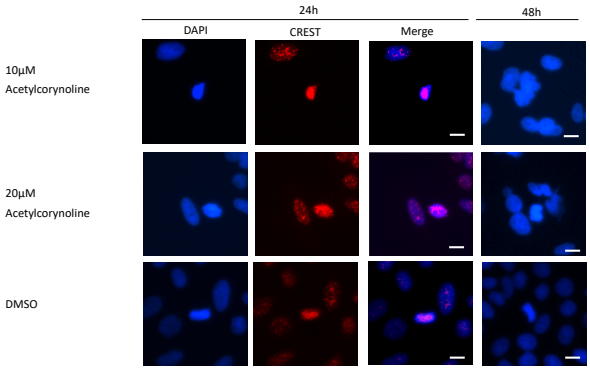
2.4 Immunofluorescent Staining of CREST

Figure 6. Result of 6 hours after treatment and DAPI staining after 48 hours.



In this experiment, we can see the abnormal localization of kinetochores in cells. RPE MBC cells were treated with CREST serum, which is represented as read in Figure 6 and Figure 7. The red dots on the diagrams represent kinetochores in cells.

Figure 7. Result of 24 hours after treatment and DAPI staining after 48 hours.



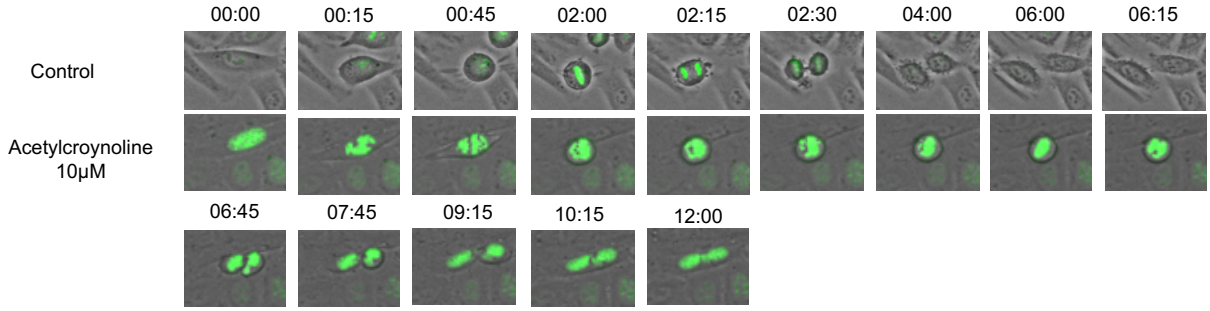
In Figure 6, DMSO group in 6 hours showed that kinetochores were located regularly in the middle

of the cell, representing that chromosomes are aligned normally in the middle of the cell. Compared to the DMSO group, after adding 10μM of acetylcorynoline, the kinetochores were scatted by a little, but could still be identified as locating in the middle of the cell regularly. However, localization of kinetochores was completely different when 20μM acetylcorynoline was added to the cells. The kinetochores were scattered and located randomly in the cell. Similar results were shown in 24 hours after the treatment. The DMSO group showed normal localization of kinetochore in the middle of the cell, compact and regular. In the cells after adding 10μM kinetochores were localized normally and regularly in the middle of the cells, just like the DMSO group. The kinetochores, yet, in cells after treated with 20μM showed irregular localization of kinetochores. The results from both time period suggested that the effect of acetylcorynoline on kinetochores depended on the concentration of the drug. If the concentration was too low, the drug would not have effect regardless of the duration of time. However, if the concentration is high enough, the drug would have its effect in a short amount of time.

DAPI staining after 48 hours showed an interesting result. In the DMSO group, there was no polyploidy formed in the cells. Even though low concentration of acetylcorynoline did not have significant effects on kinetochores, polyploidies were formed and were obvious. In 20μM, polyploidies were also formed. These elements showed that acetylcorynoline is effective on arresting mitosis.

2.5 Time Lapse on Treated Cells and Normal Cells

Figure 8. Time Lapse results used for comparison of cells after treatment and control group from prophase.



The Time Lapse showed the transition of cells from phase to phase. From Figure 8, the entire cell cycle after adding acetylcorynoline was twice as long as the control group cell. It can also be shown that prophase of the treated cell is significantly longer than the control: more than three hours longer. The diagram also showed that acetylcorynoline block the completion but not the initiation of cytokinesis.

3. Conclusion

Acetylcorynoline was never reported with the effect of anticancer activity; however, throughout my experiments, this drug has effects on preventing cancer cells to spread. Extracted from *Corydalis* longicalcarata Rhizomes, acetylcorynoline was shown with the ability to induce polyploidy. IC50 values, compared with Taxol, an anticancer drug that is already on the market, of acetylcorynoline suggested the effectiveness of the drug. Even though the values of acetylcorynoline were higher than those of Taxol, the drug still showed the potential of further development in the later experiments. Stained with Histone H3ser10P and ß-tubulin, cells treated with acetylcorynoline had irregular arrangement of spindles and spindle poles, forming monopolar or multipolar spindles. The next experiment showed that the kinetochores, giving the right amount, would be arranged disorderly. These two experiment were co-related because the spindles connect with the kinetochores. When the spindles were loosely arranged, kinetochores showed the same result too. Both of the experiments showed that

acetylcorynoline, after 48 hours, will induce polyploidy in both 10μM and 20μM. Time Lapse showed the abnormally long prophase, which resulted in longer cell cycle. It also showed that cytokinesis was initiated, but failed to complete, which resulted in polyploidy. All of these experiments have shown that acetylcorynoline has the effect of arresting mitosis, and hence anticancer activities.

4. Experimental

4.1 Cell Culture

Cells were cultured at 37 °C and 5% of CO2 in DMEM (Gibco, Cleveland, TN, USA) supplemented with 5% fetal bovine serum (Gibco), penicillin (100 U/mL)-streptomycin (100 μg/mL) (Gibco, 15140-122), 2mM L-glutamine (Gibco, 200mM solution), and 1mM sodium pyruvate (Gibco, 100mM solution).

4.2 CREST Staining

Immunofluorescent was conducted with antibodies against CREST. RPE-MBC cells were first on coverslips precoated with 0.1% gelatin in a six-well plate and allowed to adhere overnight at 37°C. The cells were then exposed to the chemicals for the indicated times(6 hours, 10 hours, 24 hours). For each time, one coverslip was taken out for immunofluorescent staining. The last coverslip was stained with DAPI at 48 hours. Once taken out from the well, the cells were washed with PBS once, fixed with 4% of paraformaldehyde (PFA) for 10 min in the presence of 0.5% of the detergent Tritonx-100, followed by washing the cells once with PBS with 1 minute. For blocking, coverslips were placed on parafilm, and then incubated with 5% BSA (each coverslip only for 50 μl) blocking buffer for 1h at room temperature, followed by washing the cells with PBS for 1 minute. The primary antibody anti-CREST-Human was performed by incubation for 2 hours at room temperature, and then followed by secondary antibody Rhodamine RedTM -X- conjugated AffiniPure Donkey Anti-Human IgG (H+L) for 1 hour at room temperature. Once reached the indicated time, the cells were washed 3 times by PBS and stood for at least 5 minutes. After washing the cells, coverslips were flipped and stained with DAPI Mounting Medium on a glass slide. Extra media was removed by tissue and glass slides were observed and analyzed under the Evos FL Auto fluorescence microscope (ThermoFisher). Once the observation is finished, slides were sealed with nail polish.

4.3 Histone H3 Ser 10 Phosphorylation and ß-tubulin Double Staining

Immunofluorescent was conducted with antibodies against Histone H3 Ser 10 Phosphorylation and ß-tubulin. RPE-MBC cells were first on coverslips precoated with 0.1% gelatin in a six-well plate and allowed to adhere overnight at 37°C. The cells were then exposed to the chemicals for the indicated times(6 hours, 10 hours, 24 hours). For each time, one coverslip was taken out for immunofluorescent staining. The last coverslip was stained with DAPI at 48 hours. Once taken out from the well, the cells were washed with PBS once, fixed with 4% of paraformaldehyde (PFA) for 10 min in the presence of 0.5% of the detergent Tritonx-100, followed by washing the cells once with PBS with 1 minute. For blocking, coverslips were placed on parafilm, and then incubated with 5% BSA (each coverslip only for 50 μl) blocking buffer for 1h at room temperature, followed by washing the cells with PBS for 1 minute. The primary antibodies ß-tubulin-Mouse and Histone H3(Ser10P)-Rabbit were performed by incubation for 2 hours at room temperature, and then followed by secondary antibody Alexa Flour 488 AffiniPure Goat Anti-Mouse and Rhodamine (TRITC) conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) for 1 hour at room temperature. Once reached the indicated time, the cells were washed 3 times by PBS and stood for at least 5 minutes. After washing the cells, coverslips were flipped and stained with DAPI Mounting Medium on a glass slide. Extra media was removed by tissue and glass slides were observed and analyzed under the Evos FL Auto fluorescence microscope (ThermoFisher). Once the observation is finished, slides were sealed with nail polish.

4.4 Time Lapse

From cell culture, cells were seeded in a twelve-well plate, then treated with acetylcorynoline. They were then put in Evos FL Auto (Thermo fisher). By using Time

Lapse, the process of cell division was shown. The cells were observed for 16 hours. Later the images were edited through Photoshop.

5. Acknowledgement

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