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Research Article

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Research of inhibition mechanism of morin on tumor cells aminopeptidase N

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ABSTRACT

In this paper, the method of enzyme inhibition kinetics was used to study the inhibition, inhibition type and inhibition kinetic constants of morin on aminopeptidase N.The leukemia cell growth inhibition test was carried out,and the mechanism of interaction between Morin and aminopeptidase was studied by fluorescence spectroscopy.At last the impact of Morin on aminopeptidase N structure was further explored. The results showed that Morin was a reversible competitive inhibitor of aminopeptidase N (half-inhibition rate IC50 was 70.85 μM, inhibition constant Ki was 21.23 μM); deactivation kinetics time course analysis showed that Morin could quickly act with aminopeptidase N and rapidly reduce the activity of the enzyme; fluorescence spectroscopy showed that Morin could significantly quench fluorescence of aminopeptidase N, and hydrophobic interaction was the main driving force of the interaction between the two; circular dichroism analysis showed that Morin induced the unfolding ofaminopeptidase conformation , so as to increase the α-helical content,which was detriment to the formation of the active center, leading to the reduction of activity of tyrosine aminopeptidase N.

Keywords: Morin; tumor cells; aminopeptidase N; inhibition; fluorescence spectroscopy; leukemia cells; secondary structure.

INTRODUCTION

The growth, invasion and metastasis of tumor is a complex multi-step process, including cell division, proliferation and extracellular matrix degradation. Tumor cells can enter the vascular system, and spread with the circulating, making cancer distant [1]; ammonia peptidase N (Aminopeptidase, APN) plays a crucial role in physiological regulation and signal transduction between cells in the body's tissues, whose function and role is different because of the different presence of positions; New research shows that aminopeptidase N have played a very important role in all aspects of malignant tumor metastasis , including the degradation of thymosin and interleukin, resulting in immune system function decreased, which provides convenient conditions for the growth of tumor, what is more, and generation of cardiovascular of tumor cells degradation of matrix is also inseparable of aminopeptidase N [2-5], which means that aminopeptidase N inhibitors can effectively prevent metastasis and metastasis of tumor.

Morin is a natural bioactive substance and widely distributed in nature. Chinese mulberry, Morusalba and mulberry are respectively from leaves, root bark and fruits of Moraceae plant. Mulberry is a food and medicine plants, which is used for medicinal purposes in both East and West. Morin can inhibit enzyme activity with the effect of anti-cancer, anti-bacterial, anti-inflammatory, anti-atherosclerotic, anti-stress and lowering blood sugar and so on [6]. Some data has indicated that Morin can inhibit the carcinogenic effects of benzopyrene on mouse skin [7], and can specifically enhance immune function; as well as destruct or inhibit morphology, proliferation of cell lines of liver cancer, esophageal squamous cell carcinoma and so on without inhibition of bone marrow cells. It is used clinically for liver cancer, esophageal cancer, gastric cardia, etc. It can also be used as a premedication or in combination with chemotherapy, and in combination with other chemotherapy drugs to improve the efficacy and reduce side effects. To explore whether the anticancer effect of Morin is related to its inhibition of aminopeptidase N this article will research inhibition mechanism of morin on tumor cells Aminopeptidase N and analysis inhibition type and

inhibition kinetic constants of Morin on aminopeptidase N, as well as explore the interaction mechanisms between Morin and aminopeptidase N and chelation with zinc ions ,and further study the affect of conformation of aminopeptidase N in order to provide basic data for the development and applications of Morin in the field of functional medicine.

EXPERIMENTAL SECTION

1.1 Inhibition of Morin on aminopeptidase N

Aminopeptidase N catalytic substrate (L-leucine-p-nitroanilide phthalate) produce Para- nitroaniline which has UV absorption at 405 nm,and concentration of Para- nitroaniline is positively correlated with the size of active enzyme;determine the activity of aminopeptidase N by testing the change of absorbance at 405 nm , which can indirectly reflect the degree of inhibition of the inhibitor on enzyme activity[8]. After Morin (AR, Aladdin reagent, Figure 1) and aminopeptidase N (Sigma Company) is incubated for 3 h at 37 ℃, pH 6.8 phosphate (50 mmol / L) buffer system, L - leucine phthalate-P-nitroaniline (AR, A Sigma) is added. Kinetics / time software of UV-2450 UV - visible spectrophotometer (Shimadzu Corporation) is used to measure change of absorbance values at 405 nm and relationship formula: Relative activity (%) = (R/R_0) × 100% is used to calculate inhibitory activity of morinonaminopeptidase N; wherein R_0 is the slope variation of absorbance change without inhibitor, R is the slope variation of absorbance change in the system containing different concentrations of morin. Bestatin (AR, Sigma Company) is set as a positive control.

1.2 Study of inhibition kinetics of Morin on aminopeptidase N

The concentration of aminopeptidaseN is fixed at 37 ℃ in the same buffer system , and enzymatic reaction rate is measured at different substrate concentrations. Lineweaver-Burk is used for picture, and analyze the inhibition kinetics of Morin on aminopeptidase N based on the apparent Michaelis constant of the enzymatic reaction (Kmapp) and the change of the maximum reaction rate (Vmax).

1.3 Cell growth inhibition test of Morin on leukemia

The succinate dehydrogenase in mitochondria of living cells can restore yellow bromide 3 - (4,5 - dimethyl-2) -2,5 - diphenyltetrazolium (MTT) to insoluble blue-purple formazan which deposites in the cell. Usually the generating amount of formazan is proportional to the number of viable cells and the cell viability; formazan can be dissolved by dimethylsulfoxide (DMSO),whose content can be determined by measuring the absorbance at 570 nm. Thus the number of the viable cell can be indirectly presumed according to the change of the absorbance measured at 570 nm to assess the ability of the inhibitor or anti-tumor[8].

Bestatin is set as a positive control (aminopeptidase N is the main target of Bestatin which can induce apoptosis of leukemia cells in acute myeloid), the blank control group is without Morin. Morin and human acute myeloid cells from leukemia cells with different concentrations are incubated for 48h at 37 ℃, 5% $CO_2$and then 0.5% MTT stain is added. After 4 h of incubation, 2500 rpm, centrifuged 30 min, the medium is discarded,and DMSO solution is added. The absorbance value (OD value) at 570 nm is measured by UV-2450 UV - visible spectrophotometer and cell growth inhibition rate is calculated as the following formula:

Inhibition rate (%) = (blank OD value - inhibitor OD value / OD value of the blank) × 100%

1.4 Determination of fluorescence spectra

The Morin solution (0-3.54 × 10-4 mol L-1) is continuously added in 3.0 mL of aminopeptidase N solution(2.0 × 10-6 mol L-1). F-7000 fluorescence spectrophotometer (Hitachi, Japan) is used at three temperatures of 298, 304 and 310 K to scan fluorescence spectra in the range of 300 - 500 nmwith excitation wavelength of 280 nm and 2.5 nm of excitation and emission slits.

1.5 Determination of circular dichroism spectra

Different concentrations of morin is added to the aminopeptidase N solution, mixed, with pH 6.8 phosphate (50 mmol / L) buffer as the blank. The circular dichroismspectroscopy of sample solution is measured at 37 ℃.

RESULTS AND DISCUSSION

2.1 Inhibition of Morin on activity of aminopeptidase N

The Figure 2 has shown that, as the concentrations of inhibitor Morin increases, the activity of aminopeptidase N decreases continuously. The relative activity of aminopeptidase N trends to be stable until the concentration of Morin reaches 150 μM, indicating that inhibition of Morin on activity of aminopeptidase N is in a concentration-dependent manner.Its 50% inhibitory concentration (IC50) is 70.85 μM, and Morin has showed some

inhibitionpositive control on tyrosinase compared with the positive control of Bestatin IC50 of 21.23 μM, which is consistent with the previous report in the literature.

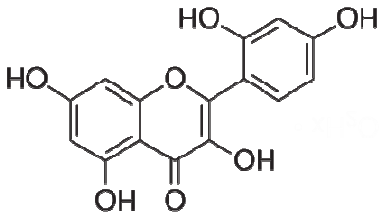

Fig 1 Structure of galangin

Figure 3 has shown the relationship between the activity of aminopeptidase N and the amount of added enzyme. In measured live system of Morin with different concentrations ,a straight line through the origin is plotted through the enzyme activity to the amount of the enzyme, and as Morin concentration increases, the slope of each line continues to reduce , indicating that the increase in the concentration of Morin inhibits aminopeptidaseN activity, rather than causing decreased enzyme activity by reducing the effective amount of enzyme, which shows that inhibition of Morin on activity of aminopeptidase N is a reversible process [9].

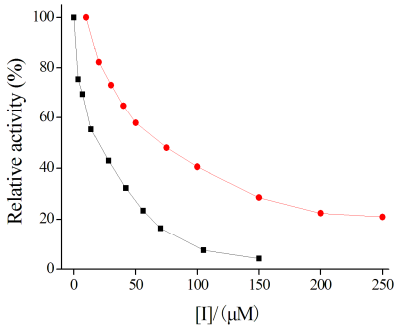

Fig.2 Inhibitory effect on APNofmorin

(■)is Bestatin，(●)is Morin，c(APN) = 4μg/mL, c(L-Leucinephthalocyanine-P-nitroaniline) = 1×10^{-3} mol/L.

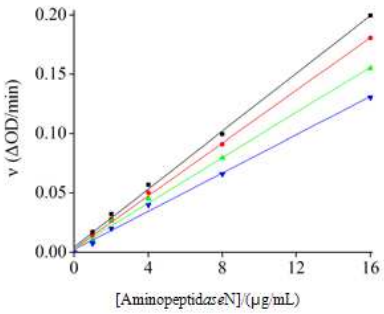

Fig 3 Inhibitory type on APN ofmorin

c (L-leucyl-P-nitroaniline phthalate) = 1 × 10-3 mol / L, c (APN) = 4 μg / mL to c (Morin)from top to bottom of the curve: 0, 25, 75 , 150 μM

2.2 Determination of the time course of inhibition of Morin on aminopeptidase N

At the same buffer system, the same volume of incubation solution is removed from different concentrations of morin - enzymes at intervals of 180 s, and then the substrate L-leucine phthalate-P-nitroanilines is added to measure the time course of loss of aminopeptidase N activity. As Figure 4 has shown, at different concentrations of Morin, the relative activity of aminopeptidase N does not change with the increasing incubation time,which has shown that Morin can easily interact with the aminopeptidase N, and quickly change the physiological structure and properties of the enzyme, ultimately inhibit the enzyme in a short time and make the relative activity do not change with the incubation time [10].

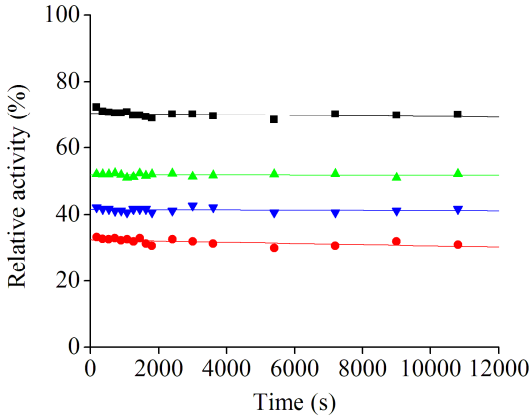

Fig 4 Time-course of APN inhibition in the presence of morin

c (APN) = 4 μg / mL, c (Morin) from top to bottom of the curve: 50, 75, 100, 150

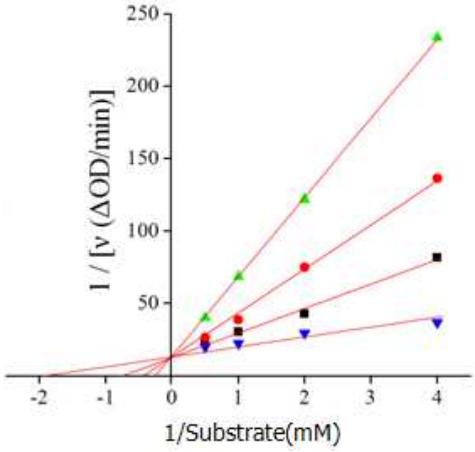

Fig 5 Lineweaver-Burk plot for inhibition ofmorin on APN

c(APN) = 4 μg/mL, c (Morin) from top to bottom of the curve:0, 75, 100, 150 μM.

2.3 Study of inhibition kinetics of Morin on aminopeptidase N

The inhibition type of Morin on aminopeptidase N is further validated. Under the same experimental conditions the inhibition of Morin on polyphenol oxidase is studied at different concentrations to research the inhibition kinetics of Morin on aminopeptidase N and the Kmapp and Vmax kinetic parameters is obtained by plotting according to Lineweaver-Burk double reciprocal equation. It can be seen from Figure 5 that Lineweaver-Burk double reciprocal plot is a straight line intersecting at the vertical axis, indicating thatVmax remains unchanged while Kmapp gradually increases in the system, which is the typical type of competitive inhibition [11]. This has shown that Morin may combine to the activity center of aminopeptidase N and the active sites of the substrate competition so as to obtain the inhabiting effect. On this basis the inhibition constant Ki = 24.83 μM is obtained by plotting for different concentrations of Morin through the different Y-axis intercepts.

2.4 Inhibition of Morin on growth of leukemia cells

In this study,inhibition of Morin on human leukemia cell is researched taking human acute myeloid leukemia HL-60 cells as the study and Bestatin as a positive control.It can be seen from Figure 6 that, inhibition rate of Morin on human leukemia cell is constantly increasing as concentration of inhibitor solution increases,and also has a concentration-dependent phenomena; half inhibitory concentration IC50 of Morin and Bestatin positive control on leukemia cells is calculated respectively as 61.23μM and 49.34μM, data has shown that ability of Morin inhabiting the growth of HL-60 cells is slightly higher than that of Bestatin, which may be due to that the inhibition on the activity of aminopeptidase N by Morin has induced apoptosis of human leukemia cells.

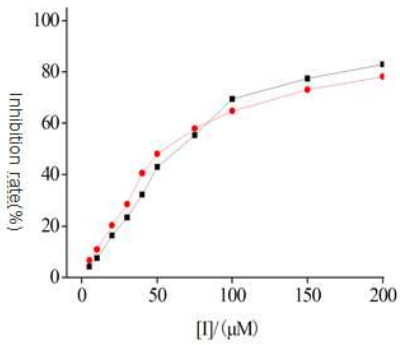

Fig 6 Inhibitory effect on the growth of leukemia cells of morin

2.5 Effect of morin on fluorescence spectra of aminopeptidase N

By measuring fluorescence spectra changes of aminopeptidase N in the presence of Morin it has been found that endogenous fluorescence of aminopeptidase N quenches in concentration-dependent manner with the increasing concentration of Morin (Figure 7). This suggests that interaction may occur between the two [12]. Binding parameters between Morin and aminopeptidase N is further obtained, using the formula:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a}$$

[13]to calculate the binding constant at 298K K ¬ a = 3.98 × 104 L moL-1;

The main driving force involved during the interaction of small molecules and biomolecules includes hydrogen bonding, hydrophobic interactions, van der Waals and electrostatic forces [14]. The type of the main force between small molecules and proteins can be determined according to the numerus of thermodynamic parameters enthalpy change (ΔH) and entropy (ΔS). When temperature changes in a small range the enthalpy during the reaction can be treated as a constant. The corresponding ΔH and ΔS is obtained by the van't Hoff equation :
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$$\lg K_a = -\frac{\Delta H}{2.303\,RT} + \frac{\Delta S}{2.303\,R}$$

Kb is the apparent binding constant at the corresponding temperature, R is the gas constant.

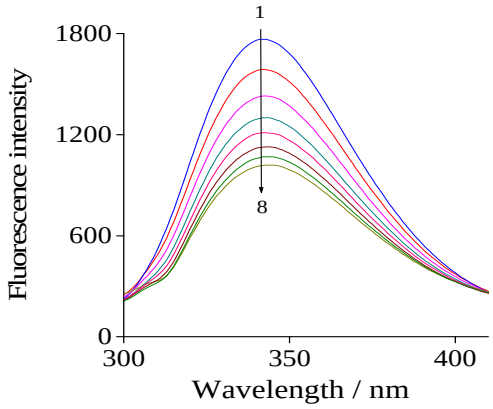

Fig 7 Effects of morin on fluorescence emission spectrum of APN

c(APN) = 4 μg/mL, c(Morin)/(μM), 1–8 : 0, 25, 50, 75, 100, 125, 150, 175.

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Kb is the apparent binding constant at the corresponding temperature, R is the gas constant.

Plot by lgKa to 1 / T, the enthalpy and entropy change of the system can be calculated by the slope and intercept of the fitted line. Free energy change ΔG can be calculated by the following equation:

$$\Delta G = \Delta H - T\Delta S$$

The thermodynamic analysis about the interaction of Morin with aminopeptidase N has been made to obtain that the binding reaction between the two is a spontaneous process. Calculate ΔH and ΔS is respectively36.58 kJ · mol-1 and 168.12 J · mol-1 · K-1, suggesting that complex formation process between Morin and aminopeptidase N is an entropy-driven endothermic reaction during which hydrophobic interactions are the main driving force.

2.6 Effect of Morin on secondary structure of aminopeptidase N

Online Dichroweb software is used to calculate the secondary structure content before and after the combination of aminopeptidase N and Morin, results are shown in Table 1. As it can be seen from the table that α-helical content of the free aminopeptidase N is 21.2%, β-sheet content is 27.2%. When the concentration of Morin put in aminopeptidase N reaches 150 μM, α-helical content increases to 32.9%, β-sheet content reduces to 20.2%, which shows that Morin induces some conformational changes in aminopeptidase N. And as the content of α-helix increases, the structure of aminopeptidase N becomes close, which is detriment to the formation of the active center of the enzyme, resulting in the decreased vitality of aminopeptidaseN [15], which may be one of the mechanisms of inhibition of morin on activity of aminopeptidase N.

Table 1 The contents of different secondary structure of tyrosinase in the presence of luteolin

[cyanidenon] (μM)	α- spiral /%	β- fold /%	β- turn /%	random coil /%
0	21.2	27.2	21.2	30.4
50	24.8	25.0	21.8	28.4
100	28.2	22.2	22.7	26.9
150	32.9	20.2	22.9	24.0

CONCLUSION

This study has shown that Morin is a reversible competitive inhibitor of aminopeptidase N (half inhibition rate IC50 of 70.85 μM, inhibition constant Ki was 21.23 μM); deactivation kinetics time course analysis has shown that Morin can quickly interact with aminopeptidase N and rapidly reduce enzyme activity; ability of Mori inhibiting the growth of HL-60 cells is stronger than that of positive control Bestatin, which shows that Morin may induce apoptosisbe of human leukemia cells due to the inhibition on aminopeptidase N activity; fluorescence spectrum analysis displays that Morin can significantly quench fluorescence of aminopeptidase N with the binding constant Ka of 3.98 × 104 L mol-1; and hydrophobic interactions is the main driving force of the interaction between the two; round dichroism analysis has suggested that Morin induces unfolding of aminopeptidase N conformation, causing the increase of α-helix content, which is detriment to the formation of activity center, leading to the reduction of the activity of aminopeptidase N; On the one hand Morin competitively binds with ammonia peptidase N active sites, on the other hand it has an impact on the secondary structure of multi-aminopeptidase N, the two aspects together form the mechanism of inhibition of morin on aminopeptidase N's.

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