

The Actions of Leech Saliva Components and Their Mechanisms in Antitumor Activity

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Abstract

Malignant tumor (hereinafter referred to as tumor) has become a major category of chronic diseases that curb human life and seriously threaten human health. For advanced tumors, chemotherapy, radiotherapy, targeted therapy and immunotherapy effects are still not satisfactory, and they are particularly urgent to find new therapeutic drugs. The medical leech therapy (MLT) can be traced back to the period of ancient Greece and ancient India for disease treatment. The discovery and appraisal of the leech saliva of hirudin aroused the research interest on the saliva of the leech. Yet of the more than 100 compounds of different molecular weights detected in leech saliva, only a small fraction have been identified as having therapeutic potential. Studies have shown that the saliva activity ingredients have certain advantages in anti-tumor metastasis, which has attracted the attention of scholars. This article reviews the role of the active ingredients of the saliva and its anti-tumor mechanism in recent years, and provides a reference for the application of the leech saliva.

Keywords: leech saliva; anti-tumor; pharmacological effect; hirudin; active components

1. Introduction

Malignant tumor (hereinafter referred to as tumor) has become a major category of chronic diseases that curb human life, and the incidence is increasing year by year, seriously threatening human health. For advanced tumors, chemotherapy, radiotherapy, targeted therapy and immunotherapy effects are still not satisfactory. With drug resistance and adverse reactions, its long-term use is limited, and it is particularly urgent to find new therapeutic drugs. Leech, is a hirudinidae annelid, the body is flat back and abdomen, the front end is narrow, there are two suction cups before and after, respectively have bite and adsorption. The earliest use of leeches in the treatment of diseases dates back to ancient Greece and ancient India, and medical leech therapy (MLT) works by releasing

leech saliva through leeches biting the human body. Since hirudin in leech saliva was discovered and identified, the mechanism of leech therapy has been gradually revealed. However, more than 100 compounds of different molecular weights detected in leech saliva, only a small fraction have been identified as having therapeutic potential^[1]. Studies have shown that the saliva active ingredients have certain advantages in anti-tumor metastasis^[2], which has attracted the attention of scholars.

2. Leech saliva active ingredients

Leech saliva contains many active components, with the deepening of research on its composition, it is found that different compounds play different roles, and the research directions and findings related to the pharmacological effects of leech saliva in recent years are summarized as follows:

2.1 Anticoagulant effect

The leech saliva components stop platelet aggregation and inhibit coagulation factors at different levels through different mechanisms, which mainly include the following compounds:

Hirudin^[3] : a peptide consisting of 65 or 66 amino acids with 3 disulfide bonds, its main feature is 63-position sulfated tyrosine, which increases hirudin and thrombin binding affinity by 10 times. It is a highly specialized thrombin inhibitor that blocks thrombin between platelets and inhibits platelet aggregation which is stimulated by thrombin, a property that leads to the disintegration of platelets.

Antistasin^[4] : derived from Mexican leeches, a peptide containing 55 amino acids that can precisely act on factor Xa, thereby preventing the conversion of prothrombin to thrombin and exerting anticoagulant effects.

Saratin^[5] : a 12 kDa molecule that binds to exposed type I and II collagen, competitively inhibits collagen binding to vWF, and also prevents platelet aggregation.

Calin^[5] : a 65 kDa protein that closely resembles Saratin's mechanism of action and binds primarily to type I collagen, thereby preventing vWF from binding to exposed collagen and platelet aggregation.

Apyrase^[5] : a small number of studies have reported it as a 45 kDa enzyme. The increase in ADP concentration can lead to increased platelet affinity for vWF, which mainly degrades ADP into AMP, thereby inhibiting the ability of platelets to adhere to the site of vascular injury.

2.2 Thrombolytic effect

Destabilizing enzyme^[6], a protein with a molecular weight of approximately 12.3 kDa, is also a highly specific endo ϵ -(γ -Glu)Lys-isopeptidase, an enzyme that catalyzes the hydrolysis of fibroin-stable linked isopeptide bonds. Destabilizing enzymes have a unique D-dimer monomerization ability, which presupposes the splitting of Lys-isopeptide bonds between the γ chains of ϵ -(γ -Glu)D-dimers. The effect can change the balance between fibrinogen and fibrin products during fibrin degradation, which is conducive to the formation of fibrin degradation products and inhibits the activation of fibrinolysis. The presence of these properties makes destabilizing enzymes a specific and effective means of stabilizing the fibrinolysis process.

2.3 Anti-inflammatory effect

Proteolytic enzyme inhibitors are present in the saliva components of leeches, a special class of proteins that have different structures and protease inhibition mechanisms that can jointly block different kinds of proteolytic enzymes^[7]. These substances are vermiptotease inhibitors, Hirustasin, trypsin inhibitors, leech inhibitors, destabilizing enzymes and certain antimicrobial peptides.

Vermiptotease inhibitors are a group of peptides and they are inhibitors of trypsin, plasmin, and acrolein. Hirustasin is a serine protease inhibitor that, unlike Antistatin, doesn't block the activity of Xa coagulation factor but inhibits trypsin, chymotrypsin, cathepsin G, and kallikrein^[8]. Trypsin^[5] is the main component of mast cell secretory cytoplasmic granules and trypsin has also been found that it has a very important pathogenic role in allergic and inflammatory diseases, which is related to mast cell dysfunction (asthma, rheumatoid arthritis, psoriasis, etc.). In addition trypsin inhibitors are first discovered and extracted in European medical leeches and they are the first protease inhibitors closely bound to human mast cell trypsin. It is hypothesized that trypsin inhibitors secreted by leech saliva block mast cell enzyme-induced defense mechanisms when they act in humans. Although trypsin inhibitors extracted from leech saliva have shown great efficacy, they have shown inconsistent effects in different studies of recombinant trypsin inhibitors, making it difficult to evaluate the actual clinical efficacy of recombinant trypsin inhibitors^[5]. Leech inhibitors are proteins with a molecular weight of about 8 Da, which are mainly found in leech saliva and have been reported to inhibit the effects of chymotrypsin, chylase, mast cell chymotrypsin, subtilis protease and human blood neutrocyte protease, elastase and cathepsin G^[7]. Among them, the most thorough study is Eglin C, which mainly inhibits human neutrophil elastase and cathepsin G (one of its strongest inflammatory mediators), thereby exerting its anti-inflammatory effect^[9].

2.4 Immunosuppressive effect

Compounds in leech saliva enter the body's internal environment, and as foreign bodies, they are supposed to undergo an appropriate response from the immune system. However, no expression of immune response was seen in the body after multiple applications of leech saliva. In addition, through the use of leeches some scholars have successfully treated patients with autoimmune diseases such as bronchial asthma, rheumatoid arthritis, systemic scleroderma and so on, which may be the credit of unknown components of saliva. The following components have been found in leech saliva that may hinder mast cell defense mechanisms: pancreatic enzyme inhibitors in mast cells and C1s protease inhibitors- preventing C1s enzymes from activating the C4 component, thereby preventing the formation and activation of C3 convertases of the C2 bond and the classical pathway of the complement system, blocking the body's immune response^[10].

2.5 Lymphatic circulation stimulating effect

This action of leeches may be related to the presence of hyaluronidase^[11], which reduces the viscosity of hyaluronic acid by breaking the C1-acetylglucosamine and C4-glucuronic acid bonds, increases the permeability of tissues, improves their nutrition, increases the elasticity of scar-modified areas, relieves the diffusion of fluid in

the tissue space.

3. Antitumor mechanisms in leech treatment

Hirudin is the largest compound in leech saliva and has been studied many times which has been confirmed that it has certain efficacy in anti-tumor, including liver cancer, lung cancer, laryngeal cancer, malignant glioma and prostate cancer, etc.

3.1 Inhibit the NF- κ B pathway

Thrombin is a serine protease that converts fibrinogen to fibrin during clotting. In the physiological state, the concentration of thrombin in the blood is very low but in the pathological state it maintains high concentration so that the body maintains a hypercoagulable state such as in tumor diseases^[12]. So in the treatment of cancer, inhibition of thrombin activity is often one of the methods. NF- κ B is a family of transcription factors that can be divided into canonical and non-canonical NF- κ B pathways, which play an important role in inflammation, cell proliferation and differentiation, immune response, etc., of which I κ B kinases and p65 proteins are important members of this pathway^[13]. Vasculogenic mimicry is a novel model in the tumor microenvironment that provides blood flow to tumors in the absence of endothelial cells and it has been shown to be associated with tumor grade, invasion, growth, and prognosis^[14]. In experiments by BingZhao^[15] et al. found that thrombin can promote the occurrence of EMT and tumor vascular mimicry (VM) through the NF- κ B pathway. The expression of E-cadherin in the experimental group of human lung cancer cells treated with thrombin and the experimental group of mice inoculated with lung cancer cells were down-regulated, the expression of vimentin and N-cadherin were upregulated, the formation of tumor vascular mimicry (VM) increased, the vascular leakage increased, the volume and weight of in situ tumors in mice increased significantly, the number of lung metastases and nodules increased, which is the obvious opposite of the results of the recombinant hirudin group. Thrombin has also been shown to promote phosphorylation of I κ B α and p65, but recombinant hirudin can reverse this effect. It suggests that hirudin may inhibit epithelial-mesenchymal transformation and tumor vascular mimicry (VM) formation by inhibiting the activation of the NF- κ B pathway. Thrombin can use the PAR-1 receptor to participate in inflammation, angiogenesis and metastatic transmission of tumor cells. In another experiment by bingzhao^[16] et al., it was demonstrated that thrombin can also promote RhoA to express by activating PAR-1, thereby inducing cytoskeletal rearrangement, letting actin stimulate fiber to form and activation of NF- κ B pathway as well as the expression of MMP9 and inflammatory marker IL-6. Recombinant hirudin can specifically combine with thrombin to block the binding of thrombin to PAR1, thereby inhibiting the activation of RhoA and NF- κ B in NSCLC cells and reducing the expression of MMP9 and IL6, which is very beneficial for inhibiting growth, invasion and metastasis. Combining a couple of experimental results, there is reason to believe that recombinant hirudin has an inhibitory effect on the NF- κ B pathway and can inhibit tumor progression through this pathway.

3.2 Regulate the cell cycle

The cell cycle regulates the occurrence and progression of tumors. The main markers of cancer are genetic variation in cell cycle regulators or checkpoints, disruption of signal transduction leading to cell cycle disorder and abnormal cell division^[17]. A growing number of studies have shown that using the regulatory mechanism of the cell cycle as the entry point for cancer treatment can inhibit the mitosis of cancer cells and even reverse cancer metabolism^[18]. At the same time, thrombin also plays a role in the cell cycle. Clinically, there are already reagents for the treatment of oncoproteins in the cell cycle, which have been approved. In the experiments of Liang Hu et al.^[19] verified that thrombin can regulate mitosis in prostate cancer cells, mainly through the upregulation of the expression of miR-222 and SPK2 by PAR-1 in thrombin and down-regulating the expression of P27^{kip1}, which promotes the transition of the cell cycle from G0-G1 phase to S phase and promotes the growth of tumor cells. However, in the study they also found that hirudin can inhibit the effect and significantly inhibit the volume and weight of tumors compared with the control group. Experiments by Yang Shen et al.^[20] showed that hirudin has an effect on the cell cycle, which can block human bladder cancer cells in the S phase, and they researched the function of exosomes released by hirudin-treated cells (hereinafter referred to as hir-exos). And flow cytometry showed that hir-exos can block the cell cycle in the G2 phase and reduce the proportion of cells in the G1 phase. The above experiments show that hirudin, as a powerful thrombin inhibitor, plays a major role in regulating cell cycle changes.

3.3 Inhibits the expression of VEGF

VEGF and its receptor (VEGF-R) are important regulators of tumor angiogenesis. In 1971, Folkman proposed the theory of "anti-angiogenesis therapy for cancer"^[21]. At present, there are clinical targeted drugs that inhibit the growth of anti-tumor blood vessels, confirming the status of vascular endothelial-derived growth factor (VEGF) in cancer treatment, which can not only promote the growth of new blood vessels, but also can change the permeability of blood vessels^[22]. In experiments by Y Q Huang et al.^[23], it was confirmed that thrombin can promote the production and secretion of VEGF in human prostate cancer cells and maintain its stable expression on cells but hirudin can significantly inhibit this effect. In Hitoshi Yamahata et al.^[24] experiments, it was shown that in human glioma cells, thrombin regulates the expression of VEGF through PAR-1 and the VEGF concentration increases by 1.5~3 times when its concentration is from 0.1U/L to 10U/L. But after treating cells with hirudin, the stimulating effect of thrombin is significantly weakened. This proves that hirudin can inhibit the expression of VEGF, reduce tumor angiogenesis, and inhibit tumor growth.

3.4 Inhibits ERK/MAPK pathways

The ERK/MAPK signaling pathway, also known as the Ras/RAF/MEK/ERK (MAPK) signaling pathway, plays an important role in cell proliferation, differentiation and metabolism, and the activation of this pathway has been shown to be associated with cancer^[25]. In the past few decades, targeted therapies targeting this pathway have been clinically available, but unfortunately these drugs haven't been able to effectively inhibit mutants. Therefore, as an anti-tumor drug, the role of hirudin cannot be ignored. Li Zhao^[26] research showed that hirudin can inhibit the viability of malignant glioma cells in a dose-dependent. With increasing concentration decreases cell viability,

LN229 and U251 hirudin 50% growth inhibition is 30 mM and 15 mM, respectively, which is mainly achieved by changing the position of ERK1/2 in cells and downregulating the expression of the EERK/MARK pathway. The experiment shows that hirudin also has an inhibitory effect on the ERK/MAPK pathway, pointing out a way for future research.

3.5 Relieve chemotherapy-induced peripheral neuropathy

Chemotherapy induced peripheral neuropathy (CIPN) is a serious adverse reaction of chemotherapy drugs, and pain is the main manifestation. Its pathogenesis is still unclear. Hirudin can not only inhibit the development of tumor, but also relieve CIPN. Yang Yang^[27] et al. used oxaliplatin (L-OHP) to establish the CIPN model. They found that chemotherapy would lead to the enhancement of tissue factor (TF), which would lead to the activation of p38/TF/HIF- α pathway, and the increase of ROS, which would lead to the overexpression of MMP-9/2, resulting in pain. They used hirudin for treatment in the experiment, which can inhibit the activation of TF-HSP70-TLR4-P38 pathway and the expression of ROS, improve microcirculation disorders, and relieve pain in mice.

4. Antitumor mechanism of other components of leech saliva

Many studies have shown that the extract of leech saliva has obvious anti-tumor effects^[28-29], but more scholars have proposed that the components of leech saliva can play an anti-tumor role as a combined anti-metastasis agent. In addition to anti-platelet and anti-thrombin activities, the anti-metastasis effect of saliva components of leech can also be exerted by inhibiting protease hydrolysis activity and inhibiting tumor cells from entering the cell matrix^[30]. In addition to hirudin, which has anti-tumor effect, active substances such as protease inhibitors, hyaluronidase, Hirustasin, Antistasin and saratin should also be included in the extracts of leech saliva.

4.1 Protease Inhibitors

Leech saliva contains protease inhibitors that inhibit protease activity in the body. The development of cancer is usually accompanied by the breakdown of extracellular matrix and tissue invasion, and protease inhibitors can interfere with these processes to prevent the spread and invasion of cancer cells^[7]. For example, Hirustasin^[8] is a serine protease inhibitor that does not block Xa clotting factor activity, but inhibits trypsin, chymotrypsin, cathepsin G, and kallikrenase. It acts by inhibiting protease hydrolysis activity and inhibiting the entry of tumor cells into the cell matrix.

4.2 Hyaluronidase

Hyaluronic acid is a polymer associated with tumor metastasis that promotes the interaction between cells and extracellular matrix. It has been reported that the presence of CD44 receptors on the cell membrane increases with the increase of destructive cancer cells, and this receptor is a precise receptor for hyaluronic acid, which is believed to promote the spread, migration, attack and metastasis of cancer cells^[8]. Hyaluronidase contained in the saliva of leeches may exert anticancer activity by inhibiting tumor-promoting substances in the tumor matrix to a certain extent, thus slowing the progression of cancer. Figure 1 illustrates the effects of hyaluronidase in leech

saliva extract (LSE) on cancer cells^[8].

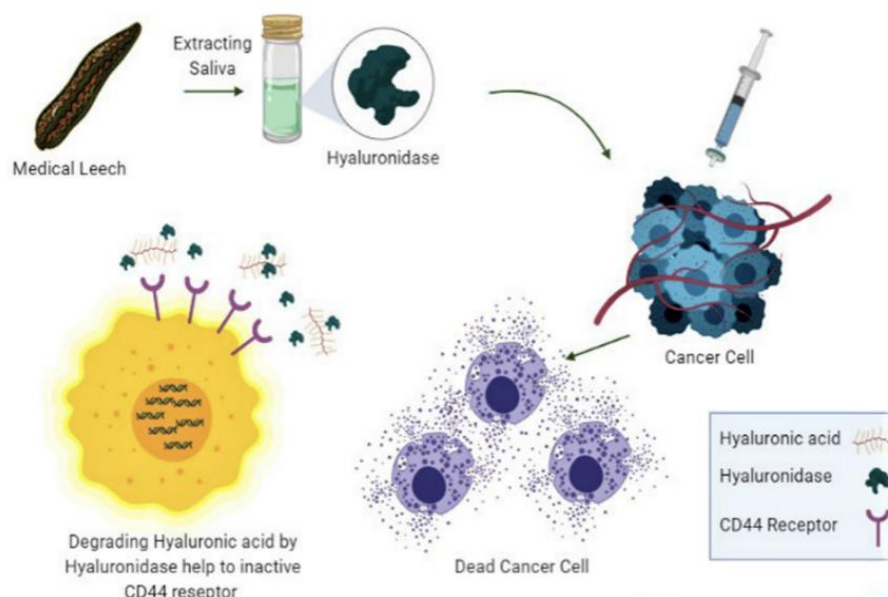


Figure 1. Activity of Hyaluronidase in Cancer. Degrading hyaluronic acid by hyaluronidase, inhibiting CD44 activation and after a while mRNA of VEGF reduced and it helps to reducing angiogenesis of cancer cells.

4.3 Other salivary proteins of leeches

As explained above, Antistasin and Saratin act as proteins extracted from leech saliva, and they exert anti-tumor effects through anti-platelet as well as antithrombin activity. Blankenship DT et al. ^[31] studies have shown that the protein Ghilantens extracted from South American leeches salivary gland cells has anticoagulation and metastasis, which can affect the migration and invasion of tumor cells, and help prevent cancer from spreading. It has been reported ^[32] that antithrombin extracted from the salivary glands of Mexican leeches, a protein with a mass of 17000 Da, can effectively inhibit the growth of T241 sarcoma cells when used in mice, and has obvious anticoagulation and anti-metastasis effects at low dose, mainly on factor X. There is no need for an anticoagulant effect as heparin relies on thrombin III. Merzouk A et al. ^[2] reported that salivary extract of medical leeches in Malaysia had a significant inhibitory effect on the metastasis and proliferation of lung cancer cell line SW1271, and the higher the concentration, the stronger the inhibitory effect. The experiment showed that salivary extract had synergistic effect with ilittecane and carboplatin, which provided a new method for cancer treatment.

4.4 Synthetic protein preparations

A study synthesizes the protein preparation with anti-tumor metastasis. These preparations can effectively inhibit the metastasis of a variety of cancer types, including lung cancer, breast cancer, bladder cancer, colon cancer, soft tissue sarcoma, leukemia and lymphoma^[33]. Some scholars can significantly inhibit the growth of breast cancer cells by targeting liposomes combined with leech protein into tumor cells. The efficiency of this combination treatment is very high, achieving a 97% kill rate^[34].

5. Conclusion

The active ingredients of leech saliva has shown strong anti-tumor effects in vitro and animal experiments, not only inhibiting tumor growth, invasion and metastasis, but also alleviating pain caused by CIPN. However, the anti-tumor mechanism and effect of leech saliva components need to be further explored and verified. A further research strategy is to correlate proteomic and transcriptomic data to study bioactive proteins and peptides derived from leech saliva^[35]. There is still a long distance between the current results and clinical application, so we still need to strengthen the in-depth study of the active ingredients of leeches.

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