Proteolysis is the most fundamental property of malignancy and its inhibition may be used therapeutically (Review)

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Abstract. The mortality rates of cancer patients decreased by ~1.5% per year between 2001 and 2015, although the decrease depends on patient sex, ethnic group and type of malignancy. Cancer remains a significant global health problem, requiring a search for novel treatments. The most common property of malignant tumors is their capacity to invade adjacent tissue and to metastasize, and this cancer aggressiveness is contingent on overexpression of proteolytic enzymes. The components of the plasminogen activation system (PAS) and the metalloproteinase family [mainly matrix metalloproteinases (MMPs)] are overexpressed in malignant tumors, driving the local invasion, metastasis and angiogenesis. This is the case for numerous types of cancer, such as breast, colon, prostate and oral carcinoma, among others. Present chemotherapeutics agents typically attack all dividing cells; however, for future therapeutic agents to be clinically successful, they need to be highly selective for a specific protein(s) and act on the cancerous tissues without adverse systemic effects. Inhibition of proteolysis in cancerous tissue has the ability to attenuate tumor invasion, angiogenesis and migration. For that purpose, inhibiting both PAS and MMPs may be another approach, since the two groups of enzymes are overexpressed in cancer. In the present review, the roles and new findings on PAS and MMP families in cancer formation, growth and possible treatments are discussed.

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1. Introduction

Research on cancer formation and treatment has finally started to reduce the mortality of cancer patients. In general, mortality rates decreased by 1.5% per year from 2001 through 2015, although these rates may differ depending on patient sex, ethnic group and type of malignancy. Nevertheless, cancer remains a significant global health problem, requiring constant search for novel treatments (1). The most common property of the malignant tumors is their ability to invade adjacent tissue and to metastasize to distant sites (2,3). This is due to increased proteolysis associated with cancer formation (4-9). For example, cysteine proteases, such as cathepsin B and cathepsin D, can take part in dissolving of connective tissue and basement membrane needed for invasion and metastasis. However, these become activated at the low pH of lysosomes, requiring transporting into regions of tumor cell invasion (10-13). Aspartate proteases and other protease enzymes also serve a role in cancer development and metastasis (14-16). Nevertheless, among all these proteolytic events, the plasminogen and metalloproteinase family members have an exceptional significance due to their ability to cleave virtually any element of the extracellular matrix and basement membrane (17-20).

The components of the plasminogen activation system (PAS) and matrix metalloproteinase (MMP) families have been found to be overexpressed in malignant tumors, revealing that tumors are hijacking these systems and using them in the local invasion, metastasis and angiogenesis (21-23). One of the hallmarks of this cancer aggressiveness is the overexpression of proteolytic enzymes (24-27). For instance, prostate cancer...
is the most common non-cutaneous malignancy in men with >160,000 new cases in the United States each year, and the median age of diagnosis is 68 years. This cancer grows slowly, and is initially confined to the gland, requiring marginal or no treatment at all. However, certain types of prostate cancer are aggressive, can spread quickly and exhibit high proteolytic activity (28-31). This is also a case for other types of cancer, such as breast, colon and oral carcinomas among others (32-36).

In the present review, new findings on the role of plasminogen activation system (PAS) and metalloproteinase families in cancer formation and growth were summarized, and the study explored whether targeting these two protein families can serve as a possible treatment strategy.

2. PAS family

Plasminogen/plasmin. Plasminogen is synthetized as a zymogen in the liver and released into the systemic circulation. In the circulation, plasminogen is resistant to activation by adopting a closed conformation. When bound to blood clots or to the cell surface, plasminogen changes to an open form that can be cleaved into active plasmin by a variety of enzymes. These enzymes are mainly plasminogen activators, namely tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), while other enzymes include kallikrein and factor XII. Plasminogen is converted into plasmin by the cleavage of the peptide bond between Arg-561 and Val-562 (37,38). Plasmin, a member of the serine protease family, was originally considered to only act to dissolve fibrin blood clots. However, in addition to fibrinolysis, plasmin cleaves proteins in numerous other systems. For instance, it activates collagenases, and cleaves fibronectin, thrombospondin, laminin and von Willebrand factor (26,35,38).

Two major forms of plasminogen are detected in humans, including type I and type II. Notably, type I plasminogen has two glycosylation moieties, namely N-linked to N289 and O-linked to T346, which is preferentially recruited to the cell surface. Type II plasminogen contains only O-linked sugar to T346, which appears to be more readily recruited to blood clots (39-41).

To prevent premature or unnecessary proteolysis, the activity of plasmin needs to be tightly controlled. Two proteins, α2-macroglobulin and α2-antiplasmin, inactivate plasmin, and the underlying mechanism involves the cleavage of α2-macroglobulin at the bait region by plasmin. This initiates a conformational change resulting in the α2-macroglobulin-plasmin complex, where the active site of plasmin is sterically shielded, decreasing the access of plasmin to its substrates (42). In addition, a major conformational change exposes a conserved COOH-terminal receptor binding domain that allows the α2-macroglobulin protease complex to bind to clearance receptors and consequently be removed from the circulation (43). Furthermore, α2-antiplasmin forms an irreversible complex with plasmin by attaching to Arg-376 in the reactive center loop, resulting in the complete loss of plasmin activity (42).

A rare disorder in humans known as plasminogen deficiency type I may lead to thrombosis, as blood clots are not effectively degraded. Plasminogen deficiency in humans is caused by numerous mutations and polymorphisms in plasminogen gene and is often manifested by ligneous conjunctivitis (44). Ligneous conjunctivitis is characterized by fibrin-rich pseudomembranous lesions that develop mainly on the underside of the eyelid, which can be sight-threatening (45). It may also influence the periodontal tissue, upper and lower respiratory tract, kidneys, middle ear and female genitalia, and affects wound healing (46-50).

Plasminogen activators. There are two main plasminogen activators, uPA and tPA (35,51,52), which are weak but specific proteolytic enzymes that activate plasminogen to plasmin by proteolytic cleavage. tPA mainly mediates intravascular thrombolysis, while uPA is involved in pericellular proteolysis during cell migration, wound healing and tissue remodeling under a variety of physiological and pathological conditions (35).

tPA. The encoded preprotein of tPA is proteolytically cleaved by plasmin or trypsin to produce heavy and light chains. These chains are linked by disulfide bridges to form an active heterodimeric enzyme that is able to activate plasminogen (53). Increased tPA activity triggers hyperfibrinolysis, which causes excessive bleeding and an increase of the vascular permeability (54-57), while decreased activity leads to hypofibrinolysis, which can result in thrombosis or embolism (58-60).

uPA. Similar to tPA, uPA is synthetized as a preprotein and is activated by cleavage at a Lys-Ile bond by plasmin to form a two-chain enzyme connected by a disulfide bond. In its active form, uPA contains the amino terminal A-chain that is catalytically active, and the carboxy-terminal B-chain. This two-chain derivative form is referred to as high molecular weight uPA (HMW-uPA). HMW-uPA can be cleaved at the A-chain into a short chain A (A1), forming low molecular weight uPA (LMW-uPA) and an amino-terminal fragment. LMW-uPA is able to activate plasminogen, however, it does not bind to the uPA receptor (uPAR) (61,62). A C/T single-nucleotide polymorphism (SNP) in codon 141 of urokinase (known as PI41L) may be associated with late onset Alzheimer's disease, decreased affinity for fibrin-binding (63) and reduced risk of Helicobacter pylori infection (64).

Urokinase receptor. uPAR, also known as cluster of differentiation 87 (CD87), was originally identified as a saturable binding site for uPA and contains three domain glycoprotein bound to the cell surface with a glycosylphosphatidylinositol (GPI) anchor. All domains of uPAR are needed for high affinity binding of the urokinase (21,27,35). Urokinase receptor anchors uPA and therefore confines plasminogen activation in the vicinity of the cell membrane. However, when uPA is bound to its receptor, it may be cleaved in the proximity of the GPI anchor, and the uPAR is released as a soluble receptor (65-67). Urokinase receptor has also been suggested to be involved in non-proteolytic processes, such as cancer, cell migration, cell cycle regulation or cell adhesion (35,65-67). A previous study reported that rs344781 (516 T/C) uPAR polymorphism was implicated in systemic sclerosis vasculopathy, impaired angiogenesis (68) and the severity of lung cancer (69).
Inhibitors of plasminogen activators. Plasminogen activator inhibitor-1 (PAI-1), also known as endothelial PAI or serine protease inhibitor E1, is a fast-acting, high-affinity, principal inhibitor of tPA and uPA. The other PAI, namely PAI-2, is only produced in physiologically significant amounts during pregnancy and secreted by the placenta. Protease nexin can also inhibit tPA and uPA, however, PAI-1 remains a major inhibitor of plasmin-driven proteolysis (35,70-72). PAI-1 is overexpressed in various diseases, such as obesity and metabolic syndrome, and has been linked to risk of thrombosis in patients with these conditions (36,73,74). Also, it has been reported that a high activity of PAI-1 is associated with recurrent pregnancy loss (75,76). By contrast, a low level of PAI-1 leads to excessive plasmin fibrinolysis that is unopposed by PAI-1 and rapid degradation of the fibrin, which may manifest in profuse bleeding. Indeed, it was reported that a life-long bleeding tendency was caused by undetectable PAI-1 activity and antigen levels in a 76-year-old man, while severe menorrhagia has been reported in patients with a low PAI-1 antigen level (77-79). Notably, in the case of low activity of PAI-1, women have achieved pregnancy without difficulty, but experienced antenatal bleeding and preterm labor (80).

The promoter polymorphisms (844 A/G and 675 4G/5G) in the PAI-1 gene yield higher plasma PAI-1 levels (81). Another SNP with substitution of A15 to T15 and possibly V17 to Ile in the signal peptide leads to lower PAI-1 activity compared with a control (78,82,83). In addition, a previous study reported that a young Amish girl and certain members of her extended family had no PAI-1 antigen and PAI-1 activity, leading to excessive bleeding. They were found to be homozygous for a dinucleotide insertion within exon 4 of PAI-1 gene, producing a truncated, non-functional protein (78,79). The diverse function of PAS, as discussed in the present study, is outlined in Fig. 1.

3. Metalloproteinase family

Matrix metalloproteinases (MMPs). MMPs also known as matrixins, are metal-dependent (Ca and Zn) endopeptidases that belong to a larger family known as the metzincin superfamily (84-87). These enzymes degrade all types of extracellular matrix proteins and are differentiated from other endopeptidases by their dependence on metal ions as cofactors (88,89). MMPs are synthesized as inactive zymogens with a domain that must be removed to activate enzymes. This domain is part of the cysteine switch, containing a cysteine residue that prevents metals binding and keeps the enzyme in an inactive form (90-93). Matrix metalloproteinases are also involved in cell proliferation, migration, differentiation, angiogenesis and apoptosis (92,94).

MMP-2 and MMP-9, two members of the 25-protein family of MMPs, are considered to be critical in local invasion and metastasis of cancer in humans (94,95). One of the most important functions of these enzymes in cancer progression is their ability of extracellular matrix degradation, which enables cancer cells to migrate out of the primary tumor site and to form metastases. Both MMP-2 and MMP-9 are capable of degrading type IV collagen, the most abundant component of the extracellular matrix.
of the basement membrane, which is an essential step for the local invasion and metastatic progression of cancer (95,96). In addition, MMP-2 and certain other MMPs can proteolytically activate transforming growth factor β, which promotes epithelial mesenchymal transition, an important step in cancer metastasis (96,97). Diverse functions of metalloproteinases are outlined in Fig. 2.

SNPs in the promoter region of MMP-2 (1306C>T and 735C>T) are associated with lower transcriptional activity, and the SNP in the promoter part of MMP-9 gene (1562C>T) amplifies the transcription of this metalloproteinase gene. Others SNPs in the coding region of the MMP-9 gene include 836A>G, 1721G>C and 2003G>A, which produce a missense amino acid sequence, reducing the substrate and inhibitor binding ability of MMP-9 (98,99).

**Tissue inhibitor of metalloproteinase (TIMP).** MMPs are inhibited by four different specific endogenous tissue inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. All MMPs are inhibited by TIMPs when in their active form, with the exception of MMP-2 and MMP-9, which can form complexes with TIMPs in the latent form (99,100). Ricci et al (101) investigated MMP-2 and MMP-9, as well as the inhibitors TIMP-1 and TIMP-2 in the urine and sera of bladder cancer patients. The data revealed that urinary TIMP-1 can be a useful, non-invasive biomarker that may serve as a decision making tool for bladder cancer disease management (101). Three SNPs were reported in TIMP-1, including 261 C>T, 328+16 C>T and 372 T>C, while six SNPs were reported in TIMP-2, including rs2277698 T>C, rs2009196 C>G, rs7342880 A>C, rs11654470 C>T, rs2003241 C>T, rs4789936 T>C (103).

### 4. Proteolysis in angiogenesis, invasion and metastasis

The hallmark of metastatic disease is an altered equilibrium between the synthesis and degradation of the extracellular matrix proteins during pathological angiogenesis, invasion and metastasis (35,102).

**Angiogenesis.** Angiogenesis is the physiological or pathological development of revascularization from existing vessels. It is a normal process during growth and development, and in wound healing; however, angiogenesis is also an essential step in malignant tumor growth (26,27,34,35). Sprouting angiogenesis occurs as angiogenic growth factors activate receptors on endothelial cells, followed by the release of proteases (PAS and MMP) that degrade the basement membrane, permitting cells to escape in tandems to or from the vessel walls (35,110). The endothelial cells then proliferate into the surrounding matrix and form solid sprouts, connecting neighboring vessels. These processes are basically the same in both normal and aberrant angiogenesis (26,35,104).
Invasion and metastasis. Lah et al (105) introduced definition of ‘degradome’ that includes proteases associated with degradation of adjacent tissue during cancer invasion and metastasis. The study stressed that invasion and metastasis were initially considered to occur as late events in cancer development. However, other studies have indicated that these processes are also highly dependent on proteases activity that takes place during early stages of cancer development (105,106).

5. Inhibition of PAS in the treatment of cancer

It has previously been reported that receptor-bound uPA on the cancer cell surface is overexpressed in final step of malignant cell transformation that is responsible for invasion and metastasis (107). In addition, uPA has been implicated in the basement membrane and interstitial protein degradation during carcinogenesis, and is responsible for the disease progression (108-110). Therefore, inhibition of uPA may be beneficial in the treatment of cancer by limiting or preventing tumor growth and angiogenesis (26,111,112). Amiloride has been introduced as an elevated PAI-1 level is a predictive factor of poor prognosis. Additionally, other studies have also revealed that these processes are highly dependent on proteases activity that takes place during early stages of cancer development.

Figure 3. Ribbon model of (A) MMP-9 and inhibitors, and (B) uPA and inhibitors. The inhibitors of MMP-9 and uPA are shown as sticks models in red, while ions in MMP-9 are shown as spheres (blue, Zn2+; light blue, Ca2+; green, Cl−). Surface of active site of (C) MMP-9 and (D) uPA. Epigallocatechin gallate is shown as a stick model in light brown. Polar contacts are shown as the yellow dotted lines [reprinted with permission from the study of Jankun et al (113)]. MMP-9, matrix metalloproteinase 9; uPA, urokinase plasminogen activator.

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Multiple novel inhibitors developed by the Bruncko et al (115) and Katz et al (116) groups, only a limited number of inhibitors were translated into clinical practice. One of these is WX-UK1, also known as ethyl 4-[3-(3-carbamidoylphenyl)-2-[[2,4,6-tri(propan-2-yl)phenyl]sulfonylamino]propanoyl] piperazine-1-carboxylate or Mesupron®, which is one of the most potent uPA inhibitors (0.41 µM), and is currently in Phase I clinical trial for pancreatic and breast cancer, with the possibility of application in ovarian and colon cancer as well (117-120).

Another possibility for inhibiting uPA is through the use of antibodies. For instance, a number of studies by Ossowski (121,122) have reported that the ability to invade the chick chorioallantoic membrane and metastasize from it to the embryo was evidently reduced for human carcinoma cells expressing a high amount of uPA during treatment with the antibody against the active site of uPA, in comparison to non-treated cells.

PAI-1 is also an attractive molecule for anticancer therapy, since it is a fast-acting, specific and high-affinity inhibitor of uPA (35,123). However, only active PAI-1 can be used to control angiogenesis, invasion and metastasis of malignant tumors. The half-life of wild-type PAI-1 is very short (t1/2 = 1-2 h), after which it converts into its inactive latent form. This transformation is associated with the insertion of part of the reactive loop (P4-P10) into the central β-sheet of the PAI-1 molecule, where P1-P1’ amino acids are not accessible for reaction with tPA or uPA (35,124). For that reason, wild-type PAI-1 cannot be used in anti-cancer therapy. Numerous engineered PAI-1 molecules with two or more mutations were developed, which exhibited a half-life extended from 2 h to >700 h, opening a possibility for their application in clinical settings (124-126). For instance, VLHL PAI-1 (t1/2 = >700 h) has been demonstrated to inhibit angiogenesis and reduce tumor size in different ex vivo and in vivo models (26,71,110).

Notably, PAI-1-deficient mice exhibited lower tumor proliferation and higher apoptosis of implanted tumors when compared with their wild-type counterparts. Additionally, an elevated PAI-1 level is a predictive factor of poor prog-
nosis in invasive breast cancer in humans (34,127,128). When PAI-1 was administered in low concentrations, it was able to increase the metastatic potential and angiogenesis (34,127-129). These observations contradict our previous statements suggesting that PAI-1 is a potent inhibitor of angiogenesis and tumor growth (26,125,128,130). This paradox can be explained by the interaction of PAI-1 with vitronectin. This protein is a multifunctional glycoprotein present in the plasma, platelets and the extracellular matrix. PAI-1 binds to vitronectin and then binds with uPA/uPAR, forming a four protein complex (namely uPAR/uPA/PAI-1/vitronectin), which can be further combined with LDL receptor-related protein. This interaction weakens the PAI-1/vitronectin binding and triggers PAI-1/uPA/uPAR internalization. PAI-1 and uPA are degraded, whereas uPAR is recycled to the cell surface. Subsequently, uPAR can bind to uPA again and form a new PAI-1/uPA/uPAR/vitronectin complex. This process clears PAI-1 in the immediate vicinity of the cell, forcing it to migrate toward increased concentration of PAI-1 (131,132). Thus, PAI-1 can have a dual function as a pro-carcinogenic and anti-cancer agent, depending on its concentration. PAI-1 in physiological concentration acts by utilizing the vitronectin pathway. However, as it has been demonstrated in our previous studies and by other groups, when PAI-1 is present in much higher supraphysiologic concentrations, it suppresses the vitronectin pathway and acts as a potent inhibitor of angiogenesis by utilizing primarily its inhibitory properties to block proteinase activity (133-136).

uPAR (or CD87) is a multidomain glycoprotein attached to the cell membrane with a glycosylphosphatidylinositol (GPI) anchor. When binding to uPA, it creates high proteolytic activity in the proximity of the cell surface (137-139). Therefore, another approach to prevent invasion or metastasis is to block the binding of uPA to its receptor on the cancer cell surface. Similarly, limiting the binding of uPA to vascular cells would reduce cancer-associated angiogenesis. An octapeptide A6 (amino acids 136-143; nonreceptor-binding domain) of uPA inhibits the interaction of uPA with its receptor uPAR, and was reported to inhibit endothelial cell motility and tumor cell invasion (117,140,141). Furthermore, the use of this polypeptide has been reported in Phase I and II clinical studies of gynecological cancer (142). Mani et al (143) synthesized a methyl 3-aminobenzoate derivative that binds to uPAR with high affinity and demonstrated that this uPAR inhibitor lowers the invasion ability of breast MDA-MB-231 cells. In addition, female NSG mice inoculated with highly malignant TMD-MDA-MB-231 cells presented impaired metastasis to the lungs when treated with this uPAR inhibitor (143). In a subsequent study by Wang et al (144), the affinity of a large number of derivatives was measured using structure-based virtual screening. The synthesis of additional and cellular studies in cell lines (A549, H460 and H1299) revealed that these compounds blocked invasion, migration and adhesion, while the effects of these compounds on invasion were consistent with their inhibition of uPAR (144). Treatment of metastatic liver cells with 1 or 4 g/ml lectin from Bandeiraea simplicifolia seeds significantly inhibited the cell migration and invasion, and downregulated MMP2, MMP9 and uPA production (145). Furthermore, the AKT/GSK-3β/β-catenin pathway, which is upstream of MMP2, MMP9 and uPA, was found to be involved in the inhibition of cell migration and invasion by lectin (145). In a similar manner, quercetin functions on the uPA/uPAR system (146).

A total of 14 clinical studies can be found in the clinicaltrials.gov registry on PAS and cancer. However, results have been posted for only one of these studies, and this study was abandoned due to limited activity of the agent (https://ccr.cancer.gov/news/clinical-trials). The diverse function of PAS is outlined in Fig. 1.

6. Inhibition of MMPs in the treatment of cancer

Inhibitors targeting MMP activity were initially intended to target the catalytic domain of these enzymes. The most clear approach was to synthetize polypeptides derived from the sequences of the amino acids of the MMP substrates, preventing hydrolysis of the scissile bond of proteins (147). Batimastat (INN/USAN, codenamed BB-94), a broad-spectrum, competitive, injectable drug was the first MMP inhibitor (MMPI) to go into clinical trials, acting as an angiogenesis inhibitor. While this agent reached Phase III trials, it was never marketed due to side effects (injection into the peritoneum caused peritonitis), poor solubility and very low oral bioavailability (147-149). Marimastat, another MMPI drug, exhibited some effects in delaying disease progression; however, it also had a dose-limiting toxicity. Patients experienced significant musculoskeletal pain and inflammation, and the clinical study was discontinued (147,150). The small molecular inhibitors of MMPs failed for a variety of reasons, despite preclinical data demonstrating their great potential for therapeutic use, leading to cancelled phase I trials on Prinomastat, and cancelled phase III trials on Batimastat, Marimastat, Tanomastat and CGS 27023A, which is a doxycycline tetracycline derivative (147).

Using hybridoma methods against the MMP-9 catalytic domain, Martens et al (151) generated the murine monoclonal antibody REGA-3G12. This antibody recognizes the W116 to K214 of the human MMP-9 catalytic domain, but not the Zn²⁺ binding site (151). The REGA-3G12 antibody inhibits MMP-9, but not MMP-2 (151,152). However, while MMPs have been recognized as a marker of poor prognosis and are one of the major classes of proteolytic enzymes involved in tumor invasion and metastasis, inhibitory monoclonal antibodies against MMPs have yet to find broad clinical applications.

**Clinical studies on MMPs in cancer.** In total, 62 clinical studies can be found in the clinicaltrials.gov registry on MMP and cancer. Several of these have been completed; however, none of the studies have posted results and numerous were abandoned (https://ccr.cancer.gov/news/clinical-trials) (135,147,148,150).

7. Conclusion

The mainstream of the presently used chemotherapeutic agents attack all dividing cells, leading to the adverse effects that are typically associated with cancer treatment, such as hair loss or loss of appetite. For future therapeutics to be clinically successful, they need to be highly selective for a specific protein or proteins, and able to act on the cancerous tissues without causing adverse systemic effects. Despite unsuccessful early clinical studies on the inhibition of proteolysis in cancer (particularly MMPs), this
approach still has great potential for therapeutic use. Inhibition of proteolysis in cancerous tissue has the ability to attenuate tumor invasion, angiogenesis and migration, which are the most fundamental properties of malignant tumors, thus deserving future development (147,153). This includes application of inhibitors at the time and stage of malignancy for which regulation of pathological proteolysis will be most beneficial. For instance, inhibiting cancer proteolytic enzymes during or after surgery can prevent cancer cell implantation and consequently cancer reemission (113). Inhibiting both uPA and MMPs is another beneficial approach, since tumors overexpress these groups of enzymes; therefore, employing this simple concept can be equally beneficial as cancer cell killing (Fig. 3). Epigallocatechin gallate, an inhibitor of uPA and MMPs, has previously been used, and this agent had a weak lethality, but prevented cancer cell implantation into the bladder wall following surgery and was slightly better in reducing cancer growth when compared with mitomycin C (Fig. 4) (113). Inhibition of PAS and MMPs simultaneously by a cocktail of inhibitors or a single inhibitor that is active against both proteolytic enzymes in all other types of cancer remains unexplored, but is a promising therapeutic approach deserving future development.

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