

Yin and yang of the plasminogen activator inhibitor

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ABSTRACT

Plasminogen activator inhibitor type one (PAI-1) is involved in many biological processes, and depending on a disease its overexpression, deficiency or normal level can sometimes produce surprising outcomes. This paper reviews low, high, and normal levels of PAI-1 in relation to diseases, their incidents, and possible treatment. Also, a role of PAI-1 in cancer is discussed with attention to "PAI-1 paradox" and explanation of the possible therapeutic applications using this protein.

In general, proteolysis is defined as directed degradation of proteins by enzymes. It plays an important role in physiological and pathological functions of the organism. Proteolysis is tightly controlled by regulation on the level of expression, activation, and inhibition. One of the members of the plasminogen activation system (PAS), plasminogen activator inhibitor type one (PAI-1), is involved in many biological processes and depending on a disease its overexpression, underexpression or normal level can sometimes produce surprising outcomes. "Yin and yang" refers to a Chinese expression describing complementary opposites within a greater whole and it comes to mind when describing functions of PAI-1.

The plasminogen activation system The PAS contains a number of elements described below.

Plasminogen Plasminogen is a pro-enzyme that is cleaved by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) to its active form called plasmin. Plasmin is a proteolytic enzyme able to digest proteins of connective tissue and basement membranes. It also activates other latent proteolytic enzymes, broadening the spectrum of proteins attacked. Pro-collagenase is activated to collagenase in this manner. Plasmin is a key enzyme in the mechanism responsible for tissue remodeling, tumor invasion, and development of distant metastasis and angiogenesis.¹

uPA and tPA activators Both uPA and tPA are weak proteolytic enzymes that activate plasminogen to plasmin by proteolytic cleavage. uPA is involved in pericellular proteolysis during cell migration, wound healing, and tissue remodeling under a variety of physiological and pathological conditions. tPA mainly mediates intravascular thrombolysis.²

Inhibitors of plasminogen activators There are four known protein inhibitors of uPA/tPA: PAI-1, PAI-2, PAI-3 and a protein called nexin. All of them are regulatory proteins mediating proteolysis on the activation level. Most relevant seems to be PAI-1, which exists in three different forms: active, nonactive-latent, and cleaved. PAI-1 has a dual function; first, it plays an important role as a direct inhibitor of the plasminogen activation system, and second, its interaction with the adhesive glycoprotein vitronectin plays a role in tissue remodeling and metastasis. This function is independent of its proteinase inhibitory properties. Unique to this serpin is the close association between its conformational and functional properties.³

uPA receptor The binding site of uPA is called the uPA receptor (uPAR). It is a glycoprotein that binds uPA to the cell surface while uPA retains its ability to activate plasminogen. High numbers of uPA receptors on the surface of cancer

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cells, if occupied by uPA, enhance the proteolytic activity in the proximity of cancer cells.⁴

PAI-1 half-life PAI-1 is a fast-acting, highly specific inhibitor of tPA and uPA, but it is not a stable molecule and converts itself into the latent form ($t_{1/2} = 1\text{--}2\text{ h}$). This conversion is associated with partial insertion of the reactive loop (P4-P10') into the central β -sheet of the PAI-1 molecule. In such a conformation, P1-P1' and other sites are not accessible for reaction with tPA or uPA.

PAI-1 mutants with extended half-life Several mutants which reduce or prevent insertion of the reactive loop into the PAI-1 molecule have been produced in the past ($t_{1/2}$ 6–170 h).^{5,6} We have developed 7 other mutants with $t_{1/2}$ of 4–700 h by replacing amino acids with cysteines to form one, two or three disulfide bridges (Cys31-Cys97, Cys192-Cys347, Cys197-Cys355). Most stable was VLHL PAI-1 mutant (Cys197-Cys355).⁷

PAI-1 deficiency PAI-1 deficiency is defined by different authors as activity of PAI-1 in blood lower than 1 to 3 IU/ml depending on report (normal: 7–21 IU/ml).^{8–11} Fay et al. reported that the molecular basis of PAI-1 deficiency was not determined with confidence; but he found that some patients were homozygous for a null PAI-1 mutation. The other individuals contained a mutation in the PAI-1 gene that resulted in defect in PAI-1 function or altered PAI-1 expression in a tissue-specific manner. Also, some individuals had a mutation within another factor that controlled PAI-1 expression or activity, such as vitronectin.⁸

Symptoms of PAI-1 deficiency Patients with PAI-1 deficiency bleed as a result of a hyperfibrinolytic hemorrhage characterized by normal primary hemostasis but extended bleeding. In these patients a normal thrombus is formed, but is quickly lysed as there is no inhibitor to moderate tPA plasmin activation.¹² Spontaneous bleeding is rarely observed, but moderate hemorrhaging of the knees, elbows, nose, and gums can be triggered by mild trauma. Additionally, prolonged bleeding after surgery is common and menstrual bleeding may be severe.^{10,12–15} Moderate PAI-1 deficiency is associated with a lifelong bleeding tendency, but severe can be life-threatening, as described by Fay et al. in the case of a serious postoperative hemorrhage of a 15-year-old patient.⁸

Incidents of PAI-1 deficiency The true incidence of PAI-1 deficiency is unknown in large part because of the lack of a standardized laboratory test and a clear-cut definition of this deficiency.¹⁰ The first case was published in 1989, when undetectable PAI-1 activity and antigen levels were noted in a 76-year-old man with life bleeding tendency.^{10,16} At that time PAI-1 deficiency was considered an extremely rare clinical entity.¹⁶

Since then more cases have been described including 7 homozygous individuals of Amish descent with complete PAI-1 deficiency and prolonged bleeding.^{8–10,16–18} Frequency estimation was given by Agren et al. were 4 out of 62 (6.45%) patients undergoing treatment for benign prostatic hyperplasia where diagnosed with low level of PAI-1 (<1 IU/ml). Bleeding complications during surgery were observed in 3 out of 4 (75%) patients with low PAI-1 levels and in 16 out of 58 (28%) patients with PAI-1 levels >1 IU/ml.¹⁹ Also, study by Agren et al. reported an incidence of low PAI-1 activity in 10–13% of healthy population.²⁰ There is no evidence that these data reflect incidence of entire population, nevertheless it seems that this condition is simply under-diagnosed rather than extremely uncommon.

Treatment of bleeding in PAI-1 deficient patients

Bleeding episodes in PAI-1 deficient patients are treated with a 5- to 7-day course of oral tranexamic acid or ϵ -aminocaproic acid.^{8,10,13} These agents bind to plasminogen and control plasmin generation subsequently minimizing bleeding.²¹ Both are considered to be safe with some side effects mainly related to the gastrointestinal tract and myalgia.^{22–24} Moreover, it has been reported that they have limited efficacy for the control of localized bleeding in the surgical setting.²⁵ It was also reported that menorrhagia in young patient did not respond well to tranexamic acid treatment.⁸

Other conditions related to PAI-1 deficiency In addition to its classic role as an inhibitor of fibrinolysis, PAI-1 has been implicated as a mediator in other processes, including fibrosis, rheumatoid arthritis, atherosclerosis, tumor angiogenesis bacterial infections, and others.^{26–28} PAI-1 deficiency can affect these conditions in different ways. For example, PAI-1 limits liver injury and mortality during acetaminophen hepatotoxicity by preventing excessive hemorrhage and facilitating tissue repair.²⁹

The best described is PAI-1 deficiency in mice with a deletion of the PAI-1 gene. The impaired host defense is manifested by enhanced lethality and increased bacterial growth of *Klebsiella pneumoniae*.²⁸ Overexpression of PAI-1 in the lung using adenoviral vector improved host defense against this infection. Authors suggest that PAI-1 protects the host against *Klebsiella pneumoniae* by promoting neutrophil recruitment to the pulmonary compartment and that PAI-1 is essential for host defense against severe Gram-negative pneumonia.²⁸ Similar findings were reported with a different strain of bacteria, i.e. *Streptococcus pneumoniae*.²⁷

These infections are a major cause of morbidity and mortality worldwide. Despite the widespread use of antibiotics, the mortality rate has not decreased significantly over the past 30 years. Patients with PAI-1 deficiency will be most likely more susceptible to infection and should be

treated with PAI-1, but not tranexamic acid or ϵ -aminocaproic acid.

Rationale for using PAI-1 to limit bleeding Tissue PA has high affinity to fibrin, fibrin-bound plasminogen, and an increased activity in presence of fibrin. These properties enhance tPA fibrinolytic potential and localize its activity at site of fibrin deposition.³⁰ Most of the known PA inhibitors are non-specific in their inhibitory activity toward tPA, uPA, and other members of the serine protease family.³¹

As opposed to the small molecule inhibitors, PAI-1 is more specific for PAs and acts by making a complex, followed by the formation of a covalent bond between the active site of the protease and its reactive center. PAI-1 is a critical regulator of the fibrinolytic system through inhibition of tPA. PAI-1 binds to the fibrin but not to fibrinogen and thus can localize itself at sites of injury.³² PAI-1 accumulated within thrombi retains its complete tPA inhibitory activity protecting clot from premature dissolution³³ making PAI-1 an attractive antifibrinolytic agent.

Therapeutic application of PAI-1 Wild-type PAI-1 (wPAI-1) inhibits fibrinolysis in a dose-responsive manner by intravenous bolus injection of active PAI-1, whereas latent, inactive PAI-1 has no effect. PAI-1 markedly inhibits fibrinolysis in vivo and delivering of PAI-1 to a developing thrombus is an important physiological mechanism for subsequent thrombus stabilization.¹⁹ PAI-1 is a fast-acting, highly specific inhibitor of tPA. But its very short half-life presents an obstacle in using wPAI-1 as a hemostatic drug.^{34,35} PAI-1 with extended half-life can answer this problem and it was successfully used to shorten total time of bleeding and total blood loss.^{36,37}

High level of PAI-1 Hyperactivity of PAI-1 PAI-1 is present in increased levels and activity in various disease states such as cancer, obesity, renal disease and the metabolic syndrome. Depending on a report, high PAI-1 activity is defined as >10–20 IU/ml but it can be as high as 68 IU/ml.^{38,39} It has been found that an insertion (5G), deletion (4G) polymorphism at position 675 of the PAI-1 gene promoter direct transcriptional activity of PAI-1.⁴⁰ Increases of concentration or activity other than that is less studied and understood.

Symptoms of hyperactivity of PAI-1 High levels of PAI-1 have been associated with an increased risk for coronary artery disease and myocardial infarction due to inhibition of fibrinolysis.⁴¹

However, PAI-1 activity plays an important role in renal fibrosis as well. In fibrotic renal diseases, PAI-1 is increased and localizes to areas of glomerulosclerosis. PAI-1 as the major inhibitor of urokinase in kidneys downregulates degradation of fibrin by plasminogen, and lack of activation of metalloproteinases by plasmin potentiates this process.⁴² Renal fibrosis causes significant

morbidity and mortality leading to the need for dialysis or kidney transplantation.

Additionally, genetic polymorphisms in PAI-1 4G/5G and consequent high level of PAI-1 are claimed to contribute to an increased risk of venous thromboembolism which is associated with the occurrence of spontaneous abortions. Authors conclude that the occurrence of PAI-1 4G/4G or 4G/5G genotypes, respectively, is clinically significant for the pathogenesis of venous thromboembolism in pregnancy, and possibly, spontaneous early abortion.⁴³

An association between high level of PAI-1 and obesity has been described, namely as contributing to the development of secondary disorders such as type 2 diabetes mellitus and cardiovascular complications. However, a causal role of PAI-1 in the development of obesity has not been established so far.⁴⁴ It has also been shown that weight loss has beneficial effects to lower PAI-1.⁴⁵

Incidents Similarly to PAI-1 deficiency, incidents of PAI-1 hyperactivity are largely unknown. Complicating factors are geographical distribution, race, and disease. For example it has been reported that 4G/5G polymorphism in the Lebanese population was found to harbor a relatively high prevalence of mutations compared to other ethnic communities.⁴⁶

Treatment Most common treatment is anticoagulation therapy which in some cases can last for life.⁴⁷ Others include indirect treatment related to a disease. For example antidiabetic drugs, such as metformin, reduce plasma PAI-1 levels in humans with type 2 diabetes.⁴⁸

However, it was reported that administration of tiplaxtinin (PAI-039), an orally active synthetic inhibitor of PAI-1, reduced its activity and significantly reduced weight in a diet-induced obesity model in mice. Crandall et al. reported similar findings.⁴⁹ Although mechanistic aspects were not addressed in these studies, the potential mechanisms of the effect of PAI-1 inhibition by tiplaxtinin on nutritionally induced obesity were most probably multifactorial including a reduction of triglycerides but an increase of low-density lipoproteins (LDL).^{44,49,50}

An interesting approach to treatment of renal fibrosis was proposed by Huang et al. using inactive PAI-1R (a new human mutant PAI-1).⁵¹ They exploit the finding that PAI-1 is localized by vitronectin found at site of injured renal tissue. Furthermore, PAI-1 complexed with vitronectin stabilizes PAI-1 in its active conformation. However, when PAI-1 binds to uPA or tPA activators, it is cleaved at reactive center loop inducing a rapid conformational change in PAI-1 that results in a reduction in PAI-1 affinity to vitronectin. It is followed by partitioning of the PAI-1 complex from vitronectin. As the complex is removed, the vitronectin becomes available to bind another PAI-1, which creates a localized area of high anti-proteolytic

activity. The noninhibitory PAI-1R has the same affinity to vitronectin as native PAI-1 but cannot bind to uPA or tPA and binds to vitronectin longer than native PAI-1. This process increases plasmin-driven proteolytic activity at that site. Authors determine that short-term administration of PAI-1R has slowed the progression of disease in the mouse model.⁵¹

It was found in a randomized clinical trial that the effect of regular physical exercise on PAI-1 activity has not changed significantly during 3 years. However, in the 4G polymorphism group the exercise reduced PAI-1 activity by 36%.⁵²

PAI-1 in cancer To achieve tissue penetration, cancer cells stimulate their proteinase machinery, overproduce and bind proteases, which allows cell migration through a degraded extracellular matrix.⁵³ One of these proteases is uPA of plasmin activation pathway.^{54,55} It was shown that inhibition of uPA activity reduced metastasis in in vitro and in vivo models.^{56,57} Also, during carcinogenesis advancing tips of capillary angiogenic vessels express a high activity of uPA. Inhibition of uPA activity results in a reduction in angiogenesis and cancer size as shown in in vivo models of breast, colon, prostate, and many other cancers.^{34,35,57} Thus, inhibitors of urokinase activity could be used as anticancer agents.

Inhibitors of uPA and metastasis The urokinase PAS (uPAS) is commonly overexpressed by many different human cancers.⁵⁸ The ability of human carcinoma cells (expressing uPA) to invade the choriallantoic membrane and metastasize from it to the embryo, while treated with the antibody against the active site of uPA, was dramatically reduced in comparison to non-treated cells in the chicken embryo model.² Cells transfected with a plasmid, causing an overexpression of uPA in prostate cancer cells, showed a marked increase in metastasis, in comparison with the parental cell phenotype in the rat model. From the same phenotype, the cells underexpressing uPA were selected and these cells displayed drastically decreased metastasis. In this model prostatic PC3 cancer cells were used and a decreased number of metastasis including skeletal metastasis was observed.⁵⁹ An increased amount or activity of uPA, or uPAR per cell, has been found in human cancer cell lines with metastatic behavior.⁶⁰ Moreover, animals injected with PC3 prostatic cancer cells expressing higher amounts of uPA and/or uPAR develop metastatic lesions including skeletal metastasis earlier and more frequently than animals injected with the same cells expressing lower amounts of uPA/uPAR.⁵⁹ Additionally, it has been reported that uPA activity is increased in metastatic tumors compared with primary tumors in experimental animals.⁶¹

Urokinase inhibitors reduce angiogenesis The tip of neovascular advancing capillary vessels surrounding tumors has been reported to contain

high amounts of uPA and its receptor. Binding of proteolytically inactive ligand to uPA receptor reduces amount of uPA on the surface of capillary endothelial cells, and reduces tumor growth.⁶² Also, our studies have shown that uPA inhibitors reduce angiogenesis in chick embryo model, and reduce the length and number of sprouts of human umbilical vascular endothelial cells. In both cases inhibition of uPA activity on tip of capillary vessel or sprout prohibits cell migration and reduces their growth.^{34,35,63} Goodson et al. has shown that binding of proteolytically inactive uPAR ligands prevents cell surface plasminogen activation, and consequently prevents angiogenesis in the mouse model.⁶⁴ They emphasize that the uPAR focuses uPA and initiates proteolytic activity on the vascular capillary cell surface, which is required for angiogenesis. Ignjatovic et al. observed inhibition of angiogenesis in the rabbit cornea while treating animals with amiloride, one of competitive inhibitors of uPA.⁶⁵

Urokinase regulates activity of hepatocyte growth factor/scatter factor Prostate cancer metastasizes preferentially to the skeleton. Its ability to invade and grow in bone marrow stroma is thought to be due in part to degradative enzymes. The formation of prostate skeletal metastases has been reproduced in vitro by growing co-cultures of prostatic epithelial cells in bone marrow stroma. Expression of urokinase plasminogen was identified to be responsible for this process.⁵⁹ It has been proposed that osseous metastatic prostate cancer cells must be osteomimetic in order to metastasize, grow, and survive in the skeleton. The reciprocal interaction between prostate cancer and bone stromal growth factors, including hepatocyte growth factor/scatter factor (HGF/SF) among others, initiates bone tropism and is enhanced by uPA.⁶¹ HGF/SF bears sequence and structural homology with plasminogen. HGF/SF exists in both an inactive single-chain form and an active two-chain form. It has been proposed that plasminogen activators could properly cleave single-chain of hepatocyte growth factor to generate the active two-chain. It has been suggested that uPA is a natural biological regulator of HGF.⁶⁶ Moreover, in a positive feedback manner HGF stimulation of cancer cells results in overproduction of proteases, including uPA, stimulating further activation of HGF.⁶⁷⁻⁷¹

Molecular basis of PAs inhibition Most of the known PA inhibitors are non-specific in their inhibitory activity toward tPA, uPA, and other members of serine protease family.^{72,73} Based on X-ray structure analysis and molecular modeling, it seems that these inhibitors are mainly inserted into the specificity pocket (residues: 87–197; 212–229) of uPA and tPA, and block recognition site preventing the binding of plasminogen activators with their substrate plasmin. This is the case of inhibition by small molecules such as benzamidine, p-benzamidine, and others.⁷⁴

Inhibition of uPA and tPA by PAI-1 is more specific. PAI-1 is a representative of serpins that are the members of the superfamily of serine protease inhibitors. Inhibitors of serine proteases act by making a 1:1 stoichiometric complex, followed by the formation of a covalent bond between the hydroxyl group of the reactive-site serine of the protease and the carboxyl group of the P1 residue at the reactive center of the serpin. Upon cleavage of an inhibitory serpin, the N-terminal end of the reactive-site loop of PAI-1 inserts into β -sheet of serine protease forming a stable serine-serpin complex.^{75,76}

PAI-1 in cancer treatment PAI-1 with extended half-life reduces angiogenesis in vitro and in vivo. In our study we have observed a reduction in tumor size while PAI-1 with extended half-life was used but not for control PAI-1 inactive mutant (administered by multiply tail vein injection).^{63,77} Additional experiments with LNCaP xenografts in severe combined immunodeficient mice using an osmotic pump to ensure continuous delivery of PAI-1 corroborate our previous findings.³⁴ Similar results were shown by others.^{78,79}

PAI-1 paradox Surprisingly PAI-1 deficient mice showed lower proliferation, higher apoptosis and different morphology of subcutaneously implanted tumors than its wild counterparts. Furthermore, PAI-1 is a predictive factor of poor prognosis in primary invasive breast cancer.⁸⁰ These findings strongly contradict our previous statement, since it has been demonstrated that PAI-1 is a potent regulator of angiogenesis and tumor growth.^{34,78} However, it has been demonstrated also that when PAI-1 is administered in low concentrations it could increase metastatic potential and angiogenesis. It has been suggested that vitronectin and PAI-1 act together to either promote or inhibit angiogenesis. Vitronectin present in the matrix might enhance angiogenesis by promoting vascular cell migration, and PAI-1 might regulate this process by controlling access to the integrin adhesion site on vitronectin.⁸¹

Vitronectin is a multifunctional glycoprotein present in plasma, platelet, and the extracellular matrix. PAI-1 is the primary vitronectin binding protein. The PAI-1/vitronectin and uPA/uPAR complexes could also interact with each other. When they bind, uPA/uPAR/PAI-1/vitronectin complex binds LDL receptor-related protein. This weakens PAI-1/vitronectin interaction and triggers PAI-1/uPA/uPAR internalization. PAI-1 and uPA are degraded but uPAR is recycled to the cell surface. Subsequently, the receptor can bind uPA again, and uPA/uPAR, PAI-1/vitronectin can form a new complex in the last step of this cyclic process. Since PAI-1 in the immediate vicinity of cell is exhausted, the cell migrates toward increased concentration of PAI-1.⁸²

However, it has been shown in our previous studies and by others, that PAI-1 added at supra-physiologic concentrations suppresses

the vitronectin pathway. PAI-1 acts then as a potent inhibitor of angiogenesis by utilizing primarily its inhibitory properties toward proteinase activity.^{34,81}

Non serpin activity of PAI-1 It was reported that PAI-1 in vitro treatment of cancer cells induced detachment of cells from vessel surface.^{26,80} The postulated mechanism is pointing to disruption of vitronectin and integrins complexes by PAI-1.⁸⁰ Our own study of this phenomenon showed that treatment of cancer cells with highly stable PAI-1 down-regulated nucleophosmin, while all forms of PAI-1 (active and nonactive) downregulated fortilin. These two proteins are implicated in important cellular processes (cell growth, cell cycle, malignant transformation).²⁶ This suggests that PAI-1, in addition to its well-known anticancer properties, plays an important role in cell signaling. This finding might lead to the development of more effective therapeutic strategies in cancer treatment.

Word of caution Knowledge on complex interaction of proteins in disease is derived mostly from animal models. Proteins of different organisms frequently differ from each other in a surprising way. For example, mouse uPA and human uPA are very similar to each other while rat uPA differs significantly from those of the mouse and human. Thus, conclusions derived from animal study must take into consideration limitation of such model and its applicability to humans as illustrated below.

Cross species reactivity of urokinase system PAI-1 binds to uPA and consequently inactivates plasmin driven proteolysis. PAI-1 binds also to the receptor bound urokinase-type plasminogen activator and this complex is internalized via LDL receptor-related protein. Interaction of vitronectin with PAI-1/uPA/uPAR and internalization of that complex regulates cell migration.⁸³ However, binding affinity of uPA to uPAR could be species specific.⁸⁴ The human uPA fails to bind to uPAR of murine cells, and murine uPA does not bind to human cells, but binding is not affected in the human- or mouse-bovine systems.⁸⁵⁻⁸⁷ The other elements of this system interact with each other from different species. For example, the angiogenic activity of purified human uPA exerts a dose-dependent angiogenic response in the chicken chorioallantoic membrane assay (CAM).⁸⁵ Our study has shown that human PAI-1 inhibits angiogenesis in CAM model.³⁴ However, human PAI-1 binds to chicken uPA or uPA/uPAR complexes with lower affinity than to all human proteins.⁸⁸ Also, there are structural differences between human and animal uPA. The most distant to human uPA is rat uPA while baboon and, surprisingly, mouse structure of uPA are very similar to human uPA.⁸⁹

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Jin i jang inhibitora aktywatora plazminogenu

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SŁOWA KLUCZOWE

inhibitor aktywatora plazminogenu (PAI), niedobór PAI-1, rak, wysoki poziom PAI-1

ABSTRAKT

Inhibitor aktywatora plazminogenu typu 1 (PAI-1) bierze udział w wielu procesach biologicznych i w zależności od choroby jego nadmierna ekspresja, brak lub normalny poziom może niekiedy prowadzić do zaskakujących rezultatów. Niniejsza praca opisuje w jaki sposób mały, duży i normalny poziom PAI-1 pozostaje w stosunku do chorób, ich wystąpienia i możliwego leczenia. Omawiana jest także rola PAI-1 w raku, ze zwróceniem uwagi na „paradoks PAI-1” i wyjaśnieniem możliwych zastosowań terapeutycznych tego białka.

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