Sys Rev Pharm 2021; 12(12): 3832-3849
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Review Article

Potential Medical Application of Plasmin-Based Therapeutics

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Article History: *Submitted: 04.11.2021* *Accepted: 18.11.2021* *Published: 25.11.2021*

ABSTRACT

Heart disease is the leading cause of death globally, and keeping hemostatic balance is essential for a healthy cardiovascular system. Disturbing the balance can cause major diseases, such as ischemic stroke, myocardial infarction, and SARS-CoV-2 caused systemic blood clotting. *In vivo*, the pathological thrombi are dissolved by the enzyme Plasmin (Plm), a serine protease derived from the proenzyme Plasminogen (Plg). Although the biological regulation of the Plg system has been intensively studied, the vast potential pharmaceutical applications of the Plm enzyme-based therapeutics are far from realized. This review focuses on the underemphasized direction of the therapeutic development of Plg, Plm, and Micro Plasmin (μ Plm), the catalytic domain of Plasmin. The major diseases can be treated with Plm-based thrombolytic therapeutics include thrombosis diseases, pulmonary fibrosis, and Alzheimer's Disease. The review also describes a new approach of using a "directional structure-based protein engineering" for μ Plasmin based therapeutic drug development. In the Future Directions section, we will analyze the long-standing problem facing thrombolytic drug development, which is the often-fatal bleeding side effect. Contrary to the conventional approach of developing more stable, longer *in vivo* half-life protein or enzyme therapeutics, here we propose a new "hit and die" strategy, in which an "ideal" thrombolytic drug hits the targeting thrombi, dissolving them, and die out, avoiding bleeding side effect resulting from the continued activity of present thrombolytic drugs. In practice, we propose to develop unstable, short *in vivo* half-life thrombolytic therapeutics to reach good drug efficacy and at the same time, avoid bleeding side effect.

Keywords: Cardiovascular diseases, Plasminogen system, Plasmin enzyme, Serine protease, Thrombolytic therapeutics

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ABBREVIATIONS

AMI: Acute Myocardial Infarction; AD: Alzheimer's Disease; α 2-AP: α 2-Antiplasmin; AHA: American Heart Association; Aβ: β-Amyloid; APP: β-Amyloid Precursor Protein; CDT: Catheter-Directed Thrombolysis; IPF: Idiopathic Pulmonary Fibrosis; μ Plm: μ Plasmin; PAD: Peripheral Arterial Disease; PAO: Peripheral Arterial Occlusion; Plm: Plasmin; Plg: Plasminogen; PAI: Plasminogen Activator Inhibitor; tPA: tissue-type Plasminogen Activator; uPA: urokinase-type Plasminogen Activator

INTRODUCTION

Keeping hemostatic balance is essential for maintaining a healthy cardiovascular system (Palta S, *et al.*, 2014), and disturbing the balance in case of pathogenic thromboembolism can cause major fatal diseases, such as ischemic stroke, myocardial infarction, and SARS-CoV-2 caused systemic blood clotting. Importantly to human health, according to AHA (American Heart Association), despite the dramatic health impact of the COVID-19 pandemic, heart disease is still the leading cause of death globally (Buzby S, 2021). *In vivo*, the pathogenic thrombi are dissolved into soluble components by the enzyme Plasmin (Plm), a serine protease that is derived from the proenzyme Plasminogen (Plg) (Lin H, *et al.*, 2020; Aisina RB and Mukhametova LI, 2014). Plg binds to both fibrin and fibrinogen, thereby incorporating into a clot as it is formed (Wu G, *et al.*, 2019). Both urokinase-type Plasminogen Activator (uPA) and tissue-type Plasminogen Activator (tPA) are exquisitely specific serine proteases which convert Plg to Plm (Gurewich V, 2000). Other major parts of the blood's intricate fibrinolytic network include the inhibitors such as Plg Activator Inhibitor (PAI), which binds to and inhibit tPA and uPA, and α 2-Antiplasmin (α 2-AP), which binds to and inhibit Plm (Bachmann F, 1994).

A critical physiological function of Plm is shown in congenital Plm deficiency, which causes a multisystemic disorder leading to deficient extravascular fibrinolysis. As a clinical consequence, the wound healing capacity of mucous membranes is markedly impaired, leading to ligneous conjunctivitis and several other manifestations (Mehta R and Shapiro AD, 2008; Klammt J, *et al.*, 2011).

In addition to dissolving blood clots, Plm can also be involved in a diverse range of physiological processes, such as cancer growth (Kwaan HC and McMahon B, 2009; Didiasova M, *et al.*, 2014), wound healing (Sulniute R, *et al.*, 2016), tissue remodeling (Miles LA and Parmer RJ, 2013), cell migration (Saksela O, 1985), inflammation (Hamilton JA, 2008), and immunity (Draxler DF, *et al.*, 2017). This review, however, will focus on few major therapeutic directions, including thromboembolism diseases such as ischemic stroke and myocardial infarction, Alzheimer's Disease, pulmonary fibrosis, and SARS-CoV-2 caused systemic thrombosis.

Although there are many therapeutic developments toward Plg activation and modulation of Plm activities (Lin H, *et al.*, 2020), and the Plasminogen Activator drugs such as tPA are the first-line treatment for thromboembolism diseases such as ischemic stroke and myocardial infarction (US National Library of Medicine, 2021), the successful development of Plm-based direct thrombolytic drugs, however, has not been successful. This review focuses on the understudied area in the therapeutic development of Plg,

Plm, and microPlm (μ Plm), the latter of which is the catalytic domain of Plm. The review will also describe a new direction of using a "directional structure-based protein engineering" approach for μ Plm based therapeutic development. In the future directions section, we will analyze major problems facing Plm-based direct thrombolytic therapy. Contrary to the conventional approach of developing *in vivo* more stable, longer half-life protein or enzyme therapeutics, here we propose a new "hit and die" strategy, in which an "ideal" thrombolytic drug hits the targeting thrombi, dissolving them, and die out, avoiding bleeding side effect resulting from the continued activity of the present thrombolytic drugs. In practice, we propose to develop *in vivo* unstable, short half-life thrombolytic therapeutics such as *E. coli* produced μ Plm and mutants, to reach good therapeutic efficacy and at the same time, avoid bleeding side effect.

Here the *in vivo* half-life for Plasmin-based therapeutics has two different definitions. The first definition is the *in vivo* enzyme activity half-life. Once in the blood, at low concentration (<1 μM, which is the serum concentration of α 2-AP), Plm is immediately neutralized by its principle inhibitor α 2-AP, and has an activity half-life of only 0.2 seconds, while μ Plm has a relatively longer activity plasma half-life of about 4 seconds (Wiman B, *et al.*, 1978). The second definition is the inherent structural stability of the protein per se in the blood. For example, recombinant μ Plm refolded from E. coli inclusion bodies is stable in the controlled buffer and temperature of laboratory conditions, but the protein itself becomes unstable once injected into the blood of a live mouse, with a structural half-life as short as 11 seconds; on the other hand, the *in vivo* structural half-life of Plg and Plm can be as long as 2-4 days, as will be discussed later. In the "hit and die" strategy, we use the second definition when describing *in vivo* stability and half-life. In our description, the "quick death" of the μPlm therapeutics is not resulting from the inhibition of protease inhibitors present in the serum, but is a situation in which at high concentration (>1 μM), after neutralizing all of the inhibitor activities and dissolving the targeting thrombi, the loss of the remaining enzyme activity from the structural disintegration of the recombinant enzyme itself.

LITERATURE REVIEW

Basic biology of the Plg/Plm system

Primary structure of Plg and its des-kringle derivatives: Plg *(Figure 1a)* is a single-chain glycoprotein, consisting of 791 amino acids, which circulates inertly in the blood, but can bind to fibrin at newly formed blood clots (Petersen TE, *et al.*, 1990). Biologically, the activation of Plg is enabled by digestion of the peptide bond between R561 and V562 by tPA trapped in the blood clot. The newly formed Plm then actively digests the fibrin in the clot, thereby dissolving it *(Figure 1b)*. The activated Plg is transformed into 2 separate subunits, but they remain interconnected through 2 disulfide bonds. The resulting longer subunit, called A chain of the Plm polypeptide, consists of 5 triple-loop disulfide Kringle (Kr) domains (approximately 78-80 amino acids each). The shorter component, called the B chain, contains a "linker" region of about 20 amino acids and a serine protease domain (approximately 228 amino acids, see *Figure 1a*). Human Plg has three major post-translational modification sites, which influence the *in vivo* activity, stability, and binding affinity to fibrinogen (Lin H, *et al.*, 2020; Aisina RB and Mukhametova LI, 2014). Clearly shown in the crystal structure of full-length Plg, the O-glycosylation site (T346, *Figure 1a*) is present in all forms of circulated Plg, and protected the zymogen from "accidental" or unwanted activation, while the N-glycosylation site (N289, *Figure 1a*) is present only in Type I Plg, which favors the more active open conformation (Law RH, *et al.*, 2012; Xue Y, *et al.*, 2012). In addition, a phosphorylation site at the S578 position has also been identified, which may influence the stability of the molecule (Wang H, *et al.*, 1990).

Through laboratory manipulations, 2 des-kringle variants of Plg with potential pharmacological applications, miniPlg (mPlg) and microPlg (μPlg), have been created. mPlg is a mini version of Plg, consisting of only Kr5, the linker, and the serine protease domains, whereas μPlg consists of only the linker and serine protease domain itself. Initially, mPlg was produced by digestion with neutrophil elastase shown in *Figure 1a* between A440 and S441 (Ney KA and Pizzo SV, 1982), while μPlg was produced by base-mediated cleavage at pH 11 (Wu HL, *et al.*, 1987; Wu HL, *et al.*, 1987). Similar to Plg activation, both mPlg and μPlg can be activated to miniPlm (mPlm) and microPlm (μ Plm) respectively, by digestion at the peptide bond between R561 and V562 by tPA, uPA, or other Plasminogen Activators (PAs), again forming 2 separate subunits interconnected by the same 2 disulfide bonds. Removal of either one or both of these disulfide bonds by mutagenesis renders the activated serine protease domain non-functional (Wang J and Reich E, 1995).

The biological activators of Plg are tPA and uPA, both of which directly activate Plg to Plm by cutting the R561-V562 *(Figure 1a)* bond. These are called direct or biological activators of Plg. Two bacterial Plg activators have also been extensively studied, including Streptokinase (SK) and Staphylokinase (SAK). SK was discovered in 1933 from hemolytic streptococci bacteria, and has been used as a thrombolytic medicine for a long time (Sikri N and Bardia A, 2007). SK, a 47 kDa protein consisting of 414 amino acid residues, has no proteolytic enzyme activity by itself, but can bind to Plg and converts the complex into a Plg activator (Wang X, *et al.*, 1998). SK, therefore, is an indirect and non-physiological Plg activator. The complex structure of μ Pm-SK shows that SK contains three domains, named α, β, and γ domain, bear-hugging the catalytic domain μ Plg, and turning the complex into a Plg activator through a forced change of conformation (Wang X, *et al.*, 1998). SAK, containing 136 amino acid residues (15 kDa), is the equivalence of the α-domain of SK. SAK does not form complex nor activate Plg by itself, and can only form a complex and turn Plm into a Plg activator (Grella DK and Castellino FJ, 1997; Loy JA, *et al.*, 2007). SAK, therefore, is a Plm dependent Plg activator (Esmon CT, Mather T, 1998; Parry MA, *et al.*, 1998).

Substrate specificity: The substrate specificity of Plm has been considered rather broad, except for the P1 position, which is either arginine or lysine. However, certain levels of P4-P2 specificity were shown in the research reported by Backes BJ, *et al.*, which is defined in the peptide level by a synthesis of positional-scanning libraries of fluorogenic peptide substrates (Backes BJ, *et al.*, 2000). A total of 6,859 peptides were screened by this strategy, revealing certain specific preferences at the P4-P2 positions. It was found that the P2 position prefers phenylalanine, tryptophan and tyrosine, while the P3 position lacks specific preference though threonine is often found at this position, and the P4 position favors a lysine residue, but valine, norleucine, isoleucine, and phenylalanine have also been found (Backes BJ, *et al.*, 2000).

Using a phage display technology, Hervio LS, *et al.* have identified potential amino acids in each position by the selection of a library of phages that can be cleaved by Plm (Hervio LS, *et al.*, 2000). They found that of the selected 111 phage clones, 69 clones showed a strong preference of arginine and lysine at P1, and 15 clones present the P2 substrate is likely to be an aromatic hydrophobic, which is either tyrosine or phenylalanine. A variety of amino acids including arginine, valine, glycine, and threonine have appeared at the P3 position. Similar to the P1 position, 3 out of 13 clones contained a P1' arginine, lysine, or serine residue, respectively, and 2 clones contained glycine at P1' position. Finally, the P2' position appears to prefer the arginine, lysine, and glycine. For natural substrates recognized by Plm, we summarize the results in *Table 1*.

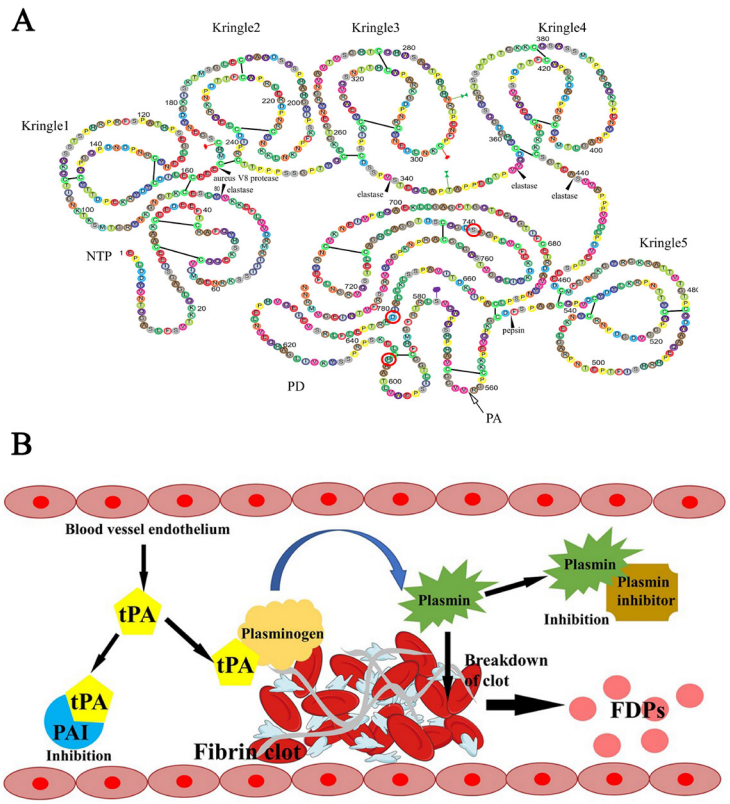

Figure 1: Primary structure of Plg and break down of a fibrin clot. A. Diagram of amino acid sequence of Plg adopted from reference (Petersen TE, *et al.*, 1990). PD: Protease Domain (residues Ala542-Asn791); Kringle1-5: Kringle domains; NTP: N-Terminal Peptide (residues 1-77); : Glycosylation sites (N289, T346); : Phosphorylation site (S578); big arrow marked as PA indicates the Arg561-Val562 bond cleaved by Plasminogen Activators (PA); small arrows are cleavage sites by other proteases; : Cysteine residues forming the S-S bond between K2 and K3; : Active site residues (H603, D646, S741) B. Schematic diagram of Plm digestion of a thrombus. The diagram shows activation of Plg by tPA (tissue-type Plasminogen Activator) in the clot and the degradation of fibrin clot by Plm, which is immediately inactivated by the Plasmin Inhibitor (α 2-Antiplasmin (α 2-AP)) when released into the blood. FDPs: Fibrin Degradation Products

Table 1: Natural substrate specificity of Plm

Consensus	P4	P3	P2	P1	P1'	P2'	P3'	P4'	References
	K/V/I/F/Nle	V/G/T/R	Y/F/W	R/K	R/K/S/G	R/K/G	X	X	
Fibrinogen α chain	K	N	N	K	D	S	H	S	
	A	N	N	R	D	N	T	Y	
β chain	Y	L	L	K	D	L	W	Q	(Repetto O, *et al.*, 2018)
	K	Q	V	K	D	N	E	N	
γ chain	S	E	V	K	Q	L	I	K	
	Q	L	I	K	A	I	Q	L	
Syndecan-1	P	D	R	R	N	Q	S	P	(Manon-Jensen T, *et al.*, 2013)
Syndecan-4	V	I	P	K	R	I	S	P	(Manon-Jensen T, *et al.*, 2013; Schmidt A, *et al.*, 2005)
	V	S	N	K	V	S	M	S	
Osteocalcin	E	A	Y	R	R	F	Y	G	(Nielsen-Marsh CM, *et al.*, 2005)
Vitronectin	K	G	Y	R	S	Q	R	G	(Chain D, *et al.*, 1991)
PAR-1 (Proteinase Activated Receptor 1)	P	E	S	K	A	T	N	A	(Kuliopulos A, *et al.*, 1999)
	L	D	P	R	S	F	L	L	
	T	E	Y	R	L	V	S	I	
	S	I	N	K	S	S	P	L	
	P	L	Q	K	Q	L	P	A	

PAR-4 (Proteinase Activated Receptor 4)	P	A	P	R	G	Y	P	G	(Xu WF, *et al.*, 1998)
Factor X	I	T	F	R	M	N	V	A	(Pryzdial EL, *et al.*, 1999)
Kininogen									
Proinsulin	P	K	S	R	R	E	V	E	(Imai S, *et al.*, 2015)
	A	Q	Q	K	R	G	I	V	
Von Willebrand Factor	L	G	P	K	R	N	S	M	(Brophy TM, *et al.*, 2015)
α S2-casein	E	T	Y	K	Q	E	K	N	(Kelly AL and McSweeney PL, 2003; Le Bars D and Gripon JC, 1989)
	K	Q	E	K	N	M	A	I	
	Q	V	K	R	N	A	V	P	
	V	F	T	K	K	T	K	L	
	F	T	K	K	T	K	L	T	
	Q	Y	L	K	T	V	Y	Q	
	Q	H	Q	K	A	M	K	P	
	I	Q	P	K	T	K	V	I	
β-casein	R	I	N	K	K	I	E	K	(Huppertz T, *et al.*, 2006; Atamer Z, *et al.*, 2017)
	M	A	P	K	H	K	E	M	
	P	K	H	K	E	M	P	F	
CDCP1 (Cub Domain Containing Protein 1)	K	Q	S	R	K	F	V	P	(He Y, *et al.*, 2010; Casar B, *et al.*, 2012)

Note: The table listed the cutting sites (P1-P1') and extended sequences from P4 to P4'. For example, in the Fibrinogen α chain (the second row after Consensus), two regions are being cleaved by Plm, which are KNNK|DSHS, and ANNR|DNTY. The Consensus row is the summary of the consensus cleavage sites

In the following, we further summarized the substrate specificity of Plm described above.

- P4 Lysine (K), Valine (V), Norleucine (Nle), Isoleucine (I), or phenylalanine (F) (Backes BJ, *et al.*, 2000; Poreba M, *et al.*, 2018).
- P3 Valine (V), Glycine (G), Threonine (T), Arginine (R), or other variety of amino acids (Hervio LS, *et al.*, 2000)
- P2 Tyrosine (Y), Phenylalanine (F), or Tryptophan (W) (Aromatic hydrophobic) (Backes BJ, *et al.*, 2000; Hervio LS, *et al.*, 2000; Gosalia DN, *et al.*, 2005)
- P1 Arginine (R) or Lysine (K) (Backes BJ, *et al.*, 2000; Hervio LS, *et al.*, 2000; Gosalia DN, *et al.*, 2005)
- P1' Arginine (R), Lysine (K), Serine (S), or Glycine (G) (Hervio LS, *et al.*, 2000)
- P2' Arginine (R), Lysine (K), or Glycine (G) (Hervio LS, *et al.*, 2000)

Pathological deficiency of Plg and Plm: Besides genetic defects, Plg deficiency during PA treatment is also a serious medical problem, which can decrease or terminate the therapeutic efficacy. Unfortunately, there are currently no FDA-approved alternative drugs to PA treatment for conditions such as ischemic stroke, myocardial infarction, peripheral artery embolism, deep vein thrombosis, and other conditions caused by thromboembolism. For example, during thrombolytic therapy with high doses of tPA, uPA, or SK, there is a depletion of Plg that may terminate the efficiency of the thrombolytic drugs (Andrew M, *et al.*, 2000; Latallo ZS,, *et al.*, 1979; Wenzel E, *et al.*, 1980). On the other hand, it has been shown in an animal model that the administration of Plg has restored the thrombolytic potential (Lijnen HR, *et al.*, 1996). In addition, decreased levels of Plg or Plm have been shown in several clinical conditions, including disseminated intravascular coagulation (Chesterman CN, 1978; Soria J, *et al.*, 1978), sepsis (Gallimore MJ, *et al.*, 1980; Smith-Erichsen N, *et al.*, 1982), leukemia (Sutor AH, 1979), hyaline membrane disease (Markarian M, *et al.*, 1967), liver disease (Biland L, *et al.*, 1978), lung fibrosis (Bauman KA, *et al.*, 2010), and Alzheimer's Disease (Dotti CG, *et al.*, 2004; Jacobsen JS, *et al.*, 2008; Ledesma MD, *et al.*, 2000; Periz G and Fortini ME, 2000). As a result, protein augmentation therapeutics with Plg and Plm based design may advance the field in many medical areas.

However, as described above, in the serum, the activity of Plm is immediately neutralized by its principle inhibitor, α 2-Antiplasmin (α 2-AP). Accordingly, one of the major hurdles in the application of Plm-based direct thrombolytic therapeutics is the decreased efficacy due to α 2-AP inhibition. Therefore, in developing Plm-based therapeutics, it is desirable to design strategies to escape or overcome inhibition by α 2-AP. Toward this direction, we have used a structure-based protein engineering strategy with the goal of increasing the catalytic activity of μ Plm, and at the same time, resisting α 2-AP inhibition. The following sections are descriptions of this approach, in which part of the results have been published (Yang D, *et al.*, 2020).

Crystal structures of Plg and Plm: *Table 2* summarized the published structures of μ Plm, μ Plg, and full-length Plg. As shown in the table, the first high-resolution crystal structure of μ Plm/SK complex was published in 1998 (Wang X, *et al.*, 1998), and almost at the same time, the structure of μ Plm/SAK/μ Plm was also published (Parry MA, *et al.*, 1998). The full-length native Plg structure was published in 2012 almost simultaneously in 2 papers (Law RH, *et al.*, 2012; Xue Y, *et al.*, 2012). Several structures of μ Plm-inhibitor complexes were published in the following years (Millers EK, *et al.*, 2013; Law RH, *et al.*, 2017; Swedberg JE, *et al.*, 2018), adding the understanding of μ Plm enzyme-inhibitor interactions and helping in further drug design and development.

Table 2: Summary of PDB (Protein Data Bank) entries for Plasminogen and Plasmin

PDB ID	Year published	Title of publications	Macromolecule name	References
1BML	1998	Crystal structure of the catalytic domain of human Plasmin complexed with Streptokinase	μ Plm/SK	(Wang X, *et al.*, 1998)
1BUI	1998	The ternary microplasmin-Staphylokinase-microplasmin complex is a proteinase-cofactor-substrate complex in action	μ Plm/SAK/μ Plm	(Parry MA, *et al.*, 1998)
1QRZ	1999	Crystal structure of the proenzyme domain of Plasminogen	μ Plg	(Peisach E, *et al.*, 1999)
1DDJ	2000	The human Plasminogen catalytic domain undergoes an unusual conformational change upon activation	μ Plg	(Wang X, *et al.*, 2000)
1L4D	2002	Effects of deletion of Streptokinase residues 48-59 on Plasminogen activation	μ Plm/SK-α domain	(Wakeham N, *et al.*, 2002)
1RJX	2004	Characterization of Lys-698 to meet substitution in human Plasminogen catalytic domain	μ Plg-mutant	(Terzyan S, *et al.*, 2004)
4DUR	2012	The X-ray crystal structure of full-length human Plasminogen	Plg-full length	(Law RH, *et al.*, 2012)
4A5T	2012	Crystal structure of the native Plasminogen reveals an activation-resistant compact conformation	Plg-full length	(Xue Y, *et al.*, 2012)
3UIR	2013	The structure of human microplasmin in complex with textilinin-1, an aprotinin-like inhibitor from the Australian brown snake	μ Plm-inhibitor	(Millers EK, *et al.*, 2013)
5UGG	2017	X-ray crystal structure of plasmin with tranexamic acid-derived active site inhibitors	μ Plm-inhibitor	(Law RH, *et al.*, 2017)
6D40	2019	Highly potent and selective Plasmin Inhibitors based on the sunflower trypsin inhibitor-1 scaffold attenuate fibrinolysis in plasma	μ Plm-inhibitor	(Swedberg JE, *et al.*, 2018)

Note: The table summarized the published structures from the very first μ Plm/SK complex structure published in 1998, to the recently published μ Plm-Inhibitor structure in 2019. These structures are essential for our understanding of the proteolytic enzyme mechanisms and protein engineering efforts to create better therapeutics

Structure-based design and selection of α 2-AP escape mutants: As described above, the *in vivo* half-life of Plm is only 0.2 seconds (Wiman B, *et al.*, 1978) because the activity of Plm is immediately neutralized by α 2-AP once free in the serum. The short serum half-life of Plm is designed by nature to avoid bleeding and other undesirable proteolytic reactions, such as cleavage of important protein molecules present in the blood but may hinder the thrombolytic drug development. To address this problem, we recently published a strategy, which is to design and select mutant μ Plm that can escape α 2-AP inhibition but also have good activity toward targeting substrates (Yang D, *et al.*, 2020). In order to design mutants capable of escaping inhibition, a three-dimensional structure of the μ Plm/α 2-AP complex is needed, which is currently not available. In order to solve the problem, we constructed a model for the complex structure according to the homologous structure of the trypsin-antitrypsin complex (Huntington JA, *et al.*, 2000), using the crystal structures of μ Plm (PDB code: 1bml) (Wu HL, *et al.*, 1987) and α 2-AP (PDB code: 2R9Y) (Law RH, *et al.*, 2008) shown in *Figure 2a*. These two structures are then superimposed to the crystal structure of the Trypsin: antiTrypsin complex (Protein Data Bank Identification Code (PDB ID): 1OPH) (Dementiev A, *et al.*, 2003). *Figure 2b* is an expanded view of the overlay of the active site structures of μ Plm and trypsin, highlighting the catalytic triad of residues S741, H603, and D646 of Plm, with backbone root mean square deviation (rmsd) of 0.6 Å. The figure shows a closely fitted central structure of the two serine proteases. *Figure 2c* displays a detailed view of the initial position of the catalytic reaction (the "suicide reaction"), where the active site serine (S741) of μ Plm assumes a pre-attacking pose to the backbone amide between R403 and M404 of a2-AP.

The molecular contact regions between μ Plm and α 2-AP are shown in *Figure 3a*. Because the crystal structure of α 2-AP (2R9Y) misses the C-terminal residues, we used the I-TASSER server (Yang J and Zhang Y, 2015) to build the missing residues 465-491 (CTT shown in *Figure 3a*). *Figure 3b* shows 6 loops in the contact surface of μ Plm, in addition to the autolysis and 70-80 loops that may have direct contact with the modeled CTT structure. To experimentally map the contribution of individual residues to the complex formation, we changed each of the amino acid residues in the μ Plm loops into alanine (alanine scanning mutagenesis (Cunningham BC and Wells JA, 1989). We made 54 alanine mutations, and 52 of them were expressed in *E. coli* as inclusion bodies, refolded, and purified. From kinetic data of the mutant proteins, we identified F587A as the most desirable mutant and performed saturation mutagenesis on the F587 position. Interestingly, the α 2-AP resistant F587A mutant is consistent with published results showing that the same mutant is resistant to certain active-site small molecule inhibitors (Law RH, *et al.*, 2017). Together we made a total of 73 mutant clones, 71 of these can be expressed and purified. Some of the results have been published. *Table 3* listed the kinetic parameters of the 9 most promising mutants selected from our mutagenesis results. In the table, we listed a column termed Kcat/Km*IC50 (Half-Maximal Inhibitory Concentration), which we defined as a mutant "escaping efficiency index"-the higher the index number, the better the mutant. The index number for F587A is 11.3, higher than any other mutants, except G739A, which is out of scale, and requires more detailed studies. Each of the mutant residues is labeled in the structures of *Figure 3*, which showed that 3 mutants are in contact with the modeled CTT (C-Terminal Tail) structure (R582A, M585A, K607A), and 5 are in contact with the reactive

center loop structure of α 2-AP (F587A, S608A, R610A, E641A, P642A). F587A is the most promising escape mutant selected, with catalytic activity and efficiency better than the WT (Wilms' Tumor gene) but highly resistant to α 2-AP inhibition. The possible molecular mechanism of the resistance based on Molecular Dynamic (MD) modeling and calculations have been described in our publication (Yang D, *et al.*, 2020). The G739 position is very close to the active site S741, and G739A has lower catalytic efficiency toward the synthetic substrate, but kinetic data show that this mutant is completely avoided of inhibition by α 2-AP. These results opened new doors for further research toward the therapeutic development of Plm-based drugs. The ultimate goal is to find engineered μPlm therapeutics that will be specific toward degrading a targeted pathogenic peptide substrate, but has no or much lower activity toward other untargeted substrates, through structure-based "directional engineering" of the enzyme (Yang D, *et al.*, 2020).

Approved drug and clinical trials of μ Plm, Plg, and Plm: Currently, there are two approved Plm-based drugs, which are recombinant μ Plm (ocriplasmin) from Thrombogenics and native Plasminogen (Ryplazim) from ProMetic Biotherapeutics Inc. The trade name for ocriplasmin is Jetrea, which is a recombinant μ Plm from a Pichia yeast expression system. The drug was approved by the US FDA for treatment of symptomatic vitreomacular adhesion on October 17, 2012 (FDA, 2012). The Ryplazim drug is a native Plasminogen purified from human serum, and has obtained the US FDA approval (June 4, 2021 (Pfaeffle V, 2021)), which was based on a single open-labeled clinical trial of 15 adult and pediatric patients with Plasminogen deficiency type 1 (Shapiro AD, *et al.*, 2018). Some of the major clinical trials cited from the clinicaltrials.gov site for μ Plm, Plg, and Plm are listed in *Table 4*.

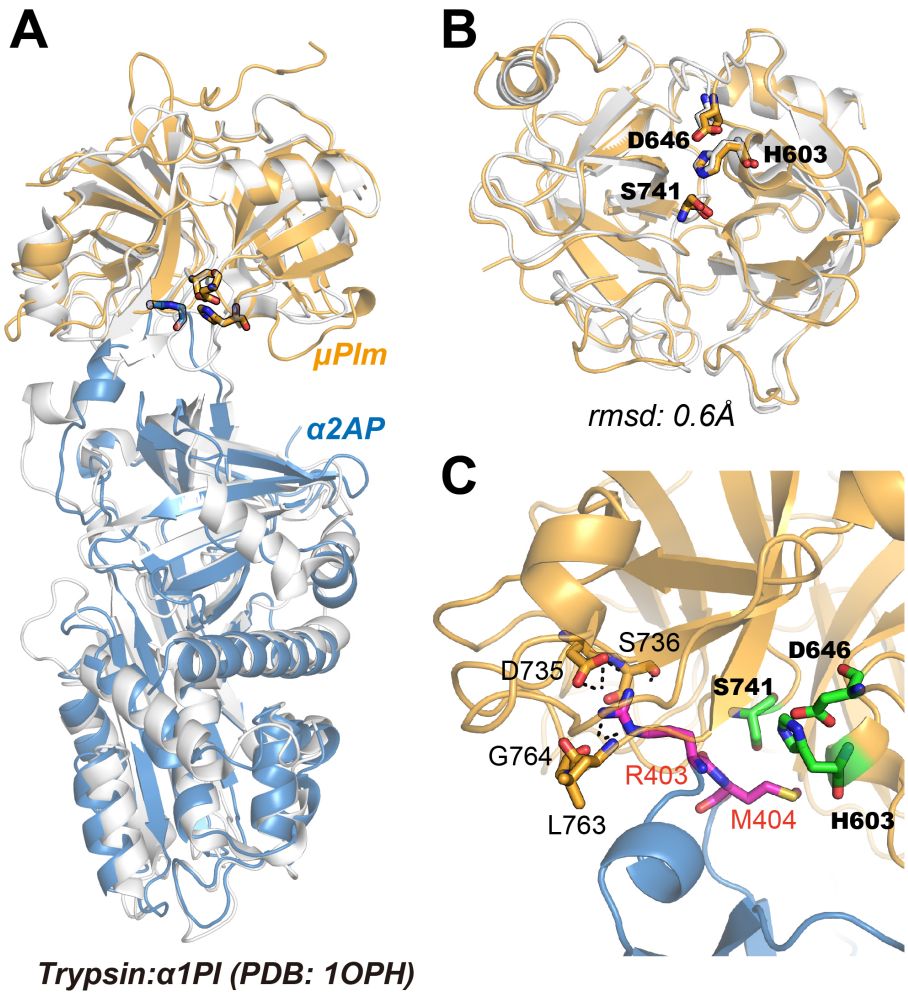

Figure 2: Stable complexes between μ Plm (highlighted in bright orange) and α 2-AP (highlighted in sky-blue) by homology modeling. The overall architecture of the μ Plm: α 2AP (2 Antiplasmin) complex is superimposed to the template Trypsin: α 1PI (Proteinase Inhibitor) (highlighted in white) crystal structure. Structural overlay of μ Plm and Trypsin with rmsd of 0.6 Å and illustration of the catalytic triad of residues S741, H603 and D646. Detailed view of interaction profile in the active site, where the catalytic triad initiates the covalent reaction with the backbone amide between R403 and M404 of α 2-AP. The active site is stabilized by extensive hydrogen bonds with the side chain of key residue R403.

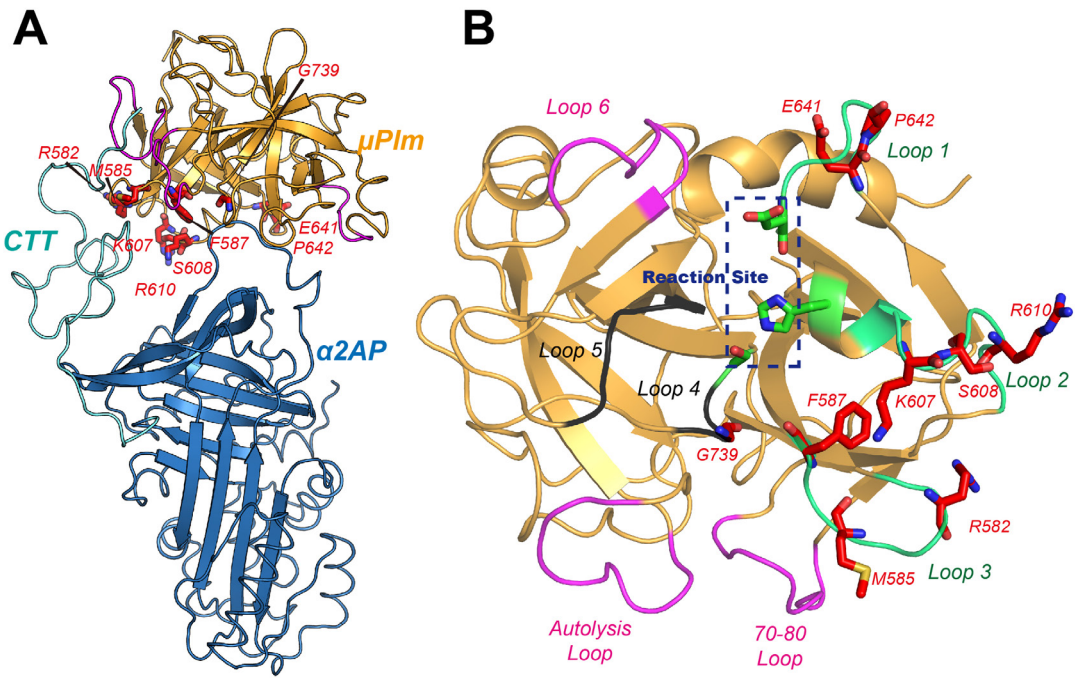

Figure 3: Distinct mutational sites map onto the complex structure and contact interface. Possible secondary interaction between the disordered C-Terminal Tail (CTT, highlighted in cyan) of α 2-AP with the autolysis loop and 70-80 loops (highlighted in magenta) of Plm, interpreting the mutational effects of these two loops. The key mutations are shown in red stick and labelled accordingly. Illustration of the interfacial loops and their mutational effects surrounding the reaction site, where mutations on loops 1-3 (highlighted in lime green) exert moderate perturbation to the active site, with the exception of F587 (highlighted in the red stick). Mutations on loops 4-5 (highlighted in black) render the protein inactive, and mutations on other distant loops (highlighted in magenta) yield the desired perturbation

Table 3: Summary of key desired mutants

Loops	Mutants	Kcat	Km	Kcat/Km	IC50	Kcat/Km × IC50
1	WT	1	1	1	1	1
	E641A	0.7	0.6	1.1	1.6	1.8
	K642A	1.6	0.7	2.2	1	2.2
2	K607A	2.9	0.9	3.3	1	3.3
	S608A	2.2	1.2	1.9	1.2	2.3
	R610A	1.6	1	1.6	1.7	2.7
3	R582A	2	1	2	1.6	3.2
	M585A	2.3	0.8	3.1	1.2	3.7
	F587A	2.6	0.9	2.9	3.9	11.3
4	G739A	0.6	4.9	0.1	∞	∞

Note: The table summarized the best mutants obtained from the alanine scanning mutagenesis data from reference (Millers EK, *et al.*, 2013). All of the kinetic values of the mutants are expressed as relative to the μ Plm Wild-Type enzyme (WT, set to be 1), which has Kcat=442 (min-1) and Km=204 μM. Kcat/Km is the catalytic efficiency, and IC50 is the Inhibition of μ Plm by α 2-AP to half of the maximum activity. In the last column, Kcat/Km × IC50 represents an artificial value to define an "escaping efficiency index". No inhibition ∞

Table 4: Clinical trials of μ Plm, Plg, and Plm

No	Phase	Year	Title of clinical trials	Enzyme	Clinical identifier	Refs
1	Phase 3	2008-2010	Non-surgical treatment of focal vitreomacular adhesion	μ Plm	NCT00798317/ NCT00781859	(Varma R, *et al.*, 2015)
2	Phase 2	2009-2011	Intravitreal microplasmin in relieving vitreo-macular adhesion in neovascular Age-related Macular Degeneration (AMD)	μ Plm	NCT00996684	-
3	Phase 2	2006-2010	Intravitreal Microplasmin Versus Sham Injection for treatment of patients with Diabetic Macular Edema (DME) (MIVI-II)	μ Plm	NCT00412451	-
4	Phase 2	2007-2009	Intravitreal Microplasmin for treatment of patients with Vitreomacular Traction (MIVI-IIt)	μ Plm	NCT00435539	-
5	Phase 2	2005-2008	Intravenous administration of microplasmin for treatment of acute ischemic stroke	μ Plm	NCT00123305	-
6	Phase 2	2006-2008	Intra-arterial Microplasmin administration in patients with Acute Intracranial Vertebrobasilar Artery Occlusion (MITI-IA)	μ Plm	NCT00123266	-
7	Phase 2	2005-2009	Intra-arterial microplasmin administration in patients with acute Peripheral Arterial Occlusion (PAC)	μ Plm	NCT00123292	-
8	Phase 3	2013-2015	A Phase III study of A01016 in subjects with symptomatic vitreomacular adhesion	μ Plm	NCT01889251	-
9	Phase 3	2011-2016	Treatment for symptomatic vitreomacular adhesion including macular hole	μ Plm	NCT01429441	(Yu TM, *et al.*, 2018)
10	Phase 1	2014-2016	Plasminogen (Human) intravenous in adults and children with Plasminogen deficiency	Plg	NCT02312180	-
11	Phase 2/3	2014-2018	Prometic Plasminogen IV infusion in subjects with hypoplasminogenemia	Plg	NCT02690714	(Shapiro AD, *et al.*, 2018)
12	Phase 2/3	2012-2020	Human Plasminogen eye drop in ligneous conjunctivitis Patients with Type I Plasminogen deficiency	Plg	NCT01554956	-
13	Phase 2	2010-2016	A Study of Intra-thrombus Plasmin (Human) In Acute Peripheral Arterial Occlusion	Plm	NCT01222117	(Comerota AJ, *et al.*, 2019)

Note: The table summarized major clinical trials of Plm-based therapeutics listed on clinicaltrials.gov site with National Clinical Trail (NCT) Identifier. Some of the references are published results, while the results of most of the trials are unpublished in the scientific literatures

Item numbers 1-9 listed in *Table 4* are μ Plm clinical trials. Most of these trials were for vitreomacular adhesion or traction-related eye diseases, leading to the approved drug Jetrea. There were also three Phase 2 clinical trials for Acute Ischemic Stroke (2005-2008), Acute Intracranial Vertebrobasilar Artery Occlusion (2006-2008), and Acute Peripheral Arterial Occlusion (2005-2009). These trials were ended more than 10 years ago, but no further Phase 3 clinical studies have been reported.

Item number 10-11 are Plg clinical trials for protein augmentation therapy in congenital Plg deficiency. These trials lead to US FDA approval on June 4, 2021. The Plg drug developed by Prometic Biotherapeutics is the first full-length, native Plg drug in the market.

The table also includes a Phase 2 trial of Acute Peripheral Arterial Occlusion using native Plm isolated from human serum. The trial ended in 2016 (2010-2016), and no further Phase 3 trial has been reported.

RESULTS AND DISCUSSION

Potential use of μ Plm, Plg, and Plm therapeutics in cardiovascular diseases

One of the potential applications of Plg and Plm based therapeutics is for Peripheral Arterial Occlusion (PAO), either alone or in combination with PAs. PAO occurs when a clot blocks artery blood flow to a distant part of the body such as the legs, arms, feet, or hands. A classical early hallmark of PAO is "intermittent claudication" or leg pain during the sustained activity which subsides after rest. Continued restriction of blood flow ultimately leads to constant pains in the leg or limb even at rest, along with ulcers, tissue death, and gangrene. Ultimately it could result in limb amputation. PAO is the result of Peripheral Arterial Disease (PAD), in which atherosclerotic plaque build-up on the artery walls leads to obstructed blood flow, leading to ischemia in blood starved limbs of the body.

Since its introduction as an alternative treatment to open balloon thrombo-embolectomy in the 1990s, thrombolytic treatment for the acute arterial occlusive disease has matured in multiple directions. Current treatments of PAO include angioplasty, stents, and thrombolytic intervention with tPA (Activase®) or uPA (Abbokinase®) (Ebben HP, *et al.*, 2019; Robertson I, *et al.*, 2013). Catheter-Directed Thrombolysis (CDT) is the most successful treatment, but is still accompanied by major bleeding complications and other treatment failures (Ebben HP, *et al.*, 2019; Robertson I, *et al.*, 2013).

Treatment of Acute Myocardial Infarction (AMI) is fundamentally different from PAO. First, the PAs do not dissolve blood clots themselves, but it generates active Plm from Plg to do so. While this can be effective in the dissolution of a thrombus in a small myocardial artery, difficulties arise in the dissolution of much larger thrombi in peripheral arteries and veins because the clots are long and retracted (Sabovic M, *et al.*, 1989; Robbie LA, *et al.*, 1996; Van-Loon BP, *et al.*, 1992). Circulation is poor near these clots

so the supply of Plg substrate is insufficient. Consequently, systemically delivered PAs will not only have difficulty infiltrating the clot, but also there is insufficient Plg substrate to enable efficient dissolution of the clot (Sabovic M, *et al.*, 1989; Robbie LA, *et al.*, 1996; Van-Loon BP, *et al.*, 1992). On the other hand, a Plm-based direct thrombolytic agent can be efficiently dissolve the clot by itself, avoiding the Plg substrate depletion problems that are encountered in the treatment of using PAs. Furthermore, once diffused into the serum, Plm activity will be immediately neutralized by α 2-AP, potentially avoiding bleeding side effects (Marder VJ, 2011).

In vitro studies have pointed to some interesting functional differences between Plm, mPlm, and μ Plm. Functionally, μ Plm is distinguished from mPlm and Plm in its inability to specifically bind to fibrin; the fibrin binding resides in Kr1-Kr3 and Kr5 domains (Wiman B, *et al.*, 1978; Thorsen S, *et al.*, 1981; Komorowicz E, *et al.*, 1998), which μ Plm lacks. While Plm and mPlm have similar catalytic rates in digesting fibrin, μ Plm is 6-fold slower than mPlm and 12-fold slower than Plm (Komorowicz E, *et al.*, 1998). The half-life of Plasmin bound to the fibrin surface is estimated to be 2-3 orders of magnitudes longer than freely circulating activated Plasmin (Wiman B, *et al.*, 1978). Once the fibrin bound Plm dissociates from the blood clot it becomes immediately accessible to its principle inactivator α 2-AP, and has a plasma half-life of only 0.2 seconds (Wiman B, *et al.*, 1978). *In vivo*, α 2-AP binds to specific lysine residues located in Kr5 and other Kr domains first and then the catalytic domain (Wiman B, *et al.*, 1978; Christensen U, *et al.*, 1996; Wiman B and Collen D, 1998). Through protein surface recognition around the active site pocket (*Figure 2* and *Figure 3* for structural modeling), α 2-AP can also directly bind to μ Plm, which has a relatively long plasma activity half-life of about 4 seconds compared to Plm *(Figures 3a and 3b)*.

Regarding to fibrin specificity, one must realize that Plm trapped into the clots resulting from Plg activation is different from Plm-based drugs injected into the blood. The trapped Plm, as described above, binds to the inside of the fibrin network, and has an activity half-life of 2-3 orders of magnitude than free Plm (Wiman B, *et al.*, 1978). In case of drug delivery, a large therapeutic dose of Plm-based drug needs to be delivered to solve an emergency situation, such as ischemic stroke and myocardial infarction, and it is unlikely that there is enough time to reach equilibrium to use the fibrin specificity to "target" the disease-causing thrombi and at the same time, to avoid α 2-AP inhibition. Practically, currently catheter-directed delivery is the main solution for fibrin targeting. We speculated that in this artificial emergency situation, fibrin specificity of the thrombolytic drug may not be as important as timing and quantity of delivered therapeutics in terms of drug efficacy. As an example, Nagai and coworkers (Nagai N, *et al.*, 2003) compared local catheter-mediated delivery of serum-derived Plm, recombinant human μPlm isolated from the yeast P. pastoris, and recombinant tPA in efficacy of dissolution of thrombi in a rabbit extracorporeal loop thrombolysis model. All three molecules were effective at dissolving thrombi in a dose-dependent manner, with similar molar doses for Plasmin and μ Plm displaying similar efficacy of thrombus dissolution. Plm and μPlm did not result in bleeding at an extra-thrombus hemostatic stable plug far away from the site of infusion at the administered doses. tPA however, did cause bleeding at all concentrations at the extra-thrombus plug site. Thus μPlm, which lacks the kringle domains of Plm and therefore does not possess the extra-fibrin binding specificity, is as effective as Plm at a similar pharmacological dose (Nagai N, *et al.*, 2003).

Potential use of μ Plm and Plm therapeutics in Alzheimer's Disease (AD)

Alzheimer's Disease (AD) was first described by the German psychiatrist Alois Alzheimer in 1901. AD is mainly characterized by extracellular plaques and intracellular neurofibrillary tangles. The extracellular plaques are primarily composed of β-Amyloid (Aβ) peptides, and the intracellular neurofibrillary tangles are composed of the cytoskeletal protein tau (Finder VH and Glockshuber R, 2007; Tiraboschi P, *et al.*, 2004). Aβ is a mixture of the peptide from 38 to 43 residues, which is generated from β-Amyloid Precursor Protein (APP) by the action of two proteases, β-secretase (Beta-Site Amyloid Precursor Protein Cleaving Enzyme: BACE-1) and γ-secretase. Results from the past research have supported the amyloid cascade hypothesis (Hardy J and Allsop D, 1991; Hardy J, *et al.*, 1998; Selkoe DJ, 1991). This hypothesis proposes that the overproduction of Aβ peptides (mostly from genetic defect), or the failure to effectively clear this peptide (most of the sporadic AD cases), leads to AD through Aβ toxicity and amyloid deposition, which is also thought to be involved in the formation of neurofibrillary tangles (Hardy J and Selkoe DJ, 2002; Leissring MA, 2008; Selkoe DJ and Schenk D, 2003). As a result, therapeutic research toward the treatment of AD has mainly aimed at blocking production, hindering aggregation, or enhancing the clearance of Aβ peptides (Citron M, 2004). One of the earliest Aβ-based therapeutic applications was immunotherapy using Aβ peptide as a vaccine (Schenk D, *et al.*, 1999), although clinical toxicity has prevented further development of this strategy (Orgogozo JM, *et al.*, 2003). Using antibodies to Aβ peptides as therapeutic agents have also been conducted in many clinical trials (Banks WA, *et al.*, 2007; Dodel RC, *et al.*, 2004; Du Y, *et al.*, 2003; Prada CM, *et al.*, 2007). However, with many clinical trial failures of anti-Aβ antibody, the amyloid hypothesis has been questioned (Cummings J, *et al.*, 2019). The field, however, has gained a renewed optimism because of an application and approval (7 June 2021) of aducanumab with the U.S. Food and Drug Administration (FDA) from Biogen Inc (Abbott A, 2019; Fillit H and Green A, 2021; Hern K, 2021; Johnson V, 2021; International ASD, 2021) and the FDA's granting of Breakthrough Therapy designation for Eli Lilly's donanemab in June 2021 based on a positive Phase 2 clinical trial (Abbott A, 2019; Fillit H and Green A, 2021; Hern K, 2021; Johnson V, 2021).

Since the discovery of β-secretase as a critical enzyme in Aβ formation (Hussain I, *et al.*, 1999; Lin X, *et al.*, 2000; Sinha S, *et al.*, 1999; Vassar R, *et al.*, 1999; Yan R, *et al.*, 1999), the β-secretase inhibitor drug studies have become a major field in AD drug development (Cole SL and Vassar R, 2008; Ghosh AK, *et al.*, 2008). The field of γ-secretase inhibitor or modulator has also been an active area of AD drug development (Czirr E, *et al.*, 2007 Harrison T, *et al.*, 2004). More importantly, the realization of the importance of *in vivo* Aβ cleavage has opened a new door to develop clearance-based therapeutic applications (Leissring MA, 2008).

In normal physiological conditions, the production of Aβ is counterbalanced by its elimination *via* multiple interrelated processes acting in concert, including proteolytic degradation, cell-mediated clearance, active and passive transport out of the brain, as well as deposition into insoluble aggregates. Although each of these processes contributes to Aβ catabolism, research results emerged have shown that proteolytic degradation is a particularly important regulator of cerebral Aβ levels and, by extension, AD pathogenesis. Saido and colleagues were the first to examine Aβ degradation in the living animal (Cummings J, *et al.*, 2019). Subsequent works have identified many different kinds of proteolytic enzymes involved in Aβ catabolism, including zinc-metalloproteases, cysteine proteases, and serine proteases (for review) (Leissring MA, 2008; Hersh LB, 2006; Leissring MA, 2006; Turner AJ and Nalivaeva NN, 2007; Wang DS, *et al.*, 2006). All of these enzymes have potential therapeutic value for treating AD. However, of all the proteases that are directly involved in degrading Aβ *in vivo*, only μPlm has been extensively studied as a therapeutic drug (FDA, 2012; Varma R, *et al.*, 2015; Chen F, *et al.*, 2007; Fu JY, *et al.*, 2007; Gandorfer A, 2008; Quiram PA, *et al.*, 2007; Rasmussen RS, *et al.*, 2008; Sakuma T, *et al.*, 2005; Sebag J, *et al.*, 2007). Therefore, presently, developing Plm-based therapeutics is a practical choice among the Aβ degradation enzymes.

Studies in cultured cells have shown that purified Plm significantly decreases the level of neuronal injuries induced by aggregated Aβ (Tucker HM, *et al.*, 2002; Tucker HM, *et al.*, 2000). In separate research, Ledesma et

al have not only shown that Plm degrades Aβ, converting it from the amyloidogenic form to a non-amyloidogenic form, but have also shown consistently that the level of Plm is reduced in brain tissues from AD patients. Published results have also shown that peripherally applied Aβ-containing inoculate induced cerebral β-amyloidosis (Eisele YS, *et al.*, 2010), further implying that clearing peripheral Aβ can be as important as cerebral clearance.

As described above, native Plasmin-based therapeutics may not be efficient enough for therapeutic application. However, a Plm-based escaping mutant may be selected such that it can specifically cleave and detoxify the β-Amyloid peptide, but at the same time, have low catalytic activities toward other common substrates such as fibrin, in addition to resisting α 2-AP inhibition. The tailor-selected mutant Pm-based therapeutics may therefore have higher efficacy toward treating AD but with reduced side effects (Yang D, *et al.*, 2020).

Potential use of μ Plm, Plg, and Plm therapeutics in pulmonary fibrosis

Reduced fibrinolysis due to Plm downregulation has been implicated in lung fibrosis (Bauman KA, *et al.*, 2010; Shetty S, *et al.*, 2008; Hattori N, *et al.*, 2004; Marudamuthu AS, *et al.*, 2015). It has been shown that mice with targeted deletion of the Plg gene have poor outcomes in pulmonary fibrosis conditions (Swaisgood CM, *et al.*, 2000). It has also been shown that mice with overexpression of a PAI-1 developed impaired systemic Plg activation to Plm, resulting in a more severe lung fibrotic response following bleomycin injury than do littermate controls, and increased Plg activation has anti-fibrotic effects (Eitzman DT, *et al.*, 1996). On the other hand, the Plg activation system is impaired in Idiopathic Pulmonary Fibrosis (IPF) (Chapman HA, *et al.*, 1986; Kotani I, *et al.*, 1985), further proving that the Plg activation system is critical for preventing the IPF disease. Direct evidence of Plm acting in the lung fibrosis system has also been published (Okunishi K, *et al.*, 2011).

In the COVID-19 section following, we will review some evidence of using Plg/Plm to treat lung fibrosis caused by SARS-CoV-2 infection. All these results clearly indicate that Plm-based therapeutic treatments could be effective for lung fibrosis, a devastating disease with no effective treatment currently. On the other hand, the major Plm inhibitor in the serum, α 2-AP, has been shown to be a pro-fibrosis, mediated by several mechanisms (Kanno Y, 2015). Besides direct dissolving fibrins, neutralization of α 2-AP by μ Plm may also have a therapeutic effect on lung fibrosis.

COVID-19 caused systematic thrombosis and the potential use of μ Plm and Plm therapeutics

Severe acquired respiratory syndrome coronavirus-2 (SARS-CoV-2) has caused a worldwide pandemic of respiratory illness (Katneni UK, *et al.*, 2020; Singhal T, 2020). The characteristics of coronavirus disease 2019 (COVID-19) associated coagulopathy are different from those that occur with severe fungal or bacterial-induced coagulopathy (Cao X, 2020; Moon C, 2020; Connors JM and Levy JH, 2020). Characterized by organ failure in patients of COVID-19 caused by widespread microclots in capillaries (Marietta M, *et al.*, 2020), an immune-triggered coagulopathy and thrombotic microangiopathy is frequently occurred (Merrill JT, *et al.*, 2020), and many studies have shown that nearly 80 percent of people who die of COVID-19 have thrombosis in the lungs (Marietta M, *et al.*, 2020; Merrill JT, *et al.*, 2020; Iba T, *et al.*, 2020).

The coagulopathy of COVID-19 patients usually shows increased fibrin degradation products (D-dimers) and fibrinogen levels, but the platelet count and prothrombin time are initially normal (Merrill JT, *et al.*, 2020; Iba T, *et al.*, 2020). The high incidence of venous thromboembolism and the importance of treating with anticoagulant thromboprophylaxis has been stated in a guidance document for treating COVID-19 (Thachil J, *et al.*, 2020). Numerous autopsy studies also discovered frequent deep vein thrombosis, such as a finding in that deep vein thrombosis occurs in 7 of 12 COVID-19 patients and 4 of them in complicating pulmonary embolism (Wichmann D, *et al.*, 2020). Significantly lower levels of antithrombin and fibrinogen were seen at the late stage in non-survivors (Tang N, *et al.*, 2020). In a study of 449 severe COVID-19 patients, the 28-day mortality is negatively correlated with platelet count, and positively correlated with D-dimers and prothrombin time (Tang N, *et al.*, 2020). Anticoagulant treatment with heparin has shown an improved prognosis for patients admitted to Intensive Care Units (ICU) (Tang N, *et al.*, 2020). An association of elevated D-dimers with in-hospital death has been reported in a study of 191 patients (Zhou F, *et al.*, 2020). High levels of D-dimers and prolonged prothrombin time were also observed in ICU patients in another report (Huang C, *et al.*, 2020).

In the advanced clinical stages of highly pathogenic human Coronavirus (hCoV), comorbidities are important factors in the occurrence of disease complications that often lead to death (Sanyaolu A, *et al.*, 2020). The most common comorbidities of severe COVID-19 patients are reported to be diabetes, hypertension, malignancies, kidney dysfunction, and Chronic Obstructive Pulmonary Disease (COPD), all of which are characterized by chronic inflammation (Jain V and Yuan JM, 2020; Qiu P, *et al.*, 2020). Blood flow abnormalities, endothelial dysfunction, hyperviscosity, and platelet activation caused by hypoxia, hypercoagulability, and immune reactions are the main factors that lead to thrombogenesis, and high doses of heparin therapy are usually applied in the treatment of COVID-19-induced thrombosis (Ahmed S, *et al.*, 2020).

A common feature in patients with underlying diseases is elevated blood Plg levels (Ji HL, *et al.*, 2020). Studies aimed at exploring new approaches both for early detection and for the treatment of severe COVID-19 patients can have a major impact on the fight against the disease. Herein, we highlight evidences that support the potential role of the Plasminogen Activator/Plasminogen Activator Receptor (PA/PAR) system in the pathogenesis of COVID-19 associated pneumonia and Acute Respiratory Distress Syndrome (ARDS). A soluble form of uPAR could be a potential biomarker for the progression of the disease, and its level correlates with comorbidities associated with the death of COVID-19 patients (D'Alonzo D, *et al.*, 2020). In a study involving 118 hospitalized COVID-19 patients, elevated levels of tPA and PAI-1 were found in COVID-19 patients and were associated with more severe respiratory conditions (Zuo Y, *et al.*, 2020).

Targeting the dysregulated PA/PAR system may represent a therapeutic approach for the treatment of severe lung injury caused by SARS-CoV-2 infections (Moore HB, *et al.*, 2020). Several studies in animal models (Hardaway RM, *et al.*, 1990; Stringer KA, *et al.*, 1998; Liu C, *et al.*, 2018) as well as a phase I clinical trial (Hardaway R, *et al.*, 2001) support the use of PA to limit Acute Respiratory Distress Syndrome (ARDS) progression and reduce ARDS-induced death. COVID-19 induced ARDS could be caused by the presence of microthrombi (Borczuk AC, *et al.*, 2020; McFadyen JD, *et al.*, 2020), which could be treated with thrombolytic enzyme therapeutics. For example, tPA treatment had shown to decrease dead space ventilation in a study involving 15 COVID-19 patients (Orfanos S, *et al.*, 2020). Another case study of three severe COVID-19 patients who were treated with tPA was reported, all of them showed improvements in their respiratory conditions (Wang J, *et al.*, 2020). In a study of 13 COVID-19 patients, Plg inhalation treatment has been reported, showing improvement in lung lesions and hypoxemia (Wu Y, *et al.*, 2020). Three more case series with a total of eight cases of ARDS caused by COVID-19 are found benefitted from tPA administration (Wang J, *et al.*, 2020; Christie DB, *et al.*, 2020; Choudhury R, et al., 2020). An anti-inflammatory drug UPARANT (Urokinase Receptor-Derived Peptide Inhibitor) has been reported to exhibit activities that regulate the uPA/uPAR system in animal models, showing a different mechanism from corticosteroids and nonsteroidal anti-inflammatory drugs (Cammalleri M, *et al.*, 2019).

The evidence clearly indicates a positive therapeutic effect for treating COVID-19 related systemic blood clotting with thrombolytic agents. The Plm-based thrombolytic therapeutics described in the following sections may provide alternative or better therapeutic options than PA-based therapeutics.

For comparison, a SARS coronavirus infection leads to lung fibrosis in late-stages of severe patients (Zuo W, *et al.*, 2009). Not surprisingly, Idiopathic Pulmonary Fibrosis (IPF) is a major risk factor for COVID-19, which in many cases can lead to long-term pulmonary fibrosis (Vasarmidi E, *et al.*, 2020; George PM, *et al.*, 2020). The resulting lung fibrosis is related, or may be a result of virus-induced coagulation, because it has been implied that SARS-CoV-2 caused lung injury can lead to extravascular coagulation in the interstitial space and lung fibrosis (Schuliga M, *et al.*, 2018; Fujii M, *et al.*, 2000; Wygrecka M, *et al.*, 2011). The fibrosis, at least in the initial stage, could be treated with Plm-based direct thrombolytic therapeutics. In fact, it has been shown in both animal model and initial clinical trials, that Plg supplementation may be a promising therapeutics for IPF treatment (Parker J, *et al.*, 2019).

FUTURE DIRECTIONS

In 2010 and 2011, Marder and Novokhatny published two review papers (Marder VJ, 2011; Marder VJ and Novokhatny V, 2010), describing the historical perspective of thrombolytic therapies and suggesting a future direction. PAs have been dominated the thrombolytic therapy but the bleeding side effects are inherent and cannot be solved easily. According to Marder's analysis (Marder VJ, 2011; Marder VJ and Novokhatny V, 2010), the future therapeutic direction should be Plm-based direct thrombolysis. Marder indicated that there are two major reasons for choosing Plm over PAs. First, Plm is the enzyme that dissolves fibrin, and Plm-based therapeutics would be more efficient and can avoid the Plg depletion problems in certain clinical conditions. Second, with the maturation and advancement of Catheter-Directed Delivery Technology (CDT), Plm can be delivered directly into the blocking thrombi, and will be neutralized by α 2-AP once diffused into the serum, potentially avoiding the bleeding side effect. Since then, there have been many thrombolytic clinical trials using the CDT technology and Plm-based therapeutics, including trials listed in *Table 4*. The table showed Phase 2 clinical trials for Acute Intracranial Vertebrobasilar Artery Occlusion and Acute Peripheral Arterial Occlusion. The latest of these trials was finished in 2009, but no further Phase 3 clinical trials have been reported after more than 10 years. Thus, after more than 10 years of research and development since Marder's review, it seems that the Plm or μ Plm-based direct thrombolytic therapeutics have not lived up to their theoretical expectations.

We speculate that the bleeding problems can still occur, even with catheter-delivered Plm/μ Plm therapeutics. The paradox is, when using Plm or μ Plm as direct thrombolytics, the drugs need to reach enough concentration to overcome α 2-AP inhibition before effectively dissolving blood clots. The normal plasma concentration of α 2-AP is about 1 μM (Collen D and Wiman B, 1978), therefore about 1 μM of Plm is required to neutralize α 2-AP before thrombolytic therapy can be effective. Even in the case of locally catheter-directed delivery, the local concentration of Plm has to reach >1 μM to be therapeutically effective. The "overdosed" Plm may reach distant hemostatic plugs and dissolve them, causing systemic bleeding, as shown in *Figure 4a*.

In the case of systemic clotting *(Figure 4b)*, such as for treating large-scale clotting in many SARS-CoV-2 infections, systemic thrombolytic delivery using Intravenous (IV) injection is required. Severe bleeding side effects occur when PAs were used, and currently, there are no easy solutions (Ebben HP, *et al.*, 2019; Robertson I, *et al.*, 2013; Daley MJ, *et al.*, 2015; Abraham P, *et al.*, 2018). In the serum, Plg is at a concentration of about 200 mg/L or 2 μM (Castellino FJ and Powell JR, 1981; Ganrot PO and Niléhn JE, 1968; Lijnen HR, *et al.*, 2004), and for a person with 5 L of blood volume, >500 mg of Plm or 145 mg of μ Pm (1 μM serum concentration) is required to neutralize α 2-AP before can be effective to dissolve blood clots. Again, the required therapeutic doses of Plm may dissolve hemostatic plugs and cause severe systemic bleeding, in addition to other damages caused by continued proteolytic activity.

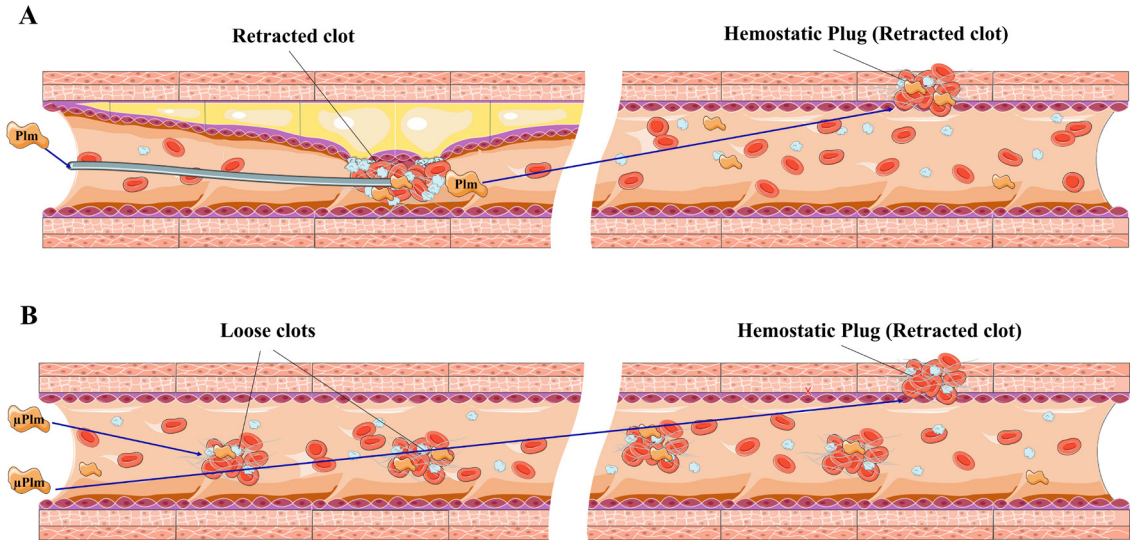

Figure 4: Schematic presentation of two possible actions of μ Plm and Plm when applied to a local and systemic blood clotting. In catheter-directed delivery of native Plm therapeutics, after dissolving the retracted blood clots such as in the case of peripheral arterial occlusion, the diffused Plm can still actively dissolve distant retracted hemostatic plugs if the applied Plm can neutralize α 2-AP and has a longer *in vivo* half-life, resulting in bleeding side effect. In acute systemic clotting, the newly formed clots are un-contracted (un-retracted) or "loose", but the hemostatic plugs formed to protect the blood wall from bleeding are usually contracted (retracted) and "denser". After neutralizing α 2-AP in the serum, excessively applied mutant μ Plm may quickly dissolve the loose clots in the serum but may not be able to dissolve the retracted hemostatic plugs because of the short *in vivo* half-life. Plm/Plm; Red Blood Cell (RBC); Platelet; Fibrin

In a recent publication, Tutwiler V, *et al.* showed that fibrinolysis of uncontracted ("loose") clots are 4-fold faster than contracted clots (Tutwiler V, *et al.*, 2019). This suggests a possibility of differentially dissolving uncontracted clots and leaving contracted clots intact. We, therefore, propose a possible "hit and die" strategy, which is to engineering a short *in vivo* half-life version of thrombolytics (such as a *E. coli* expressed mutant μ Pm) in such a way that after dissolving the newly formed "loose" clots in acute systemic bleeding (*Figure 4b*, here the concentration of delivered μ Pm is "overdosed" to neutralize all of the protease inhibitors, and "transient" because of the very short *in vivo* half-life), the mutant μ Pm may die out or lost activity quickly and unable to dissolve the contracted or "dense" hemostatic plugs, avoiding bleeding side effect resulting from the continued activity of the present thrombolytic drugs. Even in the case of local delivery shown in *Figure 4a,* the *in vivo* "fragile" mutant μ Pm may be able to dissolve the immediately accessible clots, if enough active materials are delivered, and then die out or lost activity quickly after diffusing into the blood, avoiding bleeding side effect. Again, we'd like to stress that the definition of the *in vivo* half-life here is the structural integrity of the enzyme in the blood, as already explained in the Introduction section.

In general, *E. coli* produced recombinant proteins have shorter *in vivo* half-life than mammalian-produced proteins (Du T, *et al.*, 2019). The *in vivo* half-life of Ocriplasmin, a recombinant μ Pm drug produced from a Pichia yeast strain, has an elimination half-life (t1/2) ranging from 0.72 to 7.07 hrs (Kotch L, 2012). It is well-known that post-translational modifications influence protein stability (Mittal S and Saluja D, 2015), and *E. coli* produced μ Pm may have shorter *in vivo* half-life because of the lack of post-translational modifications, such as phosphorylation at the S578 site (Wang H, *et al.*, 1997). In addition, we have produced both wild-type and mutant forms of μ Pm from *E. coli* inclusion bodies (Yang D, *et al.*, 2020), and have further tested different versions of the recombinant proteins in a mouse model of pulmonary embolism (Miao R, *et al.*, 2010).

On the other hand, native Plm may not fit the purpose because of its much longer *in vivo* half-life. In addition, recombinant full-length Plm has not been produced, excluding the possibility of further protein engineering. The *in vivo* half-life of Glu-Plg is about 2 days (Collen D, *et al.*, 1975), and a reported Plm-α 2-AP complex has an *in vivo* half-life of about 4.5 h (Chandler WL, *et al.*, 2000). Therefore comparatively, *E. coli* produced μ Pm is a better starting enzyme for engineering a short half-life version of direct thrombolytic therapeutics.

CONCLUSION

Here we considered several aspects that may help to solve the long-standing bleeding problem in thrombolytic therapy. The primary goal is to engineering short *in vivo* half-life thrombolytics that can be effective in dissolving disease-causing blood clots but leave the protective hemostatic plugs intact. With the new thrombolytic therapeutics, we may selectively dissolve clots in the immediate delivery site (i.e. catheter tip-site) but leave the distantly retracted hemostatic plugs intact (*Figure 4a* shows a potential spatial differentiation of clot-dissolution between the targeted and protective clots). In addition, the new agents may differentiate clot "toughness", and selectively dissolve newly formed "loose" clots but leave the retracted hemostatic plugs intact (*Figure 4b* shows a potential time differentiation of colt-dissolution between the newly formed "loose" clots and the protective retracted clots).

Furthermore, we have selected and engineered a mutant μ Pm (F587A, *Table 3*) that has higher catalytic activity than the wild-type enzyme, and can partially escape α 2-AP inhibition (Yang D, *et al.*, 2020), potentially requiring lower concentration for *in vivo* therapeutic effect. With deeper structure-based directional protein engineering, we may engineer an enzymatically more efficient, and shorter *in vivo* half-life version of mutants for the future development of a super-active but *in vivo* unstable "ideal" thrombolytics. Therefore, an important future direction of thrombolytic development may be based on a rational development of high-efficient, α 2-AP inhibition resistance, and short *in vivo* half-life *E. coli* produced recombinant mutant μ Plm therapeutics.

In addition, there may be broader application of the new theory on emergency drug development. Instead of the traditional approach of developing more stable, longer *in vivo* half-life emergency protein drugs, a new direction may be to develop *in vivo* unstable, short half-life medicines to avoid toxic side effects.

ACKNOWLEDGEMENTS

Consent to participate and publication

All authors consented to participate the writing and agree with the content of the paper

Funding

This work was supported in part by Shenzhen Municipal Science and Technology Innovation Commission (KQTD2017-0330155106581), Major Program of Shenzhen Bay Laboratory S201101001, National Natural Science Foundation (30901023), and the Natural Science Foundation of Shandong Province (ZR2017LC017).

Authors' contributions

X.L., W.Z. and J.G. are responsible for designing the overall concept and writing the manuscript; Y.W. contributes to the structural section; Y.C. contributes to the COVID-19 section; X.D. contributes to the substrate specificity section and overall editing; D.Y., L.L. contributes overall editing and some figures; F.T. contributes to the drug development section; Z.M. contributes some figures and editing. All authors approved the submitted version.

REFERENCES

1. Palta S, Saroa R, Palta A. Overview of the coagulation system. Indian J Anaesth. 2014; 58(5): 515.
2. Buzby S. AHA: Heart disease remains leading cause of death worldwide; trends discouraging. Healio. 2021.
3. Lin H, Xu L, Yu S, Hong W, Huang M, Xu P. Therapeutics targeting the fibrinolytic system. Exp Mol Med. 2020; 52(3): 367-379.
4. Aisina RB, Mukhametova LI. Structure and function of Plasminogen/Plasmin system. Russ J Bioorganic Chem. 2014; 40(6): 590-605.
5. Wu G, Quek AJ, Caradoc-Davies TT, Ekkel SM, Mazzitelli B, Whisstock JC, *et al.* Structural studies of Plasmin inhibition. Biochem Soc Trans. 2019; 47(2): 541-557.
6. Gurewich V. Fibrinolysis: an unfinished agenda. Blood Coagul Fibrinolysis. 2000; 11(5): 401-418.
7. Bachmann F. The Plasmin-Plasminogen Enzyme System. Haemostasis and Thrombosis: Basic Principles and Clinical Practice. 1994; 1592-1622.
8. Mehta R, Shapiro AD. Plasminogen deficiency. Haemophilia. 2008; 14(6): 1261-1268.
9. Klammt J, Kobelt L, Aktas D, Durak I, Gokbuget A, Hughes Q, *et al.* Identification of three novel Plasminogen (PLG) gene mutations in a series of 23 patients with low PLG activity. Thromb Haemost. 2011; 105(03): 454-460.

10. Kwaan HC, McMahon B. The role of Plasminogen-Plasmin system in cancer. Cancer Treat Res. 2009; 148: 43-66.
11. Didiasova M, Wujak L, Wygrecka M, Zakrzewicz D. From Plasminogen to Plasmin: role of Plasminogen receptors in human cancer. Int J Mol Sci. 2014; 15(11): 21229-21252.
12. Sulniute R, Shen Y, Guo YZ, Fallah M, Ahlskog N, Ny L, *et al.* Plasminogen is a critical regulator of cutaneous wound healing. Thromb Haemost. 2016; 116(05): 1001-1009.
13. Miles LA, Parmer RJ. Plasminogen receptors: the first quarter century. Thromb Haemost. 2013; 39(4): 329-337.
14. Saksela O. Plasminogen activation and regulation of pericellular proteolysis. Biochim Biophys Acta. 1985; 823(1): 35-65.
15. Hamilton JA. Plasminogen Activator/Plasmin system in arthritis and inflammation: Friend or foe?. Arthritis Rheum. 2008; 58(3): 645-648.
16. Draxler DF, Sashindranath M, Medcalf RL. Plasmin: A modulator of immune function. Thromb Haemost. 2017; 43(2): 143-153.
17. US National Library of Medicine. Thrombolytic therapy. Medline Plus. 2021.
18. Wiman B, Boman L, Collen D. On the kinetics of the reaction between human Antiplasmin and a low-molecular-weight form of Plasmin. Eur J Biochem. 1978; 87(1): 143-146.
19. Petersen TE, Martzen MR, Ichinose A, Davie EW. Characterization of the gene for human Plasminogen, a key proenzyme in the fibrinolytic system. J Biol Chem. 1990; 265(11): 6104-6111.
20. Law RH, Caradoc-Davies T, Cowieson N, Horvath AJ, Quek AJ, Encarnacao JA, *et al.* The X-ray crystal structure of full-length human Plasminogen. Cell Rep. 2012; 1(3): 185-190.
21. Xue Y, Bodin C, Olsson K. Crystal structure of the native Plasminogen reveals an activation-resistant compact conformation. J Thromb Haemost. 2012; 10(7): 1385-1396.
22. Wang H, Prorok M, Bretthauer RK, Castellino FJ. Serine-578 is a major phosphorylation locus in human plasma Plasminogen. Biochemistry. 1997; 36(26): 8100-8106.
23. Ney KA, Pizzo SV. Fibrinolysis and fibrinogenolysis by Val442-Plasmin. Biochim Biophys Acta. 1982; 708(2): 218-224.
24. Wu HL, Shi GY, Bender ML. Preparation and purification of microplasmin. Proc Natl Acad Sci. 1987; 84(23): 8292-8295.
25. Wu HL, Shi GY, Wohl RC, Bender ML. Structure and formation of microplasmin. Proc Natl Acad Sci. 1987; 84(24): 8793-8795.
26. Wang J, Reich E. Structure and function of microplasminogen: II. Determinants of activation by urokinase and by the bacterial activator streptokinase. Protein Sci. 1995; 4(9): 1768-1779.
27. Sikri N, Bardia A. A history of streptokinase use in Acute Myocardial Infarction. Tex Heart Inst J. 2007; 34(3): 318.
28. Wang X, Lin X, Loy JA, Tang J, Zhang XC. Crystal structure of the catalytic domain of human Plasmin complexed with streptokinase. Science. 1998; 281(5383): 1662-1665.
29. Grella DK, Castellino FJ. Activation of human Plasminogen by staphylokinase: Direct evidence that preformed Plasmin is necessary for activation to occur. Blood. 1997; 89(5): 1585-1589.
30. Loy JA, Lin X, Schenone M, Castellino FJ, Zhang XC, Tang J. Domain interactions between streptokinase and human Plasminogen. Biochemistry. 2001; 40(48): 14686-14695.
31. Esmon CT, Mather T. Switching serine protease specificity. Nat Struct Biol. 1998; 5(11): 933-937.
32. Parry MA, Fernandez-Catalan C, Bergner A, Huber R, Hopfner KP, Schlott B, *et al.* The ternary microplasmin-staphylokinase-microplasmin complex is a proteinase–cofactor–substrate complex in action. Nat Struct Biol. 1998; 5(10): 917-923.
33. Backes BJ, Harris JL, Leonetti F, Craik CS, Ellman JA. Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of Plasmin and thrombin. Nat Biotechnol. 2000; 18(2): 187-193.
34. Hervio LS, Coombs GS, Bergstrom RC, Trivedi K, Corey DR, Madison EL. Negative selectivity and the evolution of protease cascades: the specificity of Plasmin for peptide and protein substrates. Chem Biol. 2000; 7(6): 443-452.
35. Repetto O, Maiero S, Magris R, Miolo G, Cozzi MR, Steffan A, *et al.* Quantitative proteomic approach targeted to fibrinogen β chain in tissue gastric carcinoma. Int J Mol Sci. 2018; 19(3): 759.
36. Manon-Jensen T, Multhaupt HA, Couchman JR. Mapping of matrix metalloproteinase cleavage sites on syndecan-1 and syndecan-4 ectodomains. FEBS J. 2013; 280(10): 2320-2331.
37. Schmidt A, Echtermeyer F, Alozie A, Brands K, Buddecke E. Plasmin-and thrombin-accelerated shedding of syndecan-4 ectodomain generates cleavage sites at Lys114–Arg115 and Lys129–Val130 bonds. J Biol Chem. 2005; 280(41): 34441-34446.
38. Nielsen-Marsh CM, Richards MP, Hauschka PV, Thomas-Oates JE, Trinkaus E, Pettitt PB, *et al.* Osteocalcin protein sequences of Neanderthals and modern primates. Proc Natl Acad Sci. 2005; 102(12): 4409-4413.
39. Chain D, Kreizman T, Shapira H, Shaltiel S. Plasmin cleavage of vitronectin Identification of the site and consequent attenuation in binding Plasminogen Activator Inhibitor-1. FEBS Lett. 1991; 285(2): 251-256.
40. Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin desensitization of the PAR1 thrombin receptor: Kinetics, sites of truncation, and implications for thrombolytic therapy. Biochemistry. 1999; 38(14): 4572-4585.
41. Xu WF, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, *et al.* Cloning and characterization of human protease-activated receptor 4. Proc Natl Acad Sci. 1998; 95(12): 6642-6646.
42. Pryzdial EL, Lavigne N, Dupuis N, Kessler GE. Plasmin converts factor X from coagulation zymogen to fibrinolysis cofactor. J Biol Chem. 1999; 274(13): 8500-8505.
43. Imai S, Takahashi T, Naito S, Yamauchi A, Okada C, Notsu Y, *et al.* Development of a novel immunoassay specific for mouse intact proinsulin. Anal Biochem. 2015; 484: 91-98.
44. Brophy TM, Ward SE, McGimsey TR, Schneppenheim S, Drakeford C, O'Sullivan JM, *et al.* Plasmin cleaves von willebrand factor at K1491-R1492 in the A1-A2 linker region in a shear-and glycan-dependent manner *in vitro*. Arterioscler Thromb Vasc Biol. 2017; 37(5): 845-855.
45. Kelly AL, McSweeney PL. Indigenous proteinases in milk. Advanced dairy chemistry-1 proteins. 2003; 495-521.
46. Le Bars D, Gripon JC. Specificity of Plasmin towards bovine α s2-casein. J Dairy Res. 1989; 56(5): 817-821.

47. Huppertz T, Upadhyay VK, Kelly AL, Tamime AY. Constituents and properties of milk from different species. Oxford. 2006.
48. Atamer Z, Post AE, Schubert T, Holder A, Boom RM, Hinrichs J. Bovine β-casein: Isolation, properties and functionality. A review. Int Dairy J. 2017; 66: 115-125.
49. He Y, Wortmann A, Burke LJ, Reid JC, Adams MN, Abdul-Jabbar I, *et al.* Proteolysis-induced N-terminal ectodomain shedding of the integral membrane glycoprotein CUB domain-containing protein 1 (CDCP1) is accompanied by tyrosine phosphorylation of its C-terminal domain and recruitment of Src and PKCδ. J Biol Chem. 2010; 285(34): 26162-26173.
50. Casar B, He Y, Iconomou M, Hooper JD, Quigley JP, Deryugina EI. Blocking of CDCP1 cleavage *in vivo* prevents Akt-dependent survival and inhibits metastatic colonization through PARP1-mediated apoptosis of cancer cells. Oncogene. 2012; 31(35): 3924-3938.
51. Poreba M, Kasperkiewicz P, Rut W, Drag M. Screening Combinatorial Peptide Libraries in Protease Inhibitor Drug Discovery. Extracellular Targeting of Cell Signaling in Cancer. 2018; 307.
52. Gosalia DN, Salisbury CM, Maly DJ, Ellman JA, Diamond SL. Profiling serine protease substrate specificity with solution phase fluorogenic peptide microarrays. Proteomics. 2005; 5(5): 1292-1298.
53. Andrew M, Monagle PT, Brooker L. Thromboembolic complications during infancy and childhood. PMPH. 2000.
54. Latallo ZS, Teisseyre E, Lopaciuk S. Evaluation of a fibrinolytic profile of plasma using chromogenic substrates. Chromogenic Peptide Substrates. 1979; 262-266.
55. Wenzel E, Jaeger H, Pfordt L, Doenecke P. Variation in coagulation and fibrinolysis parameters due to urokinase administration. Fibrinolysis and urokinase. 1980; 57-74.
56. Lijnen HR, Carmeliet P, Bouche A, Moons L, Ploplis VA, Plow EF, *et al.* Restoration of thrombolytic potential in Plasminogen-deficient mice by bolus administration of Plasminogen. Blood. 1996; 88(3): 870-876.
57. Chesterman CN. Fibrinolysis and disseminated intravascular coagulation. Fibrinolysis. 1978; 157-172.
58. Soria J, Soria C, Samama M. A Plasminogen assay using a chromogenic synthetic substrate: results from clinical work and from studies of thrombolysis. Progress in chemical fibrinolysis and thrombolysis. 1978; 3: 337-346.
59. Gallimore MJ, Aasen AO, Erichsen NS, Larsbraaten M, Lyngaas K, Amundsen E. Plasminogen concentrations and functional activities and concentrations of Plasmin inhibitors in plasma samples from normal subjects and patients with septic shock. Thromb Res. 1980; 18(5): 601-608.
60. Smith-Erichsen N, Aasen AO, Amundsen E. Treatment of sepsis in the surgical patient evaluated by means of chromogenic peptide substrate assays. Acta Chir Scand Suppl. 1982; 509: 33-38.
61. Sutor AH. Bleeding complications in acute myeloblastic leukemia (author's transl). Klin Padiatr. 1979; 191(2): 217-224.
62. Markarian M, Githens JH, Jackson JJ, Bannon AE, Lindley A, Rosenblut E, *et al.* Fibrinolytic activity in premature infants: relationship of the enzyme system to the respiratory distress syndrome. Am J Dis Child. 1967; 113(3): 312-321.
63. Biland L, Duckert F, Prisender S, Nyman D. Quantitative estimation of coagulation factors in liver disease. The diagnostic and prognostic value of factor XIII, factor V and Plasminogen. Thromb Haemost. 1978; 39(03): 646-656.
64. Bauman KA, Wettlaufer SH, Okunishi K, Vannella KM, Stoolman JS, Huang SK, *et al.* The antifibrotic effects of Plasminogen activation occur *via* prostaglandin E 2 synthesis in humans and mice. J Clin Invest. 2010; 120(6): 1950-1960.
65. Dotti CG, Galvan C, Ledesma MD. Plasmin deficiency in Alzheimer's Disease brains: causal or casual?. Neurodegener Dis. 2004; 1(4-5): 205-212.
66. Jacobsen JS, Comery TA, Martone RL, Elokdah H, Crandall DL, Oganesian A, *et al.* Enhanced clearance of A β in brain by sustaining the Plasmin proteolysis cascade. Proc Natl Acad Sci. 2008; 105(25): 8754-8759.
67. Ledesma MD, Da Silva JS, Crassaerts K, Delacourte A, De Strooper B, Dotti CG. Brain Plasmin enhances APP α-cleavage and A β degradation and is reduced in Alzheimer's Disease brains. EMBO Rep. 2000; 1(6): 530-535.
68. Periz G, Fortini ME. Proteolysis in Alzheimer's Disease. EMBO Rep. 2000; 1(6): 477-478.
69. Yang D, Zhu W, Wang Y, Tan F, Ma Z, Gao J, *et al.* Selection of mutant μ Plasmin for Amyloid-β cleavage *in vivo*. Scientific Reports. 2020; 10(1): 1-2.
70. Millers EK, Johnson LA, Birrell GW, Masci PP, Lavin MF, de Jersey J, *et al.* The structure of human microplasmin in complex with textilinin-1, an aprotinin-like inhibitor from the Australian brown snake. PLoS One. 2013; 8(1): 54104.
71. Law RH, Wu G, Leung EW, Hidaka K, Quek AJ, Caradoc-Davies TT, *et al.* X-ray crystal structure of Plasmin with tranexamic acid–derived active site inhibitors. Blood Adv. 2017; 1(12): 766-771.
72. Swedberg JE, Wu G, Mahatmanto T, Durek T, Caradoc-Davies TT, Whisstock JC, *et al.* Highly potent and selective Plasmin inhibitors based on the sunflower trypsin inhibitor-1 scaffold attenuate fibrinolysis in plasma. J Med Chem. 2018; 62(2): 552-560.
73. Peisach E, Wang J, de los Santos T, Reich E, Ringe D. Crystal structure of the proenzyme domain of Plasminogen. Biochem. 1999; 38(34): 11180-11188.
74. Wang X, Terzyan S, Tang J, Loy JA, Lin X, Zhang XC. Human Plasminogen catalytic domain undergoes an unusual conformational change upon activation. J Mol Biol. 2000; 295(4): 903-914.
75. Wakeham N, Terzyan S, Zhai P, Loy JA, Tang J, Zhang XC. Effects of deletion of streptokinase residues 48–59 on Plasminogen activation. Protein Eng. 2002; 15(9): 753-761.
76. Terzyan S, Wakeham N, Zhai P, Rodgers K, Zhang XC. Characterization of Lys-698 to met substitution in human Plasminogen catalytic domain. Proteins. 2004; 56(2): 277-284.
77. Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. Nature. 2000; 407(6806): 923-926.
78. Law RH, Sofian T, Kan WT, Horvath AJ, Hitchen CR, Langendorf CG, *et al.* X-ray crystal structure of the fibrinolysis inhibitor α 2-Antiplasmin. Blood. 2008; 111(4): 2049-2052.

79. Dementiev A, Simonovic M, Volz K, Gettins PG. Canonical inhibitor-like interactions explain reactivity of α 1-proteinase inhibitor Pittsburgh and antithrombin with proteinases. J Biol Chem. 2003; 278(39): 37881-37887.

80. Yang J, Zhang Y. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res. 2015; 43(1): 174-181.

81. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science. 1989; 244(4908): 1081-1085.

82. FDA. Jetrea (ocriplasmin) Intravitreal Injection. Food and Drug Administration. 2012.

83. Pfaeffle V. FDA Approves First Treatment for Patients with Plasminogen Deficiency, a Rare Genetic Disorder. Food and Drug Administration. 2021.

84. Shapiro AD, Nakar C, Parker JM, Albert GR, Moran JE, Thibaudeau K, *et al.* Plasminogen replacement therapy for the treatment of children and adults with congenital Plasminogen deficiency. Blood. 2018; 131(12): 1301-1310.

85. Varma R, Haller JA, Kaiser PK. Improvement in patient-reported visual function after ocriplasmin for vitreomacular adhesion: results of the Microplasmin for Intravitreous Injection-Traction Release Without Surgical Treatment (MIVI-TRUST) Trials. JAMA Ophthalmol. 2015; 133(9): 997-1004.

86. Yu TM, Dugel PU, Haller JA, Kaiser PK, Arnold RJ. Budget impact analysis of ocriplasmin for the treatment of symptomatic vitreomacular adhesion in the USA. J Comp Eff Res. 2018; 7(12): 1195-1207.

87. Comerota AJ, Davidovic L, Hanna K, Courtney KL, Shlansky-Goldberg RD. Phase 2, randomized, open-label study on Catheter-Directed Thrombolysis with Plasmin versus rtPA and placebo in acute Peripheral Arterial Occlusion. J Drug Assess. 2019; 8(1): 43-54.

88. Ebben HP, Jongkind V, Wisselink W, Hoksbergen AW, Yeung KK. Catheter directed thrombolysis protocols for Peripheral Arterial Occlusions: A systematic review. Eur J Vasc Endovasc Surg. 2019; 57(5): 667-675.

89. Robertson I, Kessel DO, Berridge DC. Fibrinolytic agents for Peripheral Arterial Occlusion. Cochrane Database of Syst Rev. 2013; 12.

90. Sabovic M, Lijnen HR, Keber D, Collen D. Effect of retraction on the lysis of human clots with fibrin specific and non-fibrin specific Plasminogen Activators. Thromb Haemost. 1989; 62(08): 1083-1087.

91. Robbie LA, Bennett B, Croll AM, Brown PA, Booth NA. Proteins of the fibrinolytic system in human thrombi. Thromb Haemost. 1996; 75(01): 127-133.

92. Van-Loon BP, Rijken DC, Brommer EJ, Van der Maas AP. The Amount of Plasminogen, Tissue-Type Plasminogen Activator and Plasminogen Activator Inhibitor Type 1 in Human Thrombi and the Relation to IVVO-TNO Lysibility. Thromb Haemost. 1992; 67(01): 101-105.

93. Marder VJ. Historical perspective and future direction of thrombolysis research: the re-discovery of Plasmin. J Thromb Haemost. 2011; 9: 364-373.

94. Thorsen S, Clemmensen I, Sottrup-Jensen L, Magnusson S. Adsorption to fibrin of native fragments of known primary structure from human Plasminogen. Biochim Biophys Acta. 1981; 668(3): 377-387.

95. Komorowicz E, Kolev K, Machovich R. Fibrinolysis with des-kringle derivatives of Plasmin and its modulation by plasma protease inhibitors. Biochem. 1998; 37(25): 9112-9118.

96. Christensen U, Bangert K, Thorsen S. Reaction of human α 2-Antiplasmin and Plasmin Stopped-flow fluorescence kinetics. FEBS Lett. 1996; 387(1): 58-62.

97. Wiman B, Collen D. On the kinetics of the reaction between human Antiplasmin and Plasmin. Eur J Biochem. 1978; 84(2): 573-578.

98. Nagai N, Demarsin E, Van Hoef B, Wouters S, Cingolani D, Laroche Y, *et al.* Recombinant human microplasmin: Production and potential therapeutic properties. J Thromb Haemost. 2003; 1(2): 307-313.

99. Finder VH, Glockshuber R. Amyloid-β aggregation. Neurodegener Dis. 2007; 4(1): 13-27.

100. Tiraboschi P, Hansen LA, Thal LJ, Corey-Bloom J. The importance of neuritic plaques and tangles to the development and evolution of AD. Neurology. 2004; 62(11): 1984-1989.

101. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's Disease. Trends Pharmacol Sci. 1991; 12: 383-388.

102. Hardy J, Duff K, Hardy KG, Perez-Tur J, Hutton M. Genetic dissection of Alzheimer's Disease and related dementias: Amyloid and its relationship to tau. Nat Neurosci. 1998; 1(5): 355-358.

103. Selkoe DJ. The molecular pathology of Alzheimer's Disease. Neuron. 1991; 6(4): 487-498.

104. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's Disease: progress and problems on the road to therapeutics. Science. 2002; 297(5580): 353-356.

105. Leissring MA. The Abeta Cs of Abeta -cleaving proteases. J Biol Chem. 2008.

106. Selkoe DJ, Schenk D. Alzheimer's Disease: Molecular understanding predicts amyloid-based therapeutics. Annu Rev Pharmacol Toxicol. 2003; 43: 545-584.

107. Citron M. Strategies for disease modification in Alzheimer's Disease. Nat Rev Neurosci. 2004; 5: 677-685.

108. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T. Immunization with Amyloid-beta attenuates alzheimer-disease-like pathology in the PDAPP mouse. Nature. 1999; 400: 173-177.

109. Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, *et al.* Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. Neurology. 2003; 61: 46-54.

110. Banks WA, Farr SA, Morley JE, Wolf KM, Geylis V, Steinitz M. Anti-amyloid beta protein antibody passage across the blood-brain barrier in the SAMP8 mouse model of Alzheimer's Disease: An age-related selective uptake with reversal of learning impairment. Exp Neurol. 2007; 206: 248-256.

111. Dodel RC, Du Y, Depboylu C, Hampel H, Frolich L, Haag A, *et al.* Intravenous immunoglobulins containing antibodies against beta-Amyloid for the treatment of Alzheimer's Disease. J Neurol Neurosurg Psychiatry. 2004; 75: 1472-1474.

112. Du Y, Wei X, Dodel R, Sommer N, Hampel H, Gao F, *et al.* Human anti-beta-Amyloid antibodies block beta-Amyloid fibril formation and prevent beta-Amyloid-induced neurotoxicity. Brain. 2003; 126: 1935-1939.

113. Prada CM, Garcia-Alloza M, Betensky RA, Zhang-Nunes SX, Greenberg SM, Bacskai BJ, *et al.* Antibody-mediated clearance of Amyloid-beta peptide from cerebral amyloid angiopathy revealed by quantitative *in vivo* imaging. J Neurosci. 2007; 27: 1973-1980.

114. Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer's Disease drug development pipeline: 2019. 2019; 5(1): 272-293.

115. Abbott A. Fresh push for 'failed' Alzheimer's drug. Nature. 2019.

116. Fillit H, Green A. Aducanumab and the FDA-where are we now?. Nat Rev Neurol. 2021; 17: 129-130.

117. Hern K. Lilly's donanemab slows clinical decline of Alzheimer's Disease in positive phase 2 trial. Lilly. 2021.

118. Johnson V. Donanemab shows slowing of decline in alzheimer phase 2 Trailblazer-Alz Study. Neurology. 2021.

119. International ASD. FDA approves Biogen's aducanumab. Food and Drug Administration. 2021.

120. Hussain I, Powell D, Howlett D, Tew D, Meek D, Chapman C, *et al.* Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol Cell Neurosci. 1999; 14: 419-427.

121. Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J. Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-Amyloid Precursor Protein. Proc Natl Acad Sci USA. 2000; 97: 1456-60.

122. Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, *et al.* Purification and cloning of Amyloid Precursor Protein beta-secretase from human brain. Nature. 1999; 402: 537-540.

123. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, *et al.* Beta-secretase cleavage of Alzheimer's Amyloid Precursor Protein by the transmembrane aspartic protease BACE. Science. 1999; 286: 735-741.

124. Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, *et al.* Membrane-anchored aspartyl protease with Alzheimer's Disease beta-secretase activity. Nature. 1999; 402: 533-537.

125. Cole SL, Vassar R. The role of APP processing by BACE1, the beta-secretase, in Alzheimer's Disease pathophysiology. J Biol Chem. 2008; 283: 2962-29625.

126. Ghosh AK, Kumaragurubaran N, Hong L, Koelsh G, Tang J. Memapsin 2 (beta-secretase) inhibitors: Drug development. Curr Alzheimer Res. 2008; 5: 121-131.

127. Czirr E, Leuchtenberger S, Dorner-Ciossek C, Schneider A, Jucker M, Koo EH, *et al.* Insensitivity to Abeta42-lowering nonsteroidal anti-inflammatory drugs and gamma-secretase inhibitors is common among aggressive presenilin-1 mutations. J Biol Chem. 2007; 282: 24504-24513.

128. Harrison T, Churcher I, Beher D. Gamma-Secretase as a target for drug intervention in Alzheimer's Disease. Curr Opin Drug Discov Devel. 2004; 7: 709-719.

129. Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, *et al.* Identification of the major abeta1-42-degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. Nat Med. 2000; 6: 143-150.

130. Hersh LB. The insulysin (insulin degrading enzyme) enigma. Cell Mol Life Sci. 2006; 63: 2432-2434.

131. Leissring MA. Proteolytic degradation of the Amyloid beta-protein: The forgotten side of Alzheimer's Disease. Curr Alzheimer Res. 2006; 3: 431-435.

132. Turner AJ, Nalivaeva NN. New insights into the roles of metalloproteinases in neurodegeneration and neuroprotection. Int Rev Neurobiol. 2007; 82: 113-135.

133. Wang DS, Dickson DW, Malter JS. Beta-Amyloid degradation and Alzheimer's Disease. J Biomed Biotechnol. 2006; 2006: 58406.

134. Chen F, Suzuki Y, Nagai N, Sun X, Wang H, Yu J, *et al.* Microplasmin and tissue Plasminogen Activator: Comparison of therapeutic effects in rat stroke model at multiparametric MR imaging. Radiology. 2007; 244: 429-438.

135. Fu JY, Ren JP, Zou LB, Bian GX, Li RF, Lu QJ. Effect of recombinant microplasmin on acute cerebral infarction in rats. Yao Xue Xue Bao. 2007; 42: 1266-1270.

136. Gandorfer A. Experimental evaluation of microplasmin-an alternative to vital dyes. Dev Ophthalmol. 2008; 42: 153-159.

137. Quiram PA, Leverenz VR, Baker RM, Dang L, Giblin FJ, Trese MT. Microplasmin-induced posterior vitreous detachment affects vitreous oxygen levels. Retina. 2007; 27: 1090-1096.

138. Rasmussen RS, Overgaard K, Pakola S, Boysen G. Effects of microplasmin on recovery in a rat embolic stroke model. Neurol Res. 2008; 30: 75-81.

139. Sakuma T, Tanaka M, Mizota A, Inoue J, Pakola S. Safety of *in vivo* pharmacologic vitreolysis with recombinant microplasmin in rabbit eyes. Invest Ophthalmol Vis Sci. 2005; 46: 3295-3299.

140. Sebag J, Ansari RR, Suh KI. Pharmacologic vitreolysis with microplasmin increases vitreous diffusion coefficients. Graefes Arch Clin Exp Ophthalmol. 2007; 245: 576-580.

141. Tucker HM, Kihiko-Ehmann M, Estus S. Urokinase-type Plasminogen Activator inhibits Amyloid-beta neurotoxicity and fibrillogenesis *via* Plasminogen. J Neurosci Res. 2002; 70: 249-255.

142. Tucker HM, Kihiko-Ehmann M, Wright S, Rydel RE, Estus S. Tissue Plasminogen Activator requires Plasminogen to modulate Amyloid-beta neurotoxicity and deposition. J Neurochem. 2000; 75: 2172-2177.

143. Eisele YS, Obermuller U, Heilbronner G, Baumann F, Kaeser SA, Wolburg H, *et al.* Peripherally applied a β-containing inoculates induce cerebral β-Amyloidosis. Science. 2010; 330: 980-982.

144. Shetty S, Padijnayayveetil J, Tucker T, Stankowska D, Idell S. The fibrinolytic system and the regulation of lung epithelial cell proteolysis, signaling, and cellular viability. Am J Physiol Lung Cell Mol Physiol. 2008; 295: L967-L975.

145. Hattori N, Mizuno S, Yoshida Y, Chin K, Mishima M, Sisson TH, *et al.* The Plasminogen activation system reduces fibrosis in the lung by a hepatocyte growth factor-dependent mechanism. Am J Pathol. 2004; 164: 1091-1098.

146. Marudamuthu AS, Shetty SK, Bhandary YP, Karandashova S, Thompson M, Sathish V, *et al.* Plasminogen Activator Inhibitor-1 suppresses profibrotic responses in fibroblasts from fibrotic lungs. J Biol Chem. 2015; 290: 9428-9441.

147. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. Am J Pathol. 2000; 157: 177-187.

148. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, *et al.* Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine Plasminogen Activator Inhibitor-1 gene. J Clin Invest. 1996; 97: 232-237.

149. Chapman HA, Allen CL, Stone OL. Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. Am Rev Respir Dis. 1986; 133: 437-443.

150. Kotani I, Sato A, Hayakawa H, Urano T, Takada Y, Takada A. Increased procoagulant and antifibrinolytic activities in the lungs with Idiopathic Pulmonary Fibrosis. Thromb Res. 1995; 77: 493-504.

151. Okunishi K, Sisson TH, Huang SK, Hogaboam CM, Simon RH, Peters-Golden M. Plasmin overcomes resistance to prostaglandin E2 in fibrotic lung fibroblasts by reorganizing protein kinase a signaling. J Biol Chem. 2011; 286: 32231-32243.

152. Kanno Y. Alpha2-Antiplasmin: A new target for fibrotic diseases. Biochem Anal Biochem. 2015; 04.

153. Katneni UK, Alexaki A, Hunt RC, Schiller T, DiCuccio M, Buehler PW, *et al.* Coagulopathy and thrombosis as a result of severe COVID-19 infection: A microvascular focus. Thromb Haemost. 2020; 120: 1668-1679.

154. Singhal T. A review of coronavirus disease-2019 (COVID-19). Indian J Pediatr. 2020; 87: 281-286.

155. Cao X. COVID-19: Immunopathology and its implications for therapy. Nat Rev Immunol. 2020; 20: 269-270.

156. Moon C. Fighting COVID-19 exhausts T cells. Nature Rev Immunol. 2020; 20: 277.

157. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. Blood. 2020; 135: 2033-2040.

158. Marietta M, Coluccio V, Luppi M. COVID-19, coagulopathy and venous thromboembolism: more questions than answers. Intern Emerg Med. 2020; 15: 1375-1387.

159. Merrill JT, Erkan D, Winakur J, James JA. Emerging evidence of a COVID-19 thrombotic syndrome has treatment implications. Nat Rev Rheumatol. 2020; 16(10): 581-589.

160. Iba T, Levy JH, Connors JM, Warkentin TE, Thachil J, Levi M. The unique characteristics of COVID-19 coagulopathy. Crit Care. 2020; 24: 360.

161. Thachil J, Tang N, Gando S, Falanga A, Cattaneo M, Levi M, *et al.* ISTH interim guidance on recognition and management of coagulopathy in COVID-19. J Thromb Haemost. 2020; 18: 1023-1026.

162. Wichmann D, Sperhake JP, Lütgehetmann M, Steurer S, Edler C, Heinemann A, *et al.* Autopsy findings and venous thromboembolism in patients with COVID-19. Ann Intern Med. 2020; 173: 268-277.

163. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. J Thromb Haemost. 2020; 18(4): 844-847.

164. Tang N, Li D, Wang X, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. J Thromb Haemost. 2020; 18(5): 1094-1099.

165. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, *et al.* Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. Lancet. 2020; 395: 1054-1062.

166. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395: 497-506.

167. Sanyaolu A, Okorie C, Marinkovic A, Patidar R, Younis K, Desai P, *et al.* Comorbidity and its impact on patients with COVID-19. SN Compr Clin Med. 2020; 2: 1069-1076.

168. Jain V, Yuan JM. Systematic review and meta-analysis of predictive symptoms and comorbidities for severe COVID-19 infection. MedRxiv. 2020.

169. Qiu P, Zhou Y, Wang F, Wang H, Zhang M, Pan X, *et al.* Clinical characteristics, laboratory outcome characteristics, comorbidities, and complications of related COVID-19 deceased: A systematic review and meta-analysis. Aging Clin Exp Res. 2020; 32: 1869-1878.

170. Ahmed S, Zimba O, Gasparyan AY. Thrombosis in Coronavirus disease 2019 (COVID-19) through the prism of Virchow's triad. Clin Rheumatol. 2020; 39: 2529-2543.

171. Ji HL, Zhao R, Matalon S, Matthay MA. Elevated Plasmin(ogen) as a Common Risk Factor for COVID-19 Susceptibility. Physiol Rev. 2020; 100: 1065-1075.

172. D'Alonzo D, De Fenza M, Pavone V. COVID-19 and pneumonia: A role for the uPA/uPAR system. Drug Discov Today. 2020; 25: 1528-1534.

173. Zuo Y, Warnock M, Harbaugh A, Yalavarthi S, Gockman K, Zuo M, *et al.* Plasma tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 in hospitalized COVID-19 patients. MedRxiv. 2020.

174. Moore HB, Barrett CD, Moore EE, McIntyre RC, Moore PK, Talmor DS, *et al.* Is there a role for tissue Plasminogen Activator as a novel treatment for refractory COVID-19 associated Acute Respiratory Distress Syndrome?. J Trauma Acute Care Surg. 2020; 88: 713-714.

175. Hardaway RM, Williams CH, Marvasti M, Farias M, Tseng A, Pinon I, *et al.* Prevention of adult respiratory distress syndrome with Plasminogen Activator in pigs. Crit Care Med. 1990; 18: 1413-1418.

176. Stringer KA, Hybertson BM, Cho OJ, Cohen Z, Repine JE. Tissue Plasminogen Activator (tPA) inhibits interleukin-1 induced acute lung leak. Free Radic Biol Med. 1998; 25: 184-188.

177. Liu C, Ma Y, Su Z, Zhao R, Zhao X, Nie HG, *et al.* Meta-analysis of preclinical studies of fibrinolytic therapy for acute lung injury. Front Immunol. 2018; 9: 1898.

178. Hardaway R, Harke H, Tyroch A, Williams C, Vazquez Y, Krause G. Treatment of severe Acute Respiratory Distress Syndrome: A final report on a phase I study. Am Surg. 2001; 67: 377-382.

179. Borczuk AC, Salvatore SP, Seshan SV, Patel SS, Bussel JB, Mostyka M, *et al.* COVID-19 pulmonary pathology: A multi-institutional autopsy cohort from Italy and New York City. Mod Pathol. 2020; 33: 2156-2168.

180. McFadyen JD, Stevens H, Peter K. The emerging threat of (Micro) thrombosis in COVID-19 and its therapeutic implications. Circ Res. 2020; 127: 571-587.

181. Orfanos S, El Husseini I, Nahass T, Radbel J, Hussain S. Observational study of the use of recombinant tissue-type Plasminogen Activator in COVID-19 shows a decrease in physiological dead space. ERJ Open Res. 2020; 6: 455-2020.

182. Wang J, Hajizadeh N, Moore EE, McIntyre RC, Moore PK, Veress LA, *et al.* Tissue Plasminogen Activator (tPA) treatment for COVID-19 associated Acute Respiratory Distress Syndrome (ARDS): A case series. J Thromb Haemost. 2020; 18: 1752-1755.

183. Wu Y, Wang T, Guo C, Zhang D, Ge X, Huang Z, *et al.* Plasminogen improves lung lesions and hypoxemia in patients with COVID-19. QJM. 2020; 113: 539-545.

184. Christie DB, Nemec HM, Scott AM, Buchanan JT, Franklin CM, Ahmed A, *et al.* Early outcomes with utilization of tissue Plasminogen Activator in COVID-19-associated respiratory distress: A series of five cases. J Trauma Acute Care Surg. 2020; 89: 448-452.

185. Choudhury R, Barrett C, Moore H, Moore E, McIntyre R, Moore P, *et al.* Salvage use of tissue Plasminogen Activator (tPA) in the setting of Acute Respiratory Distress Syndrome (ARDS) due to COVID-19 in the USA: A Markov decision analysis. World J Emerg Surg. 2020; 15: 29.

186. Cammalleri M, DalMonte M, Pavone V, DeRosa M, Rusciano D, Bagnoli P. The uPAR system as a potential therapeutic target in the diseased eye. Cells. 2019; 8: 295.

187. Zuo W, Zhao X, Chen YG. SARS coronavirus and lung fibrosis. Molecular Biology of the SARS-Coronavirus. 2009; 247-258.

188. Vasarmidi E, Tsitoura E, Spandidos DA, Tzanakis N, Antoniou KM. Pulmonary fibrosis in the aftermath of the COVID-19 era (Review). Exp Ther Med. 2020; 20(3): 2557-2560.

189. George PM, Wells AU, Jenkins RG. Pulmonary fibrosis and COVID-19: The potential role for antifibrotic therapy. Lancet Respir Med. 2020; 8: 807-815.

190. Schuliga M, Grainge C, Westall G, Knight D. The fibrogenic actions of the coagulant and Plasminogen activation systems in pulmonary fibrosis. Int J Biochem Cell Biol. 2018; 97: 108-117.

191. Fujii M, Hayakawa H, Urano T, Sato A, Chida K, Nakamura H, *et al.* Relevance of tissue factor and tissue factor pathway inhibitor for hypercoagulable state in the lungs of patients with Idiopathic Pulmonary Fibrosis. Thromb Res. 2000; 99: 111-117.

192. Wygrecka M, Kwapiszewska G, Jablonska E, VonGerlach S, Henneke I, Zakrzewicz D, *et al.* Role of protease-activated receptor-2 in Idiopathic Pulmonary Fibrosis. Am J Respir Crit Care Med. 2011; 183: 1703-1714.

193. Parker J, Geerts L, Laverdure A, Tremblay M, Grouix B, Gagnon W, *et al.* Plasminogen supplementation therapy may be a good add-on for the treatment of IPF exacerbations. American Thoracic Society. 2019; 5251.

194. Marder VJ, Novokhatny V. Direct fibrinolytic agents: Biochemical attributes, preclinical foundation and clinical potential. J Thromb Haemost. 2010; 8: 433-444.

195. Collen D, Wiman B. Fast-acting Plasmin inhibitor in human plasma. Blood. 1978; 51: 563-569.

196. Daley MJ, Murthy MS, Peterson EJ. Bleeding risk with systemic thrombolytic therapy for pulmonary embolism: Scope of the problem. Ther Adv Drug Saf. 2015; 6: 57-66.

197. Abraham P, Arroyo DA, Giraud R, Bounameaux H, Bendjelid K. Understanding haemorrhagic risk following thrombolytic therapy in patients with intermediate-risk and high-risk pulmonary embolism: A hypothesis paper. Open Heart. 2018; 5: 735.

198. Castellino FJ, Powell JR. Human Plasminogen, in methods in enzymology. Academic Press. 1981; 365-378.

199. Ganrot PO, Niléhn JE. Immunochemical determination of human Plasminogen. Clinica Chimica Acta. 1968; 22: 335-340.

200. Lijnen HR, Pannekoek H, Vermylen J. Thrombosis and thrombolytic therapy. 2004.

201. Tutwiler V, Peshkova AD, Le Minh G, Zaitsev S, Litvinov RI, Cines DB, *et al.* Blood clot contraction differentially modulates internal and external fibrinolysis. J Thromb Haemost. 2019; 17: 361-370.

202. Du T, Buenbrazo N, Kell L, Rahmani S, Sim L, Withers SG, *et al.* A bacterial expression platform for production of therapeutic proteins containing human-like o-linked glycans. Cell Chem Biol. 2019; 26: 203-212.

203. Kotch L. Treatment of symptomatic vitreomacular adhesion including macular holes. Center for Drug Evaluation and Research. 2012.

204. Mittal S, Saluja D. Protein post-translational modifications: Role in protein structure, function and stability. Springer. 2015; 25-37.

205. Miao R, Liu J, Wang J. Overview of mouse pulmonary embolism models. Drug Discov Today. 2010; 7: 77-82.

206. Collen D, Ong EB, Johnson AJ. Human Plasminogen: *In vitro* and *in vivo* evidence for the biological integrity of NH2-terminal glutamic acid Plasminogen. Thromb Res. 1975; 7: 515-529.

207. Chandler WL, Alessi MC, Aillaud MF, Vague P, Juhan-Vague I. Formation, inhibition and clearance of Plasmin *in vivo*. Haemostasis. 2000; 30: 204-218.