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Review

Advancements in Serine Protease Inhibitors: From Mechanistic Insights to Clinical Applications

Yang Wei [1], Mingdong Huang [1,2,*] and Longguang Jiang [1,2,*]

[1] College of Chemistry, Fuzhou University, Fuzhou 350116, China; 221320296@fzu.edu.cn
[2] The National & Local Joint Engineering Research Center on Biopharmaceutical and Photodynamic Therapy Technologies, Fuzhou University, Fuzhou 350116, China
* Correspondence: hmd_lab@fzu.edu.cn (M.H.); jianglg@fzu.edu.cn (L.J.)

Abstract: Serine proteases, a significant class of enzymes comprising approximately one-third of known human proteases, are ubiquitously present across various organisms. These enzymes typically exhibit highly conserved catalytic domain structures, and their activity is stringently regulated within the body, playing a pivotal role in numerous physiological processes. Dysregulation of serine protease activity can result in severe consequences, including excessive inflammation, heightened risk of thrombosis and cancer, and even mortality. Serine protease inhibitors have emerged as critical regulators, offering a broad range of physiological functions such as maintaining the coagulation–fibrinolysis balance, modulating inflammatory responses, accelerating wound healing, promoting apoptosis, and providing antitumor and antiviral effects. As a result, the development of serine protease inhibitors has become increasingly vital. In recent years, significant progress in the study of serine proteases has led to the pivotal role of various serine protease inhibitors in clinical diagnosis and treatment. This review explores the fundamental mechanisms of serine protease inhibitors, summarizes those that have been successfully integrated into clinical practice, and discusses the challenges encountered in their development along with partial solutions. These advancements lay the groundwork for further refinement and innovation in serine protease inhibitor therapeutics.

Keywords: serine protease; serine protease inhibitor; clinical application; challenges; strategies

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1. Introduction

Serine proteases are enzymes that specifically cleave peptide bonds within proteins and are widely distributed across various organisms. They are named for the nucleophilic serine residue located at their active site. Among the known proteases in humans, nearly one-third can be classified as serine proteases. The most representative families include chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease [1,2].

The hydrolytic activity of serine proteases is driven by a "catalytic triad" composed of the amino acids aspartate (Asp), histidine (His), and serine (Ser), all of which are located at the enzyme's active site [2,3]. The hydroxyl group of the serine residue plays a key role in the catalytic process by acting as a nucleophile, often donating electrons. The catalytic triad functions through a coordinated mechanism where the imidazole ring of histidine activates the serine hydroxyl group, enabling acid–base catalysis. Meanwhile, the aspartate residue stabilizes this interaction by forming a hydrogen bond with the nitrogen atom of the histidine imidazole ring (Figure 1) [4].

There are numerous types of serine proteases widely distributed in nature, and their catalytic activity is strictly regulated within biological systems, playing important roles in many key physiological pathways. Trypsin is a very common serine protease. As a well-known pancreatic digestive enzyme, trypsin aids in the digestion of proteins in the human body and plays a significant role in the digestive process. However, when the function of trypsin becomes disrupted, it can trigger a series of gastrointestinal diseases,

posing a threat to human health [5–7]. Plasmin plays a crucial role in the fibrinolytic system by dissolving fibrin [8]. Coagulation factors are vital in the human coagulation cascade [9]. Dysregulation of the activity or expression of these proteases can have serious consequences (Figure 2). Multiple serine proteases play essential roles in the immune complement system [10–12]. However, excessive activation of serine proteases in the complement system can lead to over-inflammation and various diseases [13]. Transmembrane serine protease 2 (TMPRSS2), regulated by androgens, is a key enzyme involved in numerous pathological and physiological processes [14]. Elevated levels of TMPRSS2 are closely associated with various cancers [15,16]. Serine proteases released from activated leukocytes and mast cells, or generated through the coagulation cascade, are key components in the inflammatory response [17,18]. They are involved in producing pro-inflammatory cytokines and activating immune cells, and any dysregulation can lead to serious consequences [19–21]. Some serine proteases have been found to serve as diagnostic and therapeutic targets in clinical applications [22]. Serine proteases also play critical roles in the viral replication cycle: cellular serine proteases can mediate the entry of the viruses into the cytoplasmic membrane or facilitate their entry into cells [23,24].

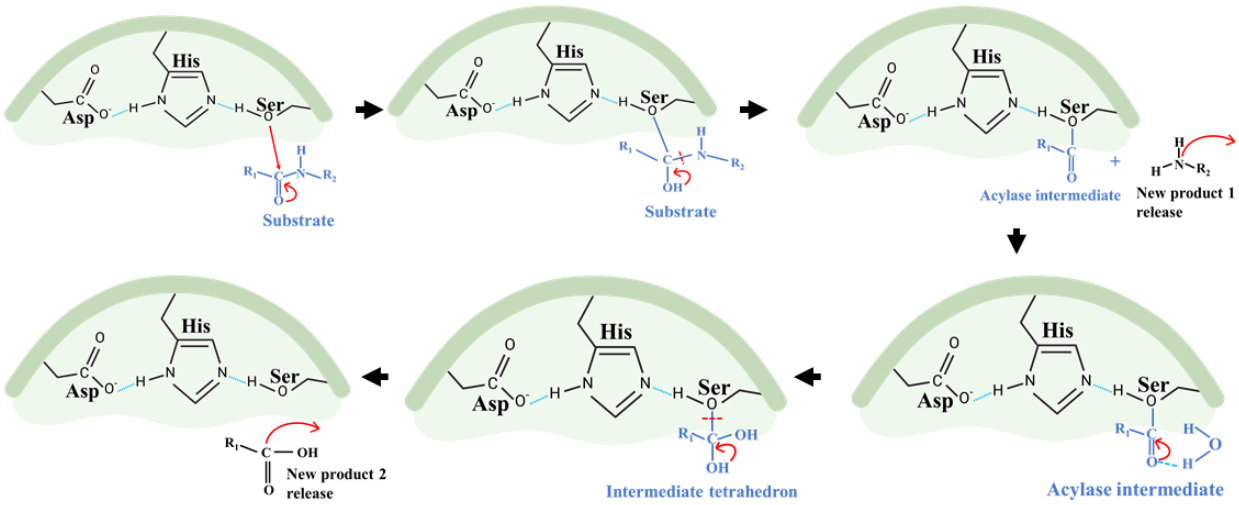


Figure 1. Simplified diagram of serine protease catalysis hydrolysis mechanism. The -OH group of Ser acts as a nucleophile, attacking the carbonyl of the substrate peptide bond, leading to peptide bond hydrolysis. A pair of electrons from the nitrogen in the imidazole ring of His can accept a hydrogen from the Ser-OH group. The carboxyl group of Asp stabilizes the tetrahedral intermediate structure through hydrogen bonding with His, followed by the formation of an acyl-enzyme intermediate. A water molecule then attacks the acyl-enzyme intermediate, producing a second tetrahedral intermediate, which releases the new product from the S1 pocket.

Serine proteases in the human body can trigger diseases and even threaten life if their activity becomes uncontrolled. Therefore, maintaining the delicate balance of serine protease activity within the body is crucial [18,25]. As indispensable regulatory factors in the fibrinolytic system, endogenous serine protease inhibitors—Plasminogen activator inhibitors (PAIs)—play a vital role. On the one hand, they effectively slow down the conversion of plasminogen to plasmin by inhibiting the activity of tPA, significantly reducing the risk of hemorrhagic complications and ensuring the stability and safety of the circulatory system. On the other hand, inhibition of uPA activity cleverly diminishes the invasiveness and metastatic ability of cancer cells, establishing a strong defense against malignant tumor invasion in the body [26]. The above research indicates that developing inhibitors capable of modulating serine protease activity has become an effective strategy for treating clinical conditions triggered by these enzymes.

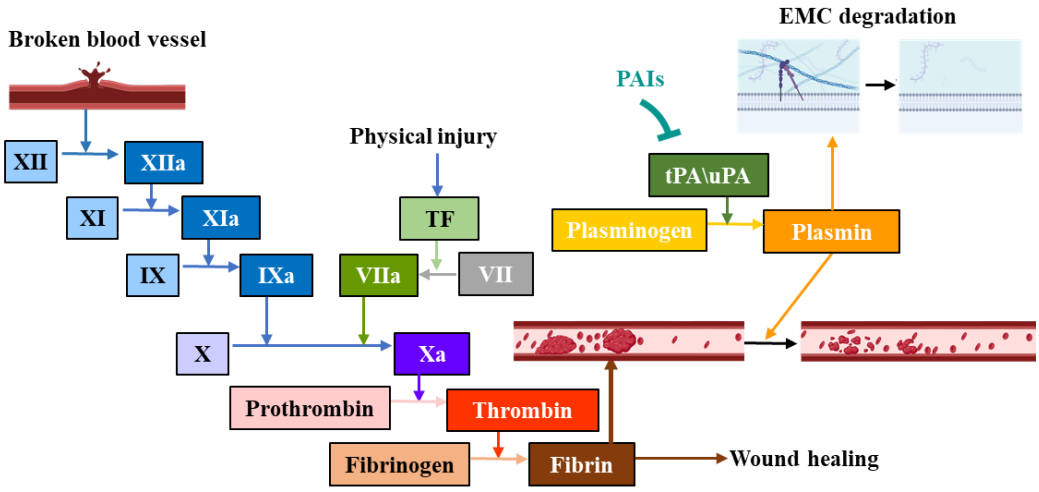


Figure 2. Schematic diagram of serine protease functions. Injury to the body, whether internal or external, activates coagulation factors, triggering the coagulation cascade, and ultimately converting prothrombin into thrombin. Thrombin activates fibrinogen, which is then transformed into fibrin, precipitating at the wound site to form a thrombus. This process promotes wound healing or can lead to thrombotic diseases. The presence of tPA and uPA in the body activates plasminogen to convert into plasmin, which dissolves fibrin and facilitates thrombus clearance, playing a crucial role in the clinical treatment of thrombosis. However, excessive activation of plasmin can lead to severe bleeding. Additionally, uPA-induced excessive plasmin activation can alter the tumor microenvironment and decrease the extracellular matrix (ECM). Therefore, high levels of uPA are positively correlated with various cancers. This indicates that uPA can be a biomarker for highly invasive cancers [22].

Given the critical role that serine proteases play in clinical diagnosis and treatment, numerous clinical trials in recent years have revealed the effectiveness of serine protease inhibitors in treating various clinical conditions caused by these enzymes. These inhibitors open new avenues for the treatment of related diseases by precisely inhibiting the activity or expression of serine proteases. This review aims to briefly outline the mechanisms of action of serine protease inhibitors, describe the current state of research and development, and categorize the serine protease inhibitors that have entered clinical trials or treatment. It also summarizes the persistent challenges faced during the development of serine protease inhibitors and proposes strategies and solutions to address these issues.

2. Mechanisms and Classification of Serine Protease Inhibitors

The goal of developing serine protease inhibitors is to prevent and treat certain diseases by modulating their activity or expression levels. Classical serine protease inhibitors interact with serine proteases through exposed loops that possess specific scaffold conformations. The binding conformation of these inhibitors to serine proteases is referred to as the "typical" conformation, and the mechanism based on this conformation is known as the standard or Laskowski mechanism (Figure 3A) [27,28]. In this mechanism, the inhibitor binds to the serine protease like a substrate and is slowly hydrolyzed by the protease. However, the hydrolysis products do not release from the active site, and the inhibitor's hydrolysis products remain bound to the protease's active site; the peptide bond is reformed, causing a decrease in or even complete loss of the protease's catalytic activity [29,30]. It is important to note that the classical mechanism is not the only mode of inhibition: non-canonical inhibitors are a class of reversible serine protease inhibitors that block the active site spatially. These inhibitors prevent other substrates from entering the active site upon binding but do not lead to any peptide bond hydrolysis (Figure 3B) [31–33]. Additionally, we reported a mechanism through which the catalytic activity of serine proteases is regulated by targeting the autolytic loop within the protease's catalytic domain. The autolytic loop is a component of the activation domain and typically undergoes significant conformational changes during

serine protease activation. Inhibitors that target the autolytic loop can disrupt not only its conformation but also the conformations of other components crucial for the catalytic activity of serine proteases, thereby interfering with their activity. This indicates that targeting the non-catalytic domains of serine proteases represents a potential mechanism for protease inhibition (Figure 4) [34]. Although some small-molecule inhibitors mimic peptide inhibitors to suppress the activity of serine proteases through the Laskowski mechanism or non-standard pathways, there exists another class of small-molecule inhibitors that exhibit a more direct mode of action. They form tight binding with the key catalytic active site Ser 195 of serine proteases or the binding sites involved in protein–protein interactions, which can be either covalent or non-covalent. By directly competing for the occupancy of the active site with the substrate, inducing changes in protein structure, or obstructing the necessary transitions in protein conformation and configuration, these inhibitors effectively inhibit the catalytic activity of proteases [35,36]. Currently, in addition to the traditional approach of directly inhibiting protease activity, there exists a more refined inhibitory mechanism: nucleotide inhibitors demonstrate their efficacy through a highly specific and precise mode of action. They can selectively block or inhibit the expression process of specific proteases while cleverly avoiding unnecessary inhibition of other enzyme activities. Carefully designed nucleotide inhibitors can accurately target and bind to the mRNA of specific proteases, affecting the normal translation of these proteases [37]. More importantly, nucleotide inhibitors regulate the expression levels of the target proteases in a precise and targeted manner, while avoiding interference with a broad range of enzyme activities.

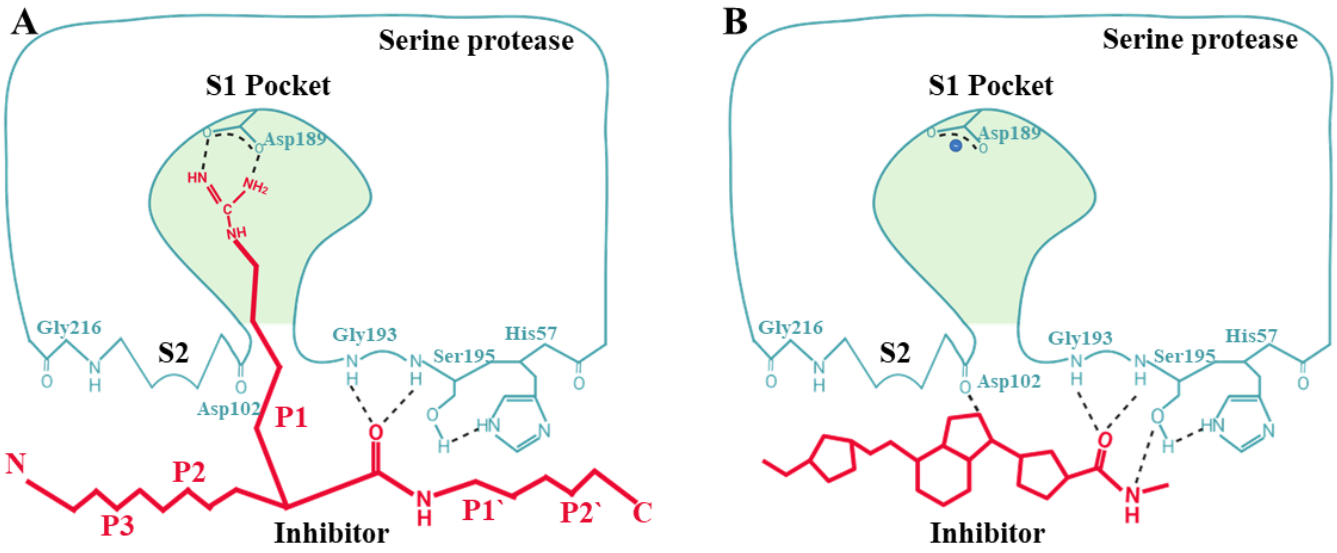


Figure 3. Mechanisms of action of serine protease inhibitors. (**A**) The mechanism of action of classic serine protease inhibitors [27]. The P1 group inserts into the interior of the S1 pocket of the serine protease. (**B**) Mechanism of action of non-standard inhibitors. Non-canonical inhibitors interact with the active site of serine proteases through their N-terminus, occupying the active site without forming new peptide bonds. This leads to the "closure" of the S1 pocket, preventing substrates from entering the S1 pocket.

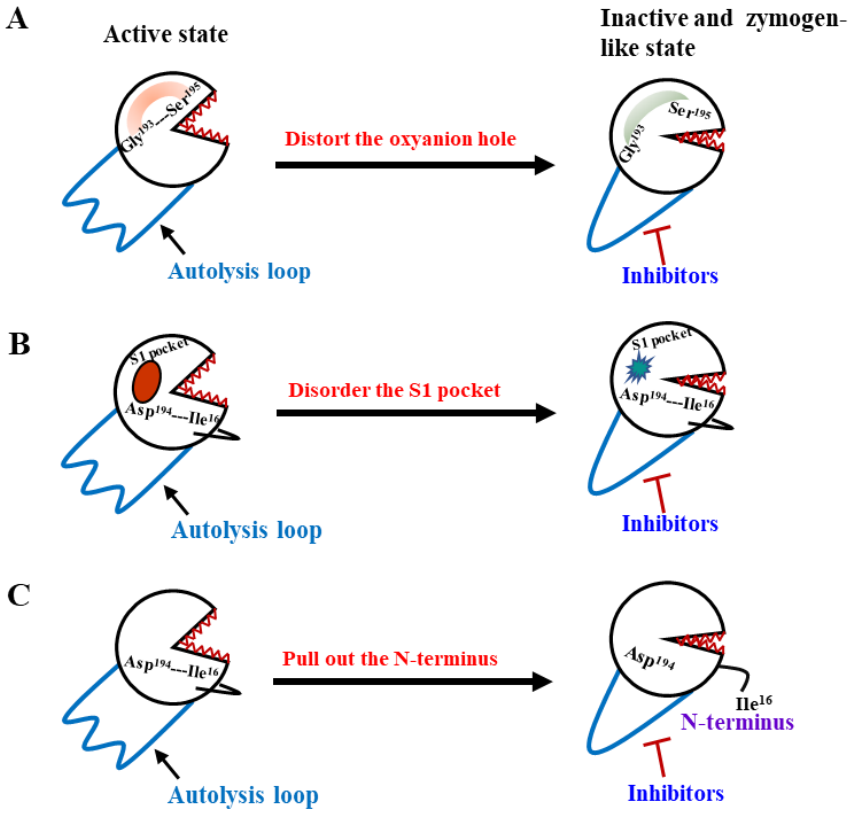


Figure 4. Three possible mechanisms for inhibiting autolysis [34]. (**A**) The binding of the allosteric inhibitor on the autolysis loop leads to the movement of the oxyanion stabilizing loop and the distortion of the oxyanion hole. (**B**) Occlusion of the S1 specificity pocket caused by the binding of the antibody to the autolysis loop. (**C**) The salt bridge between residue Asp194 and the N-terminal residue 16 is disrupted, which causes the N-terminal residue 16 to fall out of the protease and leads to an inactive and zymogen-like protease.

3. Different Types of Serine Protease Inhibitors

Serine protease inhibitors hold significant applications and research value in the fields of biology and medicine. Over decades of development, serine protease inhibitors can be broadly categorized into the following types.

3.1. Small-Molecule Inhibitors

Small-molecule inhibitors of serine protease are advantageous compared to other types of inhibitors due to their low production costs, lack of immunogenicity, long shelf life, and high formulation flexibility. When small molecules enter the active site pocket of serine proteases, they form covalent or non-covalent bonds with the catalytic active site, leading to reduced activity or inactivation of the protease.

Pradaxa® is clinically used for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation (NVAF) and one or more risk factors, with Dabigatran as its main component [38]. Dabigatran is a direct thrombin inhibitor that reduces intracranial bleeding in patients with atrial fibrillation and decreases major and clinically relevant non-major bleeding in acute venous thromboembolism (VTE) treatment. However, its use in patients with dynamic renal impairment due to dehydration may lead to bleeding complications, and the substructure of Dabigatran may cause glomerular hyperfiltration, reducing its efficacy [39]. Melagatran is also a direct thrombin inhibitor, while Ximelagatran is its oral prodrug, which is converted to Melagatran through enzymatic reactions. Clinical studies indicate that Melagatran has a rapid onset of action, stable absorption, and does not require routine monitoring, making it suitable for VTE prevention in patients undergoing hip or knee replacement surgery [40,41]. However, due to its

potential severe hepatic toxicity, Melagatran has been withdrawn from the market [42]. Argatroban binds to thrombin reversibly and is the only small-molecule oral thrombin inhibitor approved by the FDA for treating Heparin-Induced Thrombocytopenia (HIT) in the U.S. [43,44]. AZD0837, developed as a follow-up compound to Ximelagatran, has shown effects similar to Warfarin in clinical Phase II trials for preventing ventricular-fibrillation-related strokes, without the hepatic toxicity seen with Ximelagatran [45]. The developed extended-release formulation allows for once-daily dosing. Currently, AZD0837 is in Phase III clinical trials for treating chronic ventricular fibrillation [46]. Bivalirudin, as a direct thrombin inhibitor, demonstrates better efficacy in the early intervention of acute coronary syndromes but is subject to temporary, reversible thrombin inhibition due to cleavage by thrombin after binding [47,48].

Apixaban, Betrixaban, Edoxaban, and Rivaroxaban are a class of potent reversible oral inhibitors that act directly on FXa [49]. Although these medications can prevent and treat thromboembolic diseases by inhibiting FXa activity, their clinical indications for treatment differ. Apixaban inhibits free FXa and prothrombin activity while also possessing the ability to indirectly inhibit thrombin-induced platelet aggregation [50–53]. It is currently used to reduce the risk of stroke in patients with non-valvular atrial fibrillation, for thromboprophylaxis after hip or knee replacement surgery, and to treat deep vein thrombosis or pulmonary embolism, as well as to prevent recurrent deep vein thrombosis and pulmonary embolism [54]. Betrixaban is primarily used for the prevention of VTE and stroke in patients with atrial fibrillation [55–57]. Edoxaban reduces thrombin production, inhibiting clot formation and is currently indicated for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation as well as for treating deep vein thrombosis and pulmonary embolism [58]. Rivaroxaban functions by inhibiting free FXa and shows a concentration-dependent relationship with FXa bound to prothrombin and thrombus-associated FXa [59,60]. However, it has no direct effect on platelet aggregation induced by adenosine diphosphate, collagen, or thrombin [59]. Other direct oral anticoagulants targeting FXa are also entering clinical trials, including Darexaban, Eribaxaban, Otamixaban, TAK-442, and LY517717 [61].

Milvexian (BMS-986177/JNJ0033093) is a reversible, highly efficient small-molecule inhibitor that acts on the active site of FXIa, exhibiting excellent antithrombotic effects mainly for thromboembolic prevention [62,63]. LNP023 is a reversible, specific, direct small-molecule inhibitor of Complement Factor B, preventing complement activation in the serum of C3 glomerulopathy patients and hemolysis of human PNH red blood cells by inhibiting CFB [64].

Nafamostat is a broad-spectrum small-molecule inhibitor covalently binding to the active site of serine proteases, effective against multiple conditions including acute pancreatitis and disseminated intravascular coagulation. Recent clinical results indicate that Nafamostat can prevent viral entry into human cells by inhibiting the transmembrane protease serine 2 (TMPRSS2), demonstrating high antiviral efficacy against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [65]. Camostat is an oral serine protease inhibitor available for treating chronic pancreatitis and post-operative reflux esophagitis in Japan and South Korea [66,67]. Current research shows that Camostat can inhibit TMPRSS2 and prevent targeted cell infection with SARS-CoV-2, suggesting potential use in managing COVID-19 [68]. Upamostat is an oral prodrug with bioavailability that targets serine protease inhibitor WX-UK1, showing the highest activity against trypsin and closely related enzymes. Although currently under Phase II clinical trials for pancreatic and breast cancers with significant inhibitory effects on tumor metastasis [69], upamostat lacks specificity for uPA and shows similar inhibition levels against various homologous proteases [70]. Recent clinical studies suggest broad-spectrum inhibitory effects of upamostat could be applicable in treating COVID-19, gastrointestinal diseases, and malignant tumors [71].

In summary, traditional small-molecule inhibitors typically contain P1 and P1′ groups. The structures of these inhibitors include basic groups such as guanidine or ami-dine, which can be precisely and specifically recognized by the active site within the S1 pocket

of serine proteases. The hydrolysis of the peptide bond occurs precisely at the junction between the P1 and P1′ groups. Crucially, the basic group in the P1 group of small-molecule inhibitors can form strong electrostatic interactions with the highly conserved Asp189 residue deeply embedded in the S1 pocket [72,73]. Additionally, the P1 group often contains aromatic groups that can form stable interactions with the S1 pocket of serine proteases. This interaction stabilizes the P1 group inserted into the S1 pocket of the serine protease, ensuring a tight binding and effective action between the inhibitor and the enzyme. Non-traditional small-molecule inhibitors, such as flavonoids like quercetin and myricetin [74], do not necessarily contain basic or weakly basic groups like amidine or guanidine in their structures. Instead, the P1 groups of non-traditional small-molecule inhibitors feature phenyl ring groups or halogen groups that replace aromatic groups to form stable interactions with the S1 pocket [75]. Despite the numerous significant advantages of small-molecule inhibitors, their inherent drawbacks, such as off-target effects and solubility issues, cannot be overlooked. These drawbacks will be discussed and analyzed in detail in the following sections (Table 1).

Table 1. Summary of the structure, molecular weight, and targets of small-molecule inhibitors.

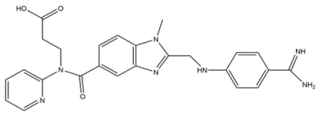
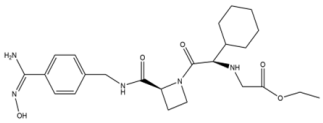
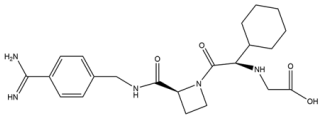
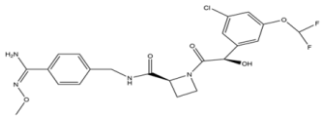
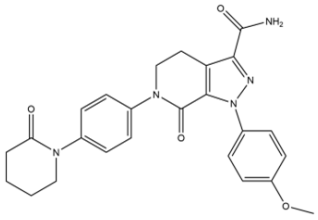
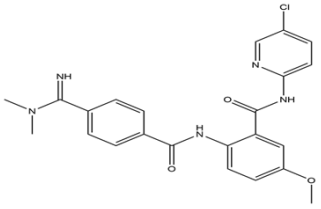
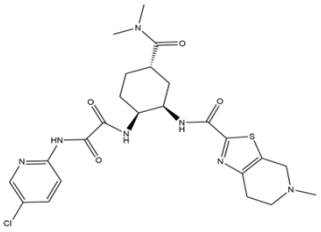
Number	Compound Names	Structures	Molecular Weight g/mol	Targets
1	Dabigatran		471.5	Thrombin
2	Ximelagatran		473.6	Thrombin
3	Melagatran		429.5	Thrombin
4	AZD0837		496.9	Thrombin
5	Apixaban		459.5	FXa
6	Betrixaban		451.9	FXa
7	Edoxaban		548.1	FXa

Table 1. *Cont.*

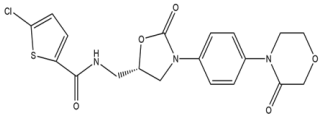
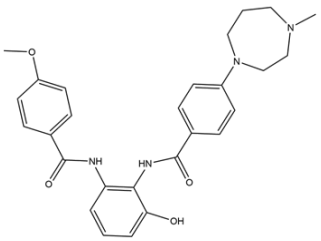
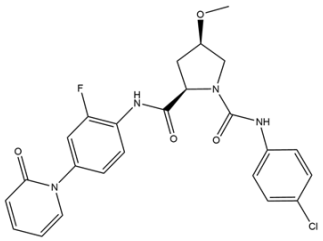
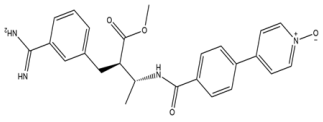
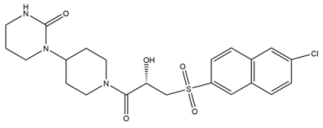
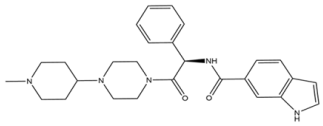
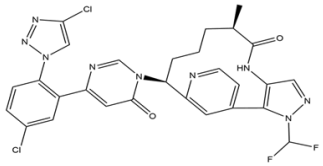
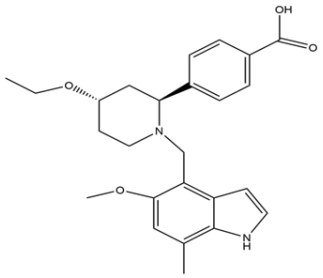
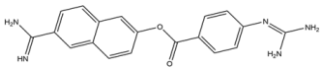
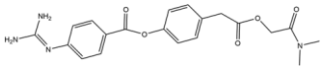
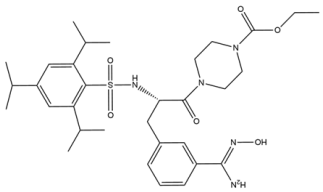
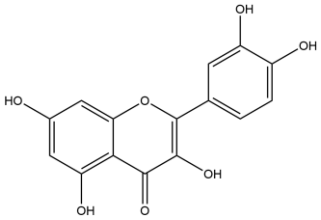
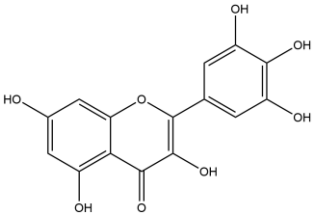
Number	Compound Names	Structures	Molecular Weight g/mol	Targets
8	Rivaroxaban		435.9	FXa
9	Darexaban		476.4	FXa
10	Eribaxaban		484.9	FXa
11	Otamixaban		446.5	FXa
12	TAK-442		480	FXa
13	LY517717		459.6	FXa
14	Milvexian		626.4	FXIa
15	LNP023		422.5	CFB
16	Nafamostat		347.4	broad-spectrum
17	Camostat		398.4	broad-spectrum

Table 1. *Cont.*

Number	Compound Names	Structures	Molecular Weight g/mol	Targets
18	Upamostat		629.8	broad-spectrum
19	Quercetin		302.23	broad-spectrum
20	Myricetin		318.23	broad-spectrum

3.2. Nucleotide Drugs

Nucleotide-based drugs are a new class of inhibitors with a broad range of applications in clinical settings. RNA-based therapeutic agents include small interfering RNAs (siRNAs), which are short double-stranded RNA molecules that mediate mRNA degradation by binding to complementary mRNA target sequences. Antisense oligonucleotides (ASOs), unlike siRNAs, are single-stranded RNA or DNA molecules, but they also bind to complementary target mRNA sequences to block protein translation.

3.2.1. Antisense Oligonucleotides (ASOs)

Through the Watson–Crick complementary pairing principles, antisense oligonucleotides (ASOs) bind to the mRNA of targeted serine protease genes, leading to the selective degradation by endogenous nucleases or inhibiting mRNA processing and/or function solely through occupancy mechanisms [37]. This effectively restricts the expression of serine proteases. Antisense technology has a high specificity for inhibiting unique targets and can be employed to suppress the synthesis of key proteases involved in diseases [76]. Successful ASOs offer advantages such as low drug design costs, high target selectivity, minimal drug–food interactions, high patient compliance, infrequent dosing, and a low incidence of clinical adverse events [77], making them a promising medical technology; selecting an appropriate ASO allows for highly specific target suppression.

AZD8233, primarily used for treating hyperlipidemia, targets PCSK9 mRNA sequences by inhibiting the synthesis of PCSK9 protein within cells [78]. Fesomersen and IONIS-FXIRX are FXI-targeting ASOs that are complementary to human FXI mRNA sequences, significantly lowering FXI mRNA levels in the liver without increasing bleeding risk, thereby demonstrating clear antithrombotic effects [79]. However, IONIS-FXIRX requires subcutaneous injections twice a week and takes 3 to 4 weeks to bring FXI levels into the therapeutic range [80]. Fesomersen, a second-generation FXI-targeting ASO, binds partially with N-acetylgalactosamine (GalNAc3) to promote its uptake by liver cells and requires administration only once a month, reducing FXI levels into the therapeutic range within a week. Compared to the first generation, IONIS-FXIRX, Fesomersen shows better efficacy and a faster onset of action [80].

3.2.2. siRNA

siRNA is a class of non-coding small RNAs that can silence target genes. siRNA induces the cleavage of specific mRNA sequences through the principle of base complementary pairing, interfering with the gene expression of the intended protein and thereby suppressing the synthesis of the corresponding protease. siRNA can inhibit gene function without causing mutations in the organism, making it an up-and-coming gene therapy technology. Inclisiran (Leqvio®) is a siRNA that requires subcutaneous administration only once every six months, which prevents the synthesis of PCSK9 in the liver, thereby lowering circulating low-density lipoprotein cholesterol (LDL-C). Clinically, it can prevent and treat atherosclerotic cardiovascular disease (ASCVD) [81]. However, the PCSK9 siRNA causes a significant reduction in PCSK9 expression, leading to the overexpression of CD36, and long-term use may trigger cancers such as acute myeloid leukemia, rodent tumors, breast cancer, and colorectal cancer [82].

4. Biomacromolecular Inhibitors

4.1. Peptides and Peptide Mimetics

Peptides as serine protease inhibitors are widely found in both animals and plants [83–86]. These inhibitors reduce the catalytic activity of serine proteases, sometimes rendering them completely inactive by forming a tight substrate-like binding with them [27]. Peptide inhibitors include families such as Kunitz, Bowman–Birk, and Kazal [87].

Kunitz inhibitors are widely occurring serine protease inhibitors with significant therapeutic potential. These inhibitors belong to two families: one derived from animals and the other from plants. The Kunitz-type inhibitor isolated from soybeans is known as the soybean trypsin inhibitor (STI), while Kunitz-type inhibitors sourced from plants other than soybeans are referred to as Kunitz trypsin inhibitors (KTIs). Research indicates that there are considerable differences in the structure and binding modes of Kunitz inhibitors from different sources [88,89]: STIs bind to proteases like substrates and are slowly hydrolyzed by the proteases. However, the products do not release from the active site, and, while the inhibitor is being hydrolyzed, peptide bonds are reformed while still bound to the active site of the protease [29,30]. Most KTIs consist of a single polypeptide chain containing 181 amino acid residues and four cysteine residues, forming two internal disulfide bonds and a unique reactive site. The amino acid residue sequence involved in protease inhibition within Kunitz-type inhibitors is known as the protease binding loop. Almost all KTIs adopt a canonical conformation in the protease binding loop, forming a complex with the active site of serine proteases. Serine proteases hydrolyze the peptide bonds between the P1–P1′ group of KTI, and the resulting products retain a structure similar to that of the intact inhibitor, trapping the enzyme in a hydrolysis/resynthesis cycle, thereby inhibiting enzyme activity [90]. Animal-derived Kunitz-type inhibitors contain one or more Kunitz domains [91], typically consisting of 50–70 amino acids. Disulfide bonds within the structure maintain the integrity of the inhibitor [92]. The presence of disulfide bonds ensures that Kunitz inhibitors possess such binding loops on their surface [92], allowing them to tightly bind to the active sites of serine proteases in a substrate-like manner, thereby inhibiting enzyme activity. This section discusses Kunitz inhibitors sourced from animals.

Aprotinin inhibits the activity of kallikrein and also of trypsin, which is why it is often named bovine pancreatic trypsin inhibitor (BPTI) and trypsin-kallikrein inhibitor (TKI); it acts as a natural serine protease inhibitor. Although Aprotinin is not an antifibrinolytic agent, it can improve hemostatic deficiencies related to elevated plasmin activity [93], thereby being useful in reducing the risk of substantial blood loss during strokes and repeat surgeries [94]. Due to its ability to inhibit the proteolytic activation of certain viruses, including the novel coronavirus, by TMPRSS2 and its binding to the ACE2 receptors on the surface of epithelial cells, it is considered a potential drug for the prevention and treatment of acute respiratory viral infections [95–97].

Desmolaris, another Kunitz-type inhibitor extracted from the vampire bat *Desmodus rotundus*, inhibits FXIa in a slow, tight, non-competitive manner, preventing FXIa from acti-

vating FXa, which leads to a dose-dependent extension of activated partial thromboplastin time (aPTT) without significant bleeding. Additionally, Desmolaris can inhibit various serine proteases such as trypsin, α-chymotrypsin, and neutrophil-derived elastase, offering great promise as a novel anticoagulant in clinical thrombotic treatment [98].

Ir-CPI is a Kunitz-type inhibitor originating from the tick *Ixodes ricinus* that selectively binds to three factors during their activation, thereby inhibiting the mutual activation of FXII, prekallikrein, and FXI in human plasma while preserving thrombotic balance. It is the first known selective inhibitor of the coagulation contact pathway capable of preventing both venous and arterial thrombosis [99].

Bowman–Birk Inhibitors (BBIs) are a different class of serine protease inhibitors derived from soybeans, distinct from SKTIs, primarily acting on trypsin, chymotrypsin, and elastase. BBIs have a molecular weight of only 6–9 kDa and consist of 60–80 amino acids. They possess a symmetrical structure with two cyclic domains located within a non-peptide region connected by disulfide bonds formed between cysteine residues. These two inhibitory domains are highly exposed, making them easily accessible to proteolytic enzymes [100,101]. As a result, BBIs can independently interact with two types of serine proteases simultaneously. The number and distribution of cysteine residues in the disulfide bonds play a crucial role in maintaining the structural stability and functionality of the domains under extreme temperature and pH conditions [100,101], indicating that BBIs have promising potential for the prevention and treatment of gastric cancer in the future. The P1 group of BBI inserts into the S1 pocket of serine proteases, forming a stable enzyme-inhibitor complex. Although the therapeutic targets and mechanisms of action of BBIs remain unknown [101], current research suggests that BBIs can exert therapeutic and preventive effects against inflammation and cancer by inhibiting serine proteases [101–104].

Kazal-type serine protease inhibitors (KTSPIs) are widely found in mammals, birds, and various invertebrates, consisting of one or multiple Kazal domains. They inhibit the catalytic activity of serine proteases from the S1 family, including trypsin, chymotrypsin, thrombin, elastase, plasmin, proteinase K, and Bacillus subtilis protease A [29]. Kazal-type inhibitors are particularly suited to act as thrombin inhibitors, as there is no spatial hindrance between Trp60 (the chymotrypsin symbol for thrombin) and the inhibitor [105]. KTSPI has been shown to modulate inflammatory responses [106], innate immunity, and antimicrobial defense [91,107,108]. AcKTSPI, derived from the venom gland of *Apis cerana*, contains a Kazal domain and exhibits inhibitory effects on Bacillus subtilis protease A and proteinase K but shows no activity against α-chymotrypsin, trypsin, factor Xa, thrombin, tissue plasminogen activator, or elastase. Additionally, AcKTSPI demonstrates antibacterial activity against Gram-positive bacteria, suggesting its potential as an effective antimicrobial agent in the future [109]. The first known Kazal-type inhibitor with anticoagulant activity, rhodiin, originated from the insect *Rhodnius prolixus* and specifically inhibits thrombin [105]. Another specific thrombin inhibitor, dipetalogastin, comes from the insect *Dipetalogaster maximus* [110]. Furthermore, infestin 1–2, found in the intestines of the hematophagous insect *Triatoma infestins*, is also a specific thrombin inhibitor [111]. Similarly, infestin-4 from the kissing bug *Triatoma infestins* inhibits not only factor XIIa but also plasmin and factor Xa [19,111,112]. AaTI, extracted from *Aedes aegypti*, is another KTSPI that can non-competitively inhibit thrombin, prolonging prothrombin time, activating partial thromboplastin time, and thrombin time [113]. In addition, the endogenous protease inhibitor serine protease inhibitor Kazal type 1 (SPINK1) in KTSPI specifically inhibits the secretion of pancreatic proteases and tumor-associated proteases, preventing the premature activation of proteases and protecting against self-digestion of the pancreas or ductal system [114–116]. In the tumor microenvironment, it plays multiple roles that facilitate cancer progression [116,117], being functionally related to processes like cell resistance, differentiation, metastasis, inflammation, and cancer spread [118]. SPINK1 is extensively researched as a cancer biomarker [119,120]. Other endogenous inhibitors will be elaborated on in the following text.

Antipain, bacitracin A, chymostatin, and leupeptin are broad-spectrum serine protease inhibitors that inactivate serine proteases by covalently or non-covalently binding to their active sites. Recent studies have found that leupeptin can form strong interactions with key amino acids in TMPRSS2, suggesting its potential as a drug for preventing and treating COVID-19 [121,122]. Hirudin, extracted from leeches, is the most effective known natural thrombin inhibitor; however, studies indicate that the bleeding incidence associated with hirudin is significantly higher than that with heparin, thus, it is not recommended for treating acute coronary syndrome and currently has no other clinical indications [123]. Desirudin, developed from modified hirudin, is used for the prevention of venous thromboembolism and is currently undergoing multiple Phase III clinical trials [44]. Additionally, PEG-Hirudin, a mixture of natural hirudin, has shown a significant extension of plasma half-life [124]. This new combination has demonstrated the ability to inhibit arterial thrombus formation in in vitro human models [125]. Although Phase II clinical trials have been completed, this medication remains experimental without any indications or recommendations.

MK-0616 is an oral macrocyclic peptide inhibitor that binds to PCSK9, inhibiting its interaction with LDLR. MK-0616 shows good inhibitory effects and selectivity against PCSK9 [126]. Sylvestin is a peptide inhibitor that can simultaneously inhibit PKal and FXIIa, demonstrating excellent efficacy against ischemic stroke by reducing thrombosis and inflammation in vivo [127]. VX-950 (LY-570310) is a highly selective, covalent reversible, potent peptidomimetic inhibitor of hepatitis C virus (HCV) NS3/4A protease, which reduces HCV RNA levels in replicating cells in a time- and dose-dependent manner, making it a potential treatment for hepatitis C infection without significant cytotoxicity at high doses [128].

Two cyclic peptides, upain-1 (CSWRGLENHRMC) and mupain-1 (CPAYSRYLDC), isolated from a phage display peptide library, exhibit potent inhibitory effects and selectivity against human-uPA and mouse-uPA, leading to the development of uPA inhibitors with comparable efficacy and specificity to monoclonal antibodies [129].

The Serine protease inhibitor (Serpins) superfamily within peptide inhibitors comprises a range of proteins that display low sequence homology but have highly conserved core structures [87,130]. The majority demonstrate significant inhibitory effects on serine proteases. Given that Serpins possess the unique ability to be autonomously secreted by human tissues, this characteristic encourages their classification as endogenous protease inhibitors for further exploration and summarization.

Neuroserpin (NS), widely expressed in the brain, and PAI-1, produced by various cells, are classic examples of SERPINE 1. These similar extracellular serine proteases, once secreted by cells, inhibit the activity of tissue-type plasminogen activator (tPA) and block the cleavage of several substrates, including plasminogen [131]. Serpin peptidase inhibitor, clade A, member 1 (SERPINA1) is primarily expressed in the liver and lungs, with expression observed in monocytes and macrophages [132]. It is a major protein in human serum, inhibiting various serine proteases such as neutrophil elastase, trypsin, chymotrypsin, and thrombin [133,134]. SERPINA1 regulates the immune system and inflammation [134], and defects in SERPINA1 can lead to severe lung and liver diseases [135].

SERPINA3, also known as α-1-antichymotrypsin (AACT, ACT), is mainly synthesized in the liver and secreted into the bloodstream [136]. It inhibits serine proteases, such as chymotrypsins, cathepsin G, and mast-cell chymase, by forming stable complexes, which diminishes their proteolytic activity and subsequently alters the extracellular matrix (ECM) [137–139]. The SERPINA3 protein plays a crucial role in tumorigenesis, with elevated levels associated with poorer prognosis in certain cancers, leading to its use as a diagnostic factor for colon, breast, lung, and gastric cancers. The serine protease inhibitor encoded by the SERPINC1 gene, antithrombin III (ATIII), possesses an exposed reactive center loop that interacts with the active site of proteases to inhibit their activity [140]. ATIII is a key inhibitor of coagulation factors, with even minute changes significantly affecting thromboembolism formation [141]. Furthermore, ATIII can inhibit inflammation through both coagulation-dependent and -independent mechanisms [142,143].

4.2. Antibody

Monoclonal antibodies consist of two heavy polypeptide chains and two light polypeptide chains, capable of targeting and binding to specific proteins anywhere in the body. The antigens bound by mAbs can elicit antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity, leading to the blockade of cell membrane receptors and inhibition of intracellular signaling [144]. Sibrotuzumab is an anti-FAP antibody that accumulates at tumor sites, aiding in the clearance of tumor cells and reducing tumor size. It is used to treat metastatic FAP-positive cancers, including breast cancer, colorectal cancer, and non-small cell lung cancer [145].

Evolocumab (AMG 145) and alirocumab (REGN727/SAR236553) are monoclonal antibodies that bind to the serine protease PCSK9 and can significantly lower plasma levels of low-density lipoprotein cholesterol (LDL-C), potentially reducing cardiovascular risk [146,147]. Narsoplimab (OMS721) is a human IgG4 monoclonal antibody that binds and inhibits mannan-binding lectin-associated serine protease-2 (MASP-2), showing significant therapeutic effects in hematopoietic stem cell transplant-associated thrombotic microangiopathy (HSCT-TMA). XIsomab 3G3 (AB023) is a humanized 14E11 antibody derived from recombinant mouse FXI, which binds to a highly conserved region of the apple 2 domain of FXI. Although 14E11 can inhibit FXIIa-mediated activation of FXI, it shows no significant inhibitory effect on FXI activated by thrombin or FXIa pathways, suggesting that XIsomab 3G3 may be specifically suitable for FXIa, functioning as a monoclonal antibody that inhibits thrombosis in a dose-dependent manner without increasing bleeding time [148]. Osocimab (BAY1213790) is a durable fully human monoclonal G1 antibody targeting FXIa, binding to a specific region near the enzyme's active site, causing significant structural changes in FXIa that reduce its binding to substrates, particularly FXI. This minimizes thrombus formation with minimal disruption to hemostasis [149], generally applicable during surgical procedures.

Based on investigations of uPA activity and inactive conformation transitions, we and collaborators developed inhibitory antibodies against huPA, mAb-112, and Fab-112. mAb-112 stabilizes the zymogen conformation of the uPA precursor and/or delays activation of the single-chain uPA precursor into its active double-chain form through steric hindrance. Fab-112 binds to the autolytic loop, causing its relocation, thereby inducing the complete conversion of activated uPA to its inactive zymogen form [150]. Additionally, recognizing features of the protease domain of muPA, we developed a set of nanobodies (Nb22) and an allosteric nanobody (Nb7) against muPA. Nb22 binds to the S1 specificity pocket of muPA in a substrate-like manner to inhibit its activity, while Nb7 can recognize both substrate-bound and unbound muPA with nearly identical affinity, indicating that Nb7 can act as a mixed inhibitor of muPA [151].

5. Challenges of Serine Protease Inhibitors in Clinical Applications

So far, serine protease inhibitors have been used clinically not only for treating diseases caused by serine proteases but also as probes in disease diagnosis. The small-molecule inhibitor FAPI-04, which inhibits FAP, shows a high affinity for FAP and exhibits excellent stability in human serum. PET/CT scan results from two patients with metastatic breast cancer revealed that after a small amount of FAPI-04 treatment, the uptake of the tracer in metastatic lesions was elevated, and pain symptoms were alleviated. Therefore, FAPI-04 holds promise as a clinical tracer for diagnostic imaging and targeted therapy of malignancies such as cancers with high levels of activated fibroblasts [152]. In terms of treatment, the endogenous inhibitor that has been studied most extensively is PAI-1, which is the primary endogenous inhibitor of tissue-type plasminogen activator (tPA) and also of urokinase (uPA). PAI-1 regulates fibrin dissolution and tumor cell invasion by inhibiting uPA and tPA. However, recent studies suggest that PAI-1 may promote tumor cell deterioration, positioning it as a critical target for novel cancer therapies [153] with the ongoing development of PAI-1 inhibitors. Furthermore, the serine protease inhibitor Kazal type 1 (SPINK1), synthesized by pancreatic acinar cells, is aberrantly overexpressed at both

mRNA and protein levels in samples of oral tongue squamous cell carcinoma (OTSCC). SPINK1 promotes the proliferation, invasion, and motility of OTSCC cells while reducing apoptosis, indicating that SPINK1 could serve as a potential prognostic biomarker for OTSCC [154].

Serine protease inhibitors have shown considerable promise in disease treatment. After decades of in-depth research, numerous compounds and biological agents exhibiting effective inhibition of serine proteases have been discovered, presenting opportunities for development into clinical therapeutic drugs. Currently, various serine protease inhibitors have entered clinical trial stages (Table 2). However, very few serine protease inhibitors have transitioned into clinical therapeutic use. The numerous challenges faced during the development of serine protease inhibitors have limited their clinical application.

Table 2. Serine protease inhibitors have entered the clinical trial stage.

Number	Compound	Therapy	Target	Phase	Indications	Usage Limitations	Solutions
1	Dabigatran	Small molecule	Thrombin	IV [155]	NVAF [38,156], VTE [157]	Emergency surgery, advanced age [158], pregnant women, severe liver and kidney dysfunction, mechanical heart valve, concomitant administration of drug classes that are strong inhibitors of CYP3A4 and P-glycoprotein [159]	Idarucizumab [160], reduce the dosage, switch to safer medications [158]
2	Ximelagatran	Small molecule	Thrombin	III	VTE [161]	Liver dysfunction [162]	No findings at this stage
3	Melagatran	Small molecule	Thrombin	III	VTE [161]	Liver dysfunction [162]	No findings at this stage
4	AZD0837	Small molecule	Thrombin	II	AF [45], Chronic ventricular fibrillation [46]	No findings at this stage	No findings at this stage
5	Apixaban	Small molecule	Thrombin	III	DVT, PE [163]	Dialysis patients, severely impaired liver function, active pathological bleeding [54], pregnant women, severe liver and kidney dysfunction, mechanical heart valve, concomitant administration of drug classes that are strong inhibitors of CYP3A4 and P-glycoprotein [159]	Switch to safer medications
6	Betrixaban	Small molecule	Thrombin	III	VTE [164]	No findings at this stage	No findings at this stage
7	Edoxaban	Small molecule	Thrombin	III	AF [165], VTE [166]	Pregnant women, severe liver and kidney dysfunction, mechanical heart valve, concomitant administration of drug classes that are strong inhibitors of CYP3A4 and P-glycoprotein [159]	Anticoagulation with intravenous unfractionated heparin, low molecular weight heparin (LMWH), or warfarin [167]

Table 2. *Cont.*

Number	Compound	Therapy	Target	Phase	Indications	Usage Limitations	Solutions
8	Rivaroxaban	Small molecule	Thrombin	III	VTE [168]	Pregnant women, severe liver and kidney dysfunction, mechanical heart valve, concomitant administration of drug classes that are strong inhibitors of CYP3A4 and P-glycoprotein [159]	Anticoagulation with intravenous unfractionated heparin, low molecular weight heparin (LMWH), or warfarin [167]
9	Darexaban	Small molecule	Thrombin	III	ACS, AF, DVT, PE [169]	No findings at this stage	No findings at this stage
10	Otamixaban	Small molecule	Thrombin	II	ACS, Non-urgent PCI [170]	No findings at this stage	No findings at this stage
11	TAK-442	Small molecule	Thrombin	II	ACS [171]	No findings at this stage	No findings at this stage
12	LY517717	Small molecule	Thrombin	II	DVT, PE [172]	No findings at this stage	No findings at this stage
13	Milvexian	Small molecule	FXIa	II	No findings at this stage [63]	No findings at this stage	No findings at this stage
14	LNP023	Small molecule	Complement Factor B	III	PNH [173], IgAN [174]	No findings at this stage	No findings at this stage
15	Nafamostat	Small molecule	broad-spectrum	IV	Acute pancreatitis, AKI, various malignant tumors [65]	No findings at this stage	No findings at this stage
16	Camostat	Small molecule	broad-spectrum	IV	Chronic pancreatitis, post-operative reflux esophagitis, COVID-19 [175]	No findings at this stage	No findings at this stage
17	Upamostat	Small molecule	broad-spectrum	II	Pancreatic cancer, breast cancer, COVID-19 [176]	No findings at this stage	No findings at this stage
18	Aprotinin	Polypeptide	broad-spectrum	IV	Anti-inflammatory and antithrombotic during operation [95]	Sensitive to Aprotinin [177,178]	Switch to safer medications
19	Antipain	Polypeptide	broad-spectrum	IV	Analgesia [179,180]	No findings at this stage	No findings at this stage
20	Bacitracin A	Polypeptide	broad-spectrum	IV	Bacterial infection [181]	No findings at this stage	No findings at this stage
21	leupeptin	Polypeptide	broad-spectrum	IV	COVID-19 [182]	No findings at this stage	No findings at this stage
22	Desirudin	Polypeptide	Thrombin	III	VTE [44]	Renal dysfunction [183]	Dose adjustment [183]
23	PEG-Hirudin	Polypeptide	Thrombin	II	No findings at this stage	No findings at this stage	No findings at this stage
24	MK-0616	Polypeptide	PCSK9	II	Hypercholesterolemia [184]	No findings at this stage	No findings at this stage
25	VX-950	Polypeptide	NS3/4A protease	I	Chronic hepatitis C [185]	Skin disease [186]	Discontinue and take oral corticosteroids [187]
26	AZD8233	ASO	PCSK9	II	Hypercholesterolemia	No findings at this stage	No findings at this stage
27	Fesomersen	ASO	FXIa	II	No findings at this stage [188]	No findings at this stage	No findings at this stage

Table 2. *Cont.*

Number	Compound	Therapy	Target	Phase	Indications	Usage Limitations	Solutions
28	IONIS-FXI$_{RX}$	ASO	FXIa	II	No findings at this stage [188]	No findings at this stage	No findings at this stage
29	Inclisiran	SiRNA	PCSK9	III	Hypercholesterolemia [81,189]	No findings at this stage	No findings at this stage
30	Sibrotuzumab	Antibody	FAP	I	Fibroblast activation protein-positive cancer [190]	No findings at this stage	No findings at this stage
31	Evolocumab	Antibody	PCSK9	III	Hyperlipidemia [147]	Liver function impairment [191]	No findings at this stage
32	Alirocumab	Antibody	PCSK9	IV	Hypercholesterolemia [192]	No findings at this stage	No findings at this stage
33	Narsoplimab	Antibody	MASP-2	III	IgAN [193]	No findings at this stage	No findings at this stage
34	XIsomab 3G3	Antibody	FXIa	II	No findings at this stage [188]	No findings at this stage	No findings at this stage
35	Osocimab	Antibody	FXIa	II	VTE [194]	No findings at this stage	No findings at this stage

The catalytic active sites of serine proteases are highly conserved, with only certain differences in the amino acid residues within and around the active pocket. Some small-molecule inhibitors of serine proteases, while exhibiting high inhibitory efficacy, primarily act through covalent or non-covalent interactions with the catalytic active sites in the active pocket. This lack of precise target identification can lead to off-target effects, potentially resulting in adverse clinical events. Consequently, the high similarity of active sites among different serine proteases poses a challenge for developing highly selective inhibitors targeting these sites. Moreover, the negative charge of the protease active sites makes the development of small-molecule competitive inhibitors challenging [195,196]. Furthermore, targeting specific serine proteases does not always guarantee successful pathological outcomes. If serine proteases possess non-catalytic domains, these domains often participate in regulating catalytic activity [197–199].

While antibody-based serine protease inhibitors exhibit strong specificity, their mechanism of action primarily relies on protein–protein interactions, which restricts the spectrum of diseases they can effectively treat. Compared to small-molecule inhibitors, antibody inhibitors are associated with higher production costs, greater susceptibility to inactivation, a limited mode of administration, and stringent storage requirements. Furthermore, antibodies have high immunogenicity, increasing the likelihood of triggering immune responses that can lead to allergic reactions, and, in severe cases, pose life-threatening risks.

Some serine protease inhibitors also face issues of instability and short half-lives in vivo. ASOs typically utilize a phosphodiester backbone during design and synthesis, which is susceptible to nuclease activity, thereby affecting the stability and efficacy of ASOs in inhibiting serine proteases [200].

Additionally, overcoming the poor solubility of small-molecule drugs is one of the greatest challenges in clinical drug development. Small-molecule inhibitors with low solubility tend to precipitate in aqueous solutions, resulting in extremely low concentrations in water that fail to reach the inhibitory levels required against serine proteases in the human body, thus hindering optimal therapeutic effects. Since solubility is a critical factor for the oral absorption of drug formulations and a necessary condition for the drugs to penetrate cell membranes and be transported within the body, serine protease inhibitors with poor solubility typically exhibit low oral bioavailability. Consequently, these inhibitors are often developed as injectables. To improve the solubility or stability of small-molecule inhibitors, those that are generally insoluble in water can often dissolve in organic solvents such as anhydrous ethanol, acetone, or tetrahydrofuran, particularly when surfactants are added to

enhance solubility. However, excessive concentrations of these organic solvents may cause irreversible alterations to the structure of serine proteases, leading to complete inactivation. Moreover, some organic solvents are metabolized very slowly within the human body, increasing the risk of bioaccumulation and resulting in solvent poisoning, which can trigger adverse reactions [201,202].

Another issue is also drawing attention. For multifunctional serine proteases, serine protease inhibitors exhibit a complex dual action: on one hand, Factor Xa and Thrombin play a crucial role in the intrinsic coagulation pathway, making the development of antithrombotic drugs targeting these two key points a hot topic in the research field [203,204]. By precisely inhibiting the activity of FXa or thrombin, unnecessary thrombus formation can be effectively curtailed, opening new avenues for the prevention and treatment of cardiovascular diseases. Although numerous preclinical trials have confirmed that some FXa inhibitors and thrombin inhibitors have achieved significant results in reducing the risk of cardiovascular events, the use of these inhibitors is also associated with an increased risk of bleeding [205,206]. This finding reveals that serine protease inhibitors, when applied to multifunctional serine proteases, demonstrate both therapeutic potential and potential safety challenges.

6. General Strategies for Overcoming Challenges in the Development of Serine Protease Inhibitors

How to address the various challenges in the development of serine protease inhibitors to ensure their widespread clinical application has long been a significant topic for consideration. The following two directions may provide ideas for the development and widespread use of serine protease inhibitors.

6.1. Structure-Based Drug Design Remains the Primary Approach for Inhibitor Development

With in-depth studies in structural biology, certain key sites on serine proteases have become references for the development of inhibitor structures. For example, ecotoin is known as a broad-spectrum serine protease inhibitor that interacts with both the active site Ser195 and the exosite of serine proteases to exert its inhibitory effects. By modifying ecotoin to enhance its interaction with serine proteases, it has been transformed into a high-affinity uPA inhibitor [207]. However, some inhibitors exhibit significant variability in their inhibitory effects on different proteins, as seen with rivaroxaban, which shows high selectivity against human FXa (Ki = 0.4 nmol/L), surpassing the selectivity for other biologically relevant serine proteases by 10,000 times [60]. The relatively conserved catalytic active site structures of serine proteases indicate that other factors may interfere with the binding of the same inhibitor to different serine proteases. We hypothesize that the S1 protease active pocket and surrounding amino acid residues may influence this effect. Therefore, attention should be paid to the S1 pocket and surrounding residues in inhibitor development, utilizing slight geometric variations in the S1 pocket and its sub-sites to achieve the development of specific serine protease inhibitors [208–210].

Small cyclized peptides have demonstrated significant potential in developing specific serine protease inhibitors. Compared to linear peptides, cyclized peptides possess greater conformational constraints, allowing them to engage with areas of serine proteases beyond the S1 pocket. These areas exhibit much larger variations among homologous enzymes, resulting in higher specificity for substrate and drug recognition, thus rendering cyclized peptides more selective toward their targets [211]. We have also reported that by altering certain key amino acid residues within cyclized peptides, it is possible to transform a specificity cyclized peptide inhibitor targeting one serine protease into a specificity inhibitor for another serine protease. Moreover, increasing the flexibility of cyclized peptides can enhance their inhibitory effects on targets [129].

Additionally, the relatively large and variable autolysis loops present across different serine proteases may serve as possible targets for highly specific serine protease activity

modulation. Therefore, we propose that targeting autolysis loops may become a general strategy for pharmacological interventions in serine protease activity in the future [34].

Structure is also an important consideration in the development of serine protease inhibitors. Research on Kunitz-STI inhibitors has shown that there are significant sequence variations among the Kunitz-STI inhibitor class, yet these sequences maintain highly similar structural conformations, which are crucial for inhibiting the catalytic activity of serine proteases [89,212]. Therefore, merely classifying based on simple amino acid residue sequences and using that as a basis for developing serine protease inhibitors is clearly inadequate; structural comparisons may provide a better starting point for this development.

ASOs have great potential for future clinical disease treatment. The stability of ASOs can be improved in several ways: modifying the ASO backbone can enhance resistance to nuclease degradation. Thiophosphoramidate and isostere backbone modifications can replace the phosphodiester backbone, increasing ASO stability [213,214]. Phosphorothioate (PS) backbone modifications not only enhance ASOs' resistance to nuclease degradation [215], ensuring that PS-ASOs can reach the target RNA in cells and tissues while recruiting RNase H enzymes to promote target RNA cleavage, but they also strengthen the attachment of ASOs to plasma proteins, facilitating ASO binding to cells and specific tissue uptake [216]. Modifications at the 2′-position of the sugar moiety can enhance the therapeutic effect of ASOs by promoting target binding. Further, 2′-O-methyl and 2′-O-methoxyethyl sugar modifications not only improve resistance to nucleases but also reduce nonspecific protein binding and lower the toxicity of ASOs [217]. Locked nucleic acids (LNAs) are another type of sugar modification; LNA-ASOs increase the binding capability of ASOs to target RNA and nuclease resistance [218], although they also enhance ASO toxicity [219]. Recent studies have shown that several LNA analogs have been synthesized, demonstrating improved activity and toxicity profiles in animal models [220,221]. However, modified ASOs have exhibited dose-dependent transient and mild to moderate toxicity, with the most common toxicities being coagulation cascade activation and increased liver enzymes observed when the plasma concentrations of PS-ASOs are high [222,223]. Current research indicates that second- and third-generation chemically modified ASOs do not have significant side effects in human patients irrespective of dose, making clinical application of ASOs in humans feasible [224]. Therefore, reducing dose-dependent toxicity or developing safer ASOs has become a pressing challenge to overcome in clinical treatments.

6.2. Drug Delivery Systems Enhance the Clinical Utilization of Poorly Soluble Inhibitors and Broad-Spectrum Inhibitors

The plasma concentrations of low-water-soluble small-molecule inhibitors of serine protease can be increased using various strategies. The primary approach typically involves chemical methods, particularly structure optimization, to enhance hydrophilic groups within the small-molecule structure, thereby improving solubility. Beyond structural optimization, solubility can also be increased through methods such as the complexation of active molecules, the use of emulsions, micelles, microemulsions, cosolvents, polymer micelle preparation, particle size reduction techniques, pharmaceutical salts, prodrugs, solid-state substitution techniques, soft gel technology, drug nanocrystals, solid dispersion methods, crystal engineering, and nanomorphology techniques [225].

Using biological methods to enhance the therapeutic effects of poorly soluble drugs is currently a research hotspot. Insoluble drugs can be covalently or non-covalently incorporated into carriers, which can then target the specific protein of interest through targeting agents [226]. The most common biological method for improving the treatment efficacy of insoluble drugs is encapsulation. In this process, the poorly soluble drug is embedded in a carrier, which then targets the relevant serine protease, releasing the drug near the target site to achieve therapeutic effects. The carrier is subsequently metabolized and eliminated by the liver. Common carriers used in clinical studies include microcapsules, microspheres, nanoparticles, liposomes, proteins (such as human serum albumin, ferritin,

low-density lipoprotein, chaperone proteins, and fibrillar proteins), as well as extracellular vehicles (EVs) [227]. Protease-activated prodrugs (PAPs) are carriers that precisely target tumor-associated proteases. The peptide precursor binds to the inhibitor, rendering the drug temporarily ineffective. When entering the human circulatory system, the prodrug is either undetected or in an inactive state. It is only when the prodrug is cleaved at specific sites by tumor-associated endopeptidases and the residual peptide segments are removed by nonspecific exopeptidases that the active agent is fully enabled to exert its inhibitory effects [228].

Common serine protease targets include uPA [229,230], plasmin [231], and FXa [232]. Pharmaceutical carriers offer several advantages in clinical disease treatment; they can deliver not only poorly soluble small-molecule drugs but also siRNA, miRNA, ASOs, proteins, small-molecule drugs, nanoparticles, and CRISPR/Cas9 gene-editing systems [227,233–235]. However, common drug carriers have limited encapsulation capacity; as the size of the drug increases, the amount that can be encapsulated decreases. Therefore, developing carriers with larger encapsulation capacities or focusing on smaller molecular weight drugs presents ongoing challenges for serine protease inhibitor delivery systems in clinical applications.

Dual-targeted combination therapy is a common treatment approach in clinical practice. While it is known that certain serine proteases are key targets for treating specific clinical indications, current research on individual proteins does not always guarantee a complete cure. Furthermore, specific inhibitors that can be used in clinical treatments targeting these key points are limited. Therefore, dual-targeted combination therapy aims to achieve precise treatment of diseases by inhibiting important serine proteases involved in disease progression. This approach not only enhances the efficacy of treatment but also reduces the toxicity risks associated with each drug, thereby minimizing the occurrence of adverse clinical events. For instance, in vitro cell experiments have shown that combinations of the three drugs Omipalisib, Remdesivir, and Tipifarnib display strong synergistic effects in inhibiting Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This drug combination has improved antiviral efficacy against SARS-CoV-2 infections while simultaneously lowering the toxicity risks of each medication [236].

Improving the specificity of serine protease inhibitors can also be achieved through the emerging and rapidly developing approach of Antibody–Drug Conjugates (ADCs). An ADC consists of three components: a monoclonal antibody, a linker, and a cytotoxic drug. By combining the precise targeting capability of monoclonal antibodies with the cancer-killing potential of cytotoxic agents, ADCs can selectively kill cancer cells [237]. The cytotoxic agent is covalently attached to the monoclonal antibody via an appropriate linker, resulting in targeted delivery of the cytotoxic agent to the target cells, which reduces toxicity compared to using the cytotoxic agent alone [238]. Compared to other treatment options, ADCs exhibit better efficacy and longer serum half-lives, reduced immunogenicity, and improved specificity toward cancer cells. Currently, there are 11 FDA-approved ADCs for tumor indications, with hundreds undergoing advanced clinical evaluations and many more in preclinical development [239]. One of the biggest challenges in ADC development is selecting the appropriate linker that effectively conjugates the cytotoxic payload to the monoclonal antibody. The chemical properties of the linker influence various characteristics of the ADC, including toxicity, specificity, stability, and efficacy [238]. uPAR has been shown to be a molecular target for ADC therapy in some cancers [240], and it is believed that ADCs may become widely used in the treatment of diseases caused by serine proteases in the near future.

We developed a drug carrier based on human serum albumin (HSA) that is fused with a targeting agent known as the Amino-Terminal Fragment (ATF) of uPA. This fusion protein, named ATF-HSA, can bind to the tumor surface marker uPAR. By loading chemotherapy drugs and photosensitizers into this carrier, we obtained a relatively stable macromolecular complex. Compared to free drugs, this macromolecular complex showed more precise targeting in vivo, significantly improved therapeutic effects, and markedly reduced side

effects [241–244]. This indicates that such HSA packaging strategies targeting tumors could also be applied to treat other disease targets. The fusion protein ATF-HSA not only serves as a therapeutic drug delivery carrier but also has been demonstrated to function as an imaging agent delivery carrier, showing specificity for tumors expressing uPAR [241]. However, preclinical studies have found that the human uPAR targeting agent typically does not bind to mouse uPAR, indicating a species-specific limitation [244]. Recently, we developed a novel fusion protein (uPARTC) that can bind to both mouse and human uPAR. The macromolecular complex formed by embedding drugs into uPARTC exhibited effective proliferation inhibition in both mouse and human uPAR-overexpressing cells, as well as potent antitumor effects in animal studies. This suggests that uPARTC is a promising tumor-targeting drug carrier capable of overcoming the challenge of species-specificity in uPAR-targeted drugs and can be used to load other cytotoxic compounds [226]. In the future, modifications of fusion proteins based on uPARTC show immense potential for enhancing the specificity of inhibitors and precise targeting for clinical treatment indications, paving the way for advancements in precision medicine.

7. Conclusions

Serine proteases play an indispensable role in human life activities, widely participating in and profoundly influencing numerous metabolic pathways. As a result, they have become important targets for clinical diagnosis and treatment of various diseases. Given their significant status, the development of serine protease inhibitors has demonstrated extraordinary value and potential in the medical field, aiming to precisely intervene and treat clinical conditions caused by abnormal activities of these enzymes. In recent years, the field of serine protease inhibitors has experienced rapid development, with increasingly in-depth theoretical research and several inhibitors successfully entering clinical application stages, marking significant progress in the translation of research outcomes into clinical practice. This review thoroughly analyzes the mechanisms of action of serine protease inhibitors and systematically summarizes the types of inhibitors that have entered clinical practice. At the same time, we acknowledge and summarize the various challenges faced in the development of serine protease inhibitors, including but not limited to issues of inhibitor specificity, efficiency enhancement, and long-term safety. In response to these challenges, we further explore feasible solutions and strategies, aiming to provide directional guidance for future research.

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Abbreviations

ACS: acute coronary syndrome; **AACT/ACT**, α-1-antichymotrypsin; **AF**, atrial fibrillation; **ADCs**, Antibody–Drug Conjugates; **AKI**, acute kidney injury; **aPTT**, activated partial thromboplastin time; **ASCVD**, atherosclerotic cardiovascular disease; **Asp**, amino acids aspartate; **ASO**, antisense oligonucleotide; **ATIII,** antithrombin III; **ATF**, Amino-Terminal Fragment; **BBI**, Bowman–Birk inhibitor; **BPTI**: bovine pancreatic trypsin inhibitor; **CAM**, drug–drug co-amorphous system; **CD36**, cluster of differentiation 36; **CFB**, Complement Factor B; **DTV**, deep vein thrombosis; **ECM**, extracellular matrix; **EVs**, extracellular vehi-

cles; **FAP**, fibroblast activation protein; **FAPI**, Fibroblast activation protein inhibitor; **FIIa**, Activated Coagulation Factor II; **FVIIa**, Activated Coagulation Factor VII; **FIXa**, Activated Coagulation Factor IX; **FXa**, Activated Coagulation Factor X; **FXIa**, Activated Coagulation Factor XI; **FAP**, Fibroblast activation protein-alpha; **GalNAc3**, N-acetylgalactosamine; **HCV**, hepatitis C virus; **His**, amino acids histidine; **HIT**, heparin-induced thrombocytopenia; **HSA**, human serum albumin; **HSCT-TMA**, hematopoietic stem cell transplant-associated thrombotic microangiopathy; **IgAN**, immunoglobulin A nephropathy; **KTI**, Kunitz trypsin inhibitor; **KTSPI**, Kazal-type serine protease inhibitor; **LDL-C**, low-density lipoprotein cholesterol; **LDLR**, low-density lipoprotein receptor; **LNA**, locked nucleic acids; **mRNA**, message RNA; **mAb**, monoclonal antibody; **NS**, neuroserpin; **NVAF**, non-valvular atrial fibrillation; **OTSCC**, oral tongue squamous cell carcinoma; **PAI-1**, plasminogen activator inhibitor 1; **PAPs**, protease-activated prodrugs; **PCI**, percutaneous coronary intervention; **PCSK9**, proprotein convertase subtilisin/kexin type 9; **PE**, pulmonary embolism; **PET/CT**, positron emission tomography/computerized tomography; **PNH**, paroxysmal nocturnal hemoglobinuria; **PS**, phosphorothioate; **SARS-CoV-2**, severe acute respiratory syndrome coronavirus 2; **Serpins**, serine protease inhibitors; **SERPINA1**, serpin peptidase inhibitor, clade A, member 1; **SERPINA3**, serine protease inhibitor A3; **SERPINC1**, serpin peptidase inhibitor, clade C, member 1; **siRNA**, small interfering RNA; **Ser**, amino acids serine; **STI**, soybean trypsin inhibitor; **SPINK1**, serine protease inhibitor Kazal type 1; **TKI**, trypsin-kallikrein inhibitor; **TMPRSS2**, transmembrane serine protease 2; **tPA**, tissue-type plasminogen activator; **uPA**, urokinase-type plasminogen activator; **uPAR**, urokinase-type plasminogen activator receptor; **VTE**, venous thromboembolism.

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