Reviewer 1's Comments

Serine proteases are validated drug targets in several pathologies and physiological processes. Using molecules that inhibit these protases is a valuable strategy to regulate the serine protease activity. The manuscript titled Advancements in Serine Protease Inhibitors: From Mechanistic Insights to Clinical Applications summarizes the serine protease inhibitors in clinical trials and describes their protease targets and clinical applications. The authors make a detailed description of the molecules, and the challenges encountered during their development as drugs. They also propose some general strategies to overcome the difficulties in the development of drugs based on these inhibitors. I consider this a valuable contribution to the efforts to develop new protease inhibitors to be used in clinical treatment and diagnosis. However, I would like to suggest some changes to improve the quality of this manuscript.

Response: Thank you sincerely for your comments on this review. Your valuable feedback has played a crucial role in enhancing the overall quality of the article. Based on your comments, we have conducted an in-depth literature review to comprehensively address your questions and made corresponding improvements to the article's content.

1. I suggest improving the quality of Figures 3 and 4.

Response: Thanks for your suggestions. We have revised Figures 3 and 4, and believe that improving the quality of them. The following are revised Figure 3 and Figure 4.

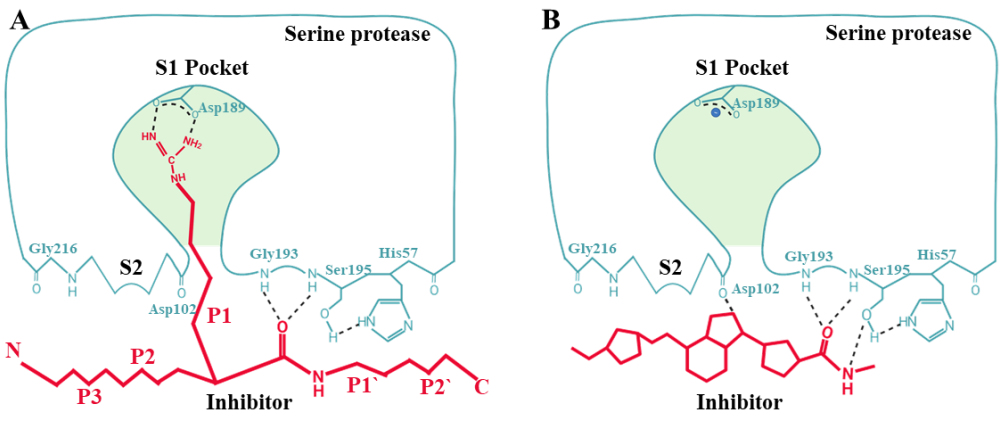

Figure 3. Mechanism of action of serine protease inhibitors. (A) The mechanism of action of classic serine protease inhibitors [27]. The P1 group inserts into the interior of the S1 pocket of the serine protease; (B) Mechanism of action of non-standard inhibitors. Non-canonical inhibitors interact with 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.

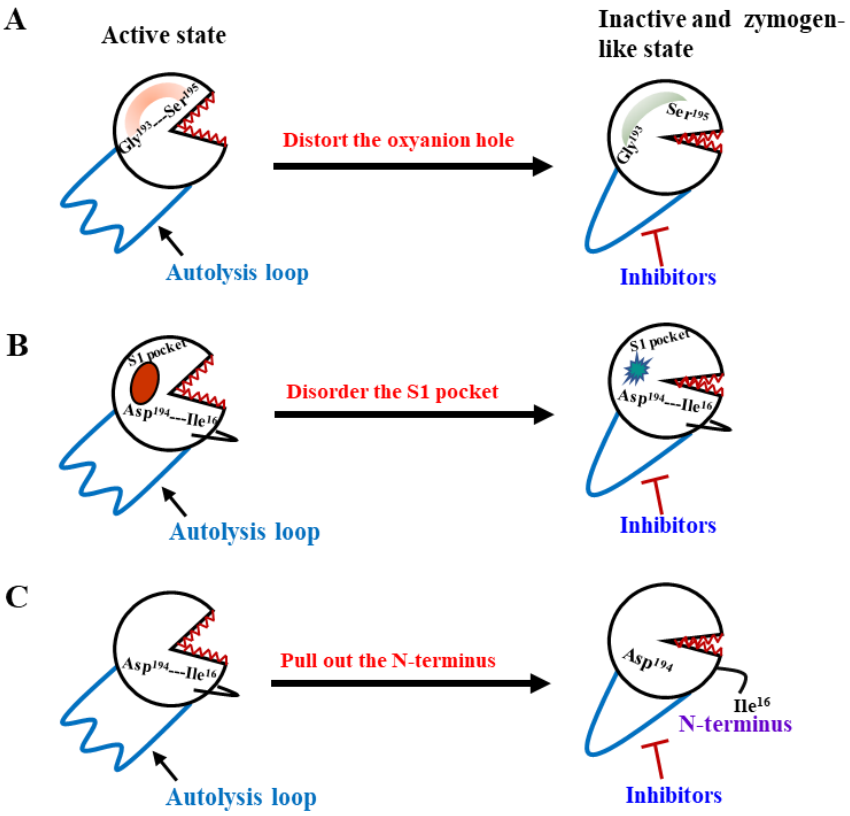

Figure 4. Three possible mechanisms for inhibiting autolysis [34]. (**A**) The binding of the allosteric inhibitor on the autolysis loop leads to the movement of the oxyanion stabilizing loop (Gly193-Ser195) and the distortion of the oxyanion hole; (**B**) Disordering of the S1 specificity pocket caused 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.

2. In section 2 "Mechanisms and Classification of Serine Protease Inhibitors", the authors describe three mechanisms of inhibition of serine proteases. Two of these mechanisms classify serine protease inhibitors as classical and non-canonical while the third is based on targeting the autolysis loop with allosteric inhibitors. However, classical (canonical) or non-canonical classification is used mainly with peptide, proteinaceous, or peptidomimetic inhibitors, not small molecule inhibitors. Some small molecules could use these mechanisms, but I suspect that some others do not.

Response: Through extensive literature review, I have supplemented and summarized the mechanisms of action of small molecule inhibitors in Section 2: apart from some small molecule inhibitors that use classical (canonical) or non-canonical classification, another group of small molecules typically binds directly to proteases, effectively inhibiting protease activity through mechanisms such as substrate competition, altering protein structure, and preventing conformational

changes. The relevant content on the mechanisms of action of small molecule inhibitors is supplemented in lines 149-157, as follows: “Although some small molecule inhibitors mimic peptide inhibitors to suppress the activity of serine proteases through the Laskowski mechanism or non-standard pathways, another class of small molecule inhibitors exhibits 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]”

3. What is the meaning of peptide inhibitors in the document? Sometimes they use the term for peptide molecules (less than 50 amino acids, eg: Leupeptin) and sometimes for polypeptides (Kunitz inhibitors).

Response: The peptide inhibitors mentioned in the original text refer to biologically active molecules composed of two or more amino acid residues. Biomacromolecules in the article are divided into two main categories: peptides and their analogs, and antibodies. This classification is primarily based on their molecular weight and the complexity of protein conformation. Although some Kunitz inhibitors, Bowman-Birk inhibitors, and Kazal-type serine protease inhibitors may have more than 50 amino acids, their molecular weight is significantly lower than that of antibodies, and structurally, they are relatively simpler than antibodies. Given these characteristics, Kunitz inhibitors are reasonably classified as peptide inhibitors.

In this discussion section about peptides, we focus on the key role that peptide inhibitors play as a whole category within biological systems, rather than delving into the subtle differences in their internal structures. Thus, we do not strictly define the distinction between peptide molecules and polypeptides.

4. Concerning Kunitz inhibitors, there is an error with their classification. Kunitz serine protease inhibitors are not a family of proteins secreted by various plants and animals. There are two families of Kunitz inhibitors: one from animals (one described in section 3.2) and another from plants (mentioned in lines 560-566). Both families have completely different

folds, sizes, and binding modes, although some inhibitors of these families use the same canonical inhibition mechanism to inhibit serine proteases. I suggest modifying this section.

Response: Thank you for pointing it out. After careful verification and revision, the errors regarding the classification of Kunitz inhibitors in the previous text have been properly corrected. In lines 325 to 348 of the article, I have rewritten the relevant content about Kunitz inhibitors. The content is as follows: "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 (SKTI), while Kunitz-type inhibitors sourced from plants other than soybeans are referred to as Kunitz trypsin inhibitors (KTI). Research indicates that there are considerable differences in the structure and binding modes of Kunitz inhibitors from different sources [88,89]: SKTI inhibitors 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 KTI P1-P1` group, 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."

In this revision, we have clearly distinguished between SKTI, derived from soybeans, and KTI from other plants, and we have provided detailed supplementary descriptions of the mechanisms of action for both SKTI and KTI inhibitors. Furthermore, to ensure the accuracy of the information in

the article, we have particularly emphasized at the end of this section that the Kunitz inhibitors discussed in this paper are of animal origin.

5. The authors mentioned (lines 276-278) that aprotinin can inhibit proteolytic activation of SARS-CoV-2 but both proteases present in this virus are cysteine proteases. Is there any evidence that aprotinin can inhibit these or other cysteine proteases?

Response: We appreciate these comments and suggestions. Through an extensive literature review, no relevant research results regarding the inhibition of cysteine proteases in viruses by Aprotinin were found. The mechanism of SARS-CoV-2 infection involves the virus's spike protein changing its spatial conformation under the action of TMPRSS2, allowing the cell surface ACE2 receptor to recognize and bind to the spike protein (*Int J Mol Sci.* 2024 Jul 10;25(14):7553). Aprotinin, as a broad-spectrum serine protease inhibitor, has been confirmed to inhibit the activity of TMPRSS2 (*Antiviral Res.* 2011 Oct;92(1):27-36.; *Biochimie.* 2017 Nov; 142:1-10). Research results from 2024 indicate that Aprotinin prevents the cleavage and activation of the SARS-CoV-2 spike protein by inhibiting TMPRSS2 (*Int J Mol Sci.* 2024 Jun 29;25(13):7209.). The errors in the original text regarding this matter have been corrected, the content of the revised manuscript is located in lines 354-357, and is as follows:

"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]."

6. In section 3.3 a different kind of protease inhibitors is introduced because they are not enzymatic inhibitors at least not in the classical or most common definition. The nucleotide drugs described act by blocking or inhibiting the expression of the proteases and not inhibiting the enzyme activity. This is quite interesting, but I was surprised by their inclusion as protease inhibitors because their mechanism of action was not included in Section 2 (Mechanisms and Classification of Serine Protease Inhibitors).

Response: Thanks for your remark. Nucleotide inhibitors suppress the expression of serine proteases through a highly specific and precise mechanism, which has become a new focus in the

design of serine protease inhibitors in recent years. Although we discussed in detail the nucleotide-based drugs developed to target serine proteases in the article, I did not address how nucleic acid drugs exert their inhibitory effects in Section 2. We have added a section in the revised manuscript that explains the mechanism by which nucleic acid drugs inhibit serine proteases, located in lines 157-166. The content is as follows:

"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."

7. The title of section 4 referred to biomacromolecular inhibitors as endogenous protein inhibitors and antibodies but some of the peptide inhibitors mentioned in previous sections are also biomacromolecular inhibitors. Besides, it is written in a way that suggests SPINK1 is a serpin inhibitor when it belongs to the Kazal family, previously described in another section of the manuscript. I recommend, checking and correcting these issues.

Response: Thanks for your suggestion. The original manuscript indeed contained multiple instances of repetitive descriptions of peptide inhibitors across different sections, which may have confused the understanding of this review. Following your suggestion, we have reorganized all content related to peptide inhibitors in the article. First, we have reclassified peptide inhibitors under the category of biomacromolecules. Secondly, we have included endogenous serine protease inhibitors under the peptide inhibitor category and reclassified the endogenous inhibitor SPINK1 into KTSPI.