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Neutrophil serine proteases

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Abstract

The identification and characterization of the four active neutrophil serine proteases (NSPs) have provided a better understanding of their roles in various physiological and pathological processes. The availability of appropriate tools such as substrates, inhibitors, and activity-based probes (ABPs) for studying their activity and functions in cells has become increasingly important. In this paper, the authors provide a comprehensive overview of the current knowledge on the tools available for studying NSPs. The substrates, inhibitors, and ABPs developed to date are described, including their strengths and limitations. The authors also discuss the potential implications of these tools for future research on NSPs, including their potential use in the development of new therapeutics for various diseases. Overall, this paper highlights the importance of understanding the activity and functions of NSPs and provides valuable information on the tools available for studying these proteases.

Keywords: Neutrophils, neutrophil serine proteases (NSPs), substrates, inhibitors, activity-based probes (ABPs)

INTRODUCTION

Neutrophils are the most abundant group of leukocytes normally occurring in human blood, which play an essential role in the innate branch of the immune system. The main functions of this type of cell are to fight infections and promote an inflammatory response to the onset of diseases caused by bacteria or fungi[1].

Neutrophils are activated by the emergence of specific danger signals such as inflammatory cytokines, molecules derived from pathogens, or a host tissue damage signal, and rapidly relocate from the peripheral blood into the inflammatory or damaged sites. As a part of the immune system's first line of defense, neutrophils employ various mechanisms in order to eliminate the invading pathogens as well as to regulate inflammatory processes, including chemotaxis, phagocytosis, the release of reactive oxygen species (ROS) generated by the NADPH oxidase, formation of extracellular chromatin filaments containing granule-derived proteins (named neutrophil extracellular traps; NETs), and degranulation[1,2]. However, these protective mechanisms can also be destructive to host cells; therefore, neutrophil production, maturation, distribution, and disposal need to be strictly regulated[3].

Neutrophil granules contain numerous proteolytic enzymes of four different classes of proteases: cysteine proteases (e.g., cathepsin C), aspartyl proteases (e.g., cathepsin D, cathepsin E), metalloproteases (e.g., collagenases, gelatinases), and serine proteases. So far, four active neutrophil serine proteases (NSPs) have been identified: neutrophil elastase (NE), cathepsin G (CatG), proteinase 3 (PR3), the recently discovered neutrophil serine protease 4 (NSP4), and azurocidin (CAP-37), an inactive serine protease displaying an antimicrobial activity[4]. The active NSPs also play an important role in the antimicrobial activity of neutrophils. They are involved in the degradation of pathogens and the regulation of inflammatory responses. NSPs are chymotrypsin-like serine proteases that contain a conserved serine residue in the catalytic triad His_{57}-Asp_{102}-Ser_{195} (chymotrypsin numbering), and the hydroxyl group of this residue is responsible for performing a nucleophilic attack on the carbonyl carbon of the scissile peptide bond[5] [Figure 1]. The proteolytic activity of release NSPs at the inflammatory sites is regulated by the presence of several endogenous inhibitors, members of the serpin family, including α2-macroglobulin (α2-MG), α1-protease inhibitor (α1-PI), secretory leucoprotease inhibitor (SLPI), leucocyte elastase inhibitor (LEI), and elafin[4,6-8].

Neutrophil serine proteases are synthesized as inactive zymogens in the bone marrow during early granulocyte development and contain a dipeptide structure at the N-terminal. These zymogens undergo maturation, which activates the enzymes and produces active neutrophil serine proteases. This N-terminal proteolytic processing of the inactive pre-form of NSPs takes place in the endoplasmic reticulum and depends on the activity of dipeptidyl peptidase I (DPPI). Dipeptidyl peptidase I, aminodipeptidase, also known as cathepsin C, is a cysteine protease located in the primary granules[5,9]. Under normal physiological conditions, the activity of NSPs must be controlled in order to avoid the development of pathological states or autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, chronic respiratory diseases (e.g., chronic obstructive pulmonary disease, emphysema, pulmonary fibrosis), and cancer[10-14].

HUMAN NEUTROPHIL ELASTASE

Human neutrophil elastase (HNE), also known as leukocyte elastase (EC 3.4.21.37), is a globular glycoprotein belonging to the chymotrypsin family. HNE is located in neutrophils, eosinophils, mast cells, monocytes, keratinocytes, and fibroblasts. This 29 kDa protease consists of a single polypeptide chain of 218 amino acids and two asparagine-linked carbohydrate chains localized at Asn95 and Asn144. The presence of four disulfide bridges and 19 arginine residues helps stabilize the structure, resulting in basic properties and an isoelectric point around 10-11 [Figure 2]. The primary structure of HNE exhibits homology with other NSPs, PR3 (57%) and CatG (37%). The substrate specificity of HNE is very similar to PR3, with both enzymes cleaving substrates after small aliphatic residues (Ala, Val, GABA, and norVal)[15].

This enzyme has a broad physiological function, including the degradation of elastin and other extracellular proteins like collagen (type I-IV), fibronectin, laminin and proteoglycans, and it has coagulation factors

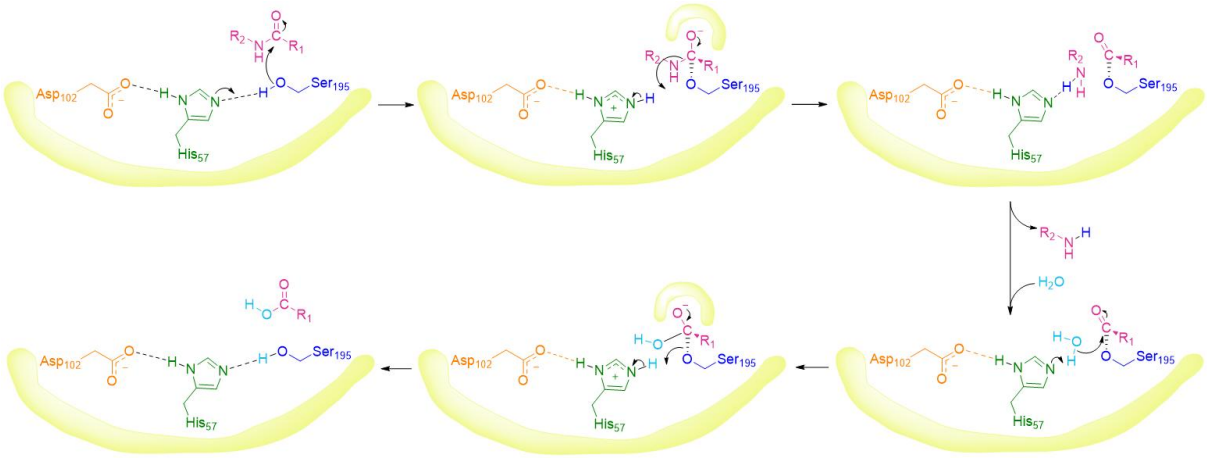

Figure 1. Mechanism of action neutrophil serine proteases.

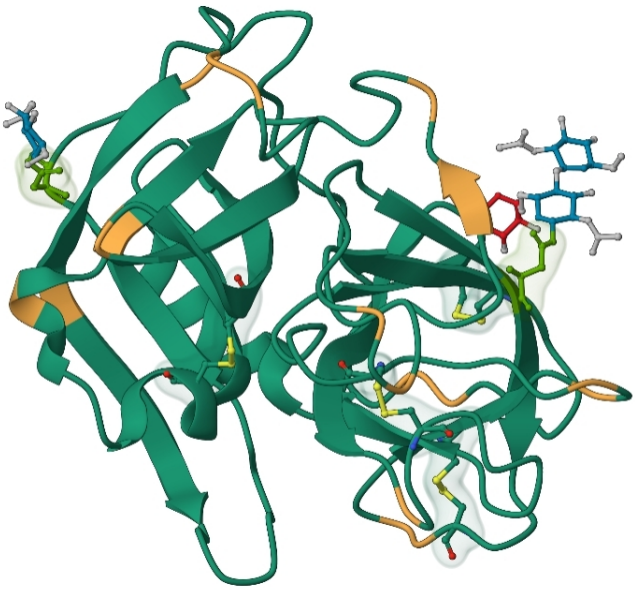

Figure 2. Structure of human neutrophil elastase; (green: Asn95 and Asn144, orange: Arg residues, yellow: disulfide bridges; based on 3Q76.pdb)[16].

(fibrinogen, factors V, VII, XII, and XIII), plasminogen, immunoglobulins (IgG, IgA, and IgM), thrombomodulin, platelet, complement factors (C3, C5), complement receptors, and the coat protein of HIV (gp120)[15]. HNE is also responsible for the degradation or activation of many essential host immune molecules, such as interleukins (IL-1β, IL-2, IL-6, IL-8, IL-12p40, and IL-12p70) or tumor necrosis factor. Furthermore, HNE processes the surface of toll-like receptors TLR2, TLR4, CD14, and tumor necrosis factor receptors. It can also degrade other neutrophil proteases and proteases inhibitors, resulting in both their activation and inactivation[17].

HNE is an integral component of NETs, which form large web-like structures containing DNA, histones, and other granular proteins such as proteinase 3, myeloperoxidase, or high mobility group protein B1[18]. NETs play an essential role as a trap for extracellular pathogens in the first-line defense response of the

innate immune system. There are forms and releases in a process known as NETosis, a unique type of cell death, after recognizing specific pathogens. NETs persistence is regulated by DNase I and DNase-like proteins, which are responsible for maintaining a balance between the formation and degradation of neutrophil extracellular traps[19,20]. Abnormal level of release NETs can be associated with the pathogenesis of different disorders, including lung injury[19,21], nephritis[22], cardiovascular disease[23], autoimmune disorders (e.g., rheumatoid arthritis, systemic lupus erythematosus, antiphospholipid syndrome[18,22]), as well as lung, breast or pancreatic cancer progression[24,25]. In the tumor microenvironment, the activity of release NE from NETs may also correlate with the formation of breast or colorectal cancer metastases[26,27]. Additionally, recent studies confirm that it is also related to COVID-19 due to SARS-CoV-2 infection can directly induce the increased formation of NETs in neutrophils[28-30].

The potent activity of NE is regulated by endogenous inhibitors, mainly by serpins such as α1-PI, α2-MG, α1-antitrypsin (α1-AT), SLPI, and elafin[15]. The uncontrolled outflow of HNE causes the degradation of extracellular matrix components and destroys alveolar epithelial cells, which may lead to the development of pulmonary disease[17]. Accumulations and excessive HNE activation in the lung are associated with the pathogenesis of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis, or pneumococcal pneumonia. HNE is also involved in rheumatoid arthritis due to the degradation of the matrix and destruction of cartilage components[12,15,17,31].

HNE Synthetic substrates

The most common substrates used for routine assay of HNE activity, which are hydrolytically stable at neutral pH, are chromogenic and fluorogenic peptide substrates. A synthesis of these simple compounds was initially described in 1979. The values of K_M, k_{cat}, and k_{cat}/K_M for the most potent chromogenic substrate MeO-Suc-Ala-Ala-Pro-Val-*p*NA (λ_{ex} = 320 nm, λ_{em} = 490 nm) are respectively 0.14 mM, 17 s^{-1}, 12 × 10^4 $M^{-1}s^{-1}$, and for fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-AMC (λ_{ex} = 370 nm, λ_{em} = 460 nm) the same values are 0.29 mM, 3.3 s^{-1} and 11 × 10^3 $M^{-1}s^{-1}$[32].

Wysocka *et al.* have described the synthesis and characterization of several fluorescent substrates displaying FRET for HNE[33]. The best parameters were obtained for PEG-FAM-Ala-Pro-Glu-Glu-Ile-Met--Asp-Arg-Gln-BAD [where PEG = 2-(2-(2-aminoethoxy)ethoxy)acetic acid, FAM = Lys(Fam)-OH, Fam = 5(6)-fluorescein, BAD = Ala(Bad)-OH], with the K_M of 24.2 ± 3.1 μM, k_{cat} of 6.2 ± 0.9 s^{-1}, and k_{cat}/K_M of 37.9 × 10^4 $M^{-1}s^{-1}$[33].

In 2013 Sun *et al.* described a design and simple synthesis of a pentafluoroethyl conjugated 7-amino-4-trifluomethylcoumarin (AFC) as the first non-peptide-based fluorescent probe and a substrate for NE[34]. This low molecular weight compound, 2,2,3,3,3-pentafluoro-*N*-(2-oxo-4-(trifluoromethyl)-2*H*-chromen-7-yl) propenamide, with high specificity (K_M = 20.45 ± 1.85 μM, k_{cat} = 21.84 ± 4.37 min^{-1}, k_{cat}/K_M = 1.07 $\mu M^{-1}min^{-1}$) was a promising candidate for tracing elastase's activity in human serum. However, the green fluorescence of this fluorophore limited its use in tests on cells and animal models[34].

HNE ABPs and Inhibitors

Prolastin®, a purified α1-antitrypsin, is the first neutrophil elastase inhibitor available on the market used in the treatment of α1-AT deficiency[15]. Elaspol®, also known as sivelestat or ONO-5046 (1), is the second NE inhibitor approved for use and the first non-peptide inhibitor. Silvelestat is applied in Japan and South Korea for the treatment of ALI and ARDS associated with systemic inflammatory response syndrome. This molecule is known as a highly specific and effective competitive NE inhibitor with IC_{50} and K_i of 44 nM and 200 nM[35]. Alvelestat, AZD9668 (2), is a reversible oral highly selective NE inhibitor with IC_{50} and K_i of 12 nM and 9.4 nM. This inhibitor is currently in phase II of clinical trials to treat patients with bronchiolitis

obliterans syndrome (BOS). Compound BAY 85-8501 (3) is a selective, reversible HNE inhibitor, with IC_{50} of 0.065 nM, and is also currently in phase II of clinical trials to treat bronchiectasis[15] [Figure 3].

Peptides chloromethyl ketone (CMK) are well-known inhibitors of HNE, with the most effective MeO-Suc-Ala-Ala-Pro-Val-CMK (4) with a $k_{obs}/[I]$ value of 922 $M^{-1}s^{-1}$[36] [Figure 4]. This group of inhibitors was useful for HNE structural studies and still serves as a cross-referent for newly developed HNE inhibitors. However, peptide-CMK has not been used in any clinical trials or therapy due to its high reactivity and potential toxicity[37].

Compounds belonging to a group of diaryl esters of 1-aminoalkylphosphonate are well-known as highly selective and specific irreversible inhibitors of several serine proteases including NSPs. Compound 61 (5) is a peptide derivative of diaryl ester of 1-aminoalkylphosphonate [Figure 5], named 2-(4-(2-((*S*)-1-((*S*)-2-((*R*)-1-(Bis(4-(methylthio)phenoxy)-phosphoryl)-2-methylpropylcarbamoyl)pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-2-oxoethoxy)phenoxy)acetic acid, and is an example of an irreversible and selective inhibitor of HNE with k_{inact}/K_I of 2,353,000 ± 89,000 $M^{-1}s^{-1}$. This group of inhibitors shows great stability in human plasma and PBS. The stability of irreversible interaction at elastase-inhibitor (compound 61) complex in time was confirmed; after 50 days of incubation, HNE recovered only 15% of initial activity[38].

Diazaborines represent a new class of inhibitors of serine proteases with boron-based (B-N) heterocycle warheads, which can be used as a boronic acid replacement with similar activity and selectivity but better stability in plasma. This group has been described as reversible covalent inhibitors with selectivity toward HNE, and without activity against urokinase, trypsin, thrombin, kallikrein, and chymotrypsin. Cytotoxicity assay based on a human cell line HEK 293T confirms non-toxicity of diazaborines in concentrations of up to 100 μM after 48 h of incubation. The best results were obtained for the *N*-*p*-toluenesulfonyl substituent compound 18 (6) (IC_{50} = 2.7 ± 0.6 μM) and compound 19 (7) (IC_{50} = 0.7 ± 0.6 μM)[39] [Figure 6].

A promising class of covalent inhibitors of HNE with high selectivity is a group of sulfur fluoride exchange (SuFEx)able derivatives. From a set of 105 synthesized compounds, a simple benzenoid compound 24 (8), 2-(fluorosulfonyl)phenyl fluorosulfate, shows the best potential to inhibit HNE [Figure 6]. In a kinetic assay using MeOSuc-AAPV-AMC as a substrate, after 10 min of incubation, compound 24 obtains the best results with an IC_{50} value of 0.24 ± 0.02 μM. 2-(fluorosulfonyl)phenyl fluorosulfate in a test against a panel of serine proteases proves selective inhibition of HNE without the inhibitory effect on CatG[40].

Compounds belonging to benzenesulfonic acid derivatives are known as competitive inhibitors of HNE. According to Xu *et al.*, from a series of synthesized *ortho*- and *meta*-substituted benzenesulfonic acids, only one, a compound 4f (9), shows moderate activity toward HNE, with an IC_{50} value of 35.2 μM[41] [Figure 7]. The cytotoxicity of the chosen compound was measured with five mammalian cancer cell lines, including MCF-7, BGC823, A549, HepG2, and HTC116, and no inherent cytotoxicity was observed[41].

Sulfonated nonsaccharide heparin mimetics are a group of novel potent, selective, non-competitive, and allosteric HNE inhibitors, without specificity toward other proteases, like plasmin, trypsin, and chymotrypsin, as well as heparin-binding coagulation proteins. Compound 3 (10), a hexa-sulfonated derivative, shows the most potent HNE inhibitor activity with an IC_{50} value of 0.22 ± 0.00 μM [Figure 8]. However, this compound does not scientifically affect the proliferation of the three human cell lines, including MCF-7, CaCo-2, and HEK-293 at a concentration of up to 10 μM[42].

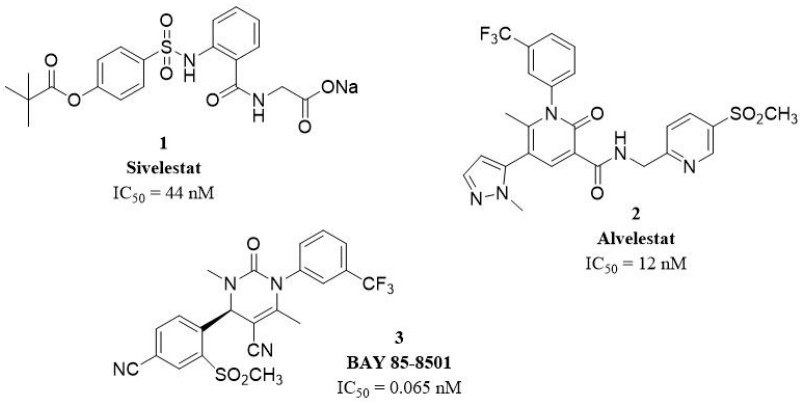

Figure 3. Structures of selected HNE inhibitors approved for use or in clinical trials[15].

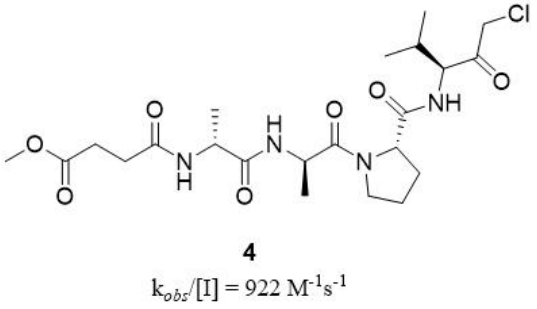

Figure 4. Structure of peptide chloromethyl ketone HNE inhibitor[37].

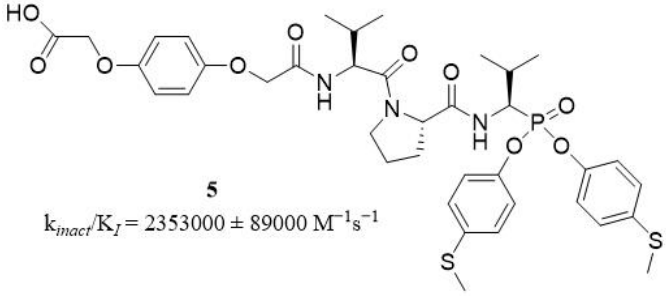

Figure 5. Structure of irreversible HNE inhibitor[38].

In 2016, a small set of 1,2,3-triazole-based 4-oxo-β-lactam derivatives was synthesized and described as potential inhibitors and ABPs of HNE. The previous study confirms that the presence of oxo-β-lactam is essential for the interaction between the inhibitor and S_1 pocket in HNE. The structure of the presented ABPs was based on the structure of the compound 2g (11), the most potent oxo-β-lactam HNE inhibitor with an IC_{50} of 14 ± 4 nM [Figure 9]. Finally, three different ABP structures were obtained - NBD-, fluorescein-, and biotin-tagged probes - respectively compound 3 (12) (IC_{50} = 56 ± 10 nM), 4 (13) (IC_{50} = 66 ± 2 nM), and 5 (14) (IC_{50} = 118 ± 10). These fluorescent small-warhead-based ABPs enable visualization of extracellular HNE and its intracellular localization[43].

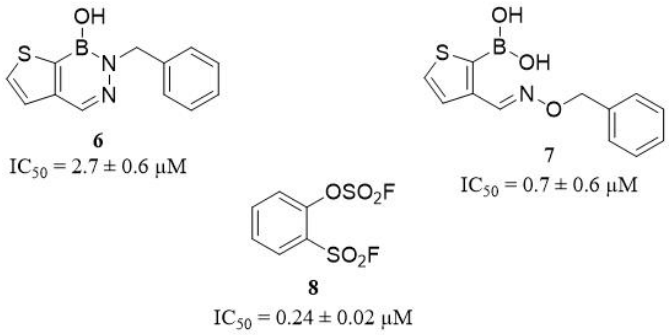

Figure 6. Structures of covalent HNE inhibitors[39,40].

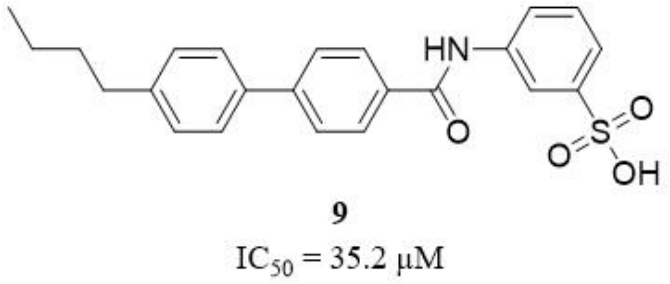

Figure 7. Structure of competitive HNE inhibitor[41].

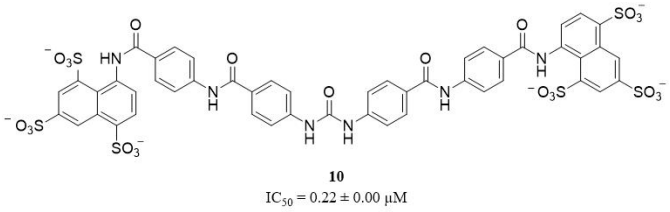

Figure 8. Structures of non-competitive allosteric HNE inhibitor[42].

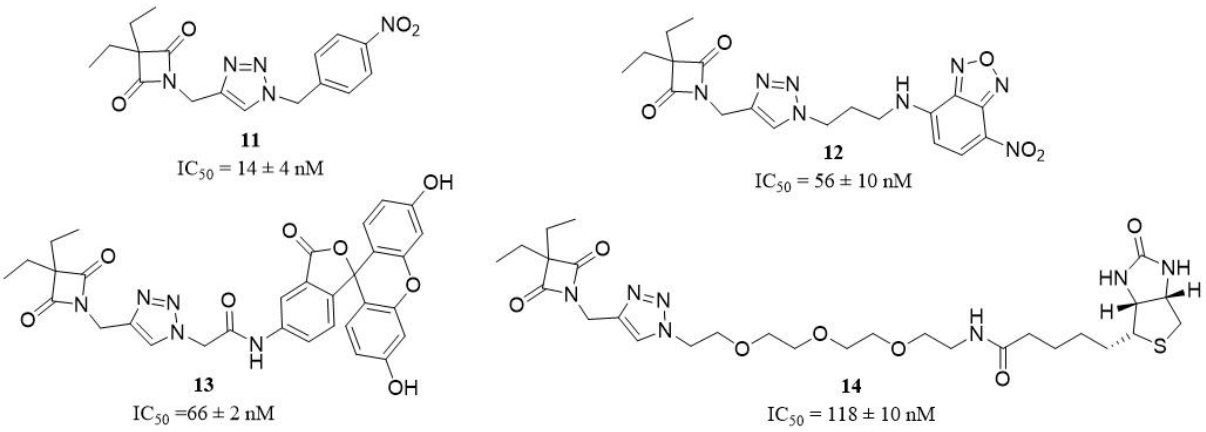

Figure 9. Structures of oxo-β-lactam HNE inhibitors[43].

In 2018, Schulz-Fincke *et al.* described a synthesis of compound 8 (15) as a potent inhibitor and ABP of HNE[44]. This compound belongs to sulfonyloxyphthalimide derivatives and contains coumarin 343 as a fluorescent tag [Figure 10]. The ability of inhibition was investigated with a spectroscopic assay with the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA. This activity-base probe shows selective activity for HNE with a significant K_i value of 6.85 ± 0.39 nM and IC_{50} value of 0.0189 ± 0.0019 μM. The application of these probes enables the detection of NE in neutrophil cell lysate, with the proviso that its selectivity to PR3 is determined[44].

PK105b (16) is a fluorescent activity-based probe that contains sulfo Cy5 as a fluorophore, a region of unnatural specific amino acid, and diphenylphosponate as a warhead [Figure 10]. According to Anderson *et al.*, this compound is an example of an irreversible HNE probe[45]. Application of PK105b facilitates the detection of NE activity in tissue lysates. Unfortunately, this APB was lack of selectivity to HNE, and cross-reactivity with other serine proteases is also observed[45].

Liu *et al.* posit that NEP (17) is a small-molecule-based near-infrared fluorogenic probe that could be used as a tool for highly specific and quick detection of HNE *in vitro* and *in vivo*[46] [Figure 10]. NEP is a molecule based on hemocyanin dye [Figure 10] with the optimum activity observed at pH 7.0 and a temperature of 37 °C in an aqueous solution, under conditions similar to physiological. The application of this tool *in vitro* and *in vivo* was tested using three different cell lines, including RBL-2H3, A549, and MDA-MB-231, as well as an ALI mice model. The detection limit of this probe is about 29.6 ng/mL. NEP shows high specificity at the long emission wavelength for HNE (λ_{ex} = 590 - 650 nm, λ_{em} = 660 - 730 nm, λ_{em}max = 700 nm) with negligible effects of chymotrypsin, trypsin, carboxypeptidase A, and carboxypeptidase B. the application of this molecule can be helpful in monitoring trafficking exogenous and endogenous NE in cells and living organisms, and may serve as a potential tool for diagnosis HNE-related disease[46].

Bacteria, plants, fungi or venomous animals are known as potential sources of new HNE inhibitors. Many of these compounds have been described in the literature; unfortunately, most of them have shown low potency of action, selectivity and stability in physiological conditions[47,48].

AvKTI is a peptide inhibitor of HNE, as well as trypsin, chymotrypsin, and plasmin, isolated from a spider *Araneus ventricosus*. This peptide with a sequence KDRCLLPKVTGPCKASLTRYYYDKDTKACVEFIYGGCRGNRNNFKQKDECEKACTDH is the first described Kunitz-type serine protease inhibitor with antifibrinolytic and anti-elastolytic activity. AvKTI shows the ability to inhibit HNE with an IC_{50} value of 446.93 nM and a K_i value of 169.07 nM. However, the inhibitory activity against plasmin was 44.4-fold stronger than against elastase (IC_{50} = 10.07 nM and K_i = 4.89 nM)[49].

ShSPI, a natural peptide isolated from venomous *Scolopendra hainanum* is a typical Kazal-type protease inhibitor of HNE. This bioactive peptide is composed of 34 amino acids and contains a cysteine-stabilized α-helix, two-stranded anti-parallel β-sheet, and two disulfide bonds. ShSPI is a non-competitive inhibitor with a K_i value of 12.6 ± 2 nM, and its equilibrium dissociation constant K_D to HNE is 4.2×10^{-8}. Due to its significant stability in physiological conditions after co-incubation with human plasma, ShSPI may be a good candidate for the design of new drugs for cardiopulmonary diseases[50].

Loggerpeptins A-C and molassamide, natural peptides derived from marine cyanobacteria, consist of 19-membered ring cyclodepsipeptides containing the modified glutamic acid residue 3-amino-6-hydroxy-2-piperidone [Figure 11]. All peptides have been tested as inhibitors of HNE and compared with Sivelestat

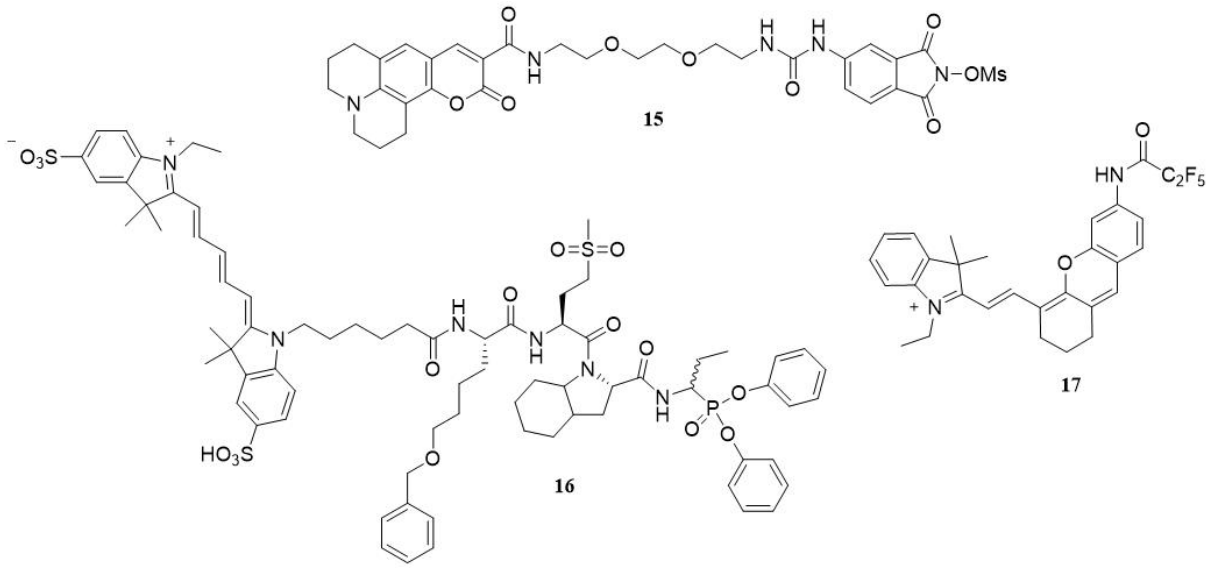

Figure 10. Structures of activity-based probes for HNE[44-46].

inhibition (IC_{50} = 0.06 μM). The kinetic test confirms the inhibitor activity of loggerpeptin A (18) (IC_{50} = 0.29 ± 0.04 μM), loggerpeptin B (19) (IC_{50} = 0.89 ± 0.09 μM), loggerpeptin C (20) (IC_{50} = 0.89 ± 0.09 μM) and molassamide (21) (IC_{50} = 0.62 ± 0.38 μM). Molassamide, the structure with the Abu unit in the cyclic core, was the most potent and selective analog against HNE[51] [Figure 11]. Lyngbyastatin 4 (22) and lyngbyastatin 7 (23) are similar cyclic depsipeptide-type structures, isolated from cyanobacteria *Lyngbya* spp., exhibit HNE inhibitory activity with an IC_{50} value of 49 nM and 29 nM, respectively[48] [Figure 11]. A synthetic methodology for gram-scale synthesis of key building blocks of lyngbyastatin 7 with improved yields was recently developed and optimized. Due to the potency, selectivity and remarkable stability in human serum of lyngbyastatin 7, this macrocyclic peptide can play a significant role in the further development of this class of HNE inhibitors[52]. Tutuilamide A (24) contains uncommon vinyl-chloride-containing residue isolated from cyanobacterium *Schizothrix* sp. This natural cyclodepsipeptide shows significant inhibitory activity against elastase with high selectivity (IC_{50} = 1.18 nM)[53] [Figure 11].

PROTEINASE 3

Proteinase 3 (PR3), also referred to as myeloblastin, azurophil granule protein-7, or p29b (EC 3.4.21.76), is the most abundant protease of the four NSPs mainly localized in azurophilic granules of neutrophils; however, it is also found in secretory vesicles. This protein is a 29 kDa chymotrypsin serine protease consisting of 222 amino acids and four disulfide bonds, which help stabilize the structure[54]. PR3 is the closest homolog of HNE; therefore, both enzymes have similar preferences at the S1 binding pocket for small aliphatic amino acid residues (Ala, Val, GABA, and norVal)[15].

One of the main functions of PR3 is the degradation of extracellular matrix components, including collagen, elastin, fibronectin, and laminin, leading to tissue remodeling and inflammation which is associated with chronic obstructive pulmonary disease (COPD) and granulomatosis with polyangiitis (GPA)[54,55]. Furthermore, PR3 is responsible for the cleavage of proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-17, IL-18, IL-32, TNF-α), receptors (e.g., C5a receptor), and heat shock protein, which results in antibacterial peptides. Proteinase 3 shows bactericidal activity through cleavage of the pro-microbicidal protein hCAP-18 into the antibacterial peptide, and by co-creating NETs[54,56].

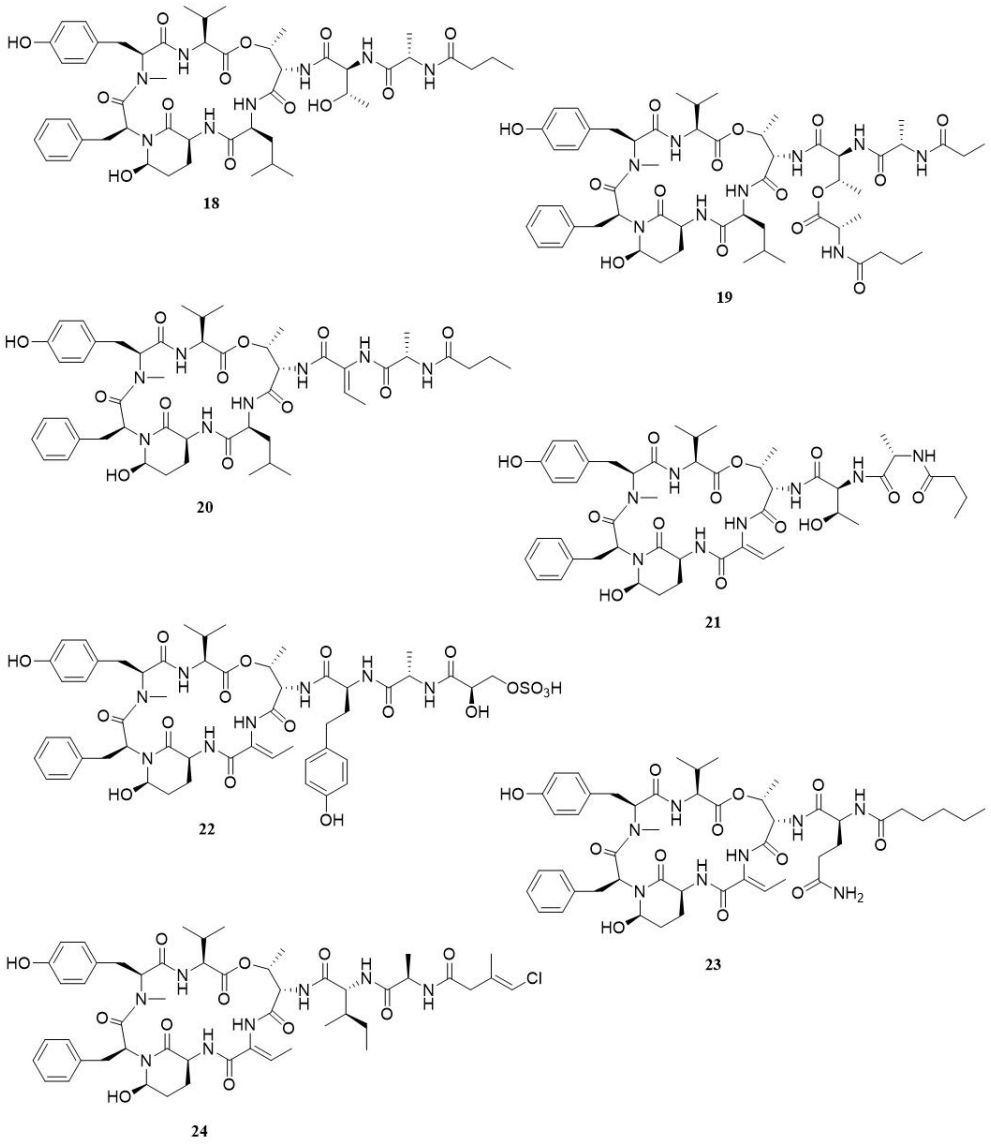

Figure 11. Structures of natural cyclodepsipeptide-type HNE inhibitors[48].

Overexpression of PR3 can be associated with the development of autonomous cell growth in leukemia. PR3 also plays a major role as an autoantigen in many diseases, including granulomatosis with polyangiitis (GPA) and idiopathic interstitial pneumonia, and is also a target of anti-neutrophil cytoplasmic antibodies (ANCA) in vasculitis. In physiological conditions, the PR3 activity is regulated by natural inhibitors, including serpins (α_1PI, monocyte neutrophil elastase inhibitor), chelonians (elafin, secretory leukocyte protease inhibitor), and α2-macroglobulin[54,55,57].

PR3 Synthetic substrates

For a long time, a synthesis of selective substrate for PR3 was difficult, mainly due to it being closely related to HNE and having a similar preference for small aliphatic amino acid residues at P1. In 2012, Epinette *et al.* published a new structure of selective FRET-type peptide substrate for PR3 without activity towards other serine proteases, especially HNE, ABZ-Val-Ala-Asp-norVal-Ala-Asp-Tyr-Gln-EDDnp with a K_M value of 1.2 μM[58]. Another structure of selective FRET-type PR3 substrate consists of ABZ and Tyr(3-NO_2)

as a pair of donor and acceptor, ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2 with a K_M value of 3.2 mM and k_{cat}/K_M value of 1596 × 10^3 $M^{-1}s^{-1}$, was reported one year later[59]. A structure of simple chromogenic PR3 peptide substrates has also been developed, including Ac-Pro-Tyr-Asp-Ala-*p*NA and Ac-Asp-Tyr-Asp-Ala-*p*NA, with a k_{cat}/K_M value of 4201 ± 29.7 $M^{-1}s^{-1}$ and 162 ± 12.8 $M^{-1}s^{-1}$ respectively[60], as well as biotinylated derivative Bt-Val-Tyr-Asp-nVal-*p*NA with a k_{cat}/K_M value of 80510 ± 2973 $M^{-1}s^{-1}$[61].

PR3 ABPs and Inhibitors

Azapro-3 (25), ABZ-Val-Ala-Asp-aza(nor)Val-Ala-Asp-Tyr-Gln-Tyr(3-NO_2), is an example of the first selective, non-covalent, reversible azapeptide inhibitor based on a structure of one of the FRET substrates for PR3 [Figure 12]. This competitive inhibitor shows high activity and selectivity for PR3 with a K_i value of 1.5 μM, with a nonsignificant inhibitory effect of HNE, and without any reactivity towards other proteases, including CatG, chymotrypsin, and granzyme B[58].

Another example of a PR3 inhibitor based on a sequence of its specific FRET substrate is ABZ-Val-Ala-Asp-(nor) Val[Ψ]($COCH_2$)-Ala-Asp-Tyr-Gln-EDDnp (26), a ketomethylene peptide derivative [Figure 12]. This compound was characterized as a competitive and reversible inhibitor with an IC_{50} value of 1.91 μM, selective for PR3 over HNE[62].

Compound 6 (27), an analog of 2-aminobenzaldehyde oxime [Figure 13], was reported as the most potent non-peptide inhibitor for PR3 from a synthesized set of compounds, with an IC_{50} value of 0.22 ± 0.02 μM. However, this inhibitor showed a lack of selectivity due to better activity toward HNE (IC_{50} = 0.05 ± 0.01 μM)[63].

Structures based on aminophosphonates constitute a well-known group of potent and selective PR3 inhibitors, which have been recently reviewed[56]. The most important advantage of this class of inhibitors is their lack of reactivity with other proteases, including threonine, cysteine, aspartyl, and metalloproteinases. The first series of peptidyl derivatives α-aminoalkylphosphonate diaryl esters as specific, irreversible inhibitors of PR3 was synthesized in 2014. This most potent covalent inhibitor, which acts as the transition state analog, was Ac-Pro-Tyr-Asp-Ala^P(O-C_6H_4-4-Cl)$_2$ (**28**) with the $k_{obs}/[I]$ value of 154 ± 3 $M^{-1}s^{-1}$. A biotinylated derivative of this compound, Bt-[PEG]$_{66}$-Pro-Tyr-Asp-Ala^P(O-C_6H_4-4-Cl)$_2$ (29), was an example of ABP, which can be used to visualize PR3 in native conditions, with $k_{obs}/[I]$ value of 1163 ± 0.1 $M^{-1}s^{-1}$ [Figure 14]. However, this Bt-ABP lacked selectivity and showed activity toward HNE ($k_{obs}/[I]$ = 46 ± 0.1 $M^{-1}s^{-1}$)[60].

Several peptidyl biotinylated derivatives of α-aminoalkylphosphonate dsiaryl esters with a potential of ABPs for NSPs, including PR3 were described by Grzywa *et al.* Five of these structures, Bt-Val-Pro-Abu^P(O-C_6H_4-4-S-CH_3)$_2$, Bt-Val-Pro-Val^P(O-C_6H_5)$_2$, Bt-Val-Pro-Val^P(O-C_6H_4-4-S-CH_3)$_2$, Bt-Val-Pro-Leu^P(O-C_6H_4-4-$COOCH_3$)$_2$, Bt-LC-Suc-Phe-Val-Thr-(4Gu)Phg^P(O-C_6H_4-4-S-CH_3)$_2$, show activity toward PR3 with a $k_{obs}/[I]$ values of 5.1 × 10^3 ± 350, 1.6 × 10^3 ± 100, 1.6 × 10^4 ± 1,200, 1.9 × 10^4 ± 2,300, 2.1 × 10^3 ± 100 [$M^{-1}s^{-1}$], respectively. However, these ABPs do not show selectivity of action over HNE or CatG[64] [Table 1].

In 2018, the same research group reported a structure of a new biotinylated PR3 inhibitor, Bt-Val-Tyr-Asp-nVal^P(O-C_6H_4-4-Cl)$_2$ (30), with $k_{obs}/[I]$ value of 73,258 ± 5,342 $M^{-1}s^{-1}$, and without significant inhibitory activity toward HNE[61] [Figure 14].

Table 1. Kinetic analysis of selected peptidyl biotinylated derivatives of α-aminoalkylphosphonate diaryl esters as inhibitors toward PR3, HNE, CatG

Compound	PR3 $k_{obs}/[I]$ [$M^{-1}s^{-1}$]	HNE $k_{obs}/[I]$ [$M^{-1}s^{-1}$]	CatG $k_{obs}/[I]$ [$M^{-1}s^{-1}$]
Bt-Val-Pro-Abu^{P}(O-C_6H_4-4-S-CH_3)$_2$	$5.1 \times 10^3 \pm 350$	$4.2 \times 10^5 \pm 7500$	5%[a]
Bt-Val-Pro-Val^{P}(O-C_6H_5)$_2$	$1.6 \times 10^3 \pm 100$	$1.8 \times 10^5 \pm 7850$	N.I.
Bt-Val-Pro-Val^{P}(O-C_6H_4-4-S-CH_3)$_2$	$1.6 \times 10^4 \pm 1200$	$5.5 \times 10^5 \pm 25000$	N.I.
Bt-Val-Pro-Leu^{P}(O-C_6H_4-4-$COOCH_3$)$_2$	$1.9 \times 10^4 \pm 2300$	$2.1 \times 10^5 \pm 750$	$1.6 \times 10^3 \pm 125$
Bt-LC-Suc-Phe-Val-Thr-(4Gu)Phg^{P}(O-C_6H_4-4-S-CH_3)$_2$	$2.1 \times 10^3 \pm 100$	N.I.	$2.4 \times 10^2 \pm 25$

[a]: Percent inhibition at 5 μm tested inhibitor. N.I.: < 5% inhibition was observed under assay conditions[64]. HNE: Human neutrophil elastase; CatG: cathepsin G; PR3: proteinase 3.

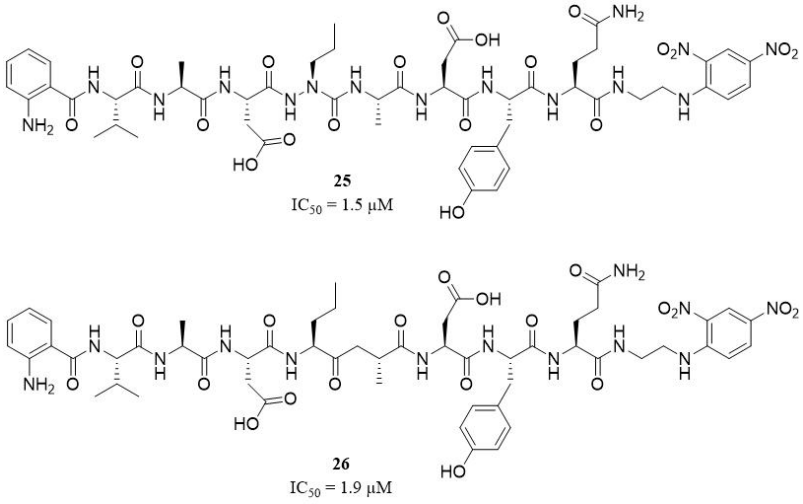

Figure 12. Structures of PR3 inhibitors based on a FRET substrate[58,62].

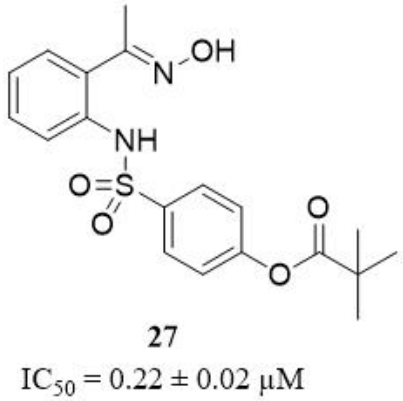

Figure 13. Structure of non-peptide PR3 inhibitor[63].

The most potent phosphonic inhibitor and fluorescent ABP against PR3 were designed by Kasperkiewicz *et al.*[65]. The structure of compound PK302 (31), with a $k_{obs}/[I]$ value of $1.4 \times 10^6 \pm 11$ $M^{-1}s^{-1}$, contains an optimal substrate sequence consisting of natural and unnatural amino acids [Figure 15]. However, this probe was not selective, and it showed weak reactivity toward HNE ($k_{obs}/[I] = 300 \pm 50$ $M^{-1}s^{-1}$)[65].

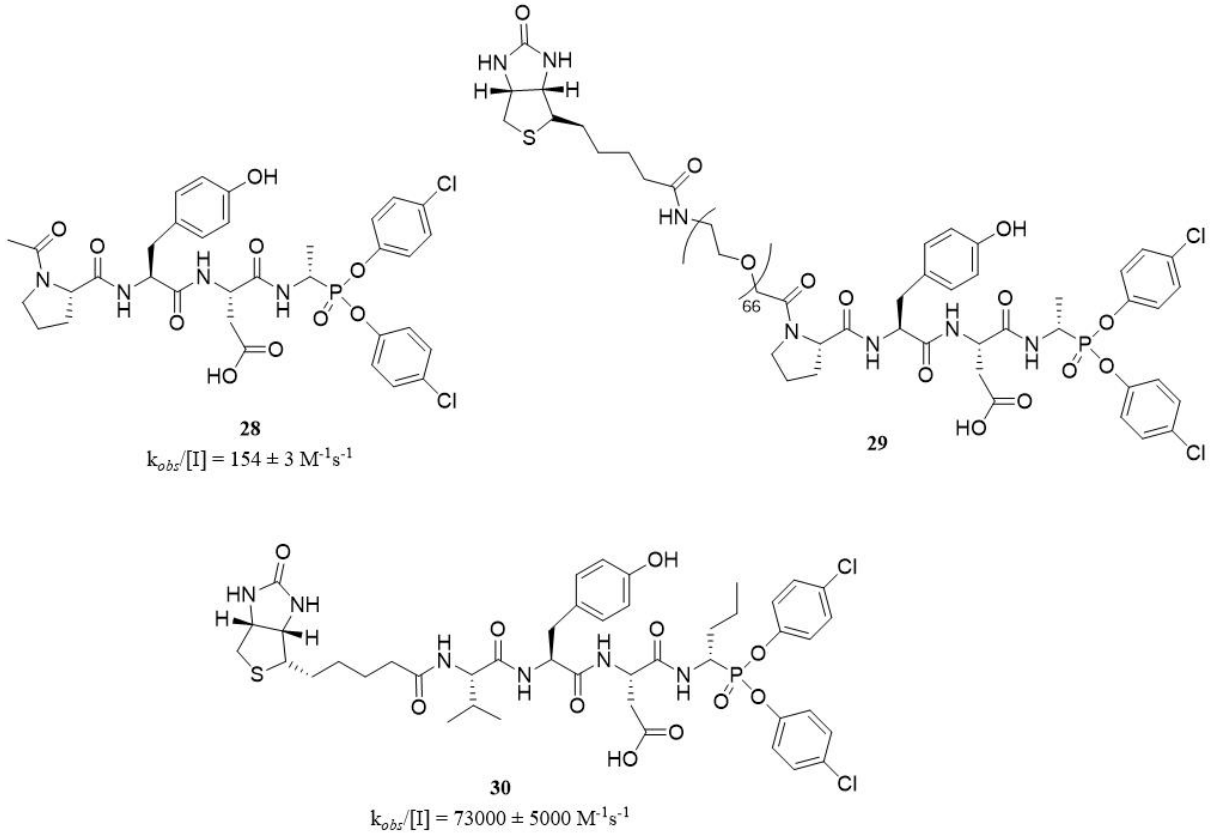

Figure 14. Structures of irreversible PR3 inhibitors and ABP[60,61].

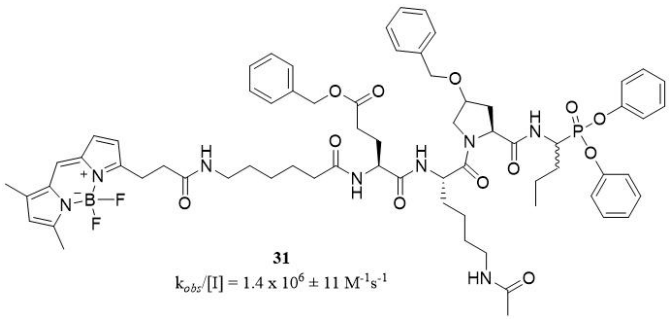

Figure 15. Structures of fluorescent PR3 activity-based probe[65].

Cyclic peptide compounds containing SFTI-variants can be an attractive group of new reversible, competitive inhibitors of PR3 with high stability in human serum. The most potent selective inhibitor toward PR3, from a synthesized set, was compound 3, c[GTCTAbuSIPPICNPN], a cyclized Gly1-Asn14 including a disulfide bond between Cys3-Cys11, with a K_i value of 9.8 ± 1.2 nM[66].

Pro3-SBP (**32**) is a near-infrared fluorescent (NIRF) substrate-based probe (SBP) designed and synthesized as a tool for monitoring active, secreted human PR3. The structure of a peptide hairpin loop consists of a specificity region (a PR3 recognition sequence) and an electrostatic zipper, driving a close vicinity of the FRET couple (sulfoCy5.5 and QSY21) [Figure 16]. Pro3-SBP (λ_{ex} = 684 nm, λ_{em} = 710 nm) was specifically

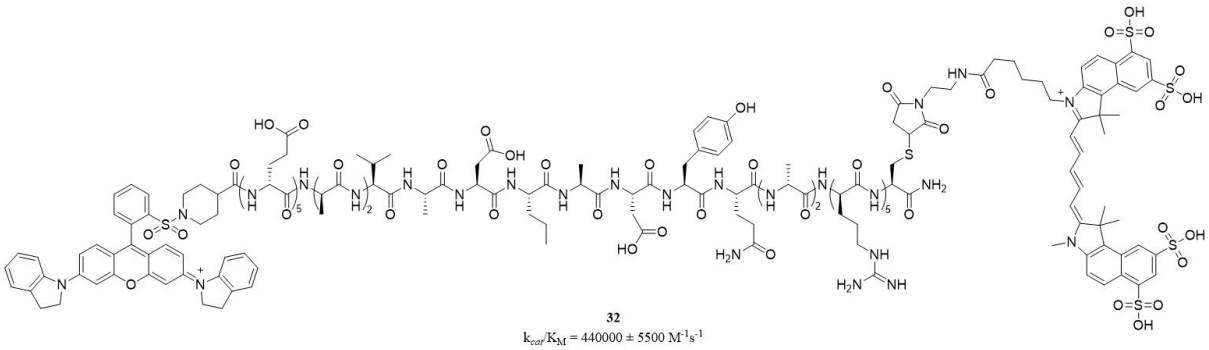

Figure 16. A structure of near-infrared fluorescent substrate-based probe of PR3[67].

hydrolyzed by extracellular PR3 with k_{cat}/K_M value of 440,000 ± 5,500 $M^{-1}s^{-1}$. Unfortunately, hydrolysis by HNE was also observed in the minority (k_{cat}/K_M = 33,000 ± 3,000 $M^{-1}s^{-1}$)[67].

CATHEPSIN G

Cathepsin G is expressed in neutrophils, mastocytes and monocytes. It is synthesized as a zymogen and is activated during packaging into granules by dipeptidyl peptidase I (DPPI) to an active form involved in several important physiological processes within the human body. CatG active site is typical of serine proteases and is composed of serine, histidine and aspartic acid. CatG structure represents the chymotrypsin fold[68]. CatG - similar to other neutrophil serine proteases - plays an important role in host defense mechanisms. CatG also mediates -platelet aggregation[69].

CatG activity also includes proteolytic processing of Ang-I and angiotensinogen and the production of angiotensin-II (Ang-II)[70]. CatG is also responsible for the activation of matrix metalloproteinases (MMPs-1, -2 and 3)[71]. CatG can activate collagenase, thus contributing to collagen and elastin degradation in human aneurysmal walls[72]. Serpin inhibitors- ACT and α1-PI are considered as the most effective endogenous inactivators of CatG[73]. The pathological effects connected with CatG activity arise when an imbalance between their levels and the levels of their major serum inhibitors, α1-PI and ACT occurs.

As CatG plays an important role in many biological events, it has become a target for the development of a set of peptides and peptidomimetics substrates, inhibitors and ABPs.

CatG Synthetic substrates

The first studies on CatG and inhibitors revealed unusual acceptance of both the bulky hydrophobic side chains of Phe or Leu, in the S1 pocket, and the basic side chains of Lys and Arg[74,75]. Solving of the crystal structure of the complex between human cathepsin G and peptidyl phosphonate inhibitor Suc-Val-Pro-PheP-$(OPh)_2$ showed that this dual specificity is caused by Glu226 residue, which is located at the bottom of the S1 pocket. This Glu226 residue interacts either with the basic side chain of Lys/Arg or with an edge of the aromatic ring of Phe[76].

In 1998, Polanowska *et al.* investigated the dual trypsin- and chymotrypsin-like specificities of CatG[74]. They synthesized a library of tetrapeptide p-nitroanilide substrates (Suc-Ala-Ala-Pro-Aaa-*p*-nitroanilide) to examine the specificity of the S1 binding pocket of human cathepsin G[74]. Their studies have shown/showed that cathepsin G can recognize both large hydrophobic and basic side chains, with a slight preference for Lys over Phe (1.3-fold) and Arg over Leu (1.1-fold). The best substrate from their studies was Suc-Ala-Ala-

Pro-Lys-*p*NA (33) (K_M = 2.75 × 10^{-3} M and k_{cat}/K_M 1,483 $M^{-1}s^{-1}$).

In 2007, Wysocka *et al.* presented studies about new chromogenic substrates of CatG[75]. The combinatorial chemistry methods enable the production of new, sensitive cathepsin G substrates. The introduction of the non-proteinogenic amino acid residue (4-guanidine-L-phenylalanine) in position P1 increases activity twice as high as in the case of Phe (Ac-Phe-Val-Thr-Gnf-Anb-NH_2 (34) K_M = 203 μM and k_{cat}/K_M 95,300 $M^{-1}s^{-1}$; Ac-Phe-Val-Thr-Phe-Anb-NH_2 K_M = 464 μM and k_{cat}/K_M 7,900 $M^{-1}s^{-1}$). The additionally obtained substrate is not cleaved by proteinase 3, human leukocyte elastase or chymotrypsin. A further modification by replacing the acetyl moiety with a residue of 7-methoxycoumarin-4-yl acetic acid (Mca) that served as a fluorescence donor and substrate elongation led to the Mca-Phe-Val-Thr-Gnf-Ser-Trp-ANB-NH_2 (35), the sequence with a specificity constant (K_M = 2 μM, k_{cat}/K_M = 252 × 10^3 $M^{-1}s^{-1}$) which is two orders of magnitude higher than that of the parent compound. The detection limit of CatG using this substrate is 70 pM[76]. Later in 2019, Groborz *et al.* developed new fluorogenic substrates of CatG based on a fluorescence quenching mechanism[77]. The most active inhibitor from their studies was OS-CG_11 (36) with k_{cat}/K_M = 206,854 $M^{-1}s^{-1}$ [Figure 17]. This substrate was also active against PR3 and displayed k_{cat}/K_M 146,824 $M^{-1}s^{-1}$[77].

CatG ABPs and Inhibitors

In 1991, two phosphonic inhibitors of CatG were reported[78]. The analog of phenylalanine: Cbz-Phe^P$(OPh)_2$ (37) and phenylglycine: Cbz-Phg^P$(OPh)_2$ (38) displays similar poor activity against CatG (k_{obs}/I = 76 $M^{-1}s^{-1}$ and k_{obs}/I = 91 $M^{-1}s^{-1}$ respectively) [Figure 18]. Further incorporation of the basic functional group into the aromatic side chain together with phenyl esters modification and peptide chain elongation resulted in the inhibitor Ac-Phe-Val-Thr-(4-guanidine)Phg^P$(OC_6H_4$-4-S-Me$)_2$ (39) with k_{obs}/I = 256,000 $M^{-1}s^{-1}$[79] [Figure 18]. Based on this inhibitor, Grzywa *et al.* developed a low-molecular-weight activity-based probe: Bt-LC-Suc-Phe-Val-Thr-(4Gu)Phg^P$(O$-C_6H_4-4-S-$CH_3)_2$ (40) [Table 2] with k_{obs}/I = 240 $M^{-1}s^{-1}$ which displayed absolute specificity toward CatG in western blotting analysis among two other neutrophil proteases- HNE and PR3[64] [Figure 18]. Another phosphonate base ABPs was reported by the Drag group in 2017[65]. From a series of compounds with a different fluorophore inhibitor 202 (41), it showed the best potency with 43,000 $M^{-1}s^{-1}$ [Figure 18]. The inhibitor 202 displayed high selectivity and did not react with other neutrophil serine proteases.

In 2015, Serim *et al.* reported quenched fluorescent activity-based probes based on mixed alkyl-aryl phosphonate esters[80] [Figure 18]. Compound 15 (42) could label the CatG in SDS-PAGE gel.

Isocoumarins equipped with detection tags were reported as CatG ABPs. Compound BIC5 showed k_{obs}/I = 59 $M^{-1}s^{-1}$ against CatG. BIC5 (43) was not specific towards CatG and could inhibit chymotrypsin and HNE (k_{obs}/I = 260 $M^{-1}s^{-1}$ and 96,000 $M^{-1}s^{-1}$ respectively)[81] [Figure 18]. In 2012, the Verhelst research group reported clickable isocumarin-based compounds that act as ABPs of serine proteases[82]. Compound IC14 (44) could label active CatG in lysates of mammalian cells (EL4 mouse lymphoma). The detection limit was 0.03% of total protein [Figure 18].

Recently, Kahler *et al.* developed aminomethyl phosphinate esters as inhibitors of serine protease. This group of compounds can interact with prime enzyme site. Primary compound testing revealed that inhibitor 13a (45) can react with CatG[83] [Figure 18].

NSP4

Human NSP4 (encoded by the gene PRSS57) is a trypsin-fold protease stored in neutrophil azurophilic granules[84,85]. Since the NSP4 discovery in 2012 by the D.E.Jenne research group, numerous studies

Table 2. Characterization of human neutrophil elastase inhibitors approved for use and in clinical trials

Name	Therapeutic application	Development phase	Features
Prolastin® (α1-antitrypsin)[15]	α1-antitrypsin deficiency and clinical evidence of emphysema	FDA approved	peptide reversible NE inhibitor recommended dose: 60 mg/kg body weight once a week (for Prolastin-C Liquid)
Elaspol® (Sivelestat/ ONO-5046)[35]	acute lung injury and acute respiratory distress syndrome associated with the systemic inflammatory response syndrome	approved for use in Japan and South Korea	non-peptide competitive NE inhibitor IC_{50} 44 nM; K_i 200 nM
Alvelestat (AZD9668)[15]	bronchiolitis obliterans syndrome	II phase clinical trials	reversible oral NE inhibitor IC_{50} 12 nM; K_i 9.4 nM
BAY 85-8501[15]	non-cystic fibrosis bronchiectasis	II phase clinical trials	reversible NE inhibitor, IC_{50} 0.065 nM; K_i 0.08 nM

NE: Neutrophil elastase.

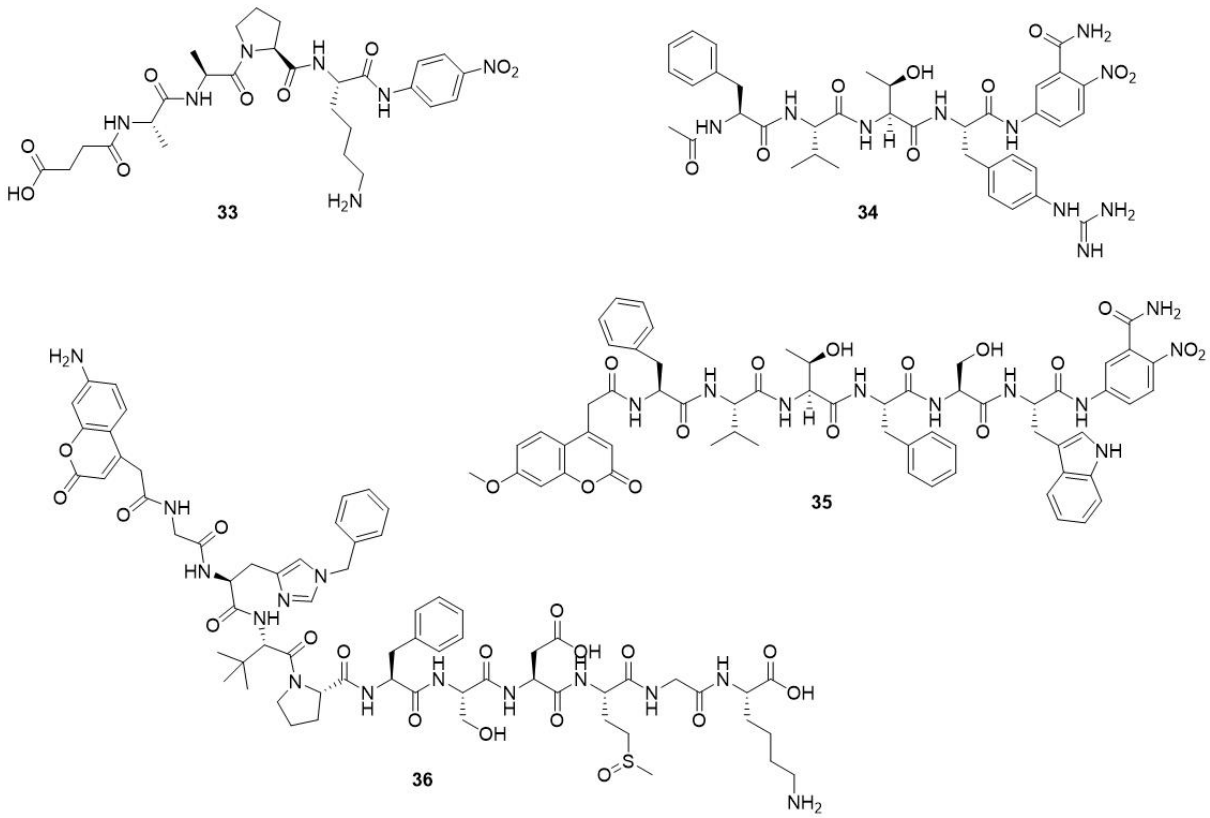

Figure 17. Synthetic substrates of CatG.

regarding NSP4 biology, substrate specificity and inhibitors development have been done. However, still, blank spots remain to be filled. NSP4, unlike other NSPs, has been conserved for over 400 million years from bony fish to humans[86]. NSP4 is the only known enzyme that cleaves substrates with post-translationally modified arginine residues, such as methylarginine and citrulline[87]. These attributes suggest that NSP4 could have another potential function, not typical of NSPs. In 2020, AhYoung *et al.* demonstrated that NSP4 plays an essential role in mast cell biology[88]. Their studies have shown/showed that NSP4 is present during early mast cell development and is critical for the regulation of levels of histamine and serotonin in the secretory granules of the developing mast cells. They discovered that NSP4 deficiency causes protection against mast cell/histamine-dependent vascular leakage. These findings open new

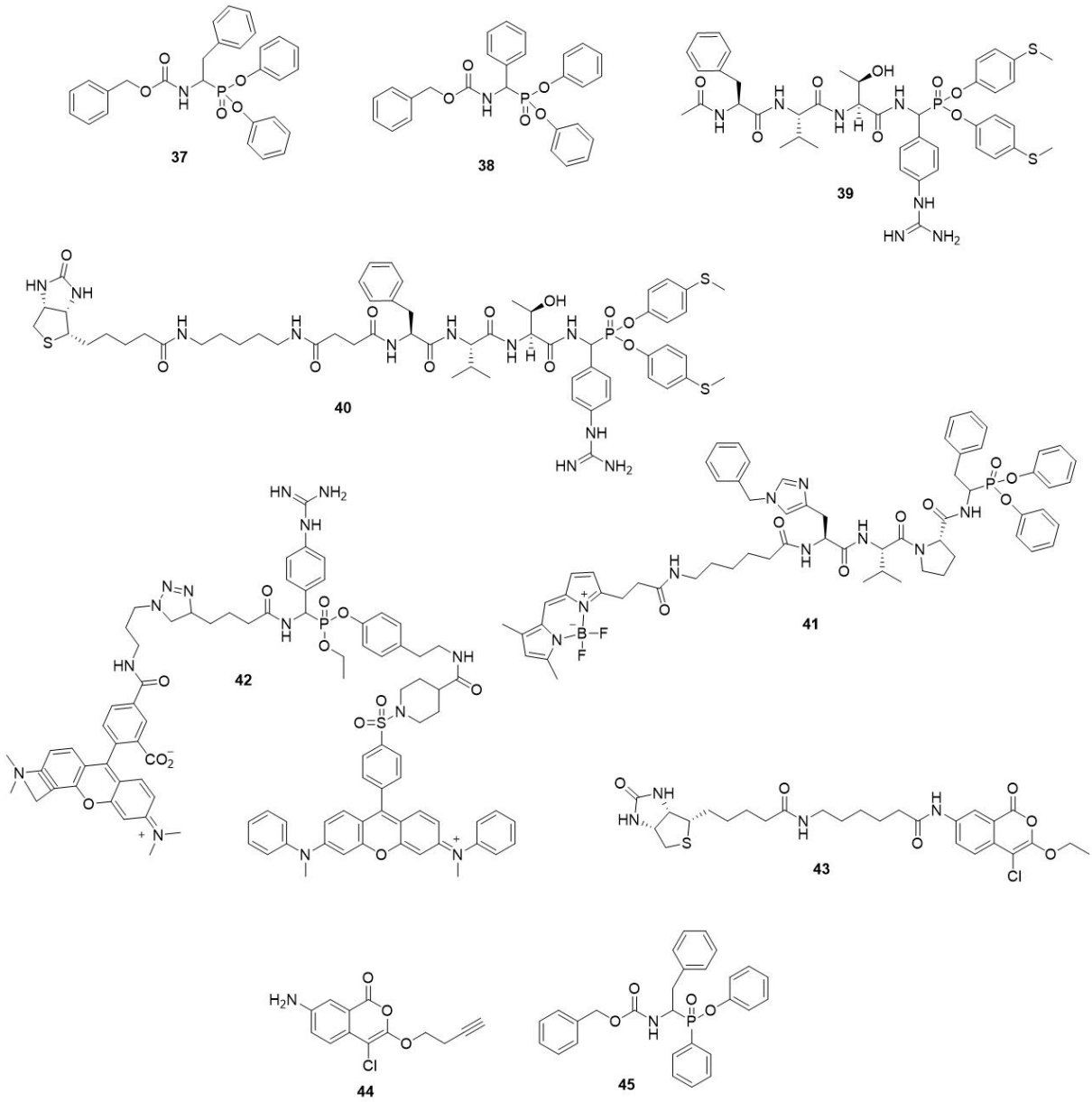

Figure 18. Inhibitors and ABPs of CatG.

opportunities for therapeutic intervention of mast cell-dependent allergic and autoimmune diseases.

Synthetic substrates

Jenne's pioneering work revealed that NSP4 can hydrolyze synthetic substrates: Boc- Ala-Pro-Nva-thiobenzyl ester -typical substrate for HNE and PR3[84]. Surprisingly, NSP4 showed/shows strong arginine preference at the P1 position. Tyr-Arg-Phe- Arg-AMC was efficiently hydrolyzed by NSP4. The replacement of arginine residue with lysine results in an almost complete lack of enzyme activity. Further studies explained the unique dual cleavage specificity of NSP4. In 2014, Lin *et al.* revealed an unusual mechanism of substrate recognition[87]. The arginine recognition by NSP4 relies on two important structural features[87]. The occluded S1 pocket and the H-bond acceptors around the P1-arginine guanidinium group. The substrate P1-arginine tits into the S1 pockets thanks to adopting a noncanonical "up" conformation,

which is stabilized by a solvent-exposed H-bond network. Interestingly, except for NSP4, all other trypsin-like proteases rely heavily on the salt bridge within a/the well-defined S1 pocket. As a result of the unusual substrate recognition mechanism, NSP4 can hydrolase substrates with methylarginine or citrulline at the P1 position. This suggests one of the possible roles played by NSP4 protease, which is processing the post-translationally modified proteins. The substrate used during the studies was MCA-PEG-Ile-Arg-Arg-Ser-Ser-Tyr-Ser-Phe-Lys (Dnp)-Lys (K_M = 13.8 μM and k_{cat}/K_M 10,000 $M^{-1}s^{-1}$).

The first studies to deeply investigate the substrate preferences of NSP4 were made by Kasperkiewicz *et al.*[89]. They synthesized the library of fluorogenic substrates with a fixed arginine residue at the P1 position. Activity studies showed that at the P2 position, substrates with proline residue were the most active among proteinogenics aminoacids. The substrates with the proline analog -Oic were the most active. P3 position revealed broad tolerance where Val, Ile, Tyr, Arg, Lys and Phe were the most active. The best non- proteinogenic amino acid was the phenylalanine derivative with a guanidine group in a/the para position (Phe(guan)). Homocyclohexylalanine (hCha) was found as the optimal amino acid at the P4 position. The most active substrate from the studies was Ac-hCha-Phe(guan)-Oic-Arg-ACC (k_{cat}/K_M = 32,000 $M^{-1}s^{-1}$) (PK421) (46) [Figure 19]. Substrate PK421 is only very weakly hydrolyzed by CatG (k_{cat}/K_M = 477 $M^{-1}s^{-1}$).

One year later, Wysocka *et al.* presented a new library of the PEGylated substrates of NSP4 protease[90]. From the library of a novel type of peptidomimetics composed of diaminopropionic acid residues modified with structurally diverse heterobifunctional polyethylene glycol chains (DAPEG), substrate 3 (47) was the most active with (K_m = 7.6 μM and k_{cat}/K_M 13,1579 $M^{-1}s^{-1}$) [Figure 19].

Inhibitors and ABPs

Jenne *et al.* found that among natural inhibitors of NSPs - NSP4 was most efficiently inhibited by antithrombin-heparin. α1-proteinase inhibitor (α1-antitrypsin) and C1 inhibitor can block enzymatic activity of NSP4[84].

The first synthetic inhibitor and ABP were reported by Kasperkiewicz *et al.*[89]. The substrate conversion into the diphenyl phosphonate probe with a biotin tag led to the Biot-Ahx-hCha-Phe(guan)-Oic-ArgP$(OPh)_2$ (48) ($k_{obs}/I = 3.8 \times 10^6$ $M^{-1}s^{-1}$ against NSP4 and $k_{obs}/I = 3.0 \times 10^3$ $M^{-1}s^{-1}$ against CatG and inactive against PR3 an HNE)[89] [Figure 19].

Further studies by Kasperkiewicz *et al.* about the development of the fluorescent ABPs of NSP lead to the compounds which are related to 401 and equipped with different N-Terminal fluorescent tags[65]. ABP with BODIPYFL fluorophore (49) was the most active with $k_{obs}/I = 3.8 \times 10^6$ $M^{-1}s^{-1}$, $k_{obs}/I = 3.0 \times 10^3$ $M^{-1}s^{-1}$ [Figure 19].

SUMMARY

NSPs are protein-degrading enzymes but also take part in a wide variety of pathophysiological processes. Thus, their inhibitors are considered potential therapeutics. Despite the fact of extensive research on substrates, inhibitors and ABPs of NSPs, there is no FDA-approved drug acting as an NSPs inhibitor.

The most advanced research was done in the field of HNE inhibitors, where inhibitors of this enzyme entered clinical trials. However, clinical trials of HNE inhibitors have faced challenges and failures. For example, a phase II clinical trial of an HNE inhibitor called AZD9668 (Alvelestat) in patients with COPD failed to show significant improvement in lung function or other clinical endpoints compared to a placebo.

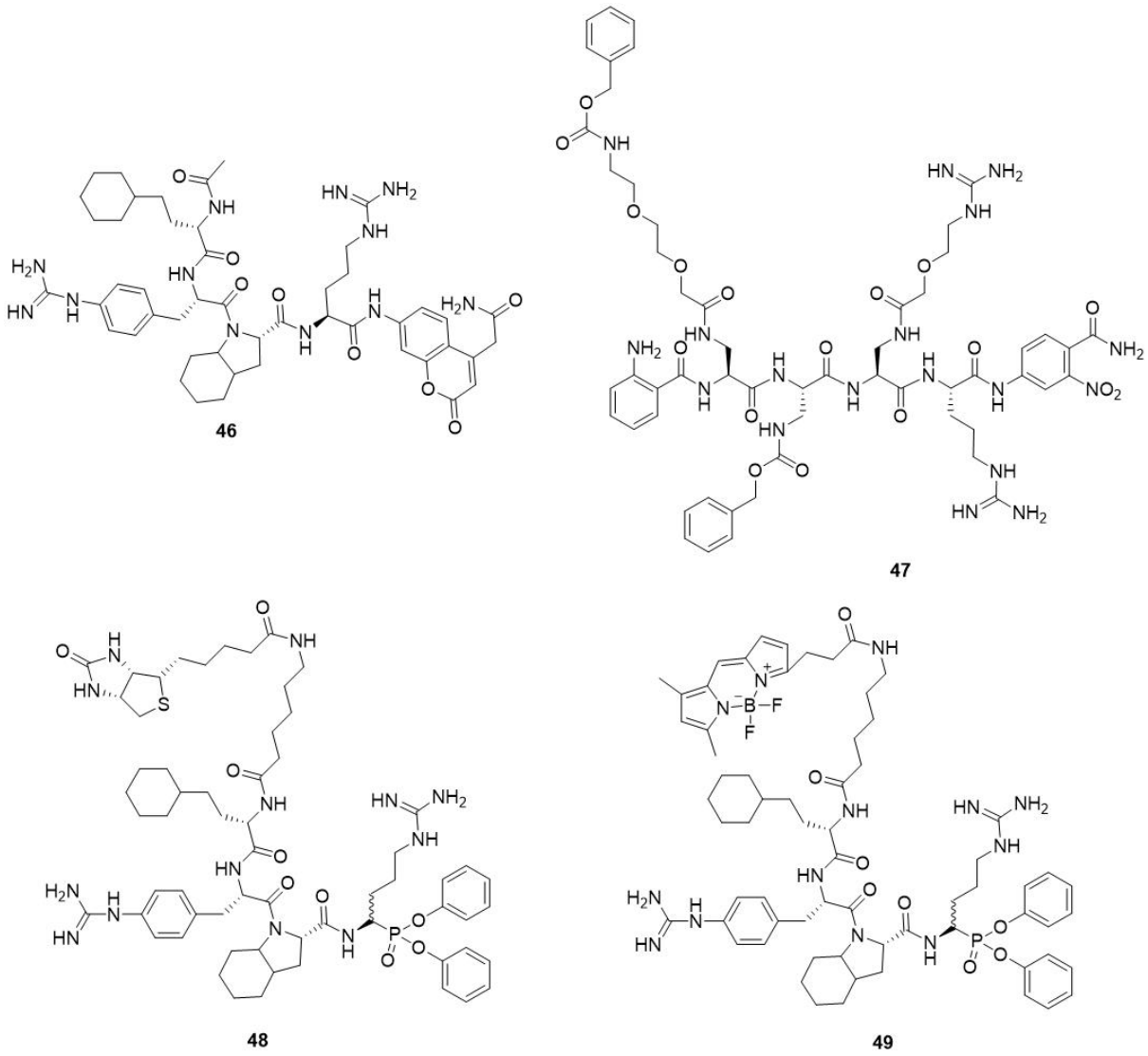

Figure 19. Synthetic substrates and ABPs of NSP4.

Another phase II clinical trial of an HNE inhibitor called ONO-6818 in patients with cystic fibrosis did not meet its primary endpoint of improving lung function compared to a placebo. A phase I clinical trial of an HNE inhibitor called M3364 in patients with advanced solid tumors showed that the drug was well-tolerated and had some anti-tumor activity, with one patient experiencing a partial response and several others having stable disease.

The reasons for the failure of HNE inhibitors in clinical trials are multifactorial. One possible explanation is the complexity and heterogeneity of the diseases that HNE inhibitors are targeting. For example, COPD and cystic fibrosis are characterized by chronic inflammation, but there are multiple underlying causes and factors that contribute to the disease pathogenesis. Therefore, targeting HNE alone may not be sufficient to achieve clinical benefits. Another challenge is the difficulty in achieving optimal pharmacokinetic and pharmacodynamic properties of HNE inhibitors. In some cases, the HNE inhibitors may not be sufficiently potent or selective to effectively inhibit HNE in vivo, or they may be rapidly metabolized or eliminated from

the body.

The latest studies have indicated the possibility of using elastase inhibitors in the treatment of acute respiratory distress syndrome (ARDS), which is a potentially life-threatening complication of respiratory infections such as COVID-19. ARDS is characterized by severe inflammation in the lungs, which can cause respiratory failure and other serious complications. Researchers have been investigating various ways to prevent or treat ARDS in COVID-19 patients, and one promising avenue of research has focused on inhibiting elastase activity. Several studies have explored the potential of elastase inhibitors as a therapeutic strategy for COVID-19-associated ARDS; however, there is no approved clinical treatment of ARDS with elastase inhibitors.

Further studies on NSPs (neutrophil serine proteases) are crucial for better understanding their role in various pathological conditions and for the development of new NSP-targeted drugs. Neutrophil serine proteases are enzymes released by immune cells, such as neutrophils, in response to infection or inflammation. They play an important role in host defense by breaking down and destroying invading pathogens. However, the excessive or uncontrolled activity of NSPs can lead to tissue damage, inflammation, and other pathological conditions. Therefore, the development of NSP-targeted drugs has emerged as a promising therapeutic strategy for various diseases, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, and inflammatory bowel disease. NSP-targeted drugs can potentially limit the harmful effects of NSPs on host tissues while preserving their beneficial antimicrobial properties.

DECLARATIONS

Authors' contributions

Conception and writing of the article: Torzyk K, Skoreński M
Manuscript preparation, final correction: Sieńczyk M

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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