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Sustainable approaches for drug repurposing in rare diseases: recommendations from the IRDiRC Task Force

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Review

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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

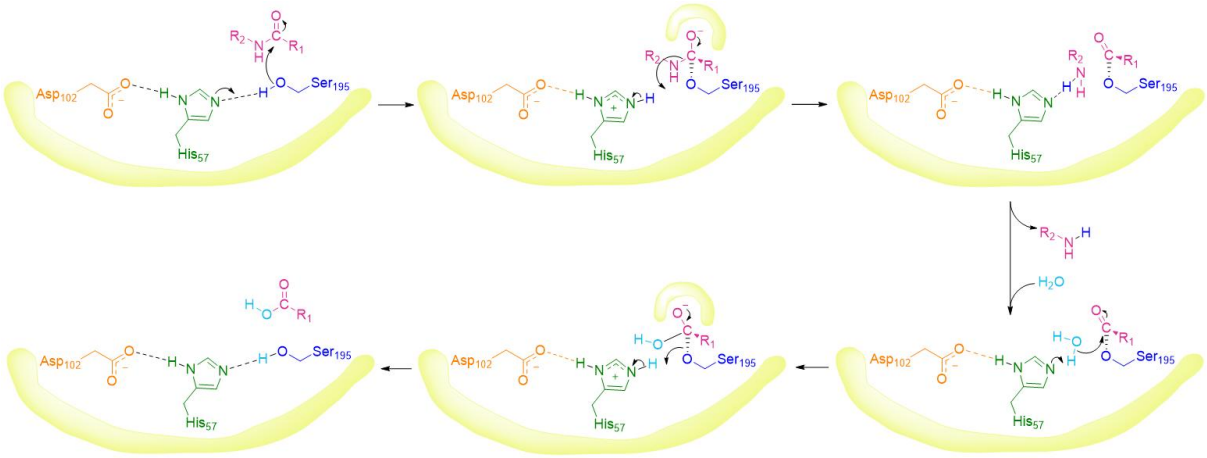

Figure 1. Mechanism of action neutrophil serine proteases.

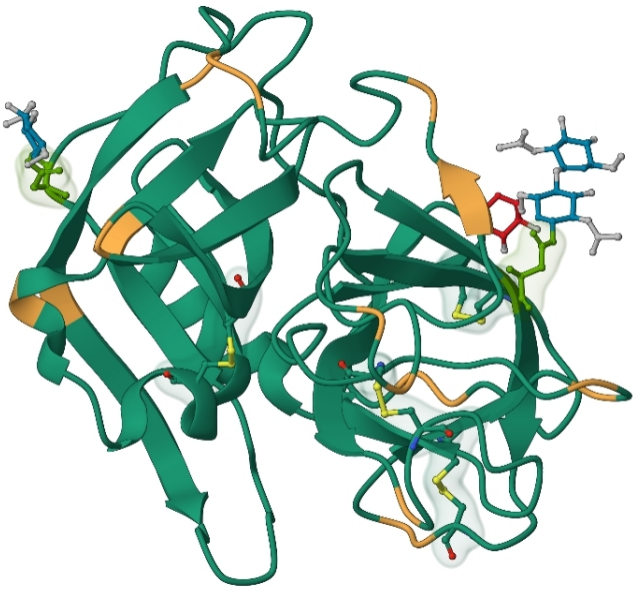

Figure 2. Structure of human neutrophil elastase; (green: Asn95 and Asn144, orange: Arg residues, yellow: disulfide bridges; based on 3Q76.pdb)[16].

(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].

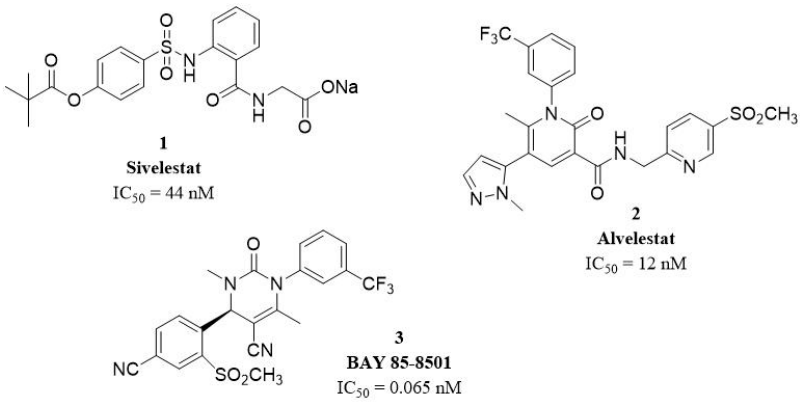

Figure 3. Structures of selected HNE inhibitors approved for use or in clinical trials[15].

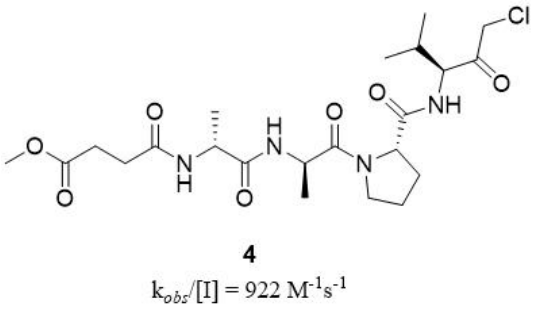

Figure 4. Structure of peptide chloromethyl ketone HNE inhibitor[37].

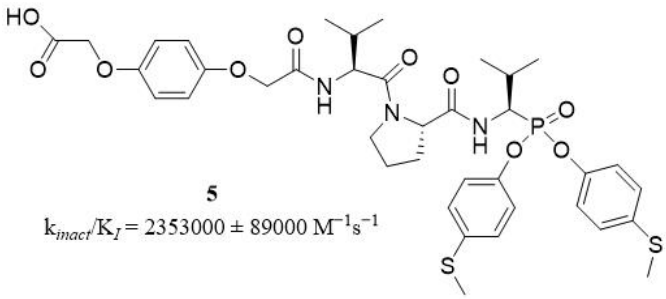

Figure 5. Structure of irreversible HNE inhibitor[38].

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].

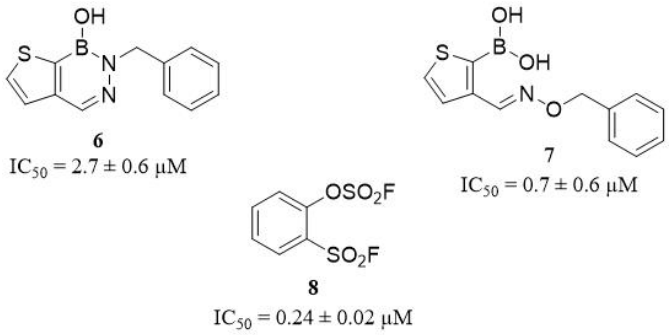

Figure 6. Structures of covalent HNE inhibitors[39,40].

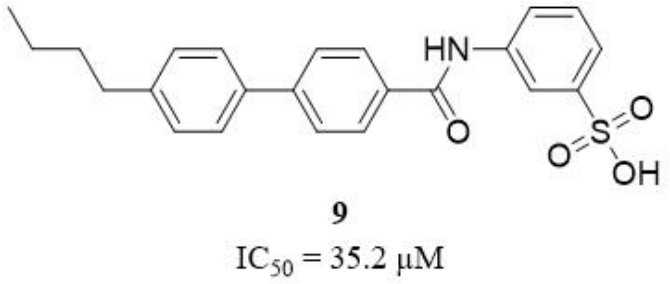

Figure 7. Structure of competitive HNE inhibitor[41].

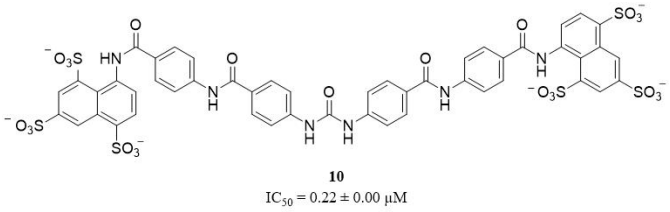

Figure 8. Structures of non-competitive allosteric HNE inhibitor[42].

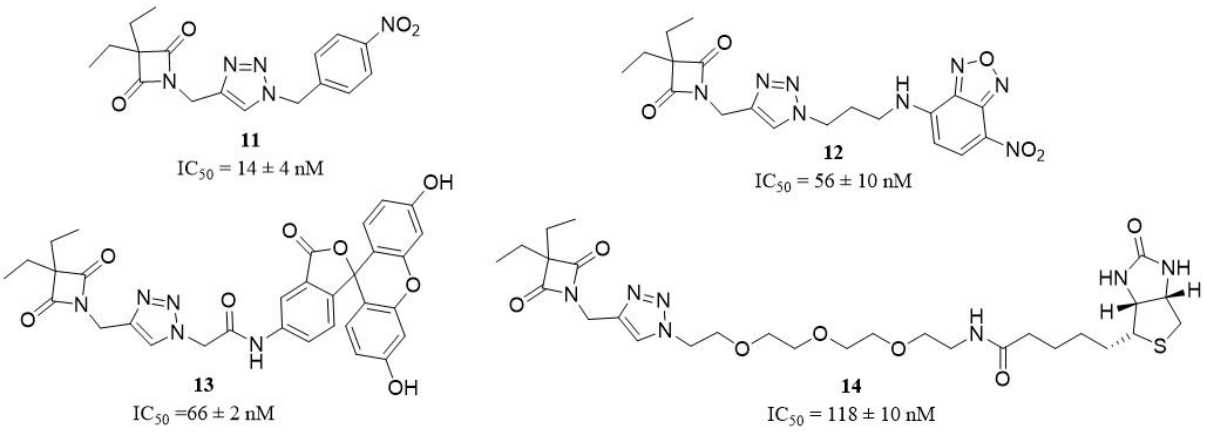

Figure 9. Structures of oxo-β-lactam HNE inhibitors[43].

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

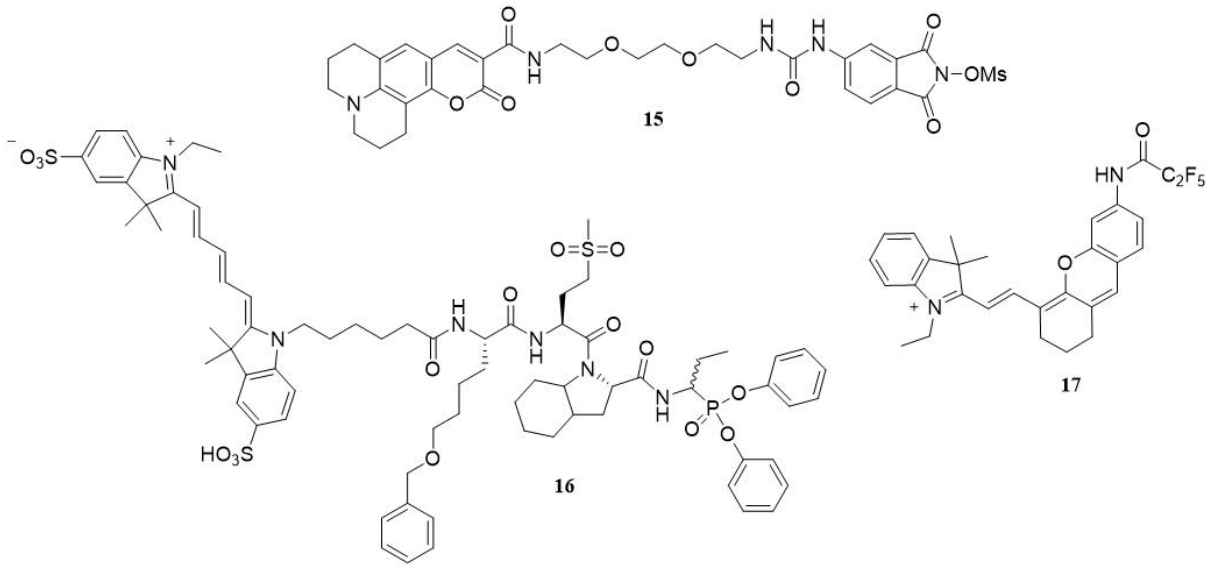

Figure 10. Structures of activity-based probes for HNE[44-46].

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].

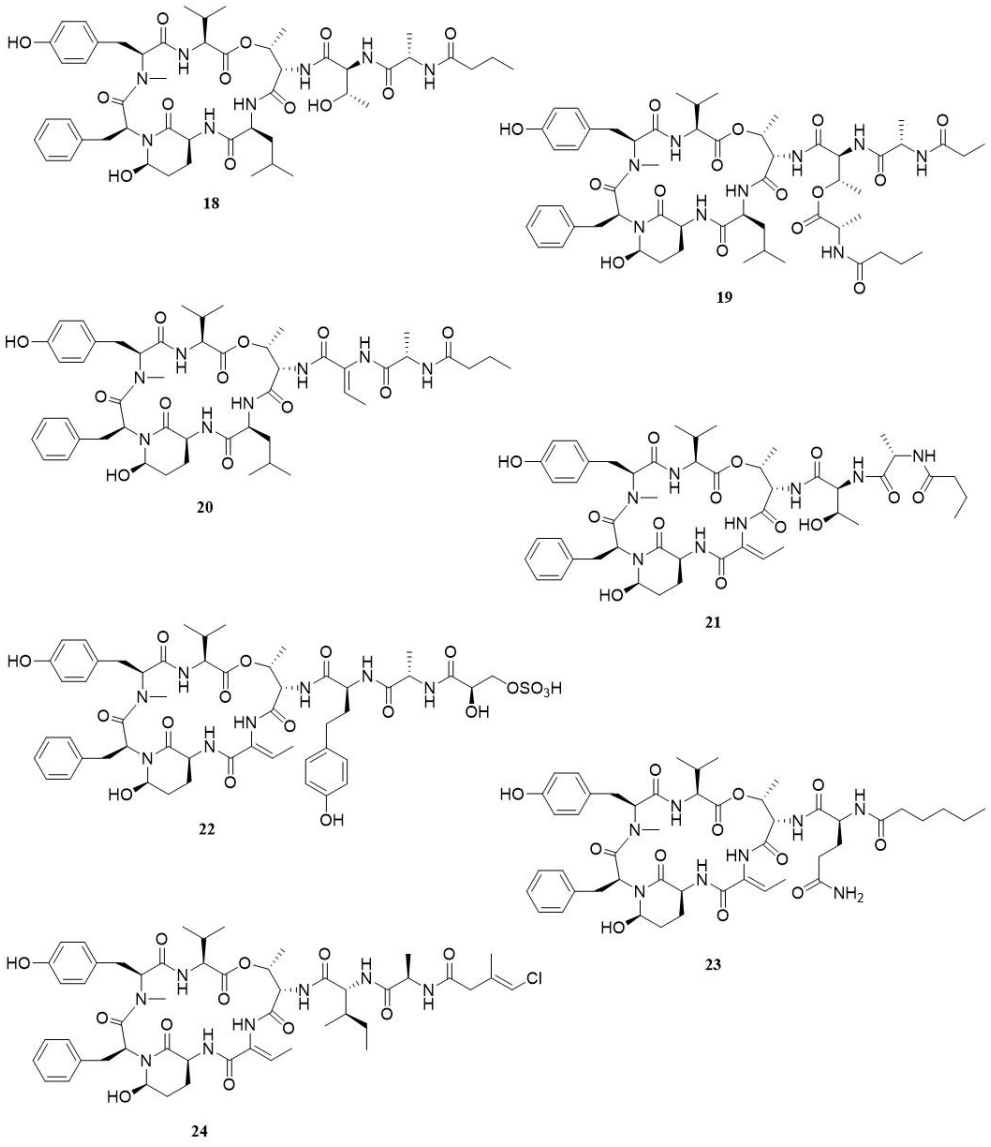

Figure 11. Structures of natural cyclodepsipeptide-type HNE inhibitors[48].

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.

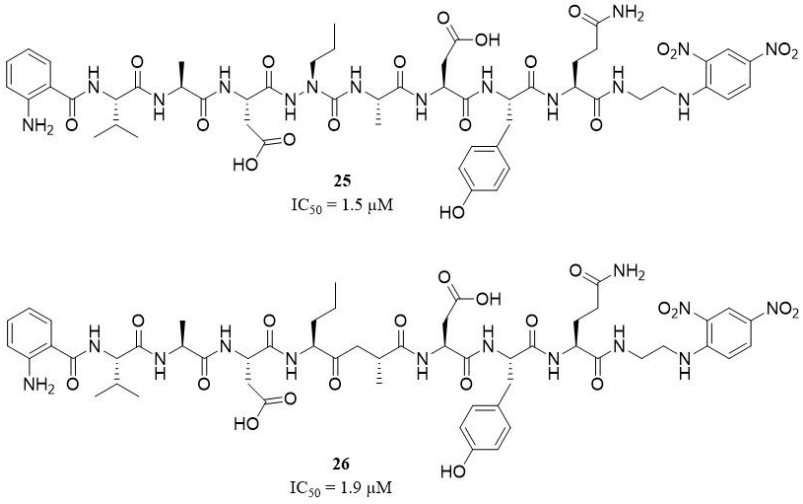

Figure 12. Structures of PR3 inhibitors based on a FRET substrate[58,62].

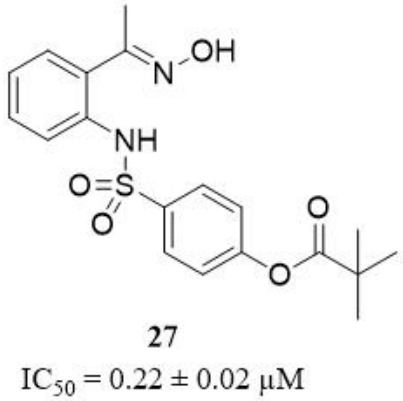

Figure 13. Structure of non-peptide PR3 inhibitor[63].

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].

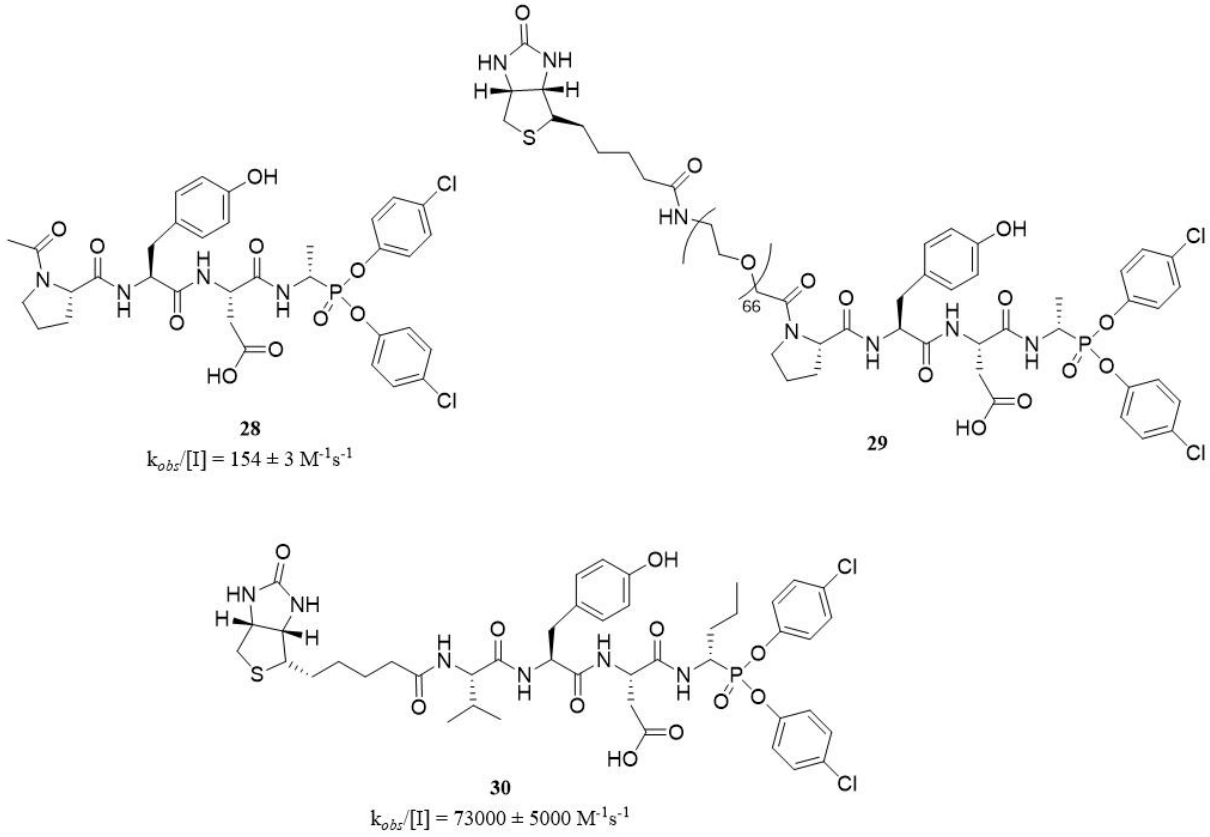

Figure 14. Structures of irreversible PR3 inhibitors and ABP[60,61].

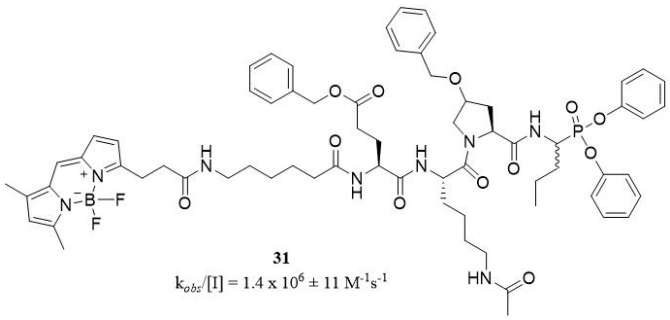

Figure 15. Structures of fluorescent PR3 activity-based probe[65].

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

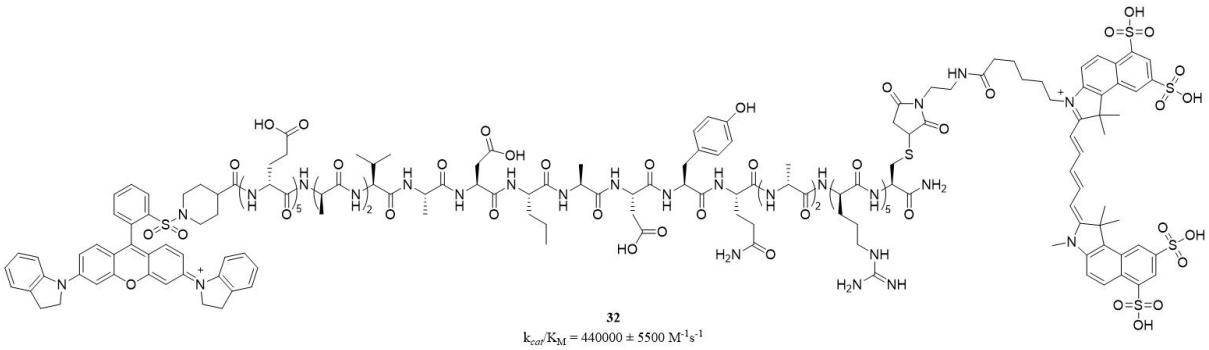

Figure 16. A structure of near-infrared fluorescent substrate-based probe of PR3[67].

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.

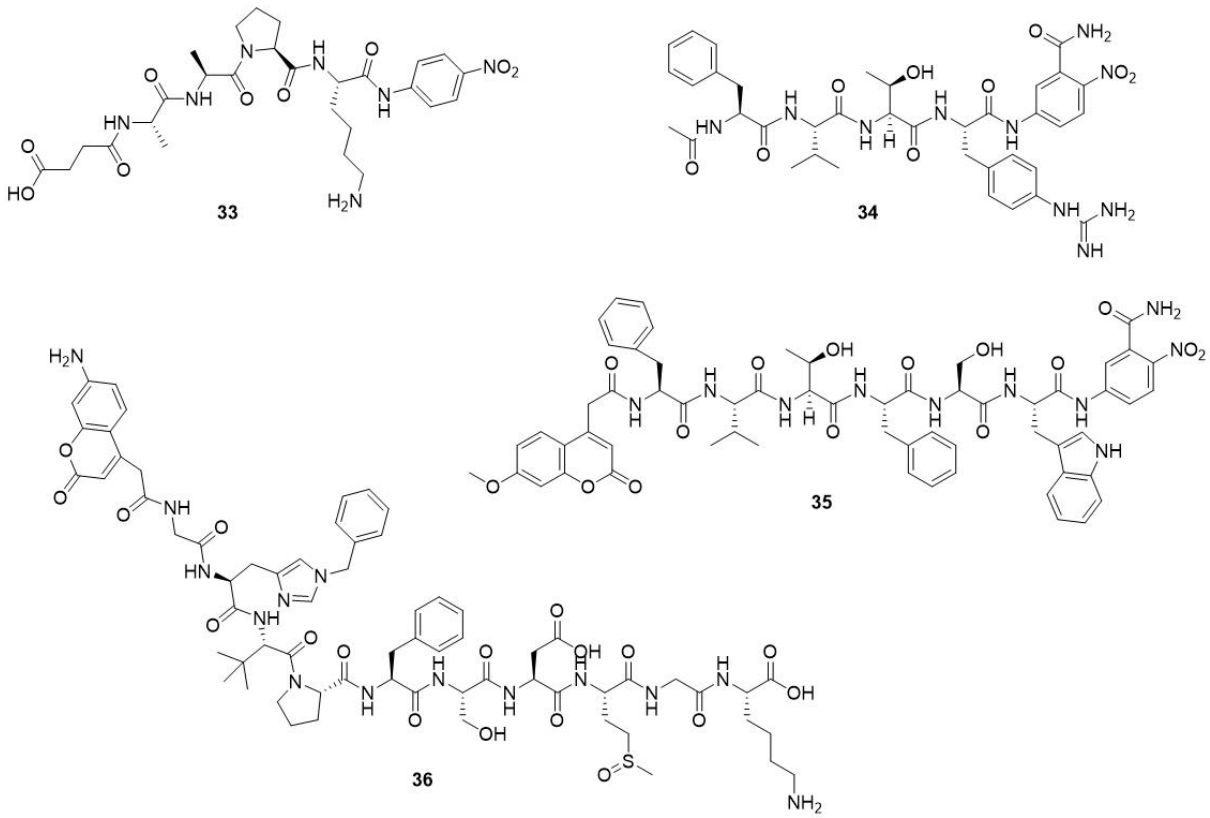

Figure 17. Synthetic substrates of CatG.

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

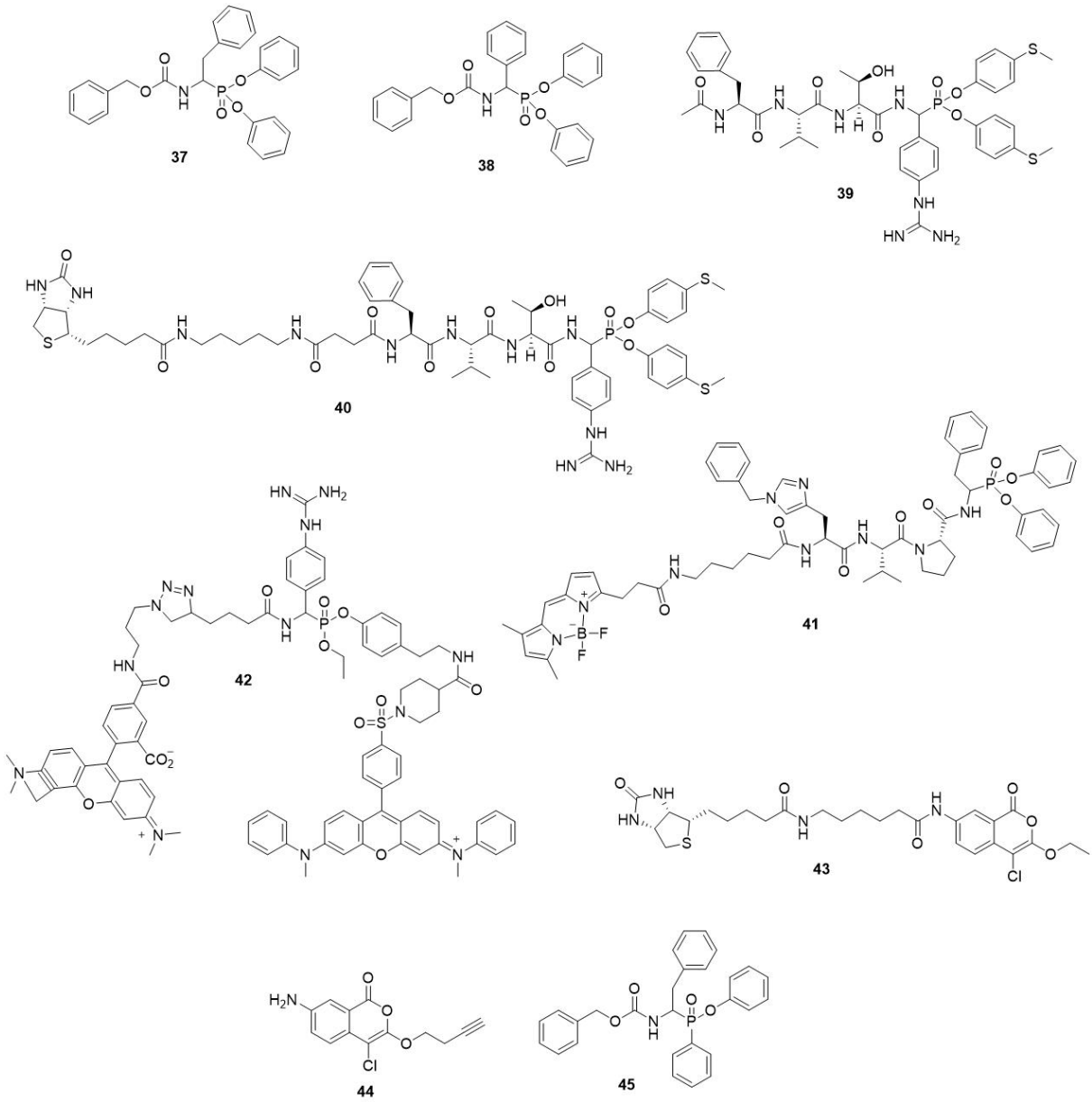

Figure 18. Inhibitors and ABPs of CatG.

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.

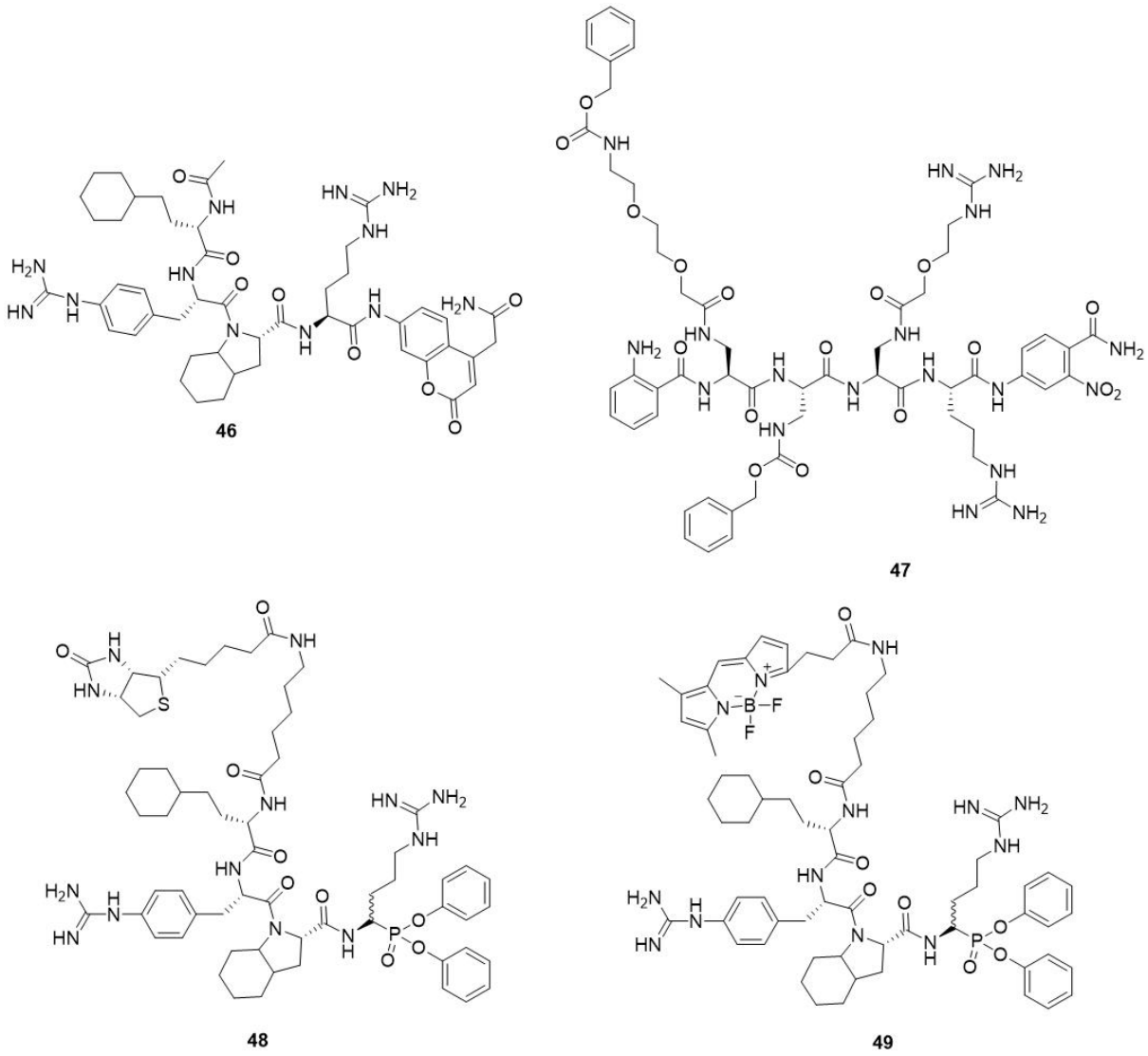

Figure 19. Synthetic substrates and ABPs of NSP4.

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.

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Authors' contributions

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REFERENCES

1. Stiel L, Meziani F, Helms J. Neutrophil activation during septic shock. *Shock* 2018;49:371-84. DOI PubMed
2. Gómez-Moreno D, Adrover JM, Hidalgo A. Neutrophils as effectors of vascular inflammation. *Eur J Clin Invest* 2018;48 Suppl 2:e12940. DOI PubMed

3. Mortaz E, Alipoor SD, Adcock IM, Mumby S, Koenderman L. Update on neutrophil function in severe inflammation. *Front Immunol* 2018;9:2171. DOI PubMed PMC
4. Majewski P, Majchrzak-Gorecka M, Grygier B, Skrzeczynska-Moncznik J, Osiecka O, Cichy J. Inhibitors of serine proteases in regulating the production and function of neutrophil extracellular traps. *Front Immunol* 2016;7:261. DOI PubMed PMC
5. Kettritz R. Neutral serine proteases of neutrophils. *Immunol Rev* 2016;273:232-48. DOI PubMed
6. Majchrzak-Gorecka M, Majewski P, Grygier B, Murzyn K, Cichy J. Secretory leukocyte protease inhibitor (SLPI), a multifunctional protein in the host defense response. *Cytokine Growth Factor Rev* 2016;28:79-93. DOI PubMed
7. Liu R, Chen L, Wu W, Chen H, Zhang S. Neutrophil serine proteases and their endogenous inhibitors in coronary artery ectasia patients. *Anatol J Cardiol* 2016;16:23-8. DOI PubMed PMC
8. Burgener SS, Leborgne NGF, Snipas SJ, Salvesen GS, Bird PI, Benarafa C. Cathepsin G Inhibition by Serpinb1 and Serpinb6 prevents programmed necrosis in neutrophils and monocytes and reduces GSDMD-Driven inflammation. *Cell Rep* 2019;27:3646-3656.e5. DOI PubMed PMC
9. Benarafa C, Simon HU. Role of granule proteases in the life and death of neutrophils. *Biochem Biophys Res Commun* 2017;482:473-81. DOI PubMed
10. Gao S, Zhu H, Zuo X, Luo H. Cathepsin G and Its role in inflammation and autoimmune diseases. *Arch Rheumatol* 2018;33:498-504. DOI PubMed PMC
11. Twaddell SH, Baines KJ, Grainge C, Gibson PG. The emerging role of neutrophil extracellular traps in respiratory disease. *Chest* 2019;156:774-82. DOI PubMed
12. Gramegna A, Amati F, Terranova L, et al. Neutrophil elastase in bronchiectasis. *Respir Res* 2017;18:211. DOI PubMed PMC
13. Lerman I, Hammes SR. Neutrophil elastase in the tumor microenvironment. *Steroids* 2018;133:96-101. DOI PubMed PMC
14. Taylor S, Dirir O, Zamanian RT, Rabinovitch M, Thompson AAR. The role of neutrophils and neutrophil elastase in pulmonary arterial hypertension. *Front Med (Lausanne)* 2018;5:217. DOI PubMed PMC
15. Crocetti L, Quinn MT, Schepetkin IA, Giovannoni MP. A patenting perspective on human neutrophil elastase (HNE) inhibitors (2014-2018) and their therapeutic applications. *Expert Opin Ther Pat* 2019;29:555-78. DOI PubMed PMC
16. Hansen G, Gielen-Haertwig H, Reinemer P, Schomburg D, Harrenga A, Niefind K. Unexpected active-site flexibility in the structure of human neutrophil elastase in complex with a new dihydropyrimidone inhibitor. *J Mol Biol* 2011;409:681-91. DOI PubMed
17. Domon H, Terao Y. The role of neutrophils and neutrophil elastase in pneumococcal pneumonia. *Front Cell Infect Microbiol* 2021;11:615959. DOI PubMed PMC
18. Wang W, Su J, Yan M, Pan J, Zhang X. Neutrophil extracellular traps in autoimmune diseases: Analysis of the knowledge map. *Front Immunol* 2023;14:1095421. DOI PubMed PMC
19. Scozzi D, Liao F, Krupnick AS, Kreisel D, Gelman AE. The role of neutrophil extracellular traps in acute lung injury. *Front Immunol* 2022;13:953195. DOI PubMed PMC
20. Zonta YR, Dezen ALO, Della Coletta AM, et al. Paracoccidioides brasiliensis releases a DNase-Like protein that degrades NETs and allows for fungal escape. *Front Cell Infect Microbiol* 2020;10:592022. DOI PubMed PMC
21. Yan S, Li M, Liu B, Ma Z, Yang Q. Neutrophil extracellular traps and pulmonary fibrosis: an update. *J Inflamm (Lond)* 2023;20:2. DOI PubMed PMC
22. Moore S, Juo HH, Nielsen CT, Tyden H, Bengtsson AA, Lood C. Role of neutrophil extracellular traps regarding patients at risk of increased disease activity and cardiovascular comorbidity in systemic lupus erythematosus. *J Rheumatol* 2020;47:1652-60. DOI PubMed PMC
23. Bonaventura A, Montecucco F, Dallegri F, et al. Novel findings in neutrophil biology and their impact on cardiovascular disease. *Cardiovasc Res* 2019;115:1266-85. DOI PubMed
24. Quail DF, Amulic B, Aziz M, et al. Neutrophil phenotypes and functions in cancer: a consensus statement. *J Exp Med* 2022:219. DOI PubMed PMC
25. Cedervall J, Herre M, Dragomir A, et al. Neutrophil extracellular traps promote cancer-associated inflammation and myocardial stress. *Oncoimmunology* 2022;11:2049487. DOI PubMed PMC
26. Yang C, Wang Z, Li L, et al. Aged neutrophils form mitochondria-dependent vital NETs to promote breast cancer lung metastasis. *J Immunother Cancer* 2021;9:e002875. DOI PubMed PMC
27. Okamoto M, Mizuno R, Kawada K, et al. Neutrophil extracellular traps promote metastases of colorectal cancers through activation of ERK signaling by releasing neutrophil elastase. *Int J Mol Sci* 2023;24:1118. DOI PubMed PMC
28. Veras FP, Pontelli MC, Silva CM, et al. SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J Exp Med* 2020:217. DOI PubMed PMC
29. Cesta MC, Zippoli M, Marsiglia C, et al. Neutrophil activation and neutrophil extracellular traps (NETs) in COVID-19 ARDS and immunothrombosis. *Eur J Immunol* 2023;53:e2250010. DOI PubMed PMC
30. Zhang R, Sun C, Han Y, et al. Neutrophil autophagy and NETosis in COVID-19: perspectives. *Autophagy* 2023;19:758-67. DOI PubMed PMC
31. Polverino E, Rosales-Mayor E, Dale GE, Dembowsky K, Torres A. The role of neutrophil elastase inhibitors in lung diseases. *Chest* 2017;152:249-62. DOI PubMed
32. Castillo MJ, Nakajima K, Zimmerman M, Powers JC. Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. *Anal Biochem* 1979;99:53-

64. DOI PubMed
33. Wysocka M, Lesner A, Gruba N, et al. Three wavelength substrate system of neutrophil serine proteinases. *Anal Chem* 2012;84:7241-8. DOI PubMed
34. Sun Q, Li J, Liu WN, Dong QJ, Yang WC, Yang GF. Non-peptide-based fluorogenic small-molecule probe for elastase. *Anal Chem* 2013;85:11304-11. DOI PubMed
35. Aikawa N, Kawasaki Y. Clinical utility of the neutrophil elastase inhibitor sivelestat for the treatment of acute respiratory distress syndrome. *Ther Clin Risk Manag* 2014;10:621-9. DOI PubMed PMC
36. Powers JC, Gupton BF, Harley AD, Nishino N, Whitley RJ. Specificity of porcine pancreatic elastase, human leukocyte elastase and cathepsin G. Inhibition with peptide chloromethyl ketones. *Biochim Biophys Acta* 1977;485:156-66. DOI PubMed
37. Jugniot N, Voisin P, Bentaher A, Mellet P. Neutrophil elastase activity imaging: recent approaches in the design and applications of activity-based probes and substrate-based probes. *Contrast Media Mol Imaging* 2019;2019:7417192. DOI PubMed PMC
38. Winiarski Ł, Oleksyszyn J, Sieńczyk M. Human neutrophil elastase phosphonic inhibitors with improved potency of action. *J Med Chem* 2012;55:6541-53. DOI PubMed
39. António JPM, Gonçalves LM, Guedes RC, Moreira R, Gois PMP. Diazaborines as new inhibitors of human neutrophil elastase. *ACS Omega* 2018;3:7418-23. DOI PubMed PMC
40. Zheng Q, Woehl JL, Kitamura S, et al. SuFEx-enabled, agnostic discovery of covalent inhibitors of human neutrophil elastase. *Proc Natl Acad Sci USA* 2019;116:18808-14. DOI PubMed PMC
41. Xu Y, Qi N, Wen H, Zhang G, Wang Y, Cui H. Synthesis and evaluation of benzenesulfonic acid derivatives as human neutrophil elastase (hNE) inhibitors. *Med Chem Res* 2021;30:387-98. DOI PubMed PMC
42. Al-Horani RA, Aliter KF, Kar S, Mottamal M. Sulfonated nonsaccharide heparin mimetics are potent and noncompetitive inhibitors of human neutrophil elastase. *ACS Omega* 2021;6:12699-710. DOI PubMed PMC
43. Ruivo EF, Gonçalves LM, Carvalho LA, et al. Clickable 4-Oxo-β-lactam-based selective probing for human neutrophil elastase related proteomes. *ChemMedChem* 2016;11:2037-42. DOI PubMed
44. Schulz-Fincke AC, Tikhomirov AS, Braune A, et al. Design of an activity-based probe for human neutrophil elastase: implementation of the lossen rearrangement to induce förster resonance energy transfers. *Biochemistry* 2018;57:742-52. DOI PubMed
45. Anderson BM, Poole DP, Aurelio L, et al. Application of a chemical probe to detect neutrophil elastase activation during inflammatory bowel disease. *Sci Rep* 2019;9:13295. DOI PubMed PMC
46. Liu SY, Xiong H, Li RR, Yang WC, Yang GF. Activity-based near-infrared fluorogenic probe for enabling *in vitro* and *in vivo* profiling of neutrophil elastase. *Anal Chem* 2019;91:3877-84. DOI PubMed
47. Ahmad S, Saleem M, Riaz N, et al. The natural polypeptides as significant elastase inhibitors. *Front Pharmacol* 2020;11:688. DOI PubMed PMC
48. Marinaccio L, Stefanucci A, Scioli G, et al. Peptide human neutrophil elastase inhibitors from natural sources: an overview. *Int J Mol Sci* 2022;23:2924. DOI PubMed PMC
49. Wan H, Lee KS, Kim BY, et al. A spider-derived Kunitz-type serine protease inhibitor that acts as a plasmin inhibitor and an elastase inhibitor. *PLoS One* 2013;8:e53343. DOI PubMed PMC
50. Luan N, Zhao Q, Duan Z, et al. Identification and characterization of ShSPI, a Kazal-type elastase inhibitor from the venom of scolopendra hainanum. *Toxins (Basel)* 2019;11:708. DOI PubMed PMC
51. Al-Awadhi FH, Paul VJ, Luesch H. Structural diversity and anticancer activity of marine-derived elastase inhibitors: key features and mechanisms mediating the antimetastatic effects in invasive breast cancer. *Chembiochem* 2018;19:815-25. DOI PubMed PMC
52. Luo D, Luesch H. Ahp-cyclodepsipeptide inhibitors of elastase: lyngbyastatin 7 stability, scalable synthesis, and focused library analysis. *ACS Med Chem Lett* 2020;11:419-25. DOI PubMed PMC
53. Keller L, Canuto KM, Liu C, et al. Tutuilamides A-C: vinyl-chloride-containing cyclodepsipeptides from marine cyanobacteria with potent elastase inhibitory properties. *ACS Chem Biol* 2020;15:751-7. DOI PubMed PMC
54. Crisford H, Sapey E, Stockley RA. Proteinase 3; a potential target in chronic obstructive pulmonary disease and other chronic inflammatory diseases. *Respir Res* 2018;19:180. DOI PubMed PMC
55. Korkmaz B, Lesner A, Guarino C, et al. Inhibitors and antibody fragments as potential anti-inflammatory therapeutics targeting neutrophil proteinase 3 in human disease. *Pharmacol Rev* 2016;68:603-30. DOI PubMed
56. Grzywa R, Lesner A, Korkmaz B, Sieńczyk M. Proteinase 3 phosphonic inhibitors. *Biochimie* 2019;166:142-9. DOI PubMed
57. Witko-Sarsat V, Thieblemont N. Granulomatosis with polyangiitis (Wegener granulomatosis): a proteinase-3 driven disease? *Joint Bone Spine* 2018;85:185-9. DOI PubMed
58. Epinette C, Croix C, Jaquillard L, et al. A selective reversible azapeptide inhibitor of human neutrophil proteinase 3 derived from a high affinity FRET substrate. *Biochem Pharmacol* 2012;83:788-96. DOI PubMed
59. Popow-Stellmaszyk J, Wysocka M, Lesner A, Korkmaz B, Rolka K. A new proteinase 3 substrate with improved selectivity over human neutrophil elastase. *Anal Biochem* 2013;442:75-82. DOI PubMed
60. Guarino C, Legowska M, Epinette C, et al. New selective peptidyl di(chlorophenyl) phosphonate esters for visualizing and blocking neutrophil proteinase 3 in human diseases. *J Biol Chem* 2014;289:31777-91. DOI PubMed PMC
61. Guarino C, Gruba N, Grzywa R, et al. Exploiting the S4-S5 specificity of human neutrophil proteinase 3 to improve the potency of peptidyl di(chlorophenyl)-phosphonate ester inhibitors: a kinetic and molecular modeling analysis. *J Med Chem* 2018;61:1858-70. DOI PubMed

62. Budnjo A, Narawane S, Grauffel C, et al. Reversible ketomethylene-based inhibitors of human neutrophil proteinase 3. *J Med Chem* 2014;57:9396-408. DOI PubMed
63. Hwang TL, Wang WH, Wang TY, Yu HP, Hsieh PW. Synthesis and pharmacological characterization of 2-aminobenzaldehyde oxime analogs as dual inhibitors of neutrophil elastase and proteinase 3. *Bioorg Med Chem* 2015;23:1123-34. DOI PubMed
64. Grzywa R, Burchacka E, Łęcka M, et al. Synthesis of novel phosphonic-type activity-based probes for neutrophil serine proteases and their application in spleen lysates of different organisms. *Chembiochem* 2014;15:2605-12. DOI PubMed
65. Kasperkiewicz P, Altman Y, D'Angelo M, Salvesen GS, Drag M. Toolbox of fluorescent probes for parallel imaging reveals uneven location of serine proteases in neutrophils. *J Am Chem Soc* 2017;139:10115-25. DOI PubMed PMC
66. Tian S, Swedberg JE, Li CY, Craik DJ, de Veer SJ. Iterative optimization of the cyclic peptide SFTI-1 yields potent inhibitors of neutrophil proteinase 3. *ACS Med Chem Lett* 2019;10:1234-9. DOI PubMed PMC
67. Saidi A, Wartenberg M, Madinier JB, et al. Monitoring human neutrophil activation by a proteinase 3 near-infrared fluorescence substrate-based probe. *Bioconjug Chem* 2021;32:1782-90. DOI PubMed
68. Pagano MB, Bartoli MA, Ennis TL, et al. Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms. *Proc Natl Acad Sci USA* 2007;104:2855-60. DOI PubMed PMC
69. Hermant B, Bibert S, Concord E, et al. Identification of proteases involved in the proteolysis of vascular endothelium cadherin during neutrophil transmigration. *J Biol Chem* 2003;278:14002-12. DOI PubMed
70. Ramaha A, Patston PA. Release and degradation of angiotensin I and angiotensin II from angiotensinogen by neutrophil serine proteinases. *Arch Biochem Biophys* 2002;397:77-83. DOI PubMed
71. Okada Y, Nakanishi I. Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ('gelatinase') by human neutrophil elastase and cathepsin G. *FEBS Lett* 1989;249:353-6. DOI PubMed
72. Chatham WW, Blackburn WD Jr, Heck LW. Additive enhancement of neutrophil collagenase activity by HOCl and cathepsin G. *Biochem Biophys Res Commun* 1992;184:560-7. DOI PubMed
73. Scott FL, Hirst CE, Sun J, Bird CH, Bottomley SP, Bird PI. The Intracellular serpin proteinase inhibitor 6 is expressed in monocytes and granulocytes and is a potent inhibitor of the azurophilic granule protease, cathepsin G. *Blood* 1999;93:2089-97. PubMed
74. Polanowska J, Krokoszynska I, Czapinska H, Watorek W, Dadlez M, Otlewski J. Specificity of human cathepsin G. *Biochim Biophys Acta* 1998;1386:189-98. DOI PubMed
75. Wysocka M, Legowska A, Bulak E, et al. New chromogenic substrates of human neutrophil cathepsin G containing non-natural aromatic amino acid residues in position P(1) selected by combinatorial chemistry methods. *Mol Divers* 2007;11:93-9. DOI PubMed
76. Lesner A, Wysocka M, Guzow K, Wiczk W, Legowska A, Rolka K. Development of sensitive cathepsin G fluorogenic substrate using combinatorial chemistry methods. *Anal Biochem* 2008;375:306-12. DOI PubMed
77. Groborz K, Kołt S, Kasperkiewicz P, Drag M. Internally quenched fluorogenic substrates with unnatural amino acids for cathepsin G investigation. *Biochimie* 2019;166:103-11. DOI PubMed
78. Oleksyszyn J, Powers JC. Irreversible inhibition of serine proteases by peptide derivatives of (alpha-aminoalkyl)phosphonate diphenyl esters. *Biochemistry* 1991;30:485-93. DOI PubMed
79. Sieńczyk M, Lesner A, Wysocka M, et al. New potent cathepsin G phosphonate inhibitors. *Bioorg Med Chem* 2008;16:8863-7. DOI PubMed
80. Serim S, Baer P, Verhelst SH. Mixed alkyl aryl phosphonate esters as quenched fluorescent activity-based probes for serine proteases. *Org Biomol Chem* 2015;13:2293-9. DOI PubMed
81. Kam CM, Abuelyaman AS, Li Z, Hudig D, Powers JC. Biotinylated isocoumarins, new inhibitors and reagents for detection, localization, and isolation of serine proteases. *Bioconjug Chem* 1993;4:560-7. DOI PubMed
82. Haedke U, Götz M, Baer P, Verhelst SH. Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases. *Bioorg Med Chem* 2012;20:633-40. DOI PubMed
83. Kahler JP, Lenders S, van de Plassche MAT, Verhelst SHL. Facile synthesis of aminomethyl phosphinate esters as serine protease inhibitors with primed site interaction. *ACS Med Chem Lett* 2020;11:1739-44. DOI PubMed PMC
84. Perera NC, Schilling O, Kittel H, Back W, Kremmer E, Jenne DE. NSP4, an elastase-related protease in human neutrophils with arginine specificity. *Proc Natl Acad Sci USA* 2012;109:6229-34. DOI PubMed PMC
85. Perera NC, Wiesmüller KH, Larsen MT, et al. NSP4 is stored in azurophil granules and released by activated neutrophils as active endoprotease with restricted specificity. *J Immunol* 2013;191:2700-7. DOI PubMed
86. Akula S, Thorpe M, Boinapally V, Hellman L. Granule associated serine proteases of hematopoietic cells - an analysis of their appearance and diversification during vertebrate evolution. *PLoS One* 2015;10:e0143091. DOI PubMed PMC
87. Lin SJ, Dong KC, Eigenbrot C, van Lookeren Campagne M, Kirchhofer D. Structures of neutrophil serine protease 4 reveal an unusual mechanism of substrate recognition by a trypsin-fold protease. *Structure* 2014;22:1333-40. DOI PubMed
88. AhYoung AP, Eckard SC, Gogineni A, et al. Neutrophil serine protease 4 is required for mast cell-dependent vascular leakage. *Commun Biol* 2020;3:687. DOI PubMed PMC
89. Kasperkiewicz P, Poreba M, Snipas SJ, et al. Design of a selective substrate and activity based probe for human neutrophil serine protease 4. *PLoS One* 2015;10:e0132818. DOI PubMed PMC
90. Wysocka M, Gruba N, Grzywa R, et al. PEGylated substrates of NSP4 protease: a tool to study protease specificity. *Sci Rep* 2016;6:22856. DOI PubMed PMC

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Review

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Pathogenesis of autoimmune and hereditary pancreatitis with a focus on neutrophil granulocytes and neutrophil serine proteases

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Abstract

Hereditary and autoimmune pancreatitis are two rare forms of inflammatory pancreatic disorders. Both share similarities with acute, acute recurrent, and chronic pancreatitis. Regarding their pathogenesis, the premature activation of the digestive protease trypsinogen and the infiltration of inflammatory cells such as polymorphonuclear leukocytes and macrophages into the pancreas are highly relevant and can reciprocally amplify inflammation. Neutrophil serine proteases are the main components of neutrophil granulocytes and have different pro-inflammatory effects in many diseases. However, their role in pancreatitis is still limited. This section focuses on known findings regarding the role of this group of enzymes in hereditary and autoimmune pancreatitis.

Keywords: Hereditary pancreatitis, autoimmune pancreatitis, neutrophil serine proteases, cathepsin C

INTRODUCTION

Acute pancreatitis is defined as an acute inflammatory condition of the pancreas and is characterized by self-digestion of the pancreatic tissue by its own digestive enzymes. It is one of the most frequent gastrointestinal diseases leading to hospitalization in Europe and the United States, with an estimated

annual incidence of 13-45/100,000 inhabitants[1]. Although acute pancreatitis is mostly characterized by a mild course with edematous changes of the organ and complete recovery, a severe disease course occurs in 10%-15% of patients, leading to organ failure and high mortality. Necrotizing pancreatitis is observed in up to 20% of patients and can lead to further complications such as secondary infections requiring further interventions[2]. Alcohol abuse and migrating gallstones are the most common causes of acute pancreatitis and account for approximately 80% of all cases. Less frequent causes include genetic factors, drug-induced effects, anatomic variants, autoimmune diseases, and metabolic disorders such as hypertriglyceridemia or hypercalcemia[3]. This review aims to provide insights into current concepts in the pathophysiology of autoimmune and hereditary pancreatitis by emphasizing the pathogenic function of neutrophil granulocytes and neutrophil serine proteases (NSPs).

HEREDITARY PANCREATITIS

The term hereditary pancreatitis classically refers to mutations in the cationic trypsinogen gene (PRSS1) that are inherited in an autosomal-dominant pattern with high penetrance[4-6]. Patients present with recurrent attacks of acute or chronic pancreatitis without an obvious underlying cause. An autosomal dominant pattern of inheritance was first reported in 1952 by Comfort and Steinberg in a family with chronic pancreatitis[7], and the genetic basis was studied intensively in the following years. A breakthrough was reached in 1996 when mutations in the *PRSS1* gene (PRSS1) leading to a gain-of-function were discovered. These led to hereditary pancreatitis[8]. Since 1996, many other genetic variants of PRSS1 have been reported, underlining the pathogenic role of trypsinogen in pancreatitis. More rarely, homozygous mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) and the serine protease inhibitor Kazal-type 1 (*SPINK1*) genes are linked to hereditary pancreatitis. Other genetic factors increase the risk of recurrent acute or chronic pancreatitis and include variants of carboxypeptidase A1 (*CPA1*), elastase 3B (*CELA3B*), and chymotrypsin C (*CTRC*) genes, as well as heterozygous *SPINK1* or *CFTR* mutations. These variants are considered to have disease-modifying functions together with environmental factors. If acute recurrent or chronic pancreatitis occurs in families with a frequency higher than expected by chance, familial pancreatitis can be suspected. These genes act in a direct or indirect way in the intracellular protease activation cascade and encode proteases and their inhibitors[9,10] [Table 1]. Patients who have hereditary pancreatitis or carry genetic susceptibility factors without Mendelian inheritance patterns mostly suffer from their first acute phase at a significantly younger age compared to patients with other etiologies, and they often have a family history of pancreatitis[11,12]. In addition, a number of genetic mutations are related to faster disease progression and a higher probability of pancreatitis-related complications such as pancreatogenic diabetes (Type 3c), exocrine pancreatic insufficiency, and pancreatic adenocarcinoma[10,11].

The best-characterized variants are localized in the cationic trypsinogen gene (*PRSS1*) and implicate different biochemical reactions: while some mutations are linked to an increase in autocatalytic activation and enhanced stability of active trypsin[4,8,13], others are connected to increased transactivation of anionic trypsinogen[14], the second major trypsinogen isoform, or even protein misfolding, which results in chronic pancreatitis mediated by increased endoplasmic reticulum stress[15].

SPINK1 is an essential part of the protective mechanism against premature and intracellular trypsin activation. The most frequently observed pancreatitis-associated genetic variant is the N34S mutation[16], but no functional change has been elucidated to date. Because this mutation is very common in the general population, it is considered to be more likely a disease-modifying mutation or a component of a polygenic model[17]. Other mutations of the *SPINK1* gene are related to reduced secretion of trypsin and increased intracellular autodigestion of acinar cells[18]. Chymotrypsin C (CTRC) forms another part of the protective machinery against intracellular trypsin-dependent digestion. This enzyme is minor human chymotrypsin

Table 1. Overview of pancreatitis-associated genetic variants

Gene	Variant	Pathophysiologic change	Publication
PRSS1	R122H	Impaired autolysis of trypsin by mesotrypsin, enzyme Y, and trypsin	Whitcomb *et al*. 1996
PRSS1	N29I	Enhanced autoactivation of trypsinogen	Gorry *et al*. 1997, Sahin-Toth 2000
PRSS1	E79K	Reduced autoactivation, but increased activation of anionic trypsinogen (PRSS2)	Teich *et al*. 2004
PRSS1	R116C	Misfolded trypsin, reduced secretion	Teich *et al*. 2006, Kereszturi *et al*. 2009
SPINK1	L14R	Enhanced intracellular autodigestion, reduced secretion	Kiraly *et al*. 2007
SPINK1	L14P	Enhanced intracellular autodigestion, reduced secretion	Kiraly *et al*. 2007
SPINK1	N34S	Unknown, probably part of a polygenic model	Witt *et al*. 2000
CTRC	R254W	Reduced secretion, reduced expression	Rosendahl *et al*. 2008
CTRC	K247_R254del	No secretion, reduced catalytic activity	Rosendahl *et al*. 2008
CFTR	Several, mostly in combination with other genetic factors	Reduction of chloride and bicarbonate secretion, detainment of trypsinogen inside of the pancreas	Chang *et al*. 2007
CPA1	Several variants	Increased protein misfolding and ER stress	Witt *et al*. 2014
CEL	CEL-HYB	Increased protein misfolding and ER stress	Fjeld *et al*. 2015 Fjeld *et al*. 2022
CELA3B	R90C	Translational upregulation resulting in uncontrolled proteolysis	Moore *et al*. 2019

and promotes the degradation of not only cationic trypsin with high specificity but also the other two trypsinogen isoforms[19]. The two best-characterized CTRC variants related to hereditary pancreatitis are R254W and K247_R254del, both cause reduced secretion and activity of this enzyme[20].

Mutations in the *CFTR* gene can influence the course and severity of pancreatitis as well. The CFTR protein functions as an adenosine monophosphate (AMP)-dependent chloride channel and regulator of transcellular bicarbonate transport[21-24]. Mutations in the *CFTR* gene lead to a defective CFTR protein and are associated with cystic fibrosis (CF) in humans, usually with impaired exocrine pancreatic function. Among CF patients without pancreas insufficiency, 20% develop acute recurrent or chronic pancreatitis[25]. The underlying reason for this large number of diseased individuals is that a critical mass of intact pancreatic acinar cells seems to be required for the development of pancreatitis, which is not the case for pancreatic-insufficient patients. More than 1,700 CFTR mutations have already been discovered in CF patients. Despite their high carrier frequency in the population, the occurrence of pancreatitis among those carrying one mutation is extremely low and suggests that multiple interacting factors, i.e. in combination with other mutations in the *PRSS1* or *SPINK1* genes, are likely to be involved in the pathogenesis of pancreatitis[26].

The entirety of the consequences of CFTR dysfunction in association with pancreatitis remains unknown, but some hypotheses have been advanced. Due to the reduced secretion of bicarbonate in the pancreatic duct, the pH increases and can promote the activation of trypsinogen and the loss of the integrity of cell-cell junctions[27,28]. Carboxyl ester lipase (CEL) is a lipolytic enzyme that is localized in pancreatic acinar cells and lactating mammary glands and is capable of hydrolysis of both water-soluble and water-insoluble lipids[29]. The *CEL* gene is highly polymorphic and contains a variable number of tandem repeat (VNTR) regions. Single base deletions cause maturity-onset diabetes of the young (MODY). The *CEL* hybrid allele (*CEL-HYB*), originating from a cross-over between *CEL* and its neighboring pseudogene *CELP*, has been found to be significantly overrepresented in chronic pancreatitis patients[30]. From a knock-in mouse model of chronic pancreatitis, where the endogenous VNTR of the mouse *CEL* gene has been substituted with the VNTR of the human *CEL-HYB1*, it could be concluded that protein misfolding with the formation of CEL

protein aggregates and ER stress appeared to be the most probable causative mechanism[31]. Single-base-pair deletions in the proximal part of the *CEL*-VNTR have been identified in a family with hereditary pancreatitis, indicating that these variants should also be considered as a cause of hereditary pancreatitis[32].

Finally, mutations in the carboxypeptidase A1 (*CPA1*) and elastase 3B (*CELA3B)* genes have also been found to be associated with hereditary pancreatitis[33,34].

AUTOIMMUNE PANCREATITIS

Autoimmune pancreatitis (AIP) is another rare type of pancreatitis that exhibits features of both acute and chronic pancreatitis. First described by Sarles *et al.* in 1961, it was termed "autoimmune pancreatitis" in 1995 by Yoshida *et al.*[35,36]. The exact numbers of incidence and prevalence of AIP are largely unclear; however, they seem to differ among countries. In Germany, the incidence has been reported to be approximately 0.29 per 100,000 people, while in Japan, there is a much higher incidence of 1.4 per 100,000 inhabitants[37,38]. AIP is classified into two subtypes: type 1, also known as lymphoplasmacytic sclerosing pancreatitis (LPSP); and type 2, idiopathic duct-centric chronic pancreatitis (IDCP). Both are associated with increased infiltration of immune cells into the pancreas and are responsive to steroid medication[39].

Each type of AIP has its own characteristics. Hallmarks of type 1 AIP are infiltration by IgG4-positive plasma cells, increased IgG4 titer in the serum, and histological abnormalities such as storiform fibrosis and lymphoplasmacytic infiltration of the pancreatic ducts[40]. In addition to the manifestation in the pancreas, other organs can be involved, including the liver, the biliary tract, the kidneys, and the lungs[41], demonstrating that type 1 AIP is one possible manifestation of the large complex of IgG4-related diseases. Often, the onset of type 1 AIP occurs in patients over 60 years of age, with a predominance of males[42], and is also related to pancreatogenic diabetes[43,44].

In comparison, type 2 AIP is characterized by the infiltration of neutrophils into the pancreatic duct and the surrounding tissue, especially the interlobular duct, with local destruction of the ductal epithelium at what are called granulocytic epithelial lesions (GELs)[45,46]. In comparison to type 1 AIP, type 2 AIP is characterized by a reduction or even complete absence of IgG4-positive plasma cells in the pancreas[46]. No reliable biomarker has been established thus far. Although no other organs are directly involved in the disease, in contrast to what is seen in IgG4-related disorders, type 2 AIP is associated with inflammatory bowel diseases[40]. Patients suffering from type 2 AIP are generally younger, and disease distribution is more gender-balanced[47]. Both types of AIP are associated with different antibodies targeting the plasminogen-binding protein of *Helicobacter pylori*, the ubiquitin-protein ligase E3 component n-recognin, SPINK1, PRSS1, and PRSS2[48,49]. Because these antibodies are also detectable in other disorders, their value in the diagnosis of AIP is limited.

NEUTROPHIL SERINE PROTEASES IN THE PATHOGENESIS OF PANCREATITIS

Under physiological conditions, pancreatic acinar cells secrete digestive proteases, which are stored as inactive precursors in zymogen granules, into the pancreatic duct. When reaching the duodenum, the serine protease trypsinogen is activated by the duodenal brush border enzyme enterokinase. Active trypsin is able to activate additional trypsinogen and can activate other zymogens secreted into the duodenum, including chymotrypsinogen, proelastase, and procarboxypeptidase A1, A2, and B1, in a cascade-like manner[50]. However, activation of trypsinogen within acinar cells is also possible and is performed either by active trypsin itself or by the lysosomal hydrolase cathepsin B (CTSB). Under normal conditions, several barriers prevent intracellular activation of trypsinogen[51,52], including a shift of intravesical pH to generate an acidic milieu and the presence of inhibiting proteins. One of these proteins is SPINK1, which acts as the major

intracellular inhibitor of trypsin[53]. Besides CTSB, the lysosomal hydrolase cathepsin L and chymotrypsin C are also capable of cleaving trypsinogen[20,54]. Moreover, the aspartic protease cathepsin D (CTSD) indirectly acts on trypsinogen as CTSD proteolytically activates pro-CTSB[55]. In contrast, under pathophysiological conditions, the activation of trypsinogen and other downstream proteases occurs inside pancreatic acinar cells, resulting in self-digestion and necrosis of the cells and the release of damage-associated molecular patterns (DAMPs). In addition, the transcription nuclear factor kappa B (NF-κB) is upregulated in pancreatic acinar cells, resulting in the overexpression and secretion of pro-inflammatory cytokines and chemokines[56]. The combination of DAMPs, cytokines, and chemokines augments the activation and infiltration of immune cells into the pancreatic tissue. Activation of trypsinogen occurs not only in acini but also within macrophages that invade the pancreas during acute pancreatitis and phagocytose zymogen-containing vesicles. This process also depends on pH as well as the lysosomal protease CTSB[57]. Neutrophils are a second major immune cell type that invades the pancreas during pancreatitis and can cause local tissue damage in different ways. In rodents with acute pancreatitis in which neutrophils were depleted by anti-neutrophil serum or anti-GR-1-antibodies, disease severity was significantly reduced[58,59,59]. Neutrophils are also able to activate trypsinogen, but the exact underlying mechanism is unknown, although it has been elucidated for macrophages[57]. One study showed that inhibition of matrix metalloproteinase-9 (MMP9) led to a reduction of trypsinogen activation by neutrophils[60], Which was NADPH-mediated[58].

Moreover, neutrophils release NSPs cathepsin G (CTSG), proteinase 3 (PR3), and neutrophil elastase (NE). The cysteine protease cathepsin C (CTSC) seems to have a higher-ranking function, as it activates NSPs[61-63]. Neutrophil serine enzymes are capable of cleaving the tight junction protein E-cadherin, but they differ in their efficiencies. The strongest cleavage effects have been observed for NE. The breakdown of E-cadherin results in a loss of cell-cell connections, facilitating the infiltration of immune cells into pancreatic tissue and the formation of edema[43]. Pharmacological inhibition of NE prevented cleavage of E-cadherin and reduced neutrophil infiltration in the pancreas *in vivo*[64].In contrast, CTSG and PR3 only have minimal E-cadherin cleavage capabilities in acinar cells, and depletion of CTSG neither decreased trypsinogen activation nor led to an amelioration of pancreatitis severity, although a transient decrease of neutrophil infiltration in the pancreas and extra-pancreatic organs was observed[65]. Furthermore, neutrophils are responsible for the extrusion of neutrophil extracellular traps (NETs), which consist of condensed chromatin and NSPs and can enhance pancreatic damage, promote platelet aggregation, and produce further NSP-associated effects[66]. NETs can also promote trypsinogen activation[67]. The main pathogenetic cellular events in the initiation of acute pancreatitis are summarized in Figure 1.

The knowledge of the role of neutrophils in general and NSPs in particular in the pathogenesis and course of hereditary and autoimmune pancreatitis remains very limited. The pathogenic role of neutrophils in chronic pancreatitis associated with genetic mutations has been reflected in two murine models. In mice carrying a heterozygous p.D23A mutation in the mouse cationic trypsinogen isoform 7 (T7D23A knock-in), strong auto-activation of trypsinogen was observed, resulting in spontaneous acute and subsequent chronic pancreatitis. Massive infiltration of inflammatory cells including neutrophils and macrophages occurred during the early disease phase. Signs of disease progression were already visible after 4-5 weeks, while immune cells became fewer in number[68]. Transgenic mice expressing the PRSS1 mutant R122H (R122H_mPRSS1) displayed early-onset acinar cell injury and inflammatory cell infiltration, followed by pancreatic fibrosis and an enhanced response to the cholecystokinin analog caerulein[69].

To the best of our knowledge, no currently available literature has specifically focused on the role of NSPs in hereditary pancreatitis, although the organ-injuring effects of neutrophils and elastase in acute pancreatitis are clear in general.

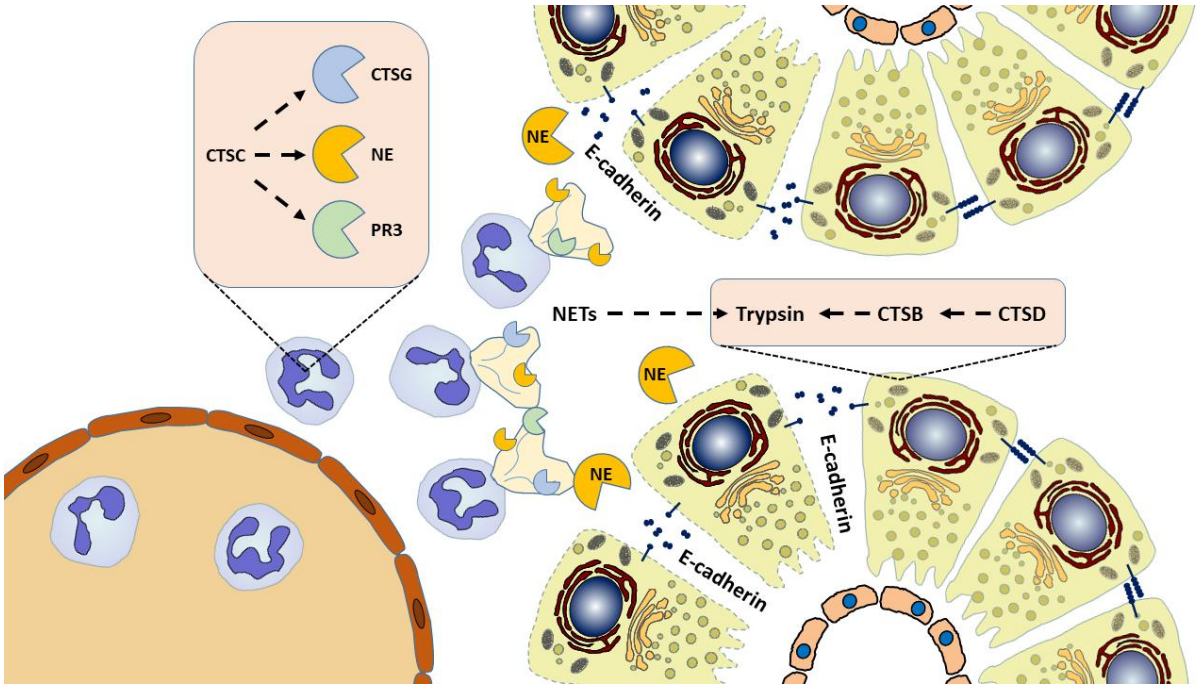

Figure 1. Roles of lysosomal and neutrophil serine proteases in acute pancreatitis. The conversion of trypsinogen into its active form is an important step in the early phase of acute pancreatitis. In addition to autoactivation, trypsinogen is activated by cathepsin B (CTSB) derived from acini and macrophages. Pro-CTSB itself is proteolytically activated by cathepsin D (CTSD). While cathepsin C (CTSC) does not directly act on trypsinogen, it activates the neutrophil serine proteases cathepsin G (CTSG), proteinase 3 (PR3), and neutrophil elastase (NE). NE readily cleaves the cell-adhesion molecule E-cadherin, leading to the dissociation of acini.

Regarding type 2 AIP, one study compared pancreatic tissue samples of patients with alcoholic chronic pancreatitis, type 1 AIP, and type 2 AIP, and investigated the extent and localization of neutrophilic infiltration. In addition, these authors analyzed the expression of the chemokines interleukin-8 (IL-8) and granulocyte chemotactic protein 2 (GCP-2), both of which can bind to neutrophilic ELR$^+$ CXC receptors and can attract and activate neutrophils. In type 2 AIP, there was higher infiltration of neutrophils into the interlobular ducts and the surrounding tissue, which was absent around the intralobular ducts. In addition, the authors found higher expression of GCP-2 around the interlobular but not around the intralobular ducts, and levels of IL-8 expression remained unaltered. They concluded that the increase of neutrophilic infiltration into the interlobular ducts in type 2 AIP depends on GCP-2 which is secreted by pancreatic epithelial cells[70].

Further knowledge of the role of neutrophils and NSPs in these two rare forms of pancreatitis remains missing. Especially regarding type 2 AIP, knowledge of the interplay between the pancreas, infiltrating neutrophils, and NSPs would be helpful for a more comprehensive understanding of this rare disorder.

MODULATION OF NEUTROPHIL SERINE PROTEASE ACTIVITY

Because NSPs are involved in the pathophysiology of acute pancreatitis, their inhibition is of therapeutic interest. Increased NE activity or an imbalance with endogenous inhibitors can cause a broad range of diseases apart from pancreatitis. Existing NE inhibitors with high inhibitory capacities and low cytotoxicity *in vitro* have not only been isolated from animal sources and fungi[71] but have also been designed synthetically, thus providing a strong basis for subsequent clinical investigations. The most prominent synthetic compound, AZD9668, has been reported to show beneficial effects in chronic lung diseases[72].

While serine protease inhibitors have attenuated disease severity by reduction of both pancreatic and extra-pancreatic injury in experimental models[73] and prophylactic administration of the serine protease inhibitor gabexate mesylate has reduced the rate of post-ERCP pancreatitis in high-risk patients[74], data regarding usefulness of NSP inhibitors in treating acute pancreatitis are limited and restricted to preclinical models.

Sivelestat, a specific inhibitor of NE, protected against pancreatic and lung injury in rats that had undergone caerulein- or taurocholate-induced acute pancreatitis[75]. Additive effects were achieved when treatment was combined with antioxidant resveratrol[76]. Lex032, an inhibitor of elastase and cathepsin G, ameliorated microcirculatory function in ischemia/reperfusion-induced acute pancreatitis by the preservation of capillary perfusion, absence of interleukin-6 increase, and preservation of mean arterial pressure during reperfusion time, indicating usefulness in preventing pancreatic tissue damage from ischemia/reperfusion[77]. Despite promising results in preclinical investigations, the effectiveness of NSP modulation in clinical use remains to be validated.

CONCLUSION

Despite advances in understanding the pathogenesis of acute pancreatitis, the precise cellular and subcellular mechanisms of the early disease phase are not entirely understood. Neutrophils are recruited to inflamed sites and drive inflammatory reactions by several processes, including the activation of NSPs. Several animal models suggest a favorable effect on acute pancreatitis when neutrophil elastase is inhibited. Recent data support the pro-inflammatory role of NSPs in acute pancreatitis; however, their pathogenic functions in autoimmune and hereditary pancreatitis, two rarer forms of pancreatitis, still need to be clarified.

DECLARATIONS

Authors' contributions

Literature research and writing: Zierke L, Gischke M, Tran QT, Aghdassi AA
Final approval of the draft: Zierke L, Gischke M, Tran QT, Aghdassi AA

Availability of data and materials

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Peery AF, Crockett SD, Murphy CC, et al. Burden and cost of gastrointestinal, liver, and pancreatic diseases in the United States: update 2018. *Gastroenterology* 2019;156:254-272.e11. DOI PubMed PMC
2. Wiese ML, Urban S, von Rheinbaben S, et al. Identification of early predictors for infected necrosis in acute pancreatitis. *BMC Gastroenterol* 2022;22:405. DOI PubMed PMC
3. Weiss FU, Laemmerhirt F, Lerch MM. Acute pancreatitis: genetic risk and clinical implications. *J Clin Med* 2021;10:190. DOI PubMed PMC
4. Gorry MC, Gabbaizedeh D, Furey W, et al. Mutations in the cationic trypsinogen gene are associated with recurrent acute and chronic pancreatitis. *Gastroenterology* 1997;113:1063-8. DOI
5. Whitcomb DC. Genetic risk factors for pancreatic disorders. *Gastroenterology* 2013;144:1292-302. DOI PubMed PMC
6. Girodon E, Rebours V, Chen JM, et al. Clinical interpretation of *PRSS1* variants in patients with pancreatitis. *Clin Res Hepatol Gastroenterol* 2021;45:101497. DOI
7. Comfort MW, Steinberg AG. Pedigree of a family with hereditary chronic relapsing pancreatitis. *Gastroenterology* 1952;21:54-63. DOI PubMed
8. Whitcomb DC, Gorry MC, Preston RA, et al. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996;14:141-5. DOI
9. Coté GA, Yadav D, Slivka A, et al; North American Pancreatitis Study Group. Alcohol and smoking as risk factors in an epidemiology study of patients with chronic pancreatitis. *Clin Gastroenterol Hepatol* 2011;9:266-73; quiz e27. DOI PubMed PMC
10. Rebours V, Boutron-Ruault MC, Schnee M, et al. The natural history of hereditary pancreatitis: a national series. *Gut* 2009;58:97-103. DOI
11. Howes N, Lerch MM, Greenhalf W, et al; European Registry of Hereditary Pancreatitis and Pancreatic Cancer (EUROPAC). Clinical and genetic characteristics of hereditary pancreatitis in Europe. *Clin Gastroenterol Hepatol* 2004;2:252-61. DOI
12. Sultan M, Werlin S, Venkatasubramani N. Genetic prevalence and characteristics in children with recurrent pancreatitis. *J Pediatr Gastroenterol Nutr* 2012;54:645-50. DOI PubMed
13. Sahin-Tóth M. Human cationic trypsinogen. Role of Asn-21 in zymogen activation and implications in hereditary pancreatitis. *J Biol Chem* 2000;275:22750-5. DOI PubMed
14. Teich N, Le Maréchal C, Kukor Z, et al. Interaction between trypsinogen isoforms in genetically determined pancreatitis: mutation E79K in cationic trypsin (PRSS1) causes increased transactivation of anionic trypsinogen (PRSS2). *Hum Mutat* 2004;23:22-31. DOI
15. Kereszturi E, Szmola R, Kukor Z, et al. Hereditary pancreatitis caused by mutation-induced misfolding of human cationic trypsinogen: a novel disease mechanism. *Hum Mutat* 2009;30:575-82. DOI PubMed PMC
16. Witt H, Luck W, Hennies HC, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet* 2000;25:213-6. DOI
17. Schneider A. Serine protease inhibitor kazal type 1 mutations and pancreatitis. *Clin Lab Med* 2005;25:61-78. DOI PubMed
18. Király O, Boulling A, Witt H, et al. Signal peptide variants that impair secretion of pancreatic secretory trypsin inhibitor (SPINK1) cause autosomal dominant hereditary pancreatitis. *Hum Mutat* 2007;28:469-76. DOI PubMed PMC
19. Szmola R, Sahin-Tóth M. Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: identity with Rinderknecht's enzyme Y. *Proc Natl Acad Sci USA* 2007;104:11227-32. DOI PubMed PMC
20. Rosendahl J, Witt H, Szmola R, et al. Chymotrypsin C (*CTRC*) variants that diminish activity or secretion are associated with chronic pancreatitis. *Nat Genet* 2008;40:78-82. DOI PubMed PMC
21. Anderson MP, Rich DP, Gregory RJ, Smith AE, Welsh MJ. Generation of cAMP-activated chloride currents by expression of CFTR. *Science* 1991;251:679-82. DOI PubMed
22. Quinton PM. Chloride impermeability in cystic fibrosis. *Nature* 1983;301:421-2. DOI PubMed
23. Reddy MM, Quinton PM. Selective activation of cystic fibrosis transmembrane conductance regulator CL- and HCO_3- conductances. *JOP* 2001;2(4 Suppl):212-8. PubMed
24. Stutts MJ, Canessa CM, Olsen JC, et al. CFTR as a cAMP-dependent regulator of sodium channels. *Science* 1995;269:847-50. DOI
25. Durno C, Corey M, Zielenski J, Tullis E, Tsui LC, Durie P. Genotype and phenotype correlations in patients with cystic fibrosis and pancreatitis. *Gastroenterology* 2002;123:1857-64. DOI PubMed
26. Ooi CY, Dorfman R, Cipolli M, et al. Type of CFTR mutation determines risk of pancreatitis in patients with cystic fibrosis. *Gastroenterology* 2011;140:153-61. DOI
27. Behrendorff N, Floetenmeyer M, Schwiening C, Thorn P. Protons released during pancreatic acinar cell secretion acidify the lumen and contribute to pancreatitis in mice. *Gastroenterology* 2010;139:1711-20, 1720.e1. DOI PubMed
28. Pallagi P, Venglovecz V, Rakonczay Z Jr, et al. Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl^- channels and luminal anion exchangers. *Gastroenterology* 2011;141:2228-2239.e6. DOI
29. Hui DY, Howles PN. Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. *J Lipid Res* 2002;43:2017-30. DOI PubMed
30. Fjeld K, Weiss FU, Lasher D, et al. A recombined allele of the lipase gene *CEL* and its pseudogene *CELP* confers susceptibility to chronic pancreatitis. *Nat Genet* 2015;47:518-22. DOI PubMed PMC
31. Fjeld K, Gravdal A, Brekke RS, et al. The genetic risk factor *CEL-HYB1* causes proteotoxicity and chronic pancreatitis in mice.

Pancreatology 2022;22:1099-111. DOI

32. El Jellas K, Dušátková P, Haldorsen IS, et al. Two new mutations in the *CEL* gene causing diabetes and hereditary pancreatitis: how to correctly identify MODY8 cases. *J Clin Endocrinol Metab* 2022;107:e1455-e1466. DOI PubMed PMC
33. Witt H, Beer S, Rosendahl J, et al. Variants in CPA1 are strongly associated with early onset chronic pancreatitis. *Nat Genet* 2013;45:1216-20. DOI PubMed PMC
34. Moore PC, Cortez JT, Chamberlain CE, et al. Elastase 3B mutation links to familial pancreatitis with diabetes and pancreatic adenocarcinoma. *J Clin Invest* 2019;129:4676-81. DOI PubMed PMC
35. SARLES H, SARLES JC, MURATORE R, GUIEN C. Chronic inflammatory sclerosis of the pancreas-an autonomous pancreatic disease? *Am J Dig Dis* 1961;6:688-98. DOI PubMed
36. Yoshida K, Toki F, Takeuchi T, Watanabe S, Shiratori K, Hayashi N. Chronic pancreatitis caused by an autoimmune abnormality. Proposal of the concept of autoimmune pancreatitis. *Dig Dis Sci* 1995;40:1561-8. DOI PubMed
37. Schneider A, Michaely H, Weiss C, et al. Prevalence and incidence of autoimmune pancreatitis in the population living in the southwest of Germany. *Digestion* 2017;96:187-98. DOI
38. Kanno A, Masamune A, Okazaki K, et al; Research Committee of Intractable Diseases of the Pancreas. Nationwide epidemiological survey of autoimmune pancreatitis in Japan in 2011. *Pancreas* 2015;44:535-9. DOI
39. Kamisawa T, Egawa N, Inokuma S, et al. Pancreatic endocrine and exocrine function and salivary gland function in autoimmune pancreatitis before and after steroid therapy. *Pancreas* 2003;27:235-8. DOI
40. Kamisawa T, Chari ST, Giday SA, et al. Clinical profile of autoimmune pancreatitis and its histological subtypes: an international multicenter survey. *Pancreas* 2011;40:809-14. DOI
41. Sandrasegaran K, Menias CO. Imaging in autoimmune pancreatitis and immunoglobulin g4-related disease of the abdomen. *Gastroenterol Clin North Am* 2018;47:603-19. DOI PubMed
42. Hart PA, Kamisawa T, Brugge WR, et al. Long-term outcomes of autoimmune pancreatitis: a multicentre, international analysis. *Gut* 2013;62:1771-6. DOI PubMed PMC
43. John DS, Aschenbach J, Krüger B, et al. Deficiency of cathepsin C ameliorates severity of acute pancreatitis by reduction of neutrophil elastase activation and cleavage of E-cadherin. *J Biol Chem* 2019;294:697-707. DOI PubMed PMC
44. Ito T, Nakamura T, Fujimori N, et al. Characteristics of pancreatic diabetes in patients with autoimmune pancreatitis. *J Dig Dis* 2011;12:210-6. DOI
45. Zamboni G, Lüttges J, Capelli P, et al. Histopathological features of diagnostic and clinical relevance in autoimmune pancreatitis: a study on 53 resection specimens and 9 biopsy specimens. *Virchows Arch* 2004;445:552-63. DOI
46. Shinagare S, Shinagare AB, Deshpande V. Autoimmune pancreatitis: a guide for the histopathologist. *Semin Diagn Pathol* 2012;29:197-204. DOI PubMed
47. Ikeura T, Manfredi R, Zamboni G, et al. Application of international consensus diagnostic criteria to an Italian series of autoimmune pancreatitis. *United European Gastroenterol J* 2013;1:276-84. DOI PubMed PMC
48. Löhr JM, Faissner R, Koczan D, et al. Autoantibodies against the exocrine pancreas in autoimmune pancreatitis: gene and protein expression profiling and immunoassays identify pancreatic enzymes as a major target of the inflammatory process. *Am J Gastroenterol* 2010;105:2060-71. DOI PubMed PMC
49. Frulloni L, Lunardi C, Simone R, et al. Identification of a novel antibody associated with autoimmune pancreatitis. *N Engl J Med* 2009;361:2135-42. DOI
50. Mayerle J, Sendler M, Hegyi E, Beyer G, Lerch MM, Sahin-Tóth M. Genetics, cell biology, and pathophysiology of pancreatitis. *Gastroenterology* 2019;156:1951-1968.e1. DOI PubMed PMC
51. Halangk W, Lerch MM, Brandt-Nedelev B, et al. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 2000;106:773-81. DOI PubMed PMC
52. Saluja A, Hashimoto S, Saluja M, Powers RE, Meldolesi J, Steer ML. Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. *Am J Physiol* 1987;253:G508-16. DOI PubMed
53. Ohmuraya M, Yamamura K. Roles of serine protease inhibitor Kazal type 1 (SPINK1) in pancreatic diseases. *Exp Anim* 2011;60:433-44. DOI PubMed
54. Wartmann T, Mayerle J, Kähne T, et al. Cathepsin L inactivates human trypsinogen, whereas cathepsin L-deletion reduces the severity of pancreatitis in mice. *Gastroenterology* 2010;138:726-37. DOI PubMed PMC
55. Aghdassi AA, John DS, Sendler M, et al. Cathepsin D regulates cathepsin B activation and disease severity predominantly in inflammatory cells during experimental pancreatitis. *J Biol Chem* 2018;293:1018-29. DOI PubMed PMC
56. Gukovsky I, Gukovskaya AS, Blinman TA, Zaninovic V, Pandol SJ. Early NF-kappaB activation is associated with hormone-induced pancreatitis. *Am J Physiol* 1998;275:G1402-14. DOI PubMed
57. Sendler M, Weiss FU, Golchert J, et al. Cathepsin B-mediated activation of trypsinogen in endocytosing macrophages increases severity of pancreatitis in mice. *Gastroenterology* 2018;154:704-718.e10. DOI PubMed PMC
58. Gukovskaya AS, Vaquero E, Zaninovic V, et al. Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. *Gastroenterology* 2002;122:974-84. DOI
59. Abdulla A, Awla D, Thorlacius H, Regnér S. Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. *J Leukoc Biol* 2011;90:975-82. DOI PubMed
60. Awla D, Abdulla A, Syk I, Jeppsson B, Regnér S, Thorlacius H. Neutrophil-derived matrix metalloproteinase-9 is a potent activator of

trypsinogen in acinar cells in acute pancreatitis. *J Leukoc Biol* 2012;91:711-9. DOI PubMed

61. Mcguire M, Lipsky P, Thiele D. Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. *Journal of Biological Chemistry* 1993;268:2458-67. DOI PubMed
62. Turk D, Janjić V, Stern I, et al. Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases. *EMBO J* 2001;20:6570-82. DOI PubMed PMC
63. Adkison AM, Raptis SZ, Kelley DG, Pham CT. Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. *J Clin Invest* 2002;109:363-71. DOI PubMed PMC
64. Mayerle J, Schnekenburger J, Krüger B, et al. Extracellular cleavage of E-cadherin by leukocyte elastase during acute experimental pancreatitis in rats. *Gastroenterology* 2005;129:1251-67. DOI
65. Aghdassi AA, John DS, Sendler M, et al. Absence of the neutrophil serine protease cathepsin G decreases neutrophil granulocyte infiltration but does not change the severity of acute pancreatitis. *Sci Rep* 2019;9:16774. DOI PubMed PMC
66. Leppkes M, Maueröder C, Hirth S, et al. Externalized decondensed neutrophil chromatin occludes pancreatic ducts and drives pancreatitis. *Nat Commun* 2016;7:10973. DOI PubMed PMC
67. Merza M, Hartman H, Rahman M, et al. Neutrophil extracellular traps induce trypsin activation, inflammation, and tissue damage in mice with severe acute pancreatitis. *Gastroenterology* 2015;149:1920-1931.e8. DOI
68. Geisz A, Sahin-Tóth M. A preclinical model of chronic pancreatitis driven by trypsinogen autoactivation. *Nat Commun* 2018;9:5033. DOI PubMed PMC
69. Archer H, Jura N, Keller J, Jacobson M, Bar-Sagi D. A mouse model of hereditary pancreatitis generated by transgenic expression of R122H trypsinogen. *Gastroenterology* 2006;131:1844-55. DOI PubMed
70. Mitsuyama T, Uchida K, Sumimoto K, et al. Comparison of neutrophil infiltration between type 1 and type 2 autoimmune pancreatitis. *Pancreatology* 2015;15:271-80. DOI
71. Marinaccio L, Stefanucci A, Scioli G, et al. Peptide human neutrophil elastase inhibitors from natural sources: an overview. *Int J Mol Sci* 2022;23:2924. DOI PubMed PMC
72. Sjö P. Neutrophil elastase inhibitors: recent advances in the development of mechanism-based and nonelectrophilic inhibitors. *Future Med Chem* 2012;4:651-60. DOI PubMed
73. Cao C, Yin C, Shou S, et al. Ulinastatin protects against LPS-induced acute lung injury by attenuating TLR4/NF-κB pathway activation and reducing inflammatory mediators. *Shock* 2018;50:595-605. DOI
74. Testoni PA, Mariani A, Masci E, Curioni S. Frequency of post-ERCP pancreatitis in a single tertiary referral centre without and with routine prophylaxis with gabexate: a 6-year survey and cost-effectiveness analysis. *Dig Liver Dis* 2006;38:588-95. DOI
75. Cao J, Liu Q. Protective effects of sivelestat in a caerulein-induced rat acute pancreatitis model. *Inflammation* 2013;36:1348-56. DOI
76. Wang H, Wang S, Tang A, Gong H, Ma P, Chen L. Combined effects of sivelestat and resveratrol on severe acute pancreatitis-associated lung injury in rats. *Exp Lung Res* 2014;40:288-97. DOI
77. Dobschuetz E, Hoffmann T, Messmer K. Inhibition of neutrophil proteinases by recombinant serpin Lex032 reduces capillary no-reflow in ischemia/reperfusion-induced acute pancreatitis. *J Pharmacol Exp Ther* 1999;290:782-8. PubMed

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Connecting academia and industry for innovative drug repurposing in rare diseases: it is worth a try

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Abstract

There are different approaches to drug repurposing (DR) depending on the status of the repurposable drug/molecule (approved, investigational, withdrawn, shelved), the context, and the stakeholders involved. The purpose of this perspective paper is to highlight the complexity of academia-industry collaborations in DR for rare diseases and go beyond stereotypes to consider realistic and mutually reinforcing cooperation among various stakeholders, including not only academia and industry but also regulators, legal experts, and payers, leading to benefits for patients with unmet medical needs. Key questions are addressed through the presentation of select DR case studies. Some ongoing and promising European and international initiatives are introduced and some recommendations are proposed.

Keywords: Drug repurposing, academia-industry collaboration, rare diseases, sildenafil, alpelisib, propranolol

INTRODUCTION

Drug repurposing (DR) can be defined as the process of identification of new uses for approved or investigational drugs that are outside the scope of the original medical indication. It is considered to have significant advantages over developing an entirely new drug: fewer risks, lower costs, and shorter timelines. Public-specific programs promote DR initiatives while pharmaceutical companies integrate them as a strategy in the life cycle management of their products. Indeed, DR represents a relevant opportunity to find

treatments, especially in the field of rare diseases, where 95% of the 7,000 to 8,000 rare diseases have no approved treatment. A few well-known success stories are good illustrations of successful academia-industry DR collaborations, which may be viewed as the tip of the iceberg. There are also bitter and anonymous failures of collaboration.

The purpose of this perspective paper is to explore academia-industry collaborations in DR of medicinal products for rare diseases through their main challenges and successes and through select DR case studies. Key questions are raised to understand why, when, and how to implement a relevant collaboration. Some recommendations are also proposed.

DEFINITION OF DRUG REPURPOSING

DR is also known as drug repositioning, reprofiling, retasking, rediscovery, rescue, *etc*. While reflecting on different types of situations encountered and described in the literature[1], all these terms are synonyms and refer to a similar process that identifies new indications for approved, investigational, failed or shelved drugs/compounds. At present, the terms are all used interchangeably. This article will refer to all terminologies as "drug repurposing" for consistency purposes. As of November 1, 2022, a search on PubMed listed 1973 results mentioning "drug repurposing" and 861 results mentioning "drug repositioning" for 2022.

DRUG REPURPOSING PROVIDES AN OPPORTUNITY FOR RARE DISEASES

Much has been said about DR over the last two years with the COVID-19 pandemic. An editorial entitled "COVID-19 and the DR Tsunami", published in July 2020, reflects an unprecedented wave to repurpose existing drugs[2]. The aim is to vastly accelerate the usually long approval process of drugs[3], which is a common goal and an objective also shared with rare disease patients desperately waiting for treatments.

Today there are an estimated 350 million people worldwide living with a rare disease. 7,000 to 8,000 rare diseases have been identified, with 250-280 new diseases discovered annually. Only about 5% of rare diseases are estimated to have approved treatments.

In Europe, between 2000 and 2021, 245 orphan drugs, so named for the rare diseases they treat, have been approved. They cover a total of 148 rare diseases[4]. About 20% of orphan drugs are repurposed drugs[5].

There is an urgent need for treatments[6] as most of the rare diseases are life-threatening. DR may be a particularly attractive option for the development of treatments for rare diseases. Of note, oncology and infection are together the most common disease areas for DR so far[7].

SELECT EXAMPLES OF SUCCESSFUL DRUG REPURPOSING

The most famous case of DR is probably the development of sildenafil which is also a good example of both investigational and approved DR[8]. Sildenafil, a phosphodiesterase-5 (PDE5) inhibitor initially explored as a treatment for angina pectoris and hypertension by Pfizer, was tested unsuccessfully in these diseases and eventually demonstrated efficacy in treating erectile dysfunction (with the trade name Viagra®) and then pulmonary arterial hypertension (with the trade name Revatio®)[9].

The clinical trials in angina, which had started in 1989, were disappointing, and sildenafil failed in the Phase II trial. However, a side effect (induction of penile erection) was serendipitously found during the Phase I and II clinical trials and redirected sildenafil to the treatment of erectile dysfunctions (ED). This indication

was approved in Europe and the United States in 1998.

After its serendipitous repurposing for erectile dysfunctions, sildenafil was then repurposed (on purpose this time) for a rare disease called pulmonary arterial hypertension[10]. This indication was approved in Europe and the United States in 2005[11].

Of note, sildenafil acts on the same target: phosphodiesterase 5 (PDE5), to treat both erectile dysfunction and pulmonary arterial hypertension. PDE5 is a key enzyme involved in the regulation of cyclic guanosine monophosphate (cGMP)-specific signaling pathways in normal physiological processes, such as smooth muscle contraction and relaxation.

Sildenafil was originally tested for angina pectoris, a chest pain associated with coronary heart disease. Since PDE5 hydrolyzes cGMP in the cardiopulmonary vasculature, researchers aimed to establish a new anti-anginal agent using PDE5 inhibitors to prolong cGMP activity and promote vasodilation of the coronary arteries. However, early unconvincing results suggesting PDE5 was minimally present in cardiomyocytes led to the abandonment of this research approach[12]. PDE5 is the predominant PDE in the corpus cavernosum. The catalytic site of PDE5 degrades cGMP, the key second messenger in the mediation of penile erection. In men with erectile dysfunction, selective inhibition of PDE5 leads to an increase of cGMP in corpus cavernosal tissue and improves erectile function.

Persistent pulmonary arterial hypertension (PAH) in newborns has been shown to be linked to PDE5 overexpression and overactivation. PAH is characterized by increased pulmonary vascular resistance due to vasoconstriction of the small pulmonary arteries and arterioles. In blood vessels, cGMP relaxes vascular smooth muscles leading to vasodilation and increased blood flow. By inhibiting PDE5 and raising the intracellular cGMP, sildenafil is an effective pulmonary vasodilator.

Understanding the mechanisms of action (MOAs) of drugs is critical not only for drug development but also for DR.

The identification of drug MOAs has been primarily based on pharmacological experiments.

Identification of MOAs through biological pathways involving a drug and its targets is a more recent alternative approach to point to diseases not currently treated by a drug, to predict new uses of existing drugs and to repurpose them. Such an approach can be of real interest also for drugs with unknown underlying mechanisms.

In fact, biological pathway analysis based on drug targets (genes and proteins) may reveal new MOAs and also new clinical functions of existing drugs[13]. Alpelisib provides a good example of innovative DR in this respect by illustrating how the analysis of a biological pathway can lead to the treatment of diseases other than those initially investigated.

Alpelisib, an inhibitor of PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), was primarily developed for the treatment of PIK3CA-mutated cancers and then repurposed for the treatment of PIK3CA-related overgrowth spectrum (PROS).

Cancer-associated PIK3CA gene mutations result in the production of an altered p110 alpha subunit. Strongly and permanently activated due to the presence of a gain-of-function mutation in PIK3CA, p110 alpha allows the Phosphatidylinositol 3-Kinase (PI3K) to signal without regulation, promoting oncogenesis and tissue overgrowth[14]. Patients with PIK3CA-mutated, hormone receptor (HR)-positive/HER2-negative tumors have poor outcomes and resistance to chemotherapy[15].

Developed by Novartis, alpelisib was approved by the United States Food and Drug Administration (FDA) as Piqray® in combination with fulvestrant for advanced or metastatic breast cancer with a PIK3CA mutation. Fulvestrant is a selective estrogen receptor antagonist which works both by down-regulating and degrading the estrogen receptor. Piqray® is used with fulvestrant after hormone treatment used alone has failed.

Activating PIK3CA mutations are also found in overgrowth syndromes, collectively known as PIK3CA-related overgrowth spectrum (PROS). PROS includes a group of genetic disorders and various clinical entities that leads to the overgrowth of various body parts due to mutations in the PIK3CA gene. The CLOVES (Congenital Lipomatous Overgrowth, Vascular malformations, Epidermal nevi, and Skeletal anomalies) syndrome, a rare disorder first described as a distinct syndrome, is, for instance, one subtype of this spectrum.

The severity of PROS is highly variable, ranging from localized overgrowth to severe, extensive, and life-threatening overgrowth affecting major vessels and/or critical organs.

PIK3CA-related cancers and PROS share the same pathogenetic mechanism and almost the same PIK3CA mutational profile[14].

PI3K/AKT/mTOR pathway is involved in many biological processes, including cell metabolism, proliferation, survival, and growth; deregulation of PI3K/AKT/mTOR pathway functioning, in the sense of overactivity, promotes oncogenesis and tissue overgrowth[16].

It is then relevant to investigate the potential of some anticancer drugs to treat rare overgrowth syndromes such as PROS.

As in cancer, PIK3CA mutations in PROS occur as post-zygotic events, but unlike in cancer, these mutations arise during embryonic development[17]. Different components of the PI3K/AKT/mTOR signaling pathway can be specifically targeted to treat PROS. The PIK3CA protein is involved in the first intracellular signal transduction step of the mTOR pathway. Alpelisib acts at the top of the PI3K/AKT/mTOR signaling pathway and sirolimus, a direct inhibitor of mTOR, at the end of the PI3K/AKT pathway [Figure 1]. Both molecules were tested in PROS[14].

However, since activation of mTOR is responsible for some but not all of the biological effects caused by PI3K gain-of-function, alpelisib proved to be a more attractive drug candidate in PROS by providing a direct outcome on affected tissues while reducing the risk of off-target effects.

Alpelisib (BYL719) was in a phase III randomized double-blind, placebo-controlled study (SOLAR-1) conducted in patients with PIK3CA-mutated, HR-positive, human epidermal growth factor receptor 2-negative advanced breast cancer with a tolerable safety profile when Canaud's research group decided to explore its therapeutic potential in PROS. After achieving impressive outcomes first on PROS mouse

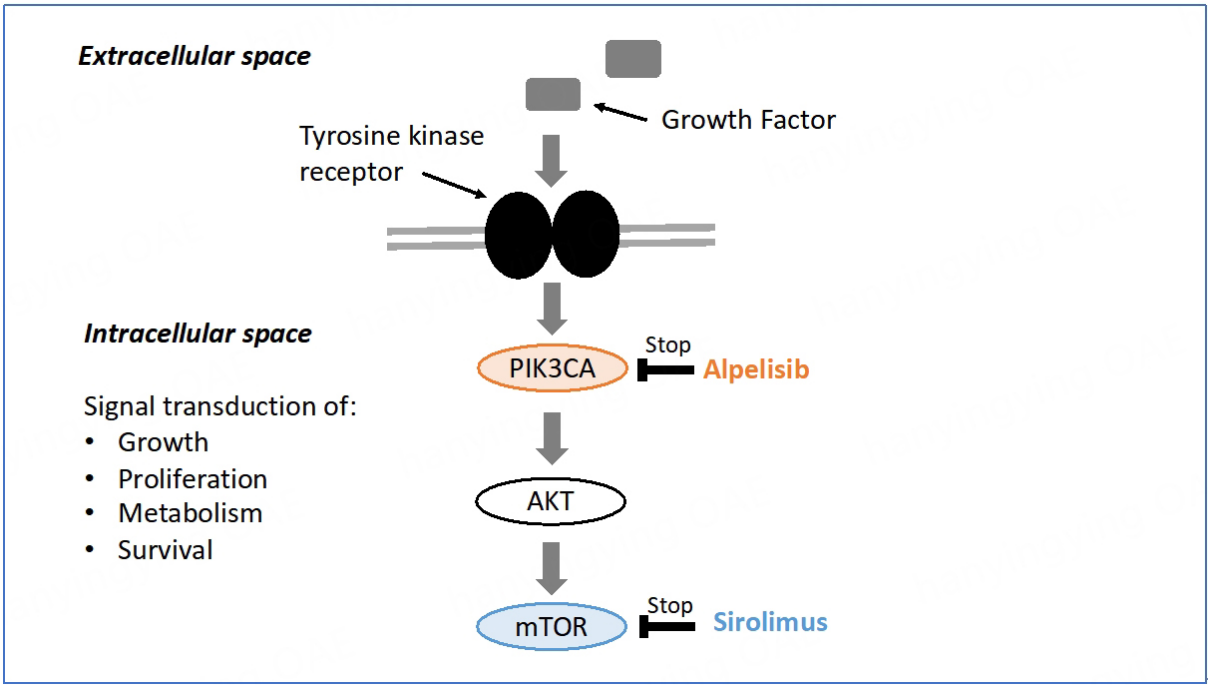

Figure 1. mTOR signal transduction pathway[15].

models and then on two patients suffering from extremely severe and life-threatening clinical manifestations of PROS/CLOVES syndrome, Canaud's group was authorized to administer BYL719 to 17 additional patients with PROS. The study was published in 2018, supporting PIK3CA inhibition as a promising therapeutic strategy in patients with PROS[18].

Four years later, in 2022, the Food and Drug Administration (FDA) granted accelerated approval to alpelisib under the brand name Vijoice® to treat severe manifestations of PROS in patients aged 2 years or older. This indication for Vijoice® is approved based on real-world response rate and duration of response findings in 57 patients in the single-arm EPIK-P1 study[19], a retrospective chart review study. Real-world data (RWD) and Real-world evidence (RWE) may be supportive for regulatory decision-making in rare and ultra-rare diseases. Medical records with chart review may be useful tools to generate RWE. They also have limitations, and continued approval will be, however, contingent upon additional placebo-controlled studies that will confirm the clinical benefit of alpelisib in PROS patients.

ACADEMIA-INDUSTRY COLLABORATION: WHY, WHEN, AND HOW

The two successful DR examples mentioned above, alpelisib in PROS but also sildenafil in ED, are good illustrations of a successful academia-industry DR collaboration. In one case, investigators reported the side effects of sildenafil that became a repurposed indication in ED, and Pfizer drove the repurposing efforts; in the other case, an academic research team attempted independent repurposing of alpelisib in PROS, a new and rare indication further developed by Novartis.

If there are remarkable DR success stories that hit the headlines, there are also bitter and anonymous failures of collaboration.

Various reasons may explain the poor cooperation between industry and academia. These reasons need to be properly understood if we want to orient efforts in appropriate directions. Key questions may be addressed: why, when, and how to implement a relevant collaboration. They deserve clear answers through a broader and more structured debate involving all stakeholders. In the meantime, some suggestions can be made, and some existing initiatives at European (for some of them) and international levels can be reported to encourage and stimulate new opportunities.

WHY

Why get academia and industry to work together? There are many reasons to support such collaborations. Even if they have different expectations and constraints, academia and industry may be complementary and bring added value to each other in a highly regulated environment.

Academia as a source of innovation

Pharma's business model fundamentally depends on product innovation to create value. Patent expiry and generic competition approximately every 10 years are real challenges for pharmaceutical companies which look to academia for valuable knowledge and innovation[20]. The contribution of academia is crucial in terms of basic research, identification and validation of new targets, and also in terms of clinical trials to evaluate the efficacy and safety of drug candidates. Academia and industry may be caricatured as the alpha and the omega as they have complementary enterprises throughout the drug value chain, from innovation to validation. Research for rare diseases starts in academia[21], development is shared between stakeholders, and commercial launch is managed by the industry.

Different expectations and constraints but working towards the same goal of improving the health of patients

It has become commonplace to highlight the gap between academia and industry in terms of cultures and practices. Although patenting is key for them both, the value of secrecy for pharma continues long after patent filing and grant. Pharmaceutical companies still consider results collected from clinical trials to be confidential information or trade secrets, even after submission to regulatory agencies. Academics, for their part, are under pressure to increase their publication count. The "publish or perish" culture is a well-known practice existing within academic institutions. But once the patent has been filed, the invention may be published. It is then crucial for a researcher to present his/her work at conferences, exchange with peers, and win grants.

Highly regulated drug development process

Academic researchers have neither the capacity nor the finance to develop a drug candidate until the launch of the finished product. Moreover, they are often unaware of what evidence needs to be submitted as part of an application for marketing approval or which complex drug development challenges will have to be addressed. Pharmaceutical companies operate in a very highly regulated area, from key nonclinical studies, Phase I-III clinical trials, chemistry, manufacturing and controls (CMC), and formulation activities to regulatory submissions, commercial launch activities, and life-cycle management[7]. Drug development requires a comprehensive and well-thought-out development strategy with a detailed roadmap.

Drug development is a risky and costly process

The time required for a new drug to be developed is about 10-15 years and the cost is around $ 2 billion. Despite such significant investments in time and money, approval rates for a new drug are about 10%. DR has been proposed as an interesting strategy with fewer risks, lower costs, and shorter timelines. Indeed, repurposed drugs are generally approved sooner (3-12 years), at reduced (50%-60%) cost and lower risk: approval rates for repurposed drugs are close to 30%[22]. However, in some cases, DR may be an expensive,

time-consuming, and risky process[23]. Furthermore, it does not always succeed. Like *de novo* drug development, DR may also fail in late-stage development. That was the case for latrepirdine, originally developed and marketed as an H1-antihistamine for the treatment of skin allergy and allergic rhinitis. Latrepirdine was repurposed as a treatment for Huntington's disease (HD). Following encouraging phase II trials and preliminary reports showing its neuroprotective functions and ability to enhance cognition in animal models, it failed to show efficacy in phase III trials in HD patients[24].

Some authors have underlined the importance of confirmatory validation studies for a successful translation of academic results to the industry before larger investments are made[25].

Such gaps between academia and industry should favor rather than discourage a move towards stronger collaboration. Both have their respective roles to play in a drug development process; there is complementarity and not duplication, and there may be mutual support learning from one another. Moreover, while repurposing previously relied on some serendipity, it has more recently evolved to continuous advances made in the field of data sciences and to approaches integrating -among others- computational assistance using Big Data and Artificial Intelligence[26].

WHEN

There is no ideal timing to start a collaboration

There are rather special circumstances leading to potential opportunities or not. For instance, one of the nightmares of a pharmaceutical company is to get safety issues during pivotal studies close to the approval stage. That was the case for alpelisib when Canaud's team asked for the molecule to be tested in PROS. Although based on an exhaustive literature review and spectacular first results in mice and patients, the alpelisib request was badly timed. The phase III trial of alpelisib in breast cancer was still ongoing. The detection of a potentially serious adverse event during a clinical trial in PROS could have jeopardized the drug development for both indications.

Academia-industry collaboration may start as early as possible within a co-development framework or should be at least anticipated to avoid unexpected issues such as the unpleasant surprise for the industry to learn one of its drugs is unexpectedly repurposed by an academic team in an out-of-scope indication, or the huge frustration for the academic team who will not be able to develop a promising candidate owned by industry and not part of its strategy. It must be noted here that large pharmaceutical companies do not have the same expectations as smaller ones. They prefer late-stage development projects (Phase II and onwards) in which they have experience and which are also a way to mitigate the risks.

Line extension or label expansion is not the right time for a collaboration

DR may be part of an anticipated strategy from the industry in the early drug development process. Rather than DR, it is considered as line extension or label expansion that is conceived from the outset of the program. It is part of life cycle management (LCM) activities whose objective is to maximize the value of the products with new indications, improved formulation, new packaging, *etc.*, and to allow for patent extension[27]. Only the marketing authorization holder of a drug can currently apply for an extension of its marketing authorization. When drugs are repurposed on the basis of on-target effects (as is the case with sildenafil and alpelisib), it means that work may be undertaken by the pharmaceutical company owning the molecule. Company data are not available to academic investigators.

Intellectual property considerations are a critical element when starting a collaboration

Drugs are protected by patents and supplementary protection certificates and may benefit from another

form of market exclusivity, as in the case of orphan drugs (7 years in the United States, 10 years in Europe).

Patents can cover the existing product, its underlying chemical or biological make-up, as well as new indications, dosage regimes, or mechanisms of delivery. Supplementary protection certificates are intellectual property rights that serve as an extension to a patent right. They all give the owner the right to prevent others from making, using, or selling the drug without permission. Patent protection is critical for developing a new therapy.

It is challenging to obtain strong patent protection when repurposing compounds. By definition, the structure of a repurposed drug is known and a novel patent claim to the active pharmaceutical ingredient is not possible. Composition of matter (COM) claims, which exclude any use of the COM, are one of the classic and powerful claim types. They cannot be proposed to be repurposed drugs in the vast majority of cases. Repurposed drugs are eligible for patent protection through use claims or method of treatment (MOT) claims. MOT patents may be perceived as second-tier to composition patents; however, it seems to be, in some cases, a good way of protection and commercial success for some companies[28].

Intellectual property (IP) position is critical in ensuring the ability to achieve a collaboration based on partnership and dialogue. IP may fall into two major categories, leading to different issues: it can be active or expired.

IP is still in force

Alpelisib is an example of a successful academia-industry DR collaboration in this category leading to a treatment for patients, although the company was not interested in its development at the very beginning. Alpelisib was being evaluated for breast cancer at that time.

If an academic researcher has discovered a new indication for an approved or investigational drug, then early engagement with the potential industry partner is highly recommended since the company is the patent owner. It will be a crucial step in the process. The objective is twofold: to know if the company is interested in the development and to obtain access to the drug/compound and potential funding of studies performed under a material transfer agreement (MTA).

Pharmaceutical companies frequently engage in preclinical research collaboration with academics. Complex knowledge transfer processes and substantial challenges in terms of IP may arise. Generating new IP has to be discussed, anticipated, and validated in a consortium agreement in order to organize the rights and obligations of all stakeholders and protect the researchers' discovery and IP[7]. If the company holding the IP agrees to provide the drug but refuses to provide funding to support preclinical or clinical programs, it will be difficult for the researcher alone to move forward with the drug, except if new patent protection can be obtained. New formulations or drug forms for a known active pharmaceutical ingredient (API) may be developed for a new indication and provide academics with an opportunity for patent protection. Academic investigators may also decide to perform clinical studies without the support of the company and with no guarantee -even if the trial is successful- of a newly approved indication. Repurposing an approved API for a new indication would only benefit the original manufacturer who owns the IP for the composition. Scientific and nonscientific understandable reasons may explain the company's refusal, such as insufficient remaining patent life, a lack of expertise and interest in the new therapeutic area, or anticipated regulatory risks. A recent study explored innovative mechanisms to fund independent clinical research initiated and led by researchers from academia with repurposed medicines[29]. Focus is put on social impact bonds, crowdfunding, or public-private partnerships to conduct expensive phase III clinical trials with, again, no

guarantee of successful achievement.

IP has expired

In this case, the drug is off-patent or generic and widely available at a low price. Propranolol is an example of a successful academia-industry DR collaboration in this category leading to a new treatment for patients [Figure 2].

Propranolol, a non-selective beta-blocker patented in 1962, was granted FDA approval in 1967 as an antihypertensive drug. When the Léauté-Labrèze team from the Bordeaux University Hospital showed its antiangiogenic therapeutic properties in 11 cases of infantile hemangioma (IH) in 2008, it was available as a generic[30]. 4.5% of birth have infantile hemangioma. Most infantile hemangiomas resolve spontaneously, but 12% are complex and severe forms[31] with functional and even life-threatening complications, such as breathing difficulties and requiring systemic treatment. Following this serendipitous discovery by the Léauté -Labrèze team, Pierre Fabre Dermatologie Laboratories and the University of Bordeaux formed a partnership in 2008 to co-develop and launch Hemangiol® in Europe and Hemangeol® (a different brand name for the same oral formulation) in the United States in 2014 for the treatment of proliferating infantile hemangioma requiring systemic therapy. For information, the brand name Hemangiol® was changed into Hemangeol® in the United States in accordance with FDA's best practices in developing proprietary names guidance, according to which "io" is used as an infix to suggest a high iodine content[32]. In terms of organization and assignment of tasks, resources, and operational skills, the company took charge of the regulatory dossier, including the development of a pediatric formulation and a pivotal study performed on 460 babies between five weeks and five months of age at treatment initiation. The objective of the study was to select the right dosage and confirm the efficacy and tolerance of propranolol in infants with proliferating IH requiring systemic therapy. The clinicians of Bordeaux University Hospital were involved in the study design and management as well as the drafting and publication of the manuscript[33].

Pharmaceutical companies may be reluctant to initiate collaborations with academia to repurpose off-patent drugs. Once drugs lose patent protection, generics can be allowed for sale, leading to off-label use and commercial risks. As it was possible to dissolve commercially available propranolol tablets or to use a non-approved preparation of liquid propranolol made up by the hospital pharmacy, off-label use was a big challenge[34] with risks of inappropriate dose and use of excipients non-recommended for infants, since the therapy was adapted from adult dosage to pediatric populations without an appropriate formulation for young children. However, different actions may be put in place to protect companies.

Patents are the first step. For propranolol in IH, new patents were granted to academia (second medical use patent in 2008) and industry partners (formulation patent in 2010), offering protection for 20 years until 2028 and 2030, respectively.

In addition to patent protection, regulatory agencies are offering incentives for the development of orphan and pediatric drugs for rare diseases, such as Pediatric-Use Marketing Authorization (PUMA) in Europe and Orphan Drug Designation (ODD) in the United States.

Thanks to PUMA, drugs can benefit from ten years of market protection, including 8 years of data exclusivity plus an additional two years of market exclusivity[35].

An Orphan Drug Designation is a status delivered by the FDA in the United States or the EMA in Europe to medicines developed for rare diseases; it may be requested by sponsors for a previously unapproved drug or

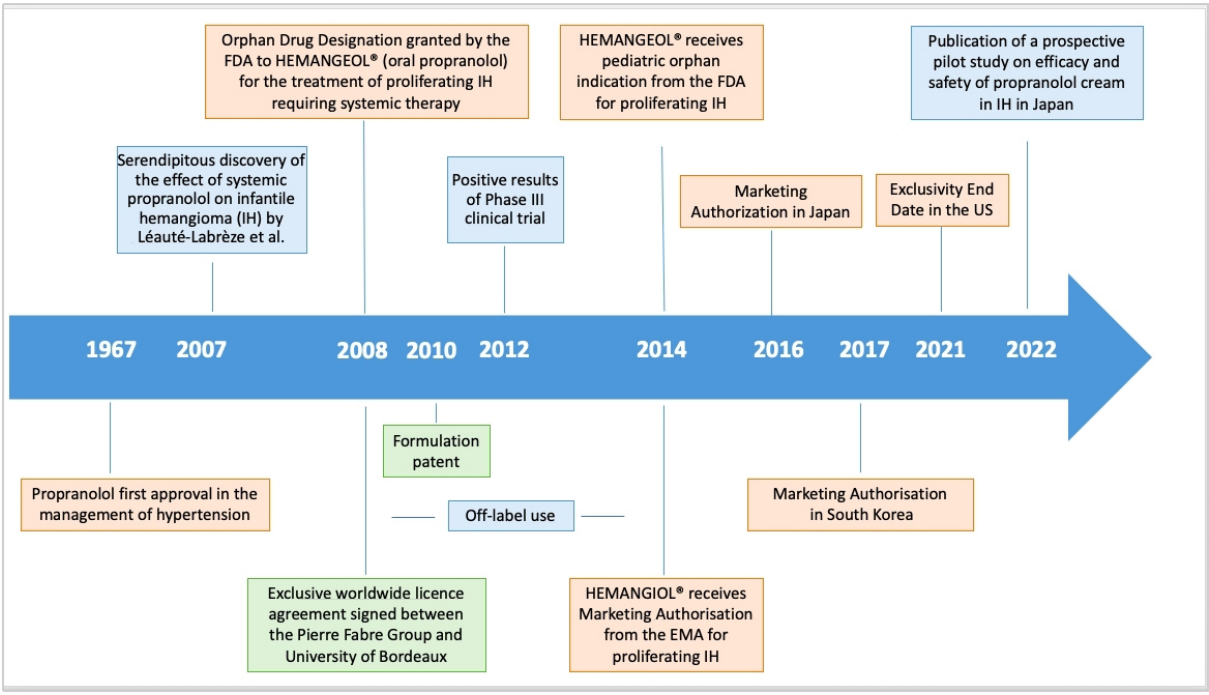

Figure 2. Repurposing propranolol in proliferating infantile hemangioma: key milestones.

for a new orphan indication for an already marketed drug. It provides incentives including tax credits for clinical trials, exemption from user fees, and market exclusivity after approval of the drug. In 2008, an ODD was granted by the FDA to Hemangeol®, which could benefit from a seven-year market exclusivity starting in 2014 when the drug was approved. ODD was not granted in the European Union for Hemangiol® because of "salami slicing" which denies the possibility of restricting the target population to severe forms only (100% of IH are taken into account in the European Union *vs.* only 12% of them in the United States).

Other Marketing Authorizations (MA) have also been granted to the drug in other countries, such as Japan in 2016 and South Korea and Australia in 2017. However, MA does not mean market access and patient access. Each country has individual requirements for pricing/reimbursement with independent Health Technology Assessments (HTAs). If the submitted data package does not support a positive decision from one country's HTA, then the product is at risk of partial reimbursement or even no reimbursement in that country.

A cost-utility analysis was conducted in 2015 in Italy to assess the costs and clinical benefits of Hemangiol® 3.75 mg/mL, an oral solution in comparison to corticosteroids considered as the standard of care in the treatment of proliferating infantile hemangiomas. Propranolol was considered cost-effective compared to corticosteroids from the Italian National Health Service perspective[36].

Propranolol is a good use case outlining the success and failure scenarios of DR for all the reasons outlined above. DR relies on a real strategy involving actors dealing with different constraints but aiming at the same final expectation.

Many lessons can be learned from it, although not always possible to be replicated. An effective IP strategy is an essential tool in identifying risks and opportunities when repurposing medicines and setting out on

new projects[37], but it is only part of the puzzle. Market access strategy is critical to therapy launch success. Landscape assessment is still more relevant in the rare disease ecosystem where literature and natural history are limited. Close collaboration with clinicians is essential to better understand the burden of the disease and patients' needs and work together on the clinical development program to optimize pediatric formulation and patient outcomes keeping in mind the expectations of regulatory agencies and payers.

A paper published in 2022 compares the price of Hemangiol® approved in IH and the price of its off-label alternative, not approved in the indication, in Germany[38]. It underlines the clinical development and registration program value taken into account by payers in the price obtained by the approved product. The price difference is significant but relatively lower for Hemangiol® compared with two other orphan drugs presented in the paper.

Although the results may not be generalized, they may foreshadow a positive return on investment for Hemangiol®. In any case, it is a success story with an approved treatment covering an unmet medical need for babies and infants with severe IH.

HOW

Implementing a relevant academia-industry collaboration requires a strategy to move forward. There are many opportunities for industry to partner with academia and vice versa. There is a wide range of forms varying in the extent of involvement and risk taken by the stakeholders. Identifying research opportunities, applying to private-public calls for proposals, forming an early-stage partnership, developing new methods for the design of small population clinical trials, understanding the natural history of the disease to better treat it, or advancing real-world evidence to support payer decisions are just a few examples of it. How to do it is the key question. A case-by-case answer is probably the best answer since there are so many different factors to consider for complete and successful achievement. Such factors are related to the stakeholders themselves, their profile, skills, interest, their willingness, and commitment, but they are also dependent on the candidate repurposing drug, its IP and freedom to operate, its safety and toxicity profile, and the data already accumulated toward gaining regulatory approval[27].

A brief overview of key suggestions and current initiatives in DR is given hereafter.

Educational program for academia

Academic researchers need to be guided and trained in IP, regulatory procedures, or market access to be able to understand/challenge the constraints and expectations of the industry. Technology transfer offices (TTOs) or neutral and trusted third parties (public or private) may support them to this end. First of all, it is extremely important for academia to understand industry needs when dealing with practical problems to which they can apply their knowledge. Academic research may be closely linked with practical implications in an industrial environment[39]. It is also important for academia to be aware of the pharmaceutical development of a drug and for the industry to be sensitized to the key role and importance of publications, grant approvals, and international networking for academia, notably for Ph.D. students and young researchers. There is a real need for mutual knowledge of expectations and constraints from both academia and industry in order to facilitate interactions and enable fruitful collaboration. Both have to learn from each other.

Pilot project from EMA and STAMP expert group

Another way of implementing relevant academia-industry collaborations is provided by some European initiatives dealing with DR. In October 2021, the European Medicines Agency (EMA) and the Heads of

Medicines Agencies (HMA) launched a pilot project to support the repurposing of drugs. That project is a follow-up to the European Commission's Expert Group on Safe and Timely Access to Medicines for Patients (STAMP) discussions. The objective is to support not-for-profit organizations, including academia, to generate sufficient evidence on the use of a well-established off-patent drug in a new indication. Academic researchers will be connected with regulatory agencies for early scientific advice and with companies for regulatory application. These investments must also be recognized in pricing and reimbursement policies to make access a reality for all patients[40,41]. Candidate projects were selected in June 2022.

Horizon Europe funding program for research and innovation

Being part of consortia involving teams from industry, academia, SMEs and patient associations may help academic partners build lasting collaborations and networks. A call for proposal "Tackling diseases (2021) (HORIZON-HLTH-2021-DISEASE-04)" was proposed under the Horizon Europe program and closed on September 21, 2021. 13 proposals among 253 submitted were related to the topic "Building a European innovation platform for the repurposing of medicinal products". Among key outcomes, proposals had to address a widened collaboration to set up a sustainable platform and an innovative repurposing model integrating different components such as IP, methodological, scientific, regulatory, and financial aspects. Particular attention was given to supporting academic-driven research[42].

IRDiRC Task Force on drug repurposing

As a brief overview, one could also mention the recent IRDiRC Task Force on DR based on the IRDiRC Orphan Drug Development Guidebook[43]. The DR Guidebook is aimed at facilitating DR for rare diseases by identifying relevant available tools and practices, defining a list of Building Blocks (tools, incentives, resources, and initiatives), and creating detailed factsheets for each Building Block. The factsheets will be based on a systematic literature review and the expertise of the Task Force members. They will also provide advice and recommendations. A web application and an article are expected by the end of 2023.

DR platforms are an example of tools and resources to be found in this DR Guidebook. The DR platform established by the FDA is still available. It can be located now at cure.ncats.io. CURE ID (Infectious Disease) is a collaboration between the FDA and the National Center for Advancing Translational Sciences (NCATS), part of the National Institutes of Health (NIH). FDA and NCATS have made critical updates to CURE ID to be a more effective tool in the COVID-19 public health emergency.

Recommendations from a recent pharmacology and translational research round table

A recent "Pharmacology and translational research" round-table of the Giens Workshops[44] formulated and published a series of recommendations to help overcome sticking points and obstacles to DR[44]. The Giens Workshops are held in Paris every year; they are a think tank whose objective is to advance thinking on current issues in pharmacology and clinical research for therapeutic innovation and health technology assessment. The meetings are organized into round tables, each covering pre-defined themes (translational research; clinical research; health technologies including diagnostics, drugs, and medical devices; health and economics; organizational and regulatory aspects, and topical issues). Each round table aims to bring together experts from various backgrounds. In 2022 one of the round tables was dedicated to DR around the following key points:

Optimizing access to and use of databases

Databases are a basis for DR methods and have, therefore, to be designed in a proper manner to achieve relevant results. They can be used to predict drug-target interactions and discover new treatment benefits of the existing drugs. Access has to be facilitated to pharmacological databases (chemical and molecule

libraries, associated pharmacological data). Although there are open-access databases that can be used for searching chemical, biological, and pharmacological data, it is not always easy to search into such specific databases. Another challenge is to have access to shelved industry compounds and their trial data when companies are reluctant to. It is then critical to simplify the procedures for accessing public databases and promote data sharing between public and private structures.

Improving awareness of intellectual property issues

DR can be done on shelved, failed compounds, live patents, and drugs sold on the market as well as generic drugs. DR always relies on a previously known drug, but each situation has its own challenges when considering collaborative research and development between pharmaceutical companies and academic researchers. If the repurposing project comes from academic bodies, they will have to discuss and negotiate with the pharmaceutical companies holders of the molecule involved in the project to convince them to agree on the methods for developing and sharing intellectual property.

It is then important to provide all researchers with better training in legal aspects of the drug development process. A proper DR project needs expert legal counsel to advise on the issues to identify risks and opportunities when setting out on new projects.

Anticipating preclinical and clinical requirements

The real feasibility of the project, the financial and human resources to be deployed, and the time needed to complete the project should always be analyzed and anticipated. There is a real need for tools or structures capable of providing advice on the model of the Scientific Advice/protocol Assistance of the EMA.

Improving the regulatory procedures for market access

Market Authorization is a regulatory process at the European level and does not necessarily mean market access in all Member States of the European Union. Some efforts should be made to get better alignment and synergies between regulatory and payers' requirements. Regulatory agencies assess new treatments' benefits and harms to the exclusion of economic considerations, whereas payers focus on effectiveness and economic consequences. In France, for instance, the doctrine of the High Authority for Health (HAS) Transparency Commission (TC) needs to evolve both regarding the methodology of potentially admissible trials and the more specific part of the so-called "relevant" clinical comparator. The HAS Transparency Commission's main task is to assess medicinal products in order to provide recommendations on reimbursement decisions made by public authorities. Access to reimbursement in France follows Marketing Authorization and requires pharmaceutical companies to submit an application file to the HAS Transparency Committee (TC)

Creating a public-private partnership support structure on a European scale

The main recommendation made during the round table was the creation of a support structure capable of managing the most complex aspects of repurposing projects. A trusted third party is useful for initiating collaborations and providing the infrastructure and resources for academia and industry to work together. Such a support structure would have dedicated financial resources and expertise at all stages of development until the Marketing Authorization. It could even become the MA holder for the repurposed drug if need be. The Innovative Health Initiative (IHI) is a public-private partnership between the European Union and the European life science industries whose main objectives are to translate research and innovation into tangible benefits for patients. Such a structure could be used as a model applied to DR[45].

CONCLUSION

DR represents a real opportunity and a cheaper and faster way to find and develop new treatments for rare diseases. But DR is also associated with misconceptions and specific barriers (IP, regulatory, market access, and lack of incentives). It makes no sense to stereotype public-private partnerships and set one community against the other. Without research, there will not be any innovation; without regulatory and commercial development, there will not be any drug on the market. Although being far away from each other in terms of culture, constraints, and objectives, academia and industry can join forces and build off each other's strengths to advance research and development of repurposed drugs for patients with rare diseases. This approach has already proved successful in several instances, but its potential is far from being fully exploited. Some fruitful collaborations have resulted in success stories that are widely publicized, but they do not fully reflect reality. The success rates of academia-industry collaboration vary depending on various factors (IP rights of the drug candidate, business strategy, Proof of Concept robustness, regulatory challenges, commercial aspects, and type of pharmaceutical company). Academic drug (re)discovery is a vital component, but the lack of knowledge of industry standards can make academics uncomfortable. Educational programs and guidelines could help them better understand industrial constraints and anticipate and adjust accordingly. There is also a need for new and sustainable DR business models involving not only academia and industry but also patient associations, regulators and payers to equitably address these questions. Some current European and international initiatives such as the pilot project from EMA and STAMP expert group, the call for proposal "Building a European innovation platform for the repurposing of medicinal products" within Horizon Europe or the IRDiRC Task Force on DR guidebook should encourage and stimulate new opportunities.

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The author contributed solely to the article.

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Ethical approval and consent to participate

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REFERENCES

1. Murteira S, Ghezaiel Z, Karray S, Lamure M. Drug reformulations and repositioning in pharmaceutical industry and its impact on market access: reassessment of nomenclature. *J Mark Access Health Policy* 2013;1:21131. DOI PubMed PMC
2. Mucke HAM. COVID-19 and the drug repurposing tsunami. *Assay Drug Dev Technol* 2020;18:211-4. DOI PubMed
3. Galindez G, Matschinske J, Rose TD, et al. Lessons from the COVID-19 pandemic for advancing computational drug repurposing strategies. *Nat Comput Sci* 2021;1:33-41. DOI
4. European Medicines Agency. Orphan medicinal product designation 2000-2020. Available from: https://www.ema.europa.eu/en/documents/other/orphan-medicines-figures-2000-2020_en.pdf [Last accessed on 7 Apr 2023].

5. European Medicines Agency. Support for development of orphan medicines. Available from: https://www.ema.europa.eu/en/documents/report/report-workshop-support-orphan-medicines-development_en.pdf [Last accessed on 7 Apr 2023].
6. Southall NT, Natarajan M, Lau LPL, et al; IRDiRC Data Mining and Repurposing Task Force. The use or generation of biomedical data and existing medicines to discover and establish new treatments for patients with rare diseases - recommendations of the IRDiRC Data Mining and Repurposing Task Force. *Orphanet J Rare Dis* 2019;14:225. DOI PubMed PMC
7. Begley CG, Ashton M, Baell J, et al. Drug repurposing: misconceptions, challenges, and opportunities for academic researchers. *Sci Transl Med* 2021;13:eabd5524. DOI PubMed
8. Shim JS, Liu JO. Recent advances in drug repositioning for the discovery of new anticancer drugs. *Int J Biol Sci* 2014;10:654-63. DOI PubMed PMC
9. Ghofrani HA, Osterloh IH, Grimminger F. Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nat Rev Drug Discov* 2006;5:689-702. DOI PubMed PMC
10. Jourdan JP, Bureau R, Rochais C, Dallemagne P. Drug repositioning: a brief overview. *J Pharm Pharmacol* 2020;72:1145-51. DOI PubMed PMC
11. Barnett CF, Machado RF. Sildenafil in the treatment of pulmonary hypertension. *Vasc Health Risk Manag* 2006;2:411-22. DOI PubMed PMC
12. Roy S, Kloner RA, Salloum FN, Jovin IS. Cardiac effects of phosphodiesterase-5 inhibitors: efficacy and safety. *Cardiovasc Drugs Ther* 2021. DOI PubMed PMC
13. Pan Y, Cheng T, Wang Y, Bryant SH. Pathway analysis for drug repositioning based on public database mining. *J Chem Inf Model* 2014;54:407-18. DOI PubMed PMC
14. Pagliazzi A, Oranges T, Traficante G, et al. *PIK3CA*-related overgrowth spectrum from diagnosis to targeted therapy: a case of CLOVES syndrome treated with Alpelisib. *Front Pediatr* 2021;9:732836. DOI PubMed PMC
15. Scherman D, Fetro C. Drug repositioning for rare diseases: knowledge-based success stories. *Therapie* 2020;75:161-7. DOI PubMed
16. Chang DY, Ma WL, Lu YS. Role of Alpelisib in the treatment of *PIK3CA*-mutated breast cancer: patient selection and clinical perspectives. *Ther Clin Risk Manag* 2021;17:193-207. DOI PubMed PMC
17. Madsen RR, Vanhaesebroeck B, Semple RK. Cancer-associated *PIK3CA* mutations in overgrowth disorders. *Trends Mol Med* 2018;24:856-70. DOI PubMed PMC
18. Venot Q, Blanc T, Rabia SH, et al. Targeted therapy in patients with *PIK3CA*-related overgrowth syndrome. *Nature* 2018;558:540-6. DOI PubMed PMC
19. Canaud G, López Gutiérrez J, Irvine A, et al. LBA23 EPIK-P1: retrospective chart review study of patients (pts) with *PIK3CA*-related Overgrowth Spectrum (PROS) who have received alpelisib (ALP) as part of a compassionate use programme. *Ann Oncol* 2021;32:S1297. DOI
20. Rosenblatt M. How academia and the pharmaceutical industry can work together: the president's lecture, annual meeting of the American Thoracic Society, San Francisco, California. *Ann Am Thorac Soc* 2013;10:31-8. DOI PubMed
21. den Berg S, de Visser S, Leufkens HGM, Hollak CEM. Drug repurposing for rare diseases: a role for academia. *Front Pharmacol* 2021;12:746987. DOI PubMed PMC
22. Hernandez JJ, Pryszlak M, Smith L, et al. Giving drugs a second chance: overcoming regulatory and financial hurdles in repurposing approved drugs as cancer therapeutics. *Front Oncol* 2017;7:273. DOI PubMed PMC
23. Fetro C, Scherman D. Drug repurposing in rare diseases: myths and reality. *Therapie* 2020;75:157-60. DOI PubMed
24. Bharadwaj PR, Bates KA, Porter T, et al. Latrepirdine: molecular mechanisms underlying potential therapeutic roles in Alzheimer's and other neurodegenerative diseases. *Transl Psychiatry* 2013;3:e332. DOI PubMed PMC
25. Prinz F, Schlange T, Asadullah K. Believe it or not: how much can we rely on published data on potential drug targets? *Nat Rev Drug Discov* 2011;10:712. DOI PubMed
26. WHO-EURO. Repurposing of medicines - the underrated champion of sustainable innovation: policy brief. Available from: https://apps.who.int/iris/handle/10665/342567 [Last accessed on 7 Apr 2023].
27. Cha Y, Erez T, Reynolds IJ, et al. Drug repurposing from the perspective of pharmaceutical companies. *Br J Pharmacol* 2018;175:168-80. DOI PubMed PMC
28. Conour J. Why method of treatment patents for repurposed drugs are worth the investment. https://www.jdsupra.com/legalnews/why-method-of-treatment-patents-for-92813/ [Last accessed on 7 Apr 2023].
29. Verbaanderd C, Rooman I, Huys I. Exploring new uses for existing drugs: innovative mechanisms to fund independent clinical research. *Trials* 2021;22:322. DOI PubMed PMC
30. Léauté-Labrèze C, Dumas de la Roque E, Hubiche T, Boralevi F, Thambo JB, Taïeb A. Propranolol for severe hemangiomas of infancy. *N Engl J Med* 2008;358:2649-51. DOI PubMed
31. Drolet BA, Frommelt PC, Chamlin SL, et al. Initiation and use of propranolol for infantile hemangioma: report of a consensus conference. *Pediatrics* 2013;131:128-40. DOI PubMed PMC
32. U.S. Department of Health and Human Services; Food and Drug Administration. Best practices in developing proprietary names for human prescription drug products guidance for industry. https://www.fda.gov/media/88496/download [Last accessed on 7 Apr 2023].
33. Léauté-Labrèze C, Hoeger P, Mazereeuw-Hautier J, et al. A randomized, controlled trial of oral propranolol in infantile hemangioma. *N Engl J Med* 2015;372:735-46. DOI PubMed
34. Phillips RJ, Penington AJ, Bekhor PS, Crock CM. Use of propranolol for treatment of infantile haemangiomas in an outpatient setting.

J Paediatr Child Health 2012;48:902-6. DOI PubMed

35. European Medicines Agency. Paediatric-use marketing authorisations. https://www.ema.europa.eu/en/human-regulatory/marketing-authorisation/paediatric-medicines/paediatric-use-marketing-authorisations [Last accessed on 7 Apr 2023].
36. Hachem M, Bonamonte D, Diociaiuti A, Mantuano M, Teruzzi C. Cost-utility analysis of propranolol versus corticosteroids in the treatment of proliferating infantile hemangioma in Italy. *PharmacoEcon Ital Res Artic* 2015:17. DOI
37. Jadeja N. The role of IP in strategies for repurposing medicines www.pinsentmasons.com/out-law/analysis/the-role-of-ip-strategies-repurposing-medicines [Last accessed on 7 Apr 2023].
38. Veldman A, Richter E, Hacker C, Fischer D. The use of off-label medications in newborn infants despite an approved alternative being available-results of a national survey. *Pharmacy (Basel)* 2022;10:19. DOI PubMed PMC
39. Ahmed F, Fattani MT, Ali SR, Enam RN. Strengthening the bridge between academic and the industry through the academia-industry collaboration plan design model. *Front Psychol* 2022;13:875940. DOI PubMed PMC
40. European Commission. Commission Expert Group on safe and timely access to medicines for patients ("STAMP") https://health.ec.europa.eu/medicinal-products/pharmaceutical-committee-veterinary-pharmaceutical-committee-and-expert-groups/commission-expert-group-safe-and-timely-access-medicines-patients-stamp_en [Last accessed on 7 Apr 2023].
41. European Medicines Agency. Proposal for a framework to support not-for-profit organisations and academia (institutions and individuals) in drug repurposing https://www.ema.europa.eu/en/documents/other/question-answers-repurposing-pilot-project-proposal-framework-support-not-profit-organisations_en.pdf [Last accessed on 7 Apr 2023].
42. European Medicines Agency. Building a European innovation platform for the repurposing of medicinal products https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/horizon-hlth-2021-disease-04-02 [Last accessed on 7 Apr 2023].
43. Hechtelt Jonker A, Hivert V, Gabaldo M, et al. Boosting delivery of rare disease therapies: the IRDiRC Orphan Drug Development Guidebook. *Nat Rev Drug Discov* 2020;19:495-6. DOI PubMed
44. Deplanque D, Fetro C, Ferry A, et al. Drug repurposing: from the discovery of a useful pharmacological effect to making the treatment available to the patient. *Therapie* 2023;78:10-8. DOI PubMed
45. Innovative health initiative. https://www.ihi.europa.eu/about-ihi [Last accessed on 7 Apr 2023].

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Sustainable approaches for drug repurposing in rare diseases: recommendations from the IRDiRC Task Force

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Abstract

Drug repurposing represents a real opportunity to address unmet needs and improve the lives of rare disease patients. It is often presented as a faster, safer and cheaper path for bringing drugs into new indications. However, several economic, regulatory and scientific barriers can impede the successful repurposing of drugs for rare diseases. The International Rare Diseases Research Consortium (IRDiRC) set up the *Task Force on Sustainable Models in Drug Repurposing* with the objective of identifying key factors for achieving sustainable repurposing approaches in rare diseases.

In order to help inform expert opinion, the Task Force investigated six cases of medicinal products repurposed into new rare indications and four cases of ongoing development programs. A questionnaire addressing the major steps of the repurposing approach was developed by the Task Force and sent to contact points of the organizations. In addition, interviews were conducted with the relevant organization representatives to conduct a deeper dive into

the sustainability of the repurposing approach for each of the selected cases.

Based on the collective experience of the members of the Task Force and the output from the questionnaires/interviews, we have identified ten key factors that should be considered by those embarking on repurposing projects. These factors include the identification of unmet patient needs and partnership with patients, collection of evidence concerning disease prevalence, patient numbers, drug pharmacology and disease etiology, drug industrial property status, off-label or compounding use, data from past clinical studies and needs for extended non-clinical and clinical studies. The development of a collaborative funding framework and early discussion with regulators and payers are additional factors to implement early in the development of sustainable drug repurposing projects.

Keywords: Drug repurposing, rare diseases, orphan drugs, sustainable approaches, medicines development, IRDiRC

INTRODUCTION

Drug repurposing is a hot topic in regulatory science and drug development. More than ever, the COVID-19 pandemic has clearly shown the importance and potential pitfalls of repurposing existing molecules in the therapeutic armamentarium[1,2].

In the context of rare diseases, a field where the majority of conditions have pediatric onset and the number of therapies is limited[3,4], drug repurposing represents an opportunity for new therapeutic alternatives by exploiting yet untapped pharmacological resources. While previous work done by the IRDiRC Data Mining and Repurposing Task Force had exemplified that the rare disease field could benefit hugely from the repurposing opportunities, the potential is not yet fully realized for a variety of reasons[5].

Repurposing is underused not because of the lack of opportunities, but because the additional resources required to demonstrate the benefit-risk profile of a new indication and sometimes to adapt formulation, posology, and mode of use, often do not translate into a sustainable return on investment via the traditional pharmaceutical industry and healthcare reimbursement models. Indeed, these models are essentially built around innovative, industrial property-protected molecules[6-8], which is defined as one of the two parts of the intellectual property field and relates to the protection of inventions and industrial or commercial creative work that includes patents for inventions, industrial designs, trademarks.

IRDiRC Task Force used the definition of drug repurposing established by the European expert group Safe and Timely Access to Medicinal Products (STAMP)[9]: "*Process of identifying a new use for an existing drug/active substance in an indication outside the scope of the original indication*". The Task Force looked at the issue of the under-utilization of repurposing with the specific aim of considering which types of development models could help to foster more successful cases of repurposing while keeping a balance between attractiveness for developers and access to therapies for rare disease patients. The scope included understanding key features of success criteria for the selected cases of repurposing projects and the subsequent reflections of the Task Force on how to optimize these features.

APPROACH AND METHODS

Task Force members were selected based on their multi-stakeholder experience and knowledge of different elements of repurposing programs. The Task Force conducted literature-based reviews to inform the conclusions of this perspective paper. In order to help further inform expert opinions from the Task Force, we selected different types of repurposed cases that would help us further identify key success factors for

sustainable repurposing models in rare diseases. Using the collective knowledge of the Task Force, we created a questionnaire that could be used to capture the major steps of each of the repurposing approaches.

Creation of the questionnaire

A targeted questionnaire addressing the following [Supplementary File 1]:

- Description of the company/organization
- Description of the drug repurposing approach and strategy
- Identification of the stakeholders
- Required evidence generation and repurposing of research studies
- Sources and mechanisms of funding
- Sustainability of the model chosen
- Patent status and exclusivity periods
- Identification of the barriers and challenges
- Measure of progress
- Result of the drug repurposing process
- Recommendations for a successful drug repurposing process

Selection of medicinal products

The Task Force extracted a list of repurposed medicinal products that have been approved for a rare indication in the United States[10,11] and/or the European Union[12]. Both oncology and non-oncology drugs were included in the analysis. The Task Force group used a set of five criteria to refine the case selection and create a list of ten repurposed drugs.

- For each selected case, the approval for the original and the repurposed indications must have been granted by the same regulatory body, which is either the United States Food and Drug Administration (FDA) or the European Medicines Agency (EMA).
- Cases related to a variation of the same indication (age, line of treatment) were excluded from the analysis.
- Cases were chosen to avoid the bias of selecting drugs approved for a second indication without the initiation of repurposing studies. This criterion was applied to maximize the probability of identifying cases no longer covered by the initial patent families and/or regulatory exclusivity.
- Diverse economic models (private funds, public funds, social bonds, and venture philanthropy) and drug developers (big pharma, SMEs, academics, patient-led groups, and public-private collaborations) were

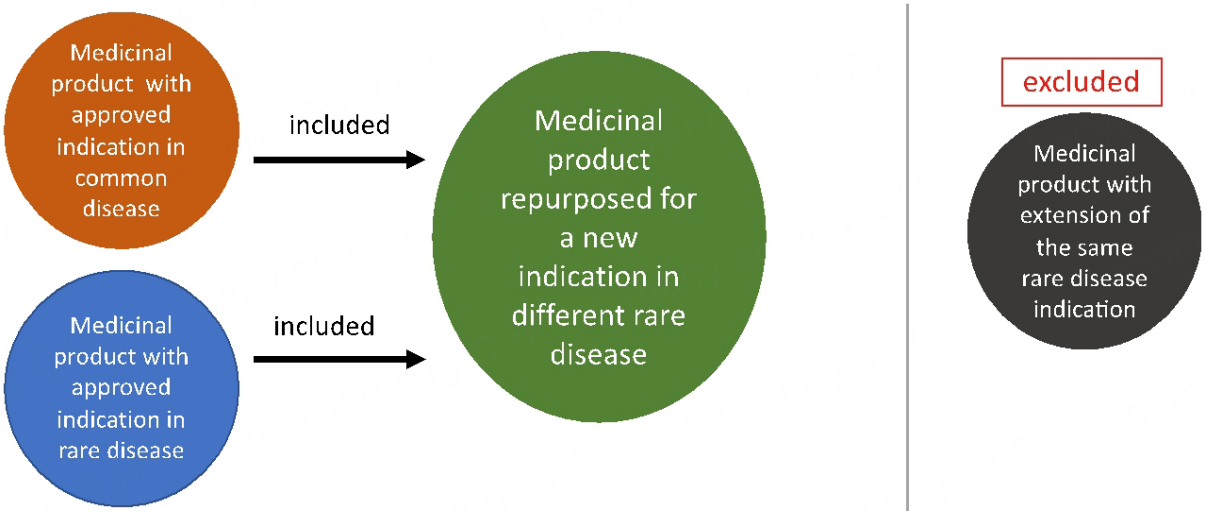

Figure 1. Selection of the Medicinal Products. The graphics represents the methods used by the Task Force group to select cases of drug repurposing in rare diseases. The medicinal products initially approved for common or rare indications were all repurposed into a new rare disease. Approvals for the original and new indication were granted by the same regulatory authority. Medicinal products with an extension of the same rare disease indication were excluded from the analysis.

considered.

• Cases were selected based on the ability of the Task Force members to identify and contact a representative from each organization involved in the repurposing of the medicinal products.

Out of the ten selected repurposed drugs, targeted invitations were sent to six identified organization representatives and six positive responses were received. The questionnaire was sent to the respondents and interviews were conducted with the relevant representatives. The six drugs had initial indications for the treatment of common or rare disorders and were all subject of a repurposed new indication for rare diseases. The selection of medicinal products is presented in Figure 1.

Case identification of ongoing development programs

Through personal knowledge of the field, the Task Force identified cases of ongoing development programs and contacted four organizations experienced in the management of drug repurposing projects (see acknowledgments section) to perform structured interviews and collect additional information on both the challenges and the key strategies implemented to increase the chance of success of a repurposing approach in rare diseases.

Information from the questionnaires and interviews was integrated into Task Force discussions and the key factors that should be considered by those embarking on repurposing projects were identified and agreed upon through consensus.

KEY FACTORS FOR CREATING A SUSTAINABLE REPURPOSING PROJECT

Alongside expert Task Force opinion, the objective of the questionnaire and interview analysis was to draw out common themes not yet considered, and to understand better the challenges and opportunities associated with different models for drug repurposing. The Task Force Group identified ten core success factors that are presented below and summarized in Figure 2.

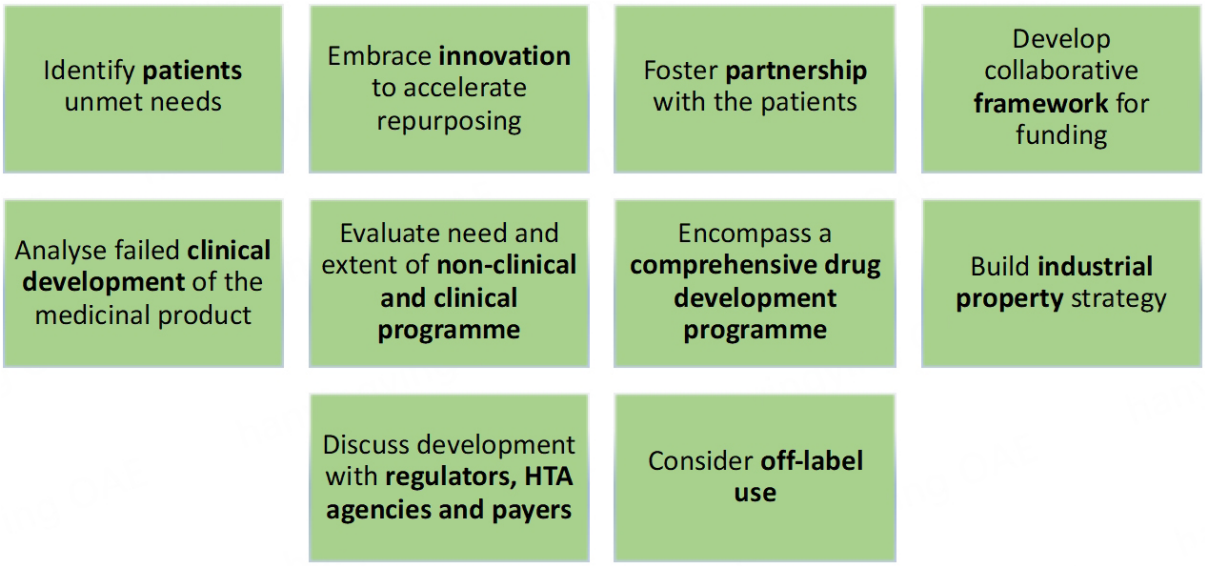

Figure 2. Ten Success Factors of Sustainable Drug Repurposing Models. The graphic represents ten key success factors to be considered by drug developers embarking on a drug repurposing project. These factors are not presented in a sequential order and form a framework of actions. The identification of unmet patient needs and patient partnerships are key components of sustainability. The collection of evidence concerning disease prevalence, patient numbers, drug pharmacology and disease etiology, drug industrial property status, off-label or compounding use, data from past clinical studies or real world evidence and the need for extended non-clinical and clinical studies is essential in designing a sustainable repurposing project and an accurate business plan. The development of a collaborative funding framework and early discussion with regulators, HTA agencies and payers are also essential factors to implement early on in the development of a sustainable project.

Repurposing is a valid approach to address the unmet needs of rare disease patients

All the respondents, without exception, mentioned that their approach was primarily serving to address the unmet medical needs of the patients, confirming the necessity of a focused and pressing need as a trigger for re-engaging in the development of old drugs. The fulfillment of the patient needs is, in many cases, not only related to the medical and/or scientific aspects of the benefit brought by the product, but also related to the increased availability of the product for the patients.

Alongside the development of therapies for new indications, it was mentioned, for instance, that, for some, the repurposing approach aims at an improvement in the availability of a drug product compared to compounded formulations, alleviating some of the risks linked to the absence of market authorization [with good manufacturing practice (GMP), pharmacovigilance], or the lack of a prescription framework.

Addressing the unmet needs of patients is seen as one of the key components of sustainability. This finding is aligned with foresight scenarios, where repurposing is often considered as an approach linked to social justice as it bears the potential for less costly products that may be accessible to a larger population than the one initially foreseen.

Repurposing is an innovative approach

At a time when the development of gene and cell therapies - and accessibility to such high-end approaches - are bringing much promise to treat or even cure cancers and rare genetic disorders, drug repurposing might not be considered as innovative within rare disease therapeutic development armamentarium.

Some of the approaches that this group, and other stakeholders, patient groups, for example, had classified as repurposing were not labeled as such by pharmaceutical representatives. There is a thin border between what some may call repurposing and the other one, extension of indication. This cannot be imputed only to commercial sensitivities but also to a genuine lack of common understanding and terminology that we should aim to overcome. One understanding of repurposing for companies involved in the development of innovative medicines is that repurposing is considered only for medicinal products that are out of industrial property (IP) and regulatory protection, while the extension of indication is a special type of variation of the initial marketing authorization when new data are submitted and assessed to add a new indication to the existing product.

In addition, repurposing in some quarters suffers from a "bad reputation" where well-known products have seen their prices skyrocketing following a repurposing project[13,14]. This negative perception is not connected to a unique category of stakeholders.

Furthermore, repurposing also brings a positive perception as it is sometimes the only way to provide a therapeutic alternative to patients. Moreover, repurposing, in many cases, is an innovation in itself, where a better understanding of the pharmacology of a medicinal product and the disease etiology reach a point of convergence to stimulate a repurposing project[8,15]. This is increasingly the case where artificial intelligence, data mining and *in silico* approaches are being used[5]. In order to advance the field, we need to encourage investment and continuous development in this field but also recognize that repurposing is a truly innovative approach.

As for every therapeutic development, patients should be considered as equal partners

Within the sample of interviews, we observed a large variety of patient involvement, from patients and patient organizations being the initiators and the drivers of the repurposing approach to patients only considered at the time of recruitment for the clinical studies.

Amongst the various activities in which patients can take part, we have enlisted: identification of the opportunity to repurpose a product, co-writing of the grant application, provision of the patient perspective in the set-up of the objectives of the therapeutic development, in the design and the planning of the clinical studies, patient identification, recruitment and retention for clinical studies, regulatory preparedness (e.g., support to orphan drug designation), and dissemination to the broader patient community of the outcomes of the development leading to better uptake of the product once on the market.

Often it appeared that the more the patients are involved as equal partners, the better, and that the positive impact is correlated to the level of patient engagement. Interviewees that have reported no or few patients involved in their development mention the identification of stakeholders as one of the main challenges in the repurposing approach, leading to difficulties at the time of recruitment and consequences on the business plan.

Respondents that have reported a high level of patient involvement, with all stakeholders (patients, clinicians, and researchers) inputting in the design and planning of the studies, as well as regulatory preparedness, mention a significant impact on the timeline of the development with faster clinical trial recruitment and better uptake of the product in the market. This is comparable to previous studies that have also emphasized the importance of patient involvement in the development and lifecycle of orphan medicinal products[16-18].

Added value of a collaborative approach in drug repurposing

From the interviews that we have conducted, there are three main patterns that emerge when it comes to funding a repurposing approach.

Firstly, when the project is initiated by a pharmaceutical company, the funding is primarily ensured by the company's own funding, with the addition of private funding such as capital risks investors on occasion.

Secondly, when the project is initiated by an academic team (researchers and/or clinicians), the project is mostly fuelled by public funding (e.g., European Union/National funding, E-Rare calls, United States federal grants, NIH grants). In that case, it can be complemented by funding from patient organizations and other not-for-profit entities, such as foundations. Additional support can be sought from pharmaceutical companies to supply the drug in clinical trials at no cost, for instance.

The third avenue which breaks the silos existing in the two previously described situations, and that the authors would encourage, is a public-private partnership, as we know that often the discovery phase is done by the public sector while the development phase is performed by the private sector. To make such a public-private approach successful, the expectations of each party and the respective contributions have to be clarified at the beginning of the collaboration, including the signature of contracts and legal agreements.

To make the link with the above item ("As for every therapeutic development, patients should be considered as equal partners"), the involvement and, to a larger extent, a true patient-led approach seem to be critical to ensure a multi-stakeholder repurposing program. The development of a framework for funding and risk in collaborative projects when there are non-commercial opportunities (patent expired) and where private partners would put the responsibility to patients above profit-motive could address some of the needs for sustainable approaches in drug repurposing. As an example, the two Consortia REPO4EU and REMEDI4ALL[19,20] selected following the call by the European Commission under the Horizon Europe program[21] might bring a piece of the answer here.

Learning lessons from failures and building on past findings

The trigger for a repurposing approach can be anything from scientific discovery, data mining or artificial intelligence (AI), clinical observation, off-label use or extensive literature evidence.

It was reported almost unanimously that the collection of scientific evidence in order to support a repurposing approach is a major obstacle. Early detection of the potential for repurposing can represent an advantage and sometimes a competitive one. However, gathering knowledge from past non-conclusive clinical studies in order to inform the new development is not always that easy (e.g., lack of responsiveness of previous investigators, failed clinical trials not published). When it has happened well, it has been highly beneficial.

According to several of the interviewees, a lot of emphases have to be put on the publication of failed clinical trials, the importance of collaboration between the research teams (e.g., the possibility of contacting the initial investigators), and the importance of data sharing which is often identified as one of the main challenges (e.g., non-clinical development information for the product that might support dosing strategies for the repurposed indication).

Generation of additional knowledge is often needed and should not be underestimated

There is a common misbelief that repurposing does not require the extensive generation of new knowledge because there is a huge amount of data that can be re-used from past investigations, clinical studies, and post-authorization generated data.

Not repeating the issue exposed in "Learning lessons from failures and building on past findings", where the information might exist but the investigator has no access to it, we shall consider two distinct situations: on-target (where the pathophysiological mechanism is known) and off-target (where the mechanism is being discovered) repurposing approaches. The latter offers fewer opportunities to re-use previously generated findings.

Without distinguishing one or the other situations, the interviewees have reported that they had to perform one or more of the following steps: toxicology testing (especially if the new indication is for the pediatric population while the initial one was in adults); non-clinical pharmacology testing (i.e., use of animal models); *in vivo* non-clinical proof of concept; development of new clinical endpoints and outcome measures, e.g., Patient Relevant Outcomes Measures, Patient Reported Outcomes, and Health-Related Quality of Life Studies.

The conduct of non-clinical research studies and clinical trials is often cited as one of the main challenges. In any case, the generation of additional knowledge is a step that should be factored into the development plan and should not be underestimated.

Repurposing is not always an easy journey, it is a true therapeutic development

To follow on from the previous item, the respondents said that being able to conduct an abbreviated program in the case of a repurposing approach is not granted. In many cases, it was not possible. It is not exceptional to have to do a Phase 1 trial and even develop an animal model.

The development of a new formulation is also often cited. A new formulation is required when reaching a new population (e.g., pediatric versus adult or targeting a specific organ), when a different dosage is needed or when a novel route of administration is explored.

On top of that, repurposing approaches encompass the need for GMP supply and pharmacovigilance follow-up (compared to compounded formulations, for example), which makes it a complete cycle and significantly increases the costs and complexity of the development estimated at first glance.

Hence a repurposing approach should not be underestimated. It is not necessarily always going to be faster than any other type of therapeutic development, and while the length can be reduced due to a certain number of factors (e.g., patient involvement, rigorous planning, extrapolation of data), this is not a given. This finding has been reported by several interviewees.

Defining industrial property strategies require strong expertise and knowledge

IP strategies must be established at the time of the discovery-based first on the patentability of the repurposed drug in its new application and then eventually on the "freedom-to-operate" analysis. The patentability analysis aims at looking into state of the art, meaning any published documents related to the invention including active, abandoned and expired patents besides the scientific literature. Once the patentability of the discovery is established, the path to developing the repurposed drug remains a fine line. Unlike the on-target approach where the pathophysiological mechanism is known, the off-target approach

allows the possibility of filing a patent in a second medical use, which can lead to the building of a patent portfolio of higher commercial value for investors. Indeed, interviewees have reported that once the patent of a second medical use is granted, the protection can be further extended via other types of patents: new formulations (i.e., pediatric formulation, medical device, and prodrug), dosage, administration route, and combination of drugs.

Additionally, the non-patentability of the discovery will likely discourage investors. IP protection is thus considered as a factor of success; the objective is to exploit it, and make good use of the advantages it provides, e.g., support the return on the investment.

Finally, IP strategy is a complex field, generating a high level of competition. Patents have to be carefully designed as they are often attacked and dismissed by competitors. For non-specialists, and especially in the case of a repurposing approach conducted by academics, the importance of Tech Transfer Offices is crucial to help navigate the field, avoid pitfalls (e.g., no publication when a patent is sought) and build a strong IP strategy.

Development/Business planning requires defining upfront the end of the journey and each step of the development path

The granting of an orphan designation does not seem to be necessarily a key element in a repurposing approach, whereas the regulatory approval, i.e., granting of a Marketing Authorization, is more often seen as a goal.

Regulatory issues have been reported, such as the need for placebo data which was not anticipated or the importance of choosing endpoints that are relevant for the patients. In those cases, the regulatory system offers tools to overcome such barriers, such as the FDA meeting or EMA Scientific Advice for example. EMA has also opened a pilot on repurposing to support not-for-profit organizations and academia[9]. FDA allows 505(b)(2) application for a not previously approved indication for a marketed product[22].

Market access is seen as a big risk for various reasons. While the availability of the product for patients and the granting of reimbursement are seen as success factors, difficulties are reported in relation to several aspects: (a) the estimate of the target population for which accurate prevalence figures are needed; (b) the anticipation of the generics & compounded products competition and the underestimation of the off-label use which leads to incorrect predictions; and (c) the commercialization costs that might be higher than expected in some countries and might represent an issue if not adequately anticipated.

All this has consequences on the selection and implementation of the economic model, which is seen as one of the main challenges in the repurposing approaches. As a mitigation measure, some interviewees mentioned the need to refine and adapt their business plan during the development path in order to better reflect the R&D costs and the commercialization costs and hence to adjust the price.

The principles expressed in the IRDiRC Orphan Drug Development Guidebook (ODDG) published in 2020[23] are applicable here, i.e., early planning of the development path, best use of all the tools available, early and continued dialogue with regulators and with Health Technology Assessment (HTA)/payers to anticipate the access phase since the beginning. However, the ODDG had not considered the aspects related specifically to repurposing and we aim to add a specific module to the existing materials. This represents a key IRDiRC activity in 2023[24].

Off-label considerations

As mentioned in the previous section, most of the respondents considered the granting of a Marketing Authorization as one of the main aims of their repurposing approach. However, this is not the case for all developers. For some, the success will be determined by the use of the repurposed drug clinically, recommended use and inclusion in well-accepted consensus treatment guidelines, and its acceptance for payment by payers. Further development could then be conducted via an observational study to track its use in the real world instead of an open-label clinical trial.

Considering that generic drugs targeting key pathways are likely to be efficacious in treating a variety of diseases, it was reported that with the appropriate pairing of drugs and untreated or undertreated diseases, drug manufacturers, health care systems, and patients stand to greatly benefit from the identification of repurposing of these medications. This model may not only provide a faster, simpler pathway to providing new therapies for underserved populations, but avoids the cost and time of developing and producing novel therapies.

This point should be balanced by the fact that off-label use is considered very differently depending on the country/region of the world, and such use lacks the advantages of regulatory review on the benefits and risks with formal pharmacovigilance oversight. In some places, it is not authorized by law; in some others, there is widespread access to the (generic) drug off-label at a relatively low cost (of note: when the drug is off-patent, it is more likely that the price will be kept low); in some others, off-label is tolerated but sometimes not reimbursed[25]. In addition, it might also bring fewer opportunities to monitor safety issues and more uncertainty for patients who are then at risk of a withdrawal from the market of the drug for reasons related to the licensed indication, leaving them with no alternative.

DISCUSSION

The over 6,000 rare diseases affect about 300-350 million people worldwide. Low disease prevalence, heterogeneous patient populations, the limited natural history of the diseases and high research and development costs are among the factors limiting the emergence of new therapeutic options for rare disease patients. Altogether, less than 6% of rare diseases have approved treatments, and therefore, drug repurposing must be supported and valued as an approach to address the unmet needs of rare disease patients. This paper aimed to provide drug developers with a framework for creating a sustainable approach to drug repurposing, helping researchers avoid recurrent pitfalls and increasing the chance of seeing medicines repurposed into new orphan indications. Through the Task Force, we have derived ten key success factors which can be considered and applied consistently by developers using repurposing approaches. Based on these ten items, the Task Force group has drawn up three main cross-cutting actionable recommendations: awareness raising, funding allocation, and operational implementation.

What is needed for sustainable approaches in drug repurposing in terms of awareness raising?

Some efforts are required in order to disentangle concerns arising around repurposing. First, there is a need for clarification of what is intended by the term "repurposing" itself. In this respect, the recent manuscript from Asker-Hagelberg *et al.* brings a welcome precision to the definition of repurposing: "*Medicine repurposing is the process of identifying and substantiating a new use for an existing medicine/active substance outside the scope of the original indications as well as the process of allowing a medicinal product to broaden its position in a relevant market (excluding the extension of an authorised indication to those of a new age group or to another genetic mutation)*". The latter part of the sentence clarifies which extensions of indications are to be considered as repurposing and which ones are not[25].

It also clarifies the scope of repurposing: "*It includes new therapeutic uses for existing medicines, different formulations of the same medicine, and/or creating new combinations of medicines or medicines with medical devices. Repurposing of medicines is part of the routine research portfolio of both the pharmaceutical industry and academic institutions in the search for solutions for those conditions with unmet medical needs including aspects related to sustainability and patient access*". This is very much aligned with the findings from this Task Force, although few considerations have been given so far to a combination of medicines or medicines with medical devices and should be included in further steps.

The second aspect that should be disentangled is the shadow put on repurposing based on pricing considerations. The authors of this paper believe that this discussion is not specific to repurposing and relates to considerations around value-based assessment versus willingness to pay, and as such, this should not hamper the opportunities offered by repurposing.

Thirdly, it came very strongly from the interviews that neither the benefits brought by repurposing nor the pitfalls that come with it should be underestimated. Despite some aspects of the therapeutic development path being easier than a development starting from scratch, careful consideration should be given to all items identified in the START checklist of the Orphan Drug Development Guidebook[23]: (a) mapping of the stakeholders; (b) analysis of the available knowledge already generated on the disease, the product and the targeted patient population; (c) resources planning in order to anticipate the gaps, the additional data to be generated, the regulatory and market access requirements; and (d) the target patient value profile to always keep the unmet needs of patients in focus.

What is needed for sustainable approaches in drug repurposing in terms of funding orientation?

Over the years, IRDiRC funding member organizations have often been referring to the Consortium recommendations when setting up their work program and calls for funding. Notably, the Horizon Europe work program from the European Commission has proposed HORIZON-HLTH-2022-DISEASE-06-04-two-stage: *Development of new effective therapies for rare diseases* & HORIZON-HLTH-2021-DISEASE-04-02: *Building a European innovation platform for the repurposing of medicinal products*[21] which are fully aligned with the roadmap of the IRDiRC Therapies Scientific Committee[26]. The latter provides provision for two consortia[19,20] that are aiming to contribute to the goal presented in this article.

Funding schemes are of utmost importance to allow for a true collaborative partnership to be formed[27-29]. In turn, public-private partnerships are crucial to help cross the so-called valley of death of therapeutic development, which exists for all types of diseases and therapeutic approaches, and which is maybe even more visible in the case of repurposing approaches in the rare disease space.

Funding is not only needed to help progress individual repurposing approaches; there is also a need for funding of pre-competitive activities in order to build infrastructures, foster common knowledge generation (e.g., validate rare disease clinical trial endpoints) and enable sharing of existing and evolving data and information. One avenue for additional investment that came out from the interviews is the lack of accurate figures of prevalence for all rare diseases and in all geographic regions. These figures are not only crucial in the development space of medicines, but also for diagnosis, care and socio-economic planning.

What is needed for sustainable approaches in drug repurposing in terms of implementation?

Access to data generated by previous researchers and developers has been clearly described as a major difficulty during the interviews. The publication of failed or non-conclusive clinical trials would be utterly helpful in the case of repurposing and should be encouraged, as well as the sharing of information amongst

researchers, clinicians and pharmaceutical companies. In the European Union, the modification of the Clinical Trials Regulation and the launch of the Clinical Trial Information System early in 2022 will facilitate access to clinical trial data and ensure more transparency to the public[30].

Another area that would need more reflection is related to off-label use. Discrepancies are so striking around the world that it is difficult to draw a single recommendation[25].

Some roadblocks to repurposing approaches do not require additional funding, but rather a better use of already-available tools, incentives and initiatives, as well as a reflection on how to make the system more efficient. For example, co-creation with patients is still not a reality in each and every therapeutic approach and a fortiori in the examples that we saw in this study, despite lots of tools being already available[31]. In addition, new frameworks have been put in place by regulatory agencies, and the opportunities need to be disseminated widely. Regulatory agencies are engaging with stakeholders in order to facilitate drug repurposing. In 2019, the FDA, in collaboration with United States National Institute of Health (NIH) and the Reagan-Udall Foundation, provided an overview of the challenges of drug repurposing while encouraging further development[32,33]. In October 2021, the European Medicines Agency (EMA), in collaboration with the Heads of Medicines Agencies (HMA), launched a pilot project to support the repurposing of medicines by providing a piece of scientific advice for the selected repurposing candidate projects[34].

Bringing repurposed medicines to rare disease patients offers a strong potential to address many unmet needs. This approach, combined with the development of innovative clinical trial designs, can accelerate the emergence of repurposed drugs targeting multiple rare diseases sharing the same molecular etiology. It would also represent a valuable option for the so-called under-researched rare diseases for which unmet needs must be urgently addressed. IRDiRC is tackling these questions with the *Shared Molecular Etiologies Underlying Multiple Rare Diseases Task Force* and the *Pluto Project*[35,36]. Drug repurposing is clearly recognized as an opportunity to accelerate the development of therapies for rare diseases and to address Goal 2 of IRDiRC[37]: "*1000 new therapies for rare diseases will be approved, the majority of which will focus on diseases without approved options*". The IRDiRC Task Force on *Data Mining and Repurposing*[5] initially addressed this topic by investigating the potential of biomedical data mining strategies to repurpose and accelerate the development of drugs for rare disease patients. More recently, IRDiRC launched *the Drug Repurposing Guidebook Task Force*[24] with the objective of describing the tools, incentives, resources and initiatives available to developers in the field. While the key principles of the Guidebook are applicable to the case of repurposing, no specific repurposing building blocks were created at the time and this will be the purpose of the creation of the "Drug repurposing Guidebook" module. This activity will apply the key principles established for the *Orphan Drug Development Guidebook Task Force*[23] and support more efficient drug development plans.

In conclusion, drug repurposing represents a real opportunity to address unmet needs and improve outcomes for rare disease patients. This IRDiRC Task Force has identified key factors for achieving sustainable repurposing approaches in rare diseases, helping developers optimize their development programs by determining the challenges and also opportunities associated with a particular drug repurposing model or approach.

DECLARATIONS

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Authors Contribution

Led the Task Force: Hivert V
Initiated the desk search analysis to identify cases of repurposed medicinal products into new indication in rare diseases: Zanello G
Initiated the redaction of the manuscript: Hivert V, Zanello G
All the co-authors revised and approved the manuscript, and all the co-authors participated in the final selection of use cases, the development of the questionnaire, the interviews and the result analysis.

Availability of data and materials

The datasets analyzed as sources for the lists are available from the corresponding author upon request.

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Conflicts of interest

All authors declared that there are no conflicts of interest. The findings and recommendations in this article are those of the contributors, who participated based on their individual expertise and are responsible for the contents, and do not necessarily represent the views of the members of the International Rare Diseases Research Consortium (IRDiRC) nor any employers of the contributors.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Bellera CL, Llanos M, Gantner ME, et al. Can drug repurposing strategies be the solution to the COVID-19 crisis? *Expert Opin Drug Discov* 2021;16:605-12. DOI PubMed
2. Venkatesan P. Repurposing drugs for treatment of COVID-19. *Lancet Respir Med* 2021;9:e63. DOI PubMed PMC
3. Giannuzzi V, Conte R, Landi A, et al. Orphan medicinal products in Europe and United States to cover needs of patients with rare diseases: an increased common effort is to be foreseen. *Orphanet J Rare Dis* 2017;12:64. DOI PubMed PMC
4. Nguengang Wakap S, Lambert DM, Olry A, et al. Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database. *Eur J Hum Genet* 2020;28:165-73. DOI PubMed PMC
5. Southall NT, Natarajan M, Lau LPL, et al; IRDiRC Data Mining and Repurposing Task Force. The use or generation of biomedical data and existing medicines to discover and establish new treatments for patients with rare diseases - recommendations of the IRDiRC Data Mining and Repurposing Task Force. *Orphanet J Rare Dis* 2019;14:225. DOI PubMed PMC
6. Breckenridge A, Jacob R. Overcoming the legal and regulatory barriers to drug repurposing. *Nat Rev Drug Discov* 2019;18:1-2. DOI PubMed
7. Begley CG, Ashton M, Baell J, et al. Drug repurposing: misconceptions, challenges, and opportunities for academic researchers. *Sci*

Transl Med 2021;13:eabd5524. DOI PubMed

8. Pushpakom S, Iorio F, Eyers PA, et al. Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discov* 2019;18:41-58. DOI PubMed
9. Member States, National Competent Authorities; EU Innovation Offices. Proposal for a framework to support not-for-profit organisations and academia (institutions and individuals) in drug repurposing. Available from: https://ec.europa.eu/health/system/files/2021-10/pharm773_repurposing_annex_en_0.pdf [Last accessed on 11 Apr 2023].
10. Miller KL, Lanthier M. Investigating the landscape of US orphan product approvals. *Orphanet J Rare Dis* 2018;13:183. DOI PubMed PMC
11. U.S. Food and Drug Administration. Search orphan drug designations and approvals. Available from: https://www.accessdata.fda.gov/scripts/opdlisting/oopd/ [Last accessed on 11 Apr 2023].
12. Orphanet Inserm US14. Lists of medicinal products for rare diseases in Europe. Available from: https://www.myobase.org/doc_num.php?explnum_id=10705 [Last accessed on 11 Apr 2023].
13. Postema PG, Schwartz PJ, Arbelo E, et al. Continued misuse of orphan drug legislation: a life-threatening risk for mexiletine. *Eur Heart J* 2020;41:614-7. DOI PubMed
14. Simoens S, Picavet E, Cassiman D, Dooms M. PHP15 What price do we pay for repurposing medicines for rare diseases? *Value in Health* 2012;15:A15-6. DOI
15. WHO/EURO. Repurposing of medicines in oncology - the underrated champion of sustainable innovation. Available from: https://apps.who.int/iris/bitstream/handle/10665/342567/WHO-EURO-2021-2807-42565-59178-eng.pdf?sequence=1&isAllowed=y [Last accessed on 11 Apr 2023].
16. Aartsma-Rus A, Vroom E, O'Reilly D. The role of patient involvement when developing therapies. *Nucleic Acid Ther* 2022;32:118-22. DOI PubMed PMC
17. Cavaller-Bellaubi M, Faulkner SD, Teixeira B, et al. Sustaining meaningful patient engagement across the lifecycle of medicines: a roadmap for action. *Ther Innov Regul Sci* 2021;55:936-53. DOI PubMed PMC
18. Hoos A, Anderson J, Boutin M, et al. Partnering with patients in the development and lifecycle of medicines: a call for action. *Ther Innov Regul Sci* 2015;49:929-39. DOI PubMed PMC
19. REPO4EU. The Euro-global platform for mechanism-based drug repurposing. Available from: https://repo4.eu/ [Last accessed on 11 Apr 2023].
20. REMEDi4ALL. The European Platform for medicines repurposing. Available from: https://remedi4all.org/ [Last accessed on 11 Apr 2023].
21. European Commission. Horizon Europe work programme 2021-2022. Available from: https://ec.europa.eu/info/funding-tenders/opportunities/docs/2021-2027/horizon/wp-call/2021-2022/wp-4-health_horizon-2021-2022_en.pdf [Last accessed on 11 Apr 2023].
22. U.S. Department of Health and Human Services; Food and Drug Administration; Center for Drug Evaluation and Research (CDER). Guidance for industry applications covered by section 505(b)(2). Available from: https://www.fda.gov/media/72419/download [Last accessed on 11 Apr 2023].
23. Hechtelt Jonker A, Hivert V, Gabaldo M, et al. Boosting delivery of rare disease therapies: the IRDiRC Orphan Drug Development Guidebook. *Nat Rev Drug Discov* 2020;19:495-6. DOI PubMed
24. IRDIRC. Drug Repurposing guidebook. Available from: https://irdirc.org/drug-repurposing-guidebook/ [Last accessed on 11 Apr 2023].
25. Asker-Hagelberg C, Boran T, Bouygues C, et al. Repurposing of medicines in the EU: launch of a pilot framework. *Front Med (Lausanne)* 2021;8:817663. DOI PubMed PMC
26. Hivert V, Jonker AH, O'connor D, Ardigo D. IRDiRC: 1000 new rare diseases treatments by 2027, identifying and bringing forward strategic actions. *Rare Dis Orphan Drugs J* 2022;1:3. DOI
27. National Institute of Allergy and Infectious Diseases (NIAID). NOT-AI-16-052: Notice of NIAID's participation in PA-16-183 "limited competition: Rare Diseases Clinical Research Network (RDCRN) Project supplements for clinical trials to repurpose drugs in collaboration with e-rare awardees (Admin Supp)". Available from: https://grants.nih.gov/grants/guide/notice-files/NOT-AI-16-052.html [Last accessed on 11 Apr 2023].
28. National Center for Advancing Translational Sciences (NCATS). NOT-TR-19-029: NCATS announces the cures acceleration network review board workshop: repurposing off-patent drugs: research & regulatory challenges. Available from: https://grants.nih.gov/grants/guide/notice-files/NOT-TR-19-029.html [Last accessed on 11 Apr 2023].
29. ERA-LEARN. E-Rare 3 call for proposals 2016 (JTC 2016): clinical research for new therapeutic uses of already existing molecules (repurposing) in rare diseases. Available from: https://www.era-learn.eu/network-information/networks/e-rare-3/e-rare-3-call-for-proposals-2016-jtc-2016-clinical-research-for-new-therapeutic-uses-of-already-existing-molecules-repurposing-in-rare-diseases [Last accessed on 11 Apr 2023].
30. European Medicines Agency. Clinical trials regulation. Available from: https://www.ema.europa.eu/en/human-regulatory/research-development/clinical-trials/clinical-trials-regulation [Last accessed on 11 Apr 2023].
31. PARADIGM. Patient engagement toolbox. Available from: https://imi-paradigm.eu/petoolbox/ [Last accessed on 11 Apr 2023].
32. U.S. Food Drug Administration. Repurposing off-patent drugs: research & regulatory challenges. Available from: https://www.fda.gov/drugs/news-events-human-drugs/repurposing-patent-drugs-research-regulatory-challenges-12052019-12062019 [Last accessed on 11 Apr 2023].

33. Reagan-Udall Foundation for the FDA. Repurposing off-patent drugs: research & regulatory challenges. Available from: https://reaganudall.salsalabs.org/repurposingoffpatentdrugsworkshop/index.html [Last accessed on 11 Apr 2023].
34. European Medicines Agency. Repurposing of authorised medicines: pilot to support not-for-profit organisations and academia. Available from: https://www.ema.europa.eu/en/news/repurposing-authorised-medicines-pilot-support-not-profit-organisations-academia [Last accessed on 11 Apr 2023].
35. IRDiRC. Shared molecular etiologies underlying multiple rare diseases. Available from: https://irdirc.org/shared-molecular-etiologies/ [Last accessed on 11 Apr 2023].
36. IRDiRC. Pluto Project. Disregarded rare diseases. Available from: https://irdirc.org/pluto-project-disregarded-rare-diseases/ [Last accessed on 11 Apr 2023].
37. IRDiRC. Vision & goals. Available from: https://irdirc.org/about-us/vision-goals/ [Last accessed on 11 Apr 2023].

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Technical Note

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Histological and biochemical methods to assess aminoacyl-tRNA synthetase expression in human post-mortem brain tissue

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Abstract

Aminoacyl-tRNA synthetases are essential, non-redundant enzymes that catalyze the charging of tRNAs with their cognate amino acids. This reaction is a prerequisite for protein translation in all cells. Mutations in human aminoacyl-tRNA synthetases are often associated with defects of the peripheral and central nervous system and are the underlying cause of many rare diseases including neuropathies and leukodystrophies. A comprehensive understanding of aminoacyl-tRNA synthetase expression domains is key to understanding these disorders and developing novel targeted treatment strategies. Here, we describe histological and biochemical methods to analyze the expression pattern of the aspartyl-tRNA synthetase AspRS in human post-mortem brain tissue. The same methods can readily be applied to other members of the aminoacyl-tRNA synthetase superfamily or, more generally, to other cytosolic proteins in the human brain.

Keywords: Aminoacyl-tRNA synthetase, aspartyl-tRNA synthetase, AspRS, *DARS1*, post-mortem brain tissue

INTRODUCTION

Protein synthesis is an evolutionarily highly conserved process that requires an intimate interplay of

specialized ribonucleic acids (RNAs) and proteins. The importance of this process is highlighted by the many diseases resulting from mutations in genes encoding the involved proteins and RNAs[1]. Before transfer RNAs (tRNAs) can transfer amino acids to the nascent polypeptide chain, they need to be charged with their cognate amino acid, a reaction catalyzed by specific aminoacyl-tRNA synthetases (ARSs). A human cell contains 37 cytosolic and mitochondrial synthetase genes, all of which are encoded on nuclear chromosomes. The correct charging of tRNAs is essential for the fidelity of protein synthesis and there is no redundancy amongst ARSs. Consequently, dysfunctional tRNA charging is not tolerated. To date, pathological mutations that cause neurological diseases have been identified in 24 ARS genes[2]. Intriguingly, most of the pathological ARS mutations primarily manifest in deficits of the peripheral and central nervous system, including encephalopathies, Charcot-Marie-Tooth neuropathies, leukodystrophies, or cerebellar ataxia. This indicates a high susceptibility of neural cells to disturbances in protein synthesis[3].

The leukodystrophy Hypomyelination with Brain stem and Spinal cord involvement and Leg spasticity (HBSL) is caused by recessive missense mutations of the *DARS1* gene encoding the cytosolic aspartyl-tRNA synthetase (AspRS)[4]. For most patients, the disease manifests from 3-36 months of age. The symptoms include leg spasticity, inability to walk unsupported or articulate, nystagmus, cognitive impairment, epilepsy, and premature death. In addition to infantile-onset patients, the disease spectrum has been broadened by the description of HBSL cases with late adolescent onset and a milder disease course[5]. Interestingly, HBSL patients present with similar symptoms as patients afflicted with Leukoencephalopathy with Brain stem and Spinal cord involvement and elevated Lactate (LBSL), which is caused by missense mutations of the *DARS2* gene encoding mitochondrial AspRS[6,7]. Although the two conditions might share a similar underlying pathophysiology, cytosolic and mitochondrial AspRS are not functionally redundant, as genetic deletion of either of the two genes in mice is embryonically lethal and cannot be compensated for by the other[8,9].

The development of accurate HBSL animal models is crucial to advance the understanding of the disease mechanism and to develop targeted and effective treatments. However, the lack of redundancy amongst ARSs and the essential nature of the AspRS enzyme have made disease modelling challenging, as complete knockout of the *DARS1* gene resulted in early embryonic lethality[9] and the introduction of patient-specific, disease-causing *DARS1* point mutations into the mouse genome has failed to replicate the full HBSL disease phenotype[10,11]. In the absence of precise animal models, detailed knowledge of the AspRS expression pattern can provide valuable insight into etiology and help define therapeutic targets. Our recent gene expression studies using mouse[9] and human[12] brain tissue from neurologically healthy individuals revealed that, despite ubiquitously expressed in all cells, the highest AspRS levels were present in neuronal lineage cells with comparably little immunoreactivity present in oligodendrocytes, astrocytes, and microglia. Anatomically, AspRS expression was highly enriched in the cerebellum, a region responsible for motor control and particularly affected in HBSL patients. Here, we describe in detail the histological and biochemical methods used to analyze the AspRS expression pattern in human post-mortem brain tissue. These protocols can readily be adapted to characterize other members of the ARS protein family or, more broadly, other cytosolic proteins in the human brain.

MATERIALS

Post-mortem brain tissue

Human post-mortem brain tissue was provided by the New South Wales Brain Bank (project no. PID391). All procedures were approved by the UNSW Sydney Human Research Ethics Advisory Panel D. The tissue samples were taken from five male subjects aged between 55 and 57 years who died from cardiovascular disease. Individuals did not suffer from neurological diseases and the brain tissue was free from overt brain

pathology. The post-mortem interval, which is the time between the death of the person and the time point at which the tissue was taken, ranged between 12 and 39 h. Brain regions analyzed included motor cortex, hippocampus, cerebellum, and brainstem (pons). Frozen tissue blocks were used for immunoblotting and quantitative real-time PCR (qRT-PCR), and formalin-fixed, paraffin-embedded, 4 µm thin sections were used for immunohistochemistry. Sections from the motor cortex, hippocampus, and cerebellum were cut in the coronal plane; sections from the brainstem were cut transversally.

Antibodies

The following primary antibodies were used for immunoblotting: mouse anti-AspRS (monoclonal; Santa Cruz Biotechnology #sc-393275; 1:500 dilution), which is specific for amino acids 170-467 of the human AspRS protein; rabbit anti-AspRS (polyclonal; Novus Biologicals #NBP1-85937; 1:500 dilution), which is specific for amino acids 1-135 of the human AspRS protein; rabbit anti-GAPDH (Cell Signaling #2118S; 1:5,000 dilution); mouse anti-β-Actin clone C4 (Sigma-Aldrich #mab1501; 1:10,000 dilution). Goat anti-mouse and goat anti-rabbit HRP-conjugated secondary antibodies were obtained from Dianova (1:10,000 dilution).

For immunohistochemistry, the following primary antibodies were used: mouse anti-AspRS (monoclonal; Santa Cruz Biotechnology #sc-393275; 1:50 dilution) and rabbit anti-NeuN (Cell Signaling #12943S; 1:40 dilution). Goat anti-rabbit Alexa555-conjugated and goat anti-mouse Alexa488-conjugated secondary antibodies were obtained from Thermo Fisher (1:300 dilution). Goat anti-mouse biotin-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (#111-065-003; 1:250 dilution).

TaqMan assays

The following TaqMan assays were used for qRT-PCR: *DARS1* (Applied Biosystems #Hs00154683_m1) and *β-Actin* (Applied Biosystems #Hs01060665_g1).

METHODS

Sample preparation for Western blotting and qRT-PCR

Before protein and RNA lysates can be prepared for Western blotting and qRT-PCR, respectively, the frozen human brain tissue from individual brain regions needs to be homogenized in liquid nitrogen using mortar and pestle until a fine powder is obtained. It is important to keep the tissue in liquid nitrogen at all times during the homogenization process. When homogenization is complete and all liquid nitrogen has evaporated from the mortar, the tissue powder is distributed into two Eppendorf tubes. The tubes are weighed before and after filling to determine the total tissue weight. Homogenized tissue can be kept on dry ice for further downstream applications or stored long-term at -80 °C. One tube is used to prepare a protein lysate for Western blot analysis; the second tube is used for RNA isolation for qRT-PCR.

Western blotting

For Western blotting, the homogenized tissue powder is resuspended in 10 µL lysis buffer [50 mM Tris-HCL pH 7.4, 1 mM EDTA pH 8.0, 250 mM NaCl, 1% v/v Triton-X, and protease inhibitors (Roche Complete #04693124001)] per 1 mg of tissue weight. Following sonication using a probe sonicator (Branson Digital Sonifier 450) at 10% sonication amplitude for 1 min, the tissue suspension is spun down at full speed in a conventional benchtop centrifuge and the supernatant is transferred to a new tube. The protein concentration of the lysates is determined by Bradford protein assay using Quick Start Bradford 1x Dye Reagent (Bio-Rad #500-0205). 30 µg of protein from each brain region is mixed with five times sample buffer (15 g SDS, 15.6 mL 2M Tris pH 6.8, 57.5 g glycerol, 16.6 mL β-mercaptoethanol) and loaded onto a 10% acrylamide gel (Bio-Rad Mini-PROTEAN gel system). Following separation by SDS-PAGE, proteins are transferred onto a PVDF membrane (Bio-Rad #162-0177). To avoid unspecific antibody binding, the

PVDF membrane is incubated in blocking solution (4% milk powder and 0.1% Tween in PBS) for at least one hour at room temperature. Primary antibodies are then applied in blocking solution overnight at 4 °C. Following three 10 min washing steps with PBS-T (0.1% Tween in PBS), the membrane is probed with secondary HRP-coupled antibodies in blocking solution for 1 h at room temperature and washed three times again in PBS-T, each 10 min. Afterwards, the membrane is developed using Clarity Western ECL substrate (Bio-Rad #170-5060) and subsequently imaged employing the Bio-Rad ChemiDoc MP imaging system. Protein bands are quantified using the densitometry function in ImageJ and AspRS protein levels in different brain regions are normalized to the housekeeping proteins β-Actin and GAPDH. A comprehensive AspRS expression study comparing protein levels across different regions of the human brain was conducted following the protocol described here[12]. This study found significantly higher AspRS levels in the cerebellum compared to the other brain regions analyzed.

Quantitative real-time PCR

For qRT-PCR analysis, the tissue powder is resuspended in 350 µL (for up to 20 mg of tissue) or 600 µL (for 20-30 mg of tissue) buffer RLT (from the Qiagen RNeasy MiniKit #74106) plus 1% β-mercaptoethanol and homogenized using a pellet pestle attached to the Kontes pellet pestle motor. Afterwards, the lysate is centrifuged for 3 min at full speed in a conventional benchtop centrifuge and the supernatant is transferred to a fresh tube. RNA extractions are performed using the RNeasy MiniKit (Qiagen #74106) following the manufacturer's instructions including an on-column DNase digest using the RNase-free DNase kit (Qiagen #79254) to get rid of any genomic DNA. At the end of the extraction procedure, the RNA is eluted from the column in 50 µL RNase free water and the concentration is determined using the NanoDrop microvolume spectrophotometer. An equal amount of RNA from each brain region is used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368813) in a conventional thermocycler. Afterwards, qRT-PCR can be performed in any suitable real-time PCR machine employing specific TaqMan probes. Data is analyzed using the comparative ΔΔCT method for relative quantification of *DARS1* expression normalized to the housekeeping gene *β-Actin*. Using this method, *DARS1* mRNA levels were analyzed across different human brain regions, revealing a significant enrichment in the cerebellum in line with the AspRS protein data[12].

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were received on glass slides from the New South Wales Brain Bank. The sections were cut at 4 µm thickness to ensure adequate penetration of antibodies. Brain sections included the motor cortex, hippocampus, and cerebellum, which were all cut in the coronal plane, and sections from the brainstem (pons), which were cut in the transverse plane.

Sections for immunofluorescent labelling [Figure 1] are incubated in a 60 °C oven for 2 h to melt the paraffin surrounding the tissue before placing the slides in xylene for 2 × 10 min to ensure complete solubilization and removal of residual paraffin. The slides are then rehydrated for 2 × 5 min in decreasing concentrations of ethanol in water, starting at 100% ethanol before moving to 96% and 70% ethanol. Finally, the slides are placed in water for a period of 5 min. The deparaffinization and rehydration process is critical to unmask epitopes for immunohistochemical labelling. To further assist the access of antibodies to antigens in the tissue, antigen retrieval can be performed. Different forms of antigen retrieval methods are described in the literature. In our case, citrate buffer antigen retrieval worked best. For antigen retrieval, slides are incubated in 10 mM citrate buffer (1.8 µM citric acid and 8.2 µM sodium citrate tribasic dihydrate in water) and placed in an RHS-1 Microwave Vacuum Histoprocessor (Milestone Medical). The microwave heats the solution to 120 °C and maintains this temperature for an additional 1 min. Slides are then removed from the microwave and allowed to cool down to room temperature. This step is critical to minimize excess background signal. Slides are washed in PBS for 5 min prior to permeabilization with 0.5% TritonX-100 for

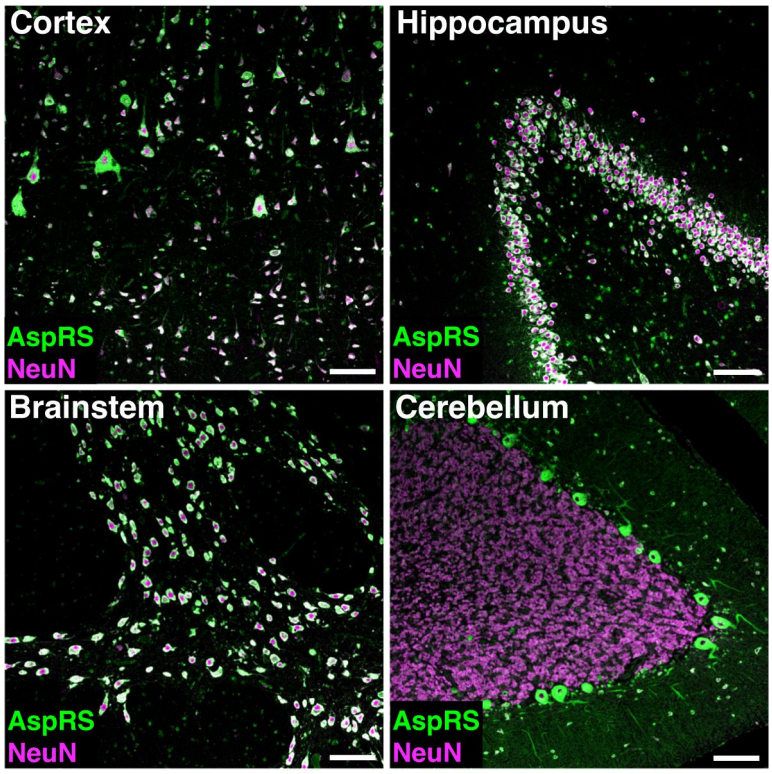

Figure 1. Representative immunofluorescent images showing co-labelling of AspRS (green) and the neuronal cell-type marker NeuN (magenta) in human post-mortem brain sections. Regions displayed include motor cortex, hippocampus, brainstem (pons), and cerebellum. Scale bar: 100 µm.

3 min. Although permeabilization with TritonX-100 is not critical, our study found that this further assisted in revealing epitopes for antibody staining. Slides are then placed in a Sequenza slide rack (Thermo Scientific), washed 3 times with PBS and blocked in 10% normal goat serum in PBS for 60 min. Primary antibodies are applied in PBS + 10% normal goat serum overnight at 4 °C. Following three washing steps with PBS, fluorophore-coupled secondary antibodies are applied in PBS + 10% normal goat serum for 1 h at room temperature. Slides are washed 3 times with PBS, incubated for 5 min with DAPI and washed again with PBS. Subsequently, slides are washed in 70% ethanol for 5 min and then a few drops of Autofluorescence Eliminator reagent (Millipore #2160) are added directly to the tissue for 3 min to reduce autofluorescence. This step is critical because perfusion of human tissue cannot be performed prior to tissue fixation, potentially resulting in strong autofluorescence signals. Slides are then washed in 70% ethanol to remove residual Autofluorescence Eliminator reagent before being washed in PBS for 5 min. Slides are mounted in ProLong Gold antifade reagent (Thermo Fischer #P101444) as this helps to preserve the fluorescent signal. Fluorescent images can be taken with any suitable confocal microscope (in our study, a Zeiss LSM710 was used). Representative immunofluorescent images depicting AspRS and NeuN (neuronal marker) immunoreactivity are shown in Figure 1. For a detailed analysis of the AspRS expression pattern in human brain tissue, including co-labelling with neuronal, oligodendroglial, astroglial, and microglial markers, please refer to Fröhlich *et al.*, 2018[12].

For immunoperoxidase labelling [Figure 2] with Diaminobenzidine (DAB), the brain sections are dewaxed and rehydrated as described above. After citrate antigen retrieval, sections are incubated with 4% H_2O_2 in 50% ethanol for 60 min. Sections are rinsed 2 × 5 min in water and 1 × 5 min in PBS. Sections are blocked for 1 h in 10% normal goat serum in PBS and incubated with the primary antibody as described above.

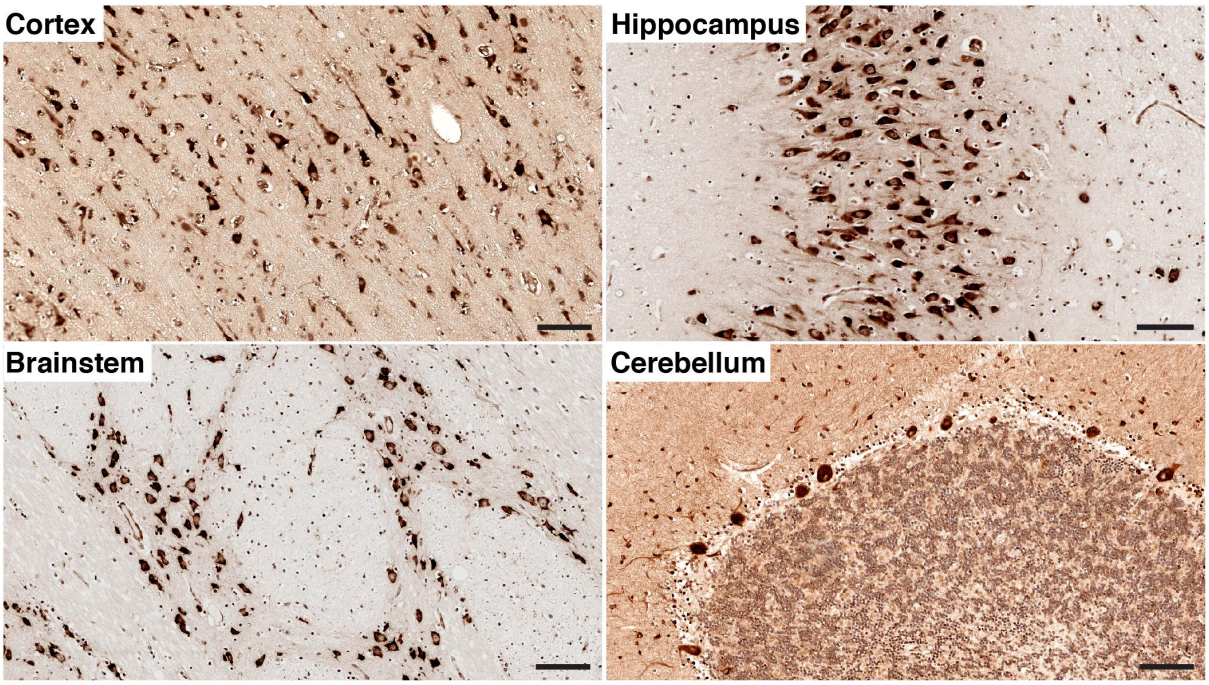

Figure 2. Representative chromogenic detection of AspRS by immunohistochemical labelling with Diaminobenzidine (DAB) in the motor cortex, hippocampus, brainstem (pons), and cerebellum of the human brain. Scale bar: 100 µm.

Sections are washed 3 times with PBS and then incubated with biotin-conjugated secondary antibodies for 2 h at room temperature (instead of fluorophore-coupled secondary antibodies). Sections are washed 3 times in PBS and incubated in ABC solution (Vectastain #PK-6105, Goat IgG) for 30 min at room temperature. Following three washes with PBS, sections are incubated with DAB solution (DAB substrate kit for peroxidase; Vector Laboratories #SK-4100) as described in the manufacturer's protocol. DAB-labelled sections can be imaged with a suitable light microscope or scanned using a slide scanner. In our study, an Aperio slide scanner (Leica) was used, followed by image processing using the Aperio ImageScope software (Leica) [Figure 2]. Representative chromogenic images showing AspRS in the cortex, hippocampus, brainstem, and cerebellum of the human brain using the DAB staining method described here are shown in Figure 2. An in-depth characterization and discussion of AspRS expression in the human brain has been described elsewhere[12].

TECHNICAL NOTES

Western blotting

The homogenization method used for protein extraction may vary depending on the kind and amount of tissue. For brain tissue, we found that a combination of sonication and grinding in liquid nitrogen works best. When grinding using mortar and pestle, make sure that the sample remains covered in liquid nitrogen to avoid thawing of the tissue. Before the pulverized tissue is transferred into an Eppendorf tube, ensure that all liquid nitrogen has evaporated from the mortar. Any liquid nitrogen transferred into an enclosed reaction tube can be hazardous as the tube might explode when warming up. During the subsequent sonication step, all cells are lysed by liquid cavitation. An advantage of sonication over other protein extraction methods is that DNA is also sheared during the process and there is no need to treat the sample with DNase. To avoid protein degradation, it is important to keep the samples on ice during sonication.

Choosing the right housekeeping protein is critical for normalization of protein levels, as some housekeepers might be regulated in certain disease conditions or tissues. Normalization against two different housekeeping proteins should yield comparable results and is a good control for the validity of the housekeeper.

Unspecific binding of an antibody - the binding of an antibody to non-target antigens, is easier to identify in Western blotting compared to immunohistochemistry as the molecular weight at which the protein of interest is detected is a strong indication of antibody specificity. Detection of multiple bands on a Western blot can indicate non-specific binding, which needs to be considered if the same antibody were to be used in immunohistochemistry. If uncertain of antibody specificity, peptide blocking should be performed to confirm the validity of the primary antibody. For this, the antibody is pre-absorbed with a blocking peptide corresponding to the epitope of the antibody, which will prevent the binding of the target antigen and consequently reduce signal intensity compared to non-blocked antibodies.

Quantitative real-time PCR

For RNA work, it is essential to keep the workplace and reagents free of RNases to avoid degradation of the RNA. It is recommended to perform all RNA work in a laminar flow hood.

Similar to protein extraction, tissue homogenization for RNA preparation is performed in two steps. Following liquid nitrogen grinding, cells are lysed using a pellet pestle homogenizer instead of a probe sonicator to avoid shearing of the RNA.

In our study, off-the-shelf TaqMan assays worked best as they provide species-specificity and low unspecific binding. Selection of the appropriate TaqMan assay should take into account that the primers are exon-exon junction spanning to avoid detection of genomic DNA. If analyzing human brain tissue from a carrier of a known ARS mutation, it is essential to select primers and TaqMan probes that anneal to cDNA sequences unaffected by the respective mutation. The specificity of qRT-PCR primer pairs or TaqMan assays should always be validated through sequencing of the amplicon.

Immunohistochemistry

Deparaffinization and rehydration of paraffin-embedded sections are critical to unmask epitopes for immunohistochemical labelling. For some antibodies, it is necessary to perform additional antigen retrieval to further assist the access of the antibody to the antigen. Which antigen retrieval method should be employed depends on the antibody used, and it is worthwhile testing them before the study commences. In our study, citrate antigen retrieval yielded the best results.

To avoid unspecific antibody binding, blocking should be performed in the serum of the host species of the secondary antibody. If this is not possible, we generally have had good experiences using normal goat serum.

For immunofluorescent labelling of antigens, we achieved the best results employing secondary antibodies coupled with Alexa fluorophores.

Human tissue samples inherently give rise to a lot of background signals, as there is no perfusion of tissue prior to fixation, resulting in autofluorescence. There are numerous contributing factors including collagen or elastin, which often illuminate blood vessels in the tissue. Historically, Sudan Black has been used to reduce autofluorescence; however, this also dampens the true fluorescent signal. We have found that the

Autofluorescence Eliminator reagent (Millipore #2160) works well on human tissue.

When choosing the most appropriate antibody, it is important to consider the specific advantages and disadvantages of polyclonal versus monoclonal antibodies[13]. Polyclonal antibodies are produced by injecting an animal with the desired antigen to elicit an immune response and produce antibodies against the antigen. Polyclonal antibodies can recognize multiple epitopes of the antigen, which often results in a higher overall affinity and sensitivity to the target protein. The same multi-epitope specificity, however, can result in cross-reactivity with other proteins leading to increased unspecific binding and background staining. In contrast, monoclonal antibodies are produced *in vitro* by a single B cell clone and recognize only one specific epitope. While this might result in lower overall sensitivity, monoclonal antibodies often show higher specificity with reduced cross-reactivity and, consequently, less background signal. If working with brain tissue from patients affected by a known ARS mutation, a monoclonal antibody that recognizes an epitope outside the mutated amino acid sequence should be used. Alternatively, a polyclonal antibody can be used to ensure multiple epitopes are recognized.

Studies involving antibody-based methods should always include appropriate controls for the specificity of antibodies, such as secondary-only stainings. A comprehensive guideline for the validation of antibodies for use in immunohistochemistry has been developed by Howat *et al.*[14]. Determination of antibody specificity is crucial in studies on human tissue when genetic manipulations such as overexpression or knockdown via RNA interference of the protein of interest cannot be performed to confirm the validity of an antibody. If positive or negative human control tissue with confirmed up- or downregulated target protein is available, this can be used for establishing antibody specificity. Western blotting can give a first indication if an antibody is specific or not (see above); however, it does not always guarantee antibody specificity in immunohistochemistry applications where the tissue has undergone different processing steps, including fixation and antigen retrieval. A good indication for the specificity of an antibody can also be obtained by ectopically expressing the human protein of interest in a cell culture model such as HEK293 cells with subsequent immunocytochemistry. Finally, peptide blocking, as described above, can be employed to rule out non-specific binding in immunohistochemistry. If the above controls are not possible or yield inconclusive results, orthogonal methods such as *in situ* hybridization of the corresponding mRNA should be applied to support the antibody-based data.

DECLARATIONS

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Authors' contributions

Led the project and manuscript preparation: Klugmann M, Fröhlich D
Conducted the research: Klugmann M, Suchowerska AK, Housley GD, Fröhlich D
All authors contributed to and approved the final version of the manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

Klugmann M was employed by the company Boehringer Ingelheim Pharma GmbH & Co. KG. All authors declared that there is no conflict of interest.

Ethical approval and consent to participate

This study was approved by the UNSW Sydney Human Research Ethics Advisory Panel D.

Consent for publication

Not applicable.

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REFERENCES

1. Abbott JA, Francklyn CS, Robey-Bond SM. Transfer RNA and human disease. *Front Genet* 2014;5:158. DOI PubMed PMC
2. Ognjenović J, Simonović M. Human aminoacyl-tRNA synthetases in diseases of the nervous system. *RNA Biol* 2018;15:623-34. DOI PubMed PMC
3. Antonellis A, Green ED. The role of aminoacyl-tRNA synthetases in genetic diseases. *Annu Rev Genomics Hum Genet* 2008;9:87-107. DOI PubMed
4. Taft RJ, Vanderver A, Leventer RJ, et al. Mutations in *DARS* cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am J Hum Genet* 2013;92:774-80. DOI PubMed PMC
5. Wolf NI, Toro C, Kister I, et al. *DARS*-associated leukoencephalopathy can mimic a steroid-responsive neuroinflammatory disorder. *Neurology* 2015;84:226-30. DOI PubMed PMC
6. Muthiah A, Housley GD, Klugmann M, Fröhlich D. The leukodystrophies HBSL and LBSL-correlates and distinctions. *Front Cell Neurosci* 2020;14:626610. DOI PubMed PMC
7. Scheper GC, van der Klok T, van Andel RJ, et al. Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. *Nat Genet* 2007;39:534-9. DOI PubMed
8. Dogan SA, Pujol C, Maiti P, et al. Tissue-specific loss of *DARS2* activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab* 2014;19:458-69. DOI PubMed
9. Fröhlich D, Suchowerska AK, Spencer ZH, et al. *In vivo* characterization of the aspartyl-tRNA synthetase *DARS*: homing in on the leukodystrophy HBSL. *Neurobiol Dis* 2017;97:24-35. DOI PubMed
10. Fröhlich D, Mendes MI, Kueh AJ, et al. A hypomorphic *Dars1D367Y* model recapitulates key aspects of the leukodystrophy HBSL. *Front Cell Neurosci* 2020;14:625879. DOI PubMed PMC
11. Klugmann M, Kalotay E, Delerue F, et al. Developmental delay and late onset HBSL pathology in hypomorphic *Dars1M256L* mice. *Neurochem Res* 2022;47:1972-84. DOI PubMed PMC
12. Fröhlich D, Suchowerska AK, Voss C, et al. Expression pattern of the aspartyl-tRNA synthetase *DARS* in the human brain. *Front Mol Neurosci* 2018;11:81. DOI PubMed PMC
13. Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. *ILAR J* 2005;46:258-68. DOI PubMed
14. Howat WJ, Lewis A, Jones P, et al. Antibody validation of immunohistochemistry for biomarker discovery: recommendations of a consortium of academic and pharmaceutical based histopathology researchers. *Methods* 2014;70:34-8. DOI PubMed PMC

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Original research, systematic reviews, and meta-analyses require structured abstracts. The abstract should provide the context or background for the study and should state the study's purpose, basic procedures (selection of study participants, settings, measurements, analytical methods), main findings (giving specific effect sizes and their statistical and clinical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations, note important limitations, and not overinterpret findings. Clinical trial abstracts should include items that the CONSORT group has identified as essential. It is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.5 Graphical Abstract

The graphical summary is optional. It should summarize the content of the article in a concise graphical form. It is recommended to use it because this can make online articles get more attention. The graphic abstract should be submitted as a separate document in the online submission system. Please provide image with a resolution greater than 300 dpi. Preferred file types: TIFF, PSD, AI, JPEG and EPS files.

2.3.1.6 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results and Discussion

This section should contain the findings of the study and discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.5 Conclusions

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be

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References should be described as follows, depending on the types of works:

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. *BJOG 2018*; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
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Figure caption is placed under the Figure;
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In some instances, a case report or case series containing information on less than three patients may not require ethical approval. However, this requirement is dependent on the institution, country or region implementing it and authors must ensure they have followed the correct regulatory requirements of their institution or country. A statement explaining this requirement must be included in the manuscript.

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Authors must keep data anonymized. If participants' details are not to be anonymized, authors must ensure that written informed consent, including consent for publication, was obtained from each participant, and consent statement must be included in the manuscript.

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9. Editorial Process

9.1 Initial check

9.1.1 Initial manuscript check

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