Therapeutic cysteine protease inhibitors: a patent review (2018–present)

Therapeutic cysteine protease inhibitors: a patent review (2018 - present)

Giulia Barchielli, Antonella Capperucci and Damiano Tanini*

University of Florence, Department of Chemistry ''Ugo Schiff'', Via della Lastruccia 3–13, I-50019 Sesto Fiorentino, Italy. *E-mail: damiano.tanini@unifi.it*

ABSTRACT

Introduction: Cysteine proteases are involved in a broad range of biological functions, ranging from extracellular matrix turnover to immunity. Playing an important role in the onset and progression of several diseases, including cancer, immune-related and neurodegenerative disease, viral and parasitic infections, cysteine proteases represent an attractive drug target for the development of therapeutic tools.

Areas covered: Recent scientific and patent literature focusing on the design and study of cysteine protease inhibitors with potential therapeutic application has been reviewed.

Expert opinion: The discovery of a number of effective structurally diverse cysteine protease inhibitors opened up new challenges and opportunities for the development of therapeutic tools. Mechanistic studies and the availability of X-ray crystal structures of some proteases, alone and in complex with inhibitors, provide crucial information for the rational design and development of efficient and selective cysteine protease inhibitors as preclinical candidates for the treatment of different diseases.

ARTICLE HIGHLIGHTS

- Cysteine proteases play a number of pivotal biological roles.
- The dysregulation of Cysteine proteases functioning can occur at different levels and may contribute to the onset and progression of different pathologies.
- Targeting cysteine proteases represents an attractive approach to develop novel therapeutics tools for the treatment of viral infections, neurodegenerative disorders, and cancer.
- Recent-years researches and patents mainly focused on the inhibition of viral cysteine proteases like PL^{pro} and M^{pro}, Cathepsins (Cats) and Ubiquitin Specific Proteases (USPs) for the treatment of viral infections, cancer and inflammatory diseases.
- Different medicinal chemistry approaches were used to develop new molecules as potential drugs, ranging from drug repurposing to the most recent library screening techniques including HTS, including also the research on natural molecules.
- Mechanistic investigations and X-ray studies provide crucial information for the rational design and development of efficient and selective cysteine protease inhibitors.
- Reported small molecule inhibitors include, amongst the others, Michael acceptors, nitriles, thioketones, α-ketoamides, chloromethyl ketones, disulfides, dithiocarbamtes, and organoselenium compounds.
- Small molecule inhibitors of SARS-CoV-2 cysteine proteases (PL^{pro} and M^{pro}), cathepsins (Cats), and Ubiquitin Specific Proteases (USPs) are discussed in the context of their potential therapeutic application.

This box summarizes key points contained in the article.

Keywords: cathepsins; ubiquitin-specific-proteases; main protease; papain-like protease; coronavirus; SARS-CoV-2; small molecules; drugs.

---

1. Introduction

Cysteine proteases are enzymes present in all living systems, plants, animals, bacteria and virus. Besides their main function of protein catabolism by hydrolysis, they have further roles in the extracellular matrix turnover, antigen presentation, digestion, immune invasion, haemoglobin hydrolysis, parasite invasion and egress, and processing surface proteins. Thus, they represent promising drug targets for several diseases including immune-related and neurodegenerative disease, cancer, atherosclerosis, osteoporosis,

viral infections, and a number of parasitic diseases including malaria and Chagas disease.[1]
Cysteine proteases activity is regulated *via* a combination of factors including localization, pH and endogenous inhibitors, whose dysregulation could be associated with diseases. The mechanism of action of cysteine proteases involves the nucleophilic attack of the thiol moiety of a cysteine residue onto the peptide carbonyl carbon. The cysteine is typically located in a catalytic dyad with a histidine residue; in some cases a triad including aspartic acid is present. The relatively low pK_a of the thiol group enables the deprotonation of the catalytic cysteine by the proximal histidine with the formation of the corresponding thiolate, which behave as an exquisite nucleophile. The cleavage of the peptide bond occurs through i) the nucleophilic reaction of the thiolate onto the peptide carbonyl carbon (nucleophilic acyl substitution), followed by ii) the hydrolysis of the thiolester intermediate.
The MEROPS database includes 14 superfamilies for cysteine proteases and, among them, clan CA and clan CD represent the bulk of these enzymes. Clan CA includes: i) papaya protease papain; ii) mammalian proteases cathepsins, mainly located in lysosomes and cellular compartments, with several roles in the biosynthesis and homeostasis in healthy tissues as well as in tumours onset and progression; iii) calcium-dependent calpains located in the cytoplasm. An extracellular activity of cathepsins has been highlighted to favour cancer progression and metastasis.[2] Clan CD includes i) caspases (mediators of apoptosis); ii) legumain (involved in protein degradation, in the major histocompatibility complex and in the pathogenesis of malignant and non-malignant diseases);[3] iii) viral proteases; iv) separase (playing key roles in the separation of sister chromatids during mitosis).[4]

1.1. Cysteine cathepsins

11 cysteine cathepsins (B, C, F, H, K, L, O, S, V, W, and X/Z), differing in their cellular and tissue localization, expression, biochemical and structural properties and activity, are encoded by the human genome.[5] Cathepsins (Cats) are briefly presented below following alphabetic criteria.
Cathepsin B (CatB) was the first lysosomal protease to be associated with breast carcinoma. [6] It also has a role in receptor-mediated apoptosis of tumour cells, particularly when caspases are inhibited. [4] A possible role of CatB in Alzheimer's disease (AD) has also been cumulatively highlighted by genetic knockout studies, chemical inhibition and RNA silencing studies in cellular and animal models. These studies confirmed that CatB inhibition improved memory deficit in AD, thus showcasing how CatB may represent a therapeutic target for the treatment of AD. [7]
Cathepsin C (CatC) – also known as DDP1 (dipeptidyl peptidase I) I – is a lysosomal cysteine protease key for the activation of granule serine peptidases in inflammatory cells, elastase and cathepsin G in neutrophils cells and chymase and tryptase in mast cells. In this way, CatC participates to inflammation and immune regulation process and it is involved in various inflammatory diseases including rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, asthma, sepsis, and cystic fibrosis, where it is responsible for tissue damage and chronic inflammation. For these reasons it is considered as an attractive target for treating the abovementioned disease. [8,9]
CatC inhibition has been widely studied - with different strategies including both peptide or nonpeptide structures - but many molecules failed as effective drugs for the off-target effects, toxicity or poor pharmacokinetics. Recently, a few non-covalent CatC inhibitors with anti-inflammatory activity have been developed, opening the route to the possible development of non-peptidyl non-covalent compounds as highly effective CatC inhibitors [8]
In 2020 the CatC inhibitor *brensocatib* has been approved for the treatment of adult non-cystic fibrosis bronchiectasis (NCFBE). Recently, the role of CatC in tumor metastasis has been investigated and the

potentiality of its inhibition as a possible therapeutic strategy both for primary cancer and metastasis has been patented. [10]

Cathepsin F (CatF) is a widely expressed lysosomal cysteine protease that has been identified as a cancer marker in cervical, gastric, lung and brain tumours and it is also considered to have a role in the apoptosis pathways. Recently, it has been proposed as a possible marker for skin aging. [11,12]

Cathepsin H (CatH) is extensively expressed in the lung, pancreas, thymus, kidney, liver, skin, and brain. CatH is active in lysosomes, where it is involved in the proteolytic functions for protein turn-over in cell metabolism. CatH is also active at neural pH levels in the extra-lysosomal and extracellular space. CatH dysregulation plays a critical role in a number of diseases, including atherosclerosis, myocardial infarction, type 1 diabetes, neuroinflammation, brain atrophy, severe pulmonary disorders, cancer and high myopia. In the past years some researches focused on the possibility to inhibit CatH as a possible therapeutic strategy. [5]

Cathepsin K (CatK) is a cysteine protease with endoproteolytic and collagenolytic activities, highly expressed by osteoclasts and activated macrophages. Its main role is the degradation of type I collagen and elastin – the major component of the organic bone matrix – in bone resorption; changes in CatK levels are associated with bone and cartilage degradation disorders including pycnodysostosis (associated with CatK deficiency), osteoporosis, and osteoarthritis (associated with CatK overexpression). CatK inhibition has been studied for osteoporosis treatment, to replace or complement traditional therapies. In postmenopausal women with osteoporosis, CatK inhibition limited bone resorption and improved bone mass and microarchitecture, preventing or decreasing bone resorption. [13,14] Some CatK inhibitors have advanced into clinical studies but the presence of CatK in other tissues than the bones/osteoclasts often caused side effects, for example increasing risk for cerebrovascular accidents. [13] On the other hand, some strategies focusing on the fine tuning of the delivery systems proved to ameliorate the bioavailability and reduce side effects. [15]

An increased secretion of CatK has been recently correlated also whit different disorders, including vascular inflammation, pneumonitis, tuberculosis, skin and CNS disorders as well as tumour progression. For this reason, its specific inhibition represents a possible diagnostic biomarker and also a therapeutic target. Good results were obtained for inflammatory arthritis and COVID-19, [16] as well as for metastatic breast cancer in animal models and in a preliminary clinical trial. [17]

Cathepsin L (CatL) is upregulated in a wide range of tumours and its inhibition was investigated as a therapeutic strategy to prevent metastasis and cancer-associated osteolysis, without the induced drug resistance observed for some currently used drugs. A proper development of intracellular CatL inhibition as a therapeutic approach was limited by the scarce specificity of inhibitors – due to the structural homology between cathepsins – that may cause side effects. Targeting extracellular CatL, specific for cancer cells, could provide a therapeutic strategy without undesired effects and some molecules have been tested in preclinical models with good results towards tumour progression and cancer-associated bone resorption. [4,18] With the outbreak of the Covid-19 pandemic, some selective inhibitors of CatL have also been investigated for their potential of blocking SARS-CoV-2 infection in humans.[19]

Cathepsin O is a polypeptide of 321 amino acids whose RNA is widely expressed in human tissues and it is believed to be involved in cellular protein degradation and turnover. [20]

Cathepsin S (CatS) is overexpressed in a variety of solid cancers, including follicular lymphoma, gastric, colon, brain, breast, and pancreatic cancer, where it promotes tumour invasion, angiogenesis and metastasis [21] and creates a favourable microenvironment [22]. Extracellular CatS overexpression could be exploited for targeting cytotoxic therapeutics to tumour cells, while CatS inhibition should be an alternative approach for cancer treatment.[23] Thus, targeting both extracellular and intracellular CatS with small molecule inhibitors could represent a possible approach for cancer treatment. Furthermore, CatS

overexpression in the tumour micro-environment (TME), could be exploited for targeted delivery of cytotoxic therapeutics to tumour cells while limiting off-target effects to healthy tissue. Non-invasive imaging and diagnosis using fluorescent activity-based probes targeted to CatS in the TME is another opportunity to be explored. [21] In this context, the use of nanocarriers that are functionalized for a directed drug delivery represents a convenient strategy. [22] In the past, CatS inhibitors have been tested in some clinical trials for treatment of different diseases, including coeliac disease, Sjogren's syndrome, systemic lupus erythematosus, psoriasis, rheumatoid arthritis, neuropathic pain and others but in many cases, there was no further progression in the trials and no additional data have been published. [21]

Cathepsin V (CatV) – also known as Cathepsin L2 – is mainly expressed in cornea, thymus, heart, brain, and skin. CatV is a lysosomal enzyme that acts as an endopeptidase and plays very different functions, including a role in immunity, in the turnover of elastin fibrils and degradation of diverse intra- and extra-cellular substrates. A possible dysregulation of CatV, whose mechanism remains to be clarified, might contribute to the onset and progression of tumours (including breast cancer, squamous cell carcinoma, colorectal cancer) and cardiovascular diseases (including atherosclerosis, aortic aneurysm and hypertension). CatV has been highlighted as a possible prognostic marker in several tumours and the use of both peptidic and non-peptidic inhibitors has also been investigated for regulating CatV activity as a possible therapeutic strategy. The strong structural similarity with cathepsin L, together with the lack of significant results in animal models, did not allow significant development in this field up to date. [24]

Cathepsin W has been recently studied for its role in immunity [25], virus infection [26] and tumour prognosis. [27]

Cathepsin X/Z is highly expressed in monocytes, T-lymphocytes, macrophages, and dendritic cells and it is thought to have roles in phagocytosis, maturation, proliferation, migration, adhesion, and signal transduction in immune cells. It is also expressed in cells of the gastrointestinal tract, non-hematopoietic bone marrow cells and brain cells. Together with CatL, CatX was suggested to have a role in some neurodegenerative diseases since both these cathepsins are involved in the degradation of the proteins causing these disorders. CatX expression is increased in the gastric mucosa infected by *Helicobacter pylory* and affected by gastric carcinoma, thus suggesting a role in chronic inflammation and tumours. [28]

1.2 Ubiquitin specific proteases

Protein ubiquitination is a pivotal post-translational modification performed by the Ubiquitin Proteasome System (UPS) involved in proteolysis, proteasomal degradation, chromatin condensation, gene expression, DNA replication and repair, cell-cycle regulation, viral infection, immune response, metabolism.[29]

Protein ubiquitination has been associated with a number of human diseases including viral infection,[30] neurodegenerative disorders [31] immune and inflammatory-diseases,[32,33] muscle and skeletal pathologies, and cancer.[34] In the past years some proteasome inhibitors approved for the treatment of lymphoma and multiple myeloma confirmed the UPS as a possible pharmacological cancer target, even though selectivity and toxicity aspects should be considered.

Deubiquitinating enzymes (DUBs) are specific proteases with the key role of removing ubiquitin or ubiquitin-like proteins from target proteins, thus preventing their subsequent degradation by the proteasome. [33,35]

Deubiquitination is finely regulated and, analogously to ubiquination, it has a role in the protein degradation, gene expression and regulation of the cell cycle, DNA repair and kinase activation. DUBs mutations have been studied for their relation with a number of diseases that range from cancer – where they are implicated in the stability of oncogenes and tumour suppressor – to neurological disorders. [34,35]

The human genome encodes nearly 100 putative DUBs belonging to five different families of which four are papain-like cysteine proteases.[35]

Among them, the Ubiquitin specific proteases (USPs) is the largest group with 58 described cysteine proteases members showing a number of conserved domains and similar catalytic sites. [35-37]

For the above-mentioned reason, some USPs (*vide infra*) have attracted growing interest in recent years and their inhibition has been studied as a possible novel therapeutic strategy.

USP7 is involved in multiple oncogenic pathways and its overexpression has been often reported in aggressive cancers. USP7 inhibition thus represents a possible therapeutic target for cancer treatment. [33,38] The inhibition of USP7 has been observed to restore the natural levels of the tumour suppressor p53 (by inhibiting its proteasomal degradation) thus resulting in anti-proliferative effects both *in vitro* and *in vivo.* [39-41] Anyway, the past identified USP7 inhibitors were weak, poorly soluble, scarcely selective and generally toxic; more potent, selective and safe USP7 inhibitors are therefore needed.

USP11 was studied for having a role in breast cancer, by interacting with the tumour suppressor gene BRCA2. A recent study highlighted that breast cancer patients with high-level USP11 expression showed higher recurrence and poorer survival. [42]

USP13 regulates the ubiquitination and degradation of parkin and alpha-synuclein, proteins playing a key role in the onset and progression of Alzheimer's disease (AD) and Parkinson's disease (PD).[43] USP13 was overexpressed in the brain of AD and PD patients while in AD and PD models USP13 knockdown ameliorated the clearance of neurotoxic proteins. According these results, the inhibition of USP13 could be a target for treating AD and PD.

USP15 is mainly expressed in endocrine tissues, gastrointestinal tract, liver, gallbladder, bone marrow, lymphoid tissues and it is upregulated in a variety of cancer cells including glioblastoma, multiple myeloma and leukemia, breast and ovarian cancer, prostate cancer, gastric cancer, pancreatic ductal adenocarcinoma. Thus, in recent times it was pointed out as s possible therapeutic target for the above-mentioned and other diseases including osteoarthritis, wound healing, Parkinson's disease and other neurodegenerative disease and human papillomavirus. [44]

USP19 has a role in apoptosis, autophagy, ERAD protein quality-control, antiviral immune response and it has been associated to cancer, neurodegeneration and degenerative diseases as well as to antiviral immune response.[45] USP19 inhibition was effective against muscle wasting in mice and USP19 silencing promoted myogenesis.[46] USP19 inhibitors could also be effective for the treatment of obesity and insulin resistance.

USP22 has been widely investigated for its roles in various conditions including different types of cancers, diabetic renal tubulointerstitialis fibrosis, cerebal ischemia, SARS-CoV-2 infection and antiviral defense.

A reduced or altered expression of USP22 has been associated with chromosomal instability and with metastatic cancers in different organs and tissues. [47]

USP28 has been highlighted to be a tumour-promoting factor, stabilizing different cancer-related proteins and modulating immune response. Its overexpression has been linked with poor prognosis in several cancers and it has been linked to several pathways, including the regulator of cell growth MYC, the histone demethylase protein LSD1 (having a role in cellular differentiation) and NOTCH signalling (that controls cellular differentiation and drives tumorigenesis in certain cancers). Generally, the inhibition of USP28 was investigated as a therapeutic target in cancers, autoimmune and inflammatory diseases, infections, and other disorders. Recent studies also reported that, in some tumours, USP28 might play an oncostatic role. [48]

USP32 was recently identified as a possible therapeutic target for breast cancer after that alterations and high transcript levels were observed both in breast cancer cell lines and in primary breast tumours. [42]

1.3 Main protease and papain-like protease of coronaviruses

In SARS-CoV-2 infection the inhibition of viral cysteine proteases (PL^{pro} and M^{pro}) – playing a pivotal role at the first stages of viral infection and replication – has been explored for the treatment of the disease.[49-51]

M^{pro} (main protease) and PL^{pro} (papain-like protease) are two highly conserved viral cysteine proteases of Coronavirus that are both crucial for viral replication and infection. In particular, M^{pro} (also known as $3CL^{pro}$ - 3C like protease) is a homodimer with a catalytic dyad at the active site in each monomer. It plays a key role in viral genome replication, transcription, and other processes, vital for the continued survival of the virus. [50,52,53] PL^{pro} active site consists in a catalytic triad and its main role is processing the viral polypeptide into functional proteins. [54] M^{pro} was largely investigated in SARS-CoV-1 and MERS and a number of effective inhibitors – both peptidomimetic and non-peptidomimetic compounds – have already been reported against SARS-CoV-1 and MERS. Thus, a number of effective SARS-CoV-2 M^{pro} inhibitors were designed using peptidomimetics from SARS-CoV1. [50] PL^{pro} inhibitors were less investigated than M^{pro} inhibitors and they were only more recently reported.

The past research on inhibitors in SARS-CoV-1 and MERS way a key point for studying new molecules active against SARS-CoV-2 and in particular small molecule inhibitors, peptidomimetics and some non-peptidomimetic molecules. [50] Both PL^{pro} and M^{pro} are promising targets for the discovery and development of anti-coronavirus drugs, including the form of protease inhibitos even dual for both the viral proteases. [55]

The inhibition of cysteine proteases has been reported to impair protozoic infections - including *Trypanosoma cruzi, Plasmodium* and *Leishmania-*, where these enzymes play critical roles including pathogenicity, nutrient accession, enzyme activation, immune-evasion.[4,56]

The comprehension of the mechanism of action of cysteine protease inhibitors (*i.e.*, covalent inhibition, multiple noncovalent interactions) is a crucial point for the development of therapeutics.[2]

Cysteine proteases inhibition generally occurs through the reaction of the catalytic cycteine with electrophiles. In this context, reported inhibitors encompass Michael acceptors, thioketone, α-ketoamides, trans-α,β-unsaturated alkyl/benzyl esters, chloromethyl ketones, hydroxymethyl ketones, nitriles, disulfides, and dithiocarbamtes. For example, the main cysteine cathepsins inhibitors known up to date are low molecular weight epoxysuccinyl derivatives, vinyl sulfones or nitriles, capable to bind the active centre of the enzyme, or antibody-based inhibitors. The non-specificity and the ocurrence of side effects often limit the practical use of such inhibitors in therapeutic protocols. Reversible proteases inhibitors have also gained interest but many of them failed in clinical trials because of their poor bioavailability or toxicity.[57]

In this paper, recent scientific and patent literature focusing on the design and study of cysteine protease inhibitors with potential therapeutic application has been reviewed. Notably, recent researches mainly explored viral cysteine proteases, ubiquitin specific proteases, and cysteine cathepsins.

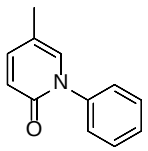
2. Cysteine proteases as drug target for the treatment of viral infections

Viral cysteine proteases have emerged as a versatile and attractive drug target for the treatment of viral infections. Additionally, besides viral enzymes, cysteine proteases present on the surface of the permissive host cells have also been demonstrated to be involved in the infection of human cells by virus. Cysteine

proteases are involved in the onset and development of several viral infections. Owing to the outbreak of the COVID-19 pandemic, most of the recently reported studies focused on the inhibition of SARS-CoV-2 cysteine proteases, namely Papain-Like protease (PL^{pro}) and Main Protease (M^{pro}).

2.1. Inhibition of SARS-CoV-2 PL^{pro}

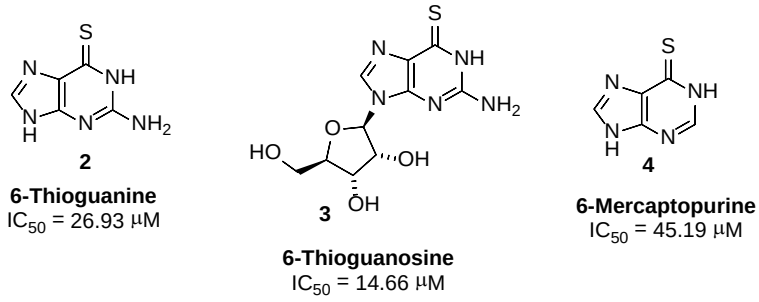
A broad variety of structurally diverse SARS-CoV-2 PL^{pro} inhibitors have been reported over the very recent years.[50,51] In 2020, pirfenidone **1** (Figure 1) – an approved drug for the treatment of idiopathic pulmonary fibrosis – was described in a patent as SARS-CoV-2 PL^{pro} inhibitor, with an IC_{50} of 5.88 ± 0.59 µM.[58] In 2022, the use of pirfenidone for the treatment of coronavirus infection was also described in a patent.[59]



1

Figure 1. Structure of pirfenidone.

In 2020, the use of 6-thioguanine and its analogues (Figure 2) as PL^{pro} inhibitors was patented. The authors described 6-thioguanosine as the most active compound within the studied series (IC_{50} = 14.66 µM). The IC_{50} value of the approved anticancer drug 6–thioguanine was found to be 26.93 µM. Notably, the structurally related guanine was showed to be inactive (IC_{50} > 100 µM).[60] In a subsequent study the PL^{pro} inhibition properties of 6-thioguanine were found to be significantly lower (IC_{50} = 72 ± 12 µM).[61] Furthermore, 6-thioguanine and 6-mercaptopurine were demonstrated to be inactive in a study where the PL^{pro} inhibition properties were determined through a FRET-based biochemical assay.[62]
The poor or moderate activity of these compounds against SARS-CoV PL^{pro}, coupled with their high cytotoxicity, renders these derivatives scarcely attractive candidates for the development of antiviral drugs.



2
6-Thioguanine
IC_{50} = 26.93 µM

3
6-Thioguanosine
IC_{50} = 14.66 µM

4
6-Mercaptopurine
IC_{50} = 45.19 µM

Figure 2. Structures of PL^{pro} inhibitors.

Other sulfur-containing organic molecules were patented for their SARS-CoV-2 PL^{pro} inhibition properties. For example, dimercaptosuccinic acid **5** (succimer, Figure 3) exhibited remarkable activity, with an IC_{50} value of 0.37 µM.[63]
The activity of the 2-imidazolyl disulfide-based compound **6** (**PX-12**) as SARS-CoV PL^{pro} inhibitor was also discussed in a patent (Figure 3; IC_{50} = 0.76 µM).[64] **6** is a thioredoxin and thioredoxin reductase inhibitor acting as cysteine thioalkylating agent and as tubuline polymerisation inhibitor,[65] which in 2006 entered

phase 2 clinical trials for the treatment of advanced pancreatic carcinoma but failed to reach the phase 3. Notably, **6** exhibited lower SARS-CoV PL^{pro} inhibitor properties when its activity was determined by a FRET-based enzymatic assay in a study peublished in 2020 (IC_{50} = 18.7 ± 2.6 μM).[66] Additionally, **6** was demonstrated to behave as a non-specific cysteine protease inhibitor and its activity against SARS-CoV M^{pro} was documented (IC_{50} = 0.9 ± 0.2 μM).[66]

Sulfur-containing small molecules with broad structural diversity have been reported to inhibit SARS-CoV-2 proteases. For example, tideglusib **7** – a glycogen synthase kinase 3 inhibitor drug candidate – was demonstrated to possess remarkable SARS-CoV PL^{pro} inhibitor activity (IC_{50} = 0.76 μM) in a patent filed in 2020 (Figure 3).[67] However, further studies based on a FRET enzymatic assay revealed a lower activity of tideglusib against SARS-CoV PL^{pro} (IC_{50} = 7.1-30.4 μM) and highlighted its non-specificity as cysteine protease inhibitor. Tideglusib was indeed demonstrated to behave as SARS-CoV M^{pro} inhibitor (IC_{50} = 2.1 ± 0.3 μM).[66] Tideglusib was found to be inactive in a successive cell-based SARS-CoV PL^{pro} inhibition study.[68]

The activity of thiamine **8** (vitamin B1, Figure 3) as inhibitor of PL^{pro} from SARS-CoV-2 (IC_{50} = 7.29 μM) was discussed in a 2022 patent.[69] The properties of the approved proton-pump inhibitor pantoprazole **9** (Figure 3) as SARS-CoV-2 PL^{pro} inhibitor were also reported in a 2022 patent (IC_{50} = 8.67 μM).[70] A patent discussing a method for the treatment of COVID-19 with pantoprazole was also filed by Curemark LLC in 2021.[71]

The researchers of the Immunologik GmbH reported a patent on the dithiocyanate **10** (**PR-619**; Figure 3), which behaved as SARS-CoV-2 PL^{pro} inhibitor at various concentrations.[72] A method of treatment patent was also filed in 2021 by Yale university.[73] The IC_{50} value of **10** against SARS-CoV-2 PL^{pro} was later published using a biochemical assay (IC_{50} = 6.1 ± 1.2 μM).[74]

Noticeably, according to a patent filed in 2021, isothiazolidinone derivatives **11a**,**b** (Figure 3) behaved as potent SARS-CoV-2 PL^{pro} inhibitors with IC_{50} values of 54 and 60 nM, respectively.[75]

Disulfiram **12** (Figure 3) – an acetaldehyde dehydrogenase inhibitor approved for the treatment of chronic alcoholism – was also described as SARS-CoV-2 PL^{pro} inhibitor in a patent filed in 2021.[76] Although no IC_{50} data were reported in the patent, the SARS-CoV-2 PL^{pro} inhibition activity was previously investigated in 2020 using a FRET-based enzymatic assay (IC_{50} ranging from 6.9 to >60 μM).[66] Disulfiram was also demonstrated to inhibit SARS-CoV-2 M^{pro} (IC_{50} = 2.1 ± 0.3 μM), thus highlighting its lack of specificity against cysteine proteases.[66]

In a patent filed by researchers of ShangaiTech University, a number of thiadiazole derivatives were described as inhibitors of PL^{pro} from SARS-CoV-2.[77] Selected examples are reported in the Figure 3. The IC_{50} value of the derivative **13a** – which was designed as a HCV helicase inhibitor in 1999 [78] and then demonstrated to behave as an allosteric modulator of G-protein coupled receptors [79-81] – was published in 2023 by the same authors (IC_{50} = 0.655 ± 0.063 μM). The inhibition properties of **13a** against M^{pro} from SARS-CoV-2 (IC_{50} = 0.185 ± 0.023 μM) were also demonstrated.[82]

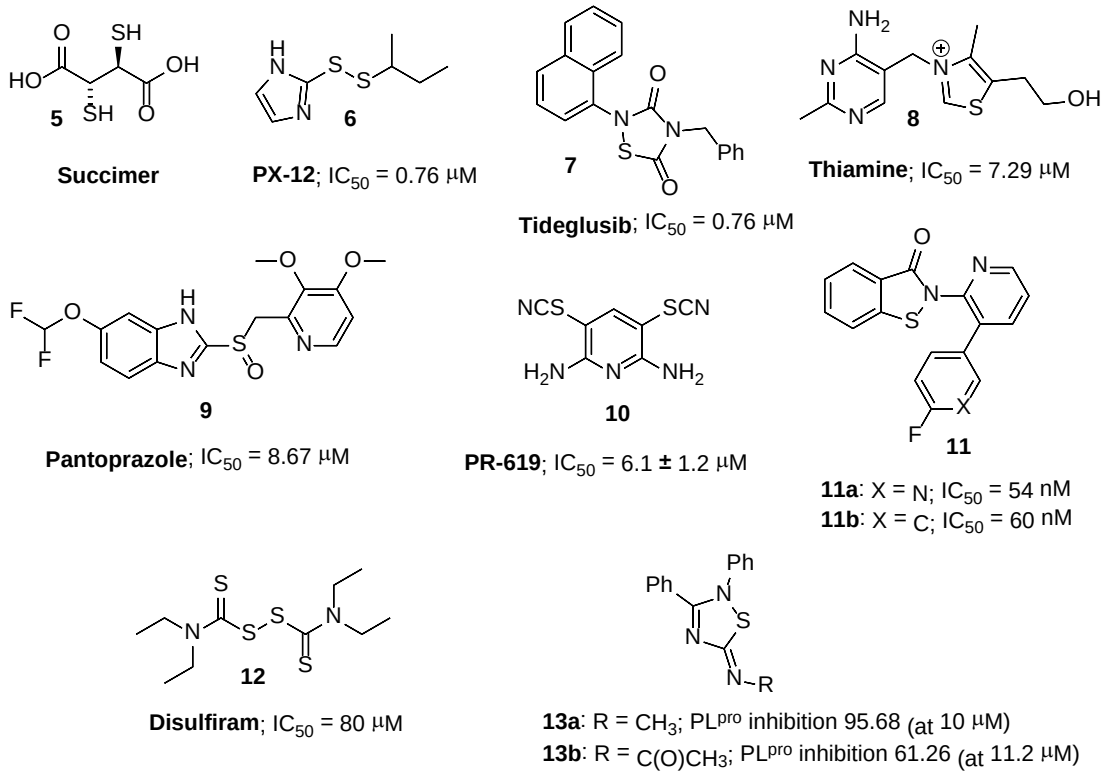


Figure 3. Sulfur-containing SARS-CoV-2 PL^{pro} inhibitors and related activities (selected examples).

Researchers from the Japanese University of Tohoku (Sendai) and Tokyo (Bio-Xcelerator) patented a drug containing an active sulfur compound as the principal component.[83] The inventors describe the action of polysulfides in inhibiting virus-derived proteases. Active sulfur compounds are $R^1S(S)_nR^2$, with R^1 and R^2 selected from amino acids. For example, glutathione trisulfide GSSSG (or polysulfide) is able to covalently bond with the thiol group of the active site of the two proteases PL^{pro} and M^{pro}, leading to their inhibition. The glutathione trisulfide is more efficient than the reduced form GSSSH as the electrophilicity of the central sulfur in GSSSG is stronger than in GSSSH. GSSSG is also able to react with nitric oxide (NO) and to enhance vasodilation and platelet aggregation, suppressing actions by NO.[83]

The role of selenium in COVID-19 infection has also been studied[84] and a number of organoselenium compounds have been investigated for their activity as SARS-CoV-2 cysteine protease inhibitors. Although research on selenium-containing drugs is still in its early stage, considerable evidence about the impact that the unique properties of selenium have on the pharmacological activity, toxicity and biotransformation pathways of organoselenium compounds has been provided. In some cases, the incorporation of selenium into small organic molecules is a rewarding strategy to modulate and increase their biological activity. [85-89] A variety of structurally diverse organoselenium compounds with chemopreventive and antioxidant activities have been described in the last four decades. The introduction of selenium into small molecules often brings about additional benefits, closely related to the modulation of the oxidative stress status of mammalian cells. [85-87] Selenium has been demonstrated to possess both protective and toxic effects on the nervous system and on the heart. Confusing effects of selenium have also been well documented in cancer biology and virus-associated diseases. [85-87]
For example, in this *scenario*, naphthyl imide-derived selenides **14a**,**b** exhibited interesting SARS-CoV-2 PL^{pro} inhibitor activity, with IC_{50} values of 1.93 and 4.09 μM (Figure 4).[90]

The inhibitor properties of diaryl diselenides **15** against SARS-CoV-2 PL^{pro} were reported in a patent filed in September 2020 by scientists of the Shandong University (Figure 4). The IC_{50} value of the 2-carboxy-substituted derivative **15b** (0.80 μM) proved to be lower than that of the related diphenyl diselenide **15a** (2.97 μM).[91]

Amongst selenium-containing compounds, ebselen **16** (Figure 4) was showed to be one of the most promising candidates to develop SARS-CoV-2 proteases inhibitor-based therapeutics. Indeed, the activity of ebselen against SARS-CoV-2 proteases – preliminary investigated in the 2020 at the beginning of the pandemic [49] – was also reported in a patent (IC_{50} against SARS-CoV-2 PL^{pro} 0.93 μM) [92] and published in a 2021 paper (IC_{50} values from 2.02 to 2.26 μM).[93] On the other hand, ebselen was later disclosed to be a non-specific protease inhibitor and its activity against *Mycobacterium tuberculosis* transpeptidase, glutamate dehydrogenase, hepatitis C virus helicase, glutathione S-transferases, and plant cysteine proteases was documented. A significantly lower SARS-CoV-2 PL^{pro} inhibitor activity of ebselen was reported in a successive FRET enzymatic assay-based study (IC_{50} 10.3 to >60 μM). Ebselen was also reported to inhibit SARS-CoV-2 M^{pro} (IC_{50} = 3.7 ± 2.4 μM).[66]

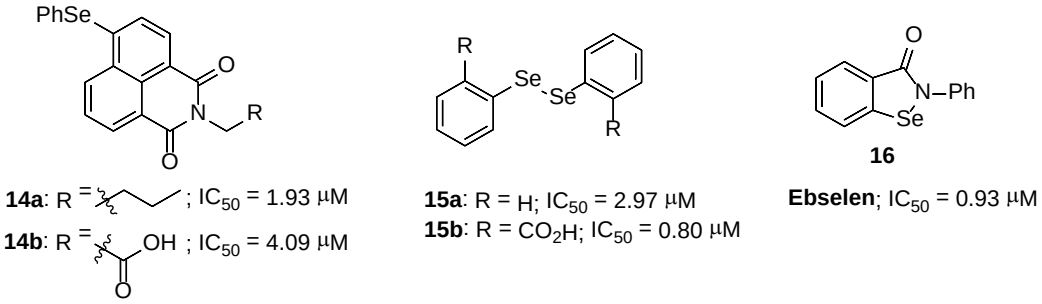


Figure 4. Structure of some selenium-containing PL^{pro} inhibitors.

The possibility to employ selenoneine (Figure 5), which likewise other organoselenium compounds behaves as inhibitor of SARS-CoV-2 proteases, for the prevention/treatment of COVID-19 infection has also been described in a patent filed at the beginning of 2022.[94]

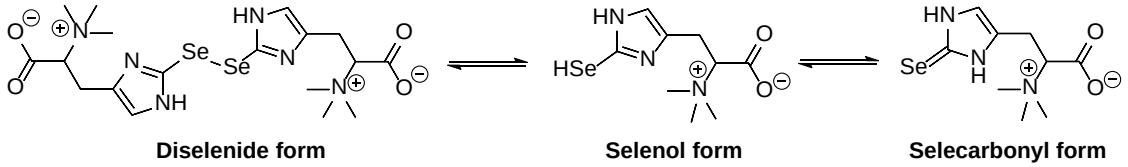


Figure 5. Equilibria between the selenol, diselenide, and selenocarbonyl forms of selenoneine.

Over the recent past years, a number of approved drugs have been investigated as SARS-CoV-2 protease inhibitors. Eflornithine **17** – an ornithine decarboxylase inhibitor used for the treatment of trypanosomiasis – was showed to inhibit SARS-CoV-2 PL^{pro} (IC_{50} = 0.63 μM, Figure 6).[95] The approved andidepressants trazodone **18** [96] and phenelzine **19** [97] were reported to have SARS-CoV-2 PL^{pro} inhibitor activity, with IC_{50} values of 3.15 and 4.56 μM, respectively (Figure 6). Vilanterol **20** – an inhalable β2 adrenergic receptor agonist employed for the treatment of chronic obstructive pulmonary disease – was also described for its activity as SARS-CoV-2 PL^{pro} inhibitor (IC_{50} = 4.05 μM, Figure 6).[98,99]

A patent filed in 2022 reported the remarkable activity of the nitrosourea derivative lomustine **21** – an approved nucleic acid alkylating agent for the treatment of brain tumours and Hodgkin's lymphoma – as SARS-CoV-2 PL^{pro} inhibitor (IC_{50} = 0.57 μM, Figure 6).[100] The main drawback of the possible use of this compound is related to its significant cytotoxicity against human cells.[101]

A patent filed in October 2021 reported the high SARS-CoV-2 PL^{pro} inhibitor activity (nM range) of two approved HCV serine protease inhibitors, namely simeprevir **22** and vaniprevir **23** (Figure 6).[102] The activity of these two compounds has been further investigated.[103-105] A cell-based study on SARS-CoV-2-infected Vero E6 cells highlighted an EC_{50} value of 15 and 51 μM for simeprevir and vaniprevir respectively.[104] Furthermore, simeprevir was showed to lack biological activity in a study published in 2022 and evaluating the effect of the drug on SARS-CoV-2-infected human angiotensin-converting enzyme 2 transgenic mice.[105]

Catechol derivatives have been described as SARS-CoV-2 cysteine protease inhibitors. The activity of norepinephrine **24** and carbidopa **25** – an aromatic L-aminoacid decarboxylase inhibitor registered for the treatment of Parkinson's disease – as inhibitors of SARS-CoV-2 PL^{pro} was described in a patent filed in 2020 (Figure 6).[106]

Tegaserod **26**, an approved drug for the treatment of irritable bowel syndrome (IBS) whose relationships with adverse cardiovascular events have been debated in the literature,[107] was also described as a SARS-CoV-2 PL^{pro} inhibitor (IC_{50} = 1.42 μM) in a patent filed on December 2020.[108]

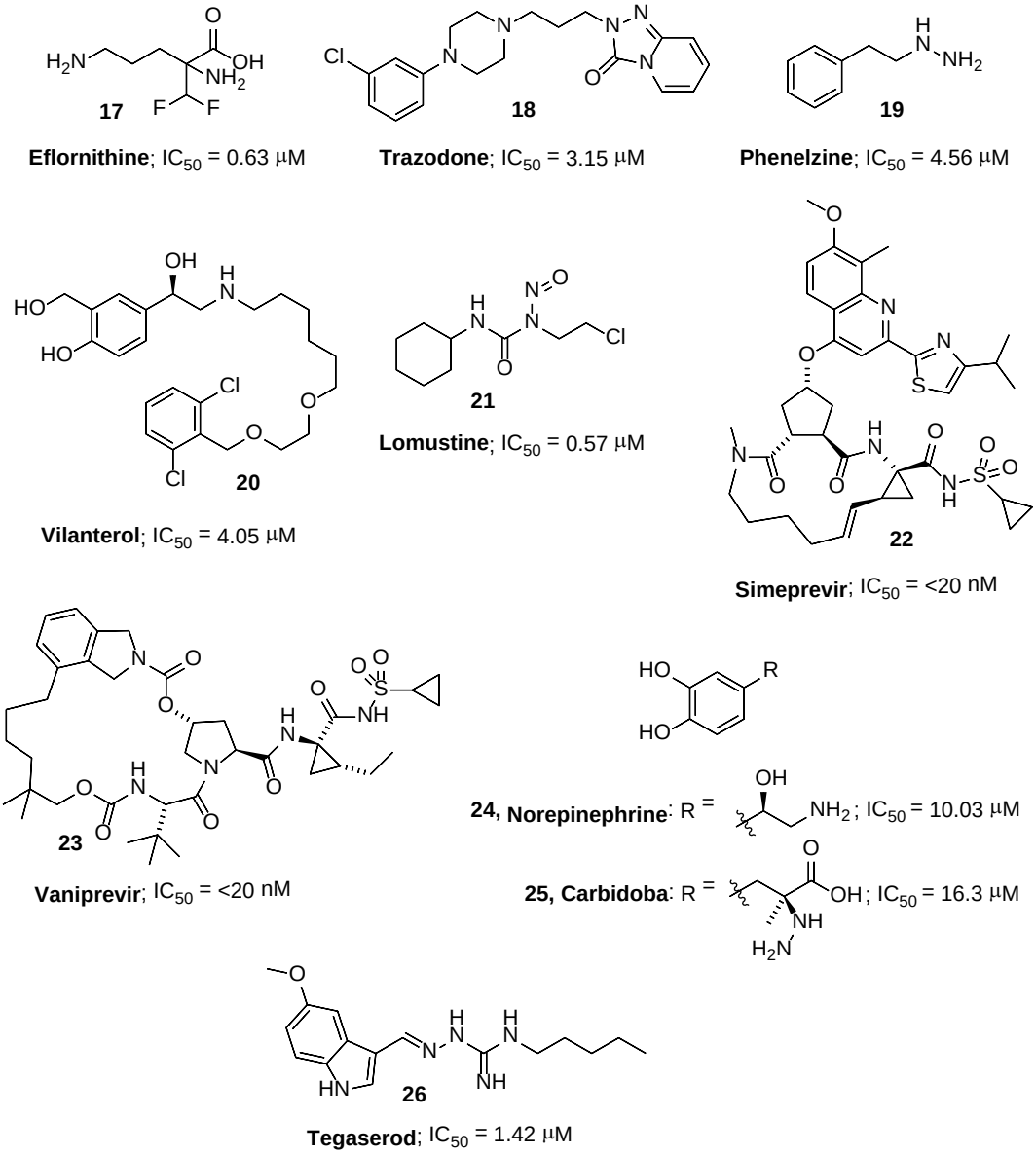
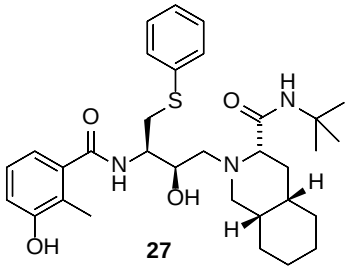


Figure 6. Some approved drugs reported as SARS-CoV-2 PL^{pro} inhibitors in recent patents.

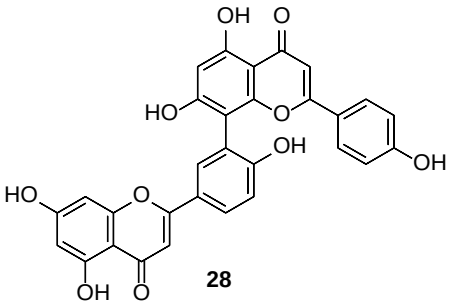
The possibility to employ Nelfinavir **27** (Figure 7) for the prevention and treatment of SARS-CoV-2-associated disease has been described. Nelfinavir is an inhibitor of HIV-1 and HIV-2 proteases which also behaves as SARS-CoV-2 M^{pro} inhibitor (IC_{50} = 3.06 μM). The administration of Nelfinavir mesylate to rhesus monkeys led to a considerable reduction of symptoms associated with COVID-19 infection and was found to improve the pathological tissue damage of major organs throughout the body.[109]



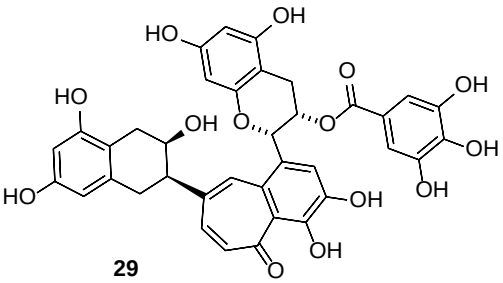
Nelfinavir

Figure 7. Structure of Nelfinavir.

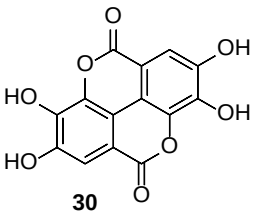
A number of natural polyphenols have also been reported to behave as SARS-CoV-2 cysteine protease inhibitors. Amongst these compounds, amentoflavone **28** exhibited the higher activity against SARS-CoV-2 PL^{pro} (IC_{50} = 11 μM, Figure 8).[110] Theaflavin-3-gallate **29** and ellagic acid **30** (Figure 8) were also shown to possess inhibitory properties against SARS-CoV-2 PL^{pro} (IC_{50} values 23.5 and 42.3 μM, respectively).[110]



Amentoflavone; IC_{50} = 11.0 μM



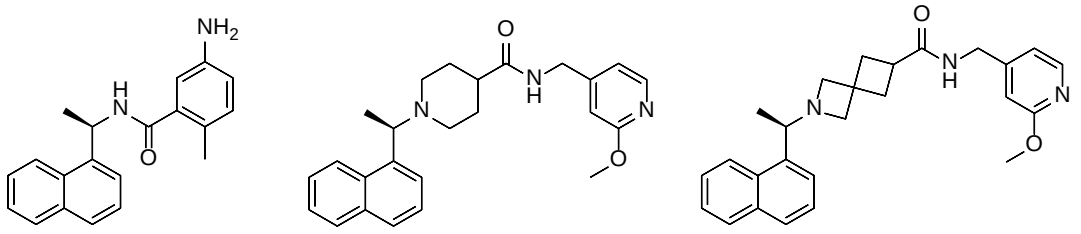
Theaflavin-3-gallate; IC_{50} = 23.5 μM



Ellagic acid; IC_{50} = 42.3 μM

Figure 8. Poliphenols as SARS-CoV-2 PL^{pro} inhibitors described in recent patents.

The SARS-CoV-2 PL^{pro} inhibitor activity of a series of naphthalene ethylamine derivatives **31** was described in 2021 by researchers of the University of Georgia; selected examples of such compounds are depicted in Figure 9 along with the related IC_{50} values.[111] The molecule **31b** was previously described as a SARS-CoV PL^{pro} inhibitor and its activity was also highlighted through a cell-based study.[112]



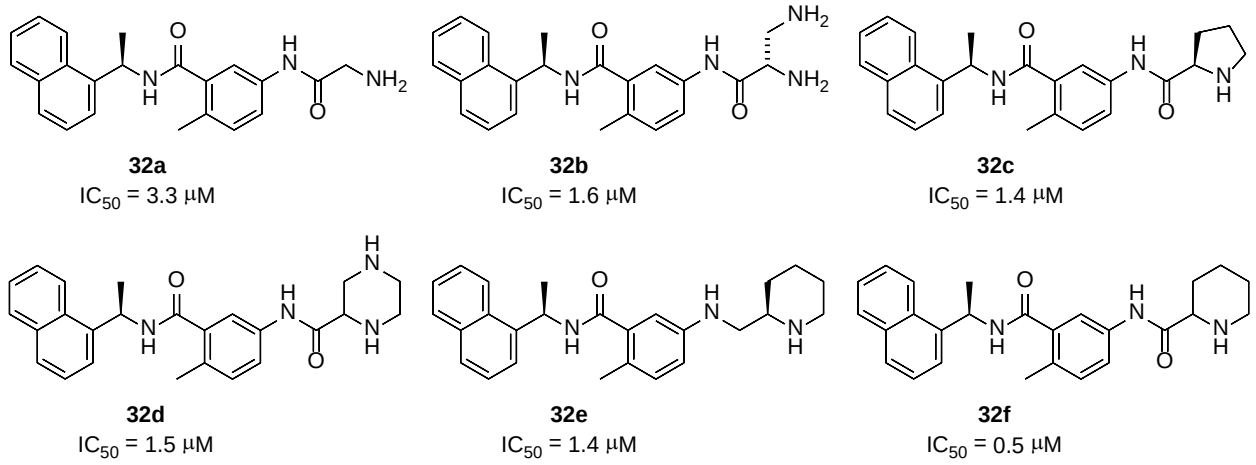
31a
IC_{50} = 24 ± 0.2 μM

31b
IC_{50} = 1.1 ± 0.1 μM

31c
IC_{50} = 5.4 ± 0.2 μM

Figure 9. Naphthalene ethylamine derivatives as PL^{pro} inhibitors. Selected examples from [111].

In this context, a patent describing the activity of a series of naphthalene ethylbenzamide derivatives was filed in 2021.[113] Selected examples are reported in the Figure 10. The compound **32f**, bearing the piperidine ring, proved to be the most potent inhibitor within the studied series, with an IC_{50} value of 0.5 μM. Notably, in a cell-based study on SARS-CoV-2-infected Vero E6 cells the EC_{50} value of **32f** was found to be 1.0 μM.[113]



32a
IC_{50} = 3.3 μM

32b
IC_{50} = 1.6 μM

32c
IC_{50} = 1.4 μM

32d
IC_{50} = 1.5 μM

32e
IC_{50} = 1.4 μM

32f
IC_{50} = 0.5 μM

Figure 10. Naphthalene ethylabenzamide derivatives as PL^{pro} inhibitors. Selected examples from [113].

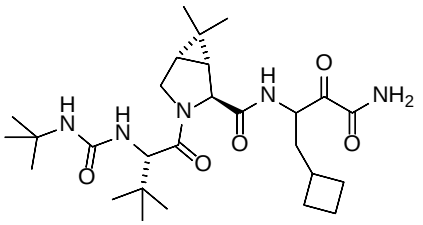
2.2. Inhibition of SARS-CoV-2 M^{pro}

As aforementioned, patents focusing on the development and the study of inhibitors of viral 3CL protease – also known as the Main Protease (M^{pro}) – have also been filed. SARS-CoV-2 M^{pro} plays a central role in viral replication and the design and/or investigation of potent and selective inhibitors of this cysteine protease represent an important step for antiviral drug discovery.[49,50]

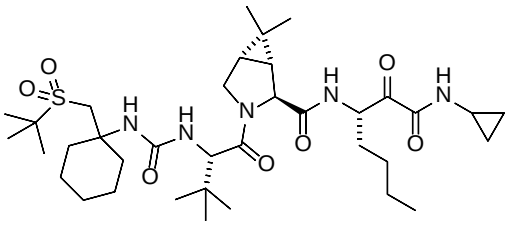
The activity of a series of small molecules with a formamido-oxoethyl-acetamide-related structure as inhibitors of SARS-CoV-2 M^{pro} has been described in a patent filed in 2021 by researchers of the University of Arizona.[114]

A library of protease inhibitors were screened by applying a FRET-based enzymatic assay for the SARS-CoV-2 M^{pro} in order to identify the most promising candidates and to investigate their mechanism of action. The results of the study enabled to highlight the high occurrence of formamide-oxoethyl-acetamide-related

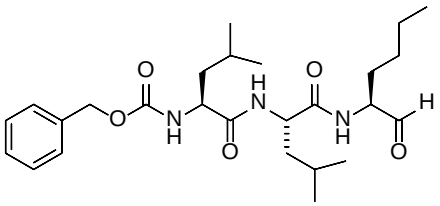
motifs within the most active compounds (Figure 11). Additionally, cathepsin/calpain inhibitors have generally demonstrated to exhibit interesting activity against SARS-CoV-2 M^{pro}, thus suggesting how cathepsin/calpain inhibitors could be considered as a source of potential SARS-CoV-2 M^{pro} inhibitors candidates.



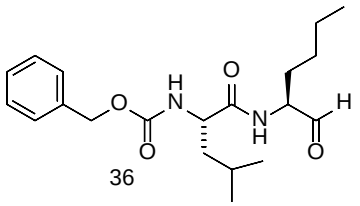
33, Boceprevir; IC_{50} = 4.13 ± 0.61 μM



34, Naraprevir; IC_{50} = 5.73 ± 0.67 μM

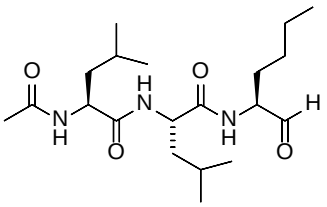


35; IC_{50} = 3.90 ± 1.01 μM

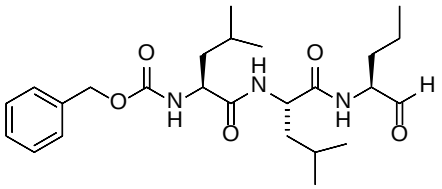


36

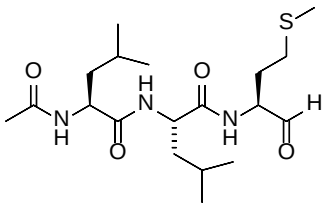
36, Calpeptin; IC_{50} = 10.69 ± 2.77 μM



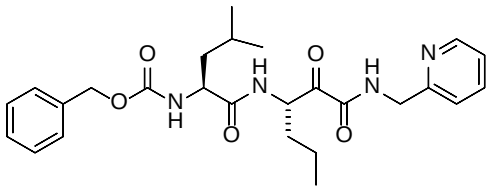
37; IC_{50} = 8.60 ± 1.46 μM



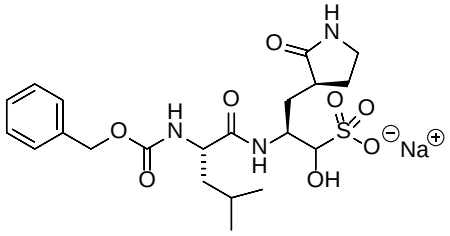
38; IC_{50} = 3.14 ± 0.97 μM



39; IC_{50} = 0.97 ± 0.27 μM



40; IC_{50} = 0.45 ± 0.06 μM



41; IC_{50} = 0.030 ± 0.008 μM

Figure 11. Some of the compounds screened as SARS-CoV-2 M^{pro} inhibitors in [114].

On the basis of the results of such a screening, a series of small molecules **42-49** characterised by a formamido-oxoethyl-acetamide-related structure was proposed as inhibitors of SARS-CoV-2 viral replication and/or SARS-CoV-2 M^{pro} inhibitors (Figure 12).[114]

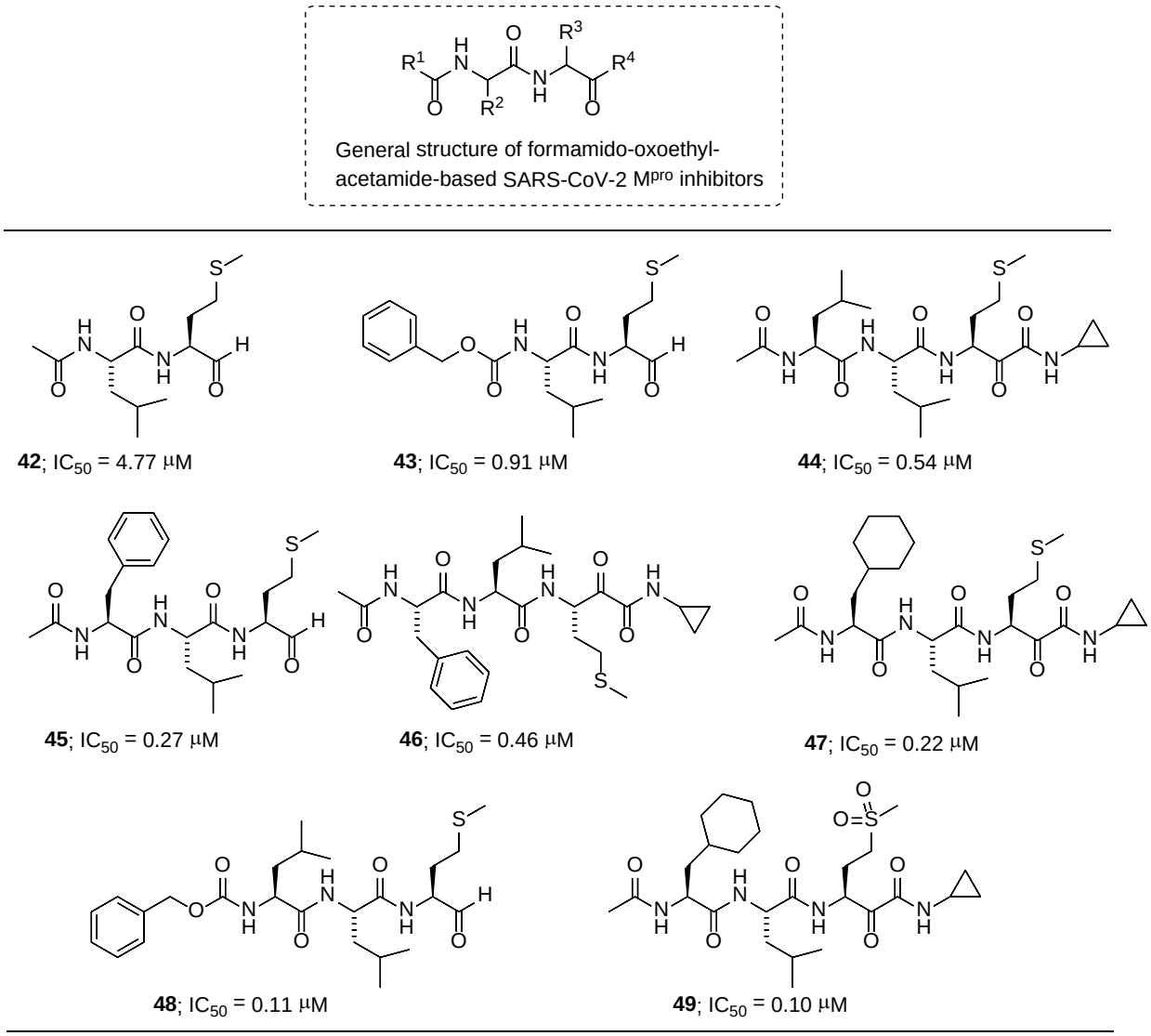


Figure 12. Selected formamido-oxoethyl-acetamide-based SARS-CoV-2 M^{pro} inhibitors developed in [114].

A method for the prevention and the treatment of SARS-CoV-2 infection was also described in a patent filed in 2021.[115] The invention is in the field of medicinal chemistry and deals with a new class of small molecules with a methyl-acetamido-propanamide structure of general formula **50**, capable to inhibit SARS-CoV-2 M^{pro} (Figure 13).

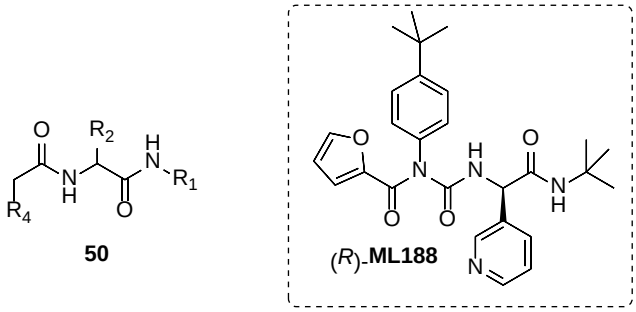


Figure 13. General structure of SARS-CoV-2 M^{pro} inhibitors from [115] and structure of compound **ML188**.

The design was based on the scaffold of **ML188**, a non-covalent inhibitor which contains a pyridyl in the P1 substitution; P2 and P4 substitutions in **ML188** were also considered. A number of compounds were synthesized and tested in the FRET-based enzymatic assay against SARS-CoV-2 M^{pro} at 20 μM. Compounds showing > 50% inhibition were then titrated to determine IC_{50} values. Molecules with IC_{50} < 5 μM were selected for cellular cytotoxicity profiling in Vero E6 cells. Additional experiments were carried out on novel cysteine reactive warheads.

Selected compounds with the highest activity are depicted in Figure 14. The most potent compound appeared **50a**, which can exist in two diastereoisomers. After HPLC separation thereof, the FRET-based enzymatic assay showed that the isomer (3*R*,23*R*)-**50a** has the highest antiviral activity, similar to **41**, while the (*23S*) isomer was inactive, with IC_{50} > 10 μM. The co-crystal structure of SARS-CoV-2 with (*23R*) showed that the P2 biphenyl and the P3/P4 α-methylbenzyl substitutions well fit into the S2 and the S3/S4 pockets. Some of the studied compounds can be considered amongst the most selective covalent M^{pro} inhibitors reported so far.

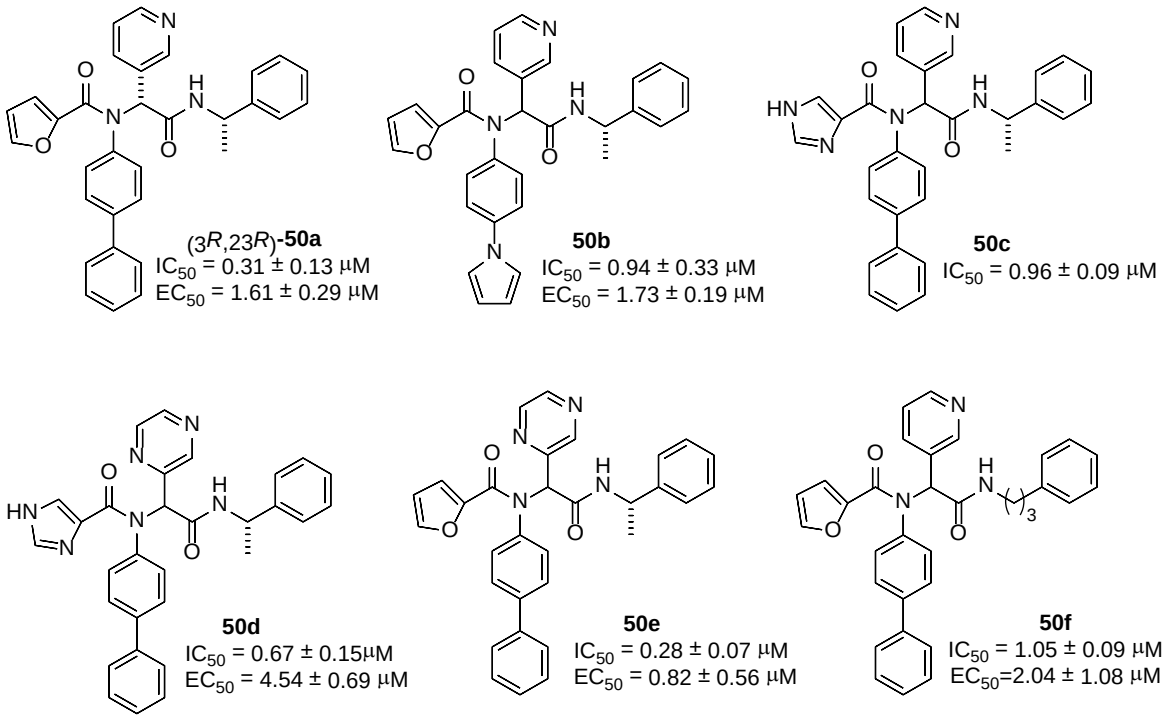
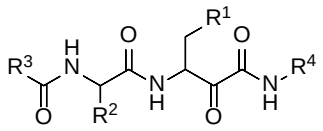


Figure 14. Selected examples of SARS-CoV-2 M^{pro} inhibitors from.[115]

Structurally-related compounds were described in an application filed in 2021 by the researchers of Anixa Biosciences and focusing on SARS-CoV-2 M^{pro} inhibitors and methods for their use in the treatment of diseases.[116] Inhibitors presented in this invention are small molecules with general formula **51** (Figure 15).

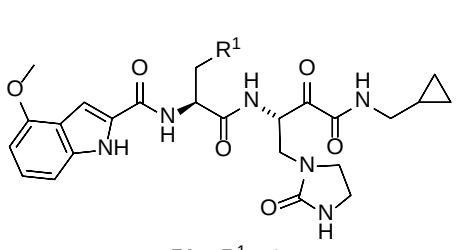


51

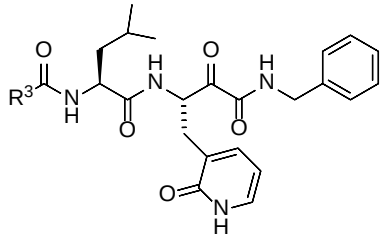
R^1: nitrogen-containing saturated and unsaturaed heterocycles
R^2: alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalky, aryl, heteroaryl
R^3, R^4: cycloalkyl, heterocycloalkyl, aryl, heteroaryl, where halogen, carbonyl group, amino group, $(SO)_2$ can be contained

Figure 15. General structure of SARS-CoV-2 M^{pro} inhibitors from [116]

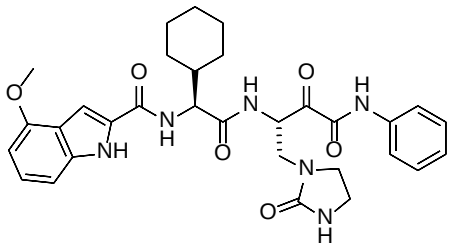
A number of variables is considered, with significant diversity of groups on the different positions of the general formula. Isotopically-labelled forms of some compounds have also been included, as for example ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{18}O, ^{32}P, ^{35}S. In some cases the use of heavy isotopes has shown positive therapeutic effects and a reduction in the required dosages. Selected compounds (Figure 16) were tested for their capacity to inhibit SARS-CoV-2 M^{pro} by using the FRET-based assay, and values of IC_{50} were determined, as depicted in Figure 16. All tested compounds resulted non-toxic for the human cells (IC_{50} > 100 μM).



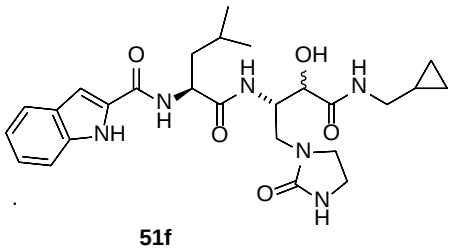
51a R^1 = *iso*-propyl
51b R^1 = cyclopropyl
IC_{50} < 2.0 μM



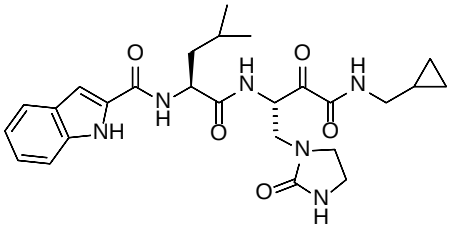
51c R^3 = 1*H*-indole
51d R^3 = 1*H*-benzo[*d*]imidazole
IC_{50} < 2.0 μM



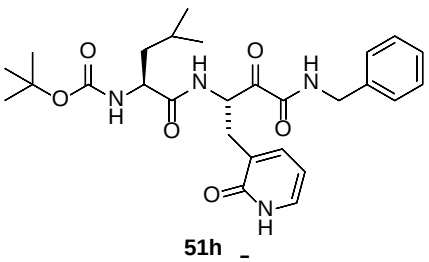
51e
IC_{50} < 2.0 μM



51f
IC_{50} < 2.0 μM



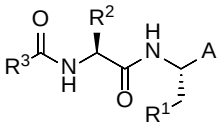
51g
2 μM < IC_{50} < 2.0 μM



51h
2 μM < IC_{50} < 2.0 μM

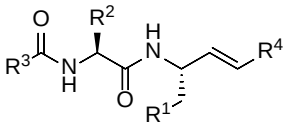
Figure 16. Selected examples of SARS-CoV-2 M^{pro} inhibitors from [116].

A series of compounds forming reversible adducts with active cysteine proteases, including SARS-CoV-2 3CL protease, were described in a patent filed in 2022.[117] General structures of these compounds **52** or **53**, known as masked aldehyde inhibitors (MAIs) and peptidomimetic vinyl heterocycles (PVHs), respectively are depicted in the Figure 17. Notably, PVHs also behave as potent, time-dependent inhibitors of cruzain; thus further optimization studies may also lead to efficient antitrypanosomal agents.



52

R^1 : substituted or unsubstituted aryl, heteroaryl or alkyl
R^2, R^3 : hydrogen, substituted or usubstituted alkyl, aryl, cycloalkyl, heteroaryl
A: aldehyde, ketone, ester, unsaturated sulfone, aryl or heteroaryl

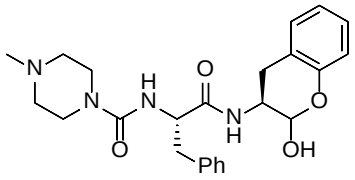


53

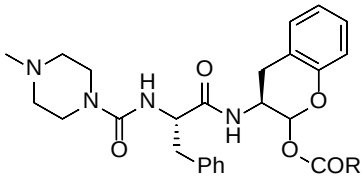
R^1 : substituted or unsubstituted aryl, heteroaryl or alkyl
R^2, R^3 : hydrogen, substituted or usubstituted alkyl, aryl, cycloalkyl, heteroaryl
R^4: aryl, heteroaryl, sulfone, amide

Figure 17. General structures of masked aldehyde inhibitors (MAIs) and peptidomimetic vinyl heterocycles (PVHs).

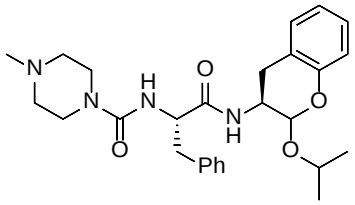
Examples of masked aldehyde inhibitors of SARS-CoV-2 3C, as well as of cruzain, cathepsins L and B are reported in the Figure 18. Compounds **54b-d** are pro-drugs of the parent masked aldehyde **54a**, and appear more active in blocking cellular infection by SARS-CoV-2 than **54a**. Compound **54e**, containing a mixed acetal, shows a lower activity.



54a



54b R = Me
54c R = Et
54d R = *i*-Pr

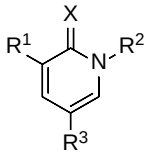


54e

Compound	Anti-CoV-2 EC_{50} (µM)	
	Vero E6 cells	A549/ACE cells
54a	7.5	n.d.
54b	2.5	0.31
54c	2.5	0.62
54d	4	0.31
54e	>20	5

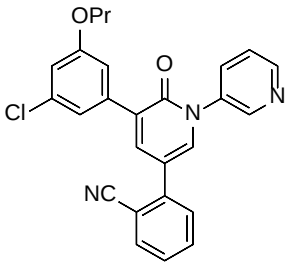
Figure 18. Masked aldehyde inhibitors from [117]. Selected examples and related activity.

A series of pyridine-2(*1H*)-one-derived structures **55** capable to act as non-covalent inhibitors of SARS-CoV-2 M^{pro} and to reduce or ameliorate symptoms associated with COVID-19 infections was described in a patent filed on January 2022.[118] General structure and selected examples of these compounds are reported in Figure 19.

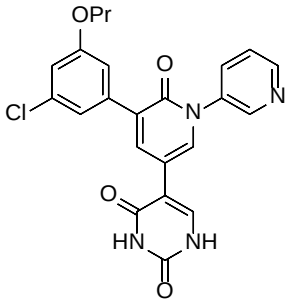


55

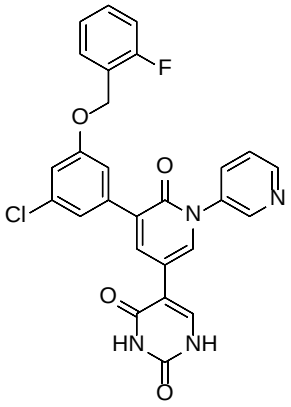
X = O, S, N-OR
R^1, R^2, R^3 = Variously substituted 5, 6, 7, 8-membered heterocyclyl substituent



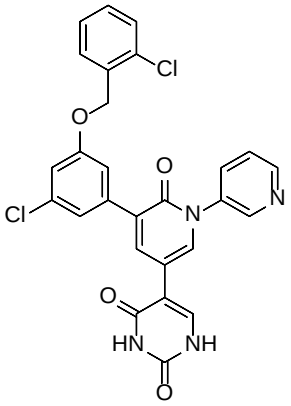
55a; IC_{50} = 0.14 ± 0.02 μM



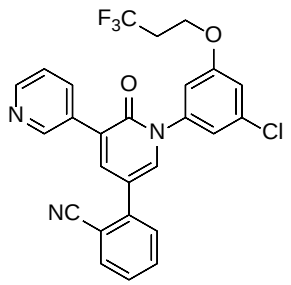
55b; IC_{50} = 0.12 ± 0.016 μM



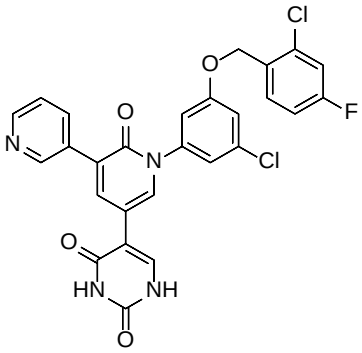
55c; IC_{50} = 0.024 μM



55d; IC_{50} = 0.018 μM



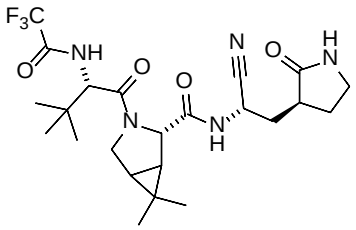
55e; IC_{50} = 0.120 μM



55f; IC_{50} = 0.036 ± 0.05 μM

Figure 19. General structure and selected examples of SARS-CoV-2 M^{pro} inhibitors from [118].

Several compounds bearing the nitrile moiety have been studied for the treatment of viral infections, including COVID-19. The compound **56**, a SARS-CoV-2 M^{pro} inhibitor bearing the cyano group (Figure 20), is used in combination with ritonavir in the novel orally available agent paxlovid, which has been approved by the FDA for the treatment of patients with moderate or severe COVID-19.



56
(Paxlovid)

Figure 20. Structure of paxlovid.

In this context, a patent filed in 2022 reported a series of differently functionalised nitriles **57**, characterised by variable molecular complexity, as inhibitors of SARS-CoV-2-M^{pro} (Figure 21).[119]

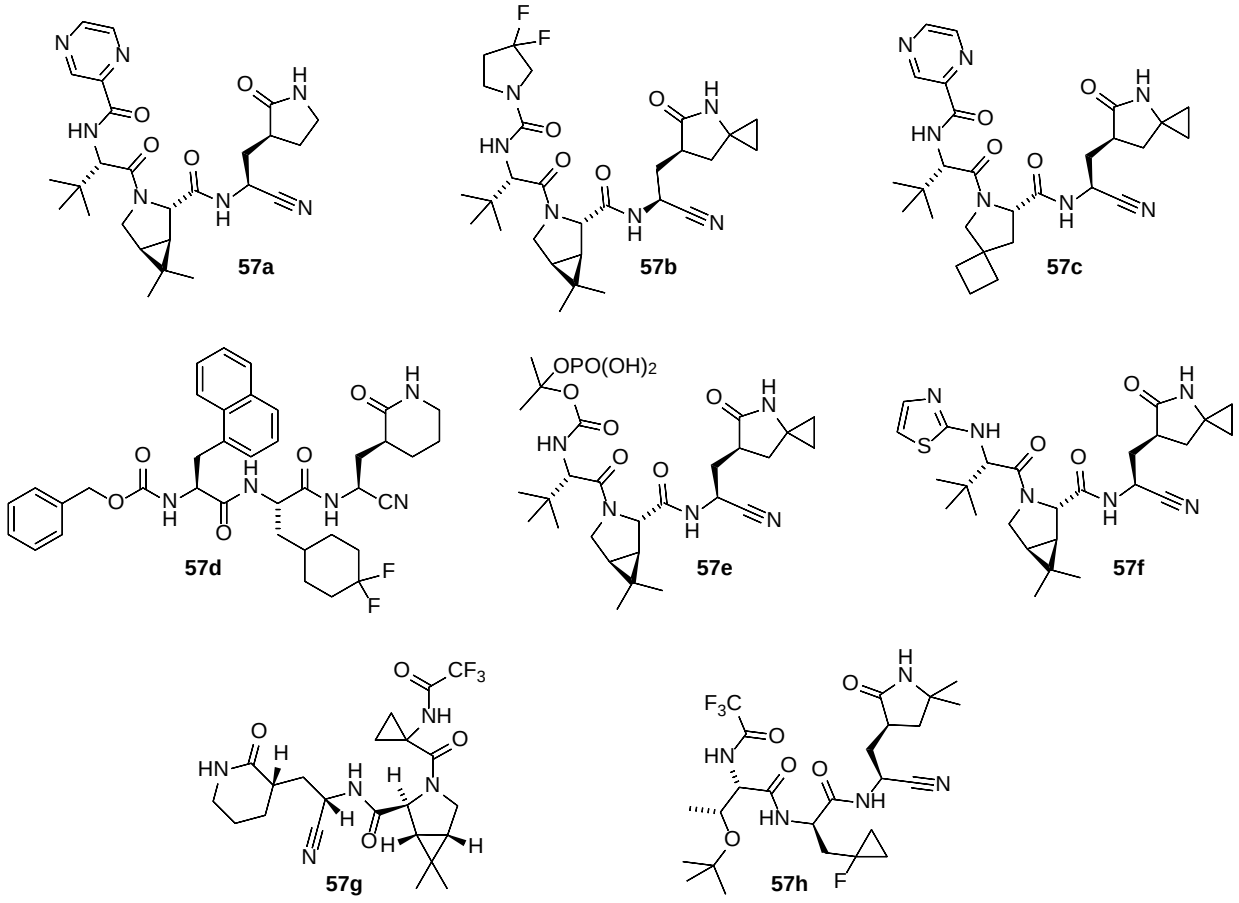
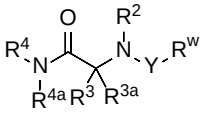


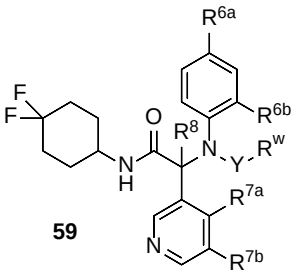
Figure 21. Structure of cysteine proteases inhibitors from [119]. Selected examples.

Similarly, a patent filed in 2022 provided a series of compounds – many of which bearing the cyano moiety – as inhibitors of different proteases and their use in treating viral infections. In Figure 22 are reported the general formula **58** and **59** of the studied compounds.[120]



58

Y: -CO, -SO, $^{-}SO_2$
R^w: cyclic and acyclic N-containing group
R^2: alkyl, cycloalkyl, aryl, heteroaryl
R^{3a}: hydrogen, deuterium, halogen, alkyl, haloalkyl
R^4: hydrogen, alkyl, cycloalkyl, aryl, heteroaryl, alkoxy, N-alkyl group

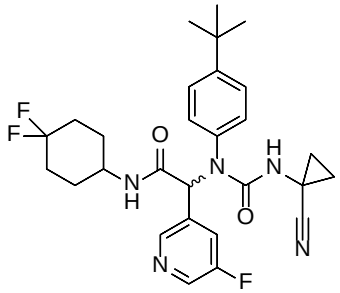


59

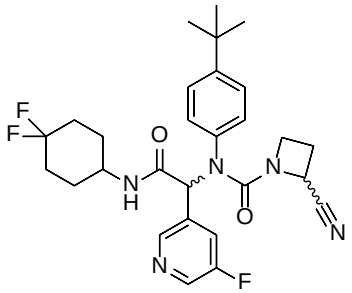
Y: -CO, -SO, $-SO_2$
R^w: cyclic and acyclic N-containing group
R^2: alkyl, cycloalkyl, aryl, heteroaryl
R^{6a},R^{6b}: hydrogen, halogen, cyano, alkyl, cycloalkyl, haloalkyl
R^{7a},R^{7b}: hydrogen, halogen, alkyl, haloalkyl
R^8: hydrogen, deuterium, halogen, alkyl, cycloalkyl

Figure 22. General structure of inhibitors described in [120].

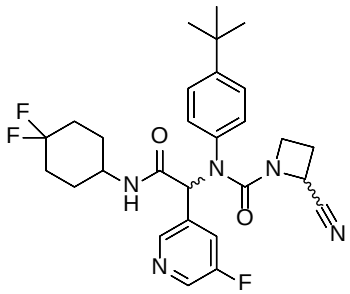
Selected compounds with the more significant activity (IC_{50} < 2 μM) against SARS-CoV-2 M^{pro} are described in Figure 23. The activity is depending also on the stereochemistry. For example, isomer 2 of compound **59b** is more active (IC_{50} < 2 μM) than the corresponding isomer 1 (IC_{50} > 30 μM).



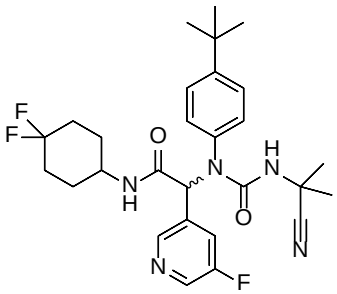
59a (isomer 1)
IC_{50} < 2 μM



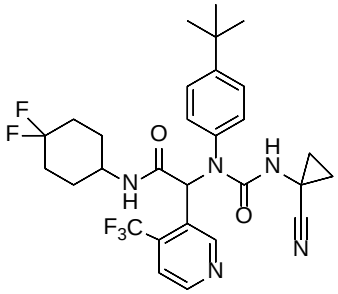
59b (isomer 2)
IC_{50} < 2 μM



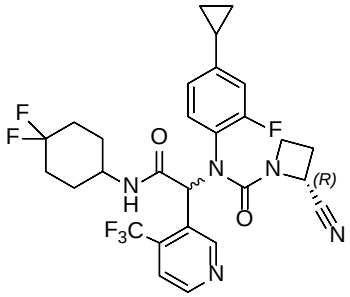
59b (isomer 1)
IC_{50} > 30 μM



59c (isomer 1)
IC_{50} < 2 μM



59d
IC_{50} < 2 μM



59e (isomer 1)
IC_{50} < 2 μM

Figure 23. Selected examples of inhibitors described in [120].

In a related field, a patent filed in 2022 described a new class of a variety of small molecules with a pyrrolidine-acetamide structure **60** (Figure 24) acting as inhibitors of SARS-CoV-2 PL^{pro} and MP^{pro}.[121] Previous screening found **41** and **33** (boceprevir, a drug used for treatment of chronic hepatitis C) as SARS-CoV-2 inhibitors (IC_{50} 0.03 μM and 4.13 μM). These results were independently validated also by

other groups.[122] Based on these findings, in the present application the hybrid inhibitors **60a** and **60b** as hybrids of **41** and telaprevir and of **41** and boceprevir, respectively, were designed (Figure 24).
The FRET-based enzymatic assay on these hybrids enabled to determine the enzymatic inhibition against the M^{pro} from seven human coronaviruses, including SARS-CoV-2; **41** was used as a control. Compounds **60a** and **60b** were found to be equally potent, with enzymatic inhibition comparable to **41** (Figure 11). Cell-based assays highlighted that **60a** was less potent than **41**, while **60b** had greater antiviral activity than **41**.

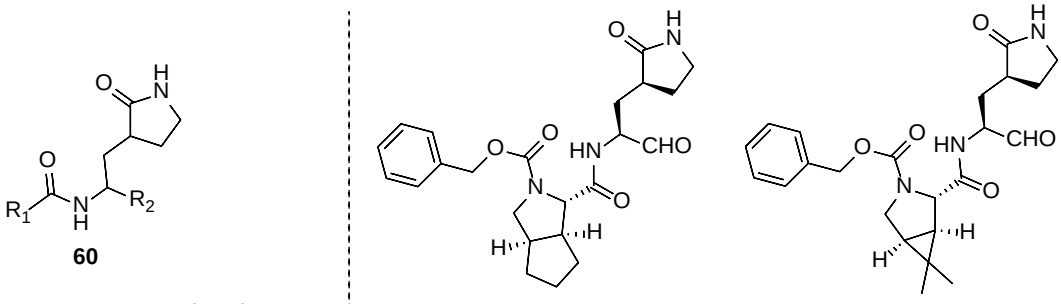


Figure 24. General structure and selected examples of pyrrolidine-acetamide inhibitors. IC_{50} values against SARS-CoV-2 M^{pro} are reported.[121]

The use of terpenes for the prevention or treatment of coronavirus infections was also reported in a patent filed in 2021.[123] Triterpenes were found to be effective in inhibition of coronavirus infections. Five triterpenes (Figure 25) were selected on the basis of FRET protease assays with the SARS-CoV-2 M^{pro}. All five compounds were significantly efficient inhibitors, and among these **61**, **62** and **63** showed the highest activity.

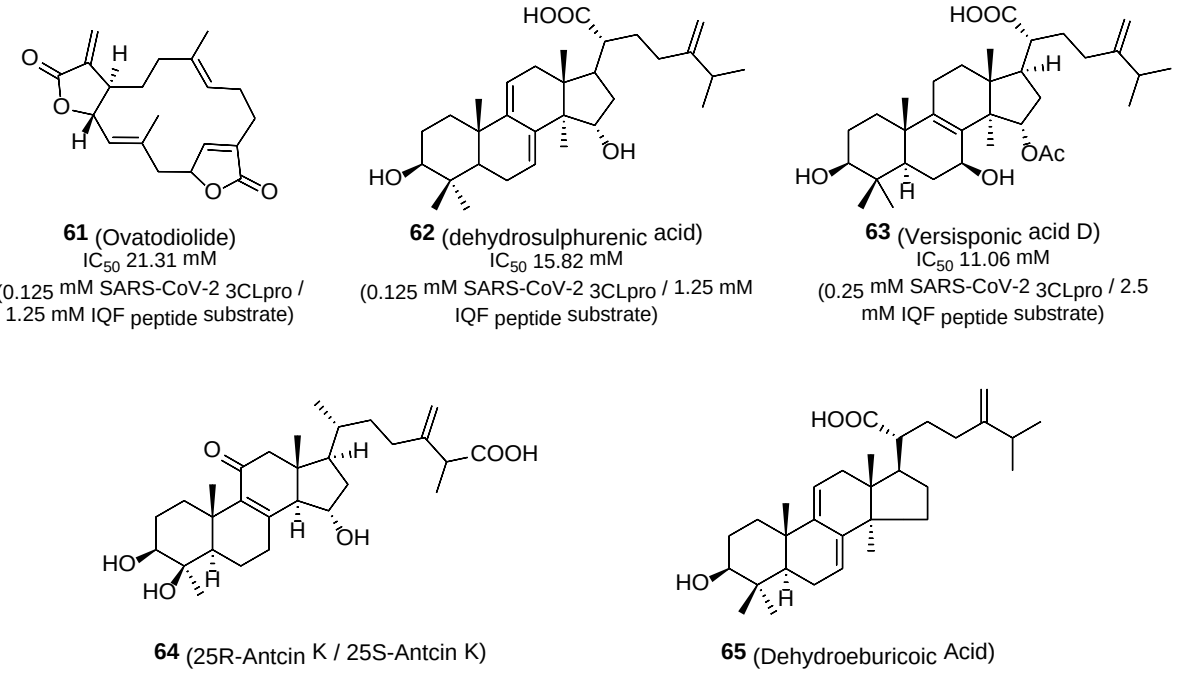
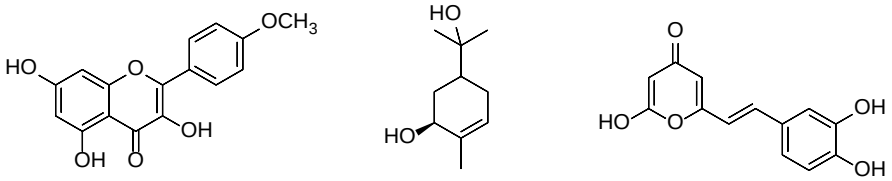


Figure 25. Structures of terpenes for the inhibition of SARS-CoV-2 M^{pro}.

A method for preventing and/or treating viral infections by inhibiting a cysteine protease in a virus and/or a sodium taurocholate cotransporting polypeptide in a cell has also been described in a patent filed in 2021 by the researchers of Arjil Biotech.[124] A group of compounds have been studied, and their inhibitory activity against SARS-CoV-2 M^{pro} was investigated. Selected structures, and their combinations, with the best activity are reported in Figure 26.



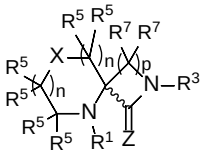
66 (3-O-methyl-kaempferol) **67** **68**

Figure 26. Structures of SARS-CoV-2 M^{pro} inhibitors **66**-**68**.

Table 1. Activity of SARS-CoV-2 M^{pro} inhibitor combinations. Selected data from [124].

Drugs	IC_{50} (μM)	Origin
62 + **67**	2.409	Combination
68	2.487	Sanghuangporus
61 + **63** (0.25p/0.6FP)	2.934	Combination
61 + **62** (0.25p/0.6FP)	3.065	Combination
61 + **66**	8.646	Combination

The use of spiro-lactam derivatives **69** (Figure 27) for the treatment of viral infections was described in a patent filed very recently by researchers of Aptinyx.[125]



X = O, NR^2
Z = O, S, NH

69

Figure 27. General structure of spiro-lactam derivatives **69**.

The results of activity against SARS-CoV-2-M^{pro} evidenced the best inhibition activity at 30 μM for compounds **70b** > **70c** > **70d** > **70a** (Figure 28); **70b** and **70c** showed IC_{50} values of 11.50 μM and 6.00 μM, respectively, with respect to **41** – used as a standard – with IC_{50} of 0.35 μM.

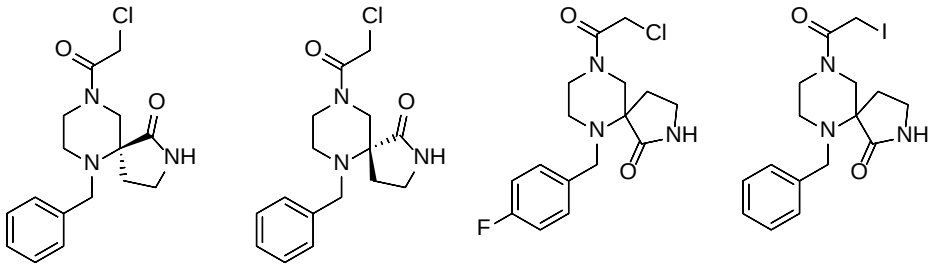


Figure 28. Structure of compounds **70a-d**.

2.3. Inhibition of host cysteine proteases for the treatment of viral infections

Pharmaceutical compositions of compounds with general formula **71**,**72** (Figure 29) were used to inhibit non-viral cysteine proteases (*i.e.*, human cathepsin L) in a patent filed on August 2021. Such a patent focuses on the treatment, prevention, amelioration, and reduction of the disease and/or symptoms associated with coronavirus infections.[126] The invention relies on the important role of cysteine proteases – both those of the host cells and those virally encoded – in the viral infection by SARS-CoV-2.

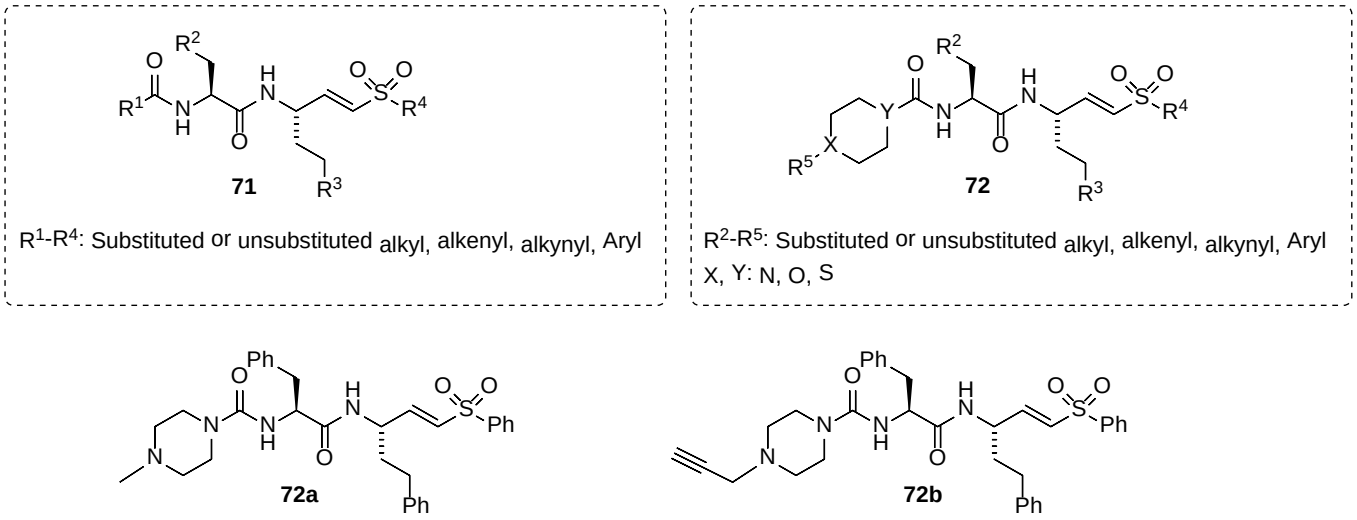


Figure 29. General structures and selected examples of cysteine proteases inhibitors reported in [126].
[a]Heteroatom-containing substituents are also included.

The use of nitroxoline **73a** (5-nitro-8-hydroxyquinoline) and its derivatives in the prevention and/or treatment of a disease caused by the virus SARS-CoV-2 has been reported in a patent filed in December 2021 (Figure 30).[127] As aforementioned, viral and host cysteine proteases plays a central role in the phase of the viral life cycle and in the disease caused by the infection from SARS-CoV-2. The role of certain cathepsins has been well investigated. Based on this, the authors of the invention found that 8-hydroxyquinoline-based cysteine protease inhibitors behave as effective agents for the prevention and/or treatment of the infection caused by SARS-CoV-2. Compounds **73a,b** – previously demonstrated to act as potent reversible cathepsin B inhibitors – exhibited the highest activity against SARS-CoV-2 in engineered

Huh7-hACE2 cells. The efficacy of these compounds is reasonably due to the activity against host and/or viral proteases involved in SARS-CoV-2 cell entry and replication. Nitroxoline **73a** is clinically used for the treatment of infections of the urinary tract and its antibiotic activity is explicated through a metal ion chelation mechanism. Thus, the synergism of metal chelation and cathepsin inhibition could lead to an increased activity of some 8-hydroxyquinoline-derived cysteine proteases inhibitors against SARS-CoV-2.

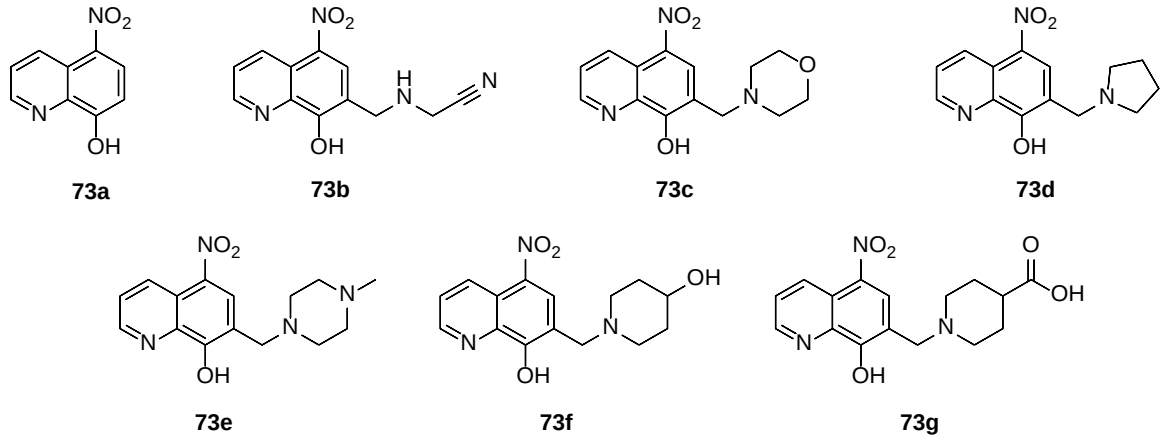


Figure 30. 5-Nitro-8-hydroxyquinoline-based cysteine protease inhibitors for use in the prevention and/or treatment of coronavirus disease. Selected examples from [127].

A method for the treatment or prophylaxis of viral diseases in animals was provided in a patent filed in March 2021.[128] The method relies on the administration of a suitable inhibitor of a cysteine protease of the cathepsin family. Relacatib **74** (Figure 31) is a potent orally available small molecule capable to effectively inhibit cathepsins K, L, and V (K_i = 41-68 pM); the selectivity of relacatib is reported to be 39-300-fold over other cathepsins. The method of treatment proposed in the patent foresees the use of relacatib for the treatment or prophylaxis of subjects with a viral disease caused by a virus including, amongst the others, poliovirus, rhinovirus, coxsackievirus, foot-and-mouth virus, influenza virus, hepatitis virus, coronavirus (SARS-CoV and SARS-CoV-2), Ebola virus, and Dengue fever virus.

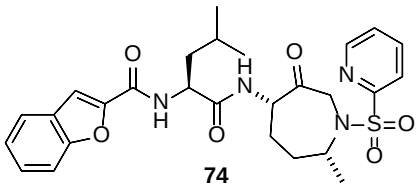


Figure 31. Structure of relacatib.

A patent dealing with the development of viral entry inhibitors and RNA polymerase inhibitors was filed in 2021.[129] Compounds discussed in the invention are also suitable for treating COVID-19 by blocking the activation of SARS-CoV-2 cysteine proteases and serine proteases. Compound **75** behaves as cysteine protease inhibitor; camostat is the serine protease inhibitor (Figure 32). EC_{50} of **75** was determined through *in vivo* assays to prevent entry of SARS-CoV-2 in various cell lines, evidencing its efficacy in preventing cellular entry. Significant results were found by the inventors using combinations of a viral

entry inhibitor and a nucleoside analogue, or a RNA polymerase inhibitor, such as Remdesivir and **76** (Figure 32).

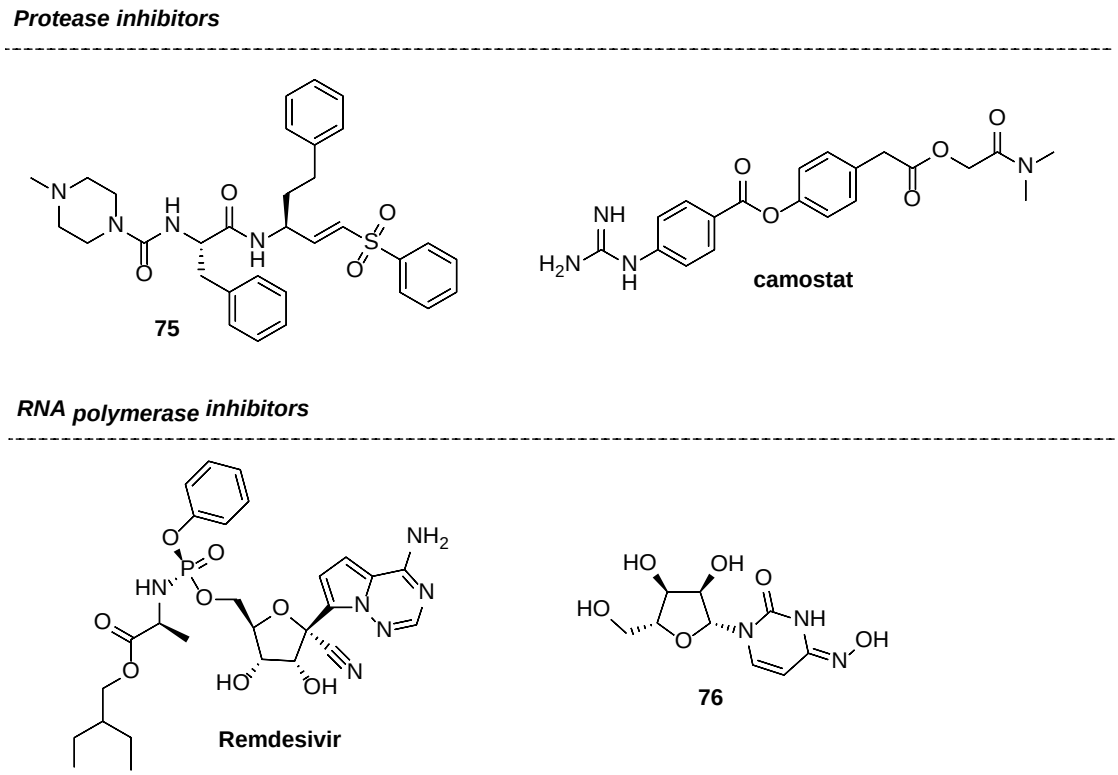


Figure 32. Structure protease inhibitors and RNA polymerase inhibitors.

3. Cysteine protease inhibition for the treatment of cancer and other diseases

As stated above, cysteine proteases (*e.g.*, USP7, USP13, cathepsins B, L, S) have a role in the onset and progression of several diseases, including, amongst the others, different types of cancer, neurogenerative disorders, osteoporosis. Thus, the inhibition of certain cysteine proteases represents an interest target in developing drug candidates for the treatment of the mentioned diseases and several studies have been reported.

With reference to cancer treatment, including both primary cancers and metastatic diseases, both cathepsins and USPs inhibitors have been investigated and some important results have been recently achieved.

3.1. Inhibition of Ubiquitin-Specific Peptidases

In a patent published in 2020, researchers of Almac Discovery Ltd claimed a series of 245 small molecule USP7 selective inhibitors as possible anticancer therapeutics or cancer preventive agents.[130] The general structure of the tested molecules is shown in the Figure 33. Such molecules can be employed – also in combination with other treatment agents – in the form of a pharmaceutically acceptable salt tautomer, stereoisomer or *N*-oxide derivative. The inhibitors of the invention were classified in three groups according to their IC_{50} values. Remarkably, 84 out of 245 compounds exhibited high activity against USP7 (IC_{50} <250 nM, selected examples of such inhibitors are depicted in the Figure 33).

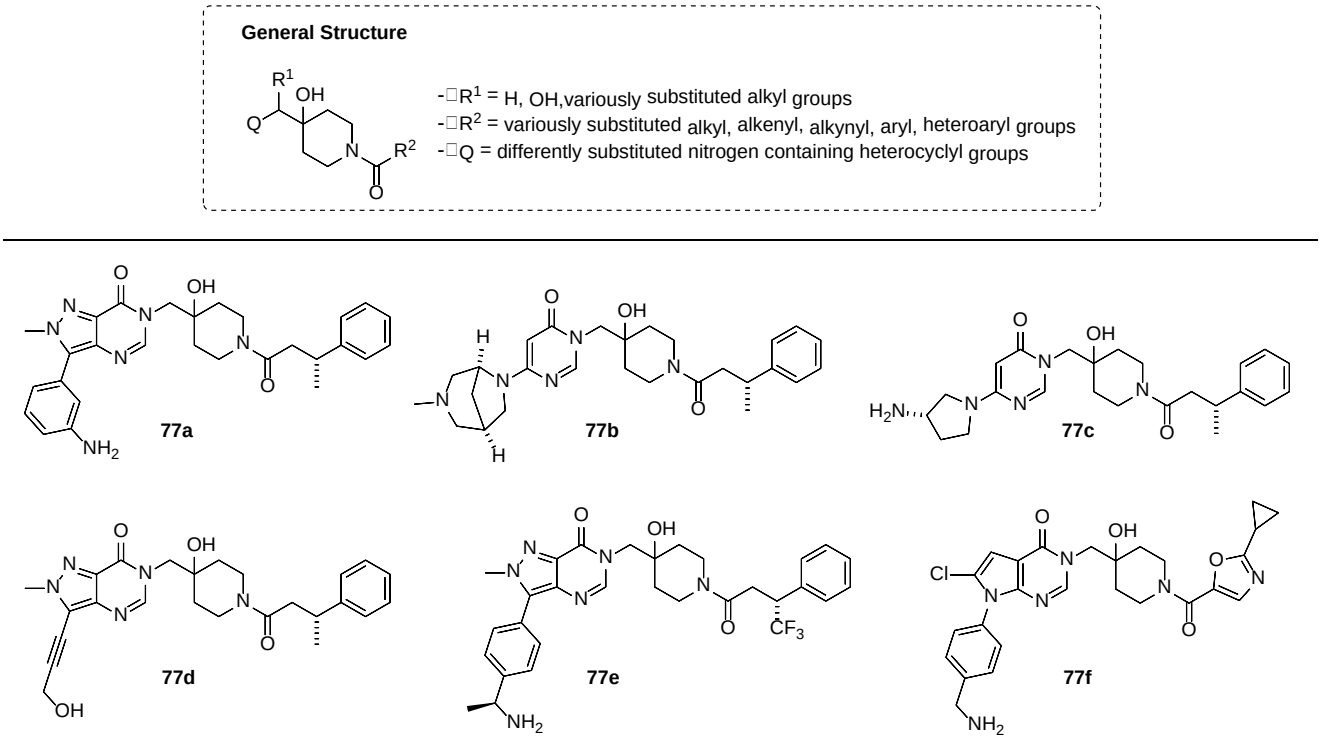


Figure 33. General structure and selected examples of the most effective USP7 inhibitors from [130].

A further experiment showed that the levels of p53, p21 and MDM2 decreased after treatment with the compound **77a** (Figure 33), when compared with vehicle (DMSO). Cell-based studies highlighted a dose-dependent p53 stabilisation, p21 induction and concomitant decrease in MDM2 levels after **77a** administration. Biochemical and cellular assays demonstrated the selectivity of the compounds of the invention for USP7 over other USPs, as well as broader classes of 38 deubiquitylating enzymes (DUBs). The high selectivity of the compounds of the invention is interesting as the overall homology between DUBs was expected to lead to cross-reactivity.

A patent filed in 2019 presented a method for treating cancer by inhibiting USP7 directly or also by modulating/inhibiting the USP7-related ETSP7, *via* the administration of a series of differently substituted and functionalised quinazolin-4(*3H*)-one-based small molecules **78** (Figure 34).[131] The patent discloses a method to inhibit USP7 that can be used as a novel treatment for a number of conditions including cancer and metastasis, neurodegenerative diseases, immunological and inflammatory disorders, cardiovascular diseases and viral or bacterial infections. The authors provided 63 examples of compounds and the activity was tested *in vitro*. Notably, thirteen compounds proved to behave as potent inhibitors (IC_{50} < 20 nM; selected examples of these derivatives are reported in the Figure 34).

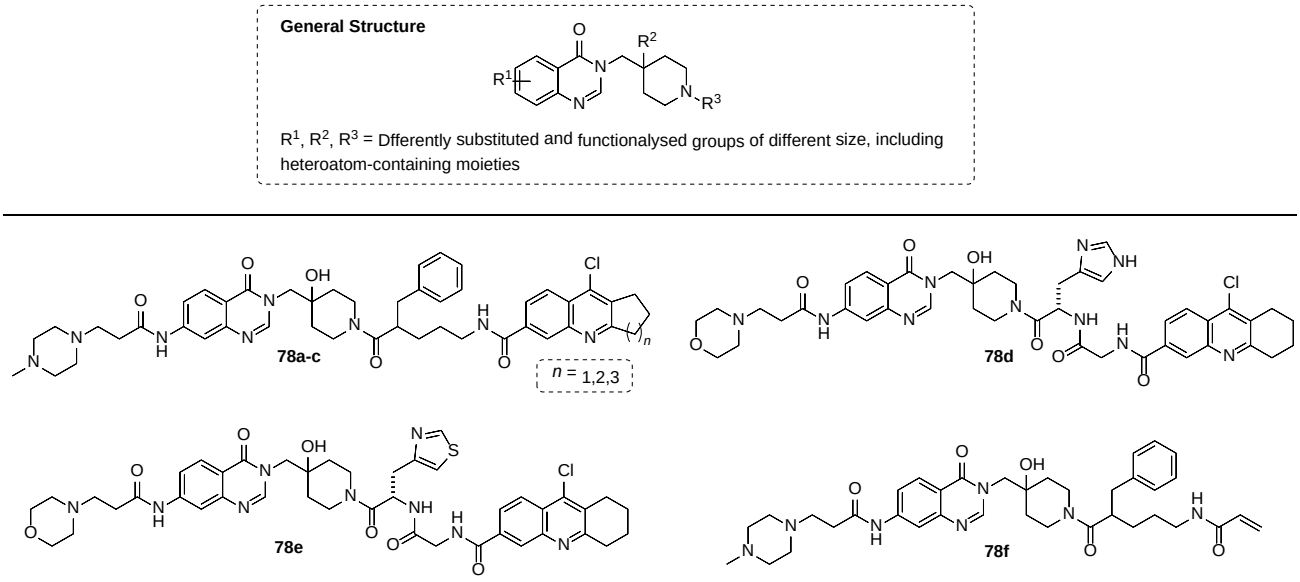


Figure 34. General structure and selected examples of the most effective USP7 inhibitors from [131].

In 2022 the researchers of the Johns Hopkins University filed a patent focusing on a series of cyclic peptides that are potent and highly specific inhibitors of deubiquitinating enzymes, including USP22.[132] The disclosed peptides are proposed for the treatment or recurrence prevention of diseases including metastatic cancer, by modulating histone H2B ubiquitination *via* the inhibition of the SAGA deubiquitinating module (DUBm), including USP22 and three scaffolding proteins. The disclosed cyclic peptides have been tested *in vitro* for their effectiveness and specificity against the Human DUB module with good results. Specificity tests against a panel of USPs showed that the inhibition for USP27 occurred at 25-fold higher inhibitor concentration while for USP51 occurred at a 2500-fold higher concentration.
The treatment with five over six inhibitors significantly increased H2B-ubiquitination in cells up to 12-15 folds. This also confirmed the ability of these compounds to cross the outer cell membrane and enter the nucleus. It is interesting to note that the effect in cells was not depending on the relative potency exhibited *in vitro*, since the weakest inhibitor tested, hD4, had a similar effect on enriching H2B-Ub in cells to the most potent inhibitor, hD1. This suggests a different capability of each molecule to penetrate the nucleus.
The researchers of Georgetown University in 2022 filed a patent on the use of USP13 inhibitors for the treatment or prevention of a series of USP13-related diseases including cancer, neurodegenerative and myodegenerative diseases and prion diseases in humans or veterinary patients. Different structures, including variously substituted heteroaromatic derivatives **79** as well as suitable amino acids *N*-functionalised with substituted 2*H*-chromen-2-one moieties **80** (Figure 35), were reported in the patent. Such compounds inhibit USP13 in cells, reducing alpha-synuclein levels both *in vitro* and *in vivo*.[133]
The IC_{50} determined on SHSY5Y neuroblastoma cell lines highlighted the potent activity of the studied molecules as UPS13 inhibitors (Figure 35). In animal models, **79c** reduced alpha-synuclein levels within the cell from 30% to 56% in a dose-dependent manner (10 mg/kg or 40 mg/kg of molecules injected respectively).

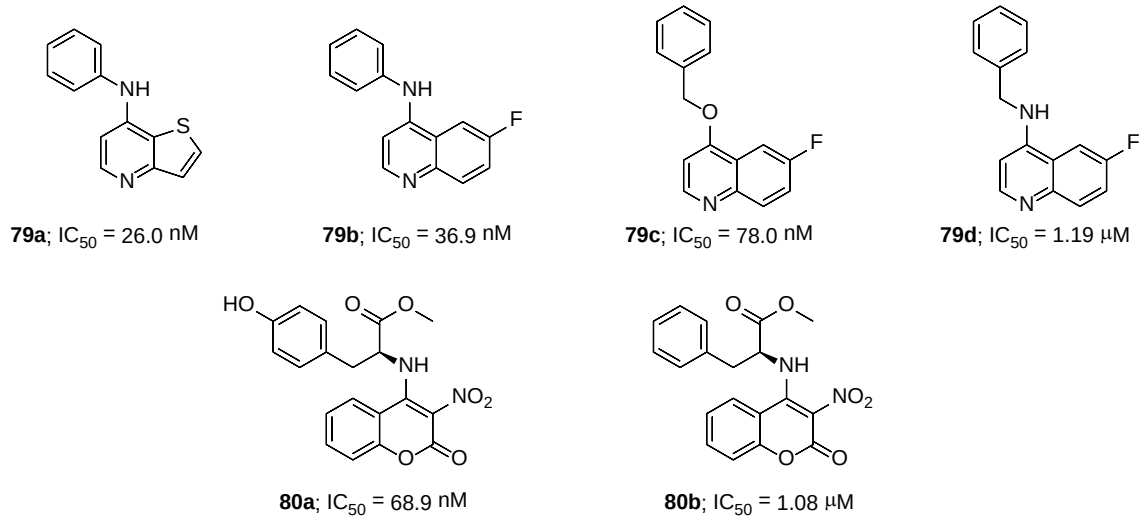


Figure 35. Structures of USP13 inhibitors from [133].

According the authors, the inhibition or reduction of activity of USP13 can be associated decreased mRNA and protein expression and/or to a decreased enzymatic activity of USP13. The viability tests showed that the reported molecules were safe and did no cause cell death up to 1mM concentration. Pharmacological parameters were evaluated in wild type mice (C57B6) injected with 10 mg/kg of deuterated **79c**. The mice tissues were analysed over 12 h after injection, highlighting a good potentiality for the tested molecules to cross the blood-brain barrier.

In a 2021 patent, the researchers from Dana-Farber Cancer Institute, claimed a compound to be used for the inhibition of USP28 – a ubiquitin-specific protease known for its capability to stabilise different oncoproteins – and the related treatment of USP28-associated diseases, particularly cancer.[134] The authors present 74 molecules having the general structure depicted in the Figure 36. The IC_{50} of 49 compounds was determined *via* a Ubiquitin-Rhodaminel 110 assay. All the synthesised molecules proved to be very effective and 37 compounds were found to have IC_{50} < 1 pM (selected examples **81a-j**are provided in the Figure 36); only one compound was found to have IC_{50} > 100 pM, whereas the IC_{50} value of remaining 11 synthesised derivatives was comprised between 1 and 100 pM.

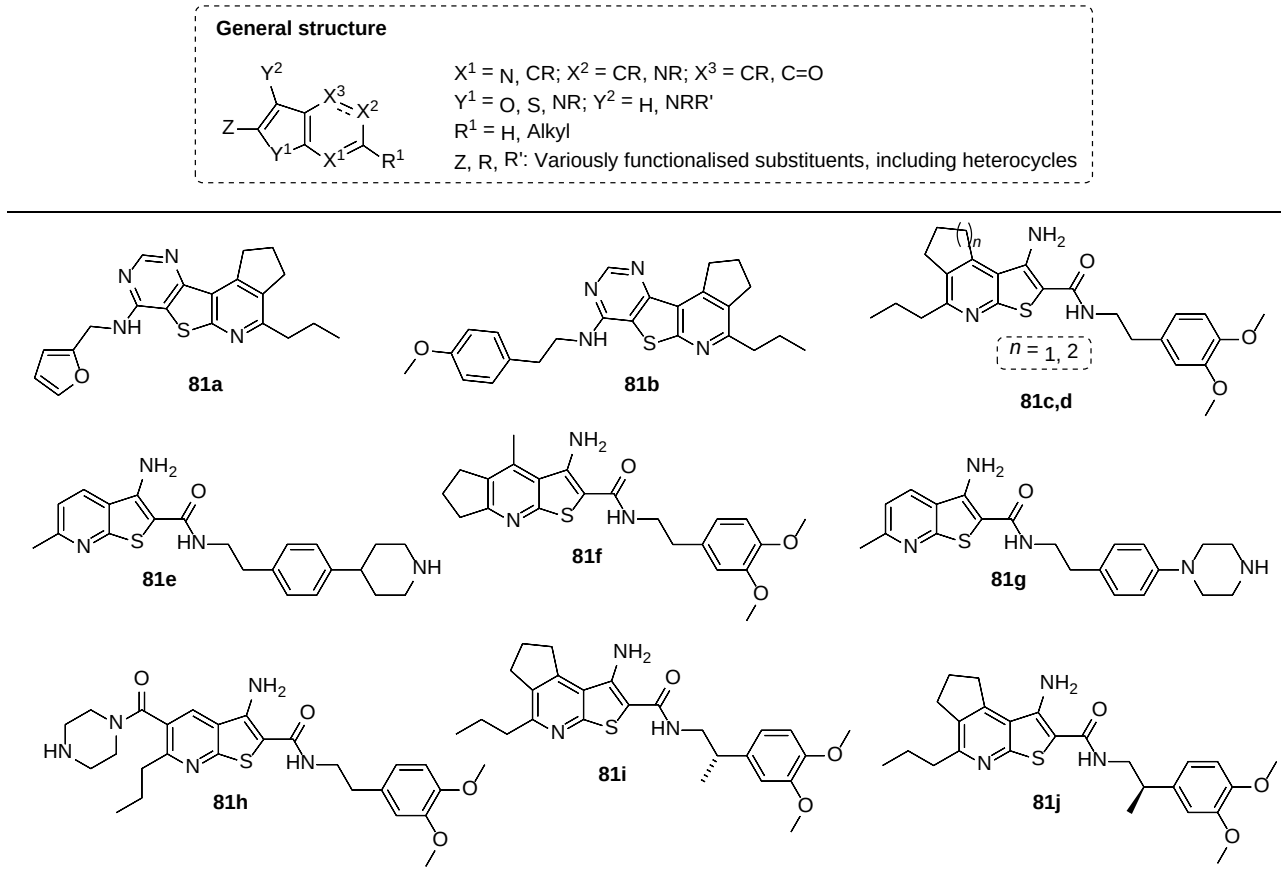


Figure 36. General structures and selected examples of small molecules as inhibitors of USP28 from [134].

The authors of a patent filed in 2022 report the use of USP19 inhibitors for the treatment of obesity, insulin resistance, type II diabetes, or muscular atrophy and reduce loss of muscle mass.[135] In the patent, 201 exemplificative compounds are discussed. The IC_{50} for the inhibitory activity was for the majority of them <0.01 µM or 0.01-0.1 µM. The general structural features of the compounds discussed in the patent, as well as selected examples of some of the most active derivatives (**82a-h**), are reported in the Figure 37. Further tests for IC_{50} were also performed on cells from a breast cancer cell line, a neuroblastoma cell line and a mouse skeletal muscle cell line. The cells were treated with a USP19 inhibitor showing a low nanomolar EC_{50} and good cell permeability. Further permeability assays were performed on human colorectal carcinoma Caco-2 cells confirming good performance. Notably, *in vivo* test on animal models also suggested that the compounds presented in this patent and a previously patented USP19 inhibitor **83**, namely ADC-141 (Figure 38), have a similar efficacy in diminishing the loss of muscle mass.

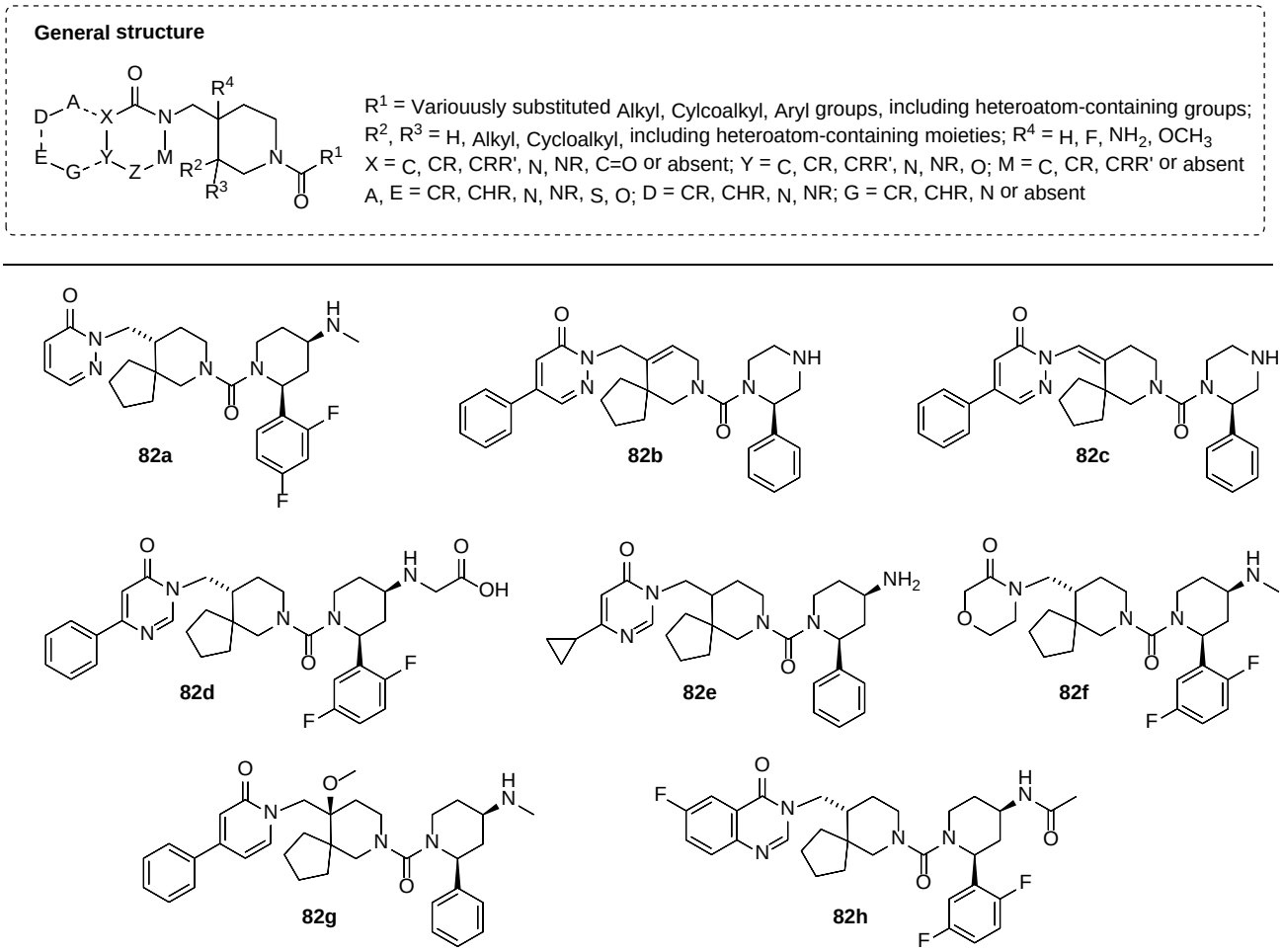


Figure 37. General structure and selected examples of USP19 inhibitors from [135].

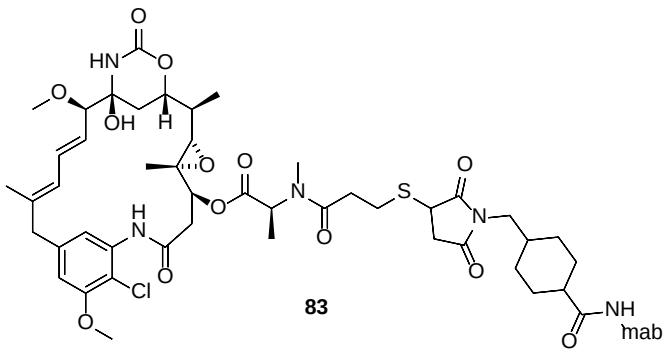


Figure 38. USP-19 inhibitor **83** (ADC-141).

3.2. Inhibition of Cathepsins

In a 2022 patent, the use of cathepsin C inhibitors **84-87** (Figure 39) for the treatment of primary cancer metastasis – including liver, lung and bone metastasis of breast cancer or bone metastasis of lung cancer – was claimed. Amongst a series of compounds, the administration of brensocatib (**88**, Figure 39) was described as particularly promising.[10]

The important link existing between cancer and CatC was highlighted via different tests. Initially, primary lesions and lung metastatic lesions from breast cancer patients were analysed by immunofluorescence

assay, showing that the enzyme is highly expressed in lung metastases. After that, serological levels of CatC were determined in breast cancer patients and correlation with traditional prognostic score was determined, indicating that CatC may be used as a marker gene to predict the risk of lung metastasis. In animal models, the overexpression of CatC corresponded to significantly more pulmonary metastatic lesions while CatC knockdown seemed to attenuate lung metastasis of breast cancer cells. CatC overexpression in lung metastases promoted and increased neutrophil recruitment and formation of Neutrophil extracellular traps (NETs), fibrous networks which protrude from the membranes of activated neutrophils found in a variety of disorder including tumours and autoimmune diseases. [136]

Brensocatib (**88**) – also known as AZD7986 or INS1007 – is a specific inhibitor of CatC already approved for the treatment of Non-Cystic Fibrosis Bronchiectasis that entered Phase II clinical trials. The administration of Brensocatib in animal models showed good results against the formation of pulmonary nodule since treated mice exhibited significantly reduced metastatic lesions and less neutrophil infiltration and NETs when compared with untreated mice.

In an animal model-based study, administration of brensocatib to mice previously spleen injected with liver and pancreatic tumour cells relevantly inhibited the primary growth of liver cancer cells HUH7 in liver and the metastatic growth of pancreatic cancer cells KP4 in liver.

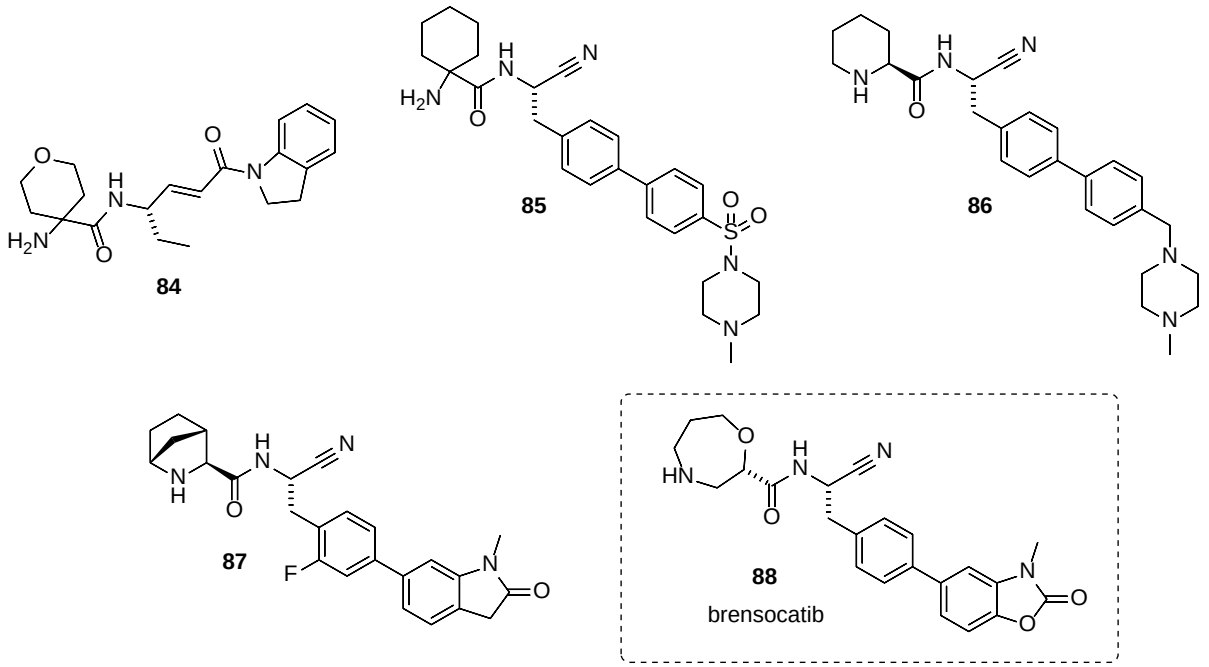
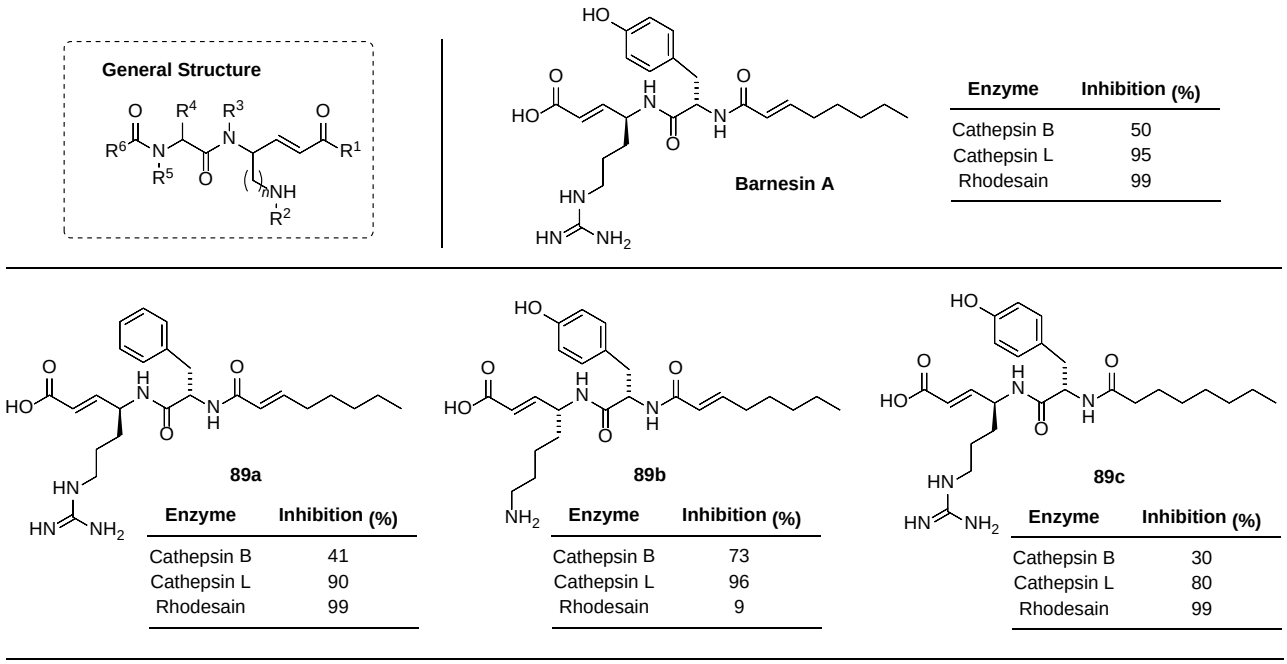


Figure 39. Selected example of CatC inhibitors and structure of brensocatib (**88**).

Barnesin A derivatives can also be used as cysteine protease inhibitors for the treatment of a wide range of diseases. A patent filed in 2019 focuses on compounds **89a-c** structurally-related to barnesin A, acting as novel potent and selective cysteine protease inhibitors (Figure 40).[137] The authors propose that pharmaceutical compositions of the reported compounds can be used for the treatment of a number of diseases where cysteine proteases are validated targets.

The molecules discussed provide an alternative therapeutic, preventive and/or curative strategy for diseases in which cysteine protease levels can be pathological. The molecules included in this patent showed good to high protease inhibition activity *in vitro* against cathepsins B and L (with higher values for

cathepsin L) and in some case good to high protease inhibition activity against rhodesain. The compounds showed good pharmacokinetic properties and high stability towards soft nucleophiles. Microsome stability assay was also performed *in vitro* to evaluate liver metabolism effects on the compounds, by measuring the intrinsic clearance and identifying the metabolites formed, with good results for all the compounds.



Enzyme	Inhibition (%)
Cathepsin B	50
Cathepsin L	95
Rhodesain	99

Enzyme	Inhibition (%)
Cathepsin B	41
Cathepsin L	90
Rhodesain	99

Enzyme	Inhibition (%)
Cathepsin B	73
Cathepsin L	96
Rhodesain	9

Enzyme	Inhibition (%)
Cathepsin B	30
Cathepsin L	80
Rhodesain	99

Figure 40. General structure and selected examples of barnesin A-related cysteine proteases inhibitors from [137].

The authors of a patent filed in 2021 claim the use of synthetic peptides as specific inhibitors of cysteine cathepsins.[138] As aforementioned, cysteine cathepsins are involved in a number of cellular pathways and their misregulation or overexpression has been related to a broad range of diseases including neurological and cardiovascular disorders, obesity and rheumatoid arthritis, and cancer. The molecules presented by the authors are tetrapeptides fluoromethyl ketone (FMK) derivatives **90** and **91** (Figure 41). Studies about the inhibitory properties of **90** and **91** were performed *in vitro* using recombinant cathepsins B and L. Further tests on renal cancer cell lines 769-P and A498 (scratch test) proved that the addition of tetrapeptides at a concentration of 20 μM slowed down the growth of tumour cells, thus showing a good potential in preventing the adhesion and the formation of intercellular contacts. The above-mentioned peptides have high affinity for the catalytic triad of cysteine cathepsins and can be used for therapeutic agents that are specific inhibitors of proteolytic processes involving cysteine cathepsins, in particular cancer.

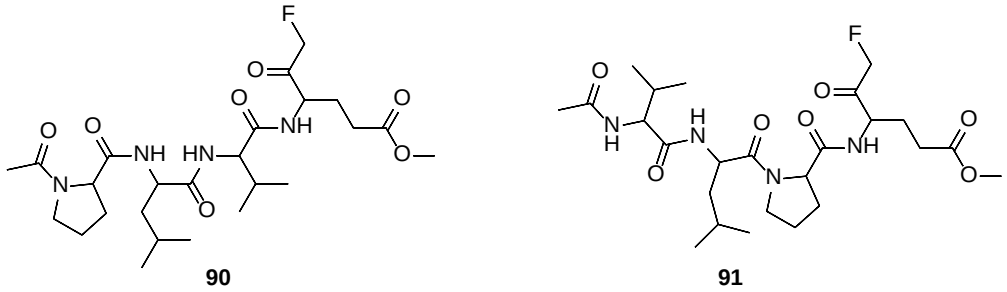
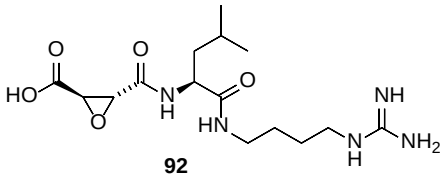


Figure 41. Structures of synthetic peptides **90** and **91** as specific inhibitors of cysteine cathepsins [138].

As mentioned above, the inhibition of cysteine protease is a possible target for the treatment of metastatic cancer. The authors of a patent filed in 2019 propose an invention for the inhibition of spontaneous metastasis, tumour cell invasion and lymph node colonization in cancer patients by trans-dermal administration of molecules that inhibit cysteine proteases.[139] Topical administration of the epoxysuccinyl-based thiol protease inhibitor **92** (Also named E-64, Figure 42) to mice highlighted a better survival rate (45%) in the animal that received the treatment with respect to the 2 control groups (20%). The topical buffering agent particle size proved to influence the efficacy of the treatment. The authors also described that the use of derivatives of **92** on mice models was demonstrated to reduce angiogenic activity and tumor growth rate with respect to the control group.

The inhibition of cathepsins activity is an interesting possible route also for the treatment of diseases where inflammation processes play a key role.
Notably, a patent a patent filed in 2020 claimed the use of known protease inhibitors, including **92**, for treatment of IBDs by inhibition of *Bacteroides spp*. proteases, including cysteine proteases. Bacteroides proteases are a source of persistent mucosal inflammation in IBDs and their inhibition reduced the disruption of epithelial barrier function at the tight junctions in Caco-2 cells. According the researchers, the use of protease inhibitors might be helpful in correcting dysbiosis, enhancing or supporting the gastrointestinal barrier and motility, up to antagonizing disease-related bacterial infections.[140]



92

Figure 42. Structure of **92**.

A patent filed in 2020 reports a number of low-molecular weight (< 3 kDa) cysteine peptidase inhibitors and cystatins of natural origin for the prevention or treatment of inflammatory conditions associated to cysteine peptidase overexpression originating from microorganism infection.[141] The claimed inhibitors were originated from natural raw material such as egg protein, and some plants including knotweed, houtt, and others. The described natural inhibitors were tested against native cathepsin B, and different tissue and organ homogenates. For example, an assay performed on gingival pocket fluids highlighted the high inhibition on gingipain both by cystatin and low molecular weight inhibitors from knotweed. For cancer, the results mainly depended on the tumour type and origin of the natural inhibitor. Low molecular weight knotweed inhibitors were the best both in lung and in colorectal cancer, with an inhibition activity up to 80% at 300 µL dose. These results further confirm the potential use of the new molecules for therapeutic purposes.
A patent filed in 2020 focused on a series of cathepsin C inhibitors for the potential treatment of inflammatory disorders associated to this enzyme. The general structure of such inhibitors is reported in Figure 43. [9] Their activity was tested against CatC on pancreas homogenate from mice with induced pancreatitis and half of the studied compounds proved to have 3 nM < IC_{50} < 300 nM.
Furthermore, in animal models, the compound **93** reduced the severity of induced acute pancreatitis in mice when the animals were administered orally twice daily at 20 mg/kg (as highlighted by pancreas histological data against control).

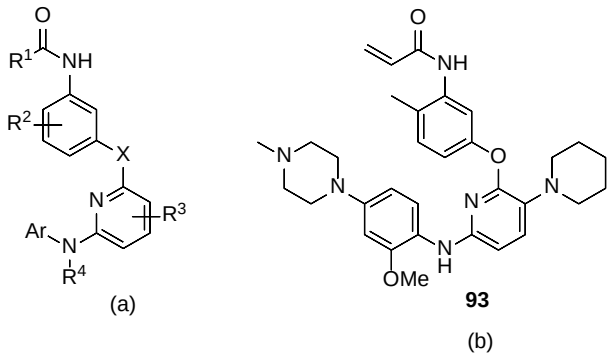


Figure 43. General structure of cathepsin C inhibitors (a) and structure of compound **93** (b) from [9].

In a patent filed in 2021 by the researchers of Ruishi Biopharmaceutical, small molecule inhibitors of cathepsin C and their possible medicinal use in inflammatory conditions were claimed (Figure 44). [142] The claimed compounds were tested *in vitro* for their capability of inhibiting CatC with respect to the control **AZD7986**. Remarkably, 12 of the 26 tested compounds showed IC_{50} values lower than that of **AZD7986** (12.21 nM). Pharmacokinetic properties were tested in mouse and beagle dog models. The claimed compounds were also tested *in vivo* on neutrophil elastase (NE), related to CatC activity in chronic inflammation and tissue damage. Compounds **94** and **95** (Figure 44) showed stronger inhibition rates of NE protease with respect to the control molecule **AZD7986**, when administered at a dose of 3 mg/kg.

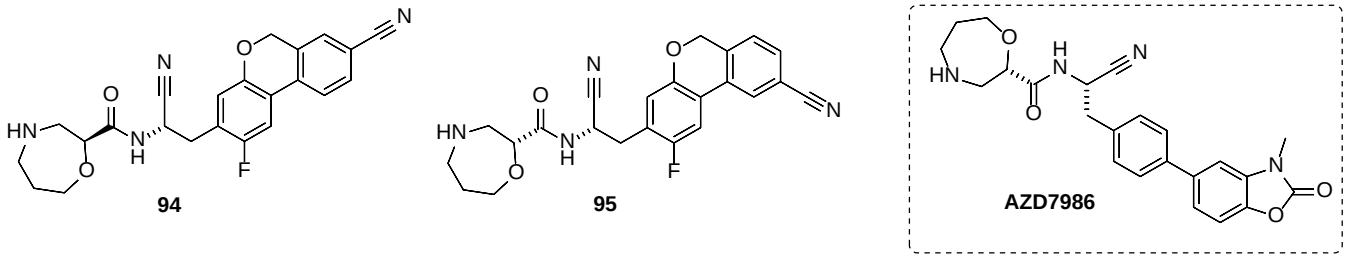


Figure 44. Structure of cathepsin C inhibitors **94**, **95** and **AZD7986**.

A patent filed in 2023 by the researchers of Boehringer Ingelheim International claims the use of compounds with the general structure **96** (Figure 45) as possible drugs for the treatment of pulmonary emphysema – and other inflammatory diseases in the lungs, involving neutrophils – by inhibiting the activity of cathepsin C. [143] Inhibition test was performed in vitro by fluorescence assay on 359 examples of the general structure **96**, which proved to be very potent (IC_{50} values in the nM range). Some compounds were also tested against neutrophil elastase NE (directly related to CatC activity in chronic inflammation and tissue damage) in U937 lymphoma cell lines, showing nM IC_{50} values. The tested compounds also showed good selectivity against other Cathepsins and good pharmacokinetic properties.

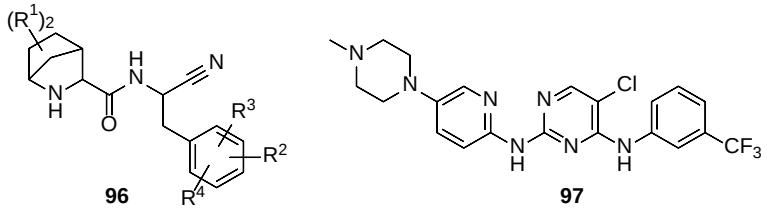


Figure 45. General structure of cathepsin C inhibitors **96** and structure of compound **97**.

In a patent filed in 2023, the use of the pyrimidine-2,4-diamine **97** (Figure 45) as a possible inhibitor of cathepsin C to be used for the treatment of inflammatory diseases was claimed.[144] The tested compounds showed low toxicity and proved to have a strong inhibitor activity *in vitro* and *in vivo* against cathepsin C. Strong anti-NSP and anti-inflammatory activities *in vivo* were also demonstrated.
When administered in a rat model of chronic obstructive pulmonary disease (COPD), **97** ameliorated histopathological changes, including alveolar haemorrhage and dilation, partial alveolar fusion, inflammatory cell infiltration, and alveolar structure destruction in a dose-dependent manner. Animals treated with **97** showed a decrement in the level of pro-inflammatory cytokines and increased anti-inflammatory Interleukin 10 (IL-10) in a dose-dependent way.

A patent claiming structurally-stabilized and/or cysteine-reactive NOXA peptides and their methods of use to inhibit selectively BFL-1, or dually BFL-1 and MCL-1 for treating cancer and autoimmune/inflammatory disorders was filed in 2018.[145] BFL-1 and MCL-1 promote mitochondrial apoptosis suppression by linking specific alpha-helical portions (BH) of pro-apoptotic proteins including NOXA. BFL-1 is an independent oncogenic driver in different human cancers; when overexpressed or mutated BFL-1 can induce chemoresistance in lymphomas and improve survival in melanomas.
MCL-1 (Myeloid Cell Leukemia-1) is a protein involved in the development, maintenance, and chemoresistance of a variety of cancers. MCL-1 is among the ten most expressed pathologic factors in human cancers.[146] Compounds interfering with BFL-1 and/or MCL-1 activity could support the treatment of cancers and autoimmune/inflammatory disorders. One of the main problems with the inhibition of anti-apoptotic protein is often the lack of specificity.
The claimed structurally-stabilized and cysteine-reactive NOXA BH3 peptides of the above-mentioned patent [145] can selectively bind covalently BFL-1 and non-covalently MCL-1, blocking their activity and reactivating apoptosis. The covalent link with the target BFL-1 protein involves a cysteine residue, as highlighted by crystallography and fluorescence studies.
The studied peptides include an electrophilic reactive group (“warhead”), a specific sequence of 5-35 or 8-18 modified amino-acids and at least one peptide structure stabilizing modification. General structure of NOXA SAHBs (stabilized α helix of BCL2) polypeptides is reported in the Figure 46 (*a*); structural modifications of the hydrocarbon cross-linkers – including epoxidation, aminohydroxylation or dihydroxylation – can also be performed (Figure 46, *b*). The epoxide moiety or one of the free hydroxyl moieties can be further functionalized, providing the possibility of attaching a therapeutic agent or an agent facilitating the polypeptide to enter the cells.

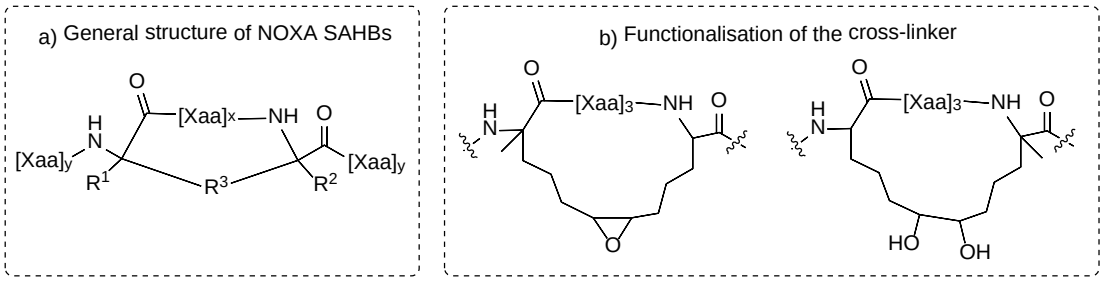
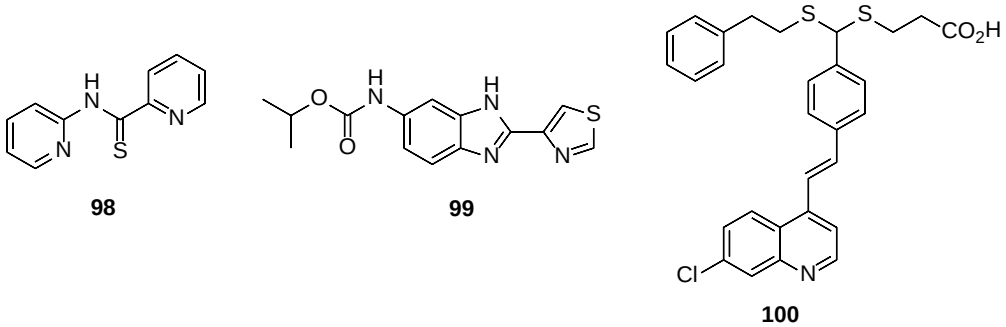


Figure 46. General structure of NOXA SAHB polypeptides and exemplificative structural modification.

A method for enhancing cytotoxic cancer therapy by using inhibitors of autophagy related 4B cysteine protease (ATG4B) has also been described in a patent.[147] Autophagy involves lysosomes and more than 30 proteins, including ATG4B. In tumour cells the autophagy mechanism helps the cancer growth and it is interesting to note that some traditional therapies including radiation therapy (RT) and the chemotherapist temozolomide (TMZ) can trigger the autophagic response thus contributing to cancer resistance. The inhibition of the activity of ATG4B might help the treatment of some kind of cancer including brain glioblastoma (GMB), breast, lung, and liver cancers.

In a patent filed in 2020, a number of novel molecules that might increase the effectiveness of traditional first-line therapies like RT and TMZ or other antineoplastic alkylating agent more than small molecule inhibitor therapy (mTOR therapy) were presented. mTOR (mammalian/mechanistic target of rapamycin) is a protein kinase that controls growth, metabolism and aging in the cell in response to nutrients and grow factors. mTOR pathways result to be over-activated in about 30 % of GBMs, increasing tumorigenicity and cancer progression. The use of allosteric inhibitors for mTOR in cancer cells has been experimented but it resulted to be not sufficient because it induced autophagy and promoted cancer cell survival. These novel molecules **98-100** that inhibit the biological activity of ATG4B (Figure 47) can be administrated to the patient before, concurrently with, or after the alkylating agent or mTOR inhibitor.

Notably, **98** suppresses the ATG4B-dependent autophagy and reduces the cytoprotective autophagic response of cancer cells, and thus the tumorgenicity, of glioblastoma. This molecule exhibited no relevant tissue toxicity in treated mice and also showed an excellent blood/brain barrier (BBB) penetration. Compared to different already known autophagy inhibitors like chloroquine (CQ) and hydroxy-CQ, **89** was highly specific against autophagy and offered better efficacy in combination treatments.



98 **99** **100**

Figure 47. Compounds used for enhancing cytotoxic cancer therapy *via* inhibition of ATG4B.

4. Expert Opinion

Cysteine proteases family includes a variety of enzymes which play a central role in several biological processes. The involvement of cysteine proteases in the onset and progression of a number of diseases, ranging from cancer to neurodegenerative disorders, going through viral infection-associated diseases has

been well documented. For example, the dysregulation of the ubiquitin-proteasome system has been associated with the pathogenesis of metabolic and neurodegenerative disorders, viral, and microbial infections, and certain types of cancer. The misregulation of cysteine cathepsins – which are mainly involved in the lysosomal proteins degradation, apoptosis, and immunity processes – can result in the onset of a broad range of diseases, including cardiovascular disorders, obesity, rheumatoid arthritis, and cancer. Particularly, in cancer cathepsins contribute to invasion, proliferation, and metastasis.

On the basis of these considerations, the selective inhibition of cysteine proteases represents an attractive and rewarding strategy to develop new therapeutic tools for the treatment of the above-mentioned diseases.

Additionally, after the COVID-19 pandemic outbreak in 2020, the key role played by cysteine proteases in coronavirus-related infections has emerged. SARS-CoV-2 encodes for several enzymes essential for its vital cycle; after entered the host cell, the viral RNA is translated into two polyproteins, whose cleavage affords the viral proteins necessary for assembling new virones. The enzymes involved in the cleavage of such polyproteins are cysteine proteases, namely main protease (M^{pro}) and papain-like protease (PL^{pro}).[49-51] Furthermore, cysteine proteases present on the surface of the permissive host cells have also been demonstrated to be involved in the infection of human cells by SARS-CoV-2. Thus, the inhibition of different cysteine proteases is an interesting target for the design and development of drug candidates to treat COVID-related infections.

Several patents dealing with the inhibition of cysteine proteases have been filed over the past five years. An analysis of such patents highlighted a rather broad structural diversity of the proposed inhibitors.

Owing to the high societal impact of COVID-19 pandemic, most of the recently reported patents focused on the inhibition of cysteine proteases for treating or preventing the disease associated with SARS-CoV-2 infections. Several effective SARS-CoV-2 M^{pro} and SARS-CoV-2 PL^{pro} inhibitors have been described in recent patents. The fact that the two proteases of SARS-CoV-2 display a high degree of conserved amino acid residues with respect to the corresponding proteases of SARS-CoV1 and MERS, coupled with the availability of resolved X-ray structures of M^{pro} and PL^{pro} from SARS-CoV-2 (alone and in complex with inhibitors), reasonably enabled a rational design and development of efficient inhibitors. In this context, both peptidomimetic and non-peptidomimetic inhibitors have been described. Their mechanism of action, which in some cases is still not elucidated, often relies on the nucleophilic attack of the cysteine thiol moiety of the enzyme onto suitable electrophilic sites of the inhibitor.

In some cases, although interesting inhibition data against SARS-CoV-2 proteases were found by enzymatic assays, additional studies related to their activity in biophysical cell-based assays should be carried out. Indeed, a number of compounds with remarkable IC_{50} values according to enzymatic assays were showed to be scarcely effective in tests conducted on SARS-CoV-2-infected cells. For example, compounds labelled as **DCPH28** and **4c** (Figures 9 and 10, *vide supra*) – which showed high potency in the enzymatic assay (IC_{50} = 0.35 and 0.5 μM, respectively) – were demonstrated to lack sufficient activity on successive studies on SARS-CoV-2-infected Vero E6 cells (EC_{50} = 9.5 and 1.0 μM, respectively).[111,113]

An additional issue related to the development of cysteine proteases inhibitors is related to their selectivity. In some cases, considering the homology between certain cysteine proteases, the design and development of selective inhibitors is quite challenging. Nonetheless, some interesting results highlighting the possibility to selectively target specific cysteine proteases (*i.e.*, USP7, SARS-CoV-2 M^{pro}, cathepsin B and L) have been recently achieved.[115,130,137] On the basis of the critical role of selectivity issues, considerable research efforts should be directed towards achieving inhibitors capable of targeting specific cysteine proteases.

A detailed comprehension of inhibition mechanisms is crucial for the improvement of the inhibitor activity as well as for its reasoned structural modification, which is critical to the successful development of drug

candidates. In this scenario, particular attention of future researches will likely be devoted to further X-ray investigations of different enzyme-inhibitor complexes.

In this *scenario*, structurally diverse small molecules acting as selective cysteine protease inhibitors has been described as promising drug candidates for cancer treatment or prevention. For example, direct selective USP7 inhibition as well as modulation/inhibition of the USP7-related ETSP7 has been achieved employing piperidine [130] and quinazolin-4(3*H*)-one derivatives.[131] Cell-based studies and in vivo tests on animal models provided interesting data on the possible use of USP19 inhibitors for the treatment of a series of diseases including cancer, obesity, insulin resistance, type II diabetes, muscular atrophy.[135]

The use of cysteine-reactive NOXA peptide-based specific inhibitors enabled the inhibition of the oncogenic driver BFL-1 and the survival protein MCL-1, which is amongst the most widely expressed pathologic factor associated with cancer growth and chemoresistance (*e.g*. in melanomas and limphomas).[145] Peptide inhibitors were also synthesised to specifically target diseases-related cathepsins [138] and the SAGA deubiquitinating module (DUBm).[132]

The design and synthesis of selective, potent, and specific inhibitors is generally highly desirable. However, in certain cases, non-specific/non-selective cysteine protease inhibitors can be successfully employed for therapeutic applications. For example, as mentioned above, compounds capable to inhibit both viral and permissive host cell cysteine proteases can be considered interesting drug candidates for the treatment of COVID-19 infection.[127]

The research of new drugs is a challenging process trat aims to find a balance between the efficacy and the toxicity, metabolic stability and cell permeability of the molecules.[148]

Different strategies are nowadays available to identify new lead compounds. High throughput screening (HTS) is currently the mainly used [149,150]**,** along with the more recent structure-based virtual screening of specific molecules libraries, that is faster and cheaper than HTS but needs a careful validation process.[148,150,151] For example, HTS has been the technique used to discover the selenium-containing cysteine protease inhibitor ebeselen, after a screening performed on 1280 molecules using fluorescence techniques.[148]

A traditional approach for the research of new drugs has been drug repurposing, which relies on the reuse of already existing drugs for new therapeutic application that presents several advantages. In drug repurposing, the use of drugs that are already known for their effectiveness on specific targets towards different ones is performed. Since already tested drugs are used, this allows to step the pharmacokinetic, pharmacodynamic and toxic studies directly to phase II or III clinical trials. Different approved drug libraries are available for this kind of research, that is a remarkably time saving approach [152] and it has been largely used for finding therapeutic strategies during the outbreak of Covid-19 pandemic (*e.g*., testing molecules that were already known for their efficacy against hepatitis C virus, Zika virus and Ebola, among the others).[151] Examples of known drugs studied as SARS-CoV-2 cysteine proteases inhibitors are reported in the Figure 6.

The research on peptidomimetics is especially interesting in the develop of protease inhibitors and wide collections of molecules can be rapidly obtained via multicomponent reactions and be later functionalized and tested.[153] Peptide-based design strategies are often based on X-ray structural analysis of the target peptide and represent a useful approach that also allows to increase stability and cell permeability.[148] This strategy has been applied also in the research field of cysteine protease inhibitors.

Also, a focus on drug conformation, with the aim of improving drug positioning in the binding pocket or finding different binding sites, strengthening the interaction (*e.g.*, *via* the introduction of multiple hydrogen bonds, covalent binding, halogen bonds, additional van der Waals forces or multivalent binding), introducing or enhancing additional interactions represents an interesting strategy to face the consequences (*e.g*., affinity decrease) of possible mutation and drug resistance.[154]

The activity screening of natural products to obtain desired derivatives as therapeutics is another favourable approach that has been recently widely explored.[148] Specific chemical modifications can be applied on lead compound discovered *via* different routes to overcome possible defects including low activity, structural instability or unfavourable pharmacokinetic and develop them into ideal drugs. These modifications include bioisosteric modification or scaffold hopping strategy. The introduction of additional functional groups can also be performed to improve the potency and drug-like properties of the studied molecules.[148] Examples of the application of this approach to the discovery of cysteine proteases inhibitors are reported in Figures 8, 25, and 40.

Overall, the design, developement and study of cysteine protease inhibitors is attracting considerable attention in medicinal chemistry-related fields and huge progresses have been made. This research topic is reasonably going through a steadily growing phase and the availability of a broad variety of inhibitors, coupled with mechanistic data, will likely bring about further achievements in a such vivacious field.

5. Conclusion

Cysteine proteases are crucially involved in a wide array of biological processes and also play a key role in the onset and progression of several diseases, including viral and parasitic infections, cancer, neurological and cardiovascular disorders. The inhibition of cysteine proteases thus represents an attractive strategy to develop new therapeutic tools, and a number of structurally diverse inhibitors have been reported in the recent scientific and patent literature. Owing to the high societal and health impact of COVID-19 pandemic, a significant number of the reported studies focused on the inhibition of viral cysteine proteases for treating or preventing the disease associated with SARS-CoV-2 infections. However, important researches on the inhibition of mammalian cysteine proteases have also been reported. Promising results have been described about the possibility to develop potent and selective inhibitors. On the basis of the reported achievements, we believe that cysteine protease inhibition may represent a great opportunity to develop novel drug candidates.

Funding

This paper was not funded.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Author contributions

Conceptualization and methodology, and classification of the collected manuscripts G.B., D.T.; analysis of the collected manuscripts and writing—original draft preparation, G.B., A.C., D.T.; writing—review and editing, G.B., A.C., D.T.; All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Verma S, Dixit R, Pandey KC. Cysteine Proteases: Modes of Activation and Future Prospects as Pharmacological Targets. Front Pharmacol. 2016;7:107. doi:10.3389/fphar.2016.00107 - **Remarkable review on Cysteine Proteases as a potential therapeutic target.**
2. Gupta SP, Gupta SD. Cancer-leading proteases: An introduction. In *Cancer-Leading Proteases - Structures, Functions, and Inhibition*. Elsevier, London, 2020, pp. 1-11 and reference therein cited
3. Solberg R, Lunde NN, Forbord KM, et al. The Mammalian Cysteine Protease Legumain in Health and Disease. Int J Mol Sci. 2022;23(24):15983. Published 2022 Dec 15. doi:10.3390/ijms232415983
4. Buttle DJ, Mort JS. Cysteine Proteases. In *Encyclopedia of Biological Chemistry*. Elsevier, Amsterdam, 2013, pp. 589-92 – **Interesting book chapter on Cysteine Proteases.**
5. Wang Y, Zhao J, Gu Y, et al. Cathepsin H: Molecular characteristics and clues to function and mechanism. Biochem Pharmacol. 2023;212:115585
6. Poole AR, Tiltman KJ, Recklies AD, et al. Differences in secretion of the proteinase cathepsin B at the edges of human breast carcinomas and fibroadenomas. Nature. 1978;273(5663):545-7. doi:10.1038/273545a0
7. Hasanbasic S, Jahic A, Karahmet E, et al. The role of cysteine protease in Alzheimer Disease. Mater Sociomed. 2016;28(3):235-38. doi:10.5455/msm.2016.28.235-238
8. Shen XB, Chen X, Zhang ZY, et al. Cathepsin C inhibitors as anti-inflammatory drug discovery: Challenges and opportunities. Eur J Med Chem. 2021;225:113818. doi:10.1016/j.ejmech.2021.113818
9. Zhang Z, Hou W, Sun H. Cathepsin C inhibitors. US021/0114983 A1
10. Hu G, Xiao Y, Cong M, et al. Methods for treating metastasis with cathepsin c inhibitors. WO2022/053019A1
11. Wei S, Liu W, Xu M, et al. Cathepsin F and Fibulin-1 as novel diagnostic biomarkers for brain metastasis of non-small cell lung cancer. Br J Cancer. 2022;126(12):1795-805
12. Takaya K, Asou T, Kishi K. Cathepsin F is a potential marker for senescent human skin fibroblasts and keratinocytes associated with skin aging. Geroscience. 2023;45(1):427-37
13. Drake MT, Clarke BL, Oursler MJ, et al. Cathepsin K Inhibitors for Osteoporosis: Biology, Potential Clinical Utility, and Lessons Learned. Endocr Rev. 2017;38(4):325-50. doi:10.1210/er.2015-1114 and references therein
14. Verbovšek U, Van Noorden CJ, Lah TT. Complexity of cancer protease biology: Cathepsin K expression and function in cancer progression. Semin Cancer Biol. 2015;35:71-84.
15. Lu J, Wang M, Wang Z, et al. Advances in the discovery of cathepsin K inhibitors on bone resorption. J Enzyme Inhib Med Chem. 2018;33(1):890-904
16. Mijanović O, Jakovleva A, Branković A, et al. Cathepsin K in Pathological Conditions and New Therapeutic and Diagnostic Perspectives. Int J Mol Sci. 2022;23(22):13762
17. Duong LT, Wesolowski GA, Leung P, et al. Efficacy of a cathepsin K inhibitor in a preclinical model for prevention and treatment of breast cancer bone metastasis. Mol Cancer Ther. 2014;13(12):2898-909. doi:10.1158/1535-7163.MCT-14-0253
18. Sudhan DR, Siemann DW. Cathepsin L targeting in cancer treatment. Pharmacol Ther. 2015;155:105-16. doi:10.1016/j.pharmthera.2015.08.007
19. Liu T, Luo S, Libby P, et al. Cathepsin L-selective inhibitors: A potentially promising treatment for COVID-19 patients. Pharmacol Ther. 2020;213:107587. doi:10.1016/j.pharmthera.2020.107587
20. Velasco G, Ferrando AA, Puente XS, et al. Molecular cloning from a breast carcinoma, production of the active enzyme in Escherichia coli, and expression analysis in human tissues. J Biol Chem. 1994;269(43):27136-42

21. McDowell SH, Gallaher SA, Burden RE, et al. Leading the invasion: The role of Cathepsin S in the tumour microenvironment. Biochim Biophys Acta Mol Cell Res. 2020;1867(10):118781
22. Bararia D, Hildebrand JA, Stolz S, et al. Cathepsin S Alterations Induce a Tumor-Promoting Immune Microenvironment in Follicular Lymphoma. Cell Rep. 2020;31(5):107522
23. Fuchs N, Meta M, Schuppan D, et al. Novel Opportunities for Cathepsin S Inhibitors in Cancer Immunotherapy by Nanocarrier-Mediated Delivery. Cells. 2020;9(9):2021. doi:10.3390/cells9092021
24. Lecaille F, Chazeirat T, Saidi A, et al. Cathepsin V: Molecular characteristics and significance in health and disease. Mol Aspects Med. 2022;88:101086
25. Li J, Chen Z, Kim G, et al. Cathepsin W restrains peripheral regulatory T cells for mucosal immune quiescence. Sci Adv. 2023;9(28):eadf3924
26. Günther SC, Martínez-Romero C, Sempere Borau M, et al. Proteomic Identification of Potential Target Proteins of Cathepsin W for Its Development as a Drug Target for Influenza. Microbiol Spectr. 2022;10(4):e0092122. doi:10.1128/spectrum.00921-22
27. Khojasteh-Leylakoohi F, Mohit R, Khalili-Tanha N, et al. Down regulation of Cathepsin W is associated with poor prognosis in pancreatic cancer. Sci Rep. 2023;13(1):16678
28. Dolenc I, Štefe I, Turk D, et al. Human cathepsin X/Z is a biologically active homodimer. Biochim Biophys Acta Proteins Proteom. 2021;1869(2):140567
29. Nandi D, Tahiliani P, Kumar A, et al. The ubiquitin-proteasome system. J Biosci. 2006;31(1):137-55. doi:10.1007/BF02705243
30. Gao G, Luo H. The ubiquitin-proteasome pathway in viral infections. Can J Physiol Pharmacol. 2006;84(1):5-14. doi:10.1139/y05-144
31. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006;443(7113):780-6. doi:10.1038/nature05291
32. Corn JE, Vucic D. Ubiquitin in inflammation: the right linkage makes all the difference. Nat Struct Mol Biol. 2014;21(4):297-300. doi:10.1038/nsmb.2808
33. Nicholson B, Suresh Kumar KG. The multifaceted roles of USP7: new therapeutic opportunities. Cell Biochem Biophys. 2011;60(1-2):61-8. doi:10.1007/s12013-011-9185-5
34. Hoeller D, Hecker CM, Dikic I. Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. Nat Rev Cancer. 2006;6(10):776-88. doi:10.1038/nrc1994
35. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem. 2009;78:363-97 - **Remarkable review on DUBs with a focus on USPs.**
36. Kitamura H. Ubiquitin-Specific Proteases (USPs) and Metabolic Disorders. Int J Mol Sci. 2023;24(4):3219. doi:10.3390/ijms24043219. – **Recent interesting review on the role of USPs in Metabolic Disorders.**
37. Cruz L, Soares P, Correia M. Ubiquitin-Specific Proteases: Players in Cancer Cellular Processes. Pharmaceuticals (Basel). 2021;14(9):848. doi:10.3390/ph14090848 - **Remarkable review on the role of USPs in Cancer.**
38. Chauhan D, Tian Z, Nicholson B, et al. A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance. Cancer Cell. 2012;22(3):345-58. doi:10.1016/j.ccr.2012.08.007
39. Cummins JM, Rago C, Kohli M, et al. Tumour suppression: disruption of HAUSP gene stabilizes p53. Nature. 2004;428(6982):1-486. doi:10.1038/nature02501
40. Reverdy C, Conrath S, Lopez R, et al. Discovery of specific inhibitors of human USP7/HAUSP deubiquitinating enzyme. Chem Biol. 2012;19(4):467-77. doi:10.1016/j.chembiol.2012.02.007

41. Chauhan D, Tian Z, Nicholson B, et al. A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance. Cancer Cell. 2012;22(3):345-58. doi:10.1016/j.ccr.2012.08.007
42. Pal, A, Donato, NJ. Ubiquitin-specific proteases as therapeutic targets for the treatment of breast cancer. Breast Cancer Res. 2014;16:461
43. Liu X, Balaraman K, Lynch CC, et al. Novel Ubiquitin Specific Protease-13 Inhibitors Alleviate Neurodegenerative Pathology. Metabolites. 2021;11(9):622.. doi:10.3390/metabo11090622
44. Li YC, Cai SW, Shu YB, et al. USP15 in Cancer and Other Diseases: From Diverse Functionsto Therapeutic Targets. Biomedicines. 2022;10(2):474
45. Lee JG, Takahama S, Zhang G, et al. Unconventional secretion of misfolded proteins promotes adaptation to proteasome dysfunction in mammalian cells. Nat Cell Biol. 2016;18(7):765-76. doi:10.1038/ncb3372
46. Bédard N, Jammoul S, Moore T, et al. Inactivation of the ubiquitin-specific protease 19 deubiquitinating enzyme protects against muscle wasting. FASEB J. 2015;29(9):3889-98. doi:10.1096/fj.15-270579
47. Jeusset LM, Guppy BJ, Lichtensztejn Z, et al. Reduced *USP22* Expression Impairs Mitotic Removal of H2B Monoubiquitination, Alters Chromatin Compaction and Induces Chromosome Instability That May Promote Oncogenesis. *Cancers (Basel)*. 2021;13(5):1043. doi:10.3390/cancers13051043
48. Ren X, Jiang M, Ding P, et al. Ubiquitin-specific protease 28: the decipherment of its dual roles in cancer development. Exp Hematol Oncol. 2023;12(1):27
49. Jin Z, Du X, Xu Y, et al. Structure of M^{pro} from SARS-CoV-2 and discovery of its inhibitors. Nature. 2020;582(7811):289-93. doi:10.1038/s41586-020-2223-y
50. Capasso C, Nocentini A, Supuran CT. Protease inhibitors targeting the main protease and papain-like protease of coronaviruses. Expert Opin Ther Pat. 2021; 31(4):309-24. doi.org/10.1080/13543776.2021.1857726. - **Remarkable review on inhibition of main proteases and papain-like proteases of coronaviruses.**
51. Brian Chia CS, Pheng Lim S. A Patent Review on SARS Coronavirus Papain-Like Protease (PL^{pro}) inhibitors. ChemMedChem. 2023; e202300216. doi.org/10.1002/cmdc.202300216 - **Remarkable review on inhibition of main proteases and papain-like proteases of SARS-CoV-2.**
52. Li G, Hilgenfeld R, Whitley R, De Clercq E. Therapeutic strategies for COVID-19: progress and lessons learned. Nat Rev Drug Discov. 2023;22(6):449-475. doi:10.1038/s41573-023-00672-y
53. Yang J, Lin X, Xing N, et al. Structure-Based Discovery of Novel Nonpeptide Inhibitors Targeting SARS-CoV-2 Mpro. J Chem Inf Model. 2021;61(8):3917-3926
54. Amin SA, Banerjee S, Ghosh K, et al. Protease targeted COVID-19 drug discovery and its challenges: Insight into viral main protease (Mpro) and papain-like protease (PLpro) inhibitors. Bioorg Med Chem. 2021;29:115860
55. Tumskiy RS, Tumskaia AV, Klochkova IN, et al. SARS-CoV-2 proteases Mpro and PLpro: Design of inhibitors with predicted high potency and low mammalian toxicity using artificial neural networks, ligand-protein docking, molecular dynamics simulations, and ADMET calculations. Comput Biol Med. 2023;153:106449
56. Rawat A, Roy M, Jyoti A, et al. Cysteine proteases: Battling pathogenic parasitic protozoans with omnipresent enzymes. Microbiol Res. 2021;249:126784. doi:10.1016/j.micres.2021.126784
57. Cianni L, Feldmann CW, Gilberg E, et al. Can Cysteine Protease Cross-Class Inhibitors Achieve Selectivity?. J Med Chem. 2019;62(23):10497-525. doi:10.1021/acs.jmedchem.9b00683
58. Wang X, Luo H, Zhang R, et al. Application of pirfenidone or pharmaceutically acceptable salt thereof in preparation of medicine for preventing and/or treating novel coronavirus inflammation. CN2020/111481547

59. Schurdak ME, Vogt A, Stern A, et al. Compounds for the treatment of a disease or disorder, methods for identifying said compounds. WO2022/159787
60. Huang H, Zhang G, Tang J, Liu S, Fu Z, Liu M. Use of a PL^{pro} protein inhibitor in a medicament for the treatment or prevention of a novel coronavirus infection. CN2020/112022855
61. Fu Z, Huang B, Tang J, et al. The complex structure of GRL0617 and SARS-CoV-2 PL^{pro} reveals a hot spot for antiviral drug discovery. Nat Commun. 2021;12(1):488. doi:10.1038/s41467-020-20718-8
62. Ma C, Wang J. Validation and Invalidation of SARS-CoV-2 Papain-like Protease Inhibitors. ACS Pharmacol Transl Sci. 2022;5(2):102-9. doi:10.1021/acsptsci.1c00240
63. Han K, Jia Y. Application of Succimer in preparation of anti-coronavirus infection medicine and medicine. CN2022/114246854
64. Han K, Jia Y, Wang Y. Application of PX-12 in preparation of coronavirus papaya-like protease PL^{Pro} inhibitor drug. CN2022/114617877
65. Huber K, Patel P, Zhang L, et al. 2-[(1-methylpropyl)dithio]-1H-imidazole inhibits tubulin polymerization through cysteine oxidation. Mol Cancer Ther. 2008;7(1):143-51. doi:10.1158/1535-7163.MCT-07-0486.
66. Ma C, Hu Y, Townsend JA, et al. Ebselen, Disulfiram, Carmofur, PX-12, Tideglusib, and Shikonin Are Nonspecific Promiscuous SARS-CoV-2 Main Protease Inhibitors. ACS Pharmacol Transl Sci. 2020;3(6):1265-77. doi:10.1021/acsptsci.0c00130
67. Han K, Jia Y, Wang Y. Application of Tideglusib in preparation of coronavirus papaya-like protease PLPro inhibitor drug. CN2022/114432306
68. Tan H, Ma C, Wang J. Invalidation of dieckol and 1,2,3,4,6-pentagalloylglucose (PGG) as SARS-CoV-2 main protease inhibitors and the discovery of PGG as a papain-like protease inhibitor. Med Chem Res. 2022;31(7):1147-53. doi:10.1007/s00044-022-02903-0
69. Han H, Wang Y, Jia Y, et al. Application of Thiamine in preparation of coronavirus papain inhibitor and coronavirus infection resistant medicine. CN2022/114469954
70. Han K, Wang Y, Jia Y, et al. Application of pantoprazole in preparation of products for inhibiting coronavirus and medicines for resisting coronavirus infection. CN2022/114504575
71. Fallon JN, Heil M, Fallon JJ, et al. Methods of prophylaxis of coronavirus infection and treatment of coronaviruses. WO2022/056103
72. Setz C, Schubert U. Inhibitors of human deubiquitinases for the treatment of coronaviral infections. WO2021/170290
73. Wilen C. Compounds and compositions for treating, ameliorating, and/or preventing sars-cov-2 infection and/or complications thereof. WO2021/247367
74. Cho CC, Li SG, Lalonde TJ, et al. Drug Repurposing for the SARS-CoV-2 Papain-Like Protease [published correction appears in ChemMedChem. 2022 Mar 4;17(5):e202200053]. ChemMedChem. 2022;17(1):e202100455. doi:10.1002/cmdc.202100455
75. Xu X, Wan S, Liu W, et al. Isothia (selenium) azolone derivative and application thereof in anti-coronavirus drugs. CN2021/113200978
76. Tsai C, Hung M, Chen Y, et al. Use of Disulfiram for Treating Infection Of SARS-COV-2. US2021/0386695
77. Bai F, Liu H, Xu Y, et al. Application of heterocyclic compound. CN2022/114014824
78. Janetka JW, Ledford BE, Mullican MD. Pentacyclic compounds useful as inhibitors of hepatitis c virus ns3 helicase. WO2000/024725
79. Fawzi AB, Macdonald D, Benbow LL, et al. SCH-202676: An allosteric modulator of both agonist and antagonist binding to G protein-coupled receptors. Mol Pharmacol. 2001;59(1):30-37. doi:10.1124/mol.59.1.30

80. van den Nieuwendijk AM, Pietra D, Heitman L, et al. Synthesis and biological evaluation of 2,3,5-substituted [1,2,4]thiadiazoles as allosteric modulators of adenosine receptors. J Med Chem. 2004;47(3):663-72. doi:10.1021/jm030863d
81. Lanzafame A, Christopoulos A. Investigation of the interaction of a putative allosteric modulator, N-(2,3-diphenyl-1,2,4-thiadiazole-5-(2H)-ylidene) methanamine hydrobromide (SCH-202676), with M1 muscarinic acetylcholine receptors. J Pharmacol Exp Ther. 2004;308(3):830-7. doi:10.1124/jpet.103.060590
82. Ren P, Yu C, Zhang R, et al. Discovery, synthesis and mechanism study of 2,3,5-substituted [1,2,4]-thiadiazoles as covalent inhibitors targeting 3C-Like protease of SARS-CoV-2. Eur J Med Chem. 2023;249:115129. doi:10.1016/j.ejmech.2023.115129
83. Akaike T, Takagi S, Sugiura H, et al. Medical agent containing active sulfur compounds as main ingredient. WO2021/235494A1
84. Barchielli G, Capperucci A, Tanini D. The Role of Selenium in Pathologies: An Updated Review. Antioxidants (Basel). 2022;11(2):251. doi:10.3390/antiox11020251. - **Recent review on the role of selenium in pathologies.**
85. Debnath S, Agarwal A, Kumar NR, et al. Selenium-Based Drug Development for Antioxidant and Anticancer Activity. Future Pharmacology. 2022; 2(4): 595-607. - **Remarkable review on the potential of selenium in drug discovery.**
86. Ali W, Benedetti R, Handzlik J, et al. The innovative potential of seleniumcontaining agents for fighting cancer and viral infections. Drug Discov Today. 2021; 26(1): 256-63. - **Remarkable review on the potential of selenium in drug discovery.**
87. Hou W, Dong H, Zhang X, et al. Selenium as an emerging versatile player in heterocycles and natural products modification. Drug Discov Today. 2022; 27(8): 2268-77. - **Remarkable review on the potential of selenium in drug discovery.**
88. Angeli A, Ferraroni M, Capperucci A, et al. Selenocarbamates As a Prodrug-Based Approach to Carbonic Anhydrase Inhibition. ChemMedChem. 2022; 17(11): e202200085
89. Tanini D, Carradori S, Capperucci A, et al. Chalcogenides-incorporating carbonic anhydrase inhibitors concomitantly reverted oxaliplatin-induced neuropathy and enhanced antiproliferative action. Eur J Med Chem. 2021; 225: 113793
90. Han K, Jia Y. Application of mono-selenoether compound in preparation of anti-coronavirus drugs and drugs. CN2022/114246867
91. Han K, Wang Y, Jia Y, et al. Application of diselenide compound as coronavirus papaya protease inhibitor, inhibitor and medicine. CN2021/112156084
92. Han K, Wang Y, Jia Y, et al. Application of Ebselen in preparation of coronavirus papain inhibitor and medicine for resisting coronavirus infection. CN2022/114469939
93. Weglarz-Tomczak E, Tomczak JM, Talma M, et al. Identification of ebselen and its analogues as potent covalent inhibitors of papain-like protease from SARS-CoV-2. Sci Rep. 2021;11(1):3640. doi:10.1038/s41598-021-83229-6
94. Suzuki N, Ogra Y, Fukumoto Y, et al. Therapeutic or prophylactic agent for covid19 which comprises selenoneine. WO2022/177029A1
95. Han K, Jia Y. Application of Eflorhithine in preparation of anti-coronavirus infection medicine and medicine. CN2022/114246855
96. Han K, Wang Y, Jia Y, et al. Application of Trazodone in preparation of coronavirus papain-like protease PL^{Pro} inhibitor drug. CN2020/112043706
97. Han K, Wang Y, Jia Y, et al. Application of Phenlzine in preparation of coronavirus papain-like protease inhibitor and coronavirus infection resisting medicine. CN2022/114469914

98. Han K, Jia Y, Wang Y. Application of Vilanterol in preparation of anti-coronavirus medicines and medicines. CN2022/114617862
99. Hanania NA, Feldman G, Zachgo W, et al. The efficacy and safety of the novel long-acting β2 agonist vilanterol in patients with COPD: a randomized placebo-controlled trial. Chest. 2012;142(1):119-27. doi:10.1378/chest.11-2231
100. Han K, Jia Y, Wang Y. Coronavirus papaya-like protease PL^{Pro} inhibitor Lomustine and application thereof. CN2022/114617864
101. Morasca L, Balconi G, Erba E, et al. Cytotoxic effect in vitro and tumour volume reduction in vivo induced by chemotherapeutic agents. Eur J Cancer (1965). 1974;10(10):667-71. doi:10.1016/0014-2964(74)90006-1
102. Montelione GT, Bafna K, Krug RM, et al. Compositions and methods utilizing protein inhibitors for synergistic inhibition and treatment of SARS-CoV-2. WO2022/076386
103. Bafna K, White K, Harish B, et al. Hepatitis C virus drugs that inhibit SARS-CoV-2 papain-like protease synergize with remdesivir to suppress viral replication in cell culture. Cell Rep. 2021;35(7):109133. doi:10.1016/j.celrep.2021.109133
104. Gammeltoft KA, Zhou Y, Duarte Hernandez CR, et al. Hepatitis C Virus Protease Inhibitors Show Differential Efficacy and Interactions with Remdesivir for Treatment of SARS-CoV-2 In Vitro. Antimicrob Agents Chemother. 2021;65(9):e0268020. doi:10.1128/AAC.02680-20
105. Muturi E, Hong W, Li J, et al. Effects of simeprevir on the replication of SARS-CoV-2 in vitro and in transgenic hACE2 mice. Int J Antimicrob Agents. 2022;59(1):106499. doi:10.1016/j.ijantimicag.2021.106499
106. Han K, Wang Y, Jia Y, et al. Application of non-peptide compound in preparation of products for inhibiting coronavirus. CN2022/114557990
107. Sayuk GS, Tack J. Tegaserod: What's Old Is New Again. Clin Gastroenterol Hepatol. 2022;20(10):2175-2184.e19. doi:10.1016/j.cgh.2022.01.024
108. Liu X, Xu X, Yuan W, et al. Use of tegaserod for the preparation of a medicament for the prevention or treatment of coronavirus infection. CN2021/112587522
109. Xu Z, Yao H, Xu Y, et al. Application of Nelfinavir in preparation of drug for preventing and treating novel coronavirus pneumonia. WO2021/164689
110. Tam HH, Kolesky DB, Wong MKC. Anti-viral compounds and methods of using same. WO2021/262799
111. Pegan S, Durie I, Freitas B, et al. Compositions and methods of treatment for sars-cov-2 through papain protease inhibition. WO2022/072975
112. Báez-Santos YM, Barraza SJ, Wilson MW, et al. X-ray structural and biological evaluation of a series of potent and highly selective inhibitors of human coronavirus papain-like proteases. J Med Chem. 2014;57(6):2393-2412. doi:10.1021/jm401712t
113. Sharma R, Agarwal S. Novel amide derivatives. WO 2022/070048
114. Wang J, Ma C. Small molecule inhibitors of SARS-CoV-2 viral replication and uses thereof. WO2021/207409
115. Wang J, Kitamura N, Ma C. Compositions and methods for inhibiting m pro protease activity and for preventing and treating Sars-Cov-2 infection. WO 2022/119756A1
116. Richter W, Abbas M, Weber L, Kumar A. M^{pro} cysteine protease inhibitors. WO 2022/040186A1
117. Meek TD, Li L, Tseng CK, et al. Inhibitors of cysteine proteases. WO 2022/187491A1
118. Jorgensen WL. Non-covalent inhibitors of the main protease of SARS-CoV-2 and methods of use. WO2022/150584A1

119. Buzard DJ, Keung W, Kumar DV, et al. Inhibitors of cysteine proteases and methods of use thereof. WO2023/023631A1
120. Arnold LD, Kerung W. Inhibitors of cysteine proteases and methods of use thereof. WO 2022/221686A1
121. Wang, J. Compositions and Methods for inhibiting M^{pro} and PL^{pro} protease activity and for preventing and treating SARS-CoV-2 infection. US 2022/0332683A1
122. Fu L, Ye F, Feng Y, et al. Both Boceprevir and GC376 efficaciously inhibit SARS-CoV-2 by targeting its main protease. Nat Commun. 2020;11(1):4417. doi:10.1038/s41467-020-18233-x
123. Lo JM, Huang C, Wu YB, et al. Compounds and methods for prevention and treatment of coronavirus infections. US 2021/0338690A1
124. Wu YB, Lo JM, Huang C, et al. Compounds and methods for prevention and treatment of virus infections. WO 2022/047147A1
125. Khan MA. Spiro-lactam compounds and methods of treating viral infections using the same. US 2023/0210851A1.
126. Meek TD, Tseng C-T, Drelich A, et al. Inhibitors of SARS-CoV-2 infection and uses thereof. WO2022/047054A2
127. Kos J, Mitrovic A, Sosoc I, et al. 8-Hydroxyquinoline cysteine protease inhibitors for use in the prevention and/or treatment of a corona virus disease. WO2022/117724A1
128. Kane CD, Soloveva V, Bavari S, et al. Cathepsin inhibitors for preventing or treating viral infections. WO2021/191875A1
129. Frueh F, Maneval D, Daley TE. Viral entry inhibitors and RNA polymerase inhibitors**.** WO 2021/203055A1
130. O'dowd C, Harrison T, Hewitt P, et al. Piperidinderivate als hemmer der ubiquitinspezifischen protease 7. EP4026832A1
131. Buhrlane S, Liu X. Usp7 inhibition. WO2020/086595A1
132. Wolberger C, Morgan MT, Suga H. Cyclic peptide inhibitors of USP22. WO2022/232567A2
133. Moussa C, Wolf, C Kaluvu B. Usp13 inhibitors and methods of use thereof. WO2022/155427A1
134. Buhrlage S, Varca A, Hu B. Fused tricyclic pyrimidine-thieno-pyridine small molecule inhibitors of ubiquitin-specific protease 28. WO2022/035806A1
135. Rountree JSS, Whitehead SK, Sheperd SD, et al. Pharmaceuticals compounds as inhibitors of ubiquitin specific protease 19 (usp19). WO2022/200523A1
136. Lee KH, Kronbichler A, Park DD, et al. Neutrophil extracellular traps (NETs) in autoimmune diseases: A comprehensive review. Autoimmun Rev. 2017;16(11):1160-73
137. Beemelmanns C, GUO H, Höfgen S, Rischer M, RAGUŽ L, Keiff F, Goris T. Barnesin a, derivatives and uses thereof. WO2019/170705A1
138. Zamyatnin AA, Rudzinska M, Parodi A, et al. Specific peptide inhibitors of cysteine cathepsins. WO2021/251850A1
139. Beal R, Sand B, Brunner S, et al. Inhibition of spontaneous metastasis via protein inhibitors of cysteine proteases. WO2019/055884A2
140. Gonzalez DJ, Dulai P, Mills R. Therapeutic approach for treating inflammatory bowel disease. US202/2395489A1
141. Siewinski MZ. Inhibitors of cysteine peptidases isolated from natural raw materials and use of the inhibitors in medicine and veterinary medicine. WO2020/167152A1
142. Hao Z, Gong S, Shen D, et al. Small molecule inhibitor of cathepsin c and medicinal use thereof. WO2022/117059 A1

143. Anderskewitz R, Binder F, Grauert M, et al. Methods for treating pulmonary emphysema using substituted 2-aza-bicyclo[2.2.1]heptane-3-carboxylic acid (benzyl-cyano-methyl)-amides inhibitors of cathepsin c. US2023/0310373 A1
144. Walensky LD, Bird GH, Guerra R, et al. Selective targeting of apoptosis proteins by structurally-stabilized and/or cysteine-reactive noxa peptides. WO2019/118719A1
145. Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. Nature. 2010;463(7283):899-905. doi:10.1038/nature08822
146. Liu X, Chen X, Shi J, et al. Pyrimidine-2,4-diamine compound and prepareation method and application thereof. US2023/0150947 A1
147. Methods for enhancing cytotoxic cancer therapy through inhibition of ATG4B. US2021069202A1
148. Xu S, Sun L, Huang B, et al. Medicinal chemistry strategies of targeting HIV-1 capsid protein for antiviral treatment. Future Med Chem. 2020;12(14):1281-4
149. Ren Y, Ma Y, Cherukupalli S, et al. Discovery and optimization of benzenesulfonamides-based hepatitis B virus capsid modulators via contemporary medicinal chemistry strategies. Eur J Med Chem. 2020;206:112714
150. Zhang L, Wei F, Zhang J, et al. Current medicinal chemistry strategies in the discovery of novel HIV-1 ribonuclease H inhibitors. Eur J Med Chem. 2022;243:114760
151. Hou L, Zhang Y, Ju H, et al. Contemporary medicinal chemistry strategies for the discovery and optimization of influenza inhibitors targeting vRNP constituent proteins. Acta Pharm Sin B. 2022;12(4):1805-24
152. Gao S, Huang T, Song L, et al. Medicinal chemistry strategies towards the development of effective SARS-CoV-2 inhibitors. Acta Pharm Sin B. 2022;12(2):581-99
153. Ding D, Xu S, da Silva-Júnior EF, et al. Medicinal chemistry insights into antiviral peptidomimetics. Drug Discov Today 2023;28(3):103468.
154. Ma Y , Frutos-Beltrán E , Kang D , et al. Medicinal chemistry strategies for discovering antivirals effective against drug-resistant viruses. Chem Soc Rev. 2021;50(7):4514-40