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REVIEW

CATHEPSIN C INHIBITION AS A POTENTIAL TREATMENT STRATEGY IN CANCER

**Brice Korkmaz^{1*}, Anne-Sophie Lamort^{2§}, Roxane Domain^{1§}, Céline Beauvillain³,
Artur Gieldon⁴, Önder Yildirim², Georgios T. Stathopoulos², Moez Rhimi⁵,
Dieter E. Jenne^{2,6} and Ralph Kettritz^{7,8}**

¹INSERM UMR-1100, “Research Center for Respiratory Diseases” and University of Tours, 37032, Tours, France

²Comprehensive Pneumology Center (CPC) and Institute for Lung Biology and Disease (iLBD), Helmholtz Center Munich-German Research Center for Environmental Health (HMGU) and Ludwig-Maximilian-University (LMU), Munich, Bavaria, 81377, Germany. A member of the German Center for Lung Research (DZL), Gießen, Hesse, 35392, Germany

³University of Angers, University of Nantes, Angers University Hospital, INSERM UMR-1232, CRCINA, Innate Immunity and Immunotherapy, SFR ICAT, F-49000 Angers, France

⁴Faculty of Chemistry, University of Gdansk, 80-308 Gdansk, Poland

⁵Microbiota Interaction with Human and Animal Team (MIHA), Micalis Institute, AgroParisTech, Université Paris-Saclay, INRAE, Jouy-en-Josas, France.

⁶Max Planck Institute of Neurobiology, 82152 Planegg-Martinsried, Germany

⁷Experimental and Clinical Research Center, Charité und Max-Delbrück-Centrum für Molekulare Medizin in der Helmholtz-Gemeinschaft (MDC), Berlin, Germany

⁸Nephrology and Intensive Care Medicine, Charité-Universitätsmedizin, Berlin, Germany

[§]Equal contribution

***Corresponding author:** Brice Korkmaz

INSERM UMR-1100 “Research Center for Respiratory Diseases”,

University of Tours, Faculty of Medicine

10 Bld. Tonnellé, 37032, Tours, France

e-mail: brice.korkmaz@inserm.fr

Tel: 0033 2 47 36 63 86

Abstract

Epidemiological studies established an association between chronic inflammation and higher risk of cancer. Inhibition of proteolytic enzymes represents a potential treatment strategy for cancer and prevention of cancer metastasis. Cathepsin C (CatC) is a highly conserved lysosomal cysteine dipeptidyl aminopeptidase required for the activation of pro-inflammatory neutrophil serine proteases (NSPs, elastase, proteinase 3, cathepsin G and NSP-4). NSPs are locally released by activated neutrophils in response to pathogens and non-infectious danger signals. Activated neutrophils also release neutrophil extracellular traps (NETs) that are decorated with several neutrophil proteins, including NSPs. NSPs are not only NETs constituents but also play a role in NET formation and release. Although immune cells harbor large amounts of CatC, additional cell sources for this protease exists. Upregulation of CatC expression was observed in different tissues during carcinogenesis and correlated with metastasis and poor patient survival. Recent mechanistic studies indicated an important interaction of tumor-associated CatC, NSPs, and NETs in cancer development and metastasis and suggested CatC as a therapeutic target in a several cancer types. Cancer cell-derived CatC promotes neutrophil recruitment in the inflammatory tumor microenvironment. Because the clinical consequences of genetic CatC deficiency in humans resulting in the elimination of NSPs are mild, small molecule inhibitors of CatC are assumed as safe drugs to reduce the NSP burden. Brensocatib, a nitrile CatC inhibitor is currently tested in a phase 3 clinical trial as a novel anti-inflammatory therapy for patients with bronchiectasis. However, recently developed CatC inhibitors possibly have protective effects beyond inflammation. In this review, we describe the pathophysiological function of CatC and discuss molecular mechanisms substantiating pharmacological CatC inhibition as a potential strategy for cancer treatment.

Abbreviations: BAL, broncho-alveolar lavage; Cat, cathepsin; IL, interleukin; MMP, matrix metalloproteinase; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NSP, neutrophil serine protease; PDB, Protein Data Bank; PLS, Papillon-Lefèvre syndrome; PR3, proteinase 3.

Contents

1. Introduction
 2. Neutrophil serine proteases in cancer
 3. Neutrophil cathepsin C
 4. Tumor cell-derived cathepsin C
 5. Pharmacological inhibition of cathepsin C
 6. Conclusion
- Acknowledgments
- Author contributions
- Conflict of interest
- Support statement
- References

1. Introduction

Cancer represents a global health problem and is ranked as the second most common cause of death by the World Health Organization. Among the different cancer types, lung cancer contributed worldwide to more than 2 million new cases in 2020 with an unfavorable survival rate [1, 2]. Substantial efforts have been made to better characterize lung cancer subtypes by the identification of key mutation patterns [3, 4] and deciphering molecular disease mechanisms [5] particularly with respect to metastases. Upon adhesion molecule loss, cancer cells from the primary tumor disseminate outside the original tissue where they can remain dormant and clinically undetectable for many years before they generate secondary tumors or metastases [6]. In addition to harboring primary tumors, the lungs are commonly affected by metastases originating from different primary cancer locations including kidney, stomach, pancreas, bladder, male and female genitourinary tract, breast, liver, head and neck [7]. Evolution and progression of several cancer types correlate with smoking, inflammation and tumor-associated immune cells [5, 8]. These immune cells are involved in both pro-tumorigenic and anti-tumorigenic roles. Among these immune cells, neutrophils present in the tumor microenvironment are referred to as tumor-associated neutrophils (TANs), which are further categorized as anti-tumoral and pro-tumoral. Mimicking the dichotomy of macrophages into M1 and M2, a nomenclature of N1 and N2 has been proposed to define anti- or pro-tumor neutrophils respectively. However, given the complexity of the activation and differentiation states of the TAN population that includes also granulocytic myeloid-derived suppressor cells (G-MDSCs), the population is best summarized as pro- and anti-tumor TANs [5, 9]. Antitumor neutrophils inhibit tumor cell proliferation and survival both directly and indirectly by recruiting additional immune cells. On the contrary, pro-tumoral neutrophils induce immunosuppression and promote tumor invasion, metastasis and angiogenesis through their release of various compounds such as MMP9, elastase, HMGB1, S100A8, S100A9 and BV8 [8, 10-13]. Smoking leading to chronic lung inflammation is also associated with higher risk of recurrence and death in breast cancer patients. Epidemiological studies revealed that the increased risk of cancer recurrence after a period of clinical remission may be linked to inflammation [14-16]. Neutrophil recruitment to the tumor site *via* chemokine interactions with

their corresponding receptors leads to poor patient outcome [17, 18]. These myeloid cells then generate a microenvironment and biological niche that favors cancer progression. Yet, the exact chemokine network and the pathways sustaining recruitment of these cells to the tumor remains largely unknown [19]. Among recruited immune cells, neutrophils were identified as critical actors in experimental models for cancer cell awakening [8] as well as cancer-associated thrombosis [20]. The latter causes significant morbidity and mortality in cancer patients. Activated neutrophils release neutrophil extracellular traps (NETs) that are decorated with several neutrophil proteins, including neutrophil serine proteases (NSPs) matured by cathepsin C (CatC, (EC 3.4.14.1)) [21] (**Figure 1**) and tissue factor [22]. Consequently, NET formation by blood neutrophils exerts procoagulant and prothrombin effects suggesting that NETs may provide a therapeutic target to inhibit cancer-related thrombosis. Enhanced susceptibility of neutrophils to form NETs is observed in several preclinical cancer models suggesting systemic and local cancer effects, which stimulate neutrophils to release NETs that in turn promote the inflammatory and prothrombotic environment. These observations suggest a pivotal role for neutrophils in cancer biology. Proteases released from activated or dying neutrophils provide candidates promoting both primary tumors and metastasis.

Cancer development and metastasis rely on increased expression and proteolytic activity of several proteases. Cathepsin D, an aspartyl protease, was shown to promote breast cancer metastasis [23]. Cysteine proteases are additional actors in the metastatic process [24, 25]. For example, cathepsin B expression is increased in a variety of human tumors and is involved in tumor invasion and metastasis, potentially by degrading basement membranes and extracellular matrix. Moreover, recent data implicating NSPs and their maturing cysteine protease CatC (**Figure 1**) in cancer development raised a new interest in exploring these proteases both as disease mediators and pharmacological targets. In addition, plasminogen and metalloprotease family members are of importance because of their ability to degrade basement membrane and extracellular matrix components [26-29]. Inhibition of MMPs (matrix metalloproteases) in cancer tissue resulted in reduced tumor invasion, angiogenesis and migration [24, 25]. These experimental data that clearly established the importance of proteases in cancer, together with the fact that existing protease inhibitors, mainly metalloprotease inhibitors, failed in previous clinical studies [30], underscore the

need for new, more effective protease inhibitors suitable for preclinical and clinical exploration. Additional research aspects include inhibitor specificity and determination of the optimal disease stage for inhibitor administration.

We next focus on NSPs and CatC, and their mechanistic involvement in cancer biology and discuss the potential of pharmacological CatC inhibition for cancer treatment.

2. Neutrophil serine proteases in cancer

The neutrophil serine protease family includes neutrophil elastase (NE, EC 3.4.21.37), proteinase 3 (PR3, EC 3.4.21.76), cathepsin G (CatG, EC 3.4.21.20) [31, 32], and recently discovered neutrophil serine protease-4 (NSP-4) [33, 34]. NSPs belong to the chymotrypsin family and are synthesized as inactive precursors in promyelocytes containing an N-terminal prodipeptide to avoid unwanted protein degradation in the endoplasmic reticulum or Golgi apparatus (**Figure 1**). N-terminal propeptides are removed in post-Golgi organelles by CatC. The X-ray three-dimensional structures of mature NSPs have the two six-stranded β -barrel domains typical of chymotrypsin-like serine proteases. The active-site residues are located in a crevice between the two β -barrels [31, 35] (**Figure 2**).

Activated neutrophils release NSPs either as soluble molecules or attached to extracellular traps (NETs). NETs are involved in killing of invading microorganisms [21] but also in thrombosis [36, 37] and tissue injury in autoimmune vasculitis [38]. NE not only decorates NETs but is also essential for NETs formation by promoting nuclear chromatin decondensation [39, 40]. The proteolytic activities of NSPs are mainly regulated by serpin (serine protease inhibitors) and chelonianin families [31, 32]. NSPs participate in intracellular and extracellular pathogen killing, but also shape the inflammatory response [41, 42] by cleavage of extracellular matrix proteins and processing of inflammatory mediators as shown in inflammatory and autoimmune diseases [31, 43-45]. Additionally, activated neutrophils transfer proteolytically active NSPs to neighboring cells, as shown for endothelial cells, resulting in cleavage of intracellular substrates and subsequently

endothelial cell injury [46]. Thus, neutrophils create an microenvironment that, at least in part, depends on NSPs and NETs.

Recent data suggest that the neutrophil-associated inflammatory microenvironment, including NSPs and NETs, is not only involved in inflammatory diseases but also in cancer [5]. Extracellular proteolytically active NSPs were detected in several cancer types and correlate with poor prognosis. Zoidakis *et al.* identified PR3 as an antigenic marker of bladder cancer by urinary proteome analysis [47]. PR3 was frequently elevated in the urine of bladder cancer patients but not detected in the urine of healthy controls. Guarino *et al.* showed that urinary PR3 is proteolytically active and originates from cancer-associated neutrophils as NE and myeloperoxidase (MPO), additional markers of neutrophil activation, were also present in the urine of patients with bladder cancer [48]. Urine samples from patients with a bladder cancer were concentrated, and both PR3 protein as well as proteolytic PR3 activity was monitored using a selective PR3-FRET substrate. Active PR3 concentration was detected in the cancer patient samples and estimated to be 0.1-1 nM based on the rate of substrate hydrolysis (**Figure 3**). NE is detected in broncho-alveolar lavage (BAL) fluids from patients with lung cancer [49]. Furthermore, the amount of NE and MPO in the BAL fluid was significantly higher in patients with lung cancer compared to COPD (chronic obstructive pulmonary disease) patients. Recently, Lerman and Hammes reviewed the current evidence for NE involvement in primary tumor growth and secondary organ metastasis [50]. As highlighted in several studies, pharmacological inhibition or gene-deletion of NE reduced cancer progression in *in vivo* mouse models [11, 50]. Furthermore, the cleavage pattern of peptides found in cancer tissue revealed a strong preference for small hydrophobic residues at P1 residues, making NE the most likely candidate involved in the observed cancer-specific proteolytic processing [51]. Albrengues *et al.* showed that sustained experimental lung inflammation induced by lipopolysaccharide (LPS) administration or tobacco smoke exposure promotes NET-formation and that these NETs in turn awaken dormant malignant mammary cancer cells [8]. Mechanistic analysis in murine models revealed that inflammation-activated neutrophils drive cancer cell awakening by laminin processing *via* NET-associated NE and MMP-9 [8]. The neo epitopes generated upon proteolytic laminin cleavage induced transformation of the previously dormant cancer cells into aggressively growing metastases by

activating integrin signaling. Furthermore, Teijeira *et al.* showed that NETs secreted by inflammation-activated neutrophils surrounded cancer cells and blocked the cytotoxic anti-cancer effects of immune cells, which impaired tumor clearance [52, 53]. Inhibition of NE reduced the extension of cancer cell-induced NETs and NETs-mediated cancer cell invasion. Thus, NE and NETs promote pro-tumorigenic actions of neutrophils and are therefore considered as therapeutic targets for multiple cancer types [8, 50].

In addition to their validated functional roles in tumor biology discussed above, it appears that *CTSC*, *ELANE*, *PRTN3*, *CTSG*, and *PRSS57* genes are frequently co-mutated in a significant proportion (6.1%) of human cancers from the cancer genome atlas (TCGA) pan-cancer dataset [54, 55]. Most changes were copy number alterations, either deletions or amplifications (**Figure 4**). Interestingly, NSP gene-alterations are enriched in some tumor types, like ovarian carcinomas, sarcomas, melanomas, and esophageal carcinomas that feature alteration frequencies of more than 10%. A number of additional cancers, like endometrial, bladder, cervical, and lung carcinomas show alterations in 5-10% of the cases, whereas other cancers, like mesothelioma, thyroid, renal, and pancreatic carcinomas have virtually none of these abnormalities (**Figure 5**). In addition to copy number alterations, single nucleotide variants (SNPs) are seen in all five genes (*CTSC*, *ELANE*, *PRTN3*, *CTSG*, and *PRSS57*), which do not feature mutational hotspots (**Figure 6**). Interestingly, patients with NSP-alterations were of a younger age at diagnosis, had higher indices of genomic alteration, aneuploidy, and hypermutation, as well as elevated hypoxia scores compared to patients without such changes (not shown). These findings suggest that cancers with NSP mutations represent a subclass of particularly aggressive tumors. This conclusion is also supported by clinical observations, since NSP-altered TCGA pan-cancer patients display higher relapse rates, higher tumor grade, and decreased progression-free survival. Moreover, specifically *CTSC* and *PRTN3*-altered patients also exhibit decreased overall survival (**Figure 7**). While all the above observations need to be confirmed in additional cohorts, and while the variable distribution of NSP alterations across human cancer types may confound these results, a number of future research questions arise. For example, do the observed NSP gene mutations occur in tumor or stromal cells? The first possibility of NSP gene mutations occurring in cancer cells calls for studies exploring intracellular (cell-autonomous) versus

paracrine NSP effects on tumor biology. No such studies have been reported so far. The second, more unlikely but not yet excluded possibility for NSP gene mutations in tumor-infiltrating stromal cells also warrants further investigation. Conceivably, neutrophils with abundant transcription of NSP genes also acquire mutations in these genes similar to what has been discovered for epithelial tissues [56]. Together, these clinical and experimental data establish mechanistic NSP implications in a variety of cancers and suggest that reducing NSPs has beneficial anti-tumor effects.

3. Neutrophil cathepsin C

CatC, also known as dipeptidyl peptidase 1 (DPP1, EC 3.4.14.1), is a ubiquitously expressed (**Figure 8**) lysosomal aminopeptidase belonging to the C1 family of papain-like cysteine peptidases [57]. CatC catalyzes the removal of dipeptides from the N-terminal end of peptides and proteins. CatC is highly, but not exclusively, expressed in immune cells and has a role in shaping innate immune responses. The best-characterized physiological function of CatC is the activation of immune cell-associated serine proteases, including NSPs [58, 59], mast cell chymase [60] and lymphocyte granzymes [61, 62]. CatC is a unique member of the cysteine cathepsins that functions as a tetrameric protease [57].

CatC is initially synthesized as a single chain ~60-kDa monomer (**Figure 8C**) that spontaneously associates to form proteolytically inactive proCatC homodimers [63] (**Figure 9**). The sequence of proCatC (residues: 1-439) is partitioned into three instead of two domains, namely a ~16-kDa N-terminal exclusion domain (1-119), a ~11-kDa internal propeptide (120-206), and a ~30-kDa C-terminal catalytic domain (207-439) with a papain-like structure. The proteolytically active homotetrameric CatC is generated by the proteolytic removal of its propeptide segment and the processing of the catalytic domain into a heavy (207-370) and light chain (371-439) [57]. Cathepsin-like proteases are identified as proCatC processing and activating proteases *in vitro* [63, 64]. The propeptide of proCatC is involved in the formation of a stable dimer and in two consecutive steps of the proCatC maturation process. The removal of the propeptides occurs during the first step. The processing of the catalytic domain generating heavy and light chains during the second step is

essential for the final homo-tetrameric active conformation [64]. Molecular modeling analysis suggests that the tetrameric structure of CatC stabilizes the exclusion domain in the correct position conferring CatC its aminopeptidase activity [64]. The carboxylic group of the Asp1 side chain of CatC activation domain sequences, is responsible for the anchoring of the N-terminal amino group of CatC substrates [65] (**Figure 10**).

Mature CatC converts proNSPs into their proteolytically active forms during neutrophil differentiation in the bone marrow [58, 59]. After liberation of their N-terminal propeptides by CatC, NSPs undergo an important conformational change and adopt a stable mature conformation [41]. Once matured, CatC and active NSPs are stored together in cytoplasmic granules [66]. Activated neutrophils secrete mature CatC into the extracellular milieu together with other granule-associated proteases. In patients with neutrophilic lung inflammation, mature CatC is found in sputa from cystic fibrosis and asthma patients [66], in tracheal aspirates from mechanically ventilated patients with pneumonia [67], and in the BAL fluid from patients with non-small lung cancer, even when containing only low neutrophil numbers [66]. Because proCatC but not mature CatC, is constitutively secreted by bronchial epithelial cells present in lung secretions from healthy individuals, mature CatC is considered as a biomarker of active pulmonary neutrophilic inflammation [66]. However, the functional role of extracellular CatC originating from neutrophils at the inflammatory sites remains unknown.

Loss of function mutations in the CatC gene *CTSC* cause PLS characterized by severe pre-pubertal periodontitis and palmoplantar keratoderma without marked immunodeficiency [68, 69]. PLS white blood cell lysates from a cohort of 24 patients with established missense, frameshift or nonsense *CTSC* mutations did not contain CatC activity or immunoreactive CatC protein [70]. The missense mutation Leu172Pro within the propeptide segment altered the proCatC stability as well as the *in vitro* maturation of the recombinant zymogen [64]. Mutated CatC with missense mutations is suspected to be degraded during neutrophil differentiation [70, 71]. The lack of CatC activity reduces both the activity and the amount of NSPs in neutrophils from PLS patients [34, 59, 70]. Using a highly sensitive and selective PR3 substrate, PR3 activity in a cohort of 20 PLS patients amounted to only 1% to 4% compared to those of healthy controls [70] indicating that CatC is the major but not unique

proNSPs maturing protease. Furthermore, proteomic analysis of azurophil granule proteins in healthy versus PLS patient samples showed that approximately 15 proteins were differentially expressed [71]. Importantly, two different teams showed that PLS neutrophils are incapable of forming NETs due to lack of NE activity [71, 72].

These data establish the importance of neutrophil CatC for NSP maturation and suggest that NSPs abrogation does not cause severe infections.

4. Tumor cell-derived cathepsin C

Although neutrophils and other immune cells harbor large amounts of CatC, additional sources for this protease exist. Upregulation of CatC expression was observed in different tissues during carcinogenesis. Ruffell *et al.* investigated the function of CatC in breast cancer and squamous cell carcinoma in mice and found increased CatC protein and proteolytic activity in both types of these tumor tissues compared to healthy control tissues [73]. Results from *Ctsc*^{-/+} and *Ctsc*^{-/-} mice revealed a tissue-specific role of CatC in squamous cell but not breast cancer development. Moreover, immune cell and fibroblast CatC was reported to be critical for promoting angiogenesis and tumor growth [73]. Zhang *et al.* investigated the differential expression of CatC in hepatocellular carcinoma (HCC) and normal liver tissues by bioinformatic tools and tissue microarrays [74]. CatC was upregulated in HCC patients and correlated with poor prognosis. Using gain/loss-of-function assays, the authors identified CatC as an oncogenic protein that promoted the proliferation and metastasis of HCC cancer cells. Mechanistically, overexpression of CatC activated the TNF- α /p38 MAPK pathway that assisted HCC growth and metastasis. The tumor-promoting role of CatC was also shown for renal carcinoma cells by Chiang *et al.* [75]. Kim *et al.* [76] and Pal Khalet *et al.* [77] reported the role of CatC in the regulation of autophagy-mediated cancer cell proliferation.

Recently, Xiao *et al.* highlight the potential of tumor cell-secreted mature CatC to promote lung metastasis of breast cancer cells [78]. The authors first identified proteins that were regulated in human breast cancers and murine breast cancer cell lines with lung metastatic capacities. CatC was

amongst the highest regulated proteins and its expression and secretion were elevated in lung metastases of breast cancer. Moreover, high CatC expression in primary tumors was negatively correlated with patients' overall survival. Hence, tumor-derived CatC provides a prognostic survival marker. In addition, in experimental murine breast cancer models, a strong correlation was observed between CatC expression and infiltrating neutrophils that deposited NETs in both primary tumors and lung metastasis. The authors reported the dual function of tumor-secreted mature CatC in neutrophil recruitment to metastatic niches and NETosis induction. To elucidate how mature CatC affects neutrophil functions, Xiao *et al.* explored the possibility that neutrophil membrane-bound NSP zymogens were proteolytically activated by tumor cell-derived CatC. They suggest that indeed a conversion of membrane-bound PR3 (PR3^{mb}) from the zymogen into the mature form by tumor derived CatC activity occurs, and that this effect provides a mechanistic link to neutrophil infiltration and NET formation. The authors proposed that the PR3^{mb} zymogen, predominantly detected as a 34-kDa molecule that is distinct from the 27-kDa mature granule-stored form, was the result of differential post-translational modifications. The authors argue that the tumor CatC-mediated activation of PR3^{mb} resulted in IL-1 β processing and NF κ b activation in the neutrophils. In the proposed scheme, this activation leads to the secretion of IL-6 and CCL3 further enhancing neutrophil recruitment to metastatic niches. The CatC-PR3-IL-1 β axis was also suggested to contribute to p38 activation and ROS (reactive oxygen species) production resulting in NET formation that in turn support metastatic growth of tumor cells. In a final pursuit to confirm that proteolytic PR3 activity is essential for neutrophil IL-1 β production, recombinant active *human* PR3 was added to conditioned medium from *CTSC*-silenced cancer cells supplemented with fetal bovine or horse serum. The authors observed that active PR3 indeed restored IL-1 β generation even in the absence of tumor cell-derived CatC.

The data obtained by Xiao *et al.* identified CatC as a potentially important actor in breast cancer. However, a note of caution is warranted with respect to the proposed molecular mechanisms in neutrophils. The authors suggest that the main action of tumor-secreted mature CatC on local and systemic neutrophil functions is caused by the small pool of inactive pro-PR3 on the neutrophil

surface that is activated by CatC. In our view, this mechanistic concept is highly speculative and needs further critical evaluation. For example, using mice with myeloid cells deficient in PR3 as tumor recipients would reveal additional information. PR3-deficient neutrophils are expected to show significantly less IL-1 β generation in response to tumor cell-derived CatC thereby strengthening the specific role of PR3 in this process. Additional concerns pertain to the suggested role of proPR3 on the neutrophil surface. We agree with the authors that most PR3^{mb} on quiescent human neutrophils is proteolytically inactive. In fact, we and others suspected previously that constitutively expressed PR3^{mb} on quiescent human neutrophils could be the zymogen form of PR3 [79, 80] and, in addition, PR3 becomes inactivated by binding to the neutrophil-specific CD177 membrane receptor [81]. In contrast, large amounts of active PR3 are translocated from the intracellular granules to the cell membrane during neutrophil activation. Unlike constitutive PR3^{mb}, induced PR3^{mb} exposed on activated neutrophils is proteolytically active [79, 80]. However, we are concerned firstly, about the high molecular weight of murine PR3^{mb} observed by Xiao *et al.* Granule-associated PR3 and PR3^{mb} have similar molecular weights and no major post-translational differences despite their different destinations after sorting and targeting of PR3 to either the outer leaflet of cellular membranes or to granules. Secondly, only a very small fraction of the total neutrophil PR3 pool is exposed and accessible on the cell surface of human neutrophils [70]. Activation of such a small amount of PR3^{mb} by tumor-derived CatC leading to the observed tumor-associated activities *in vivo* is implausible. Importantly, almost no PR3^{mb} is exposed on the surface of murine neutrophils because the hydrophobic patch of human PR3 that mediates membrane association is not conserved in murine PR3 [82, 83]. Thirdly, in our hands, it is not easy to activate pro-PR3 and related pro-NSPs by CatC, even under controlled laboratory conditions in solution. High concentrations of the substrates (at least 0.3 mg/ml) and optimal acidic conditions are required (personal observation by D.E. Jenne). It is unlikely that such conditions exist in the tumor environment. Moreover, it is very difficult to distinguish mouse PR3 from mouse NE at low concentrations using activity measurements, as mouse PR3 is more similar to mouse NE than human PR3 is to human NE [31, 41]. In fact, the PR3 substrate used in the Xiao *et al.* study was designed to be specific for human PR3 and it is not cleaved by mouse PR3 [84, 85]. A better approach would be to employ catalytically active and inactive murine PR3 and murine pro-PR3

as a control. Finally, recombinant active PR3 was added to cell cultures containing fetal bovine or horse serum rich in the main physiological PR3 inhibitor alpha-1 antitrypsin, and exogenous active *human* PR3 was administered intravenously. In both circumstances, the presence of high alpha-1 antitrypsin concentrations rapidly inhibits the proteolytic PR3 activity and we cannot see how it possibly can exert its functions in the neutrophil. Thus, we believe that tumor-derived CatC within the inflammatory tumor microenvironment employs other molecules than PR3^{mb} to activate neutrophils that in turn promote lung metastasis.

Together these data support the notion that tumor-derived CatC promotes growth and metastasis of several cancer types and that CatC inhibition has beneficial anti-tumor effects.

5. Pharmacological inhibition of cathepsin C

CatC is increasingly recognized as a pharmacological target for blocking NSP activity and NETosis in neutrophil-driven inflammatory and autoimmune diseases [31, 44]. The observation that CatC knock-out mice were protected in NSPs-driven experimental inflammation models supports the therapeutic strategy of CatC inhibition in these diseases [43, 44, 58]. Because of the discussed role of CatC, NE, and NETs in cancer, pharmacological CatC inhibition can also be envisioned as a therapeutic strategy to prevent primary tumor growth and metastasis [86]. While several CatC inhibitors have been discovered and tested in preclinical models (including IcatC_{XPZ-01}, BI-9740 (Jerke *et al.*, *in preparation*), EGFR-derived inhibitor [87], non-peptidyl non-covalent CatC inhibitor [88], GSK-2793660 [89] and brensocatib [90] have advanced the furthest in clinical development.

Two dipeptidyl nitrile inhibitors IcatC_{XPZ-01} ((*S*)-2-amino-*N*-((1*R*,2*R*)-1-cyano-2-(4'-(4-methylpiperazin-1-ylsulfonyl)biphenyl-4-yl)cyclopropyl)butanamide), IC₅₀.CatC = 15 nM) [91] and brensocatib (formerly INS1007/AZD7986, (*S*)-*N*-((*S*)-1-Cyano-2-(4-(3-methyl-2-oxo-2,3-dihydrobenzo-[d]oxazol-5-yl)phenyl)ethyl)-1,4-oxazepane-2-carboxamide, IC₅₀.CatC = 22 nM) [92] were recently shown to effectively block the maturation of NSP zymogens *in vitro* and *in vivo* (**Figure 10**). Nitrile compounds are the best investigated group of CatC inhibitors [44]. The nitrile group of these compounds reacts with the active Cys234 site resulting in the reversible formation of a

thioimidate adduct (**Figure 11**). In rodents, IcatC_{XPZ-01} [91] and brensocatib [92] reached sufficient levels in the bone marrow to inhibit CatC and downstream NSPs activation. In a murine model of rheumatoid arthritis, prolonged administration of IcatC_{XPZ-01} resulted in sustained anti-arthritic activity [91]. IcatC_{XPZ-01} was also evaluated in an orthotopic mouse lung transplantation (LTx) model after 18 h cold storage of the graft [93]. Recipient mice treated with IcatC_{XPZ-01} prior to LTx showed improved early graft function. Furthermore, prolonged administration of IcatC_{XPZ-01} in *Macaca fascicularis* resulted in almost complete elimination of NSPs in white blood cells confirming that a reduction in NSPs, comparable to that seen in PLS patients, is possible using pharmacological CatC inhibitors [94] (**Figure 11**). Zymogen conformations of NSPs are susceptible to proteolysis leading to their proteolytic degradation within intracellular granules at very early stages of neutrophil maturation [70, 94]. Xiao *et al.* showed that systemic administration of brensocatib effectively inhibited breast cancer lung metastasis in their mouse model, preserved body weight, and enhanced overall animal survival time [78]. This effect is encouraging, even though the underlying molecular mechanisms need to be better characterized.

Brensocatib was the first nitrile CatC inhibitor that was assessed in clinical trials [92]. The safety, tolerability and pharmacokinetics/pharmacodynamics of single and multiple oral doses were evaluated in a randomized, placebo-controlled, phase 1 study started in 2014. Daily doses of 10, 25 and 40 mg brensocatib in 81 healthy subjects treated for 28 days resulted in significant reduction of neutrophil NE activity without serious side effects [95]. Safety, efficacy and pharmacokinetics of brensocatib were then evaluated in the WILLOW phase 2 study, an international, randomized, double-blind placebo-controlled trial [96]. 256 adults with non-cystic fibrosis bronchiectasis, a chronic inflammatory lung disease defined as a permanent dilatation of the bronchi [97], were treated once daily for 24 weeks with 10 mg or 25 mg brensocatib or placebo. Brensocatib reduced the primary endpoint, namely the time to first pulmonary exacerbation, and a key secondary study endpoint, namely the rate of pulmonary exacerbations, and significantly reduced sputum NE by approximately ~85% [96]. The efficacy, safety, and tolerability of brensocatib is currently being evaluated in a global, randomized, double-blind, placebo-controlled phase 3 trial termed ASPEN (NCT number: NCT04594369). Patients with non-cystic fibrosis bronchiectasis were randomized to receive

brensocaticib 10 mg, 25 mg, or placebo once daily for 52 weeks. Acute respiratory distress syndrome (ARDS), a severe outcome of COVID-19 is also associated with neutrophilic inflammation. Elimination of NSPs may reduce lung injury and thereby prevent the irreversible pulmonary failure in COVID-19 patients [67, 98]. To explore this effect further, brensocaticib is currently being evaluated in the STOP-COVID19 (Superiority Trial of Protease inhibition in COVID-19, EudraCT Number: 2020-001643-13) trial in 300 hospitalized patients with COVID-19 pneumonia [98].

6. Conclusion

The best-characterized function of CatC is the activation of immune cell-associated serine proteinases. However, additional CatC roles, particularly in non-immune cells, are beginning to emerge. Recent analyses in cancer patients revealed increased CatC expression in some malignant cells that correlated with metastasis and poor survival implicating CatC as a biomarker. Mechanistically, tumor-derived CatC was suggested to regulate immune cell functions in the tumor microenvironment thereby promoting tumor growth and metastasis. Specifically, tumor CatC, in addition to neutrophil CatC, may control NSPs and NETs formation – both implicated in cancer biology. Synthetic compounds are now available to inhibit CatC in neutrophils, tumors and other host tissues. Brensocaticib is a CatC inhibitor that is currently being evaluated in a phase 3 clinical trial in patients with non-cystic fibrosis bronchiectasis. If effective in preclinical cancer models, brensocaticib could possibly be repurposed for treatment of cancer patients.

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Author contributions

All authors contributed to the writing and revision process of the manuscript. B. Korkmaz supervised the work.

Conflict of interest

B. Korkmaz has been paid for the time spent as a committee member for advisory boards (INSMED Inc., NJ, USA), other forms of consulting (Neuprozyme Therapeutics Aps (Denmark), Santhera Pharmaceuticals (Switzerland)), symposium organization (INSMED Inc., NJ, USA) and travel support, lectures or presentations, outside the submitted work. R.K served on the INSMED advisory board.

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FIGURE LEGENDS:

Figure 1. NSPs and CatC in the inflammatory tumor microenvironment. (*Left*) Maturation of NSPs. NSP zymogens are processed and converted to active enzymes by CatC during the promyelocytic stage of neutrophil differentiation in the bone marrow. Pro-dipeptides of NSPs, removed by CatC, are shown in orange. (*Right*) Release of NSPs and CatC from activated neutrophils. Mature NSPs and CatC stored in intracellular granules are released from activated neutrophils following degranulation. NSPs are also detected in the inflammatory tumor microenvironment on neutrophil extracellular traps (NETs) consisting of extracellular nuclear DNA from activated neutrophils. Expression and release of cancer cell-derived CatC. Cancer cells can secrete mature and/or proCatC.

Figure 2. Three-dimensional structures of human NSPs. (*Left*) Solvent accessible surfaces and (*Right*) ribbon plots of NSPs. The depicted 3D structures are based on the 3D atomic coordinates of PR3 [Protein Data Bank (PDB) code: 1FUJ, [99]], NE in complex with inhibitor GW475151 [PDB code: 1H1B [100]], CatG in complex with the peptidyl phosphonate inhibitor Suc-Val-Pro-Phe^P-(OPh)₂ [PDB code: 1CGH, [101]] and NSP4 in complex with the chloromethylketone inhibitor Phe-Phe-Arg-CMK [PDB code: 4Q7Z, [102]]. The structures on the left show the positive (blue) and negative (red) electrostatic surface potential. Ribbon plots of NSPs show the characteristics of the trypsin/chymotrypsin family, the two asymmetric β -barrels, and the C-terminal α -helix. Positions of the subsites S4 to S2' in the active site region of PR3 are shown in orange [103]. Bound inhibitors are shown as green stick models.

Figure 3. NSP expression in human bladder cancer. (A) Schematic representation of different bladder cancer stages progressing from Ta to T4. Ta: This stage refers to noninvasive papillary carcinoma; T1: The tumor has spread to the connective tissue called the lamina propria that separates the lining of the bladder from the muscles beneath, but does not involve the bladder wall muscle;

T2: The tumor has spread to the muscle of the bladder wall; T3: The tumor has grown into the perivesical tissue that surrounds the bladder; T3A: Microscopic perivesical tissue infiltration; T3B: Macroscopic perivesical tissue infiltration; T4: The tumor has spread to any of the following: the abdominal or pelvic wall, prostate or seminal vesicles, uterus, or vagina. **(B)** Western blot detection of secreted PR3 in 10x concentrated urine samples of patients with bladder cancer using an anti-PR3 antibody (*left*). Selective labeling of PR3 using the activity-based probe biotin-Pro-Tyr-Asp-Ala^P-(O-C₆H₄-4-Cl)₂ [48] in concentrated (10x) urine samples. **(C)** Activity determination of secreted PR3 in the urine of patients with different stages of bladder cancers. Rate of hydrolysis of selective PR3 substrate ABZ-VAD(nor)VADYQ-EDDnp in concentrated (10x) urine from patients with bladder cancer. Proteolytically active PR3 in unconcentrated urine was 0.1-1 nM based on the rate of substrate hydrolysis. Immunodetection and PR3 activity quantification in urine according to cancer stages (numbers of patients displaying benign lesions, n=11, and early stages of bladder cancer; Ta, n = 10, T1, n = 12, and T2, n = 12) [48] **(D)** Western blot analyses of NE (*upper panel*) and myeloperoxidase (MPO) (*lower panel*) in concentrated (10x) urine samples from healthy volunteers and patients with bladder cancer.

Figure 4. Alteration of NSPs in human cancers. Shown are *CTSC*, *ELANE*, *PRTN3*, *CTSG*, and *PRSS57* mutation frequencies in the cancer genome atlas (TCGA) pan-cancer dataset (n = 10,189 patients), in form of a clinical and molecular data plot with alteration frequencies **(A)** and co-mutation matrix **(B)**. Columns represent patients and rows clinical/molecular features. Data were accessed at <https://www.cbioportal.org/> on March 1st 2021. TCGA acronyms were from <https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations> accessed on the same date. *P*, probability, hypergeometric test; FDR *q*, probability, false discovery rate.

Figure 5. NSP gene-alteration in cancer types. Cumulative *CTSC*, *ELANE*, *PRTN3*, *CTSG*, and *PRSS57* mutation frequencies across cancer types from the TCGA pan-cancer dataset (n = 10,189) **(A)**, **(B)**. Data were accessed at <https://www.cbioportal.org/> on March 1st 2021. TCGA acronyms were from

<https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations> accessed on the same date. *P*, probability, χ^2 test.

Figure 6. NSPs mutations in human cancers. Lollipop plots of *CTSC* (A), *ELANE* (B), *PRTN3* (C), *CTSG* (D), and *PRSS57* (E) mutations across cancer types from the TCGA pan-cancer dataset (n = 10.189). Data were accessed at <https://www.cbioportal.org/> on March 1st 2021. TCGA acronyms were from <https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations> accessed on the same date.

Figure 7. Frequency distributions of CatC/NSP mutations and progression-free patient survival.

(A) Frequency distributions of *CTSC*, *ELANE*, *PRTN3*, *CTSG*, and *PRSS57* mutant cancers compared with non-altered cancers from the TCGA pan-cancer dataset (n = 10.189) according to relapse (*top*) and cancer grades G1 (considered as a low grade) to GX (considered as a high grade) (*bottom*). G1: well differentiated (low grade); G2: moderately differentiated (intermediate grade); G3: poorly differentiated (high grade); G4: undifferentiated (high grade); GB: grade borderline; GX: grade cannot be assessed (undetermined grade). (B, C) CatC and NSP mutations characterize high-grade human cancers that tend to relapse. Progression-free survival of patients with *CTSC*, *ELANE*, *PRTN3*, *CTSG* and *PRSS57* mutant cancers compared with patients with non-altered cancers (B) and overall survival of patients with *CTSC*, *ELANE*, *PRTN3*, *CTSG*, *PRSS57*, or none of the above mutations (C) in the TCGA pan-cancer dataset (n = 10.189). *CTSC* and *PRTN3*-mutant cancers are associated with decreased patient survival. Shown are Kaplan-Meier survival estimates. Data were accessed at <https://www.cbioportal.org/> on March 1st 2021. *P*, probability, χ^2 test (A) and log-rank test (B, C).

Figure 8. Physiologic CatC expression in different tissues. (A) *CTSC* mRNA (blue) and protein (brown) expression were ranked as low (little square), medium (middle-long bar), and high (long horizontal bar). Low mRNA, <25 normalized transcripts per million; medium, 25-50 normalized transcripts per million; high, >50 normalized transcripts per million. Low protein, expression by <25%

of individuals tested; medium, protein expression by 25–50% of individuals tested; high, protein expression by >50% of individuals tested. RNA-seq and immunohistochemistry data were from The Human Protein Atlas (<https://www.proteinatlas.org/>). **(B)** Immunoperoxidase detection of CatC (brown) in diverse healthy organs lung, gallbladder, testis, kidney) using anti-CatC sc-74590 HRP. Images provided by Charline Bubel (Santa Cruz Biotechnology). **(C)** Western blot analysis of secreted CatC in 10x concentrated urine samples of seven healthy donors using an anti-CatC antibody (sc-74590) directed against the 23 kDa CatC heavy chain. The samples are concentrated 10 times [104]. The unprocessed pro-CatC single chain and the heavy chain liberated upon processing are detected in urine samples. The diagram on the right illustrates the processing of the 60 kDa proCatC into the mature CatC. The arrow indicates the CatC heavy chain.

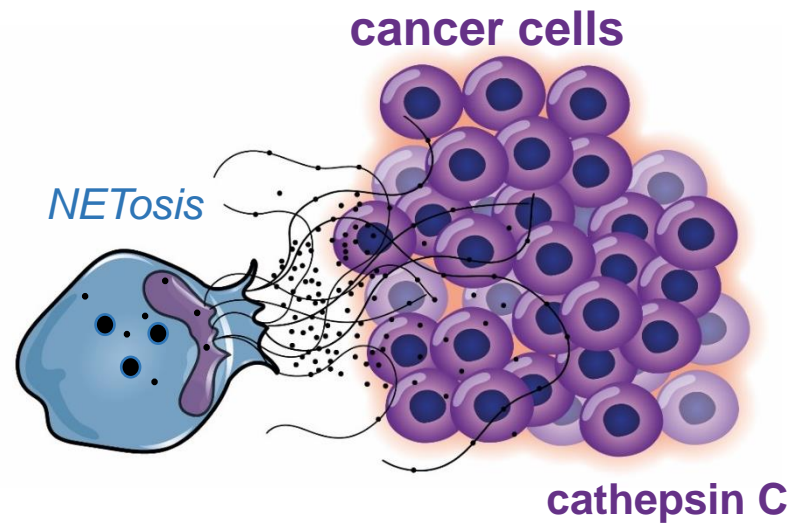
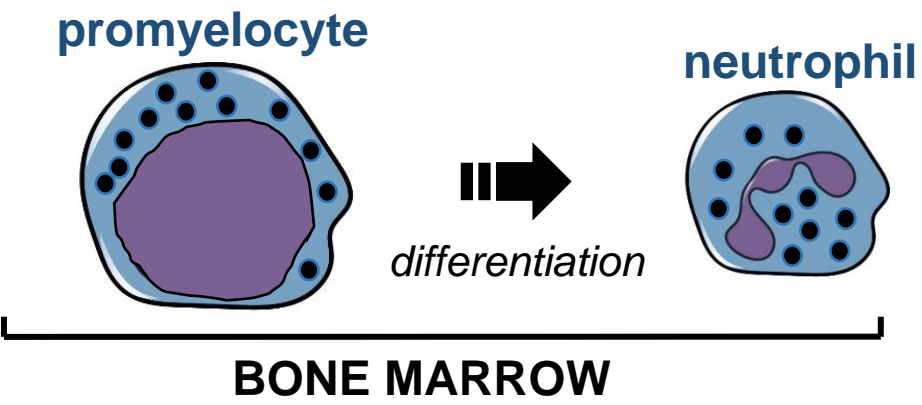
Figure 9. Human proCatC and mature CatC structures. **(A)** Model structure of human proCatC monomer. The surface representation of the exclusion domain and papain-like catalytic domain is shown and colored in grey. The ribbon plot shows the topology of the propeptide (residues ¹²⁰Thr-His₂₀₆) in orange. Leu, Arg and Asp (₃₆₉Leu-Arg/Asp₃₇₁, the sequence containing the cleavage sites of proCatC maturing proteases between the heavy and light chains) are colored in yellow, blue and red respectively. **(B)** ProCatC homodimer constituted of monomers A and B in surface representation with the same color coding as in A is shown. **(C)** Processing by CatL-like proteases of two homodimers and their association to form proteolytically mature tetrameric CatC. The models [64] were based on the crystal structures of human CatC (PDB code: 3PDF [105]), and the crystal structures of trypanosome brucei proCatB (PDB code: 4HWY [106]).

Figure 10. NSP degradation as a result of CatC inhibition. Structure of two CatC inhibitors, IcatC_{XPZ-01} [91] and brensocatib [92].

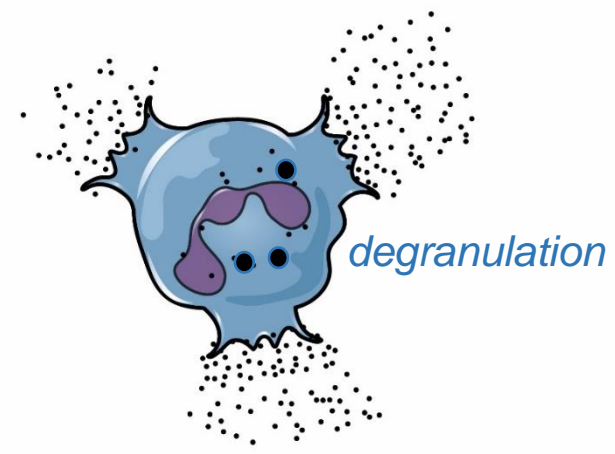
Figure 11. Model structure of the complex formed between IcatC_{XPZ-01} or a FRET substrate and human CatC. (*Left*) Solvent accessible surface of CatC colored according to its positive (blue) and negative (red) electrostatic potential. (*Right*) The carbon atoms of CatC and compounds are shown in turquoise and green, respectively. Oxygen, nitrogen, sulfur atoms are colored in red, blue and yellow, respectively. Critical residues in the vicinity of the active site involved in binding e.g. Asp-1 (D1) and Pro-3 (P3) are labeled in black. The position P2 according to the nomenclature of Schechter and Berger [107] is highlighted by the violet label “P2” and shows residues of the compounds interacting with CatC. **(A)** The coordinates of CatC complex with amino-N-((1*R*,2*R*)-1-cyano-2-(4'-((4-methylpiperazin-1-yl)sulfonyl)-[1,'-biphenyl]-4-yl)cyclopropyl)cyclohexane-1-carboxamide (PDB code: 6IC7 [91]) was used for molecular modeling. **(B)** The model of internally-quenched fluorescent substrate Thi-Ala(Mca)-Ser-Gln-Tyr(3-NO₂)-NH₂ in complex with CatC [108] (based on PDB code: 2DJF [65]). The presence of the exclusion domain blocks the enzyme active site beyond the S2 subsite, making it only accessible to the N-terminus of its substrates. The CatC S2 subsite is the deepest site and it has the shape of a pocket.

Processing by cathepsin C

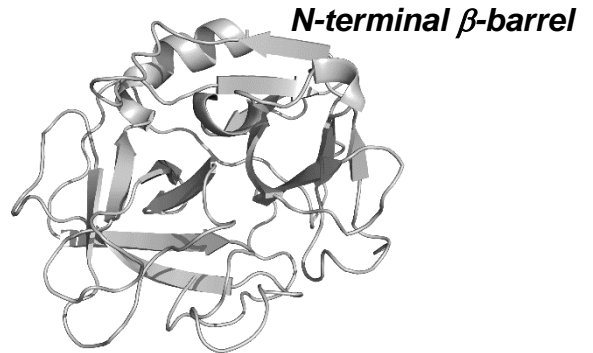
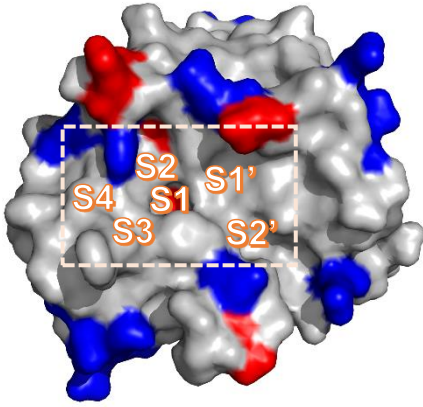
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AQ IIGGHE.....	pro-NSP4



proteinase 3, elastase,
cathepsin G, NSP-4, cathepsin C

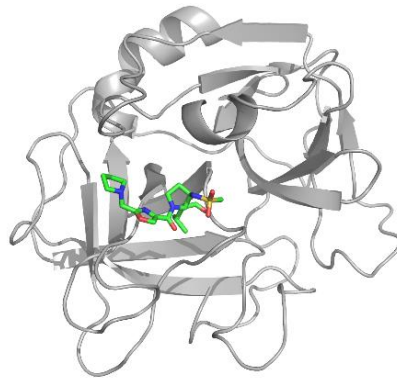
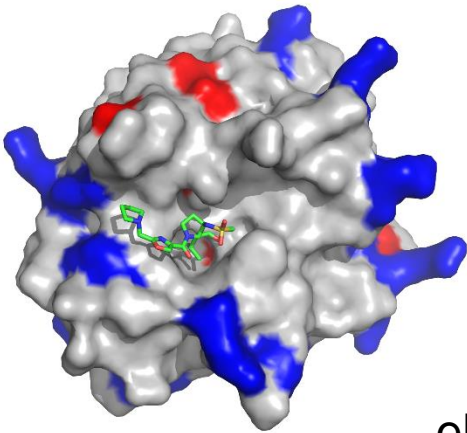


INFLAMMATORY MICROENVIRONMENT

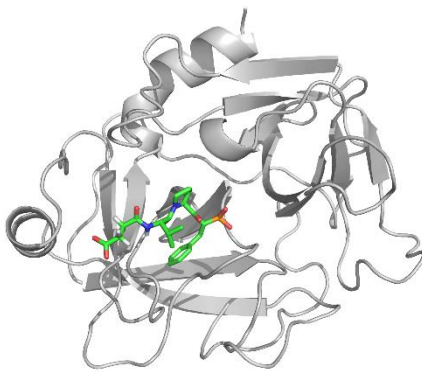
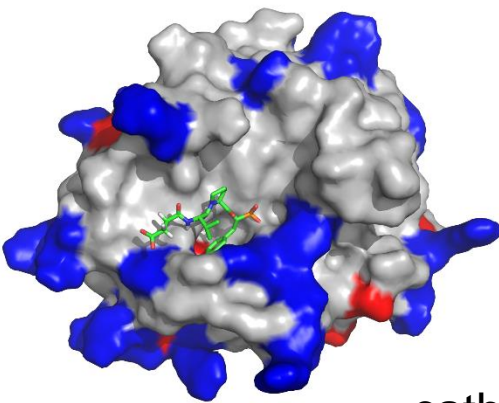


C-terminal β -barrel

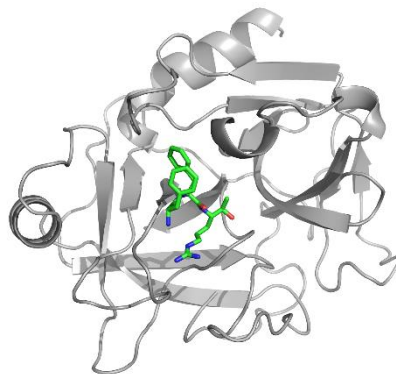
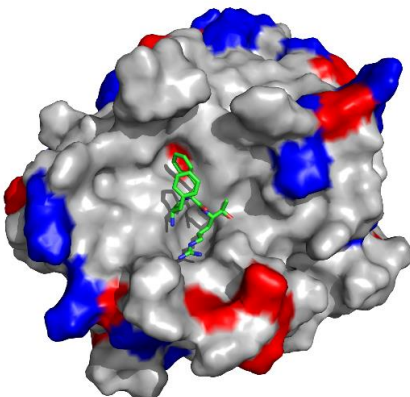
proteinase 3



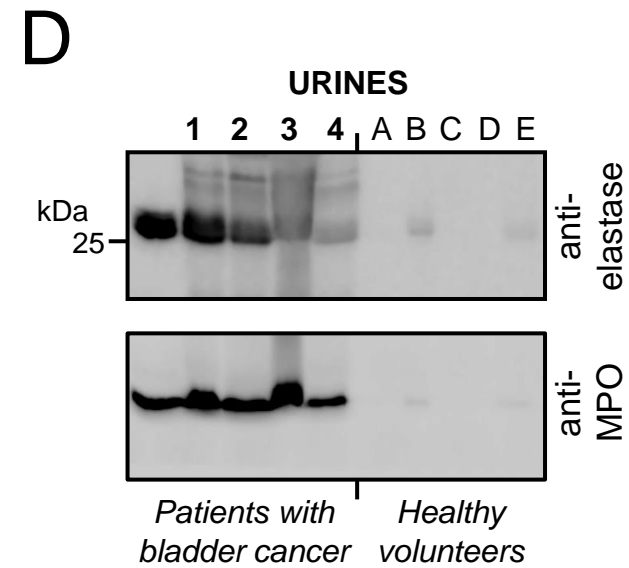
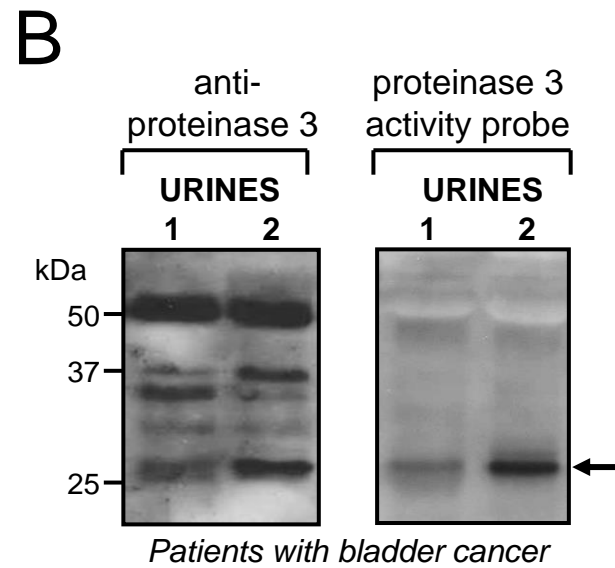
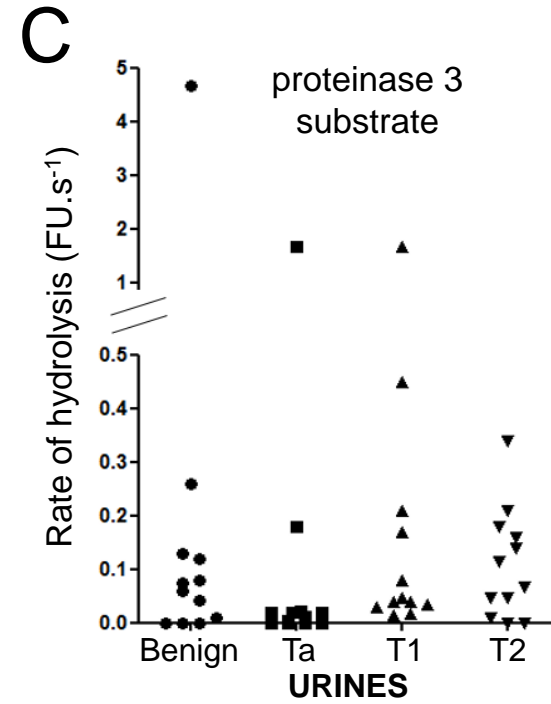
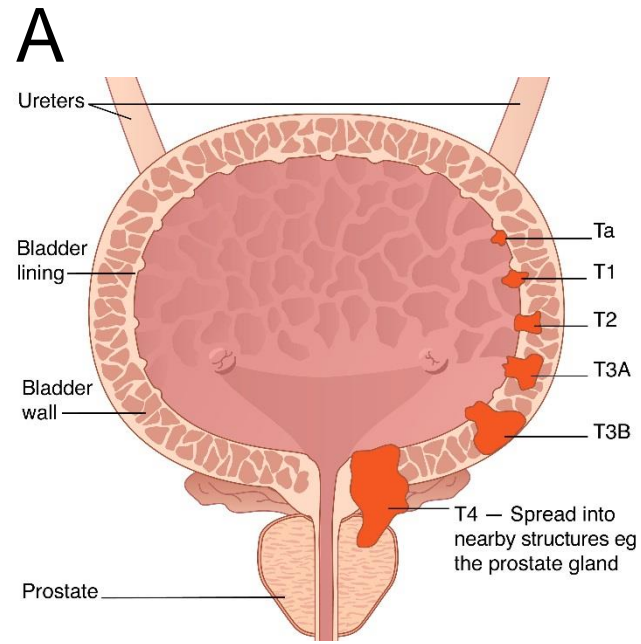
elastase



cathepsin G

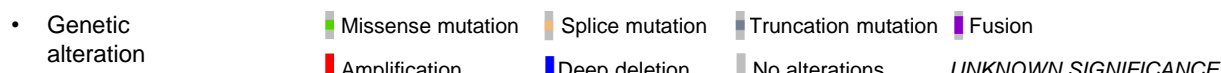
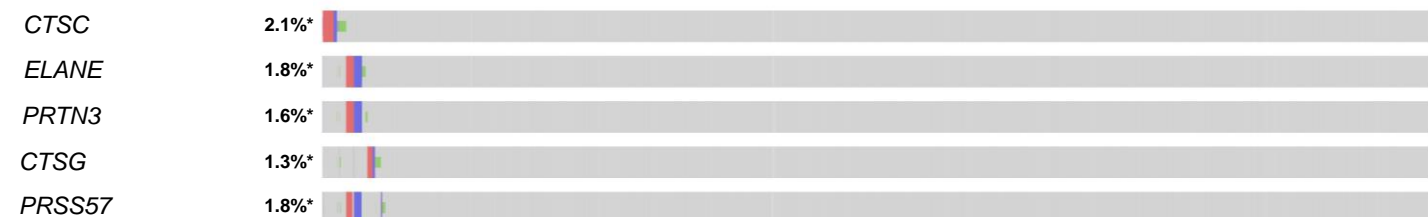
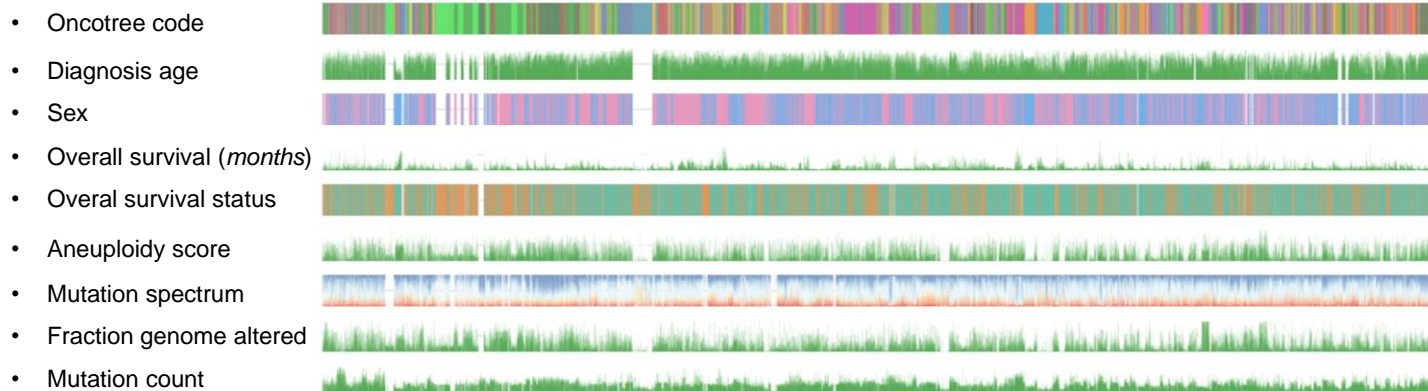


NSP-4

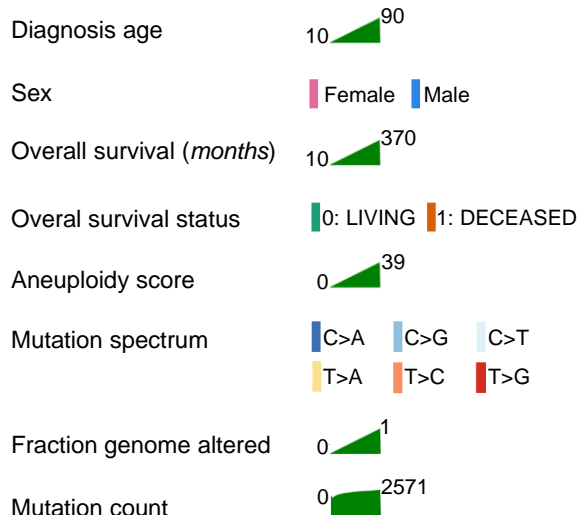
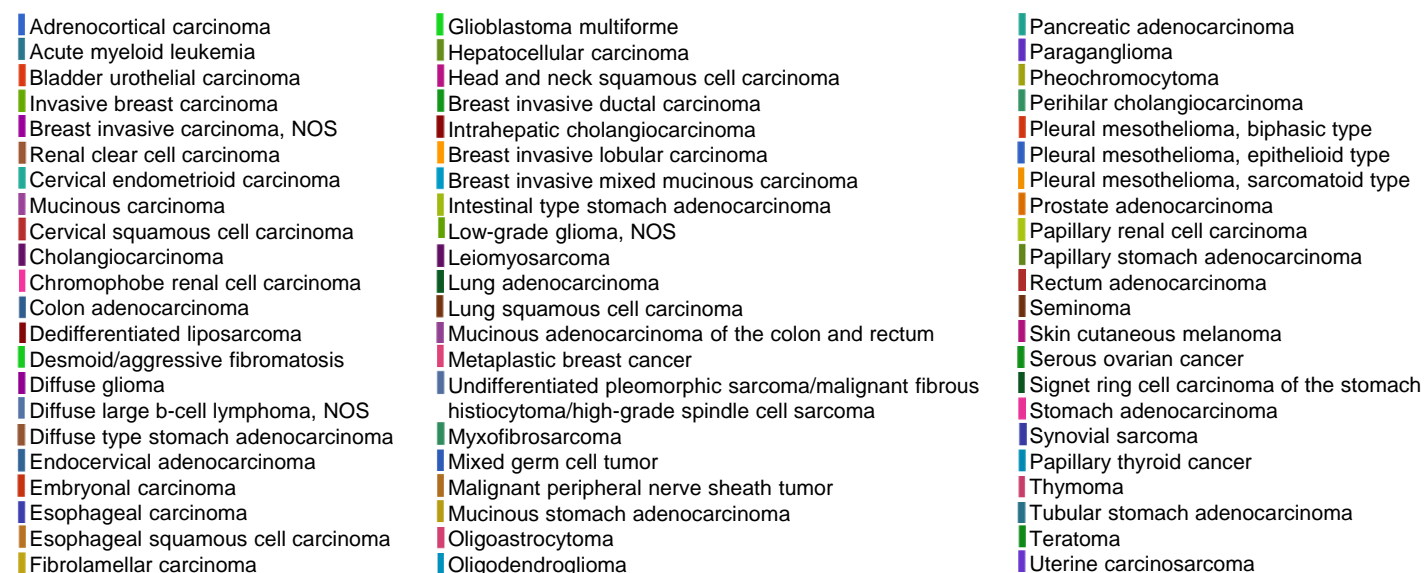


A

Total alteration frequency = 619 of 10189 patients = 6.1%

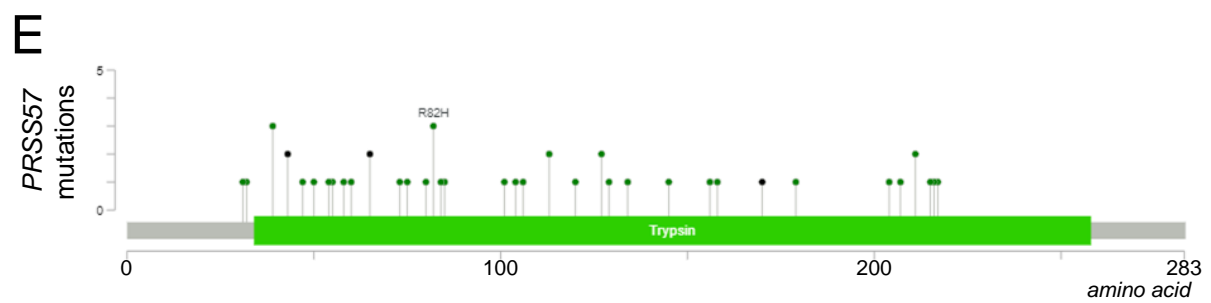
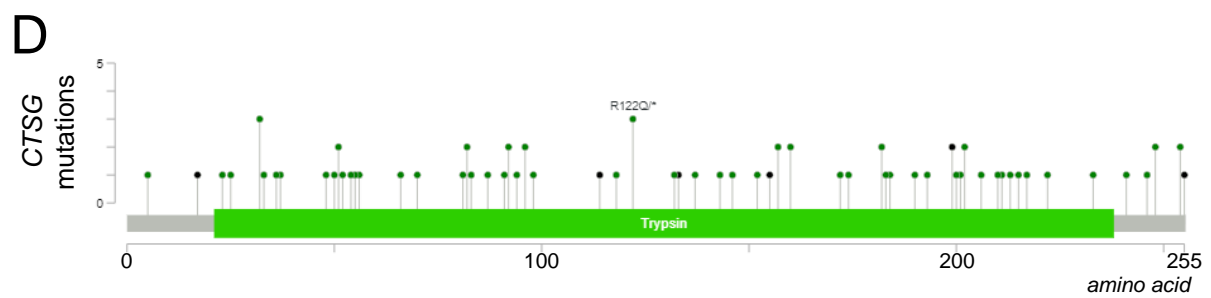
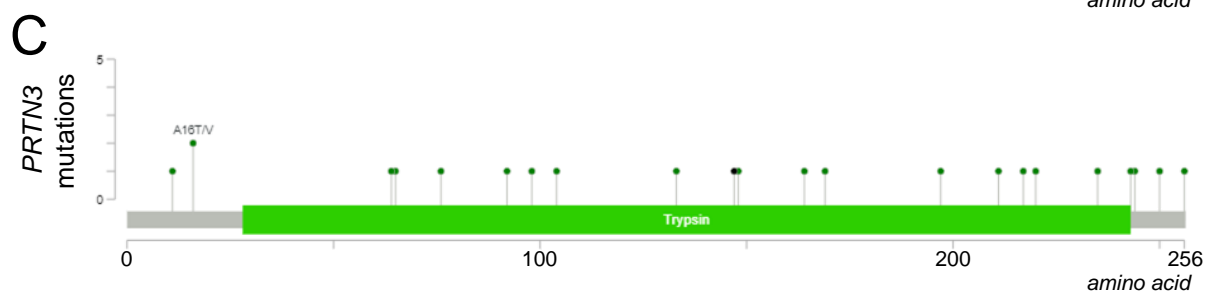
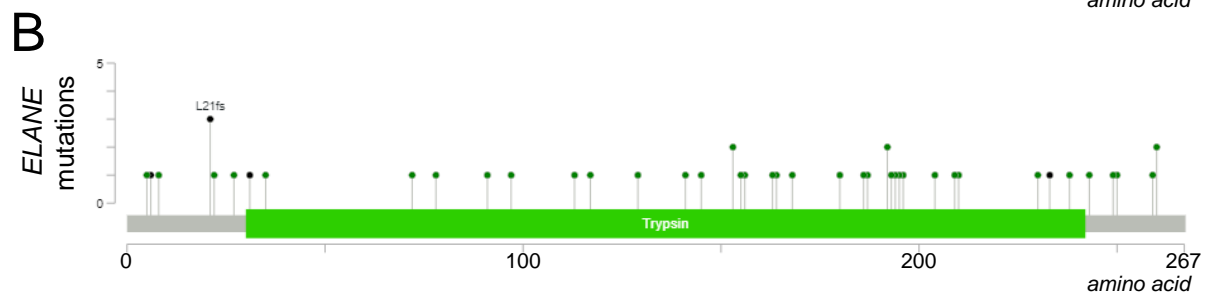
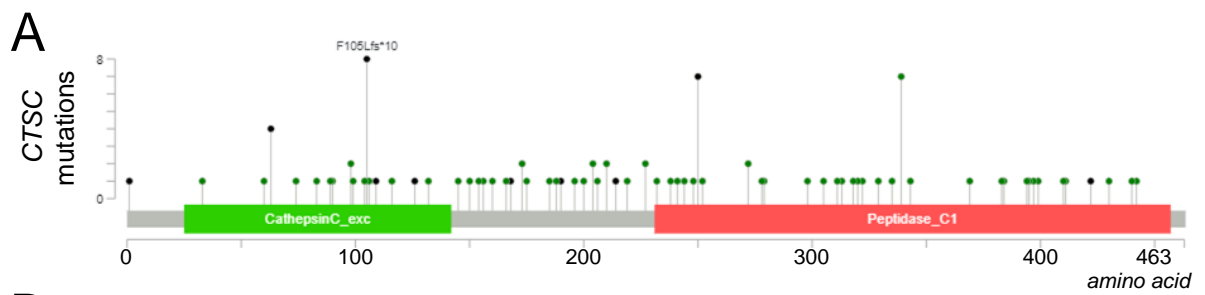


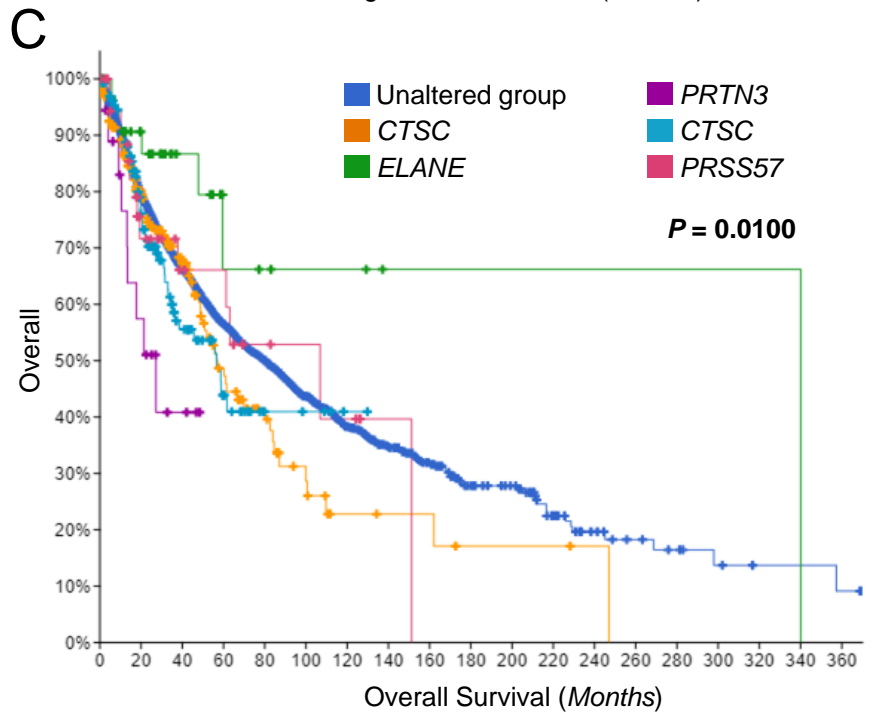
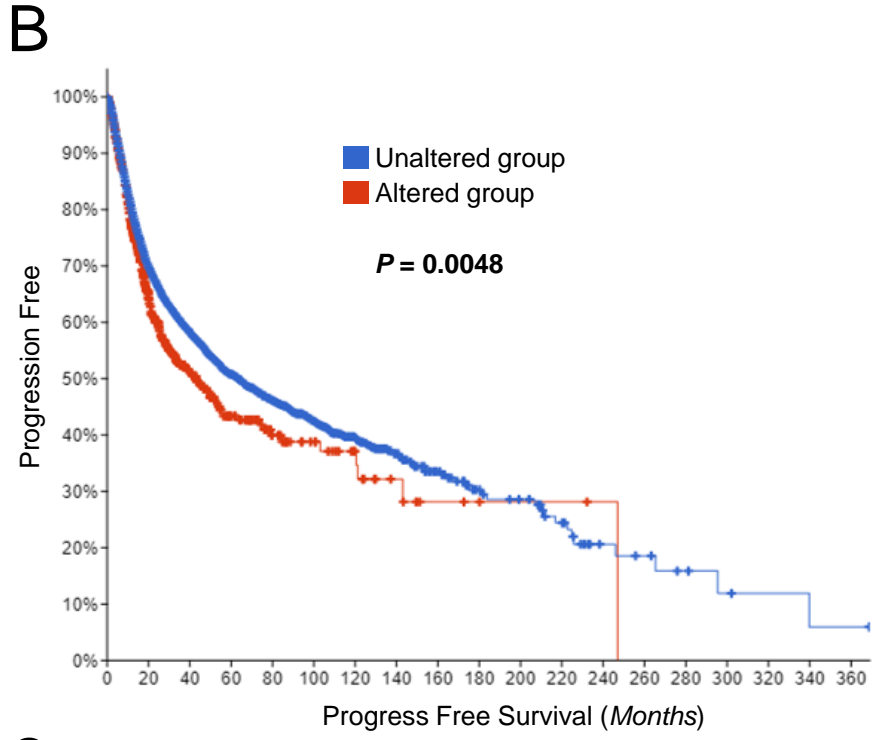
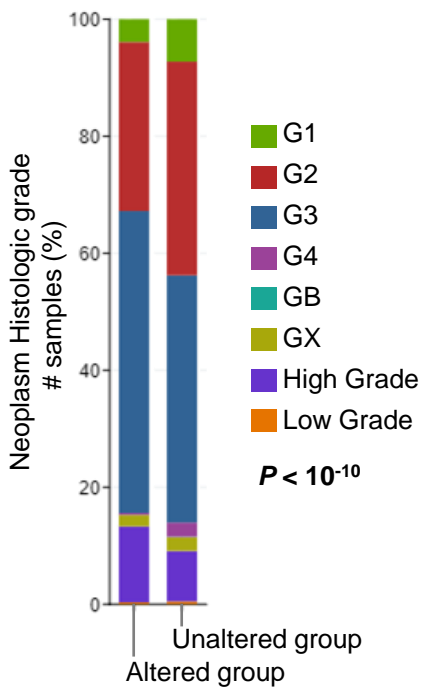
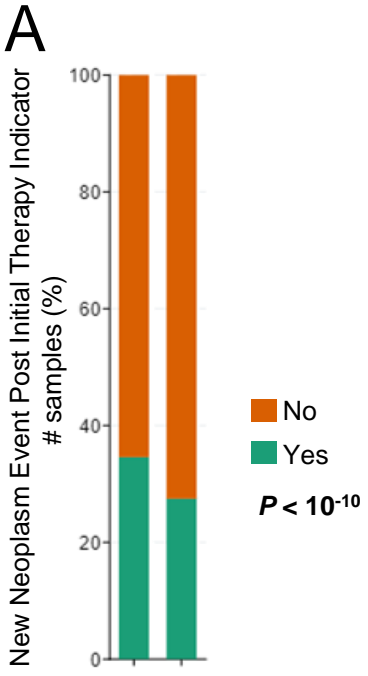
Oncotree code



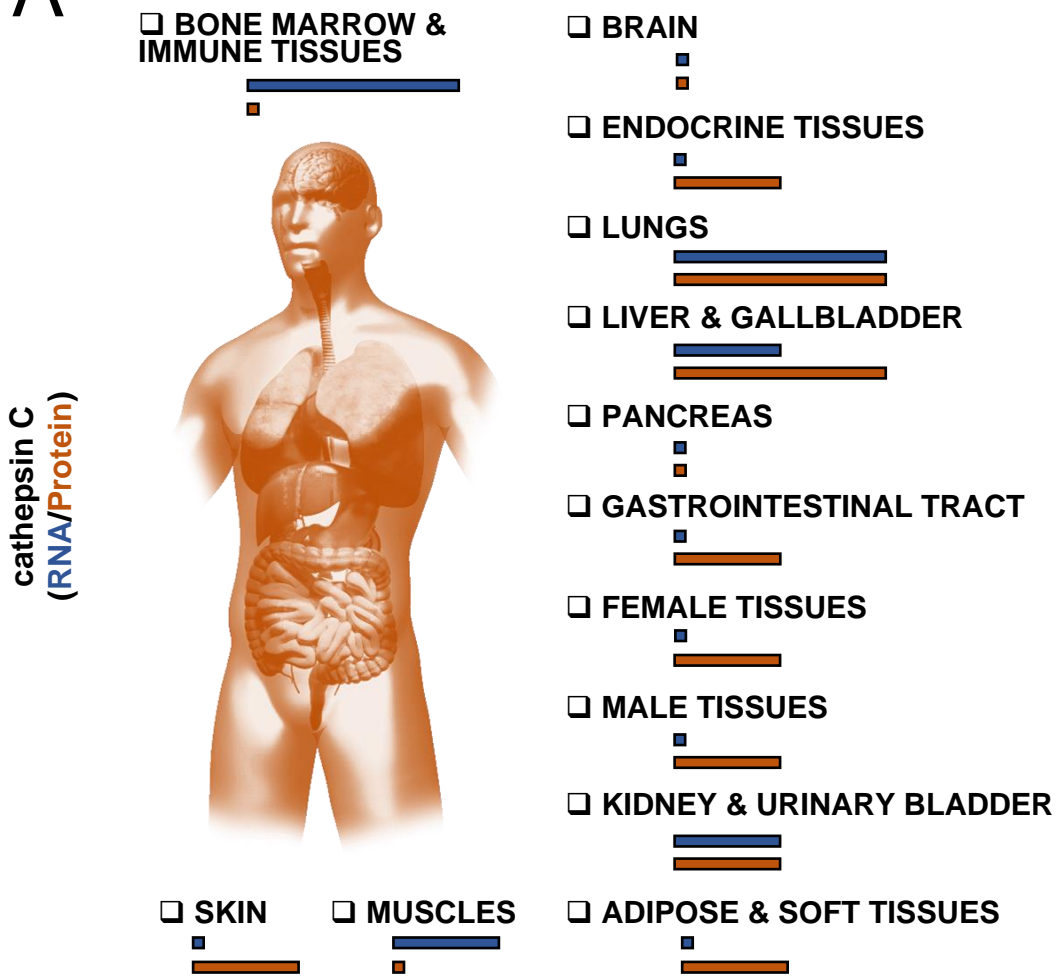
B

Gene A	Gene B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	P	FDR q
<i>ELANE</i>	<i>PRTN3</i>	9973	42	21	155	>3	<0.001	<0.001
<i>PRTN3</i>	<i>PRSS57</i>	9965	41	50	135	>3	<0.001	<0.001
<i>ELANE</i>	<i>PRSS57</i>	9945	61	49	136	>3	<0.001	<0.001
<i>CTSC</i>	<i>CTSG</i>	9831	216	132	12	2.049	<0.001	<0.001
<i>CTSC</i>	<i>PRSS57</i>	9791	215	172	13	1.783	<0.001	<0.001

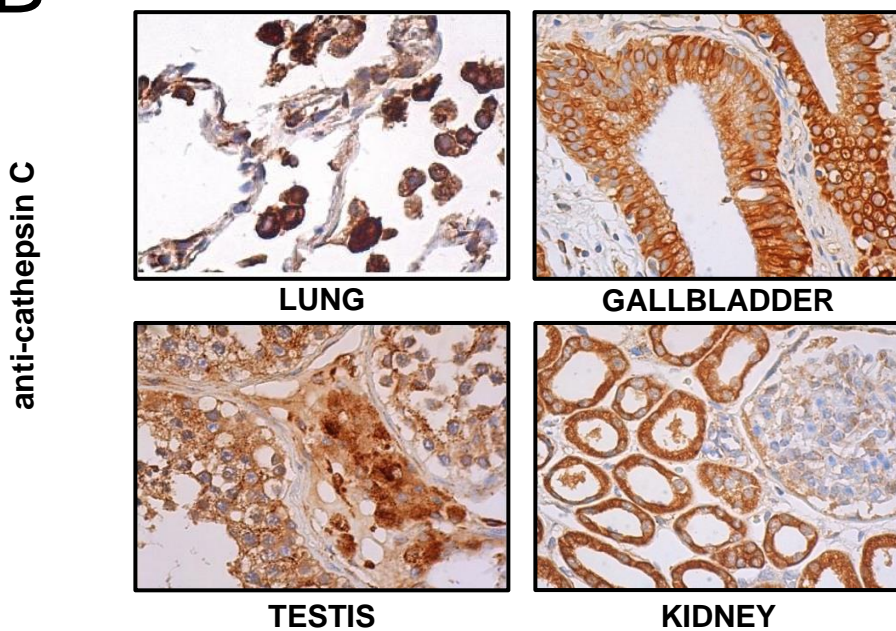




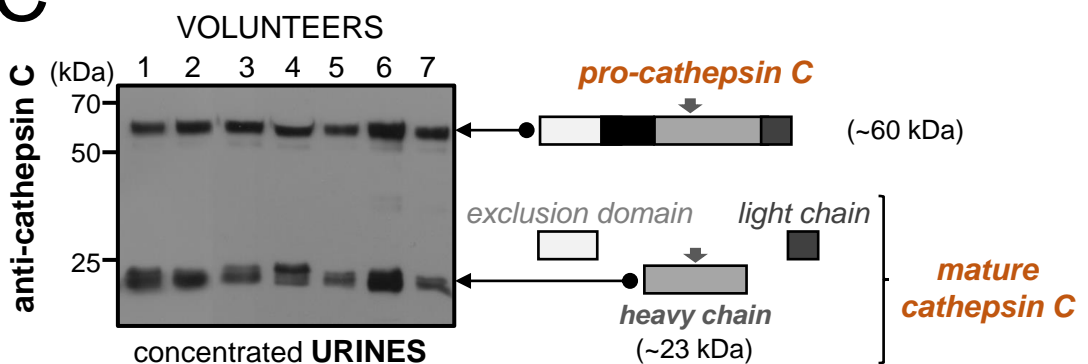
A

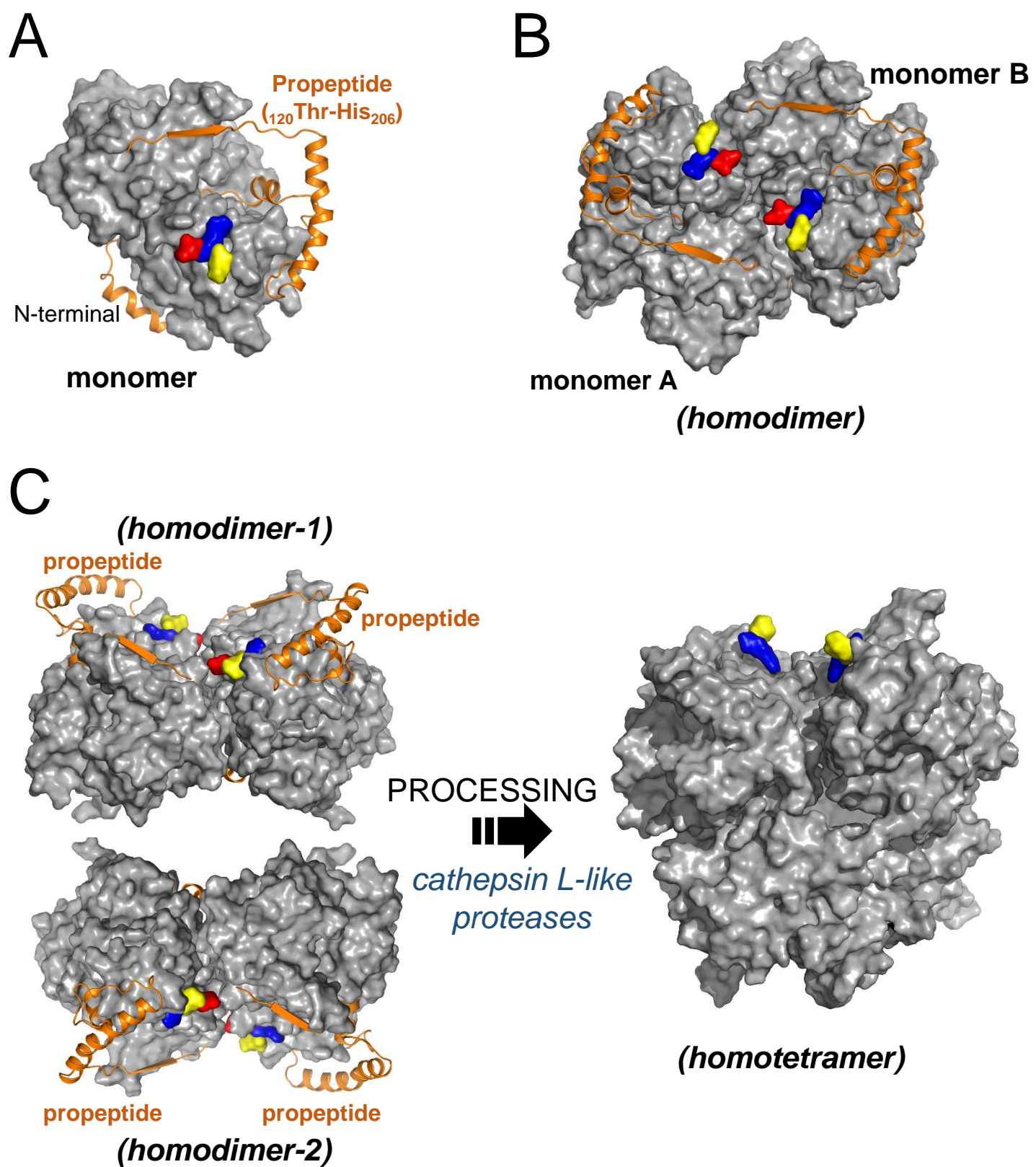


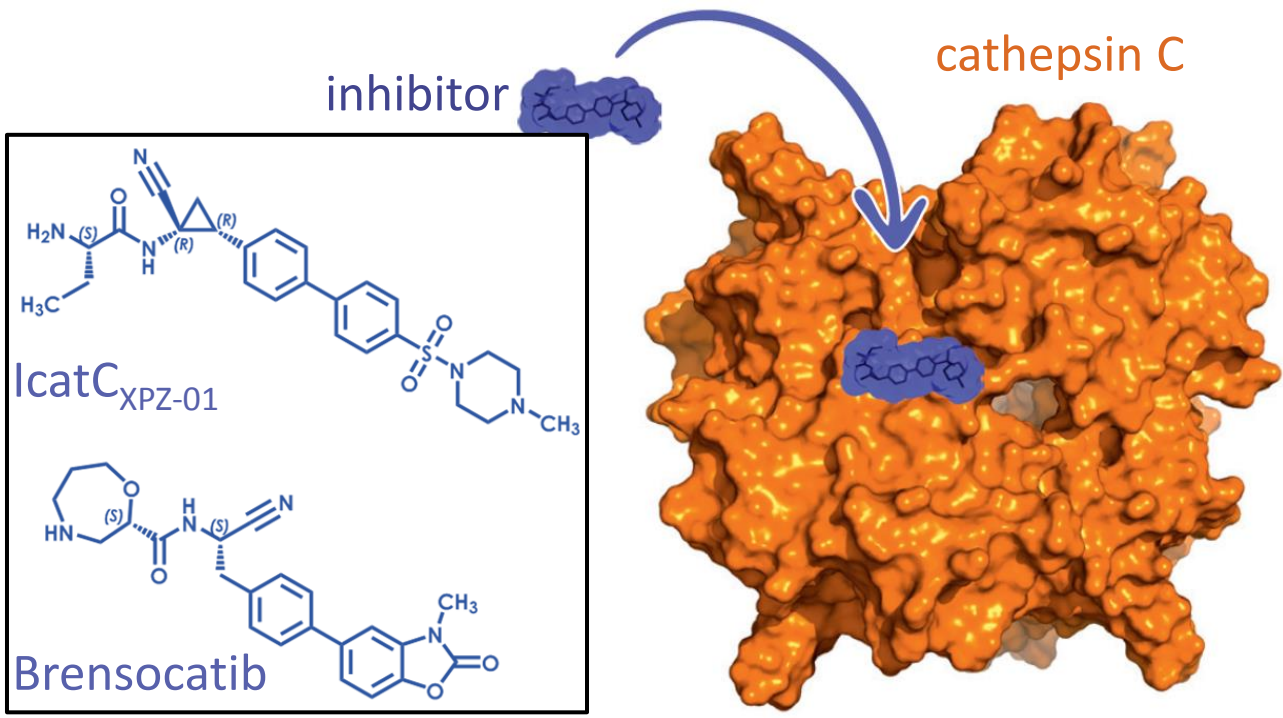
B



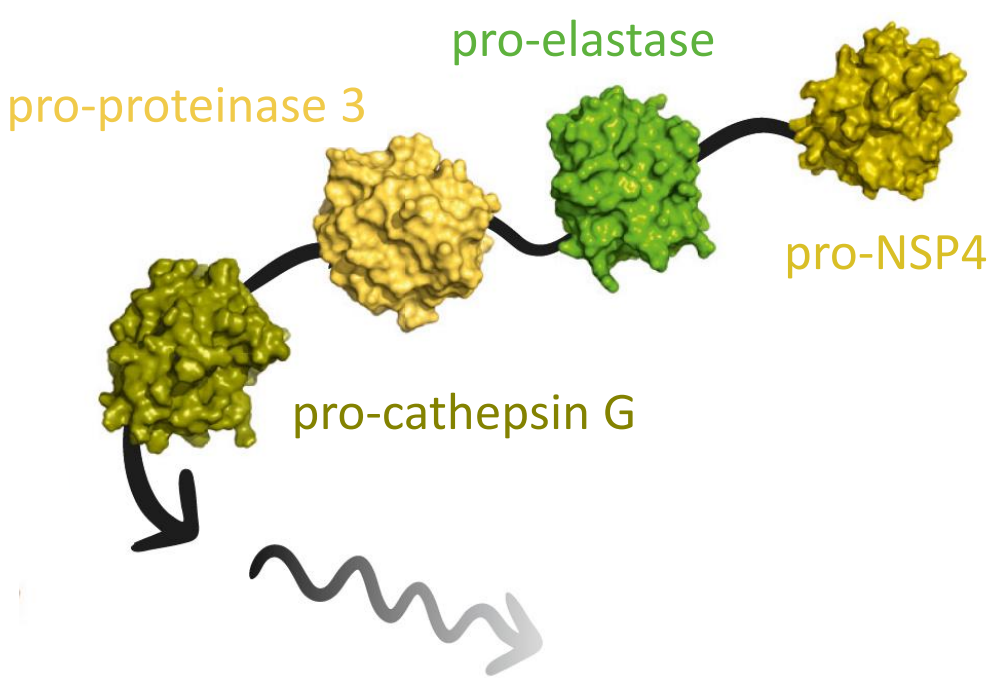
C





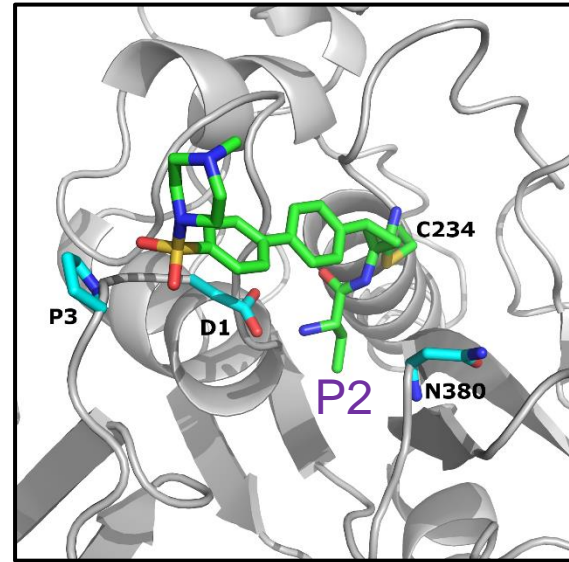
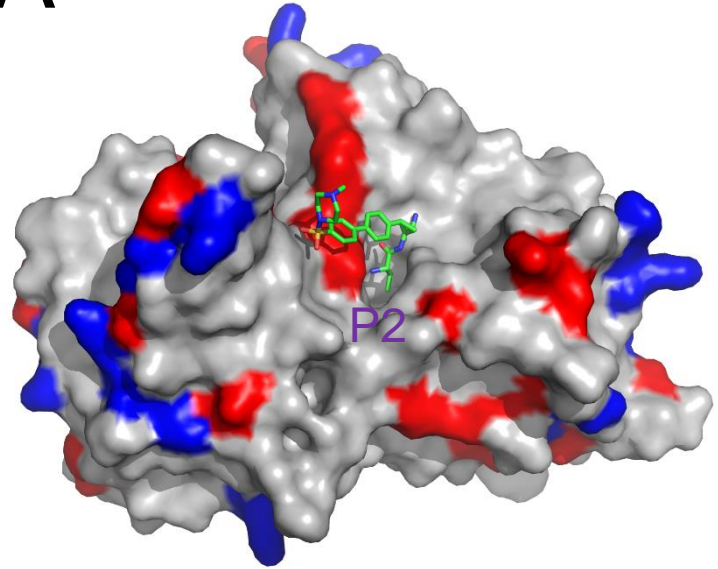


MATURATION

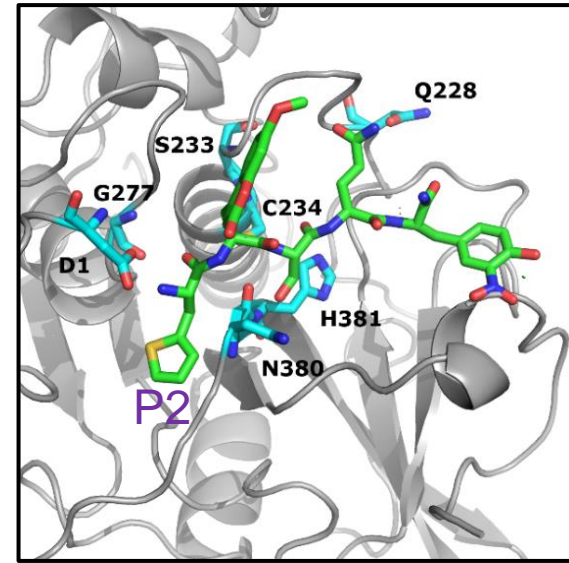
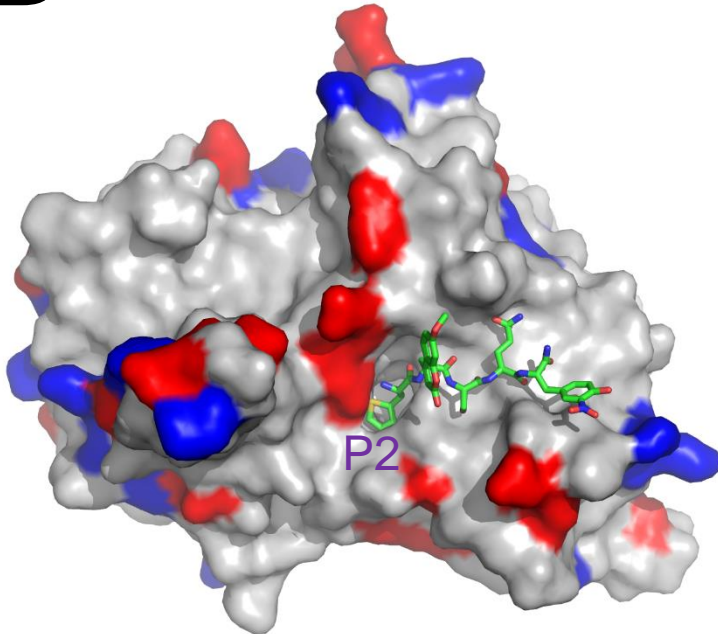


DEGRADATION

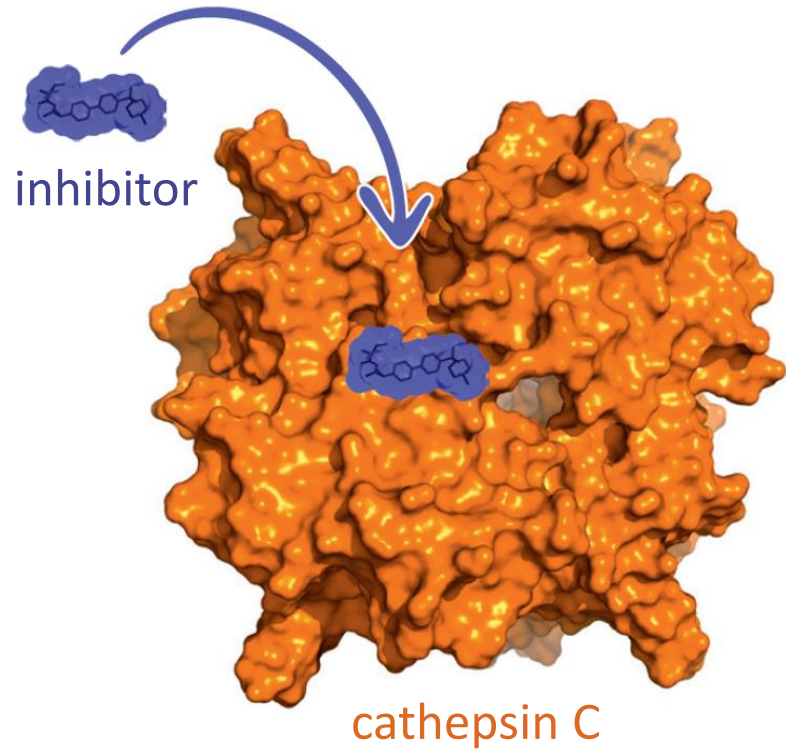
A



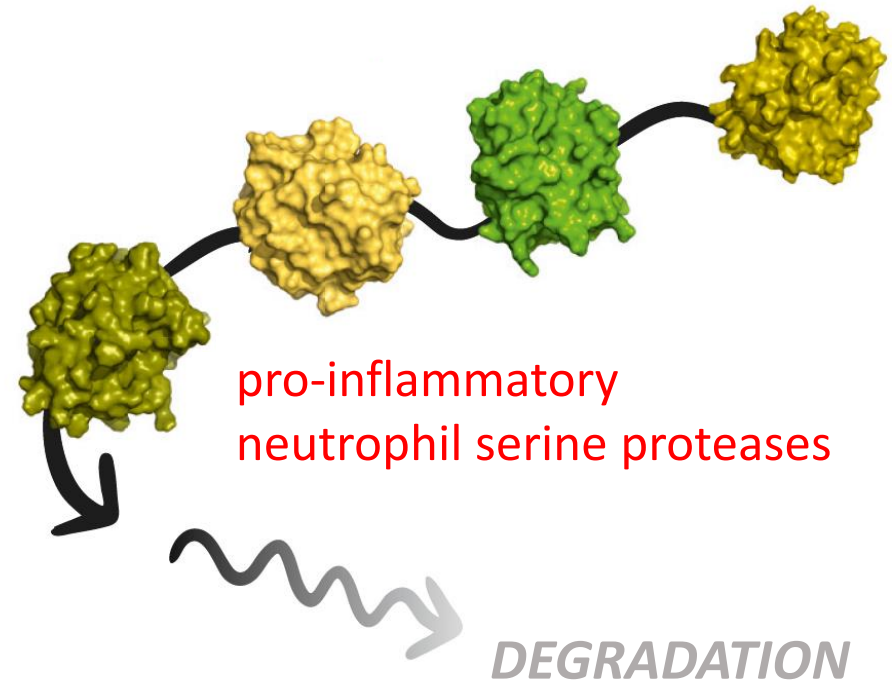
B



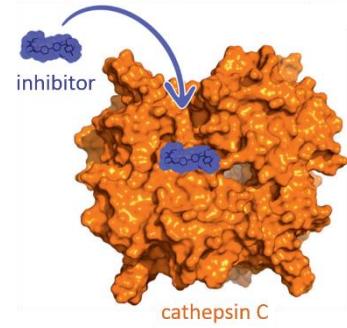
#targetingcancerpromotinginflammation



MATURATION



#targetingcancerpromotinginflammation



 MATURATION

