The proteasome as a target for cancer therapy

Peter Larsson

Department of Oncology
Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2023

Cover illustration by Peter Larsson
The proteasome as a target for cancer therapy
© Peter Larsson 2023
peter.larsson.3@gu.se

ISBN 978-91-8069-303-5 (PRINT)
ISBN 978-91-8069-304-2 (PDF)
http://hdl.handle.net/2077/75897

Printed in Borås, Sweden 2023
Printed by Stema Specialtryck AB

“Nothing is impossible. Impossible just takes a bit longer”
Dan Brown

ABSTRACT

The main objective of this PhD thesis was to evaluate the significance of proteasome genes as prognostic markers for different cancer types and identify specific cancer forms that respond to proteasome inhibition. The proteasome (PSM) plays an important role in maintaining cellular proteostasis and degrades the majority of proteins that require breakdown in the cell. Elevated PSM activity has been detected in most cancer types, making it an interesting target for cancer treatment. PSM inhibitors interfere with protein degradation, leading to a decrease in free amino acids, increased accumulation of proteins, and apoptosis. This doctoral thesis is based on five studies focusing on the proteasome and cancer.

In **Study I**, pan-cancer data from The Cancer Genome Atlas (TCGA) was used to study the relationship between PSM gene expression patterns and genetic changes, as well as expression and patient survival. Several PSM genes (e.g., *PSMB4-5*, *PSMD2*) were identified and shown to affect patient survival. In **Study II**, comprehensive bortezomib sensitivity, genomic and transcriptomic data from >800 cell lines were used to investigate the effect of PSM inhibition on e.g., cancer cell survival. We identified 33 genes involved in bortezomib resistance and cancer types that were sensitive to bortezomib. In **Study III**, we developed a strategy to identify and minimize the influence of environmental and experimental factors to improve the replicability and reproducibility of cell viability analysis. Several confounding factors (e.g., number of solvent controls, drug storage, plate construction) were shown to have an impact on the replicability and reproducibility of the resazurin-based cell viability assay. In **Study IV**, extensive data from e.g., CMap were used to study the transcriptomic signature of compounds to identify putative proteasome inhibitors similar to known proteasome inhibitors (bortezomib, MG-132, and MLN-2238). Six possible proteasome inhibitors were identified with a high affinity for the chymotrypsin-like catalytic domain (β5) of the proteasome. In **Study V**, a high-throughput drug screen was performed to identify chemotherapeutic agents (conventional breast cancer chemotherapy and proteasome inhibitors) used as single agents or in combination that can potentially improve the treatment of triple-negative breast cancer (TNBC). We identified potent drugs e.g., bortezomib and cisplatin (as single) or e.g., bortezomib+nedaplatin (in combination) that had an adverse impact on the survival of TNBC cells.

In summary, several cancer types demonstrated an association between PSM gene expression and clinical outcome, as well as sensitivity to proteasome inhibitors. Therefore, the proteasome is an attractive target for cancer treatment.

SAMMANFATTNING PÅ SVENSKA

Huvudsyftet med denna doktorsavhandling var att utvärdera betydelsen av proteasomgener som prognostiska markörer för olika cancertyper och identifiera specifika cancerformer som svarar på proteasomhämning. Proteasomen (PSM) spelar en viktig roll för att upprätthålla cellulär proteostas och bryter ner majoriteten av proteiner som kräver nedbrytning i cellen. Förhöjd PSM-aktivitet har upptäckts i de flesta cancertyper, vilket gör det till ett intressant mål för cancerbehandling. PSM-hämmare stör proteinnedbrytningen, vilket leder till en minskning av fria aminosyror, ökad ackumulering av proteiner och apoptos. Denna doktorsavhandling bygger på fem studier med fokus på proteasomen och cancer.

I **Studie I** användes pan-cancerdata från The Cancer Genome Atlas (TCGA) för att studera genetiska förändringar, genuttryck av PSM-gener och patientöverlevnad. Flera PSM-gener (t.ex. *PSMB4-5*, *PSMD2*) identifierades och visade sig påverka patientens överlevnad. I **Studie II**, omfattande data om bortezomib känslighet, genomiska och transkriptomiska data från >800 cell liner användes för att undersöka effekten av PSM-hämning på t.ex. cancercellöverlevnad. Vi identifierade 33 gener, involverade i bortezomib känslighet och identifierade cancertyper som var känsliga för bortezomib. I **Studie III**, utvecklade vi en strategi för att identifiera och minimera påverkan av miljö- och experimentella faktorer för att förbättra replikerbarheten och reproducerbarheten av cellviabilitetsanalysen. Flera faktorer identifierades (t.ex. antal lösningsmedelskontroller, läkemedelslagring, plattkonstruktion) som hade inverkan på replikerbarheten och reproducerbarheten av den resazurinbaserade cellöverlevnadsanalysen. I **Studie IV** användes omfattande data från bl.a. CMap för att studera föreningars transkriptomiska signatur för att identifiera okända proteasomhämmare liknande den för kända proteasomhämmare (bortezomib, MG-132, och MLN-2238). Sex möjliga proteasomhämmare identifierades och dessa hade hög affinitet till den kymotrypsin-liknande katalytiska domänen på proteasomen. I **Studie V** utfördes en multipel läkemedelstestning för att identifiera cytostatika (konventionell bröstcancerkemoterapi och proteasomhämmare) både ensamt och i kombinationer som har potential att förbättra behandlingen av trippelnegativ bröstcancer (TNBC). Vi identifierade flertalet potenta läkemedel t.ex. bortezomib och cisplatin (ensamma) eller t.ex. bortezomib+nedaplatin (kombination) som hade en negativ inverkan på överlevnaden på TNBC-celler.

Sammanfattningsvis identifierades ett samband mellan cancertyper, PSM-genuttryck och kliniskt utfall, samt känslighet för proteasomhämmare. Därför är proteasomen ett attraktivt mål för cancerbehandling.

LIST OF ORIGINAL STUDIES

Studies included in the thesis

I. **Larsson P**, Pettersson D, Engqvist H, Werner Rönnerman E, Forssell-Aronsson E, Kovács A, Karlsson P, Helou K, and Parris TZ,
Pan-cancer analysis of genomic and transcriptomic data reveals the prognostic relevance of human proteasome genes in different cancer types.
BMC Cancer 22, 993 (2022)

II. **Larsson P**, Olsson M, Fäldt Beding A, Forssell-Aronsson E, Kovács A, Karlsson P, Helou K, and Parris TZ,
Multi-omics analysis identifies repurposing bortezomib in the treatment of kidney-, nervous system-, and hematological cancers.
Submitted (2022)

III. **Larsson P**, Engqvist H, Biermann J, Werner Rönnerman E, Forssell-Aronsson E, Kovács A, Karlsson P, Helou K, and Parris TZ,
Optimization of cell viability assays to improve replicability and reproducibility of cancer drug sensitivity screens.
Sci Rep 10, 5798 (2020)

IV. **Larsson P**, De Rosa MC, Righino B, Olsson M, Forssell-Aronsson E, Kovács A, Karlsson P, Helou K, and Parris TZ,
Integrated transcriptomics- and structure-based drug repositioning identifies putative proteasome inhibitor-like compounds.
Submitted (2022)

V. **Larsson P**, Pettersson D, Olsson M, Ittner E, Forssell-Aronsson E, Kovács A, Karlsson P, Helou K, and Parris TZ,
Repurposing proteasome inhibitors for improved treatment of triple-negative breast cancer.
Submitted (2023)

Studies not included in the thesis

i. Engqvist H, Parris TZ, Biermann J, Rönnerman E, **Larsson P**, Sundfeldt K, Kovács A, Karlsson P, Helou K,
Integrative genomics approach identifies molecular features associated with early-stage ovarian carcinoma histotypes.
Sci Rep 10, 7946 (2020)

ii. Fäldt Beding A, **Larsson P**, Helou K, Einbeigi Z, and Parris TZ,
Pan-cancer analysis identifies BIRC5 as a prognostic biomarker.
BMC Cancer 22, 322 (2022)

iii. **Larsson P**, Parris TZ,
Cell viability assay.
In press (Book chapter-Springer Protocols)

ABBREVIATIONS

ATCC	American Type Culture Collection
BRCA	Breast cancer
EMA	European Medicines Agency
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
GDSC1	Genomics of Drug Sensitivity in Cancer 1 (Sanger)
GDSC2	Genomics of Drug Sensitivity in Cancer 2 (Massachusetts General Hospital)
HER2	Human epidermal growth factor receptor 2
MCL	Mantel cell lymphoma
MM	Multiple myeloma
PD-L1	Programmed cell death ligand 1
PR	Progesterone receptor
PSM	Proteasome
TCGA	The Cancer Genome Atlas
TNBC	Triple-negative breast cancer
UPS	Ubiquitin-proteasome system
QCM	Quality Control Metrics

AIMS

The main objective of this PhD thesis was to assess the prognostic relevance of proteasome genes in different cancer types and identify specific cancer forms that respond to proteasome inhibition.

This was achieved through the following specific objectives:

Study I

To (1) investigate the prevalence of genetic alterations in the proteasome gene family in a pan-cancer dataset from The Cancer Genome Atlas (TCGA) and (2) analyze the impact of dysregulated proteasome genes on clinical outcome.

Study II

To (1) evaluate the sensitivity of cancer cell lines to proteasome inhibitors and (2) identify a correlation between genomic or transcriptomic factors and sensitivity/insensitivity to proteasome inhibition.

Study III

To improve the replicability and reproducibility of 2D high-throughput viability screens for cancer cell lines by identifying and minimizing the influence of assay-associated confounding factors.

Study IV

To identify putative proteasome inhibitors based on (1) drug-induced transcriptomic profiles and (2) the binding affinity to the β5 proteasome catalytic site.

Study V

To determine (1) the chemosensitivity of triple-negative breast cancer (TNBC) cell lines to proteasome inhibitors and clinically relevant chemotherapy in mono- and combination settings, and (2) the potential synergistic effect of combination therapy with proteasome inhibitors and common chemotherapy.

CONTENT

ABSTRACT 4
SAMMANFATTNING PÅ SVENSKA 5
LIST OF ORIGINAL STUDIES 6
Studies included in the thesis 6
Studies not included in the thesis 7
ABBREVIATIONS 8
AIMS 9
STUDY CONTRIBUTIONS 12
PhD student contribution in each study 12
INTRODUCTION 13
Cancer 13
Cancer statistics 14
Cancer genetics 14
Breast cancer 15
Triple-negative breast cancer (TNBC) 16
TNBC treatment 17
The proteasome 18
Proteasome inhibitors 20
Drug repurposing 22
MATERIALS AND METHODS 23
Data sources 23
Study I 23
Study II 24
Study III 26
Study IV 27
Study V 29
Data analysis 33
Resazurin-based cell viability assay 33
Flow cytometry (Annexin V and cell cycle analysis) 33
Proteasome activity assay 34
Immunoblot 35
Gene expression analysis (qPCR) 36
Computational analysis 37
Study I 37
Study II 37
Study III 38
Study IV 38
Study V 38

RESULTS AND DISCUSSION 39
Study I 39
Study II 41
Study III 42
Study IV 44
Study V 46
ETHICAL COMMENTARY 49
STUDY LIMITATIONS 50
Study I 50
Study II 50
Study III 50
Study IV 51
Study V 51
CONCLUSIONS 52
FUTURE PERSPECTIVES 55
ACKNOWLEDGEMENTS 56
REFERENCES 57

STUDY CONTRIBUTIONS

PhD student contribution in each study

Study I

In this study, I was responsible for analyzing the data and performing statistics, interpreting the results, manuscript writing, submission of the manuscript to journals, as well as contact with the reviewers and editors.

Study II

In this study, I was involved in designing the study concept, data collection, data and statistical analysis, manuscript writing, and contact with the journals. I also performed all of the lab work for the validation studies using the A-172 (glioblastoma cell line), A-375 (melanoma cell line), BT-474, MCF-7, and ZR-75-30 (breast cancer cell lines).

Study III

In this study, I was involved in the study design, planning the study, planning and performing the lab work, manuscript writing, manuscript submission, and contact with the reviewers and editors.

Study IV

In this study, I was involved in the study design, planning the study, planning and performing the lab work, manuscript writing, manuscript submission, and contact with the reviewers and editors.

Study V

In this study, I was involved in the study design, planning the study, data analysis, planning and performing the lab work, manuscript writing, manuscript submission, and contact with the reviewers and editors.

INTRODUCTION

Cancer

In 2020, about 19.3 million people were diagnosed with some type of cancer and 10 million cancer-related deaths were reported worldwide [1,2]. However, the number of unreported cases is expected to be large because some countries have no working reporting system. The number of new cancer cases is expected to increase to about 25-28 million by the year 2040 [1,2]. Today it is expected that every fourth person will develop cancer during their lifetime [3].

Cancer is a collective term for many diseases where the accumulation of genetic and epigenetic alterations causes cells to divide and spread uncontrollably in the body [4-7]. The specific type of cancer is named after the tissue of origin, e.g., breast cancer originates in the breast [5,8]. Approximately 200 primary cancer types have been identified in the human body [8]. As some cancer types can be further stratified by cell type (subtypes), there are possibly more than 200 different cancer types [8]. The main cause of cancer development is environmental factors (e.g., tobacco smoking and radon), while only about 5% is caused by internal factors e.g., genetic predisposition [9,10]. Cancer develops when a single cell change normal behavior to abnormal behavior due to accumulation of genomic alterations (e.g., DNA amplification and other mutations; **Figure 1**) and can therefore not control its own processes and begins to divide and spread uncontrollably in the body [7,8,11-14]. These events lead to genomic instability [4,6,7,15]. This cancer cell will give rise to a new population of cancer cells by clonal expansion [7]. The molecular characteristics of a cancer cell are e.g., genomic instability, while the phenotypical characteristics are e.g., abnormal growth rate, division immortality, drug resistance, escaping the immune system, increased metabolism, and invasion capabilities [9,16-21].

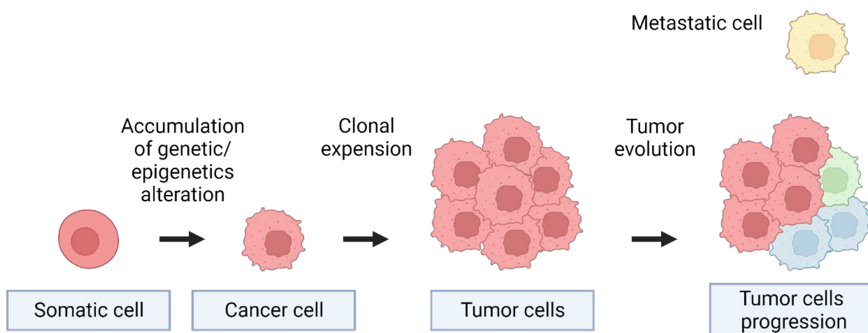

Figure 1. Cancer development. Cancer is initiated by the accumulation of genetic and epigenetic alterations in a single cell. This cell divides (clonal expansion) and give rise to a population of cancer cells. Genomic instability causes diversity between cells in the cancer population and invasion and/or metastasis can occur. The figure was created in BioRender.com.

Cancer statistics

Cancer was the most common cause of mortality in 2020 [2]. Approximately 18.1 and 19.3 million new cancer patients were reported worldwide in 2018 and 2020, respectively [2,22]. This is an increase of around 1 million cases during a 2-year period [2,22]. In 2020, the cancer forms (including both sexes) with >1 million newly diagnosed cases were cancers of the breast (2.3 million diagnosed; 700,000 deaths), lung (2.2 million diagnosed; 1.8 million deaths), prostate (1.4 million diagnosed; 400,000 deaths), nonmelanoma of skin (1.2 million diagnosed; 64,000 deaths), colon (1.1 million diagnosed; 600,000 deaths), and stomach (1.1 million diagnosed; 800,000 deaths) [2,23]. Moreover, the cancer types with a mortality rate ≥90% were cancers of the pancreas (94%), liver (92%), and esophagus (90%) [2]. Lung cancer is the leading cause of cancer-related death (18% of all cancer deaths) [2,23]. In women, the most common cancers are breast-, stomach-, colorectal-, and esophageal cancer, while lung-, prostate-, and colorectal cancer are most common among men [2,24,25].

In Sweden, approximately 68,810 individuals (36,015 [52%] men and 32,795 [48%] women) were diagnosed with malignant tumors in 2021 [26]. Breast cancer was the most common cancer for women (8,619 individuals) and prostate cancer for men (10,199 individuals) [26]. Compared to the three years (2017-2019) prior to the Covid-19 pandemic, the incidence of female breast cancer cases during 2021 had increased with 3.7% (average [2017-2019] 160.4/100,000 citizens and 166.6/100,000 citizens in 2021) and prostate cancer incidence had decreased with 8.9% (average 212.0/100,000 citizens [2017-2019] and 194.6/100,000 citizens [2021]) [26]. The type of cancer afflicting an individual and corresponding survival rates have been shown to depend on gender [27]. Hormones can either have a stimulatory or inhibitory effect on different cancer types. For example, right-sided colon cancer is more common in women, while the left side is more common in men. Tumor sidedness in colon cancer is dependent on estrogen, which in turn is linked to patient survival as right-sided colon cancer is more severe [27,28].

Cancer genetics

Despite improved cancer treatment in recent decades, treatments for some cancers (e.g., colorectal and pancreas) have not improved survival rates, especially in later stages [29]. Cancer types (e.g., breast- and hematological cancer) have improved their treatments in the recent three decades and experience increased survival rates [29,30]. Furthermore, treatment response is linked to genomic heterogeneity, which leads to patients

diagnosed with the same type of disease and despite similar treatment regimens having different survival rates [31]. In 2003, the Human Genome Project provided us with a better understanding of the different molecular mechanisms associated with cancer and their use in diagnostics, risk assessment, and prognosis [10]. This knowledge also provided the opportunity to use personalized treatment strategies for individual cancer patients [31]. The Cancer Genome Atlas (TCGA) is a comprehensive dataset of >30 cancer types and >11,000 tumor samples screened for e.g., gene expression and genetic alterations that has enhanced our knowledge about genetic variability between different cancer types and the differences between patients diagnosed with same type of cancer [31,32].

It is known that various molecular events (e.g., DNA amplifications [≥5 gene copies], insertions, deletions, translocations, and methylation) lead to genetic instability and increase the risk of cancer [4,6,12]. Several types of genes controlling the phenotype of cells are susceptible to genetic alterations such as proto-oncogenes (*ERBB2*), tumor suppressors (*TP53*), as well as apoptosis and DNA repair genes (*BRCA1* and *BRCA2*) [10]. Genetic alterations can induce activation or inactivation of genes [14]. A common genetic alteration in cancer is DNA amplification. Amplification of a genomic region often occurs where oncogenes (e.g., *ERBB2*) are located and can span a wide range of genes that may also be involved in cancer progression [12,14]. Amplification events have also been associated with drug resistance [12]. In contrast, functional DNA repair is critical for maintaining genomic integrity [33]. Impairment of the DNA repair system increases genomic instability and is likely to cause the accumulation of mutations with subsequent cancer development and progression [4,6,15]. The identification of genomic and transcriptomic markers is crucial for detecting genes involved in drug resistance, choice of treatment, prediction of side effects, prognosis, diagnosis, and novel targets for improved cancer treatment [31]. These biomarkers can also be used in cancer prevention programs to detect the early development of cancer and improve survival in high-risk patients [29].

Breast cancer

Breast cancer (BRCA) is an heterogenous disease characterized by several intrinsic molecular subtypes (luminal A, luminal B HER2-, luminal B HER2+, HER2 enriched, and triple-negative breast cancer [TNBC]) with varying biologic features (e.g., gene expression patterns) and prognosis (**Figure 2**) [34,35]. Surrogate subtyping can be performed with immunohistochemistry using antibodies for three receptors (estrogen [ER], progesterone [PR], and human epidermal growth factor receptor 2 [HER2]) and a cell proliferation marker (Ki67) [36,37]. In addition to other patient (e.g., age) and tumor characteristics (e.g., tumor size, histological grade, and metastatic spread), subtyping can help clinical teams to design treatment plans for each patient with BRCA. If the breast cancer is local, surgery with adjuvant therapy (e.g., radiation) is used. Commonly is also

to use systemic treatment in the adjuvant or neoadjuvant settings. Furthermore, endocrine therapy and HER2-targeted therapy have been used successfully for decades to treat BRCAs showing positivity for the hormone receptors and HER2 receptor, which has significantly prolonged the life of these patients [38]. However, which therapy is used is determined whether the cancer is local or, if it is spread, and what subtype it is [39]. Due to the lack of ER, PR, and HER2 expression, TNBCs do not respond to endocrine therapy or HER2-targeted therapy [40].

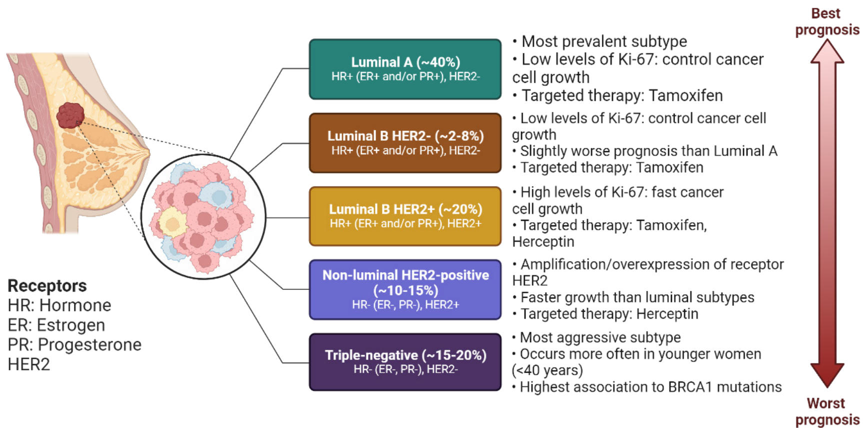

Figure 2. Breast cancer classification. Breast cancer can be classified into four distinct subtypes (Luminal A, Luminal B HER2-, Luminal B HER2+, Non-luminal HER2-positive, and triple-negative [TNBC]) according to receptor expression (estrogen [ER], progesterone [PR], and human epidermal growth factor receptor-2 [HER2]). Luminal A has the best prognosis, while TNBC has the worst prognosis. TNBC can be further divided into four subtypes (basal-like 1 [BL1], basal-like 2 [BL2], mesenchymal-like [M], and luminal androgen receptor [LAR]) according to molecular analysis. The figure was created in BioRender.com.

Triple-negative breast cancer (TNBC)

About 15-20% of all diagnosed breast cancers are classified as TNBC, which is one of the breast cancer subtypes with the worst prognosis [41-45]. Approximately 70% of basal-like BRCAs are classified as TNBC [46]. Omics analysis is commonly used to further stratify TNBC into four subtypes (basal-like 1 [BL1], basal-like 2 [BL2], mesenchymal-like [M], and luminal androgen receptor positive [LAR]) [46]. Patients with M and BL2 subtypes are more likely to have relapses within 3-years and probably hence the poorer survival rate [47,48]. In 2020, 2.3 million individuals were diagnosed with breast cancer, of which approximately 400,000 were TNBC [2]. In Sweden, 8,619 women were diagnosed with breast cancer in 2020, with around 1,000 TNBC diagnoses [26]. Despite lacking expression of ER, PR, and HER2, TNBCs have previously been associated with another receptor (folate receptor alpha [FRα], epidermal growth factor receptor (EGFR) and androgen receptor (AR)) [49,50]. Recently, malignant tumors (bladder and pancreatic) have been

associated with elevated FRα expression, which in turn is correlated with cell growth and unfavorable prognosis [49-51]. In primary breast cancer, ER-negativity has been correlated with elevated FRα expression. Astvatsaturyan *et al*. revealed that expression levels of EGFR and AR were associated with prognosis (disease-free survival; DFS) in TNBC [52]. Cells with high levels of EGFR and low of AR were associated with more unfavorable prognosis [52]. The LAR subtype in TNBC has low EGFR and high AR, which is associated with better survival [52]. However, this needs further evaluation [52].

TNBC is common in premenopausal women (40 years of age or younger), certain ethnicities (African American and Hispanic origin) and obese women [44,46,53,54]. In contrast, non-TNBC is more common in elderly women (>55 years of age) [39,54]. Approximately 20% of all TNBCs harbor *BRCA1* or *BRCA2* mutations [46,54], which can lead to genomic instability, the accumulation of mutations, and tumor progression [4,6,46,54]. The 5-year survival rates for patients with TNBC are approximately 75% [41,42,46,55,56]. For breast cancer in general the 5-year survival rate is approximately 90% [57]. Tumor size is significantly larger for TNBCs than non-TNBCs, often above 2 cm [55]. Risk of locoregional recurrence is also common for TNBCs in comparison with non-TNBCs, but lymph node-positivity is more common in HER2+ breast tumors than in TNBC [42,58]. Holleczek *et al.* recently showed an increase in locoregional recurrence or distant metastasis in TNBCs (23%) compared to non-TNBCs (8% and 11%, respectively) [59]. For patients with TNBC, distant recurrence frequently occurs within 3 years of diagnosis, but seldom after 8 years [42,46,55,58]. The tumors are larger and the time for recurrence risk (within 3 years) is shorter than non-TNBC shows that TNBC is a more aggressive breast cancer subtype [42,46,54,55,58]. Distant metastases to the brain and lungs are mostly found [42,46].

TNBC treatment

Standard treatment for TNBC is surgery, chemotherapy (neoadjuvant and/or adjuvant settings), and radiation. But other therapies for TNBC can also be chosen according to BRCA mutations and programmed cell death ligand 1 status (PD-L1 positive or negative) [39,60,61]. If the tumor harbors *BRCA1* or *BRCA2* (*BRCA1/2*) mutations, PARP inhibitors (e.g., olaparib) alone or in combination with carboplatin can be administered [60]. *BRCA1/2* are involved in DNA repair. If this process is deficient, the enzyme poly(adenosine diphosphate ribose) polymerase (PARP) will step in and help repair the DNA damage [62]. Inhibition of PARP will decrease the efficiency of the DNA repair system, thereby leading to an accumulation of unfixed DNA damage and apoptosis [62]. By adding platinum agents (e.g., carboplatin) to PARP treatment, DNA damage will increase, and the cell will undergo apoptosis [39,62,63]. Moreover, if the tumor is positive for PD-L1 marker, immunotherapy alone or in addition with a taxane (e.g., docetaxel) can be administered [39,60,62,63]. However, if the status for *BRCA1/2* mutations and PD-L1 is unknown, chemotherapy is commonly recommended [60]. Stage II and III disease are commonly

treated with chemotherapy using taxanes (e.g., docetaxel), platinum agents (e.g., carboplatin), or anthracyclines (e.g., epirubicin) [60,64,65].

The proteasome

The 26S proteasome (PSM; 2,500 kDa) is an evolutionarily conserved protein complex with protease degradation properties that is responsible for regulating multiple fundamental processes (cell cycle checkpoints, apoptosis, and metabolism) and recycling of amino acids for the production of new proteins, controlling protein quality, and protein homeostasis in the cell [66-68]. In the human body, there are five different PSMs (constitutive proteasome, immunoproteasome, spermatoproteasome, thymoproteasome, and intermediate proteasome) found in different cell types with varying functions [69-73]. Only the constitutive proteasome is present in all cell types [69,73,74]. The PSM complex is divided into one 20S core subunit (700 kDa) with catalytic activity and one or two 19S regulatory particles (RP; 900 kDa). The RP consists of two subunits (base and lid) [67,75-80] that regulate which proteins enter the core particle for degradation [67]. The PSM is part of the ubiquitin-proteasome system (UPS) [67]. Other important components involved in the UPS include ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) which are involved in initiating and tagging proteins targeted for degradation with ubiquitin molecules [67,75]. Ubiquitin-tagged proteins are degraded to small peptide fragments of <25 amino acids (**Figure 3**) [73,75,79].

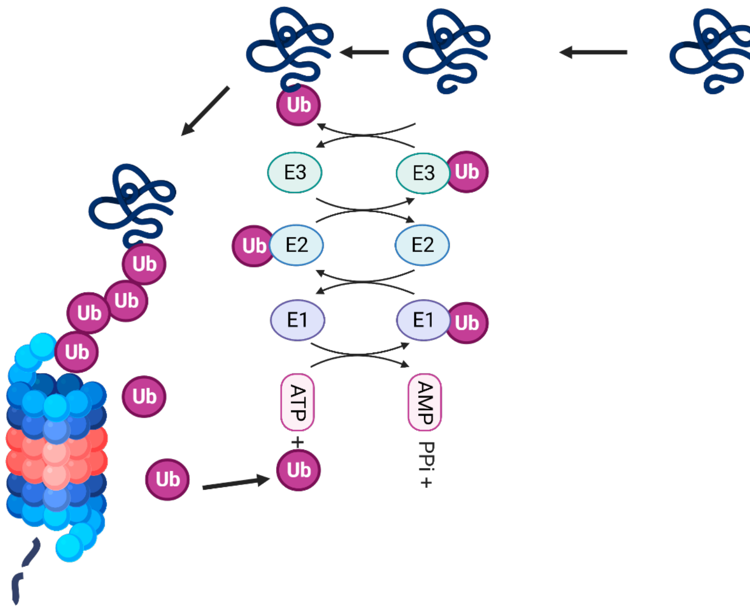

Figure 3. Ubiquitin-proteasome system (UPS) workflow. The protein degradation process starts when a protein is tagged with ubiquitin (Ub) molecules for degradation. Three enzymes (E1, E2, and E3) help to attach Ub to proteins, an ATP-driven process (when pyrophosphate (PPi) is separated from ATP the energy is used to attach UB to protein). When the protein is polyubiquitinated, it will be transported to the proteasome for degradation. The Ub molecules will be detached and recycled, while the protein will be cut into fragments less than 25 amino acids in length. The figure was created in BioRender.com.

Proteins chosen for degradation are ubiquitinated with at least four ubiquitin molecules which will activate transport to the PSM for degradation [66-68,75,77,79,81]. The destiny of ubiquitinated proteins can change, with the detachment of ubiquitin by deubiquitylating enzymes (DUBs) [67,68,75,82]. These DUBs are also involved in the recycling of ubiquitinated proteins [67,82]. About 80% of amino acids that make up new proteins in the human body are from recycled proteins [78]. The UPS system is important for degradation of these proteins (e.g., misfolded and temporary proteins) in the cell, accounting for about 80% of all degraded proteins and the rest are broken down with other systems such as proteases [75,78,83-85].

The PSM consists of six catalytic sites, with two of each type (chymotrypsin-like [β5], caspase-like [β1], and trypsin-like [β2]) in the core particle. β5 is the most important catalytic site, which is responsible for degradation of 11-50% of total protein degradation in comparison with the β1 and β2 sites that are responsible for 12-22% and 3-35% of degraded proteins, respectively [86]. A previous study demonstrated no or only

very slight changes to the cellular phenotype and growth following disruption of the β1 and β2 processes, but disruption of β5 caused phenotypical defects, reduced cell growth, and accumulation of proteins in the cell [86-90]. Although inhibition of the β1 and β2 sites has little to no effect on the phenotype, they may play an important role in the cell [88].

In total, 49 PSM genes are involved in the PSM complex but not all are involved in same type of PSM [67]. There are five different classes of PSM genes, α- and two β in the 20S core particle (class I), 26S subunit with ATPases and non-ATPases (class II), activators (class III), inhibitor (class IV), and assembly chaperones (class V) involved in PSM assembly and function [72]. The 20S core subunit consists of four rings (two α- and two β-rings) [73,78,91]. The α-rings are encoded by *PSMA1-8* and β-rings by *PSMB1-11*. The six PSM catalytic sites are located (β1, β2, and β5) in the β-rings [73], with different catalytic sites depending on the type of PSM. The RP base contains six ATPases (Rpt1-6; *PSMC1-6*) that form a ring attached to the core particle [80]. The chaperones *PSMG1-4* are responsible for attaching the regulatory particle (RP) to the core particle. Individual chaperones are responsible for specific parts of the RP for accurate assembling and attachment to the core particle [80,92].

A non-functional protein degradation process performed by the PSM will induce e.g., neurodegenerative- or cardiovascular disease (e.g., Alzheimer's disease) because of the accumulation and aggregation of damaged proteins [93,94]. A recent study using a neuronal model of Huntington's disease showed that an increase in the expression of PSM activators (PA28) will lead to an increase in cell survival [95,96]. Elevated *PSMC1-6* expression has previously been associated with poor prognosis in breast cancer [97]. Other PSM genes have been associated with increased risk of colorectal cancer and autoimmune diseases (e.g., *PSMB8-9*) when mutated [71,91]. Mutations in genes in the catalytic site of the constitutive proteasome (*PSMB5*, *PSMB6*, and *PSMB7*) are uncommonly observed, which are most likely deleterious events for the cell [71]. A non-functional PSM is correlated with the accumulation of damaged proteins, which leads to cell death [88,93,94,98]. PSM inhibitors exploit this advantage in cancer treatment to disrupt the degradation process by inducing protein accumulation in the cell followed by apoptosis (**Figure 4**) [88,93,94,98].

Proteasome inhibitors

An impaired PSM protein degradation process leads to instability of protein homeostasis and amino acid starvation, resulting in disease development or apoptosis [94]. Disruption of PSM activity has emerged as a target for inducing apoptosis [94,98]. Bortezomib (BTZ; Velcade®) was the first PSM inhibitor to be developed and approved (U.S. Food and Drug Administration [FDA] and European Medicines agency [EMA]) for medical use [94] in the treatment of multiple myeloma (MM). It has since been approved for use in the treatment of mantel cell lymphoma (MCL) [94]. Both cancers are hematological

malignancies [94]. As single agents, proteasome inhibitors have not been fully successful in clinical studies. Twelve metastatic breast cancer patients were treated with bortezomib as single agent and no response was observed [99]. But Petrocca *et al*, showed that basal-like TNBC cell lines were sensitive for proteasome inhibition [100]. Interestingly, bortezomib in combination with docetaxel or carboplatin, phase 1 studies in lung cancer and ovarian cancer have shown increased antitumoral activity with a response rate of 47% [101-103]. It has also been shown that proteasome inhibitors have higher antitumoral activity when IKK/NF-kB is dysregulated as in ER-negative breast cancer [104,105]. The primary target of most PSM inhibitors is to disrupt the activity of the chymotrypsin-like catalytic site (*PSMB5*, [β5]) in the core particle [94,98]. BTZ resistance has been observed when treating some patients with MM and MCL, likely due to e.g., mutations in the *PSMB5* gene [106]. To overcome resistance, second-generation PSM inhibitors have been developed (e.g., carfilzomib, delanzomib, epoxomicin, and MLN-2238) [85,106]. Carfilzomib is now also approved for the treatment of MM [107]. Increased PSM activity has been observed in cancer, which makes these cells more vulnerable to PSM inhibition (**Figure 4**) [108,109]. This shows that proteasome inhibitors in single or combination therapy can improve survival of cancer patients. More studies need to be conducted to reveal potent combinations and cancer patients that will benefit from that treatment.

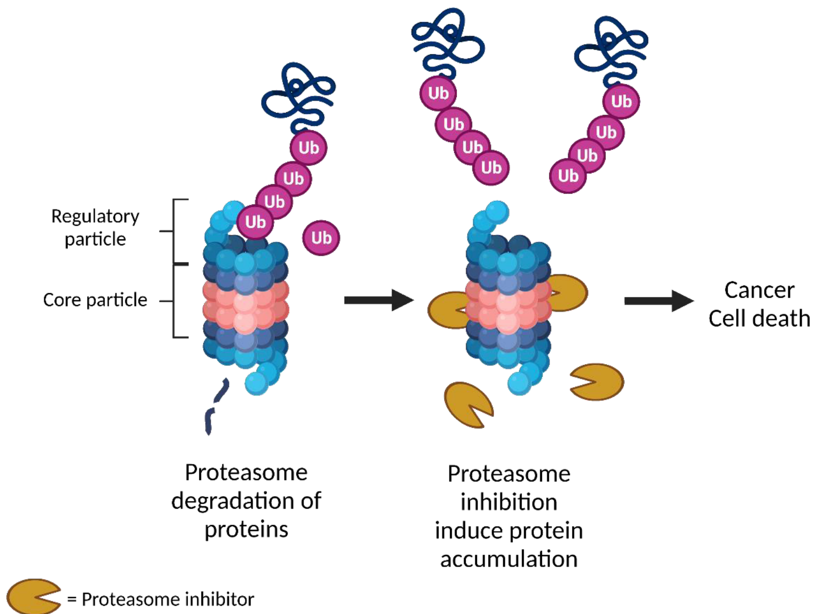

Figure 4. Proteasome inhibition. The proteasome is responsible for the degradation of 80-90% of proteins, thereby controlling protein homeostasis. When this process is disrupted by e.g., proteasome inhibitors, there will be an accumulation of proteins in the cell and the cell will undergo programmed cell death (apoptosis). The figure was created in BioRender.com.

Drug repurposing

Drug repurposing is a drug discovery approach where existing FDA/EMA-approved drugs are used to treat a different disease for which it was originally intended [110]. Traditional drug development is a high-risk and expensive process that may not pass clinical trials [111]. Few drugs tested in clinical trials are eventually FDA/EMA-approved, while costing around $1-3 billion (repurposing a tenth of that cost) and taking up to 15 years from target identification to drug approval (5-7 years longer than drug repurposing) [110,112-114]. Between 2000 and 2015, success rates for newly developed oncology drugs were as low as 3.4% [115]. The success rates for a drug improves if patients have been selected according to biomarkers [115].

Several drug repurposing approaches have been used in research and clinical trials, e.g., transcriptome-based (drug-disease similarity or drug-drug similarity) and molecular docking (computational chemistry) [112]. By using drug-induced transcriptomic profiles (before and after treatment) to identify novel, potent alternative drugs with the same or similar mechanism-of-action to the query drug, treatment resistance and/or side effects could be prevented [116]. To identify novel drugs for a disease, drug signatures and disease signatures can be matched [117]. Experimental drug repurposing studies frequently use large drug libraries and cells in high-throughput screens (HTS) [110,114]. In recent years, drug repurposing has been used to improve treatment for a number of diseases e.g., Ebola virus disease, Alzheimer's disease, COVID-19, breast cancer (e.g., tamoxifen), and colorectal cancer (e.g., aspirin) [110,111,118-120]. The advantage of repurposing existing FDA/EMA-approved drugs is they have already passed safety measures and if drug dose and treatment strategy is nor changed, they can therefore be tested in simple clinical trials [110,121].

MATERIALS AND METHODS

Data sources

Study I

In this study, we used transcriptomic and genomic data for the 49 PSM genes and 11,000 TCGA patient tumor samples derived from 33 cancer types representing 11 organ systems (e.g., gynecologic and thoracic) and normal samples (16 cancer types representing eight organ system). PSM genetic alterations (e.g., DNA amplification and missense mutation) were downloaded from cBioPortal (https://www.cbioportal.org/), which contained data for 10,967 tumor samples (10,953 patients) representing 30 cancer types and 10 organ systems (e.g., gastrointestinal and head and neck). GISTIC2 copy number and RNA sequencing data for the PSM genes were downloaded from Broad GDAC Firehose (https://gdac.broadinstitute.org/). Focal and/or arm level DNA amplification of the PSM genes and the resulting impact of these events on gene expression was evaluated using UNC RNASeqV2 level 3 expression (normalized RSEM; mRNA; **Figure 5**). Using previously published lists of cancer drivers, we correlated DNA amplification, genomic location, and their effect on PSM gene expression [122,123]. We performed multivariable Cox regression analysis using clinical data from UCSC Xena browser (https://xena.ucsc.edu/) and Genomic Data Commons (GDC; https://portal.gdc.cancer.gov/). Using FPKM log2 (RNA-seq) gene expression data and median expression as a reference, high or low expression was determined. External validation was performed using in-house genomic profiling data (SNP genotyping and RNA-seq) for 229 breast cancers and mutation data for 23/229 samples [124,125]. Survival was interpreted using overall survival (OS, time from diagnosis to death of any cause) and progression-free interval (PFI, life span during and after treatment without worsening disease). The multivariable analysis was adjusted using age and/or tumor grade, when available. The correlation between PSM gene expression and OS was accessed with RNA microarray data for breast-, gastric-, lung-, and ovarian cancer and RNA-seq data for liver cancer using the interactive Kaplan-Meier plotter site (KM plotter; https://kmplot.com/analysis/).

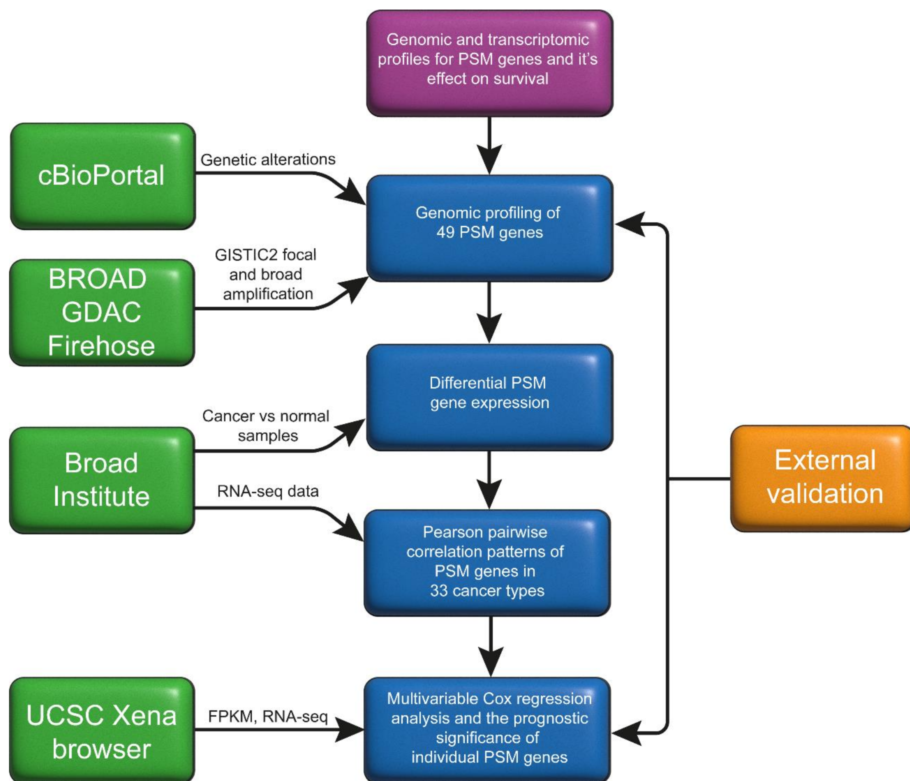

Figure 5. The workflow for the evaluation of genetic changes, gene expression of PSM genes, and patient survival. Genetic alteration (e.g., amplification) frequency of PSM genes was determined in all 33 cancer types and the amplification on the broad or focal levels was investigated. Using data from Broad institute, differences in gene expression of PSM genes between cancer and normal tissue and also if there was a positive or negative correlation between PSM genes were evaluated. Multivariable Cox regression analysis was used to determine if PSM gene expression levels were correlated with patient survival. External validation of the findings in genetic alteration and prognosis analyses was performed using an in-house breast cancer dataset and KM plotter.

Study II

We retrieved drug sensitivity data for cell lines treated with BTZ from two publicly available datasets (https://www.cancerrxgene.org/), i.e., Sanger (GDSC1; 791 cell lines) and Massachusetts General Hospital (GDSC2; 756 cell lines) that were merged and narrowed down to 860 cell lines after removing duplicates from the GDSC2 dataset. The 860 cell lines represented 30 cancer types and were derived from 13 organ systems. Sensitivity data for the half maximal inhibitory concentration (IC50) and area under the curve (AUC) were stratified into two BTZ-sensitivity groups (insensitive and sensitive)

using thresholds for BTZ-sensitivity (natural logarithm IC50 [LNIC50] < -6 and AUC < 0.6) and BTZ-insensitivity (LNIC50 > -3 and AUC > 0.9). Sanger COSMIC Cell Lines Project (970 cell lines [mutation data] and 1,007 cell lines [gene expression data]) and Cell Model Passport (1,318 cell lines) supplied data for mutations, gene expression, and mutated cancer drivers for the BTZ-sensitive and insensitive groups. Mutation data for the NCI-H2595 and SK-MEL-1 cell lines were missing, while cancer driver data were missing for the EHEB and QIMP-WIL cell lines. Gene expression levels were determined using Z-score values, where a score <2 corresponded to underexpression and >2 was overexpression. Five cell lines (A-172 [glioblastoma], A-375 [melanoma], and three breast cancer cell lines [MCF-7, BT-474, and ZR-75-30]) were used to validate BTZ sensitivity in the GDSC1 and GDSC2 datasets, BTZ-induced gene expression, and mechanism-of-action. Over- or underexpressed genes in >1/5 of cell lines in either group were taken into account (**Figure 6**). Cell culture information is listed in **Table 1**.

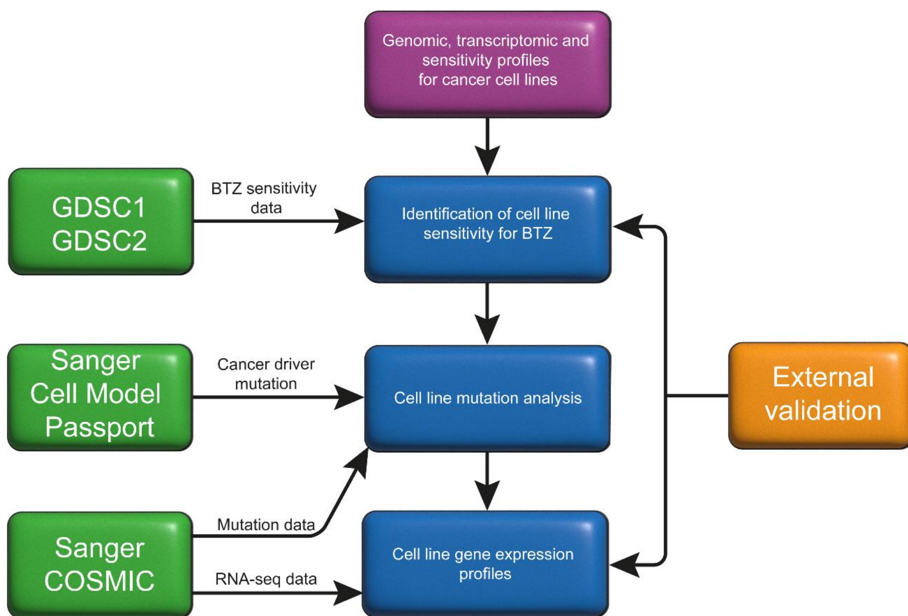

Figure 6. Genomic and transcriptomic events confer resistance to bortezomib (BTZ). Two comprehensive datasets (GDSC1 and GDSC2) were used to evaluate BTZ sensitivity in different cancer types. The mutation frequency of cancer drivers was investigated using datasets from Sanger Cell Model Passport. Further analysis on the influence of mutations and gene expression on sensitivity were evaluated using the Sanger COSMIC dataset. The findings for BTZ-sensitivity and induced gene expression were validated using *in vitro* studies of the A-172, A-375, MCF-7, BT-474, and ZR-75-30 cell lines.

Study III

The reproducibility and replicability of cell viability screens was evaluated using three breast cancer cell lines (HCC38, MCF-7, and MDA-MB-436) and one control breast fibrocystic disease cell line (MCF-10A) treated with bortezomib, carboplatin, cisplatin, and DMSO (**Figure 7**). The following parameters of the drug screens were evaluated:

(1) seeding density (5.0 x 10^3, 7.5 x 10^3, and 1.0 x 10^4 cells per 96-well),
(2) drug solvent (DMSO concentrations 0.33-30%),
(3) medium type (growth medium + 0-15% FBS or HuMEC serum-free medium),
(4) medium volume (100-240 µL),
(5) medium/drug renewal every 24h (only for 48h and 72h treatment),
(6) antibiotics (penicillin/streptomycin [with or without]),
(7) solvent controls (concentration matched or single),
(8) resorufin detection (absorbance or fluorescence),
(9) resazurin exposure time (1-6h),
(10) resazurin concentration (5-20%),
(11) compound cross reaction (no cells).

We also evaluated the impact of evaporation on the drug- and cell plates. Our results from the optimized cell viability assay were validated using drug sensitivity data for bortezomib and cisplatin from the PharmacoDB database (https://pharmacodb.ca/) [126] and Hafner *et al* [127]. Cell culture information is listed in **Table 1**.

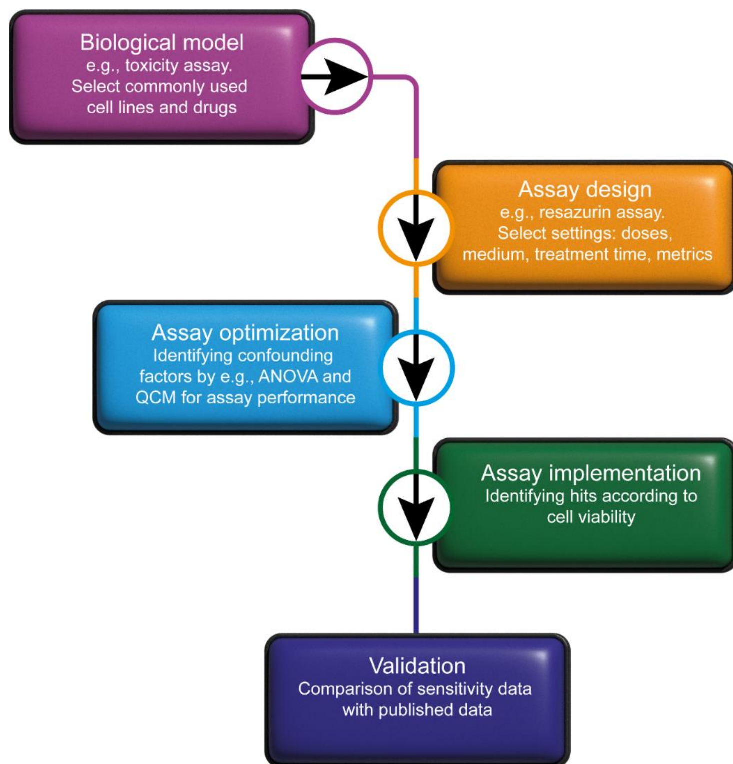

Figure 7. Optimization of the cell viability assay. First, a biological model (e.g., cell line and drugs) was chosen that could be validated after optimization. The assay setup was determined according to previously published performance. Confounding factors were identified and evaluated according to their influence on viability using statistical analysis and quality control metrics (QCM) to validate the assay performance after optimization. The optimized setup was run three times to verify replicability and reproducibility before comparing the results to previously published data.

Study IV

To identify novel PSM inhibitors, we used gene signature data, structure-based virtual screening, and PSM activity perturbation analysis. In the gene signature analysis, we used three known PSM inhibitors (BTZ, MG-132, and MLN-2238) as query in Broad Institute Connectivity Map (CMap; https://clue.io/about and Library of Integrated Network-based Cellular Signatures (iLINCS; http://www.ilincs.org/ilincs/), which are platforms with comprehensive gene expression profiling data for cell lines before and after treatment with clinically relevant drugs and other perturbagens (**Figure 8**) [128-130]. Using bortezomib as query, iLINCS data were retrieved for 60 cell lines representing cancers of the breast, central nervous system, colon, large intestine, large intestine

epithelial, leukemia, lung, melanoma, ovarian, prostate, and renal. A threshold was set to identify the top 100 differentially expressed genes per perturbation, giving an output of 5,448 genes. From these genes, the top 250 genes were selected. CMap uses the L1000 technology where expression for 978 landmark genes is used to predict the expression of 11,350 genes in nine cell lines (A-375, A549, HA1E, HCC515, HEPG2, HT29, MCF-7, PC3, and VCAP). The MG-132 and MLN-2238 PSM inhibitors were used as query (BTZ was missing in the CMap dataset). The 250 differentially expressed genes were identified by transcriptTool R package (version 0.0.0.9000) [131]. Hierarchical clustering of the expression patterns for the identified genes was performed using pheatmap R package (version 1.0.12) using the Manhattan distance metric and Ward's minimum variance method (Ward.D2) to identify concordant genes for further Gene Ontology analysis using Reactome (https://reactome.org/) [132].

To identify perturbagens with similar transcriptomic profiles as known PSM inhibitors, CMap was used with BTZ, MG-132, and MLN-2238 as input compounds. Further analysis was performed on perturbagens with *tau*-score ≥ 95, indicating strong similarity (similar drug-induced gene expression patterns). The identified novel PSM inhibitors were analyzed using L1000 fireworks plot with The L1000 fireworks display (L1000FWD; https://maayanlab.cloud/l1000fwd/#) [116], followed by analysis of docking score (binding affinity) to the PSM β5 catalytic site. Novel PSM inhibitors with (1) similar transcriptome profiles to known PSM inhibitors and (2) high binding affinity were further evaluated to test drug potency (cell viability assay) and inhibition of PSM activity for the three catalytic sites (β5, β1, and β2). Differential expression of *DNAJB1* and *HMOX1* (geometric mean of the *HPRT1*, *PPIA*, and *PUM1* endogenous controls) was validated using MCF-7 cells treated with 10 µM BTZ for 1, 6, and 24h with quantitative real-time PCR and the ΔΔCt method.

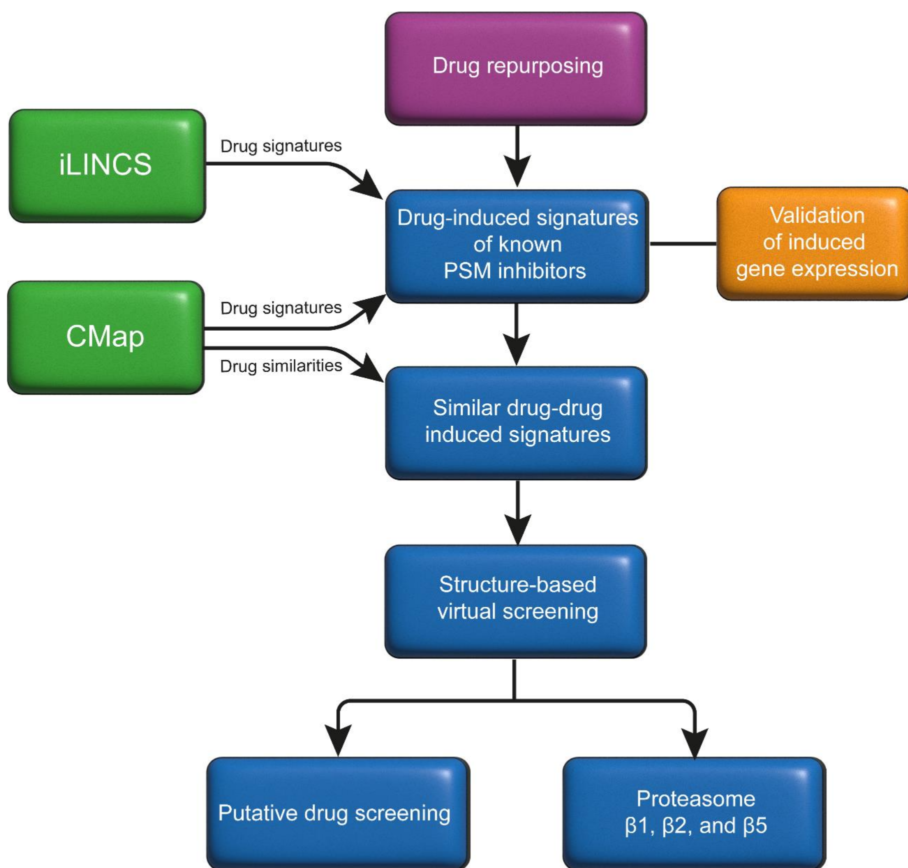

Figure 8. Drug repurposing. Known proteasome inhibitors (BTZ, MG-132, and MLN-2238) were used as query to identify genes that were differentially expressed in connection with drug treatment. Validation of differentially expressed genes were done using BTZ and the MCF-7 cell line. Drugs with similar gene signatures were identified and further evaluated according to their binding affinity to the β5 catalytic site. The compounds with the highest affinity to the β5 catalytic site were evaluated for drug potency and their ability to inhibit the β5 catalytic site in MCF-7 cells.

Study V

A drug sensitivity screen was performed for eight TNBC cell lines, representing the four TNBC subtypes (BT-549, CAL-148, HCC1806, HCC38, HCC70, MDA-MB-436, MDA-MB-453, MDA-MB-468) and three control cell lines (BT-474, MCF-10A, and MCF-7) treated with 11 PSM inhibitors and 7 clinically relevant drugs (2 mitosis inhibitors, 2 topoisomerase inhibitors, and 3 platinum agents; **Figure 9** and **Table 2**). The cell lines were treated with the 18 drugs as monotherapy, while combination treatment was performed for 13 drug combinations predicted by IDACombo (version 1.0.2) using the

monotherapy data [133,134]. SynergyFinder (version 3.2.10) [135] was used to determine the sensitivity and synergistic effect of 13 drug combinations in treatment of four TNBC cell lines (CAL-148, HCC1806, HCC38, and MDA-MB-468) and one control cell line (MCF-7). Western blot (immunoblot) was used to assess androgen receptor (AR) expression for the eight TNBC cell lines and one control cell line (BT-474). Analysis of PSM activity was performed using the MCF-7 cell line treated with the 11 PSM inhibitors to evaluate their ability to inhibit the β5 catalytic site. Cell culture information is listed in **Table 1.**

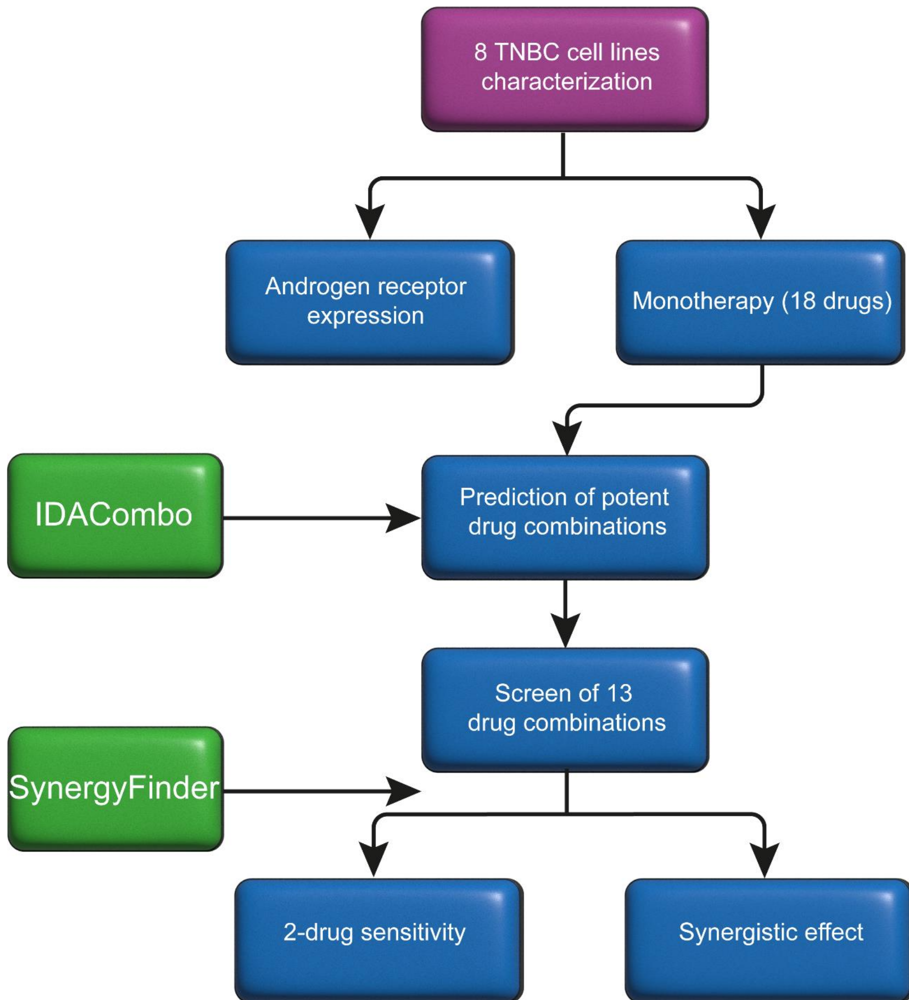

Figure 9. Drug sensitivity analysis in TNBC cell lines. The eight TNBC cell lines were evaluated for expression of androgen receptor (AR). We used 10 cell lines and determined their sensitivity to 18 drugs (11 proteasome inhibitors, 3 platinum agents, 2 mitosis inhibitors, and 2 topoisomerase inhibitors) in monotherapy. The monotherapy results were used to predict possible 2-drug combinations with IDACombo. In addition to cell viability, the synergistic effect of the 13 selected 2-drug combinations on four TNBC cell lines and one control cell line was determined using SynergyFinder.

Table 1. Cell lines and culture conditions

Cell-line	Cancer type	Subtype[a]	Medium	Supplier[b]	Study
A-172	Glioblastoma		DMEM + 10% FBS, suppl. with 2 mM L-glutamine and 1% sodium pyruvate	MJ	II
A-375	Melanoma		DMEM + 10% FBS	JN	II, IV
BT-474	Breast cancer	ER+, PR+, HER2+	DMEM + 10% FBS	ATCC	II, V
BT-549	Breast cancer	TNBC***	RPMI + 10% FBS	ATCC	V
CAL-148	Breast cancer	TNBC****	DMEM + 10% FBS	DSMZ	V
HCC1806	Breast cancer	TNBC**	RPMI + 10% FBS	ATCC	V
HCC38	Breast cancer	TNBC***	RPMI + 10% FBS	ATCC	III, V
HCC70	Breast cancer	TNBC*	RPMI + 10% FBS	ATCC	V
MCF-10A	Breast cancer	Breast fibrocystic disease	RPMI + 10% FBS suppl. with Hydrocortisone (0,5 mg/mL), EGF (20ng/mL), Cholera toxin (100 ng/ml), insulin (10µg/mL)	ATCC	III, V
MCF-7	Breast cancer	ER+, PR-, HER2-	DMEM + 10% FBS	JG	II, III, IV, V
MDA-MB-436	Breast cancer	TNBC**	DMEM + 10% FBS	ATCC	III, V
MDA-MB-453	Breast cancer	TNBC****	DMEM + 10% FBS	ATCC	V
MDA-MB-468	Breast cancer	TNBC*	RPMI + 10% FCS + 1% sodium pyruvate + 1% L-glutatmine + 1% penstrep	GL	V
ZR-75-30	Breast cancer	ER+, PR-, HER2+	RPMI + 10% FBS	ATCC	II

[a] TNBC = triple-negative breast cancer (ER-, PR-, HER2-) * = BL1 subtype, ** = BL2 subtype, *** = M subtype , **** = LAR subtype, [b]ATCC = American Type Culture Collection, DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen, MJ = Group, Martin Johansson, University of Gothenburg, JN = Group, Jonas Nilsson, University of Gothenburg, JG = Julie Grantham, University of Gothenburg, GL = Group, Göran Landberg, University of Gothenburg.

Table 2. Pharmaceutical drugs, solvents, and working concentrations

Drug	Solvent	Working concentration	Supplier	Study
Proteasome inhibitors				
Bortezomib	DMSO	1-10000 nM	Selleckchem	II, III, IV, V
Carfilzomib	DMSO	1-10000 nM	Selleckchem	IV, V
Celastrol	DMSO	1-10000 nM	Selleckchem	IV, V
Delanzomib	DMSO	1-10000 nM	Selleckchem	IV, V
Epoxomicin	DMSO	1-10000 nM	Selleckchem	IV, V
MG-132	DMSO	1-10000 nM	Selleckchem	IV, V
MLN-2238	DMSO	1-10000 nM	Selleckchem	IV, V
MLN-9708	DMSO	1-10000 nM	Selleckchem	IV, V
Oprozomib	DMSO	1-10000 nM	Selleckchem	IV, V
PI-1840	DMSO	1-10000 nM	Selleckchem	IV, V
VR-23	DMSO	1-10000 nM	Selleckchem	IV, V
Mitosis inhibitors				
Docetaxel	DMSO	1-10000 nM	Selleckchem	V
Paclitaxel	DMSO	1-10000 nM	Selleckchem	V
Topoisomerase inhibitors				
Doxorubicin	DMSO	1-10000 nM	Selleckchem	V
Epirubicin	DMSO	1-10000 nM	Selleckchem	V
Platinum agents				
Carboplatin	Milli-Q water	2-1024 µM	Selleckchem	III, V
Cisplatin	0.9% NaCl	3-768 µM	Selleckchem	III, V
Nedaplatin	Milli-Q water	2-1024 µM	Selleckchem	V
Putative proteasome inhibitors				
kinetin riboside	DMSO	1-10000 nM	Sigma-Aldrich	IV
Manumycin-A	DMSO	1-10000 nM	Sigma-Aldrich	IV
Puromycin dihydrochloride	Milli-Q water	1-10000 nM	Sigma-Aldrich	IV
Tegaserod maleate	DMSO	1-10000 nM	Sigma-Aldrich	IV
Thapsigarin	DMSO	1-10000 nM	Sigma-Aldrich	IV
Resistomycin	DMSO	1-10000 nM	Cayman Chemicals	IV

DMSO = Dimethylsulfoxide

Data analysis

Resazurin-based cell viability assay

The resazurin-based cell viability assay was used to validate the chemosensitivity of cell lines treated with bortezomib in the GDSC1 and GDSC2 datasets (**Study II**). In **Study III**, the assay was used to determine cell viability at different culturing conditions and identify optimal settings that improve the replicability and reproducibility of the drug screen. In **Study IV**, it was used to identify cell lines showing sensitivity to putative PSM inhibitors in comparison with known PSM inhibitors. In **Study V**, the sensitivity of cell lines to 18 compounds was determined for both monotherapy and 13 selected 2-drug combinations in TNBC cell lines.

Method

The resazurin-based cell viability assay is a powerful tool with many applications e.g., cell proliferation, compound cytotoxicity screening, and optimal seeding density. The workflow for compound cytotoxicity screening includes: First, the cells were plated on a 96-well plate (cell plate) at an appropriate seeding density per well ($4 - 7.5 \times 10^3$ cells/well), secondly, after 24h incubation the cells were treated at increasing concentrations of the drug and corresponding controls that were prepared on a 96-well PCR plate before treatment (drug plate) and third, after 24h treatment, resazurin was added to the cells and viability (resorufin absorbance) was measured with a Wallac 1420 VICTOR2^{TM} plate reader (Perkin Elmer; 560 nm excitation filter and a 615 nm emission filter) after 4h incubation.

Flow cytometry (Annexin V and cell cycle analysis)

Flow cytometry was used to evaluate BTZ-induced cell cycle arrest in cell lines. In **Study II**, the A-172, A-375, BT-474, and ZR-75-30 cell lines were evaluated, while the HCC38 cell line was evaluated in **Study III**. In **Study II**, BTZ-induced apoptosis (Annexin V) was analyzed in the A-172, A-375, BT-474, and ZR-75-30 cell lines.

Method

In the cell cycle and apoptosis analyses (Annexin V), the cells were first incubated at 37°C in a humidified 5% CO_2 for 24h. Second, the cells were harvested after treatment for 24h or 72h. In the cell cycle analysis, the cells were fixed with 70% ethanol and then stained with propidium iodide (DNA staining). The wavelength of 510 nM with the green laser

was used. The samples were run in a LSRFortessa™ flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). In the apoptosis analysis (Annexin V), the cells were suspended in Annexin V Binding Buffer followed by Annexin V-FITC Conjugate. DNA was stained with propidium iodide and diluted with more Annexin-V Binding Buffer. The cells were run in the BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) with the FL1 (Annexin V-FITC) and FL3 channels (propidium iodide). The results were analyzed and visualized using FlowJo™ software v10.8.1 (BD Life Sciences).

Proteasome activity assay

The PSM activity assay was used in **Study II** and **III** where PSM activity of the β5 catalytic site was evaluated in cell lines (**Study II** [A-172, A-375, BT-474, and ZR-75-30] and **Study III** [HCC38, MCF-10A, MCF-7, and MDA-MB-436]) treated with BTZ. This assay was also used in **Study IV** and **V** with MCF-7 cells to validate the catalytic activity (β1, β2, and/or β5) of the identified putative PSM inhibitors (manumycin-A, kinetin riboside, puromycin dihydrochloride, resistomycin, thapsigargin or tegaserod maleate) and known PSM inhibitors (bortezomib, carfilzomib, celastrol, delanzomib, epoxomicin, MG-132, MLN-2238, MLN-9708, oprozomib, PI-1840, and VR-23).

Method

The cells were seeded in white (luminescence) or black (fluorescence) flat-bottom 96-well plates (ThermoFisher Scientific) at 7.5×10^3 cells per well. The cells were incubated for 24h and then the cells were treated with drugs for 2 - 6h and corresponding controls before PSM activity markers (Promega kit [Proteasome-Glo-Chymotrypsin-Like Cell-Based Assay]) or (Z-Leu-Leu-Glu-AMC [caspase-like; Enzo, Cat. BML-ZW9345], Ac-Arg-Leu-Arg-AMC [trypsin-like; Enzo, Cat. BML-AW9785] or Suc-Leu-Leu-Val-Tyr-AMC [chymotrypsin-like; Enzo, Cat. BML-P802]) were added to the cells for 30 min or 2h, and the activity was determined by measuring the fluorescence or luminescence intensity with a Wallac 1420 VICTOR2™ microplate reader plate reader (Perkin Elmer), which correlated to activity.

Immunoblot

Immunoblot (also called Western blot) technique was used in **Study V** to determine the protein levels of androgen receptor (AR) across eight TNBC cell lines (BT-549, CAL-148, HCC1806, HCC38, HCC70, MDA-MB-436, MDA-MB-453, and MDA-MB-468); two cell lines from each TNBC subtype (BL1, BL2, M, and LAR). The BT-474 cell line was used as control and beta-actin was used as a loading control. In **Study IV**, we studied the accumulation of poly-ubiquitinated proteins in MCF-7 cells caused by treatment with six putative proteasome inhibitors with a working concentration of 10,000 nM.

Method

Firstly, Benzonase® Nuclease, protease inhibitor, and phosphatase inhibitor were added to the Qproteome Mammalian Lysis Buffer (Qiagen, Hilden, Germany), followed by cell lysis and protein isolation. Second, Bradford Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA, USA) was used to determine the protein concentration with a Wallac 1420 VICTOR2™ plate reader (Perkin Elmer) set at 595 nm. Third, 50 µg of protein was loaded in each well on a NuPAGE 4-12% Bis-Tris gel (ThermoFisher Scientific) and two ladders (Novex® Sharp Pre-stained Protein Standard [3.5 – 260 kDa] and the MagicMark™ XP Western Protein Standard [20–220 kDa]; ThermoFisher Scientific). The proteins were separated by molecular weight with gel electrophoresis and then transferred to a nitrocellulose membrane. Unspecific sites on the membrane were blocked with non-fat dry milk overnight in the refrigerator and then the protein of interest was probed with the primary antibody and then the secondary antibody (**Table 3**). SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher) was used as detection reagent. Images were taken with the Fujifilm LAS-1000 Luminescent Image Analyzer and the protein band concentration on the membrane was quantified using ImageJ version 1.53t software.

Table 3. Primary and secondary antibodies for immunoblot

Antibody	Dilution	Host	Cat. nr	Supplier	Note	Study
Primary antibody						
Anti-androgen receptor (AR)	1:250	Rabbit	ab133273	Abcam		V
Anti-ubiquitin	1:200	Mouse	VU-0101	LifeSensors		IV
Anti-beta-actin	1:2000	Mouse	ab6276	Abcam	LC	IV, V
Secondary antibody						
Anti-mouse HRP-linked	1:2000	Sheep	NA931V	Amersham		IV, V
Anti-rabbit HRP-linked	1:2000	Donkey	NA934V	Amersham		V

LC = loading control

Gene expression analysis (qPCR)

In **Study II** and **Study IV,** gene expression analysis was performed using quantitative real-time PCR to evaluate the genes induced by BTZ treatment in different cell lines (**Study II** [BT-474, MCF-7, and ZR-75-30] and **Study IV** [MCF-7]).

Method

Both untreated (DMSO) and treated (1, 6, and 24h with 10 µM BTZ) cells were used. RNA was extracted from cells using the RNeasy Lipid Tissue Mini Kit (Qiagen). RNA integrity and concentration were evaluated with TapeStation (Agilent) and Qubit (ThermoFisher Scientific), respectively. Superscript III First-Strand Synthesis for qRT-PCR kit (ThermoFisher Scientific) was used to construct cDNA and TaqMan Gene Expression Assays were used to assess gene expression for genes of interest and three endogenous controls (**Table 4**).

Table 4. Primers for qPCR

Gene	Assay ID	Supplier	Note	Study
SHARPIN	HS00229642_m1	ThermoFisher Scientific		II
VPS28	HS01598026_m1	ThermoFisher Scientific		II
HPRT1	HS02800695_m1	ThermoFisher Scientific	EC	II, IV
PPIA	HS99999904_m1	ThermoFisher Scientific	EC	II, IV
PUM1	HS00472881_m1	ThermoFisher Scientific	EC	II, IV
HMOX1	HS01110250_m1	ThermoFisher Scientific		IV
DNAJB1	HS00428680_M1	ThermoFisher Scientific		IV

EC = Endogenous controls (geometric mean was used)

Computational analysis

Statistical analyzes were performed using Bioconductor in R (versions; 3.6.1 [Study I], 4.0.3 [Study II and Study III] and 3.14.0 [Study V]) and Microsoft Excel 2016/2019 (Study II). A p-value < 0.05 was statistically significant.
Manhattan Distance Metric (MDM) and Ward's Minimum Variance Method (Ward.D2) were used together with the pheatmap package (version 1.0.12) [136] to compare and visualize PSM gene expression between normal and cancer tissues (Study I; Log2 relative RNA-seq) and gene expression (Study II; Z-Score).

Study I

Wilcoxon test and Benjamin-Hochberg adjusted p-value (ns = not significant (P > 0.05); *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001) was used to determine the amplification effect on expression and individual PSM gene expression between cancer and normal samples and were visualized using boxplots (ggpubr [version 0.2.4.999] [137] and rstatix [version 0.4.0.999]) [138]. Co-expression of PSM genes were determined using pairwise Pearson's correlation coefficient (r) (0 < r < 0.4 (weak); 0.4 < r < 0.7 (moderate); r > 0.7 (strong)), clustered using Ward.D2, and it was visualized with R package corrplot (version 0.84) [139]. Multivariable Cox regression analysis (age and tumor grade) was used to determine overall survival (OS) and progression-free interval (PFI) of individual PSM genes and their expression pattern.

Study II

Two sensitivity groups (insensitive and sensitive) were stratified by AUC and IC50 (LNIC50) values for bortezomib. MDM and Ward.D2 along with pheatmap package (version 1.0.12) [136] were used to visualize gene expression (Z-score) in cell lines. The Limma R package (version 3.50.0) [140] was used to assess the differentially expressed genes between sensitivity groups. R-package ggstatsplot (version 0.9.1) and ggbetweenstats[141] were used to evaluate and visualize the mutation frequency between the sensitive groups. Stratification of the 50 most mutated genes across in each sensitive groups were visualized using maftools (version 2.10.0) and GenVisR[142,143]. Cell cycle distribution and apoptosis were assessed after treatment with bortezomib (concentration range 1-100nM) using FlowJo™ software v10.8.1. Statistically significant differences between concentrations were assessed using one-way ANOVA and dplyr in R-package (version 1.0.8) [144].

Study III

Quality control metrics (QCM; signal window [SW], Z-factor [Z], coefficient of variation [CV]) [100,145] were used to assess the performance of the optimized cell viability assay. The Wilcoxon test was used to determine the evaporative effect on the inner or outer well of a 96-well plate. R-packages ggpubr (version 0.2.1.999) [137] and rstatix (version 0.1.1.999) [138] were used to create bar charts and statistical analysis using t-test or Wilcoxon test with Benjamin-Hochberg adjusted p-value.

Study IV

To determine whether the data were normally distributed, the Shapiro-Wilks test and the R-package dplyr (version 1.0.8) [146] were used. The result of this test tells us what test to use, normally distributed = parametric test and if not normally distributed = Wilcoxon test. R-packages ggpubr (version 0.4.0) [137] and ggplot2 (version 3.3.6) [144] were used to generate bar charts and violin charts respectively and Benjamin-Hochberg statistical analysis corrected p-values in bar charts.

Study V

The metrics GR50, GRmax, IC50 and AUC mean, and standard deviation were used to determine the efficacy of the drugs on cell lines. R-package IDACombo was used to predict possible potent drug combinations and was visualized using R-package pheatmap (version 1.0.12) [136] and the MDM and Ward.D2. R-package ggplot2 (version 3.3.6) [144] was used to create scatterplots, dotplots and used in correlation analysis between GR50 and band quantification of western blot (AR).

RESULTS AND DISCUSSION

Study I

Analysis of genomic and transcriptomic alterations reveals PSM genes involved in patient overall survival

Genomic changes in the cell have been associated with cancer progression [147]. These events can alter gene expression and are used to classify tumors and identify pathways for cancer progression genes, which can then be used as therapeutic targets to improve the survival of cancer patients [147,148]. To identify genetic alterations and transcriptomic profiles for PSM genes associated with clinical outcome in **Study I**, we therefore used datasets from The Cancer Genome Atlas (TCGA) project with its comprehensive genomic, transcriptomic, and survival data for approximately 11,000 samples [32,149-151]. The TCGA data were retrieved from various online databases, including mutational and copy number alteration data (cBioPortal and Broad GDAC Firehose), gene expression data for normal and cancer samples (Broad Institute), and patient survival data (UCSC Xena browser). To correlate between PSM gene expression and overall survival, external validation of our findings was performed with an in-house breast cancer cohort and the interactive Kaplan-Meier plotter website (KM plotter; RNA microarray data for breast-, gastric-, lung-, and ovarian cancer as well as RNA-seq data for liver cancer).

By analyzing the rate of DNA amplification (≥5 gene copies of the same gene), fusion, multiple alterations (e.g., fusions and amplification), and mutation events in all 49 PSM genes, this analysis showed that DNA amplification was most prevalent across the different cancer types. Moreover, esophageal carcinoma (ESCA; 67%) and lung squamous carcinoma (LUSC; 66%) had the highest proportion of samples with altered PSM genes (**Figure 10**). Both cancer types have low 5-year survival rates in the US of 19% (ESCA) and 20% (LUSC), indicating that a high rate of genetic alterations may have an adverse effect on patient survival [152]. For specific PSM genes, the cancer samples most frequently harbored genetic alterations in *PSMD2* (6%), *PSMB4* (4%), and *PSMD4* (4%) (**Figure 10**). It is then not a surprising that DNA amplification was most prevalent in these genes. The *PSMD2* gene was altered in 37% of all LUSC samples, while >400 amplification events were found in the *PSMB4*, *PSMD2*, and *PSMD4* genes in the evaluated samples. It is suggested that amplicons are developed in connection with DNA double strand breakage, which occurs in deficient cells (e.g., cancer cells) that continue in the cell cycle without arrest [153]. Gene amplification has previously been associated with elevated gene expression, treatment resistance, and tumor evolution [153].

Amplification events often occur at the focal (0.5 kb-85 Mb) or broad level (chromosome arm to whole chromosome) and span a wide range of genes [154]. DNA

amplification regions frequently span oncogenes, leading to their activation and e.g., treatment resistance [12,149]. Some of the PSM genes were also co-amplified due to their proximity on the same cytoband (e.g., *PSMB4* and *PSMD4*; 1q21.3). In contrast, other PSM genes span the same cytoband as known oncogenes (*ERBB2* and *BRCA1*), i.e., *PSMB3* (17q12) and *PSME3* (17q21.31), respectively. Focal amplifications of the *PSMB4*, *PSMD2*, and *PSMD4* genes could be detected in about 10 cancer types. Subsequent amplification of PSM genes correlated with significant levels of overexpression. Although amplification was the most common alteration in PSM genes, we identified other PSM genes containing primarily mutational events (e.g., *PSME4*). Of the 312 mutations found in *PSME4*, 243 were missense mutations (a base pair substitution) that likely results in amino acid changes, but how the function of the protein is affected is unknown [155].

Due to missing gene expression data for 17 normal tissue types, the gene expression analysis could only be performed for 16 cancer types and their corresponding normal tissues. Nevertheless, most PSM genes had increased expression in cancer compared to normal tissue. Interestingly, 7/49 PSM genes (e.g., *PSMA1, PSMA4, PSMC1, PSMC3IP*, *PSMD13*, and *PSMG2-3*) were only overexpressed in cancer. *PSMB11*, which encodes for the thymoproteasome, was the least frequent differentially expressed PSM genes in only 2/16 cancer types (KICH and THCA), while *PSME3* was most frequently differentially expressed (15/16 cancer types). *PSME3* is involved in activation of the PSM, which could be an interesting target for cancer treatment. Furthermore, high levels of *PSMC* genes have also previously been associated with poor prognosis in breast cancer [97]. The overexpression of PSM genes most likely correlates with the essential features of the PSM process that the cell needs for survival [156,157].

Analysis of co-expressed PSM genes revealed a cluster containing *PSMB8-10* across all cancer types that was generally correlated with positive co-expression with *PSME1-2*. The *PSMB8-10* genes encode for the immunoproteasome and are involved in the immune system. Interestingly, high expression of these genes has previously been associated with prolonged survival in cancer patients, except for hematological cancers [150]. Thus, the role of the immune system and gene expression patterns for *PSMB8-10* in particular need further investigation for its role in cancer.

Several PSM genes (e.g., *PSMA4*, *PSMB4*, *PSMB8*, and *PSMB10*) were associated with more unfavorable overall survival (OS) when overexpressed and only two genes (*PSMA1* and *PSMD11*) with progression-free interval (PFI). Interestingly, these genes (*PSMA1* and *PSMD11*) were identified in both analyses (OS and PFI). Some of these genes (e.g., *PSMA4* and *PSMB5*) have previously been associated with reduced survival when overexpressed [151,158]. In summary, by using large datasets containing cancer patient samples in **Study I**, we were able to identify PSM genes involved in unfavorable survival when they were dysregulated. We were also able to identify genes (e.g., *PSMB8*) in a cluster that had positive correlation and are known to be involved in poor cancer

prognosis [159]. Further research needs to be done to identify putative prognostic biomarkers and selection of treatment for improved patient survival.

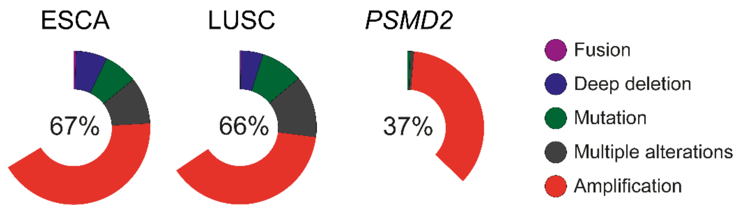

Figure 10. Genomic alterations of proteasome genes (PSM) in cancer. Esophageal carcinoma (ESCA) and lung squamous carcinoma (LUSC) had the highest percentage of altered PSM genes. The PSM gene *PSMD2* was altered in 37% of the LUSC samples. Amplification was most prevalent.

Study II

Analysis of sensitivity to bortezomib reveals genomic and transcriptomic events involved in sensitivity

Genomic instability and cancer progression are synonymous with each other [147]. Increased frequency of genomic alterations (e.g., DNA amplification) has previously been associated with treatment resistance [9,16-18]. Evaluation of genetic alterations, transcriptomic profiles, and response to treatment with bortezomib can identify cancer types that will benefit from treatment with this PSM inhibitor. In **Study II**, we performed a pharmacogenomic analysis of publicly available data to investigate how effective bortezomib is in treating different types of cancer. We also identify genomic and transcriptomic events associated with BTZ-sensitivity. For this analysis, we used drug sensitivity, genomic, and transcriptomic data for 860 cell lines (GDSC1 and GDSC2 cell line datasets) from different sources (e.g., Genomics of Drug Sensitivity in Cancer [GDSC] database, Sanger COSMIC, and Sanger Institute Cell Model Passport). We were able to stratify this cell line cohort into a small number of cell lines that were insensitive (38 cell lines) or sensitive (49 cell lines) to bortezomib.

Bortezomib is an effective drug that counteracts several important biological processes in the cell following treatment. In prostate cancer, bortezomib has been shown to inhibit e.g., cell cycle progression and induction of apoptosis [160,161]. In line with these findings, we also observed that bortezomib treatment of our validation cell lines (A-172, A-375, BT-474, and ZR-75-30) resulted in cell cycle arrest in G2/M phase and induction of apoptosis. Analysis of genomic and transcriptomic events involved in treatment resistance was also investigated. *TP53* is a tumor suppressor gene that

exhibits an apoptotic and treatment resistance phenotype when mutated [162]. We were able to identify an increased number of mutated genes in cell lines classified as bortezomib-insensitive in comparison with bortezomib-sensitive. Some of the genes (e.g., *FSIP2*, *MUC12*, and *TP53*) were concordant between the insensitive cell lines and linked to treatment resistance. The mutated genes were involved in e.g., DNA repair.

Gene expression profiles for cancer cells have previously been associated with treatment resistance and tumor progression, which could be due to e.g., epigenetic changes leading to the underexpression of tumor suppressor genes and overexpression of oncogenes [163]. However, 33 dysregulated genes between the two sensitivity groups, and especially the *LIFR* gene, have been associated with cancer progression and treatment resistance when overexpressed [164]. Interestingly, hierarchical clustering of the differentially expressed genes between the sensitivity groups demonstrated that the bortezomib-sensitive cell lines clustered together (sorted into their organ system) more frequently than bortezomib-insensitive cell lines. This could be due to intra organ system variability, suggesting that specific cancer subtypes are insensitive to bortezomib [100]. Our comprehensive analysis consisted of almost 1000 cancer cell lines derived from 13 organ systems, each represented by 14 to 163 cell lines. Therefore, some organ systems were represented by relatively few cell lines e.g., thyroid (n=14) and soft tissue (n=19).

In summary, this comprehensive *in silico* drug sensitivity, genomic, and transcriptomic analysis reveals the importance of identifying therapeutic biomarkers before deciding on appropriate treatment strategies for patients. We identify dysregulated genes (e.g., *ALDH18A1*, *LIFR*, *PUF60*) and mutated genes (e.g., *FSIP2*, *MUC12*, and *TP53*) involved in sensitivity to bortezomib that can be used when choosing patient treatment with PSM inhibitors and especially bortezomib. These findings need further evaluation to identify patients that will benefit from treatment with PSM inhibitors, particularly bortezomib, to improve therapy response rates for patients with cancer.

Study III

Identification of confounding factors improves replicability and reproducibility of cell viability screening assays

The inability to reproduce and replicate results from *in vitro* drug screens due to intra and interlaboratory variability is a major problem in biomedical research [165-168]. This means that the failure rate during the later stages of the drug discovery process (*in vivo* and clinical trials) is tangible. Inconsistent *in vitro* drug screening is likely, in part, due to the lack of assay optimization to reduce the effect of confounding factors, e.g., cell seeding density, evaporation, and the use of multiple controls that produce inconsistent results, but perhaps also due to incorrectly performed lab work [169]. Therefore,

experimental factors that could potentially influence the performance of a cell viability assay must be identified and the performance of an assay must be validated to minimize the risk of subsequent failure during further preclinical and clinical tests and ensure that drug candidates produce reproducible and replicable results [170].

In **Study III**, we used established cell lines (HCC38, MCF-10A, MCF-7, and MDA-MB-436) and clinically relevant anticancer drugs (bortezomib, carboplatin, and cisplatin) to identify factors that can affect cell viability assays. We initially utilized commonly used settings for drug concentrations, culture medium composition, seeding density, and a single drug solvent control (dimethyl sulfoxide; DMSO). These settings affected reproducibility and reproducibility and hence the interpretation of drug potency. So, we identified possible confounding factors (e.g., evaporation on the drug and cell plates [edge effect], cell seeding density, drug solvent interference, medium type, medium volume, number of controls, resazurin [viability marker] incubation time, and resazurin concentration) that could affect the performance and interpretation of drug potency. Moreover, we set up a drug screen with different experimental parameters. One-way ANOVA was used to evaluate the influence of these experimental parameters on cell viability, with viability as the dependent variable and multiple independent variables (i.e., cell type, drug type, drug concentration, culture medium type, medium volume, solvent controls, resazurin concentration, and cell seeding density). Not surprisingly, e.g., cell type, drug type, and drug concentrations affect cell viability the most. Moreover, evaporation on 96-well microplates has previously been associated with affecting cellular metabolic activity [171]. Here, all independent variables were found to affect cell viability to some extent, which in turn will affect the outcome of a drug screen [166,172].

We investigated how evaporation affects (1) the initial medium volume in standard 96-well flat-bottom microplates for cells (i.e., cell plate) stored in an incubator (humidified environment, 5% CO_2 and at 37°C) and (2) diluted drugs (i.e., drug plate) stored in a -20°C freezer for 48h and sealed with parafilm. We observed a significantly ($P \leq 0.001$), higher degree of evaporation in the perimeter wells (rows A and H, and columns 1 and 12), which not surprisingly had a substantial effect on viability [171] since drug concentrations will be higher if added to a lower medium volume. We also revealed a significantly lower degree of evaporation when using 96-well PCR plates for the diluted drugs sealed with aluminum tape when stored in the freezer ($P \leq 0.0001$), which is preferable to storage in the refrigerator. Drug solvent (DMSO) concentration was also evaluated, showing an adverse effect on viability at low (1%) concentrations. Therefore, the effect of the drug solvent on cell viability should be minimized so that the assay will show the effect of the drug only. Since drug dilutions have different concentrations of the drug solvent, the solvent concentration should be matched to that found in each drug concentration. This can be time demanding but will ultimately minimize over or under estimation of drug potency.

After optimizing the resazurin assay, we performed the optimized assay three times to ensure its replicability and reproducibility. We were therefore able to recommend the following experimental parameters for HCC38, MCF-10A, MCF-7, and MDA-MB-436 cell lines treated with bortezomib and cisplatin: medium type for each cell line, 100 µL medium volume, no drug renewal, multiple matched solvent-concentration controls (DMSO), 10% resazurin exposure for 4h, and seeding 7,500 cells/well. The assay results were compared to publicly available data at PharmacoDB which provides comprehensive drug screening data on cell lines from multiple pharmacogenomics projects [126], which were in agreement with our data. However, the experimental parameters suitable for other viability assays and/or cell types should always be optimized. The performance of our drug screen was evaluated using quality control metrics (QCM; Z-factor, signal window [SW], coefficient of variation [CV]) with thresholds set to Z-factor > 0.4, SW > 2, and CV < 20%), which showed that assay optimization improved reproducibility and replicability.

In summary, we established that unoptimized viability assays will likely produce inconsistent data that are difficult to compare between labs. We have highlighted which factors can affect the assay results, thereby making it both difficult to replicate results within a lab and form an idea of which drugs are "good" for a specific disease. Therefore, the risk of rejecting "good" drugs increases, but also of overestimating the potency of potentially unsuitable drugs.

Study IV

PSM inhibitor-induced transcriptomic signatures reveals other compounds with similar mechanisms-of-action

Repurposing FDA/EMA approved drugs is beneficial because it is cheaper, less time consuming, low risk of failure, and low risk of unknown side effects for patients [112]. This approach for identifying drugs for a disease or identifying similar potent drugs for the same disease could be performed in several different ways. Drug-induced signatures can identify similar drugs with the same mechanism-of-action or drug- and disease signatures together can reveal suitable drugs for a disease [117]. In **Study IV**, we evaluated publicly available drug-induced gene signatures (iLINCS and CMap) and the structure of the human 20S proteasome (Protein Data Bank) to identify compounds with previously unknown features as PSM inhibitors. First, compounds inducing similar transcriptomic profiles as known PSM inhibitors (bortezomib, MG-132, and MLN-2238) were identified. The list of compounds was then limited to those with docking scores revealing their ability to bind and inhibit the chymotrypsin catalytic site (β5) in the PSM. To identify genes that were dysregulated following treatment, we used the iLINCS dataset where cell lines were treated with MG-132 or MLN-2238 for 6h or 24h. This analysis revealed

11 genes (e.g., *BAG3*, *DNAJB1*, and *HMOX1*) that were upregulated in the tested cell lines [130]. Using CMap, 8 upregulated genes (e.g., *BAG3*, *DNAJB1*, and *HMOX1*) were found to be connected with treatment and likely correlated with the drug mechanism-of-action [173]. The identified genes were found to play a role in e.g., cellular response to stress. Using the MCF-7 cell line to validate these findings, we confirmed elevated expression of *DNAJB1* and *HMOX1* following ≥6h treatment with ≥100nM bortezomib.

Furthermore, a query for PSM inhibitors (MG-132 and MLN-2238) in CMap was performed to search for perturbagens with induced gene expression patterns (median *tau* score ≥95) similar to MG-132 and MLN-2238. This analysis revealed 113 perturbagens similar to MG-132 and 152 for MLN-2238. However, 96 compounds (e.g., manumycin-A and thapsigargin) and 11 gene knockdowns (e.g., *PSMB5*) were concordant in both queries. Furthermore, knockdown of PSM genes has a similar cellular effect as inhibition of PSM activity [174,175], which in turn causes protein accumulation in the cell and apoptosis. To further evaluate the potency of the identified compounds, we used the online GR Metric Calculator (http://www.grcalculator.org/grcalculator/). However, only 18/96 compounds were available (missing data for 78 compounds). Nevertheless, we were able to identify compounds (e.g., thapsigargin) with similar potency in cell lines as bortezomib.

We then used molecular docking to evaluate the 96 compounds and their ability to bind to and inhibit the β5 catalytic site [176]. Bortezomib binds to the threonine (Thr1) which is proposed to be the chymotrypsin active site (β5 site) in the core particle on proteasome [177,178]. We therefore evaluated whether the putative PSM inhibitors bind to this site by e.g., hydrogen bonds to that amino acid. We could therefore identify eight compounds with high binding affinity to the Thr1 residual [177,178]. Moreover, 6/8 compounds (kinetin riboside, manumycin-A, puromycin dihydrochloride, resistomycin, tegaserod maleate, thapsigargin; two compounds were not available for purchase) were evaluated for their ability to inhibit the activity of the three PSM catalytic sites (chymotrypsin-like [β5], caspase-like [β1], and trypsin-like [β2]). Here, we could conclude that inhibition of these sites by the six compounds was poor. These results were surprising, so we also evaluated the PSM activity for 11 known PSM inhibitors (e.g., bortezomib, carfilzomib, and delanzomib) and evaluated their ability to inhibit the activity of the important β5 catalytic site. This analysis showed that bortezomib and delanzomib were strong inhibitors of the β5 site, while the remaining PSM inhibitors showed poor activity inhibition. We then assessed whether drug treatment resulted in accumulation of polyubiquitinated proteins, thereby showing a significant accumulation of polyubiquitinated proteins after treatment with manumycin-A. So, there must be some other underlying mechanisms-of-action connecting the known and putative PSM inhibitors together, such as inhibition of similar proteases in the cells [175].

In summary, by using transcriptomic profiling and docking scores we could identify putative PSM inhibitors (kinetin riboside, manumycin-A, puromycin

dihydrochloride, resistomycin, tegaserod maleate, and thapsigargin). We evaluated their ability to inhibit all three PSM catalytic sites, thereby showing that the six putative PSM inhibitors had a weak ability to inhibit these and their potency on A-375 and MCF-7 cell lines showing that puromycin dihydrochloride was the most potent drug of the six putative PSM inhibitors (**Figure 11**). So, it is likely not inhibition of the PSM catalytic sites that these compounds have in common with known PSM inhibitors (bortezomib, MG-132, and MLN-2238). Instead, these compounds most likely have another unknown target(s) in common with PSM inhibitors that warrants further investigation.

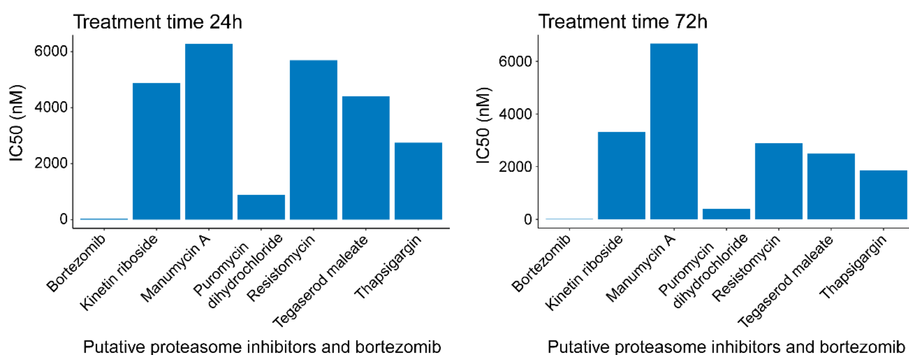

Figure 11. Potency of putative proteasome inhibitors on the A-375 cell line. Sensitivity analysis of putative proteasome inhibitors in comparison with a known proteasome inhibitor (bortezomib). The A-375 cell line was treated for 24h or 72h. Treatment time of 72h showing decreased cell survival in comparison with 24h. The most potent putative proteasome inhibitor was puromycin dihydrochloride at 24h and 72h treatment.

Study V

Drug screening identifies promising potent single drugs and 2-drug combinations to improve TNBC therapy

Cell viability assays can be used to investigate phenotypic characteristics (e.g., cell proliferation and drug cytotoxicity) of cells at different conditions [179]. The most useful is to screen large libraries of drugs on cell lines to determine their sensitivity to treatment, thereby identifying potentially novel treatment alternatives for a disease [179]. In **Study V**, we evaluated whether PSM inhibitors could improve *in vitro* treatment of TNBC, which is a cancer form that relies on chemotherapy [99,180,181]. We also (1) compared their ability to inhibit the β5 catalytic site to their drug potency and (2) investigated whether a synergistic effect occurs between 2-drug combination treatment. We used 8 TNBC cell lines and 2 control cell lines that were exposed to 11 PSM inhibitors and 7 clinically relevant drugs representing mitosis inhibitors, topoisomerase inhibitors, and platinum

agents. These analyses were therefore able to identify drugs that were potent as solitary treatment but most interestingly, we identified drug combinations that had a very high negative impact on the survival of TNBC cells.

As single agents, we identified bortezomib, carfilzomib, cisplatin, delanzomib, docetaxel, epoxomicin, MLN-2238, MLN-9708, and nedaplatin as highly potent drugs according to AUC values for the tested cell lines (**Figure 12**). The AUC is suitable to use when the different drugs have different concentration range. Surprisingly, no topoisomerase inhibitors (doxorubicin) were classified as potent drugs for TNBC though they are currently used in the clinical management of TNBC [181]. Not surprisingly, the most potent drugs were bortezomib and cisplatin across cell lines, which have previously been associated with TNBC anticancer activity [100,182,183]. The least potent drugs were the PSM inhibitors PI-1840 and VR-23. The LAR subtype was least sensitive to the tested drugs.

Given the varied cytotoxic effect of the 11 PSM inhibitors in the tested cell lines, we analyzed the PSM activity to determine their ability to inhibit the important 20S PSM chymotrypsin-like (β5) catalytic site [184]. This analysis showed that PSM inhibitors vary in their ability to inhibit the β5 site. Bortezomib and delanzomib strongly inhibited the β5 site, while PI-1840 and VR-23 inhibited the β5 site poorly which was not surprising according to their drug potency *in vitro*. The analysis of how the different proteasome inhibitors could cause accumulation of poly-ubiquitinated proteins in connection with treatment showed that all proteasome inhibitors except PI-1840 and VR-23 caused increased numbers of poly-ubiquitinated proteins. However, it was surprising that highly potent drugs like epoxomicin showed low ability to inhibit the β5 site. Therefore, we need to develop a better understanding of the mechanism-of-action for epoxomicin and why this drug was potent in TNBC cell lines. We hypothesize that epoxomicin might share another mechanism-of-action with PSM inhibitors.

Before setting up the combination treatment analysis, we used the IDACombo pipeline to predict potentially potent 2-drug combinations based on the data from the single drug treatments. This analysis resulted in 11 combinations; carboplatin + docetaxel and carboplatin + paclitaxel was also included as controls, resulting in 13 combinations. This screen revealed at least two (bortezomib + nedaplatin and epoxomicin + epirubicin) potent combinations that kill almost 100% of cells. The combination with bortezomib + nedaplatin showed a high degree of potency on TNBC cell lines, but lower on control cell lines which indicates that these combinations were very suitable for TNBC cell lines though not TNBC subtype-dependent. These results need to be further evaluated in more TNBC cell lines, their subtypes, *in vivo* models, and patient samples. In summary, we identified potent drugs as monotherapy (e.g., bortezomib and delanzomib) and even higher efficiency as 2-drug combinations (bortezomib + nedaplatin and epoxomicin + epirubicin) in TNBC cell lines. These findings need further evaluation using more TNBC cell lines and TNBC patient samples to confirm

their potency *in vitro* and *in vivo*. Surprisingly, 2-drug combinations with carboplatin + taxane showed the highest synergistic effect but have very weak potency in TNBC cell lines. Combination treatment with carboplatin and taxanes has previously been shown to improve treatment of TNBC [39,60,64]. However, it shows that high synergistic effect does not necessarily equate to high drug potency. The ability of known PSM inhibitors to inhibit the PSM β5 site was sometimes weak, indicating unknown shared mechanisms-of-action between PSM inhibitors that need more attention to better understand their effect on drug potency.

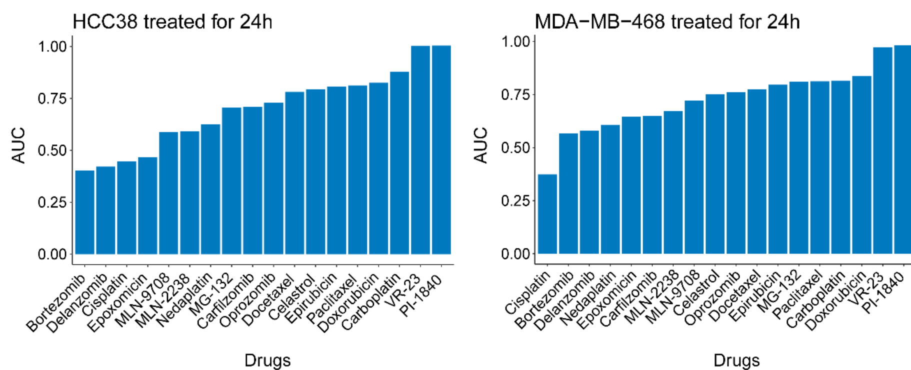

Figure 12. Drug screen on the HCC38 and MDA-MB-468 cell lines. Potency was interpreted by area under the curve (AUC). In HCC38 and MDA-MB-468 cells, bortezomib, cisplatin, and delanzomib were the most potent drugs. The least potent drugs in both analyses were carboplatin, doxorubicin, PI-1840, and VR-23.

ETHICAL COMMENTARY

Study I

The results of the genomic and transcriptomic analysis were validated using previously published data for breast cancer from our research group. Ethical approval to use patient tumor samples in the biobank was approved in 2002 (application S 164-02).

STUDY LIMITATIONS

Study I

- Some cancer types had relatively small sample sizes (e.g., DLBC [n = 48], PAAD [n = 56], and UCS [n = 57]) compared to others, making the results statistically less robust than those with larger sample size
- The differential gene expression analysis was limited to only 16 out of 33 cancer types due to missing data for corresponding normal tissue
- There was no data available on whether the patients had received neoadjuvant treatment, which could have affected the results

Study II

- Some organ systems contained relatively few cell lines, such as thyroid (n = 14), soft tissue (n = 19), and kidney (n = 23), which can reduce the statistical power of the analysis. In addition, information on the passage number and culturing conditions of the cell lines was lacking, which may impact the results as high passage numbers may affect gene expression and cell proliferation
- It is challenging to determine the clinical relevance of *in vitro* experiments performed with cell lines, so further investigation in other models (e.g., spheroids, patient-derived organoids, and animal model) is needed

Study III

- We did not test other types of 96-well plates which may differ in their ability to prevent evaporation
- We did not investigate the effect of different types of drug solvents on cell survival
- We did not determine whether the cells can be affected by the frequency of putting the 96-well plate in and out of the incubator
- We did not investigate how the cells are affected by the duration they are kept at room temperature during e.g., plating and application of treatment
- We did not explore how drug sensitivity for different cell lines is affected by different culturing conditions before the analysis starts
- We did not investigate how passage numbers affect the analysis
- We did not test cell lines derived from different organ systems

Study IV

- We did not investigate whether the putative PSM inhibitors have better permeability in solid tumors than, for example, bortezomib
- We did not perform studies in spheroids, patient-derived organoids, or animal models to determine whether the drug may be effective in patients
- We did not test the drugs as combination therapy
- We did not analyze the effect of the tested drugs on signaling pathways or inhibition of proteases in the cell, which could help in determining their similarity to PSM inhibitors
- We did not analyze whether the known PSM inhibitors affected other PSM catalytic sites (β1 and β2)
- Analysis of PSM activity was only performed once but should at least be performed in three independent experiments

Study V

- We have limited knowledge about how individual PSM inhibitors function and their effects on different processes in the cell
- The number of cell lines used was insufficient to establish a link between sensitivity and specific TNBC subtypes
- The identified drugs and drug combinations need to be tested in more comprehensive studies such as e.g., spheroids, patient-derived organoids, and animal models to determine their potency and toxicity

CONCLUSIONS

In this project, we performed a comprehensive investigation of PSM genes at the genomic and transcriptomic levels and explored their implications in cancer. We therefore demonstrated that proteasome inhibition could be a promising therapeutic approach in the treatment of several cancer types, including TNBC. However, more research is needed to establish suitable treatments and gain a better understanding of genomic and transcriptomic events to select the best treatment for individual patients.

Study I

The aim of this study was to investigate the prevalence of genetic alterations in the proteasome gene family in a pan-cancer dataset from The Cancer Genome Atlas (TCGA) and analyze the impact of dysregulated proteasome genes on clinical outcome.

Conclusions:

The frequency of genetic alteration varied among the 49 PSM genes, with *PSMB4* (4%), *PSMD2* (6%), and *PSMD4* (4%) having the most alterations in the 10,000 samples. The most frequently altered genes were found in ESCA and LUSC. Cancer types with the highest frequency of altered PSM genes were ESCA (67%) and LUSC (66%). Amplification was the most common alteration in PSM genes and was linked to overexpression in comparison with normal tissue. Furthermore, overexpression of PSM genes is commonly found in cancer and dysregulated PSM genes are associated with poor patient prognosis.

Study II

This study aimed to evaluate the sensitivity of cancer cell lines to proteasome inhibitors and identify a correlation between genomic or transcriptomic factors and sensitivity/insensitivity to proteasome inhibition.

Conclusions:

Identification and selection of bortezomib-insensitive (38 cell lines) and sensitive (49 cell lines) cell lines was based on their sensitivity to bortezomib from a comprehensive library of 860 cell lines. Mutated and or dysregulated genes involved in bortezomib resistance were identified. Several organ systems (blood, kidney, and nervous system) were sensitive to bortezomib, while e.g., lung was insensitive. Mutated genes involved in bortezomib resistance were e.g., *MUC12*, *RYR1*, and *SPTA1* and 33 dysregulated genes

(e.g., *ATP9A*, *CCNH*, and *PARP2*) were associated with bortezomib resistance. Further research is needed to investigate the involvement of mutations and gene expression profiles in bortezomib resistance.

Study III

The aim of this study was to improve the replicability and reproducibility of 2D high-throughput viability screens with cancer cell lines by identifying and minimizing the influence of assay-associated confounding factors.

Conclusions:

Several confounding factors such as drug and cell plate evaporation, drug storage, drug solvent controls, and medium volume were found to influence the outcome of the resazurin cell viability assay. Failing to minimize the influence of such factors may lead to a rejection of suitable drugs and overestimation of the potency of potentially unsuitable drugs that could ultimately fail in subsequent preclinical and clinical studies.

Study IV

The aim of this study was to identify putative proteasome inhibitors based on drug-induced transcriptomic profiles and drug binding affinity to the β5 proteasome catalytic site.

Conclusions:

Using drug-induced transcriptomic signatures, drugs with similar mechanism-of-action to known proteasome inhibitors were identified. We identified 96 compounds (putative proteasome inhibitors) with similar transcriptomic signatures as three proteasome inhibitors (bortezomib, MG-132, and MLN-2238), which were further investigated for their binding affinity to the β5 site. Eight compounds with high affinity to the β5 site were identified, but only six (heliomycin [resistomycin], kinetin-riboside, manumycin-A, puromycin dihydrochloride, tegaserod maleate, and thapsigargin) were further investigated *in vitro*. However, all six compounds showed poor inhibition of the proteasome activity at all three catalytic sites. Further research is therefore warranted to investigate why these compounds have similar transcriptomic signatures and their mechanism-of-action.

Study V

This study aimed to determine (1) the chemosensitivity of triple-negative breast cancer (TNBC) cell lines to proteasome inhibitors and clinically relevant chemotherapy in mono- and combination settings, and (2) the potential synergistic effect of combination therapy with proteasome inhibitors and common chemotherapy.

Conclusions:

We identified the most effective drugs in monotherapy, including mitotic inhibitors (docetaxel), platinum agents (cisplatin and nedaplatin), and proteasome inhibitors (bortezomib, carfilzomib, delanzomib, epoxomicin, MLN-2238, and MLN-9708). In addition, bortezomib + nedaplatin was the most effective 2-drug combination for CAL-148, HCC1806, MDA-MB-468, and epoxomicin + epirubicin as the most effective combination for HCC38 cells. However, further evaluation of these combinations is needed in similar or different research models (e.g., animals, patient tumors, and additional TNBC cell lines).

FUTURE PERSPECTIVES

- A panel of drugs was used on several cell lines to determine the chemosensitivity of specific cell lines to different drugs and drug efficacy after 24h treatment. However, some cells survived treatment. It was also not determined how these cells were affected by the 24h treatment phenotypically and whether they could continue to divide and give rise to new populations. To investigate the consequences of treatment on surviving cells, a follow-up study using clonogenic assay is required.

- Bortezomib treatment was found to be effective on TNBC cell lines, but *in silico* analysis showed that other types of cancer could also benefit from treatment with this drug. Further studies with bortezomib and the other cancer types identified in **Study II** are required**.**

- Different subtypes of TNBC responded differently to the same treatments, suggesting that despite classification into the TNBC subtypes, there are still significant differences within each subtype. To offer the best possible treatment for each patient, disease signatures need to be linked to the response to treatment with each drug. This could be achieved by using more TNBC cell lines and/or animal models.

- Further investigation is needed to determine how PSM inhibitors affect other cellular processes. It was found that different cell lines responded differently to PSM inhibitors. These inhibitors, in turn, inhibited the β5 catalytic site differently. The effects of different PSM inhibitors on different processes, such as cell cycle and DNA repair, needs to be assessed.

- Drugs that had a major impact on TNBC cells in mono drug- and 2-drug screens need further evaluation in more TNBC cell lines, spheroids, patient-derived organoids before potentially moving on to animal models and clinical trials with TNBC patients.

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor **Toshima** who gave me the opportunity to do my PhD with her and with an interesting project that is close to my heart. I would also like to thank **Toshima** for all the help and guidance through the difficult but educational doctoral studies. You are fantastically talented, helpful, and are very result-oriented person. I would also like to thank my co-supervisors **Khalil**, **Anikó**, **Eva**, and **Per** for your help during my doctoral studies.

A special thank I would like to address to my Master/Bachelor students **Daniella**, **Maxim**, and **Sesan**. It has been developing and fun to have you as students. I have learned a lot from you and hope you have learned a lot from me.

It has been a pleasure to work in the lab and in the office with all the nice people that have been around me there **Ella**, **Hanna**, **Daniella**, **Michelle**, **Gülay**, **Dorota**, **Agnieszka**, **Toshima**, **Maxim**, **Nishte**, **Charlotte, Sesan**, **Lucas**, **Hugo and others.** I would like to wish you good luck with your further research.

I will miss you all!

I also want to thank my family, friends and especially my wife Lotta for all the understanding when I had to work late evenings and weekends but also for the help with the everyday chores. It wouldn't have been possible without you.

There are many teachers (Adult Education Vänersborg/Trollhättan and later on the biology/molecular biology program at the University of Gothenburg) that I would like to thank, who have contributed and inspired me to continue to develop into a researcher.

REFERENCES

1 Carbone, A. Cancer Classification at the Crossroads. *Cancers (Basel)* **12**, doi:10.3390/cancers12040980 (2020).

2 Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**, 209-249, doi:10.3322/caac.21660 (2021).

3 Roy, P. S. & Saikia, B. J. Cancer and cure: A critical analysis. *Indian J Cancer* **53**, 441-442, doi:10.4103/0019-509X.200658 (2016).

4 Yao, Y. & Dai, W. Genomic Instability and Cancer. *J Carcinog Mutagen* **5**, 1000165, doi:10.4172/2157-2518.1000165 (2014).

5 Torpy, J. M., Lynm, C. & Glass, R. M. Cancer: The Basics. *JAMA* **304**, 1628-1628, doi:10.1001/jama.304.14.1628 (2010).

6 Negrini, S., Gorgoulis, V. G. & Halazonetis, T. D. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* **11**, 220-228, doi:10.1038/nrm2858 (2010).

7 Feinberg, A. P., Ohlsson, R. & Henikoff, S. The epigenetic progenitor origin of human cancer. *Nature reviews. Genetics* **7**, 21-33, doi:10.1038/nrg1748 (2006).

8 Song, Q., Merajver, S. D. & Li, J. Z. Cancer classification in the genomic era: five contemporary problems. *Hum Genomics* **9**, 27, doi:10.1186/s40246-015-0049-8 (2015).

9 Anand, P. *et al.* Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res* **25**, 2097-2116, doi:10.1007/s11095-008-9661-9 (2008).

10 Parsa, N. Environmental factors inducing human cancers. *Iran J Public Health* **41**, 1-9 (2012).

11 Uhlen, M. *et al.* A pathology atlas of the human cancer transcriptome. *Science* **357**, doi:10.1126/science.aan2507 (2017).

12 Matsui, A., Ihara, T., Suda, H., Mikami, H. & Semba, K. Gene amplification: mechanisms and involvement in cancer. *Biomol Concepts* **4**, 567-582, doi:10.1515/bmc-2013-0026 (2013).

13 Grade, M., Difilippantonio, M. J. & Camps, J. Patterns of Chromosomal Aberrations in Solid Tumors. *Recent Results Cancer Res* **200**, 115-142, doi:10.1007/978-3-319-20291-4_6 (2015).

14 Albertson, D. G., Collins, C., McCormick, F. & Gray, J. W. Chromosome aberrations in solid tumors. *Nature Genetics* **34**, 369-376, doi:10.1038/ng1215 (2003).

15 Hao, D., Wang, L. & Di, L.-j. Distinct mutation accumulation rates among tissues determine the variation in cancer risk. *Scientific Reports* **6**, 19458, doi:10.1038/srep19458 (2016).

16 Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S. & Baradaran, B. The Different Mechanisms of Cancer Drug Resistance: A Brief Review. *Adv Pharm Bull* **7**, 339-348, doi:10.15171/apb.2017.041 (2017).

17 Housman, G. *et al.* Drug resistance in cancer: an overview. *Cancers (Basel)* **6**, 1769-1792, doi:10.3390/cancers6031769 (2014).

18 Emran, T. B. *et al.* Multidrug Resistance in Cancer: Understanding Molecular Mechanisms, Immunoprevention and Therapeutic Approaches. *Front Oncol* **12**, 891652, doi:10.3389/fonc.2022.891652 (2022).

19 Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov* **12**, 31-46, doi:10.1158/2159-8290.CD-21-1059 (2022).

20 Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab* **23**, 27-47, doi:10.1016/j.cmet.2015.12.006 (2016).

21 Romero-Garcia, S., Lopez-Gonzalez, J. S., Baez-Viveros, J. L., Aguilar-Cazares, D. & Prado-Garcia, H. Tumor cell metabolism: an integral view. *Cancer biology & therapy* **12**, 939-948, doi:10.4161/cbt.12.11.18140 (2011).

22 Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394-424, doi:10.3322/caac.21492 (2018).

23 Thandra, K. C., Barsouk, A., Saginala, K., Aluru, J. S. & Barsouk, A. Epidemiology of lung cancer. *Contemp Oncol (Pozn)* **25**, 45-52, doi:10.5114/wo.2021.103829 (2021).

24 Pernar, C. H., Ebot, E. M., Wilson, K. M. & Mucci, L. A. The Epidemiology of Prostate Cancer. *Cold Spring Harb Perspect Med* **8**, doi:10.1101/cshperspect.a030361 (2018).

25 Sheikhalipour, Z., Ghahramanian, A., Fateh, A., Ghiahi, R. & Onyeka, T. C. Quality of Life in Women with Cancer and Its Influencing Factors. *J Caring Sci* **8**, 9-15, doi:10.15171/jcs.2019.002 (2019).

26 *Cancer statistics Sweden 2021 Socialstyrelsen*, <https://www.socialstyrelsen.se/> (2021).

27 Kim, H. I., Lim, H. & Moon, A. Sex Differences in Cancer: Epidemiology, Genetics and Therapy. *Biomol Ther (Seoul)* **26**, 335-342, doi:10.4062/biomolther.2018.103 (2018).

28 Kim, S. E. *et al.* Sex- and gender-specific disparities in colorectal cancer risk. *World J Gastroenterol* **21**, 5167-5175, doi:10.3748/wjg.v21.i17.5167 (2015).

29 Turnbull, C., Sud, A. & Houlston, R. S. Cancer genetics, precision prevention and a call to action. *Nat Genet* **50**, 1212-1218, doi:10.1038/s41588-018-0202-0 (2018).

30 Nordenskjold, A. E. *et al.* Breast cancer survival trends in different stages and age groups - a population-based study 1989-2013. *Acta Oncol* **58**, 45-51, doi:10.1080/0284186X.2018.1532601 (2019).

31 Oates, J. T. & Lopez, D. Pharmacogenetics: An Important Part of Drug Development with A Focus on Its Application. *Int J Biomed Investig* **1**, doi:10.31531/2581-4745.1000111 (2018).

32 Tomczak, K., Czerwinska, P. & Wiznerowicz, M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol (Pozn)* **19**, A68-77, doi:10.5114/wo.2014.47136 (2015).

33 Hakem, R. DNA-damage repair; the good, the bad, and the ugly. *Embo j* **27**, 589-605, doi:10.1038/emboj.2008.15 (2008).

34 Jin, Y. H. *et al.* Diagnostic Value of ER, PR, FR and HER-2-Targeted Molecular Probes for Magnetic Resonance Imaging in Patients with Breast Cancer. *Cellular Physiology and Biochemistry* **49**, 271-281, doi:10.1159/000492877 (2018).

35 Schnitt, S. J. Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. *Modern Pathology* **23**, S60-S64, doi:10.1038/modpathol.2010.33 (2010).

36 Yersal, O. & Barutca, S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World J Clin Oncol* **5**, 412-424, doi:10.5306/wjco.v5.i3.412 (2014).

37 Kondov, B. *et al.* Presentation of the Molecular Subtypes of Breast Cancer Detected By Immunohistochemistry in Surgically Treated Patients. *Open Access Maced J Med Sci* **6**, 961-967, doi:10.3889/oamjms.2018.231 (2018).

38 Tremont, A., Lu, J. & Cole, J. T. Endocrine Therapy for Early Breast Cancer: Updated Review. *Ochsner J* **17**, 405-411 (2017).

39 Waks, A. G. & Winer, E. P. Breast Cancer Treatment: A Review. *JAMA* **321**, 288-300, doi:10.1001/jama.2018.19323 (2019).

40 Mahtani, R. *et al.* Advances in Therapeutic Approaches for Triple-Negative Breast Cancer. *Clin Breast Cancer* **21**, 383-390, doi:10.1016/j.clbc.2020.12.011 (2021).

41 Almansour, N. M. Triple-Negative Breast Cancer: A Brief Review About Epidemiology, Risk Factors, Signaling Pathways, Treatment and Role of Artificial Intelligence. *Front Mol Biosci* **9**, 836417, doi:10.3389/fmolb.2022.836417 (2022).

42 Baranova, A. *et al.* Triple-negative breast cancer: current treatment strategies and factors of negative prognosis. *J Med Life* **15**, 153-161, doi:10.25122/jml-2021-0108 (2022).

43 Bissanum, R., Chaichulee, S., Kamolphiwong, R., Navakanitworakul, R. & Kanokwiroon, K. Molecular Classification Models for Triple Negative Breast Cancer Subtype Using Machine Learning. *J Pers Med* **11**, doi:10.3390/jpm11090881 (2021).

44 Lee, K. L., Kuo, Y. C., Ho, Y. S. & Huang, Y. H. Triple-Negative Breast Cancer: Current Understanding and Future Therapeutic Breakthrough Targeting Cancer Stemness. *Cancers (Basel)* **11**, doi:10.3390/cancers11091334 (2019).

45 Zhu, X. *et al.* The prognostic and predictive potential of Ki-67 in triple-negative breast cancer. *Sci Rep* **10**, 225, doi:10.1038/s41598-019-57094-3 (2020).

46 Lebert, J. M., Lester, R., Powell, E., Seal, M. & McCarthy, J. Advances in the systemic treatment of triple-negative breast cancer. *Curr Oncol* **25**, S142-S150, doi:10.3747/co.25.3954 (2018).

47 Masuda, H. *et al.* Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 5533-5540, doi:10.1158/1078-0432.Ccr-13-0799 (2013).

48 Yin, L., Duan, J. J., Bian, X. W. & Yu, S. C. Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res* **22**, 61, doi:10.1186/s13058-020-01296-5 (2020).

49 Norton, N. *et al.* Folate receptor alpha expression associates with improved disease-free survival in triple negative breast cancer patients. *npj Breast Cancer* **6**, 4, doi:10.1038/s41523-020-0147-1 (2020).

50 O'Shannessy, D. J., Somers, E. B., Maltzman, J., Smale, R. & Fu, Y. S. Folate receptor alpha (FRA) expression in breast cancer: identification of a new molecular subtype and association with triple negative disease. *Springerplus* **1**, 22, doi:10.1186/2193-1801-1-22 (2012).

51 Kelemen, L. E. The role of folate receptor alpha in cancer development, progression and treatment: cause, consequence or innocent bystander? *International journal of cancer* **119**, 243-250, doi:10.1002/ijc.21712 (2006).

52 Astvatsaturyan, K., Yue, Y., Walts, A. E. & Bose, S. Androgen receptor positive triple negative breast cancer: Clinicopathologic, prognostic, and predictive features. *PLoS One* **13**, e0197827, doi:10.1371/journal.pone.0197827 (2018).

53 Pierobon, M. & Frankenfeld, C. L. Obesity as a risk factor for triple-negative breast cancers: a systematic review and meta-analysis. *Breast Cancer Res Treat* **137**, 307-314, doi:10.1007/s10549-012-2339-3 (2013).

54 Feng, Y. *et al.* Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. *Genes Dis* **5**, 77-106, doi:10.1016/j.gendis.2018.05.001 (2018).

55 Dent, R. *et al.* Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 4429-4434, doi:10.1158/1078-0432.Ccr-06-3045 (2007).

56 Aysola, K. *et al.* Triple Negative Breast Cancer - An Overview. *Hereditary Genet* **2013**, 001, doi:10.4172/2161-1041.S2-001 (2013).

57 Guo, F., Kuo, Y. F., Shih, Y. C. T., Giordano, S. H. & Berenson, A. B. Trends in breast cancer mortality by stage at diagnosis among young women in the United States. *Cancer* **124**, 3500-3509, doi:10.1002/cncr.31638 (2018).

58 Lin, N. U. *et al.* Clinicopathologic features, patterns of recurrence, and survival among women with triple-negative breast cancer in the National Comprehensive Cancer Network. *Cancer* **118**, 5463-5472, doi:10.1002/cncr.27581 (2012).

59 Holleczek, B., Stegmaier, C., Radosa, J. C., Solomayer, E. F. & Brenner, H. Risk of loco-regional recurrence and distant metastases of patients with invasive breast cancer up to ten years after diagnosis - results from a registry-based study from Germany. *BMC Cancer* **19**, 520, doi:10.1186/s12885-019-5710-5 (2019).

60 Loibl, S., Poortmans, P., Morrow, M., Denkert, C. & Curigliano, G. Breast cancer. *The Lancet* **397**, 1750-1769, doi:https://doi.org/10.1016/S0140-6736(20)32381-3 (2021).

61 Li, Y. *et al.* Recent advances in therapeutic strategies for triple-negative breast cancer. *Journal of Hematology & Oncology* **15**, 121, doi:10.1186/s13045-022-01341-0 (2022).

62 Miglietta, F., Bottosso, M., Griguolo, G., Dieci, M. V. & Guarneri, V. Major advancements in metastatic breast cancer treatment: when expanding options means prolonging survival. *ESMO Open* **7**, 100409, doi:10.1016/j.esmoop.2022.100409 (2022).

63 Tong, C. W. S., Wu, M., Cho, W. C. S. & To, K. K. W. Recent Advances in the Treatment of Breast Cancer. *Front Oncol* **8**, doi:10.3389/fonc.2018.00227 (2018).

64 Hong, R. & Xu, B. Breast cancer: an up-to-date review and future perspectives. *Cancer Commun (Lond)* **42**, 913-936, doi:10.1002/cac2.12358 (2022).

65 Agostinetto, E., Gligorov, J. & Piccart, M. Systemic therapy for early-stage breast cancer: learning from the past to build the future. *Nature Reviews Clinical Oncology* **19**, 763-774, doi:10.1038/s41571-022-00687-1 (2022).

66 Varshavsky, A. Regulated protein degradation. *Trends Biochem Sci* **30**, 283-286, doi:10.1016/j.tibs.2005.04.005 (2005).

67 Tanaka, K. The proteasome: overview of structure and functions. *Proc Jpn Acad Ser B Phys Biol Sci* **85**, 12-36, doi:10.2183/pjab.85.12 (2009).

68 Tai, H.-C. & Schuman, E. M. Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nature Reviews Neuroscience* **9**, 826-838, doi:10.1038/nrn2499 (2008).

69 Thibaudeau, T. A. & Smith, D. M. A Practical Review of Proteasome Pharmacology. *Pharmacol Rev* **71**, 170-197, doi:10.1124/pr.117.015370 (2019).

70 Kaur, G. & Batra, S. Emerging role of immunoproteasomes in pathophysiology. *Immunol Cell Biol* **94**, 812-820, doi:10.1038/icb.2016.50 (2016).
71 Gomes, A. V. Genetics of proteasome diseases. *Scientifica* **2013**, 637629-637629, doi:10.1155/2013/637629 (2013).
72 Budenholzer, L., Cheng, C. L., Li, Y. & Hochstrasser, M. Proteasome Structure and Assembly. *J Mol Biol* **429**, 3500-3524, doi:10.1016/j.jmb.2017.05.027 (2017).
73 Abi Habib, J., Lesenfants, J., Vigneron, N. & Van den Eynde, B. J. Functional Differences between Proteasome Subtypes. *Cells* **11**, doi:10.3390/cells11030421 (2022).
74 Kimura, H., Caturegli, P., Takahashi, M. & Suzuki, K. New Insights into the Function of the Immunoproteasome in Immune and Nonimmune Cells. *J Immunol Res* **2015**, 541984, doi:10.1155/2015/541984 (2015).
75 Zhang, X., Linder, S. & Bazzaro, M. Drug Development Targeting the Ubiquitin-Proteasome System (UPS) for the Treatment of Human Cancers. *Cancers (Basel)* **12**, doi:10.3390/cancers12040902 (2020).
76 Weyburne, E. S. *et al.* Inhibition of the Proteasome β2 Site Sensitizes Triple-Negative Breast Cancer Cells to β5 Inhibitors and Suppresses Nrf1 Activation. *Cell Chem Biol* **24**, 218-230, doi:10.1016/j.chembiol.2016.12.016 (2017).
77 Vilchez, D., Saez, I. & Dillin, A. The role of protein clearance mechanisms in organismal ageing and age-related diseases. *Nat Commun* **5**, 5659, doi:10.1038/ncomms6659 (2014).
78 Rousseau, A. & Bertolotti, A. Regulation of proteasome assembly and activity in health and disease. *Nat Rev Mol Cell Biol* **19**, 697-712, doi:10.1038/s41580-018-0040-z (2018).
79 Nunes, A. T. & Annunziata, C. M. Proteasome inhibitors: structure and function. *Semin Oncol* **44**, 377-380, doi:10.1053/j.seminoncol.2018.01.004 (2017).
80 Nahar, A., Sokolova, V., Sekaran, S., Orth, J. D. & Park, S. Assembly checkpoint of the proteasome regulatory particle is activated by coordinated actions of proteasomal ATPase chaperones. *Cell Reports* **39**, 110918, doi:https://doi.org/10.1016/j.celrep.2022.110918 (2022).
81 Sharon, M., Taverner, T., Ambroggio, X. I., Deshaies, R. J. & Robinson, C. V. Structural organization of the 19S proteasome lid: insights from MS of intact complexes. *PLoS Biol* **4**, e267, doi:10.1371/journal.pbio.0040267 (2006).
82 Sha, Z. & Goldberg, A. L. Proteasome-mediated processing of Nrf1 is essential for coordinate induction of all proteasome subunits and p97. *Curr Biol* **24**, 1573-1583, doi:10.1016/j.cub.2014.06.004 (2014).
83 Bouzat, J. L. *et al.* Phylogenomic analysis of the alpha proteasome gene family from early-diverging eukaryotes. *J Mol Evol* **51**, 532-543, doi:10.1007/s002390010117 (2000).
84 Jang, H. H. Regulation of Protein Degradation by Proteasomes in Cancer. *J Cancer Prev* **23**, 153-161, doi:10.15430/JCP.2018.23.4.153 (2018).
85 Park, J. E., Miller, Z., Jun, Y., Lee, W. & Kim, K. B. Next-generation proteasome inhibitors for cancer therapy. *Transl Res* **198**, 1-16, doi:10.1016/j.trsl.2018.03.002 (2018).
86 Kisselev, A. F., Callard, A. & Goldberg, A. L. Importance of the Different Proteolytic Sites of the Proteasome and the Efficacy of Inhibitors Varies with the Protein Substrate. *Journal of Biological Chemistry* **281**, 8582-8590, doi:10.1074/jbc.M509043200 (2006).
87 Arendt, C. S. & Hochstrasser, M. Identification of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation. *Proceedings of the National Academy of Sciences* **94**, 7156-7161, doi:doi:10.1073/pnas.94.14.7156 (1997).
88 Britton, M. *et al.* Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem Biol* **16**, 1278-1289, doi:10.1016/j.chembiol.2009.11.015 (2009).
89 Chen, P. & Hochstrasser, M. Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. *Cell* **86**, 961-972, doi:10.1016/s0092-8674(00)80171-3 (1996).
90 Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U. & Wolf, D. H. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J Biol Chem* **272**, 25200-25209, doi:10.1074/jbc.272.40.25200 (1997).
91 L. Hopper, J., Begum, N., Smith, L. & A. Hughes, T. The role of PSMD9 in human disease: future clinical and therapeutic implications. *AIMS Molecular Science* **2**, 476-484, doi:10.3934/molsci.2015.4.476 (2015).

92 Hanssum, A. *et al.* An inducible chaperone adapts proteasome assembly to stress. *Molecular cell* **55**, 566-577, doi:10.1016/j.molcel.2014.06.017 (2014).

93 Chondrogianni, N., Sakellari, M., Lefaki, M., Papaevgeniou, N. & Gonos, E. S. Proteasome activation delays aging in vitro and in vivo. *Free Radic Biol Med* **71**, 303-320, doi:10.1016/j.freeradbiomed.2014.03.031 (2014).

94 Dou, Q. P. & Zonder, J. A. Overview of proteasome inhibitor-based anti-cancer therapies: perspective on bortezomib and second generation proteasome inhibitors versus future generation inhibitors of ubiquitin-proteasome system. *Current cancer drug targets* **14**, 517-536 (2014).

95 Seo, H., Sonntag, K. C., Kim, W., Cattaneo, E. & Isacson, O. Proteasome activator enhances survival of Huntington's disease neuronal model cells. *PLoS One* **2**, e238, doi:10.1371/journal.pone.0000238 (2007).

96 Huang, L. & Chen, C. H. Proteasome regulators: activators and inhibitors. *Curr Med Chem* **16**, 931-939, doi:10.2174/092986709787581860 (2009).

97 Kao, T.-J. *et al.* Prognoses and genomic analyses of proteasome 26S subunit, ATPase (PSMC) family genes in clinical breast cancer. *Aging (Albany NY)* **13**, 17970-17970, doi:10.18632/aging.203345 (2021).

98 Fricker, L. D. Proteasome Inhibitor Drugs. *Annual Review of Pharmacology and Toxicology* **60**, 457-476, doi:10.1146/annurev-pharmtox-010919-023603 (2020).

99 Yang, C. H. *et al.* Bortezomib (VELCADE) in metastatic breast cancer: pharmacodynamics, biological effects, and prediction of clinical benefits. *Ann Oncol* **17**, 813-817, doi:10.1093/annonc/mdj131 (2006).

100 Petrocca, F. *et al.* A genome-wide siRNA screen identifies proteasome addiction as a vulnerability of basal-like triple-negative breast cancer cells. *Cancer Cell* **24**, 182-196, doi:10.1016/j.ccr.2013.07.008 (2013).

101 Rinnerthaler, G. *et al.* Ixazomib in combination with carboplatin in pretreated women with advanced triple-negative breast cancer, a phase I/II trial of the AGMT (AGMT MBC-10 trial). *BMC Cancer* **18**, 1074, doi:10.1186/s12885-018-4979-0 (2018).

102 Edelman, M. J. *et al.* Phase I trial of carboplatin/paclitaxel/bortezomib and concurrent radiotherapy followed by surgical resection in Stage III non-small cell lung cancer. *Lung Cancer* **68**, 84-88, doi:10.1016/j.lungcan.2009.05.003 (2010).

103 Aghajanian, C. *et al.* Phase I trial of bortezomib and carboplatin in recurrent ovarian or primary peritoneal cancer. *J Clin Oncol* **23**, 5943-5949, doi:10.1200/JCO.2005.16.006 (2005).

104 Tapia, M. *et al.* Activity of bortezomib, a proteasome inhibitor, in breast cancer cells: Association with negative estrogen receptor and IKK/NF-κB expression. *Journal of Clinical Oncology* **23**, 3169-3169, doi:10.1200/jco.2005.23.16_suppl.3169 (2005).

105 Awada, A. *et al.* Bortezomib/docetaxel combination therapy in patients with anthracycline-pretreated advanced/metastatic breast cancer: a phase I/II dose-escalation study. *Br J Cancer* **98**, 1500-1507, doi:10.1038/sj.bjc.6604347 (2008).

106 Barrio, S. *et al.* Spectrum and functional validation of PSMB5 mutations in multiple myeloma. *Leukemia* **33**, 447-456, doi:10.1038/s41375-018-0216-8 (2019).

107 Xin, B. T. *et al.* Structure-Based Design of Inhibitors Selective for Human Proteasome β2c or β2i Subunits. *J Med Chem* **62**, 1626-1642, doi:10.1021/acs.jmedchem.8b01884 (2019).

108 Morozov, A. V. & Karpov, V. L. Proteasomes and Several Aspects of Their Heterogeneity Relevant to Cancer. *Front Oncol* **9**, 761, doi:10.3389/fonc.2019.00761 (2019).

109 Arlt, A. *et al.* Increased proteasome subunit protein expression and proteasome activity in colon cancer relate to an enhanced activation of nuclear factor E2-related factor 2 (Nrf2). *Oncogene* **28**, 3983-3996, doi:10.1038/onc.2009.264 (2009).

110 Trombetta, R. P., Dunman, P. M., Schwarz, E. M., Kates, S. L. & Awad, H. A. A High-Throughput Screening Approach To Repurpose FDA-Approved Drugs for Bactericidal Applications against Staphylococcus aureus Small-Colony Variants. *mSphere* **3**, doi:10.1128/mSphere.00422-18 (2018).

111 Rudrapal, M., J. Khairnar, S. & G. Jadhav, A. in *Drug Repurposing - Hypothesis, Molecular Aspects and Therapeutic Applications* (2020).

112 Pushpakom, S. *et al.* Drug repurposing: progress, challenges and recommendations. *Nat Rev Drug Discov* **18**, 41-58, doi:10.1038/nrd.2018.168 (2019).

113 DiMasi, J. A., Hansen, R. W. & Grabowski, H. G. The price of innovation: new estimates of drug development costs. *Journal of Health Economics* **22**, 151-185, doi:https://doi.org/10.1016/S0167-6296(02)00126-1 (2003).
114 Ashburn, T. T. & Thor, K. B. Drug repositioning: identifying and developing new uses for existing drugs. *Nature Reviews Drug Discovery* **3**, 673-683, doi:10.1038/nrd1468 (2004).
115 Wong, C. H., Siah, K. W. & Lo, A. W. Estimation of clinical trial success rates and related parameters. *Biostatistics* **20**, 273-286, doi:10.1093/biostatistics/kxx069 (2019).
116 Wang, Z., Lachmann, A., Keenan, A. B. & Ma'ayan, A. L1000FWD: fireworks visualization of drug-induced transcriptomic signatures. *Bioinformatics* **34**, 2150-2152, doi:10.1093/bioinformatics/bty060 (2018).
117 Cheng, J., Yang, L., Kumar, V. & Agarwal, P. Systematic evaluation of connectivity map for disease indications. *Genome Medicine* **6**, 95, doi:10.1186/s13073-014-0095-1 (2014).
118 Quirke, V. M. Tamoxifen from Failed Contraceptive Pill to Best-Selling Breast Cancer Medicine: A Case-Study in Pharmaceutical Innovation. *Front Pharmacol* **8**, 620, doi:10.3389/fphar.2017.00620 (2017).
119 Jordan, V. C. Tamoxifen: a most unlikely pioneering medicine. *Nature Reviews Drug Discovery* **2**, 205-213, doi:10.1038/nrd1031 (2003).
120 Garcia-Albeniz, X. & Chan, A. T. Aspirin for the prevention of colorectal cancer. *Best Pract Res Clin Gastroenterol* **25**, 461-472, doi:10.1016/j.bpg.2011.10.015 (2011).
121 agency, S. m. p. *Repurposing – projekt för att hitta ny användning för äldre läkemedel*, <https://www.lakemedelsverket.se/sv/tillstand-godkannande-och-kontroll/radgivning/innovationskontoret/repurposing--projekt-for-att-hitta-ny-anvandning-for-aldre-lakemedel> (2022).
122 Futreal, P. A. *et al.* A census of human cancer genes. *Nature reviews. Cancer* **4**, 177-183, doi:10.1038/nrc1299 (2004).
123 Dietlein, F. *et al.* Identification of cancer driver genes based on nucleotide context. *Nat Genet* **52**, 208-218, doi:10.1038/s41588-019-0572-y (2020).
124 Parris, T. Z. *et al.* Genome-wide multi-omics profiling of the 8p11-p12 amplicon in breast carcinoma. *Oncotarget* **9**, 24140-24154, doi:10.18632/oncotarget.25329 (2018).
125 Parris, T. Z. *et al.* Clinical Implications of Gene Dosage and Gene Expression Patterns in Diploid Breast Carcinoma. *Clinical Cancer Research* **16**, 3860, doi:10.1158/1078-0432.CCR-10-0889 (2010).
126 Smirnov, P. *et al.* PharmacoDB: an integrative database for mining in vitro anticancer drug screening studies. *Nucleic Acids Res* **46**, D994-D1002, doi:10.1093/nar/gkx911 (2018).
127 Hafner, M. *et al.* Quantification of sensitivity and resistance of breast cancer cell lines to anti-cancer drugs using GR metrics. *Sci Data* **4**, 170166, doi:10.1038/sdata.2017.166 (2017).
128 Subramanian, A. *et al.* A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles. *Cell* **171**, 1437-1452 e1417, doi:10.1016/j.cell.2017.10.049 (2017).
129 Pilarczyk, M. *et al.* Connecting omics signatures and revealing biological mechanisms with iLINCS. *Nat Commun* **13**, 4678, doi:10.1038/s41467-022-32205-3 (2022).
130 *iLINCS a platform for omic data*, <http://www.ilincs.org/ilincs/signatures/search/bortezomib> (2022).
131 Pearce, D. transcripTools: Collection of functions for analysing gene expression. (2022).
132 Gillespie, M. *et al.* The reactome pathway knowledgebase 2022. *Nucleic Acids Research* **50**, D687-D692, doi:10.1093/nar/gkab1028 (2022).
133 Ling, A. & Huang, R. S. Computationally predicting clinical drug combination efficacy with cancer cell line screens and independent drug action. *Nature Communications* **11**, 5848, doi:10.1038/s41467-020-19563-6 (2020).
134 Fan, K., Cheng, L. & Li, L. Artificial intelligence and machine learning methods in predicting anti-cancer drug combination effects. *Brief Bioinform* **22**, doi:10.1093/bib/bbab271 (2021).
135 Zheng, S. *et al.* SynergyFinder Plus: Toward Better Interpretation and Annotation of Drug Combination Screening Datasets. *Genomics, Proteomics & Bioinformatics* **20**, 587-596, doi:https://doi.org/10.1016/j.gpb.2022.01.004 (2022).
136 Kolde, R. *pheatmap, pretty heatmap*, <https://CRAN.R-project.org/package=pheatmap > (2019).
137 Kassambara, A. R package "ggpubr": 'ggplot2' Based Publication Ready Plots. (2019).

138 Kassambara, A. R package "rstatix": Pipe-Friendly Framework for Basic Statistical Tests. (2019).
139 R package 'corrplot': Visualization of a Correlation Matrix (2021).
140 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* **43**, e47-e47, doi:10.1093/nar/gkv007 (2015).
141 Patil, I. Visualizations with statistical details: The 'ggstatsplot' approach. *Journal of Open Source Software* **6**, doi:10.21105/joss.03167 (2021).
142 Skidmore, Z. L. *et al.* GenVisR: Genomic Visualizations in R. *Bioinformatics* **32**, 3012-3014, doi:10.1093/bioinformatics/btw325 (2016).
143 Mayakonda, A., Lin, D. C., Assenov, Y., Plass, C. & Koeffler, H. P. Maftools: efficient and comprehensive analysis of somatic variants in cancer. *Genome Res* **28**, 1747-1756, doi:10.1101/gr.239244.118 (2018).
144 Wickham, H. R package "ggplot2": Elegant Graphics for Data Analysis. (2016).
145 O'Brien, J., Wilson, I., Orton, T. & Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* **267**, 5421-5426, doi:10.1046/j.1432-1327.2000.01606.x (2000).
146 Wickham, H., François, R., Henry, L. & Müller, K. *dplyr: A Grammar of Data Manipulation*, <https://CRAN.R-project.org/package=dplyr> (2022).
147 Garnis, C., Buys, T. P. H. & Lam, W. L. Genetic alteration and gene expression modulation during cancer progression. *Molecular Cancer* **3**, 9, doi:10.1186/1476-4598-3-9 (2004).
148 Stickeler, E. Prognostic and Predictive Markers for Treatment Decisions in Early Breast Cancer. *Breast Care (Basel)* **6**, 193-198, doi:10.1159/000329471 (2011).
149 Tanaka, H. & Watanabe, T. Mechanisms Underlying Recurrent Genomic Amplification in Human Cancers. *Trends Cancer* **6**, 462-477, doi:10.1016/j.trecan.2020.02.019 (2020).
150 Rouette, A. *et al.* Expression of immunoproteasome genes is regulated by cell-intrinsic and – extrinsic factors in human cancers. *Scientific Reports* **6**, 34019, doi:10.1038/srep34019 (2016).
151 Li, Y. *et al.* The transcription levels and prognostic values of seven proteasome alpha subunits in human cancers. *Oncotarget* **8**, 4501-4519, doi:10.18632/oncotarget.13885 (2017).
152 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2020. *CA Cancer J Clin* **70**, 7-30, doi:10.3322/caac.21590 (2020).
153 Albertson, D. G. Gene amplification in cancer. *Trends Genet* **22**, 447-455, doi:10.1016/j.tig.2006.06.007 (2006).
154 Beroukhim, R. *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* **463**, 899-905, doi:10.1038/nature08822 (2010).
155 Zhou, X., Iversen, E. S., Jr. & Parmigiani, G. Classification of Missense Mutations of Disease Genes. *J Am Stat Assoc* **100**, 51-60, doi:10.1198/016214504000001817 (2005).
156 Naujokat, C. & Hoffmann, S. Role and Function of the 26S Proteasome in Proliferation and Apoptosis. *Laboratory Investigation* **82**, 965-980, doi:10.1097/01.LAB.0000022226.23741.37 (2002).
157 Schröter, F. & Adjaye, J. The proteasome complex and the maintenance of pluripotency: sustain the fate by mopping up? *Stem Cell Research & Therapy* **5**, 24, doi:10.1186/scrt413 (2014).
158 Wang, C. Y. *et al.* PSMB5 plays a dual role in cancer development and immunosuppression. *Am J Cancer Res* **7**, 2103-2120 (2017).
159 Chen, D. *et al.* Pan-cancer analysis of the prognostic and immunological role of PSMB8. *Sci Rep* **11**, 20492, doi:10.1038/s41598-021-99724-9 (2021).
160 Wang, Z. *et al.* Bortezomib prevents oncogenesis and bone metastasis of prostate cancer by inhibiting WWP1, Smurf1 and Smurf2. *Int J Oncol* **45**, 1469-1478, doi:10.3892/ijo.2014.2545 (2014).
161 Rastogi, N. & Mishra, D. P. Therapeutic targeting of cancer cell cycle using proteasome inhibitors. *Cell Div* **7**, 26-26, doi:10.1186/1747-1028-7-26 (2012).
162 Zhu, G. *et al.* Mutant p53 in Cancer Progression and Targeted Therapies. *Front Oncol* **10**, 595187, doi:10.3389/fonc.2020.595187 (2020).
163 Jeong, H. M., Kwon, M. J. & Shin, Y. K. Overexpression of Cancer-Associated Genes via Epigenetic Derepression Mechanisms in Gynecologic Cancer. *Front Oncol* **4**, 12, doi:10.3389/fonc.2014.00012 (2014).

164 Viswanadhapalli, S., Dileep, K. V., Zhang, K. Y. J., Nair, H. B. & Vadlamudi, R. K. Targeting LIF/LIFR signaling in cancer. *Genes Dis*, doi:10.1016/j.gendis.2021.04.003 (2021).
165 Stransky, N. *et al.* Pharmacogenomic agreement between two cancer cell line data sets. *Nature* **528**, 84-87, doi:10.1038/nature15736 (2015).
166 Niepel, M. *et al.* A Multi-center Study on the Reproducibility of Drug-Response Assays in Mammalian Cell Lines. *Cell systems* **9**, 35-48.e35, doi:10.1016/j.cels.2019.06.005 (2019).
167 Haibe-Kains, B. *et al.* Inconsistency in large pharmacogenomic studies. *Nature* **504**, 389-393, doi:10.1038/nature12831 (2013).
168 Baker, M. 1,500 scientists lift the lid on reproducibility. *Nature* **533**, 452-454, doi:10.1038/533452a (2016).
169 Xue, Z. *et al.* Proteomics reveals that cell density could affect the efficacy of drug treatment. *Biochem Biophys Rep* **33**, 101403, doi:10.1016/j.bbrep.2022.101403 (2023).
170 Wright Muelas, M., Ortega, F., Breitling, R., Bendtsen, C. & Westerhoff, H. V. Rational cell culture optimization enhances experimental reproducibility in cancer cells. *Scientific Reports* **8**, 3029, doi:10.1038/s41598-018-21050-4 (2018).
171 Mansoury, M., Hamed, M., Karmustaji, R., Al Hannan, F. & Safrany, S. T. The edge effect: A global problem. The trouble with culturing cells in 96-well plates. *Biochem Biophys Rep* **26**, 100987, doi:10.1016/j.bbrep.2021.100987 (2021).
172 Hatzis, C. *et al.* Enhancing reproducibility in cancer drug screening: how do we move forward? *Cancer Res* **74**, 4016-4023, doi:10.1158/0008-5472.Can-14-0725 (2014).
173 Rees, M. G. *et al.* Correlating chemical sensitivity and basal gene expression reveals mechanism of action. *Nat Chem Biol* **12**, 109-116, doi:10.1038/nchembio.1986 (2016).
174 Motosugi, R. & Murata, S. Dynamic Regulation of Proteasome Expression. *Front Mol Biosci* **6**, 30, doi:10.3389/fmolb.2019.00030 (2019).
175 Tanahashi-Hori, T., Tanahashi, N., Tanaka, K. & Chiba, T. Conditional knockdown of proteasomes results in cell-cycle arrest and enhanced expression of molecular chaperones Hsp70 and Hsp40 in chicken DT40 cells. *J Biol Chem* **278**, 16237-16243, doi:10.1074/jbc.M301331200 (2003).
176 Jang, G. *et al.* Predicting mechanism of action of novel compounds using compound structure and transcriptomic signature coembedding. *Bioinformatics* **37**, i376-i382, doi:10.1093/bioinformatics/btab275 (2021).
177 Chen, D., Frezza, M., Schmitt, S., Kanwar, J. & Dou, Q. P. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Current cancer drug targets* **11**, 239-253, doi:10.2174/156800911794519752 (2011).
178 Chen, D. & Dou, Q. P. The Ubiquitin-Proteasome System as a Prospective Molecular Target for Cancer Treatment and Prevention. *Current protein & peptide science* **11**, 459-470 (2010).
179 Riss, T. L. *et al.* in *Assay Guidance Manual* (eds G. S. Sittampalam *et al.*) 353-377 (Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004).
180 Sunwoo, J. B. *et al.* Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **7**, 1419-1428 (2001).
181 Bianchini, G., De Angelis, C., Licata, L. & Gianni, L. Treatment landscape of triple-negative breast cancer — expanded options, evolving needs. *Nature Reviews Clinical Oncology* **19**, 91-113, doi:10.1038/s41571-021-00565-2 (2022).
182 Meissner, T. *et al.* Metastatic triple-negative breast cancer patient with TP53 tumor mutation experienced 11 months progression-free survival on bortezomib monotherapy without adverse events after ending standard treatments with grade 3 adverse events. *Cold Spring Harb Mol Case Stud* **3**, doi:10.1101/mcs.a001677 (2017).
183 Baek, D. W., Park, J. Y., Lee, S. J. & Chae, Y. S. Impressive effect of cisplatin monotherapy on a patient with heavily pretreated triple-negative breast cancer with poor performance. *Yeungnam Univ J Med* **37**, 230-235, doi:10.12701/yujm.2019.00423 (2020).
184 Kisselev, A. F., van der Linden, W. A. & Overkleeft, H. S. Proteasome inhibitors: an expanding army attacking a unique target. *Chem Biol* **19**, 99-115, doi:10.1016/j.chembiol.2012.01.003 (2012).