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Invited review

Applied techniques for mining natural proteasome inhibitors ☆

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ABSTRACT

In eukaryotic cells, the ubiquitin–proteasome-system (UPS) is responsible for the non-lysosomal degradation of proteins and plays a pivotal role in such vital processes as protein homeostasis, antigen processing or cell proliferation. Therefore, it is an attractive drug target with various applications in cancer and immunosuppressive therapies. Being an evolutionary well conserved pathway, many pathogenic bacteria have developed small molecules, which modulate the activity of their hosts' UPS components. Such natural products are, due to their stepwise optimization over the millennia, highly potent in terms of their binding mechanisms, their bioavailability and selectivity. Generally, this makes bioactive natural products an ideal starting point for the development of novel drugs. Since four out of the ten best seller drugs are natural product derivatives, research in this field is still of unfathomable value for the pharmaceutical industry. The currently most prominent example for the successful exploitation of a natural compound in the UPS field is carfilzomib (Kyprolis®), which represents the second FDA approved drug targeting the proteasome after the admission of the blockbuster bortezomib (Velcade®) in 2003. On the other hand side of the spectrum, ONX 0914, which is derived from the same natural product as carfilzomib, has been shown to selectively inhibit the immune response related branch of the pathway. To date, there exists a huge potential of UPS inhibitors with regard to many diseases. Both approved drugs against the proteasome show severe side effects, adaptive resistances and limited applicability, thus the development of novel compounds with enhanced properties is a main objective of active research. In this review, we describe the techniques, which can be utilized for the discovery of novel natural inhibitors, which in particular block the 20S proteasomal activity. In addition, we will illustrate the successful implementation of a recently published methodology with the example of a highly potent but so far unexploited group of proteasome inhibitors, the syrbactins, and their biological functions. This article is part of a Special Issue entitled: Ubiquitin–Proteasome System. Guest Editors: Thomas Sommer and Dieter H. Wolf.

1. Introduction

The ubiquitin–proteasome-system (UPS) selectively labels and destroys short-lived, misfolded and abnormal proteins, therefore playing a crucial role in protein homeostasis [1]. Moreover, it is involved in important biological pathways and signaling processes via the degradation of cellular key players such as cyclins [2] or the tumor suppressor p53 [3]. Besides, not only the digestion of substrates, but also the generation of peptide fragments is exploited in vertebrates for the generation of antigens that are presented on MHC-I complexes at the cell surface [4,5]. Due to the entanglement with these vital processes, the UPS is directly linked to diseases as diverse as cancer, autoimmunity or neurodegeneration [6,7], which can in turn be correlated with aberrant proteasomal activity or increased expression of genes involved in the UPS [8]. Hence, the pharmaceutical manipulation of the UPS activity is a promising principle for the treatment of various diseases. Despite the vast potential, the medical application of UPS inhibitors is still limited to only certain types of blood cancer [9]. Nevertheless, the discovery of various natural substance classes acting on the UPS demonstrates the tremendous importance of this pathway for biological systems and raises hopes to also apply similar compounds to a broader spectrum of medical indications [10]. These secondary metabolites feature distinct modes of action, carry unique lead structures and fulfill a specific biological function such as the attenuation of the immune system or the general debilitation of a host organism [11,12]. In contrast to most synthetic compounds, they do not only show excellent *in vitro* effects, but are also able to selectively mingle with the UPS in living cells [13], which is largely due to their outstanding properties in terms of cell penetration, clearance rates, metabolism and binding kinetics. Thus, natural products represent a perfect starting point for further drug development. However, not all of them can be used because of their complex structure or their high reactivity, which potentially causes detrimental side effects. Therefore, the search for novel compounds remains an important field of future research. Although recent studies demonstrate that many components of the UPS pathway are equally *drugable*, the

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search for natural inhibitors has mainly focused on the 20S proteasome (core particle; CP) upon its validation as a cancer drug target. Due to the diversification of the UPS in higher vertebrates, the exploitation of its different branches emerges as a new principle for UPS inhibitors. Consequently, all components of the UPS are of high interest to the pharmaceutical industry, especially with regard to other clinical pictures than multiple myeloma or mantle cell lymphoma for which bortezomib received its primary admission [14].

2. Chapter 1: The UPS pathway

2.1. Ubiquitination

The *in vivo* stability of a given protein in pro- and eukaryotes is determined by the so called N-end rule [15]. It defines particular N-terminal amino acids such as lysine or arginine as degradation signals that considerably abbreviate the protein's half-life time. In nucleated cells, the UPS is majorly responsible for the substrate selective digestion of proteins [16,17]. The pathway consists of two parts, the covalent ubiquitination of target proteins and their successive decomposition by the 26S proteasome to defined oligopeptides [18] (Fig. 1). Ubiquitin (Ub) is a comparatively small but vital 8.5 kDa protein that can be posttranslationally attached to the ε-NH_2 moiety of an exposed lysine residue by an isopeptide bond [23]. Further Ub molecules can then be coupled to the first Ub via any of its seven lysine amino acids to form differently linked chains with particular signaling functions such as translocation or degradation [24,25]. Due to their high cellular abundance, K48 linked poly-Ub chains are the best characterized type of ubiquitination and have been shown to mark proteins for proteasomal fragmentation [26]. The overall ubiquitination of the proteome is a highly dynamic process that has been likened in its complexity and cellular function to phosphorylation [27]. It is balanced and controlled by a system of substrate specific Ub ligases on the one hand side and deubiquitinases on the other that represent an entire posttranslational regulation level. The molecular mechanism of ubiquitination involves three successive enzymes called E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin-protein ligase), which activate, hand over and attach a Ub moiety to a target protein [25] (Fig. 1a). These enzymes are arranged in a cascade like reaction setup, starting with two E1 enzymes that interact with about fifty E2 proteins [28]. The E2:Ub complexes are then able to specifically select their target E3 ligase among more than 1000 E3enzymes [28,29], hereby reflecting the enormous variety of proteins that are posttranslationally labeled for disposal. Hereby, Ub gets ATP-dependently activated by its C-terminal carboxyl-group via E1 to yield a highly energetic Ub–E1 thioester bridged metabolite [30], which in turn transmits the Ub moiety to an E2 protein [30,31]. Suitable UPS substrate proteins are either recognized by E3 alone or in a trimeric complex with E2, which then provides the activated ubiquitin for the catalyzed ligation to the substrate [32]. Once ubiquitinated, repeated cycles of these steps lead to attachment of further Ub moieties to the first Ub molecule [33], thus resulting in proteins with isopeptide bridged oligo-Ub chains that are recognized and degraded by the 26S proteasome.

2.2. The 26S proteasome

Once a substrate molecule is successfully polyubiquitinated, it is caught by the 26S proteasome (Fig. 1b). This 2.6 MDa huge multimeric particle is composed of the proteolytically active 20S proteasome (core particle, CP) and the 19S regulatory particle (RP) [18]. The RP recognizes ubiquitinated substrate proteins, unfolds them in an ATP dependent manner and translocates them into the CP. Although the dynamic and flexible structure of the RP has thwarted attempts of crystal structure analysis to date, recent electron microscopy studies have shed new light on its functional and molecular organization [22,34]. In a cap-like structure, the RP is perched on both ends of the barrel shaped CP, thus gating entry for substrate molecules into the catalytic sites [35–39] (Fig. 1b). Its 19 different proteins are categorized into Rpt (Regulatory particle ATPases) as well as Rpn subunits (Regulatory particle non-ATPases) and were historically further subdivided into two multimeric complexes called “base” and “lid” according to their presumed location within the RP [40]. Computational, structural and biochemical studies, however, have made these categories superfluous as the lid is not located on top of the RP but rather on its side [34]. Moreover, subunits that had been assigned to the base such as Rpn 10 and Rpn 13 [34,40] that

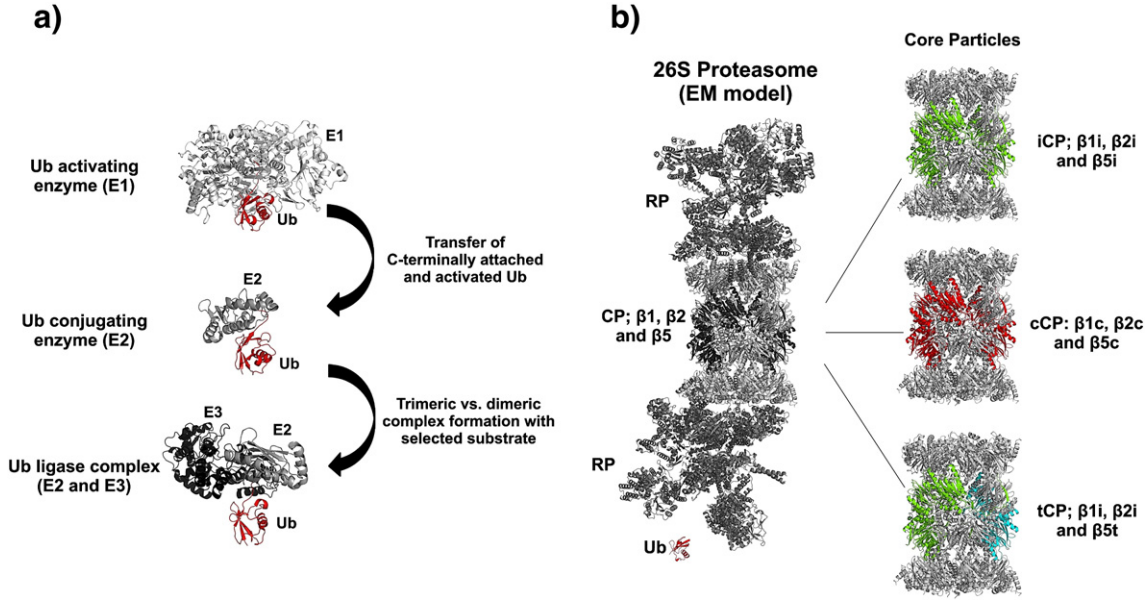

Fig. 1. The UPS pathway. A) Ubiquitinylation involves three enzymes, which activate (E1, upper) [19], hand over (E2, middle) [20] and conjugate (E3, lower) [21] the Ub molecule (red) to a cellular substrate protein. B) The 26S proteasome with a molecular mass of 2.600 kDa is huge compared to the 8.5 kDa Ub molecule. It contains the proteolytic CP (light gray), which is flanked by two 19S RP's (gray); coordinates were provided by Edward Morris [22]. In eukaryotes, only three β-type subunits are endowed with catalytic activities (black). Diversification of the UPS in vertebrates has led to three distinct particles with altered β-subunit configuration. The proteolytically active subunits β1, β2 and β5 are color-coded for the each CP type: iCP (green), cCP (red) and tCP (β1i and β2i in green; β5t in blue).

have been shown to interact with Ub [41–43] are located on the far side of the RP [22,34]. These two proteins are the primer receptors to recognize tetra-Ub chains. Once bound to the RP, the labeled substrates are guided to the catalytically active zink-metallaprotease Rpn11 [44] that is located in the palm of the horseshoe like setup formed by the residual subunits Rpn 3, 5–7, 9 and 12 [45]. Rpn11 then cleaves off the prearranged Ub molecules en bloc, thus rescuing them from proteasomal degradation. Subsequently, the substrate protein gets (i) actively unfolded by the six AAA^+ ATPases (Rpt 1–6) [46–50], which are located in a pseudohexameric spiral staircase arrangement adjacent to the CP [22,34] and (ii) is further translocated into the hydrolytic chamber of the 20S proteasome [38,39].

2.3. The core particle

Contrary to the dynamic RP, the CP is a rigid body framed by the RP [17] or other adaptor proteins [51–53] (Fig. 1b). It forms the downstream end of the UPS and exerts the catalytic activity that cleaves proteins into defined peptide fragments [54,55]. Unlike for the RP, high resolution data is available for the 20S proteasomes from prokaryotes [56] and eukaryotes [35,57,58]. This allowed the elucidation of its molecular structure, including the details of substrate binding and conversion, as well as extensive studies with inhibitory molecules [59]. The C_2 symmetrical CP cylinder is composed of four stacked heptameric rings containing α- and β- subunits in an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ stoichiometry [56]. The outer α subunits actively participate in CP assembly and play scaffolding and functional roles in the maturated particle. By spanning an interdigitating network with their N-termini, the α-subunits form a gate and actively lock the central proteasomal pore, thus protecting the cell from uncontrolled self-digestion by free 20S particles [35–37]. Once the RP is docked on the α-subunits, however, this regulatory function is passed on to the ubiquitin related branch of the UPS and the gate is opened by a distinct interaction with the Rpt-proteins [39,60]. The proteolytic activities of the CP that in turn degrade an introduced unfolded protein are conferred to subunits within the equatorial β-rings [61]. During the catalytic reaction, the nucleophilic Thr-$1O^\gamma$ attacks the carbonyl carbon of the scissile peptide bond, thereby forming a stabilized oxyanion [35,62]. Next, the C-terminal substrate fragment is released, which results in a peptide–enzyme–acyl intermediate that is subsequently attacked by a prearranged water molecule in the Bürgi–Dunitz trajectory to complete peptide bond hydrolysis [63,64]. With regard to this mechanism including the role of the free N-terminus as proton acceptor, the CP is assigned to the family of Ntn (N-terminal nucleophilic) hydrolases [65]. As the proteolytic machinery is entirely unspecific, the CP can be compared to a molecular shredder that has to be strictly compartmented *in vivo* [18]. However, although it is not selective with respect to the digested protein, it is endowed with cleavage specificity by its peptide binding channels [59]. These harbor distinct pockets for the amino acid side chains of substrate molecules, thereby prolonging the mean residence time of peptides with suitable primary sequences [35]. In turn, proteins are preferentially hydrolyzed at defined positions, eventually resulting in a reproducible cleavage pattern with respect to a defined substrate [54,55]. Interestingly, whereas most simpler organisms such as archaea get along with identical α- and β-subunits [56], eukaryotes harbor distinct subunits with only three catalytically active β-type subunits that are named β1, β2 and β5 according to their position within the seven-membered rings [35] (Fig. 1b). Due to their distinct substrate specificities, the activities exerted by these subunits are termed caspase-like (CL), trypsin-like (TL) and chymotrypsin-like (ChTL) [61,66].

This divergent evolution of subunits present in eukaryotes conduces to the functional diversification of the UPS and its involvement in intracellular signaling [2,3,67]. Moreover, in vertebrates, the active β-subunits have further evolved to several subsets with particular substrate selectivities [6,66]. Due to their assigned function or the tissue in which they are produced, they are incorporated into nascent CP's, which are consequently named constitutive (cCP), immuno (iCP) or thymus core particles (tCP) [68,69] (Fig. 1b). This further specialization enables a defined participation of the CP in the various biological pathways inherent in higher organisms. In 2012, the crystal structure of the murine iCP, as well as the corresponding cCP, were elucidated [58]. Comparative analysis of the exchanged subunits revealed, that the overall folding of the immuno- and constitutive subunits matches almost perfectly. The molecular details for the altered catalytic properties were disclosed, hereby opening the opportunity for the rational design of cCP or iCP selective inhibitors. Interestingly, the β2c and β2i subunits both preferentially cleave after basic residues and exhibit a broad range of substrate specificity. Thus, the functional and biological background of this replacement still remains elusive to date [6]. Contrarily, the substrate selectivity of β1i is changed from acidic (CL) towards small branched amino acids, which are better suited to bind to MHC I complexes [58,70]. Although the thymoproteasome is linked to various autoimmune diseases, little is known about the cleavage preferences of the β5t subunit [71]. Structural predictions and initial experiments, however, suggest that its peptide binding channel is lined by hydrophilic residues [68]. Therefore, the production of peptides with hydrophobic residues is attenuated in β5t, thus affinity of these ligands for MHCI presentation is limited. As T-cell development is impaired in β5t knockout mice, the thymus specific subunit has been connected to positive $CD8^+$ T-cell selection [68]. In contrast, the cleavage preference of β5c towards small neutral amino acids has been shown to be altered in β5i to large hydrophobic residues, which enhances antigen presentation [58].

Therefore, β1i and β5i exhibit a converged and directed evolution towards a concerted production of antigenic peptides, thus generating a broader spectrum of possible epitopes most suitable for MHC I binding [70]. The further adaptation and specialization of the CP in different cell types reflects the increased complexity in higher eukaryotic organisms and might offer a chance to selectively address different pathways by medicinal chemistry [6]. Today, the CP is in the focus of the pharmaceutical industry within the UPS. This is due to its far downstream position and straightforward addressable enzymatic activity, but also to its signaling function in cell proliferation, immune response and inflammatory processes such as the NF-κB pathway [6,72]. For the same reasons, the CP is also exploited by pathogenic microorganisms via small molecules during infection.

2.4. Natural and synthetic CP inhibitors and their potential applications

To date, many entirely different classes of natural CP inhibitors have been described [10] (Fig. 2). They all display distinct modes of action and selectively block proteasomal β-subunits to address the various proteasomal functions [59,83]. After the discovery of the rather unspecific peptide aldehydes [56,66], the β-lactone omuralide was among the first natural proteasome inhibitors identified [74,84] (Fig. 2a–b). Lactones belong to the original antibiotic agents discovered and consequently this class of natural products represents the model compound in the field of small molecule CP inhibitors [35,64,85]. Upon CP binding, the strained β-lactone ring in omuralide undergoes a nucleophilic ring opening reaction with Thr-$1O^\gamma$ to form an irreversible acyl–enzyme product [35] (Fig. 2b), thus blocking the proteasomal active sites. It was shown that omuralide is quite specific for the proteasome and selectively blocks the ChTL activity [35,84]. Moreover, treatment with the natural metabolite causes cell cycle arrest and neurite outgrowth in cell culture experiments by inhibition of cyclin degradation [84], therefore assigning a defined biological mode of action. As tumor cells exhibit high protein turnover rates in combination with chromosomal instability and abbreviated cell division intervals [86], CP inhibitors stepped into the limelight of cancer therapy. Although the pharmaceutical exploitation of omuralide was abandoned due to its high off-target activities, a closely related natural compound named Marizomib (Fig. 2d) is currently under clinical trials [87].

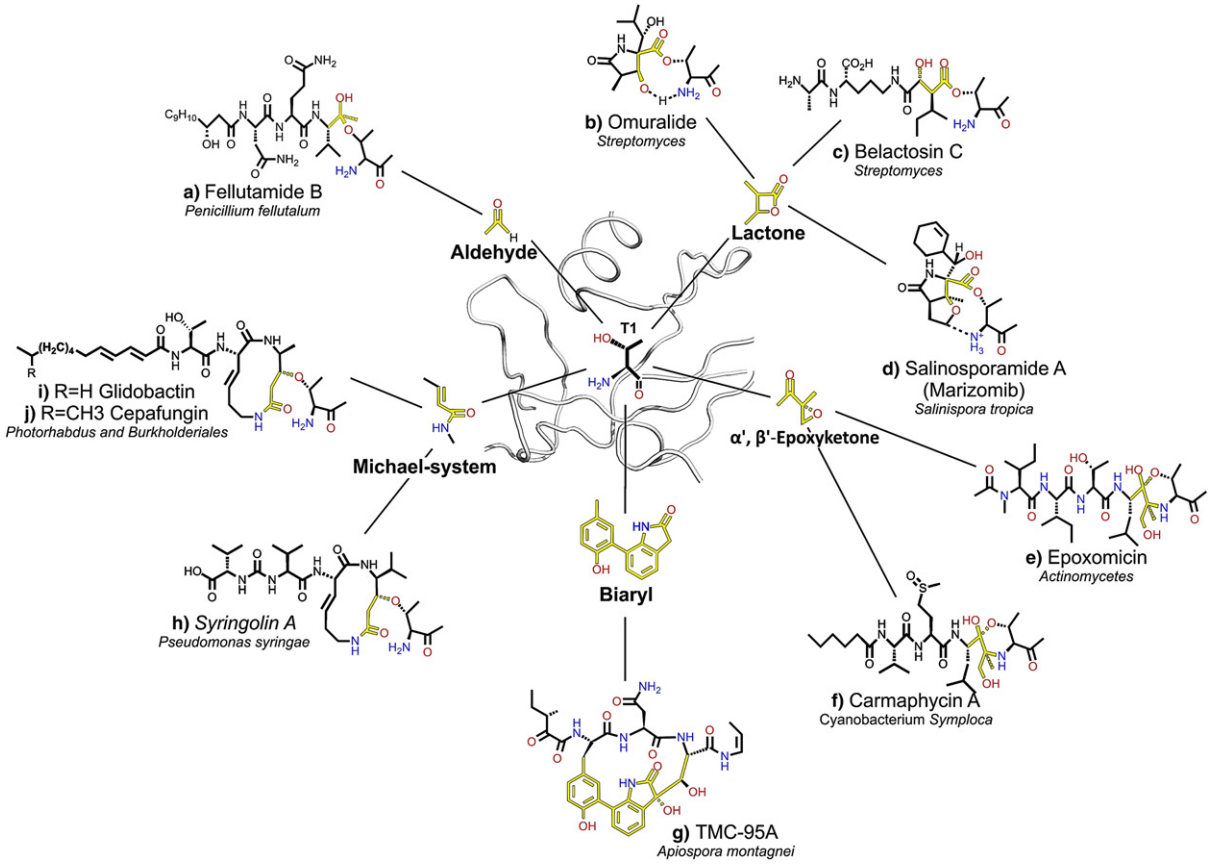

Fig. 2. Natural product inhibitors of the proteasome. a) The peptide aldehyde Fellutamide B and b) the β-lactone omuralide belong to the first natural proteasome inhibitors identified [73,74]. c) Belactosin C [75] and d) salinosporamide A (marizomib) [76] join the class of β-lactones, whereby the hydroxyl moiety of the latter reacts in a second step to produce an irreversible tetrahydrofuran ether. e) Epoxomicin carries a bifunctional α', β'-expoxyketone pharmacophore [77]. The newly released carfilzomib as well as ONX 0914 are derived from this natural compound. f) Carmaphycin A is another member of this highly potent inhibitor class that combines the reactive pharmacophore with a distant lipophilic tail [78]. g) TMC-95A is the only natural non-covalent CP inhibitor identified to date [79]. Its biaryl-bridged (oxindol–phenyl clamp) peptide scaffold carries many decorations and chiral centers that shape the compounds' three-dimensional structure, thus balancing the enthalpic loss by enhanced entropic properties. h) Syringolin A, as well as i) glidobactin A and j) cepafungin I belong to the class of syrbactins [80–82]. They all harbor a constrained Michael-system in a twelve-membered macrolactam ring attached to either a carboxylic acid or a fatty acid tail, respectively. CepI is the most powerful natural inhibitor identified to date. Only those compounds are displayed for which crystallographic data are available.

These initial successes finally led to the development of the first synthetic blockbuster drug bortezomib, which is approved for relapsed multiple myeloma and mantle cell lymphoma [88–90]. The boronic acid pharmacophore of the peptide inhibitor binds to the nucleophilic Thr-1O$^{\gamma}$ to form a covalent tetrahedral adduct that mimics the oxyanion during peptide cleavage [91]. The prescriptive application of bortezomib as a line-one drug against multiple myeloma has validated the CP within the UPS as a promising target for cancer therapy [9]. Besides, laboratory experiments have demonstrated reduced rejection rates in transplanted and bortezomib treated mice, thereby highlighting the clinical importance of the immunological role of the CP including autoimmune disorders [92,93]. On the other hand, the application of bortezomib suffers from severe side effects like peripheral neuropathy or myelotoxicity as well as limited tissue penetration and short bioavailability [94–96]. All these effects are assigned to the extremely reactive boronic acid warhead, which causes substantial off-target activities [97]. It is therefore currently of central interest to develop equally potent compounds with reduced cross reactivity and improved pharmacokinetic properties. The admission of carfilzomib in 2012 [98], which is based on the highly potent natural product epoxomicin from an *Actinemyces* strain [77,99,100] (Fig. 2e), now heralds the era of second generation proteasome inhibitors. The bivalent and irreversible α′,β′-epoxyketone compound reacts with the hydroxyl- and with the amine moiety of Thr-1 [101], which considerably contributes to target specificity towards only those few members of the Ntn-hydrolase family [10] (Fig. 2e–f). In clinical studies, carfilzomib sports high potency in treatment of multiple and refractory multiple myeloma with response rates of 52% in single-arm phase II studies [102], whereas complimentary applications with other established compounds such as the thalidomide derivative lenalidomide or the glucocorticoid dexamethasone even yielded rates of 92% [103]. Due to the orthogonal toxicological side effects of these substances, treatment could be performed over several years without dose reduction [103]. Another derivative of epoxomicin, which is currently in medicinal research focus, is the first-in-class immunoproteasome inhibitor ONX 0914 [104]. The compound holds promising applications in various autoimmune diseases, transplantation therapy as well as treatment of several cancer subtypes with abnormal iCP expression patterns [6,105]. Currently, ONX 0914 is tested in preclinical studies for treatment of immune disorders. However, both carfilzomib and ONX 0914 still resemble bortezomib in their highly reactive pharmacophore, which complicates their application in medical treatment e.g. due to premature hydrolysis in the blood plasma [106].

2.5. Substrate toxication of the CP by cyclic peptides

In contrast to bortezomib and carfilzomib, the cyclic peptide TMC-95A, which was isolated from cultures of *Apiospora montagnei* [79,107] (Fig. 2g), belongs to the class of reversible and non-covalent inhibitors [108]. However, with an IC_{50} of 5 nM its inhibitory strength is in the same order as covalently reacting substances, thus demonstrating that binding affinity is dependent on both enthalpy and entropy [109,110]. Since reversible inhibitors only temporarily block the

proteasome, they exhibit decreased toxicity towards benign cells [83,111]. Moreover, they are hypothesized to penetrate further into the tissue due to the dynamic equilibrium of their binding, thus making TMC-95A an interesting candidate for pharmaceutical research. However, attempts to synthesize TMC-95A did not lead to satisfactory results due to its demanding stereochemistry [110,112–115]. Therefore, the complex structure was reduced to the crucial functional determinants, however none of the derived molecules reached the inhibitory potential of TMC-95A [109,116,117]. Moreover, the search for other non-covalent inhibitors did not lead to the discovery of new compounds. Major reasons therefore are the dominance of irreversible inhibitors, but also to the lack of appropriate assay techniques for CP activity evaluation.

2.6. Proteasomal inhibitors from pathogenic sources trigger virulence

Eventually, the syrbactin compounds, which are named after their most prominent examples syringolin A (SylA), glidobactin A (GlbA) and cepafungin I (CepI) represent the latest group of natural CP inhibitors identified to date [11,12,81,118,119] (Fig. 2h–j). They share the common feature of an 18-membered macrolactam scaffold containing a Michael-system as electrophilic group that reacts with the Thr-1O$^{\gamma}$ to produce an irreversible ether product [12]. Interestingly, analog synthetic compounds such as vinyl esters or vinyl sulfones exhibit the same reaction mechanism [120–123]. Yet, their IC_{50} values range in the micromolar range, whereas the syrbactins reach nanomolar values [11,124]. Comparative structural studies suggest that the rather unreactive Michael-system has to be prepositioned to unfold its true potential, which is achieved in the syrbactin family by the restrained macrolactam scaffold [13,125,126]. Furthermore, binding is entropically favored towards elongated and flexible peptide chains, which lose considerable degrees of freedom compared to their state in free solution. Furthermore, the intracyclic carbonyl oxygen simulates the oxyanion in substrate digestion and is therefore stabilized by hydrogen bonds with Gly-47NH [124]. Due to the hydrophobic character of the macrolactam ring, all syrbactins preferentially bind to the β5 subunit. However, the decoration pattern between SylA and GlbA differs considerably in the S1 site, as well as the acyl-chain attached to the cyclic system [13,80,81]. These alterations account for the tremendously improved IC_{50} values of glidobactin compared to syringolin. Whereas the isopropyl moiety of syringolin is spatially demanding for the cCP, thereby shifting Met-45 out of its native state, the methyl group in glidobactin perfectly fits into the P1 pocket [11,12]. Besides, binding of the P3 threonine next to the mactolactam ring is improved due to characteristic hydrogen bond formations. More importantly, however, is the exchange of the very polar free carbonic acid tail of SylA by a partially unsaturated fatty acid chain in GlbA, which was shown to bind into a hydrophobic grove in the adjacent proteasomal subunit β6 [12]. Remarkably, the highly potent GlbA with an IC_{50} value of 17 nM was still improved during evolutionary optimization, which resulted in CepI [11,82] (Fig. 2j). Recent results elucidated that CepI, which exhibits an IC_{50} value of 4 nM, currently represents one of the strongest proteasomal inhibitor described so far. X-ray structure elucidation of the yeast CP in complex with CepI revealed the molecular details of its enhanced binding properties, which are assigned to the increased hydrophobic interactions of the branched fatty acid tail with the respective lipophilic proteasomal grove [11]. Therefore, SylA, GlbA and CepI follow a common mode of action, but differ from each other by a directed optimization of individual side chains with major impacts on their respective binding affinities (Fig. 2h–j).

Contrary to most covalent inhibitors characterized to date, the syrbactins carry a rather unreactive electrophilic headgroup, thus suggesting decreased cross reactivity and adverse side effects. Hence, the syrbactins are likely to follow carfilzomib and marizomib [98] as second generation CP inhibitors in the future. The high versatility of the currently identified CP-ligands gives a first insight into the vast repertoire that nature has developed to interact with the UPS by small molecule elicitors [10]. Therefore, many other hitherto undescribed compounds are still waiting for their discovery to join the field of natural proteasome inhibitors. Considering the unexploited applications of UPS modulating agents in cancer therapies and also the recently disclosed area of selective iCP and tCP inhibition [6], the search for novel lead structures is of utmost importance for both academia and industry.

Unfortunately, natural product research in our days is in a severe dilemma. The number of newly identified substances is steadily declining, whereas at the same time the effort for their discovery surpasses economic efficiency criteria, which has led to the withdrawal of pharmaceutical industry from many screening programs [127]. Besides, the analysis of the increasing number of available genomes has led to the conclusion that even though many natural products have been isolated over the last decades, the majority of secondary metabolites is still undiscovered [128]. Yet, the respective gene clusters responsible for their synthesis are silent under common growth conditions, as they must be activated by so far unknown environmental triggers [129,130]. Especially toxins, whose synthesis is exclusively directed against the respective host organism or nutrition competitors, are strictly regulated to avoid detrimental or self-destructive effects [11]. Since most natural proteasome inhibitors fall into that category, this principle may be the rationale for the identification of novel compounds. In order to discover the correct environmental conditions for their synthesis, it is necessary to screen extracts or secretions by a robust, quick and unambiguous methodology on the presence of inhibitory substances. Once the trigger for biosynthesis is found, the downstream isolation and characterization of the small molecule is a straightforward process relying on standard purification techniques (Fig. 3). Many methods have been developed for assaying the proteasome activity *in vitro* and *in vivo*, but they all act within certain principle boundaries (Table 1).

3. Chapter 2: Methods for proteasomal inhibitor detection

3.1. UV–VIS techniques

The most popular and commonly applied proteasome assay uses unnatural substrate peptides, which carry a C-terminal chromophor that is internally quenched by the attachment to the peptide scaffold. 2-Naphtylamine (pNA) or 7-amino-4-methyl-coumarin (AMC) are the most widespread and commercially available head groups, but also 4-methoxy-2-naphtylamine (MNA) has been described in literature [131]. The primary sequence of the peptide tail determines the affinity for the appropriate substrate binding channel [132], thus allowing the selective analysis of a single active site within the CP. The proteasomal activities hydrolyze the chromophor, which then develops its absorptive or fluorogenic properties [133] (Fig. 3d). Hereby, the signal is directly proportional to the enzymatic turnover, which makes the assay suitable for quantitative measurements. The UV–VIS technique is among the most applied methodologies in high-throughput-screenings for compound identification [134], but suffers from several severe drawbacks that lead to high numbers of false positive and false negative results [11]. As the assay uses the UV–VIS spectrum of light, it is highly susceptible to quenching and diffraction in suspensions or colorful solutions, which are, however, typical for many microorganisms after prolonged incubation periods [135–138]. Moreover, some organisms produce fluorogenic substances that mimic proteolytic activities [129,139]. Hence, the background for this methodology is too high to reliably measure inhibitor secretion in culture broth or other heterogeneous conglomerates [11,118,119].

3.2. Site specific activity probes (SSAP)

A more advanced approach has been developed that relies on the irreversible interactions of a labeled ligand with the active sites of the CP [13,133,140,141]. The inhibitory molecule, which has to equally bind to all proteasomal active sites, is chemically linked to a fluorescent dye, e.g. DANSYL, rhodamine and BODIPY derivatives, and added to an

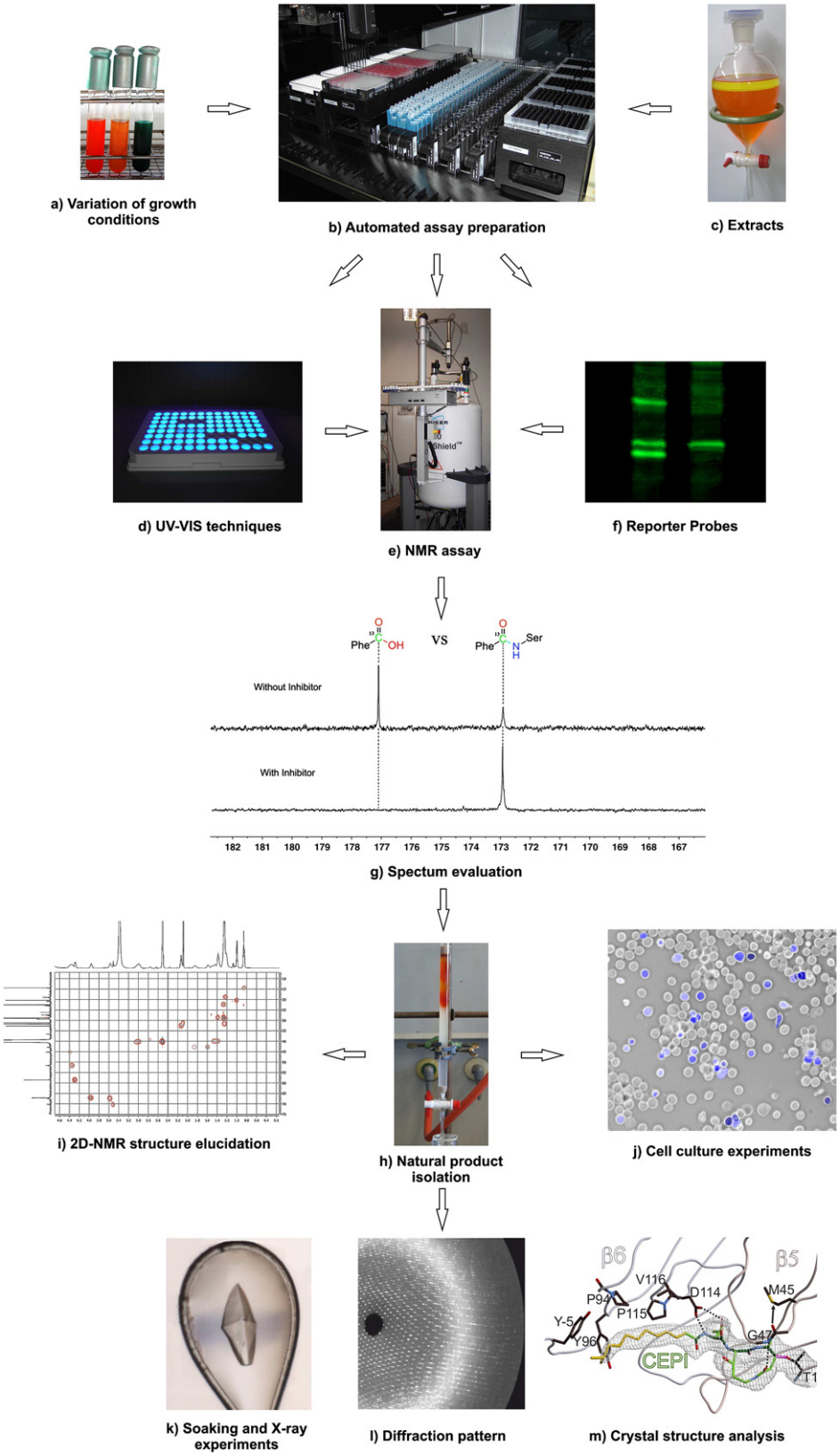

Fig. 3. Screening and isolation of natural CP inhibitors. a–c) By variation of the fermentation conditions, silent biosynthesis machineries can be switched-on, which can be detected in automated high-throughput screens. Suitable environmental triggers are often deducible from the natural life-cycle of the respective organism. However, target-directed assays that are based on the UV–VIS spectrum of light (d) often result in high numbers of false positives due to the intense color of fermentation media and organic extracts, which often contain fluorescent polyphenols and a whole array of pigments. Alternative techniques include the NMR approach (e, g) as well as the SSAP labeling of active subunits (f). Once the triggering conditions are found, the downstream chromatographic purification of the inhibiting compound (h) as well as its structure elucidation by mass spectrometry and 2D-NMR studies (i) are a straightforward process. The evaluation of cell permeability and the affection of distinct cellular pathways are accomplished by cytotoxicity measurements (j) and substrate accumulation assays. The final characterization of the compound *in vitro* is performed by IC_{50} measurements and co-crystallization with the CP (k–m).

assay mixture containing eukaryotic cells, whole cell lysate or purified proteasomes [13,141–143]. The inhibitor moiety of the probe molecule then covalently binds to the respective proteasomal sites, thereby co-tagging them with the fluorophore [133]. After submission to SDS-PAGE, the three active β-subunits can be visualized in fluorescence gel scanning devices (Fig. 3f). Alternatively, cells can also be assessed by confocal microscopy or flow cytometry [144]. The assay was initially developed to assess the cross reactivity of novel CP inhibitors, but it can also be applied for the standard analysis of complex mixtures or uncharacterized compounds [133,144]. The sample to be examined is added to the proteasome before labeling it with the fluorescent probe. If it contains an inhibitory substance, the active sites are blocked before the probe can bind, thus suppressing the labeling and in turn the signal after SDS-PAGE development. This linear dependence results in a good quantifiability of inhibition of all activities [141,144,145]. Moreover, by using cells that co-produce several proteasome particle subtypes such as lymphocytes, it is possible to determine selectivities for either the cCP or the iCP in a competitive assay. However, the biggest advantage of this method is its application in crude culture broth, since disturbing chromogenic substances in the sample are removed during gel electrophoresis. Furthermore, whole cells can be used in this assay and therefore preparation of purified CP is not necessary [146]. The method also allows co-evaluation of cell penetration of the inhibitory compounds, thus making it a valuable alternative to immunological methods [143]. Yet, the difficult preparation of the probe, which must be derived from defined proteasome inhibitors synthesized beforehand, is rather complicated and therefore the technique is restricted to only advanced research laboratories with an attached organic chemistry department. A major drawback of the approach is that the probe molecule has to react covalently and irreversibly with the respective subunit [13,99] and eventually displaces all non-covalent CP inhibitors. Since TMC-95A is the only non-covalent natural product identified to date and due to the high expectations towards such compounds, this is a poignant disadvantage of the method.

3.3. Ub accumulation assays

Contrary to the techniques described above, the UPS activity can also be assessed on the level of the proteome. A straightforward approach takes advantage of the accumulation of ubiquitinated proteins after treatment of mammalian cells with inhibitory compounds [147]. After lysing the cells and submission of the crude extract to SDS-PAGE, ubiquitinated proteins can be visualized by western blot analysis [147,148]. The assay is able to detect all kinds of cell-penetrating UPS inhibitors and exclusively uses commercially available compounds, thus making it an ideal methodology for broad applications. However, the technique is rather indirect as it does neither primarily detect the activity of the core particle, nor the digestion of an intracellular substrate, but the accumulation of Ub labels. Their respective levels are determined by the complex dependencies within the ubiquitination system and can be influenced by a broad variety of mechanisms, e.g. stress response reactions, deubiquitination or altered abundances of regulatory particles [149–152]. Furthermore, the ultimate result is merely an ubiquitination fingerprint of the proteome, thus precluding any form of quantification. The approach is also neither specific for any proteasomal subunit, CP type or even the whole UPS [153,154]. Plus, selective inhibition of only one subunit does not necessarily lead to decreased degradation of substrate proteins [55,155]. An additional disadvantage of this method is its complicated performance and time consuming execution: Every cell extract has to be standardized in its concentration, the blot has to be co-stained against at least one housekeeping protein and the concentration of ubiquitinated proteins must differ considerably to get resolved in the western blot analysis. To conclude, the approach is not suitable for screenings but might support hit identifications to complement other methodologies.

3.4. Pathway specific accumulation assays

Similar to the Ub monitoring technique, it is also possible to quantify intracellular proteasome substrates [156–158]. The concentration levels of short-lived proteins like cyclins or the tumor suppressor protein p53 are most affected by proteasome inhibition [15,159]. One of the first assays using western blot analysis focuses on the NF-κB pathway [156]. The eponymous transcription factor is inactively sequestered in the cytosol by binding to its inhibitory counterpart IκBα [72]. Upon cellular stimulation by TNFα or IL-1, IκBα is phosphorylated and subsequently submitted to UPS degradation [72]. The generated NF-κB protein enters the nucleus and starts transcription of various, mostly inflammation related genes [160]. However, if the CP is blocked by a cell-penetrating inhibitor, IκBα accumulates and can be detected by semi-quantitative immunoblotting analysis [156]. The method was initially designed for evaluation of pathways that are disturbed by CP blockage, as well as analysis of different tumor subtypes [161,162], but it can also be applied for detection of inhibitors in culture medium. Contrary to the Ub accumulation assay, it uses a distinct cellular and natural substrate of the CP, thus making this direct approach less prone to disruptive factors. Furthermore, its pathway specificity directly connects the target modulation exerted by the inhibitors on the proteasome with a biological effect such as the down-regulation of inflammatory cyclins by the shutdown of the immunoproteasomal activities [104,163]. The technique is suitable for detection of all kinds of CP inhibitors and in contrast to the difficult-to-access activity probe molecules, antibodies against IκBα are commercially available. In agreement with the Ub monitoring approach, this approach does neither distinguish between the different subunits affected nor CP subtypes [11,164]. Thus, it can be misleading in that IκBα may still be degradable by a CP that is selectively blocked only in one activity [54]. Moreover, it is not specific towards the proteasome, but may reflect inhibition of previous steps, for example ubiquitination or phosphorylation of IκBα [165]. In addition, the execution of this assay variant is time-consuming and the results can be ambiguous due to the semi-quantitative character of western blot analyses.

3.5. Translational fluorescent fusion substrates

Due to the difficult performance of the substrate monitoring assays, an alternative methodology has been developed using transfected cell lines with stable expression of a fusion construct such as Ub–Luciferase [166,167] or Ub-green fluorescent protein (GFP) [155]. Although the reporter protein is not linked to Ub via an isopeptide bond, the fusion protein is further ubiquitinated and subsequently degraded by the proteasome. In order to prevent deubiquitination, the C-terminal glycine residue of Ub is mutated to valine, thus inevitably leading to a linear degradation of the reporter protein by the UPS [155]. If the cells are treated with culture medium that contains UPS inhibitors, GFP accumulates and can easily be quantitatively measured by either fluorescence microscopy or spectrometry [155]. Once a suitable cell line is established, the experiment can easily be transferred to any cell culture facility. Detrimental effects by colorful or fluorescent culture broths that would affect the signal-to-noise ratio can be vastly eliminated by rinsing the cells before the measurement. However, this methodology again lacks specificity towards the 20S proteasome, since alterations of the RP functionality will lead to a positive assay result as well [153]. Additionally, the intracellular abundance of the 26S proteasome may be submitted to fluctuations caused by impaired assembly or feedback regulations during treatment of the cells with culture broth [152]. The approach is also neither specific for a particular proteasome particle nor a single active subunit as the overall proteolytic activity is measured [155,168–170]. In agreement with the substrate accumulation assays, inhibition of one or even two proteasomal activities does not necessarily lead to a significantly reduced GFP degradation [54,171]. Finally, the detection limits in cell culture based approaches are usually far higher than *in vitro*, thus neglecting compounds that are produced only in

Table 1
Pros and cons of the presented methodologies in natural product screenings.

Methodology	Advantages and disadvantages in natural product screenings for the CP	
1) UV–VIS	- Commercially available substrates - Quantitative and linear readout - Applicable for IC_{50} and kinetics - Easy performance on fermentation screenings - Monitoring of selected activities	- Unspecific *in vivo* - Signal-to-noise ratio affected by quenching, absorption and auto-fluorescence
2) SSAP	- Co-evaluation of cell permeability - Subunit selectivity of distinct CP types - Applicable for fluorescent microscopy - Quantifiable - Reliability in crude conglomerates	- Sophisticated probe preparation - Complex quantification - Comprehensive UPS analysis impossible - Detection of reversible inhibitors impossible
3) Ub accumulation	- Commercially available - Co-evaluation of cell permeability - Analysis of the whole UPS	- Insensitive readout - Unspecific - Time-consuming and error-prone - Ambiguous results - Indirect assay - Not quantifiable
4) Pathway specific accumulation	- Commercially available - Co-evaluation of cell permeability - Pathway affection in various cell lines - Analysis of cellular function - Direct assay	- Complex handling and improper quantification - Ambiguous results - False-negative on selective inhibition - Interference by pathway specific determinants - Insensitive readout
5) Translational fluorescent fusion substrates	- Easy readout and quantifiability - Co-evaluation of cell permeability - Sensitive of detection - Applicable in FACS and fluorescent microscopy - Reliability in crude conglomerates	- Lack of proteasome specificity - False-negative on selective inhibition - Off-target effects
6) NMR spectroscopy	- Native proteasomal substrate - Easy peptide synthesis - Reliability in crude conglomerates - Applicable in high-throughput-screenings - High sensitivity - Subunit specific, quantifiable, unambiguous readout	- Substrate amount - Restricted to *in vitro* analysis

trace amounts, which however is a typical characteristic for toxic substances.

3.6. NMR spectroscopy mining for CP inhibitors in conglomerates

Recently an orthogonal methodology was established to directly measure the proteolytic activity exerted by the CP [11]. Contrary to previously described techniques, it is not based on UV–VIS but on NMR signal detection and is therefore not affected by any diffraction or quenching effects in crude mixtures. The assay uses a native proteasomal peptide substrate, which is derived from the digestion pattern of the murine JAK-1 kinase [54]. Labeling of the peptide probe, which can be generated by standard peptide chemistry in high amounts, occurs at the scissile peptide bond with a ^{13}C-carbonyl carbon [11]. Upon hydrolysis, a distinct shift is observed in the high field area of the recorded NMR spectrum that is assigned to the altered chemical environment of the ^{13}C-probe in the free carbonic acid product versus the bound amide educt [11] (Fig. 3e, g). Although other substances in a given sample also give rise to the NMR signals, most of them are in the range of 0 to 150 ppm, whereas the probe atom is detected at 170 ppm. Furthermore, as the abundance of the ^{13}C probe is 100% versus 1.1% in natural compounds, the signal-to-noise ratio is abundantly increased [11]. Similar to conventional fluorogenic peptide assays, the sequence of the peptide can be adapted towards a defined CP activity, thus allowing to selectively measure inhibition of a distinct active site [132]. Therefore, it might be a rather simple approach to alter the primary sequence to produce specific substrate molecules for the ChTL, TL and CL activities, thus allowing simultaneous measurement of all proteolytic centers at once [132,172]. Furthermore, the assay is able to detect covalent, non-covalent, reversible and irreversible inhibitors. However, a drawback of the method is the large amount of peptide substrates, which have to be used in millimolar concentrations, as the sensitivity of recorded NMR spectra in general lacks far behind fluorescence approaches. On the other hand, the technique is suitable for screening of a vast number of samples, as the processing of one assay can be accomplished within 15 min on a 500-MHz NMR spectrometer [11]. Therefore the NMR assay still allows the performance of a standard amount of approximately 100 samples of crude extracts or conglomerates containing thousands of different compounds per day. Plus, the results yielded by this assay are unambiguous. The introduction and a proof of principle of this recently developed methodology were reported for the insect pathogenic bacterium *Photorhabdus luminescens* [11], which was suspected to produce a proteasome inhibitor at a defined state during its pathogenic life-cycle [12,173].

4. Chapter 3: Application of proteasome assays for natural product mining on syrbactins

4.1. Triggering secretion of natural CP inhibitors during defined life stages

All assays described in chapter 2 are valuable tools for the detection of proteasome inhibiting substances in extracts and crude culture broths of microorganisms, plants or fungi. Furthermore, they can be used to screen different fermentations in order to initiate the biosynthesis of secondary metabolites that are silenced under common growth conditions [174]. Matching environmental triggers can often be inferred from the natural life stages of the respective organisms and the objective for which the compound is produced. In the field of CP inhibitors, the first substance to which a biological role could be assigned was the syrbactin compound SylA (Fig. 2h) [12]. It is produced by the phytopathogenic bacterium *Pseudomonas syringae*, which causes the brown spot disease in bean plants and other

economic crops [80,175,176]. By deletion of the corresponding gene cluster responsible for the synthesis of SylA, the infectivity of *P. syringae* towards the host organism was decreased by 70%, thus highlighting the role of the CP inhibitor as a virulence factor [12]. Interestingly, the compound is secreted by the bacterium only under specific growth conditions that simulate the chemical composition of plant leaves by the addition of fructose and phenolic sugars [80,177]. This finding corresponds to the general secretion pattern of many toxic compounds that are only produced after their initiation by environmental conditions resembling those in the corresponding host organism, thus ensuring a directed and selective application.

Subsequent to the discovery of SylA, a similar substance named GlbA (Fig. 2i) was identified in a database search and determined to be a proteasome inhibitor as well [12,81]. Its characterization revealed that GlbA was the strongest CP inhibitor among all previously described compounds with extraordinary properties in cell culture experiments [12]. These results fuelled the search for further members of the syrbactin family. The disclosure of the genes responsible for the synthesis of SylA and GlbA led to the identification of many species with similar clusters among the genera *Burkholderia* and *Photorhabdus*, which intriguingly both comprise human pathogenic organisms like *P. asymbiotica*, *B. pseudomallei* or *B. mallei* [178,179]. Curiously, the genetic analysis also led to the conclusion that *B. mallei* is probably not able to produce the corresponding proteasome inhibitor because it carries a transposon mediated intron in the decisive gene cluster [178]. Notably, *B. mallei* displays a significantly reduced pathogenicity compared with *B. pseudomallei*, which even has been classified as weapon of mass destruction [180]. This finding again alludes to the biological role of CP inhibiting compounds as virulence factors.

In order to discover and characterize the effector molecules produced by the identified organisms on a chemical level, extensive studies were carried out to find suitable fermentation procedures for the induction of the respective biosynthetic machineries [11,118]. As shown for the insect pathogen *Photorhabdus luminescens*, the combination of different methodologies was investigated on the biosynthesis of a syrbactin-like compound [11]. Distinct growth media of the bacteria were initially monitored by common fluorescence and UV–VIS based methods. Yet, due to the autofluorescence and the intensive color of *Photorhabdus* cultures, which range from light blue to dark shades of red (Fig. 3a), the results were too ambiguous to infer the presence of an inhibitory compound [11,133]. Contrary to the UV–VIS methodology, the implementation of the NMR technique for the screening of culture conditions yielded unambiguous results (Fig. 3e, g). Surprisingly, the performed experiments revealed the absence of any CP modulating compound, hence proving that the corresponding biosynthetic machinery is silent under standard growth conditions [11,181]. Therefore, *Photorhabdus* was hypothesized to require an equivalent molecular trigger as *Pseudomonas syringae* to induce its virulent phase and in consequence the production of the predicted proteasome inhibitor. Screening of defined media with various additives finally disclosed the prerequisite to trigger inhibitor biosynthesis in *Photorhabdus* [11]. In a low osmolarity medium, which was inoculated by a saturated *Photorhabdus* culture cultivated at high sodium chloride concentrations, the bacteria instantly started to secrete a powerful inhibitory compound, which was detectable by the NMR assay within few hours after the environmental change. Upon addition of conditioned and induced medium to an assay mixture containing purified yeast proteasome and labeled peptide, the digestion of the substrate was prevented as displayed by the ^{13}C NMR spectra [11] (Fig. 3g).

4.2. Biological background of sensory systems exemplified on *P. luminescens*

Similar to *P. syringae*, this switch from the quiescent to the pathogenic phase after the transfer to an inducing medium reflects the bipolar life-cycle of the entomopathogenic bacterium. During the first life stage of *Photorhabdus*, the bacteria hibernate in the gut of their symbiont nematode *Heterorhabditis*, which searches the soil for insect larvae [182,183]. When the worm detects its prey, it penetrates the insect to reach the hemocoel, where it regurgitates the bacteria. Together, they kill the larvae in a concerted action and feed on the tissue to start reproduction. Once the carcass is exploited, the nematodes devour the bacteria, and both organisms leave the larvae to search for a new prey [183]. During its virulent phase, *Photorhabdus* produces a plentitude of bioactive molecules to kill the insect and to protect it against other scavenging organisms [184]. This is reflected by the observation that *Photorhabdus* occurs in two distinct phases *in vitro* [173]. Whereas it is non-infective in phase I, it develops full activity and biosynthesis of various secondary metabolites in phase II [12,182]. The induction of the pathogenic phase and in turn the secretion of the CP inhibitor was shown to be strictly controlled in *Photorhabdus* and must be induced by the identified environmental conditions [11,181]. Besides, the compound cannot be detected under the growth conditions for SylA biosynthesis in *P. syringae* or GlbA production in the respective *Burkholderiales* strain K481-B101 [11]. This demonstrates the adaptation of the systems of these bacteria to their respective host organisms. Furthermore, the involvement of a proteasome inhibitor in a symbiotic relationship has not been observed before. The bacterium presumably suspends its biosynthesis until it is released into the insect larvae. *Photorhabdus* then has to safeguard and foster the second generation of nematodes by the selective application of its toxins [11,182].

After the establishment of the induction procedure, Stein et al. demonstrated the straightforward downstream processing of the inhibiting substance by its isolation and identification as CepI [11,82] (Fig. 3h–i). Surprisingly, the molecular structure matches GlbA except from one methyl moiety at the distal terminus from the Michael-system that binds covalently to the active proteasomal subunits (Figs. 2h–j, 3m). This was unexpected, as the gene clusters between the organisms share only moderate sequence homology. Therefore the high overall similarity of the small molecules was unforeseen, which illustrates the unpredictability of a secondary metabolite on the basis of the primary DNA sequence. In contrast to the inhibitory compounds, the sensory systems diverge significantly because they are optimized towards the circumstances during the respective life-cycles of the producing microbes. Thus, also virulence factors that are not primarily directed against humans are suitable for development of novel pharmaceutical compounds because their lead structures are not altered between the targeted organisms.

However, the characterization of CepI determined the compound to range among the most potent proteasome inhibitors described so far [11]. While its isolation was achieved by the application of the NMR assay, the *in vivo* characterization of CepI was performed by cell viability assays and the NF-κB analysis [11] (Fig. 3j), thereby verifying its cell penetration and the affection of the UPS. The crystal structure analysis revealed that the increased hydrophobic surface is crucial for the enhanced binding properties of CepI compared with GlbA [11] (Fig. 3k–m). Yet, the prediction of the extent of this effect is definitely not possible by computational methods to date. Since also mammalian pathogenic organisms have been identified with gene clusters similar to those in *P. luminescens* [12,178] future analysis will show whether they are capable to produce analogous compounds with optimized inhibitory properties for the human cCP, iCP or tCP.

5. Conclusions

The UPS is a key player in crucial cellular processes [1]. Its dysregulation is closely linked to various malignancies including cancer and autoimmune diseases [6]. In contrast to this vast applicative scope of UPS modulating agents, the CP inhibitor bortezomib is only approved for treatment of the blood cancers multiple myeloma and

mantle cell lymphoma [88–90]. This is largely due to the high reactivity of the boronic acid inhibitor, which considerably deteriorates its pharmacokinetic effects e.g. in treatment of solid tumors [91]. The development and release of second generation therapeutic agents now announce the implementation of more specific and less reactive compounds with broader applications in this important field of research [6]. Yet, the broad range of possible medical indications, as well as primary and secondary resistances in cancer therapy, fuels the search for novel UPS and especially CP modulating compounds. A huge spectrum of substances acting on the proteasome has been identified already, thus making the discovery of other compound classes most likely [83,185]. Albeit, natural product research stagnates or is even reduced in pharmaceutical industry in our days, the lead structures derived from these substances remain a central focus due to their enhanced pharmacological properties [10]. Although genome analyses have demonstrated that only a minor share of compounds produced by the sequenced organisms has been isolated to date, the majority is inaccessible by common isolation procedures because biosynthesis only occurs under distinct environmental conditions that reflect the cognate life stages of the analyzed species [11]. However, these compounds belong to the most fascinating group of natural compounds since they often serve as molecular keys to the initiation of important cellular processes such as immune response modulation in a host organism. Therefore, the disclosure of suitable molecular triggers to initiate the change of life stages is a promising means to expand the limits of natural compounds research [10].

Yet, in order to detect the right environmental circumstances, suitable methods for the assessment of compound production in crude culture broth or raw extracts must be available. In the case of the CP, several approaches have been developed and are comparatively presented in this review. Although UV–VIS and fluorescent techniques using unnatural peptide substrates are very popular and easy to perform, they bear disadvantages and are error prone in complex mixtures. More advanced methods have been deceived *in vitro* and *in vivo* that account for these drawbacks that, however, also hold specific disadvantages according to their nature. Cell based techniques for example, suffer from their lack of selectivity for the UPS, the crosstalk between several pathways and a difficult performance. On the other hand, they are suitable to co-assess the cell penetration or even the affection of a distinct pathway by a given compound [161]. Hence these methods have a limited application for high-throughput approaches but are essential in the characterization of an isolated compound. Contrarily, SSAP based methods are suitable to address the intracellular affection of the CP by a straightforward and direct approach [142], but they require the synthesis of a radioactively or fluorescent labeled proteasome inhibitors, which are challenging to obtain. A new promising technology represents the recently developed NMR technique [11], which combines the advantage of a direct, specific and robust assay with excellent detection limits and a good applicability for high-throughput screenings of conglomerates, culture broths and cell extracts. Its successful application was instantly demonstrated with the isolation and characterization of CepI, one of the strongest proteasome inhibitors identified to date, thus demonstrating the vast potential of the approach. However, it is the combination of different methods with their respective pros and cons and their varying kinds of information (Table 1) that must be applied to expand the spectrum of natural proteasome inhibitors during the next decade.

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