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Immunoproteasomes

Inhibitors for the Immuno- and Constitutive Proteasome: Current and Future Trends in Drug Development

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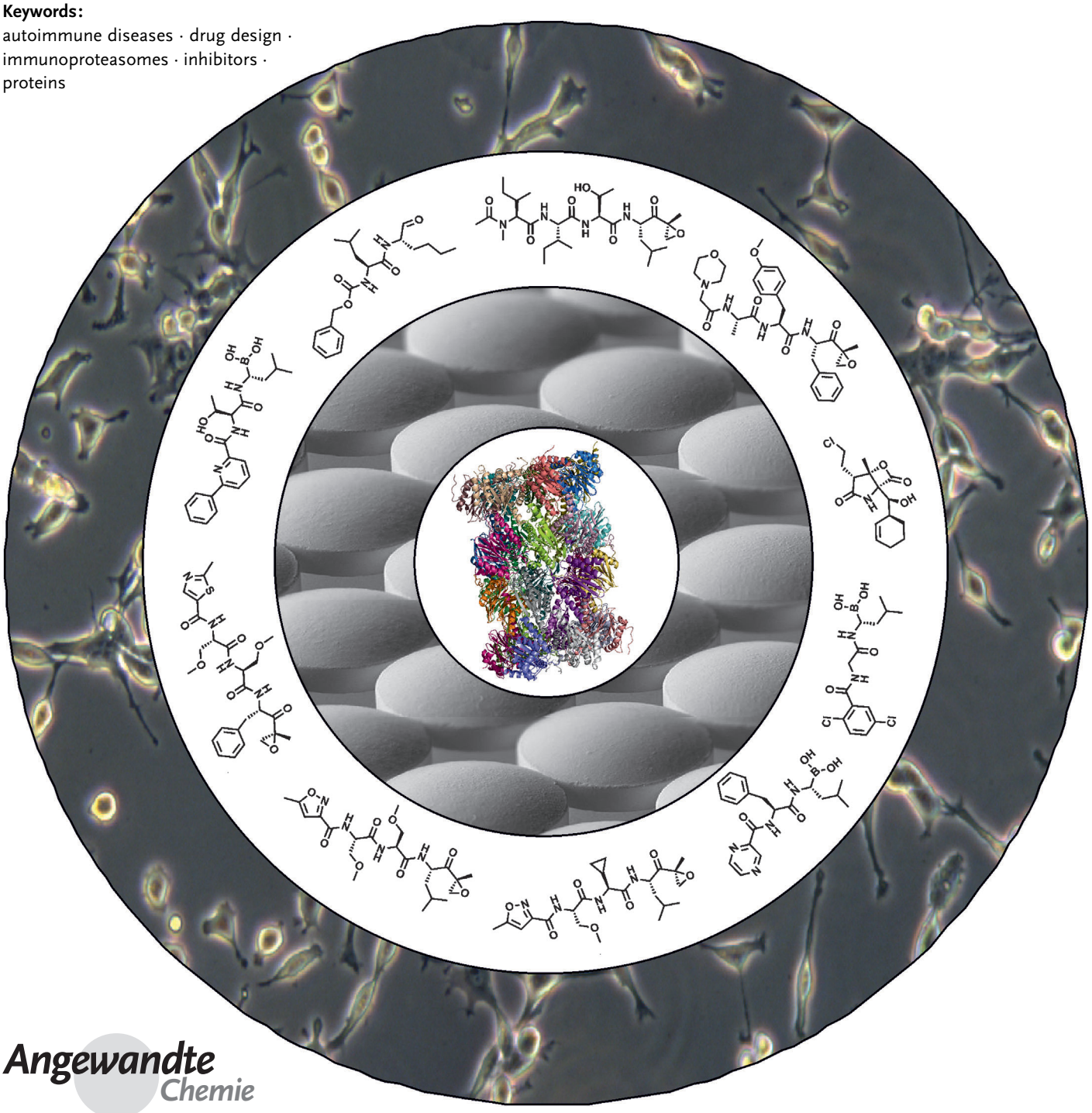

Proteolytic degradation is an essential cellular process which is primarily carried out by the 20S proteasome core particle (CP), a protease of 720 kDa and 28 individual subunits. As a result of its central functional role, the proteasome represents an attractive drug target that has been extensively investigated during the last decade and validated by the approval of bortezomib by the US Food and Drug Administration (FDA). Currently, several optimized second-generation proteasome inhibitors are being explored as anticancer drugs in clinical trials, and most of them target both constitutive proteasomes (cCPs) and immunoproteasomes (iCPs). However, selective inhibition of the iCPs, a distinct class of proteasomes predominantly expressed in immune cells, appears to be a promising therapeutic rationale for the treatment of autoimmune disorders. Although a few selective agents have already been identified, the recently determined crystal structure of the iCP will further promote the development and optimization of iCP-selective compounds.

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1. Introduction

The 20S proteasome core particle (CP) constitutes the key player of the nonlysosomal protein degradation pathway and mediates the hydrolysis of proteins to peptides of 2–23 amino acids.[1] Thus, a plethora of different cellular processes, among them cell-cycle progression, signaling, and antigen processing, rely on the proteasomal activity. In vertebrates, three major classes of CPs shape the antigenic repertoire: the thymoproteasome (tCP) is exclusively found in cortical thymic epithelial cells, the immunoproteasome (iCP) predominantly in mono- and lymphocytes, and the constitutive proteasome (cCP) in most other tissues (Figure 1 a).[2] However, during inflammation, cytokines such as interferon-γ and tumor necrosis factor (TNF) α also induce iCP formation in cells of non-haematopoietic origin.[3] In contrast to cCPs, iCPs enhance the production of oligopeptides with hydrophobic C termini. After N-terminal trimming of the cleavage products down to 8–10 amino acids, they are readily loaded on major histocompatibility complex class I (MHC-I) receptors and presented to cytotoxic T cells to trigger immune responses.[4] Hence, the proteasome determines the C-terminal anchor residue of MHC-I ligands and their affinity, whereas other proteases in the cytosol or endoplasmic reticulum define the N-terminal end and overall length of these peptides.[4c] Although MHC-I antigens generated by cCPs can trigger cytotoxic T-cell responses, studies on mice devoid of partial or total iCP activity show strongly impaired and altered MHC-I epitope presentation, thus indicating the outstanding importance of iCPs for antigen processing.[5] Furthermore, iCPs were also demonstrated to be involved in T-cell differentiation, as well as in the synthesis of proinflammatory cytokines and to play a preservative role during oxidative stress.[6]

cCPs, iCPs, and tCPs exert various biological functions, as they harbor unique sets of catalytic β-type subunits, and thus lead to distinct but overlapping peptide repertoires (Figure 1 a): cCPs incorporate the subunits β1c, β2c, and β5c, iCPs the subunits β1i, β2i, and β5i, and tCPs the subunits β1i, β2i, and β5t. The cleavage preferences of these catalytically active subunits are determined by the chemical nature of their substrate specificity (S) pockets, which accommodate the ligand's side chains (P sites) and, thus, influence the mean residence time of ligands in the substrate binding channel (Figure 1 b). In agreement with ligand docking experiments to the yeast 20S proteasome (yCP), which demonstrate the importance of the P1 side chain,[7] proteasomal cleavage specificities were assigned according to the preferred P1 amino acid. Although β2 subunits have been attributed to trypsin-like (TL) activity, their rather large substrate binding pockets endow them with a broad substrate specificity. The cCP subunit β1c cleaves peptide bonds after acidic side chains, which equates to caspase-like (CL) activity. In contrast, the hydrophobic lining of the β1i substrate binding channel gives rise to a branched chain amino acid preferring (BrAAP) activity.[8] Similarly, the active sites of both β5c and β5i are surrounded by nonpolar environments and, hence, their substrate specificity was termed chymotrypsin-like (ChTL) activity. However, the crystal structures of mouse cCP and iCP revealed that the S1 specificity pocket of β5c is significantly smaller than the β5i counterpart. Therefore, β5c exerts an elastase-like or small neutral amino acid preferring activity (SnAAP) instead of a ChTL activity.[7,8] Unlike β5c, subunit β5i exerts ChTL activity by preferentially hydrolyzing oligopeptides on the C-terminal side of bulky hydrophobic amino acids.[7,9] In summary, β1i, β5c, and β5i generate high-affinity epitopes for MHC-I receptors, whereas subunit β5t of tCPs is suggested to produce low-affinity MHC-I ligands, as the bottom of the

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S1 specificity pocket formed by Thr45 is of hydrophilic nature.[2b,10] These different cleavage preferences of β5 subunits predominantly result from modifications within the substrate binding channel and not at the active site, as all active β subunits share the same catalytic mechanism of N-terminal nucleophilic (Ntn) threonine hydrolases.[7,11]

During the last 15 years the CP has been extensively explored as a drug target, and many inhibitory compounds were developed based on the empirically found substrate specificities and on the crystal structures of the 20S proteasome from *Thermoplasma acidophilum*,[12] *Saccharomyces cerevisiae*,[13] and *Bos taurus*.[14] Most agents, both natural and synthetic ones, proved to have antitumor and anti-inflammatory activity and are regularly reviewed in the literature.[15] However, new studies on the selective inhibition of the iCP demonstrated a therapeutic benefit, particularly in autoimmune diseases, and put the iCP in the limelight as a novel drug target.[6a,16] This Review covers the current advances in the field of proteasome inhibitors, with a particular focus on iCP- and cCP-selective compounds and their therapeutic potential.

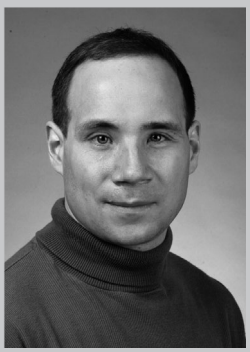
2. 20S Proteasomes as Drug Targets

The 20S proteasome is of outstanding importance for intracellular protein homeostasis. Inhibition of the CP results in the accumulation of misfolded proteins as well as reactive oxygen species, thereby giving rise to the induction of endoplasmatic reticulum stress and the unfolded protein response.[18] Furthermore, proteasome inhibitors prevent degradation of tumor suppressors such as the cyclin kinase inhibitor $p27^{kip1}$ [19] and downregulates proinflammatory pathways, such as the nuclear factor-κB signaling cascade as well as their antiapoptotic target genes. Ultimately, proapoptotic factors accumulate and cause cell-cycle arrest and apoptosis.[15b] Numerous studies have proved the therapeutic window of proteasome inhibition in the treatment of cancers. Rapidly dividing malignant cells turned out to be much more susceptible to the blockage of CPs, as their accelerated cell cycle necessitates increased breakdown capacities of cyclins. In addition, cancer cells essentially require the proteasome to cope with accumulating misfolded proteins, which result from their chromosomal instability and their imbalanced protein synthesis. Thus, inhibition of the CP primarily induces cell death in neoplastic cells, but healthy quiescent cells tolerate this stress.[15b,20]

Whereas increased proteasome concentrations are a general feature of tumor cells, abnormal iCP levels have been associated with the development and progression of neurodegenerative diseases, autoimmune disorders, and certain types of cancers.[21] However, it remains to be further investigated if elevated iCP levels really drive disease progression or only represent a consequence of excessive cytokine synthesis or cellular stress. Alzheimer's and Huntington's disease[21a,22] as well as macular degeneration[23] are currently characterized by increased concentrations of proinflammatory markers and iCPs. In rheumatoid arthritis,[24] inclusion body myositis, myofibrillar myopathy,[25] amyotrophic lateral sclerosis,[21c,26] Crohn's disease,[21d] and dextran sulphate sodium (DSS) induced colitis[27] only the β1i levels are augmented. Furthermore, in malignancies such as multiple myeloma,[28] feline primary fibrosarcoma,[29] as well as colon,[30] lung,[30] and prostate[21b] cancer cell lines, abnormal levels of iCP subunits have been detected. Overexpression of iCP positively correlates with chronic inflammation dependent tumor pathogenesis, cardiovascular inflammation, and cytokine production.[6a,25b,30] Thus, selective inhibition of the iCP represents a promising novel therapeutic strategy for the above diseases;[6a,16] however, only a few selective inhibitors of the iCP have been identified so far. The knowledge available now on the structural differences between iCP and cCP in the presence and absence of the iCP-selective inhibitor ONX 0914 (Onyx Pharmaceuticals; 4.3.3.) provides the basis for molecular modeling and ligand docking studies as well as structure-guided drug design.

3. Proteasome Inhibitors—A Mechanistic Description

The presence of substrate binding channels with non-primed and primed specificity pockets, located in the N- and C-terminal directions of the scissile peptide bond, classifies the proteasome as an endoprotease (Figure 1b). The nucleophilic $Thr1O^{\gamma}$ of the proteasome attacks the carbonyl carbon atom of peptide bonds, thus resulting in an acyl–enzyme intermediate and a peptide fragment with a newly generated N terminus. Subsequent hydrolysis of the acyl–enzyme species by addition of the nucleophilic water molecule restores



Michael Groll studied chemistry and received his PhD for crystallographic and biochemical studies on the yeast 20S proteasome. After postdoctoral studies in the groups of Prof. Huber (Munich), Prof. Finley (Boston), Prof. Neupert (Munich), and Prof. Kloetzel (Berlin) he became assistant professor at the Charité (Berlin) in 2006. Since 2007, he has been full professor of biochemistry at the Technische Universität München. His major research interest focuses on the structural and functional characterization of multiprotein complexes.

Eva Huber studied biochemistry at the Technische Universität München and in 2009 completed her MSc, which was funded by the Studienstiftung des deutschen Volkes. She joined the group of Prof. Groll for postgraduate studies on the immunoproteasome. Her major current achievements are the elucidation of the crystal structures of the mouse immuno- and constitutive proteasome alone and in complex with the immunoproteasome-selective inhibitor ONX 0914.

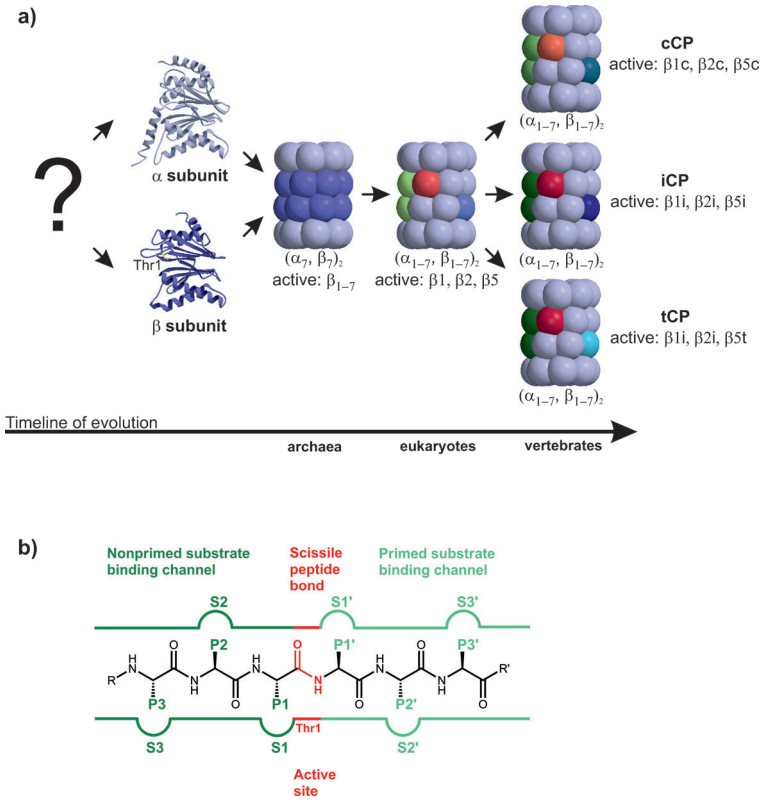


Figure 1. Evolution of 20S proteasomes (CPs). a) CPs and derivatives thereof are present in all three kingdoms of life. Although bacteria, with the exception of actinomycetes, are devoid of 20S proteasomes, they harbor CP-like complexes termed HslV that consist of two six-membered rings of one type of β subunit.[17] As a rule, archaea possess CPs that are built up by two different types of monomers, namely inactive α (gray) and proteolytically active β subunits (blue), which presumably originated by divergent evolution from a common but unknown precursor protein. Whereas *Thermoplasma acidophilum* assembles only one type of α and β subunit into their CPs,[12] other representatives use different types of α and β subunits. Eukaryotes, such as *Saccharomyces cerevisiae*, encode seven different α and seven different β subunits. Interestingly, only three out of the seven β subunits are proteolytically active: β1 (green), β2 (red), and β5 (blue).[13] For clarity the catalytically inactive β subunits are colored like α subunits in gray. Vertebrates emerged as the most recent step on the timeline of evolution and with their adaptive immune system three major classes of 20S proteasomes evolved: cCPs, iCPs, and tCPs. Their distinct sets of catalytically active β subunits give rise to different substrate-cleavage specificities.[7,14] In particular, β5 subunits, which originated by divergent evolution, seem to be perfectly adapted to the requirements of adaptive immune responses and possibly other biological pathways. b) Schematic representation of the proteasomal substrate binding channel. The primed (S′) pockets and the P′ sites of the ligand are colored in green, and the unprimed specificity (S) pockets and the corresponding substrate residues (P) are highlighted in dark green. The active site including Thr1 and the scissile peptide bond are shown in red.

the active site and completes the reaction by release of the C terminus of the N-terminal cleavage product.[13]

Most currently investigated drug candidates are peptide-like compounds that mimic the binding mode of natural substrates through the formation of an antiparallel β sheet in the substrate binding channel of the active site cleft.[15a] As the mode of action of pharmacophores is identical for all proteasomal subunits, irrespective of the type of CP, subunit selectivity of peptidomimetics can predominantly be achieved by modification of their side chains.[7] However, to confer not only specificity but also inhibitory potency, the majority of compounds are endowed with a functional reactive head group, which either irreversibly or reversibly modifies the Thr1 residue of active proteasomal β subunits by formation of a covalent bond. Covalently acting compounds can be grouped into seven classes, depending on their pharmacophore type, which are briefly described below: aldehydes, vinyl sulfones, vinylamides (syrbactins), boronates, α′,β′-epoxyketones, α-ketoaldehydes (glyoxals), and β-lactones (Scheme 1).

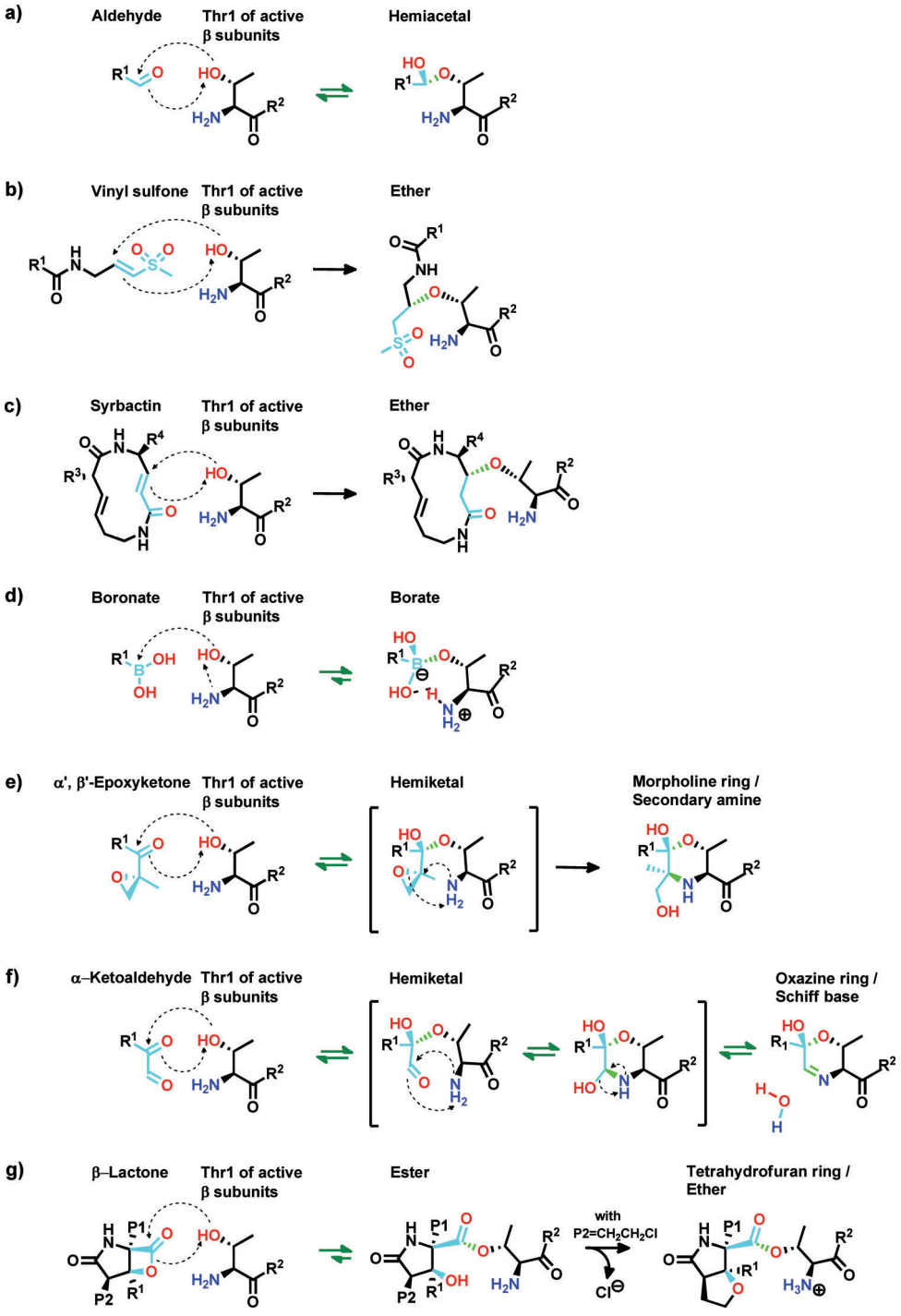
a) Aldehydes represent the first inhibitors developed for the proteasome.[31] Their electrophilic carbonyl carbon atom reacts with the N-terminal Thr1O$^{\gamma}$ residue of proteolytically active β subunits, thus forming a rapidly reversible tetrahedral hemiacetal (Scheme 1 a).[12] This reaction mechanism enables aldehydes to also target serine and cysteine proteases.[31] As they can be easily inactivated by oxidation, aldehydes have only limited therapeutic potential.[32]

b) Peptides with a vinyl sulfone head group react through a Michael-type 1,4-addition with the proteasomal Thr1O$^{\gamma}$ residue, ultimately resulting in the irreversible formation of an ether bond (Scheme 1 b).[15a,33] However, by their unspecific reaction mechanism vinyl sulfones also target the functional thiol group of cysteine proteases.[15a]

c) The Michael-type 1,4-addition has also been exploited by nature: syrbactins, natural products consisting of a 12-membered macrolactam ring system, react through their vinylamide functional group with Thr1O$^{\gamma}$ in a manner similar to vinyl sulfones (Scheme 1 c).[34]

d) As a consequence of the Food and Drug Administration (FDA) approval of bortezomib, the boronic acid pharmacophore is the most prominent class of proteasome inhibitors. Boronates form a reversible tetrahedral transition state with the nucleophilic Thr1O$^{\gamma}$ residue, which is stabilized by hydrogen bonds between the boronate hydroxy groups and the oxyanion hole Gly47NH as well as Thr1N.[35] These interactions and the Lewis hard/soft acid base principle explain bortezomib's enhanced affinity for Ntn hydrolases compared to other proteases (Scheme 1 d; Table 1; Section 4.1).[35,36]

e),f) Both α′,β′-epoxyketones and α-ketoaldehydes react in a two-step mechanism with the active site nucleophilic Thr1O$^{\gamma}$ residue (Scheme 1 e,f):[37] reversible formation of a hemiketal beween Thr1O$^{\gamma}$ with the ketone group is followed by a nucleophilic attack of Thr1N on the epoxide group of α′,β′-epoxyketones, or in the case of α-ketoaldehydes on the aldehyde function. Whereas α′,β′-epoxyketones irreversibly cycle with Thr1, thereby resulting in a morpholine ring system through the formation of a secondary amine, α-ketoaldehydes



Scheme 1. Head groups of proteasome inhibitors and their reaction mechanisms. Chemical structures of pharmacophores that covalently react with the nucleophilic Thr1O$^{\gamma}$ residue of active proteasome subunits: a) aldehydes, b) vinyl sulfones, c) vinylamides (syrbactins), d) boronates, e) α′,β′-epoxyketones, f) α-ketoaldehydes, and g) β-lactones are marked in cyan. The variable part of the compounds is designated R^1. The proteasomal active site Thr1 is colored in black. R^2 represents the corresponding active β subunit. Oxygen atoms involved in catalysis are shown in red and the amine group of Thr1 in blue. Bonds formed during the reaction mechanism are highlighted in green. P1 and P2 as well as R^3 and R^4 designate the ligand side chains of β-lactone inhibitors and syrbactins, respectively. Arrows of reversible reactions are colored in dark green.

reversibly form a cyclic carbinolamine intermediate with Thr1, which undergoes a condensation reaction and transforms into a 5,6-dihydro-2*H*-1,4-oxazine ring with a Schiff base bond.[37a,b,38] As a consequence of their unique bivalent mode of action, involving Thr1O$^{\gamma}$ and Thr1N of the proteasomal active site, α′,β′-epoxyketones and α-ketoaldehydes are the most specific functional head groups for CPs known today.

g) β-Lactones represent a further promising pharmacophore, which currently is the focus of drug development.[39] Attack of Thr1O$^{\gamma}$ on the carbonyl carbon atom of the functional ester group opens the β-lactone ring system, thereby generating a hydroxy group and an acyl–enzyme ester. Although the nucleophilic water molecule is displaced from its normal position in the active site during this reaction, the catalytically active Thr1O$^{\gamma}$ can be restored by slow hydrolysis of the acyl–enzyme complex.[40] Interestingly, in the case of marizomib, the most potent β-lactone-based inhibitor, a second reaction step follows the formation of the acyl–enzyme complex: the P2 chloroethyl side chain of marizomib is attacked by the newly formed hydroxy group and irreversibly cycles with release of chloride and formation of a tetrahydrofuran ring (Scheme 1 g).[40a,c] As a result of the formation of this ether bond covalent modification of Thr1O$^{\gamma}$ by marizomib cannot be reversed by the addition of water.[15a,40c]

Some of the highly reactive functional head groups listed above are associated with increased inhibitory potency and cytotoxicity, but reduced subunit selectivity. In agreement, in 2010 Screen et al. showed that exchange of the α′,β′-epoxyketone func-

Table 1: Inhibitory potency of compounds toward the proteasomal subunits. IC_{50} values are given in [nM]; n.r. designates IC_{50} values not reported.

	β1c	β1i	β2c	β2i	β5c	β5i	Ref.
Bortezomib	74	n.r.	4200	n.r.	7	4	[64]
MLN9708	31	n.r.	3500	n.r.	3.4	n.r.	[58]
CEP-18770	<100	n.r.	>100	n.r.	3.8	n.r.	[57]
Carfilzomib	2400	n.r.	3600	n.r.	6	33	[64]
ONX 0912	n.r.	n.r.	n.r.	n.r.	36	82	[63a]
Marizomib	330	n.r.	26	n.r.	2.5	n.r.	[40a]
ONX 0914	ca. 7000	ca. 500	ca. 3000	ca. 1000	236	28	[6a]
PR-924	>30000	8200	>30000	>30000	2900	22	[42b]
IPSI-001	239000[a]	1450[a]	n.r.	n.r.	105000[a]	1030[a]	[44a]
PR-893	2800	221	8900	3100	17	357	[42b]
PR-825	n.r.	n.r.	n.r.	n.r.	9	238	[6a]

[a] For IPSI-001 only K_i values [nM] are published.

tional group by the less-reactive vinyl sulfone group enhances the β5-specificity of ligands and, therefore, decreases their toxicity to cells.[41] So far, most proteasome inhibitors target the β5 active sites of both cCPs and iCPs, and it appears that this simultaneous inhibition is required to efficiently induce apoptosis in tumor cells.[42] Although β2- or β1-selective compounds alone cause no cytotoxic effects, they were shown to sensitize malignant cells to β5-targeting drugs such as bortezomib or carfilzomib.[42a,43] However, as inhibitors of single iCP subunits were reported to exert anti-neoplastic effects in transformed cells (see Sections 4.3.2 and 4.3.3),[21b,44] further experimental data will be required to assess the therapeutic benefit of the selective inhibition of single iCP subunits in different types of malignancies. However, specific iCP inhibitors are potent aspirants for the treatment of autoimmune diseases (see Section 4.3.3).

4. Prominent Inhibitory Compounds—Pros and Cons

4.1. Bortezomib

Intensive research efforts over the last decade cumulated in the FDA approval of the dipeptide boronate proteasome inhibitor bortezomib (Velcade, PS-341; Millenium Pharmaceuticals; Figure 2a: compound 1) for the treatment of multiple myeloma as well as relapsed or refractory mantle cell lymphoma.[45] In addition, bortezomib is currently being evaluated in clinical studies for solid tumors, including non-small cell lung cancer,[46] and has demonstrated therapeutic efficacy in kidney transplantation.[47] Bortezomib inhibits the subunits β5c and β5i equally, with IC_{50} values of 7 nM and 4 nM, respectively, and with a lower affinity this drug also targets the CL active site of the proteasome (IC_{50}: 74 nM).[18e,48] This nonspecificity regarding the subunits β5c and β5i is explained by favorable interactions of the N-terminal pyrazine moiety of bortezomib with the amino acids surrounding the S3 pocket.[7]

Despite its success on the market, bortezomib therapy has several disadvantages, including intravenous administration and noteworthy side effects such as thrombocytopenia and neutropenia as well as gastrointestinal disorders.[49] More than 30% of bortezomib-treated patients also suffer from severe but reversible neurodegenerative effects,[50] including neurotoxicity, tremor, and reduced nerve conduction velocity.[50c,51] Only recently, scientific studies proved that bortezomib triggers nerve degeneration by exhibiting substantial off-target activity. In particular, in 2011 Arastu-Kapur et al. reported that the proteasome and serine proteases such as cathepsin G, cathepsin A, chymase, dipeptidyl peptidase II, and HtrA2/omi, required for neuronal cell survival, are equally inhibited by bortezomib.[50c]

An additional major concern is the large fraction of nonresponding newly diagnosed patients and the high probability of relapse after treatment with bortezomib.[51c,52] While primary resistance to bortezomib is conveyed, for example, by elevated concentrations of heat shock protein Hsp 27,[53] the reason for nonresponsiveness acquired during therapy is currently a matter of debate. Exposing cell cultures to stepwise increasing bortezomib concentrations and subsequently analyzing their DNA, led to the identification of a number of adaptive mutations, such as Met45Val, Met45Ile, Ala49Thr, Ala49Val, Cys52Phe, and Cys63Phe, in the β5c substrate binding channel.[49e,54] Molecular modeling studies suggest that these mutations impair the affinity of bortezomib for the active site and, thus, lead to reduced inhibition and therapeutic efficacy.[54c] Along with the reported mutations in subunit β5c, a reduction in the catalytic activity, which is proposed to be overcome by increased expression levels of subunit β5, is often described.[54c] Interestingly, mutant β5c subunits were found in vitro in malignant cells from various tissues, but the clinical relevance of these mutations is still lacking, since so far none of the reported mutations have been detected in specimens from bortezomib-resistant patients. Moreover, these mutations in the β5c subunits seem to confer cross-resistance to other classes of inhibitory compounds, such as peptide aldehydes and epoxyketones.[49e,55] However, it remains controversial whether acquired resistance to bortezomib is also mediated by multidrug-resistance (MDR) transporters such as P-glycoprotein or the MDR-related protein 1.[49e,56] Ultimately, the propensity of bortezomib to kill nontumor cells and the resulting toxicities frequently require a reduction of the dosage or termination of the therapy and, hence, the development of second generation proteasome inhibitors is of great demand.

4.2. Second Generation Proteasome Inhibitors

4.2.1. Next Generation Boronate Inhibitors

Currently, there are two novel peptide boronic acids under investigation, namely delanzomib (CEP-18770; Cephalon; Figure 2a: compound 2)[57] and MLN9708 (Millenium Pharmaceticals), a prodrug which is hydrolyzed to the bioactive compound MLN2238 upon administration (Figure 2a: compound 3).[58] Both candidates display improved pharmacokinetics and -dynamics as well as enhanced anti-

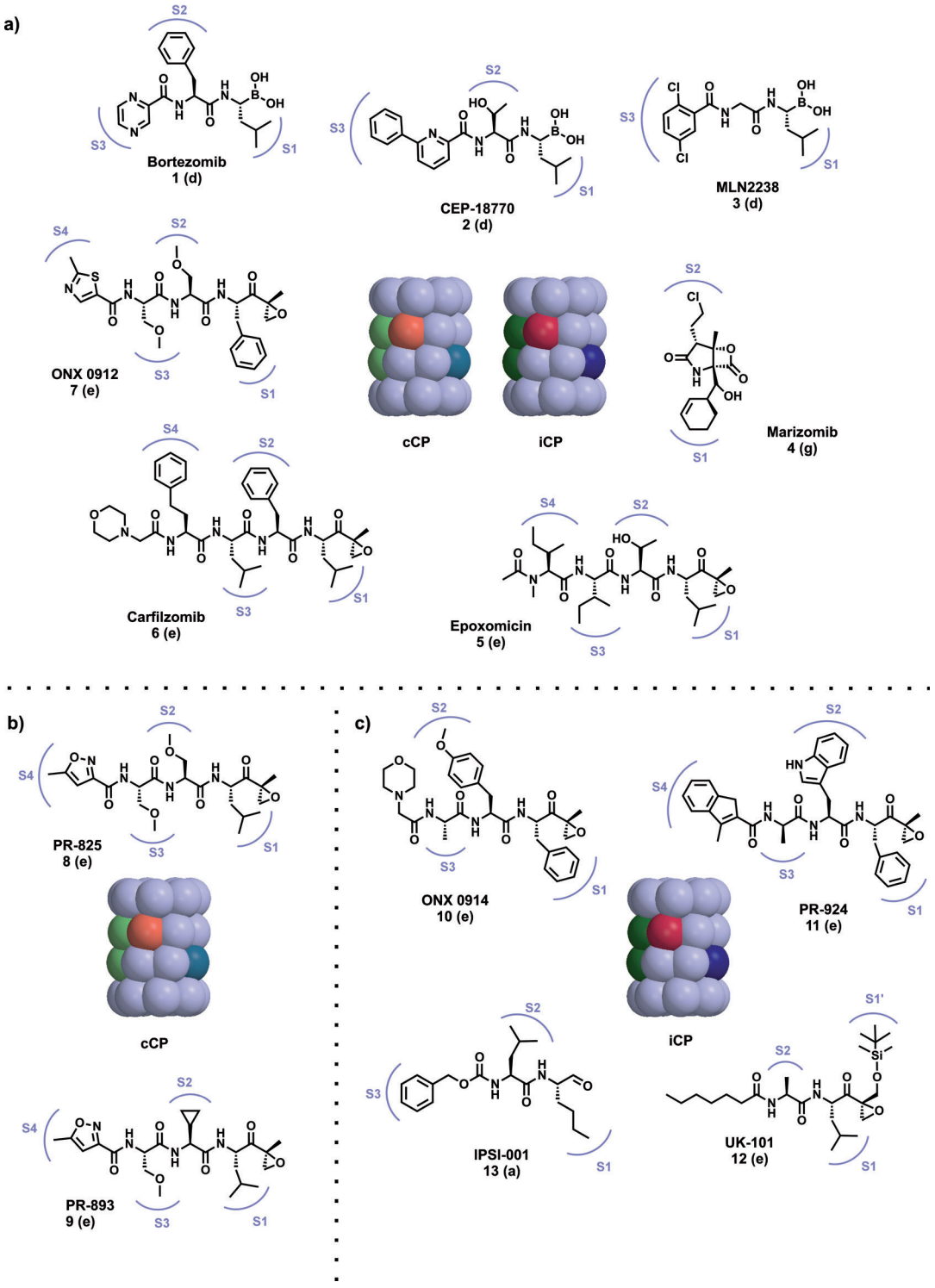


Figure 2. Proteasome inhibitors and their targeted CP. The chemical structures of prominent inhibitory compounds are numbered from 1 to 13; letters in brackets refer to the reaction mechanisms shown in Scheme 1. The specificity pockets of the proteasomal substrate binding channel that are occupied by the ligand side chains are indicated in gray and termed S1–S4 according to standard nomenclature. a) Compounds that inhibit cCP and iCP equally. b) Proteasome inhibitors specific for cCPs. c) iCP-selective compounds for the subunits β1i and β5i. Their subunit-specificity can be deduced from the IC_{50} values given in Table 1.

tumor activity compared to bortezomib, and are currently under investigation for hematologic and solid tumors.[58] The fact that CEP-18770 and MLN9708 can be administered orally is an advantage over bortezomib.[57,58] Interestingly, MLN2238 has a higher dissociation rate than bortezomib and, thus, is more widely spread throughout the body. However, it remains to be elucidated if these second generation boronates cause less adverse effects and if they are able to overcome

bortezomib resistance. As treatment with proteasome inhibitors also abrogates DNA repair, polytherapy with DNA-damaging substances may represent an effective alternative to deal with bortezomib resistance.[59]

4.2.2. Carfilzomib and ONX 0912

For a long time boronates were considered as highly specific compounds, but prolonged treatment with bortezomib revealed several disadvantages (see Section 4.1). These unwanted side effects encouraged academia and pharmaceutical industry to develop new but equipotent proteasome inhibitors with less off-target activity. One of the most promising drug candidates is carfilzomib (PR-171; Onyx Pharmaceuticals; Figure 2a: compound 6), a tetrapeptide derivative of the natural product epoxomicin (Figure 2a: compound 5),[60] which targets the subunits β5c and β5i of the proteasome with much more subunit-selectivity than bortezomib (Table 1).[42b] In agreement with the crystal structures of the cCP and iCP, carfilzomib slightly favors subunit β5c over β5i, as it bears a leucine side chain in P1, which preferentially binds to the S1 pocket of subunit β5c.[7] However, IC_{50} values cannot be deduced from the P1 residue alone, since the P2, P3, and P4 side chains also significantly contribute to the affinity of a peptide ligand.

As a result of its ability to induce cell death in a number of tumor cell lines, carfilzomib is currently being evaluated in clinical trials for the treatment of multiple myeloma (Phase III) and solid tumors (Phase I). Similar to bortezomib, the dose-limiting effects in carfilzomib therapy are neutropenia and thrombocytopenia, but carfilzomib does not induce peripheral neurotoxicity.[49d] The α′,β′-epoxyketone head group of carfilzomib results in higher specificity for the proteasome and less off-target effects than bortezomib.[50c] Since modification of proteasomal active sites by epoxomicin analogues follows a unique binding mechanism that results in the formation of a morpholine ring system (Scheme 1e),[37b] carfilzomib and other epoxyketones are selective for Ntn hydrolases. Hence, peripheral neuropathy is not generally associated with proteasomal inhibition as previously thought, but rather with the unselective reaction mechanism of bortezomib.[50c] Contradictory results exist on the ability of epoxyketone inhibitors such as carfilzomib, ONX 0912 (see below), and ONX 0914 (see Section 4.3.3) to overcome bortezomib resistance.[53b,55,61] Additionally, speculations arose that epoxyketone inhibitors may be affected by drug efflux mediated by the ATP binding cassette transporter.[55,62] Known drawbacks of carfilzomib are its poor oral bioavailability, which necessitates intravenous administration, as well as its rather short half-life of less than 30 minutes.[45a]

To address these disadvantages structure–affinity studies were performed, which led to the design of an orally available tripeptide epoxyketone inhibitor termed ONX 0912 (PR-047; Figure 2a: compound 7).[63] ONX 0912 is now being evaluated in Phase I clinical trials as monotherapy for solid tumors and hematologic malignancies, and the first results indicate that ONX 0912 is as potent as carfilzomib at inducing apoptosis in myeloma cells.[63b] According to the crystal structures of the cCP and iCP, ONX 0912, with its bulky phenyl side chain as the P1 residue, should preferentially bind to subunit β5i.[7] However, ONX 0912 is reported to target the subunits β5c and β5i with IC_{50} values of 36 nM and 82 nM, respectively.[63a] Since the S3 pocket of the β5i substrate binding channel is smaller than the β5c counterpart, it might create a steric barrier to the P3 Ser(OMe) group of ONX 0912.[7] In contrast, the P3 Ser(OMe) side chain perfectly fits into the S3 pocket of subunit β5c. This illustrates that the selectivity and affinity of inhibitors represent the sum of favorable and unfavorable interactions of all of their P sites with the proteasomal substrate binding pockets.

4.2.3. Marizomib

A further promising proteasome inhibitor currently being tested in clinical phase studies is the natural compound marizomib (NPI-0052; Salinosporamide A; Figure 2a: compound 4), developed by Nereus Pharmaceuticals.[39,65] The secondary metabolite from the marine actinomycete *Salinispora tropica* represents the smallest proteasome inhibitor known to date. The unique chemical structure of marizomib leads to a more sustained inhibition of the proteasome (predominantly of the subunits β2 and β5) compared to other β-lactones (Table 1)[40a] and, thus, more probably to apoptosis. Currently, marizomib shows promise in bortezomib-resistant cell lines and in Phase I clinical trials for the treatment of multiple myeloma as well as leukaemia and solid tumors.[15b,65b,66] Even though oral and intravenous application of marizomib is well-tolerated, its rather short half-life of less than five minutes and its ability to penetrate the blood–brain barrier may limit its therapeutic application.[49d,67]

So far, marizomib has not been explored for the inhibition of the iCP, but in agreement with the crystal structures of cCP and iCP, engineered derivatives of omuralide and marizomib with a phenyl moiety in P1 have a significantly reduced affinity toward the ChTL activity of yCP and cCP compared to the natural products.[68] Therefore, testing these derivatives for their affinity toward the iCP might reveal interesting new results. As peptidomimetics are associated with many poor pharmacological characteristics, marizomib (the only non-peptide-based inhibitor currently in clinical trials) in particular could occupy a niche by showing significantly different pharmacokinetic and -dynamic properties.

4.3. Subunit-Specific Inhibitors

4.3.1. β2i-/β2c-Selective Compounds

The selective inhibition of the TL activity of CPs by epoxyketone inhibitors has been reported only recently.[43] Although these compounds alone do not exert antitumor activity, they were shown to sensitize multiple myeloma cells to exposure to bortezomib and carfilzomib. Remarkably, co-inhibition of the ChTL and TL active sites appears to be more cytotoxic than simultaneous inhibition of the ChTL and CL activities.[43] So far, selective inhibitors for the TL activities of cCPs and iCPs are not available, as the high structural similarity of the β2i and β2c substrate binding channels[7] makes it rather difficult to develop compounds specific for

one of the TL active sites. In addition, the exact biological impact of the subunits β2c and β2i is still elusive, in particular the reason for the exchange of subunit β2c by β2i in iCPs, the only catalytically active iCP subunit that is not encoded in the MHC-class I cluster region.[7] Despite the so far unknown physiological role of β2 subunits, β2i-knockout mice were shown, similar to β5i-knockout mice, to be protected from DSS-induced colitis,[16a] thus suggesting also a potential therapeutic benefit from inhibition of the catalytic activity of subunit β2i. However, further studies on β2-selective compounds have to be carried out to clarify the physiological role of β2 subunits in general and to assess the therapeutic benefit from their inhibition in more detail.

4.3.2. *β1i-/β1c-Selective Compounds*

UK-101, the chemical structure of which is based on dihydroeponemycin, was the first reported β1i-specific inhibitor (Figure 2c: compound 12).[21b] Interestingly, UK-101 potently induces cell death in prostate tumor cells that overexpress the immunosubunit β1i,[21b] because its linear hydrophobic heptanoic tail at the P3-position conveys β1i-selectivity.[69] Modeling studies on the binding of UK-101 to the cCP and a simulated iCP revealed that its linear hydrocarbon group fits perfectly into the β1i–β2i interface that forms the S3 pocket of the β1i active site.[70] In agreement with the iCP structure, the β1i–S1 pocket accommodates the leucine side chain. Further modeling studies indicated that the *tert*-butyldimethylsilyl group in position P1′ of UK-101 does not interact with the protein surroundings.[70]

More recently, a second β1i-specific inhibitor, IPSI-001, has been reported. This compound, also known as calpeptin, is a peptide aldehyde inhibitor that solely targets the β1i subunit of the iCP with 100-fold binding selectivity over β1c (Figure 2c: compound 13; Table 1).[44a] In agreement with structural results and previous reports that C-terminal hydrophobic amino acids lead to high selectivity for the β1i subunit of the iCP, IPSI-001 has a carbobenzoxy-leucyl-norleucinal (Z-L-nL-CHO) structure. However, IPSI-001 also targets the ChTL activity of the iCP, with even a lower K_i value than for the BrAAP activity (Table 1).[44a] IPSI-001 was shown to induce apoptosis in haematological malignancies, which express elevated iCP levels, as well as in bortezomib-resistant cells, and appears to be less cytotoxic to nonmalignant cells.[30,44a] However, in 2009, Parlati et al. claimed that the observed anticancer effect of IPSI-001 does not result from selective inhibition of subunit β1i, as IPSI-001 was used for these experiments in concentrations that also target the subunits β5c and β5i.[42b] The well-known cross-reactivity of aldehydes towards cysteine proteases such as calpain I and II cannot yet be excluded from additionally contributing to the cytotoxic effects of IPSI-001.[31] With respect to the low metabolic stability of aldehydes, fusion of the peptide portion of IPSI-001 to an irreversibly acting functional head group may enhance its potency for the proteasome and prevent unwanted co-inhibition of other enzymes, but might also reduce its subunit selectivity.[44a]

4.3.3. *β5i-/β5c-Selective Compounds*

ONX 0914 (PR-957; Figure 2c: compound 10), the first β5i-selective compound developed, is an irreversibly acting α′,β′-epoxyketone. By selectively targeting the iCP with high affinity (Table 1), it lowers the production of proinflammatory cytokines such as interleukin-6 (IL-6), IL-23, and TNF.[6a] In conclusion, the differentiation of T_H1 and T_H17 cells, which are associated with autoimmune diseases, is impaired, while the development of regulatory T cells is not affected.[4a,6a] Moreover, ONX 0914 reduces the expression of MHC-I receptors on the surface to 50% as well as the generation of β5i-dependent MHC-I peptides, thus modulating cytotoxic T-cell responses.[6a] ONX 0914 has so far been shown to successfully prevent the progression of inflammatory disorders, such as rheumatoid arthritis, experimental colitis, and systemic lupus erythematosus.[6a,16] In contrast to unselective inhibitors, ONX 0914 is therapeutically effective at concentrations far below the maximum tolerated dose and is even more potent than the soluble TNF-α antagonist etanercept, which is in use for the treatment of multiple autoimmune disorders.[6a] As a consequence of its exquisite therapeutic effects in mouse models, ONX 0914 rapidly translated into preclinical studies, which are currently ongoing.

The molecular reason for the β5i-selectivity of ONX 0914 has recently been elucidated by crystallographic studies.[7] These X-ray structures reveal that subunit β5i harbors a more spacious S1 pocket than β5c because of the different orientation of Met45. In contrast to subunit β5i, the covalent reaction of ONX 0914 with the Thr1O^{γ} residue of subunit β5c requires the dislocation of the side chain of Met45. The rotation of Met45 causes the reorientation of Ile35 and triggers additional major structural changes, thereby resulting in an offset of the protein backbone by up to 1.7 Å between the amino acids 34 and 76. As Met45 of subunit β5c projects into the S1 pocket of the substrate binding channel, it sterically hinders the docking of the phenyl P1 residue of ONX 0914 or any other aromatic side chain to the active site.[7] This is in agreement with studies on engineered derivatives of β-lactone inhibitors with a bulky and rigid phenyl moiety in P1, which were reported to exert notably reduced inhibitory potency towards the ChTL activity of yCP and cCP compared to the natural product.[68] Further support is provided by bortezomib variants with a *m*-CF_3-Phe group as the P1 residue displaying a 220-fold elevated IC_{50} value for the human cCP.[71]

Additional investigations of the reaction mechanism of epoxyketone inhibitors indicate that the initial binding step of ONX 0914 to the proteasomal active site involves only the reactive functional group and the P1 side chain of the ligand.[7] These observations highlight the importance of the interactions between the P1 site of the ligand and the S1 pocket of the substrate binding channel, and are in line with non-peptidic proteasome inhibitors such as marizomib, which solely target the S1 pocket of the substrate binding channel.

As deduced from the crystal structures of cCP and iCP, compounds with a small hydrophobic P1 residue and a large apolar side chain in P3 should favor subunit β5c, whereas β5i-selective agents preferentially harbor an aromatic P1 side

chain and a tiny, more polar P3 site.[7] These structural requirements concur with the structures of the selective fluorogenic substrates Ac-WLA-AMC and Ac-ANW-AMC for the subunits β5c and β5i, respectively.[9,71] In terms of the anti-inflammatory activity of ONX 0914 and the active site architecture of subunit β5i, cytokine synthesis regulating factors might be selectively processed by β5i-mediated cleavage at the C-terminal side of bulky hydrophobic residues.[6a,7]

Apart from ONX 0914, PR-924 (Figure 2c: compound 11) is another β5i-selective epoxyketone inhibitor developed by Onyx Pharmaceuticals.[42b] In contrast to previous data from Parlati et al., who in 2009 demonstrated that neither β5c- nor β5i-selective compounds have antitumor activity,[42b] PR-924 was recently reported to selectively cause cell death of human multiple myeloma cell lines.[44b] However, PR-924 has not yet entered clinical trials. In addition to β5i-selective agents, two β5c-specific inhibitors have so far been reported: PR-825[6a] (Figure 2b: compound 8) and PR-893[42b] (Figure 2b; compound 9), which are both epoxomicin-analogues.[60a] Except for their P2 side chains, the chemical structures of PR-825 and PR-893 are identical, thus giving rise to similar subunit specificity and potency (Table 1). Consistent with the features of the β5c and β5i subunits described in this section, leucine in P1 and Ser(OMe) in P3 confer selectivity for subunit β5c. PR-825 and PR-893 were used as controls for experiments with ONX 0914 and carfilzomib.[6a,42b] These studies show that PR-825, in contrast to ONX 0914, has neither anti-inflammatory activity nor therapeutic potential in autoimmune diseases.[6a] In agreement, PR-893 cannot provoke apoptosis in multiple myeloma cells, whereas concomitant inhibition of the subunits β5c and β5i by carfilzomib causes anti-neoplastic effects.[42b] β5c-selective compounds still appear to have no medicinal indication, but inhibitors selective for the iCP are promising drug candidates, which might gain future FDA approval.

5. Future Prospects

On the basis of a sequence identity of more than 90% between mouse and human proteasome subunits, the available structural information on the murine cCP and iCP will support the design of novel specific and selective inhibitory compounds by enabling molecular modeling studies and ligand docking simulations.

After years of negligence, β2 subunits of cCPs and iCPs have now been discovered to be potential drug targets.[43] However, the physiological difference between the structurally similar subunits β2c and β2i has not yet been identified and requires further experimental studies. Two selective inhibitors have been characterized for subunit β1i, but further developments toward more selectivity and potency are necessary.[21b,44a] This will be facilitated by the knowledge of the substrate binding channel architectures of subunit β1c, which primarily accommodates negatively charged side chains in the S1 pocket, and β1i, which favors nonpolar branched moieties in position P1 and small, preferentially polar groups in P3.[7]

Current experimental results indicate that β5i-selective inhibitors have a huge therapeutic potential for the treatment of autoimmune diseases. Apart from iCP-selective agents, future investigations will focus on the development of compounds specific for tCPs, which appear to be crucial for the development of T-cells.[2b] Additionally, proteasome inhibitors in general might also be explored in more detail for other therapeutic applications, such as prevention of allo- and xenograft rejection.

Although known proteasome inhibitors have a huge inhibitory and therapeutic potential, most of them have to be ameliorated in terms of stability, half-life, bioavailability, clearance rates, convenience of application, efficacy, safety, and toxicity profiles. In this regard, medicinal chemists might consider the recently investigated α-ketoaldehyde pharmacophore.[37a,c,38] As the reversibly acting α-ketoaldehydes, similar to α′,β′-epoxyketones, exploit the unique catalytic mechanism of the proteasome Thr1, they might be promising candidates for future drug developments. With the exception of bortezomib, inhibitors with a reversible mode of action are expected to exhibit less undesired side effects than other agents. Thus, future drug development efforts will also focus on noncovalently acting compounds. Since these inhibitors do not form an acyl–enzyme complex with Thr1O^{γ}, their affinity solely depends on their entropic and enthalpic stabilization in the substrate binding channel, and their selectivity and specificity is gained by rigidity and optimized interactions with the substrate binding pockets. The chemical structures of formerly investigated compounds, such as the natural TMC-95 cyclic peptides,[72] decarboxylated peptides,[73] or hydroxyureas,[74] might serve as starting points for the design of iCP-selective noncovalent inhibitors. However, it remains to be seen whether these or other agents can overcome the drawbacks of compounds currently being applied or investigated. In conclusion, the herein presented status quo offers the possibility of many further interesting stories on proteasome inhibition and its clinical application.

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