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NMR-Bioassay Guided Isolation of the Natural 20S Proteasome Inhibitors from Photorhabdus Luminescens

A Novel NMR-Tool for Natural Product Detection

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Martin Lorenz Stein

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A Novel NMR-Tool for Natural Product Detection

Doctoral Thesis accepted by
Technical University of Munich, Germany

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ISSN 2190-5053 ISSN 2190-5061 (electronic)
ISBN 978-3-319-07913-4 ISBN 978-3-319-07914-1 (eBook)
DOI 10.1007/978-3-319-07914-1
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014941117

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Parts of this thesis have been published in the following journal articles:

Modulating the reactivity of the proteasome using a chimeric enone inhibitor based on the natural products carmaphycin and syringolin.
Trivella D, Pereira A, Stein ML, Byrum T, Kasai Y, Valeriote F, Tantillo D, Groll M Gerwick W, (**2014**) Moore B. Chem. Biol. *In press*

Systematic comparison of peptidic proteasome inhibitors highlights the α-ketoamide electrophile as an auspicious reversible lead motive.
Stein ML, Cui H, Beck P, Dubiella C, Voss C, Krüger S, Schmidt B, Groll M, (**2014**) Angew. Chem. Int. Ed. 3;53(6):1679–83

Applied techniques for mining natural proteasome inhibitors.
Stein ML, Groll M (**2014**), Biochem. Biophys. Acta. 1843(1):26–38

One-shot NMR analysis of microbial secretions identifies highly potent proteasome inhibitor.
Stein ML, Beck P, Kaiser M, Dudler R, Becker CF, Groll M (**2012**). Proc. Natl. Acad. Sci. 109, 18367–71

Activity enhancement of the synthetic syrbactin proteasome inhibitor hybrid and biological evaluation in tumor cells.
Archer CR, Groll ML, Stein ML, Schellenberg B, Clerc J, Kaiser M, Kondratyuk TP, Pezzuto JM, Dudler R, Bachmann AS (**2012**). Biochemistry. 51, 6880–8

Elucidation of the α-keto-aldehyde binding mechanism: a lead structure motif for proteasome inhibition.
Gräwert MA, Gallastegui N, Stein ML, Schmidt B, Kloetzel PM, Huber R, Groll M. Angew. Chem. Int. Ed. (**2011**) 10;50(2):542–4.

The work in this thesis was conducted from August 2010 fo December 2013 under the supervision of Prof. Dr. Michael Groll, Chair of Biochemistry, TUM.

To my family

Supervisor's Foreword

The ubiquitin-proteasome system is of fundamental importance to many cellular pathways and is nowadays an established target for the treatment of malignant blood tumors in the pharmaceutical industry. Furthermore, the development of the first immunoproteasome-specific inhibitors has recently disclosed the vast field of immunosuppressive therapies. The numerous future applications of proteasome inhibitors, however, decisively depend on the identification of highly selective small molecule elicitors that modulate the proteolytic reactions of the various proteasomal active sites. The release of carfilzomib, the first natural-product-based-compound, has impressively demonstrated the advantages of these evolutionarily optimized compounds by a vastly reduced record of side effects compared to the first FDA-approved and fully synthetic inhibitor bortezomib. The identification of further lead structures is therefore of vital importance to answer the urging need for new compounds in the oncological and immunological sectors.

The outstanding Ph.D. thesis of Dr. Martin Lorenz Stein describes the establishment of a novel methodology to detect such bioactive natural products from the highly heterogeneous matrices present in raw organic extracts or culture broths. Compared with previously applied screening techniques, the developed tool is highly robust and produces unambiguous and reproducible readout. Moreover, due to the standard preparation of the peptidic substrate and the straightforward execution of the method, it is accessible to a broad scientific community. These features allow both the analysis of samples produced by standard protocols as well as the screening of individual environmental growth conditions during fermentation. Due to the silencing of many biosynthesis assembly lines of natural products in microorganisms, this is vital to determine a suitable molecular trigger to initiate biosynthesis and secretion of highly toxic, but likewise interesting secondary metabolites. The present work depicts the identification and isolation of applicable syrbactin proteasome inhibitors from the bacterium *Photorhabdus luminescens*. Noteworthy, it was shown that the secretion of these natural products is strictly regulated and thus only gets induced by a defined and adequate environmental habitat, dependent on the partially symbiotic life cycle of the producing bacteria. However, even after inducing the pathogenic phase, the syrbactins are produced in trace amounts, which is typical for toxic secondary metabolites. As a consequence, the isolation of the secondary metabolites was

only feasible by the application of the newly developed NMR-technique both in the screening and isolation approaches.

With regard to the rich assortment of natural proteasome inhibitors available nowadays, the new tool will pave the way to identify even more and diverse molecules in future screening approaches. It has again proven its strengths in the first subsequent studies in our lab by the identification of promising organisms for further investigations. Hereby, it forms the basis of all analytical steps during our robot-assisted automated screenings and is applied for the bioassay-guided isolation of the active compounds. Hence, the transferability of the assay principle to many other target enzymes will certainly stimulate the research on natural products and their distinct implementations in the near future.

Garching, March 2014 Prof. Michael Groll

Acknowledgments

First of all, I would like to thank my supervisor, Prof. Michael Groll, for giving me the opportunity to write my Ph.D. thesis at his chair and for his tremendous support during these 3 years. The highly interesting topics together with his fierce enthusiasm for even the slightest progress made me bear even the times when my projects did not proceed as desired. His positive leadership has encouraged me to enjoy even challenging experiments and helped the whole team to strive even harder. Eventually, his ingenious ideas and perseverance let me achieve the high goals during this thesis.

I am also especially grateful for the support of Prof. Robert Dudler, University of Zürich, and Prof. Christian F. W. Becker, University of Wien. Both did not only supply crucial material for the establishment of the NMR assay, but constructively supported my project during all stages.

Furthermore, I would like to acknowledge PD. Dr. Wolfgang Heinemeyer for his advice and support in yeast genetics. He is a true expert and I would argue that there are not many scientists in this field who match his practical and theoretical competence. Due to his experience and calm character he was always one of my first contact persons for difficult questions.

I strongly want to thank Richard Feicht for his tremendous effort in proteasome isolation and his advice to all questions concerning protein purification. I have never met anyone with comparable and sometimes seemingly supernatural skills with regard to native protein purifications. His steady supply with crystals and material has facilitated the work of everyone in the team working on the proteasome

A special thank you goes to all members of the Groll group. I have always enjoyed the friendly atmosphere in our lab and often laughed heartily about the funny moments during our everyday work. Our synchrotron trips, which were often extraordinarily exhausting, were only bearable due to our mutual personal and professional appreciation that in some cases developed into friendships. I will never forget the legendary moments during our shifts.

Last but not least, I would like to thank my family for enabling my studies and their interest and personal support for my work. I doubt that I would have achieved this high goal without them.

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Abbreviations

AAA	ATPases associated with diverse cellular activities
ACN	Acetonitrile
AMC	7-Amino-4-methyl-coumarin
APS	Ammonium persulfate
BODIPY	Boron-dipyrromethene
BSA	Bull serum albumin
CD8	Cluster of differentiation 8
Cdk	Cyclin dependent kinase
ChTL	Chymotrypsin-like
CL	Caspase-like
COSY	Correlated spectroscopy
cCP	Constitutive 20S proteasome
CP	Core particle
DCM	Dichloromethylene
DIPEA	*N,N*-Diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
ECL	Electrochimiluminescence
EDTA	Ethylenediaminetetraacetic acid
ESI	Electron spray ionization
FBS	Fetal Bovine Serum
Fmoc	Fluorenylmethyloxycarbonyl
GADPH	Glycerinaldehyd-3-phosphat Dehydrogenase
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate
HMBC	Heteronuclear multiple bond correlation spectroscopy
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single bond correlation spectroscopy
iCP	20S immunoproteasome
MeOH	Methanol
MDa	Mega Dalton
MHC-I	Major histocompatibility complex I

MPD	2-methyl-2, 4-pentanediol
NMR	Nuclear magnetic resonance (spectroscopy)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDB	Potatoe dextrose broth
pNA	Para-naphtylamine
PTFE	Polytetrafluoroethylene
RP-C18	Reversed Phase capped with linear C18 alkyl chains
SDS	Sodium dodecyl sulfate
tCP	20S thymo proteasome
TEMED	Tetra methylethylenediamine
TB	Terrific broth
TBS	Tris buffered saline
TFA	Trifluoro acetic acid
TL	Trypsin-like
Tris	Tris(hydroxymethyl)aminomethane
Trt	Triphenylmethyl
TWEEN 20	Polysorbate 20
Ub	Ubiquitin
WT	Wild type

Chapter 1
Introduction

1.1 The Ubiquitin-Proteasome-System

The ubiquitin-proteasome system (UPS) is responsible for the directed disposal of unfolded, misfolded and short-lived proteins in eukaryotic cells and is therefore a key player in protein homeostasis. Moreover, it participates in crucial biological pathways by the degradation of signaling factors such as cyclins or the tumor suppressor p53. Eventually, not only the degradation of substrate proteins, but also the generation of peptide fragments is exploited in vertebrates for MHC-I epitope presentation and subsequent T-cell activation. Due to the involvement in these vital processes, the UPS is linked to diseases as diverse as cancer, autoimmunity or neurodegeneration and has been in the limelight of pharmaceutical industry for the last decade. Being an evolutionarily conserved target, many bacteria and fungi have developed secondary metabolites that attenuate the activity of their host's or competitor's UPS. Due to their gradual optimization over millions of years, these compounds are highly potent and versatile in terms of binding mechanism, target specificity and bioavailability, which makes them a treasure trove for the rational design of new agents. Although several classes of natural proteasome inhibitors have been discovered, the identification of novel lead structures is an ongoing challenge especially with regard to the discrepancy between the exploited applications of the commercially available drugs and their potential scope for various indications.

1.1.1 Ubiquitinylation

Similar to small molecule metabolites, proteins are in a permanent dynamic equilibrium that is determined by their ribosomal synthesis rate and their degradation by proteases such as cathepsins or the proteasome [1, 2]. Thus, the rates for their (re-)formation and destruction can be used as a control system for various processes just like transcriptional and translational regulation, which is exemplified

M. L. Stein, *NMR-Bioassay Guided Isolation of the Natural 20S Proteasome Inhibitors from Photorhabdus Luminescens*, Springer Theses, DOI: 10.1007/978-3-319-07914-1_1,
© Springer International Publishing Switzerland 2014

by the periodically fluctuating levels of cyclins during the cell cycle [3]. Generally, the intracellular stability of a random protein in pro- and eukaryotes can be evaluated by the so-called N-end rule [4]. It defines degradation signals, whose detrimental effects accumulate to curtail a protein's half-life time. Apart from hydrophobic patches, distinct N-terminal amino acids such as lysine and arginine lead to an accelerated recognition by the respective degradation system [5].

In eukaryotic cells, the ubiquitin-proteasome system (UPS) represents the main non-lysosomal pathway for the selective decomposition of proteins [3]. It is structured into two successive processes, the covalent ubiquitinylation of target proteins and their subsequent break down by the 26S proteasome to defined cleavage products [6].

Ubiquitin (Ub), a comparatively small 8.5 kDa protein, can be posttranslationally attached by an isopeptide bond to the ε-amine moiety of surface-exposed lysine residues via its free glycine C-terminus [7]. Further Ub molecules can then be linked in a similar way to any of its seven lysine residues, thereby generating a great variety of polyubiquitinylated products [8]. Although often referred to as a black spot that dooms a protein to degradation, only K-48 linked Ub chains of at least 4 molecules definitely lead to proteasomal digestion, while other configurations have been shown to rather convey a translocational information or contribute to signaling [9, 10]. The overall ubiquitinylation of the proteome is therefore an exceptionally dynamic process that has been compared in its complexity and versatility with other post-translational modification like phosphorylation or glycosylation [8]. It is controlled by a highly specific system of ubiquitin ligases on the one hand and deubiquitinating enzymes on the other, thereby representing an entire posttranslational level of regulation [4, 11].

The biochemical reaction of ubiquitinylation includes three different classes of enzymes denominated as E1 (Ub-activating enzymes), E2 (Ub-conjugating enzymes) and E3 (Ub-ligating enzymes), which successively activate, transmit, and tag the Ub molecule to a substrate protein (Fig. 1.1) [11]. Resembling a pyramid scheme reaction setup, there exist only two rather promiscuous E1 enzymes in the human proteome that interact with around 50 E2 proteins, which in turn bind to approximately 1,000 E3 ligases [13, 14]. Due to the high degree of substrate specificity of the latter, their number also reflects the vast amount of proteins that are thus labeled for proteolytic degradation.

In detail, Ub is ATP-dependently activated by the E1 enzyme to form a thermodynamically rich Ub-E1 thioester intermediate [19]. Subsequently, E1 hands over its Ub moiety to an E2 protein [20], where it is again covalently bound as a thioester. Eventually, the substrate protein is recognized by the Ub ligase, which transfers the Ub molecule from E2 to the target protein [18]. Repeated cycles of this process finally yield isopeptide-bridged polyubiquitin chains that can be recognized by the 19S regulatory particle of the 26S proteasome [21].

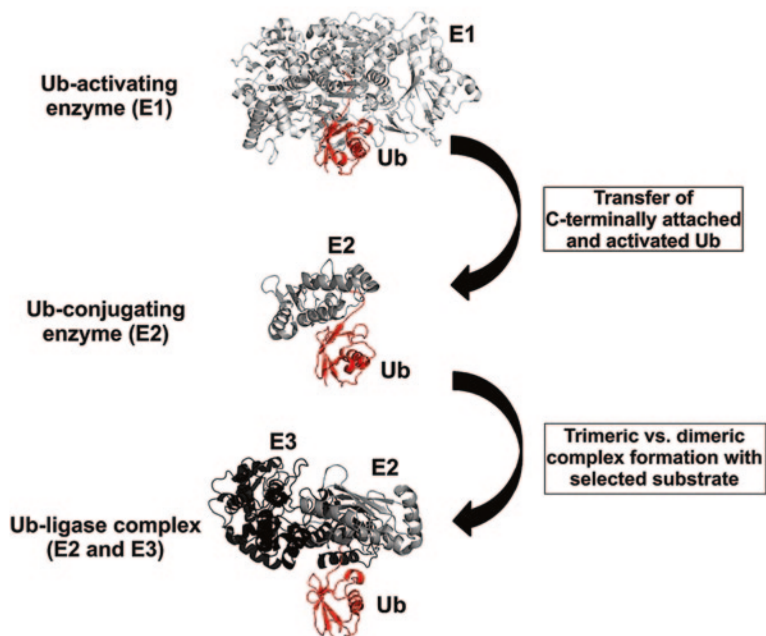

Fig. 1.1 Ubiquitinylation involves three enzymes in a cascade-like reaction setup, which activate (E1, *upper*) [15], hand over (E2) [16] and ligate (E3) [17] an Ub molecule to a growing chain attached at a suitable substrate molecule. Substrate recognition can be achieved by a E3 alone or in complex with the E2 enzyme [18]. Figure adapted from Stein and Groll [12]

1.1.2 The 26S Proteasome

This huge 2.6 MDa heteroprotein complex is composed of the catalytically active 20S proteasome (core particle; CP) and the 19S regulatory particle (RP) [22, 23]. After the detection of suitably Ub-tagged substrates, the RP removes the Ub molecules and simultaneously unfolds and threads the substrate into the proteolytic chamber of the CP [23, 24]. Although the inherent flexibility of the RP has hampered attempts of crystallization to date, recent biochemical, bioinformatical and largely electron microscopical analyses have contributed to understand the subunit location, the precise function and dynamics of this macromolecular machinery [21, 25, 26]. Gating the entry into the proteolytic CP, the RPs are perched on both lateral sides of the barrel-shaped 20S proteasome (Fig. 1.2). Due to their ability to hydrolyze ATP, the 19 different proteins that build up the RP were classified into Regulatory particle ATPases (Rpt) and Regulatory particle non-ATPases (Rpn)

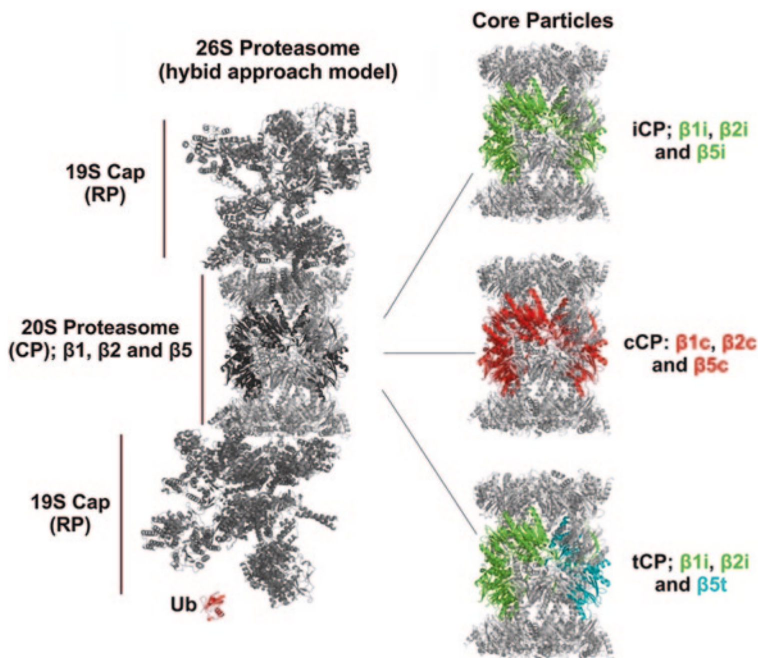

Fig. 1.2 The 26S proteasome with an approximate molecular weight of 2.6 MDa is huge compared to the small Ub tag (*red*) of marked substrate proteins. Vertebrates have developed subspecies of the central catalytic machinery residing in the CP. In these particles, the proteolytic subunits are exchanged to reshuffle the cleavage activities and hence adapt to the physiological requirements. Electron microscopical model received from Morris [26]. Figure adapted from Stein and Groll [12]

subunits [27]. Furthermore, they were historically assigned to the modules "base" and "lid" due to biochemical association studies and their assumed location in the macroscopic subunits present in low resolution electron microscopy data [27]. However, the lid has meanwhile been shown to not be located top of the RP but rather on its side [28], with contact areas even to the CP subunits. Making the base and lid categories even more superfluous, subunits that had been assigned to the base, such as Rpn10 or Rpn13, are situated at the far side of the RP [25, 27, 29].

These two Ub-binding receptors are responsible for the selective docking of suitably tagged substrate proteins [30–32]. After this initial recognition step, the substrate is deubiquitinylated by the catalytically active zink-metallaprotease Rpn11 [33], which resides in the palm of the horseshoe-like setup formed by the subunits Rpn 3, 5–7, 9 and 12 [34]. Explaining the minimum Ub chain length, structural data have shown that only four Ub molecules are able to span the distance between the receptor proteins and Rpn11, which is required to prevent dissociation of the substrate from the RP [21]. Moreover, comparison of Rpn11, as well as the residual lid subunits, in the free and the bound state in complex with the remaining RP subunits revealed the regulatory mechanism of Rpn11, which is

only activated by structural rearrangements in the matured form of the RP, thereby protecting the cell from futile deubiquitinylation activity in the cytosol [29].

Subsequently, the six AAA+ ATPases Rpt1-6 [35, 36], which are arranged in a pseudohexameric spiral staircase above the outer subunits of the CP, exert tethering forces on the substrate protein [26]. Recent electron microscopical studies in complex with a substrate protein suggest that the Rpt subunits already engage with flexible and unstructured regions of the substrate before the Ub chain is cleaved off [21]. However, only after deubiquitinylation, which restores the Ub molecules for attachment to other client proteins, the Rpt subunits can gradually unfold and translocate the substrate into the hydrolytic chamber of the CP [21].

1.1.3 The Core Particle

The 20S proteasome represents the downstream end of the UPS that performs the hydrolytic digestion of unfolded protein substrates to defined cleavage products [12, 37, 38]. Unlike the highly dynamic 19S cap, the CP is a rigid body that is framed by either the RP or other adapter proteins such as PA28 and Blm10, which tune its catalytic activity (Fig. 1.2) [39–41]. Its cylinder-shaped overall structure is composed of four heptameric rings following a C_2-symmetrical $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ arrangement [42].

As high resolution data is available for both the prokaryotic and eukaryotic CP, the molecular details including the regulatory and catalytic mechanisms of this molecular shredder have been elucidated [42, 44]. The α-subunits that form the outer rings hold both structural and functional roles during assembly and in the maturated particle [22]. Apart from forming the interface for binding to adapter proteins, they seal the central longitudinal pore through the CP by a thin interdigitating network composed of their N-termini, thus protecting the cell from detrimental self-digestion [24, 44]. As the proteolytic activity is completely unspecific with regard to the substrate proteins, it must be strictly compartmented in vivo. Therefore, the gate is only unlocked if a regulator such as the RP docks onto the outer α-ring and in consequence takes over the regulatory function [24]. Hereby, three Rpt subunits containing a C-terminus with a discrete recognition sequence bind into pockets between the adjacent α-subunits α1–α2, α3–α4 and α5–α6 to trigger the movement of a lever-like structure that lifts the N-terminus of the respective α-subunit, thus breaking the network over the central pore [44, 45].

On the other hand, the β-subunits within the equatorial rings exert the catalytic activities within the CP [46]. Whereas in prokaryotic organisms all α- and β-subunits are equal in structure, primary sequence and function [42], they have evolved in nucleated cells to individual proteins in order to reflect the advanced requirements in terms of signaling and sequence specificity [43, 47]. In consequence, the human proteasome contains only three proteolytically active subunits called β1, β2 and β5 that are endowed with distinct proteolytic specificities denominated as caspase-like (CL), trypsin-like (TL) and chymotrypsin-like