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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.

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 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,

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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 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

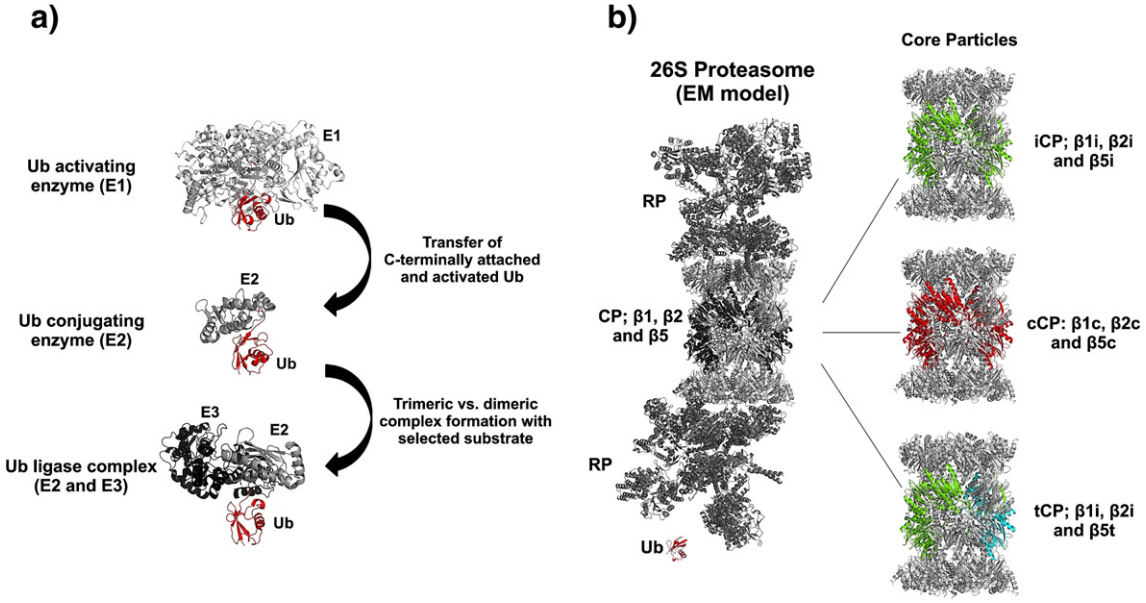

Fig. 1. The UPS pathway. A) Ubiquitinylation involves three enzymes, which activate (E1, upper) [19], hand over (E2, middle) [20] and conjugate (E3, lower) [21] the Ub molecule (red) to a cellular substrate protein. B) The 26S proteasome with a molecular mass of 2.600 kDa is huge compared to the 8.5 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).

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