Rapamycin Allosterically Inhibits the Proteasome

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ABSTRACT

Rapamycin is a canonical allosteric inhibitor of the mammalian target of rapamycin (mTOR) kinase with immunosuppressive and proapoptotic activities. We found that in vitro rapamycin also regulates the proteasome, which is an essential intracellular protease of the ubiquitin-proteasome pathway. Rapamycin inhibits proteinase and selected peptide activities of the catalytic core proteasome at low micromolar concentrations. Moreover, the drug interferes with binding of the 19S cap essential for processing of polyubiquitylated substrates and with the PA200 proteasome activator to the 20S catalytic core proteasome. These protein complexes are known to bind to specific grooves on the α face region of the 20S core. Treatment with rapamycin affects the conformational dynamics of the proteasomal gate, which is centrally positioned within the α face and allosterically regulated element responsible for the intake of substrates. We showed that rapamycin shares all the proteasome targeting properties not only with other two-domain, closed-ring analogs (rapalogs) but also with its single domain mimics and seco-rapamycin, which is the first in vivo open-ring metabolite of rapamycin that does not affect mTOR. We hypothesize that rapamycin and related compounds bind to the α face and allosterically impact proteasome function. This article discusses the implications of our findings for the mechanism of in vivo actions of rapamycin and for the design of novel allosteric drugs targeting the proteasome.

Introduction

The phenomenon of allostery, broadly defined as the coupling of conformational changes between distant sites, is one of the fundamental regulatory mechanisms of enzymatic catalysis (Goodey and Benkovic, 2008). Therefore, it comes as no surprise that allosteric ligands are rapidly gaining recognition as attractive drug candidates. In fact, allosteric inhibitors exhibit many benefits over the commonly used competitive inhibitors. They provide a much broader range of mechanisms to interfere with catalysis; they are more specific and less likely to induce drug resistance. Still, the structural and functional complexity of many enzymes poses a special challenge in finding or designing allosteric regulators. One of such enzymes is proteasome, the essential protease of the ubiquitin proteasome pathway, which plays critical regulatory and housekeeping functions in every eukaryotic cell (Ciechanover, 2012). Inhibition of proteasome leads to apoptosis, a feature already applied for cancer treatment (Adams, 2004). Several competitive proteasome inhibitors are used in humans, including two drugs approved by the Food and Drug Administration: bortezomib (Velcade; Millennium Pharmaceuticals, Inc., Cambridge, MA) and carfilzomib (Kyprolis; Onyx Pharmaceuticals, Inc., South San Francisco, CA) (Dick and Fleming, 2010). Apart from cancer, the proteasome is considered a target for anti-inflammatory drugs (Tan et al., 2006).

The proteasome is multifunctional and modular protease (Groll et al., 1997; Unno et al., 2002). Three pairs of active sites are concealed inside the tube-shaped catalytic core [core particle (CP) 20S] (Fig. 1A). The sites exhibit chymotrypsin-like (ChT-L), trypsin-like (T-L), and postacidic [postglutamyl peptide hydrolyzing (PGPH)] specificities cleaving polypeptides after hydrophobic, basic, and acidic amino acid residues, respectively. The activity of the CP is controlled by attachment of additional protein modules to the external surface on both sides of the 20S. The surface (α face) harbors the gate guarding access to the catalytic chamber and accepts the 19S regulatory particle (RP), 11S [proteasome activator/regulator (PA28/REG)], or PA200 activators (Fig. 1, B–D). A complex of the core with one or two (26S proteasome) 19S “caps” recognizes and processes substrates tagged for degradation by polyubiquitinylation (Da Fonseca et al., 2012; Huber and Groll, 2012; Lander et al., 2012; Lasker et al., 2012). Proteins

ABBREVIATIONS: AFM, atomic force microscopy; AMC, 7-amino-4-methylcoumarin; ChT-L, chymotrypsin-like or posthydrophobic residues activity; CP, core particle; DMSO, dimethylsulfoxide; FK-506, (1R,9S,12S,13R,14S,17R,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatri-cyclo[22.3.1.0³⁷]octacos-18-ene-2,3,10,16-tetrone; FKBP12, FK-binding protein 12; Hb, hydrophobic amino acid residue; MCA, 7-amido-4-methylcoumarin; mTOR, mammalian target of rapamycin; NVP-Bez235, 2-methyl-2-(4-(3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydroimidazo[4,5-c]quinolin-1-yl)phenyl)propanenitrile; PA, proteasome activator; PGPH, postglutamyl peptide hydrolyzing or postacidic residues activity; PI-103, 3-[4-(morpholin-4-yl)pyridol[2,3]furo[2,4-b]pyrimidin-2-yl]phenol; PR, Pro- and Arg-rich peptides; REG, regulator; RP, regulatory particle; Rpt, regulatory particle ATPases; Suc, succinylic acid; T-L, trypsin-like or postbasic residues activity.
On the other hand, Pro- and Arg-rich (PR) peptides hydroxyquinoline, binding inside the antechamber (Li et al., 2000). There is an example of an allosteric inhibitor, 5-amino-8-hydroxyquinoline, which can inhibit degradation of selected substrates and display antitumor activities in vitro (Gaczynska et al., 2003). In vivo, PR peptides gate and the RP-CP interactions and affect peptidase activities (Bajorek et al., 2003; Rechsteiner and Hill, 2005). However, gate opening is mediated not only by signals from the grooves on the α face but also by signals from the catalytic chamber (Whitby et al., 2000; Rabl et al., 2008; Osmulski et al., 2009). Besides gate regulation, the allosteric path between active sites and the α face is used for modulation of stability of the 26S assembly (Kleijnen et al., 2007).

Unlike competitive inhibitors, the small-molecule allosteric ligands of the proteasome are much less explored. There is an example of an allosteric inhibitor, 5-amino-8-hydroxyquinoline, binding inside the antechamber (Li et al., 2010). On the other hand, Pro- and Arg-rich (PR) peptides bind to the α face, likely on its outer edge, destabilize the gate and the RP-CP interactions and affect peptidase activities in vitro (Gaczynska et al., 2003). In vivo, PR peptides inhibit degradation of selected substrates and display anti-inflammatory and proangiogenic properties (Gao et al., 2000; Li et al., 2000).

The phenomenon of regulation of the core properties by attachment of dedicated proteins to specific allosteric sites inspired us to search for small-molecule allosteric ligands affecting protein-protein interactions. A straightforward case of such interference is provided by short peptide sequences derived from C termini of the modules, blocking the grooves and mimicking some allosteric effects of their parent proteins (Jankowska et al., 2010). The anchor peptide fragments of selected regulatory particle ATPases (Rpt) subunits of the 19S, and of PA200 activator, are equipped with the Hb-YX motif (hydrophobic amino acid-Tyr-any amino acid) (Ortega et al., 2005; Rabl et al., 2008). We hypothesized that a canonical hydrophobic allosteric ligand such as rapamycin may interfere with the docking of protein modules to the α face and may provide an alternative to the short peptides.

Indeed, rapamycin (sirolimus) is one of the oldest examples of a successful allosteric drug. This natural macrocyclic binds the FK-binding protein 12 (FKBP12) with its FKBP binding domain, induces dimerization of FKBP12 and mammalian target of rapamycin (mTOR), and inhibits the latter with its effector domain binding to the allosteric site adjacent to the kinase domain (Fig. 2) (Liang et al., 1999; Banaszynski et al., 2005). The mTOR kinase regulates translation, autophagy, response to hypoxia, and glucose metabolism (Dowling et al., 2009). Rapamycin is an immunosuppressive drug used to prevent transplant rejection. High doses of the drug are proapoptotic, and close synthetic analogs of rapamycin (rapalogs) are used as anticancer agents (Dowling et al., 2009). In addition, animal studies revealed surprisingly strong antiangiogenic effects of prolonged treatment with low doses of rapamycin (Harrison et al., 2009).

Here we report a discovery that rapamycin, rapalogs, and a rapamycin metabolite are allosteric regulators of the core proteasome. They interfere with substrate gating and with interactions between the 20S core and 19S components. The discovery opens an opportunity to design a new class of allosteric proteasome inhibitors targeting allosteric sites on the α face and possessing potential anticancer properties.

**Materials and Methods**

**Proteasome Activity Measurements.** Human purified proteasome complexes were purchased from Enzo Life Sciences, Inc. (20S and 26S; Farmingdale, NY) or Boston Biochemicals, Inc. (19S complex; Cambridge, MA). BODIPY-casein (Invitrogen/Molecular Probes (Life Technologies), Carlsbad, CA) was used as a model protein substrate, with increasing fluorescence of BODIPY-labeled peptide products of degradation monitored for up to 8 hours at 37°C. The peptidase activity of the enzyme was measured as arbitrary intensity units of the released fluorescent group 7-amino-4-methylcoumarin (AMC) from peptide substrates, as described. The common substrates specific for the three kinds of active sites, succinyl-LeuLeuValTyr-7-amido-4-methylcoumarin (Suc-LLVY-MCA; for the ChT-L activity; Bachem Bioscience, Inc., Philadelphia, PA), butoxycarbonyl-LeuArgArg-MCA (for T-L; Bachem Bioscience), and carbobenzoxy-LeuLeuGlu-MCA (for PGPH; Enzo Life Sciences, Inc.) were used at 100 μM final concentrations.
unless stated otherwise (Gaczynska and Osmulski, 2005). Everolimus, FK-506, NVP-BEZ235, rapamycin, and temsirolimus were obtained from LC Laboratories (Woburn, MA). Pimecrolimus, PI-103 and ridaforolimus were purchased from Selleck Chemicals, LLC (Houston, TX). Proteasome substrates, rapamycin, its derivatives, and competing peptides were stocked in dimethylsulfoxide (DMSO) and diluted 100-fold in the reaction mixtures. The Rpt5 and PA200 C-terminal peptides were synthesized in the Departmental Peptide Synthesis Core using standard solid-phase peptide chemistry. To activate the latent 20S proteasome, 0.005% (final concentration) of SDS was used. The reactions were carried out in 96 well plates, with 2.3 nM proteasome and other components, as indicated, dissolved in 50 mM Tris-HCl buffer (pH 8.0), and incubated at 37°C for up to 1 hour. To study reconstruction of the 26S proteasome with 20S and 19S complexes, the reaction buffer was supplemented with 2 mM ATP, 1 mM MgCl2, and 1 mM dithiothreitol. The fluorescence of released AMC was monitored every 2 minutes with a Fluoroskan Ascent plate reader (Thermo Fisher Scientific Inc., Waltham, MA). Reaction rates were calculated from smoothed linear segment of kinetic curves using OriginPro 8.6 (OriginLabs, Northampton, MA). For determination of an inhibition type, at least six distinct substrate concentrations and two inhibitor concentrations were used. The kinetic parameters of inhibition were analyzed in terms of the Michaelis-Menten formalism using the enzyme kinetic module of SigmaPlot version 12 (Systat Software, Inc, San Jose, CA) to perform the respective calculations.

Atomic Force Microscopy Imaging. Atomic force microscopy (AFM) imaging of the 20S proteasomes was performed in tapping (oscillating) mode in liquid, as previously described (Osmulski et al., 2009; Gaczynska and Osmulski, 2011). In short, 3 µl of proteasome preparations diluted to nanomolar concentration was deposited on a freshly cleaved muscovite mica surface. After 2 minutes of incubation allowing electrostatic attachment of the protein particles to mica, the droplet was overlaid with 30 µl of 50 mM Tris-HCl buffer (pH 7.0) and mounted in the wet chamber of a MultiMode NanoScope IIIa (Bruker Corporation, Billerica, MA). Oxide-sharpened silicon nitride tips on cantilevers with a nominal spring constant 0.32 N/m (Bruker Corporation) were used to image 1-μm2 fields in the height mode; scan rate was 3.05 Hz. The frequency was manually tuned to 9 to 10 kHz, with a drive voltage of 200–500 mV and a relatively high set point (1.6–1.9 V) to ensure tapping with low, nondestructive force. Trace and retrace images were collected with resolution of 512×512 pixels, which resulted in a digital (apparent) resolution of 2 nm in x and y directions. As we established previously, such resolution was sufficient to detect distinct conformations of the α face, covered by six scan lines. Multiple fields were scanned for each sample to collect images of hundreds of particles. Selected fields were repeatedly scanned to monitor changes in topography of the same particles for prolonged time. Inhibitors and the SucLLVY-MCA substrate were diluted in 10 µl of the imaging buffer and directly injected into the chamber. Raw images are presented, with a standard plain-fit and flattening [NanoScope software version 5.12; Scanning Probe Image Processor (SPIP) version 6.02; Image Metrology, Hørsholm, Denmark] used as the only processing tools. For display purposes, the brightness and contrast of the images were adjusted with the NanoScope software or with Adobe Photoshop (Adobe Systems Inc., San Jose, CA), and outlier scan lines were occasionally manually removed (NanoScope software). Top-view (“standing,” rounded) proteasomes were distinguished from the minor population of side-view (“lying,” rectangular) particles, as described, by comparison of their length-to-width ratios (Osmulski et al., 2009). The dimensions of particles were approximated, and a shape of the α face in top-view proteasomes was judged with the help of a section tool in the NanoScope version 5.12 or SPIP software (Image Metrology).
Results

Rapamycin Noncompetitively Inhibits Proteolytic Activity of the 20S Proteasome. The CP particle is capable of cleaving short peptides and poorly structured proteins, for example, certain transcription factors with intrinsically disordered domains, or proteins partially unfolded by stressors. The free CPs are believed to exist in a cell where they are involved in ubiquitin-independent degradation of selected disordered or damaged substrates (Liu et al., 2003; Pickering et al., 2010). In vitro, the poorly folded protein, such as casein, can be used as a model substrate for the 20S proteasome. We tested the influence of rapamycin on the degradation of fluorescently labeled casein by the latent proteasome. Addition of 2 or 5 μM rapamycin slowed down the release of fluorescent products 2- to 3-fold (Fig. 3). Moreover, for the drug-treated proteasome, the release of new products almost ceased after 1 hour of incubation, whereas for the control enzyme, accumulation of products continued for at least 8 hours. Since degradation of protein engages all active sites of the proteasome, to gain insight into the effects of rapamycin on the performance of individual active sites, we tested the degradation of site-specific short-peptide substrates. Rapamycin inhibited posthydrophobic and postacidic peptidase activities of the purified latent human proteasome in submicromolar to low micromolar concentrations, with an IC50 concentration of an inhibitor inducing a 50% decrease of the maximal enzyme reaction rate of 1.9 and 0.4 μM, respectively (Table 1). Titration curves of ChT-L peptidase inhibition obtained for housekeeping and immunoproteasome were undistinguishable (not shown); consequently, we used the housekeeping 20S in all subsequent experiments. Consistent with previously reported data (Meyer et al., 1997), the detergent-activated CP was refractory to rapamycin up to concentration of about 5 μM, with only a weak inhibition of posthydrophobic cleavages noted at higher drug concentrations. The interactions between 20S and rapamycin were fully reversible. Incubation of CP with 2 μM rapamycin lowered the ChT-L peptidase activity to 51% ± 2%. After a 10-fold dilution, the ChT-L peptidase was 100% ± 12% active compared with the control treated with DMSO. The inhibition effect was reversible in the case of the PGPH peptidase as well; namely, incubation of CP with 0.5 μM rapamycin lowered the PGPH peptidase activity to 49% ± 6% and the activity rebound to 91% ± 6% (means ± S.D.; n = 3) of the control after a 10-fold dilution of the reaction mixture.

Analysis of peptide degradation in the presence of rapamycin indicated a mixed type of inhibition mechanism for the ChT-L peptidase (Fig. 4A). On the other hand, pure noncompetitive inhibition was found for the PGPH peptidase (Fig. 4B). Similar to certain other small noncompetitive ligands of the proteasome, the actions of rapamycin were not restricted to inhibition of the peptidases (Jankowska et al., 2010). The T-L peptidase was moderately activated by rapamycin. The activation of postbasic cleavages was of nonessential type, with a nearly 2-fold increase in activity and the dissociation constant in the range of 0.1 μM (Fig. 4C; Table 1).

Rapamycin Derivatives and a Rapamycin Metabolite Affect the Activities of 20S Proteasome. As a next step in our analysis, we tested the effect of rapamycin-derived small ligands known as mTOR inhibitors. Modifications of rapamycin introduced on carbon-40 [temsirolimus (Torisel; Pfizer, New York, NY), everolimus (Afinitor; Novartis Pharmaceuticals Corporation, East Hanover, NJ), and ridaforolimus (Ariad; ARIAD Pharmaceuticals, Inc., Cambridge, MA)], which is not directly involved in interactions with mTOR or FKBP12 (Fig. 2A), did not abolish the inhibition of the proteasome. The IC50 values for rapalogs did not differ much from the IC50 values recorded for rapamycin. They tend to be slightly higher than for rapamycin in the case of the ChT-L peptidase and very similar in the case of PGPH peptidase (Table 1). It is worth mentioning that temsirolimus and everolimus are in clinical trials as anticancer drugs (Vignot et al., 2005). The rapalogs modified at the C-40 position retain the binding and effector domains that are two pharmacophores characteristic for rapamycin (Fig. 2A). In contrast, single-domain rapamycin mimics such as pimecrolimus and FK-506 [(1R,9S,12S,13R,14S,17R,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxyxyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxo-4-azatricyclo[22.3.10.04,9]octacos-18-ene-2,3,10,16-tetrone] preserve only the FKBP binding site and the dissociation constant in the range of 0.1 μM.

Figure 3. Rapamycin (RAPA) inhibits the degradation of casein by 20S proteasome. Casein fluorescently labeled with BODIPY was incubated with the human housekeeping core particle for up to 8 hours, and progress of its digest was monitored by measuring the increase of BODIPY fluorescence.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 ChT-L</th>
<th>IC50 PGPH</th>
<th>Kd T-L</th>
<th>Bmax T-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>1.9 ± 0.2</td>
<td>0.41 ± 0.03</td>
<td>0.1</td>
<td>83</td>
</tr>
<tr>
<td>Temsirolimus</td>
<td>2.1 ± 0.2</td>
<td>0.38 ± 0.03</td>
<td>0.19</td>
<td>81</td>
</tr>
<tr>
<td>Everolimus</td>
<td>2.5 ± 0.7</td>
<td>0.48 ± 0.11</td>
<td>0.07</td>
<td>93</td>
</tr>
<tr>
<td>Ridaforolimus</td>
<td>2.9 ± 0.5</td>
<td>0.43 ± 0.11</td>
<td>0.11</td>
<td>114</td>
</tr>
<tr>
<td>Pimecrolimus</td>
<td>&gt;20</td>
<td>0.57 ± 0.12</td>
<td>1.82</td>
<td>483</td>
</tr>
<tr>
<td>FK-506</td>
<td>&gt;20</td>
<td>0.93 ± 0.04</td>
<td>1.14</td>
<td>257</td>
</tr>
<tr>
<td>Seco-rapamycin</td>
<td>&gt;20</td>
<td>6.45 ± 0.78</td>
<td>5.33</td>
<td>77</td>
</tr>
</tbody>
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domain (Fig. 2B). Nevertheless, both compounds inhibited the two peptidase activities of the proteasome, albeit less efficiently than rapamycin or rapalogs (Fig. 4D; Table 1). The inhibition of postacidic cleavages was the least affected by the lack of effector domain, with the IC50 increasing by only 40% in the case of pimecrolimus compared with rapamycin (Table 1). At low concentrations of the drugs, up to about 1 μM, the two-domain (rapamycin) and single-domain (FK-506) derivatives inhibited ChT-L activity to a similar extent (Fig. 4D). However, treatment with 10 μM rapamycin lowered the activity to 30% or less, whereas the effects of 10 μM FK-506 and pimecrolimus remained at the 50–60% level. On the other hand, the PGPH activity was routinely lowered to 10–15% by all the drugs at 10 μM concentration (Fig. 4E).

The two single-domain mimics activated the T-L peptidase even stronger than did rapamycin. However, they reached the maximal effects at relatively high concentrations (Fig. 4F; Table 1).

In addition to two-domain analogs and single-domain mimics, we tested seco-rapamycin, the open-ring first product of rapamycin metabolism in humans (Fig. 2C). Seco-rapamycin was reported not to affect the mTOR function (Cai et al., 2007). Surprisingly, the metabolite did affect the proteasome activities at the low micromolar concentrations, with the PGPH and T-L peptidases most affected. The efficiency of inhibition or activation by seco-rapamycin was lower than that by rapamycin, but still only 5 μM of the former was sufficient to induce nearly a 50% inhibition of the postacidic cleavages or almost a 2-fold activation of postbasic (T-L) cleavages (Fig. 4F; Table 1). In contrast, all three proteasome peptidase activities were refractory to the treatment with up to 10 μM PI-103 (3-(4-morpholin-4-ylpyrido[2,3-furo[2,3-b]pyrimidin-2-yl]phenol) or NVP-BEZ235 (2-methyl-2-(4-(3-methyl-2-oxo-8-(quinolin-3 -yl)-2,3-dihydroimidazo[4,5-c]quinolin-1-yl)phenyl)propanenitrile); the mTOR kinase inhibitors that blocked its ATP-binding pocket are structurally distinct from rapamycin (Fig. 2D; Table 1).

Comparison of the IC50 values revealed interesting trends in the inhibition potency among the rapamycin-related compounds. The posthydrophobic (ChT-L) activities were inhibited much better by the two-domain compounds than by the single-domain and linear derivatives (Fig. 4D; Table 1). As for postacidic (PGPH) cleavages, both the two-domain and single-domain drugs were comparably good inhibitors, leaving the linear metabolite as a sole example of a weak inhibitor (Fig. 4E; Table 1). On the other hand, the significantly better maximal activation of the postbasic (T-L) cleavages was induced by the single-domain compounds. An unexpected finding was that the maximal T-L activation effect was observed at much lower concentrations of the two-domain drugs (Fig. 4F; Table 1).

Summarizing, all the tested rapamycin-related compounds exerted diverse effects on the peptidase activities of human catalytic core proteasome; however, the efficiency of inhibition or activation was clearly related to the structural constrains of the rapamycin derivatives.

Fig. 4. Rapamycin (RAPA) inhibits (A and D) the posthydrophobic (ChT-L) and (B and E) PGPH peptidases, and (C and F) activates the T-L peptidase of the human core proteasome. (A–C) The Lineweaver-Burk plots of the control and rapamycin-treated proteasome. The data followed the mixed inhibition model for the posthydrophobic cleavages, with R² = 0.958. The corresponding Michaelis constant (Km) was 55.3 μM and inhibition constant (Ki) = 0.49 μM. For the PGPH activity, the corresponding values were R² = 0.978, Km = 173 μM, and K = 0.25 μM. The activation of the postbasic peptidase was of nonessential type. (D–F) The dose-response curves. The single-domain derivative of rapamycin, FK-506, and the linear metabolite of rapamycin, seco-rapamycin, inhibit the ChT-L (D) and PGPH (E) peptidases, and activate the T-L (F) peptidase. The corresponding IC50 values are listed in Table 1. Means ± S.D. (n = 3–5) or results of representative experiments are presented.
Rapamycin and Its Derivatives Affect Conformation of the Proteasome α Face. The noncompetitive nature of inhibition by rapamycin prompted us to search for the compound-induced structural changes in the core proteasome. For this purpose, we used the noninvasive tapping mode AFM in liquid, the imaging technique suitable for analysis of surface topography of macromolecules in their native state with a nanometer-scale practical resolution. We established before that AFM imaging is an indispensable tool for studying structural dynamics of the 20S α face (Gaczyńska et al., 2003; Osmulski et al., 2009; Gaczyńska and Osmulski, 2011). Namely, we demonstrated that the gate of the latent, free-core proteasome exists in a state of conformational equilibrium between the prevailing closed-gate state and the less populous open-gate state (Osmulski and Gaczyńska, 2002). We assume that the open-gate conformation enables substrates to enter the central channel and to reach the catalytic chamber, and thus the AFM-detected sporicid gate opening accounts for the detectable catalytic activity of the latent 20S proteasome. We acquired and analyzed images of hundreds of single native, fully active 20S molecules. Most of the particles were in top-view (“standing”) position, conveniently allowing for imaging of their α faces. A closer analysis of the zoomed-in images of control human 20S proteasomes revealed the presence of two clearly distinguishable conformations: one with a smooth, cone-shaped α face, another with a crater-shaped dip in the middle of the α face, where the gate to the proteasome catalytic chamber is located. After our previous extensive studies, we refer to the two forms as “closed-gate” and “open-gate” proteasomes, respectively (Osmulski et al., 2009). As described previously, we used the shape of sections carried out in four directions through the top portion of the surface topography of the α ring to distinguish between the two forms. In short, a particle was classified as closed if all four sections were cone shaped. In contrast, the particle was classified as open if all sections presented a dip surrounded by a rim (Fig. 5A) (Osmulski et al., 2009; Gaczyńska and Osmulski, 2011). The same particles imaged in consecutive scans were able to assume either closed or open conformations; however, the cone-shaped particles were always more abundant than were the crater-shaped particles and accounted for about three-quarters of the imaged molecules (Fig. 5, B and C). Addition of rapamycin to the imaged particles remarkably changed the partition of forms. In the presence of rapamycin at concentrations as low as 0.2 μM, the closed forms accounted for 61% of proteasomes, a statistically significant (P < 0.001) difference, with the 75% closed CP registered for control proteasomes (Fig. 5D). The abundance of closed molecules decreased with increasing concentration of rapamycin, reaching 40% at 10 μM, and was paralleled by decreasing activities of the ChT-L peptidase (Fig. 5D). The rapamycin-treated particles retained their ability to switch between forms, similarly to the control particles treated with DMSO (Fig. 5C). The derivatives of rapamycin followed the parent drug in the ability to change the conformational equilibrium. Exposure to 10 μM concentration of any of the three compounds—the linear metabolite (seco-rapamycin), the one-domain mimic (pimecrolimus), or the two-domain rapamycin—resulted in a very similar ultimate partition of conformers, reaching about 60% open and 40% closed proteasomes (Fig. 5E). The conformational shift from the 1:3 ratio in controls to the 3:2 partition of open to closed particles, albeit highly significant, was still less pronounced than a shift to the 3:1 partition we observed before for eukaryotic proteasomes engaged in catalytic action under the steady-state conditions (Osmulski et al., 2009). Therefore, we tested whether the presence of rapamycin would affect the catalysis-related changes in the partition of conformers. We added a model substrate for the posthydrophobic peptidase to the 20S proteasomes already pretreated with rapamycin or its derivatives. Remarkably, the partition of conformers did not change significantly, in sharp contrast to control proteasomes, which conformed to the expected 3:1 (open:closed) partition under the same conditions (Fig. 5E). We also checked the response of CP topography to a treatment with PI-103, which as a nonallosteric mTOR kinase inhibitor does not significantly affect the proteasome activities in vitro. Proteasomes treated with PI-103 were undistinguishable from the DMSO-treated control and followed the response of control particles to the treatment with the substrate (Fig. 5E; Table 1).

All the compounds at 10 μM concentration induced almost the identical ultimate partition of the CP conformers, which would suggest that CP achieved the maximum structural response to the presence of the ligands detectable with AFM. In contrast, at this concentration, each of the compounds also produced maximal but clearly distinct level of peptidase inhibition (Fig. 5F).

Rapamycin and Related Compounds Interfere with Activation of the 20S Core by 19S but Not by 11S Regulators. As demonstrated, rapamycin interfered with dynamics of the proteasome gate located on the α face. This result inspired us to test whether the drug would affect interactions of the 20S core with the 19S regulatory particle, which binds to the α face. De novo assembly of 19S from subunits is a complex process assisted by chaperones. In vitro, and likely also in cells, the 26S can also be reconstructed from 20S and the already assembled 19S particle in the presence of ATP (Smith et al., 2005). Enhancement of the peptidase activities of the core proteasome is an established test for efficiency of the in vitro reconstruction. Addition of RP to CP at the 1:1 molar ratio resulted in a 2-fold increase in ChT-L activity. In contrast, pretreatment of 20S with 5 μM rapamycin before adding ATP and 19S totally abrogated the activation, leaving the proteasome inhibited by almost 40% instead (Fig. 6). The activities of already assembled 26S proteasomes were not significantly affected by rapamycin (see later) and related compounds (compounds listed in Table 1; unpublished data) at up to 10 μM concentrations. For example, the relative ChT-L activity of the 26S treated with 1, 2, 5, or 10 μM rapamycin was 103% ± 14%, 103% ± 6%, 105% ± 9%, 111% ± 4%, respectively (mean ± S.D., n = 3 or 4). In a representative experiment, the relative ChT-L activity of the 26S treated with 10 μM rapamycin derivatives was 107% (temsirolimus), 101% (everolimus), 104% (ridaforolimus), 108% (FK-506), 94% (pimecrolimus), and 96% (seco-rapamycin) in comparison with the activity of the solvent only (DMSO)-treated 26S proteasome (100%). The specific activity of the 26S preparation used in the experiments was 0.74 ± 0.09 nM AMC/mg per second (n = 5) and about 4-fold higher than the specific activity of the 20S (Fig. 6).

Interestingly, the interference of rapamycin with the peptidase activation was not restricted to the interactions of CP with the entire 19S particle. It is established that activation of CP by RP can be reproduced by C-terminal peptides derived
Fig. 5. Rapamycin and its derivatives affect the conformational dynamics of the proteasome α face. (A) AFM images of 20S proteasomes reveal the presence of two conformations: with smooth, convex α face (“closed”) or with a dip (a darker spot) in the central area of α face (“open”). The leftmost panel presents a fragment of a field with imaged control proteasomes. Enlarged images of two top-view particles from the panel are presented on the right. Below the zoomed-in images are corresponding sections through the topmost 1-nm part of the α ring, as marked on the contour of the core proteasome on the bottom right. The diagrams between the field fragment and the single-molecule images demonstrate how the central sections in four directions (a–d) were carried out through the images to distinguish between closed and open conformers. In short, a particle is classified as “closed” if all four sections are convex, as in the case of particle 1. If all four sections are concave instead of convex, a particle is classified as “open,” as in the case of particle 2. The grayscale bar on the far right represents the height of the particles, from the baseline (black) to the top (white). The same height scale applies to single molecule images in (B). (B) A gallery of zoomed-in images of control, DMSO-treated human proteasomes (top) and proteasomes treated with 0.2–5.0 μM rapamycin (bottom). The last three control images and the last six images of rapamycin-treated proteasomes represent particles in open conformation. (C) Treatment with rapamycin (rapa) promotes changes in conformational dynamics of proteasome particles. Single-particle analysis was applied to images of proteasomes in continuously scanned fields, with a single scan lasting for nearly 3 minutes. Open and closed conformers are represented by open and black-filled circles, respectively. Each row in the diagrams represents a single proteasome particle imaged in consecutive scans of the same area. Each column represents particles from a single field. The top diagram represents four particles treated with 10 μM rapamycin. The bottom diagram represents four particles treated with 10 μM rapamycin and then with the model substrate for the ChT-L peptidase (SucLLVY-MCA; 100 μM). All the particles retain the ability to switch between open and closed conformations. (D) Treatment of proteasomes with increasing concentrations of rapamycin results in decreasing ChT-L peptidase activity and decreasing content of closed conformers. Mean values ± S.D. are presented for n = 3 experiments (activity) or n = 10–14 fields with 100–300 proteasome particles (partition of conformers). Differences in the percent of closed conformers
from the selected ATPase subunits of the 19S. It was determined previously that a 10-residue-long peptide derived from the C-terminal segment of Rpt5 (tRpt5; Fig. 2D) exhibits the strongest activating effect. We decided to test the influence of rapamycin on the activation imposed by tRpt5. The addition of 10 μM tRpt5 resulted in a more than 2-fold increase in the core proteasome activity. However, when the CP was pretreated with 5 μM rapamycin before the addition of 10 μM tRpt5, the activation was totally abolished and the CP was mildly inhibited instead (Fig. 6). The tRpt5 was not the sole core-activating peptide derived from protein ligands attaching to the α face. The 10-residue C-terminal fragment of the activator protein PA200 (tPA200; Fig. 2B) shares with tRpt5 the capability to activate CP. Again, rapamycin abolished activation by the 10 μM tPA200 peptide. The addition of 10 μM rapamycin left the proteasome 50% inhibited instead of 2-fold activated (Fig. 6). These trends were observed for the single-domain rapamycin mimic (FK-506) and the open metabolite (seco-rapamycin) as well. Their effects were most pronounced in the presence of tPA200; addition of the 10 μM derivatives caused about 40% inhibition of the proteasome, regardless of the presence or absence of the 10 μM tPA200. FK-506 and seco-rapamycin interfered with activation of the core by the tRpt5 peptide, albeit not as efficiently as rapamycin. On treatment with either derivative (10 μM), activation by the peptide (10 μM) was still well detectable; however, it was about 30% lower than without the inhibitors. To the contrary, no detectable effects on activation of 20S with 19S were observed on treatment with 5 μM PI-103.

Not all α face ligands were sensitive to the presence of rapamycin. Incubation of the CP with the heterohexameric PA29αβ/RGαβ (11S) at the 1:1 molar ratio increased the ChT-L peptidase activity 8-fold. The activation level remained unaffected on the addition of 5 μM rapamycin before fortifying the CP with the 11S complex (Fig. 6). Summarizing, rapamycin and its derivatives influenced the functional effects of selected ligands of the α face, including the most physiologically relevant 19S regulatory particle.

Discussion

Here we report that rapamycin, a canonical inhibitor of the mTOR kinase, affects in vitro performance of the 20S proteasome, the major intracellular protease in human cells. Rapamycin compromises degradation of the model protein, attenuates two of three major peptidase activities, and interferes with interactions of the 20S with its physiologic regulators. Rapamycin and all related compounds, regardless of their macrocyclic structure, productively interact with the proteasome. It was rather surprising that the effector domain of rapamycin, missing in the single-domain derivatives, is only a beneficial but not essential factor for proteasome targeting. Even more remarkable, the presence of the closed macrocyclic structure, essential for targeting the mTOR pathway, is dispensable in the case of proteasome targeting. Nevertheless, the macrocyclic likely enforces the conformation, supporting the most productive interactions with CP. Since the effects of each rapamycin-related compound on the individual activities are qualitatively similar, it is plausible that they all use the same binding sites. Taken together, the data demonstrate the possibility to design rapamycin-inspired compounds that target the proteasome without directly influencing the mTOR pathway, a case represented here by seco-rapamycin.

Our data strongly support the hypothesis that a significant part of the observed functional effects of the rapamycin-related compounds on proteasome are allostery driven. The diversity of effects exerted on the peptidase activities (i.e., pure noncompetitive inhibition, mixed-type inhibition, or moderate activation) provides the first culprit. We noted similarly diverse effects before with allosteric ligands of the 20S core: PR peptides and peptide fragments of proteins binding to the proteasome (Gaczynska et al., 2003; Jankowska et al., 2010). The AFM-detected rapamycin-induced changes in the dynamics of the gate offer an additional line of evidence for the allosteric nature of rapamycin actions. We already used AFM imaging of the proteasome α face to identify gate movements allosterically driven by structural changes in the active centers or to detect conformational destabilization of the α face induced by PR peptides (Gaczynska et al., 2003; Osmulski et al., 2009).

Analysis of AFM images revealed that treatment with rapamycin induces in a dose-dependent manner a shift of the conformational equilibrium toward moderately elevated incidence of the open-gate state. The frequency of the open gate CP is significantly higher than that in the latent proteasome but substantially lower than in the proteasome engaged in catalytic action. It is important to note that the abundance of the open conformers in rapamycin-treated proteasomes is refractory to an addition of the excess of a peptide substrate. The lower abundance of the open conformers likely compromises substrate gating and prevents the CP from reaching its full catalytic potency. Rapamycin and its allies thus emerge as unique regulators that affect the conformational dynamics of the target enzyme.

However, it seems that the perturbations of substrate gating account for just part of the inhibitory effects of the agents. The same partition of proteasome conformers induced by the high dose of distinct rapamycin derivatives results in distinct levels of peptidase inhibition that cluster according to the number of the domains and preservation of the macrocycle.
pharmacophore domains are required to reach the strong and PGPH activities by rapamycin-related compounds induce the maximal conformational shift, which results in compromised gating of substrates. The mixed-type inhibition of posthydrophobic cleavages may stem from interactions of two pharmacophores. The pure noncompetitive inhibition of postacidic cleavages may involve allosteric interactions. In vitro, they interfere with the gating of substrates and with binding of the physiologically critical 19S assembly. Two important questions concerning the practical implications of these findings remain. First, does the effect have any significance in vivo in humans or in animals treated with rapamycin or rapalogs? Second, can the unique mechanism of action be of pharmacological use? The in vitro affinity of rapamycin and rapalogs to mTOR is much higher than to the proteasome, with an IC50 difference in a range of at least two orders of magnitude. We could expect that the in vivo consequences of mTOR inhibition could be sufficient to reach the strong inhibition of the ChT-L peptidase. On the other hand, the presence of only FKBP binding domain is sufficient for the effective inhibition of the PGPH peptidase. We hypothesize that interactions of the proteasome with all the tested rapamycin-related compounds induce the maximal conformational shift, which results in compromised gating of substrates. These effects manifest in weak-to-moderate inhibition of ChT-L.

Moreover, the “maximal inhibitory effects” were in perfect agreement with the drugs’ IC50 values. Overall, the two canonical pharmacophore domains are required to reach the strong inhibition of the ChT-L peptidase. Without additional structural studies, it is impossible to determine whether the compounds bind to one or more sites and whether binding to a single site affects one or more allosteric routes. Nevertheless, the data point at the importance of protein dynamics in biologic catalysis, an emerging concept of great implications for rational drug design and protein engineering.

Identification of binding sites of rapamycin and its derivatives with the proteasome core demands extensive structural studies. However, we found that rapamycin and related compounds abrogate activation of the core by protein and peptide ligands known to anchor in the grooves on the α face. Therefore, we speculate that rapamycin directly competes with canonical ligands for the same binding grooves, which are already allosterically connected to the gate and, putatively, to the catalytic chamber. These links can be used by rapamycin. Indeed, the molecular modeling data fully support effective docking of rapamycin to at least selected grooves (Bohmann, Gaczynska, Osmulski, unpublished observations). Rapamycin binding apart from the grooves or even apart from α face still cannot be excluded; however, it would entail the presence of new potential binding sites and yet-unknown allosteric connections.

An interesting finding is that rapamycin does not interfere with activation of the core by the 11S particle, thus limiting the effective competition to ligands using the Hb-Y-X motif for binding. The PA28/REG anchors in the grooves with C-termini devoid of Hb-Y-X; however, the interactions are stabilized by activation loops (Whitby et al., 2000). We may envision that the 11S heptamer efficiently blocks access to all grooves on the occupied α face, unlike the peptide ligands, or, possibly, the wobbling-prone 19S cap. The results, however, suggest a more complex effect than a simple outcompeting of the rapamycin by 11S activator. Even when 11S was added to the 20S in a 1:1 molar ratio, which is not sufficient to saturate the CP, the peptidase activity was not affected by rapamycin. This result can be explained by the following nonexclusively phenomena. First, binding the 11S may allosterically lower the affinity of the core to rapamycin or abrogate the inhibitory effects of rapamycin already bound to the core. Second, adding rapamycin may result in a partition of 20S, 20S-11S, and 11S-20S-11S complexes, which is distinct from that established in the absence of the drug. The shifts in partition resulting in specific alterations in activity may effectively obscure the inhibition by rapamycin. Experiments to determine potential rapamycin-induced changes of 20S affinity to the protein regulators are under way.

The data presented here demonstrate that rapamycin, its derivatives, and its metabolite are unexpected inhibitors of the CP. They likely use a novel molecular mechanism of action involving allosteric interactions. In vitro, they interfere with the gating of substrates and with binding of the physiologically critical 19S assembly. Two important questions concerning the practical implications of these findings remain. First, does the effect have any significance in vivo in humans or in animals treated with rapamycin or rapalogs? Second, can the unique mechanism of action be of pharmacological use? The in vitro affinity of rapamycin and rapalogs to mTOR is much higher than to the proteasome, with an IC50 difference in a range of at least two orders of magnitude. We could expect that the in vivo consequences of mTOR inhibition.

**Fig. 6.** Rapamycin interferes with activation of the 20S core by selected protein and peptide ligands of the α face. The relative ChT-L peptidase activity is presented as percent of the control (20S with DMSO solvent). Specific activity of the control proteasome was in the range of 0.15 to 0.26 nM of the AMC product released by mg of the 20S per second (0.20 ± 0.03 nM of AMC/mg per second; n = 20). Values of mean ± S.D. from n = 3–7 independent experiments are presented. Five micromolar rapamycin (RAPA) was used, except in experiments with tPA200 where 10 μM RAPA was applied. tRpt5 and tPA200 peptides were both used at 10 μM concentration. 19S or 11S protein complexes were used in the 1:1 molar ratio with 20S. Differences between samples without and with rapamycin added were statistically significant (P < 0.01) except for the samples liganded with 11S activator (green columns).

The data presented here demonstrate that rapamycin, its derivatives, and its metabolite are unexpected inhibitors of the CP. They likely use a novel molecular mechanism of action involving allosteric interactions. In vitro, they interfere with the gating of substrates and with binding of the physiologically critical 19S assembly. Two important questions concerning the practical implications of these findings remain. First, does the effect have any significance in vivo in humans or in animals treated with rapamycin or rapalogs? Second, can the unique mechanism of action be of pharmacological use? The in vitro affinity of rapamycin and rapalogs to mTOR is much higher than to the proteasome, with an IC50 difference in a range of at least two orders of magnitude. We could expect that the in vivo consequences of mTOR inhibition...
will be evident at a much lower dose of the drug than any direct effects on the proteasome. However, our observation that seco-rapamycin exerts significant effects on the proteasome in vitro without affecting mTOR in vivo opens a venue for design of compounds inspired by the structure of rapamycin and seco-rapamycin but specifically targeting the proteasome with the new allosteric mechanism. We speculate that such compounds will have distinct intracellular effects than bortezomib and other common inhibitors binding to the proteasome active centers and rapidly blocking all forms of the protease. The rapamycin-like inhibitor will affect activity of free cores and the formation of new 26S assemblies. The cellular effects of such a compound on the ubiquitin-dependent degradation will likely unfold slowly, which may help at least to delay drug resistance. Summarizing, the allosteric inhibitors affecting the proteasomal α face may constitute important tools to control proteasome catalytic activity and useful probes to test its molecular mechanism. These properties may put rapamycin-based compounds in the forefront of the search for pharmacologically useful allosteric regulators of the ubiquitin proteasome pathway.

**Authorship Contributions**

**Participated in research design:** Osmulski, Gaczynska.

**Conducted experiments:** Osmulski, Gaczynska.

**Performed data analysis:** Osmulski, Gaczynska.

**Wrote or contributed to the manuscript:** Osmulski, Gaczynska.

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