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Silybins are stereospecific regulators of the 20S proteasome

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ABSTRACT

A reduced proteasome activity tiles excessive amyloid growth during the progress of protein conformational diseases (PCDs). Hence, the development of safe and effective proteasome enhancers represents an attractive target for the therapeutic treatment of these chronic disorders. Here we analyze two natural diastereoisomers belonging to the family of flavonolignans, Sil A and Sil B, by evaluating their capacity to increase proteasome activity. Enzyme assays carried out on yeast 20S (y20S) proteasome and in parallel on a permanently "open gate" mutant ($\alpha 3\Delta N$) evidenced that Sil B is a more efficient 20S activator than Sil A. Conversely, in the case of human 20S proteasome (h20S) a higher affinity and more efficient activation is observed for Sil A. Driven by experimental data, computational studies further demonstrated that the taxifolin group of both diastereoisomers plays a crucial role in their anchoring to the $\alpha 5/\alpha 6$ groove of the outer α -ring. However, due to the different stereochemistry at C-7" and C-8" of ring D, only Sil A was able to reproduce the interactions responsible for h20S proteasome activation induced by their cognate regulatory particles. The provided silybins/h20S interaction models allowed us to rationalize their different ability to activate the peptidase activities of h20S and y20S. Our results provide structural details concerning the important role played by stereospecific interactions in driving Sil A and Sil B binding to the 20S proteasome and may support future rational design of proteasome enhancers.

1. Introduction

Protein misfolding and aggregation are considered common pathogenic mechanisms underlying the development of many incurable human disorders termed as protein conformational diseases (PCDs) and including Alzheimer's disease (AD), Parkinson's disease (PD), and type II diabetes mellitus (T2DM) and retinal diseases such as glaucoma. Originally, it was suggested that targeting abnormal protein aggregation would have been a resolutive therapy against PCDs,¹ but recent results have demonstrated that drugs designed to solely inhibit protein aggregation are mostly ineffective; therefore, the development of successful PCDs therapies needs a more comprehensive understanding of the biochemical pathways supervising excess protein clearance.²⁻³

The ubiquitin proteasome system (UPS) is the main cytosolic proteolytic pathway responsible for the hydrolysis of misfolded or damaged proteins.⁴ The UPS is ignited by a sequential process controlled by three

enzymes, E1, E2 and E3 which couple ubiquitin (Ub) polymers to a protein substrate.⁵ In the first step, an ATP-dependent E1 ubiquitin-activating enzyme forms a high-energy thioester bond with Ub.⁶ Next, activated Ub is moved to the E2 ubiquitin-conjugating enzymes which promote the growth of Lys-linked poly-ubiquitin chains that are, eventually, conjugated to the substrate by an E3-ligase.⁷ The endpoint of the UPS is the proteasome, a 2.5 MDa multimeric assembly that degrades the Ub-tagged proteins. The proteasome is composed by a catalytic unit termed core particle (CP) or 20S, coupled with one (26S) or two (30S) 19S regulatory particles (RPs).⁸⁻⁹ The 20S unit has a barrel-like shape, made of 28 subunits arranged in four heptameric rings, stacked in an α - β - α form.⁸ The two inner β -rings include six proteolytic sites endowed with peptidylglutamyl-peptide hydrolyzing (PGPH), trypsin-like (T-L), and chymotrypsin-like (ChT-L) activities and located in the $\beta 1$, $\beta 2$, and $\beta 5$ subunits, respectively.¹⁰

The outer α -rings bind the RPs and regulate substrate entry to the

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catalytic chamber.¹¹ Due to their considerable flexibility the *N*-terminal tails of α subunits play a major role in 20S gating mechanisms.¹² The RPs anchor to the α -rings leading to a reorganization of *N*-termini by the α -subunits, particularly the α -grooves. This reorganization induces the

displacement and repositioning of Pro17 reverse turn loop, promoting the opening of the gate and allowing the entry of substrate into the catalytic chambers.^{13–14} In addition to RPs, it has been reported that also small peptides, such as, proline-rich (PR) peptides,¹⁵ Tat1- and Tat2-

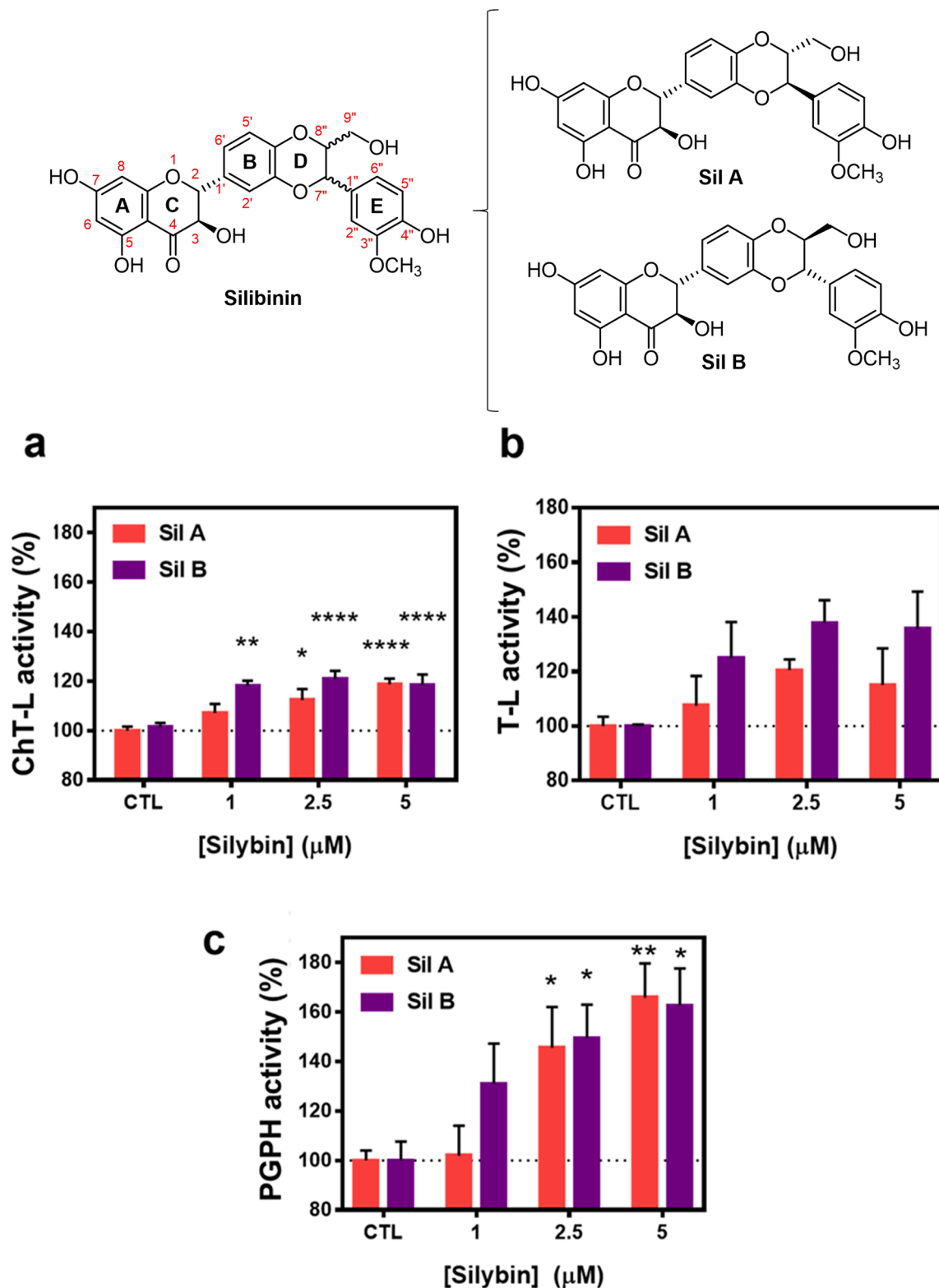


Fig. 1. Upper panel: chemical structure of silibinin and the two diastereoisomers Silybin A and Silybin B. Central and lower panels: ChT-L, (a) T-L (b) and, PGPH (c) activities of γ 20S measured at increasing concentrations of Sil A (red bars) and Sil B (purple bars). Assay buffer: 50 mM Tris buffer (pH 8), 37 °C. Results are reported as normalized percentage of residual activity ($\pm$ SEM); analysis of variance has been performed by One-way ANOVA followed by statistical Dunnett's test (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

derived peptides (fragments of the basic domain of HIV-1 Tat protein),^{16–17} Blm-10¹³ and human Rpt5-derived peptides (containing the YYA C-terminal motif),¹⁸ as well as some small molecules as porphyrins,^{19–21} imidazolines,²² fluspirilene,²³ pipercolic esters,²⁴ or pyrazolones²⁵, are able to modulate the CP activity by interacting with the grooves present on the α -surface.

The 26S proteasome mostly degrades globular proteins thanks to the support of 19S particles which deubiquitinate and unfold polyubiquitinated substrates.^{26–27} Unstructured proteins are instead preferentially degraded by the 20S without the assistance of the Ub-conjugating machinery.²⁸

Notably, a decrease of UPS activity and consequent accumulation of misfolded proteins has been suggested to play a role in the development of PCDs including AD²⁹ and T2DM.³⁰ For this reason, many studies are focusing on the design of novel molecules able to enhance proteasome activity. Due to their antioxidant and anti-inflammatory properties and superior tolerability by the organisms, small molecules extracted from natural sources are generally considered as attractive candidates to be assayed for AD and T2DM therapy.^{8,31}

In particular, many reports suggest that silibinin, a component of the milk thistle extract known as silymarin,^{32,33} regulates glucose levels in blood³⁴ and exhibits neuroprotective effects.^{35–37} However, despite these promising results would suggest a development of these compounds in AD³⁸ and T2DM therapy,³⁹ the molecular mechanisms underlying the protective effects of silibinin and, in particular its effects on UPS function, are still unknown. Silibinin is an equimolar mixture of two flavonolignans epimeric at positions C-7'' and C-8'', named silybin A (Sil A) and silybin B (Sil B) (Fig. 1, upper panel).^{40–41} Many reports suggest that there are significant metabolic differences between Sil A and Sil B^{42–44} but there are still few studies that clarify the role of stereochemistry in driving their biological activity.⁴⁵

Inspired by these premises, here we investigate the ability of Sil A and Sil B to affect cell-free polyubiquitination reactions and 20S proteasome activity, providing atomic-molecular models concerning SilA / Sil B binding modes to proteasome α -rings.

2. Results

2.1. Effects of Sil A and Sil B on ubiquitin chain growth and 20S peptidase activities

UPS malfunctioning may be related not only to proteasome failure, but also to the impairment of the ubiquitin conjugation machinery. Indeed, accumulation of ubiquitinated amyloid proteins and the occurrence of amyloid/19S complexes have been observed in affected tissues, thus suggesting that Ub-dependent protein tagging may also play a significant role in amyloid removal.⁴⁶ Having observed only slight effects of Sil A and Sil B on ubiquitination⁴⁷ (see Fig. S1 of Supporting Information) and ATP-consuming Ub activation⁴⁸ (see Fig. S2 of Supporting Information), we focused on the ability of the two molecules to enhance 20S proteasome activities.

We measured the effects of increasing concentrations of Sil A and Sil B on the ChT-L, T-L and PGPH activities of the isolated yeast 20S proteasome (y20S) (Fig. 1, central and lower panel). We observed that micromolar concentrations of both ligands somewhat enhanced all three peptidase activities of the 20S, suggesting that both Sil A and Sil B are likely to activate proteasome by interfering with α -rings dynamics.²⁵ This interpretation arises from the evidence that small molecules may enhance 20S activity by two distinct mechanisms, namely either (i) as “gate-openers” by preferentially binding and stabilizing the open-gate 20S conformation (thus facilitating substrate entry into the catalytic channel), or (ii) as “stimulators” by activating allosterically one or more peptidase activities of the 20S.⁴⁹ However, albeit Sil A and Sil B enhanced by about 20% the activity of the y20S at a concentration of 5 μ M (see Fig. S3 of supporting information), the two diastereoisomers exhibited different dose-response profiles. In fact, ChT-L peptidase

activity of y20S increased almost linearly with Sil A concentrations over the 0–5 μ M concentration range, whereas for Sil B the full effect was already obtained at 1 μ M concentration, envisaging a higher affinity than for Sil A (see Fig. 1, central and lower panel).

To confirm whether silybins may activate the core particle by promoting gate opening, we probed the effect of the two ligands on the ChT-L peptidase activity of a mutant yeast 20S proteasome (α 3 Δ N) which shows a constitutive “open gate” conformation because of the deletion of the α 3-subunit N-terminal tail and the mutation of the α 5- and α 6-loop (Fig. 2).^{11,21,50} In contrast to the activation obtained in wild type y20S proteasome, silybins did not further activate the already very active α 3 Δ N mutant. As a matter of fact, the lack of any activation effect of the α 3 Δ N mutant by silybins, as compared to their ability to activate the wild type y20S, confirms the role of silybins in stabilizing the open-gate conformation of y20S.

We next investigated the effects of the two diastereoisomers on the ChT-L activity of human 20S (h20S). Sil A enhanced the ChT-L activity of h20S proteasome when added at concentrations ranging from 0.1 to 5 μ M (Fig. 3). In the same concentration range, the effect of Sil B was almost negligible.

A different mode of interaction between the two diastereoisomers emerges from the evidence that in the case of h20S Sil A exhibited a higher affinity than for y20S (see Figs. 1 and 3), while Sil B, which was very effective on y20S, exerted only a negligible activation effect on h20S (see Fig. 3).

2.2. Modeling Silybin/h20S interactions

To investigate the molecular mechanism rooting proteasome activation by silybins we endeavored a structural characterization of the ligand-enzyme complexes. Due to the low water solubility of the two compounds and, in turn, the minimal ligand concentration in the crystallization drop, any attempt to run x-rays structural analysis by soaking proteasome crystals with silybins would result challenging.¹⁹ Therefore, we assembled several Sil/h20S interaction models by performing docking simulations.

First, according to the experimental data published by Bai et Al.,⁵¹ the two silybin ligands were built in their anionic form (i.e., deprotonated hydroxyl group at C-5) and subjected to a conformational analysis by using the BEST stochastic conformational search algorithm (see the Materials and methods section for details). It resulted that both ring C and ring D of both Sil A and Sil B showed a conformational preference for the flip presenting the substituents in a pseudo equatorial disposition (Tables S1 and S2). Accordingly, the global minimum energy (GM) conformers of the two diastereoisomers presented the same flip (namely flip I) of ring C, while, due to the inversion of chirality at C-7'' and C-8'', they showed the opposite flip of ring D (I in Sil A and II in Sil B, respectively, Fig. S4).

Once sampled the conformational space of the ligands, we proceeded with the generation of the docking starting complexes. Experimental results concerning peptidase activities suggest that Silybin/20S interactions may occur at the α -rings, likely mimicking the interactions of RPs with the catalytic core.

All the so far reported crystal structures of RPs in complex with the 20S proteasome exhibit a close interaction of the RP C-termini tails with the 20S α -grooves. For example, the C-terminal tails (Rpt1-6) of the hexameric ATPase ring of 19S anchor the 20S α 1/ α 2, α 2/ α 3, α 3/ α 4, α 4/ α 5, and α 5/ α 6 grooves.⁵² In particular, the Rpt2 (α 3/ α 4) and Rpt5 (α 5/ α 6) of 19S contain a conserved HbYX motif (HbYX; hydrophobic, Hb; Tyrosine, Y; any amino acid, X) at their C-terminal tails proved to be able to activate the 20S catalytic activities (Rpt2-peptide: T-L > PGPH > ChT-L; Rpt5-peptide: PGPH > T-L > ChT-L).⁵³ On the other hand, the C-terminal tail of the monomeric PA200, which also contains the HbYX motif, can only bind to the 20S α 5/ α 6 groove.⁵⁴ The C-terminal tails of PA200 and 19S Rpt5, both showing a higher activation of the PGPH activity with respect to the other two ones,^{53,55} share the same C-

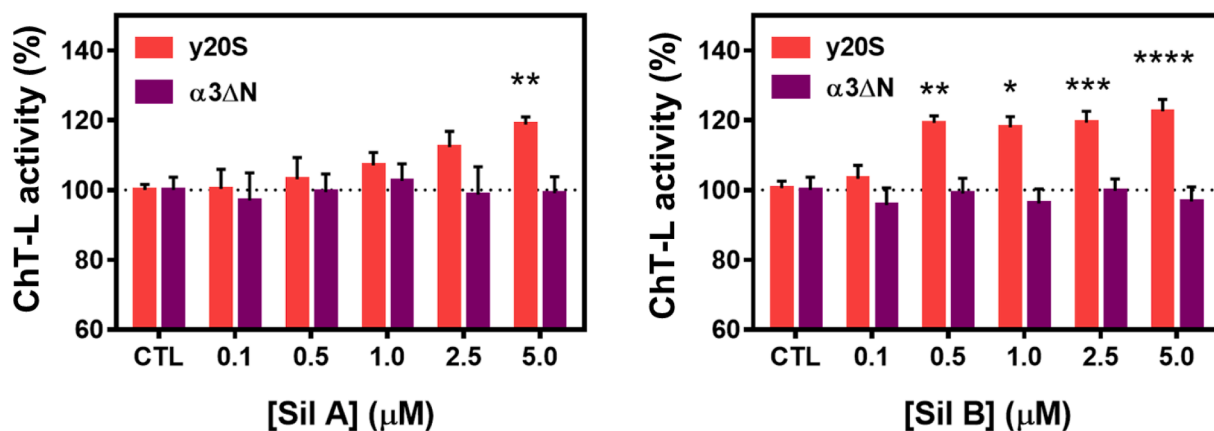


Fig. 2. ChT-L activity of wild type y20S (red bars) and the $\alpha 3\Delta N$ mutant (purple bars) at increasing concentrations of Sil A or Sil B. Assay buffer: 50 mM Tris-HCl buffer (pH 8), 37 °C. Results are reported as the normalized percentage of residual activity of the latent (y20S) and mutant ($\alpha 3\Delta N$) proteasome, respectively ($\pm$ SEM); analysis of variance has been performed by One-way ANOVA followed by statistical Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

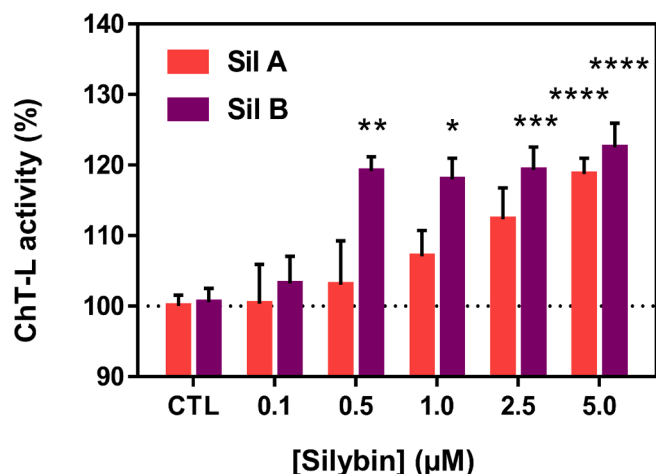


Fig. 3. ChT-L activity of h20S measured at increasing concentrations of Sil A (red bars) or Sil B (purple bars). Assay buffer: 50 mM Tris-HCl buffer (pH 8), 37 °C. Results are reported as the normalized percentage of residual activity of the h20S ($\pm$ SEM); analysis of variance has been performed by One-way ANOVA followed by statistical Tukey's test (* $p < 0.05$; ** $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

terminal sequence (YYA) interacting with the 20S $\alpha 5/\alpha 6$ groove. Since the structures and the effects of silybins resemble those shown by the C-terminal tails of 19S Rpt5 and PA200, then, we selected the $\alpha 5/\alpha 6$ groove as a starting point for h20S/silybins docking studies. A molecular model of the full-length h20S proteasome in the open conformation was built as reported in the experimental section. This model and the previously developed model of h20S in the closed conformation²⁰ were used for dynamic docking calculations. Docking simulations were performed by applying a MonteCarlo/Metropolis based procedure, which considers the whole system (i.e., the ligand and the protein) as flexible, and by defining the whole 20S structure as possible binding site (see the Materials and methods section for details). The complexes with the best nonbond interaction energies obtained by Monte Carlo calculations (Tables S3–S6) were selected as the structures representing the most probable binding modes. The quality of the selected complexes was assessed by using Molprobit structure evaluator (Table S7).⁵⁶

When silybins were docked to the closed structure of h20S, one complex was selected for each ligand (hereafter referred to complex Sil A-C and Sil B-C; Fig. S5). On the other hand, when the silybins were

docked to the open structure of h20S, one complex was selected for Sil A (hereafter referred to complex Sil A-O; Fig. 4) while two almost isoenergetic complexes ($\Delta E = 0.4$ kcal/mol) were obtained for Sil B (hereafter referred to complex Sil B-O1; Fig. 5 A-B and complex Sil B-O2; Fig. 5 C-D).

Sil A showed a more favorable binding energy when docked to the open h20S structure (Sil A-O = -43.980 kcal/mol vs Sil A-C = -40.256 kcal/mol; Tables S3–S4). On the contrary, Sil B presented a more favorable binding energy when docked to the closed h20S structure (Sil B-C = -44.835 kcal/mol vs Sil B-O1 = -38.559 kcal/mol and Sil B-O2 = -38.955 kcal/mol; Tables S5–S6). Indeed, in this case, Sil B interacts with a larger number of residues with respect to Sil A (Fig. S5 A-B vs C-D), five of which are charged, involving the ligand in an ionic interaction and six charge-assisted hydrogen bonds. These outcomes are in accordance with our experimental data showing a significant h20S activation only by Sil A and support the hypothesis that silybins can act as “gate openers” by stabilizing the 20S open conformation.

To properly analyze the results obtained with open h20S and rationalize the experimental data obtained in the case of y20S, we performed a bioinformatics and structural analysis on the experimentally determined structures of the closed and the open states of human and yeast 20S in complex with 19S. The comparison between the closed and the open states of 20S in complex with 19S put in evidence the molecular interactions leading to h20S and y20S activation by the Rpt5 C-terminal HbYX motif of h19S (YYA) and y19S (FYA), respectively (Fig. 6). The h20S substructures involved in the activation by 19S Rpt5 are the $\alpha H0$ helix (E25 side chain) and the N-terminal reverse turn (L21 carbonyl oxygen) of the $\alpha 5$ subunit, together with the $\alpha H1$ helix (L77 and T78 side chains) and the $\alpha S2$ - $\alpha S3$ loop (K62) of the $\alpha 6$ subunit (Fig. 6 A). In particular, the interactions involving E25 and L21 are coupled with the change in the backbone conformation of the $\alpha 5$ N-terminal reverse turn. This leads to the repositioning of the Pro17 reverse turn loop, promoting the open-gate conformer and allowing the entry of substrate into the catalytic chamber.⁵⁷ In the y20S/y19S complexes this changing in the backbone conformation between the open and closed states is not present and the interactions with E25 and L21 are replaced by the hydrogen bond with G19 (Fig. 6 B). Additional hydrogen bonds interactions involve the side chains of T163 and Y165 on the $\alpha S7$ strand, Y165 corresponding to V163 in h20S (Fig. 6 C). Finally, in the activated (open) complexes of both y20S/y19S and h20S/h19S the aromatic ring of the C-terminal residue contacts L77 ($\alpha H1$ of $\alpha 6$) establishing a π -alkyl interaction.

As above reported, the docking simulations performed on the h20S open structure produced the Sil A-O complex for Sil A (Fig. 4), and the two almost isoenergetic complexes Sil B O-1 (Fig. 5 A-B) and Sil B O-2

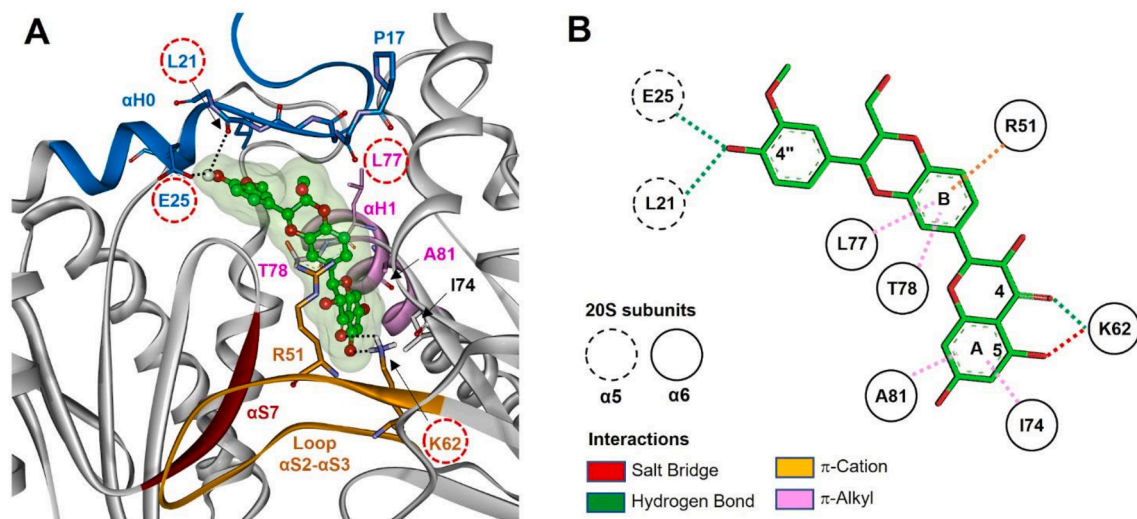


Fig. 4. Selected docked complex of Sil A bound to human 20S (open conformation). A: for sake of clarity only the $\alpha 5$ and $\alpha 6$ subunits are displayed as ribbons. On the $\alpha 5$ subunit, the *N*-terminal tail (aa1-22) and the $\alpha H0$ helix (aa23-29) are evidenced in light blue while the $\alpha S7$ strand (aa161-165) in brown. On the $\alpha 6$ subunit, the loop between the $\alpha S2$ - $\alpha S3$ strands (aa50-62) and the $\alpha H1$ helix (aa77-87) are evidenced in orange and in pink, respectively. The interacting residues, P17 on $\alpha 5$ and the backbone of the $\alpha 5$ *N*-terminal reverse turn (aa17-22) are displayed in stick. The interacting residues crucial for the 20S activation are evidenced with red circles. Sil A is displayed in ball&stick and colored by atom type (C = green, O = red, H = white). Sil A solvent accessible surface is showed. Hydrogens are omitted for clarity, except those involved in hydrogen bond interactions (black dashed lines). B: 2D representation of the interactions between Sil A and h20S. The Sil A atoms and rings involved in the interaction with h20S are labeled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 5 C-D) for Sil B.

In complexes Sil A-O and Sil B-O1 the two diastereoisomers share the same interactions with the $\alpha 6$ subunit while they establish different interactions with the $\alpha 5$ subunit. Common interactions with the $\alpha 6$ subunit include: π -alkyl interactions of ring A with both I74 and A81, cation- π and π -alkyl interactions of ring B with R51, L77 and T78, a salt bridge between the anionic oxygen at C-5 and K62, and a hydrogen bond between this latter and the carbonyl oxygen at C-4. Importantly, the interactions with L77 ($\alpha H1$) and K62 (loop $\alpha S2$ - $\alpha S3$) reproduce those formed by the HbYX motif of h19S Rpt5 with the $\alpha 6$ subunit of h20S (Fig. 6 A). However, Sil A and Sil B differed in the interactions with the $\alpha 5$ subunit, which involve the functional groups placed in a different spatial disposition in the two diastereoisomers. In particular, the 4''-OH group of Sil A is involved in hydrogen bonds with L21 backbone (*N*-terminal reverse turn) and E25 side chain ($\alpha H0$), thus reproducing the interactions of the C-terminal YYA motif of h19S Rpt5 crucial for h20S activation (Fig. 6 A). On the contrary, Sil B does not interact with any of the $\alpha 5$ residues involved in the h20S activation by the HbYX motif of RPs (i.e., E25 and L21). Indeed, the only interaction of Sil B with the $\alpha 5$ subunit is a hydrogen bond between the hydrogen atom of the 9''-OH hydroxyl group and E18 backbone carbonyl oxygen (*N*-terminal reverse turn). It is noteworthy that also in the y19S/y20S complex, the interactions with E25 and L21 are absent and the binding of the FYA motif to the $\alpha 5$ *N*-terminal reverse turn is achieved only by a hydrogen bond with G19 backbone carbonyl oxygen (Fig. 6 B).

Complex Sil B-O2 (Fig. 5 C-D) is slightly favored in terms of nonbond interaction energy compared to Sil B-O1 ($\Delta E = 0.4$ kcal/mol). This binding mode is characterized by a very different ligand-protein interaction pattern compared either to Sil B-O1 or to the result obtained with the h20S closed conformation. Sil B 3-OH and 9''-OH hydroxyl groups form hydrogen bonds with R51 ($\alpha 6$) side chain and backbone, respectively. Also ring B establishes a π -cation interaction with the protonated side chain of R51. Further interactions with the $\alpha 6$ subunit include i) a hydrogen bond between O-1 oxygen and the backbone of T78, ii) a π -alkyl interaction between ring A and L77, and iii) a π -donor hydrogen bond between ring E and Q53 side chain. On the other hand, Sil B interacts with the $\alpha 5$ subunit through a charge-assisted hydrogen bond between the 4''-OH group and the guanidine group of R168, and a

π -alkyl interaction between ring A and L21. In conclusion, assuming this binding mode, the only interaction between the h19S HbYX motif and h20S that Sil B is able to reproduce is the π -alkyl interaction with L77 ($\alpha H1$ helix of the $\alpha 6$ subunit). However, Sil B 9''-OH group point towards V163 (placed on the $\alpha S7$ strand of the $\alpha 5$ subunit), which corresponds to Y165 in y20S, a key residue involved in gate opening by the Rpt5 C-terminal YFA motif of y19S (Fig. 5 C vs Fig. 6 B).

3. Discussion

Currently, our knowledge about the cytosolic factors that may counteract amyloid toxicity is still far to be complete. However, an increasingly large number of reports suggest that a reduced protein turnover⁵⁸ and, more specifically, an age-related decline of the UPS may play a crucial role in the development of diseases characterized by amyloid deposition.³⁰ Further in vitro tests demonstrated that proteasome inhibitors may accelerate intracellular accumulation of protein deposits thus suggesting that proteasome is a focal point responsible for amyloid clearance.⁵⁹ Therefore, there is increasing interest in the development of small molecules able to stimulate proteasome activity. However, the design of new proteasome activators is hampered by our incomplete knowledge of the ligand-induced proteasome structural rearrangements underlying enzyme activation. In recent years, bioactive molecules extracted from natural sources have been experiencing a renaissance in many drug-discovery projects targeting proteasome activation.⁶⁰ Although they do not behave as strong antioxidants,⁶¹ silibinin as well as Sil A and Sil B have attracted special attention due to their anti-aggregating and protective properties.^{38,47,62} Unfortunately, due to poor ligand solubility and the highly dynamic nature of proteasome assemblies, an elucidation of ligand/target structures at a sufficient resolution remains challenging. More importantly, most investigations overlook the stereochemistry of the compounds. Indeed, testing for silybins biological activity should be done after a proper purification process to exclude interference with accompanying stereoisomers.

To establish whether silybins may also activate Ub-dependent degradation pathways, we have run Ub-chain growth reactions in tube tests. Although Sil A and Sil B slightly augmented ATP consumption

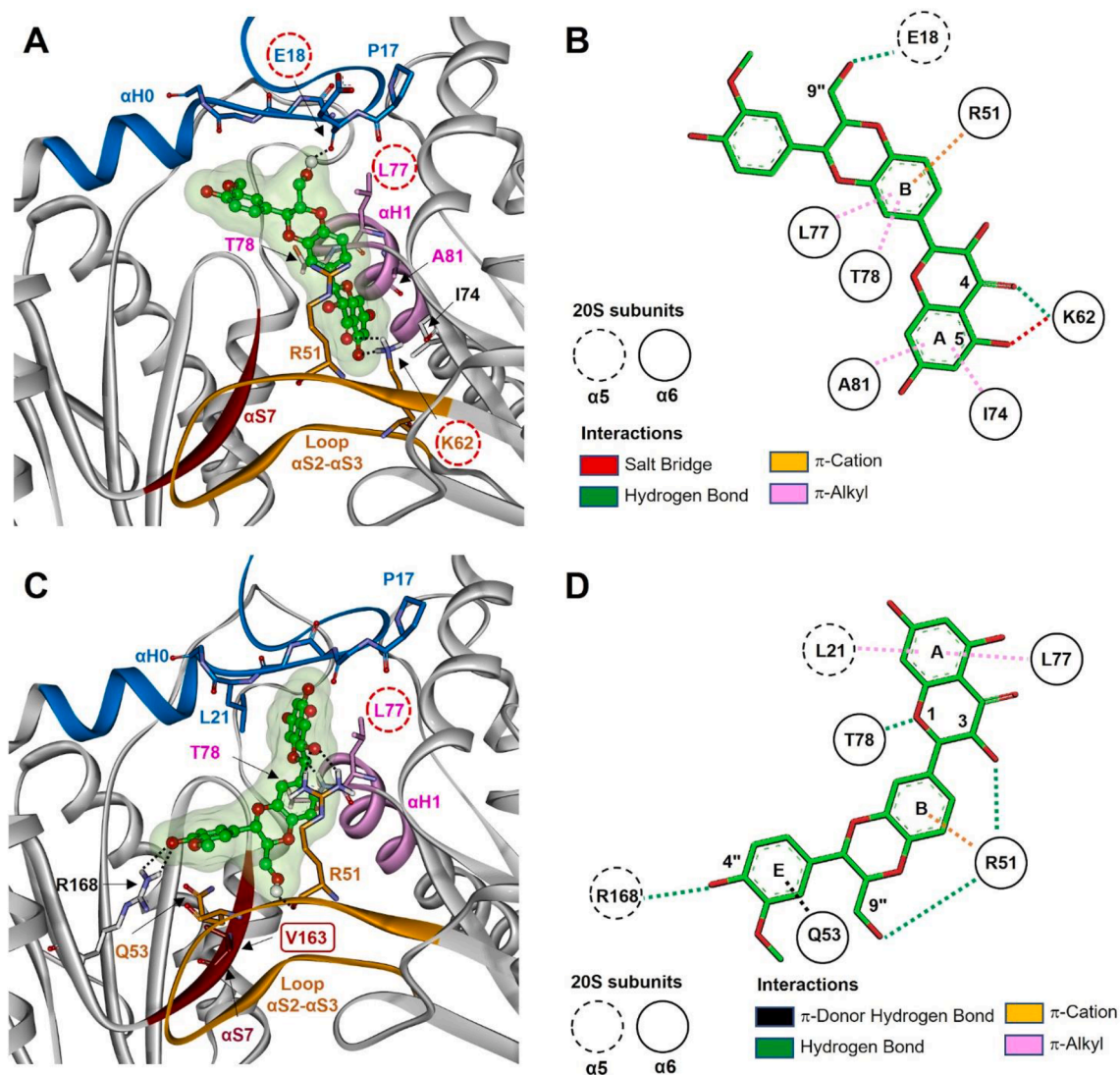


Fig. 5. Selected docked complexes of Sil B bound to human 20S (open conformation): complex Sil B-O1 (A-B) and complex Sil B-O2 (C-D). A and C: for sake of clarity only the $\alpha 5$ and $\alpha 6$ subunits are displayed as ribbons. On the $\alpha 5$ subunit, the N-terminal tail (aa1-22) and the $\alpha H0$ helix (aa23-29) are evidenced in light blue while the $\alpha S7$ strand (aa161-165) in brown. On the $\alpha 6$ subunit, the loop between the $\alpha S2$ - $\alpha S3$ strands (aa50-62) and the $\alpha H1$ helix (aa77-87) are evidenced in orange and in pink, respectively. The interacting residues, P17 and V163 (evidenced with a brown rectangle) on $\alpha 5$ as well as the backbone of the $\alpha 5$ N-terminal reverse turn (aa17-22) are displayed in stick. The interacting residues crucial for the 20S activation and E18 are evidenced with red circles. Sil B is displayed in ball&stick and colored by atom type (C = green, O = red, H = white). Sil B solvent accessible surface is shown. Hydrogens are omitted for clarity, except those involved in hydrogen bond interactions (black dashed lines). B and D: 2D representation of the interactions between Sil B and h20S. The Sil B atoms and rings involved in the interaction with h20S are labeled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during the E1-mediated Ub activation step, they did not significantly enhance the production of Ub chains. This implies that silybins mostly activate Ub-independent proteasomal digestion pathways. Remarkably, the effects of silybins on T-L and PGPH enzyme activities are more evident than on ChT-L hydrolysis. Indeed, some reports suggest that age and oxidative stress may differentially affect the three peptidase activities of the 20S proteasome. As an example, some early reports put in evidence that a decline in PGPH activity plays a major role in age-related proteasome inactivation in human subjects.^{63–64} Moreover, HNE (4-hydroxy-2-nonenal), an age-related lipid oxidation product, selectively inactivates 20S proteasome T-L activity in vitro.⁶⁵ Therefore, a molecule that enhances these two proteasome activities may be of particular interest for the development of drug candidates to the treatment of age-related neurodegenerative disorders.

Based on our experimental results and similarities with the effects of peptides containing the C-terminal HbYX motif of PA200⁵⁵ and 19S Rpt5,⁵³ here we provide a high resolution description of Silybins/h20S

predicted docked complexes in which each diastereomer occupies a binding pocket located in the groove between $\alpha 5/\alpha 6$ subunits by assuming a different binding mode. The two ligands share identical structures at the taxifolin group (A and C rings) responsible for their interactions with the $\alpha 6$ subunit. However, the molecular interactions driving the binding of Sil A and Sil B to the $\alpha 5$ subunit are significantly different because of the involvement of functional groups which are in a different spatial disposition in the two diastereoisomers.

Remarkably, the different binding modes of Sil A and Sil B within the $\alpha 5/\alpha 6$ groove are in agreement with their effects on peptidase activities of both h20S and y20S. Indeed, our experimental data evidenced that Sil A is a more effective h20S enhancer than Sil B, and, accordingly, it showed a more favorable binding energy for the open than the closed h20S conformation, while the opposite result has been obtained in the case of Sil B; further, this last point might actually account for the weak inhibitory activity by SilB on h20S at higher concentrations (see Fig. 3). Moreover, only Sil A is able to reproduce the key molecular interactions

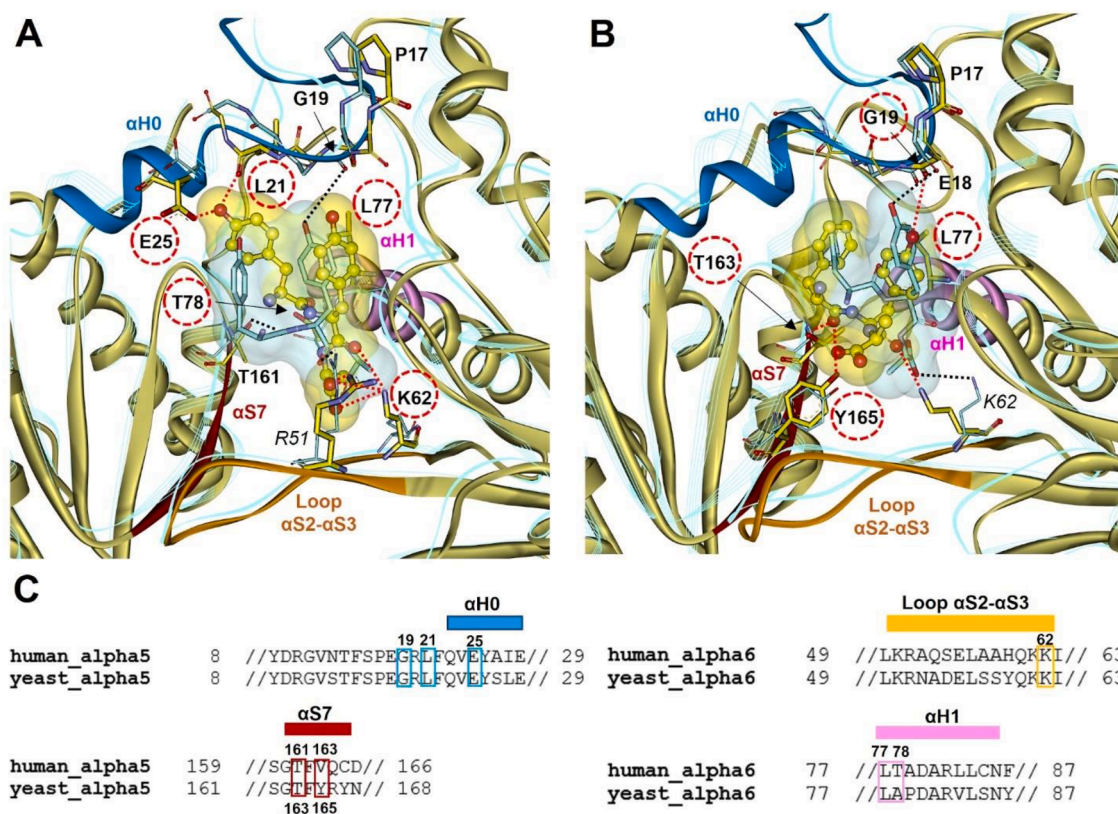


Fig. 6. A: Structural overlap (by fitting the C α atoms of the α 5 and α 6 subunits) of: A) h26S in the open state (solid ribbon colored in light yellow; PDB ID: 6MSK) with h26S in the closed state (line ribbon colored in light blue; PDB ID: 6MSB); B) y26S in the open state (solid ribbon colored in light yellow; PDB ID: 6FVW;) with y26S in the closed state (line ribbon colored in light blue; PDB ID: 6FVT). YYA and FYA motifs are evidenced in ball&stick in the open state and their solvent accessible surfaces are shown. For sake of clarity, only the α 5 and α 6 subunits are displayed. On the α 5 subunit of the 20S open state, the N-terminal tail (aa8-22) and the α H0 helix (aa23-29) are evidenced in light blue while the α S7 strand (aa161-165) in brown. On the α 6 subunit of the 20S open state, the loop between the α S2- α S3 strands (aa50-62) and the α H1 helix (aa77-87) are evidenced in orange and in pink, respectively. The interacting residues, P17 on α 5 and the backbone of the α 5 N-terminal reverse turn (aa17-22) are displayed in stick. The interacting residues crucial for the 20S activation are evidenced with red circles. The interacting residues conserved between closed and open state are labeled in italics. The residues are colored by atom type (O = red, N = blue). Hydrogen bonds are highlighted by red (open conformation) and dark (closed conformation) dashed lines. Hydrogens are omitted for clarity. C: sequence alignments of the N-terminal tail and α H0 helix, the α S7 strand, the loop between the α S2- α S3 strands and the α H1 helix of h20S with the corresponding regions of y20S. The interacting residues crucial for the 20S activation are evidenced and labeled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the α 5/ α 6 groove of h20S observed upon Rpt5-induced activation (Fig. 7), in line with the observed higher affinity for h20S with respect to Sil B.

On the contrary, Sil B was more effective on y20S exerting only a negligible activation effect on h20S. Accordingly, Sil B did not reproduce the interactions responsible for h20S activation by the YYA C-terminal sequence of human Rpt5 while the results of our structural analysis suggested that it is able to reproduce the positioning and the interactions of the activating FYA motif of the yeast Rpt5 subunit with y20S.

Our findings suggest that the mechanisms at the root of Sil A-induced activation of h20S mimic some of the RP/CP contacts triggering gate opening.

Conclusively, we provide structural details about the stereospecific interactions of Sil A and Sil B with the 20S with the aim of supporting future rational design of proteasome enhancers. Our data corroborate the hypothesis that highly specific interactions occurring at the inter-subunit grooves are needed for proteasome activation.

4. Materials and methods

Chemicals. High purity MeCN and MeOH were purchased from Carlo Erba Reagents. All chemical reactants, including silibinin and Ub, were purchased from Sigma-Aldrich. Sil A and Sil B were obtained by purification of commercial silibinin by HPLC according to a protocol reported elsewhere.⁶⁶ Purified human 20S proteasome (h20S), and the

fluorogenic peptide substrates Suc-LLVY-AMC, Z-Leu-Leu-Glu-AMC, and Ac-Arg-Leu-Arg-AMC used to assay the ChT-L, T-L, and C-L activity obtained from Boston Biochem, Inc. (Cambridge, MA). 20S proteasome from *Saccharomyces cerevisiae* (y20S) and its fully open mutant form, α 3 Δ N, were isolated as previously reported.¹¹ Human recombinant Ub activating enzyme (E1), UbcH13/Uev heterodimer complex, and E2-25 K were purchased from Boston Biochem. Standard solutions of di-ubiquitin (Ub2), tri-ubiquitin (Ub3), and tetra-ubiquitin (Ub4) chains and Lys63-linked Ub were purchased from ENZO Life Sciences. For SDS-PAGE, NuPAGE Novex Bis – Trisgels and 2-(N-morpholino) ethane-sulfonic acid buffer were obtained from Invitrogen. All solutions were prepared with Milli-Q water.

Ub activation colorimetric assays. The production of Pyrophosphate (PPi) during Ub activation by E1 in presence of 1, and 5 μ M Sil A or Sil B, and the E225K (E2) conjugating enzyme was monitored by a colorimetric assay at λ = 790 nm with a time interval of 5 min using a 96 well plate reader Varioskan (Thermo). Reaction mixtures contained 1 mM E225K, 0.5 mM E1, 15 mM Ub. The colorimetric reaction was started by adding 80 μ L of a solution of ascorbic acid (500 mM) in 2 M HCl (60% AcN/W). In each well, 10 μ L of 400 mM EDTA solution were used to quench all reactions. Then, solutions were incubated for 10 min with 200 μ L of 3 mM ammonium molybdate in 0.6 M HCl (60% acetonitrile/water) and immediately assayed by a UV spectrophotometer.

Cell-free Lys48 and Lys63 self-polyubiquitination reactions. Poly-ubiquitination reactions were carried out in the presence of 1 and 5 μ M

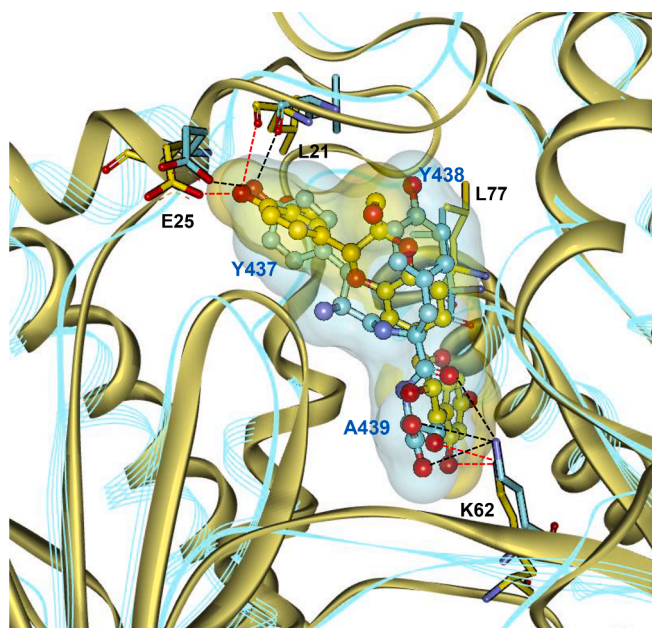


Fig. 7. Structural overlap of the YYA motif of Rpt5 with Sil A obtained by fitting the C α atoms of the α 5 and α 6 subunits of the Sil A-O complex (solid ribbon colored in light yellow) on the C α atoms of the α 5 and α 6 subunits of the h26S structure (open state; PDB ID: 6MSK; line ribbon colored in light blue). For sake of clarity, only the α 5 and α 6 subunits of h20S structures are displayed. Sil A (light yellow) and YYA motif (light blue) are displayed in ball&stick and their solvent accessible surfaces are showed. Residues interacting with both Sil A and YYA motif are displayed in stick and labelled. The residues and Sil A are colored by atom type (O = red, N = blue). Hydrogen bonds are highlighted by red dashed lines for Sil A and black dashed lines for YYA motif. Hydrogens are omitted for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sil A or Sil B. Lys48-linked polyubiquitination reactions were performed at pH 7.4 ($T = 25^\circ\text{C}$) in small volumes (40 μL) of ligation buffer (50 mM MOPS, 5 mM MgCl_2 , 30 μM DTT and 2 mM ATP) containing Ub (10 μM), UBA1 (500 nM) and E2-25 K (1 μM) and the ligand. The same procedure was followed for K63-linked polyubiquitination reactions, using the ligation buffer containing Ub (5 μM), UBE1 (100 nM), UbcH13/Uev enzymes (500 nM). The reactions were quenched after 3 h incubation as described above and size-fractionated by SDS-polyacrylamide gel electrophoresis and silver staining.

Proteasome assays. Proteasome activity was assayed by incubating yeast or human 20S proteasome (2 nM) with increasing concentrations (0.1–5 μM) of Sil A or Sil B in 50 mM TrisHCl, pH 8.0 for 30 min at 37°C . Next, 100 μM of fluorogenic peptides Suc-LLVY-AMC, Z-LLG-AMC or Ac-RLR-AMC, specific for chymotrypsin-like (ChT-L), trypsin-like (T-L) and PGPH-L peptidase activities respectively, were added to the proteasome solutions. The fluorescence signal which originates from the cleavage of AMC was recorded at 440 nm (excitation at 360 nm) for 60 min in a 384 multi-well black plate using a fluorescence plate reader (Varioskan, Thermo). A minimum of three replicates were performed for each data point. In these experimental conditions we observe an almost linear fluorescence vs time plot. Data are expressed as the difference between the enzyme activity recorded in the presence of proteasome enhancers and the latent 20S activity considering the slope of the control (fluorogenic peptide/proteasome in the absence of molecules) as 100% of proteasome activity. Results are reported as the normalized percentage of residual activity of the h20S ($\pm\text{SEM}$); analysis of variance has been performed by One-way ANOVA followed by statistical Tukey's test.

Computational studies. Molecular modelling calculations were performed on E4 Server Twin 2 $\times$ Dual Xeon 5520, equipped with two nodes. Each node: 2 $\times$ Intel Xeon QuadCore E5520, 2,26Ghz, 36 GB

RAM. The molecular modeling graphics were carried out on a personal computer equipped with Intel(R) Core(TM) i7-4790 processor and SGI Octane 2 workstations.

Conformational analysis of Silybin A and B. According to the experimental data published by Bai et al.,⁵¹ the molecular models of Sil A and Sil B were built considering the compounds in their anionic form (i.e., deprotonated at C-5 hydroxyl group) (Small Molecule tool of Discovery Studio 2017; Dassault Systèmes BIOVIA, San Diego). Resulting structures were subjected to molecular mechanic (MM) energy minimization ($\epsilon = 80^*\text{r}$) until the maximum RMS derivative was $<0.001 \text{ kcal}/\text{\AA}$, using Conjugate Gradient⁶⁷ as minimization algorithm. Atomic potentials and charges were assigned using the CFF forcefield.⁶⁸ The conformers obtained for each compound were used as starting structure for the subsequent conformational analysis (Search Small Molecule Conformations; Discovery Studio 2017). The conformational space of the compounds was sampled using the stochastic conformation search algorithm BEST for the random generation of a maximum of 300 conformations. Finally, to ensure a wide variance of the input structures to be fully minimized, an energy threshold value of 10^6 kcal/mol was used as selection criteria. The generated structures were then subjected to MM energy minimization until the maximum RMS derivative was $<0.001 \text{ kcal}/\text{\AA}$, using Conjugate Gradient as minimization algorithm. The resulting conformers were ranked by their potential energy values (i.e., energy difference from the global minimum (ΔE_{GM})). The conformers showing a $\Delta E_{\text{GM}} \leq 5 \text{ kcal/mol}$ were selected and classified into four families (named 1–4) according to the combination of the C and D ring flips, i.e., positive ($\sim 60^\circ$; flip I) or negative ($\sim -60^\circ$; flip II) values of the torsion angles τ_1 (ring C) and τ_4 (ring D).

Modeling of full-length human 20S proteasome in the open conformation. The molecular model of full-length h20S proteasome in the open conformation was built starting from the experimentally determined structure of h20S proteasome (PDB ID: 6MSK), which lacks the N-terminal regions ($\alpha 1$: aa1-5; $\alpha 2$: aa1-4; $\alpha 3$: aa1-3; $\alpha 4$: aa1; $\alpha 5$: aa1-7; $\alpha 6$: aa1-3; $\alpha 7$: aa1-5), C-terminal regions ($\alpha 1$: aa245-246; $\alpha 3$: aa252-261; $\alpha 4$: aa241-248; $\alpha 6$: aa242-263; $\alpha 7$: aa246-255; $\beta 1$: aa192-205; $\beta 2$: aa221-234; $\beta 4$: aa200-201; $\beta 5$: aa202-204; $\beta 7$: aa216-219), one loop region ($\alpha 5$: aa128-133), and the side chains of several residues ($\alpha 1$: aa45, 46, 55, 59, 63, 146, 181, 186–188, 196, 223; $\alpha 2$: aa18, 36, 52–55, 60, 139–141, 143, 163, 176–178, 184, 193, 195, 196, 199, 200, 202, 226, 227, 230, and 233; $\alpha 3$: aa13, 37, 51, 52, 54, 177, 184, 194, 201, 205, 210, 222, 229–231, 250, and 251; $\alpha 4$: aa27, 40, 42, 46–50, 53, 136–138, 141, 157, 163, 166, 169, 170, 174, 181–183, 185, 187, 189, 192, 193, 195–199, 204, 207, 208, 212–213, 216, 218, 223, 225–227, 229, 232–237, 239 and 240; $\alpha 5$: 8, 86, 187, 192, 208, 209, and 231; $\alpha 6$: aa208, 217, 218, 237, 238, and 241; $\alpha 7$: aa110, 144, 204, 206, 208, and 224; $\beta 2$: aa9, 180, 206, and 214; $\beta 3$: aa47; $\beta 4$: aa41, 95, 109, 185, 198, and 199; $\beta 5$: aa106, 150, and 185; $\beta 6$: aa45, 161, 166, and 200; $\beta 7$: aa44, 156, and 195). The sequence of 6MSK was aligned with the sequences of human 20S proteasome downloaded from the UniProtKB/Swiss-Prot Data Bank (<https://www.uniprot.org>; entry P60900 ($\alpha 1$); P25787 ($\alpha 2$); P25789 ($\alpha 3$); O14818 ($\alpha 4$); P28066 ($\alpha 5$); P25786 ($\alpha 6$); P25788 ($\alpha 7$); P28072 ($\beta 1$); Q99436 ($\beta 2$); P49720 ($\beta 3$); P49721 ($\beta 4$); P28074 ($\beta 5$); P20618 ($\beta 6$), and P28070 ($\beta 7$)) by using the Multiple Alignment algorithm (Homology module, Accelrys, San Diego). Subsequently, the secondary structural prediction of human 20S proteasome was performed using the Structure Prediction and Sequence Analysis server PredictProtein (<https://www.predictprotein.org/>). The coordinates of the structurally conserved regions ($\alpha 1$: aa6-244; $\alpha 2$: aa5-234; $\alpha 3$: aa4-251; $\alpha 4$: aa2-240; $\alpha 5$: aa8-127, 134–241; $\alpha 6$: aa4-241; $\alpha 7$: aa6-245; $\beta 1$: aa1-191; $\beta 2$: aa1-220; $\beta 3$: aa1-204; $\beta 4$: aa1-199; $\beta 5$: aa1-201; $\beta 6$: aa1-213; $\beta 7$: aa1-215) were accordingly assigned by the Structurally Conserved Regions (SCRs)-AssignCoords procedure (Homology Module, Insight 2005) using 6MSK as template structure. The lacking loop segment in the $\alpha 5$ subunit (aa128-133) was inserted by using the Generate Loops procedure. With the Generate Loops procedure, a peptide backbone chain is built between two conserved peptide segments

using randomly generated values for all the loops' φ 's and ψ 's. The chain was defined starting from the N-terminal end of the loop being built; the Start and Stop Residues were defined as the SCR residues of the model protein at either end of the loop itself. The geometry about the base was described by the four distances between C α and N-termini of the Start residue and the C α and C-termini of the Stop Residues. In the process of closing the loop, the values for the generated φ 's and ψ 's are adjusted until the four distance criteria are met. Specifically, a function was defined for the distances in terms of the dihedral angles (Scale Torsions: 60). The differences between the desired distances and their current values were minimized using a linearized Lagrange multiplier method. After a series of 1000 iterations, the loop was closed, except in the case where the distances between the ends of the loop were not respected (Convergence = 0.05). The geometry at the base of the loop is then checked for proper chirality. Finally, the loops were screened on the basis of steric overlap violations. All loops that are found to have unacceptable contacts were rejected. Since successive calculations can correct some bad contacts, a fairly large overlap factor was used (Internal and External overlap = 0.6). A bump check of the 10 generated loops together with the evaluation of their conformational energy were used as selection criteria. The lowest conformational energy loop presenting no steric overlap with the rest of the protein, was selected. Finally, the coordinates of the lacking N-terminal and C-terminal amino acids were assigned using the EndRepair command (Homology Module, Insight 2005). The obtained homology model was completed inserting the missing residue side chains by using the Replace command (Biopolymer module, Accelrys, San Diego). Atomic potentials and partial charges were assigned using the CVFF force field.⁶⁹

The obtained homology model was then subjected to a full energy minimization within Insight 2005 Discover_3 module (Steepest Descent algorithm, maximum RMS derivative = 0.5 kcal/Å; ϵ = 80*r). During the minimization, only the whole disordered N- and C- terminals and the SCRs side chains were left free to move, whereas the SCRs backbone were fixed to avoid unrealistic results. Each step of refining procedure was followed by a structural check by using the Struct_Check command of the ProStat pulldown in the Homology module to verify the correctness of the geometry optimization procedure before moving to the next step. Checks included φ , ψ , χ_1 , χ_2 , χ_3 , and ω dihedral angles, C α virtual torsions, and Kabsch and Sander main chain H-bond energy evaluation. The quality of the resulting complexes was then checked using Molprobit structure evaluator software⁵⁶ and compared to that of the reference PDB structure. The obtained homology model was used for successive dynamic docking studies.

Docking studies on human 20S proteasome. The lowest energy minimum conformers of each of the 1–4 conformational families of Sil A and Sil B as well as the corresponding conformers presenting the A-C bicyclic system (and the anionic oxygen at C-5) rotated by 180° were placed in the $\alpha 5$ - $\alpha 6$ groove taking into account the positioning of the side chains of the C-terminal HbYX activating motif of Rpt5 (i.e., ⁴³⁷YYA⁴³⁹) both in the closed and open conformational state (PDB IDs: 6MSB and 6MSK). In particular, for each selected conformer two superimpositions were performed. In the first superimposition, i) the anionic oxygen at C-5 of the ligand was superimposed on the centroid of the C-terminal carboxyl group of Rpt5 (considering the two oxygen atoms), ii) the centroids of rings B and E of silybins were superimposed on the centroids of the phenyl rings of Y437 and Y438, respectively. In the second superimposition, ring B was fitted on Y438 and ring E on Y437. The conformers with the lowest potential energy and the lowest value of RMSD that did not show steric overlap with the protein backbone were selected as starting conformations for the docking calculations. Docking calculations were performed by using our previously developed atomic model of human 20S in the closed conformational state²⁰ and the new developed atomic model of human 20S in the open state. The starting complexes were then subjected to dynamic docking studies (Affinity, SA_Docking; Insight2005, Accelrys, San Diego). In particular, a docking methodology, which considers all the systems flexible (i.e., ligand and

protein), was used. Flexible docking was achieved using the Affinity module in the Insight 2005 suite, setting the SA_Docking procedure⁷⁰ and using the Cell Multipole method for non-bonded interactions.⁷¹ The binding domain area was defined as a subset including all residues of human 20S proteasome. Thus, all proteasome atoms were left free to move during the entire course of docking calculations, whereas, in order to avoid unrealistic results, a tethering restraint was applied on the SCRs of the protein (see below). To identify SCRs, the human 20S proteasome sequences were analyzed using the Structure Prediction and Sequence Analysis server PredictProtein (<https://www.predictprotein.org/>). In $\alpha 1$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa23-33; $\alpha 2$, aa85-104; $\alpha 3$, aa111-128; $\alpha 4$, aa172-183; $\alpha 5$, aa191-207; $\alpha 6$, aa232-243; $\beta 1$, aa13-16; $\beta 2$, aa38-43; $\beta 3$, aa48-53; $\beta 4$, aa68-72; $\beta 5$, aa76-80; $\beta 6$, aa135-143; $\beta 7$, aa150-154; $\beta 8$, aa160-168; $\beta 9$, aa215-222; $\beta 10$, aa226-229). In $\alpha 2$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa20-30; $\alpha 2$, aa81-100; $\alpha 3$, aa107-124; $\alpha 4$, aa167-178; $\alpha 5$, aa184-198; $\alpha 6$, aa223-231; $\beta 1$, aa9-13; $\beta 2$, aa34-39; $\beta 3$, aa44-49; $\beta 4$, aa66-68; $\beta 5$, aa72-76; $\beta 6$, aa131-139; $\beta 7$, aa145-149; $\beta 8$, aa155-163; $\beta 9$, aa208-214; $\beta 10$, aa219-220). In $\alpha 3$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa18-29; $\alpha 2$, aa80-100; $\alpha 3$, aa107-124; $\alpha 4$, aa168-178; $\alpha 5$, aa186-200; $\alpha 6$, aa230-248; $\beta 1$, aa10-12; $\beta 2$, aa33-38; $\beta 3$, aa43-48; $\beta 4$, aa66-68; $\beta 5$, aa72-76; $\beta 6$, aa131-139; $\beta 7$, aa146-150; $\beta 8$, aa157-164; $\beta 9$, aa211-217; $\beta 10$, aa224-227). In $\alpha 4$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa17-27; $\alpha 2$, aa78-97; $\alpha 3$, aa104-121; $\alpha 4$, aa165-176; $\alpha 5$, aa183-198; $\alpha 6$, aa222-243; $\beta 1$, aa6-10; $\beta 2$, aa31-36; $\beta 3$, aa41-46; $\beta 4$, aa62-65; $\beta 5$, aa69-73; $\beta 6$, aa128-136; $\beta 7$, aa143-147; $\beta 8$, aa154-161; $\beta 9$, aa206-212; $\beta 10$, aa217-219). In $\alpha 5$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa22-32; $\alpha 2$, aa83-102; $\alpha 3$, aa109-120; $\alpha 4$, aa174-185; $\alpha 5$, aa191-206; $\alpha 6$, aa231-240; $\beta 1$, aa11-15; $\beta 2$, aa36-41; $\beta 3$, aa46-51; $\beta 4$, aa66-70; $\beta 5$, aa74-78; $\beta 6$, aa138-146; $\beta 7$, aa152-156; $\beta 8$, aa162-170; $\beta 9$, aa215-221; $\beta 10$, aa226-228). In $\alpha 6$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa19-30; $\alpha 2$, aa79-98; $\alpha 3$, aa105-122; $\alpha 4$, aa165-176; $\alpha 5$, aa184-199; $\alpha 6$, aa226-236; $\beta 1$, aa10-13; $\beta 2$, aa35-39; $\beta 3$, aa45-49; $\beta 4$, aa62-66; $\beta 5$, aa70-74; $\beta 6$, aa129-137; $\beta 7$, aa143-147; $\beta 8$, aa154-161; $\beta 9$, aa210-216; $\beta 10$, aa221-223). In $\alpha 7$ subunits, 6 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa22-32; $\alpha 2$, aa83-102; $\alpha 3$, aa109-124; $\alpha 4$, aa170-180; $\alpha 5$, aa187-202; $\alpha 6$, aa229-245; $\beta 1$, aa13-15; $\beta 2$, aa36-41; $\beta 3$, aa46-51; $\beta 4$, aa67-70; $\beta 5$, aa74-78; $\beta 6$, aa133-141; $\beta 7$, aa148-152; $\beta 8$, aa160-166; $\beta 9$, aa212-219; $\beta 10$, aa224-227). In $\beta 1$ subunits, 5 α -helix and 11 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa52-69; $\alpha 2$, aa76-90; $\alpha 3$, aa132-143; $\alpha 4$, aa149-166; $\alpha 5$, aa191-200; $\beta 1$, aa2-8; $\beta 2$, aa12-16; $\beta 3$, aa25-27; $\beta 4$, aa34-37; $\beta 5$, aa41-45; $\beta 6$, aa95-103; $\beta 7$, aa110-114; $\beta 8$, aa120-122; $\beta 9$, aa124-128; $\beta 10$, aa174-180; $\beta 11$, aa185-189). In $\beta 2$ subunits, 5 α -helix and 12 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa52-67; $\alpha 2$, aa76-90; $\alpha 3$, aa131-142; $\alpha 4$, aa148-165; $\alpha 5$, aa194-201; $\beta 1$, aa2-8; $\beta 2$, aa12-17; $\beta 3$, aa25-27; $\beta 4$, aa33-37; $\beta 5$, aa41-45; $\beta 6$, aa95-103; $\beta 7$, aa109-113; $\beta 8$, aa123-127; $\beta 9$, aa173-179; $\beta 10$, aa184-186; $\beta 11$, aa210-213; $\beta 12$, aa215-225). In $\beta 3$ subunits, 4 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa56-76; $\alpha 2$, aa83-97; $\alpha 3$, aa142-152; $\alpha 4$, aa159-175; $\beta 1$, aa9-14; $\beta 2$, aa18-24; $\beta 3$, aa32-34; $\beta 4$, aa41-44; $\beta 5$, aa48-52; $\beta 6$, aa103-111; $\beta 7$, aa119-123; $\beta 8$, aa134-138; $\beta 9$, aa184-190; $\beta 10$, aa194-199). In $\beta 4$ subunits, 5 α -helix and 10 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa32-35; $\alpha 2$, aa50-70; $\alpha 3$, aa77-93; $\alpha 4$, aa136-147; $\alpha 5$, aa153-170; $\beta 1$, aa3-8; $\beta 2$, aa12-17; $\beta 3$, aa26-27; $\beta 4$, aa36-38; $\beta 5$, aa42-46; $\beta 6$, aa99-107; $\beta 7$, aa114-118; $\beta 8$, aa129-132; $\beta 9$, aa178-184; $\beta 10$, aa189-190). In $\beta 5$ subunits, 6 α -helix and 11 β -sheet secondary structures were predicted to be highly conserved ($\alpha 1$, aa31-34; $\alpha 2$, aa49-69; $\alpha 3$, aa76-91; $\alpha 4$, aa132-143; $\alpha 5$, aa149-166; $\alpha 6$, aa189-200; $\beta 1$, aa2-8; $\beta 2$, aa12-16; $\beta 3$, aa25-26; $\beta 4$, aa35-37; $\beta 5$, aa41-45; $\beta 6$, aa96-104; $\beta 7$, aa110-114; $\beta 8$, aa120-122;

β9, aa124-128; β10, aa174-180; β11, aa185-186). In β6 subunits, 4 α-helix and 11 β-sheet secondary structures were predicted to be highly conserved (α1, aa58-77; α2, aa85-99; α3, aa142-152; α4, aa168-184; β1, aa5-6; β2, aa11-16; β3, aa20-26; β4, aa34-36; β5, aa42-46; β6, aa50-54; β7, aa105-113; β8, aa120-124; β9, aa134-138; β10, aa193-199; β11, aa203-210). In β7 subunits, 6 α-helix and 10 β-sheet secondary structures were predicted to be highly conserved (α1, aa39-41; α2, aa57-77; α3, aa85-100; α4, aa143-154; α5, aa162-179; α6, aa207-216; β1, aa10-16; β2, aa20-25; β3, aa33-35; β4, aa42-45; β5, aa49-53; β6, aa107-115; β7, aa121-125; β8, aa136-139; β9, aa187-193; β10, aa198-199).

Within the identified SCRs, the distance between backbone hydrogen bond donors and acceptors in the α-helices was restrained within 2.5 Å. On the other hand, the φ and ψ torsional angles of the β-sheets were restrained to −119° and +113°, or −139° and +135°, respectively, according to the presence of a parallel or anti-parallel structure. According to the reliability index values obtained from the secondary structure prediction analysis, we applied restraints with a quadratic form and the following set of force constants: i) 1 kcal/mol/Å² (maximum force: 10 kcal/mol/Å²) for reliability index values from 0 to 3, ii) 10 kcal/mol/Å² (maximum force: 100 kcal/mol/Å²) for reliability index values from 4 to 6, and iii) 100 kcal/mol/Å² (maximum force: 1000 kcal/mol/Å²) for reliability index values from 7 to 9.

The docking protocol included a Monte Carlo based conformational search of the ligand within the obtained homology models of human 20S proteasome (i.e., closed and open conformation) for the random generation of a maximum of 20 acceptable complexes. During the first step, in the starting structures, the ligand was moved by a random combination of translation, rotation, and torsional changes to sample both the conformational space of the ligand and its orientation with respect to the protein (MxRChange = 3 Å; MxAngChange = 180°). During this step, van der Waals (vdW) and Coulombic terms were scaled to a factor of 0.1 to avoid very severe divergences in the vdW and Coulombic energies. If the energy of a complex structure resulting from random moves of the ligand was higher by the energy tolerance parameter than the energy of the last accepted structure, it was not accepted for minimization. To ensure a wide variance of the input structures to be successively minimized, an energy tolerance value of 10⁶ kcal/mol from the previous structure was used. After the energy minimization step (conjugate gradient; 2500 iterations; ε = 1), the energy test, with an energy range of 50 kcal/mol, and a structure similarity check (rms tolerance = 0.3 kcal/Å) was applied to select the 20 acceptable structures. Each subsequent structure was generated from the last accepted structure. Following this procedure, the resulting docked structures were ranked by their conformational energy and were analyzed considering the non-bonded interaction energies between the ligand and the enzyme (vdW and electrostatic energy contribution; Group Based method⁷²; CUT_OFF = 100; ε = 2°r; Discover_3 Module of Insight2005). The complexes with the best non-bonded interaction energies obtained by Monte Carlo calculations were selected as the structures representing the most probable binding modes.

The protein structural quality in the selected complexes was then checked using Molprobiy structure evaluator software⁵⁶ and compared to that of the reference PDB structure.

Bioinformatics and structural analysis. Structural analysis of the selected docked complexes and of the experimentally determined structures of 20S proteasome were performed using Macromolecules, Simulation and Receptor-Ligand Interaction tools of Discovery Studio 2017 (Dassault Systèmes BIOVIA, San Diego, 2017).

The following experimentally determined structures: i) h20S proteasome (PDB ID: 4R3O), ii) h20S in complex with the regulatory particle 19S (PDB IDs: 6MSB (closed state) and 6MSK (open state)), iii) y20S proteasome (PDB ID: 1RYP), and iv) y20S in complex with the regulatory particle 19S (PDB IDs: 6FVT (closed state) and 6FWW (open state)) were downloaded from the Protein Data Bank (PDB; <https://www.rcsb.org/pdb/>).

Sequence alignments of yeast and human 20S proteasome α5 and α6

subunits were performed using PROMALS3D server (<https://prodata.swmed.edu/promals3d/promals3d.php>).⁷³ Structural comparisons were performed by superimposing the Cα atoms of the α5 and α6 subunits. The residues of h20S and y20S involved in the interactions with the C-terminal tail of Rpt5 as well as those of h20S involved in the interactions with Sil A and Sil B, were identified by creating a subset around the ligand including all 20S residues having at least one atom within a 5 Å radius from any given ligand atom.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

SGV, AMS, GRT and DS performed proteasome assays; VL runned Ub activation and ubiquitination reactions; VR, AZ, and GDF separated, purified and characterized silybin diastereoisomers; MP performed molecular simulations; CF supervised, analysed molecular simulations and wrote the paper; MC designed enzyme assays and wrote the paper; DM designed the research and wrote the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2022.116813>.

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Corrigendum

Corrigendum to “Silybins are stereospecific regulators of the 20S proteasome” [Bioorgan. Med. Chem. 66 (2022) 116813]

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The authors regret that in the abovementioned article, Fig. 3 (activity on human 20S) had inadvertently been replaced with Fig. S3 (activity on yeast 20S) during the revision of the manuscript, this was caused by a

clerical error. The figure has been corrected in the online article, and can be found below.

The authors would like to apologise for any inconvenience caused.

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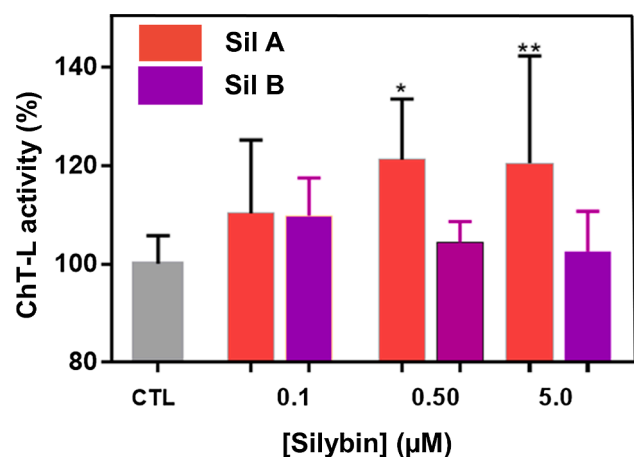


Fig. 3. Residual ChT-L activity of h2OS measured at increasing concentrations of Sil A (red bars) or Sil B (purple bars). Assay buffer: 50 mM Tris-HCl buffer (pH 8), 37 °C. Results are reported as the normalized percentage of residual activity of the h2OS ($\pm$ SEM); analysis of variance has been performed by One-way ANOVA followed by statistical Tukey's test (* $p < 0.05$; ** $p < 0.01$).