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Discovery and Development of Cyclic Peptide Proteasome Stimulators

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Abstract

The proteasome degrades proteins, which is essential for cellular homeostasis. Ubiquitin independent proteolysis degrades highly disordered and misfolded proteins. A decline of proteasomal activity has been associated with multiple neurodegenerative diseases due to the accumulation of misfolded proteins. In this work, cyclic peptide proteasome stimulators (CyPPSs) that enhance the clearance of misfolded proteins were discovered. In the initial screen of predicted natural products (pNPs), several cyclic peptides were found to stimulate the 20S core particle (20S CP). Development of a robust structural activity relationship led to the identification of potent, cell permeable CyPPSs. *In-vitro* assays revealed that CyPPSs stimulate degradation of highly disordered and misfolded proteins without affecting ordered proteins. Furthermore, using a novel flow-based assay for proteasome activity, several CyPPSs were found to stimulate the 20S CP *in-cellulo*. Overall, this work describes the development of CyPPSs as chemical tools capable of stimulating the proteasome and provides strong support for proteasome stimulation as a therapeutic strategy for neurodegenerative diseases.

Graphical Abstract

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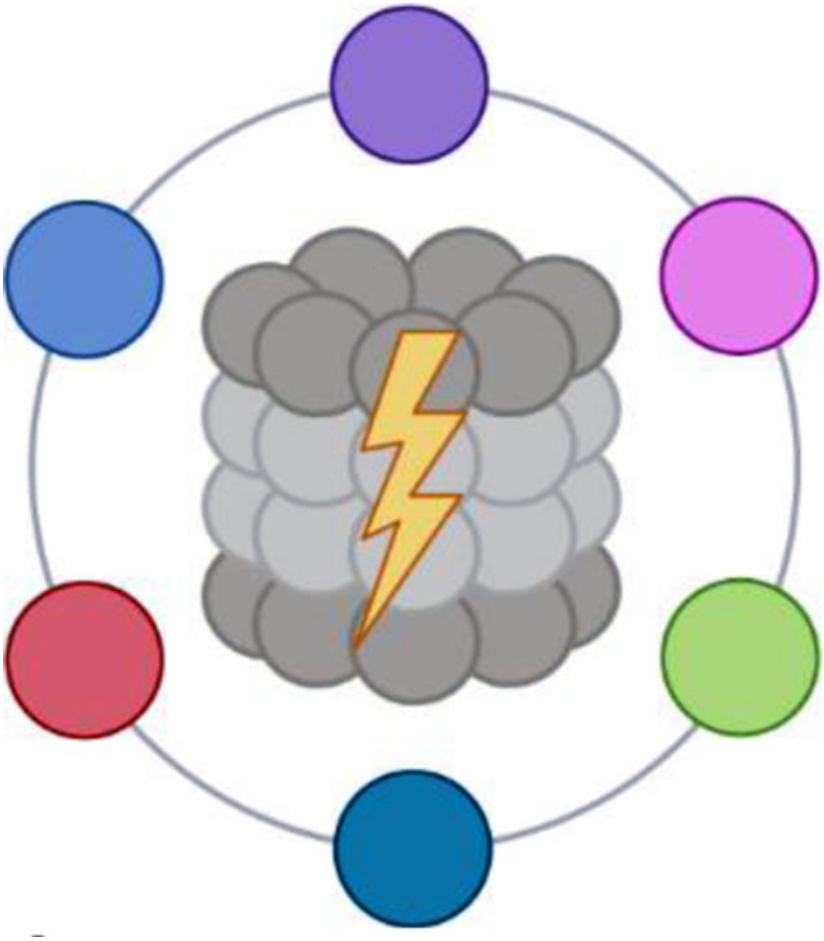
Author Contributions

S.N., E.I.P., and D.J.T. designed the experiments. S.N., C.S., B.B., and C.N. synthesized CyPPSs. S.N., C.S.M., and M.E.M. performed TAS-1 screen. S.N. performed purified protein degradation and confocal microscopy. T.J.H. synthesized epoxomicin warhead and performed flow cytometry. S.N. performed cytotoxicity and hemolysis assays. S.N., T.J.H., E.I.P, and D.J.T. wrote manuscript.

Conflicts of interest.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Trader is a shareholder and consultant for Booster Therapeutics, GmbH. Other author declares no conflict of interest.



Cyclic peptide proteasome stimulators: Cyclic peptides inspired by natural products activate ubiquitin independent degradation of disordered proteins. Development and utilization of a flow-based proteasome assay enabled confirmation of the activity of cyclic peptides in cells, making them valuable chemical tools for studying proteasome activation.

Keywords

macrocycles; peptides; proteasome; stimulation

Introduction

The proteasome is essential for the degradation of cellular proteins[1–3] and maintenance of normal cellular functions[3–11]. Protein degradation via the proteasome is done by either a ubiquitin-proteasome system (UPS)[12–14] or a ubiquitin-independent proteasome system (UIPS)[15–18]. The 26S and 30S isoforms[1,19,20] of the proteasome, which consist of the 19S regulator particle (19S RP) and the 20S core particle (20S CP), are responsible for UPS[12,21,22] (Figure 1A). The 20S CP can also degrade proteins alone via UIPS. However, the proteins must be at least partially disordered to pass through the gate formed by the α-subunits[15,23–27]. Homeostasis between the UPS and UIPS is essential for the maintenance

of healthy cells[28]. During the aging process the production of the 19S RP diminishes[29,30], leaving UIPS as a major pathway for protein degradation. This decline in UPS activity, along with other mechanisms of UPS inactivation, has been associated with many neurodegenerative diseases including Huntington's Disease[31], Alzheimer's Disease[32,33], and Parkinson's Disease[34,35]. These proteopathies are hypothesized to be caused by the accumulation of misfolded proteins[36–38]. Literature suggests that the associated proteins (e.g. α-synuclein and tau) can be degraded by the proteasome[23]. However, the UIPS is not strongly activated, limiting its ability to clear these proteins. For this reason, there is an effort to identify UIPS activators that can increase the rate of clearance of misfolded proteins. Multiple small molecules and a handful of peptides (Figure 1B) have been observed to stimulate the proteasome[8,39–43]. While these small molecules and peptides have been useful for answering foundational questions about the mechanism of proteasome stimulation[39,40,44,45], the majority of proteasome stimulators reported to date either have low potency (*e.g.* AM-404 and ursolic acid), are not selective (*e.g.* cyclosporine), or have limited activity in cell-based assays (*e.g.* betulinic acid, chloropromazine, and MK-866). Target-engagement studies with these molecules have also proven challenging, with little-to-no evidence of their direct interaction with the proteasome. A cyclic peptide stimulator could provide regions to easily append cross-linking moieties or other pull-down handles for these future important studies.

Recently, we reported synthetic natural product inspired cyclic peptides (SNaPP), a useful tool for predicting novel natural product-like molecules with biological activity[46]. Given that linear peptides have previously been identified as proteasome stimulators, we hypothesized that our cyclic peptide predicted natural products (pNPs) might be capable of stimulating the 20S CP. Cyclic peptides have several advantages over linear peptides, including their generally greater stability to proteases[47–49], their improved cell permeability[47–51], and their rigidity[47–49,52,53], which allows for increased affinity for their targets. Described herein, we utilized pNPs in our previously developed *in vitro* proteasome stimulation assay[41] and found several cyclic pNPs that efficiently stimulate the 20S CP. Derivatives were synthesized to evaluate any structure activity relationship and revealed more potent cyclic pNPs. *In vitro* protein degradation assays revealed that these molecules stimulate proteasomal degradation of disordered proteins (*e.g.* α-synuclein) while having little to no effect on ordered proteins (*e.g.* lysozyme and GAPDH). Finally, cell-based assays revealed cyclic pNPs stimulate the 20S CP.

Results and Discussion

Development of Cyclic Peptide Proteasome Stimulators

The TAS-1 biochemical assay[54] was utilized to analyze the 20S CP proteasome stimulation. This assay relies on a fluorescent-based probe, which incorporates a rhodium fluorescent probe conjugated to a proteasome peptide substrate on one side and a peptoid for solubility on the other. Active proteasome cleaves the peptide, resulting in fluorescence of the rhodium probe. This enables monitoring of the real-time activity of the proteasome, with greater intensity of fluorescence corresponding to higher proteasome activity.

Utilizing the TAS-1 biochemical assay, we screened a library of 45 cyclic peptides that were generated by SNaPP[46] (Figure S1). In our library, we had nine peptides that stimulated the proteasome 20% more than the DMSO control, giving us a hit rate of 20%. This is in stark contrast to many other proteasome stimulator screens, which typically have hit rates of less than 1%[40,55]. Of the hits identified in the initial screen, six cyclic peptides stimulated the proteasome greater than 140%. These top compounds all contained an arginine, a polar uncharged amino acid, and an aromatic amino acid. Furthermore, four of the six with the highest percent stimulation contained six amino acids (Figure 2). Analogous to the peptides in Figure 2, many peptide proteasome stimulators have an arginine[56]. Another common motif throughout peptide proteasome stimulators is a tyrosine, which has shown to open or stabilize the gate of the 20S CP[45,57]. Although, none of the top pNP stimulators have tyrosine, they do have a conserved phenylalanine, which may be acting similarly.

Because pNP-40 (renamed Cyclic Peptide Proteasome Stimulator 1, CyPPS1) had the highest percent stimulation of the identified hits, we decided to pursue derivatives of CyPPS1 to develop a structural activity relationship (SAR). An alanine scan was first performed to determine the residues that were necessary for activity (CyPPS2–6, Figure 3A). Each amino acid was substituted with an alanine, except for arginine. Unfortunately, the derivative where arginine is replaced with alanine is insoluble and thus could not be evaluated. For this reason, we utilized a derivative that switched the position of arginine with alanine (CyPPS6). The stimulatory activity for CyPPS6 was preserved, suggesting that the position of the arginine is not essential. When each amino acid was substituted with an alanine, the activity decreased, suggesting that all the amino acids are essential for the stimulatory affect (Figure 3A–B). Specifically, replacing either the D-leucine (CyPPS3) or the D-phenylalanine (CyPPS5) with D-alanine resulted in the greatest loss of stimulatory activity, suggesting that these amino acids are particularly important for activity. Additionally, a linear version of CyPPS1 (PPS1) was explored and found to have little-to-no stimulatory effect. This provides strong evidence for the necessity of cyclizing these peptides for activity and suggests that cyclization likely holds them in an active conformation.

The SAR was further explored by substituting other amino acids at each position (Figure 3C–E, Table S1–2). The threonine at position 1 was substituted with lysine (CyPPS7), aspartic acid (CyPPS8), and valine (CyPPS9). The lysine substitution decreased activity, suggesting basic amino acids are not tolerated at position 1. Both CyPPS8 and CyPPS9 maintained stimulatory activity, suggesting that position 1 tolerates both acidic and aliphatic amino acids, in addition to the polar threonine. The D-leucine at position 2 was substituted with D-aspartic acid (CyPPS10), D-lysine (CyPPS11), D-isoleucine (CyPPS12), and D-valine (CyPPS13). Only CyPPS13 retained activity, with the other derivatives having decreased activity. While the lack of activity with CyPPS12 is surprising, these results overall suggest that an aliphatic amino acid with branching is preferred. Position 2 was also substituted with D-propargylglycine (CyPPS14), which also retained stimulatory activity, supporting the necessity for an aliphatic side chain. All substitutions at position 3 decreased activity except for 4-fluorophenylalanine (CyPPS15). Tyrosine (CyPPS16/CyPPS17) was also substituted for position 3 and 4. Interestingly, a decline in stimulatory activity was observed, indicating CyPPSs are likely not acting at the

same location as the previously studied tyrosine-containing peptides. Substitutions of D-4-fluorophenylalanine (CyPPS18) at position 4 decreased activity. Other substitutions such as D-3-flurophenylalanine (CyPPS19) and D-3,4-difluorophenylalanine (CyPPS20) retained activity, suggesting that electron-withdrawing groups are tolerated. We also substituted this position for benzophenone (CyPPS21) and found that the 20S CP stimulatory activity was maintained. Substitution of the D-alanine at position 6 with D-serine (CyPPS22) and D-diaminopropionic acid (CyPPS23) maintained stimulatory activity, suggesting that small polar substitutions are tolerated. We were unable to substitute position 5 with any amino acids other than basic amino acids due to solubility issues. The basic amino acid diaminopropionic acid (CyPPS24) maintained stimulatory activity. Finally, a cyclic pentapeptide (CyPPS25) was explored but had decreased activity, demonstrating the importance of ring size for activity. Of the 37 derivatives tested, 4 derivatives were found to have similar or increased stimulatory activity compared to the parent CyPPS1 (Figure 3C–D). These were selected to undergo further testing.

Dose response and activity with purified proteins

Initially, the dose-response relationship was determined for the top five hits (Figure 3F). CyPPS13 and CyPPS23 had the lowest EC_{50} values (4.0 μM and 4.1 μM, respectively), suggesting they are the most potent compounds. However, their maximal response was lower compared to CyPPS8 and CyPPS14 (Table S3 for E_{max} and EC_{50} values). While the reasoning for the differences in EC_{50} and E_{max} for these molecules is currently unclear, the structural similarity of the molecules suggests they likely have similar binding sites.

Given the potency of the CyPPSs in the TAS-1 assay, we chose to explore their abilities to degrade proteins using an *in vitro* degradation assay. The 20S CP typically degrades proteins that are highly disordered. To ensure the selectivity of the CyPPSs for disordered proteins, the ability of these molecules to induce degradation of disordered proteins (e.g. α-synuclein) and ordered proteins (*e.g.* GAPDH and lysosyme) was explored. CyPPS1, CyPPS14, and CyPPS23 greatly enhanced proteasomal degradation of α-synuclein while having little-to-no effect on GAPDH and lysozyme (Figure 4 and Figure S2). The lack of activity of CyPPS8 in this assay is unsurprising given that molecules were tested at 10 μM, a concentration where CyPPS8 has little effect (Figure 3F). Overall, this suggests that CyPPS1, CyPPS14, and CyPPS23 are excellent leads for proteasome stimulation. The top hits were also tested for toxicity in mammalians cells. Based on their selectivity for disordered proteins, we would not expect these molecules to be toxic. Gratifyingly, no toxicity was observed in HEK293 cells (Table S2). Additionally, no hemolysis was observed with human red blood cells, further supporting these as excellent lead molecules (Table S2).

Cell-Based Activity

While many cyclic peptides are cell permeable, it is challenging to predict *a priori* the peptides that are capable of entering cells. Because the proteasome is an intracellular target, we decided to investigate the ability of the CyPPSs to enter cells. To do this, a BODIPY-tagged CyPPS (CyPPS26) was synthesized from the alkyne derivative CyPPS14 (SI Scheme 2). A549 cells were then dosed with CyPPS26 and analyzed for cell permeability via confocal microscopy. Furthermore, we investigated the mechanism by which CyPPS26

was entering the cell. Cellular uptake of cyclic peptides usually occurs either via passive diffusion[58,59] or endocytosis[60–65]. The puncta present suggest that CyPPSs may enter the cell via endosomal uptake. This possibility was further supported by the overlap of the BODIPY and LysoTracker signal (Figure 5). However, further experimentation is needed to confirm this. As some cytosolic fluorescence is observed, it is hypothesized that at least some of the CyPPSs are capable of escaping the endosome, and thus likely capable of engaging with the cytosolic proteasome. However, it does appear like the majority of the peptide remains within the endosome, at least at this timepoint. This is an area that will be optimized in the future. Cytosolic protein accumulation is linked to many neurodegenerative diseases, including α-synuclein accumulation in Parkinson's disease[66]. Given that CyPPSs can degrade highly disordered proteins and are cell permeable, we chose to investigate their abilities to stimulate the proteasome *in cellulo* using a newly established flow cytometry assay.

Flow cytometry allows for the study of proteasomal activity in physiologically relevant conditions and presents a high-throughput alternative to traditional gel electrophoresis and western blotting techinques[67–69]. To test the assay, a covalent fluorescent-based probe[70,71] was applied in HEK293T cells to quantify proteasome stimulation of CyPPSs, a known proteasome stimulator (miconazole [MO]) and proteasome inhibitor (MG132) (Figure S3). The covalent fluorescent-based probe is based on the proteasome inhibitor epoxomicin, which interacts with the β5 subunit of the proteasome, with a fluorophore appended to the N-termini. Upon incubation with the proteasome, it forms a covalent bond, thus fluorescently tagging the proteasome[72]. This probe has previously been shown to act in cells, enabling the current flow-based studies with higher fluorescence indicating higher proteasome activation. The addition of CyPPSs induces a significant shift in the intracellular fluorescence (Figure 6A). This shift validates that CyPPSs are capable of stimulating cytosolic proteasome *in cellulo*. Interestingly, the same compounds that degrade purified α-synuclein (CyPPS1, CyPPS14, and CyPPS23) show significant cellular stimulation of the proteasome. However, their activity is lower than what would be expected based on their *in vitro* activity. This may be due to incomplete cellular uptake or escape from the endosomes. Unsurprisingly, the linear PPS1 does not stimulate the proteasome. This is likely due to a combination of effects including the inability to stimulate the proteasome (Figure 3B–D) as well as the generally poor cell permeability and proteolytic stability of linear peptides.

Conclusions

Described herein, CyPPSs were developed as stimulators of UIPS. Exploration of derivatives enabled development of a robust SAR, resulting in identification of three CyPPSs that selectively degrade highly disordered proteins (i.e. α-synuclein). CyPPSs were found to enter the cell via endocytosis and demonstrated significant endosomal escape into the cytosol. A flow-based proteasome stimulation assay—which allows for high throughput evaluation of proteasome stimulation—was developed and revealed that CyPPSs efficiently stimulate the proteasome in cells. Overall, the CyPPSs are promising leads for potent, cell-active proteasome stimulators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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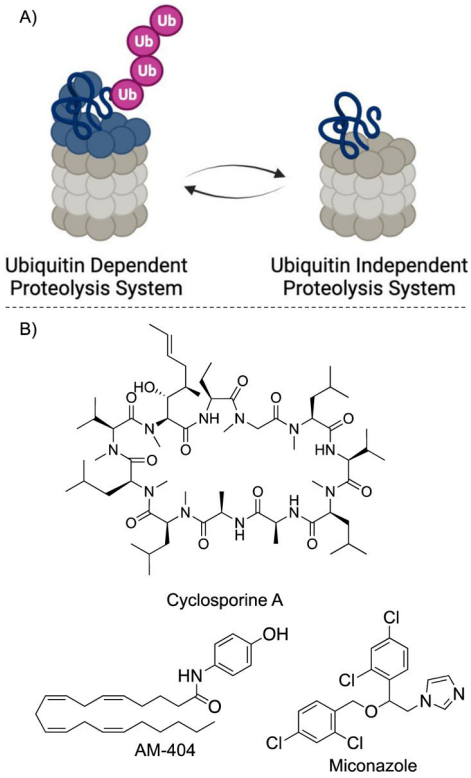

Figure 1.

A) The 26S proteasome (left) consists of the 19S regulatory particle (blue) and the 20S core particle (gray). Ubiquitin independent proteolysis (right) only consists of the 20S core particle. **B)** Known 20S core particle proteasome stimulators.

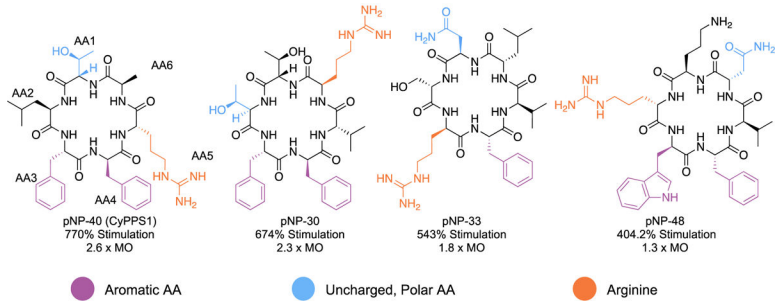

Figure 2.

Structural activity relationship of the initial screen of the SNaPP library. Each peptide is a hexamer that consists of at least one aromatic amino acid (purple), uncharged polar amino acid (blue), and arginine (orange).

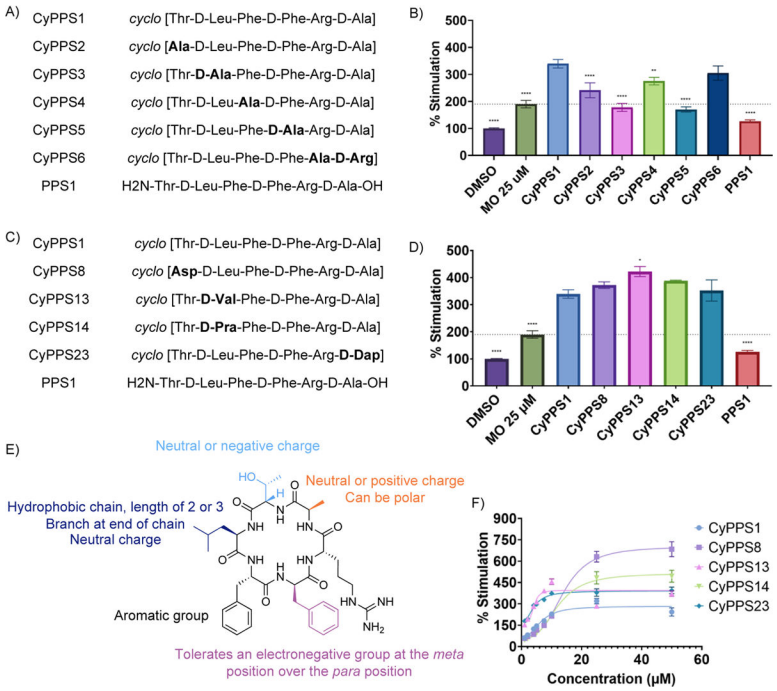

Figure 3.

A) Structures of the peptides in Figure 3B. **B)** TAS-1 20S CP stimulation of the alanine scan of CyPPS1. All compounds except miconazole (MO) were tested at 10 µM. 2-way ANOVA analysis performed comparing to CyPPS1 (n=3 with S.E.M. indicated) **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05, and ns P > 0.05. Average of 3 independent replicates. Abbreviations: MO = miconazole. **C)** Structures of the peptides in Figure 3D. **D)** TAS-1 20S CP stimulation of the top five 20S CP stimulators and the linear of CyPPS1. All compounds except MO were tested at 10 µM. 2-way ANOVA analysis performed comparing to CyPPS1 (n=3 with S.E.M. indicated) **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05, and ns P > 0.05. Average of 3 independent replicates. Abbreviations: MO = miconazole. **E)** Structural activity relationship of CyPPS1**. F)** Dose response curve of the top 5 20S CP stimulators.

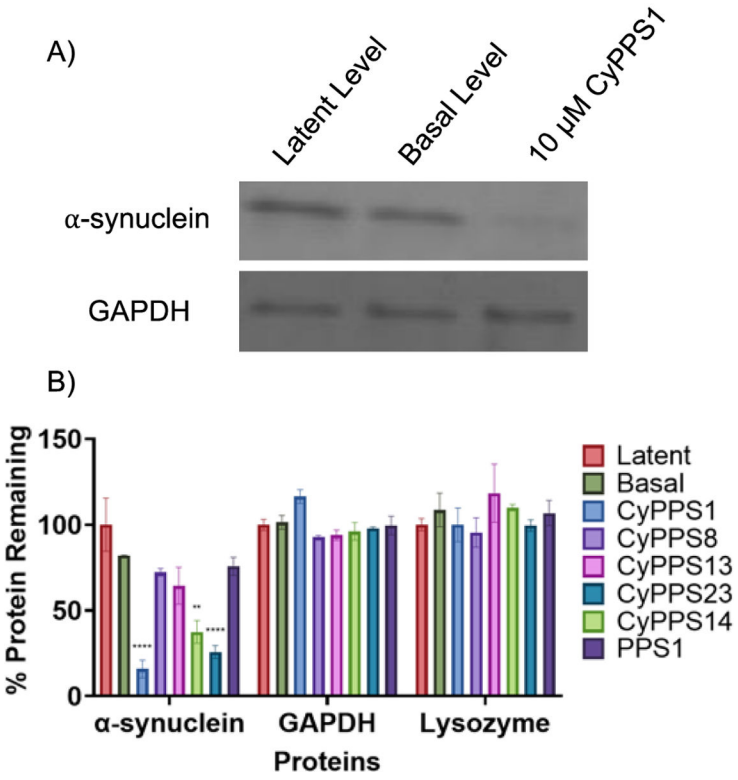

Figure 4.

A) Coomassie gel (representative of 3 independent replicates) and **B)** quantification of the degradation of highly disordered (α-synuclein) and low disordered (GAPDH and lysozyme) proteins. All compounds were tested at 10 μM. 2-way ANOVA performed comparing to basal activity (n=3 with S.E.M. indicated) **** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05, and ns P > 0.05. Average of 3 independent replicates.

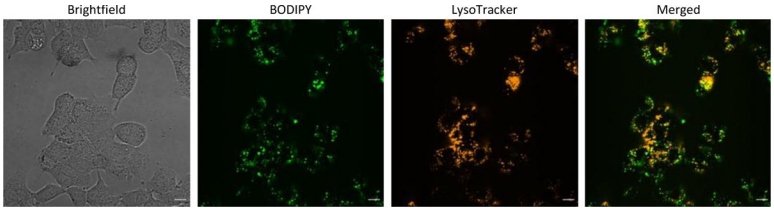

Figure 5.

Cell permeability and mechanism of cell uptake of CyPPSs was determined with 15 μM of CyPPS26 in A549 cells after 1.5 hours (representative of 3 independent replicates). Scale bar is 10 μm.

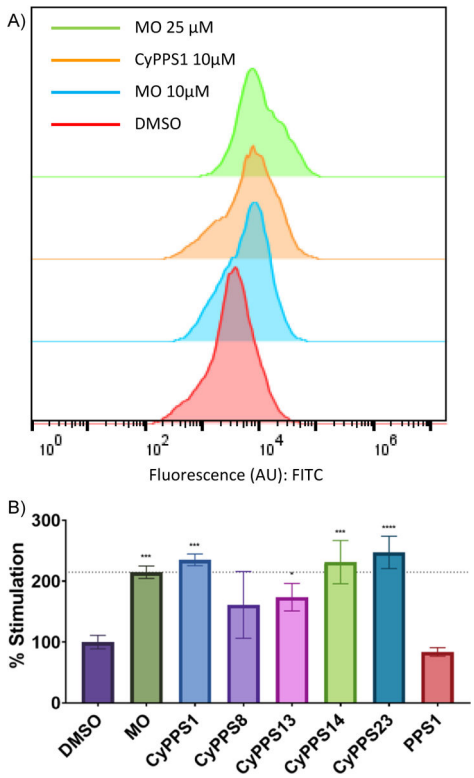

Figure 6.

A) Flow cytometry plots showing shifts in fluorescence signal (FITC) in HEK293 T cells (representative of 3 independent replicates). Abbreviations: MO = miconazole. **B)** Flow cytometry analysis of CyPPSs at 10 µM in HEK293 T cells. 2-way ANOVA performed ((n=3 with S.E.M. indicated) **** P<0.0001, *** P<0.001, ** P<0.01, *<0.05, and ns P > 0.05. Average of 3 independent replicates. P<0.05, and ns P > 0.05. Average of 3 independent replicates. Abbreviations: MO = miconazole.