HHS Public Access

Author manuscript

Chembiochem. Author manuscript; available in PMC 2022 June 02.

Published in final edited form as:

Chembiochem. 2021 June 02; 22(11): 1961–1965. doi:10.1002/cbic.202100023.

Approaches to Evaluate the Impact of a Small Molecule Binder to a Non-catalytic Site of the Proteasome

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Abstract

Proteasome activity is crucial for cell survival and proliferation. In recent years, small molecules have been discovered that can affect the catalytic activity of the proteasome. Rather than targeting the active sites of the proteasome, it may be possible to affect ubiquitin-dependent degradation of proteins by limiting the association of the 19S regulatory particle (19S RP) with the 20S core particle (20S CP) of the proteasome. We recently described the discovery of TXS-8, a peptoid that binds to Rpn-6. Rpn-6 is a proteasome associated protein that makes critical contacts with the 19S RP and the 20S CP. Here we present a general workflow to evaluate the impact of a small molecule binder on proteasome activity using TXS-8 as an example. This workflow contains three steps where specific probes or overexpressed proteins in cells are utilized to determine if hydrolysis activity of the proteasome is affected. While in our case, TXS-8 did not affect proteasome activity, our work flow is highly amenable to studying a variety of small molecule-proteasome subunit interactions.

Introduction:

The proteasome is a part of the ubiquitin-proteasome system (UPS), which is responsible for the degradation of misfolded and/or damaged proteins. It degrades more than 80% of proteins in cells, which makes proteasome activity crucial for survival and proliferation.[1,2] The proteasome consists of two major components, the 19S regulatory particle (RP) and the 20S core particle (CP) (Figure 1). [3,4] The 19S RP recognizes poly-ubiquitinated proteins, removes the poly-ubiquitin chain, unfolds the protein, and shuttles the protein into the 20S CP. The 20S CP is composed of four heptameric rings that arrange in an α, β, β, α fashion. The β1, β2 and β5 subunits contain catalytic active sites are and exhibit “caspase-”, “trypsin-” and “chymotrypsin-like” activities, respectively and are responsible for hydrolyzing unfolded proteins into small peptide fragments. These fragments are further degraded into single amino acids by other cellular proteases, which can be used to synthesize new proteins.[5]

Associated Content:

Please refer to the supporting information for detailed experimental procedures.

Conflict of Interest:

Prof. Trader is a scientific advisor and shareholder of Booster Therapeutics, GmbH. Other authors declare no conflicts of interest.

Modulating proteasome activity as a therapeutic method has been extensively studied in cancers, as certain types of cancer cells rely more heavily on proteasome activity for survival.[6] Inhibiting proteasome activity has proven successful in triggering apoptosis in several hematological cancer cell types, although off-target toxicity in healthy cells can also occur. Three proteasome inhibitors, Bortezomib, Carflizomib and Ixozomib have been approved by the FDA for multiple myeloma treatment, highlighting that the proteasome is a viable target for therapeutic intervention.[7–11] Meanwhile, recent studies have indicated that small molecule stimulation of the proteasome could be a method to decrease levels of aggregated or toxic proteins in a variety of diseases including Parkinson's and pre-mature aging.[12–15] Discovery of these inhibitors and stimulators can be accomplished by using a variety of the small molecule probes that can actively monitor the hydrolysis activity of the β-subunits of the 20S CP. Utilizing techniques or assays to determine if a small molecule binder to a non-catalytic subunit can affect proteasome activity is more complicated.

We recently described the discovery of a small molecule binder to Rpn-6, Figure 2 and S1.[16] Rpn-6 serves as the molecular hinge that connects the 19S RP and the 20S CP to both maintain the structural integrity of the 26S proteasome and fully activate its function.[17,18] We initially hypothesized that a molecule such as TXS-8 could affect the association of the 19S RP and 20S CP or could prevent post-translational modifications, such as phosphorylation by PKA, that are needed to stabilize protein-protein interactions with Rpn-6 for maximum proteasome activity.[19] While TXS-8 was shown to be cytotoxic to Ramos B-cells in the low micromolar range, which require significant proteasome activity to survive, and not HEK-293T cells, it was unclear if the toxicity was related to a decrease in proteasome activity. Here we describe the development and application of assays that can be used to evaluate molecules such as TXS-8, which can bind non-catalytic proteasome subunits, to determine if they can affect ubiquitin-dependent or -independent proteasome activity.

Results and discussion:

We first sought to determine if proteasome activity in cells is decreased if there is reduced amount of Rpn-6 expression. HEK-293T cells were transfected with silencing RNA for Rpn-6 knockdown (siRNA-Rpn-6) that was previously demonstrated to reduce Rpn-6 expression.[20] Rpn-6 knockout is not possible as the 26S proteasome could not form, which would lead to unwanted cell death.[21]

HEK-293T cells were transfected with the siRNA-Rpn-6 or a scrambled version of the siRNA (scram-siRNA) as a control. Two days after transfection, the cells were lysed and the amount of Rpn-6 present in the lysate was quantitated using western blot analysis (Figure 3A). We observed a 60% decrease in the amount of Rpn-6 present in the siRNA-Rpn-6 treated cells as compared to the mock transfected cells (Figure 3B and S2). The scrambled version of the siRNA did not significantly affect Rpn-6 levels compared to the mock sample.

We have previously developed a suite of cell permeable probes that can monitor proteasome activity in real-time.[22] These probes can be used in a variety of live-cell types, including HEK-293T cells and A549 in a 96-well plate using a fluorescent plate reader assay.

Cleavage of the probe by proteasomes in cells is monitored over time, and the increase in fluorescence can be observed to obtain the rate of proteasome cleavage activity (change in relative fluorescent units per minute (ΔRFU/min)). We chose to use the probe TAS-2 for these Rpn-6 related experiments (Figure 4A). This probe has an unnatural amino acid preceding the rhodamine moiety where the proteasome cleaves. Exchanging the typical tyrosine for 4-chlorophenylalanine slows down the rate of cleavage of the probe, allowing us to observe small changes in proteasome activity. After transfecting cells with the siRNA-Rpn-6 or scram-siRNA for 48 hours, proteasome activity was monitored by dosing the cells with TAS-2. The cells with knocked down Rpn-6 expression showed a significant decrease in the overall amount of proteasome activity as compared to the two controls (Figure 4B). This result indicates that decreasing Rpn-6 expression also leads to a decrease in proteasome activity. This observation also validates previous literature that Rpn-6 is crucial to maintain the proper function of the proteasome.[18,23,24]

We next wanted to determine if dosing with TXS-8, our Rpn-6 binder, could also decrease overall proteasome activity in a similar fashion to the siRNA-Rpn-6 knockdown. From our previous results, we knew that HEK-293T cells were not especially susceptible to TXS-8 toxicity, but we needed to establish the maximum concentration of TXS-8 that could be used in the control and siRNA-transfected cells (Figure S3A). The transfected HEK-293T cells could be treated with up to 30 μM of TXS-8 for seven hours before significant decrease in viability was observed (Figure S3B). This was important, as we did not want the confounding variable of cell death to contribute to changes in proteasome activity in response to TXS-8 dosage. With the conditions for Rpn-6 knockdown and TXS-8 dosing established, we could begin to compare proteasome activity.

Cells were pre-treated with 30 μM of TXS-8 or TXS-14 (negative control peptoid) for 3.5 hours and washed with PBS (Figure S4). TXS-8 or 14 was added again at 30 μM concentration to the cells with TAS-2 in KRBH buffer and fluorescence generated by cleavage of TAS-2 was monitored over time. We were surprised to observe that TXS-8 did not affect proteasome activity as observed in cells that were knocked down for Rpn-6 (Figure 5 and S5). It is possible that TXS-8 could be only affecting ubiquitin-dependent degradation of p roteins. Since TAS-2 is a small probe compared to a full-length protein, it can be cleaved in a ubiquitin-independent manner, *i.e.* without the association of the 19S RP with the 20S CP, for which Rpn-6 is critical. Therefore, we next wanted to evaluate if TXS-8 could affect the ubiquitin-dependent activity of the proteasome.

To investigate if TXS-8 could affect the 26S proteasome activity, we wanted to monitor the degradation of a full-length, folded protein. We decided to used green fluorescent protein (GFP), as it is easy to transfect in HEK-293T cells and previous literature demonstrates that GFP is degraded through the ubiquitin-dependent proteasome pathway.[25]

We first tried co-transfecting HEK-293T cells with the GFP plasmid and siRNA-Rpn-6, but the cells were not viable.[26] Instead, we decided to transfect GFP first overnight then the siRNA-Rpn-6 for 48 hours (Figure S6).

Cell viability and GFP degradation was investigated after the transfected cells were treated with 30 μM of TXS-8 for 3.5 hours (Figure S7). We discovered that GFP degradation was not affected by TXS-8 treatment when Rpn-6 was knocked down by siRNA transfection or at normal levels (Figure 6). This result agrees with the TAS-2 activity experiments that the interaction between TXS-8 and Rpn-6 does not affect proteasome activity.

Since we were unable to detect significant changes in proteasome activity in response to dosing HEK-293T cells with TXS-8, we next turned our attention to using a primary cell line, as the impacts of TXS-8 may be more pronounced. We selected the primary cardiomyocyte cell line, AC16 for further study. Recent studies have indicated that prolonged exposure to the proteasome inhibitor bortezomib results in cardiac cell damage and reduced proteasome activity.[27,28]

Since cardiomyocytes are reported to be sensitive to proteasome inhibition, we sought to determine if TXS-8 could modulate their proteasome activity. Lysate from AC16 cells was subjected to western blot analysis to ensure that the Rpn-6 subunit was detectable in this cell line (Figure 7A). A band corresponding to the molecular weight of Rpn-6 (49 kDa) was observed. We then established viability of the cells in response to dosing with varying concentrations of TXS-8 using the CellTiter-Glo reagent (Figure 7B). The AC16 cells appeared to be more sensitive to TXS-8 than HEK-293T, as reflected by significant cell death after dosing with 25 μM of the compound while HEK-293T cells were still viable at this concentration. Since the AC16 cells were still about 90% viable after dosing with 12.5 μM of TXS-8, this concentration was selected for further study.

After establishing the proper dosing conditions, we next sought to determine if dosage of TXS-8 resulted in alterations of proteasome activity in these cardiomyocytes. Cells were dosed with TXS-8 at 12.5 μM for 3.5 hours. As a control, cells were also dosed with DMSO or 5 μM of MG-132, a known proteasome inhibitor.[29] Cells were then washed, and the corresponding compounds and TAS-2 were diluted in KRBH buffer and this solution was added to the wells. Fluorescence intensity was monitored over time to determine if TXS-8 altered the ability of the proteasome to cleave the TAS-2 probe (Figure 8). TXS-8 did not appear to impact proteasome activity, as the rate of probe cleavage in TXS-8 dosed cells was similar to that of the DMSO control cells. As expected, dosing with MG-132 resulted in reduced proteasome activity. This data suggests that although AC16 cells are more sensitive to TXS-8 than HEK-293T cells, TXS-8 does not alter proteasome activity in this cell line and agrees with our previous results.

Conclusion

Here we describe the workflow for investigating the impact of a small molecule on proteasome activity using TXS-8 as an example (Figure 9 & S8). Although our results suggest that TXS-8 does not affect proteasome activity at non-lethal concentrations, the workflow we propose for elucidating the impacts of a small molecule on proteasome activity could be used to study other small molecule-proteasome interactions. The workflow is designed in three steps, all of which are performed in cells. The TAS-2 probe used in step 1

in Figure 9 is significantly more sensitive to changes in proteasome activity as compared to the traditional aminomethyl coumarin probes.

TXS-8 was previously discovered as a binder to Rpn-6, a subunit of the 26S proteasome. Using our described assays, it does not appear that TXS-8 does affect proteasome activity molecule at non-lethal concentrations. We hypothesize that Rpn-6 may be involved in other cellular process that do not involve the proteasome as literature has pointed out Rpn-6 has a recognition helix that could be placed in the major groove of DNA.[18] Future studies with TXS-8 include cellular localization studies in HEK-293T and Ramos B-cells.

Small molecules that affect proteasome activity through interactions beyond inhibiting the active sites could be of significant interest. We were able to develop critical assays that could be broadly applied to study future Rpn-6 small molecule binders and investigate their impact on proteasome activity. These assays should be amenable for the evaluation of small molecule binders to other proteasome subunits of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This work was supported through a start-up package from Purdue University School of Pharmacy and the Purdue University Center for Cancer Research NIH grant (P30 CA023168) Phase II grant. MEM was supported by a fellowship from the Purdue Cancer Center. Figure 9 was generated using Bio-render.

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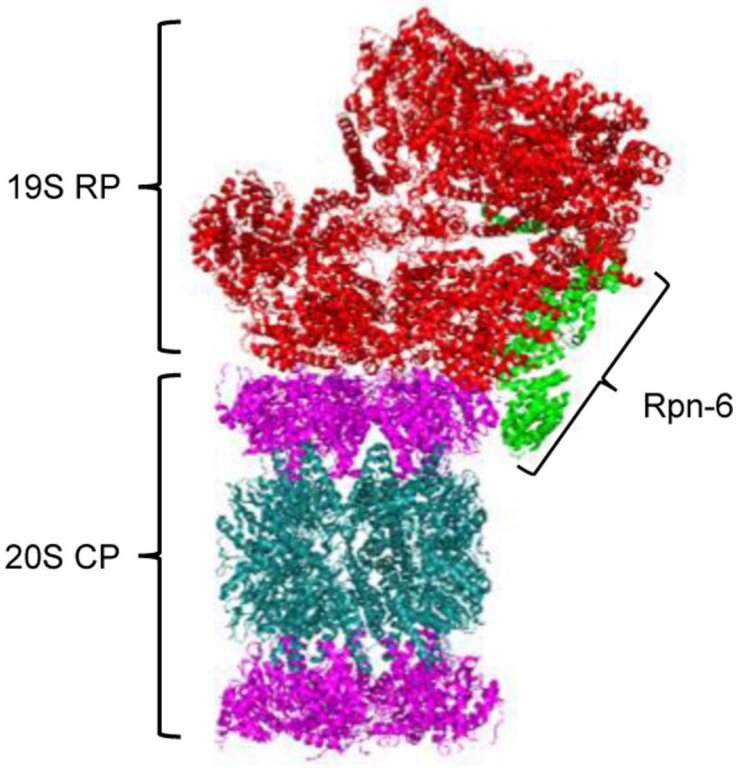

Figure 1.
The 26S proteasome is made up of the 19S regulatory particle (RP) and the 20S core particle (CP). Rpn-6 (green) is an essential proteasome subunit that helps to maintain the structural integrity of the 26S proteasome.

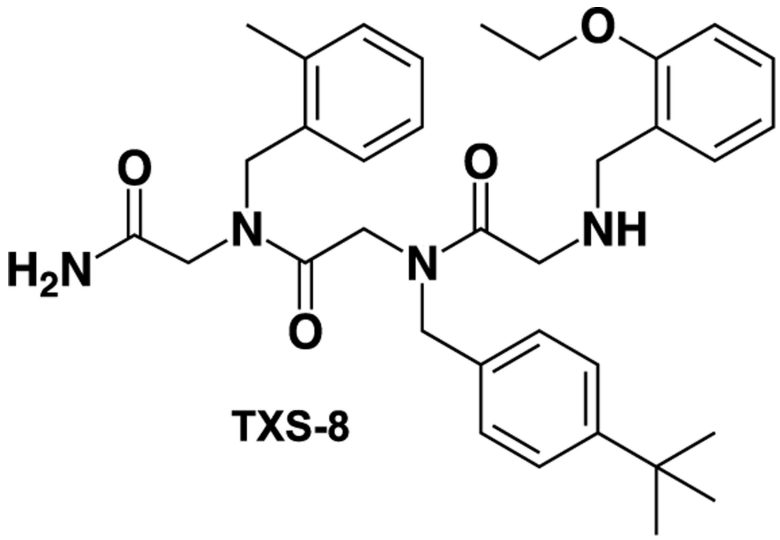

Figure 2.

TXS-8 was discovered by screening a peptoid library utilizing a thermal shift assay against purified Rpn-6. Validation of the binding of TXS-8 to Rpn-6 was performed using fluorescent polarization assays and cross-linking experiments. The K_d of TXS-8 to Rpn-6 is ~14 μM.

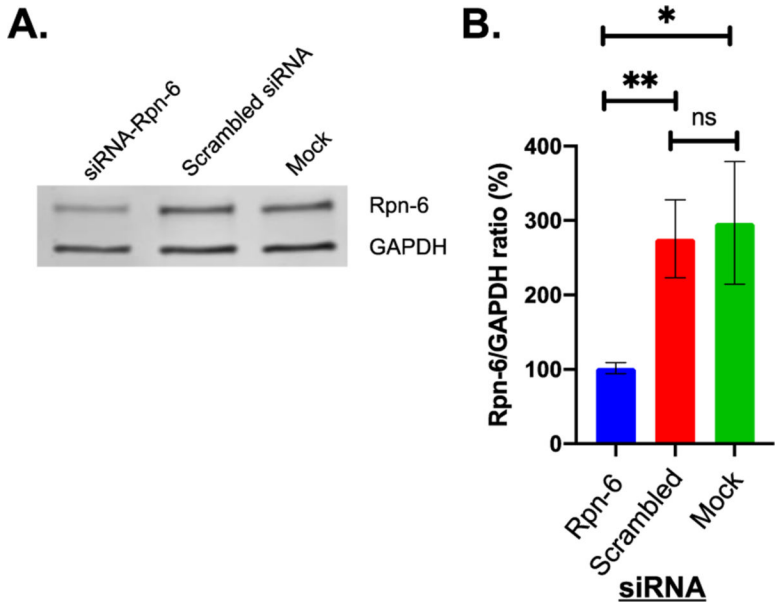

Figure 3.

(A) Western blot analysis with anti-Rpn-6 and anti-GAPDH antibodies of HEK-293T lysate after transfection for two days. (**B**) Rpn-6 expression was reduced by 60% when HEK-293T cells were treated with siRNA to knockdown expression of Rpn-6. The scrambled siRNA and the mock transfection conditions did not affect Rpn-6 levels. Error bars represent SEM and n=3. *p<0.05, **p<0.005

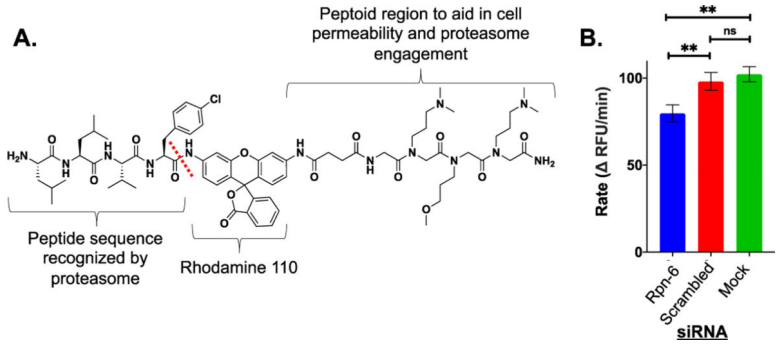

Figure 4.

(**A**) The TAS-2 probe can be used to monitor proteasome activity in live cells. The 20S CP/26S can cleave the probe between the peptide and rhodamine, produces a fluorescent signal. **(B)** Hydrolysis rate comparison of proteasome activity in transfected HEK-293T cells. The Rpn-6 knockdown HEK-293T cells exhibited a 15% decrease on the proteasome activity. Error bars represent SEM and n=4. **p<0.005, ns= p>0.05.

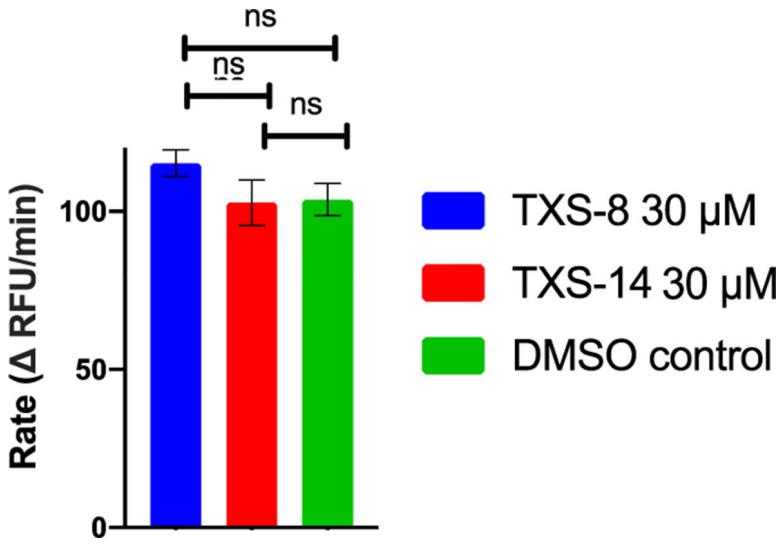

Figure 5.

Proteasome hydrolysis rate comparison in HEK-293T cells. No significant changes were observed with TXS-8 treatment. TXS-14 was a derivative of TXS-8 with the second amine exchanged for *N*-methylamine (Figure S1A). TXS-14 does not bind Rpn-6, thus serves as a negative control. Error bars represent SEM and n=4, ns= $p>0.05$.

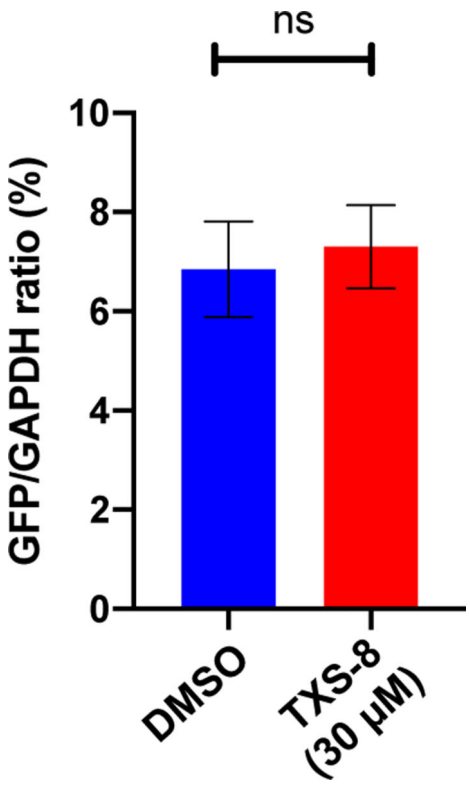

Figure 6.

GFP level comparison in HEK-293T cells with 30 μM TXS-8. The amount of GFP was not altered after dosing cells with TXS-8, indicating that 26S proteasome activity was not impacted. Error bars represent SEM and n=4, ns= $p>0.05$.

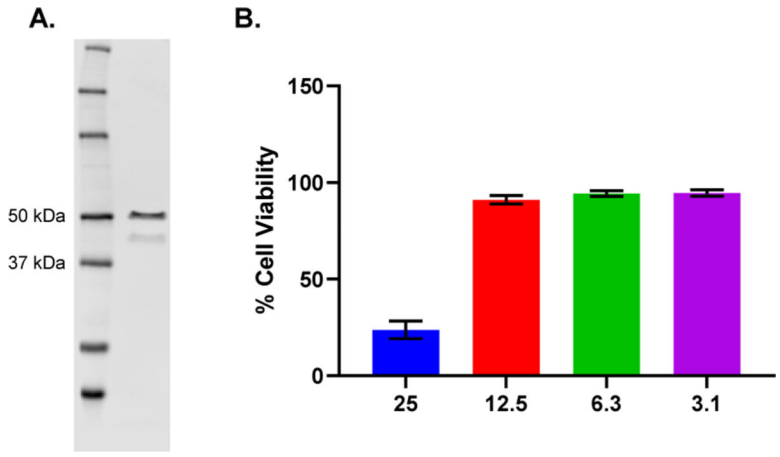

Figure 7.

(**A)** Western blot of AC16 lysate for Rpn-6. **(B)** Cell viability of AC16 cardiomyocytes treated with various concentration of TXS-8 for 3.5 hours. Significant cell death was observed when TXS-8 concentration is above 12.5 μM. Error bars represent SEM and n=3

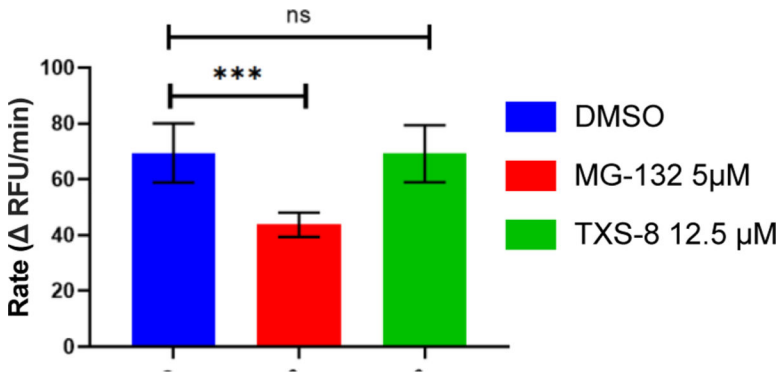

Figure 8.

Hydrolysis rate comparison in AC16 cells was monitored with TAS-2. No significant change in proteasome activity was observed with TXS-8 treatment while the proteasome inhibitor MG-132 significantly decreased the hydrolysis rate of the TAS-2 probe. Error bars represent SEM and n=5, ns= $p>0.05$, ***$p<0.0005$.

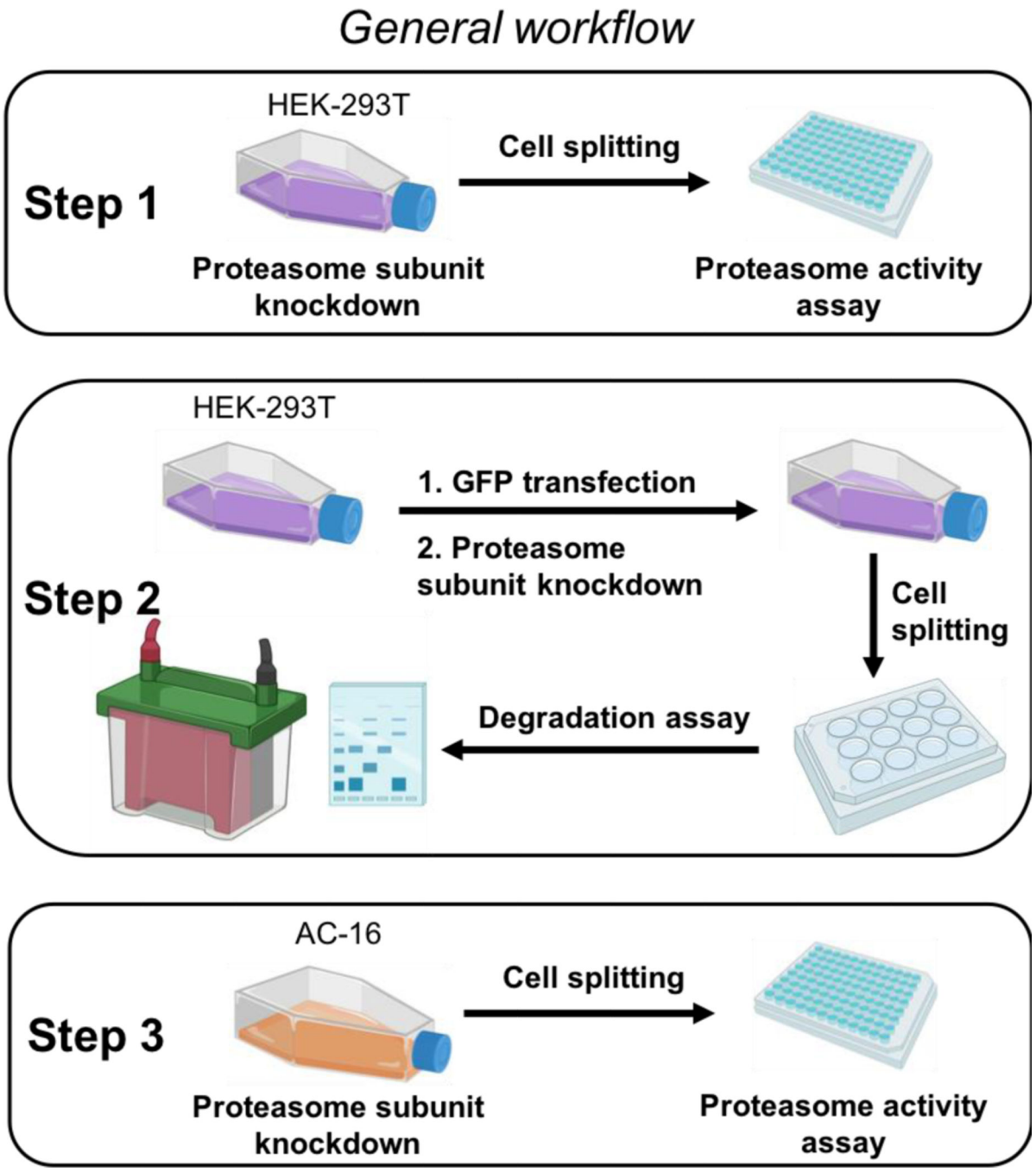

Figure 9.
General workflow of for the investigation of the impact of a small molecule on proteasome activity.