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Oleic amide derivatives as small molecule stimulators of the human proteasome's core particle†

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A series of oleic acid amide derivatives were synthesized based on our previous and continuing endeavors towards stimulation of the 20S core particle of the proteasome (20S CP) with the goal of increasing the protein degradation rate *via* the ubiquitin-independent pathway. The designed compounds were tested in a variety of biochemical and cell-based assays to assess their ability to increase the rate of hydrolysis of the 20S CP, and compared to a known fatty acid amide stimulator of the 20S CP, AM-404. AM-404 was previously described to stimulate the activity of the 20S CP, however, it does negatively affect viability of cells after prolonged dosing. Here we report the development of several small molecules with a similar ability to enhance the activity of the 20S CP as AM-404. While one molecule (**17**) was just as potent as AM-404, it still caused significant unwanted cytotoxicity. Molecules such as these are compatible with biochemical assays and short-term cell-based proteasome activity assays, but their unwanted toxicity limits their use in prolonged cell assays or *in vivo* studies.

Introduction

The synthesis and degradation of proteins are two essential cellular processes. Proteins are typically degraded through a multi-step process that ends with the ubiquitinated proteins being hydrolyzed into peptides *via* the 26S or 30S isoform of the proteasome.[1] The uncapped isoform of the proteasome, known as the 20S core particle (20S CP), has a preference to degrade proteins that have enough disorder to fit through the gate formed by the N-termini of the alpha-ring subunits.[1,2] We and others have been interested in how small molecules can be used to increase the rate at which the 20S CP can degrade proteins, as many proteins associated with neurodegenerative diseases can be hydrolyzed by the 20S CP.[3–5]

We recently have been interested in small molecules that may be able to dissociate the protein–protein interactions of the N-termini of the alpha subunits, leading to a more open gate conformation which could enable greater amounts of protein to enter and be degraded. One example is AM-404, that has been previously demonstrated as an effective stimulator of the 20S CP, Fig. 1A.[6]

Given that the polyunsaturated fatty acid (PUFA) chain of AM-404 is prone to oxidation and isomerization,[7,8] we have in a previous communication described studies that sought to determine the minimum chain length and unsaturation of the lipid chain of AM-404 required to elicit 20S CP stimulation.[9] Our results indicated that a *cis* alkene between carbons nine and ten was required to generated significant

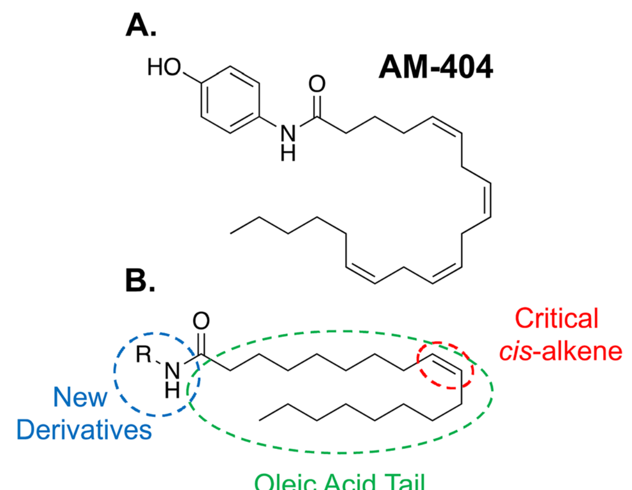

Fig. 1 (A) AM-404, a previously discovered 20S CP stimulator. (B) General structure of oleic acid amide derivatives synthesized by conjugating respective amines to the oleic acid explored in this work for their ability to stimulate the 20S CP.

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stimulation. Unlike PUFA's, mono-unsaturated fatty acids do not readily undergo isomerization or oxidation since an isolated alkene is far more stable towards oxidation or isomerization under except under harsh conditions. With this result in mind, we wanted to explore if modifying the other end of the carbon chain (in the case of AM-404 where the amino phenol is located, which we refer to as the head group) could result in an increase in 20S CP activity and minimize the cell toxicity that has been observed with AM-404.

Here in we report here the conjugation of oleic acid with a variety of amines, Fig. 1B, and evaluated their ability to increase the ability of the 20S CP to degrade a fluorescent peptide reporter and alpha-synuclein, an intrinsically disordered protein that has been associated with Parkinson's disease.[4,10–12] When compared with AM-404, our best molecule, **17**, exhibited a similar *in cellulo* stimulatory activity of the 20S CP, as well as toxicity profile.

Results and discussion

AM-404, a derivative of arachidonic acid, was discovered in 2017 as a 20S CP stimulator following a high throughput screen, and then being validated in a cell-based assay.[6] Previously, we investigated the structure activity relationship between the aliphatic chain of AM-404 and its 20S stimulatory activity.[9] In this study, it was concluded that molecules with a *cis*-alkene and an extended saturated chain produced the most potent stimulators.

In the work presented here, we extended our structure–activity relationship (SAR) efforts on the AM-404 scaffold by replacing the amino-phenol headgroup. Using oleoyl chloride, we could quickly access a range of structures through amide bond formation with various amines. As such, we chose to explore four categories of head groups with commercially available amines. These structures were obtained in sufficient yields for biochemical testing, with minimal purification required (see ESI† Appendix A for characterization and Fig. S1). Using our previously developed FRET peptide probe,[9,13] we measured the effects of each molecule on the 20S CP activity. Briefly, in a 384-well plate a sample containing purified 20S CP, the FRET reporter and the experimental compound dissolved in DMSO was prepared for each of the synthesized analogs, and the change in fluorescence was monitored over time in a microplate reader. The fluorescence was recorded every two minutes, and the rate of increase in signal was compared to the basal level (20S CP treated with DMSO) to evaluate the increase in activity in the presence of the newly synthesized molecules (ESI† Fig. S2). All molecules were screened at 25 μM and compared to DMSO (basal level of activity) and AM-404 at 25 μM (positive control). Molecules were tested in triplicate to generate an average increase in proteasome activity. The error in the % stimulation was no more than ±10% for all compounds.

Our initial set of molecules consisted of *para*-substituted anilines conjugated to oleic acid, Table 1. A variety of stimulatory profiles were observed for these compounds. Across the Table 1, the data suggests that the presence of a H-bond acceptor (delta minus) at the *para* position of the phenyl ring has an agonistic effect on stimulation. Molecule **6**, which contains the amino phenol, was the most effective, but elicited only 50% of the stimulation achieved with AM-404 at 25 μM *in cellulo*.

Table 1 Aromatic headgroups and their respective stimulatory activity at 25 μM conc. using a FRET probe. Evaluation of anilines conjugated to oleic acid. Molecule **6**, which has the same head group as AM-404 showed the best stimulation activity

Molecule	Structure	% stimulation
AM404	HO, O, N, H	219%
Molecule #	**R**	**% stimulation**
1		159%
2	O	241%
3	O	145%
4	Cl	144%
5		68%
6	HO	247%

The next set of tested molecules incorporated a benzyl amines, rather than an aniline, with a variety of substitutions on the aromatic ring, Table 2. We hypothesized that this will impart a slightly higher conformational flexibility to the molecules compared to their aniline counterparts, and might result in better ligand docking. Looking at the pairwise comparison of the two aforementioned series, we see a moderate increase in stimulation for the pair (**1**, **7**), (**5**, **13**) and (**6**, **17**). The pair (**4**, **12**) however shows a slight decrease in stimulation. Across the series in Table 2, adding a larger substitution at the *para* position, such as methoxy (**11**) and *t*-butyl (**13**), or a nonpolar substitution, such as a methyl (**10**), trifluoromethyl (**14**), decreases 20S CP stimulation. This pattern is also evident in Table 1. Next, comparing molecules **7**, **8**, and **9**, we concluded that a methyl substitution at the benzylic carbon made little difference in the molecules' ability to stimulate the 20S CP. From this set, the best molecule was **17**, which contains a phenolic OH at the *para* position. This derivative **17** and molecule **6** are identical except for the extra methylene between the aromatic ring and the amide in **17**.

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Table 2 Benzylic headgroups and their respective stimulatory activity at 25 μM conc. using a FRET probe. Benzylamines were conjugated to oleic acid and evaluated for their ability to stimulate the 20S CP

Molecule #	R	% stimulation
7		208%
8		195%
9		225%
10		167%
11		116%
12	Cl	138%
13		106%
14	F F F	92%
15		107%
16		183%
17	HO	361%

To further explore the tolerated substitutions of the amide, we replaced the aryl ring with alkyl rings of a variety of sizes, Table 3. We envisioned that we could use this information to explore if perhaps a different ring size off the aliphatic chain would be of interest. Interestingly, compared to having just a phenyl head group (molecule **1**), aliphatic rings of size 4–6 (molecules **18**–**20**) portrayed a higher stimulatory activity. This might result from a better space filling in the binding pocket due higher conformational flexibility of the smaller rings compared to a phenyl group. Once a ring size larger than six was included, the stimulatory activity decreased significantly (molecules **21**–**23**), possibly due to increased steric crowding in the binding pocket. Additionally, molecules with linear substitution also had weak stimulatory results (Table 4). Considering all these results, there is a clear preference to have a phenyl ring with a small H-bond acceptor functionality at the *para* position to the amide. Molecule **6** (Table 1) was the most effective in the FRET assay, but elicited only 50% of the stimulation achieved with AM-404 at 25 μM *in cellulo*. So,

Table 3 Aliphatic cyclic headgroups and their respective stimulatory activity at 25 μM conc. using a FRET probe. The weak stimulation of these molecules indicates that a phenol group is preferred at the head group position

Molecule #	R	% stimulation
18		202%
19		218%
20		204%
21		136%
22		148%
23		118%

we moved forward with **2** instead. **17** was chosen from Tables 2 and 3 (with cyclic head groups), and **25** was chosen from Table 4 to evaluate them in additional assays.

To evaluate if these molecules were able to stimulate the 20S CP in cells, we used a probe developed by the Ovaa group that can covalently bind the beta-5 active site of the 20S CP.[14,15] This probe also contains a BODIPY fluorophore that can be used to quantitate how much of the 20S CP was able to react with the probe. Cells are pre-dosed with the stimulator for 2 h followed by the addition of 500 nM of the probe, incubated for an additional hour, lysed, and the cell lysate was subjected to SDS-PAGE. The amount of proteasome activity correlates with the amount of BODIPY signal observed at the molecular weight for beta-5 (ESI† Fig. S3). HEK-293 T cells were dosed with three different concentrations of molecules **2**, **17**, **25**, and AM-404, Fig. 2. Both molecules, **17** and **25**, were nearly identical in their ability to stimulate the 20S CP as compared to AM-404 at 25 μM. Molecule **17** at all three concentrations maintained similar levels of stimulation as compared to AM-404, which decreases in a dose–response manner.

Next, we wished to determine if these three compounds affect viability to a lesser extent as compared to AM-404. HEK-

Table 4 Simple aliphatic headgroups and their respective stimulatory activity at 25 μM conc. using a FRET probe. Conjugation of aliphatic amines to oleic acid yielded the weakest stimulators

Molecule #	R	% stimulation
24	H_3C	132%
25		154%
26		138%
27		124%
28		139%

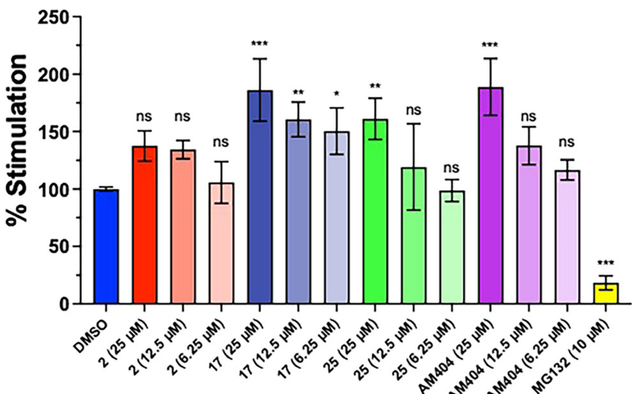

Fig. 2 HEK-293T cells were treated with DMSO (basal level of activity), three different concentrations of each molecule, and MG-132 (a proteasome inhibitor which serves as a negative control). All molecules showed an expected dose–response, with molecule **17** being as effective at AM-404. Significance between the DMSO treated and stimulator treated was determined using one-way ANOVA analysis. ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 6$.

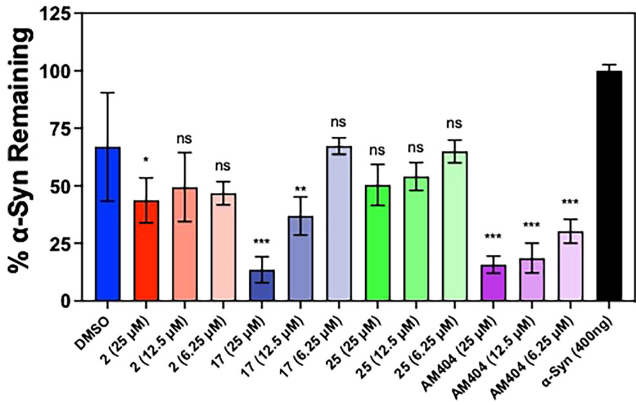

Fig. 4 Degradation of α-Syn using purified 20S CP. **17** and AM-404 (25 μM) led to the greatest 20S mediated degradation of alpha-synuclein. Significance between the DMSO treated and stimulator treated was determined using one-way ANOVA analysis. ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 6$.

293T cells were dosed with three concentrations of all molecules for 24 h. The viability of the cells was determined using CellTiter-Glo® and the amount of luminescent signal of the treated samples was compared to those treated with DMSO. As shown in Fig. 3, molecule **17** affected viability to a similar extent as AM-404 at all concentrations. Molecule **2** was the least toxic, but also had the least amount of stimulation (Fig. 2).

Despite the disappointing viability results that our new molecules were just as toxic as AM-404, we wanted to determine if they were still able to aid the 20S CP in degrading alpha-synuclein. The accumulation of alpha-synuclein is a major contributing factor in Parkinson's disease and there is a hypothesis that increasing 20S CP activity could decrease the available monomers of alpha-synuclein that aggregate to form the toxic oligomeric species.[2,11,12] Monomeric alpha-synuclein was incubated with

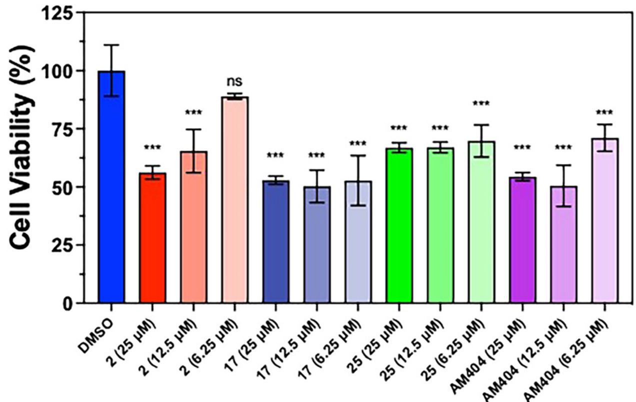

Fig. 3 Cell viability data: HEK-293T cells were treated for 24 hours with each of the molecules at various concentrations. Cells treated with stimulator were compared to those treated with DMSO (set to be 100% viable). Significance between the DMSO treated and stimulator treated was determined using one-way ANOVA analysis. ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 6$.

purified 20S CP for 1 h at 37 °C. The three molecules and AM-404 were tested at three concentrations. After incubation, the 20S CP activity was quenched by the addition of Laemmli sample buffer that contained beta-mercaptoethanol. The sample was loaded into a SDS-PAGE gel and stained with coomassie. The 20S CP is in such low quantities that it cannot be detected, therefore, the only band detected will be the remaining alpha-synuclein.[10] The amount of degradation was determined by comparing the band intensity from loading the initial amount of alpha-synuclein (400 ng) and comparing the band intensity from the samples treated with a stimulator (ESI† Fig. S4). The 20S CP without a stimulator can degrade alpha-synuclein as indicated by the sample treated with only DMSO. The addition of the stimulators increased the amount of degradation, with AM-404 still being the best to stimulate the 20S CP, Fig. 4. Molecule **17** at 25 μM showed an ability to degrade alpha-synuclein at the same level as AM-404, but rapidly lost potency. Molecules **2** and **25** were able to increase alpha-synuclein degradation, but not as significantly as compared to **17** and AM-404.

Conclusion

20S CP stimulators represent an exciting class of molecules that can increase the degradation of proteins through the ubiquitin-independent pathway. It is likely within the class of 20S CP stimulators, there will be several mechanisms of action. These include stabilization of the open-gate conformation as pioneered by the Tepe research group,[4,16] molecules that can interact with the active sites aiding in the turnover rate of substrates, and molecules such as those described here that can disrupt the protein–protein interactions of the 20S CP gate allowing substrates to enter the catalytic core more easily. These protein–protein interaction inhibitors have been of interest to us as not only 20S CP stimulators, but may also be able to help disrupt protein aggregates, which can then be degraded by the 20S CP.

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AM-404 is an effective 20S CP stimulator, but can cause a decrease in viability with prolonged dosing. The set of molecules we present here were designed to evaluate if the shorter and more saturated aliphatic chain of oleic acid with different head groups could be as effective as AM-404, while being more stable. While molecule **17** had similar stimulation capabilities as AM-404, it still negatively affected cell viability. Despite this result, molecule **17** has better stability properties than AM-404 as the poly-unsaturated carbon chain of *cis*-alkenes can easily isomerize to *trans*-alkenes, as well degrade *via* aerial/photo oxidation.[7,8] This reduces its ability to stimulate the 20S CP and can lead to irreproducible results, depending on the amount of degradation. Therefore, it might be better to use molecules such as **17** for more consistent results when evaluating if this mechanism of action is effective.

Conflicts of interest

Prof. Trader is a shareholder and consultant for Booster Therapeutics, GmbH. Other authors declare no conflict of interest.

Acknowledgements

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