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Inhibition of the purified 20S proteasome by non-heme iron complexes

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Abstract

Polypyridyl pentadentate ligands N4Py (**1**) and Bn-TPEN (**2**), along with their respective iron complexes, have been investigated for their ability to inhibit the purified 20S proteasome. Results demonstrated that the iron complexes of both ligands are potent inhibitors of the 20S proteasome ($IC_{50} = 9.2 \mu M$ for $[Fe^{II}(OH_2)(N4Py)]^{2+}$ (**3**) and $4.0 \mu M$ for $[Fe^{II}(OH_2)(Bn-TPEN)]^{2+}$ (**4**)). Control experiments showed that ligand **1** or Fe^{II} alone showed no inhibition, whereas **2** was moderately active ($IC_{50} = 96 \mu M$), suggesting that iron, when bound to these ligands, plays a key role in proteasome inhibition. Results from time-dependent inactivation studies suggest different modes of action for the iron complexes. Time-dependent decay of proteasome activity was observed upon incubation in the presence of **4**, which accelerated in the presence of DTT, suggesting reductive activation of O_2 and oxidation of the 20S proteasome as a mode of action. In contrast, loss of 20S proteasome activity was not observed with **3** over time, suggesting inhibition through direct binding of the iron complex to the enzyme. Inhibition of the 20S proteasome by **4** was not blocked by reactive oxygen species scavengers, consistent with a unique oxidant being responsible for the time-dependent inhibition observed.

Introduction

The ubiquitin–proteasome system is an important target in cancer chemotherapy. Proteasome inhibitors induce apoptosis in cancer cells while at the same time showing reduced toxicity to normal cells. Examples of medicinal agents that target the proteasome include peptidomimetics such as boron-based inhibitor bortezomib,¹ recently approved for the treatment of multiple myeloma and mantle cell lymphoma, as well as a series of transition metal complexes based on nickel, copper, zinc, gold and gallium that are in preclinical development and show anticancer activities in live cells and animals.^{2–6} Most of these agents inhibit the proteasome by binding to the enzyme's catalytic subunits. However, recent studies have revealed an exciting strategy towards enzyme inhibition, where rather than blocking the activity by binding alone, metal-based reagents diminish enzyme activity through oxidation of the target enzyme. This strategy has proven effective against medically relevant targets,^{7,8} uses biological oxidants such as H_2O_2 and O_2 ,^{7,9} and can be carried out selectively in live cells.¹⁰ Enzyme oxidation has great potential because of its irreversible nature, as well as its power to reduce the concentration of an inhibitor that is needed to provide the desired effect.¹⁰ In this essay, we describe a new iron-based approach

towards the targeting of the proteasome that occurs spontaneously in the presence of a non-heme iron complex and air. This chemistry was established against the purified 20S proteasome *in vitro*.

Polydentate nitrogen-containing ligands such as N4Py (**1**) and Bn-TPEN (**2**) (Fig. 1), developed by bioinorganic chemists for modeling non-heme iron active sites,^{11–14} demonstrate many properties that are promising for biological applications. These ligands bind iron tightly with dissociation constants in the pM–fM range and mobilize Fe^{II} from ferritin, the major intracellular storage site for iron, suggesting that such ligands could access iron in cells.¹⁵ Iron complexes derived from N4Py (**1**), Bn-TPEN (**2**) and other ligands of this class activate dioxygen and also generate high-valent iron species such as ferryls (Fe^{IV}O) that are potent oxidants of organic molecules.^{12,16–18} Recent studies from our laboratory established that iron complexes based on pentadentate ligands have applications in the area of amino acid, peptide and protein oxidation.^{19–21} Because previous work with the proteasome demonstrated that the enzyme is inactivated by oxidants,^{22–24} we examined the ability of these iron complexes to inhibit the 20S proteasome.

Materials and methods

Materials

N4Py (**1**),¹¹ Bn-TPEN (**2**)²⁵ and TPEN²⁶ were synthesized according to literature methods. Iron(II) perchlorate hydrate and dithiothreitol (DTT) were purchased from Sigma-Aldrich and Acros chemicals, respectively. Purified human 20S proteasome and fluorogenic substrates, Suc-LLVY-AMC and Bz-VGR-AMC (for proteasomal chymotrypsin- and trypsin-like activities, respectively), were from Boston Biochem (Cambridge, MA) whereas the fluorogenic substrate Z-LLE-AMC (for proteasomal peptidylglutamyl peptide hydrolyzing-like activity) was from Calbiochem. Human 20S proteasome was aliquoted in buffer (1 M HEPES, 3 M NaCl, final concentration: 25 nM). The iron complexes [Fe^{II}(OH₂)(N4Py)]²⁺ (**3**) and [Fe^{II}(OH₂)(Bn-TPEN)]²⁺ (**4**) were generated *in situ* by treating ligands **1** and **2** in 100 mM MOPS buffer (pH 7.4) containing 1% DMSO with 1.0 equiv. of Fe^{II}(ClO₄)₂·xH₂O.

Purified 20S proteasomal inhibition assay

Purified human 20S proteasome (0.5 nM, final concentration) was incubated with 20 μM of a fluorogenic substrate, Suc-LLVY-AMC or Bz-VGR-AMC or Z-LLE-AMC (for proteasomal chymotrypsin-, trypsin- and peptidylglutamyl peptide hydrolyzing-like activities, respectively), in 100 μL assay buffer (100 mM MOPS buffer, pH 7.4) for 30 min at 37 °C in the presence of inhibitor molecules at different concentrations (0–1000 μM). After incubation, proteasomal activities (production of hydrolyzed AMC) were measured with a Wallac Victor multilabel counter (PerkinElmer, Waltham, MA) with an excitation filter of 365 nm and emission filter of 460 nm. Background fluorescence was subtracted from the blank reaction. Activities were plotted against log [inhibitor] and fit to a sigmoidal curve to calculate IC₅₀ values.

Time-dependent 20S proteasomal inactivation assay

Purified human 20S proteasome (0.5 nM, final concentration) was incubated at room temperature with 10 μM inhibitor in the presence or absence of DTT (250 μM) for different time periods (0–165 min) in assay buffer (100 mM MOPS buffer, pH 7.4). A fluorogenic substrate, Suc-LLVY-AMC (20 μM), was added and the mixture was further incubated for 15 min at 37 °C. Proteasomal activities (production of hydrolyzed AMC) were measured with a Wallac Victor multilabel counter with an excitation filter of 365 nm and emission filter of 460 nm. In control experiments, reactive oxygen species scavengers D-mannitol (20

mM) and NaN_3 (10 mM) were added as aqueous solutions prior to treatment with the iron complexes.

Results and discussion

Studies began by evaluating the ability of ligands **1** and **2** and their ferrous complexes $[\text{Fe}^{\text{II}}(\text{OH}_2)(\text{N4Py})]^{2+}$ (**3**) and $[\text{Fe}^{\text{II}}(\text{OH}_2)(\text{Bn-TPEN})]^{2+}$ (**4**), to inhibit the chymotrypsin-like activity of the purified 20S proteasome. The purified 20S proteasome (0.5 nM) was treated with varied concentrations (0–1000 μM) of **1–4**, along with $\text{Fe}^{\text{II}}(\text{ClO}_4)_2 \cdot x\text{H}_2\text{O}$ as a control, in 100 mM MOPS buffer (pH = 7.4). IC_{50} values were calculated (Table 1). The results indicated that ligand **1** was ineffective in inhibiting the chymotrypsin-like activity of the proteasome ($\text{IC}_{50} > 1000 \mu\text{M}$) but its ferrous complex **3** was a more potent inhibitor with an IC_{50} value of 9.2 μM (entry 2). Similar results were observed for ligand **2** where its ferrous complex **4** was more potent than ligand **2**. Ligand **2**, unlike ligand **1**, also inhibited the proteasome, albeit at a 25-fold higher concentration than its ferrous complex **4** (Table 1, entries 3 and 4). Despite the fact that $\text{Fe}^{\text{II}}(\text{ClO}_4)_2$ had no effect on proteasome activity (Table 1, entry 5), the ferrous complexes **3** and **4** showed more potent inhibition than the ligands alone. These results confirm that iron and the ligand together play a key role in proteasome inhibition. Surprisingly, the trypsin- and PGPH-like activities of the proteasome were not affected by these ferrous complexes, indicating a high level of selectivity by these ferrous complexes toward inhibition of chymotrypsin-like activity of the proteasome, which is consistent with oxidative inactivation.²⁷

Based on literature data, two possible pathways were considered for proteasome inhibition, either direct binding of the iron complexes to the α_5 subunits (responsible for the chymotrypsin activity) of the 20S proteasome,^{2,4} or alternatively oxidation of the enzyme (Fig. 3).^{10,22,23,28} In order to gain insight into the mode of action, the 20S proteasome was incubated with ferrous complexes **3** and **4** (10 μM) at room temperature under an aerobic atmosphere and the activities were determined as a function of incubation time. Proteasome activity did not change over time with **3**, suggesting that direct binding of the iron complex to the enzyme contributed to the observed inhibition (Fig. 2a). In contrast, time-dependent inactivation was observed in the presence of **4**, where 20S proteasome activity was diminished from ~70% to ~10% over the course of 165 min at room temperature (Fig. 2b). Time-dependent loss of proteasome activity was not observed with ligand **2** or with $\text{Fe}^{\text{II}}(\text{ClO}_4)_2$ alone, both in the presence and absence of the reductant dithiothreitol (DTT) (results not shown). However, proteasome activity was lowered, albeit slightly, with **4** in the presence of DTT, consistent with the reductive activation of O_2 playing a role in enzyme inactivation (Fig. 2d).⁷ This mode of action was further corroborated by the fact that time-dependent inhibition was not observed with $[\text{Fe}^{\text{II}}(\text{TPEN})]^{2+}$ (Fig. 2c), in which all six coordination sites around the iron center are occupied (Fig. 4). Blocking all six sites would inhibit access of O_2 to the iron center with respect to **4**, which contains a labile H_2O , presumably allowing **4** to reach a coordinatively unsaturated state easier than $[\text{Fe}^{\text{II}}(\text{TPEN})]^{2+}$. It is important to note that the timescale for the decay of proteasome activity observed with **4** is too long to result from simple binding of the iron complex to the enzyme. Rates for association of small molecules with proteins are extremely fast ($k = 10^6$ – $10^8 \text{ M}^{-1} \text{ s}^{-1}$),²⁹ much faster than the time scale of the experiments (165 min). Taken together, these results are consistent with **4** oxidizing the 20S proteasome over time, leading to its inactivation.

In order to gain further insight into the nature of the oxidant, control experiments were performed in the presence of reactive oxygen species (ROS) scavengers. When the inactivation of the 20S proteasome with **4** was performed in the presence of NaN_3 (10 mM), a potent singlet oxygen ($^1\text{O}_2$) scavenger, 20S proteasome inhibition was still observed,

precluding a major role for $^1\text{O}_2$ (Fig. 5). Similar results were obtained in the presence of D-mannitol (20 mM), a potent scavenger of the hydroxyl radical and superoxide,³⁰ confirming that another oxidant apart from ROS is responsible for the time-dependent proteasome inhibition observed with **4**. These results suggest that a metal-based oxidant derived from **4** may be responsible for the loss of proteasome activity over time. Although the exact nature of this oxidant has not been confirmed, activation of O_2 by non-heme iron complexes is known to generate Fe^{III} -superoxo, -hydroperoxo and Fe^{IV} -oxo species.^{31–34} Our own work proved that iron-based oxidants of this class inactivate the serine proteases trypsin and chymotrypsin efficiently.⁹ Furthermore, we have observed that **3** decomposes much slower than **4** under aerobic conditions, which agrees well with **4** activating O_2 at a faster rate than **3**, and with differences in time-dependent inhibition between **3** and **4** (Fig. 2a and b). Therefore, faster oxidation of **4** may lead to a more rapid loss of the purified 20S proteasome activity over time under aerobic conditions.

Studies were conducted to gain insight into modes of action for compounds **1–4** in cells. PC-3 cells were incubated with these compounds and cell proliferation data were collected. These data, reported in another manuscript,³⁵ indicate that compounds **1–4** elicit apoptotic effects and give rise to potent cell death. Proteasome inhibition, however, was not observed, presumably because cell death occurs at lower concentrations than proteasome inhibition. However, even though proteasome inhibition was not observed in these live cell assays, it is important to note that the ligands and complexes **1–4** used in this study do not contain a proteasome binding motif. Directing these compounds to the proteasome by attachment of a binding motif could lead to more potent proteasome inhibition, giving the possibility of inhibiting the proteasome before cell death occurs.

Conclusions

In conclusion, non-heme ligands **1** and **2** and their iron complexes **3** and **4** were investigated for their ability to inhibit the purified 20S proteasome. Ferrous complexes **3** and **4** were more potent in inhibiting the proteasome than the ligands and iron salts alone. Control experiments confirmed a key role for iron and ligands in enzyme inhibition. The time-dependent inactivation studies suggested that the ferrous complexes of N4Py and Bn-TPEN have different modes of action, either binding to the target enzyme, or alternatively enzyme oxidation. Investigations precluded the role of ROS and suggested that an iron-based oxidant was responsible for loss of proteasome activity observed. Studies are now underway to understand the biological activities of these complexes further and to extend this method of enzyme targeting towards achieving anticancer activities.

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Fig. 1.
Polypyridyl ligands.

Fig. 2.

Time-dependent inactivation of the purified 20S proteasome. The reaction was performed by incubating the proteasome (0.5 nM) with 10 μM of ferrous complexes **3** (a), **4** (b and d) (blue) without DTT, d (red) with DTT, 250 μM) and $[\text{Fe}^{\text{II}}(\text{TPEN})]^{2+}$ (c) in 100 mM MOPS buffer at pH = 7.4. The fluorescence intensity was converted to %CT-like activity, with 100% activity equal to fluorescence of the blank reaction containing proteasome, in the absence of the inhibitor at $t = 0$.

Fig. 3.
Binding and oxidation mode of action of iron complexes for 20S proteasome inactivation.

Fig. 4. Model illustrating the coordination sphere around iron centers of the ferrous complexes derived from (a) Bn-TPEN and (b) TPEN. The sixth coordination site in model a is vacant for redox chemistry.

Fig. 5.

Inactivation of the purified 20S proteasome by ferrous complex **4** in the presence of ROS scavengers (azide and D-mannitol). The reactions were performed by incubating proteasome with **4** (10 μ M) in the presence/absence of DTT (250 μ M) for 80 min at rt in 100 mM MOPS buffer at pH = 7.4. The fluorescence intensity was converted to %CT-like proteasome activity, with 100% activity equal to fluorescence of blank reaction containing proteasome in the absence of inhibitor and additive. Data points are averages from three independent experiments, errors equal to the standard deviation of the data set.

Table 1

IC₅₀ values (μM) for inhibition of chymotrypsin-like (CT), trypsin-like (T) and peptidylglutamyl peptide hydrolyzing-like (PGPH) activity of 20S proteasome by ligands **1** and **2** and their respective ferrous complexes (**3** and **4**) along with iron metal ion control

Entry	Compound	CT ^a	T ^a	PGPH ^a
1	N4Py (1)	>1000	>1000	>1000
2	[Fe ^{II} (OH ₂)(N4Py)] ²⁺ (3)	9.2	>1000	>1000
3	Bn-TPEN (2)	96	>1000	>1000
4	[Fe ^{II} (OH ₂)(Bn-TPEN)] ²⁺ (4)	4.0	>1000	>1000
5	Fe ^{II} (ClO ₄) ₂	>1000	nd ^b	>1000

^aThe %proteasome activity at different concentrations was determined as the average from three independent experiments, with 100% activity equal to the fluorescence of the proteasome in the absence of inhibitor. Proteasome concentration was 0.5 nM; fluorogenic peptide substrates, Suc-LLVY-AMC (for proteasomal chymotrypsin-like activity) and Bz-VGR-AMC (for proteasomal trypsin-like activity), concentrations were 20 μM. The reactions were conducted at pH = 7.4 in 100 mM MOPS buffer.

^bnd = not determined.